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Pesticide Impact on Excretory Physiology of the Common Frog, *Rana tigrina* (Daud) Tadpoles

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Application of pesticides has become inevitable to protect crop plants. Such agricultural loss due to pests and diseases was estimated around 50% of total production in developing tropical countries (FAO 1975). Pesticides reach water bodies either by direct application or indirectly. The indirect sources include run-off from agricultural fields, spray drifts, rain water, sewage and effluent from industries which are manufacturing the pesticides or using them in their processes (Bhaskaran 1980). The effect of pesticides on aquatic life is often acute resulting in mass mortality of animals or chronic changes in behaviour and/or reduction in the rates of survival, growth and reproduction. Chronic effects of pesticides may induce histological and physiological changes in aquatic animals (Konar 1981). Pesticide pollution also poses a constant threat to the frog population in India. Ecologists warn that the diminishing frog population can upset the ecological balance, resulting in an abundance of vector and pests (Abdul Ali 1985). *Rana tigrina* (Daud) is commonly seen in paddy fields. Water bodies adjacent to paddy fields are directly exposed to different kinds of pesticides and hence a study on *R. tigrina* tadpole with reference to adundantly used pesticide like carbamates (eg., carbaryl) and organophosphates (eg., methyl parathion) has become imperative. The present paper reports on the comparative study of toxic effects of carbaryl and methyl parathion on excretory physiology of *Rana tigrina* tadpoles.

MATERIALS AND METHODS

Rana tigrina tadpoles were produced in the laboratory following the technique of hypophysation (Rugh 1934). Tadpoles were fed with pellet feed (40% protein) and feeding of tadpoles commenced 2 days after hatching (Pandian and Marian 1986). Static, renewal bioassay tests were conducted to determine tadpole 96 h LC50 values for carbaryl (CA) and methyl parathion (MP). Ten healthy, three days old tadpoles (15 $\pm$ 2 mg) were separately exposed to 8 different concentrations of CA and MP. A control was also maintained in pesticide-free water. The experiment was conducted in circular plastic troughs containing 6 l of test media. Test animals were not fed during the bioassay test. Fresh concentrations were prepared daily to maintain the constancy of the pesticide in the test media. Mortality of the tadpoles was observed for 96 h and median lethal concentrations were calculated following the method of Finney (1971).

After determining 96 h LC50, three levels of CA and MP, 10, 20 and 30% of 96 h LC50, were taken as the test concentrations. A control was also maintained.

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Ten tadpoles (15 ± 2 mg) were reared in each concentration and control group until they reached the froglet stage (31 days). The experiment was conducted in circular plastic troughs containing 6 l of test media and triplicates were maintained for each group.

Tadpoles undergo distinct morphological changes during metamorphosis (growth). In the present study, tadpoles were classified into four types based on their morphological indices following the method of Gosner (1960). They are (I) appearance of hind limb, (II) disappearance of external gill, (III) appearance of fore limb and, (IV) disappearance of tail. The experimental tadpoles were fed *ad libitum* with pellet feed and uneaten food was removed after 2 hr. Strings of faecal matter and the left out faecal matter were pipetted out and filtered respectively while changing the water on alternate days. The hydrobiological parameters DO, temperature, pH, hardness and salinity of different experimental exposures did not vary much and averaged to 4.40 mlO_2^{-1}, 26 ± 1°C, 7.28, 70 mg $CaCO_3^{-1}$ and 0.11 ppt respectively.

Immediately after feeding, tadpoles of selected stage were transferred to separate containers having 500 ml (for initial stage smaller tadpoles) to 1000 ml (for larger final stage tadpoles) well-aerated tap water for 24 h period, where the respective CA and MP concentrations were maintained. Three water samples (5 ml) from each container were analysed at the end of 24 h period for ammonia and urea contents. Ammonia excreted by the tadpoles was estimated by the phenol hypochlorite method (Solorzano 1969). To 5 ml of sample (test medium), 1 ml phenol, 1 ml sodium nitroprusside and 2.5 ml of oxidizing reagent were added successively, mixed well and kept undisturbed for color development. Intensity of the resulting blue color was measured at 640 nm in a colorimeter using a red filter and the concentration was directly read from a standard ammonia curve. By the same method, urea-N was also determined after incubating the sample with 15µg urease for 20 minutes at 30°C. A control (distilled water and urease) was also used.

