Synergistic Effect of Cow Urine And Ocimum Sanctum Leaves on The Haematology of Oreochromis Mossambicus (Peters)

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Abstract-*The herbal cow urine decoction obtained by distillating Occimum sanctum in cow urine was investigated for its effect on haematologial responses (total erythrocyte count, total leukocyte count, haemoglobin concentration, MCV, MCH, MCHC) and survival rate in Oreochromis mossambicus against immunization with heat killed Aeromoans hydrophila. The fishes were exposed to different concentration of O. sanctum cow urine decoction (OCUD) (Control-with distilled water; T1-0.01%; T2-0.1%; T3-0.25%; T4-0.5% and T5-1% (v/v)) for a week. All experimental groups were immunized with heat killed A. hydrophila through intraperitoneal administration of the pathogen (0.2 ml of 1x 107 cells/fish-1) on day 8 post immunostimulation with cow urine O.sanctum treatment. The haematological parameters and survival rate were assessed on 7, 14, 21 and 28 days. Administration of 0.01% (v/v) of the O. sanctum cow urine decoction enhanced maximum survival as well as increased leukocytes, erythrocyte and haemoglobin concentration level. However, at 0.5 and 1% (v/v), concentrations of herbal cow urine decoction recorded 100% mortality. This study reports that administration of 0.01% and 0.1% (v/v) cow urine decoction could positively influence the haematological response to A. hydrophila and enhance the health status of tilapia with respect to this microbe.*

Keywords-Gir, herbal Cow urine decoction, Ocimum sanctum, RBC, WBC, Oreochromis mossambicus, Organic Aquaculture

I. INTRODUCTION

Aquaculture consists of a broad spectrum of systems, from small ponds to large-scale, highly intensified commercial systems. Fish has always been an important source of protein in the human diet and on a global scale, fish and fish products are the most important source of protein and it is estimated that more than 30% of fish for human consumption comes from aquaculture (Hastein *et al* 2005). India is one of the leading fish producing nations in the world with an average annual production of 6.1 million tonnes of fish and shell fish from capture and culture fisheries in 2001 (Ayyappan and Biradar 2004). Fisheries play a very significant role in the Indian economy by providing employment to nearly 7 million people directly or indirectly, supplying rich protein food and earning valued foreign exchange. The inland fish production has registered steady increase during the past two decades and the share of inland fisheries sector in the total fish production of the country is about 50% in 1999 -2000 (Keremane and Naik, 2001).

Blood is the most important fluid in the body and its composition often reflects the total physiological condition of an organism. Haematological parameters are nowadays not only used for clinical diagnosis of physiology but also help in addressing the effects of toxic substances on the fish (Wendlaar, 1997). Studies have shown that when the water quality is affected by toxicants, any physiological changes will be reflected in the values of one or more of the haematological parameters (Van Vuren, 1986). In fish, exposure to chemical pollutants can induce either an increase or decrease in haematological levels depending on fish species, age and cycle of sexual maturity (Luskova, 1997).

The use of haematological parameters in assessment of fish physiology was proposed by Hesser (1960). Since then haematology has been used as an index of fish health status in a number of fish species to detect physiological changes, as a result of exposure to different stressful conditions such as handling, pollutants, pesticides, metals, hypoxia, anaestehtics and acclimation. Blood cell responses are important indicators of changes in the internal and/or external environment of animals. Bouck and Ball (1966) stated that hematology may be an useful tool in monitoring stress levels of aquatic pollution on fish. In this investigation this tool is used to study the wellness of the fish after administration of herbal extract.

