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Optimization of Concentration of *Bos indicus* Cow Urine Distillate (CUD) in *Oreochromis mossambicus* (Peter) for Immunostimulatory activity

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Abstract

Cows were regarded as wealth and were the backbone of the economy of ancient Indians. The sacred Indian cow, *Bos indicus,* is believed to be a "mobile hospital" for the treatment of many diseases. Wars were fought for acquiring cows. Cattle were one of the most frequently used animals described in Vedas. Cows were regarded as mother ("*Gau-mata*") and referred to as *Aghnya*. Atharvaveda provides interesting information about ailments of animals, herbal medicines, and cure of diseases. Urine was also considered as an antidote to poisons (Sushrut Samhita). From the ancient period, cow's urine has been used as a medicine. In Veda, cow's urine was compared to the nectar. The present study is aimed at investigating the immunostimulatory effect of cow urine distillate of *Bos indicus* (Gir breed) in *Oreochromis mossambicus* (Peter). Since cow urine is employed for curing many human ailments and also its immunomodulatory potential was well reported in literature it was thought worthy to find its optimal concentration for its application in aquaculture. Aquaculture is an ecofriendly, socially sound and economically viable innovative technology to manage water resources on low capital input basis. However there is a continuous threatening for the industry due to fish pathogens. The results revealed that 0.001% of cow urine distillate (CUD) is the optimal concentration for its effect on neutrophil activity.

Key words: CUD, *Bos Indicus*, *Oreochromis mossambicus*, Neutrophil activity, Immunomodulation, Aquaculture.

Introduction

Cow urine therapy and all traditional practices from Indian systems of medicine have a strong scientific base. Traditional systems in medicines, whether from Ayurveda or Siddha or the use of cow urine distillate as immunomodulator are based on classical texts , systems, practices and products handed down over generations going back to Charaka, Sushrutha, Vagabhatta, the Ashtangahridaya and the Samhitas. Cow urine has been described in 'Sushrutha Samhita' and 'Ashtanga Sangraha' to be the most effective substance/secretion of animal origin with innumerable therapeutic values. In Ayurveda cow urine is suggested for improving general health (Khanuja 2007). According to a recent online report of 'Love4Cow Trust' by researchers at Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow have identified a fraction of cow urine distillate as a bioenhancer of commonly used antibiotics and anti-cancer drugs. It increases life span and purifies blood from all sorts of impurities.

Cow urine is entirely sterile after secretion and has antiseptic effect. It acts like a disinfectant and prophylactic agent. Most of the medicines are made by distilling urine and collecting Vapours termed as ark (distillate). It is proved as a universal curer of blood disorders, leucorrhoea and even leprosy. This therapy has been reported to be beneficial for dreaded diseases like Cancer, AIDS and diabetes (*Chauan R.S* 2004). Literature shows that cow urine has enormous enhancing properties on biological systems. Cow urine one of the ingredients in panchagavya is believed to have therapeutic value. In India cow urine is used by majority of rural population as Folklore remedy in almost all the states. Urine therapy was not only used in India, but for several Centuries in many parts of the Globe. However, cow urine has not been examined for their effect on disease resistance & new immunostimulant promotion in aquaculture. Aquatic ecosystems are well utilized for aquaculture in recent decades. The use of unutilized resources and modern technology hiked aquaculture production. However, the number of outbreaks of bacterial diseases in cultured fish has also increased and it causes decline in aquaculture production. Fish health is closely associated with characteristics of aquatic environment.

Bos indicus

Bos indicus are the hardiest of high yielders in the world. The *Bos indicus* are famous milk cattle breed of India. It has been used in the improvement of other breeds including the Red

Sindhi and the Sahiwal. Cattle of the breed are famous for their tolerance to stress conditions and resistance to various tropical diseases. The breed has been exported to other parts of the world also. This is most important milk breed of country mainly kept for milk production. Under good management conditions the Gir cow produces between 1150 – 1600 kg of milk lactation. The *Bos indicus* are famous milk cattle breed of India for their tolerance to stress conditions and resistance to various tropical diseases.

