CHAPTER IV

RESULTS

Acute toxicity tests of neem *Azadirachta indica* seed extract on Nile tilapia *Oreochromis niloticus*

In the definitive test, an average percentage of mortality of Nile tilapia *O. niloticus* exposed to concentrations at 30, 35, 40, 45, 50, 55, 60, 65 and 70 ppm neem seed extract over 96 hr at four time intervals was estimated. Exposure to neem seed extract to a concentration of 30 and 35 ppm were unable to kill the Nile tilapia within 24 hr but the mortality could be observed at higher concentrations of neem seed extract. Conversely, the death of fish was not found in the control group all time of the bioassay. After 24 hr of exposure to 40, 45, 50, 55, 60, 65 and 70 ppm of neem seed extract, the percent of death was 53.3, 53.3, 40, 46.7, 100, 100 and 100%, respectively. Fourty-eight hours later, the percent fish killed was 26.67, 73.33, 80.00, 66.67, 86.67, 100, 100 and 100% for concentration at 35, 40, 45, 50, 55, 60, 65 and 70 ppm, respectively. Exposure to 35, 40, 45, and 50 ppm neem seed extract for 72 hr, the percent of death was 40.00, 73.3, 86.67 and 80.00 %, respectively. For the concentrations of 55 to 70 ppm, the percent mortality was equal to 100 % (Table 4.1).

After 96 hr of exposure, the percent mortality was 46.67 and 80.00% at the concentrations of 35 and 40 ppm, respectively (Table 4.1). All fish were killed at concentrations of 45, 50, 55, 60, 65 and 70 ppm of neem seed extract. The LC_{50} values of neem seed extract for 24, 48, 72 and 96 hr were 47.71, 40.71, 38.92 and 36.25 ppm, respectively (Table 4.2). Interpretations of the 24, 48, 72 and 96 h LC_{50} values (Fig.4.1 and Fig.4.2) were calculated by Probit program (Finney, 1971) and summarized in Appendix C.

Table 4.1 Percent mortality of Nile tilapia at various concentrations of neem *A. indica* seed extract over 96 hours.

Neem seed extract (ppm)	Number of fish	Number of replication	Percent mortality											
			24-hour			48-hour			72-hour			96-hour		
			No. of dead	Death (%)	Mean death (%)	No. of dead	Death (%)	Mean death (%)	No. of dead	Death (%)	Mean death (%)	No. of dead	Death (%)	Mean death (%)
control	10	1	0	0		0	0		0	0		0	0	
	10	2	0	0	0	0	0	0	0	0	0	0	0	0
	10	3	0	0		0	0		0	0		0	0	
30	10	1	0	0		0	0		0	0		0	0	
	10	2	0	0	0	0	0	0	0	0	0	0	0	0
	10	3	0	0		0	0		0	0		0	0	
35	10	1	0	0		2	20		4	40		4	40	
	10	2	0	0	0	6	60	26.67	6	60	40.00	6	60	46.67
	10	3	0	0		0	0		2	20		4	40	
40	10	1	6	60		10	100		10	100		10	100	
	10	2	4	40	3.33	4	40	73.33	4	40	73.33	6	60	80.00
	10	3	6	60		8	80		8	80		8	80	
45	10	1	6	60		8	80		8	80		10	100	
	10	2	4	40	53.33	6	60	80.00	8	80	86.67	10	100	100.00
	10	3	6	60		10	100		10	100		10	100	

Table 4.1 Percent mortality of Nile tilapia at various concentrations of neem *A. indica* seed extract over 96 hours (continued).

Neem seed extract (ppm)	Number of fish	Number of replication	Percent mortality											
			24-hour			48-hour			72-hour			96-hour		
			No. of dead	Death (%)	Mean death (%)	No. of dead	Death (%)	Mean death (%)	No. of dead	Death (%)	Mean death (%)	No. of dead	Death (%)	Mean death (%)
50	10	1	2	20		6	60		6	60		10	100	
	10	2	4	40	40.00	4	40	66.67	8	80	80.00	10	100	100.00
	10	3	6	60		10	100		10	100		10	100	
55	10	1	4	40		8	80		10	100		10	100	
	10	2	6	60	46.67	10	100	86.67	10	100	100.00	10	100	100.00
	10	3	4	40		8	80		10	100		10	100	
60	10	1	10	100		10	100		10	100		10	100	
	10	2	10	100	100.00	10	100	100.00	10	100	100.00	10	100	100.00
	10	3	10	100		10	100		10	100		10	100	
65	10	1	10	100		10	100		10	100		10	100	
	10	2	10	100	100.00	10	100	100.00	10	100	100.00	10	100	100.00
	10	3	10	100		10	100		10	100		10	100	
70	10	1	10	100		10	100		10	100		10	100	
	10	2	10	100	100.00	10	100	100.00	10	100	100.00	10	100	100.00
	10	3	10	100		10	100		10	100		10	100	

All the Nile tilapia died during the acute toxicity test showed the progressive sign in the same manners e.g., air gulping, agitation, accelerated ventilation with rapid mouth and opercular movements which became arrhythmic and convulsive, gill hemorrhage, loss of stability and finally sank to the bottom of the glass jars.

Calculated LC_{50} of neem seed extract to Nile tilapia *O. niloticus* from data in Table 4.1 are shown in table 4.2.

Table 4.2 Calculated LC_{50} values (ppm) of neem *A. indica* seed extract to Nile tilapia *O. niloticus* using probit analysis.

Duration of exposure (hour)	LC_{50} (ppm)	95% confidence limits (ppm)	R^2
24	47.71	39.37 - 55.49	0.77
48	40.71	34.50 - 45.17	0.82
72	38.92	36.13 - 41.28	0.78
96	36.25	34.47 - 37.94	0.62

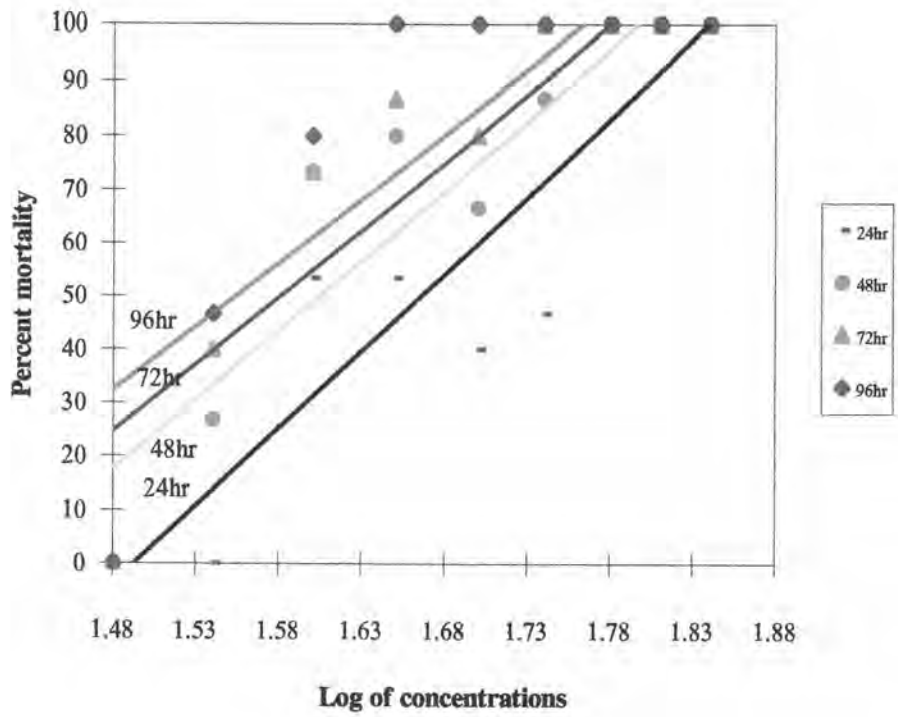

Fig. 4.1 Percent mortality of Nile tilapia *O. niloticus* after acute toxicity test.

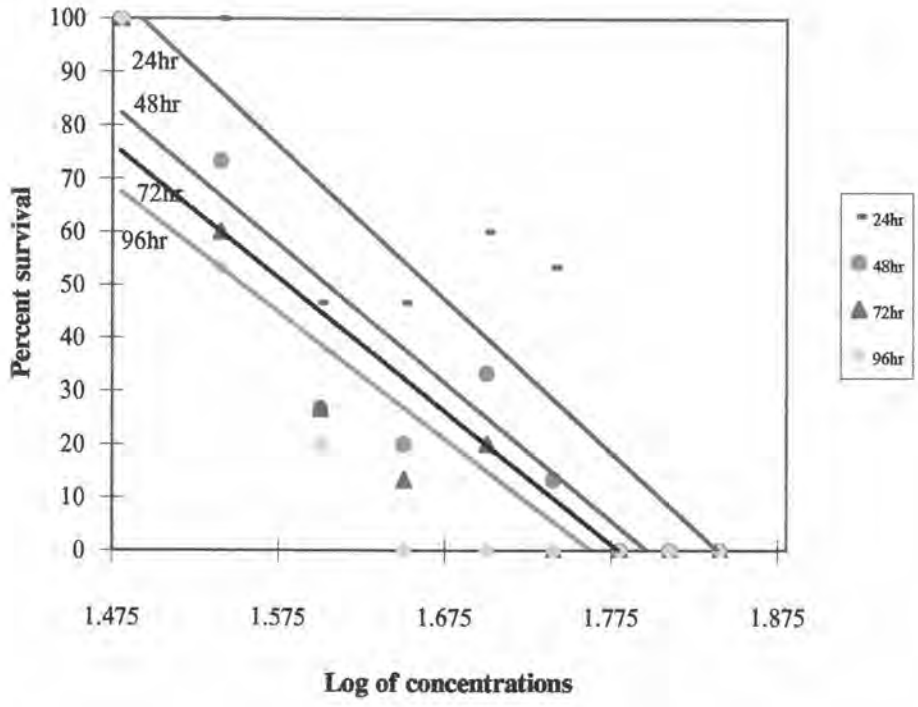

Fig. 4.2 Percent survival of Nile tilapia *O. niloticus* after acute toxicity test.

Determination of application factor (AF)

From Table 4.2, the 96-hr LC_{50} value of neem *Azadirachta indica* seed extract was 36.25 ppm. Also, the range defined between the highest concentration tested at which no significant deleterious effect (NOEC) and the lowest concentration tested at which some significant deleterious effect (LOEC) of neem seed extract was selected from this calculation and the LC_4 was chosen as a maximum acceptable toxicant concentration (MATC). Therefore, the LC_4 or MATC was 30.21 ppm (this value was shown in Appendix C). Thereafter, the application factor (AF) was calculated from these values. It followed as:

$$AF = MATC / LC_{50}\ 96hr$$

$$AF = 30.21 / 36.25$$

$$AF = 0.83$$

Thus, the AF for determining the sublethal concentration of neem seed extract in the long-term study was determined at 0.83. From this value, the sublethal concentration was estimated by multiplying 0.83 by the 96-hr LC_4 value. The calculation followed as:

$$AF \times LC_4 = 0.83 \times 30.21$$

$$= 25.07 \text{ ppm}$$

The sublethal concentration of neem seed extract for this long-term study (sublethal toxicity) was determined at 25.07 ppm.

Sublethal toxicity effect of neem seed extract

1. Morphological alteration of blood cells

1.1 Normal blood cells

Blood cellular elements of Nile tilapia *O. niloticus* in this study were observed from blood smear. Cells were classified according to their morphological features. Seven types of cell were distinguished including erythrocyte, thrombocyte and five different leukocyte types. They consisted of lymphocyte, monocyte, neutrophil, basophil and eosinophil.

Erythrocytes Size of cell is about 9x6 μm. It is an ellipsoidal cell with the nucleus centrally located with its long axis to that of the cell, the densely clumped chromatin staining purple. The cytoplasm stains a buff color (Fig.4.3). The less mature cells are larger and slightly purple to pink in color with Giemsa stain. Usually these cells appear a few number in peripheral blood.

Thrombocytes Most of the cells are variable in shape, sometimes round, spindle- or teardrop-shaped. These cells have a deep purple-staining nucleus and a relatively scanty, pink-staining rim of cytoplasm (Fig.4.4, 4.5 and 4.6).

Lymphocytes The lymphocyte is the most numerous leukocyte in Nile tilapia blood. These cells vary in size appearance from the very small cells (about 4 μm ϕ) with a large nucleus and narrow cytoplasmic margin, to the larger cells (about 7 μm ϕ), with numerous fine pseudopodia extending from plasma membrane. The round nucleus stains purple blue, with a dense finely-clumped chromatin. The large lymphocyte has a greater amount of pale-blue cytoplasm and large nuclei (Fig.4.7).

Monocytes Usually, monocytes are round cells with a diameter of 8-12 μm. These cells have the magenta-colored eccentrically-located nucleus which is polymorphic and varies in shape from round, bilobed or kidney shaped. The abundant cytoplasm stains pale blue. The occurrence of vacuoles in the cytoplasm is often seen (Fig.4.8).

Neutrophils Neutrophils are round to oval in shape, 9-12 μm in diameter. They contain an eccentric nucleus of variable form which is sometimes round, indented, kidney-shaped or lobed. The cytoplasm of the neutrophils stains pale blue and the numerous fine granules stain dark violet with Giemsa stain (Fig.4.9).

Basophils These cells are found infrequently in the peripheral blood. Basophil is about 7-11 μm ϕ. The large nucleus is eccentrically located and is somewhat oval, round or lobed in shape. The basophilic cytoplasmic granules stain purplish-blue (Fig.4.10).

Eosinophils Eosinophils are round to oval in shape, approximately 10 x 12 μm to 15 x 20 μm in diameter, with an eccentric round to oval nucleus. The relatively small nucleus stains deep purple and the diameter is about 5 μm to 8 μm ϕ. The eosinophilic red-orange granules are smaller than 1 μm in diameter. They are dispersed throughout the colorless cytoplasm (Fig.4.11). Eosinophil is rarely found in the peripheral blood.