Ocimum tenuiflorum, previously known as *Ocimum sanctum* (*Tulsi*, vernacular) is one of the indigenous plant used extensively in India to treat various ailments. Pharmacological studies conducted on the plant have shown that its leaves and seeds possess many medicinal properties like hypoglycemic, adaptogenic, antifertilty, antimicrobial and hepatoprotection (Gupta *et al.*, 2012). It has been implicated that different mechanisms like free radical scavenging metal chelation, GSH stimulation as well as immunomodulation might act at

different levels individually or in combination for adaptogenic, radioprotective and cancer preventive action (Uma Devi, 2000). *O. tenuiflorum* leaf and seed extracts were found to exhibit antiviral activity against bovine herpes virus-1 *in vitro* (Shynu *et al*., 2006). Leaf powder was found to have enhancing effect on humoral and cell mediated immune responses in immuno-suppressed poultry (Sadekar *et al.*, 1998a, 1998b). Butanolic extract of leaves had an immuno-restorative effect in immuno-suppressed mice (Khajuria *et al.*, 1998). *O. sanctum* water extract was shown to enhance the differential leukocyte counts (Venkatlakshmi and Michael, 2000).

Cows were regarded as wealth and were the backbone of the economy of ancient Indians. Cattle were one of the most frequently used animals described in Vedas (Chauhan., 2002). The ayurvedic medicines of animal origin are mainly prepared from *Panchgavya* (five things from Indian cow *viz.*, urine, dung, milk, butter oil and curd), which boosts up the body's immune system and makes body refractory to various diseases (Chauhan *et al*., 2001). The specificity of immune system depends upon the number and activity of lymphocytes. Chauhan *et al.* (2001) studied the immunomodulatory effect of cow urine in mice and found that cow urine enhances both T- and B-cell blastogenesis and also increases the level of IgG. Kumar (2004) and Chauhan *et al.* (2003) reported enhanced cellular and humoral immune responses in cow urine treated animals. Hence the synergistic effect of cow urine and *O. sanctum* could be explored for getting maximum benefits out of them. In the present study the effect of exposure of herbal cow urine decoction on the blood parameters of *O. mossambicus* against *A. hydrophila*, was monitored to the fix the optimum dose of herbal cow urine decoction.

II. MATERIALS AND METHODS

Fish and their maintenance

Fingerlings of *Oreochromis mossambicus* weighing (8.0 ±0.2 g) and length (12.01±0.34 cm) of both sexes were procured from S.M fisheries, Swamimalai, Kumbakonam. Fishes were brought to the wet laboratory and acclimatized for one week prior to experimentation. Chlorine free water was used throughout the course of the experiment. Plastic tubs were washed and then sundried to avoid fungal contamination. Healthy fishes were then transferred to plastic tubs (Vol 70 L). They were regularly fed with formulated feed and the medium was changed frequently to avoid stress due to ammonia accumulation. Faeces and food remnants, were removed by siphoning daily.

Mode of feeding

Each of the treatment was fed with prepared laboratory feed *ad libitum* total body weight (Venktalakshmi and Ebanaser 2012). The fish were fed twice a day for an hour between 9.00 am to 10.00 am and 4.00 pm to 5.00 pm.

Collection of Cow urine

Cow urine was collected from six disease free tagged Gir cows throughout the study (Sattanathan and Venkatalakshmi, 2015). The early morning (4.00-5.00am) first urine of Gir, was collected from Goshala, Sri Vittal Rukmini Samsthan, Govindhapuram, near Kumbakonam. The urine was pooled, separately and transported to laboratory in sterile airtight containers.

Collection of Medicinal plant

Mature leaves of *O. sanctum*leaves were collected from local market, Kumbakonam, Thanjavur. These were authenticated by Department of Botany, of the college and the specimen samples were deposited in the laboratory herbarium. Fresh leaves were used for the study (Logambal et al, 2000).

Preparation of Distillate Cow Urine Decoction

100 gram of *O. sanctum*fresh leaves were surface sterilized with sterile distilled water thrice. These leaves were cut into small pieces and add to 200 ml of fresh Gir cow urine and was distilled at 50-60° C using by glass multiple distillation apparatus (Edwin Jarald *et al* 2008).

