UNIVERSITY GRANT COMMISSION
BAHADUR SHAH ZAFAR MARG
NEW DEHLI - 110 002

Final Report of the work done on the Major Research Project (Report to be submitted within six weeks after compilation of work)

1. Project report no.
1st /2nd /3rd /final : Final

2. UGC Reference No. : F.No.36-210/2008 (SR), 26th March 2009.

3. Period of the project : From 01/05/2009 to 30/04/2012.

4. Title of research project : Comparative study of metal toxicity and molecular mechanism of immune defense with circulatory, excretory and neurophysiology in mollusca.

5. a) Name of the Principal investigator : **Dr. Nitin Anandrao Kamble,**
Assistant Professor,
Department of Zoology,
Shivaji University,
Kolhapur, 416 004.

b) Dept. /University /College Where the work has progressed : Department of Zoology,
Shivaji University,
Kolhapur- 146 004.

6. Effective date of starting the project: 01/05/2009

7. Grant approved and expenditure incurred during the period of the report:

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a) Total amount approved	: Rs. 6, 17,160/-
b) Grant released as first installment	: Rs. 3, 57,800/-
c) Grant released as second installment	: Rs. 2, 59,360/-
d) Total grant sanctioned	: Rs.6, 17,160/-
e) Total expenditure	: Rs. 6, 01,124/-
f) Total balance remained	: Rs. 16036/-

8. Report of the work done: (Please See Appendix A)

9. Objectives of the proposal:

a) Analysis of mortality rate of animals under different metal concentrations in laboratory.

b) Accumulation and mobilization of metals in targeted tissues.

c) Analysis of oxygen consumption with different concentration in relation to physiology of respiration.

d) Study of cellular mechanism with circulatory, excretory and neurophysiology.

e) Study of histological and histochemical alterations in the circulatory, excretory and neuronal cells at molecular level.

f) Study of homeostasis and defense mechanism at cellular level.

10. Has the progress been according to original plan of work and towards achieving the objectives if not, state reason: Yes, it is as per original plan of work.

11. Please indicate the difficulties, if any, experienced in implementing the project: No.

12. If project has not been completed, please indicate approximate time by which it is likely to complete: NA.

13. If the project has been completed, please enclose the summary of the findings of the study: (Please See Appendix A)

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14. Any other information which would help in the evaluation of the work done on the project. At the completion of project, the second report should indicate the output, such as:

A) Manpower trained:

Attended workshops.

i. Workshop on "Recent Advances in Applied Zoology" at Department of Zoology, Shivaji University, Kolhapur.

ii. National Workshop on "Biological control of pests" at Department of Zoology, Shivaji University, Kolhapur.

B) Ph.D. Awarded:

Project fellow has been registered for Ph. D. and the work is in progress.

Title of Ph. D. work:

i. Effect of Cadmium and Copper on nephrocytes and hemocytes of *Bellamya bengalensis* and *Semperula maculata*

ii. Toxicity assessment of metals on neuronal and respiratory physiology of some molluscan species.

C) Publications of the results:

a. Paper presented in conference - 02

b. Conferences/Seminar Attended - 06

c. Papers Published – 09

(National – 03 International – 06)

d. Paper Accepted - 01

e. Papers Communicated - 02

a. Paper presented in Conference:

i. Research paper entitled "Histomophology of neuronal and neurosecretory cells in the two molluscan species *B. bengalensis* and *S. maculata*" presented in International Symposium on Current Trends in Endocrine and Reproductive Health And 29th Annual Meeting of society for Reproductive Biology and Comparative Endocrinology – SRBCE -2011, held on the 10th - 12th feb. 2011 at

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Department of studies in Zoology, University of Mysore, Manasagangotri, Mysore- 570 006, India.

ii. Research paper entitled "Induced toxicity of lead acetate on mortality of freshwater snail *Bellamya bengalensis* (Lamarck)", presented in National Conference on Global Warming and Conservation Strategies on 21st and 22nd December 2010. Sponsored by UGC & Organized by Department of Zoology & Drongo Environmental movement, Satara, Lal Bahadur Shastri College Satara.

b. Conferences/Seminar Attended:

i. International Conference on Environmental Pollution, Water conservation and Health. July 29th to 31st 2010. Cenetary Celebration of Department of Zoology, (1909- 2009), Organized by Department of Zoology, Bangalore University, Bangalore, In association with: Indian Society of Comparative Animal physiology (ISCAP), Tirupati India.

ii. National Workshop on Environment Science and Problems" on 1-2 Sept 2010Sponsored by UGC & Organized by Department of Zoology & Drongo Environmental movement, Satara. Lal Bahadur Shastri College Satara.

iii. National Conference on Recent Trends in Life Science with Special Reference to Animal Physiology, Biotechnology and Biodiversity Sponsored by UGC & Organized by Department of Zoology, Sadguru Gadage Maharaj College, Karad on 20th Octomber 2010.

iv. National Seminar on Recent Trends and Techniques in Bio- Science Sponsored by UGC & Organized by Department of Zoology, Arts, Commerce and Science college, Palus on 30th November – 1st December.

v. National Seminar on Preparation of Skillful Research Papers at National and International Scenario, Sponsored by DST – Purse, Organized by Department of Zoology, Shivaji University, Kolhapur. On 6th February. 2012.

vi. National Seminar on Biodiversity: Conservation and Protection, Sponsored by UGC & Organized by Department of Zoology, Bharati Vidyapeeth, deemed University, Pune (India) on 13th and 14th March, 2012.

