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The Use of Oxolinic Acid to Enrich *Artemia urmiana* from Urmia Lake, Iran

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Abstract: The live food enrichment technique using emulsions for preventing fish diseases was investigated as a tool for transferring therapeutics through the food chain. Nauplii and adult of the brine shrimp, *Artemia urmiana* were enriched with Oxolinic Acid (OXA) as an antibacterial drug to determine its content after different enrichment intervals. Various concentrations of OXA ranging from 25, 50, 75 and 100 mg L^{-1} were mixed with emulsion oil. Enriched nauplii and adult were sampled at 2, 4, 6, 8 h intervals. An analytical method for the quantification of OXA in a biological matrix, *A. urmiana* nauplii and adult, was developed using High Performance Liquid Chromatography (HPLC). The highest and lowest accumulation rate of OXA in enriched nauplii body tissue occurred at 100 and 75 mg L^{-1} at intervals 6 and 8 h, respectively and for adult at was 50 and 25 mg L^{-1} at 4 and 8 h enrichment period, respectively. The average amounts of this were estimated to be 39 and 2.5 μg g^{-1} WW, respectively for nauplii and 192 and 3 μg g^{-1} WW, respectively for adult of *A. urmiana*. The obtained results showed that there is a significant outcome between enrichment dosage and period with content of OXA in both *A. urmiana* nauplii and adult ($p<0.001$). However, a direct correlation could not be observed between enrichment dosage and period with OXA level in all enriched groups.

Key words: *Artemia urmiana*, oxolinic acid, enrichment dosage and period

INTRODUCTION

Various bacterial diseases produce many problems in fish culture such as massive mortalities and economic loses. Therefore, antimicrobial agents are widely used to prevent and treat bacterial diseases in fish cultures (Trust, 1986; Alderman, 1988). Treating microbial infections in fish and shrimp larvae is most often done by dissolving relatively high doses of broad spectrum antibiotics in the culture water or or mixing them with food (Brown, 1989; Yanong, 2006). A disadvantage of this method is the large amounts of expensive drugs which are used and discharged in the environment, placing animal and human health at risk (Cabello, 2006). Traditional methods to control microbial fish diseases are inefficient and impose high costs of the drug waste and treatment repetitions on farmer (Leger *et al*., 1986; Alderman, 1988; Samuelsen *et al*., 1999).

Nowadays, a direct treatment is available through the food chain, which uses much smaller quantities and is far more effective to control fish disease and safer for the environment than other traditional methods. Among live food organisms, the brine shrimp *Artemia* as filter feeder has successfully been used as a biological carrier for transferring essential nutrients to predator larvae (Leger *et al*., 1986; Stottrup and McEvoy, 2004). This is achieved through enrichment or bioencapsulation techniques (Gapasin *et al*., 1996; Aguilar-Aguila *et al*., 1994). This technique is a suitable and controlled method to deliver pharmaceutical agents into cultured organisms and to prevent the manifestation of many problems which occur when using traditional methods (Nelis *et al*., 1991; Duis *et al*., 1995; Touraki *et al*., 1999).

Artemia nauplii are used as an essential food source in fish and crustacean larviculture and *Artemia* biomass is used in adult fish and shrimp cultures (Sorgeloos, 1973). Noshirvani *et al*. (2006) reported enriching freshly hatched *Artemia urmiana* nauplii with Ascorbic Acid (AA) and the AA concentration was determined during various enrichment dosage and periods. Further, *Artemia*

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enrichment with pharmaceutical agents and its application in bacterial diseases inhibition was reported by Verpraet *et al.* (1992), Dhert *et al.* (1993) and Chair *et al.* (1996).

Oxolinic acid belongs to the quinolons group, which is more effective on gram negative bacteria and is widely used to control infectious disease in the aquaculture industry (Varvarigos, 2003). The aim of the present study was to enrich *A. urmiana* nauplii and adult with Oxolinic Acid (OXA) as antibacterial pharmaceutics and to determine the variation of OXA content in *A. urmiana* body during various enrichment dosages and periods.

MATERIALS AND METHODS

Artemia enrichment: *A. urmiana* cysts were hydrated and decapsulated according to the method proposed by Van Stappen (1996). Decapsulated cysts were incubated in a 2-L conical incubator containing natural filtered sea-water (30 g L^{-1}) at 28±1°C under strong aeration and illumination (2000 lux) according to the method suggested by Sorgeloos (1997). After 24 h, Instar I nauplii were harvested and separated from hatching debris and thoroughly rinsed. A part of the freshly nauplii were transferred into conical beakers and remainder were reared till adult stage (density 400 individuals mL^{-1}, salinity 30 g L^{-1}) and kept for 2, 4, 6 and 8 h enrichment. The enrichment medium consisted of a commercial live food (INVE®) into which various levels (25, 50, 75 and 100 mg L^{-1}) of oxolinic acid (Sigma®) was incorporated. The 25 individual mL^{-1} of nauplii and adults were enriched with aeration at 28±1°C for various intervals (2, 4, 6 and 8 h). Sampling was done at the end of each enrichment period. Nauplii and adults were taken after each interval, rinsed in ambient freshwater to remove excess surface oxolinic acid from their bodies.

