http://www.egejfas.org
Ege J Fish Aqua Sci 31(3): 137-143 (2014)

Su Ürünleri Dergisi (2014)
DOI:10.12714/egejfas.2014.31.3.04

RESEARCH ARTICLE ARAŞTIRMA MAKALESİ

Acute toxicty of several esential oils on *Daphnia magna* (Straus, 1816)

Uçucu yağların *Daphnia magna* (Straus, 1816) üzerine akut toksisitesi

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How to cite this paper:
Arslan, Ç.Ö., Parlak, H., Boyacıoğlu, M., Karaaslan, A.M., 2014. Acute toxicty of several esential oils on *Daphnia magna* (Straus, 1816). *Ege J Fish Aqua Sci* 31(3): 137-143. doi: 10.12714/egejfas.2014.31.3.04

Özet: Bu çalışmada, beslenme, tıbbi tedavi ve kozmetik endüstrisinde kullanımı söz konusu olan 6 çeşit bitkisel yağın *Daphnia magna* akut hareketsizlik testi ile etkili konsantrasyoları (EC_{50}) belirlenmiştir. Toksisite testlerinde Biberiye, Okaliptüs, Üzüm Çekirdeği, Kekik, Çay Ağacı, Buğday özü yağları test maddesi olarak kullanılmıştır. Tüm yağların etki konsantrasyonları karşılaştırıldığında (EC_{50}) Kekik yağının diğerlerine göre daha toksik olduğu belirlenmiştir.

***Anahtar kelimeler*:** Uçucu yağlar, Akut toksisite, *Daphnia magna*, Hareketsizlik testi.

Abstract: In this study, effective concentrations (EC_{50}) of six natural essential oils as Rosemary, Eucalyptus, Grape Seed, Thyme, Tea Tree, Wheat Germ Oil. This oils are used in supplement in diet, medical treatments and cosmetics industries. In this study, the aquatic toxicity of the oils were examined by using *Daphnia magna* acute immobilization test. According to the toxicity tests results, Thyme Oil were found to be more toxic among the other oils when comparing the EC_{50} values.

***Keywords*:** Essential Oil, Acute toxicity, *Daphnia magna*, Immobilization test

INTRODUCTION

In this study, aquatic toxicity of the essential oils as Rosemary oil (RO), TeaTree oil (TTO), Wheat Germ Oil (WGO), Grape seed Oil (GSO), Thyme oil (TO) and Eucalyptus Oil (EO) were tested. Essential oils have been widely used for bactericidal, virucidal, fungicidal, antiparasitical, insecticidal, medicinal and cosmetic applications, especially recently in pharmaceutical, sanitary, cosmetic, and agricultural and food industries. Essential oils are volatile, natural, complex compounds and are formed by aromatic plants as secondary metabolites (Reuveni *et al.* 1984, Bakkali *et al.* 2008, Evandri *et al.* 2005).

Essential oils are extracted from various aromatic plants generally localized in temperate to warm countries like Mediterranean and tropical countries. They are liquid, volatile, limpid and rarely colored, lipid soluble and soluble in organic solvents with a generally lower density than that of water. They can be synthesized by all plant organs, i.e. buds, flowers, leaves, stems, twigs, seeds, fruits, roots, wood. (Bakkali *et al.* 2008, Miresmailli *et al.* 2006). Essential oils have been largely employed for their antibacterial, antifungal and insecticidal activities (Özcan and Arslan 2011). At present, approximately 3000 essential oils are known, 300 of which are commercially important especially for the pharmaceutical, agronomic, food, sanitary, cosmetic and perfume industries. Essential oils or some of their components are used in perfumes and make-up products, in sanitary products, in dentistry, in agriculture, as food preservers and additives, in perfumes, creams, soaps, as flavor additives for food, as fragrances for household cleaning products and as industrial solvents and as natural remedies, (Bakkali *et al.* 2008, Perry *et al.* 2003). Moreover, essential oils are used in massages as mixtures with vegetal oil or in baths but most frequently in aromatherapy. Some essential oils appear to exhibit particular medicinal properties that have been claimed to cure one or another organ dysfunction or systemic disorder (Silva *et al.* 2003, Hajhashemi *et al.* 2003, Perry *et al.* 2003).

This literature knowledges shows that the essential oils used widely and discharged and accumulated in aquatic environment. The lack of information about the toxicity of these essential oils on aquatic organisms courage we to study and test of their toxicity using *Daphnia magna* immobilization test.

