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TOXICITY ASSESSMENT OF *NEPHROLEPIS EXALTATA* (L.) SCHOTT, FAM. NEPHROLEPIDACEAE

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Abstract: The fern *Nephrolepis exaltata* (L.) Schott, fam. Nephrolepidaceae, has little traditional medicinal use. In order to estimate its potential safety, in the present study we have investigated the phytotoxicity (on *Triticum aestivum* L.) and toxicity on brine shrimp of extracts from plants grown hydroponically. The species identity was confirmed by macroscopic and microscopic examinations on rhizomes, rachises, pinnae and runners, using bright field and fluorescent microscopy. Biological assays were performed on aqueous and ethanol solutions of the fronds. The brine shrimp lethality assay was performed on *Artemia franciscana* Kellog and a phytobiological bioassay on *Triticum aestivum* L. Lethality, root elongation and karyokinetic film modifications were evaluated, and LC_{50} and IC_{50} values were calculated. The microscopic analysis revealed the main histo-anatomic elements: polystelic structure and hypodermis (rhizome, rachis, runners), trichomes (rachis), homogenous structure, trichomes and diacytic/anisocytic stomata (leaves). The ethanol and aqueous extracts showed low cytotoxic effects on both *Triticum aestivum* roots and *Artemia franciscana* nauplii.

Keywords: *Nephrolepis exaltata*, microscopy, *Triticum* bioassay, *Artemia* bioassay, hydroponics.

1. Introduction

The fern *Nephrolepis exaltata* (L.) Schott, Nephrolepidaceae, has been studied for its soil phytoremediation properties (Sultana et al., 2014), the effects of its volatile oil (El-Tantawy et al., 2016), its possible hormonal and cytotoxic effects on human cancer cells (Bobach et al., 2014), and its air purifying capabilities (Wolverton and Wolverton, 1993).

The genus name comes from the Greek words *nephros* meaning a kidney and *lepis* meaning a scale, in reference to its kidney-shaped indusia. The specific epithet (*exaltata*) means very tall or lofty.

The species, *Nephrolepis exaltata* (L.) Schott, is a perennial plant, terrestrial or epiphytic in its native state (both forests and open habitats) (Nauman, 1993), mostly found in the tropics as it prefers relatively high temperatures and high humidity, but no direct sunlight. Originally it is from the south of the USA, Central and South America, but it has become naturalized in different parts of the

world such as the Canary Islands, Africa, Asia, India, Polynesia and New Zealand (Large and Farrington, 2016). Its fronds are alternatively pinnate with deltate-oblong, falcate to different degrees and minutely serrated pinnae, with different degrees of inequality, a truncated to auriculated base, having deltate or acute, acroscopic lobes (Nauman, 1993; Hovenkamp and Miyamoto, 2005). Its indusia are kidney to U-shaped attached at a sinus, described as "narrow or broad" by Nauman (1993), although in a more recent revision it is claimed that typically the species has a "wide" sinus (Hovenkamp and Miyamoto 2005). It propagates usually asexually through long thin and green runners (but also through spores) and is a common houseplant commercially found in florist shops. It closely resembles *Nephrolepis cordifolia* (L.) C. Presl, but presents no tubers (Nauman, 1993; Hovenkamp and Miyamoto, 2005) and its fronds are not erect, but arching. Because of its sword-like fronds it received the name sword fern (Langeland, 2001).

The phytochemistry of *Nephrolepis exaltata* (L.) Schott has previously been investigated qualitatively only, and the following chemical constituents have been found: saponins, flavonoids, tannins and reducing sugars (Oloyede et al., 2014).

The species is traditionally used in the island of Fiji to treat women's menstrual disorders (Cambie and Ash, 1994).

The more studied species, *Nephrolepis cordifolia* (L.) C. Presl, has the following folkloric uses: in India it is used to stop the bleeding of wounds, as a treatment for coughs, for stomach and intestinal disorders (Singh and Upadhyay, 2014).

