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THE USE OF HYDROPONIC TECHNIQUES FOR THE CULTIVATION OF PLANTS WITH MEDICINAL PROPERTIES AND SOIL PHYTOREMEDIATION POTENTIAL

PHD THESIS ABSTRACT

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Contents of the doctoral thesis

Introduction

Hydroponic plant cultivation techniques have been known in their modern form for more than a century and, although most applications and research have been oriented towards plants for food or industrial use, for at least several decades there has been a growing interest in the use of these unconventional cultivation techniques in the field of medicinal plants as well [1-3]. In this context, we have been interested in exploring this method of culture in obtaining plant products from three medicinal plant species from very diverse taxonomic groups: a fern (*Nephrolepis exaltata* (L.) Schott, hereafter abbreviated NE), a dicotyledon (*Taraxacum officinale* (L.) Weber ex F.H. Wigg, TO) and a monocotyledon (*Iris germanica* (L.), IG).

NE is the least studied of the three species: its phytochemical composition is only superficially known and its therapeutic potential has remained almost untouched by pharmacological research. However, some hydroponic cultivation studies have been carried out [4-6], so there is some ground from which to start our studies. In contrast to NE, both TO and IG have been extensively investigated, with the current researcher benefiting from many phytochemical or experimental pharmacology data, summarized also in a few review synthesis evaluations [7-12]. Neither the impact of the hydroponic environment on the growth rate and productivity of these plant species, nor the effect of growth factors such as gibberellic acid, humic substances, salicylic or acetylsalicylic acid in the concentrations tested on them when grown in hydroponic systems has been studied so far. Hydroponic culture offers unique possibilities to investigate the phytoremediation potential of heavy metals such as vanadium, strontium or barium, and I wanted to exploit these possibilities in my PhD studies.

As with phytochemistry, there is no data in the scientific literature on the toxicity of NE, nor on its potential pharmacologic effects. Therefore, in the framework of our doctoral research, we also aimed to perform toxicity tests on monocotyledons, dicotyledons and invertebrates on polar and non-polar extracts obtained from the 3 species. We did this, in a comparative approach, for extracts prepared from specimens grown in hydroponic and geoponic environments, respectively. Similarly, we made comparisons of such extracts in terms of their antioxidant capacity, total polyphenols and flavonoids concentration. We have also taken steps to explore the pharmacological potential of the three species, in particular by performing, in collaboration with colleagues from the Pharmacology department of the

faculty, a screening of the activity of some extracts from NE on the central nervous system, as well as by using QSAR technique to identify the chemical compounds in IG potentially responsible for the hypocholesterolemic effect of an extract obtained from the latter species. For the NE species, further studies are needed to determine the chemical composition and to further evaluate the therapeutic potential, in particular the phytoestrogenic potential.

Chapter 1. Overview of hydroponic techniques

The general part contains overviews on the definition, history of hydroponics, hydroponic systems in use, parameters influencing plant growth in hydroponic systems, advantages, disadvantages of hydroponics and the comparison between hydroponic and geoponic growth systems.

Chapter 2. Data on the plant species selected and included in the hydroponic/geoponic study

The second chapter of the general part summarizes the known scientific data on the 3 selected plant species: *Nephrolepis exaltata* (L.) Schott, *Taraxacum officinale* (L.) Weber ex F.H. Wigg, *Iris germanica* (L.).

Chapter 3. Study hypothesis and general aims

Potential phytochemical, anatomical or toxicological differences in our selected species grown in geoponic versus hydroponic environments have not been explored so far. Our hypothesis is that such differences are minimal, but at the initiation of the PhD research experimental evidence in this respect was lacking. Therefore, we set out to comparatively investigate the morphological (by macroscopic and microscopic evaluations) and phytochemical characters of the three species grown in geoponic and hydroponic environments.

There are also epistemic gaps in the scientific literature regarding the impact of hydroponic cultivation on the improvement of the growth rate and crop quality of these plants as well as the effect of certain growth factors such as gibberellic acid, humic substances, salicylic acid or acetylsalicylic acid in the tested concentrations on the above mentioned hydroponic cultivation. We therefore set out to investigate these aspects through a series of designed experiments.

These three species have not been studied so far for their potential usefulness in the

phytoremediation of toxic metals such as vanadium, strontium or barium, nor has the effect of these heavy metals on the cultivation of these three plant species been studied. In addition, the hydroponic environment offers superior possibilities to investigate this issue by eliminating the statistical confounding effect of the soil and its properties, including the soil microbiome, in conventional cultivation [13], and also to avoid bioavailability problems that occur in geoponic experiments [14].

