Effect of a plant-derived nutraceutical on the tolerance of axenically-cultured *Artemia* towards abiotic or biotic stressor

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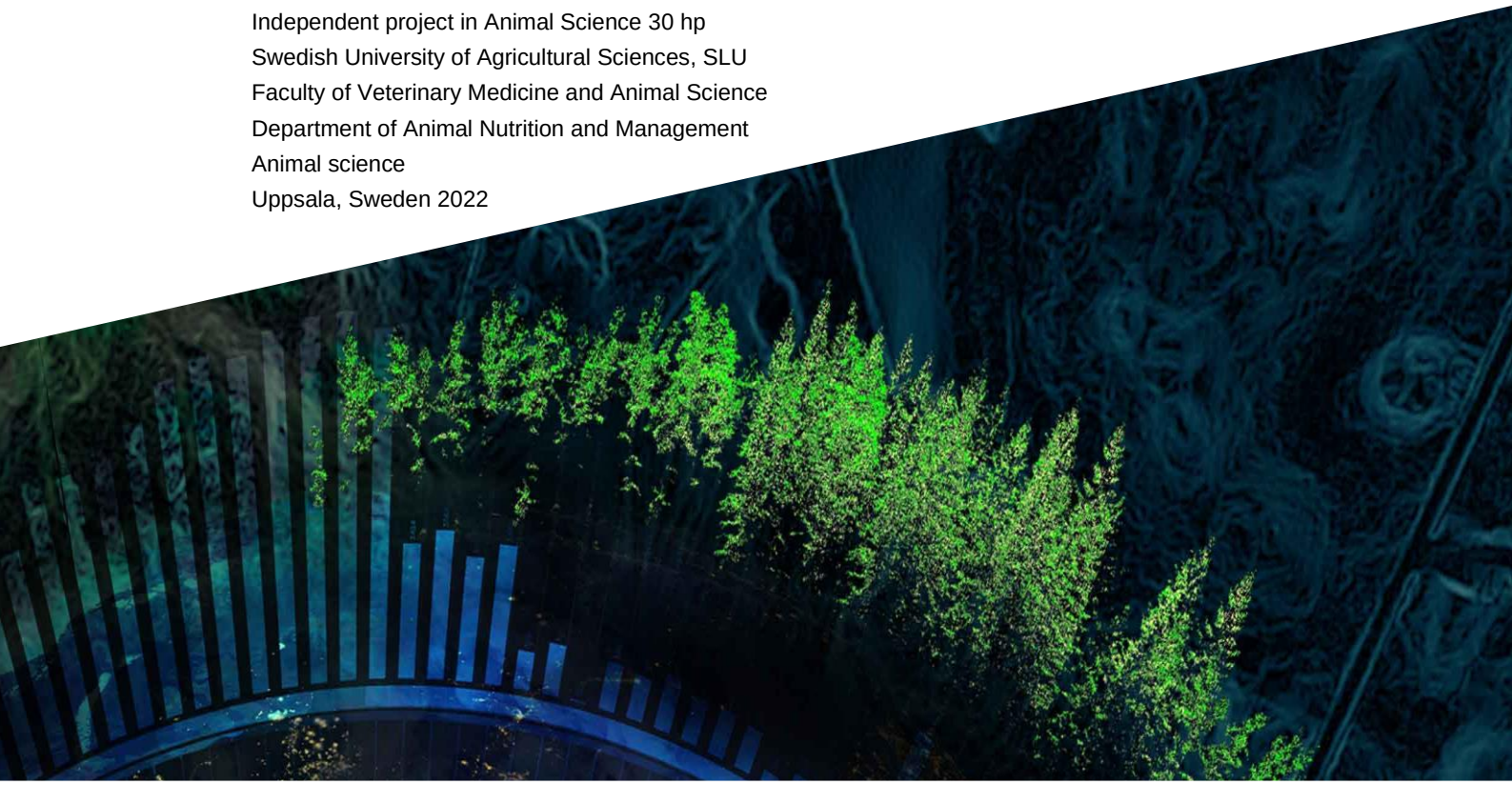

Effect of a plant-derived nutraceutical on the tolerance of axenically-cultured *Artemia* towards abiotic or biotic stressor

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Artemia.

Abstract

Aquaculture is one of the fastest-growing food-production industries worldwide accounting for almost half of fish used for human consumption. Super-intensive, intensive, and semi-intensive farming practices used to produce large farm fish are increasing the frequency of disease outbreaks which is a major problem affecting sustainable aquaculture growth. Antibiotics used to overcome disease have led to the development of resistant strains which is becoming a global public concern. Over the past few years, there has been a growing interest in using plant-derived nutraceuticals, especially those that possess heat shock protein Hsp70-inducing bioactivities as an alternative to antibiotics. In this study using brine shrimp *Artemia* as a model organism, an Asian ginseng extract (referred to as Ash01) was studied to examine it's potential as an Hsp70 inducer for controlling diseases in aquaculture animals. First, a range-finding test for the extract was carried out in *Artemia* and was found between 200 and 1000 mg/l. Using this dose range, subsequent studies were conducted to determine whether the extract could confer protection to the host *Artemia* against an aquaculture pathogen *Vibrio campbellii* LMG 21363, or a wide range of abiotic stressors. Results showed that pre-treatment of *Artemia* nauplii with Ash01 at a dose of 1000 mg/l caused a significant improvement in the resistance of the nauplii towards pathogenic *V. campbellii* challenge as well as towards a wide range of environmental stressors like thermal shock, salinity stress, and low pH. This salutary effect of Ash01 appeared to be associated with an increase in the level of heat shock protein Hsp70 and/or possibly other family members of stress proteins. An upregulation of *hsp70* gene by a fold of 3.6 although was observed in the Ash01-treated group. The increase however was not significantly different from the untreated control group. Further studies should be carried out using an extract from Ash01 to analyze Hsp70 both at the gene and protein levels as it is likely that the production of Hsp70 in response to Ash01 pre-treatment in *Artemia* occurred at the protein level. Overall results add new information about the functional properties of Ash01 and advance our knowledge of this herbal plant as a potential prophylactic agent for controlling infection stress in aquaculture animals.

Keywords: Artemia, Hsp70, *Vibrio campbellii*, plant extract, aquaculture, abiotic stressor.

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Abbreviations

PSU	Practical salinity unit
ASW	Autoclaved sea water
Hsp	Heat shock protein
NLHS	Non-lethal heat shock

1. Introduction

Aquaculture is one of the fastest-growing food-production sectors in the world providing 50% of fish for human consumption (Stankus, 2021). The sector is important and necessary in the provision of quality and affordable food in the world food system. With global supply and apparent per capita consumption of fish and fish products increasing faster than the population growth, the need to fulfill the demand for quality proteins and lipids had led to the intensification of aquaculture. Stress is one major challenge in the sustainable production of farmed fish which has caused high mortality rates and economic losses to farm species. Stress can be caused by environmental factors like changes in temperature, pH, ammonia, and salinity or by biotic factors in form of pathogens. Water temperature is the most common stress factor affecting aquatic life. In crustaceans, high temperature affects the immune system by decreasing the levels of hemocytes and prophenoloxidase activity. Change in the temperature has also a negative influence on the growth, survival, and reproduction of shrimp (Tropea *et al*., 2015). Salinity change is common in a marine aquatic environment, especially with rising climate changes across the world which surpasses the ability of aquatic life to cope. In white shrimp *Litopenaeus,* low salinity levels of 2 PSU and 10 PSU caused a reduction in the growth performance and transcript levels of Na^+-K^+-ATPase α- subunit, carbonic anhydrase in gills, and chymotrypsin, and trypsin levels in the hepatopancreas (Iryani *et al.,* 2022). The pH of water is another factor affecting the well-being of aquatic life. When pH is low, the metabolic activity reduces the ionic balance of gills affecting the growth, production, and survival of fish (Freda and McDonald, 1988)