The plants in this study were cultivated in a hydroponic deep water culture system as opposed to regular soil. Hydroponics is a method of growing plants using no soil and a nutrient solution that contains all the macro and micro-elements needed for the plants to grow. Deep water culture is one of the simplest methods of cultivating plants in a pure hydroponic medium, as the plants have their roots permanently submerged in an aerated solution which provides dissolved oxygen to the roots. This hydroponic setup has the advantages of eliminating the possible diseases related to soil pathogens, the mineral composition and the pH of the solution can be fully controlled in order to better suit the plants nutrient needs, thus eliminating potential nutrient deficiencies. Also, there are no water pumps that can become clogged in time. Plants usually grow faster in hydroponics than in geoponics. There are, in counterpart, a few downsides: a regular maintenance is needed, as well a constant control of the possible pH fluctuations; the water temperature is hard to maintain consistently as it directly influences dissolved oxygen concentrations. The water must be constantly changed in order to prevent growth of possible algae and other potentially pathogenic microorganisms (anaerobic bacteria and fungi). If not, the roots can become subject to rotting, regularly induced by the parasite *Pythium* spp. (Owen-Going et al., 2003). Plants grown hydroponically can more easily be handled and the roots examined, compared to plants grown in soil. Also, the nutrient solution can be used to test substances on the plants during scientific experiments.

Our objectives in this study were to check the identity of the species and establish its specific histo-anatomical elements of rhizomes, rachises, pinnae and runners through microscopic analysis of cross-sections and surface preparations using bright field and fluorescent microscopy. In order to estimate its potential safety for human use, in the present study we have investigated its phytotoxicity on *Triticum aestivum* L. and its lethality on the invertebrate brine shrimp, *Artemia franciscana* Kellog.

2. Materials and Methods

2.1 Hydroponic growth conditions

The plants were cultivated in an in-house laboratory hydroponic deep water growth system. They were obtained commercially (Dedeman, Bucharest, Romania, imported from the Netherlands), potted, having at the time of purchasing about 30 cm in height and 12 cm in diameter. The roots were cleaned of earth, washed and planted in net pots filled with hydrocorn. The grow tent used was a HL 100 V2.0 (HOMEbox, Berlin, Germany). The water was constantly aerated using a Hailea ACO 9602 air pump that had an output of 7.2L of air per minute. The tubing had 4 mm in diameter and the airstone was a round ceramic 150 mm Hailea airstone (pepika.ro). The plants had a 12 hour day/night light cycle and were grown under a 250W growth spectre MH lamp (GIB lighting, Germany). The photosynthetic active radiation (PAR) was measured with a MQ-500 full-spectrum quantum sensor (Apogee Instruments, terra-preta.ro) and had 10 µmol m^{-2} s^{-1} used to simulate household conditions. Inside the tent the average day temperature was 26.39ºC (s.d.1.18) and the average relative humidity 65.32% (s.d.4.41). The hydroponic solution was made using Flora Series (General Hydroponics, USA), was kept at a concentration used for cuttings or seedlings, had an average pH of 6.51 (s.d.0.10) and an average temperature of 24.22ºC (s.d.1.24). Measurements were made using an ADWA AD31 total dissolved solids (TDS) meter (ADWA, Hungary) and a Testo 206-pH1 pH meter (Testo, Germany).

2.2 Macroscopic and microscopic examination

The species' identity was confirmed by macroscopic and microscopic examinations on rhizomes, rachises, pinnae and runners using bright field and fluorescent microscopy. We used a Labophot-2 microscope equipped with a Nikon digital camera; an Euromex Oxion microscope equipped with a CMEX 5 camera; an Optika B-383FL fluorescence microscope equipped with a Nikon digital camera. We have examined surface preparations from the pinnae, clarified with NaOH 5% and manually obtained cross-sections from rhizomes, rachises, pinnae and runners clarified with Javel water and stained with iodine green and carmine alum double stain, FABIL (Dinu et al., 2013) and calcofluor (Herth and Schnepf, 1980).