1.2 Anomalies of blood cells in experimental fish

Erythrocytes The alteration of red blood cell was seen in both immature and mature erythrocytes.

Immature erythrocytes showed a numerical increase in circulation and were found in the peripheral blood of fish exposed to 25.07 ppm neem seed extract from 1month through 7 months of experiment (Fig. 4.12). The most numerous of this kind of evidence was observed in the third until the seventh month. However, the most severity change was found in the sixth month of exposure (Table 4.3 and 4.4).

Extrusion of nuclear material was also shown in immature erythrocyte. This anomaly was found in all groups of treated fish (Fig.4.13). But severity was marked in blood of fish exposed for 3 months. In other group was relatively mild in alteration (Table 4.3 and 4.4).

Mature erythrocytes showed morphological alteration in eight patterns.

1. Poikilocytosis including spindle-, spherical- and irregular -shaped was noted in the peripheral blood of all groups of fish exposed to neem seed extract. Spindle-shaped erythrocyte was severely changed in the third month of exposure (Fig.4.14). For spherical-shaped was markedly alterated in the first and second month (Table 4.3 and 4.4).

2. Extrusion of nuclear material was relatively severely occurred in the second month of exposure (Fig. 4.15). However, the occurrence of this kind of anomalous erythrocyte was observed in all treated groups. From five months to seven months of experiment, this phenomenon tended to be rare observed in the peripheral blood of treated fish (Table 4.3 and 4.4).

3. Increase nuclear interchromatin spaces was shown in mature erythrocytes of all treated fish (Fig.4.16). An appearance of increase nuclear interchromatin spaces was markedly found in the fouth and fifth month of exposure. However, this occurrence was relatively severe in all groups of treatment (Table 4.3 and 4.4).

4. Vacuolated erythrocyte was observed in all groups of treated fish (Fig. 4.17). The fourth month of exposure was the most severity alteration. In other group, vacuolated erythrocyte was relatively mild in changes(Table.4.3 and 4.4).

5. Hypochromic erythrocytes were exhibited in treated fish throughout the experiment (Fig.4.18). But in the fourth month, the effect was the most severe while treated group in other month showed relatively mild effect(Table 4.3 and 4.4).

6. Gray or basophilic cytoplasm was occurred in all groups of treatment (Fig.4.16). While severity was observed in blood of fish exposed to neem seed extract for 4 months. In other group, this incidence was mild effect (Table 4.3 and 4.4).

7. An appearance of a "ragged" cytoplasmic membrane or echinocyte was found in all groups of treatment (Fig.4.19). These evidences are mostly found in the first and second month of exposure. Thereafter, it tended to be less severe (Table 4.3 and 4.4).

8. Dividing erythrocytes was initially observed in the peripheral blood of the first, second and third month of treated fish (Fig.4.20). After that there was not found in the fourth, fifth and sixth month of exposure. However, it was seen again in the seven month (Table 4.3 and 4.4).

White blood cells Alteration of white blood cells are seen in only monocyte and neutrophil.

- Monocytes with nuclear hypertrophy was occasionally found in the blood smear of treated fish, particularly from the fourth to the seventh month of exposure (Fig.4.2.1). But there was no alteration in the first three month of exposure (1, 2 and 3 month) (Table 4.3 and 4.4).

- Neutrophils showed vacuolization in cytoplasm in the peripheral blood. This cell was sometimes found in fish blood exposed for 3-7 months. However, this abnormality of cell was relatively mild in these study (Table 4.3 and 4.4).

The frequency of incidences in observing the anomalous erythrocytes and white blood cells are shown in Table 4.3.

Table 4.3 Incidence of morphological anomalies of blood cell in Nile tilapia after exposure to a sublethal concentration (25.07 ppm) of neem seed extract from 1-7 months.

Morphological alteration	Duration of exposure (month)						
	1 n=50	2 n=50	3 n=50	4 n=50	5 n=50	6 n=50	7 n=50
Immature erythrocyte							
(a) Numerical increase in circulating blood	19	27	32	27	33	39	34
(b) Extrusion of nuclear material	5	5	26	1	3	9	10
Mature erythrocyte							
(a) Poikilocytosis							
- Spindle-shaped	24	20	26	6	19	17	14
- Spherical-shaped	41	41	31	30	22	24	40
- Irregular-shaped	21	38	40	4	17	18	6
(b) Extrusion of nuclear material	12	46	28	31	15	4	15
(c) Increase of nuclear interchromatin spaces	40	43	27	49	49	44	45
(d) Vacuolated erythrocyte	5	8	10	10	10	11	9
(e) Hypochromic erythrocyte	28	26	19	40	14	20	14
(f) "Ragged" cytoplasmic membrane	32	31	17	7	9	4	9
(g) Gray cytoplasm	9	9	10	28	24	22	5
(h) Dividing erythrocyte	16	2	3	0	0	0	2
Anomalous monocyte (nuclear hypertrophy)	0	0	0	3	1	1	4
Anomalous neutrophil (vacuolization)	0	0	5	4	3	5	1

Table 4.4 Severity of morphological alteration of blood cell in Nile tilapia after exposure to sublethal concentration (25.07 ppm) of neem seed extract from 1-7 months.

Morphological alteration	Duration of exposure (month)						
	1	2	3	4	5	6	7
Immature erythrocyte							
(a) Numerical increase in circulating blood	+	++	++	++	++	+++	++
(b) Extrusion of nuclear material	+	+	+++	+	+	+	+
Mature erythrocyte							
(a) Poikilocytosis							
- Spindle-shaped	++	++	+++	+	+	++	+
- Spherical-shaped	+++	+++	++	++	++	++	++
- Irregular-shaped	++	+++	+++	+	+	+	+
(b) Extrusion of nuclear material	+	+++	++	++	+	+	+
(c) Increase of nuclear interchromatin spaces	++	++	++	+++	+++	++	++
(d) Vacuolated erythrocyte	+	+	++	+++	++	++	+
(e) Hypochromic erythrocyte	++	++	++	+++	+	++	+
(f) "Ragged" cytoplasmic membrane	+++	+++	++	+	+	+	+
(g) Gray cytoplasm	+	+	+	+++	++	++	+
(h) Dividing erythrocyte	++	+	+	-	-	-	+
Anomalous monocyte (nuclear hypertrophy)	-	-	-	+	+	+	+
Anomalous neutrophil (vacuolization)	-	-	+	+	+	+	+

- : no change (0%)
+ : mild in alteration (25%)
++ : moderate in alteration (50%)
+++ : marked in alteration (75%)

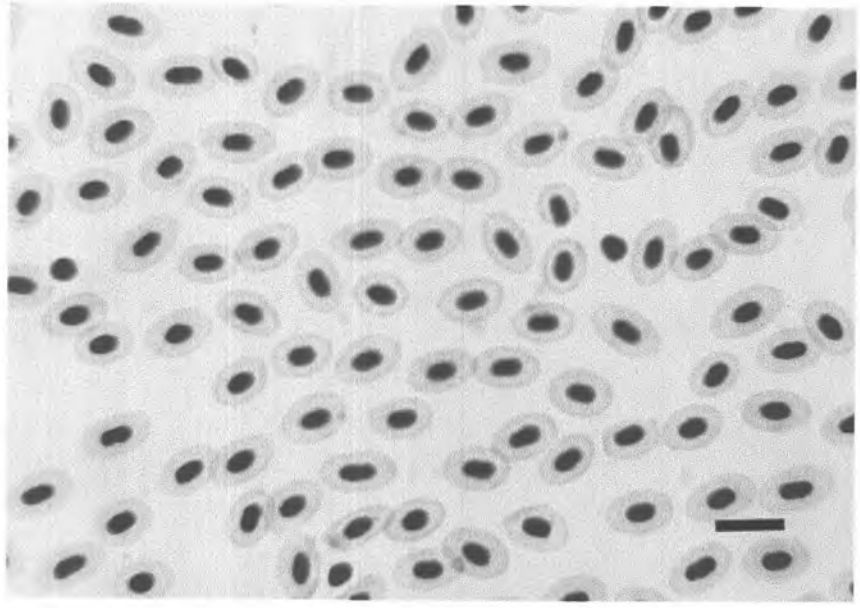

Fig.4.3 Micrograph of normal erythrocyte of 2 months old Nile tilapia *Oreochromis niloticus* shows ellipsoidal cell with the oval nucleus centrally located. Scale bar = 10 μm.

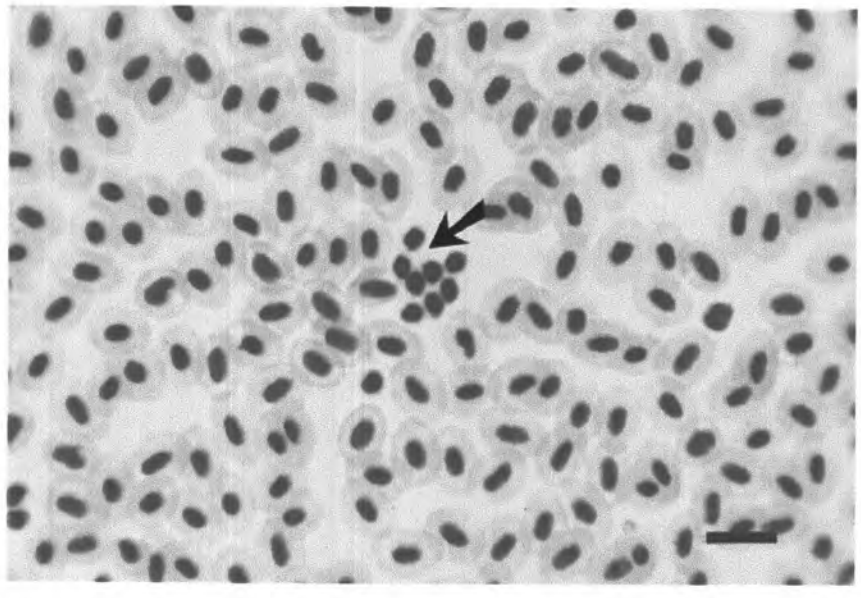

Fig.4.4 Micrograph of the normal round shaped thrombocyte with a thin rim of cytoplasm (arrow).Scale bar = 10 μm.

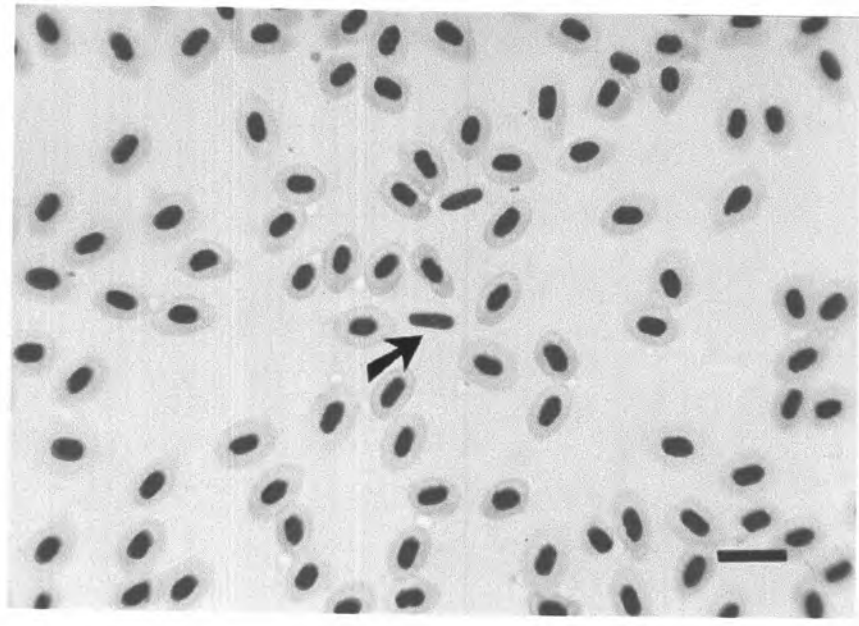

Fig.4.5 Micrograph of normal spindle shaped thrombocyte with elongated nucleus centrally located (arrow). Scale bar = 10 μm.

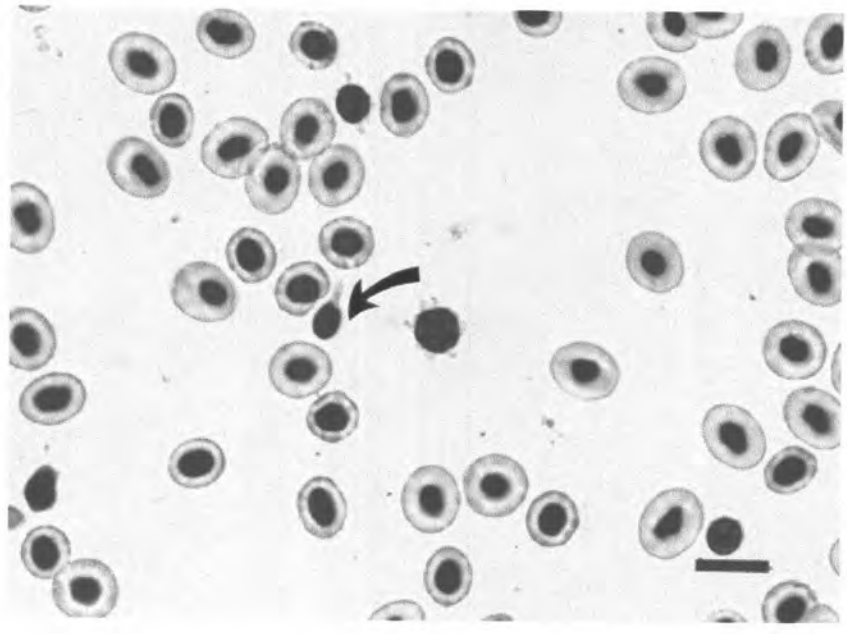

Fig.4.6 Micrograph of the characteristic of teardrop-shaped thrombocyte (arrow). Scale bar = 10 μm.