Daphnia magna is one of the most important fresh water species widely employed in ecotoxicity testing procedures through the world. Except for fish and more recently algae, chronic and acute tests with *D. magna* are among the most frequently performed standardized studies in aquatic toxicology (OECD 2004). *D. magna* has been used extensively to determine the toxicity of chemicals, effluents, and water and sediment samples and has been demonstrated to be sensitive to many environmental contaminants (Le Blanc 1980, Parlak *et al.* 2010). The choice of *D. magna* for use as a standard test species was strongly influenced by the following factors: reproduction is normally parthenogenetic, which allows the maintenance and testing of clones; it can be cultured in the laboratory; it represents the zooplankton community, a major element of the freshwater food chain; as a species of worldwide occurrence, the ecological relevance of the test results is recognized. Daphnids are important invertebrate species in aquatic food webs. Most daphnids are cyclic partheogenetic species capable of both asexual and sexual reproduction. Laboratory cultures of Daphnids are typically maintained in partenogenetic state (Le Blanc 1980, Parlak *et al.* 2010).

Ecotoxicology can be loosely defined as the effects of pollutants on natural ecosystems. Although data from acute toxicity testing of single animal may be regarded as overly simplistic, they are often the starting point for assessing ecotoxicity (Hammer *et al.* 2006). Data describing the aquatic ecotoxicity of essential oils are very limited.

In our study, considering the knowledge above, we have attempted to determine the acute toxic effects of RO, TTO, WHO, GSO, TO and EO using *D.magna* and taking immobilized individual into account. As it is well known the toxicity of chemicals were ranged according to species. Although the toxic effects of six essential oils on some acari and bacteria species examined (Laborda *et al.* 2013), there are lack of information about the toxic effect on aquatic organisms and Daphnids.

MATERIAL AND METHODS

We used several essential oils which are Rosemary oil (RO), Tea Tree oil (TTO), Wheat Germ Oil (WGO), Grape seed Oil (GSO), Thyme oil (TO) and Eucalyptus Oil (EO) for tests. Stock solutions were prepared as 10 ppm of all essential oils by dissolving the chemicals with Dimethylsulphoxide (DMSO) (Sigma, Cat. No: 67-68-5). From the stock solutions, five experimental concentrations of five essential oils and four concentration of thyme oil (Table 1) were used by dilutions from stock solutions in order to determine the acute toxicity.

Controls accompanying the experiments consisted of untreated negative controls (Filtered Fresh Water: FFW), solvent control (DMSO: 1ml/10ml) and 10^{-4} M $CdCl_2$ (100µl/10ml: Sigma, Cat no: 7790-78-5) as a positive control.

Daphnia magna is one of the most important fresh water species employed in ecotoxicity testing for effluents, water and sediment samples, chemicals and complex mixture (Parlak *et al.*, 2010). *D. magna* is a species of zooplankton. Due to its worldwide distribution and adaptability to laboratory culture, Daphnia has long been employed as a representative species for testing chemicals (OECD 2004).

The acute toxicity tests were carried out in accordance with the guidelines of the Organization for Economic Cooperation and Development (Test no. 202, *Daphnia* sp., Acute Immobilization Test) (OECD, 2004). *D. magna* cultures consisted of glass beakers containing culture medium and 20 daphnids. Culture medium was renewed and offspring produced discarded twice weekly. Brood daphnids were discarded after 4 weeks in culture and replaced with neonatal organisms. Cultures were maintained at 21 ± 0.5 °C under 18-h light: 6-h dark photoperiod. The Daphnia were fed 75x106/ml cells the unicellular green algae *Selenastrum copricornutum* was cultured in Bristol medium. These culture conditions maintained the daphnids in the parteogenetic reproductive stage.