2.3 Toxicity assay

For both the phytotoxicity and the lethality assays, the extracts were prepared as follows: 5g of dried and pulverized fronds were extracted with 50ml ethanol 96% at reflux (about 70ºC, for 30 minutes): after cooling, the solution was filtered in a 50 ml volumetric flask and filled to the mark with solvent in order to obtain a 10% ethanol extractive solution. This solution was then evaporated on a hot plate in order to obtain a dry extract which was then suspended in 50ml of distilled water using an ultrasonic bath. The 10% aqueous extractive solution was obtained from leaves in a similar manner, using a temperature of 100ºC, for 30 minutes.

2.3.1 Phytotoxicity assay

The assessment of the phytotoxicity was done on embryonic wheat roots (*Triticum aestivum* L. - the Constantinescu method) (Dinu et al., 2012). The ecological Spelta wheat was obtained commercially from Solaris Plant. We examined the influence on the embryonic root elongation and the modifications suffered by the mitotic film in root tips.

The wheat caryopses were germinated in laboratory conditions (kept for 24h in distilled water at room temperature in Linhardt dishes). After germination 11 caryopses with a 1 cm root tip were collected in separate Petri dishes

(ten to be used for the root elongation assay, one for the acetic orcein staining and microscopic investigation). For both extracts five samples were prepared: for the ethanol extracts: E1 (10%), E2 (5%), E3 (1%), E4 (0.5%), E5 (0.1%) and for the aqueous extracts: A1 (10%), A2 (5%), A3 (1%), A4 (0.5%), A5 (0.1%) (the concentration is expressed as grams of dried plant material for 100 ml of solvent). The control sample (M) consisted of distilled water. The root elongation was observed at the same time of day for three consecutive days. For the microscopic study of the nuclei from the embryonic root, we used the acetic orcein stain and squash method. The mitosis was observed under the 100X objective lens after immersion in cedar oil. Root elongation and karyokinetic film modifications were evaluated (Dinu et al., 2012).

2.3.2 Lethality assay

The brine shrimp lethality assay was performed on *Artemia franciscana* Kellog. The cysts were commercially obtained from Ocean Star International (USA), repackaged by S.K. Trading (Thailand), originating from Great Salt Lake (USA). Artificial seawater was prepared dissolving a commercial salt mixture (Coral Marine, Grotech) in distilled water, by sonication, at a concentration of 33.5 g/L. The hatching was initiated about 48 hours before the initiation of the test, as it takes cca 36-48 hours. The test was carried out in a 24 (6 x 4) multiwell test plate, the extract solutions of different concentrations being placed in triplicate in the wells. The concentrations used were 10%, 5%, 1%, 0.5%, 0.25%, 0.125%, and 0.0625% for both the aqueous and the ethanol extracts. As a control, artificial seawater was used.

The hatched nauplii were transferred to the wells with a micropipette, counting between 10 and 20 nauplii per well. All dead and alive nauplii at 24h and 48h were counted and recorded (Ancuceanu et al., 2016).

2.4 Statistical analysis

The statistical comparisons between multiple groups for the concentration and the type of extract for the *Triticum* assay were done by using robust and parametrical models of multiple regression (equivalent to bidirectional ANOVA models). The statistics were performed with the R packs “car”, “robustbase” and “robust” (Fox and Weisberg, 2011; Maechler et al., 2017; Wang et al., 2017) on the values measured at 48h.We have evaluated the lethality and calculated the IC_{50} and LC_{50} by using non-linear regression (4-parameter logistic models, as implemented in the R pack “dr4pl”) (Landis et al., 2018).

3. Results and discussions

3.1 Macroscopic and microscopic examinations

The identity of the species has been confirmed by macroscopic findings consistent with the literature data: the sessile pinnae can be recognized macroscopically by their pale green color and by their deltoid shapes, with unequal bases, the anterior part being auriculated, the edges slightly serrated, the indusia kidney shaped, the spores ellipsoidal or spherical with an uneven surface. (Kramer et al., 1990; Nauman, 1993; Ancuceanu, 2013). Based on our microscopic examination, the following histological characteristics (see Fajuke et al., 2018 for surface preparations) have been highlighted:

Similarly to other fern species (Dinu et al., 2013; Dinu and Ancuceanu, 2016), the rhizomes show a thick lignified hypodermis, cortical parenchyma and a polystelic structure with hadrocentric vascular bundles of the meristele type.