Experimental data on the toxicity of the species included in the study is generally poor, and for NE there is no data at all. In this context, we were interested in carrying out phytotoxicity tests and assessing the toxicity on invertebrates (nauplii of *Artemia franciscana* Kellog) of polar and non-polar extracts obtained from them. These evaluations were done comparatively, in order to detect (indirectly, via toxicity) possible differences in phytochemical content properties between hydroponically and geoponically grown specimens. Similarly, we aimed to comparatively evaluate the antioxidant capacity, total polyphenol and flavonoid concentration of a series of lyophilized extracts obtained from specimens cultivated in the two media.

Given the very poor data on the pharmacology of the NE species, we proposed (in collaboration with the Pharmacology department of the faculty) to screen the central nervous system activity of extracts from this plant species. We were also interested in investigating whether compounds biosynthesized by IG have the ability to inhibit HMG-CoA-reductase, using a series of regression-based QSAR models for inhibitors of this enzyme.

Chapter 4. Establishing the identity of the studied species

As a first step in our doctoral research, we were concerned with the identification of the studied species. In addition, for the three species examined, we aimed to assess the impact of hydroponic cultivation on the morphology and anatomy of their vegetative organs. For *Nephrolepis exaltata* (L.) Schott, we did not find, in the literature consulted, data on histo-anatomical changes in the vegetative organs below and/or above ground following hydroponic culture. We assumed that there would be no significant differences, but we wanted to evaluate these aspects experimentally.

Materials and methods

Both for the studies reported in this and the following chapters, the fern specimens were obtained commercially (Dedeman, imported from the Netherlands), were potted, of approximately 30 cm tall and 12 cm in diameter at the time of purchase. The dandelion was

grown either directly from seeds collected in the county of Dâmbovița, or from rhizomes (with roots) collected from the surrounding areas of Bucharest and Ilfov. The rhizomes of *Iris germanica* L. were obtained courtesy of the staff of the Botanical Garden "Dimitrie Brândză" in Bucharest.

The identities of the studied species were confirmed by macroscopic and microscopic examinations of the roots, rhizomes, leaves, rachis, pinnules and stolons, using light and fluorescence microscopy and by comparison with the literature of reference (Flora of North America [15] and World Flora Online [16]). We examined the surface preparations of pinnules (NE) and leaves (TO, IG), clarified with 5% NaOH, and manual cross sections of rhizomes, rachis (NE), pinnules (NE), leaves and stolons (NE) clarified with potassium hypochlorite (bleach) and double stained with iodine green and carmine alum, fabil (NE) [17] or calcofluor white [18].

Results and discussions

Macroscopic examination of *Nephrolepis exaltata* (L.) Schott

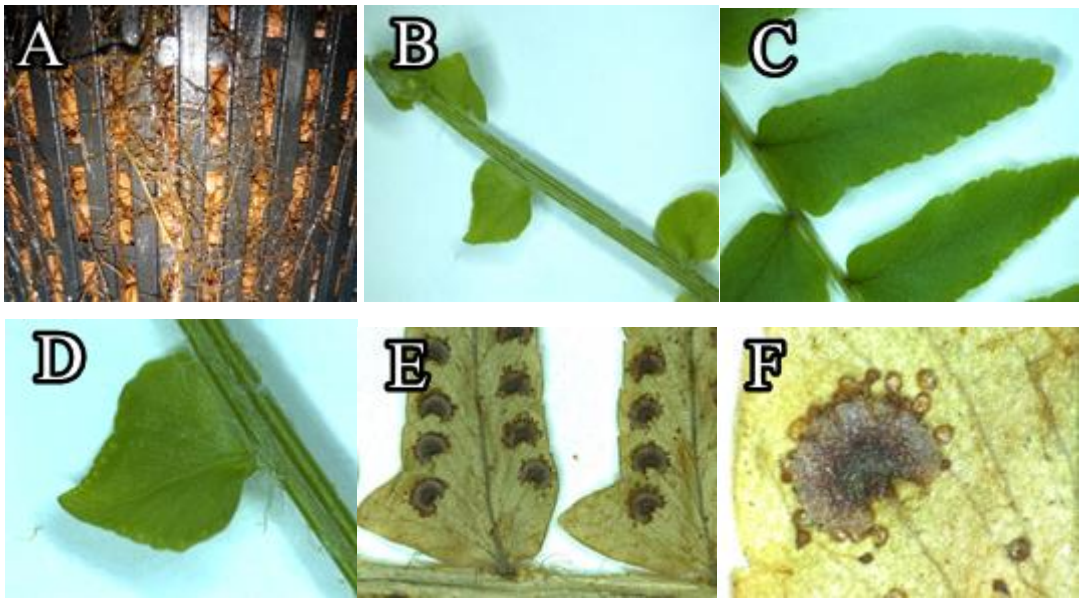


Fig. 4.1 Photographs of NE organs grown hydroponically and geoponically (B-F digital microscope):

A - rhizomes with fine roots; B - crutch-shaped young frond; C - frond, pinule with a serrate-crenate and a typical, auriculate lobe; D - young frond and trichomes on the rachis; E - underside of a mature frond with indurated sori and sporangia, geoponic specimen; F - sori with reniform indusium and pedicellate sporangia (digital microscope), geoponic specimen.

