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Ecotoxicity assessment of aqueous extract of *Tetraclinis articulata* on green alga *Pseudokirchneriella subcapitata.* Comparison with toxicity on *Daphnia magna*

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Abstract

The use of *Tetraclinis articulata's* aqueous extract as an alternative to conventional insecticides was carried out in wetland ecosystems in Morocco. In order to evaluate the ecotoxicological risk of aqueous extract of *Tetraclinis articulata* to freshwater populations, an aquatic bioassay was used. In previous work, toxicity of aqueous extract was investigated in our laboratory on the juvenile Cladoceran *Daphnia magna*. The results revealed a high level of acute and chronic toxicity on immobilization and reproduction of *D. magna*. The 48h-EC50 value registered was 6.490 ± 0.541 mg/L. The No Observed Effect Concentration (NOEC) and Low Observed Effect Concentration (LOEC) values were 0.49 and 0.83 mg/L, respectively for number of neonates. To investigate effects of this extract on another non target organism, we have choose a green alga *Pseudokirchneriella subcapitata* population. Our results showed that the growth of alga was gradually decreased with increased of concentrations. Based on 72h cell count, the EC50 value was 13.695 ± 0.021 mg/L. The 72-CE50 was 10.64 ± 1.074 mg/L and 24.75 ± 1.046 mg/L, respectively for biomass and growth rate. The NOEC and LOEC values were 23.01 and 29.92 mg/L respectively, for algal cell density, biomass and growth rate. This study demonstrated that aqueous extract have ecotoxicity effect on aquatic organisms. Our results also demonstrated that *P. subcapitata* was considerably less sensitive to *T. articulata* than *D. magna.*

1. Introduction

In Morocco, the use of plants as an alternative to insecticides is becoming more widespread due to its biocidal activity and its low toxicity to the environment. Thuya (*Tetraclinis articulata* (Valh) Masters) is present in northwestern Africa (Morocco, Algeria and Tunisia). Many studies have investigated its ecological and botanical characteristics and also biochemical composition of its essential oil [1-2-3].

Although widely used for antimicrobial, antifungal [4-5], antioxidant [6-7], analgesic [8] and biocidal activities [9], very few data on effects on aquatic organisms are available for *Tetraclinis articulata* (Valh) Masters. In previous study, we have demonstrated that this plant has been showed an acute and chronic effect on mobility and reproduction parameters against cladoceran *Daphnia magna* species. As part of ecotoxicological approach and for constant biomonitoring of aquatic ecosystems, we aimed to investigate the effects of aqueous extract of *T. articulata* on another non-target aquatic organism; *Pseudokirchneriella subcapitata.* This aquatic species represents chlorophyceae's population. Given the importance and role of alga, pollutants are accumulated in this organism, enter in the food chain and may engender serious damage to human and animals health through biomagnifications.

D. magna and *P. subcapitata* are the most widely used in aquatic ecotoxicity assessment and recommended by several international organizations (ISO, OCDE, USEPA) as ecotoxicological bioindicator species because of their key role in aquatic ecosystems through energy transfer, but also their high sensitivity to pollutants through the trophic chain.

Pseudokirchneriella subcapitata is a unicellular green freshwater microalga considered one of the most sensitive indicators of toxicity. Because of its easier mode of culture in laboratory, microalga has long been used

as test organism to assess several substances including heavy metals [10], pesticides [11], effluents [12] and drugs [13]. Several studies underline the difference of sensitivity between *D. magna* and *P. subcapitata* and suggest the use of bioassay batteries to evaluate toxic effects at various biological levels. The toxicity of pollutants on *P. subcapitata* can be evaluated in a short term test (72h and/ or 96h).

The objective of this study was to add information regarding the ecotoxicity of aqueous extract of *T. articulata* on *P. subcapitata* based on several parameters such as algal biomass, growth rate and cell density inhibition. The 72h-CE50, LOEC and NOEC for each parameter was calculated. Finally, based on our results, the sensibility of microalga to aqueous extract will be compared by results of *D. magna* founded in previous work [14].

