

Effects of oregano (*Origanum vulgare*) leaf extract on early life stages of *Artemia salina*

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Abstract. This study aims primarily to describe the effect of *Origanum vulgare* leaf extract on the early developmental stages of *Artemia salina*. Four different concentrations of the leaf crude extract: 25 μL , 100 μL , 500 μL , and 1000 μL were prepared. *A. salina* larvae were exposed to different extract concentration at increasing exposure time (12 hours, 24 hours, 48 hours, and 96 hours). The results showed that exposure to high crude extract concentration (100 μL , 500 μL and 1000 μL) inhibited the normal development of *A. salina*. Degeneration of the second antennae (which is responsible for the filtrations of the ingested foods) was apparent after 48-hour exposure to 500 μL concentration. 100% mortality rate was observed at 1000 μL concentration, both on its 48th hours and 24th hours of exposure. These results suggest that increasing the time of exposure to high concentration of the extract would increase the rate of both morphological defects and mortality.

Key Words: *Artemia salina*, exotoxicological studies, nauplii, *Origanum vulgare*.

Introduction. *Artemia salina* (brine shrimp) is an aquatic crustacean that inhabits hypersaline environments and is utilized as an excellent food source for recently-hatched fish larvae. *A. salina* produce cysts which are able to remain in hibernation-like state and are inactive as long as they are kept off from water. Once placed in salt water with proper conditions, the *A. salina* cyst will hatch within twelve to forty-eight hours and will measure less than 0.5 mm. At this point, series of developmental stages will occur in consecutive order: cyst, nauplius, semi-adult, and full adult. The nauplius stage takes around one week to complete, and will reach full development usually within three weeks (Schumann 1995). The nauplii of *A. salina* are often used for the toxicity evaluation of environmental pollutants and in ecotoxicological studies.

The easy hatching from dry cysts and year-round worldwide availability of these organisms can be used in determining the plant toxicity through estimating the medium lethality concentration LC_{50} . Since the nauplii of *A. salina* do not require any maintenance or stock culturing ergo, it has a unique advantage over all other zooplankters (Vanhaecke et al 1987) and are considered as the most convenient test organisms for toxicity studies, due to their way of non-selective filter feeding that makes them vulnerable to the attack of toxic substances (Dong & Zhou 2012).

Brine shrimp lethality test (BSLT) is a simple, high throughput cytotoxicity test of bioactive chemicals and natural products (Meyer et al 1982). This test is also a convenient monitor for screening and fractionation in the discovery of bioactive natural products (McLaughlin & Rogers 1998) and this also represents a rapid, inexpensive and simple bioassay for testing the plant extract lethality which in most cases correlates reasonably with cytotoxic properties (Baravalia et al 2012).

Lamiaceae is one of the most diverse and widespread plant families in terms of ethnomedicine and its medicinal value is based on the volatile oils concentration (Sarac & Ugur 2007). Phytochemicals such as alkaloids, saponins, flavonoids, glycosides and phenols are deemed to be the basis for the therapeutic usage of the plants. *Origanum vulgare*, also locally known as *oregano* or *kalabo*, is a perennial herb with highly aromatic

leaves under Lamiaceae family. Aromatic plants, such as *O. vulgare*, are sources of bioactive compounds (Christaki et al 2012). This plant has been widely studied for its antioxidant, antifungal, antibacterial and antimicrobial properties (Raduđienė et al 2005; Cleff et al 2010; Saeed & Tariq 2009).

Although *A. salina* is one of the most valuable and widely used for ecotoxicity testing (Rajabi et al 2015), the impact of the *O. vulgare* leaf extract on the development of these organisms is poorly understood. Hence, the present study was undertaken to evaluate the larvicidal effect of crude leaf extract of *O. vulgare* on the development of *A. salina*. This study also aims to describe morphological effect of this medicinal plant on the early developmental stages of *A. salina*.

Materials and Methods

Plant extract preparation. The study was conducted from May 1 to 5, 2015. About 500 grams of fresh and clean leaves of the *O. vulgare* were ground using mortar and pestle. The extract was filtered using filter paper and stored in vials until needed for the lethality testing.

***A. salina* hatching.** *A. salina* eggs were obtained from the Chemistry department of Mindanao State University – Iligan Institute of Technology (MSU-IIT). The eggs were rehydrated with distilled water for 30 minutes and were transferred to a glass container containing filtered sterile seawater. The hatching chamber has two partitions: dark (covered) and light areas. Shrimp eggs were added into the dark side of the chamber. The other side was lighted with lamp to attract the hatched nauplii. The nauplii were subjected to strong aeration until needed for the toxicity test.

