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Effect of Phytocee™ (a Phytogenic feed additive) on survivability of Zebra fish subjected to ammonia stress

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Abstract
Stress is a common factor among all animals and aquatic species suffer wide variety of stresses. Adaptogens are plant substances that have been known to mitigate stress in animals. Phytocee™ is a phytogenic feed additive known to alleviate stress in animals. In the current study, Phytocee™ was studied to know the survivability of the model fish, Zebra fish (*Danio rerio)* in an ammonia stress model. The test groups consisted of two groups; a) Normal control: 20 fishes received the normal feed without Phytocee™ b) Experimental group: 20 fishes received feed added with Phytocee™ @ 1Kg/ton (0.1%). The fishes were fed on these diets for 15 days. On day 16, the fishes were challenged with ammonium acetate at 40mM and were observed continuously and the mortality at every hour (time in hrs) was recorded till 100% mortality. Results showed that Phytocee™ afforded the protection against ammonia toxicity in Zebra fish. This study shows that Phytocee™, a herbal additive may offer antistress effect to aquatic species and improve their survival.

Keywords: Zebra fish, Phytocee™, anti-stress, ammonia stress

1. Introduction
Stress is universal in animals and it can be defined as the nonspecific response of the body to any demand made upon it. Stress can cause adverse effects in animals like low productivity, susceptibility to disease, organ damage, retarded growth and death [1]. The physiology of stress was described by Selye H as ''General Adaption Syndrome'' (GAS). As per the General adaption syndrome there are three stages in stress, namely 1) The Alarm stage: This is a stage of ''flight or fight'' mode where in the organism's reaction to stress is heightened to combat the induced stress. It is characterized by increase in sympathetic -adrenocortical activity, which includes increase in heartbeat, increase in alertness, increased blood flow to muscles and possibly reduction of blood flow to digestive system, increase in blood pressure and rate of respiration. 2) The Stage of Resistance: In this stage the organism continues to defend against the stress by elevated physiological responses, Example, elevated cortisol or corticosterone levels. The organism's resources are utilized to maximum to fight the stressor, if it continues for a short term, the animal returns to normalcy. 3) The stage of Exhaustion: In this stage the organisms would have nearly exhausted the resources and would be susceptible for disease, immune depression, loss of energy, organ damage or possibly death [2].
There are detailed accounts on effect of stress in aquatic species like fishes, disturbed level of cortisol is one of the hallmarks of stress in fishes. The stressors in fishes can be broadly categorized as 1) Genetic factors: Fishes exhibit a wide variation in stress response. The cortisol release in fishes can range from 30 to 300 ng/ml and stress response results in approximately two-fold increase in many types of fish species. 2) Developmental factors: Stress response in fishes vary as per their developmental stages like just after hatching, fingerling stage and adult stages. The metamorphosis stage of the fishes appears to be most sensitive to stressors. 3) Environmental factors: a wide variety of environmental factors have been known to cause stress in fishes, they include: salinity of the water, temperature of the water, illumination levels in the water (including the wavelength of light). Presence of disease, dissolved ammonia levels, pH of the water etc. [3]. The negative effects of stress on fish can be varied particularly larval stages are more susceptible. The effect of stress on fish include: susceptibility to disease, decreased immune competence was manifested due to increased levels of cortisol, decreased amounts of lysozyme, reduced numbers of B-lymphocytes, decreased antibody response and complement hemolytic activity [4].

A variety of disease can strike during stress, the notable infections in hatcheries include Vibriosis, infection due to *Aeromonas hydrophila*, enteric red mouth (due to *Yersinia ruekeri*), bacterial gill disease (*Flavobacterium branchiophilum*). Stress is known to trigger parasitic diseases as well as predisposing factor for noninfectious conditions such as swim bladder stress syndrome, coagulated yolk disease and various skeletal disorders. The stressors obviously affect growth and productivity of the fishes in aquaculture [4]. Adaptogens are plant substances known to mitigate the burden off stress in animals, various plants like *Withania somnifera*, Ocimum *sanctum*, *Panax ginseng*, *Tinospora cordifolia*, *Emblica officinalis*, *Bacopa monnieri*, *Rhodiola species*, *Terminalia chebula* etc… have been reported to be adaptogenic [5]. With these viewpoints the current study was conducted with the objective to evaluate a botanical formulation Phytocee™ for its adaptogenic/antistress response on Zebra fish (*Danio rerio*) in ammonia induced toxicity test model.

2. Materials and Methods

2.1 Test material used

The polyherbal formulation Phytocee™ (batch number: 18T112, Mfd: 08/2018) was used for the study. Phytocee™ composed of: Dried powder of *Withania somnifera* stems, fruits of *Emblica officinalis* and whole plant of *Ocimum sanctum*. The formulation was standardized to contain a known quantity of polyphenols and gallic acid.