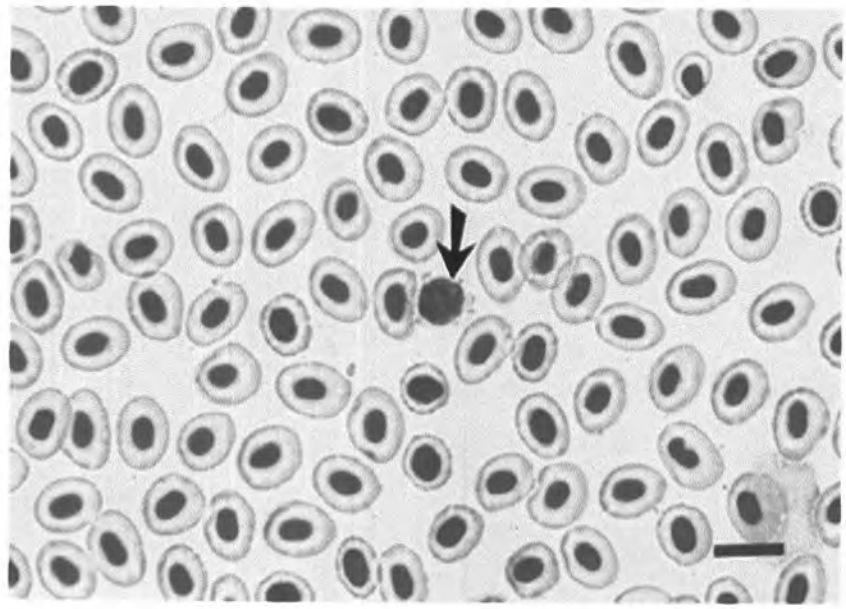

Fig.4.7 Micrograph of normal lymphocyte with numerous fine pseudopodia extending from cell membrane and a thin rim of light.pale blue cytoplasm (arrow). Scale bar = 10 μm.

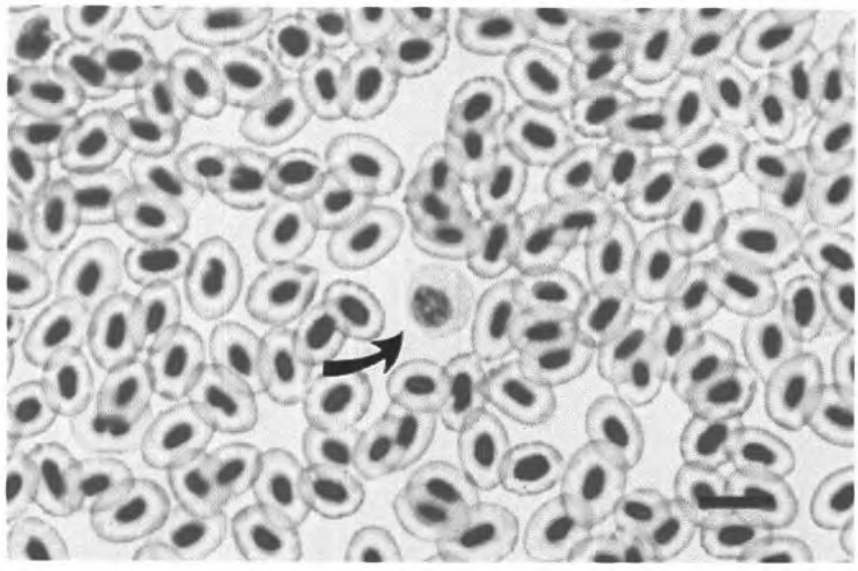

Fig.4.8 Micrograph of normal monocyte shows a magenta-colored eccentrically located nucleus in the vacuolated cytoplasm (arrow). Scale bar = 10 μm.

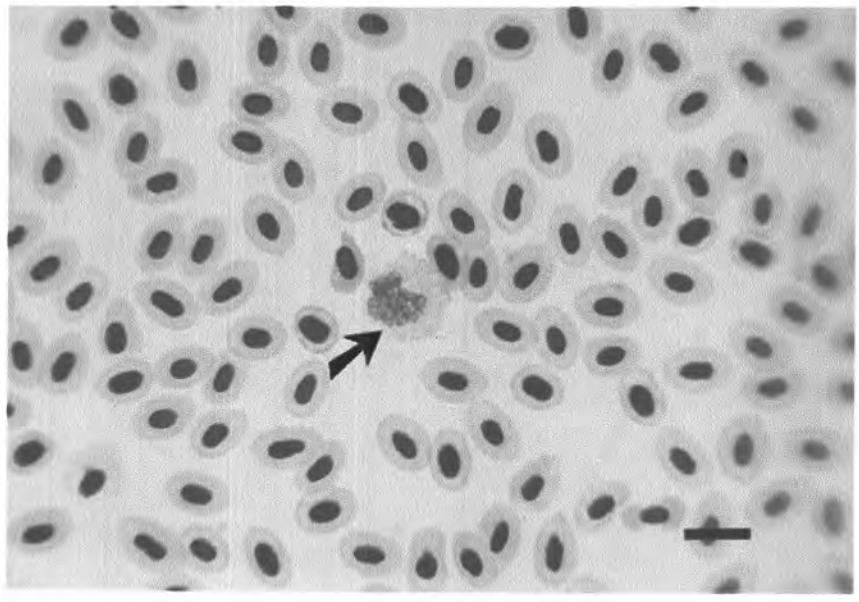

Fig.4.9 Micrograph of normal neutrophil with numerous fine dark violet granules shows an intended nucleus eccentrically located (arrow).Scale bar = 10 μm.

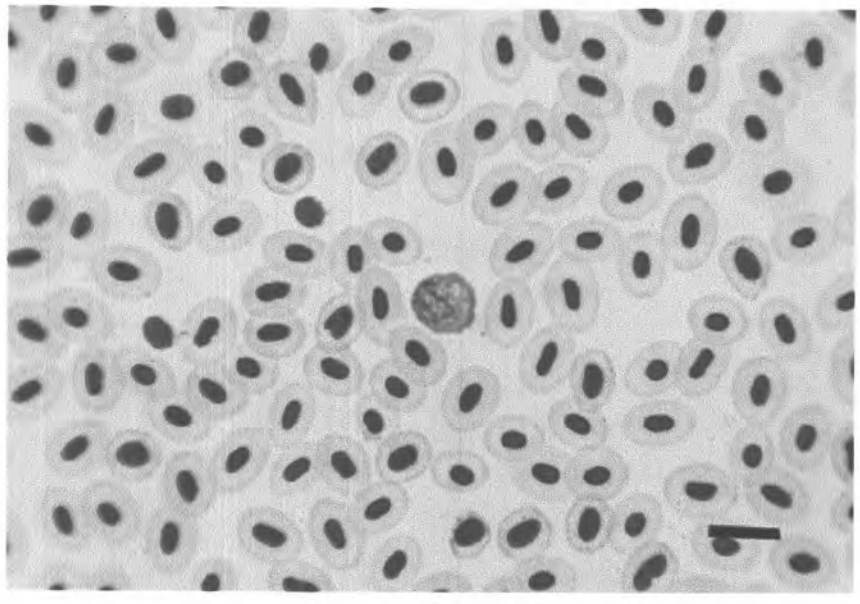

Fig.4.10 Micrograph of normal basophil shows the large oval, eccentric nucleus and the cytoplasm stains small darkly granules. Scale bar = 10 μm.

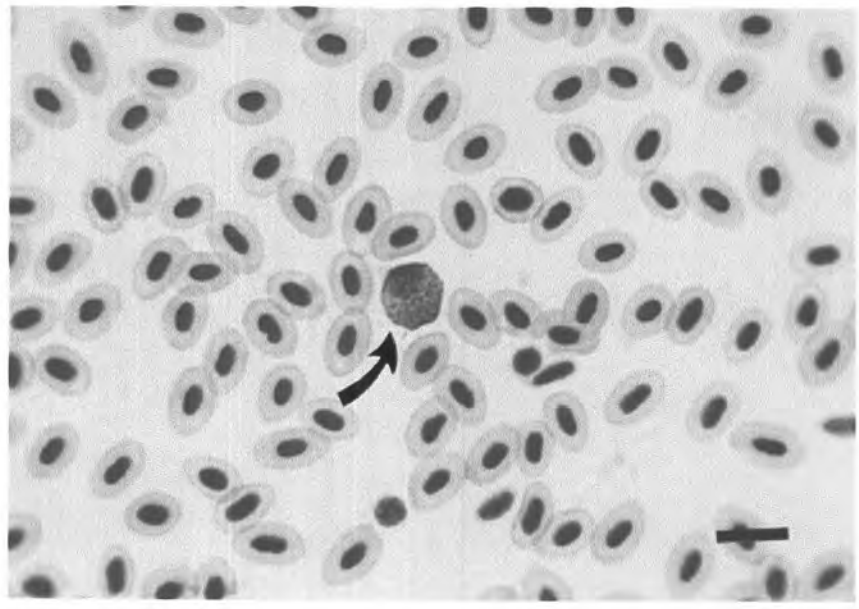

Fig.4.11 Micrograph of eosinophil with an eccentric nucleus shows refractile eosinophilic red-orange granules (arrow). Scale bar = 10 μm.

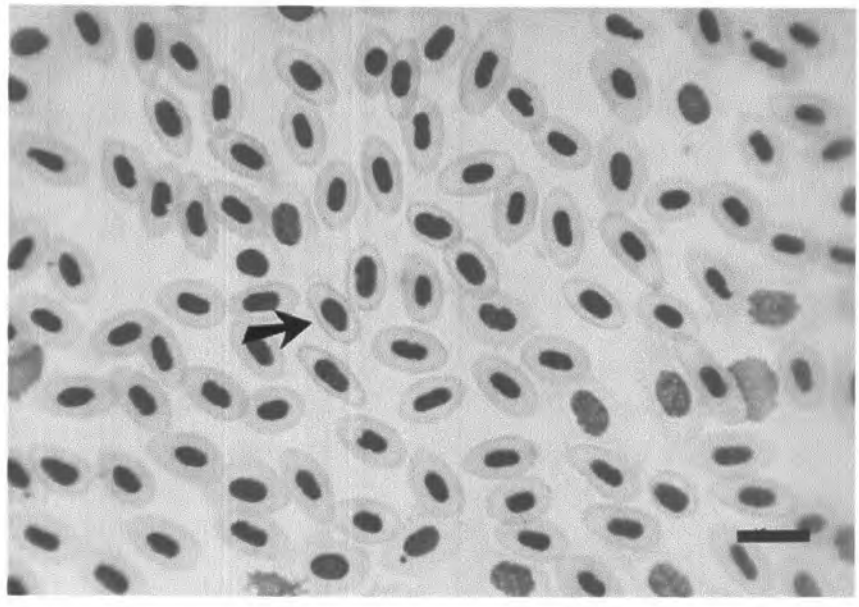

Fig.4.12 Micrograph shows immature erythrocyte and a "ragged" appearance of cytoplasmic membrane (arrow) of Nile tilapia blood after exposure to 25.07 ppm neem seed extract for 1 month. Scale bar = 10 μm.

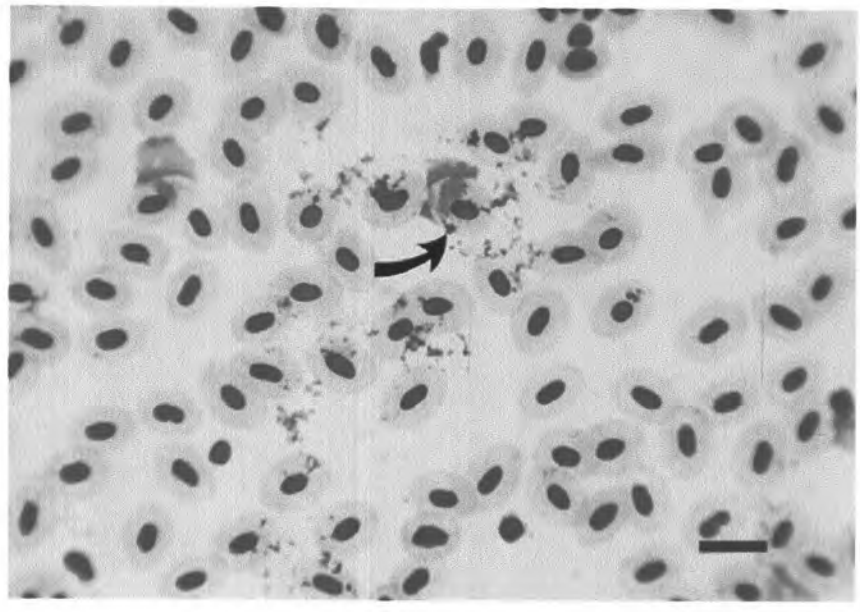

Fig.4.13 Micrograph shows extrusion of nuclear material in immature erythrocyte (arrow) after exposure to 25.07 ppm of neem seed extract for 2 months. Scale bar = 10 μm.