Microscopic examination of *Nephrolepis exaltata* (L.) Schott (NE)

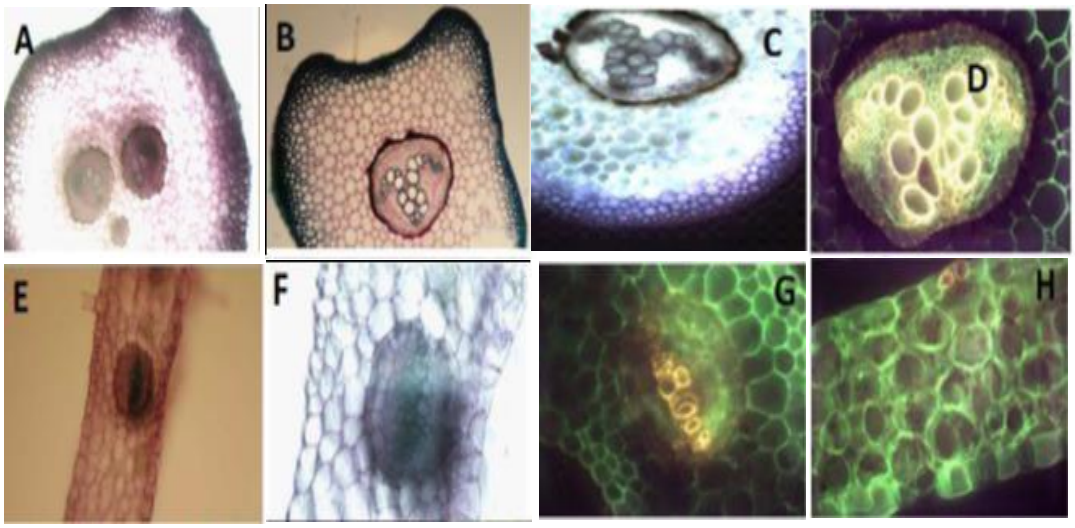


Fig. 4.2. Cross sections through the rhizome, rachis and NE pinula:

A - Rhizome - polystelic structure, pluristratified and lignified hypodermis, hadrocentric vascular bundle (ob. 4x, double stain); B - Rachis - pluristratified, lignified epidermis and hypodermis, uniseriate pluricellular trichomes, hadrocentric vascular bundles (ob. 4x, double stain); C - Rachis - lignified, pluristratified epidermis and hypodermis, hadrocentric vascular bundle (ob. 4x, fabil); D - Rachis - hadrocentric vascular bundle, endodermis with Caspary bands (ob. 10x, calcofluor); E - Pinula - homogeneous structure, basal trichomatous cell, hadrocentric vascular bundle, endodermis with Caspary bands (ob. 4x, double stain); F and G - Pinula - hadrocentric vascular bundle, endodermis with Caspary bands (ob. 10x, fabil F and calcofluor G); H - Pinula - epidermis with cuticle, stomata, substomal chamber, homogeneous structure (ob. 10x, calcofluor).

The identity of the NE species was confirmed by macroscopic observations that are in agreement with the literature data: the pale green sessile pinules are deltoid with unequal bases, auriculate anterior part, slightly serrate margins, reniform indusium, ellipsoidal or spherical spores with uneven surface [19-21].

Also for the other two species, macroscopic and microscopic observations were in agreement with the literature.

Comparative macroscopic and microscopic examinations (using a variety of microscopy techniques and stains) revealed no significant differences between specimens grown in the hydroponic versus the geoponic medium.

Chapter 5. Comparative geoponic and hydroponic cultivation of the species of interest

Introduction

Some studies have been published in the scientific literature comparing the efficacy of conventional (geoponic) and hydroponic cultivation processes, especially for plant species of food interest (e.g. *Spinacea oleracea* L. [22], *Telfairia occidentalis* Hook.f. [23]). Since no studies have been conducted on the impact of growing the NE, TO, IG species in the hydroponic compared to the geoponic medium, we aimed to conduct this study to determine whether or not there are differences of the two growing media on the cultivated plants, whether these species grow faster and larger in hydroponic compared to geoponic medium.

Materials and Methods

For the geoponic medium, an equal mixture of three commercial substrates was used: garden substrate, orchid substrate and universal soil for flowering plants (Agro CS, Klasman-Deilman Gmbh). The water used came from tap water and had 7 German degrees of hardness corresponding to 70 mg/L CaO or 124.936 ppm.

