EFFECT OF COW URINE DISTILLATE ON THE GROWTH AND BIOCHEMICAL COMPOSITION OF CLADOCERA, DIAPHANOSOMA EXCISUM

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Abstract
Diaphanosoma excisum, the fresh water cladocera was cultured with different concentrations of cow urine distillate (CUD) to evaluate its growth performance, nutritional composition and the antibacterial potential of hemolymph. The *D.excisum* showed a dose dependent increase in growth and concentration of biomolecules with the treatment of CUD. The LC50 concentration of CUD against *D.excisum* was 0.683 µg/ml. The hemolymph isolated from the *D.excisum* culture with CUD showed effective antibacterial activity. The study thus suggests that CUD could be utilized for the enhanced of growth and nutritional composition of *D.excisum* under optimized growth conditions and thus can be mass cultured to provide the live food in aquaculture.

Keywords: *D.excisum*; Zooplanktons; Cow urine distillate; Antibacterial activity; Nutritional composition.

DOI: 10.48047/ecb/2023.12.si10.0051

Introduction

Zooplankton organisms have been identified as critical components of aquatic ecosystems. Through grazing, they aid in the regulation of algal and microbial productivity as well as the transfer of primary productivity to fish and other consumers (1). They help to improve water quality by grazing on phytoplankton and bacteria. As a result, zooplanktons are regarded as water quality indicators (2). Furthermore, zooplanktons provide vital live feed for fish and crustaceans, which are the primary sources of animal protein in developing countries. Thus, the development of aquaculture and inland fisheries has been phenomenal in the last two decades (3). There is a growing demand for live zooplankton of appropriate size and quality to serve as prey for crustaceans and fish larvae. *Artemia* (4) and rotifer species (5) are common prey, but cladocerans (6-7) and copepods are gaining popularity (8).

Imported cysts of marine Artemia were the only live feed for commercially cultured fish larvae for several decades. The high cost of Artemia has been a main constraint in the commercial production of fish larvae and fingerlings in fish hatcheries since the 1980s. This issue is particularly prevalent in developing countries intending to import the commodity (6,9-10). Scientific research was initiated to identify and culture suitable cost-effective local zooplankton to replace imported *Artemia*. Cladocera are a type of zooplankton found in freshwater. Daphnia is the dominant representative in the temperate region. *Diaphanosoma*, on the other hand, is the most diverse pelagic genus in the tropical region (11). They are an important link between primary production and higher trophic levels because they are filter feeders and a food source for planktivorous fish and other predators(11). *D. excisum* is a pan-tropical species that can be found in Australia, Africa, and Asia (12).

Diaphanosoma (13) species exhibit potential aquaculture characteristics (14). *Diaphanosoma excisum*, a common freshwater zooplankton, has significant potential as a starter diet for fish larvae (6, 9). *Diaphanosoma*, also known as 'tropical Daphnia,' is widespread in the tropics and subtropics (11, 15-16). *Diaphanosoma excisum* and *Diaphanosoma dubium* are the two most common and dominant *Diaphanosoma* species. They are parapatrically distributed in warmer waters and rarely coexist in the tropics-subtropics transition zone (17-18). *D. excisum* is frequently the only Cladoceran present in the tropics (19). Previous research concentrated on the effect of cow urine distillate on the growth of the copepod *Mesocyclops leukarti* in the laboratory (20).