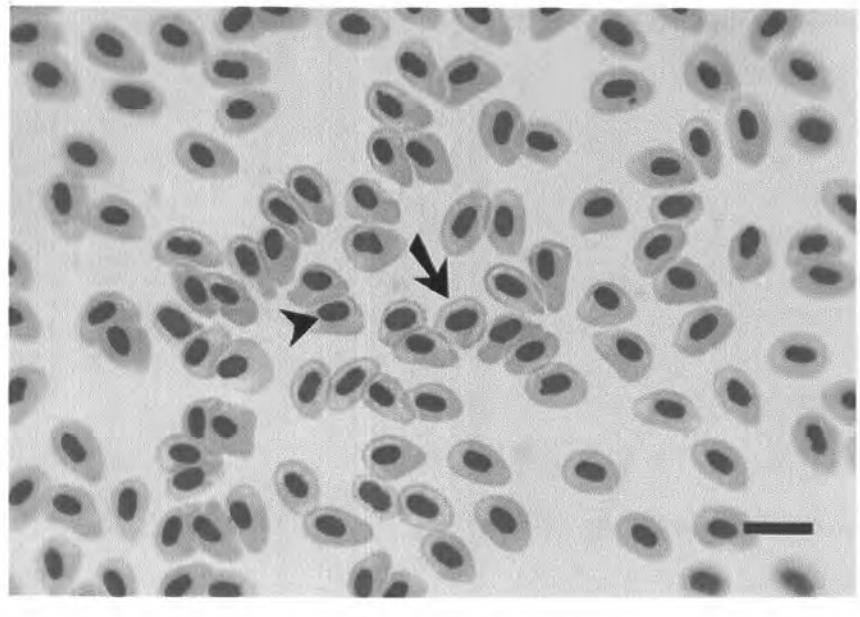

Fig.4.14 Micrograph of anomalous erythrocyte shows poikilocytosis (arrow) and an increase of nuclear interchromatin spaces (arrow-head) in fish exposed to a sublethal concentration (25.07 ppm) of seed extract for 1 month.
Scale bar = 10 μm.

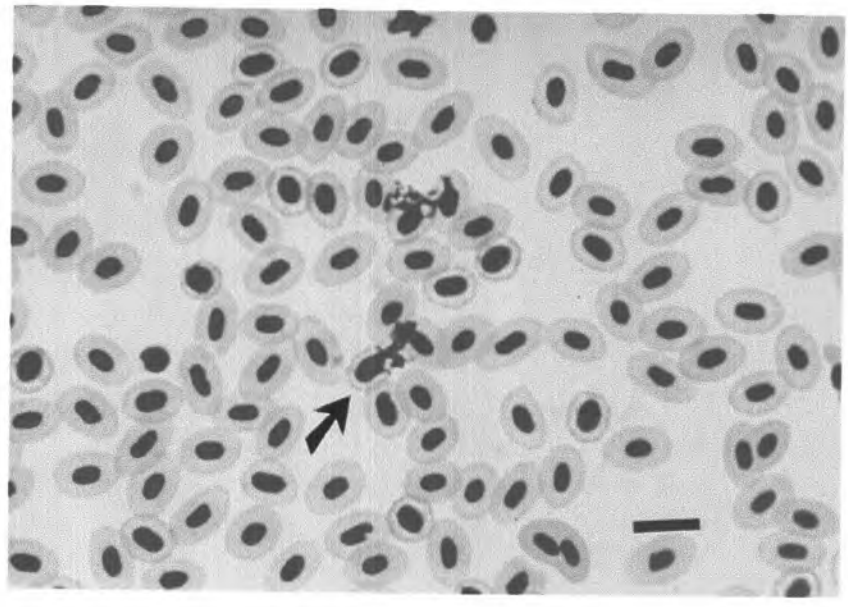

Fig.4.15 Micrograph shows the extrusion of nuclear material in mature erythrocyte of fish (arrow) after exposure to a sublethal concentration (25.07 ppm) of neem seed extract for 1month. Scale bar = 10 μm.

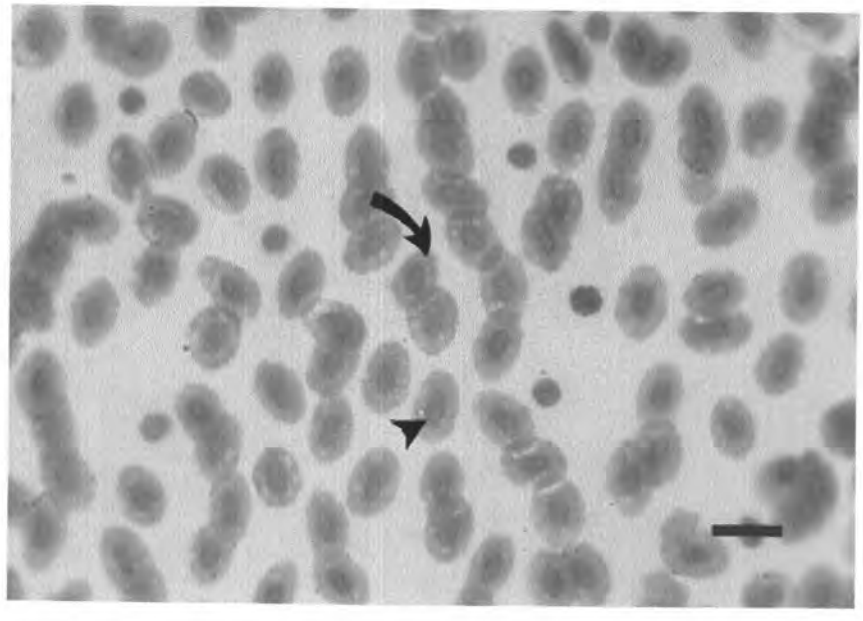

Fig.4.16 Micrograph of anomalous erythrocyte of Nile tilapia shows gray cytoplasm (arrow) and an increase of nuclear interchromatin space (arrow-head) in the fourth month of fish exposure to a sublethal concentration of neem seed extract. Scale bar = 10 μm.

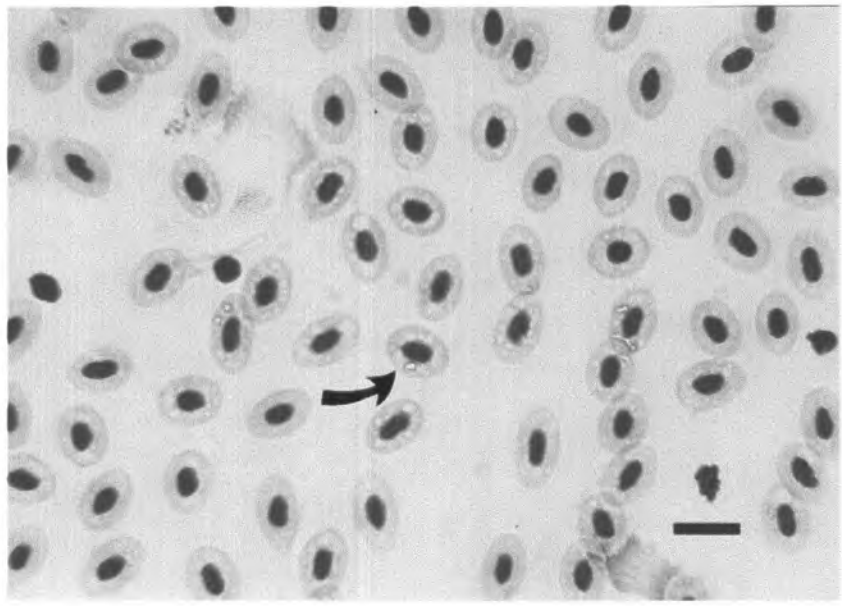

Fig.4.17 Micrograph shows vacuolated erythrocyte (arrow) after exposure to a sublethal concentration (25.07 ppm) of neem seed extract for 3 months. Scale bar = 10 μm.

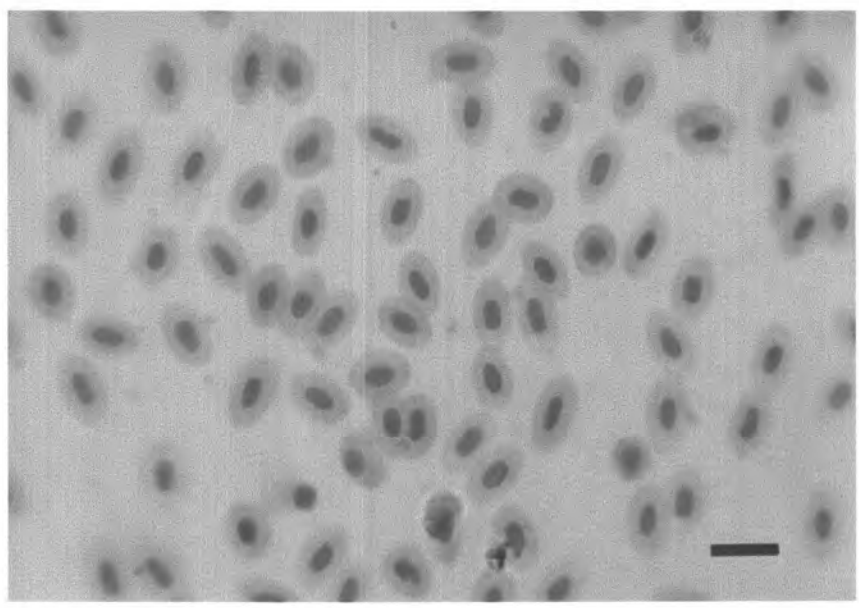

Fig.4.18 Micrograph of hypochromic erythrocyte after exposure to a sublethal concentration (25.07 ppm) of neem seed extract for 4 months. Scale bar = 10 μm.

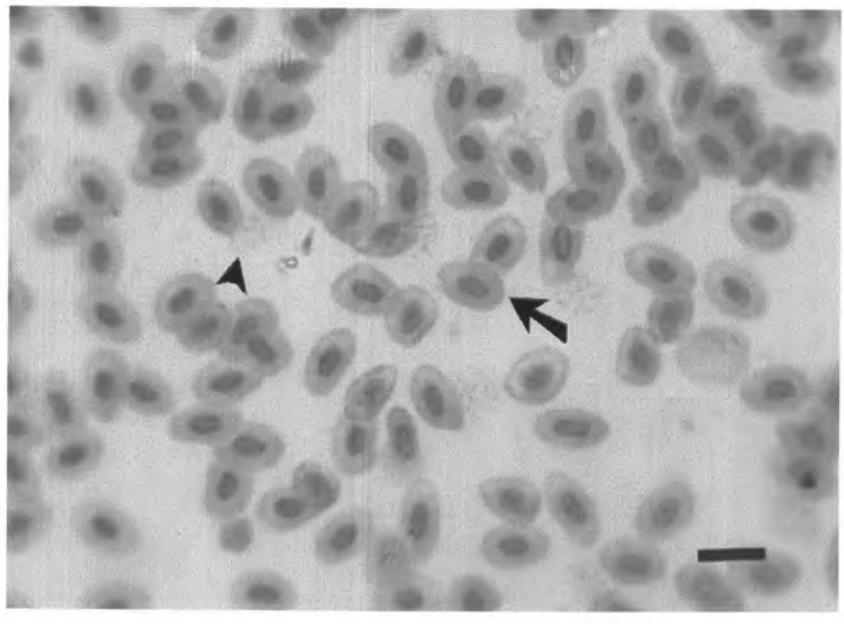

Fig.4.21 Micrograph of anomalous monocyte shows nuclear hypertrophy after caused by exposure to a sublethal concentration (25.07 ppm) of neem seed extract for 2 months.Scale bar = 10 μm.

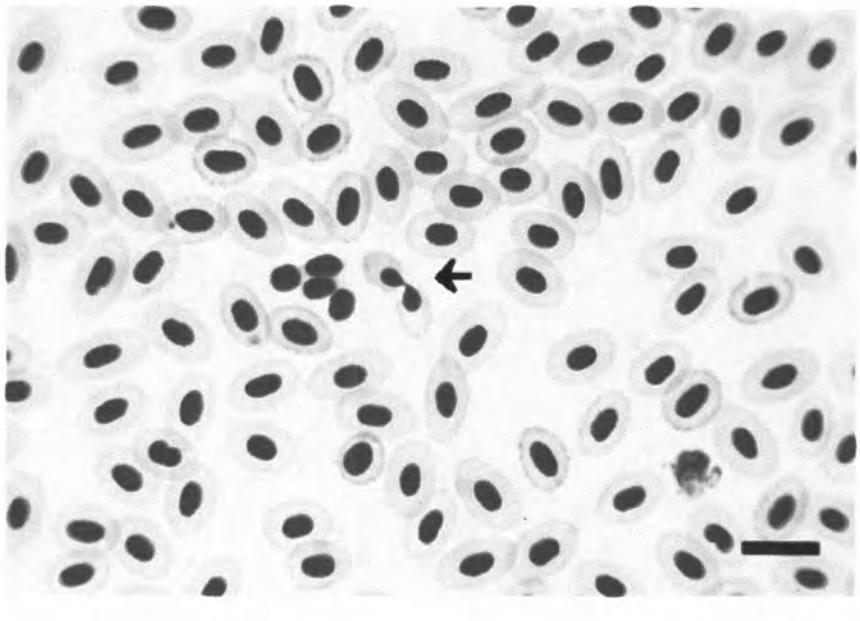

Fig.4.22 Micrograph shows anomalous vacuolated neutrophil of Nile tilapia after exposure to a sublethal concentration (25.07 ppm) of neem seed extract for 5 months. Scale bar = 10 μm.

