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EFFECTS OF ACUTE DOSE OF *THEVETIA PERUVIANA* EXTRACT ON THE HAEMATOCYES OF A FRESH WATER EDIBLE SNAIL *BROTIA CAUSTULA*.

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ABSTRACT

Edible Snail *Brotia costula* were treated with molluscicide the extract of *Thevetia peruviana* seed and EC_{50} value were measured. In the same time both acute and long term and lethal and sub lethal doses were applied. To study the total count of haematocyte and differential count against those dose. The result shows that number of haematocyte varies significantly with exposure hour and doses of *Thevetia peruviana.* In the first few hour the blood cell count decreases remarkably. But after the critical period the snail try to their best to increase or maintain the normal T.C. Snails are able to produce as many as 19800 count haematocyte under stressed condition contrast to only 5315 in normal state.

Key Words: *Brotia costula*, *Thevetia peruviana*, haematocyte, total count, acute and long term, $EC_{50.}$

INTRODUCTION

Application of pesticides to control the pests are common practice. Since synthetic pesticides remain viable for a considerable lengths of time in environment it is now-a-days not being considered safe to use them at large. Accordingly, attention is being paid to use the naturally occurring toxic materials in controlling the pests (Baalawy, 1972; Gedon, 1983; Sing and Agarwal, 1987; Protorius, 1988; Cruz-Reyes et al., 1989; Lo and Ayele, 1990; Parashar et al., 1990; Mendes et al., 1992). As the toxic property of *Thevetia peruviana* is well established (Wealth of India, 1976) in killing the terrestrial snails and slugs (Panigrahi and Raut, 1994) it is expected that the same could be applied against freshwater vector molluscs due course of time. *Brotia costula* is a widespread species in south and southeast Asia, including much of the Ganges-Brahmaputra systems. Whilst there are known threats (from pollution and habitat degradation, amongst other threats), it is at present assessed as Least Concern due to its very wide distribution. This species is widely distributed across southeast Asia from the Ganges region in India to the island of Sumatra (Kohler and Glaubrecht 2001). The occurrence of this species is conceived to range from North East India (Ganges, Assam, Manipur, Meghalaya, Mizoram, Skiim, West-Bengal), Bangladesh, Burma, Thailand, Cambodia and Viet Nam to the Malaysian Peninsula (abundant in the Pahang River system) and Sumatra. It is taken as food by people in India (Subba Rao 1989) and some ethnic groups particularly Tharu, Mushar, Danuwar in Nepal (Budha pers. obs. 2010). *B. costula* is also used as food in Arunachal Pradesh, Manipur, Meghalaya and North Bengal (Ramakrishna and Dey 2007).
This species is also an edible, market species, research has also shown this species to have accumulations of As (Arsenic), Cu (Copper), Fe (Iron), Se (Selenium) and Zn (Zinc) in individual specimens tissues (Lau *et al.*, 1998).This species is also listed as a consumed species in the regions of North and West Bengal .

Since the edible snails *Brotia costula* are the member of the same community to which the vector snail species belong it is most likely that the said toxic material may harmfully affect the living process of that desirable species. In view of the idea the present programme was designed to study the impact of *Thevetia peruviana* on the haemocytes of *Brotia costula* and the result are presented in this treaties.

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MATERIALS AND METHODS

Ripe *Thevetia peruviana* fruits were collected from the local gardens. Kernals removed from these were then ground and the resulting paste was used to execute experimental studies. The snails *Brotia costula* between 20.4-40.8 mm (shell length) size group were collected from the local ponds from time to time as per need of experiments. These snails were acclimatized for a period of five days under laboratory conditions before they are used in experiments. Required number of aquaria (each 15 x 15 x 15 cm in size) containing 2.5l. pond water were taken for experimentations. In each aquarium 20 snails were released at a time to study the effect of *Thevetia peruviana* doses on the haemocytes of *Brotia costula*. Since EC_{50} values are essential for any kind of experimental studies with toxicants the first experiment was designed to note the effect of *Thevetia peruviana* on the density of haemocytes occurring in the haemolymph of *Brotia costula* in respect to doses and exposure time period.