To overcome disease problems and issues associated with poor growth in farm fishes, antibiotic has traditionally been used either through administration in feed or by directly adding to the rearing water. However, their indiscriminate use has led to the accumulation of the compound in the environment and within animal tissue which has resulted in the development of multiple-resistant strains of bacteria detrimental to human and farmed animal health (Shimizu *et al.,* 2013). Owing to this, there is an increasing interest in developing antimicrobial strategies as an alternative to chemotherapeutics to improve health and control diseases in aquaculture animals. Over the past few years, several anti-infective strategies or nature-based preventive measures to protect aquaculture animals from pathogenic

microbes have been developed, and some of them have been applied successfully. For example, microbes-associated feeding strategies i.e., prebiotics, probiotics and synbiotics, and the use of natural immunostimulants are some of the promising alternatives (Verschuere *et al.*,2000; Huang *et al.*,2015; Munir *et al.*, 2016; Jami *et al.*, 2019; Dawood *et al.*,2020). Because of the magnitude of the challenges that the industry has faced as it grows, more novel disease control strategies are becoming urgently needed since no anti-infective treatment appears to be capable of solving every problem.

One potential approach is offered by the discovery of heat shock proteins (Hsps) with their multifunctional health-beneficial features. Hsps, also referred to as stress proteins, are a group of evolutionarily conserved proteins of varying molecular weight (16 – 100 kDa) produced in all organisms from prokaryotes to eukaryotes (Welch, 1993). Their expression in the cell is constitutive or inducible under different conditions. Functionally, they play essential roles in protein biogenesis and homeostasis including maintaining the structure of the protein, repairing damaged cytoskeleton, and folding proteins and hormone receptors. Additionally, these proteins, particularly those belonging to 70 kDa protein (i.e. Hsp70s), have been shown to elicit protective immune responses in many aquaculture animals under different pathological conditions (Tsan and Gao, 2009; Chen and Cao, 2010). In a variety of experimental models, early induction of Hsp70 was shown to induce resistance against thermal stress, osmotic stress as well as pathogen challenge (Sung *et al*., 2008; De la Vega *et al*.,2006; Delaney and Klesius, 2004). In the *Artemia* model, for instance, induction of Hsp70 by exposure of the animals to a classical stress inducer, i.e. non-lethal heat shock (NLHS) led to a significant improvement in the survival of the animal challenged with pathogenic vibrios or environmental stressor like thermal stress (Norouzitallab *et al*.,2015; Sung *et al*.,2007). Another study demonstrated that feeding *Artemia* with Hsp70 protected them against vibrio challenges and the increased protection was associated with the increase in the production level of phenol-oxidase, a major immune molecule in crustaceans, in *Artemia* (Baruah *et al*.,2011). Classical Hsp inducer i.e. NLHS is not a feasible, convenient and non-invasive approach to induce Hsp70 within aquaculture animals. It is because a slight change in temperature might give a detrimental effect on the physiological balance of animals causing mortality of cultural organisms such as happened during (lethal) temperature shock. It would therefore be needed to develop another safe, useful, and beneficial inducer of Hsps. One alternative could be the use of plant extracts or plant-based compounds such as phloroglucinol (Kumar *et al.*, 2018), pyrogallol (Defoirdt *et al.*,2013), and carvacrol (Baruah *et al*.,2017), which had shown to induce Hsps in aquaculture animals and to have positive effects in boosting the immune system against infection stress.

The herbal plant Indian ginseng is an evergreen shrub that grows in India, the Middle East, and parts of Africa. Many pharmacological studies have been carried

out to describe the multiple biological properties of Indian ginseng. These studies have shown that the plant preparation has anti-inflammatory, antistress and immunomodulatory, and adaptogenic activities (Kulkarni and Dhir, 2008). The plant is known to reduce lipid peroxidation and increased superoxide dismutase and catalase activity in animal models, thus possessing a free radical scavenging property (Panda and Kar, 1997). Owing to the multi-functional properties of the plant, I selected this herbal plant to test the following hypothesis that the herbal plant is a safe modality to control disease in aquaculture animals. To address the research question, I used the germ-free brine shrimp *Artemia*, an aquatic invertebrate, as a model organism. The germ-free system allows for avoiding the interference of the microbial communities present in the experimental system on the functional properties (e.g. Hsp induction) of the plant extract under study.

Hypothesis

Indian ginseng extract (hereafter referred to as Ash01) induces Hsp70 protecting *Artemia* from biotic and abiotic stress factors.

Aim

Using *Artemia* as a model organism, this thesis aimed to investigate:

1) Whether pre-treatment of germ-free *Artemia* with Ash01 could induce resistance in the animal against subsequent stress caused by pathogenic *Vibrio* challenge or environmental stressors.
2) Whether the potential protective effect of Ash01 was associated with the induction of Hsp70 protein in *Artemia.*

2. Literature review

An unhealthy diet is among the modifiable factors contributing to stress in farmed animals. Organisms with stress become fragile and prone to the effects of diseases caused for example by opportunistic pathogens like vibrios (Sung *et al.* 2011). Feed and feed supplements can thus be used as a tool for preventing these diseases.

2.1 Bioactive active compounds and nutrients

Bioactive compounds are nutrients and non-nutrients present in vegetal and animal sources that produce physiological effects beyond their classical nutritional properties. They are present in all foods (fruits, vegetables, and whole grain) providing health benefits beyond nutritional value like anti-inflammatory, antioxidant, antibacterial, antifungal, and antimalarial activities. Bioactive compounds such as saponins, flavonoids, amides, alkaloids, glycosides, terpenoids and phenolic compounds have been reported to be present in leaves, stems, roots, and seeds of piper species (Scott *et al.*, 2005).

Studies indicate consumption of bioactive compound-rich foods including vitamins, phenolic compounds such as flavonoids, and carotenoids have a positive effect on both human and animal health and could reduce the risk of numerous diseases, such as cancer, and diabetes (Siriwardhana *et al*., 2013). Bioactive compounds are capable of inhibiting receptor activities, and gene expression (Carbonell-Capella *et al.,* 2014).

Chemical structures of bioactive compounds affect bioavailability while antinutritional factors reduce the bioavailability of some compounds (Septembre-Malaterre *et al.,* 2018). Utilization of bioactive compounds is dependent on the digestion process as this affects their stability and availability affecting health (Carbonell-Capella *et al*., 2014).

2.2 Heat shock proteins

Ritossa in 1962 was the first to discover heat shock proteins in the salivary gland of *Drosophila* when larvae were exposed to elevated temperatures. The nature of Hsp was verified to reflect the transcriptional induction of heat shock protein genes (Tissiéres *et al.,* 1974). Hsps are found in the nucleus, mitochondria, chloroplast, endoplasmic reticulum, and cytosol of all cell types in different sub-cellular compartments. Hsps are referred to as stress proteins which up-regulate in response to stress especially heat stress (Roberts *et al.,* 2010) and other cellular stressors such as hypoxia, osmotic stress, ultraviolet and gamma radiation damaging molecular structurers of cells (Wange *et al*., 2009). Quick exposure of organisms trigger heat shock protein (De la Vega *et al.,* 2006). Hsp chaperones support the folding and translocation of nascent proteins and sorting proteins into subcellular compartment (Roberts *et al.,* 2010).

2.2.1 Induction and regulation of Hsp gene

Hsp expression is regulated by the interaction of transcription factor heat shock factors (HSFs) with heat shock elements in promoter regions Hsp genes. (Pockley., 2003). Plants and animals HSFs share structural homology (Feder and Hofmann., 1999). Vertebrates have four HSF (Pirkkala *et al.,* 2001), while *Drosophila* have one HSF (Clos *et al.,* 1990). HSF3 is specific to avian (Morimoto., 1998) whereas HSF1 and HSF2 are in all species (Sarge *et al.,* 1993). HSF2 is selective to induction during differentiation and early development whereas HSF1 is the main transcription factor regulating responses to physiological and environmental stress (Pirkkala *et al.,* 2001).