Experimental setup

After two weeks of acclimatization five groups of fish were treated with different concentration of herbal cow urine decoction (T_1 –0.01%; T2-0.1%; T3-0.25%; T4-0.5% and T5-1% (v/v) for seven days. A control group was maintained separately without cow urine decoction treatment. All fish groups were immunized with i.p injection of 0.2 ml of $1x10^7$ cells/fish heat killed *A. hydrophila* on the 8^{th} day post treatment.

Preparation of Heat killed *A. hdyrophila* vaccine

A virulent strain of *A. hydrophila* (MTCC-1769) was obtained from the IMTECH, Chandigarh, India, was cultured on Tryptic Soy Broth (Himedia, India) for 24 h at 37° C. Overnight cultured cells were subjected to 60°C for one hour in a water bath (Catherine *et al.*, 2010). The sterility was checked by inoculating a sample on nutrient agar plates. The

heat killed bacterial culture was centrifuged at 3000 rpm for 15 minutes Then the packed cells were collected and required dose (10^7 cells/fish) was prepared by adjusting the optical density of bacterial suspension to 0.5 at 456 nm which corresponds to $1x10^7$ cells/0.2 ml.

Serial bleeding

The fish were bled serially using one ml tuberculin syringe (Glass van) with 26-gauge needle from the common cardinal vein situated just below the gills, at regular intervals of seven days after immunization (Michael et al 1998). The blood drawn was collected in small micro centrifuge tubes (Torson). The herbal cow urine decoction treatment and serial bleeding were done between 2 to 4 pm throughout the investigation to avoid the possible influence of circadian rhythmic variation on the immune response (Hurshesky, 1984; Michael and Priscilla, 1994).

III. HEMATOLOGICAL PARAMETERS

Hemoglobin level

Total haemoglobin estimation in blood was done by using Sahli Haemometer (Marienfeld, Germany). In this method, the measuring tube was first filled with 0.1N hydrochloric acid up to the mark 2, and then 20 µl of blood was added (Schaperclaus, 1991). After proper mixing, the colour of the sample was compared with the standard coloured rod (Marienfeld, Germany) following dilution of the sample by adding distilled water drop wise. Reading of the haemoglobin content was determined from the scale given in the measuring tube.

Total Erythrocyte count and leukocyte count

The total red blood cell counts were determined in a 1:200 dilution of the blood sample in Hayem's solution and total white blood cell counts in a 1:20 dilution of the blood sample in Turck's fluid with a Neubauer hemocytometer (Blaxhall and Daisley, 1973).

Determination of mean corpuscular haemoglobin (MCH)

To calculate MCH, expressed in picograms (pg), the following formula was used (Dacie and Lewis, 1991)

$$\text{MCH (pg)} = \frac{\text{Hemoglobin(g / dL)} \times 10}{\text{RBC Count}}$$

Survival rate

Survival rate was calculated by following formula:

$$\text{Survival rate} = \frac{\text{Initial number of fish – mortality}}{\text{Initial number of fish}} \times 100$$

IV. RESULTS

Haemoglobin content

The haemoglobin content of T1 group was higher (9.1 g/dL) than the control, T2 and T3 group on 14th day post immunization (Fig 1). The effect was significantly ($p<0.05$) different among the treatment groups (Table 1). The minimum haemoglobin content was found to be in control (6.1 g/dL).

Total erythrocyte count

The highest total erythrocyte count was recorded in T1 group ($1.4x10^6/mm^3$) and least value was recorded in T3 groups ($1.1x10^6/mm^3$; Fig 2) on 14th day post immunization. However, the differences between treatments were statistically not significant ($p>0.05$; Table 2).

Total leukocyte count

The total leukocyte count of T1 group was higher ($2.46x10^3/mm^3$) than the T2 and T3 group. The result was statistically significant among the groups (Fig 3) (Table 3).