After 30 days, a number of variables of interest were measured to assess differences in the developmental status of the two samples grown in different media: stomatal conductance, leaf number, leaf length, leaf area and dry plant mass. Stomatal conductance was measured using a steady-state diffusion-based leaf porometer, model SC-1 (Decagon Devices), on several leaves from each specimen (depending on the number of leaves of each specimen). Leaf length was estimated using a measuring roller and leaf area was estimated using the software program ImageJ v. 1.53 [24], following photography.

All statistical analyses were performed in the R computing and programming environment, version 4.4.1 [25].

Results and discussions

The experimental data indicate that the differences between the parameters analyzed for specimens grown in hydroponic and geoponic environments do not differ significantly, and any differences that could not be detected due to insufficient statistical power are generally small, so that any undetected differences are unlikely to be practically relevant, at least for the variables of interest analyzed. These make hydroponic cultivation of NE, TO and IG a viable and reasonable alternative to traditional, geoponic cultivation. However, we did not obtain in our experiments any evidence of a clear superiority of one of the cultivation methods over the other (but it is possible that further optimization may reveal such

superiority, as the literature often indicates the superiority of the hydroponic method [22], [23]).

Chapter 6. Hydroponic growth factor tests

Introduction

In view of the particularly simple possibility offered by hydroponic cultivation systems to apply growth factors in the growing medium without the challenges that soil brings, we were interested in assessing the extent to which the administration of gibberellic acid (GA3), salicylic acid (SA), acetylsalicylic acid (AS) and humic substances (HS) in the water culture can influence the development of the cultivated plants.

Materials and methods

Two HL 100 V2.0 culture tents with 2 culture pots in each were used for hydroponic cultivation experiments with growth factors (and for phytoremediation experiments). The plants had a 12 h day/night light cycle and were grown under a 250 W growth spectra MH lamp (GIB lighting, Germany). Measurements were carried out using an ADWA AD31 total dissolved solids (TDS) meter (ADWA, Hungary) and a Testo 206-pH1 pH meter (Testo, Germany).

The deep water culture system used was a proprietary one, each tank was made of 25L opaque PVC boxes, six 12 cm mesh pots, expanded clay and each pot was filled with a 15L solution made with hard water and 30 - 40 ml of concentrated liquid fertilizer for the vegetative growth cycle of green plants (SC Amia International). The water was constantly aerated using a Hailea ACO 9602 air pump, which had a flow rate of 7.2L of air per minute. The tubing was 4 mm in diameter and the 150 mm air stones were ceramic. pH was adjusted to between 5.5 and 6.5 with citric acid and 20 mL of 3% hydrogen peroxide was added for the disinfecting and oxygenating effect on the submerged roots.

Photosynthetically active radiation (PAR) was measured with an MQ-500 full-spectrum quantum full-spectrum sensor (Apogee Instruments, terra-preta.ro). This was 47.33 µmol/m2s ± 11.16 for pot 1; 56.50 µmol/m2s ± 11.74 for pot 2; 56.83 µmol/m2s ± 4.91 for pot 3 and 52.33 µmol/m2s ± 5.35 for pot 4.

The **gibberellic acid** (GA3) used in the experiment was obtained from a commercial source (GibbA3 T, 20% x 5g tablets, S.C. RomSoft SRL). It was used at a concentration of 0.02, 0.1 and 0.5 ppm for the first three culture pots. The growth factor **salicylic acid** (SA; Fagron) was used in the first three pots at the following concentrations: 10 µM, 100 µM and

150 µM. The growth factor **acetylsalicylic acid** (AAS, synthesized in the faculty) was used in the first three pots at a concentration of 50 µM for the first pot, 100 µM for the second and 10 µM for the third. The **humic substances** (HS) used in the experiment were obtained from a commercial product called Diamond black (General Organics), which contained humic acid derived from lignin. This was used at a concentration of 240 ppm humic acids for the first pot, 360 ppm for the second and 40 ppm for the third.

The plants were kept in contact with the growth factors for two weeks and the solution was changed weekly to prevent degradation of the growth hormone. The same final evaluation criteria were followed as in the previous chapter.

Results and discussions

None of the mentioned phytohormones have been evaluated so far in hydroponic systems of the three species we evaluated.

In our experiments we did not observe any significant effect of **GA3** (on any of the three species) and we assume that this phenomenon is a consequence of the too low concentrations used by us (maximum 0.5 mg/L).

We did not observe significant effects on leaf, stomatal conductance or leaf mass, underground organs or total dry mass for the evaluated species. At the higher concentration levels tested by us (100 and 150 µM AS), there were trends towards inhibitory effects on leaf number, leaf area and a significant inhibitory effect on stomatal conductance. Since our samples were limited to six plants per group, a type I error is not impossible, but it warrants repeating the experiment (and using higher concentrations) to assess whether this is a specific effect or a randomized observation.

No significant effects of the acetylsalicylic acid were observed on the development of the three species evaluated.