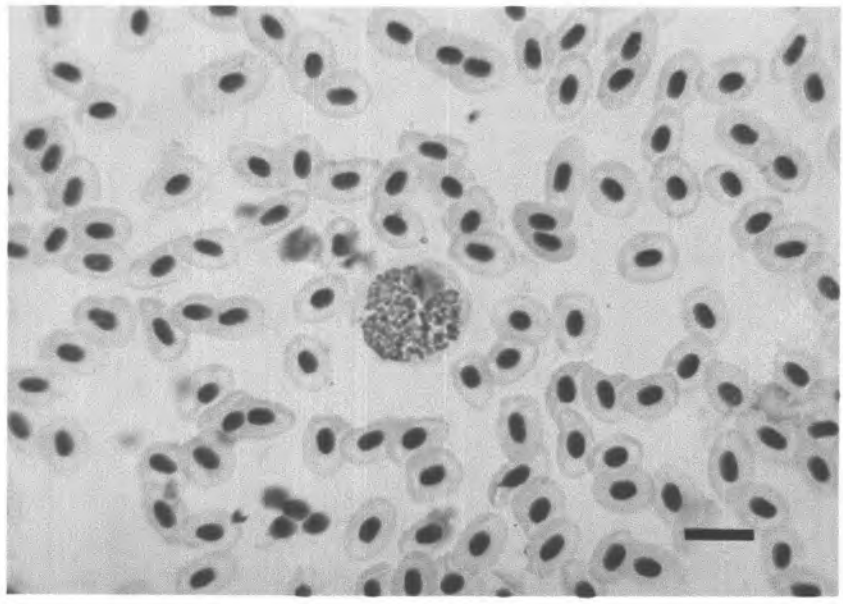

Fig.4.19 Micrograph of hypochromic erythrocyte shows gray cytoplasm (arrow) and mildly extrusion of nuclear material (arrow-head) after exposure to neem seed extract for 4 months. Scale bar = 10 μm.

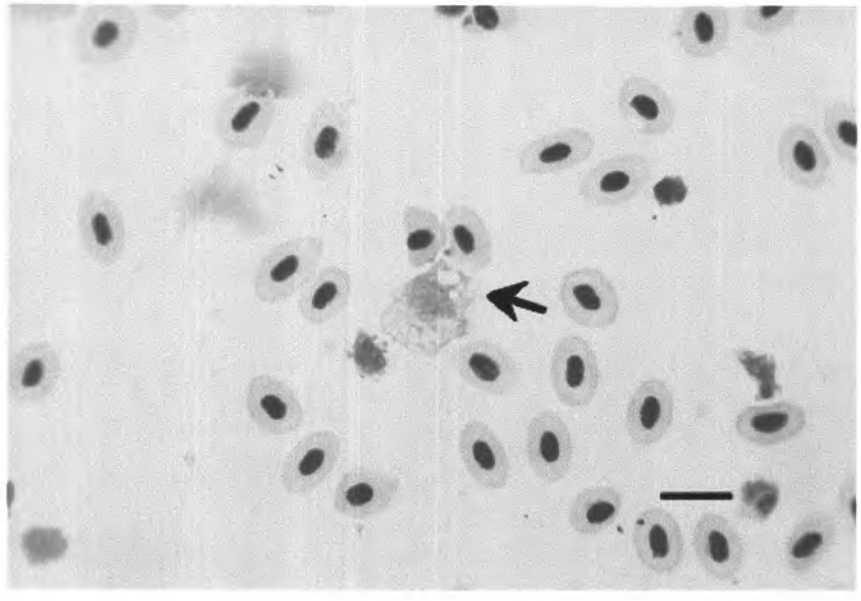

Fig.4.20 Micrograph of dividing erythrocyte (arrow) after exposure to a sublethal concentration of neem seed extract for 1 month. Scale bar = 10 μm.

2. Differential leukocyte count

From the total of a hundred leukocytes were counted for each sample and the percentage number of different types of leukocyte including lymphocyte, monocyte, neutrophil, basophil and eosinophil of Nile tilapia *Oreochromis niloticus* at the age of 2, 3, 4, 5, 6, 7 and 8 months in both control and treated groups were recorded (Fig.4.23 and Table 4.5)

The percentage of lymphocyte In control fish, the average percentage of lymphocyte at seven time intervals was 85.10 ± 1.58, 95.68 ± 0.53, 94.74 ± 0.65, 95.86 ± 0.54, 96.84 ± 0.48, 95.28 ± 0.50 and 93.74 ± 1.09 % for 1, 2, 3, 4, 5, 6, and 7 months of experimentation, respectively. For treated fish, it was 93.38 ± 0.91, 90.34 ± 1.20, 97.56 ± 1.20, 97.04 ± 0.43, 97.00 ± 0.44, 95.50 ± 0.60 and 93.66 ± 1.05 % for 1, 2, 3, 4, 5, 6, and 7 months of experimentation, respectively (Table 4.5 and Fig.4.24). Statistical significance ($p \leq 0.05$) between control and treatment was analysed, the result showed a significant increase in number of lymphocyte in fish exposed to neem seed extract for 1, 2 and 3 months.

The percentage of monocyte In control group, the average percentage of monocyte at seven time intervals was 11.96 ± 1.37, 3.54 ± 0.50, 4.44 ± 0.54, 2.60 ± 0.40, 1.82 ± 0.31, 3.30 ± 0.42 and 4.74 ± 0.94 % for 1, 2, 3, 4, 5, 6, and 7 months of experimentation, respectively. For treated group, it was 5.18 ± 0.80, 7.94 ± 1.10, 1.50 ± 0.20, 1.92 ± 0.30, 2.20 ± 0.40, 3.22 ± 0.49 and 4.76 ± 0.91 % for 1, 2, 3, 4, 5, 6, and 7 months of experimentation, respectively (Table 4.5 and Fig.4.25). Statistical significance ($p \leq 0.05$) between control and treatment was analysed, the result showed a significant increase in number of monocyte in fish exposed to neem *A. indica* seed extract for 2 months. A significant decrease in this cell number was observed in the first , third and fourth month of exposure to neem seed extract.

The percentage of neutrophil In control fish, the average percentage of neutrophil at seven time intervals was 2.04 ± 0.40, 0.60 ± 0.13, 0.72 ± 0.19, 0.56 ± 0.14, 0.30 ± 0.08, 0.66 ± 0.12 and 0.90 ± 0.20 % for 1, 2, 3, 4, 5, 6, and 7 months of experimentation, respectively. For treated fish, it was 1.08 ± 0.19, 1.46 ± 0.31, 0.68 ± 0.16, 0.26 ± 0.08, 0.30 ± 0.09, 0.36 ± 0.12 and 0.46 ± 0.15 % for 1, 2, 3, 4, 5, 6, and 7 months of experimentation, respectively (Table 4.5 and Fig.4.26). Statistical significance ($p \leq 0.05$) between control and treatment was analysed, the result showed a significant increase in number of neutrophil in fish exposed to neem *A. indica* seed extract for 2 months and a significant decrease in this cell number in the fourth month exposure fish.

The percentage of basophil In control group, the average percentage of basophil at seven time intervals was 0.58 ± 0.12, 0.16 ± 0.07, 0.12 ± 0.05, 0.86 ± 0.20, 1.00 ± 0.25, 0.74 ± 0.15 and 0.60 ± 0.15 % for 1, 2, 3, 4, 5, 6, and 7 months of experimentation, respectively. For treated fish, it was 0.38 ± 0.10, 0.18 ± 0.06, 0.22 ± 0.07, 0.72 ± 0.17, 0.48 ± 0.12, 0.90 ± 0.21 and 0.95 ± 0.16 % for 1, 2, 3, 4, 5, 6, and 7 months of experimentation, respectively (Table 4.5 and Fig.4.27). Statistical significance ($p \leq 0.05$) between control and treatment was analysed, the result showed a significant increase in number of basophil in fish exposed to neem *A. indica* seed extract for 3 months.

The percentage of eosinophil In control fish, the average percentage of eosinophil at seven time intervals was 0.10 ± 0.04, 0.02 ± 0.02, 0, 0.08 ± 0.04, 0.02 ± 0.02, 0.02 ± 0.02 and 0.02 ± 0.02 % for 1, 2, 3, 4, 5, 6, and 7 months of experimentation, respectively. For treatment, it was 0.08 ± 0.04, 0.08 ± 0.04, 0.04 ± 0.03, 0.06 ± 0.06, 0.02 ± 0.02, 0.02 ± 0.02 and 0.02 ± 0.02 % for 1, 2, 3, 4, 5, 6, and 7 months of experimentation, respectively (Table 4.5 and Fig.4.28). Statistical significance ($p \leq 0.05$) between control and treatment was analysed, the result showed a significant increase in number of eosinophil in fish exposed to neem *A. indica* seed extract for 3 months.

Table 4.5 Differential leukocyte count of Nile tilapia *O. niloticus* exposed to a sublethal concentration (25.07 ppm) of neem *A. indica* seed extract for 7 months. Values are means of percent count from fifty observations ± values indicate the standard errors.

Duration of exposure (month)	Control					Treatment				
	%Lymphocyte	%Monocyte	%Neutrophil	%Basophil	%Eosinophil	%Lymphocyte	%Monocyte	%Neutrophil	%Basophil	%Eosinophil
1 (n=50)	85.10 ± 1.58	11.96 ± 1.37	2.04 ± 0.40	0.58 ± 0.12	0.10 ± 0.04	3.38 ± 0.92*	5.18 ± 0.80*	1.08 ± 0.19	0.38 ± 0.10	0.08 ± 0.04
2 (n=50)	95.68 ± 0.53	3.54 ± 0.50	0.60 ± 0.13	0.16 ± 0.07	0.02 ± 0.02	90.34 ± 1.20*	7.94 ± 1.10*	1.46 ± 0.31	0.18 ± 0.06	0.08 ± 0.04
3 (n=50)	94.74 ± 0.65	4.44 ± 0.54	0.72 ± 0.19	0.12 ± 0.05	0	97.56 ± 1.20*	1.50 ± 0.20*	0.68 ± 0.16	0.22 ± 0.07*	0.04 ± 0.03*
4 (n=50)	95.86 ± 0.54	2.60 ± 0.40	0.56 ± 0.14	0.86 ± 0.20	0.08 ± 0.04	97.04 ± 0.43	1.92 ± 0.30*	0.26 ± 0.08*	0.72 ± 0.17	0.06 ± 0.06
5 (n=50)	96.84 ± 0.48	1.82 ± 0.31	0.30 ± 0.08	1.00 ± 0.25	0.02 ± 0.02	97.00 ± 0.44	2.20 ± 0.40	0.30 ± 0.09	0.48 ± 0.12	0.02 ± 0.02
6 (n=50)	95.28 ± 0.50	3.30 ± 0.42	0.66 ± 0.12	0.74 ± 0.15	0.02 ± 0.02	95.50 ± 0.60	3.22 ± 0.49	0.36 ± 0.12	0.90 ± 0.21	0.02 ± 0.02
7 (n=50)	93.74 ± 1.09	4.74 ± 0.94	0.90 ± 0.20	0.60 ± 0.15	0.02 ± 0.02	93.66 ± 1.05	4.76 ± 0.91	0.46 ± 0.15	0.92 ± 0.16	0.02 ± 0.02

* Significant difference between the means of control and treated fish ($P \leq 0.05$)

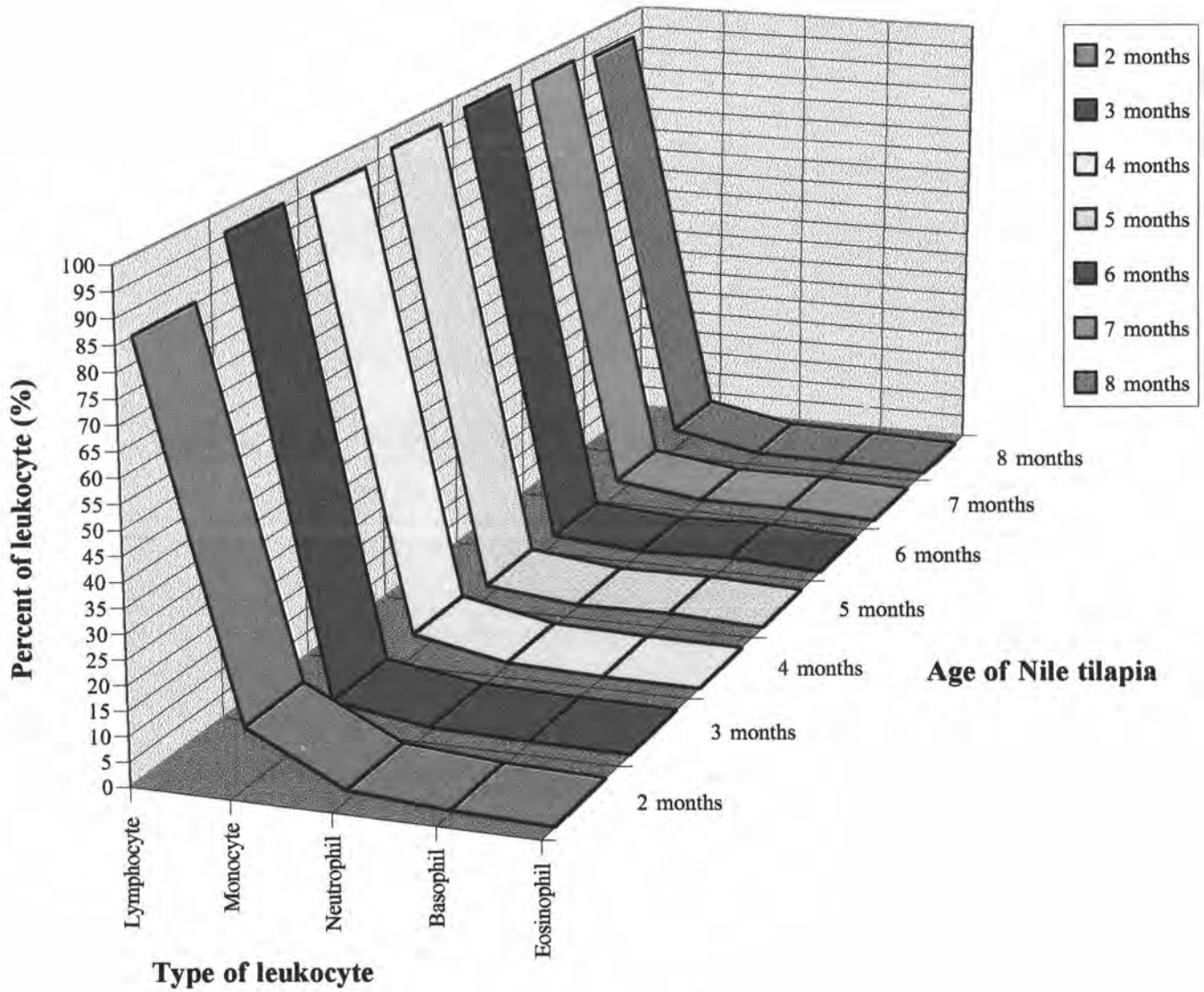

Fig.4.23 Differential leukocyte count of Nile tilapia *O. niloticus* in control group at the age of 2-8 months.