Cow urine distillate has the potential to stimulate the growth of zooplanktons. The composition of essential elements in urine influences the different components of urine (21). Among the components obtained from urine, 75-90% of nitrogen excreted is primarily in the form of urea, with the remainder released as uric acid, creatinine, and amino acid (22). Urine also contains ions such as Na^{+}, Cu^{2+}, Mg^{2+}, and Cl, which are essential for plant growth (23). Because of the abundance of these substances in urine, it is critical to thoroughly review how these substances can be captured and reused. Cow urine is useful for a variety of therapeutic purposes due to the presence of various constituents. For example, Allantoin in cow urine makes it an excellent substrate for wound healing (24). Because cow urine contains copper and gold hydroxide, it acts as an antipoison and is used to eliminate the toxic effects of medicine residues (25). Furthermore, the presence of hippuric acid (a common component of urine that increases with increased consumption of phenolic compounds such as tea, fruit juices, and wine), phosphates, and uric acid makes it useful as a diuretic agent. Because of its high nitrogen content, it is beneficial in stimulating the kidney. In fact, cow urine taken orally has been shown to boost the immune system, improve memory, relieve tension, and lower cholesterol levels (24-25). In this context, our goals were to examine *Diaphanosoma excisum* growth capacities in monospecific cultures under optimised laboratory conditions, as well as the effect of cow urine distillate on *Diaphanosoma excisum* population density and nutrient composition.

Materials and Methods

Collection of Plankton Samples

Plankton samples were collected from the subsurface layer of Banapureeshwar temple at a depth of 50 to 100 cm (26) and were stained with Eosin for the morphological identification with a compound microscope (27). The isolated cladocera were maintained in 500 ml of tap water contained within a beaker.

Culture survivability

The cultivated cladocera were subjected to survival tests to determine their suitability for further research in accordance with Nandakumar, (28). 10 healthy adults of dominant cladocera species were hand-selected and placed in a 500 ml

glass beaker containing tap water filtered with a 1 filter bag. They were fasted for twenty-four hours before to the trial. The survival of the cladocera was inspected and tallied daily. The studies were conducted in duplicate for a total of fourteen days. The number of surviving cladocera on the final day was counted to determine their survival (28).

Pure culture preparation

The morphological characteristics of *Diaphanosoma excisum* was recognised under a microscope (10x) using practical guide (29). Then, the cladocera species were isolated, inoculated, and fed with yeast media in a 2-liter container (28)

Collection cow urine and preparation of CUD

Six disease-free cows (Tag Nos. 0206, 0177, 0184, 0468, 0133, and 0201) from Goshala, Sri Vittal Rukmini Samsthan, Govindapuram, near Kumbakonam, were selected for urine collection. Six cows' first urine was collected between 4:00 and 5:00 a.m., pooled and transported to the laboratory in airtight, sterile containers (30). The obtained cow urine samples were distilled for 5 to 6 hours at 50 to 60ºC using a distillation device (31). It was utilised immediately for treatment without storage.

***D.excisum* susceptibility to CUD**

For the toxicity tests, only actively swimming adult animals from the stock culture were chosen. The concentrations of CUD used were 200, 400, 600, 800, and 1000 µl/ml. Twenty animals were utilised for each of the five concentrations and the control group. Animals were divided into two groups of 10 for each concentration (in duplicate). In all tests, well-aerated tap water was used. Each test lasted 24 hours, and deaths were recorded every 6, 12, 18 and 24 hours. The LC_{50} concentration of CUD was determined by probit analysis using IBM SPSS software.

Effect of CUD on *D.excisum*

D.excisum (500 nos) were introduced into tanks containing 20 ml of water. Different concentrations (0.01, 0.025, 0.05 and 0.1 µl/ml) of CUD based on the LC_{50} value were added to the experimental tanks. The control tank was maintained by adding an equal volume of tap water without CUD. After 60 days, biochemical composition of CUD-treated plankton and untreated plankton were evaluated.

Isolation of hemolymph

Using a hand lens 20 mature *Diaphanosoma excisum* adults were separated and homogenized with sterile physiological saline solution in a tissue homogenizer. The homogenate was centrifuged for 10 min at 6000 rpm. The resulting supernatant was collected that contained the hemolymph (20).

Biochemical analysis

The quantitative estimation of carbohydrates was determined using the method of Roe, (32). The Lowry's method was adapted for the estimation of protein content. The total lipid was extracted following Folch et al (33) and estimated using Banes and Blackstock's (34) method. All the experiments were carried out in triplicates.