2.2.2 Heat shock protein 70

Heat shock protein 70 (Hsp70) is among the most preserved stress protein in the Hsp families (Baruah *et al.,* 2012). Hsp70 protects cells from stress and against folding (Mayer and Bukau., 2005). Studies have shown that Hsp70 aid strong adaptive immune responses against various diseases (Robert *et al.,*2003). Hsp70 has protected of cells from thermal stress or oxidative stress and prevented denaturing proteins (McDonough and Patterson., 2003)

2.2.3 Inducers of heat shock responses

Initial chemical inducers of Hsp70 are protein synthesis inhibitors puromysin and azetidine an amino acid analogue (Hightower *et al.,* 1980; Lee *et al*.,1987). These compounds result in the expression of amino-acid analogue-containing misfolded proteins prematurely which signals the induction of heat shock proteins during

stress. Additionally, stress signals from protein misfolding come from inhibitors of protein degradation of proteasome inhibitors and lactacystin and the serine protease inhibitors inducing expression of Hsp70 and other Hsps (Rossi *et al.*,1998). On the other hand, molecules that regulate inflammation activate heat shock responses by inducing Hsp70. The inflammation response is induced by signalling surge involving arachidonic acid stimulating it's release by phospholipase A_2 activating heat shock responses (Jurivich *et al.*,1996).

Vitamin C

Vitamin C also known as ascorbic acid is an important nutrient normal functioning of fish as regards to health, reproduction, and growth (Xie *et al.,* 2006). Absence of L-gulonolactone has made it impossible for teleost to synthesis vitamin C (Fracalossi *et al*., 2001). Vitamin C improves harmful effects of stress by increasing antioxidant activity induced by high temperature, improves immunity and resistance to pathogens. In fish, vitamin C lowers feed conversion ratio, and stimulates growth. This has been shown in Nile Tilapia where immunity and growth rate were positively affected after supplementation with Vitamin C (Ibrahem *et al*., 2010).

Curcumin

Curcumin is a biologically active hydrophobic polyphenol derived from rhizome of curcuma with an exceptional structure capable of trapping radical ability as an antioxidant, anti-inflammatory against metabolic, neurodegenerative, and autoimmune diseases (Aggarwal and Harikumar.,2009). It has a strong effect on cell-mediated immunity and cytokine production and has been used as a supplement in Pacific White shrimp to control diseases and improve immunity (Vanichkul *et al.,* 2010). Curcumin lessened quorum sensing-dependent virulence by 88% drop in the bioluminescence of *Vibrio harveyi* (Packiavathy *et al.*,2013) and enhanced *Artemia* survival by 67% against *Vibrio harveyi* infection (Packiavathy *et al*., 2014).

Phloroglucinol

Phloroglucinol is a polyphenol compound with anti-inflammatory and antioxidant activities. Phlorotannin compounds are oligomeric compounds of phloroglucinol units found in brown algae responsible for biological activities in Ecklonia (Yoon *et al.,* 2009). Phloroglucinol is responsible for preventing chronic diseases related to oxidative stress and inflammation (Kim *et al.,* 2010). They have also been reported to promote the expression of antioxidant enzymes such as superoxidase dismutase, glutathione in lung fibrioblasts (kang *et al*., 2006).

Inducers and co-inducers of Hsp and their co-chaperones could be a hope for protection against biotic and abiotic stressors in aquaculture although studies on their effect on aquaculture animals are still very limited.

Indian ginseng

Indian ginseng is an important herbal plant of the Indian subcontinent. It is widely used, singly or in combination, with other herbs against many diseases in humans since time immemorial and also in farmed animals for the past few decades. The plant contains a spectrum of diverse phytochemicals enabling it to have a broad range of biological implications. Several *in vitro* and *in vivo* studies have shown anti-microbial, anti-inflammatory, anti-tumor, anti-stress, neuroprotective, cardioprotective, and anti-diabetic properties (Dar and Ahmed, 2015). Additionally, it has demonstrated the ability to reduce reactive oxygen species, modulate mitochondrial function, regulate apoptosis, and reduce inflammation and enhance endothelial function (Chandrasekaran *et al.,* 2013; Grover *et al.,* 2012). In view of these pharmacologic properties, Indian ginseng is a potential healthcare candidate to treat various disease conditions in the farmed aquatic animals, particularly related to those caused by infection stress.

2.3 Brine shrimp *Artemia*

2.3.1 *Artemia* in aquaculture

Artemia is essential in commercial hatchery operations as live prey for fish in improving fish nutrition (Sorgeloos *et al.,* 2001). During the early life stages of most marine fish, *Artemia* as live feed is indispensable (Kolkovski *et al.,* 2004). *Artemia* is not only highly nutritious but also easy to use. (Sorgeloos *et al.,* 2001). *Artemia* cyst consumption has increased drastically by over 1,500 metric tonnes worldwide for both fresh water and marine feed inform of dry cysts. (Lim *et al.,* 2003). Marine fish larviculture and the ornamental industry are the main consumers of *Artemia* cyst with about 85% the rest taken up by the latter (Lim *et al.,*2003).

2.3.2 Characteristics of *Artemia*

Brine shrimp *Artemia* is an arthropod with a segmented body naturally found in salt lakes, coastal lagoon with hyper salinity. It is tolerant to salinities between 45 g/L and 200 g/L, adapts to changing temperatures ranging between 6°C to 35°C, and pH varying from neutral to highly alkaline. (Van stappen *et al.,*2002). Their ability to survive in environments with extreme temperatures and high radiations

makes it unique compared to other life forms. In unfavourable harsh conditions, *Artemia* can produce brown chorion cyst covered with strong shell capable of remaining dormant if dry. Under favourable conditions of sea water, temperature, and aeration, cysts rupture within 24 hour releasing free-swimming larvae at instar I stage which depend on reserves of the yolk. After 8 h, larvae are at Instar II where they are capable of taking up exogenous particles. Males have a larger modified antennae than females used for clasping during mating. The length of female *Artemia* range between 10-12mm while males 8-10mm as the maximum length. *Artemia* can utilize nutrients in feed ranging from 1 to 50µm such as corn bran, whey powder, rice bran etcetera (Sorgeloos *et al.,* 2001). Brine shrimp feeding characteristics makes it suitable to be used in administering nutrient, vaccines to aquatic organisms.

2.3.3 *Artemia* as a model organism

Artemia as a model organism is highly recommended by researchers to study the biology of infections, host-microbes relationship against diseases affecting crustaceans like lobsters and shrimp (Marques *et al.,* 2006). The stability of dry cyst all year round, ability to be cultured under axenic conditions, short life cycle, fast growth from nauplius to adult within 8 days makes it more advantageous to other animals for research in biology (Marques *et al.,* 2005). *Artemia* are used for studies on stress (MacRae 2003), the feed quality analysis (Marques *et al.,* 2004), interaction of host microbes (Marques *et al.,* 2006), nutrition and immune-stimulatory properties of yeast (Soltanian *et al.,* 2007), and quorum sensing (Defoirdt *et al.,* 2007).

3. Materials and Methodology

3.1 Reagents preparation

3.1.1 Sea water

A half cup of Instant Ocean® was dissolved in 15 liters of distilled water by high aeration for proper mixing overnight. To attain 35 parts per thousand (ppt) seawater, the concentrated solution was further dissolved in distilled water and thoroughly mixed in 2-L jars. A salinometer was used to obtain the required salinity of 35 ppt and the final solution was placed in 1-liter glass tubes and autoclaved at 121°C for 20 minutes. After the solution was cooled and crystals settled, the clear supernatant is transferred to autoclaved glass tubes of 40 ml under the laminar floor hood.