2. Material and Methods

2.1. Aqueous extract preparation

Aqueous extract was prepared by adding 10 g of sawdust of *T. articulata* to 100 mL of boild distilled water. This solution was filtered, pH was adjusted to 7.8 and extract was used immediately in the toxicity tests.

2.2. Alga and culture media

The freshwater green microalga *P. subcapitata* were maintained in our laboratory in sterile ISO medium culture under controlled temperature (23 ± 2°C) and constant light (3500 Lux, cool white fluorescent lamps) with rotary agitation at 100 rpm. After 7 days, algal cell was inoculated in fresh medium. To identify the exponential growth phase, the batch cultures were monitored daily by removing a small aliquot (1 mL). Cell density was determined using a counter cell (Malassez cell) [15].

2.3 Chronic ecotoxicity test

After approximately 4 days, cultures in exponential growth were used in the toxicity tests. The algal cultures were exposed to 40 mL of test medium with an initial density of 10^4 cells/mL. Experiences were conducted according to NF T 90-375, (1998) [15]. Algal chronic toxicity tests were conducted using concentration of aqueous extract ranged between 4.8 mg/L and 38.8 mg/L and flaks were incubated as the same culture conditions during 72h. All concentrations and control were conducted in triplicate. Potassium dichromate ($K_2Cr_2O_7$) was used as a reference substance and 72 h-EC50 value should be in the range between 0.45 and 1.01 mg/L as required by NF T 90-375, (1998). Three parameters revealed the evolution of toxicity response were determined including cell density, biomass and growth rate.

At the end of the test, the algal growth in each flak was determined by counting the number of cells using a particle counter; the percentage inhibition values for each concentration were calculated using the formula:

$$\% \, I = \frac{Nc - Nt}{Nc} \times 100$$

Where *% I* = percentage inhibition after 72h; *Nc* = mean value for cell inhibition in control after 72h and *Nt* = mean value for cell inhibition in treatment after 72h.

For each concentration and for each replicate, the biomass was calculated using the following formula:

$$Ai = \frac{N1 - N0}{2} \times t1 + \frac{N1 + N2 - 2N0}{2} \times (t2 - t1) + \frac{N(n-1) + Nn - 2N0}{2} \times (t_n - t_{n-1})$$

Where *Ai* = area; N_0 = nominal number of cells per milliliter at time t_0; N_1 = measured number of cells per milliliter at t_1, N_n = measured number of cells per milliliter at t_n; t_1 = time of first measurement after beginning of test; t_n = time of nth measurement after beginning of test.

The mean value of Ai for each treatment and for control was calculated. From these mean values, the biomass inhibition was calculated as follows:

$$\% \, A = \frac{Ac - At}{Ac} \times 100$$

Where *A*= the percentage inhibition of biomass; *Ac* = the mean value for biomass in the control; *At* = the mean value for biomass in the treatment replicate.

The specific growth rate for each period was obtained following equation:

$$\mu_{i\text{-}j} = \frac{\ln Nj - \ln Ni}{tj - ti} \times 100$$

Where μ_{i-j} = the average specific growth rate from time *i* to *j*; N_i = cell density at time i; N_j = cell density at time j; t_i = the time (*d*) of i^{th} cell density measurement after beginning the exposure; t_j = the time (*d*) of j^{th} cell density measurement after beginning the exposure.
Percentage inhibition of growth rate was calculated using following equation:

$$\% I_{\mu} = \frac{\mu c - \mu t}{\mu c} \times 100$$

Where I_{μ} = the percentage inhibition in average specific growth rate; μ_c = the mean value for average specific growth rate (μ) in the control group; μ_t = the average specific growth rate for the treatment replicate.

2.4 Statistical analyses
EC50's toxicity endpoints (biomass, growth rate and cell density) for *P. subcapitata* were estimated using the bootstrap method in the REGTOX Excels macro. An analysis of variance using single factor one-way analysis of variance (ANOVA) was performed. A non-parametric Kruskal-Wallis test was used and the multiple comparisons with Dunn's method was conducted to detect significant differences between control and treatment cultures ($P < 0.05$: STATISTICA version 6 for Windows, Statsoft, Tulsa, OK, USA).