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c. Papers Published: (Total - 09)

i. **N.A. Kamble and V. V. Potdar (2010).** Study of copper sulphate induced mortality to freshwater snail *Bellamya bengalensis* (Lamarck). **Uttar Pradesh Vol. 30(3): 399-405.**

ii. **N.A. Kamble** and **V. V. Potdar (2010).** Hematological analysis of molluscan species *Bellamya bengalensis* and Lamellidens *marginalis.* **Biological forum: An International Journal, Vol. No. 2(1): 70-72(2010).**

iii. **N.A.Kamble and S.R. Londhe (2011).** Histomorphology of Neuronal and Neurosecretory Cells in Two Molluscan Species *B. bengalensis* and *S. maculata.* **Bionano Frontier, Vol. 4 (2): 270-272, Impact factor: 0.233.**

iv. **N.A. Kamble** and **V. V. Potdar (2011).** Impact of seasonal variations on the metal pollution of Rajaram tank Kolhapur, (Maharashtra). **Uttar Pradesh Journal of Zoology, Vol..31 (2): 221-225.**

v. **N.A.Kamble and S.R. Londhe (2011).** Copper Sulphate Induced Neuronal Histological Changes in the Freshwater snail *Bellamya bengalensis.* **The Bioscan: An International Quarterly Journal of Life Sciences, Vol. 6 (2): 351-354.**

vi. **N.A. Kamble** and **V. V. Potdar (2011).** Metallic concretions in the nephrocytes of freshwater snail *Bellamya bengalensis.* **Bionano Frontier: an International Journal of Sciences and Technology, Vol. 4 (2): 264-266, Impact factor: 0.233.**

vii. **N.A. Kamble** and **V. V. Potdar (2011).** Histopathological alterations and metal accumulation in the mantle epithelial cells of slug *Semperula maculata.* **Journal of experimental zoology India, Vol. 14(2): 531-535, Citation Index: 1.**

viii. **N.A. Kamble and S.R. Londhe (2012).** Mercuric Chloride Induced Neuropathology and Physiological Responses in slug *Semperula maculata"* has been published in **International journal of zoological Research, Science Alert, Vol. 8 (1): 33-42.**

ix. **N.A.Kamble and S.R. Londhe (2012).** Epileptic Activity in Neuronal cells, Induced by HgCl2 and CdCl2 in Terrestrial slug *Semperula maculata*: Fine structure investigated by Histology and Histochemistry. **Toxicology and Environmental Chemistry, Taylor and Francis, Vol.94 (1): 109-120.**

d. Paper Accepted: (Total - 01)

i. **N.A.Kamble and S.R. Londhe (2012).** Neurohistochemistry in molluscan species: focus on extracellular matrix" has been accepted for publication in **Invertebrate neuroscience, Springer.**

e. Papers Communicated: (Total - 02)

i. **N.A.Kamble and S.R. Londhe (2012).** Metal intoxicated immunoreactivity of gastrin cholecystokinin in the neuronal system of terrestrial slug *Semperula maculata* (Semper), **Indian Journal of Experimental Biology, Impact factor- 0.702**

ii. **N.A. Kamble** and **V. V. Potdar (2012).** Oxidative metabolism and protein profiling of terrestrial slug *Semperula maculata* after cadmium chloride induction **Bionano Frontier- An International Journal of Science and Technology,**

d) Other impact if any: No.

Principal Investigator

Dr.N.A.Kamble
Principal Investigator
U.G.C.Major Research Project
Department of Zoology
Shivaji University,Kolhapur

Registrar/Principal

Registrar,
Shivaji University, Kolhapur

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Appendix A

Report of the work done;

A. Methodology:

a. Material:

i. Selection of animal:

For the present toxicological study two molluscan species was selected namely, freshwater snail *Bellamya bengalensis* and terrestrial slug *Semperulla maculata.* Freshwater Snail *B. bengalensis* was collected from Rajaram tank, near the Shivaji University, Kolhapur (Maharashtra) and terrestrial slug *S. maculata* was collected from Miraj, Malgoan and Bedag, Dist. Sangli (Maharashtra).

ii. Toxicity Assessment:

For the toxicological study three heavy metals have been selected, Cadmium chloride, Copper sulphate and Mercuric chloride.

Cadmium chloride:-

Cadmium chloride ($CdCl_2.H_2O$) is nonessential, water miscible, soft bluish white coloured chemical compound. Pollution of drinking water with cadmium can occur as a result of leaching from solder containing the metal in the fitting of water heater, coolers and taps. About one third of the cadmium found absorbed and stored in the kidney, which constitute the principal target organ in the body of animals. Lung or gills are the major target organ affected by airborne and aquatic cadmium; and proved to be immunosuppressive as indicated by IgG mediated rosette formation in immune mechanism.

Copper Sulphate:-

Copper sulphate ($3CuSo_4.5H_2O$) is water miscible, blue coloured and occured primarily as in its oxide and sulphide ores. Next to Iron and Aluminum, copper is the third most important metal used in the industries. Because of thermal and electrical conductivities, it is used as conductor in many alloys. It has main sources as, wood combustion, steel production, paints, etc. Copper mining and metallurgical operations contribute majority in contamination of aquatic environment. Disposal of industrial or municipal sewage waste represent other resources of copper in the environment.

Mercury chloride:

Mercury chloride or Mercuric chloride ($HgCl_2$) is white crystalline, solid laboratory reagent. It is gradually used in industrial processes through which it comes in to the water. Due to its total solubility in water, it mixed into aquatic bodies and becomes toxic to flora and fauna. Higher concentration causes death of the animals.

iii. Dose and calculation of LC_{50}:

Bellamya bengalensis:

Healthy and adult snails having same size and weight (23-26 mm, shell height and 2.8-3.5 g weight) were selected for experiment. Experiments were carried out in plastic troughs having 5 liters of capacity. Water miscible Copper sulphate ($3CuSO_4.5H_2O$) and Cadmium chloride ($CdCl_2.H_2O$) was used as toxicant. Snails were divided into six groups. First as control group, remaining five groups of were intoxicated with toxicants for 24, 48, 72 and 96 hrs of exposure. In each group 10 snails were used. All the experiment was repeated thrice, for the correct evaluation and calculation. After completion of each exposure period, percent mortality was calculated and was graphically presented.

Semperula maculata::

Adult herbivorous terrestrial slug *S. maculata* (Semper) (Pulmonata: Stylomatophoram) of (4-5 cm length, 1- 1.5 cm width, weighing 3-4 g) were selected for experiment. LC_{50} of terrestrial slug was determined by using method of Otitoloju *et al.*, (2009). Stock solution of known strength was prepared for intoxication. The stock solution was then serially diluted for to obtain solution of required concentration. For each of the test concentration 1000 g of the loamy humus soil was placed into the bioassay container to form a thick layer of substrate. 100 g of Mulberry leaves were then placed in each bioassay container. Each test medium was then sprinkled with 150 mls of solution containing predetermined concentration of test compound. Experiment for control group of slug was similarly carried out. The trough was covered with wire mesh and labeled accordingly.

iv. Calculation of LC_{50} values:

The per cent mortality during intoxication, the probit values of mortality (Y) and concentration of metals (X), LnX, LnX^2, LnXY, Y, LnX^2, and LnXY were calculated.