3.1.2 Sodium thiosulphate

Five grams of sodium thiosulphate reagent 99% (Sigma-Aldrich®) was dissolved in 500 ml of distilled water and autoclaved at 121°C for 20 minutes. The stock solution was stored at room temperature.

3.2 Ash01 extraction

Water-based extraction was employed to extract the bioactive compounds from the Ash01 plant. 90 milligrams of the powder were dissolved in 30ml of seawater within a 50ml falcon tube. The solution was vortexed for 2 mins and placed on a rotor at 4 rotations per minute for 48 h at 28°C in the dark. The solution was then filter sterilized with a non-pyrogenic sterile-R of 0.20µm using a syringe of 10ml under the laminar flow.

3.3 Bacterial culture media

Marine Broth and Marine Agar (Difco™ 2216) containing nutrients for marine bacteria were prepared by dissolving 37.4 and 55.1g respectively in 1 liter of

distilled water and thoroughly mixed. A magnetic stirrer was put in the bottle and then placed on a magnetic heater for heating until the powder was dissolved. Marine agar plates are prepared by pouring mild warm marine agar on Petri dishes until it covered the bottom. The plates were left to solidify under the laminar flow hood and then stored at 4°C.

3.4 Bacteria stock preparation

50 µl of *Aeromonas hydrophila* strain LVS3, and *Vibrio campbellii* LMG 21363 were each cultured in a 50 ml Erlenmeyer flask containing 20 ml of marine broth. Flasks were covered with a cotton cap and placed on a shaker for overnight incubation at 120 rpm at 28°C. On the following day, 1 ml of the subcultures of LVS3 and LMG21363 were separately added in a 50 ml Erlenmeyer flask containing 20 ml of marine broth. The cultures were placed on a shaker and incubated further at 28°C for 6 h. To prepare the stock solution, 6 ml of bacteria culture and 4 ml of autoclaved glycerol were added to a 50 ml falcon tube and vortexed for 1 minute. The solution was distributed in 0.5 ml Eppendorf tubes and stored at -80°C for further use (Marques *et al.,* 2005). LVS3 was used as a feed in this study (Marques *et al.,* 2005). LMG21363 is an aquaculture pathogen that was used to challenge *Artemia*.

3.5 Axenic *Artemia*

Using a procedure described by Marques *et al*. (2005), germ-free *Artemia* were obtained following decapsulation and hatching. Chorion surrounding the cysts were removed by hydrating 80 mg of cyst in distilled water for an hour using 0.22µm filtered aeration. Sterile cysts and nauplii were obtained via decapsulation using 330 µl sodium hydroxide (NaOH; 32%) to maintain pH, then shortly exposing the cysts to an aqueous solution of 5 ml sodium hypochlorite (50%) for 2 minutes. The reaction was stopped with an equal amount of 5 ml sodium thiosulfate ($Na_2S_2O_3$) when 80% of the orange cyst sank. All were carried out under the laminar flow hood and the pieces of equipment used were previously autoclaved at 121°C for 20 minutes. Rinsing was carried out by repeated washing using 2 liters of sterile seawater. A required quantity of decapsulated embryo was transferred to 50 ml falcon tubes containing 30 ml of autoclaved seawater. The tubes containing the embryos were placed on a rotor, rotating at 4 rotations per minute. The embryos were incubated for 28 h under constant illumination (2000 lux) in a temperature-controlled room. After hatching (28 hours), counting was carried out on a petri dish using a lamp where active *Artemia* at instar stage II that moved towards light were selected to make up 20 nauplii and transferred to autoclaved 40 ml glass tubes

containing autoclaved seawater. The nauplii were fed with autoclaved LVS3 and treated with different concentrations of herbal powder.

3.5.1 Axenic Hatching verification

To ensure that hatching was not contaminated by microorganisms, 100 ml of the hatching water was spread on a Marine Agar plate (Baruah *et al*., 2010) and incubated for 5 days at 28°C. Positive results were discarded.

3.6 Toxicity test

The cytotoxic effect of Ash01 was first determined in axenic *Artemia* as previously described (Baruah *et al*.,2015). Twenty germ-free *Artemia* larvae were transferred to a 40 ml sterile glass tube containing 20 ml of seawater. Larvae were pre-treated with an increasing concentration of Ash01 (200, 300, 400, 500, 1000, 1500, and 2000 mg/l) for 2 h at 28°C. Negative control was maintained without the addition of Ash01. After pre-treatment, larvae were rinsed repeatedly with autoclaved seawater (ASW) to wash away the extract as much as possible and then allowed to recover for 3 h at 28°C. After recovery, the larvae were fed with autoclaved LVS 3 at a density of 10^7 cells/ml. Five replicates were maintained for each treatment and control. The toxicity of the extract was determined by scoring the percentage of survivors after 48 h of the recovery period as previously described (Baruah *et al.*, 2015).

3.7 *Vibrio* challenge test

Based on the outcome of the toxicity test, the doses of 200, 300, 400, 500, and 1000 mg/l of Ash01 extract were selected to carry out the subsequent studies. Hatched *Artemia* nauplii at instar 2 were pre-treated with Ash01 for 2 hours. *Artemia* larvae were rinsed with autoclaved seawater at 35 ppt to wash away the extract, then allowed to recover for 3 h. The experimental group of *Artemia* (200,300,400,500, and 1000 mg/l) with 5 replicates each containing 20 larvae was fed with autoclaved LVS 3 at a density of 10^7 cells/ml and simultaneously challenged with LMG 21363 at a density of 10^6 cells/ml. The tubes were placed on a rotor at 4 rotations per minute for incubation in a temperature-controlled (28°C). Survival of the larvae was scored after 48 h of the challenge.

3.8 Thermal challenge

Germ-free *Artemia* larvae were pre-treated with Ash01 as described in the above section. After the recovery period, 20 larvae were distributed into 40 ml glass tubes each containing 20 ml autoclaved seawater. Larvae that did not receive Ash01 pre-treatment were maintained as control. The larvae were given a thermal challenge by exposure to a temperature of 41°C for 15 min. Survival of the larvae was scored 6 h after the thermal challenge Fig. 1.

Figure 1. The larvae were pre-treated with different doses of Ash01 (200,300,400,500,1000 mg/l), rinsed to wash away the compound then allowed to recover for 3 h. The larvae were subsequently challenged with lethal heat at 41°C for 15 min.

3.9 pH Challenge

Artemia nauplii were incubated at 28°C with 35 g/l of autoclaved water at a pH of 6.5 as described in the methodology. Germ-free nauplii at instar II were pre-treated with different concentrations of Ash01 as described above. Twenty *Artemia* from each treatment and control (*Artemia* that did not receive Ash01 pre-treatment) groups were distributed in 40 ml glass tubes each containing 20 ml autoclave seawater. Five replicates were maintained for all the experimental groups. A lethal pH of 5.5 was obtained by using sodium hydroxide (NaOH) or hydrochloric acid (HCl) as described by Iryani *et al*. (2022). A pH of 5.5 was used to challenge *Artemia* nauplii. *Artemia* survival was determined after 24 hours by counting live *Artemia*.

3.10 Salinity stress test

Here, the dose of Ash01 that conferred the best protective effects in the above challenge tests was used. *Artemia* nauplii. Twenty *Artemia* from each treatment and control (*Artemia* that did not receive Ash01 pre-treatment) groups were distributed in 40 ml glass tubes each containing 20 ml autoclave seawater. Five replicates were maintained for all the experimental groups. The salinity of the autoclaved seawater was maintained at 100 ppt to induce salinity stress conditions. After 48 h, the number of survived nauplii was counted.