The concentrations of NH_3–N and urea-N were converted into energy values using 24.86 J/mg for NH_3–N and 23.06 J/mg for urea-N (Elliott 1976).

$$\text{Rate of } NH_3\text{-N/urea-N excretion (J/g/day)} = \frac{NH_3\text{-N/urea-N excretion (J/day)}}{\text{Weight of tadpoles (g)}}$$

$$\text{Rate of total nitrogen excretion (J/g/day)} = \frac{\text{Total } (NH_3\text{-N+urea-N}) \text{ excretion (J/day)}}{\text{Weight of tadpoles (g)}}$$

Two way ANOVA was applied to determine the significance of interaction between pesticide concentrations and developmental stages of tadpoles on the excretion of NH_3-N and urea-N. Students 't' test was used to determine the significance of mean values between control and experimental groups. Correlation was applied to determine the concentration dependence of tested parameters (Zar 1974).

RESULTS AND DISCUSSION

The 96 h LC50 values of tadpoles exposed to CA and MP were 5.68 and 4.86 ppm respectively. The effect of CA and MP on excretion of NH_3-N and urea-N in various selected stages of *R. tigrina* tadpoles are presented in Table 1. Excretion of

both NH_3-N and urea-N was inversely proportional to all the stages of the tadpole. For instance, the excretion of NH_3-N decreased from 2.1 J/g/day to 0.6 J/g/day whereas the urea-N increased from 0.5 to 1.6 J/g/day as tadpoles developed from stage I to IV in the control group. A similar trend was also obtained in tadpoles exposed to CA and MP but the amount of excretion of NH_3-N and urea-N was concentration and stage dependent. The NH_3-N excretion in control tadpoles at stage I was 2.10 J/g/day and it increased to 2.60 or 3.20, 3.0 or 3.50 and 3.20 or 4.10 J/g/day in tadpoles exposed to 10, 20 and 30% of CA or MP respectively. The results obtained for stages II, III and IV were also similar to those of first stage (Table 1). Urea-N excretion was 144 or 290% higher in the third stage tadpoles exposed to the 30% levels of CA or MP as compared to control tadpoles of same stage. Two way ANOVA revealed that both developmental stage and concentration of CA and MP significantly ($P<0.05$) influenced NH_3-N excretion and urea-N excretion.

The rate of total excretion of NH_3-N and urea-N was greater in tadpoles exposed to the pesticides and it was concentration dependent (Table 1). The total excretion rate of control tadpoles at stage I was 2.6 J/g/day and it increased to 3.1 or 3.9, 3.6 or 4.3 and 3.9 or 4.9 J/g/day in tadpoles exposed to 10, 20 and 30% CA or MP respectively. A similar trend was obtained in other stages also. Tadpoles exposed to MP showed higher rate of total excretion than those exposed to CA. The change in total excretion rate was highly significant ($P<0.01$) and positively correlated with pesticide concentrations. ANOVA tests showed that both the concentration and the developmental stage had a significant ($P<0.01$) effect on total excretion.

Students 't' test revealed no significant change in the proportion of NH_3-N and urea-N excretion between control and the sublethal concentrations of CA and MP in stages I, II and IV. However, the lowest level of CA or MP (10%) significantly ($P<0.05$) changed the proportion of NH_3-N and urea-N excretion in stage III of experimental tadpoles. The urea excretion in control tadpoles was 47.7% and it increased to 55.9 or 67.5, 60.3 or 66.8 and 67.2 or 76.7% in tadpoles exposed to 10, 20 and 30% of CA or MP respectively. MP exposed tadpoles showed a higher percentage urea-N excretion than those exposed to CA (Table 2) and NH_3-N excretion elicited the opposite trend as compared with that of urea-N excretion.