$$LC_{50} = \frac{5 - a}{b}$$

The values of 'b' and 'a' of the above equation were calculated by the following formula:

$$b = \frac{LnXY - \frac{LnX.Y}{n}}{LnX^2 - \frac{(LnX)^2}{n}}$$

n is total number of concentrations used.

$$a = Y - bLnX$$

Where, X is concentration of heavy metal and Y is mortality.

v. Electrophoretic separation:-

Plasma proteins of control and experimental animals were carried out by electrophoresis technique (Andrews 1981; Davis 1964 and Orstein 1949).

vi. Haematological analysis -

The differential haemocyte count (DHC) of experimental and control animal was determined by Praful (1994).

vii. Histological staining techniques:-

1) Harris Haematoxylene (1900) and Eosin technique (HE)
2) Mallory's triple staining technique (MT)

viii. Histochemical staining techniques –

- **Neutral mucosubstances-**

  Periodic Acid – Schiff (PAS) technique (Mc Manus, 1946; Hotchkiss, 1948)

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- **Acidic mucosubstances -**

  Alcian blue (AB) at pH 1.0 (Lev and Spicer 1964)

  Alcian blue (AB) at 2.5 (Lev and Spicer 1964)

ix. Immunohistochemistry:-

Immunohistochemical study of nervous system was carried out by Using Pisu *et al.*, (2000) and Sternberger *et al.*, (1970).

B) Results:

A. Atomic absorption spectrophotometer:

Analysis of water samples from Rajaram tank, (near the Shivaji University, Kolhapur) was carried out by Atomic Absorption Spectrophotometer (AAS). AAS analysis of water sample showed that, over all in the water body Zn content was found in highest concentration, whereas Cu and Pb were in moderate amount. The Cd content was found very less or sometime it was negligible and remained undetectable in AAS. The relative metals in the water body were Zn>Cu>Pb>Cd, 0.094, 0.016, 0.012, 0.005 ppm, respectively (Fig.1, Table- 1, 2 and 3).

Throughout the year, water was found alkaline in the range of 7.8 to 9.4 pH with mean of 8.2 pH. The lowest pH was observed in the rainy season, may be due to the dilution effect of incoming rain water. Significant difference was not noted in pH during summer, rainy and winter, but the variation to change in sampling location was highly significant. The highest concentration of copper, lead and cadmium was observed in winter, because low pH increases the competition between metal and hydrogen ions for cellular binding sites.

Seasonal variations showed high concentration of zinc during the summer season. It was observed that, concentration of contaminants begin to recover, reaching to its maximum in the summer due to sulphide-oxidising bacterial activity, increasing with the temperature, and simultaneously, concentrating effect of the dissolved pollutants in the water due to water evaporation.

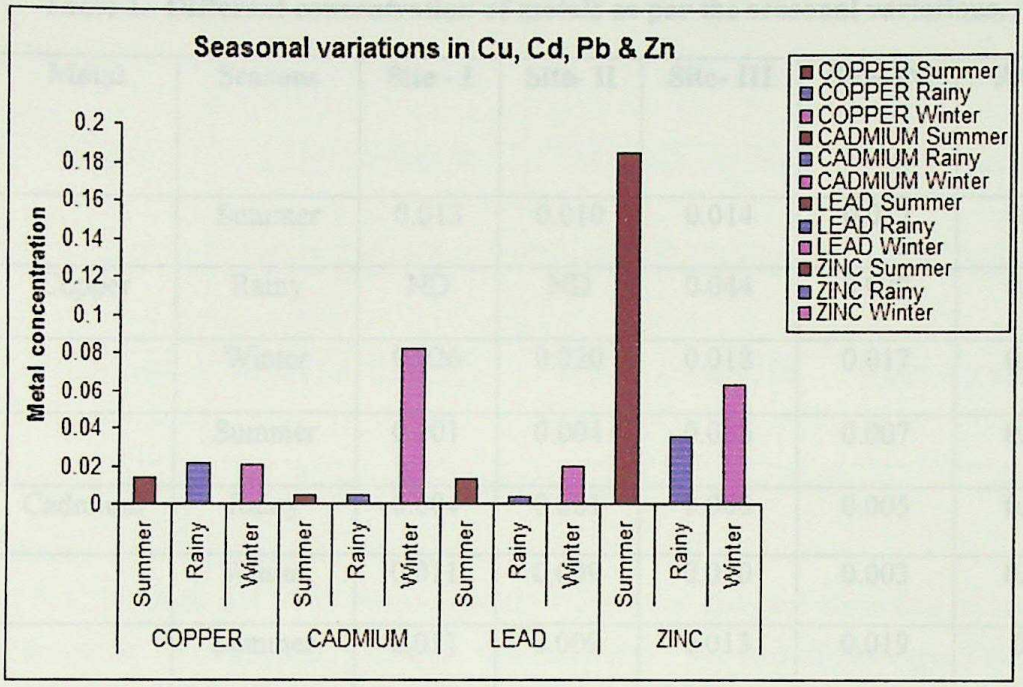

Seasonal variations in Cu, Cd, Pb & Zn
Metal concentration
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Summer
Rainy
Winter
COPPER
CADMIUM
LEAD
ZINC
COPPER Summer
COPPER Rainy
COPPER Winter
CADMIUM Summer
CADMIUM Rainy
CADMIUM Winter
LEAD Summer
LEAD Rainy
LEAD Winter
ZINC Summer
ZINC Rainy
ZINC Winter