Experiment- I: Determination of EC_{50} value in respect to a given exposure period

Fifteen aquaria as per above specifications were taken and the snail individuals were released into the same. Of these, one was left as such for control experiment while the remaining 14 aquaria were prepared ready for experiments with different doses of *Thevetia peruviana.* A total of 14 doses starting from 250 mg up to 6 g were considered for studies. The defined amount *Thevetia peruviana* paste was added to the water of each aquarium. Thus the snails were subjected to 14 different doses of *Thevetia peruviana*. Attention was given to note the number of snail individuals died by the end of 3, 6, 24, 48, 72 and 96 hours of exposure. The Snail individuals fallen down on the bottom of the aquaria were collected, washed thoroughly with fresh pond water and then left undisturbed in fresh pond water in separate containers in respect to dose treatment for a period of 48 hours as per method described by de Villiers and Mackenzie (1963), Shiff and Ward (1966) and Boyce et al. (1967). The number of snail individuals found dead after such treatment was counted and depending upon the dead of just 50% individuals the EC_{50} value of *Thevetia peruviana* in respect to the time was determined. In all cases, a single dose at the time of initiation of experiment was applied. To be sure of the said EC_{50} value, experiments were repeated thrice with same dose of *Thevetia peruviana* in respect to exposure time.

Experiment II: To study the effect of *Thevetia peruviana* on haemocytes.

A. Treatment with EC_{50} dose.

(a) Single dose.

As per result of experiment-I the snails were exposed to EC_{50} doses in respect to time periods as considered in experiment-I. In all cases the single dose was given at the initiation of experiment. One or two individuals were considered for studies by the end of each hour until it was coincided with the EC_{50} hour.

(b) Fresh dose at every hours period.

Only two EC_{50} doses viz. 1000 ppm and 2500 ppm were considered for experiments. By the end of each hour the snail were transferred to the another aquaria kept ready with the fresh dose of *Thevetia peruviana*. One or two individuals were considered for studies by the end of each hour until it was coincided with EC_{50} hour.

(c) Single dose but transferred to freshwater at certain intervals.

The Snails were exposed to EC_{50} doses viz.1000 ppm and 2500 ppm given only once at the initiation of experiment. Then, a few individuals not less than five were collected from these containers by the end of 1^{st} , 2^{nd} , and 3^{rd} hour in case of 2500 ppm dose and continued for the 4^{th} , 5^{th} and 6^{th} hours in case of 1000 ppm dose to release them into the freshwater of course, after washing them thoroughly. From them, one or two individuals were taken at an interval of 90, 180, 240, 270 and 360 minutes for studies.

(d) Fresh dose at every hour period and exposure to freshwater.

Only one dose i.e. 2500 ppm (EC_{50} for 3 hours time period) was considered for equipment. Other than initial dose fresh doses were given just by the end of 1^{st} hour and 2^{nd} hour of exposure to initial dose. Few individuals (not less than five) of *Brotia costula* were transferred to fresh water by the end of 1^{st}, 2^{nd} and 3^{rd} hour following initial exposure

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time. One or two individuals were taken from these freshwater containers by the end of 90, 180, 240 and 270 minutes for studies.

In all cases the snails were provided with foods (freshwater needs) ad libitum. Adequate attention was given to keep the experimental stock clean and hygienic. Frequently, in some cases waters of the containers were replaced by fresh pond waters.