The excretory rate of NH_3-N declined and urea-N enhanced in both control and experimental tadpoles as they developed. This trend indicates the shifting from ammonotelism to ureotelism during development (Schultheiss 1973; Marian *et al.* 1983; Chandran 1991). The control tadpoles excreted about 20% urea in stage I and it reached a maximum proportion of 72% during metamorphosis. The present study closely agrees with the reports of Chandran (1991) who reported that *R. tigrina* tadpoles excreted 30% urea in stage I and it increased to 70% in stage IV tadpoles. Sublethal levels of pesticides significantly enhanced the NH_3-N and urea-N excretion, ultimately elevating the total nitrogen considerably in all stages. This observation is well substantiated in the snail. Total protein and free amino acid levels decreased while ammonia and pyruvic acid showed elevation in the body fluids of the snail, *Pila globosa*, when exposed to methyl parathion (Satya Prasad *et al.* 1982).

It is very interesting to note that there was a dramatic increase in the rate of

Table 1. Effect of different sublethal concentrations of carbaryl and methyl parathion on the rate of NH_3-N and urea-N and total excretion in different developmental stages of *R. tigrina*. Each value represents the average ($\bar{X}\pm SD$) performance of three observations at 26 ± 1°C. Values are given in J/g/day.

Stage	Sublethal concentration (% of LC50)											
	Control			10			20			30		
	NH_3-N	Urea-N	Total	NH_3-N	Urea-N	Total	NH_3-N	Urea-N	Total	NH_3-N	Urea-N	Total
Carbaryl												
I	2.10 ± 0.14	0.50 ± 0.09	2.60 ± 0.22	2.60 ± 0.10	0.50 ± 0.07	3.10 ± 0.10	3.00 ± 0.12	0.60 ± 0.05	3.60 ± 0.08	3.20 ± 0.05	0.70 ± 0.06	3.90 ± 0.01
II	1.40 ± 0.10	0.70 ± 0.07	2.10 ± 0.05	1.70 ± 0.09	0.80 ± 0.02	2.50 ± 0.07	1.70 ± 0.07	0.80 ± 0.06	2.60 ± 0.13	2.00 ± 0.07	0.90 ± 0.02	2.90 ± 0.07
III	1.00 ± 0.07	0.90 ± 0.05	1.90 ± 0.07	1.10 ± 0.07	1.40 ± 0.08	2.50 ± 0.14	1.10 ± 0.05	1.60 ± 0.03	2.70 ± 0.07	1.20 ± 0.01	2.20 ± 0.06	3.30 ± 0.16
IV	0.60 ± 0.04	1.60 ± 0.10	2.20 ± 0.08	0.70 ± 0.03	1.80 ± 0.09	2.50 ± 0.09	0.80 ± 0.06	1.90 ± 0.10	2.70 ± 0.15	1.10 ± 0.06	2.20 ± 0.10	3.30 ± 0.15
Methyl parathion												
I	2.10 ± 0.14	0.50 ± 0.09	2.60 ± 0.22	3.20 ± 0.10	0.70 ± 0.02	3.90 ± 0.15	3.50 ± 0.08	0.80 ± 0.05	4.30 ± 0.06	4.10 ± 0.08	0.80 ± 0.04	4.90 ± 0.11
II	1.40 ± 0.10	0.70 ± 0.07	2.10 ±0.05	1.60 ± 0.06	0.80 ± 0.07	2.40 ± 0.12	2.20 ± 0.11	0.90 ± 0.05	3.10 ± 0.08	2.40 ± 0.01	1.00 ± 0.06	3.40 ± 0.07
III	1.00 ± 0.07	0.90 ± 0.05	1.90 ± 0.07	1.00 ± 0.12	2.00 ± 0.14	3.00 ± 0.25	1.00 ± 0.04	2.20 ± 0.06	3.20 ± 0.09	1.10 ± 0.04	3.50 ± 0.01	4.60 ± 0.14
IV	0.60 ± 0.04	1.60 ± 0.10	2.20 ± 0.09	0.90 ± 0.05	2.10 ± 0.01	3.00 ± 0.10	1.00 ± 0.08	2.30 ± 0.04	3.30 ± 0.16	1.10 ± 0.06	2.50 ± 0.13	3.60 ± 0.10

Table 2. Effect of different sublethal concentrations of carbaryl and methyl parathion on the percentage of NH_3-N and urea-N excretion (100%) in relation to the developmental stages of *R. tigrina*. Each value represents the average ($\bar{X}\pm SD$) performance of three observations at 26 ± 1°C.