Fig. 1

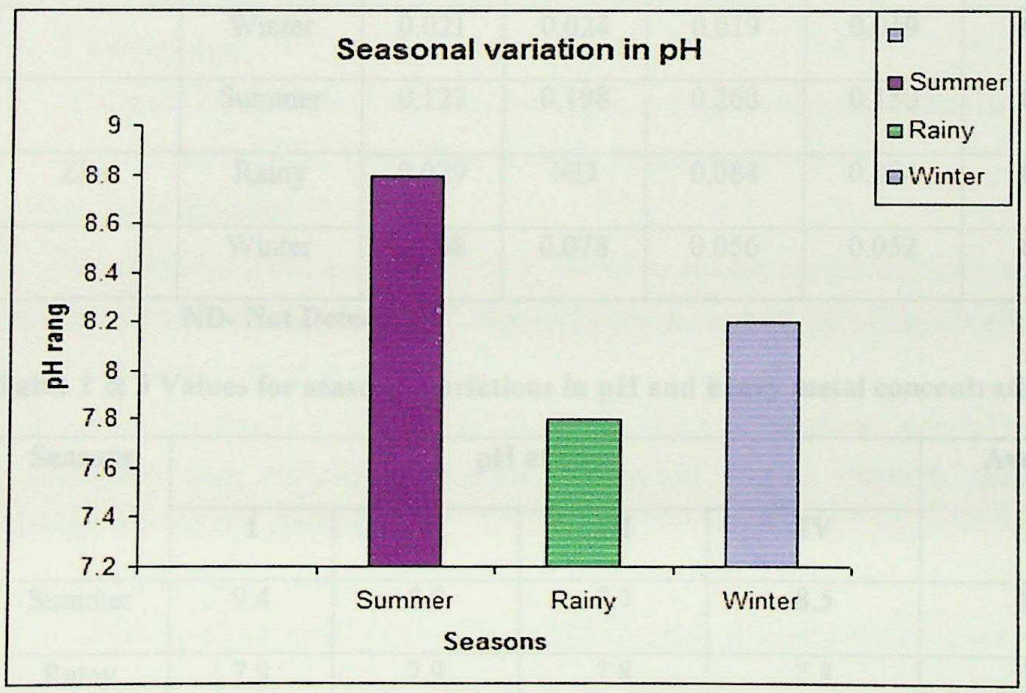

Seasonal variation in pH
pH rang
9
8.8
8.6
8.4
8.2
8
7.8
7.6
7.4
7.2
Summer
Rainy
Winter
Seasons
Summer
Rainy
Winter

Fig. 2

Table 1. Different concentration of metals as per the seasonal variations.

Metal	Seasons	Site - I	Site- II	Site- III	Site- IV	Average
	Summer	0.013	0.010	0.014	0.017	**0.0135**
Copper	Rainy	ND	ND	0.044	0.027	**0.0177**
	Winter	0.026	0.020	0.018	0.017	**0.0202**
	Summer	0.001	0.004	0.006	0.007	**0.0045**
Cadmium	Rainy	0.004	0.001	0.008	0.005	**0.0045**
	Winter	0.011	0.009	0.010	0.003	**0.0820**
	Summer	0.011	0.009	0.013	0.019	**0.013**
Lead	Rainy	ND	ND	0.016	ND	**0.004**
	Winter	0.021	0.024	0.019	0.019	**0.020**
	Summer	0.127	0.198	0.268	0.150	**0.185**
Zinc	Rainy	0.029	ND	0.084	0.084	**0.035**
	Winter	0.068	0.078	0.056	0.052	**0.063**

ND- Not Detectable

Table 2 & 3 Values for seasonal variations in pH and heavy metal concentration

Seasons	pH at sites				Average
	I	II	III	IV	
Summer	9.4	8.9	8.7	8.5	8.8
Rainy	7.9	7.9	7.8	7.8	7.8
Winter	8.2	8.2	8.3	8.1	8.2
Average	8.5	8.3	8.2	7.9	

Metal	Summer	Rainy	Winter
Zinc	0.0133 ± 0.000158	0.0175 ± 0.000158	0.0198 ± 0.0002
Copper	0.183 ± 0.000158	0.033 ± 0.001581	0.062 ± 0.001581
Lead	0.011 ± 0.001581	0.0021 ± 0.001327	0.018 ± 0.001581
Cadmium	0.0043 ± 0.000158	0.0043 ± 0.000158	0.008 ± 0.000158

Seasonal Variation for all the metals is highly significant (P<o.oool)

Paper published related to this study.

i) N.A. Kamble and **V. V. Potdar (2011).** "Impact of seasonal variations on the metal pollution of Rajaram tank Kolhapur, (Maharashtra)". **Journal of Zoology, Vol. 31 (2): 221-225.**

B. LC $_{50}$ Values for each metal in both molluscan species:

***i) B. bengalensis*:**

The mean LC_{50} value of *B. bengalensis* against Copper sulphate, Cadmium chloride and Mercuric chloride was 3ppm, 30 ppm, and 1.5 ppm respectively.

***ii) Semperula maculata*:**

The calculated mean LC_{50} value of *S. maculata* against copper sulphate, cadmium chloride and mercuric chloride was 1600ppm, 1400ppm, and 107ppm respectively.

Paper published related to this study.

i) N.A. Kamble and V. V. Potdar (2010). "Study of copper sulphate induced mortality to freshwater snail *Bellamya bengalensis* (Lamarck)". **Uttar Pradesh Journal of Zoology. Vol. 30(3): 399-405.**

C. Haematological analysis:

In gastropod molluscs *B. bengalensis* and *S.maculata* mainly two different types of blood cells were found i.e. Granulocytes and Agranulocytes. Haematocytes were found an important in defense mechanism. They were extremely effective in transporting foreign particles including biotic and abiotic materials as fungi and bacteria, metals, pesticides, through the process of phagocytosis. During toxicity, we found increased cell

count of granulocytes at the 24 and 48hrs of intoxication. But after 72 hrs of intoxication decrease in number of granulocytes was observed as compared to the control group of animal. In case of Cadmium chloride and Mercuric chloride sudden increase in granulocytes and agranulocytes was observed, but after 72 hrs of intoxication there was reduction in number of granulocytes and agranulocytes was found and finally greatly reduced after 96hrs of toxicity leading to lower down the defense mechanism of animal, as a result we found mortality in the experimental animals.

Paper published related to this study.

i) N.A. Kamble and **V. V. Potdar (2010).** "Hematological analysis of molluscan species *Bellamya bengalensis* and *Lamellidens marginalis*". **Biological forum: An International Journal Vol. No. 2(1): 70-72 (2010).**

D) Electrophoretic analysis:

Plasma protein composition varies in relation to physiological and functional status of the animal, which in turn may depends on exposure to pollutants and pathogens with suitable/favorable environmental factors relating to its reproductive cycle. Term protein profiling refers to the extraction, separation and characterization of different proteins expressed in an individual.