***A. salina* lethality test.** A 10,000 μL stock solution was obtained from the pure extract. In triplicate, serial dilutions (1000 μL , 500 μL , 100 μL , 50 μL) and control (sterilized seawater) were made for each test tube. Ten nauplii were collected and transferred to the test tubes using glass dropper; sterile seawater was then added to each test tube to produce a 5 mL total volume. The number of nauplii survivors was counted after 12h, 24h, 48h and 96h and percent mortality was calculated. Morphological changes in the *A. salina* were also observed using stereomicroscope. Photographs of *A. salina* were taken.

Results and Discussion

Larvicidal activity (LC_{50}) of *O. vulgare* leaf extracts on *A. salina*. As shown in Table 1, the level of toxicity of *O. vulgare* was directly proportional to the concentration of the extracts. Mortality rate of the *A. salina* were found to be concentration-dependent. The percentage of *A. salina* mortality increases as the concentration and time increases. After 12-48-hour exposure, the highest mortality was observed at 500 μL and 1000 μL while the lowest mortality was observed at 25 μL . After 96-hour exposure, 100% mortality was observed at 100 μL , 500 μL and 1000 μL . Results showed that the pure extract has LC_{50} value of 193.87 μL at 12-hour exposure and LC_{50} value of 39.11 μL at 24-hour exposure.

Mortality rate of *A. salina* is directly proportional to the level of leaf extract concentration over time. The most number of *A. salina* death was observed at high concentration, which increases as the time of exposure increases. Investigation by Raduđienė et al (2005) on the essential oils found in the inflorescence and leaves of *O. vulgare* was reported to have inhibitory effect on microorganisms. Another study by Teixeira et al (2013) also showed that extract of *O. vulgare* inhibited the growth of bacterial strains. These results are correlated with the study of Oliveira et al (2009), where essential oils from *O. vulgare* presented significant inhibitory effect on the cell viability of the assayed bacterial strains showed bactericidal effect. The inhibitory effect on the bacterial cell viability was noted on 1 hour of exposure and this effect was enhanced on longer exposure. The antimicrobial mechanism of essential oils such as in *O. vulgare* is the disruption of cell homeostasis leading to growth inhibition and cell death (Devi et al 2010). Savini et al (2009) also investigated the effect of *O. vulgare* ethanolic

extracts on redox balance, cell proliferation, and cell death in colon adenocarcinoma Caco2 cells and found out that oregano extract leads to growth arrest and cell death in a dose- and time-dependent manner.

Table 1

Mortality rate *Origanum vulgare* pure extract on *Artemia salina*

Extract (μL)	Mortality (%)
12 hrs	
25 μL	17%
100 μL	50%
500 μL	100%
1000 μL	100%
24 hrs	
25 μL	27%
100 μL	67%
500 μL	100%
1000 μL	100%
48 hrs	
25 μL	50%
100 μL	90%
500 μL	100%
1000 μL	100%
96 hrs	
25 μL	83%
100 μL	100%
500 μL	100%
1000 μL	100%

Morphological effect of *O. vulgare* leaf extracts on *A. salina*. Figure 1 shows the development of *Artemia* nauplii after 96 hours under a normal condition. Adult *Artemia* have an elongated body with two stalked complex eyes, a linear digestive tract, sensorial antennula and pairs of functional thoracopods. Paired lobular appendages are appearing at the trunk region and differentiate into thoracopods.

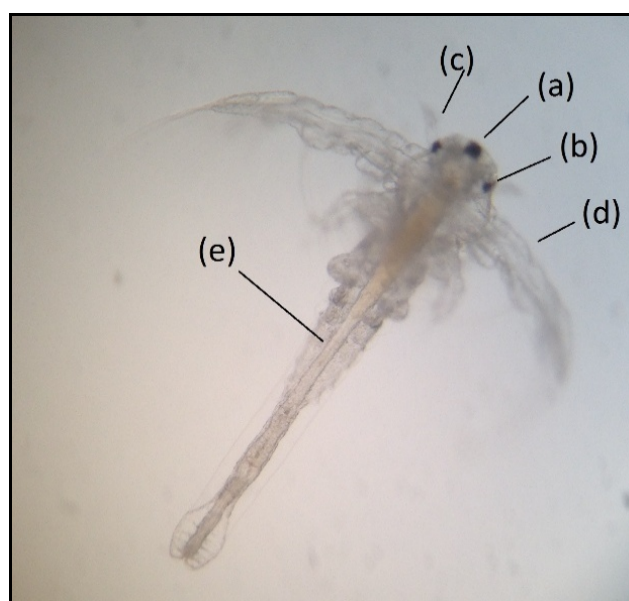


Figure 1. Head and thoracic area of *Artemia* nauplii after 96 hours: (a) nauplius eye; (b) lateral complex eye; (c) antennula; (d) antenna; (e) digestive tract.