3. Results and discussion

In several previous studies, *T. articulata* has been primarily reported to have antimicrobial, antifungal and biocide effects on different species. In this work, we have used green freshwater microalga *P. subcapitata* as model organism to investigate aqueous extract toxicity by assessing growth cell inhibition. The sensibility of green alga was verified using the recommended reference substance (potassium dichromate) and the 72h-EC50 was 0.661 ± 0.025 mg/L. This value is ranged between 0.45 and 1.01 mg/L according to NF T 90-375, (1998) and confirmed the sensibility of the used alga species.
To satisfy NF T 90-375, (1998) validation's condition, we also determined the growth ratio between initial density incubated and measured cell density after 72h of incubation in control flaks. The number of cells was increased from initial cell number 10^4 cell/ mL to 70.133×10^4 cell/ mL. This result satisfied conditions for NF T 90-375 (1998) test.
P. subcapitata in exponential growth phase was exposed following standardized protocols NF T 90-375, (1998) at concentrations of aqueous extract's *T. articulata* ranging from 4.8 to 38.8 mg/L and effects on the growth cell were measured. After 72h, we observed that algal cell density decreasing progressively with increased of concentrations of aqueous extract (Figure 1).
After 72h of exposure, the percentage of inhibition was 6.83 % at 4.8 mg/L. Cell density inhibition passed from 15.35% at 8.05 mg/L to 52.25% at 13.62 mg/L. At concentration 17.7 mg/L, the percent of inhibition was 75.06% and increased to 86.11% and to 95.16%, respectively at 23.01 and 38.8 mg/L. The statistical analysis of these results was determined. The 72h-EC50 was 13.695 ± 0.021 mg/L. Algal cell density was significantly reduced in the 29.92 and 38.88 mg/L. The No Observed Effect Concentration (NOEC) value was 23.01 mg/L.

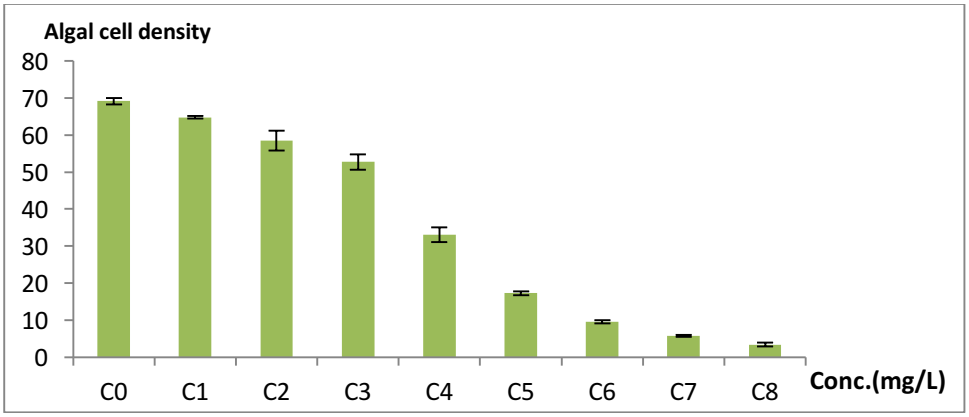

Figure 1: Effect of aqueous extract of *T. articulata* on the cell density of *P. subcapitata* after 72h. Data are mean values of three replicates and error bars indicate standard deviations

The following two parameters were calculated from cell density number. Decrease of biomass (Figure 2) and growth rate (Figure 3) were also calculated at 72h for all conducted experiments. After 72h of exposure, a significant decreased of biomass compared with control was observed showing clearly a dose-response relationship (Figure 2). In the lowest concentration 4.8 mg/L, *P. subcapitata* biomass inhibition was 18.92%

while at 10.47 mg/L, the percentage of inhibition was 46.85%. At concentrations 13.62 and 23.01 mg/L, the percentage of biomass inhibition was 63.70% and 88.90% respectively, after 72h of exposure. At 38.8 mg/L, the percentage of inhibition was 98.30%. The 72h-EC50 calculated for biomass was 10.64 ± 1.074 mg/L. No significant difference was observed at 23.10 mg/L. Consequently, the LOEC was 29.92 mg/L.