Mean corpuscular haemoglobin

The maximum value of MCH was recorded in T1 group (82.54 pg) and least was recorded in control (61.37 pg) on 21st day post immunization (Fig 4). However, there was no significant difference ($p>0.05$) between control, T1, T2 and T3 groups.

Survival rate

The highest survival rate was recorded in T1 treated group (83.3%) and least survival rate was recorded in T3 and control (Fig 5). T4 and T5 groups showed 100% mortality in the beginning of the experiment itself.

Discussion

The immunomodulatory, anti-inflamatory, anti-analgesic, anti-tumor, anti-pyretic, anti-biotic properties of holy basil was proved by various researchers (Singh et al., 1995). The Indian system of Ayurveda is the result of codified system of medicine developed through ages documenting over 25,000 herbs.

Commercial immunostimulants are no longer attractive because of resistant development, less economical and environmental hazardous. Herbs on the other hand have many therapeutic effects with little or no side effects. India is the rich source of medicinal plants that all indigenous or cultivated. Harikirishnan et al., (2011) the Inidan system of Ayurveda is the result of codified system of medicine development thorugh ages documenting over 25,0000 herbs.

Occiumum sanctum the queen of herbs have been subjected to various researches especially by the Indian researchers and scientists in the last two decades to prove its versatile beneficial nature (Pandey Govind, 2011). Different preparations like dried leaf powder methanolic, acetonic and petroleum ether extracts were studied in Gas chromatography and mass spectrophotometry (GC-MS). The dried leaf powder was shown to have 49 compounds (Aswar and Joshi, 2010).

Cow urine has natural disinfectant and anti-septic including many therapeutic properties. It contains 24 types of salts and minerals it has been found to stimulate immune mechanism (Padmapriya and Venkatalakshmi, 2014; Priya and Venkatalakshmi, 2016). Hence, it was attempted to check whether new combination of plant derived phytochemicals with mineral derived cow urine distillate have any improved effect on the hematology and survival of *Oreochromis mossambicus*.

The results obtained are promising that *O. sanctum* leaves distillated with cow urine is effective in increasing the blood health in terms of increased RBC, WBC, and hemoglobin levels. The increase in health status reflects in the increase in survival of the fish during the experimental period. Sattanathan and Venkatalakshmi, 2015, showed that cow urine distillate is effective at 0.1% concentration. Padmapriya and Venkatalakshmi (2014), Priya and Venkatalakshmi, (2016) showed 0.1% as the optimum concentration. However, in the present study 0.01% is found to be effective. Nahak and Sahu (2014) showed that the crude extract of *O. sanctum* is effective in enhancing RBC, WBC, and Hb at 5% concentration. Logambal et al. (2000) showed that *O. sanctum* water extract is effective at 8 µg concentration through intra peritoneal route and at 0.01 % through oral route. Venkatalakshmi and Michael (2001) showed similar results in *O. mossambicus* through intraperitoneal route at 0.01% concentration. The present study proves the synergistic effect of CUD and *O. sanctum* and the maximum effect is obtained at a lower dose of 0.01% concentration itself through medium route. The primary function of the red blood cells is to carry oxygen from lungs to body tissues. This functions is accomplished via haemoglobin. The blood supplies nutrients to all parts of the body. Mean corpuscular hemoglobin measures the amount of hemoglobin present in one RBC. Hence, the normal values of RBC, Hb, MCH is an index for general health. The present study records significant influence of herbal CUD on these parameters ($p<0.05$). Similarly the WBC count is a preliminary index for immune status. The herbal CUD at very less concentration of 0.01% proves it as a potential candidate for immunostimulant. Based on this further studies are designed to explore the effect of this herbal CUD on the non-specific and adaptive immunity of tilapia.

The results obtained in the present study give confident foundation for organic aquaculture initiatives with less cost, eco-safe and eco-friendly ways to improve profit through increased disease resistance and survival.