There were no visible morphological changes observed both under control (Figure 2 a-c) and under 25 μ L concentration (Figure 2 d-f) of *O. vulgare* extract. *A. salina* under 25 μ L concentration may be able to endure the larvicidal effect of the leaf extract and thus, are able to survive and continue its development. Nevertheless, morphological defects such as deterioration of antennula and antennae were observed with increasing concentration of the leaf extract (Figure 2 g-m).

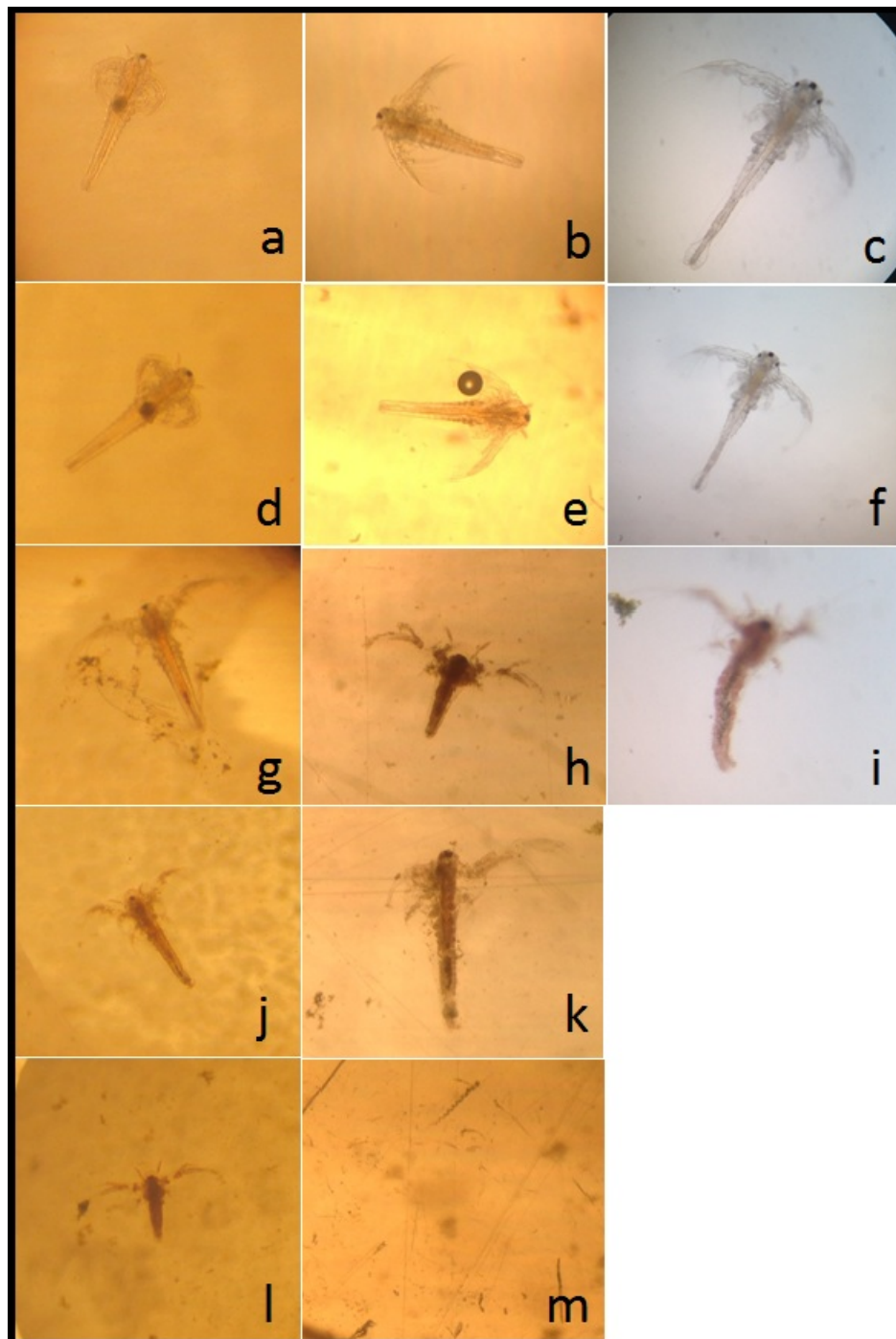


Figure 2. Morphological development of *Artemia salina* in five different levels of concentrations of *Origanum vulgare* extract. (a) Control, 24th hour; (b) Control, 48th hour; (c) Control, 96th hour; (d) 25 μ L, 24th hour; (e) 25 μ L, 48th hour; (f) 25 μ L, 96th hour; (g) 100 μ L, 24th hour, loss of antennula; (h) 100 μ L, 48th hour, loss of antennula; (i) 100 μ L, 96th hour, damage of antennae; (j) 500 μ L, 24th hour; ; (k) 500 μ L, 48th hour; (l) 1000 μ L, 24th hour; (m) 1000 μ L, 48th hour, *A. salina* is no longer visible.