At the concentrations used in the experimental studies (40 mg/L), humic substances had no significant beneficial effects on the development of the three species in hydroponic systems. Limited experimental data suggested that higher levels might be beneficial (in agreement with the manufacturer's recommendation), but this needs experimental confirmation.

Chapter 7. Hydroponic phytoremediation tests

Introduction

Cleaning up soils or waters contaminated with toxic metals can be done economically through phytoremediation. This involves using plants to extract and accumulate contaminating metals from soil or water.

Neither NE, TO or IG have been tested to assess their potential for phytoremediation of the toxic metals vanadium, strontium and barium. Also, the toxic effect of these metals on the three plant species when grown in hydroponic medium is unknown. In addition, the hydroponic medium provides better possibilities to evaluate these aspects, as it eliminates the confounding effect of soil characteristics and soil microbiome in geoponic cultivation [13]. The hydroponic medium also avoids the mass transfer (bioavailability) problems that characterize experiments carried out in a geoponic system [14].

Materials and methods

The culture method was identical to that used for the growth factor tests. Ammonium metavanadate (meta puriss., Schering) was used at the following concentrations: 10 ppm, 20 ppm, 40 ppm and none for the control. Strontium nitrate (Schering) was used in concentrations of: 50 ppm, 100 ppm, 200 ppm and barium peroxide (Schering) was used in concentrations of: 10 ppm, 65 ppm, 170 ppm.

The plant matter was brought to a constant weight using an oven and measured on an analytical scale (Partner PS 600/C/2). The analyses were performed by inductively coupled plasma mass spectrometry (ICP-MS) for IG and NE, simultaneously for V and Sr. Atomic absorption spectrometry (AAS) was used for the rest of the analyses. Data on the accumulation of Ba in vegetative organs of TO are unavailable due to an error in the analysis apparatus and the destructive nature of the technique, which led to the destruction of the samples to be analyzed.

Results and discussions

All three species evaluated tolerate V, Sr and Ba ions very well without showing signs of wilting or other signs of toxicity. All three species accumulate V, Sr, and Ba ions, but not all, and not for all metals evaluated, meet the criteria to qualify as hyperaccumulators.

NE withstands high levels of vanadium while being unaffected, accumulates relatively high amounts in underground organs, but accumulation in leaves is much lower. **NE concentrated more Sr in aerial than in underground parts and in very high amounts, indicating that it is a hyperaccumulator and highly recommended for phytoremediation, especially since it did not wilt at any of the concentrations tested.**

TO accumulates large amounts of V in the rhizome, but has the disadvantage that it accumulates little V in the leaves. It also concentrated Sr more in underground than in

aboveground parts, but Sr accumulation was in small amounts, much lower than the other species tested.

IG showed good tolerance when grown in the presence of vanadium, but it accumulated the metal in large amounts only within roots and not in rhizomes or leaves, being resistant to V, but not suitable for phytoremediation.

IG accumulated large amounts of strontium in the leaves, higher than some hyperaccumulators mentioned in the literature, being a potentially useful species for phytoremediation of this metal.

Although all evaluated species accumulated modest amounts of barium, none of them corresponded to a hyperaccumulator [26].

Chapter 8. Phytotoxicity and lethality tests on *Artemia franciscana* Kellog nauplii

Introduction

The acute toxicity of substances or mixtures of substances (including plant extracts or other products of natural origin) can be tested on a variety of organisms, from prokaryotes to mammals. Inexpensive test methods, particularly useful for preliminary assessments, include testing on monocotyledonous plant species such as *Triticum aestivum* L. [27] or dicotyledonous plant species, e.g. *Lactuca sativa* L. [28] and testing on invertebrates, e.g. *Artemia franciscana* Kellog [29].

In the context of investigating the three plant species grown hydroponically, we were interested in testing their toxicity in order to assess, to what extent this cultivation method influences the phytochemical profile and hence the toxicity of the respective species (it is assumed that the differences are minimal). In addition, the toxicity of NE has not been previously assessed, so for this one, toxicity assessment is of additional interest.

Materials and methods

For all three species, aqueous and alcoholic extracts were obtained from the leaves, rhizomes, roots from geoponically and hydroponically grown plants. Each plant material was dried, finely ground (500 μm mesh sieve) and two extracts of 5% concentration were obtained: one with absolute methanol and one with distilled water, by heat. For the hydroponically grown NE fronds, ethanol was used instead of methanol and the extracts were evaluated for phytotoxicity at the following concentrations 10%, 5%, 1%, 0.5%, 0.1%. For all other plant products, methanol was used as the alcohol and the following concentration levels were used to assess phytotoxicity: 5%, 1%, 0.5%, 0.5%, 0.25% and

0.125% (10% concentration having total inhibitory effects on plants). The *Lactuca* test was performed only for IG because difficulties were encountered with seed germination.