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The runners are covered in trichomes, and show an internal structure similar to the rhizome, but with less lignification. The rachis is covered with few pluricellular uniseriate trichomes and internally shows a lignified epidermis with cuticle and a pluristratified lignified hypodermis, a large hadrocentric vascular bundle in the stele encircled by an endodermis which shows Casparian strips. Internally they show a homogenous structure, hadrocentric vascular bundle, lobed cell epidermis (puzzle-shaped), stomata with 2 to 3 diacytic or anisocytic accessory cells, pluricellular uniseriate trichomes with a rounded tip. The clarified and stained cross sections are shown in **Fig. 1**, the surface preparations are shown in **Fig. 2** and the observations for the surface preparations are similar with those found in the literature (Fajuke et al., 2018; page 23).

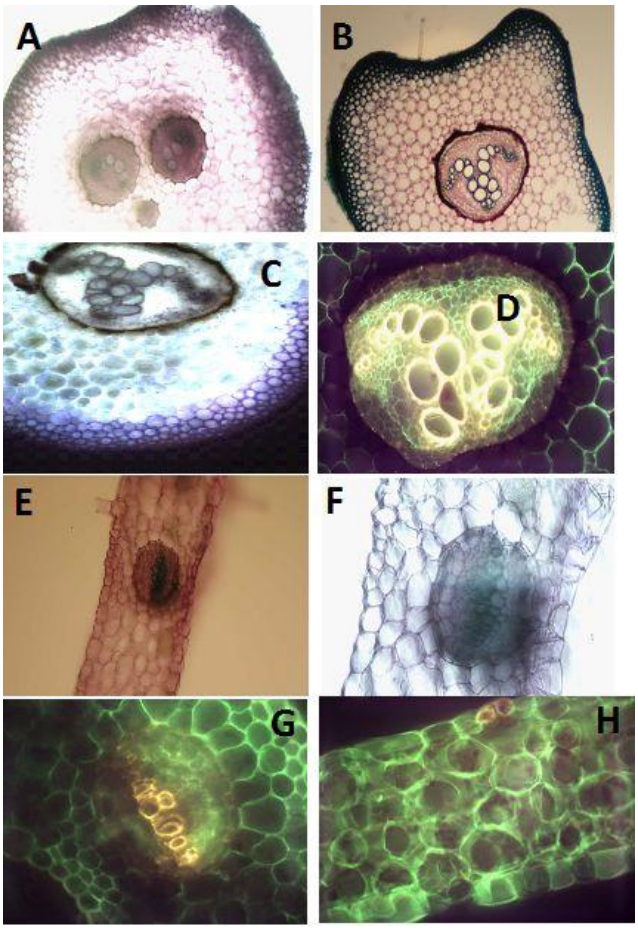

Fig. 1. Cross sections of the rhizome, rachis, and pinna of *Nephrolepis exaltata*:
A. Rhizome - polystelic structure, lignified pluristratified hypodermis, hadrocentric vascular bundles (meristeles) (rhizome, ob. 4x, ig+ca); **B.** Rachis - epidermis and lignified pluristratified hypodermis, pluricellular uniseriate trichome, hadrocentric vascular bundle (ob. 4x, ig+ca);
C. Rachis - epidermis and pluristratified lignified hypodermis, hadrocentric vascular bundle (ob. 4x, f); **D.** Rachis - hadrocentric vascular bundle in the stele, endodermis with Casparian strips (ob. 10x, c); **E.** Pinna - homogenous inner structure, basal trichome cell, hadrocentric vascular bundle, Casparian strips on the endodermis (ob. 4x, ig+ca); **F.** and **G.** Pinna - hadrocentric vascular bundle, Casparian strips present on the endodermis (ob. 10x, f and c); **H.** Pinna - Epidermis with cuticle, stomata, substomatal cavity, homogenous inner structure (ob. 10x, c). Abbreviations: ig – iodine green; ca – carmine alum; f – FABIL; c – calcofluor.