Antimicrobial activity of hemolymph

Using the well diffusion method, the antimicrobial activity of hemolymph of *D.excisum* was determined against *Escherichia coli*, *Staphylococcus.aureus*, *Pseudomonas.aeruginosa* and *Rhodococcus rhodochrous*. Sterilization of Nutrient agar media was used. After the Nutrient agar had solidified, wells were cut with a well borer. The bacterial pathogens under test were swabbed on the surface of Nutrient agar plates. 25µl of the test sample was injected into each well. For 30 min, the plates were incubated to allow the samples to diffuse into the medium. The plates were incubated at 37ºC for 24 hours before measuring the diameters of the zones of inhibition in millimetres. Each antibacterial assay was carried out in triplicate, and the mean results were reported (35).

Result and Discussion

The most important factor in considering zooplanktons as live feed in aquaculture is the nutritional value of the selected zooplankton species and its sustainability for large scale synthesis (16). Cladocerans offers the mass production in a short span of time under optimized growth conditions (36-37) Previous literatures reported the use of horse manure, chicken droppings, cow dung and fish faces in the culture of cladocerans (38-39). The cladocerans holds high values in aquaculture and thus are the most used live feeds. Hence, the present study involved in the identification of cladoceran species from the fresh water Banapureeshwar temple pond and used cow urine distillate for the laboratory rearing of *D.excisum* for the evaluation of their nutritional values. The dominant species of cladocera identified in the fresh water pond includes *D.excisum*, *D.brachyurum*, *D.sarsi* and *D.magna* was assessed for their survivability under laboratory conditions. The survival test was

carried out for a period for 14 days and the population density of the species at the end of experimental period was determined (Fig.1). The survival of cladocera species based on population density was observed to be in the order *D.excisum* > *D.brachyurum* > *D.magna* > *D.sarsi*. Based on the survival test, the highly populated species *D.excisum* was considered for further evaluation.

To determine the toxicity of cow urine distillate as well as the tolerance of *D.excisum*, different concentrations of CUD (200, 400, 600, 800, and 1000 µl/ml) were used. The mortality of *D.excisum* was recorded after 24 h of treatment. The percentage mortality and the derived LC_{50} value were presented in Table 1. 96.67% mortality was recorded after 24 h of treatment with 1000 µg/ml and the LC_{50} value was 369.735 µl/ml. Hence, the CUD concentrations ranging below the LC_{50} value was considered for further evaluation of its effects on the growth and biochemical composition of *D.excisum*.

In cladocerans, lipids and proteins are considered to be good indicators of the nutritive state (36-37). At low food concentration, lipid reserves, mainly triacylglycerides, are metabolised while proteins are only catabolised under severe starvation (38) Under laboratory conditions, the lipid content of *D. magna* ranges from 128 to 197 mg/g (DW) in well-fed animals and drops to about 60 mg/g (DW) in starved, hatching or senescent specimens (38). Fig.2 presents the effect of different concentrations of CUD on the biochemical composition of *D.excisum*. The zooplankton grown with CUD concentrations of 0.01µl/ml, 0.025µl/ml and 0.05µl/ml showed a carbohydrate content of 54, 67 and 60 µl/ml respectively compared to the control organism. But with increase in concentration of CUD a decrease in the carbohydrate content was observed. A dose dependent reduction in the total protein was observed compared to control. Increase in the concentration of CUD (0.1 µl/ml) significantly reduced the level of total protein (13 µl/ml). The CUD concentration of 0.05µl/ml showed an increase in the total lipid content (13.33 µl/ml) compared to control. But the total lipid was found to be decreased with increase in the concentration of CUD. The low content of protein and lipids observed in this study in *D.excisum* might be attributed to the age and feed received by the zooplankton species during their growth period (40). Farkas et al., (41) reported a protein content of 223 mg/g and 346 mg/g of lipids in cladocera. Cauchie et al., (42) reported that the protein and lipid content of cladocera ranged from 186 to 397 mg/g and 65 to 171 mg/g respectively. The protein content in Daphnia species was found to be 62.6% and *D.excisum* was reported to contain 57.3% of protein. The study by Praveena et al., (2020) showed a low protein content of 96 µg/dl in *Mesocyclops leukarti*. From the earlier reports it was generally found that the cultured organism had low content of biomolecules compared to wild type (42) . Praveena et al., (20) reported an optimal concentration of CUD (0.5%) effectively enhanced the growth of copepod culture, *Mesocyclops leukarti.* The present study witnessed an optimal concentration of 0.025 µl/ml concentration of CUD had a effective role in enhancing the biochemical composition of *D.excisum* hemolymph.