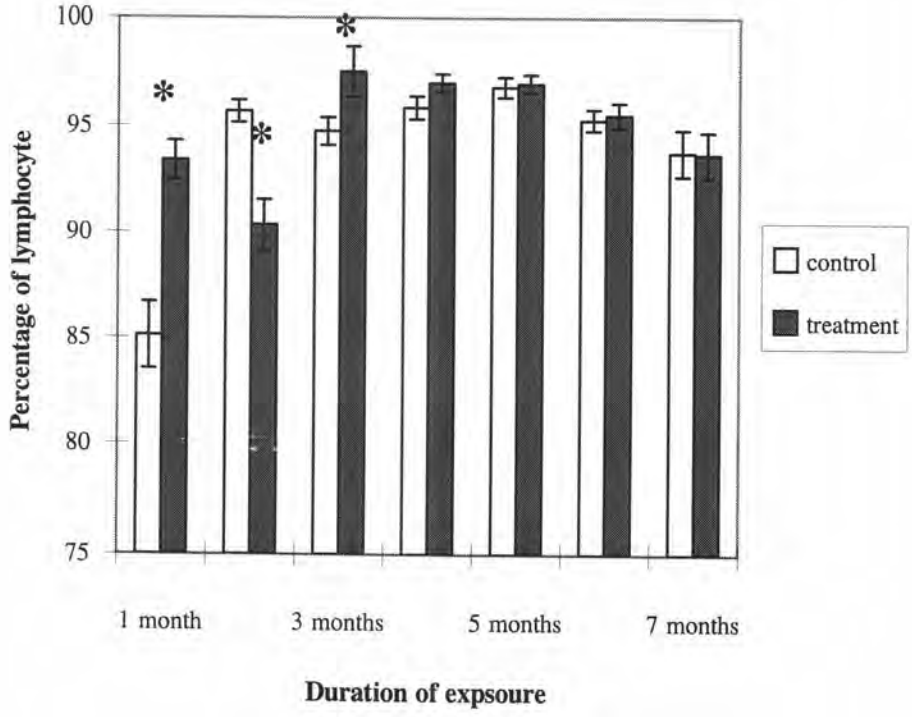

Fig.4.24 Percentage of lymphocyte in control and treatment group at each time interval of exposure, * significantly different at $p \leq 0.05$ (n=50).

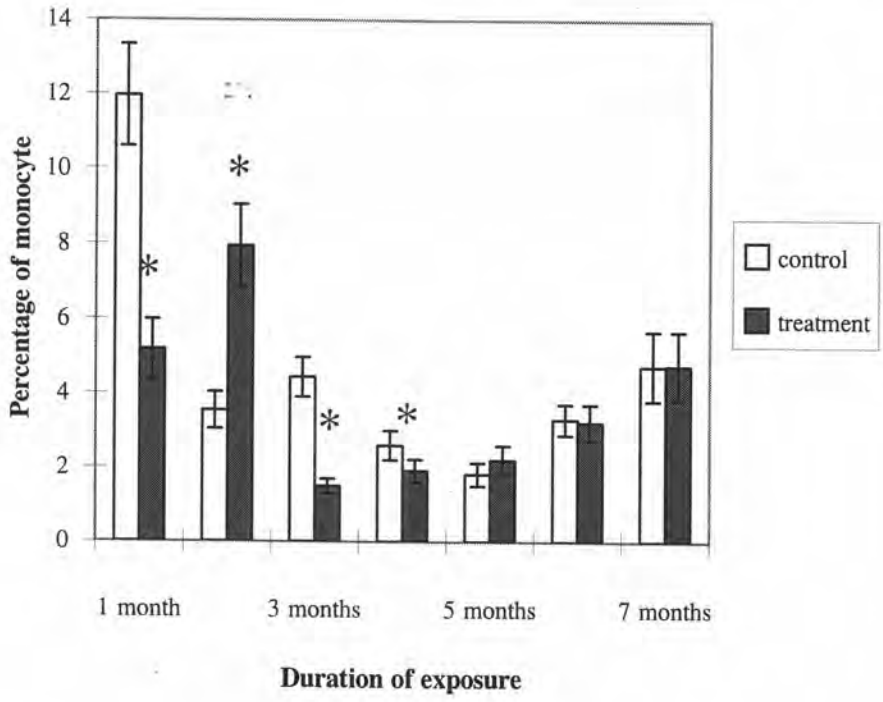

Fig.4.25 Percentage of monocyte in control and treatment group at each time interval of exposure, * significantly different at $p \leq 0.05$ (n=50).

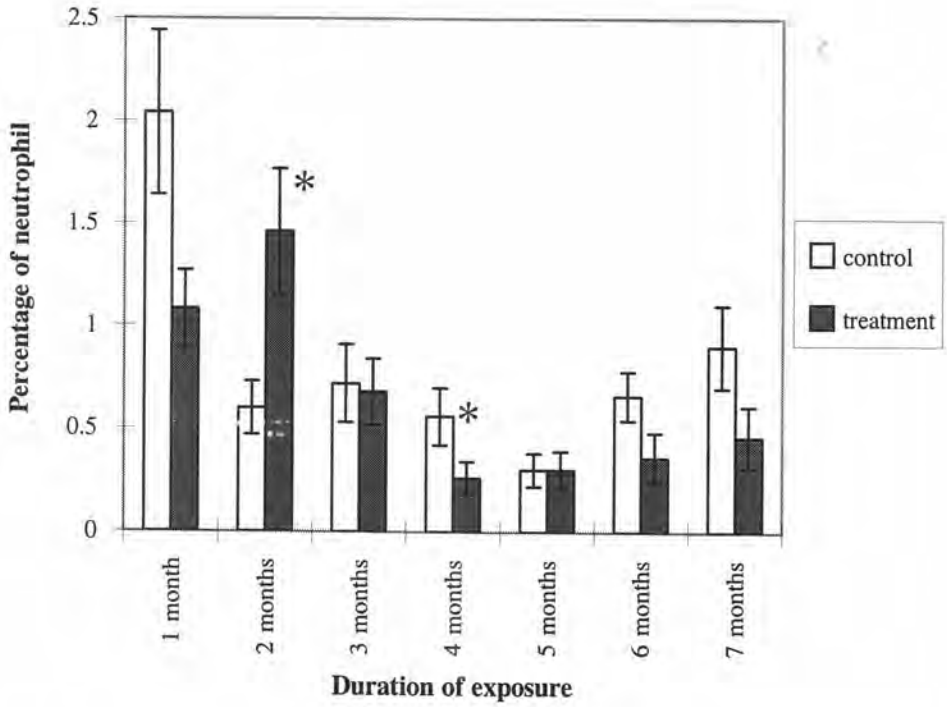

Fig.4.26 Percentage of neutrophil in control and treatment group at each time interval of exposure, * significantly different at $p \leq 0.05$ (n=50).

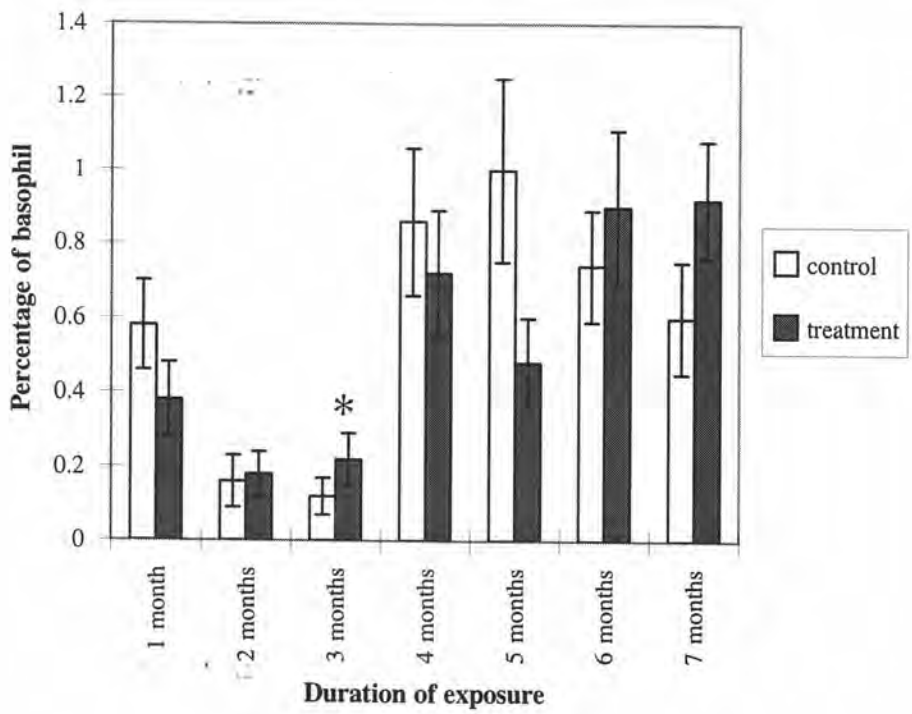

Fig.4.27 Percentage of basophil in control and treatment group at each time interval of exposure, * significantly different at $p \leq 0.05$ (n=50).

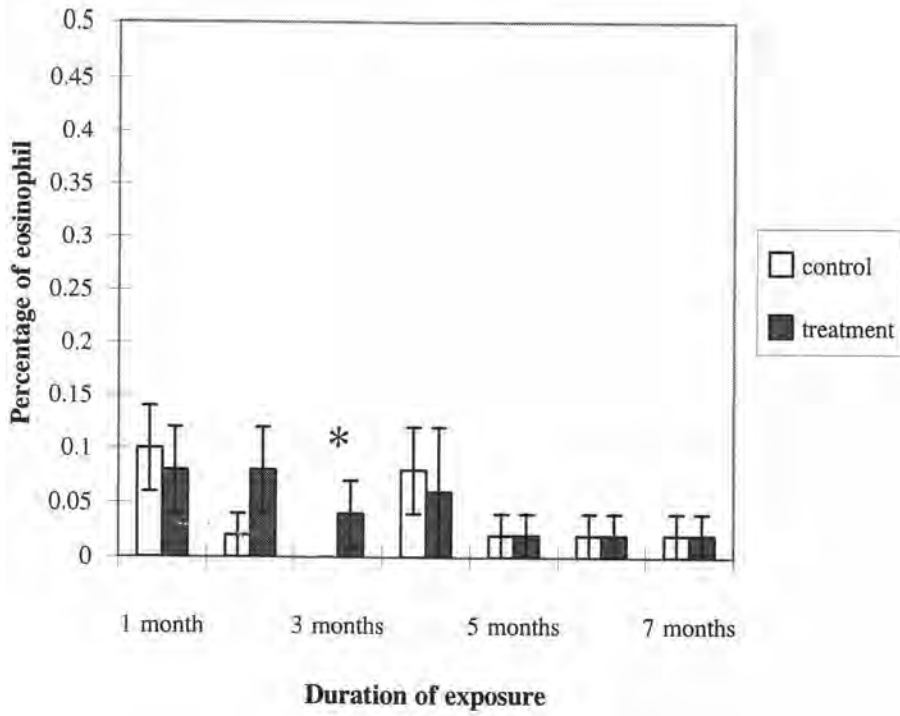

Fig.4.28 Percentage of eosinophil in control and treatment group at each time interval of exposure, * significantly different at $p \leq 0.05$ (n=50).

3. Haematological parameters

The haematological observation presented in Table 4.6 showed the values of total number of leukocytes, red blood cell count (RBC), haematocrit and red cell indice or mean cell volume (MCV) in the experimental groups.

Total leukocyte count In control group, the total number of leukocytes was 22.73 ± 3.57, 72.68 ± 15.63, 155.65 ± 20.99 and 65.95 ± 13.24 $x10^2$ cell /cm^3 for 4, 5, 6 and 7 months of experimentation period, respectively. For treatment, it was 224.45 ± 49.68 , 217.18 ± 55.26, 216.10 ± 30.93 and 299.68 ± 25.92 $x10^2$ cell /cm^3 for 4, 5, 6 and 7 months of experimentation period, respectively (Table 4.6 and Fig.4.29). A significant increase of this value was shown in the fourth, fifth and seventh month of experimentation.

Total red blood cell count In control group, the total number of erythrocytes was 197.80 ± 22.55, 120.10 ± 11.53, 138.05 ± 8.62 and 190.81 ± 17.15 $x10^6$ cell /cm^3 for 4, 5, 6 and 7 months of experimentation period, respectively. For treatment, it was 140.05 ± 20.62, 127.90 ± 14.30, 141.40 ± 10.18 and 147.15 ± 12.83 $x10^6$ cell /cm^3 for 4, 5, 6 and 7 months of experimentation period, respectively (Table 4.6 and Fig.4.30). There was no significant difference in all groups of experimentation.