3.11 RNA extraction and cDNA synthesis

Germ-free *Artemia* nauplii were exposed to the best dose of Ash01 as described above. After 3 h recovery period, the nauplii were sampled, rinsed in distilled water, immediately frozen in liquid nitrogen and stored at -80 °C for hsp70 gene expression analysis. Unexposed *Artemia* nauplii were used as a control group. Three replicates were maintained for each group. Qiagen RNeasy plus Mini kit was used to extract RNA from the sampled *Artemia* samples according to the manufacturer's instructions. Confirmation of the quality and quantity of RNA was done by NanoDropTM 8000. Using RevertAid H Minus First Strand cDNA synthesis kit (Thermo Scientific, Sweden), reverse transcription from 1 µg total RNA was done following the manufacturer’s guidelines.

3.11.1 Assay of *hsp70* gene

The expression of *hsp70* gene in the sampled *Artemia* nauplii was analysed by RT-qPCR using a pair of specific primers (see Table 1). RT-qPCR assay was performed on step one Real-Time system (Applied Biosystems) in a total volume of 25 µl containing 0.4 µl of both forward and reverse primers, 12.5 µl of SYBR green master mix, 9.7 µl of nuclease-free water and 2 µl cDNA. A four-step amplification protocol was followed: initial denaturation (10 min at 95℃); 40 cycles of amplification and quantification (15 s at 95°C, 30 s at 60° C, and 30 s at 72 °C); melting curve (55–95°C with a heating rate of 0.10°C s-1 and a continuous fluorescence measurement) and cooling (4°C). The *β-actin* gene was used as a reference gene. Master mixes were prepared in two technical replicates for three biological replicates of each sample and quantitative PCR for target and reference genes was performed. To analyze the level of expression of target genes, the comparative CT method ($2^{-\Delta\Delta Ct}$ method) following Livak and Schmittgen (2001) was used.

Table 1. primers used in RT- PCR

(Primer name)	Primer sequence (5′-3′)
actin	F: AGCGGTTGCCATTTCTTGTT R: GTCGTGACTTGACGGACTATCT
hsp70	F: CGATAAAGGCCGTCTCTCCA R: AGCTTCAGGTAACTTGTCCTTG

3.12 Statistical analysis

Data were subjected to a one-way analysis of variances followed by Duncan's multiple range tests using IBM statistical software SPSS version 27.0 to determine significant differences between the treatments. The expression level in the control group was regarded as 1.0 and thereby the expression ratio of the treatments was expressed in relation to the control. Analysis for significant differences in the gene expression levels between the control and treatment was performed using an independent sample's T-test. The expression levels were expressed concerning the control with the control group as 1.0. *P* values <0.05 were considered significant.

4. Results

4.1 Toxicity of Ash01 on *Artemia* nauplii

In the first experiment where we investigated whether Ash01 has cytotoxic effects on axenic *Artemia* larvae, the larvae were exposed for 48 h to an increasing concentration of Ash01. The nauplii were not subsequently challenged with any biotic or abiotic stressor. There was a significant difference between the negative control and the treatment groups exposed to a dose of Ash01 higher than 1500 mg/l, with the latter groups having lower survival compared to the control. No significant difference in survival was noted between the negative control and the groups exposed to Ash01 in the dose range between 200 and 1000 mg/l. In all the groups survival of more than 80% was recorded (Figure. 2).

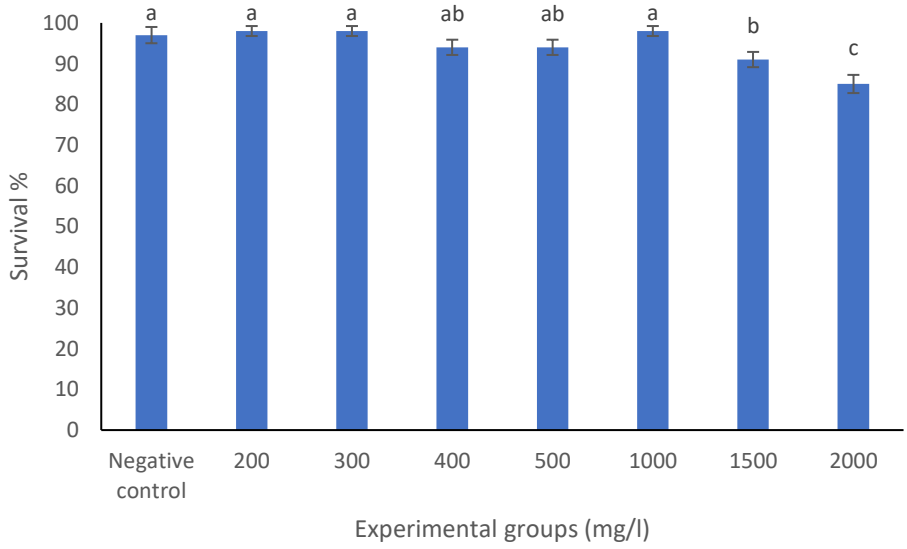

Figure 2. Toxicity of Ash01 to Artemia larvae. The larvae were pre-treated with Ash01 at different doses (200, 300, 400, 500, 1000, 1500, 2000 mg/l), rinsed to wash away the compound then allowed to recover for 3 h. The larvae were subsequently fed with LVS 3 at 10^7 cells/ml of rearing water. Non-pretreated larvae served as control. Survival was scored 48 h after the recovery period. Error bars represent the standard error of five replicates. Bars with the same alphabet letters are not different significantly (P> 0.05, Duncan's new multiple range test).

4.2 Effect of Ash01 on the survival of *Artemia* challenged with *V. campbellii* LMG 21363

In the second experiment, we investigated whether Ash01 could confer protection prophylactically against LMG 21363 to *Artemia* larvae. Larvae were pre-treated with Ash01 at different concentrations as shown in Figure.3. In comparison to the positive control that received no Ash01 pre-treatment but was challenged, groups that were exposed to Ash01 with a dose higher than 300 mg/l, all had significantly higher survival. *Artemia* nauplii that received the dose of 1000 mg/l of Ash01 had the highest percentage of survival.

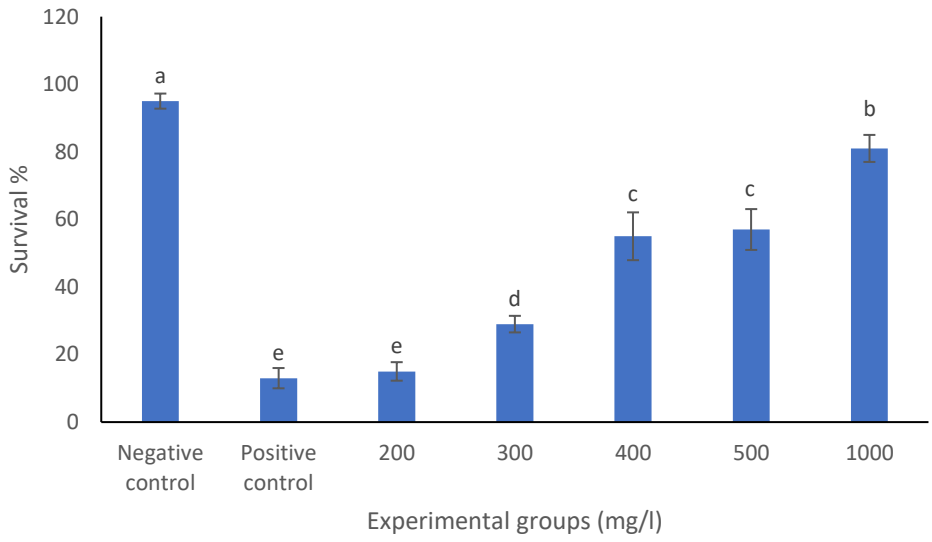

Figure 3. Effect of Ash01 pre-treatment on survival of Artemia after 48 h challenge with V. campbellii LMG 21363. The larvae were pre-treated with different doses of Ash01 as described in Fig. 1. Non-pretreated larvae served as control. The larvae were subsequently fed with LVS 3 at 10^7 cells/ml and challenged with V. campbellii LMG 21363 at 10^6 cells/ml of rearing water. Non-pre-treated larvae that were either challenged with V. campbellii LMG 21363 (positive control) and those not challenged with V. campbellii LMG 21363 (negative control) served as controls. Error bars represented the standard error of five replicates. Bars with the same alphabet letters are not different significantly (P> 0.05, Duncan's new multiple range test).