Oreochromis mossambicus

Oreochromis mossambicus often used as a good experimental model and is extensively used in genetic and physiological studies in relation to pollution, stress, or growth promotors (Baskaran *et al*, 1989; Pratap *et al*, 1989) , Tilapia is selected as the experimental model because of its economical importance worldwide and also Tilapia eat a wide range of natural food organisms; tolerate poor water quality and easily spawned. Tilapia is a good fish for warm water aquaculture. Consumers like tilapia for their firm flesh and mild flavor. Worldwide harvest of farmed tilapia has now surpassed 800,000 metric tons and of the most widely farmed fresh water fish in the world, tilapias are second only to carps. (Popma and Masseri, 1999).

METHODOLOGY

Animal maintenance:

Oreochromis mossambicus a common fresh water cichlid fish was used for the study. Fish procured from local fish form were stocked in large fiber tanks. The experiments were carried out in plastic tubs of 70 lit capacities. Fish of both sexes weighing 10-25gm were used in the study. Water was changed frequently to avoid stress due to ammonia accumulation. The animals were fed *ad libitum* with a balanced fish diet prepared in our laboratory.

Cow urine collection

Cow Urine was collected from Gir breed in selected six healthy cows. These cows were selected after obtaining certificate from veterinary doctor they are disease free. Cow Urine was collected sterile container in the early morning from each cows, and then total three litters (collected from six cows) were pooled together for the distillation. The cows in the Gosala (cow farm) at Govindapuram , in Kumbakonam have been considered for the sample collection.

CUD Preparation

The collected urine samples were distillated at 50^0C - 60^0 C using distillation apparatus for 5 – 6 hours (Arunkumar Sathasivam *et,al 2010*). The cow urine distillate (CUD) was stored in sterile glass containers and was used for treatment on the same day without storage.

Aeromonas hydrophila

It is a gram negative, facultative rod shaped bacteria belonging to the family Vibrionaceae. It causes hemorrhagic septicemia in warm water fishes like channel catfish, tilapia (Amin *et al*., 1985; Leung *et al*., 1995). It may become an opportunistic pathogen (Grizzle and Kirya, 1993) infecting fish under stressful conditions or in concern with other pathogens (Noga *et al*., 1991). *Aeromonas hydrophila* is a causative agent for one of the major fish diseases in India. It was cultured in Tryptic soy broth (Himedia, India).

Preparation of heat killed whole cell vaccine

Single colony of *A. hydrophila* from the agar plate was inoculated in the tryptic soy broth. After 24 hrs, the bacterial cells in the broth were subjected to 60°C for one hour in a water bath. 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. The supernatant was discarded and the pellet was re-suspended in sterile PBS .Then the bacterial number was calculated by measuring optical density (OD) in a spectrophotometer and also confirmed by plate count method. The final bacterial concentration was adjusted to $1x10^8$ cfu/ml by serial dilution (Rajesh Kumar *et.ai.,2008).* The experimental fishes were challenged intraperitoneally with the bacterial suspension of 0.2ml ($1x10^8$ cfu ml^{-1}).

Exposure to CUD

After acclimatization, three groups of fish were treated with cow urine distillate in different concentrations for seven days (0.1%.0.01%,0.001% concentration). A control group was kept separately. On the seventh day immunization was done with 10^8 of cells of heat killed *A.hydrophila.* The neutrophil activity was assessed on the 4^{th}, 8^{th},$12^{th.}$ and 16^{th} days of post immunization.

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.,* 1994). The blood drawn was collected in heparinzed heamatological tubes. Great care were taken to avoid foaming when drawing the blood into micropipette as this readily resulted in hemolysis.(P. Sivanatarajan and T. Sivaramakrishnan, *2013*).