The snails considered for studies were crashed carefully and the shell fragments were removed. The fluid and or water were soaked by the blotting paper from over the visceral mass. The needle (22 G X 1") of a sterilized syringe (1 ml volume) was pushed in to the heart haemolymph was drawn into it. Then, two to three drops of haemolymph was drawn into it. Then, two to three drops of haemolymph were put on a clean, greeze free haemocytometer slide. A cover glass was placed over the same. Then the haemocytes were observed carefully and total counting was done at a magnification 10 x 45 x of a compound microscope. Similarly, one or two drops of haemolymph were put on a greeze free glass slide and the smear was prepared. The smear was allowed to dry and then the slide was dipped into methanol (CH_3OH) for fixation. Giemsa stain was used to stain the haemocytes. Stock concentrated Giemsa solution was diluted at 1:24 by adding distilled water. The diluted stain solution was added to the smear and the slide was undisturbed for a period of 8 to 10 minutes. After this, the slide was washed with distilled water. The haemocytes in respect to their types were observed at 10 x 45 x and/or 10 x 100 x and counted.

RESULTS

Experiment I: The snail required 2500, 1000, 380, 6, 3.5 and 2.5 ppm dose of *Thevetia peruviana* to ensure 50% death at 3, 6, 24, 48, 72 and 96 hours exposure time respectively.

Experiment II: The normal average T.C. of haemocytes of *Brotia costula*was 5315 / mm^3. The normal average percentage of neutrophil, basophil, eosinophil, lymphocytes, hyalinicytes and megahaemocytes were 3.14, 3.32, 9.97, 80.97, 1.35 and 1.21 respectively.

(a) The number of haemocytes varied with the doses and exposure hours (Table 1 -6). It is revealed that the number of haemocytes attained is peak at the end of 2^{nd} hour both in cases of 3 (6150/mm^3) and 6 hours (8258/mm^3) exposure doses. In cases of 24 h, 48 h, 72 h and 96 h exposure doses the peak was attained by the end of 16^{th} hours (10011/mm^3), 36 hour (17345/mm^3), 54^{th} hour (18200/mm^3) and 78^{th} hour (198800/mm^3) respectively. The number of haemocytes recorded, at the end of respective exposure hours was 3758/mm^3 (3 hr), 2808/mm^3 (6 hr), 3256/mm^3 (24 hr), 4280/mm^3 (48 hr), 2830/mm^3 (72 hr) and 2960/mm^3 lymphocytes and eosinophilic cells by the end of EC_{50} hours of the respective doses of *Thevetia peruviana* (Tables: 1-7).

(b) At 2500 ppm dose the number of haemocytes gradually increased with the progress of hours and attained peak at the end of 3^{rd} hour i.e. EC_{50} hours (Table- 7). In case of 1000 ppm dose the increasing tendency was noted up to the end of 4^{th} hours (also peak hours) which was followed by a gradual decline by the next two hours. (Table-7).

(c) The snails examined after specified time period following expose to freshwater regained their normal status between 270 and 360 minutes irrespective of the doses 1000 ppm and 2500 ppm (Tables-8 and 9).

(d) The snails regain the normal status of haemocytic density between 240 and 270 minutes (Table 10).

Table 1. TC of haemocytes of *Brotia costula* after 4 hours exposure in the pesticide (2500 ppm).

Exposure time (hr)	1	2	3	4
TC	3875 ±288	6150 ±318	3758 ±380	1633 ±101

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Table 2. TC and haemocytes of *Brotia costula* after 6 hrs exposure in *Thevetia peruviana against 1000 ppm* ($LC_{50}$for 6 hrs)

Exposure time (hr)	1	2	3	4	5	6
TC	4365 ±236	8258±512	6820 ±428	6455 ±521	5876 ±219	2808 ±322

Table 3. TC of haemocytes of *Brotia costula* after 24 hours exposure in the pesticide obtained from *Thevetia peruviana* (380 ppm).

Exposure time (hr)	4	8	12	16	20	24
TC	7131 ±219	5788 ±546	7783 ±621	10011 ±441	4620 ±528	3256 ±593

Table 4. TC of haemocytes of *Brotia costula* after 48 hours exposure in the pesticide obtained from *Thevetia peruviana* (6 ppm)

Exposure time (hr)	6	12	18	24	30	36	42	48
TC	7205 ±441	5150 ±528	6730 ±871	4225 ±288	6220 ±721	17345 ±219	6890 ± 198	4280 ±329

Table 5. TC of haemocytes of *Brotia costula* after 72 hours exposure in the pesticide obtained from *Thevetia peruviana* (3.5 ppm).