The primary objective of the 48-hour toxicity test using *Daphnia magna*. Is to evaluate the acute toxicity of essential oils on freshwater crustaceans. The test is conducted in small beakers or standard test tubes, and often incorporates replication (*e.g.*, 5 organisms in each of 4 replicates). The measurement endpoints generally evaluated are the 48-hour LC_{50} (for survival), and the 48-hour EC_{50} (for immobility). The acute test was performed in accordance to the standard protocol for *D. magna* immobilization test (OECD 2004). 5 neonates aged less than 24 h, divided in to four groups were exposed to each concentration of essential oils for 48 h in a static test. The test containers used were 20-ml glass beakers filled with 10 ml of test solution. The test was performed at 20 ± 2°C under 18-h light: 6-h dark photoperiod. Numbers of mobile and immobile specimens were registered after 24 and 48 h; pH and oxygen were measured in the controls and at the highest test concentrations. During the experiments, these physical and chemical variables were measured daily. At the end of the acute toxicity tests, the organisms were observed under a stereomicroscope, and the number of dead neonates in the four replicates was counted and used to determine the 48-h LC_{50}.

The LC*50*'s were calculated by probit analysis using Toxicologist 1.00 (1990) statistical software. All data were tested for statistical significance using a Student-t test.

RESULTS

Essential oil (RO, TTO, WHO, GSO, EO) bioassay with daphnia 5 concentration was used and 4 concentration was used for TO (Table 1). The summary of 24-h and 48-h acute toxicity of essential oils of 6 essential oils presented in Figures. In this study the 95% confidence limits overlap and dose-response relationship was defined.

Concentrations of Thyme oil (TO) were found to be more toxic in the test. The number of Immobilize Daphnids rates was increasing parallel to increasing concentrations in all test medium. In the meantime, any death was found in the control group (Table 2, Figure 3).

Table 1. Concentrations of esential oils.

Essential Oils	Physical State and Appearance	Molecular Weight (d)	Concentrations (mg/L)
Rosemary Oil (RO)	Liquid	0.8015	4.01, 8.02, 20.04, 48.09, 100.19
Tea Tree Oil (TTO)	Liquid	0.8043	4.02, 8.041, 12.061, 20.1013, 40.205
Wheat Germ Oil (WGO)	Liquid	0.8057	4.03, 12.08, 20.1, 48.3, 100.7
Grape Seed Oil (GSO)	Liquid	0.7456	3.728, 11.184, 18.64, 44.74, 93.2
Thyme Oil (TO)	Liquid	0.8136	3.051, 5.085, 12.204, 54.425
Eucalyptus Oil (EO)	Liquid	0.8446	22.53, 33.78, 45.05, 56.31, 67.57

Table 2. Acute test results of essential oils.

Essential Oil Concentrations mg/L	Total Number of Individual	Number of Immobilization	% Number of observed Immobilization
Control	20	0	0
Negative (DMSO)		0	0
Positive (CdCl2)	20	20	100
Rosemary Oil (RO)			
4.01	20	4*	20
8.02	20	8*	40
20.04	20	13*	65
48.09	20	17*	85
100.19	20	20*	100
Tea Tree Oil (TTO)			
4.02	20	2*	10
8.041	20	3*	15
12.061	20	7*	35
20.1013	20	11*	55
40.205	20	15*	75
Wheat Germ Oil (WGO)			
4.03	20	7*	35
12.08	20	18*	90
20.1	20	18*	90
48.3	20	20*	100
100.7	20	20*	100
Grape Seed Oil (GSO)			
3.728	20	3*	55
11.184	20	6*	50
18.64	20	7*	60
44.74	20	14*	60
93.2	20	20*	75
Thyme Oil (TO)			
3.051	20	4*	20
5.085	20	5*	25
12.204	20	11*	55
54.425	20	20*	100
Eucalyptus Oil (EO)			
22.53	20	0	0
33.78	20	6*	30
45.05	20	11*	55
56.31	20	17*	85
67.57	20	20*	100

*p<0.005

Mobilized Daphnids was 100% in the control group, while in the solvent substance DMSO, which has no toxic effect, it was 100%. Percentage of immobilized daphnids, depending on the toxicant, began to increase from the first concentrations onwards (Figure 1-3), a 20 % increased being observed at the first concentration of 4.01mg-RO/L ($p<0.005$). At first concentrations of TTO containing 4.02 mg-TTO/L percentage of immobilize daphnids observed as 10% (Table 2, Figure 1).

However immobilized daphnids was not observed at 22.53 mg-EO/L, immobilized daphnids was observed as 35% for WGO (4.03 mg-WGO/L), 15% for GSO (3.728 2 mg-GSO/L) and 20 % for TO (3.051 mg-TO/L). The rise in the dose-response curve continued until the final concentration of 100.19 mg-RO/L, 40.205 mg-TTO/L, 100.7 mg-WGO/L, 93.2 mg-GSO/L, 54.425 mg-TO/L and 67.57 mg-EO/L (Figure 1-3).