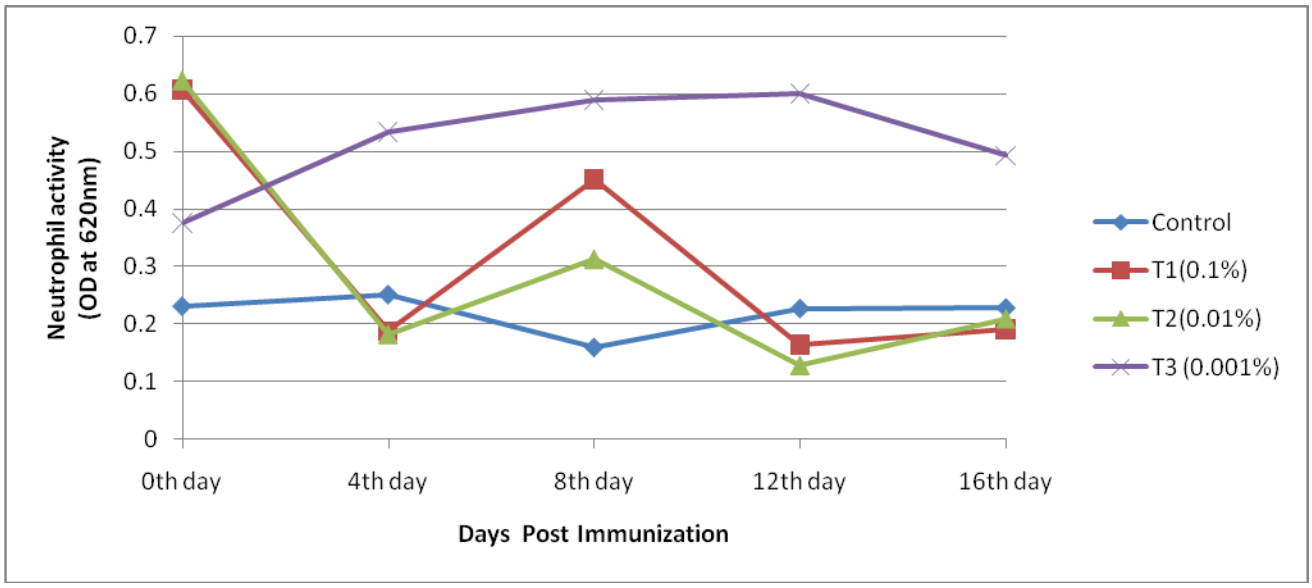
Neutrophil activity

The NBT assay was assessed by following modified Stasiack and Bauman method. 50µl samples of blood were taken from the experimental fish with a 1ml Tuberculin heparinized syringe. Collected blood was transfer into the flat bottom ELISA plate. The plate was incubate at 37ºC in water bath for 1 hour (to facilitate cell adherence).The incubating plate was washed 3 times in PBS (100µl) to remove non-adherence cells. Add 100µl of 0.2% NBT as coloring agent and Incubate for 1 hr at 37º C. After incubation fix with 100% methanol (100µl) for 2-3 min after 3min discard. Wash with 70% methanol (100µl).After washing plates was dry. Add 120 µl of 2N KOH and 140 µl of DMSO-mix properly. Take absorbance at 620 nm in ELISA reader. The NBT assay was done on 4^{th}, 8^{th}, 12^{th} and 16^{th} days post stimulation with cow urine and after immunization.

Result and Discussion

The neutrophil activity (OD at 620 nm) of experimental groups in different concentration of Gir breed Cow Urine Distillate (CUD) is compared to control. On the whole the minimum value occurred in T_2 (0.01%) on the 12^{th} day of post immunization and the maximum value occurred in T_3 (0.001%) on the same day. The high OD value in T_3 indicates the high level of neutrophil activity. Compared to control the effect of T_3 on the neutrophil activity is better. The control group shows no modulation in the neutrophil activity. T_1 group & T_2 group is affected by immunization of *A.hydrophila* . Hence a drop in neutrophil activity occurred. Since on the 12^{th} day of post immunization, peak value of 0.601 is occurred in T_3 group (0.001%) it is considered as the optimum concentration for immunostimulation of neutrophil activity.

Effect of different concentration of Gir CUD on the neutrophil activity of *Oreochromis mossambicus*



It has been already reported that 0.1% concentration as the optimal dose for CUD treatment in literature for growth enhancement (Padmapriya and Venkatalakshmi, 2014) (Sattanathan and Venkatalakshmi., 2015). This different in the optimal concentration for the same product on difference effects like growth and neutrophil activity could be attributed to the fact that for any biological activity modulators need higher does for gross effects. Whereas very minimal dose is required for a fine effect like neutrophil activity enhancement. Similar report was observed by Venkatalakshmi & Michael (2001) and (Dinakaran Michael *et,al.*,1998) in *Ocimum sanctum* leaf water extract and Ascorbic acid respectively. It has been shown that higher concentration caused immunosuppression and still higher concentration as toxic. But lesser doses were proved to be immunostimulative. Hence the observation of the present is in line with the previous reports.

The requirement of cow urine distillate for giving disease protection is very less (0.001%). This gives a scope for cost effective, eco friendly, Organic way of Aquaculture management practices.

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