2.2 Test organism used

Adult Zebra fish (*Danio rerio*) procured from a pet store in a local market, Bangalore. Adult zebrafish with an average standard length of (2.00 ± 0.20 cm) and an average weight of (0.40 ± 0.18 g) was used.

2.3 Preparation of test feed

50mg of Phytocee™ in 50g of ‘’Tetra Bits Complete Fish Food’’ (a commercial pet fish food) was blended in a mixer grinder for 3 min to get fine powder, a little water was added to prepare a dough and rubbed on a fine mesh to get uniform pellets. The pellets were dried in an oven at 50^0C for 2 hrs. The dried pellets were stored in an air tight container. A control feed was made in the same manner without Phytocee™.

2.4 Test protocol

The Zebra fish were acclimatized to laboratory conditions at 12 light and 12 dark photo period. The fishes were maintained in dechlorinated tap water contained in beakers. The water was oxygenated uniformly using an air bubbler. The fishes were divided into two groups: a) Normal control: 20 fishes received the normal feed without Phytocee™ b) Experimental group: 20 fishes received feed added with Phytocee™ @ 1.0 Kg/ton (0.1%). The fishes were fed on these diets for 15 days. On day 16, the fishes were challenged with ammonium acetate at 40mM and were observed continuously. The mortality at every hour was recorded. The end point (time in hours) for 100 % mortality was evaluated.

2.5 Ethical approval

The study was conducted by scientists and technicians in compliance with the guidelines laid down by the Institutional Animal Ethics Committee (IAEC). (vide NRPL /06/02.) All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

3. Results

In normal control group the cumulative mortality (%) at 0 hr, 1 hr, 2 hr and 3 hr was 00%. 15%, 60% and 100% respectively. Whereas in experimental group, the cumulative mortality (%) reveal that mortality rate was gradually increased and 100% mortality was recorded only at 14 hrs. This implies that the survivability of Zebra fish (*Danio rerio*) was better in experimental group as compared to normal control group (Table 1 and Figure 1)

Table 1: Effect of Phytocee™ on Survivability Rate of Zebra Fish to Ammonia Induced Toxicity

	0 hr	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr	7 hr	8 hr	9 hr	10 hr	11 hr	12hr	13hr	14hr
Normal Control Group (NH4AC induced at 40mM on 16th Day)															
Live (No.)	20	17	08	00	--										
Mortality (No.)	00	03	09	08	--										
Cumulative Mortality (%)	00	15	60	100	--										
Experimental Group (Phytocee™ for 15 days + NH4AC induced at 40mM on 16th Day)															
Live (No.)	20	20	19	12	12	12	10	09	07	06	04	03	02	01	00
Mortality (No.)	00	00	01	07	00	00	02	01	02	01	02	01	01	01	01
Cumulative Mortality (%)	00	00	05	40	40	40	50	55	65	70	80	85	90	95	100

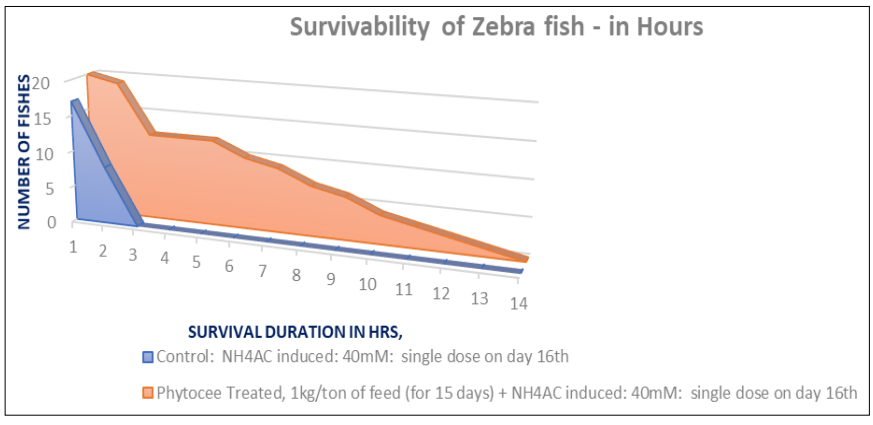

Fig 1: Effect of Phytocee™ on Survivability Rate of Zebra Fish to Ammonia Induced Toxicity

4. Discussion

In the present study 100% mortality of Zebra fish (*Danio rerio*) was recorded within 3 hrs in the normal control group, however, in experimental group supplemented with Phytocee™ at the dose of 1.0 Kg/ton 100% mortality was recorded only at 14 hrs. This depicts that Phytocee™ afforded protection to Zebra fish (*Danio rerio*) in the experimental group against the ammonia induced toxicity. The afforded protection of Phytocee™ could be attributed to the phytoconstituents present in the product.