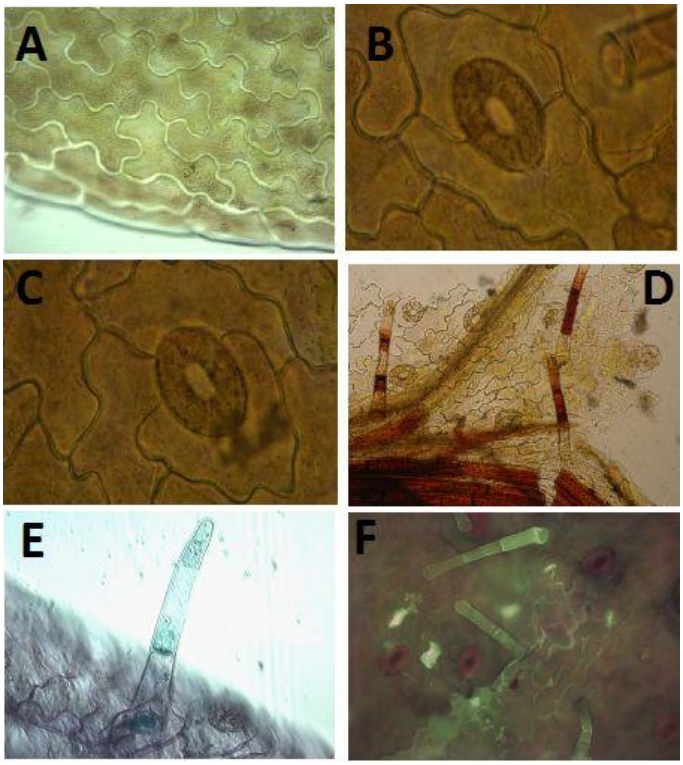

Fig. 2. Surface preparations of the *Nephrolepis exaltata* pinnae, clarified with NaOH 5%: **A.** Epidermis with cuticle, lobed cells (ob. 10x, no staining); **B.** Diacytic stoma (ob. 40x, no staining); **C.** Anisocytic stoma (ob. 40x, no staining); **D.** Stomata, uniseriate pluricellular trichomes, lobed cells (ob. 10X, no staining); **E.** Uniseriate pluricellular trichome (ob. 40x, FABIL); **F.** Stomata, uniseriate pluricellular, trichomes, lobed cells (ob. 10x, calcofluor).

3.2 The phytotoxicity assay

The analyzed extracts have a moderate dose-dependent mitoinhibitory effect, ($p<0.0001$) on the *Triticum aestivum* embryonic tips, producing a mitodepressive and stathmokinetic effect. No statistically significant difference was seen between the aqueous and the ethanol extracts with respect to the inhibitory effect on the root elongation ($p>0.64$) both in the parametric models of multiple regression and on the robust regression models. The IC_{50} for the aqueous extract was of 0.93% (95%CI 0.70-17.96%). Strong inhibition of root elongation occurs only at high concentrations (10%, 5%), which suggests that the phytotoxic effect of the extract is relatively low. The results for the *Triticum* assay are presented in **Fig. 3**.

The microscopic examination of the acetic orcein and squashed wheat root tips revealed the following for both the ethanol and the aqueous extracts: At high concentrations (10%) the mito-depressive effect was pronounced: elongated prophases, interphases characterized by nuclei with hypertrophied nucleoli, quasi-normal metaphases, anaphases, very frequent telophases with slight tropokinesis. At 1% and 0.5%, interphases with 2-3 hypertrophied nucleoli, metaphases showing tropokinesis, anaphases with bridges, frequent telophases with bridges and slight tropokinesis; metaphases and telophases are predominant.

At 0.1%, numerous divisions have been observed; interphase cells with nuclei having two to four hypertrophied nucleoli, numerous prophases, metaphases in tropokinesis and numerous telophases (normal, in tropokinesis, and with a simple bridge indicating delayed chromosomes) (**Fig. 4**).