Haematocrit value In control group, haematocrit value was 30.80 ± 1.17, 30.35 ± 0.73, 29.50 ± 0.83 and 33.25 ± 0.45 % for 4, 5, 6 and 7 months of experimentation period, respectively. For treatment, it was 29.65 ± 1.73, 22.30 ± 1.58, 28.55 ± 1.02 and 29.20 ± 1.02 % for 4, 5, 6 and 7 months of experimentation period, respectively. Significance of differences between control and treatment was showed in the seventh month of experimentation (Table 4.6 and Fig.4.31).

Mean cell volume (MCV) In control group, mean cell volume (MCV) was 1.72 ± 0.17, 2.73 ± 0.26, 2.20 ± 0.12 and 1.88 ± 0.18 μm^3 for 4, 5, 6 and 7 months of experimentation period, respectively. For treatment, it was 2.00 ± 0.19, 1.84 ± 0.14, 1.92 ± 0.08 and 2.15 ± 0.22 μm^3 for 4, 5, 6 and 7 months of experimentation period, respectively (Table 4.6 and Fig.4.32). Significance of the differences between control and treatment was not exhibited in all groups of experimentation.

4. Biochemical parameters

Blood glucose level Measurement of blood glucose was done in whole blood from caudal peduncle of fish exposed to a sublethal concentration of neem *A. indica* seed extract for 2, 3, 4, 5, 6 and 7 months by One Touch Basic Blood Glucose Meter (a Johnson & Johnson company). In control, the average value of blood glucose was 44.18 ± 2.29, 53.84 ± 1.65, 58.02 ± 2.94, 80.28 ± 4.84, 74.86 ± 3.94 and 43.44 ± 1.38 mg/ml, respectively. For treatment, it was 30.86 ± 1.13, 35.22 ± 1.55, 81.90 ± 3.62, 77.10 ± 4.06, 66.08 ± 3.85 and 76.44 ± 3.70 mg/l, respectively (Table 4.7 and Fig.4.33). Significance of the differences between control and treatment was showed in Table 4.7.

Table 4. 6 Haematological parameters of Nile tilapia *O. niloticus* exposed to 25.07 ppm neem *A. indica* seed extract from the 4th- 7th month. Values are means ± values indicate the standard errors.

Duration of experiment	Parameters			
	WBCs ($x10^2/cm^3$)	**RBCs ($x10^6/cm^3$)**	**Haematocrit (%)**	**MCV (μm^3)**
4 months (control)	22.73 ± 3.57 (10)	197.80 ± 22.55 (10)	30.80 ± 1.17 (10)	1.72 ± 0.17 (10)
5 months (control)	72.68 ± 15.63 (10)	120.10 ± 11.53 (10)	30.35 ± 0.73 (10)	2.73 ± 0.26 (10)
6 months (control)	155.65 ± 20.99 (10)	138.05 ± 8.62 (10)	29.50 ± 0.83 (10)	2.20 ± 0.12 (10)
7 months (control)	65.95 ± 13.24 (10)	190.81 ± 17.15 (10)	33.25 ± 0.45 (10)	1.88 ± 0.18 (10)
4 months (treatment)	224.45 ± 49.68* (10)	140.05 ± 20.62 (10)	29.65 ± 1.73 (10)	2.00 ± 0.19^{ab} (8)
5 months (treatment)	217.18 ± 55.26* (10)	27.90 ± 14.30 (10)	22.30 ± 1.58 (10)	1.84 ± 0.14^{ab} (10)
6 months (treatment)	216.10 ± 30.93 (10)	141.40 ± 10.18 (10)	28.55 ± 1.02 (10)	1.92 ± 0.08^{a} (9)
7 months (treatment)	299.68 ± 25.92* (10)	147.15 ± 12.83 (10)	29.20 ± 1.02* (10)	2.15 ± 0.22^{b} (10)

* Significant difference between the means of control and treated fish, letters a, b mean significant difference within treated group ($P \leq 0.05$). Number of observations is shown in parentheses.

Table 4.7 Blood glucose levels in Nile tilapia *O. niloticus* exposed to neem *A. indica* seed extract at a sublethal concentration (25.07 ppm) from the 2nd- 7th month.

Duration of exposure	Blood glucose level (mg/ml)	
	Control (mean ± SE)	**Treatment** (mean ± SE)
2 months	44.18 ± 2.29	30.86 ± 1.13*[a]
3 months	53.84 ± 1.65	35.22 ± 1.55[b]
4 months	58.02 ± 2.94	81.90 ± 3.62[c]
5 months	80.28 ± 4.84	77.10 ± 4.06*[c]
6 months	74.86 ± 3.98	66.08 ± 3.85[c]
7 months	43.44 ± 1.38	76.44 ± 3.70*[c]

* Significant difference between the means of control and treated fish, different letter (a, b and c) means significant difference within treated group ($P \leq 0.05$), (n= 50)

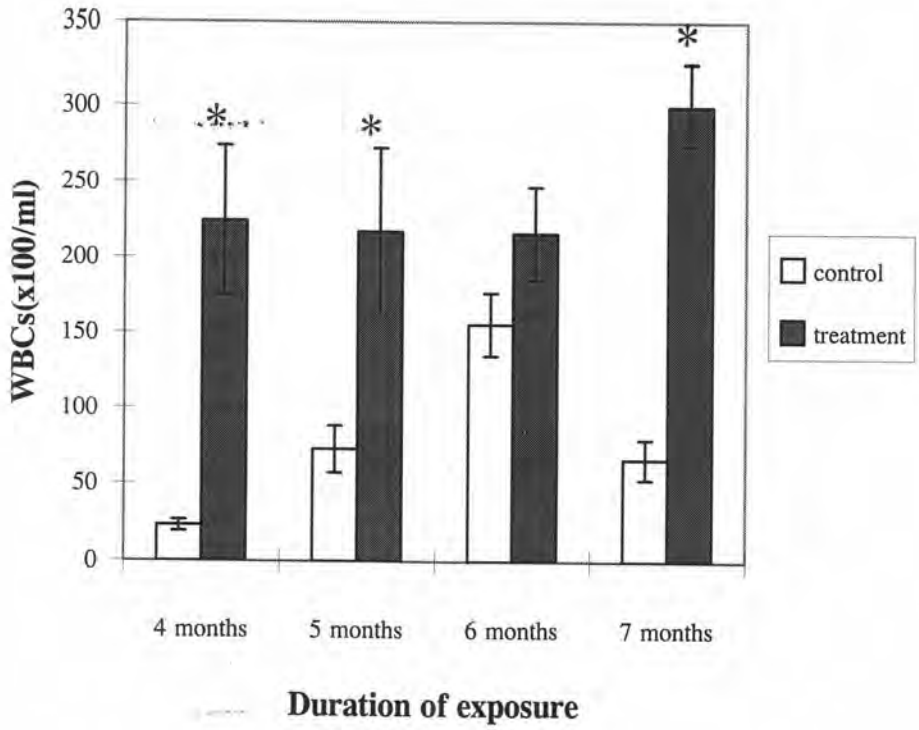

Fig.4.29 Total leukocyte count in control and treatment group at each time interval of exposure to a sublethal concentration of neem *A. indica* seed extract, * significantly different at $p \leq 0.05$ (n=50).

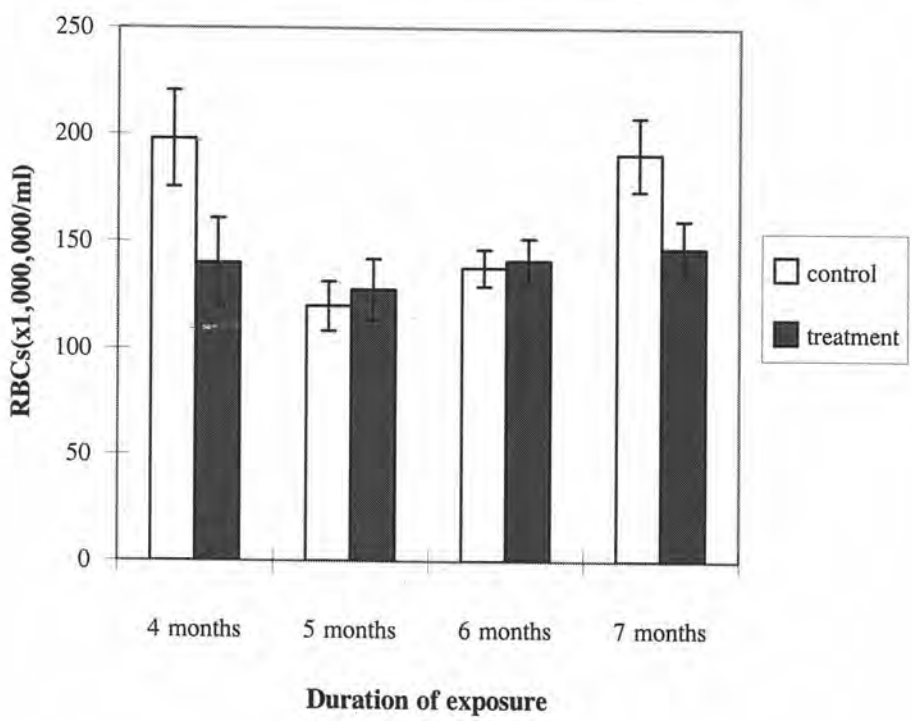

Fig.4.30 Total red blood cell count in control and treatment group at each time interval of exposure to a sublethal concentration of neem *A.indica* seed extract (n=50).

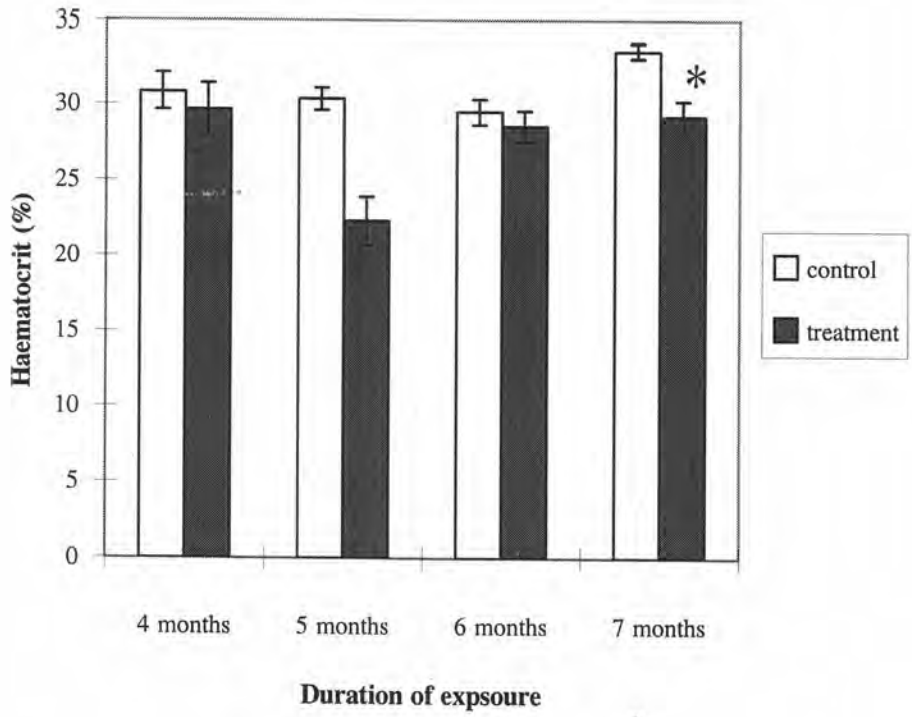

Fig.4.31 Haematocrit in control and treatment group at each time interval of exposure to a sublethal concentration of neem *A. indica* seed extract, * significantly different at $p \leq 0.05$ (n=50).

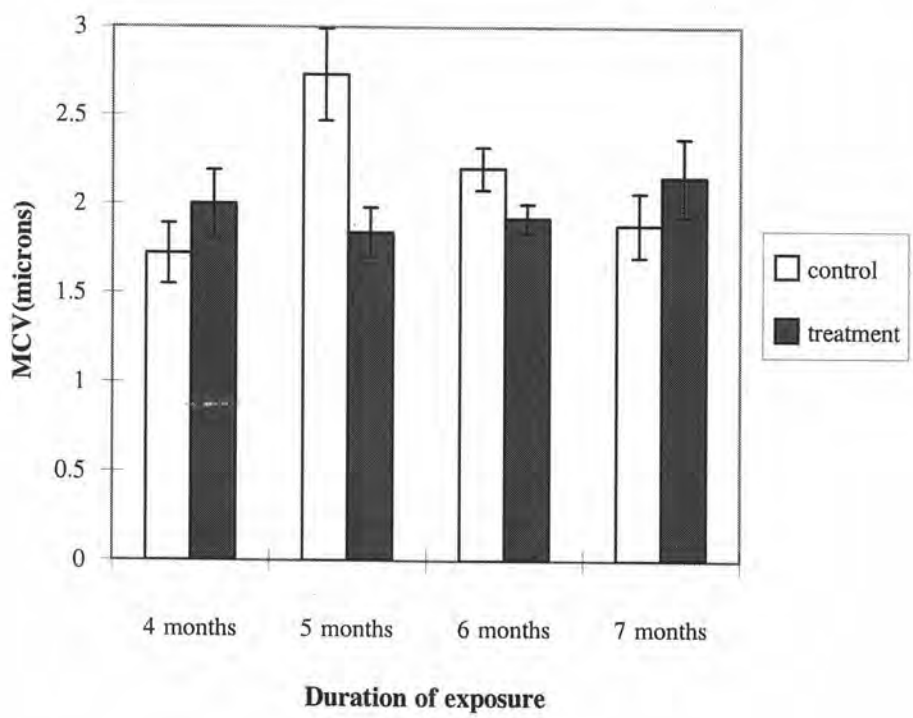

Fig.4.32 Mean cell volume (MCV) in control and treatment group at each time interval of exposure to a sublethal concentration of neem *A.indica* seed extract (n=50).