Oxolinic acid analysis (OXA analysis): A total mass of 500 mg of each nauplii and adult from each enrichment and interval was weighed; all specimens were homogenized in methanol. After centrifugation the residue was separated from the methanol fraction. This step was repeated twice on the residue. The three methanol fractions were combined. The interfering lipids and caretenoids were removed from the methanol fraction by a double hexane extraction at pH 7.0. Samples were kept frozen (-20°C) until examination for OXA determination. Using the autoinjector, 20 μL of aqueous extract was injected into the column. A Knauer® High Performance Liquid Chromatography (HPLC) system was used to determine the OXA levels in *A. urmiana* nauplii and adults. The system consisting of a K-1001 pump, an injection valve model D-14163 fitted with a 20 μL injection loop. A variable wavelength UV-Vis detector 2501 and Model V 7566 version 0696 interface box. The system was driven by a personal computer using EuroChrom® 2000 HPLC software for Windows®. The analytical cartridge (Knauer® 125×4 mm I.D.) was packed with 5 μm Spherimage® 80 and ODS as end capping sorbent (Merk®). The mobile phase (PH 4.01) used comprised of 30% methanol and 70% water and detection of OXA was made at a wavelength 254 nm. Oxolinic acid contents were expressed per gram Wet Weight (WW). Three replicates from each enrichment and intervals were examined and OXA levels in all specimens determined as an average of obtained values for each treatment. The statistical differences between means and homogeneity of variance were evaluated by one-way ANOVA and Laven`s test. All analysis was performed using the Statistical Package for the Social Sciences (SPSS® V.11.5).

RESULTS AND DISCUSSION

The role of feeding aquacultural organisms with antibacterial pharmaceutics, vaccines and hormones incorporated into the live foods especially via *Artemia* nauplii and adults facilitates the remedy of bacterial diseases in fish species (Joosten *et al.*, 1995; Campbell *et al.*, 1993; Skjermo *et al.*, 1995). Enrichment of *A. franciscana* nauplii with Sarafloxacin at various dosages (1, 5, 15, 20 and 40%) at 2-24 h intervals to control four *Vibrio* strain through Antibiotic Susceptibility Test was carried out by Dixon *et al.* (1995). They concluded that 15% dosage at 6 h enrichment period was effective against four Vibrio strains. Dhert *et al.* (1993) demonstrated the efficiency of sulphadrugs agents against *Vibrio angillarum* through feeding sea-bass and trout larvae with enriched *Artemia* nauplii. The oral feeding of larvae fish after 4 h was effective against *V. angillarum*.

As, no evidence has been published on *A. urmiana* to explain the variation of OXA content during enrichment, then, the present study was concentrated on to introduce optimum dosage of OXA as antibacterial agent to control bacterial diseases in fish species through enrichment of it at various dosage and intervals with *A. urmiana* nauplii and adults. In this research, *A. urmiana* has shown a different process of variation in OXA concentration during enrichment periods. The highest accumulation rate of OXA in nauplii occurred in the 100 mg mL^{-1} dosage with 6 h enrichment time. In contrast, the lowest accumulation rate of OXA in nauplii was in the 75 mg mL^{-1} dosage with 8 h enrichment time. The accumulated amounts of OXA in 6 and 8 h enrichment time were estimated to be 39 and 2.5 μg g^{-1}

WW, respectively. Mohney *et al.* (1990) studied the *A. franciscana* nauplii enriched with Romet-30® and they concluded that the higher absorbance rate of this drug in nauplii occurred at the 3 mg L^{-1} dosage with 4 h interval to be 0.1 μg $nauplii^{-1}$. In comparison, the highest and lowest OXA accumulation rate in adults body tissue was in dosage 50 and 25 mg mL^{-1} at intervals 4 and 8 h, respectively. The accumulated amounts of OXA in 4 and 8 h enrichment time were estimated to be 192 and 3 μg g^{-1} WW, respectively.

The results of the present study revealed that there is a significant outcome between enrichment dosages and intervals with OXA concentration in *A. urmiana* nauplii and adult of ($p<0.001$). Although, the obtained results was not in accordance to the findings of Aguilar-Aguila *et al.* (1994) in which there was a dependence among accumulation rate of Romet-30® with enrichment dosage and periods. Their results suggest that there is a possible saturation threshold for OXA in *A. urmiana* nauplii under certain enrichment dosages and periods. As many quantitative analysis methods have been developed to assay different pharmaceutical agent's concentrations in *Artemia* nauplii (Nelis *et al*., 1991) then, we can indicate that pharmaceutical agents accumulation rates not only depend on enrichment time and period, but also involves other parameters such as nauplii density, species and enrichment condition. Therefore, further investigations regarding using of different enrichment methods to detect OXA inhibitory concentration via *Artemia* as a biologic carrier against fish and shrimp bacterial diseases are recommended.

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