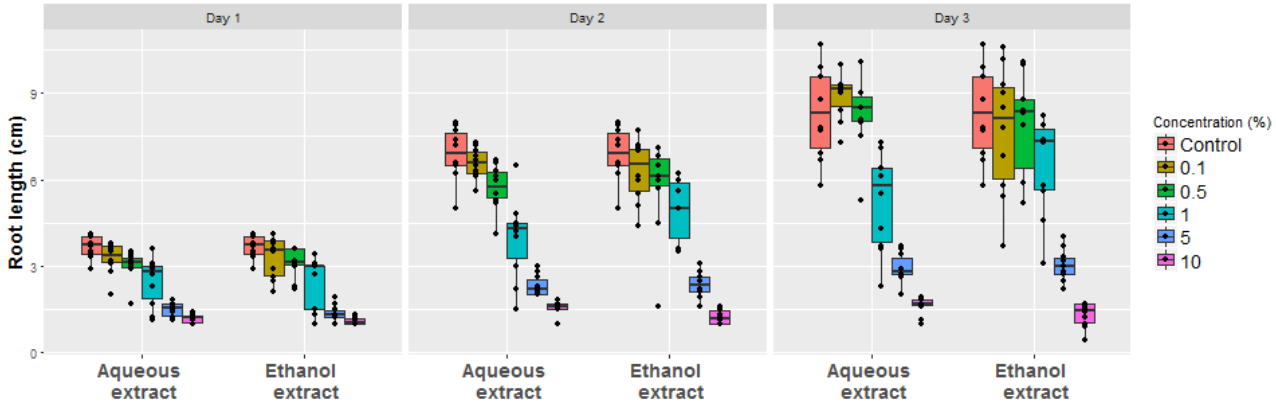

Fig. 3. Boxplot and point type graphs illustrating the root elongation (*T. aestivum* L.) variation according to the *Nephrolepis exaltata* extract, concentration of extract and day of measurement

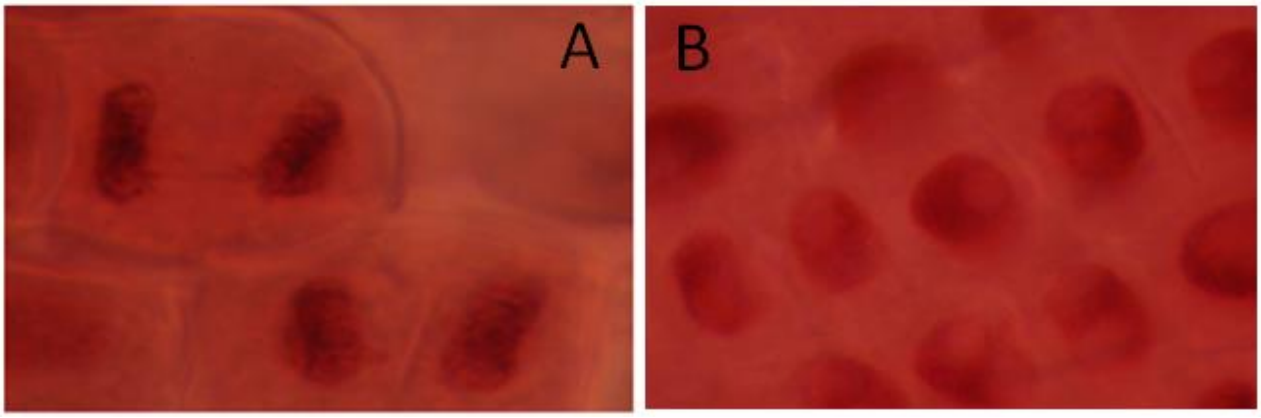

Fig. 4. Acetic orcein stained nuclei of the embryonic root tips of *Triticum aestivum* tested with aqueous and ethanol extract of *Nephrolepis exaltata*: **A.** Telophase in tropokinesis and simple bridge. **B.** Nuclei with hypertrophied nucleoli (ob. 100x).

3.3 The lethality assay

In the *Artemia* assay, LC_{50} for the aqueous extract was 1.093% (95% CI 0.869-1.374%), and the LC_{50} for the ethanol extract was 0.686% (95% CI 0.400-1.172%). In the literature, LC_{50} values higher than 0.05% have been stated to be "practically non-toxic" (Moshi et al, 2010). The lower margin of the 95% CI of the values computed by us for the two extracts are much higher than the 0.05%, and thus one should conclude that the extracts of *N. exaltata* are virtually devoid of acute toxicity. The concentration-lethality curves for the two extracts are shown in **Fig. 5**.