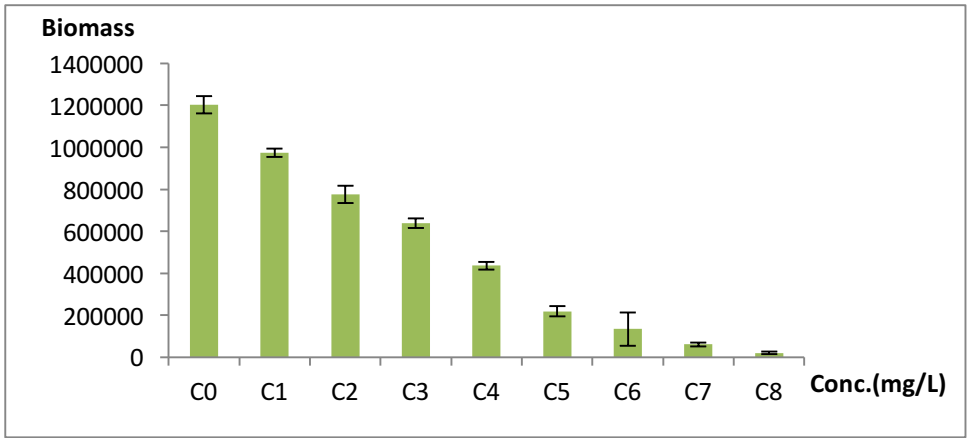

Figure 2: Effect of aqueous extract of *T. articulata* on the biomass of *P. subcapitata* after 72h. Data are mean values of three replicates and error bars indicate standard deviations.

P. subcapitata growth rate (μ) after 72h at different concentrations of aqueous extract's *T. articulata* ranging from 4.8 to 38.8 mg/L are showing in Figure 3. At lowest concentrations 4.8 and 8.05 mg/L, inhibitory effect was less pronounced. The growth rate was 1.41 in control. At 13.62 mg/L, growth rate was 1.16 attesting a factor of inhibition of 11.23%. At concentration 23.03 and 29.92 mg/L, the growth rate was 0.74 and 0.57, respectively. The growth rate inhibition for these two concentrations was 47.09% and 59.17% respectively. Growth rate inhibition of 71.93% was registered after 72h at 38.8 mg/L. The 72h-EC50 was 24.75 ± 1.046 mg/L. Growth rate was significantly reduced in 29.92 mg/L and 38.88 mg/L in comparison to the control ($p \leq 0.05$). The NOEC and LOEC values were 23.01 and 29.92 mg/L, respectively.

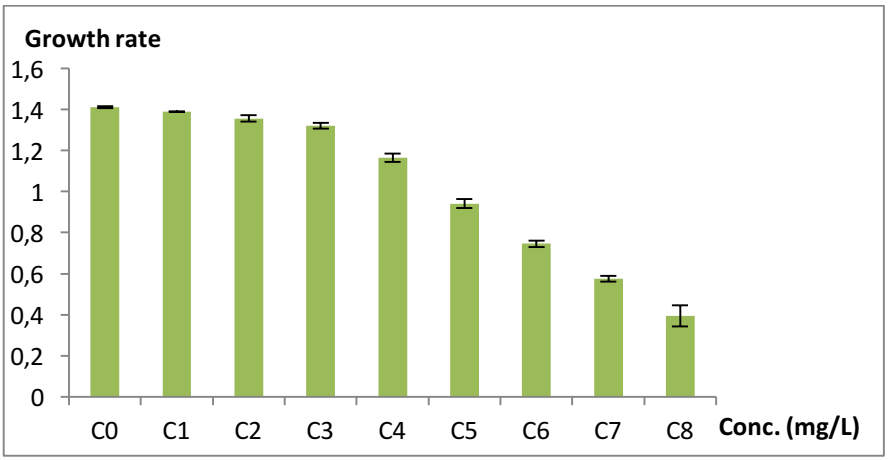

Figure 3: Effect of aqueous extract of *T. articulata* on the growth rate of *P. subcapitata* after 72h. Data are mean values of three replicates and error bars indicate standard deviations.