The hemolymph of *D.excisum* cultured with CUD was estimated for its antibacterial activity against the gram positive and gram negative bacterial pathogens such as *R.rhodochrous S.aureus, E.coli* and *P.aeruginosa*. Table 2 and Fig.3 shows the inhibitory activity of *D.excisum* against pathogenic bacterial species. From the results obtained it could be concluded that gram positive bacterial species were more susceptible compared to gram negative species. Maximum zone of inhibition was observed with *S.aureus* (23.9±0.7) and minimum inhibitory activity was recorded against the gram negative strains (18.5±0.9) without any significant difference in activity among the two negative strains examined in this study. In a previous study, utilizing the hemolymph of *D.excisum*, the negative bacterial species including *E.coli* and *K.pneumoniae* were reported to be effectively inhibited compared to gram positive strains. This antagonist finding with the present study might be attributed to difference in species response towards bacterial pathogens (20).

Conclusion

Increasing global population and the subsequent demand for animal proteins are majorly compensated by the rapid development of aquaculture. Mass production of zooplanktons as live food in aquaculture thus becomes inevitable. The present study signifies the use of cow urine distillate as a valuable resource in the enhanced production of zooplankton, particularly cladocera, *Diaphanosoma excisum*. The species showed sustainable growth with increased biochemical composition with cow distillate under optimized conditions. Hence, the study recommends the use

of cow urine distillate in the culture of *D.excisum* to be used as live feed in aquaculture.

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Fig. 1 . Survivability of different Cladocera species in culture