The plasma proteins of controlled slug *S. maculata* have showed total five protein bands, where first two bands were sharply stained. However 3rd, 4th and 5th bands were intensely stained representing its high concentration of protein in the plasma of *S. maculata*. In the experimental animals, banding pattern of 24 hrs exposure, found similar to the control group showing the low metallic stress over animal, with the less oxidative reaction. At the 48 hrs of intoxication the 5th band was found diminished indicating that, during toxicity this protein may be utilized to overcome the metallic stress of the cadmium up to 72 hrs of exposure period. In the protein bands, it was found that after 96 hrs of exposure period maximum alteration in band pattern was found. Two bands were diminished i.e. 1st and 2nd with three less intensified bands, indicating utilization of amino acid by protein oxidation in metabolism. The protein bands which appeared may be because of its binding with metal ion to form metalloproteins during the transportation through plasma.

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In control snail, *B. bengalensis*, nine bands were observed. At the 24 hrs of toxicity found that, banding pattern was similar as that of control group of animals. After the 48 hrs of intoxication only six bands were observed with reduction in band intensity of 2^{nd} and 3^{rd} bands. During the 72 hrs of intoxication, band intensity was found deceased. At the 96 hrs only five bands were observed, where as remaining four bands was diminished. This may be because of utilization of amino acid to overcome the toxicity effect.

D. Histological study/ observations:

a. Kidney (Excretory system)/ Nephrocytes:

Molluscan kidney provides an opportunity to study the relationship between metal binding pattern in toxicity assessment since, these organs not only concentrates metals to a great extent, but also carry out two important functions with high affinity cytosolic proteins and mineral concretions within it.

i. Histology in general:

Molluscan kidney consists of a tubule enclosing its luminal cavity derived from the coelome. Histomorphologically it was observed that, the tubules were internally lined by circular and columnar epithelial cells: both were found taking part in excretion process by filtering the metabolic substances, referred as nephrocytes. Microscopically, each nephrocyte was enclosing the nucleus having round or oval shape in basaly arranged position. The presence of vacuole in the nephrocyte indicated that, these cells are involved in the excretion of waste and resorption of essential metabolites from urinary fluid taking part in homeostasis mechanism of body fluids.

ii Metalic concretions:

In the metalic concreation, we have observed that, after the exposure of 24 hrs of Copper sulphate, Cadmium chloride and Mercury chloride induction, there was appearance of crystalline metallic concretions with light refractive, rounded bodies having central core which appeared yellowish-brown in Haematoxylene-Eosin staining technique. The nephrocytes extrudes the concretion into the lumen of the kidney, from were metals excreted. After 48 hrs of exposure shrinkage in the tubule was found with a significant enlargement. Extended exposure period up to 72 hrs; showed magnified and destructed epithelial lining of tubule with increased quantity of metallic concretions in it.

At last after 96 hrs, destructed tubular lining were found with luminal spaces filled by metallic concretion as well as cellular debris. The nephrocytic enlargement and accumulation of metallic crystalline material indicated that, throughout the biochemical reaction the metal binding proteins carry the induced copper sulphate up to nephrocyte for temporary storage and then, if excess, excreted through the excretory mechanism.

Paper published related to this study.

i) N.A. Kamble and V. V. Potdar (2011). "Metallic concretions in the nephrocytes of freshwater snail *Bellamya bengalensis*". **Bionano Frontier: an International Journal of Sciences and Technology Vol.** 4 (2): **264-266.**

b. Nervous system:

i. Organization of CNS:

The central nervous system of Prosobranch, *B. bengalensis* and Stylommatophoran *S. maculata* consist of 11 ganglia i.e. of paired buccal, cerebral, pedal, pleural and parietal ganglia and of single visceral ganglia. The cerebral ganglia found connected with very short connective. The ganglia were surrounded by perinerium, a capsule of connective tissue containing smooth muscle cells. Each ganglion in both the molluscs *B. bengalensis* and *S. maculata* having natural light pink and white creamy colour respectively. Histomorphologically, the cerebral ganglion at the periphery contained neurosecretory cells secreting neurohormones, neromodulators or neurotransmitters to make control over of body mechanism as well as to maintain homeostasis as per the environmental conditions.

ii. Cerebral ganglia in *B. bengalensis* and *S. maculata*:

Histologicaly, the neurons of cerebral ganglia were classified into four main types giant, large, medium and small sized cells. Each neurons found oval with well defined nucleus. The neuropil (fibrous mass) was found centrally. Gilal cells were found between the neuronal cells. The cells were distinguished by their dense elongated small nuclei, along with few gilal cells and large number of nerve axons that was found in different directions in the section. Some variable sized neurons were also found at the border layer of the cerebral ganglia. The nerve cells were found mostly unipolar with one axonal process which was often found divided into collaterals.

Paper published related to this study.

i) N.A.Kamble and S.R. Londhe (2011). "Histomophology of Neuronal and Neurosecretory Cells in Two Molluscan Species *B. bengalensis* and *S. maculata*". **Bionano Frontier: an International Journal of Sciences and Technology. Vol. 4 (2): 270-272, Impact factor: 0.233.**

iii. Histopathology of cerebral ganglia in *B. bengalensis* and *S. maculata.*

Light microscopic study showed histopathological changes in the cerebral ganglia of snail *B. bengalensis* and *S. maculata after* treated with LC_{50} of heavy metals Copper sulphate, Cadmium chloride and Mercuric chloride after intoxication for 24, 48, 72 and 96 hrs.

After 24 hrs of intoxication, neurons were exhibited multinuclear and showed eccentric and lysis of chromatin. In 48 hrs of exposure to Copper sulphate, Cadmium chloride and Mercuric chloride lysis of neuronal perinerium were found with severe pyknosis in it. The gaint neurons showed a shrunken appearance with poor nuclear integrity. Other cells showed shrinkage of the cell bodies along with migration of deformative nuclei. After 72 hrs of exposure gaint neurons became swollen and showed dark staining with eccentric karyosomes. It was observed that, the counters of cell membrane and nuclear envelopes were found irregularly arranged with dissolved perinerium. Complete degeneration of axonal process with lysis of nerve fiber was found in the neuropil. The large and medium sized nerve showed extreme shrinkage. Few cells were found completely degenerated in cell cytoplasm. Several neurons were appeared to be disturbed with peripheral extracellular space after 96 hrs of exposure.