The micro-crustacean *Artemia franciscana* Kellog, AF (Ocean Star International) [30] was used for the lethality test. The concentrations used were 0.50%, 0.25%, 0.125%, 0.0625% and 0.03125% for both aqueous and methanolic extracts. The protocol wais similar to the *Artemia salina* brine shrimp Brine Shrimp Lethality Assay [31].

Organic wheat was locally procured from the Călărași county and *L. sativa* was commercially bought (s.c. Agrosem Impex s.r.l.). The influence of extractive solutions on the root elongation of germinated seeds was evaluated for both wheat (*Triticum* test) and lettuce (*Lactuca* bioassay), as well as their impact on the mitotic film in the root tips of wheat roots, by the Constantinescu method [32,33]. For the *Lactuca* bioassay, the protocol was identical to that of the *Triticum* bioassay, except that only lettuce seeds with a root length of 1 mm were chosen and acetic orcein staining and microscopic observations were not performed [34].

Results and discussions

The low inhibitory effect of hydroponically grown NE leaves observed in the *Triticum* assay and the low lethality observed in the *Artemia* bioassay suggest that the plant can 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 (rhizome) to treat menstrual disorders, female infertility and as a facilitator of childbirth [35,36]. *In vitro* data obtained on LNCaP and PC-3 prostate cancer cell lines indicated that the leaf fractions might have potential antiandrogenic effects [37]. Such data from folk medicine, together with the apparently low toxicity observed in our experiments, indicate that further investigations on both the chemical composition and pharmacological properties of extracts prepared from rhizomes and leaves are of interest and should be carried out.

Intriguingly, our experimental data indicated that an aqueous extract from the underground organs from specimens grown in geoponic culture exhibited higher phytotoxicity to *Triticum aestivum* compared to the aqueous extract from leaves (but on some days, the relationship was reversed between the two). In contrast, for extracts obtained from specimens grown in hydroponic culture, the reverse was true: the aqueous extract from leaves was more phytotoxic than that from underground organs. In the case of methanolic extracts, irrespective of the culture medium (hydroponic or geoponic), those obtained from the leaf were more active than those obtained from underground organs. Toxicity data on nauplii of *Artemia franciscana* Kellog showed a slightly higher toxicity of extracts obtained

from the subterranean organs (compared to the leaf), regardless of the solvent used. However, both tests (*Triticum* and *Artemia*) showed relatively low levels of toxicity. In rats, a hydro-ethanolic extract from the subterranean organs of TO was estimated to have an LD50 of 1100 mg/kg in the rat, which was classified by the study authors as a mixture with relatively low acute toxicity [38]- a conclusion in agreement with our findings in the *Triticum* and *Artemia* tests. Other studies have estimated much higher acute LD50 values of 36.8 and 28.8 g/kg [39] for other extracts, indicating an even lower acute toxicity.

All toxicity assessments performed by us indicated that the extracts obtained from IG, both from leaves and underground organs, have very limited or no acute toxicity. There was good agreement between the *Artemia* tests and the phytotoxicity tests applied in our experimental setting (on *Lactuca* and *Triticum* species). There was a small difference between extracts obtained from specimens grown in geoponic and hydroponic environments. Toxicity was somewhat higher for extracts obtained with alcohol than with water, but in both cases it was very low.

Chapter 9. Total antioxidant capacity, polyphenol and flavonoid tests

Introduction

Total antioxidant capacity, CAT measures the efficiency of phytoextracts to scavenge free radicals and to stop undesirable effects of ROS on biological macromolecules. CAT is derived from total polyphenols (P) and flavonoids (F) [40].

In the context of our PhD research program, we aimed to test the hypothesis that plants grown in hydroponic medium exhibit similar, if not more varied and rich phytochemistry/chemical composition in phytochemical components than conventionally grown plants. In this regard, we comparatively studied CAT, P and F in lyophilized hydro-alcoholic extracts prepared from the organs of hydroponically and geoponically grown plants.

Materials and methods

The dried plant material of the three species was separated into four batches: leaves and underground organs of plants grown in the hydroponic medium and leaves and underground organs of plants grown in the geoponic medium, respectively. These were finely ground and weighed, then treated with 50% methanol. Each final extractive solution was distilled in a rotavap until the methanol was completely distilled off. The remaining

aqueous solution was frozen and lyophilized using a Scanvac Coolsafe 55-4 lyophilizer. Extracts were stored at (-) 20 °C until use.

The determination of the total polyphenol content of the samples was carried out with Folin-Ciocâlteu reagent according to the method previously described by Singleton et al. (1999) [41]. Total flavonoid content was determined using the spectrophotometric method described by Chang et al. (2002) [42]. Determination of the antioxidant activity was carried out by the DPPH [43], ABTS[44], CUPRAC [45] methods.