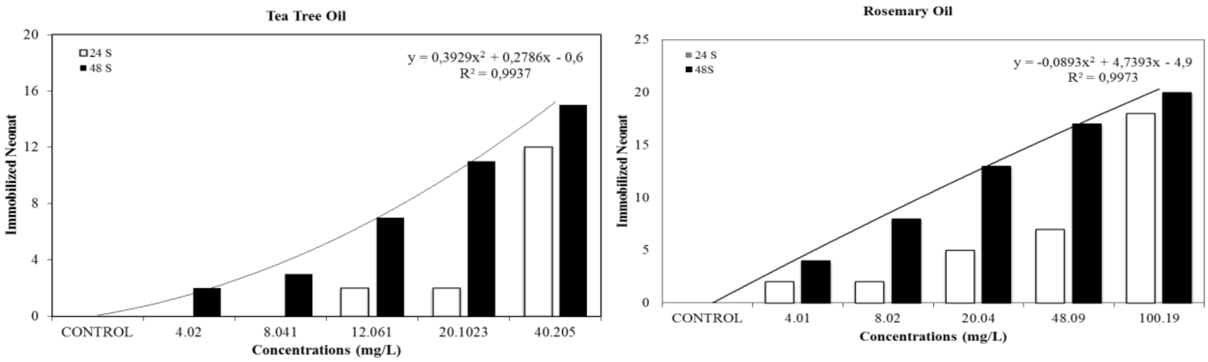

Figure 1. Dose response curve of Rosemary and Tea Tree Oil for immobilized neonate.

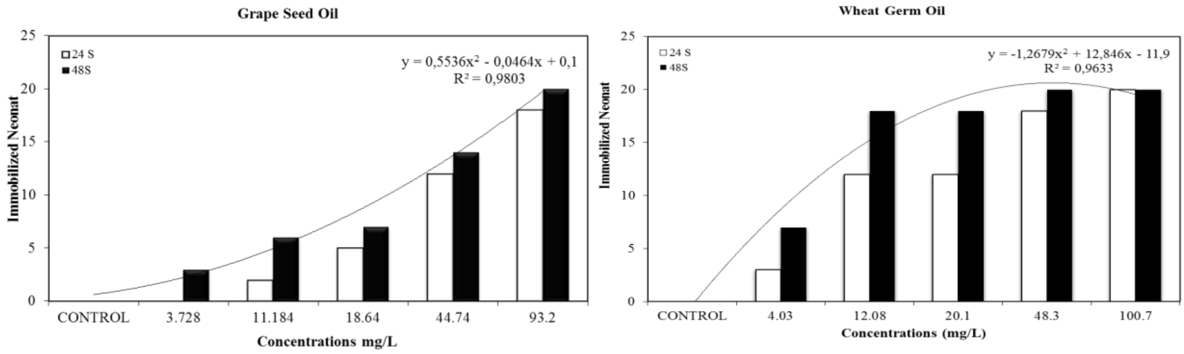

Figure 2. Dose response curve of Wheat Germ and Grape Seed Oil for immobilized neonate.

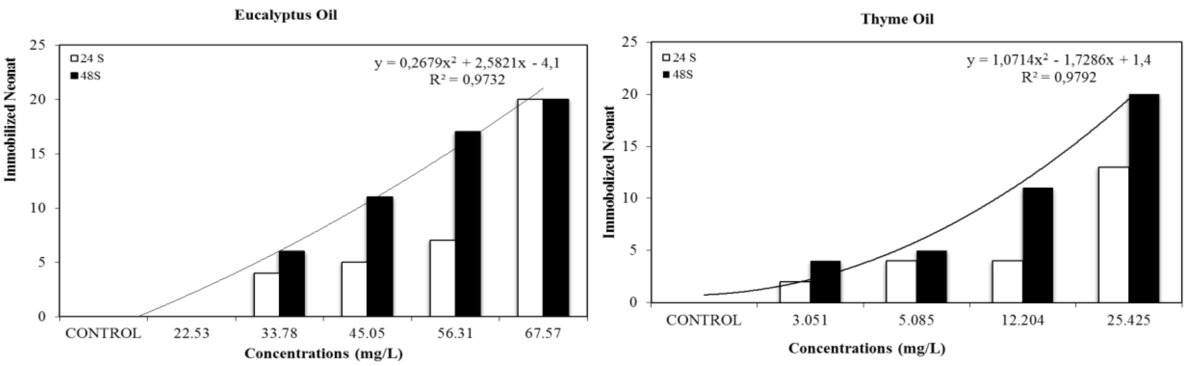

Figure 3. Dose response curve of Thyme and Eucalyptus Oil for İmmobilized neonate.