Paper published related to this study.

i) N.A.Kamble and S.R. Londhe (2011). "Copper Sulphate Induced Neuronal Histological Changes in the Freshwater snail *Bellamya bengalensis*". **The Bioscan: An International Quarterly Journal of Life Sciences, Vol. 6 (2): 351-354.**

ii) N.A. Kamble and S.R. Londhe (2012). "Mercuric Chloride Induced Neuropathology and Physiological Responses in slug *Semperula maculata*" **International journal of zoological Research, Science Alert, Vol. 8 (1): 33-42.**

E. Histochemical analysis:

a. Kidney (Excretory system):

Molluscs possess a wide range of complexes mucosubstances in the cells, which preassembly play an important role in the vital metabolic functions of the animal. Histochemically, acidic mucosubstances of nephrocytes were found directly proportional to exposure duration. Where in the experiment, the glycogen moiety in the nephrocytes was significantly decreased.

The histochemical study of nephrocytes showed significant increase in nucleus – cytoplasm ratio with enlargement of vacuole in the cytoplasm. The morphometric changes in cell diameter were more prominent in Mercuric chloride toxicity than in the Copper sulphate and Cadmium chloride. Based on the histochemical composition obtained from AB and PAS staining techniques, data indicated that, glycoproteins were more interacted in Extra Cellular Matrix (ECM) of nephrocytes. The glycoconjugates from different areas of nephrocytes found to be decreased as the time of exposure increased. During exposure period, glycoproteins and neutral mucosubstances such as glycogen were found decreased with elevated level of acidic mucosubstances. This may be for to overcome the toxicity stress produced by toxicants as, Mercuric chloride, Copper sulphate and Cadmium chloride.

b. Nervous system:

i. Mucin zones in the cerebral ganglia of the *B. bengalensis* and *S. maculata*.

In our study we have found that, mucosubstances were found in the ECM with three different anatomical ganglionic units as; Periganglionic connective tissue sheath (PS) - include the membrane lamina, muscle fibers, axon and glial processes. Interperikaryonal space (IS) - include the perineuronal net and neuropil (N) - contained glial elements, with the mass of axon projection. Acidic mucopolysaccahrides viz; chondrotin, chondrotin sulphate, kerato sulphate, hyaluronic acid, heparin, heparin sulphate in ECM of neuronal cells were analyzed, when stained moderate turquoise with Periodic acid Schiff (PAS) staining method. These mucopolysaccahride found crucial role in the movement, guidance and synaptic formation of neuronal cells during developments. In PAS staining method, ECM of neuronal cells showed intensive hexoes containing neutral mucosubstances. High percentage of hexoes or glycogen indicated its

low concentration with less weakly acidic hyaluronic and sialomucins within. It was found that, well balanced mucosubstances in the cells were maintained for normal functions of neuronal cells in the controled animal.

Paper accepted related to this study:

i. N.A.Kamble and S.R. Londhe (2012). "Neurohistochemistry in molluscan species: focus on extracellular matrix" **Invertebrate neuroscience, Springer, (International journal).**

ii. Neutral– Acid mucins from ECM after intoxication of Copper sulphate, Cadmium chloride and Mercuric chloride.

The cerebral neurophil and ECM of neuronal cells was found filled with acid mucopolysaccahride viz; chondrotin, chondrotin sulphate, kerato sulphate, hyaluronic acid, heparin and heparin sulphate with small amount of weakly acidic mucopolysaccahride. Initially carbohydrate moiety found decreased in the different time of exposure when intoxicated with $CuSO_4$, $CdCl_2$ and $HgCl_2$. The neutral mucosubstances like glycogen polymers was found utilized during excess stress of $CuSo_4$, $HgCl_2$ and $CdCl_2$. The majority of glycosylated component as amino acid with several linkages as W- linked glycoprotein, oligosaccharide, acetylglucosamine and other monopoly residues with N- linked glycoprotein, N- acetyl glycosamine, proteoglycans of the ECM were found depleted. These mucopolymers were minimized with increased time of exposure.

Paper published related to this study:

i) **N.A.Kamble and S.R. Londhe (2012).** "Epileptic Activity in Neuronal cells, Induced by $HgCl_2$ and $CdCl_2$ in Terrestrial slug *Semperula maculata*: Fine structure investigated by Histology and Histochemistry". **Toxicology and Environmental Chemistry, Taylor and Francis, (International journal), Vol.94 (1): 109-120.**

F. Immunohistochemical study.

a. Nervous system:

i. Cerebral ganglia: Organization and immunoreactivity in controled slug

Morphologically, cerebral ganglia of experimental slug, *S. maculata* consist of three regions, procerebrum, mesocerebrum and metacerebrum found connected with a short commissure. Cerebral ganglia from control group of slug showed pattern of gastrin

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/CCK 8 positive neuronal cells. The positive immunoreactive neuronal cells were found scattered all over in the regions of cerebral ganglia. The detailed specification of procerebrum, mesocerebrum and metacerebrum as-

Procerebrum

Different types of neuronal cells were found associated in the procerebrum of cerebral ganglia. Medium and small sized (diameter 5-16 µm) from anterior region of procerebrum were found immunoreactive. Immunoreactive mechanism was not observed in controlled staining sections. Total 10 % of cells were found immunoreactive out of complete cell population. The nerve fibers of perikaryal and neuropil regions were showed moderate response to the immunoreactive mechanism of gastrin/CCK 8.

Mesocerebrum

Perikaryal fibers of mesocerebrum showed immunoreactive in the neuronal cells. Total 12 % of cells including large and small sized neurons (diameter 8-20 µm) were found positively reacted. Gastrin/CCK 8 immunoreactivity in neuropil nerve fibers from mesocerebrum was not found. The sections stained with normal goat serum instead of primary antibodies showed the absences of immunoreactivity.

Metacerebrum

Anatomically all the types of cell were found in metacerebrum of cerebral ganglia. As compared with procerebrum and mesocerebrum, metacerebrum showed low quantity of positively reacted neuronal cells against vertebrate antiserum. Only 8 % of neuronal cells were found positive immunoreactivity. Nerve fibers from neurophile regions showed moderate immunoreactive as compared with controlled immunoreactive staining mechanism.

ii. Metal induced immunoreactivity:

Effects of heavy metal Mercuric chloride on immunoreactive mechanism of gastrin/CCK 8 activity of slug *S. maculata* was observed and were documented as per time intervals of 24h, 48h, 72h and 96h in the toxicity study.