Nephrolepis exaltata (L.) Schott was previously considered to belong to the families Oleandraceae, Davalliaceae or Lomariopsidaceae and has recently somewhat provisionally classified to its own family, Nephrolepidaceae due to uncertainty in its phylogenetic placement, (Kramer, 1990) until the accumulation of further data. Our microscopic data is consistent with the close relationship between the genus Nephrolepis and these families, although they are not very specific. Oleandraceae, although the anatomy has variations, are usually characterized by a peripheral sclerified sheet, parenchyma and a dictyostele. Similar features are observable in *N. exaltata* (Hovenkamp and Ho, 2012). However, the dictyostele seen in the rhizome of *N. exaltata* seems quite different in morphology from the one seen in a published image for *Oleandra musifolia* (Nopun, 2016).

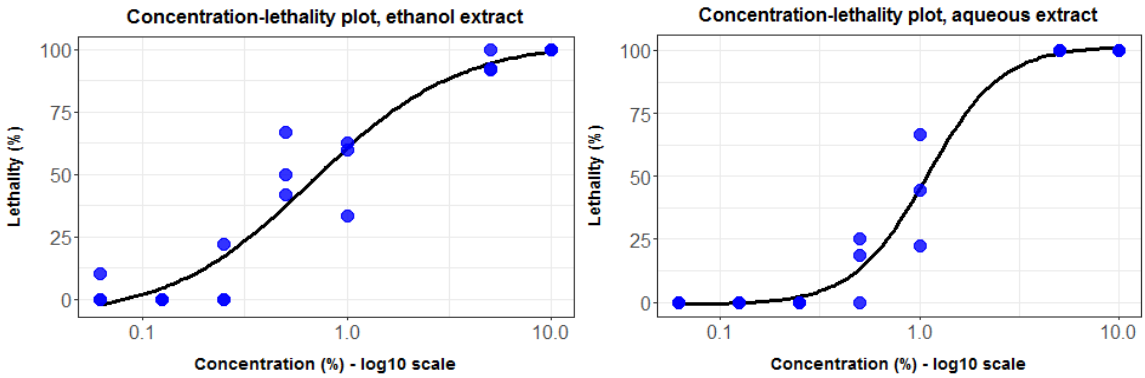

Fig. 5. Dose-response graphs and modeling for the *Artemia* assay with *Nephrolepis exaltata* extracts

A dictyostelic structure of the long-creeping rhizomes has also been described for the Davalliaceae species (Smith et al., 2006). The dictyosteles of Lomariopsidaceae have elongated ventral meristeles, (Chen et al., 2017) unlike those seen in our *Nephrolepis* sections.

The low inhibitory effect seen in the *Triticum* test, as well as the low lethality observed in the *Artemia* bioassay suggests that the plant may be safely used for therapeutic purposes. There is little ethnopharmacological knowledge on the use of this species. However, data from Fiji and India show that it is traditionally used (the rhizome) for the treatment of menstrual disorders, women's sterility and as an aid in childbirth (Cambie and Ash, 1994; Singh and Singh, 2012). In vitro data on prostate cancer cell lines LNCaP and PC-3 indicated that fractions from leaves might have potential antiandrogenic effects (Bobach et al., 2014). Such folk medicine data together with the apparent low toxicity seen in our experiments indicate that further investigation on both its chemical composition and pharmacological properties of extracts prepared from rhizomes and leaves are of interest and should be carried out.

Conclusions

In this study we have characterized the microscopic histo-anatomical elements of identification of the species by using cross-sections of plant parts and surface preparations. The ethanol and aqueous extracts of *Nephrolepis exaltata* (L.) Schott have reduced toxic effects both on the roots of *Triticum aestivum* L. and on the *Artemia franciscana* Kellog nauplii. Further research is needed to elucidate fully the chemical composition of this fern and its potential pharmacologic activity.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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