Stage	Sublethal concentration (% of LC50)							
	Control		10		20		30	
	NH_3-N	Urea-N	NH_3-N	Urea-N	NH_3-N	Urea-N	NH_3-N	Urea-N
Carbaryl								
I	80.20 ± 2.16	19.80 ± 2.16	82.60 ± 2.90	17.40 ± 2.09	83.30 ± 1.60	16.70 ± 1.60	82.90 ± 1.01	17.10 ± 1.01
II	66.70 ± 3.56	33.30 ± 3.56	67.10 ± 1.50	32.90 ± 1.50	68.50 ± 0.85	31.50 ± 0.85	70.00 ± 1.10	30.00 ±1.00
III	52.30 ± 2.00	47.70 ± 2.00	44.10 ± 0.17	55.90* ± 0.17	39.70 ± 0.65	60.30 ± 0.65	32.80 ± 1.50	67.20 ± 1.50
IV	27.40 ± 2.40	72.60 ± 2.40	26.70 ± 1.40	73.10 ± 1.40	29.30 ± 1.40	70.70 ± 1.40	32.70 ± 0.60	67.30 ± 0.60
Average	56.60 ± 2.20	43.40 ± 2.56	55.10 ± 1.32	44.90 ± 1.32	55.20 ± 0.77	44.80 ± 0.77	54.60 ± 0.32	45.40 ± 0.32
Methyl parathion								
I	80.20 ± 2.16	19.80 ± 2.16	84.10 ± 1.20	15.90 ± 1.20	81.30 ± 1.20	18.70 ± 1.20	82.70 ± 1.50	17.30 ± 1.50
II	66.70 ± 3.56	33.30 ± 1.50	66.10 ± 1.50	33.90 ± 1.50	71.10 ± 1.97	28.90 ± 1.97	70.60 ± 2.02	29.40 ± 2.02
III	52.30 ± 2.00	47.70 ± 2.00	32.50 ± 3.60	67.50* ± 3.60	33.20 ± 0.62	66.80 ± 0.62	23.30 ± 0.46	76.70 ± 0.46
IV	27.40 ± 2.40	72.60 ± 2.40	29.80 ± 0.85	70.20 ± 0.85	30.40 ± 1.40	69.60 ± 1.40	30.40 ± 2.10	69.60 ± 2.10
Average	56.60 ± 2.20	44.00 ± 2.20	53.10 ± 1.14	46.90 ± 1.14	54.02 ± 0.76	45.98 ± 0.76	51.80 ± 0.92	48.20** ± 0.92

Carbaryl:
* Control Vs 10% CA t = 4.01 n = 6 ($P<0.05$)

Methyl parathion:
* Control Vs 10% CA t = 6.8 n = 6 ($P<0.01$)
** Control Vs 30% MP t = 3.94 n = 6 ($P<0.05$)

urea-N excretion due to pesticide stress particularly in the stage III during which the tadpoles are known to develop mesonephric kidneys (Fox 1981; Turpen and Knudson 1982). It appears that the tadpoles make use of the well developed kidney in that particular stage. The maximum urea-N excretion was observed in tadpoles exposed to 30% of both carbaryl (144% of control) and methyl parathion (290%) treated groups in stage III. This steep increase in urea-N excretion indicates its specific stress on excretory physiology of tadpoles. Reports on excretory physiology show that anuran tadpoles enhance urea excretion under various stress conditions. The larvae of aquatic *Xenopus laevis* excreted more urea when subjected to osmotic stress (Seiter *et al.* 1978) and temperature (Balinsky and Baldwin 1961). Tadpoles exposed to salt water reportedly increased urea excretion (Ireland 1973).