The impact of RO on mobilization of exposed neonate was determined as EC_{50} 15.5875 mg/L RO concentration by probit analyses Table 3. The acute toxicity of TTO on mobilization are shown in Table 2. And EC_{50} value of TTO was estimated as 18.614 mg/L (Table 3). The EC_{50} values of other 5 essential oils are shown in Table 3. It can be concluded that comparing EC_{50} levels of the essential oils concentrations the toxicity order is Eucalyptus Oil< Grape Seed Oil<Tea Tree Oil<Rosemary Oil<Wheat Germ Oil<Thyme Oil (Table 3).

Table 3. Estimated LC/EC values and confidence limits for immobilized neonate.

Esential oil	Exposure Conc. (mg/L)	95% Confidence Limits		Probit Curve
Rosemary Oil	15.5875	9.965	22.775	3.7984+1.007 x log(conc.)
Tea Tree Oil	18.614	7.937	30.144	2.490+1.977 x log(conc.)
Wheat Germ Oil	15.059	10.66 3	20.401	2.261+2.326 x log(conc.)
Grape Seed Oil	23.001	12.62 3	55.981	2.689+1.697 x log(conc.)
Thyme Oil	11.787	7.342	169.844	3.135+1.741 x log(conc.)
Eucalyptus Oil	83.8111	Too	wide	2.194+1.459 x log(conc.)

DISCUSSION

Essential Oils contain a complex mixture of volatile aroma compounds from secondary plant metabolism. In general antifungal and insecticidal activity of essential oils are well documented (Reuveni *et al.*, 1984). Although researchers describe the in-vivo and in-vitro effects of some essential oil on various animal species. A limited data have been found on the acute toxicity of Rosemary, Tea Tree, Wheat Germ, Greap Seed, Thyme and Eucalyptus Oil, that makes difficult to have comparisons of data's.

However, the acute toxicity of some essential oils to aquatic organism has been assessed by a few researcher (Le Blanc, 1980, Stroh et al,. 1998, Park *et al.*, 2011). The lack of data on the acute toxic effects of essential oils on the physiology, reproduction and development is particularly evident in some aquatic invertebrate taxa, such as Clodoceran. In the study by Pavela (2014), using *Culex quinquefasciatus* and non-target organism *Daphnia magna*, in order to determine the acute toxicity of selected essential oil *Pimpinella anisum* fruits were used. LC50 for instar larvae was estimated as 15-19 µl/L and besides that essential oil were toxic for *D. magna* (62-92 % mortality) and significantly reduced fertility. The oil used in this study was not used in our study. But the *D. magna* mortality average were compared our study essential oil we tested are less toxic to *D. magna.*

The essential oil Tea Tree Oil is widely investigated as alternative antimicrobial, anti-inflammatory agent (Hammer *et al.*, 1999, 2006) limited data are available on safety and the toxicity of oil. The mutagenic activity of Tea Tree Oil was determined by Evandri *et al.*, (2005). This researcher reported that essential oils tea tree and lavender have no mutagenic activity either in the TA 98 and TA 100 *Salmonella typhimirium*. In the study of researcher tea tree oil dilutions were tested (0.80, 0.66, 0.50, 0.40, 0.25 and 0.13 mg/plate). According to test result of this study tea-tree oil proved toxic on both TA strains (at 0.28 mg/plate on TA98 and at 0.88 mg/plate on TA100): when the S9 mixture was added, tea-tree oil was less toxic (toxicity began at 2.78 mg/plate). Although, Tea-tree oil, with and without metabolic activation, induced no antimutagenic activity, on TA98 or TA100. In our study, Tea Tree oil were toxic at 18.614 mg/L. Comparison of The test results of Evandri *et al.*, (2005) and present study showed that strains of Bacteria are more sensitive to Tea Tree oil.