All previous studied parameters showed a drastic decreased underline a dose-response relationship. However, a difference of percentage of inhibition was observed between each parameter while for growth rate inhibition, the response was slow especially for the lowest concentrations. The ecotoxicological values of cell density (EC50, NOEC and LOEC) (Table 1) will be used for comparison between the sensitivity of *D. magna* and *P. subcapitata.*

In our previous work, acute and chronic toxicity of *T. articulata* on *D. magna* was evaluated. Acute toxicity results were expressed as the estimated concentration able to immobilize 50% (EC50) of the neonates exposed to aqueous extract during 48h. This study showed that 48h-EC50 value was 6.490 ± 0.541 mg/L for immobilization [14]. By comparing 72h-EC50 for algal cell density inhibition (13.695 ± 0.021) induced by aqueous extract, we observed that this value is twice more than 48h-EC50 (6.490 ± 0.541 mg/L) for *D. magna's*

immobilization. This latter attested that *P. subcapitata* are less sensitivity to aqueous extract of *T. articulata* in acute conditions. The same observation was registered when we consider the NOEC and LOEC values. In fact, the NOEC and LOEC values for cell density of *P. subcapitata* were 23.01 and 29.92 mg/L showing a high sensitivity of *D. magna* (Table 1).

Table 1: Comparative values of EC50, NOEC and LOEC (mg/L) for *D. magna* and *P. subcapitata* exposed to aqueous extract of *T. articulata*

Species	**EC50** mg/L	**NOEC** mg/L	**LOEC** mg/L
P. subcapitata	13.695 ± 0.021	23.01	29.92
D. magna	6.490 ± 0.045	0.49	0.83