Phytocee™ was a proven adaptogen, it was found to improve swim endurance in mice. It was found to mitigate the negative effects of chronic variables of stress and reduce stress induced corticosterone levels in rats. *In-vitro* studies revealed, the product was found to reduce corticosterone release in forskholin stimulated H295R cells an, adenocarcinomas cell line. Phytocee™ downregulated the gene expression of 11-beta hydroxysteroid dehydrogenase -1(11-beta HSD-1) in H295R cells. 11-beta HSD-1 is involved in conversion of 11 -dihydrotestosterone (inactive state of the stress hormone corticosterone) to corticosterone. Phytocee™ was found to act as an antagonist at CRHR1 receptor. This receptor found in anterior pituitary cells binds to the endogenous agonist corticotrophin releasing hormone (CRH) which results in the release of adrenocorticotrophic hormone (ACTH). ACTH in turn acts on adrenal cortex to release the stress hormone corticosterone [6]. In a study by Chandrasekaran CV *et al.*, Phytocee™ was found to offer cellular antioxidant effect in AAPH induced oxidative stress in HepG2 cells [7].

Phytocee™ has been shown to exhibit anti-oxidant effect in a rat model of carbon tetra chloride induced toxicity. The product was found to reduce CCL4 induced increase in serum AST, ALT and hepatic MDA levels, whereas the levels of hepatic superoxidase dismutase (SOD), catalase were increased. Since stress is known to induce free radicals, the antioxidant effect of Phytocee™ may partially explain its antistress effects [8]. Phytocee™ was also demonstrated to have adaptogenic effect in heat induced stress in broiler chickens. In this study, Phytocee™ improved the heterophil-lymphocyte ratio which is used as an index of anti-stress effect. Phytocee™ also reduced the stress induced elevation of serum corticosterone in these birds. Taken together, there is substantial evidence for the antistress property of Phytocee™ in rodent and avian models [9].

The adaptogenic effect of Phytocee™ could be understood by examining its ingredients. *Withania somnifera*, one of the key ingredients has been shown to have anti-stress effects in a variety of models. *Withania somnifera* is known to improve swim endurance, reduce stress induced cortisol concentrations, prevent stress induced ulcers, and improve immunity in animals [10]. Interestingly, *Withania somnifera* has been shown to improve growth, haemato-biochemical response and disease Exhaustion of *Labeo rohita* against *Aeromonas hydrophila* infection [11]. In another study by Laltlanmawia C *et al.*, inclusion of 1.0% *Withania somnifera* (Ashwagandha) root extract and dietary L-ascorbic acid (Vitamin C) combination in diet had a immunostimulatory effect and attenuated the effect of multiple stressors namely, low pH and waterborne iron toxicity in *Labeo rohita* fingerlings [12]. Another ingredient of Phytocee™, *Ocimum sanctum* is also a well-known adaptogen and proven in many experimental models that the anti-stress active principles of this plant are believed to be Ocimumosides A and B and Ocimarin [13, 14, 15]. Nahak G *et al.* reported the beneficial role of *Ocimum sanctum* on a common fish *Clarias batrachus (Linn.)* in augmenting the immunity, growth and survivality as evident from the enhanced haematological and biochemical parameters [16].

Reddy VD *et al.* reported amelioration effect of *Emblica officinalis* against the alcohol induced oxidative stress in rat models. The *Emblica officinalis* fruit extract administration at the dose of 250 mg/kg body wt./day to alcohol-administered rats causes normalization of plasma lipids and lipoprotein patterns, nitrite/nitrate, total bilirubin, creatinine, plasma protein, uric acid levels and A/G ratio [17]. In an another study, Sharma *et al.* reported that *Emblica officanlis* reduces the number of free radicals and extent of cellular damage and this antioxidant potential of *Emblica officanlis* could be attributed to the polyphenolic contents present in the extracts facilitate conjugation with free radical species [18]. Several authors reported the multiple effects of pharmacological and immunomodulatory properties of *Emblica officinalis* [18, 19]. Mandal AB reported dietary supplementation of *Emblica officinalis* at the dose of 0.2% improves the cell mediated immune responses and at 0.3% improves HSP-70 gene expression in extreme summer condition in broiler chickens helps to alleviate the summer stress [20]. Furthermore, previous studies reveal that dietary addition of *Emblica officinalis* enhances the performance of broiler chickens through its anti-stress properties during high environmental temperature as *Emblica officinalis* is rich source of ascorbic acid [21, 22, 23]. Minomol M reported *Emblica officinalis* augmented the immune system and reduced the microbial infection in goldfish *Carassius auratus* [24].

5. Conclusions

In conclusion, the animal feed additive Phytocee™ (phytogenic feed additive) supplementation at the dose of 1 kg/ton of feed could enhance the survivability of Zebra fish challenged with ammonia induced toxicity. The amelioration of ammonia induced toxicity effect of Phytocee™ could be attributed to the antioxidant, anti-stress and immunomodulatory properties of phytoconstituents present in Phytocee™. Usage of Phytocee @ 1.0 Kg /ton of feed may protect the aquatic species against myriad stressors found in production environment.

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