39 % of neuronal cells from cerebral ganglia were showed intensive and moderate positive immunoreactivity against metal induction. About 16 % of large, medium and small sized cells were found immunoreactive in procerebrum regions. Neuropil and perikaryal nerve fibers were found high immunoreactivity. Mesocerebrum and

Metacerebrum showed slight immunoreactivity against induced metal toxicity in the neuronal cells. Overall about 13 % and 10 % of neuronal cells were found positively reacted against Mercuric chloride in the cerebral ganglia. After 48 hours of exposure, immunoreactive tissue sections were increased gastrin/ CCK 8 reactivity in nerve fibers and neuronal cells in cerebral ganglia. Sectional view of procerebrum showed that, about 19% of neuronal cells were found immunoreactive after 48 hours of exposure to metal $HgCl_2$. Higher immunoreactivity of nerve fibers in neuropil and perikaryal regions was found in the intoxicated neuronal cells as compared with 24 hours of exposure. In general 18 % of mesocerebral region with 14 % of metacerebrum neurons were found immunoreactive against metal induction

Immunohistochemical observation after 72 hours of exposure period, showed enhanced immunoreactivity in the cell of cerebral ganglia against vertebrate antiserum gastrin/ CCK 8. Comparatively it was observed that, immunoreactivity found in mesocerebrum and metacerebrum was less than immune reactions of remaining neuronal cells in procerebrum. Out of the cell population, 32 % immunoreactivity was found in large, medium and small sized neurons in cerebral ganglia. 27% cells in mesocerebrum and 20% neuronal cells in metacerebrum was showed immunoreactive mechanism against induction of $HgCl_2$. Immunoreactivity of perikaryal and neuropil region was found more after 72 hours of exposure when intoxicated with metal $HgCl_2$. After 96 hours, almost 50 % of cerebroganglial neuronal cells were found immunoreactive with few exceptions of the gaint neurons within. 52 % of neurons from sectional view of procerebrum showed immunopositive reaction. Neuropil fiber was found intensively involved in immunoreactivity, in which 39% and 23 % of neurons were showed immunoreactive mechanism of mesocerebrum and metacerebrum respectively. The neuropil and perikaryal nerve fibers showed extreme immunoreactivity after 96 hours of intoxication of mercuric chloride. The immunoreactivity of neuronal cells including all the procerebrum, mesocerebrum and metacerebrum of cerebral ganglia showed its protective mechanism against metal induction in the neuronal cells. The immunity of ganglionic neuronal cells was found altered as per induction period and bioconcentration of metallic content in the neuronal cells. The accumulation of metals in the neuronal cells were enhanced the immunoreactive mechanism, as a result the cells of ganglia showed

dark/ intensive immune reaction or mechanisms to the gastrin / CCK 8 content in the cells. The immunity of the cells was found altered as per LC_{50} concentration of each of the metal in the intoxication study.

Paper considered for publication related to this study:

i) N.A.Kamble and S.R. Londhe (2012). "Metal intoxicated immunoreactivity of gastrin cholecystokinin in the neuronal system of terrestrial slug *Semperula maculata* (Semper)", **Indian Journal of Experimental Biology, Impact factor- 0.702**

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UNIVERSITY GRANT COMMISSION

BAHADUR SHAH ZAFAR MARG

NEW DELHI – 110 002

Utilization certificate

Certified that the grant of Rs. 6, 17,160/- (Six lakh seventeen thousand one hundred and sixty only) received from the university Grants Commission under the scheme of support for Major Research Project entitled "Comparative study of metal toxicity and molecular mechanism of immune defense with circulatory excretory and neuro physiology in mollusca." vide letter no. F.No.36-210/2008 (SR) dated 26th March, 2009. From the sanctioned amount Rs. 6, 01, 124/- (Six lakh one thousand and one hundred twenty four only) has been fully utilized for the purpose for which it was sanctioned and accordance with the terms and conditions lay down by the University Grant Commission. The remaining balance of Rs. 16,036/- (Sixteen thousand and thirty six only) from non-recurring and recurring will be submitted to UGC.

Principal Investigator

Dr.N.A.Kamble
Principal Investigator
U.G.C.Major Research Project
Department of Zoology
Shivaji University,Kolhapur

Registrar

Registrar,
Shivaji University, Kolhapur

Statutory Auditor

For Sankpal Kulkarni & Associates
Chartered Accountants

(A. N. Sankpal)
Partner, M. No. 104718
FRN 121256W

SANKPAL KULKARNI & ASSOCIATES
Chartered Accountants
KOLHAPUR

13.6.12

UNIVERSITY GRANT COMMISSION

BAHADUR SHAH ZAFAR MARG

NEW DELHI – 110 002

Statement of expenditure in respect of Major Research Project

1. Name of Principal Investigator - Dr. Nitin Anandrao Kamble
   Assistant Professor,
   Department of Zoology,
   Shivaji University,
   Kolhapur, 416004

2. Department of University - Zoology Department
   Shivaji University,
   Kolhapur, 416004

3. UGC Approval No. and date - F.No.36-210/2008 (SR),
   26th March 2009.

4. Title of research project - Comparative Study of Metal Toxicity and Molecular Mechanism of Immune Defense With Circulatory Excretory and Neuro Physiology in Mollusca.

5. Effective date of starting the project: 01/05/2009

b) Staff: Date of Appointment: 07 /07/2009 and 14/06/2010

Expenditure done: (From 01/05/2009 to 30/04/2012)

Sr. No.	Expenditure Incurred (Rs.)	Amount Approved (Rs.)	Expenditure Incurred in 2009-2010 (Rs.)	Expenditure incurred in 2010-2011	Expenditure incurred in 2011-2012 Up to 30th April 2012	Total expenditure	Balance remained
1	Honorarium to PI (Retired Teachers) 10,000/-p.m.	----	----	----	----	----	----
2.	Project Associate Fellowship @, Rs.8, 000/- p.m.	2,70,400/-	61,935/-	89,333/-	1,04,000/-	2,55,268/-	15, 132/-
3.	Project Fellow Consolidated Salary, @ Rs. 6000/- p.m.	----	----	----	----	----	----

Item	Total Amount Approved (Rs.)	Expenditure Incurred in 2009-2010 (Rs.)	Expenditure incurred in 2010-2011 (Rs.)	Expenditure incurred in 2011-2012 Up to 30th April 2012	Total Expenditure of three years (Rs.)	Balance remained (Rs.)
Books and Journal	1. First installment: **3, 57,800/-** 2. Second installment: **2,59,360/-**	16,412/-	-	7,805/-	24,217/-	783/-
Equipment		74,880/-	-	-	74,880/-	120/-
Contingency		11,648/-	12,752/-	44,943/-	69,343/-	01/-
Field Work/Travel		3,185/-	12,877/-	25,726	41,788/-	nil
Hiring Services		nil	nil	nil	nil	nil
Chemicals and glassware		22,114/-	-	74,714/-	96,828/-	nil
Overhead		12,933/-	12, 933/-	12, 934/-	38, 800/-	nil
Total	**6,17,160/-**	**2,03,107/-**	**1, 27, 895/-**	**2,70,122/-**	**6,01,124/-**	**16,036/-**

1. It is certified that the appointment(s) have been made in accordance with the terms and conditions laid down by the Commission.