4.3 Effect of Ash01 on the survival of *Artemia* challenged with thermal shock

Next, I tested how *Artemia* nauplii pre-treated with different Ash01 concentrations would respond to a temperature shock that is lethal for *Artemia* grown at standard

temperature. It was found that on exposure to a typically lethal stress (41°C for 15 min), *Artemia* nauplii that were pre-treated with Ash01 at 1000 mg/l concentration had a survival of 28% compared to positive control with a survival of 17% although the difference between the two experimental groups was not significantly different (Figure. 4).

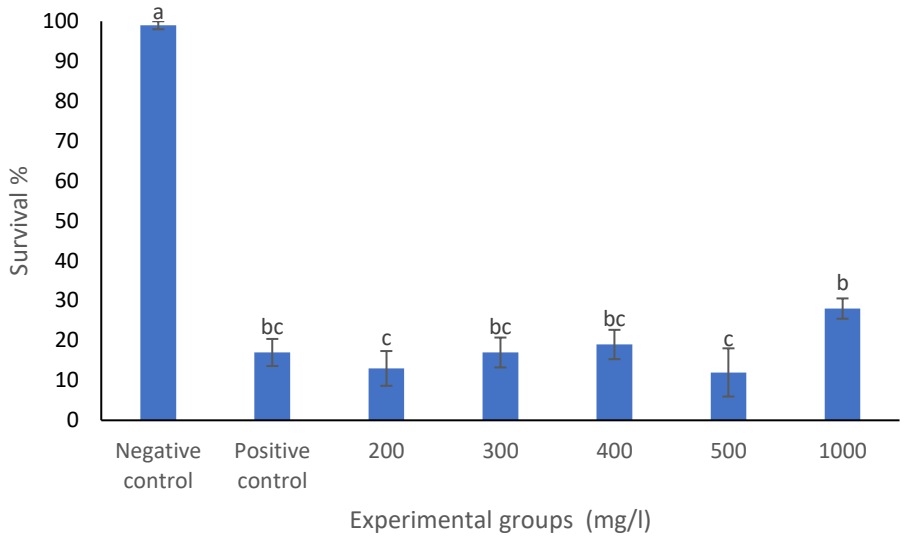

Figure 4. Effect of Ash01 pre-treatment on survival of Artemia after 6 h of lethal heat stress. Ash01 pre-treatment and thermal challenge. The larvae were pre-treated with different doses of Ash01 as described in Fig. 1. The larvae were subsequently challenged with lethal heat stress at 41°C for 15 min. Larvae that served as controls were either challenged with thermal stress (positive control) or not (Negative control). Non-pre-treated larvae that were either challenged with lethal heat stress (positive control) and those not challenged with lethal heat stress (negative control) served as controls. Error bars represent the standard error of five replicates. Bars with the same alphabet letters are not different significantly (P> 0.05, Duncan's new multiple range test).

4.4 Effect of Ash01 on the survival of *Artemia* reared in a low pH condition

In the next experiment, we used a pH of 5.5 as a challenge to determine whether Ash01 could induce tolerance of *Artemia* nauplii in a low pH condition. Different doses as shown in Figure.5. Results after 24 hours of challenge showed a significant increase in the survival of *Artemia* nauplii in response to pre-treatment with Ash01 dose of 300 mg/l and higher. *Artemia* nauplii exposed to 1000 mg/l Ash01 dose exhibited the highest survival.

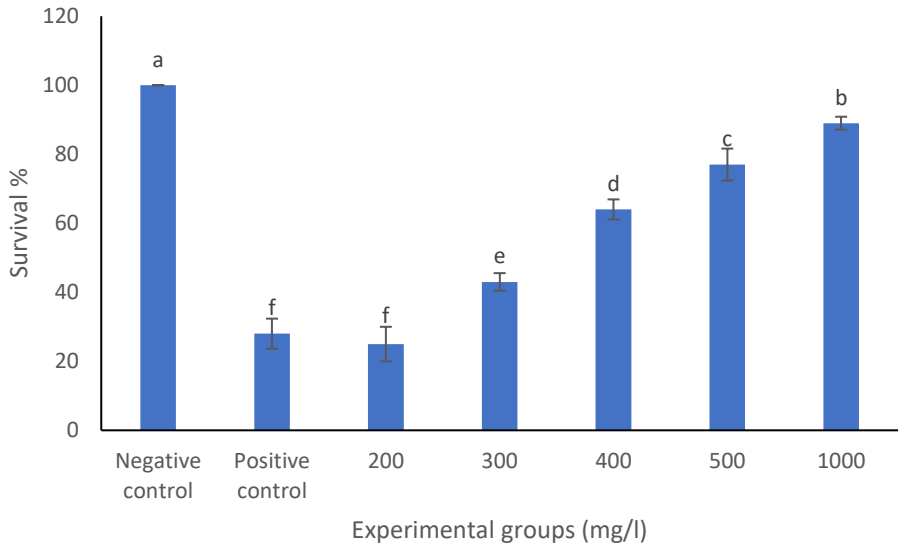

Figure 5. Effect of Ash01 pre-treatment on survival of Artemia after 24 h pH challenge. The larvae were pre-treated with different doses of Ash01 as described in Fig. 1. Replicates were challenged by adjusting the pH of the water to 5.5. Larvae that were in rearing water with a pH of 5.5 (positive control) and in rearing water with pH 6.5 (Negative control). The standard error of 5 replicates is represented by error bars in alphabet letters. Error bars represent the standard error of five replicates. Bars with the same alphabet letters are not different significantly (P> 0.05, Duncan's new multiple range test).

4.5 Effect of Ash01 on the survival of *Artemia* challenged with salinity stress

Results from the above three experiments (LMG challenge, thermal shock, and pH challenge) showed that 1000 mg/l provided maximum protection to *Artemia*. Next, the best Ash01 dose of 1000 mg/l was selected to verify if could confer protection to *Artemia* against salinity stress(100ppt). As shown in Figure 6, Ash01 offered protection to *Artemia* against salinity stress. The dose of 1000 mg/l significantly improved the survival of *Artemia* nauplii by 1.7 folds compared to the positive control

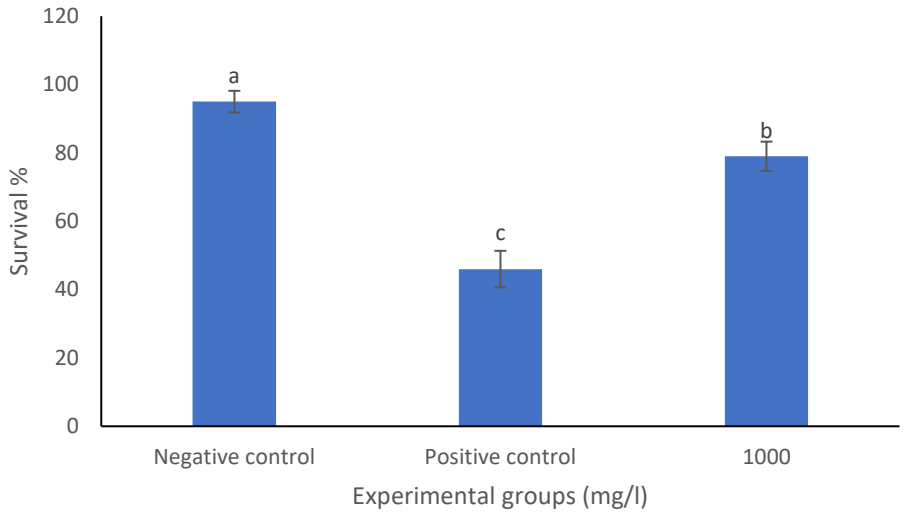

Figure 6. Effect of As-01 pre-treatment on survival of Artemia after 24 h challenge with salinity. The larvae were pre-treated with different doses of Ash01 as described in Fig. 1. Replicates were challenged with salinity at a pH of 2/l. The larvae were subsequently challenged in rearing water with a salinity of 100 ppt. Larvae that were reared in water at 100 ppt (positive control) and those reared in water at 35 ppt (negative control). Error bars represent the standard error of five replicates. Bars with the same alphabet letters are not different significantly (P > 0.05, Duncan's new multiple range test).