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Table 1: One-Way Analysis of Variance showing the effect of cow urine decoction on haemoglobin level in *Oreochromis mossambicus*

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Analysis of Variance for Haemoglobin
Source     DF        SS        MS        F        P
factor      3   13.4692    4.4897   199.54    0.000
Error       8    0.1800    0.0225
Total      11   13.6492
                                 Individual 95% CIs For Mean
                                 Based on Pooled StDev
Level       N      Mean     StDev  --------+---------+---------+--------
C           3    6.4667    0.0577   (-*-)
T1          3    9.1333    0.1155                            (-*-)
T2          3    8.4667    0.2517                      (-*-)
T3          3    7.1000    0.1000         (-*-)
                                 --------+---------+---------+--------
Pooled StDev =   0.1500               7.0       8.0       9.0
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Table 2: One-Way Analysis of Variance showing the effect of cow urine decoction on total erythrocyte count in Oreochromis mossambicus

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Analysis of Variance for total erythrocyte count
Source     DF        SS        MS        F        P
factor      3    0.1420    0.0473     3.65    0.063
Error       8    0.1037    0.0130
Total      11    0.2458
                                 Individual 95% CIs For Mean
                                 Based on Pooled StDev
Level       N      Mean     StDev  ---+---------+---------+---------+---
C           3    1.2333    0.1528         (-------*------)
T1          3    1.4000    0.1000                 (-------*-------)
T2          3    1.3100    0.0854             (------*-------)
T3          3    1.1033    0.1060   (------*-------)
                                 ---+---------+---------+---------+---
Pooled StDev =   0.1139             1.00      1.20      1.40      1.60
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Table 3: Effect of one way Analysis of Variance showing the effect of cow urine decoction on total leukocyte count in Oreochromis mossambicus

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Analysis of Variance for WBC
Source     DF        SS        MS        F        P
factor      3    0.2939    0.0980     8.46    0.007
Error       8    0.0926    0.0116
Total      11    0.3865
                                 Individual 95% CIs For Mean
                                 Based on Pooled StDev
Level       N      Mean     StDev  -----+---------+---------+---------+-
C           3    2.3000    0.1000              (------*------)
T1          3    2.4667    0.0577                     (------*------)
T2          3    2.3667    0.1528                 (------*------)
T3          3    2.0433    0.0981  (------*------)
                                 -----+---------+---------+---------+-
Pooled StDev =   0.1076             2.00      2.20      2.40      2.60
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Table 4: One-Way Analysis of Variance showing the effect of cow urine decoction on mean corpuscular haemoglobin level in Oreochromis mossambicus

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Analysis of Variance for MCH
Source     DF        SS        MS        F        P
factor      3     329.6     109.9     3.14    0.087
Error       8     279.6      35.0
Total      11     609.3

                                 Individual 95% CIs For Mean
                                 Based on Pooled StDev
Level       N      Mean     StDev  ----+---------+---------+---------+--
C           3    52.951     6.340  (---------*---------)
T1          3    65.495     5.388                (---------*---------)
T2          3    64.896     6.095               (---------*---------)
T3          3    64.720     5.782               (---------*---------)
                                 ----+---------+---------+---------+--
Pooled StDev =    5.912            48.0      56.0      64.0      72.0
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Fig 1: Effect of cow urine decoction on heamoglobin content in Oreochromis mossambicus post immunization.