Results and discussions

For all of the three medicinal plants the P, F and CAT values were generally **lower for extracts from the organs of plants grown hydroponically** than those from geoponically grown plants **with a few exceptions. Thus:**

For **NE**, the values of polyphenols and antioxidant capacity were lower for extracts obtained from underground organs of hydroponically grown species than those grown geoponically, but for leaves the situation was reversed. The level of flavonoids was lower in extracts from hydroponically grown specimens (compared to geoponically grown), both in the underground organs and leaves. Antioxidant capacity values tended to be lower for underground organs from hydroponically grown specimens, but higher for leaves obtained from hydroponically grown specimens. Thus, in terms of polyphenols and antioxidant capacity, the effect was more pronounced for leaf extracts from hydroponically grown specimens and less pronounced for underground organs from hydroponically grown specimens. In contrast, the flavonoid content is lower in both underground organs and leaves from hydroponically grown specimens.

For **TO**, P and CAT values are **extremely low** and F values are low for all extracts obtained from hydroponically grown specimens, both for underground organs and leaves.

For **IG**, all P, F and CAT values **are lower for extracts obtained from hydroponically grown specimens**, both those derived from underground organs and those obtained from leaves.

Chapter 10. Pharmacological studies

Introduction

TO and IG are medicinal plants with a long tradition of use, whereas NE, not being native to Europe, has been used less in traditional medicine. Only the traditional use of the species in the island of Fiji to treat menstrual disorders [35] and possible hormonal and

cytotoxic effects on human cancer cells [37] are known. Given the limited data on the toxicity of the two species, in a preliminary investigation, we aimed to **evaluate the toxicity** of plant extracts of the three species grown in geoponic and hydroponic systems on rodent species. In addition, taking into account the fact that **NE** has hardly been investigated pharmacologically so far, we also proposed to perform **a pharmacological screening** of the Central Nervous System (CNS) to detect the effects of the active principles contained in the lyophilized hydro-methanolic extracts (1:1) obtained from this species.

10.1. Acute toxicity testing of *Nephrolepis exaltata* (L.) Schott, *Iris germanica* L. şi *Taraxacum officinale* (L.) Webb

OECD guideline 420 (1992) [46] recommends testing for acute toxicity by observing clear signs of toxicity following the administration of fixed doses (this avoids death of experimental animals). The method allows the assignment of substances according to the Globally Harmonized System (GHS) classification of acutely toxic chemicals into 5 categories [ENV/JM/MONO(2001)6, 2001] [47]. *We aimed to evaluate the **acute toxicity** of lyophilized extracts of Nephrolepis exaltata (L.) Schott., Iris germanica L. and Taraxacum officinale (L.) Webb grown in geoponic and hydroponic environments.*

The principle of the method

The method consists in administering to groups of animals of the same sex, fixed doses of 5, 50, 300 and 2000 mg/kg (exceptionally an additional dose of 5000 mg/kg) in steps.

A dose of 2000 mg/kg (or, exceptionally, a dose of 5000 mg/kg) is administered to a single animal, followed by subsequent administration to 4 other animals if no deaths are recorded [OECD 420] [46].

Materials and methods

A collective of 60 male, white mice, belonging to the NMRI breed, with an initial weight of 36.97 ± 2.67, were purchased from INCDMI "Cantacuzino" (National Institute for Medico-Military Research and Development "Cantacuzino", Bucharest, Romania) was used. The animals were housed in plexiglas cages with transparent walls and access to food and water *ad libitum* (constant test conditions: temperature 21-230C, humidity 45-60%).

The bioethical rules for research on experimental animals for scientific purposes, according to Law 43/2014 amended and supplemented by Law no. 199/2018 on the protection of animals used for scientific purposes, were followed.

The animals were fasted with water ad libitum before single dose administration. The mice were weighed and then they were administered the tested extract (food was put 1-2 hours post administration).

The lyophilized hydro-methanolic (1:1) plant extracts were obtained from the leaves and underground parts of *Nephrolepis exaltata* (L.) Schott., *Iris germanica* L. and *Taraxacum officinale* (L.) Webb species grown in geoponic and hydroponic environment.

The preliminary study

Twelve batches (one mouse/lot) were formed, which received the dried extracts, dispersed in distilled water, as a 20% aqueous suspension in a single dose, p.o.

The main study

Forty-eight hours after the first administration, 12 further batches (4 mice/mouse/lot) were made up and given the same extracts at the same dose in a single dose, p.o. Animals were observed individually after administration, at least once in the first 30 minutes and then periodically over 24 hours. Weight was determined initially before administration, at 7 days, 10 days and 14 days after the start of the experiment.

Signs of toxicity were monitored: pathological changes in integument and mucous membranes, changes in external appearance, changes in other organs and systems (respiratory system, central nervous system) or changes in behavior. In particular, signs such as tremor, convulsions, hypersalivation, diarrhea, lethargy, sleepiness and coma were observed.