4.6 Mechanisms behind the protection of *Artemia*

I sampled *Artemia* that were given 1000 mg/l of Ash01 pre-treatment and measured Hsp70. There was a numeric increase in the expression of *hsp70* genes by 3.6 folds (mean = 3.6767) although it was not significantly different P > 0.05 (figure. 7).

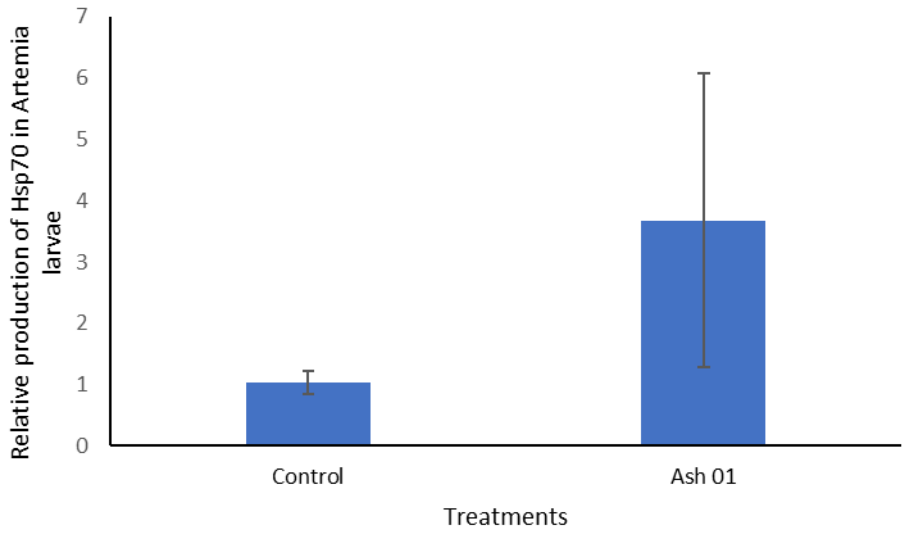

Figure 7. Induction of HSP70 protein in Artemia larvae. Artemia larvae were pretreated with Ash01 at a dose of 1000 mg/l as described in fig1. Artemia samples were immediately frozen in liquid nitrogen and then stored at -80°C. Unexposed Artemia were used as control. Three replicates were maintained for each group. Qiagen RNeasy Plus Mini kit was used to extract RNA from the sampled Artemia samples according to the manufacturer's instructions. Confirmation of the quality and quantity of RNA was done by NanoDropTM 8000. Using RevertAid H Minus First Strand cDNA synthesis kit (Thermo Scientific, Sweden), reverse transcription from 1 µg total RNA was done following the manufacturer's guidelines.

5. Discussion

Stress caused by environmental factors and pathogenic biotic agents is one of the major problems in the sustainable production of farmed fish. All these factors interact together to cause disease outbreaks which lead to significant economic losses. Owing to the multifunctional properties of Hsp70, this stress protein has become a molecular target for the development of an anti-infective strategy in aquaculture animals. In my study, I focused on a potential Hsp70 inducer from natural sources as has been done previously by other researchers who in their studies found a strong positive correlation between Hsp production and stress tolerance in terrestrial (Segal *et al.*, 2006; Pockley 2003; Moseley 2000) and aquatic animals (Deane & Woo 2005; Ackerman & Iwama 2001; Forsyth *et al.*, 1997). In the *Artemia* model, for instance, the bioactive compound pyrogallol was shown to induce Hsp70 by its prooxidant action (e.g., generating peroxide like H_2O_2). It was also noted that the compound-mediated Hsp70 elicits the prophenoloxidase and transglutaminase immune responses in *Artemia* against *V. harveyi* (Baruah *et al.*, 2015). Similar responses were reported when an extract from prickly pear fruit was added to the rearing water of *Artemia* triggering factors responsible for causing Hsp70 induction within *Artemia* (Baruah *et al.*, 2014). When the same cactus extract was used on carp fingerlings, an increase of Hsp70 in the gills and muscles resulted in the protection of fish from ammonia stress (Sung *et al.*, 2012). The extract from the prickly pear also showed similar results on *Artemia* with the enhancement of Hsp70 which protected against temperature stress. (Baruah *et al*., 2012).

In my study, I used germ-free *Artemia* as a biological model to explore the Hsp70-inducing ability of the herbal plant of interest originating from Asia - Indian ginseng, designated as Ash01 in our study. It is because *Artemia* can be reared under germ-free conditions (allowing full control over the host-associated microbial communities, (Marques *et al*., 2006), and that allows to distinguish direct effects of Ash01 on the host [by (pre) exposing axenic *Artemia* larvae to Ash01 for a certain duration] from indirect effects. It also eliminates any possible microbial interference during the exposure period and hence facilitates the interpretation of the results in terms of a cause-effect relationship (Baruah *et al.,* 2014). In this study, it was found that Ash01 tend to increase the expression of hsp70 gene in *Artemia* by a fold of 3.5 in comparison to the control. The difference in the expression level

between the treated and control groups, however, was not significant. There is a possibility that *hsp70* gene has already been translated into protein by the time there is an expression of the gene (Silver and Noble, 2012). However, in my study, we did not measure protein.

The inducible Hsp70 was reported to play a key role in defining the tolerance of organisms to several stressors. For instance, in previous studies, induction of Hsp70 in aquatic animals upon exposure to a classical Hsp70 inducer, i.e., non-lethal heat stress or bioactive components significantly improved the resistance of the animal toward subsequent environmental or pathogenic biotic stress (Baruah *et al*., 2012, 2014, 2015b; Boerrigter *et al*., 2014; Sung *et al*. 2012). A similar increased tolerance to extreme temperatures or pathogens, following induction of Hsp70, has also been demonstrated in other organisms, such as bacteria, coelenterates, molluscs, fish, shrimp, echinoderms, and humans (Iryani *et al.*,2022; Baruah *et al*., 2013; Iwama *et al*., 1998; Roberts *et al.*, 2010). This improved resistance was because Hsp70 plays a crucial function as a molecular chaperone and is involved in protein biogenesis and protein homeostasis in the cells or it contributes to the generation of protective immune responses in the host (*Baruah et al*., 2014; Clegg *et al*., 2000). In my study, the results showed that Ash01 tends to induce some level of protective effect in *Artemia* against a lethal temperature of 41°C for 15 min. However, it should be noted that this effect appeared to be dose-dependent, with maximum protection at 1000 mg/l Ash01. These results suggest that Hsp70 is behind the moderate improvement in the survival of heat-stressed *Artemia*. In a similar study on shrimp where a combination of non-lethal heat shock (37°C) followed by a lethal heat shock (42°C) for 30 mins was applied, Hsp70 synthesis was induced in *Artemia* larvae and that resulted in an increased thermotolerance (Sung *et al*.,2008). A similar increased tolerance to a variety of environmental stress as well as pollutants in response to exposure to non-lethal heat exposure has been reported in *Artemia* as well as other farmed aquatic organisms including white-leg shrimp, common carp, pacific oysters, and Asian green mussels (Iryani *et al.*, 2022; Pestana *et al.*, 2016; Aleng *et al.*, 2015; Sung *et al*., 2014; Baruah *et al.*, 2012). The increased tolerant phenotypes was associated with the increased in the production of Hsp70 in the organisms (Iryani *et al.*, 2022; Pestana *et al.*, 2016).