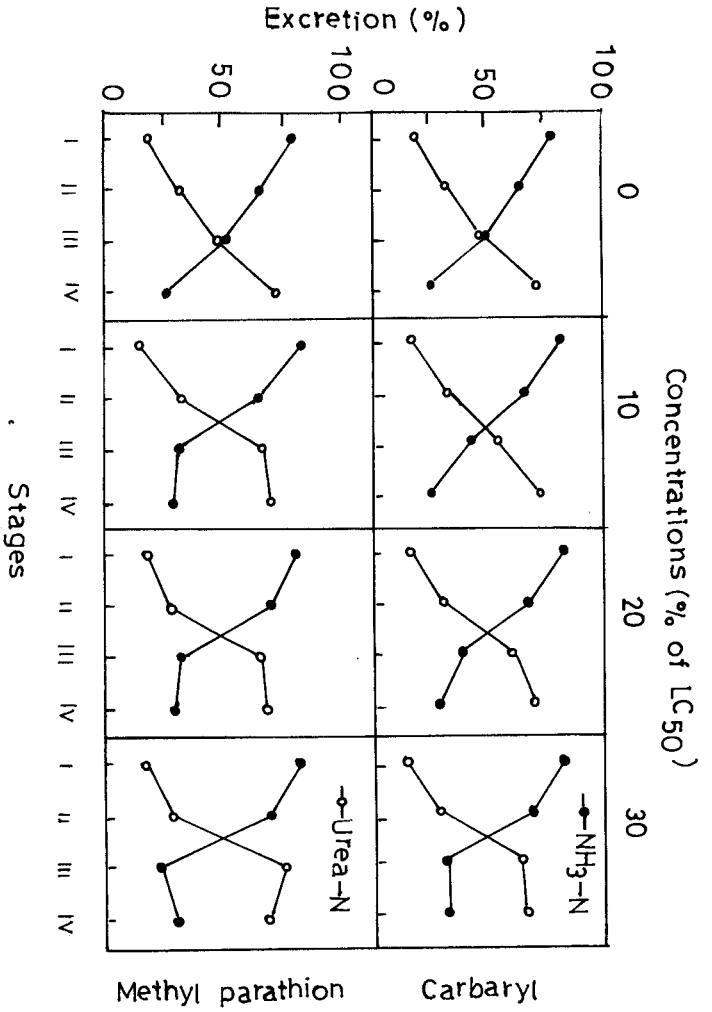

Figure 1 Effect of different sublethal concentrations of carbaryl and methyl parathion on the percentage of NH_3-N and urea-N excretion on total excretion (100%) in relation to developmental stages of *R. tigrina*.

The stage at which urea-N excretion exceeded the 50% level of total excretion could be considered as the critical stage of switch-over to ureotelism (Chandran 1991). In the present study, the switching occurred when the tadpole crossed from stage II to III, as the percentage of urea excretion reached 47.7% in this stage in control. It is interesting to note that though the 30% concentration of CA and MP caused 67.2 and 76.7% urea excretion respectively, against 47.7% in the control group at stage III; the percentage share of urea excretion did not reach the 50% mark in stage II and it remained around 30% in both control and all experimental groups (Fig.1). This indicates that even though the pesticide impact was remarkable on urea excretion at stage III, the stress did not play a role in switching over.

Obviously, switching over to ureotelism depends on structural development (Balinsky and Baldwin 1961).

The average percentage of NH_3-N and urea-N excretion was remained 56.6 and 43.4% respectively in control tadpoles. These values are fairly comparable to those published for *R. tigrina* tadpoles by Chandran (1991) who reported an average of 56 and 44% of NH_3-N and urea-N respectively, when the animals were fed with the 20.9 KJ/g dietary energy and 45% protein. Carbaryl did not alter the average percentage of excretion. Balinsky *et al.* (1967) reported that the percentage of NH_3-N and urea-N excretion remained constant during development and supports the results of control and carbaryl exposed tadpoles in the present study. However, the significant alteration in the ratio of NH_3-N and urea-N excretion in stage III tadpoles exposed to the highest concentration of MP is due to pesticide stress on excretory phsyiology.

The 96 hr LC50 values of *R. tigrina* tadpoles exposed to CA and MP were 5.68 and 4.86 ppm respectively. However, long term exposure of animals to sublethal concentrations of pesticides affect the physiological functions. The present study showed that, even the lowest concentrations of CA and MP (10% of 96 hr LC50) significantly influenced the excretory physiology of *R. tigrina* tadpoles and there is a need to calculate safe levels. Based on a test with larvae and early juveniles of freshwater fishes, Verma *et al.* (1979) suggested that a value of about 40 times lower than the LC50 would be physiologically safe for the animals. Working on *R. tigrina* tadpoles, Kennedy (1997) arrived at a value of 0.06 and 0.03 ppm as safe concentrations for CA and MP and emphasized the need to maintain these pesticides below the safe levels in the environment to protect and conserve the dwindling amphibian populations. These low safe concentrations values will absolutely safe level for most of the fish species which co-inhabit with the amphibian in nature.