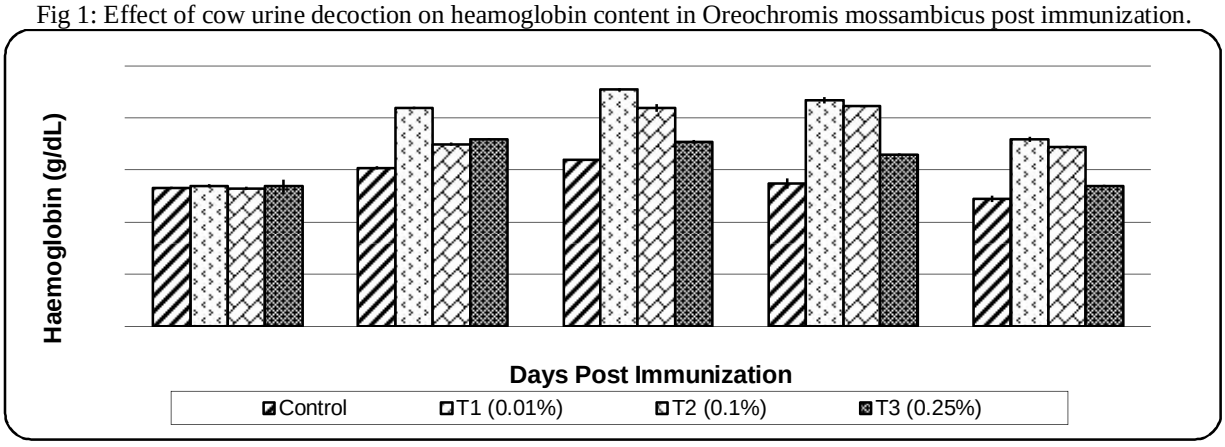

Fig 2: Effect of cow urine decoction on total erythrocyte count in Oreochromis mossambicus post immunization.

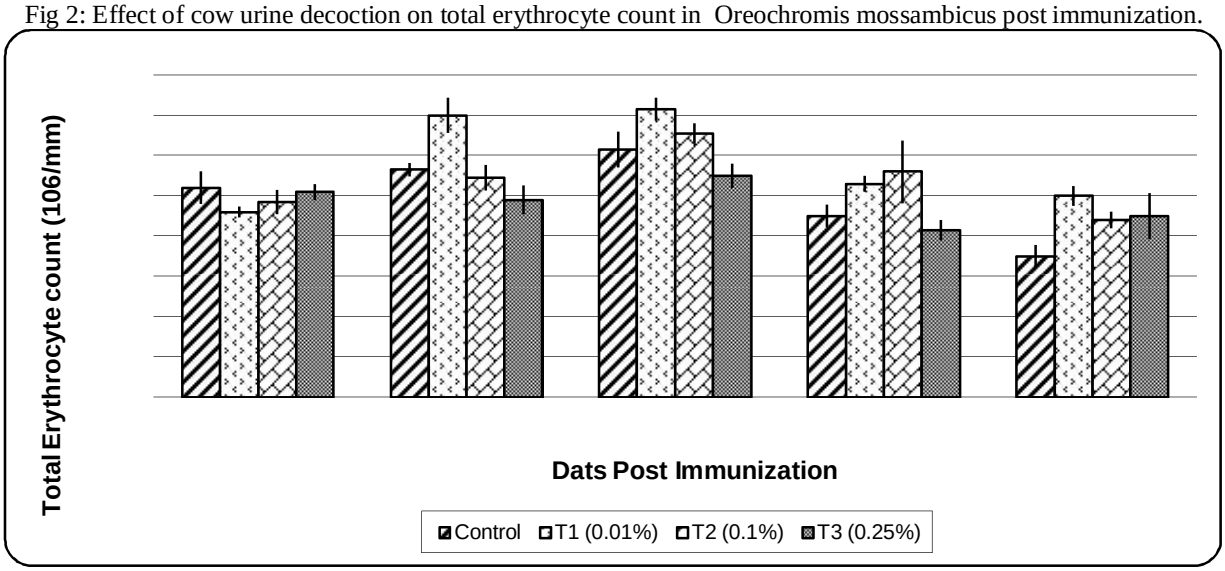

Fig 3: Effect of cow urine decoction on total leukcoyte count in Oreochromis mossambicus post immunization.