Alkaline phosphatase (ALP) level ALP quantity was determined from each blood sample pooled from five specimens at the fourth, fifth, sixth and seventh month of experimentation by the colorimetric method using commercial kits (Sigma Chemical Company). The results of ALP measurement in control group were 1.01 ± 0.48, 1.39 ± 0.77, 0.79 ± 0.45 and 0.36 ± 0.11, IU/L respectively. In treated fish, they were 0.93 ± 0.36, 1.20 ± 0.55, 1.20 ± 0.50 and 1.13 ± 0.62 IU/L, respectively (Table 4.8 and Fig.4.34). Significance of the differences between control and treatment was no exhibited.

Glutamic oxaloacetic transaminase (GOT) level Also, this enzyme was determined from each blood sample pooled from five specimens at the fourth, fifth, sixth and seventh month of experimentation by the colorimetric method using commercial kits (Sigma Chemical Company). The average quantity of GOT in control was 3240.57 ± 201.84, 3146.76 ± 163.81, 3722.96 ± 173.59 and 2147.76 ± 169.72 IU/L, respectively. In treated fish, they were 3154.70 ± 272.76, 3604.03 ± 187.98, 3360.66 ± 192.94 and 2852.51 ± 114.45 IU/L, respectively (Table 4.8 and Fig.4.35). Significance of the differences between control and treatment was no exhibited.

Glutamic pyruvic transaminase (GPT) level GPT level was determined from each blood sample pooled from five specimens at the fourth, fifth, sixth and seventh month of experimentation by the colorimetric method using commercial kits (Sigma Chemical Company). The average GPT level in control group was 648.34 ± 116.25, 521.89 ± 94.79, 385.27 ± 72.89 and 1235.78 ± 167.03 IU/L, respectively. In treated fish, it was 623.46 ± 51.10, 1275.50 ± 252.18, 806.09 ± 142.81 and 1612.89 ± 299.73 IU/L, respectively (Table 4.8 and Fig.4.36). There was a significant increase in GPT levels in fish exposed to a sublethal concentration of neem *A. indica* seed extract for 5, 6 and 7 months.

Table 4. 8 Blood biochemistry of Nile tilapia *O. niloticus* exposed to a sublethal concentration of neem *A. indica* seed extract from the 4^{th}-7^{th} month. Values are means ± values indicate the standard errors.

Duration of experiment	Parameters		
	ALP (IU/L)	GOT (IU/L)	GPT (IU/L)
4 months (control)	1.01 ± 0.48 (10)	3240.57 ± 201.84 (10)	648.34 ± 116.25 (8)
5 months (control)	1.39 ± 0.77 (10)	3146.76 ± 163.81 (10)	521.89 ± 94.79 (10)
6 months (control)	0.79 ± 0.45 (10)	3722.96 ± 173.59 (10)	385.27 ± 72.89 (8)
7 months (control)	0.36 ± 0.11 (10)	2174.76 ± 169.72 (10)	1235.78 ± 167.03 (10)
4 months (treatment)	0.93 ± 0.36 (10)	3154.70 ± 272.76^{a} (10)	623.46 ± 51.10^{a} (10)
5 months (treatment)	1.20 ± 0.55 (10)	3604.03 ± 187.98^{ab} (10)	1275.50±252.18*^{bc} (10)
6 months (treatment)	1.20 ± 0.50 (10)	3360.66 ± 192.94^{ab} (10)	806.09 ± 142.81*^{b} (10)
7 months (treatment)	1.13 ± 0.62 (10)	2852.51 ± 114.45^{b} (10)	1612.89±299.73*^{c} (10)

* Significant difference between the means of control and treated fish, different letter (a,b) means significant difference with in treated group ($P \leq 0.05$). Number of observations is shown in parentheses.

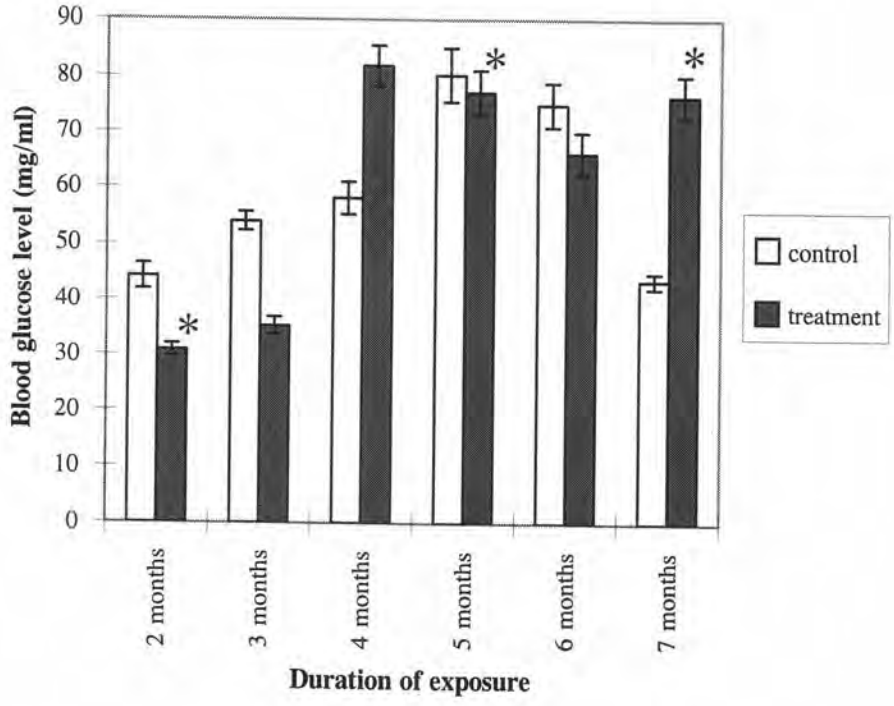

Fig.4.33 Blood glucose level in control and treatment group at each time interval of exposure to a sublethal concentration of neem *A. indica* seed extract, * significantly different at $p \leq 0.05$ (n=50).

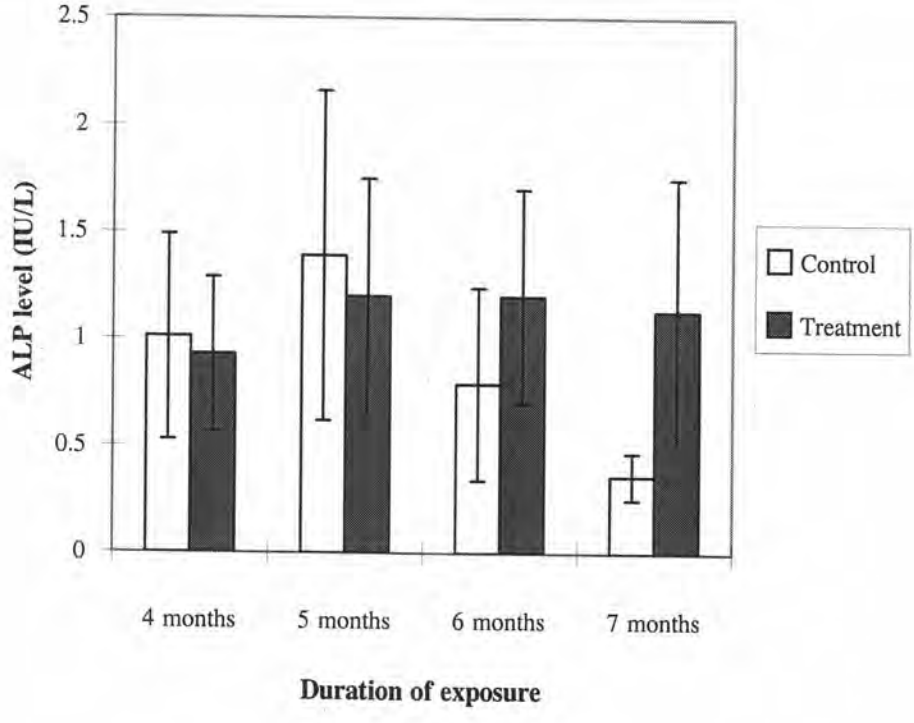

Fig.4.34 ALP level in control and treatment group at each time interval of exposure to a sublethal concentration of neem *A. indica* seed extract (n=50).

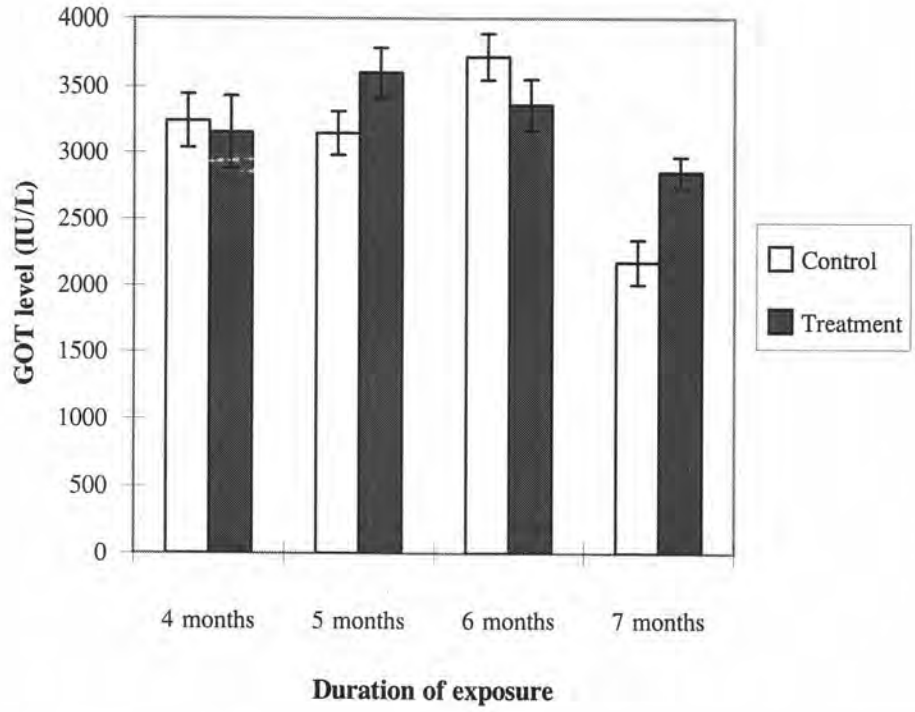

Fig.4.35 GOT level in control and treatment group at each time interval of exposure to a sublethal concentration of neem *A. indica* seed extract (n=50).

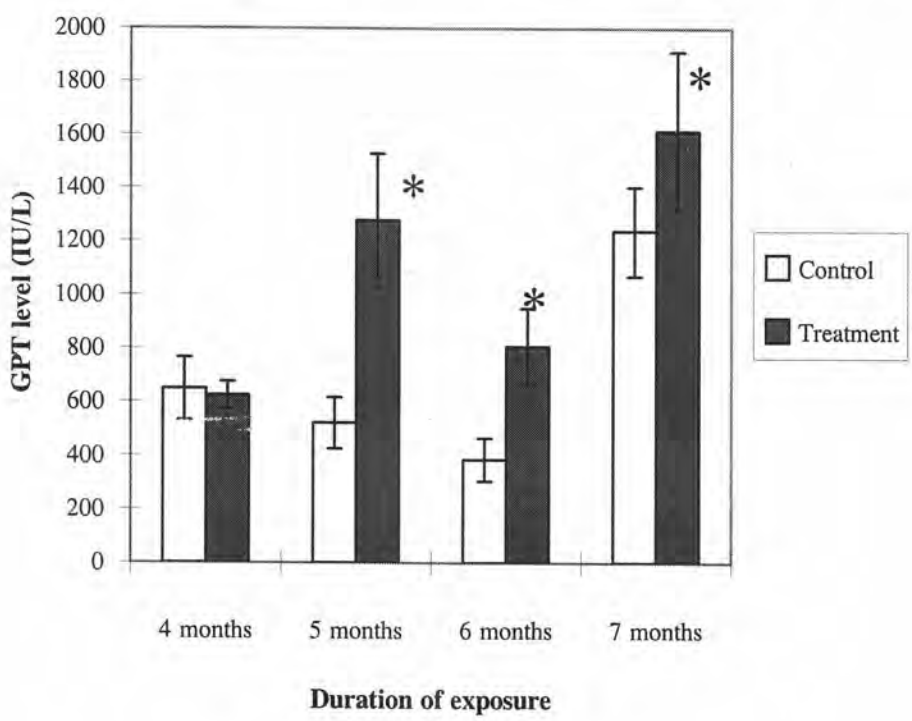

Fig.4.36 GPT level in control and treatment group at each time interval of exposure to a sublethal concentration of neem *A. indica* seed extract, * significantly different at $p \leq 0.05$ (n=50).

5. Water quality

The water quality data that including temperature, dissolved oxygen (DO), pH and hardness was reported throughout the experiment.

Water temperature was in the normal range of 24-27 C° and 24-27 C° for control and treatment. Also, dissolved oxygen was measured about 7.0-7.38 mg/l and 7.0-7.4 mg/l in control and treatment, respectively. In case of the result of pH determination was in the range of 5.4-5.9 and 5.4-5.8 for control and treatment, respectively. Furthermore, hadrness value was in the range of 66-112 mg/l in both control and treatment (Table 4.8). All data demonstrated that water quality ought not be concerned in any changes in haematology and biochemistry of Nile tilapia *O. niloticus* during exposure to neem *A. indica* seed extract at sublethal concentration from 1-7 months.

Table 4.9 Water quality during a sublethal toxicity test of neem *A. indica* seed extract on Nile tilapia *O. niloticus*.

Parameters	Range	
	Control	Treatment
Water temperature (C°)	24.0-27.0	24.0-27.0
Dissoved oxygen (mg/l)	7.0-7.38	7.0-7.4
pH	5.4-5.9	5.4-5.8
Hardness (mg/l)	66.0-112.0	66.0-112.0