It is noteworthy to mention that in the first experiment, I conducted a range-finding test to investigate the dose range at which Ash01 that are safe for application for potentially inducing protective responses in the host *Artemia* against infection stress. Results showed that *Artemia* larvae that received pre-treatment of Ash01 in the dose range of 200 – 1000 mg/l, the survival and swimming activity of the larvae were not significantly affected when compared with the control (Figure. 1). The results suggested that exposure to Ash01 with doses between 200 – 1000 mg/l was not lethal to *Artemia* at least in the described experimental conditions. These doses

were therefore selected to verify whether Ash01 could confer protection against biotic and abiotic stress factors.

Using this range, I also tested the protective effect against pH stress in *Artemia* and found that Ash01 at 1000 mg/l dose was the best to confer maximum protection to *Artemia* against pH stress. Additionally, the same dose of Ash01 was also shown to induce maximum protection in *Artemia* against salinity stress as well as *V. campbellii* challenge. From this compendium of evidence, it can be suggested that the production of Hsp70 during pretreatment is at least in part responsible for inducing tolerance in *Artemia* against a wide range of environmental stressors as well as *V. campbellii* challenge. It is also likely that other members of Hsps (Hsp40, Hsp60, Hsp90) are induced by Ash01, and these proteins in conjunction with Hsp70 are responsible for causing the observed protective effects. In a study carried out on angel fish, Hsp70 and Hsp90 levels in the liver, muscles, and gills of pre-exposed fish with cactus extract raised (Camilleri, 2002) which implies that there is a possibility that other Hsps might be induced by Ash01. Further studies should be carried out to determine the production of these stress proteins both at the gene and protein levels.

6. Conclusion

In conclusion, the results from my study provided evidence suggesting that Ash01 is a potential enhancer of Hsp70, and this natural functional ingredient was shown to be effective in generating protective responses against both biotic and abiotic stressors, suggesting that such kind of nutraceutical can be a part of a disease management strategy. Further studies should be carried out to analyze the potential roles of other Hsp molecules by measuring both at the gene and protein levels, and verify whether the interaction of these Hsp molecules is involved in conferring protection to Ash01 pretreated *Artemia* against a wide range of stressors. Also, the immune sysem of *Artemia* could be influenced by Ash01 as Hsp70 has a positive influence on the generation of immune response an assumption that warrants further verification by measuring effector molecules in *Artemia*.

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Popular science summary

Stress is a major problem in aquaculture causing diseases and affecting the sustainable growth of the aquaculture industry. This stress can be abiotic or biotic. Antibiotics are traditionally used to overcome disease problems in aquaculture. However, their indiscriminate uses have led to the development of resistant strains, which is becoming a global public health concern. Over the past few years, there has been a growing interest in using nutraceuticals derived from plant sources capable of inducing immunogenic Hsp70 molecule as an alternative to antibiotics. Nutraceuticals are natural biological components that possess health-beneficial properties in addition to the basic nutritional value. Previous studies have shown a positive correlation between inducible Hsp70 and robustness phenotypes in farmed animals. In my study, Indian ginseng extract (Ash01) was used to examine whether it could be used as a natural strategy to induce Hsp70 in aquaculture animals to subsequently improve tolerance towards a variety of stressors aquaculture animals commonly encounter. I used the brine shrimp *Artemia* as a model organism to address my research question.

First, I used different doses of the extract to test for its toxicity. It was found that the dose range between 200 and 1000 mg/l of Ash01 was not toxic to *Artemia* host. Using this dose range, I carried out subsequent study to test whether Ash01 could give protection to *Artemia* host against a biotic stressors or abiotic stressors. I selected temperature, pH, and salinity as abiotic stressor because they are often responsible for triggering stress, which result in causing disease in aquaculture animals. Results showed that pretreatment of *Artemia* with Ash01 caused a significant improvement in the tolerance of the animals towards biotic and abiotic stressors. It is likely that inducible Hsps are involved in inducing robustness in the host *Artemia*. These results add new information about the functional properties of Ash01 and advance our knowledge of this herbal plant as potential prophylactic agent for controlling diseases in aquaculture animals.

Further studies should be carried out to analyze the potential roles of other Hsp molecules by measuring both at the gene and protein levels and verify whether the interaction of these Hsp molecules is involved in conferring protection to Ash01-pretreated *Artemia* against a wide range of stressors. Also, the immune system of *Artemia* could be influenced by Ash01 as Hsp70 has a positive influence on the

generation of the immune response, an assumption that warrants further verification by measuring the immune effector molecules in *Artemia*.

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Appendix 1

Toxicity		
Percentage		
Final-active	Final-NA	Total survival
95	5	100
90	5	95
95	5	100
90	10	100
90	0	90
95	0	95
100	0	100
95	0	95
100	0	100
100	0	100
100	0	100
100	0	100
95	0	95
90	10	100
95	0	95
100	0	100
95	0	95
90	0	90
80	10	90
90	5	95
95	0	95
90	0	90
85	5	90
95	0	95
100	0	100
100	0	100
95	0	95
100	0	100
100	0	100
95	0	95
90	0	90
95	0	95
80	5	85
95	0	95
85	5	90
65	15	80
90	0	90
75	10	85
80	0	80
85	5	90

Appendix 2

	Themal challenge	
Percentage		
Final-active	Final-NA	Total survival
100	0	100
100	0	100
100	0	100
100	0	100
95	0	95
10	5	15
15	10	25
5	0	5
15	5	20
15	5	20
15	5	20
5	15	20
5	0	5
10	10	20
0	0	0
5	10	15
5	5	10
5	5	10
10	20	30
0	20	20
15	15	30
5	20	25
10	0	10
10	5	15
15	0	15
20	15	35
0	0	0
5	0	5
10	0	10
5	5	10
20	5	25
10	10	20
25	5	30
15	15	30
15	20	35

Vibrio Challenge		
Percentage		
Final-active	**Final-NA**	**Total survival**
90	0	90
90	0	90
95	0	95
100	0	100
100	0	100
0	5	5
0	10	10
0	10	10
5	15	20
5	15	20
5	15	20
10	20	20
5	10	15
5	20	15
0	5	5
10	25	35
20	5	25
15	10	25
15	10	25
30	5	35
35	15	50
35	25	60
40	30	70
20	45	65
20	10	30
25	10	35
40	15	55
45	20	65
30	30	60
45	25	70
70	10	80
60	15	75
55	15	70
70	20	90
70	20	90

Appendix 3

pH challenge		
Percentage		
Final-active	Final-NA	Total survival
100	0	100
100	0	100
100	0	100
100	0	100
100	0	100
10	35	45
10	15	25
5	15	20
10	10	25
10	15	25
10	15	25
10	20	30
10	10	20
10	30	40
15	5	10
20	30	50
20	5	45
15	10	40
15	10	45
15	5	35
35	25	60
35	35	70
50	20	70
45	20	65
35	20	55
50	25	75
50	35	85
55	35	90
45	25	70
45	20	65
60	30	90
75	20	95
55	35	90
55	30	85
65	20	85

Salinity stress test		
Percentage		
Final-active	Final-NA	Total survival
85	0	85
90	0	90
100	0	100
100	0	100
100	0	100
20	35	55
15	15	30
25	35	60
20	25	45
20	20	40
55	20	75
55	25	80
45	30	75
65	30	95
50	20	70

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