Exposure time (hr)	6	12	18	24	30	36	42	48	54	60	66	72
TC	5850 ±458	3990 ±784	4965 ±422	7905 ±659	7165 ±823	5365 ±732	5295 ±661	5350 ±522	18200 ±549	4220 ±284	3980±6 27595	2830 ±251

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Table 6. TC of haemocytes of *Brotia costula* after 96 hours exposure in the pesticide obtained from *Thevetia peruviana* (3.5 ppm).

Exposure time (hr)	6	12	18	24	30	36	42	48	54	60	66	72	78	84	90	96
TC	5745± 239	4685± 342	4940± 735	8395± 388	7170± 533	4970± 451	6990± 290	7800± 565	5010± 572	5151± 401	5410± 256	15555± 876	19800± 822	6730 ±761	6440 ±221	2960 ±321

Table 7. TC of haemocytes of *Brotia costula* after when exposed in 2500 and 1000 ppm pesticides (*Thevetia peruviana*) by single dose and freshly made new dose in every hour.

Exposure time (hr)	TC of haemocytes exposed on 2500 ppm pesticides		TC of haemocytes exposed on 1000 ppm pesticides	
	Single dose in every hour	Fresh dose in every hour	Single dose in every hour	Fresh dose in every hour
1	4875±347	5389±579	4365±447	5600±356
2	6150±478	5780±562	8259±534	7065±546
3	3758±523	6480±178	6820±556	7160±612
4	NA	NA	6455±267	16450±408
5	NA	NA	5876±489	4115±370
6	NA	NA	2808±497	2880±511

Table 8. TC of haemocytes of *Brotia costula* after when exposed in pesticide (2500 ppm) and then transferred into freshwater until T.C. will come normal level.

Exposure time (hr)	TC of haemocytes of *Brotia costula* after				
	After pesticide treatment	After pesticide & Fresh Water Treatment			
		1.5 hr.	3 hr.	4 hr	4.5 hr
1	5765±218	3875±547	4195±426	5430±762	NA -
2	6360±365	6100±573	6000±465	5630±543	5430±532
3	6115±378	6085±442	5855±478	5345±177	NA

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Table 9. TC of haemocytes of *Brotia costula* after when exposure in pesticide (1000 ppm) and then transferred into freshwater.

Exposure time (hr)	T.C. after Pesticide Exposure (Single dose)+ fresh water treatment for the time of (hr)				
	1 hr	1.5 hr	3 hr	4 hr	4.5 hr
1	5360±529	5015±781	5670±623	5495±421	NA
2	8830±412	7905±623	6990±352	5835±439	5420±428
3	6070±527	5805±633	5305±590	5555±399	NA
4	5800±621	5720±482	5650±480	5420±386	NA
5	5005±533	5265±491	5080±483	5515±471	NA
6	3475±422	4780±729	5380±±399	5600±363	NA

Table 10. TC of haemocytes of *Brotia costula* after when exposure into single dose and fresh dose in every hour, and release into fresh water after freshly prepared pesticide dose treatment (2100 ppm).

Time of exposure (hr)	T.C. with single dose	T.C. with changed dose	T.C. with changed dose & fresh water treatment for the period (hr)			
	0	1.5	3	4	4.5	NA
1	3875±657	5390±542	4195±213	3875±187	5430±534	NA
2	6150±353	5780±345	5230±654	4495±390	5600±498	NA
3	3758±±645	6480±543	6215±346	5760±632	5715±392	5570±523

DISCUSSION

That *Thevetia peruviana* is effective in controlling the terrestrial pest slugs and snails has been reported earlier by Panigrahi and Raut (1994). The result of the present studies conforming the effectiveness of the said toxicant in killing the freshwater snails. Both these findings strongly advocate the use of *Thevetia peruviana* as molluscicide irrespective of the habitats concerned.