2. It as result of check or audit objective, some irregularly is noticed, later date, action will be taken to refund, adjust or regularize the objected amount.

3. Payments@ revised rates shall be made with arrears on the availability of additional funds.

4. It is certified that the grant of Rs. 6, 17,160/- (Six lakh seventeen thousand one hundred and sixty only) received from the university Grants Commission under the scheme of support for Major Research Project entitled "Comparative study of metal toxicity and molecular mechanism of immune defense with circulatory excretory and neuro physiology in mollusca." vide letter no. F.No.36-210/2008 (SR) dated 26th March, 2009. From the sanctioned amount Rs. 6, 01, 124/- (Six lakh one thousand and one hundred twenty four only) has been fully utilized for the purpose for which it was sanctioned and accordance with the terms and conditions lay down by the University Grant Commission. The remaining balance of Rs. 16,036/- (Sixteen thousand and thirty six only) from non-recurring and recurring will be submitted to UGC.

Principal Investigator

Dr.N.A.Kamble
Principal Investigator
U.G.C.Major Research Project
Department of Zoology
Shivaji University,Kolhapur

Registrar / Principal

Registrar,
Shivaji University, Kolhapur.

UNIVERSITY GRANT COMMISSION

BAHADUR SHAH ZAFAR MARG

NEW DELHI – 110 002

Statement of expenditure incurred on field work

Name of Principal Investigator: **Dr. N.A. Kamble**
Assistant Professor,
Department of Zoology,
Shivaji University,
Kolhapur, 416004

Name of the place visited	Duration of the visit		Mode of the journey	Expenditure Incurred
	From	To		
Kolhapur-Ankali-Malgoan-Arag bedag-Kolhapur.	9 am. on 06/03/2010	6 pm. on 06/03/2010	Rented vehicle Tata-sumo	Rs.1,351/-
Mumbai-Kolhapur-Mumbai.	7.20am 20/06/2009	8.20pm. 20/06/2009	By train	Rs.1,834 /-
Kolhapur-Ankali-Malgoan-Arag bedag-Kolhapur	8 am. on 18/07/2010	6 pm. on 18/07/2010	Indica car	Rs. 1287/-
Kolhapur-Ankali-Malgoan-Arag bedag-Kolhapur	8 am. on 18/09/2010	6 pm. on 18/09/2010	Indica car	Rs. 1100/-
Kolhapur-Ankali-Malgoan-Arag bedag-Kolhapur	7 am. on 02/11/2010	6 pm. on 02/11/2010	Indica car	Rs. 1100/-
Kolhapue-pune-Delhi-Delhi- pune-Klhapur	11.00am 22/03/2011	10.00pm 26/03/2011	By air	Rs.9,390/-
Kolhapur-Sangli- -Arag -Bedag-Miraj-Kolhapur	7am 13/06/2011	6pm 13/06/2011	Indica car	Rs.1400/-

Kolhapur-Sangola-Miraj- Kolhapur	6am 27/06/2011	10 pm 27/06/2011	Own two wheeler	700/-
Kolhapur-Sangli- -Arag -Bedag-Miraj-Kolhapur	7am 11/08/2011	6pm 11/08/2011	Own two wheeler	391/-
Kolhapur-Narsobawadi-Sangli-Kolhapur	7am 04/10/2011	6pm 04/10/2011	Own two wheeler	391/-
Kolhapur-Narsobawadi-Sangli--Arag -Bedag-Kolhapur	7am 12/10/2011	6pm 12/10/2011	Indica car	1505/-
Kolhapur-solapur-Kolhapur	6am 11/11/2011	8pm 11/11/2011	Indica car	3274/-
Kolhapur-Narsobawadi-Sangli--Arag -Bedag-Kolhapur	7am 19/11/2011	6pm 19/11/2011	Indica car	1770/-
Kolhapur-Sangli-Bedag-Palus-Islampur- Kolhapur	6am 30/11/2011	8pm 30/11/2011	Indica car	1858/-
Kolhapur- Arag - Bedag- Kolhapur	6am 03/12/2011	6pm 03/12/2011	Own two wheeler	374/-
Kolhapur-Narsobawadi-Sangli--Arag -Bedag-Kolhapur	6am 11/01/2012	6pm 11/01/2012	Own two wheeler	459/-
Kolhapur-Narsobawadi-Sangli--Arag -Bedag-Kolhapur	6am 12/02/2012	6pm 12/02/2012	Indica car	1800/-
Kolhapur-Ratnagiri-Sawantwadi-Kolhapur	6am 27/02/2012	10pm 27/02/2012	Indica car	3488/-
Kolhapur-Ratnagiri-Sawantwadi-Kolhapur	6am 12/03/2012	10pm 12/03/2012	Indica car	3802/-
Kolhapur-Narsobawadi-Sangli--Arag -Bedag-Kolhapur	6am 19/03/2012	6pm 19/03/2012	Indica car	2100/-

Kolhapur-Narsobawadi-Sangli--Arag -Bedag-Kolhapur	6am 20/03/2012	6pm 20/03/2012	Indica car	1190/-
Kolhapur-Narsobawadi-Sangli--Arag -Bedag-Kolhapur	6am 05/04/2012	6pm 05/04/2012	Own two wheeler	510/-
Kolhapur-Sangola-Bedag-Miraj-Kolhapur	6am 08/04/2012	10pm 05/04/2012	Own two wheeler	714/-
			Total	**Rs. 41,788/-**

Certified that the above expenditure is in accordance with the UGC norms for Major Research Project.

Principal Investigator

Dr.N.A.Kamble
Principal Investigator
U.G.C.Major Research Project
Department of Zoology
Shivaji University,Kolhapur

Registrar / Principal

Registrar,
Shivaji University, Kolhapur