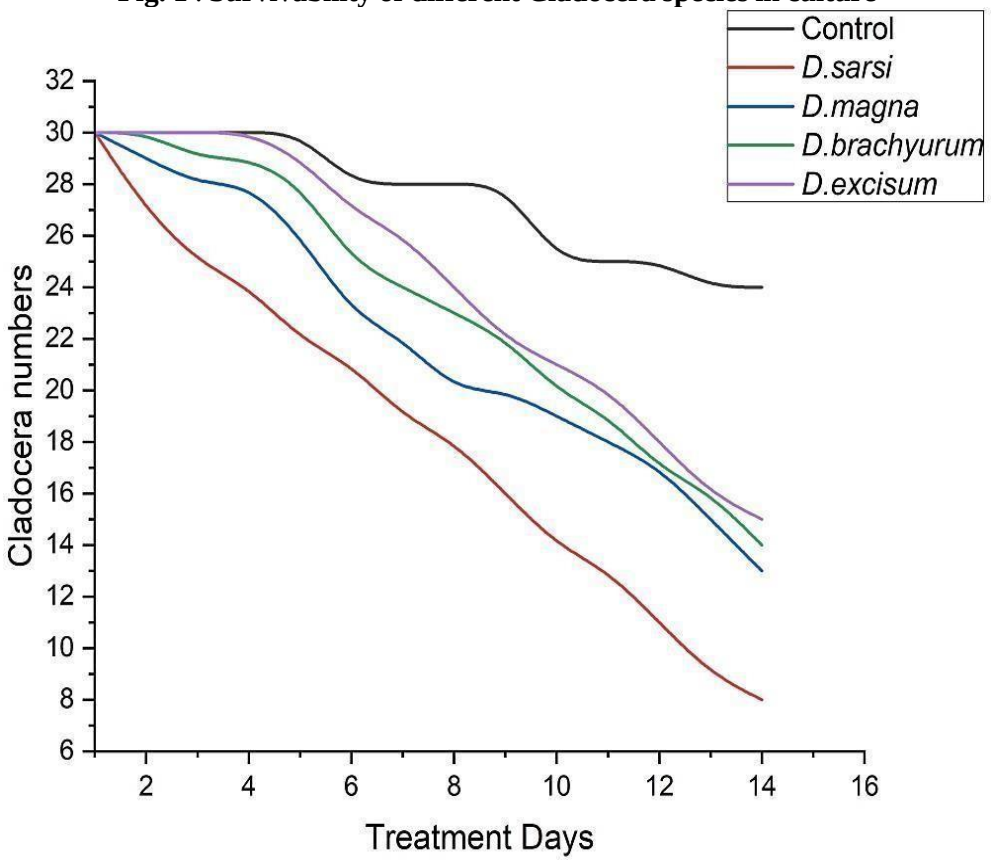

Table1. Toxicity bioassay with the treatment of CUD on *D.excisum*

Hours	Percentage Mortality/ CUD (µl/ml)					LC 50 (LCL-UCL)	LC 90 (LCL-UCL)	Regression Equation
	200	400	600	800	1000			
6	10.00±10.00	26.67±5.77	36.67±5.77	50.00±20.20	70.00±10.00	598.494 (501.584-722.841)	1614.154 (1189.944-2898.294)	Y= 6.667X+.087
12	20.00±10.00	40.00±10.00	50.00±10.00	60.00±10.00	76.67±5.77	517.515 (397.860-663.346)	1867.949 (1222.073-5053.396)	Y= 5.667X+.075
18	23.33±5.77	46.67±5.77	56.67±5.77	66.67±5.77	86.67±5.77	482.741 (380.860-594.812)	2175.428 (1425.142-3345.285)	Y= 14.667X+.063
24	36.67±5.77	56.33±5.77	56.67±5.77	73.33±5.77	96.67±5.77	369.735 (235.134-483.094)	2465.242 (1416.634-10724.634)	Y= 26.667X+.053

Fig. 2 Effect of CUD on the biochemical composition of *D.excisum* hemolymph

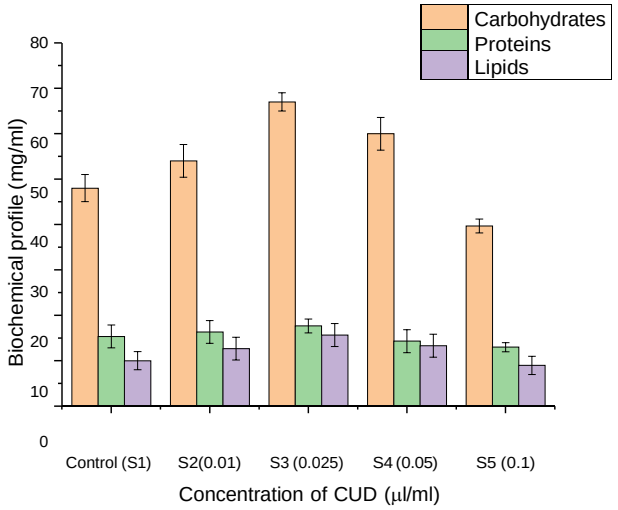

Table 2 Antibacterial activity of *D.excisum* hemolymph

Sl.No	Bacteria	Zone of Inhibition (mm)	
		Antibiotic	Hemolymph
1	*R.rhodochrous*	20.6±0.5	14.5±0.04
2	*S.aureus*	24.0±0.3	23.9±0.7
3	*E.coli*	21.3±0.6	20.6±0.5
4	*P.aeruginosa*	20.4±0.4	18.5±0.4

Fig. 3 Antibacterial activity of *D.excisum* hemolymph