REFERENCES

Abdul Ali H (1985) On the export of frog legs from India. J Bombay Nat Hist Soc 18: 347-375

Balinsky JB and Baldwin E (1961) The mode of excretion of ammonia and urea in *Xenopus laevis*. J Exp Biol 38: 695-705

Balinsky JB, Choritz EL, Coe CGL and Van Der Schans GS (1967) Aminoacid metabolism and urea synthesis in naturally restivating *Xenopus laevis*. Comp Biochem Physiol 22: 59-68

Bhaskaran R (1980) Biological studies on chosen thermoconformers (*Channa striatus*). Ph.D. Thesis, Madurai Kamaraj University, Madurai, India, Pp.135

Chandran (1991) Protein-energy interactions on the nutritional requirements of *Rana tigrina* tadpoles. Ph.D. Thesis, Madurai Kamaraj University, Madurai, India Pp.110

Elliott JM (1976) Energy losses in the waste products of brown trout (*Salmo trutta* L.) J Anim Ecol 45: 561-580

FAO (1975) The monographs FAO Rome Proceeds Publ AGP/1973/M/491

Finney DJ (1971) Probit analysis. 3rd Edn. Cambridge, Cambridge University Press Pp.333

Fox H (1981) Cytological and morphological changes during amphibian metamorphosis. In: Gilbert LI and Friden E (Ed.) Metamorphosis: A problem

in Developmental Biology, Plenum, New York, Pp.327-362
Gosner KL (1960) A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16: 183-190
Ireland MP (1973) Studies on the adaptation of *Xenopus laevis* to hyperosmotic media. Comp Biochem Physiol 46A: 469-476
Kennedy IJJ (1997) A comparative study of toxic effects of an organophosphate (methyl parathion) and a carbamate (sevin) pesticides on some physiological and biochemical aspects in *Rana tigrina* tadpoles. Ph.D. Thesis submitted to Manonmaniam Sundaranar University, Tirunelveli, India Pp.89-90
Konar SK (1981) Pollution of water by pesticides and its influence on aquatic ecosystem. Indian Rev Life Sci 1: 139-165
Marian MP, Arul V and Pandian TJ (1983) Acute and chronic effects of carbaryl on survival, growth and metamorphosis in the bull frog (*Rana tigrina*). Arch Environ Contam Toxicol 12: 271-275
Pandian TJ and Marian MP (1986) Prediction of absorption efficiency from food nitrogen in amphibians. Proc Indian Acad Sci 95: 387-395
Rugh R (1934) Induced ovulation and artificial fertilization in the frog. Biol Bull 66: 22-29
Satya Prasad K, Prasada Rao KS, Ahamad Sahib IK, Sailatha D, Sivaiah S, Basha SM, Ramana Rao KV (1982) Effect of methyl parathion on the body fluid of the snail, *Pila globosa* (Swainson). Comp Physiol Ecol 7: 251-252
Schultheiss H (1973) Der Emflup Von ACTH und corticosteroiden auf den stick stoff-stoffweehsel wahrend der metamorphose des maxikanischen Axolod (*Ambystoma mexicanum* Cope). Zool J Physiol 77: 199-227
Seiter P, Schultheiss H, Hanke W (1978) Osmotic stress and excretion of ammonia and urea in *Xenopus laevis*. Comp Biochem Physiol 61A: 571-576
Solorzano L (1969) Determination of ammonia in natural waters by the phenyl-hypochlorite method. Limnol Oceanogr 14: 799-801
Turpen JB and Knudson CM (1982) Ontogeny of hermatopoietic cells in *Rana pipiens*: Precursor cells migration during embryogeneis. Dev Biol 89: 138-151
Verma SR, Bansal SK, Gupta AK, Pal N, Tyagi AC, Bhatnagar MC, Kumar K, Dalela RC (1979) Acute toxicity of twenty three pesticides to a freshwater teleost, *Saccobranchus fossilis* Proc Symp Environ Biol In: Verma SR, Tyagi AK, Bansal SK (Eds.) The Academy of Environmental Biology, Muzaffarnagar, India, Pp.481-497
Zar JH (1974) Biostatistical analysis. Prentice Hall, New Jersey, Jersey. Pp.620