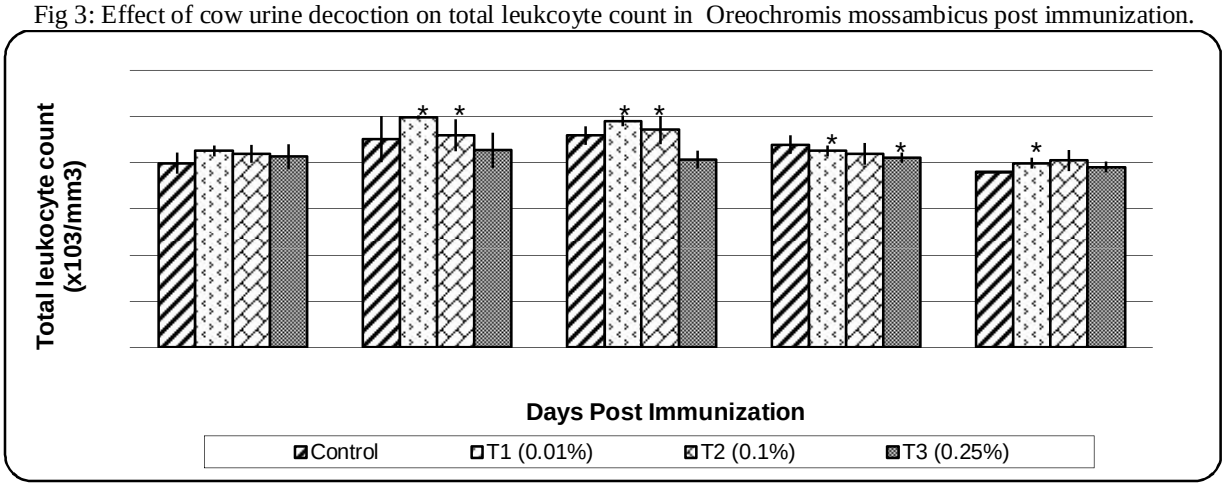

Fig 4: Effect of cow urine decoction on mean corpuscular haemoglobin in Oreochromis mossambicus post immunization.

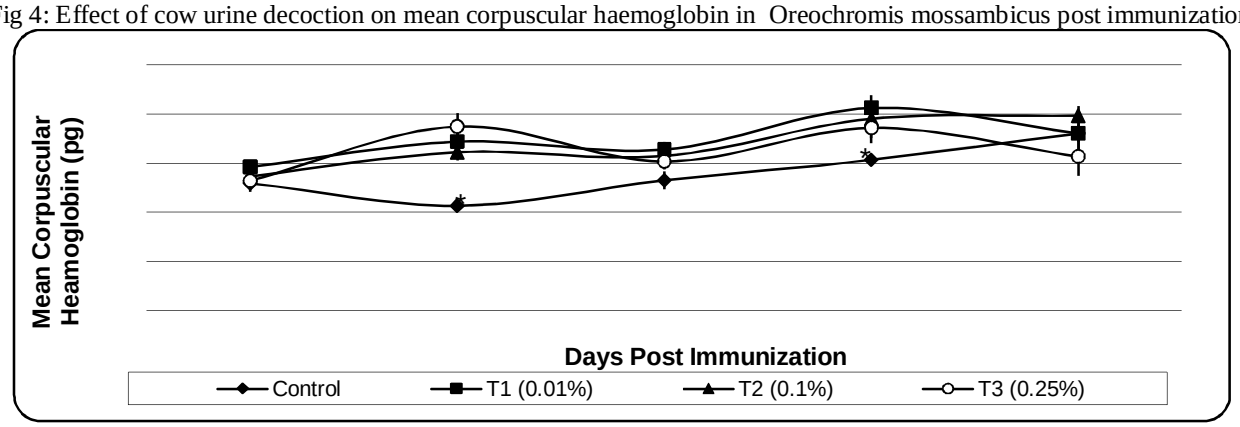

Fig 5: Effect of cow urine decoction on survival rate in Oreochromis mossambicus post immunization.