Each part of an organism's body has its own designated function, such as the antennula of *A. salina* (for sensorial function, Figure 2c), its second antenna (for locomotory and filter-feeding, Figure 2d), and its mandible (for food uptake) (van Stappen 1996). Hence, normal development, growth and differentiation are necessary. Figure 2a shows the first larval stage (instar I) of the *A. salina*, which will then lead to the second larval stage (instar II). Although the digestive system is not yet functional in the latter stage, it becomes operative in the former stage for the ingestion of small food particles (van Stappen 1996; Schumann 1995).

It has been suggested that morphological and functional defects (loss of locomotory function of the antennae) in *A. salina* will occur at 10th instar stage and onwards (van Stappen 1996). However, the present result showed that the development, growth and differentiation of the larvae were inhibited after 24th hour exposure to 100 μ L pure extract of the *O. vulgare* leaves (Figure 2 g-m). The observed larvicidal activity (Table 1) of the plant demonstrates its high toxicity level which may have contributed to the morphological and functional defects of *A. salina*. Observable morphological alterations of *A. salina* and leaf extract residues accumulation in the digestive tract were detected in concentrations 100 μ L, 500 μ L and 1000 μ L, in contrary to control and 25 μ L concentration (Figure 2 h-l). The second antennae were defected/impaired at 100 μ L after 24-hour exposure showing a high toxicity level of the extract. Antennae degeneration (Figure 3b) was apparent after 48-hour exposure at 500 μ L concentration. Moreover, a 100% mortality was observed at 1000 μ L concentration after 48-hour exposure. This result suggests that increasing the time of exposure to high concentration of the extract would increase the rate of both morphological deterioration (Figure 2 l & k) and mortality.

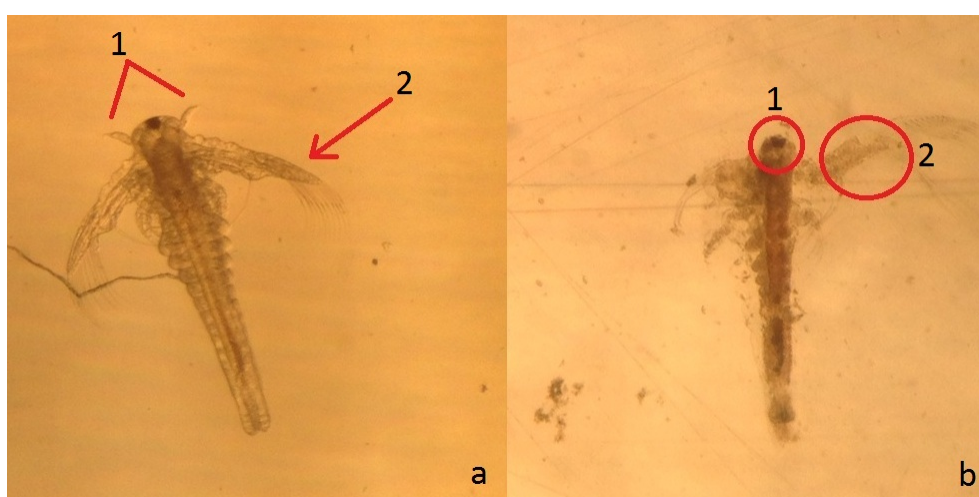


Figure 3. The morphological alterations of *Artemia salina* at 100 μ L after 48 hours. (a) normal, 1- antennule, 2 - antennae; (b) impaired, 1 - loss of antennule, 2 - damage of antennae.

Conclusions and recommendations. The pure *O. vulgare* leaf extract has LC₅₀ value of 193.87 μ L at 12-hour exposure and LC₅₀ value of 39.11 μ L at 24-hour exposure on *A. salina*. Exposure to *O. vulgare* extract (1000 μ L concentration) for 24 hours caused 100% mortality of *A. salina*, suggesting a larvicidal effect against *A. salina*. Exposure of *A. salina* to low concentration (100 μ L) of *O. vulgare* extract still caused defect/impairment and degeneration of the organism's antennule and antennae. Increasing the time of exposure to high concentration of the extract has increased the rate of both morphological defects and mortality. In particular, loss of antennula after 24-48 hour exposure to 100 μ L, damage of antennae after 96-hour exposure to 100 μ L, degeneration of the second antennae after 48-hour exposure to 500 μ L *O. vulgare*, and degeneration of *A. salina* larvae after 48-hour exposure to 1000 μ L *O. vulgare* extract were observed. The present result may imply that *O. vulgare* leaf extract may also have a growth inhibitory potential on pathogenic organisms at cellular level. Since the study conducted mainly focuses on the morphological changes of *A. salina*. It is recommended

to also check the molecular changes of the said species to show clearer evidence of the effect of *O. vulgare* extract.

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