Pimenta *et al.*, (2000) evaluated that acute toxicity of total samples of eucalyptus pyrolysis liquids and the phenolic compounds by *D. magna* bioassay and microtox assay. They are reported that total eucalyptus pyrolysis liquids were immobilize 50% of the *D. magna* population at concentrations 68- 170 mg/L and also showed that eucalyptus has no genotoxic effects. In this study, eucalyptus pyrolysis liquids tested while eucalyptus oil were tested in our study. There are no studies with Eucalyptus Oil to determine the effects on aquatic organisms. These results are compared with our study, eucalyptus oil is more toxic than eucalyptus pyrolysis (55% immobilize Daphnid at 45, 05 mg/L; 50% at 40, 95 mg/L).

The study of Pavela (2008) were determined the insecticidal activity of 34 essential oils, extracted from plants, was screened against the house fly, *Musca domestica* L. under laboratory conditions. Results of these study showed that Essential oils from *Pogostemon cablin* (Patchouili) proved to be the most efficient at a lethal dose of 3 µg/fly after topical application. Eight oils (*Pelargonium roseum*, Origanum *vulgare, Origanum compactum, Mentha pulegium, Ocicum basilicum, Origanum majorana, Thymus vulgaris* and *Palergonium graveolens*) were lethal in doses ranging from 10 to 20 µg (µg/fly, respectively). The lethal doses of another 13 oils were ascertained in the range (including *Rosmerinus officinalis*) 20–50 µg/fly, nine oils had lethal doses of 50–100 µg. Results of these study showed that Thyme Oil and Rosemary Oil more toxic to house fly *Musca domestica* L. When comparing these results with our study similar effects were observed according to lethal concentrations.

Khater *et al.* (2011) using *Lucilla sericata* (Diptera) exposed essential oils lettuce, chamomile, rosemary and annise, at larval stage it was reported that lethal concentrations (LC50) of 0.57%, 0.85%, 2.74%, and 6.77% for lettuce, chamomile, anise, and rosemary oils, respectively. The Study by Seo *et al.*, (2012) were evaluated larvicidial activity of 20 plant essential oils and also tested the acute toxicity of five of these oils. According to their results, Peru balsam, ajowan balsam, benzyl benzoate and thymol were more toxic to *D. magna*. And also reported LC_{50} value as 5.94 mg/L for thymol, 3. 89 mg/L for Peru balsam. Although in our study LC_{50} value measured as 11,787 mg/ L for Thyme oil. The acute toxicity of some Myrtaceae plant essential oils or their components to aquatic organisms has been assessed. Tea Tree Oil and Eucalyptus oils were classified as nontoxic (EC50 > 10 mg/L) (Stroh et al,. 1998, Park *et al.*, 2011). Duringer *et al.*, (2010) were evaluated acute aquatic toxicity of two essential oils (Western juniper and Port orford) using *Daphnia magna, Oncorhynchus mykiss* and *Selenastrum* *copricornutum* in order to gauge the relative toxicological risk. Results of this study showed that Western juniper oil not toxic for D. *magna* and O. *mykiss* although toxic for *S. copricornutum* at 5.0 mg/L. Besides that researcher reported that Port orford oil EC_{50} value (48 hour) for *D. magna* as 1.9 mg/L.

The purpose of Khalifa *et al.*, (2011) study was to assess the antioxidant role of wheat germ oil (WGO) and grape seed oil (GSO) in chlorpyrifos-induced oxidative stress, biochemical and histological changes in liver in male albino rats. The results showed that the enzyme activities were significantly increased in rats administrated only by chlorpyrifos. Besides that Wheat germ oil and grape seed oil supplementation caused significant improvement in different biochemical parameters of all rat groups. Toxic effects of Wheat Germ and Grape Seed Oil on aquatic organisms are not available.

Our results and the results of the other related research studies showed that essential oils cause immobilization/mortality of daphnia at low concentrations. As with RO, TTO, WGO, GSO, TO and EO was determined according to Zucker's (1985) criteria that this chemical was slightly toxic for daphnia.

As it is well known that the effects of toxic chemicals on early developmental stages of aquatic organisms are of great importance in the protection of the natural population's health. This results bring us a conclusion that the essential oils has toxic effects on neonate and decreased mobilization percentage of exposed neonates became more important from the ecotoxicological point of view. Besides that considering the increasing use of this essential oils for personal care and another uses, their safety profile must be carefully determined.

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