Through the action path-way of *Thevetia peruviana* in mollusk is not known to us the presence of cardio-active glycosides viz. cerberocide (Thevetin-B) ($C_{42}H_{66}O_{18}$), 2-O-Acetylcerberocide ($C_{44}H_{68}O_{19}$), Nerafolen ($C_{30}H_{46}O_8$), Cerberin ($C_{32}H_{48}O_9$), Thevetin-A ($C_{42}H_{46}O_{19}$), peruvoside ($C_{30}H_{44}O_9$), thevenerin ($C_{30}H_{46}O_9$) and Peruvosidic acid ($C_{30}H_{44}O_{16}$) has been reported by Besset (1961). It is also stated that the toxic manifestation of *Thevetia* poisoning mainly involves with the cardio vascular system and gastrointestinal tract (Arora and Rangaswami, 1965; Bisht, 1965). The results of the present study clearly indicate that the number of haemocytes varies significantly with the exposure of the snails into different doses of *Thevetia peruviana.* Since a marked decrease in number of haemocytes after one, two or a few hours of application of the toxicant in respect to the dose of the same is well documented it

can be said that these animals, at the first phase are unable to regulate their physiological mechanism at the desired level. Thereafter, they do their best to fight against the severity of the toxicity of *Thevetia peruviana* which has been resulted in production of possible maximum of haematocytes after different hours of exposure to different doses of *Thevetia peruviana*. It is also evident that the production of the maximum number of haematocytes noted by the end of 2nd exposure hours in case of 2500 ppm and 1000 ppm doses but the number of haematocytes was 2108 more in later than the amount noted in former case. Subsequent results revealed that the snails exposed to gradually lower doses of *Thevetia peruviana* are able to fight with the toxic materials for a longer period and finally attain that 'alarming state' which is judged from the fact of production of haemocytes in maximum number. It is also evident the snails are able and bound to produce haemocytes as many as 19800 under such circumstances contrast to only 5315 in normal state. This indicates that the snails are highly adopted to cope up with the conditions to which they are exposed, but the action process is dependent on the (intensity of the factor) degree of reacting power of the organisms of the reaction inducing factor(s). It appears that, if the snail fails to overcome the hazards induced by the factor concerned then, they would manifest a condition which could be considered as the death signal with the progress of time. Once the individual attains that status it could not be possible to save its life from such toxic effect. This could be well judged from the fact of increase of haematocytes in small number within a period of one hour following application of fresh dose (2500 ppm) at an interval of one hour until the experiment was coincided with the LC_{50} hour in respect to same but single dose. But in case of 1000 ppm dose the increasing tendency was maintained with the application of successive doses but up to the end of 4th hours when the number was increased to 16450. Thereafter a sharp decline is well marked. So, it appears that the organisms try to combat with the hazardous condition in the same manner in respect to the intensity of the dose applied. If we compare the data obtained following application of single and recurring dose of 1000 ppm *Thevetia peruviana* then the picture will be clear. In single dose the haemocytes come down to 4365 by the end of 1st hour and to 8258 by the end of 2nd hour, which was followed by a gradual decline and the minimum (2808) was at the end of EC_{50} time period (6 hrs) contrast to this, in cronic dose (at 1 hr interval) the number was almost same by the end of 2nd (7065) and 3rd (7160) hour but then jumped to 16450 (by the end of 4th hour). So, in single dose the peak was noted by the end of 4th hour but the number of haemocytes regaining the normal state.So far the number of haemocytes are concerned the death of these snails having 5735 haemocytes are surprising. The probable explanations of such phenomenon require further study as expected to through some lights on the variations in the percentages of haemocytes after treatment with different dosages of *T. peruviana*.

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