Several authors worked on aqueous plant extract and compared their effects on different species.
Junčula *et al.,* [16], tested effects of aqueous extract of five species from *Papaveraceae* family on green alga *P. subcapitata* and cladoceran *D. magna*. They registered a 96h-EC50 ranged from 21.27 mg/L to 868.09 mg/L for *P. subcapitata* and from > 400 to > 1000 mg/L after 48h for *D. magna*. These values showed the high sensitivity of *P. subcapitata* compared with *D. magna.*
Mousa *et al.,* [17] tested effect of *Azadirachta indica's* aqueous extract on cladoceran (*Daphnia sp*.), copepods (*Cyclops sp*,), Nile tilapia (*Oreochromis niloticus*) and African cat fish (*Clarias gariepinus*). The 96h-LC50 values for each species were 0.1, 0.2, 1.8 and 4 g/L, respectively. These values showed the high sensitivity of *D. magna* compared to others species tested and also confirmed that *D. magna* was the most sensitive species.
In another study, the same plant was tested using alga species *Scenedesmus quadricauda.* The results showed an algicidal effect which the growth rate was reduced with 100% of inhibition at highest concentration tested. A decrease of chlorophyll *a* and altered activity of Catalase and Peroxidase were also observed [18].
The Bioneem of *Azadirachta indica* was also used to evaluate its effects on zebrafish *Danio rerio* and *D. magna.* Authors founded a 96h-LC50 = 0.22 mL/L for *D. rerio* and 48-EC50 = 0.17 mL/L for *D. magna.* They also observed an inhibition of size and a reduction in number of *D. magna*'s neonates [19].
Duringer *et al.,* [20], assessed effects of Juniper foliage oil and Port Orford cedar heartwood oil on *D. magna*, *P. subcapitata* and fish *Oncorhynchus mykiss.* After an exposure to Juniper foliage oil, the 72h-EC50 values for algal cell density was 1.7 mg/L. For *D. magna*, the 48h-EC50 was > 5.0 and for *Oncorhynchus mykiss,* the 96h-LC50 was > 5.0 mg/L. These values showing that *P. subcapitata* was more sensitive to Juniper foliage oil than *D. magna* and *O. mykiss.* In contrast, an exposure to Port Orford cedar heartwood oil showed a similar sensibility between *P. subcapitata* (72h-EC50 = > 5.0 mg/L) and *O. mykiss* (96h-LC50 = > 5.0 mg/L). However for *D. magna,* the 48h-EC50 was 1.9 mg/L showing that this species was the most sensitive for Port Orford cedar heartwood oil.
Ribeiro *et al.,* [21] have used *P. subcapitata*, *D. magna* and *D. rerio* as model organism to investigate the toxicity of silver nanoparticles (AgNP) and silver nitrate ($AgNO_3$). For AgNP, the 72h-EC50 was 32.4 µg/L for *P. subcapitata*, the 48h-EC50 was 10.2 µg/L for *D. magna* and the 48h-LC50 was 128.54 µg/L for *D. rerio*. For $AgNO_3$, the 72h-EC50 was 33.79µg/L for *P. subcapitata*, the 48h-EC50 was 1.05 µg/L for *D. magna* and the 48h-LC50 was 78.32 µg/L for *D. rerio.* These results showed the high sensitivity of *D. magna* to heavy metals compared to *P. subcapitata* and *D. rerio*. For *P. subcapitata*, the toxicity was the same for both substances tested. These results suggests that the toxicity of both $AgNO_3$ and AgNP differs significantly based on the test species with a major difference in toxicity for *D. magna*, a small difference for *D. rerio* and no differences in toxicity for *P. subcapitata.*
Several pesticides was assessed including organophophates, phenyluereas and thiocarbamate using three standardized bioassays based on *D. magna, P. subcapitata* and the bioluminescent bacteria *Vibrio fischeri.*
In case of organophosphates such as Diazinon, *D. magna* (EC50 = 0.6 µg/L) was more sensitive than *P. subcapitata* (EC50 = 10 mg/L) and *V. fischeri* (EC50 = 84 mg/L). However, in case of phenyluereas such as Diuron, alga *P. subcapitata* (EC50 = 2.4 µg/L) was more sensitive than *D. magna* (EC50 = 8600 µg/L) and *V. fischeri* (EC50 = 43240 µg/L). The same observation was registered for thiocarbamate, which *P. subcapitata* (EC50 = 70 µg/L) was more sensitive to Molinate than *D. magna* (EC50 = 1830 µg/L) and *V. fischeri* (EC50 = 2500 µg/L) [22]. Compared to these pesticides, aqueous extract are certainly less toxic on aquatic population.
Based on all these results, we can conclude that using *T. articulata* such an alternative to insecticides against mosquitoes and their presence in aquatic environment generate an ecotoxicological effects to freshwater organisms especially on the primary consumer *D. magna.* These results suggest that *T. articulata* has an ecotoxic effects against species possessing a nervous system such as mosquitoes and cladocerans, in contrast to

plants playing role of herbicides. This latter well known to inhibit transport of electron between photosystem I and II. In some case, pollutants can inhibit respiration, photosynthesis and enzyme activity. It also cause the disturbance of normal metabolism and growth, cause cellular mutation and even death in alga.

Conclusion

In conclusion, we found that aqueous extract of *T. articulata* has great inhibitory effect on *D. magna's* immobilization and reproduction. The growth cell of *P. subcapitata* populations is also strongly affected by this aqueous. The observed results clearly demonstrated differences in sensitivity to aqueous extract between *D. magna* (CE50 = 6.490 ± 0.541 mg/L) and *P. subcapitata* (CE50 = 13.695 ± 0.021 mg/L). In fact *D. magna* was more sensitive whatever the parameter studied (mobility and reproduction) than freshwater green alga *P. subcapitata.* This difference of sensitivity between to tested population (*D. magna* and *P. subcapitata*) highlights the necessity to use a battery of bioassays to evaluate the impact and risk of pollutant substances on aquatic ecosystems.

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