Results and discussions

The preliminary study

After administration of 2000 mg/kg body of extract, there was no lethality and no changes in the parameters followed.

The main study

Forty-eight hours after the first tests, 2000 mg/kg body of extract was administered again. **There was no lethality and no change in the parameters monitored.**

10.2. *Central Nervous System Pharmacological Screening* for effects of the active principles contained in *Nephrolepis exaltata* (L.) Schott extracts.

Since this fern species has been little studied from a therapeutic point of view, the data in the literature concerning its chemical composition and possible therapeutic uses being almost nonexistent, we investigated the possible effects of the species on the CNS by a pharmacological screening in behavioral tests for four lyophilized extracts of *Nephrolepis exaltata* (L.) Schott. leaves and rhizomes, harvested from plants grown in hydroponic or geoponic environments.

Materials and methods

The extracts were administered orally in a daily dose of 500 mg/kg. Evaluation was performed after the single dose and after one week of treatment.

A total of 57 male mice, the NMRI breed, purchased from INCDMI 'Cantacuzino' (National Institute for Medico-Military Research and Development 'Cantacuzino', Bucharest, Romania) were used. The animals were housed in plexiglass cages with transparent walls and with access to food and water ad libitum, in compliance with the test conditions mentioned under acute toxicity.

The bioethical rules for research on experimental animals for scientific purposes, according to Law 43/2014 amended and supplemented by Law no. 199/2018 on the protection of animals used for scientific purposes, were followed.

Animals were divided into 7 batches (8 animals/lot) and for 7 days were administered the test substances. The batches were treated with: distilled water 0.1 mL/10g (control/control, M); diazepam 1.5 mg/kg (negative reference, DZ); caffeine 20 mg/kg (positive reference, CF); *Nephrolepsis exaltata* rhizome hydroponic extract 500 mg/kg (NERH) and geoponic extract 500 mg/kg (NERG); *Nephrolepsis exaltata* leaf hydroponic extract 500 mg/kg (NEFH) and geoponic extract 500 mg/kg (NEFG);

Behavioral pharmacological tests

We assessed ***the spontaneous locomotor activity, motor coordination*** *as well as* ***anxiogenic or anxiolytic effects.***

Results and discussions of the parameters and anxiety index in the suspended plus maze test

A statistically significant anxiogenic effect, compared to the control group, was revealed after 7 days of treatment, for the groups treated with *Nephrolepsis exaltata* extract obtained from rhizome grown in hydroponic and geoponic medium and for the extract obtained from leaf grown in hydroponic medium. The tested extracts did not significantly modify the time spent in the central zone nor the anxiety index.

Results and discussions on spontaneous locomotor activity (Activity Cage, Ugo Basile)

Vertical motor activity was statistically significantly decreased after 7 consecutive doses in the groups of animals treated with extracts obtained from the leaves of *Nephrolepsis exaltata* grown in hydroponic and geoponic environments. It can be concluded that the extracts obtained from the leaves of *Nephrolepsis exaltata* grown in hydroponic and geoponic medium exhibit a depressant effect on the CNS.

Results and discussion on coordinated motor activity (Rotarod test)

Research results revealed the following: stimulation of motor performance for caffeine-treated animals both after single and repeated doses. Also, **extracts obtained from rhizome and leaves of *Nephrolepsis exaltata*** grown in **hydroponic environment stimulated the motor performance of the tested animals.**

Chapter 11. Evaluation of the HMG-CoA reductase inhibitory potential of the studied species using the QSAR method

Introduction

QSAR is a computational approach that is based on building models that describe the relationship between biological activity and certain structural properties (descriptors) of ligands that bind to a specific biological target (or have a specific biological effect) [48].

Iqbal Choudhary et al. (2005) reported that an ethanolic extract of IG (rhizomes) significantly reduced all lipid components including LDL-cholesterol [49]. The authors neither identified nor discussed the chemical compounds responsible or the mechanism of action involved, and we were unable to identify any other published research to clarify this issue. Therefore, we were interested in assessing whether compounds biosynthesized by IG have the ability to inhibit HMG-CoA-reductase, using a series of regression-based QSAR models for inhibitors of this enzyme.

Materials and methods

A set of 1170 human HMG-CoA reductase inhibitors, whose activity was evaluated based on half-maximal inhibitory concentration (IC50) values, which were downloaded from the ChEMBL database (target ID CHEMBL402) [50], was used. The MACCS fingerprints calculated with the "Rcpi" package [51] were used as descriptors, and 3874 2D molecular descriptors, grouped into 18 blocks (constitutional indices, ring descriptors, topological indices, etc.) were calculated with the AlvaDesc software [52].

We downloaded the chemical structures of all chemical compounds reported to be identified in *Iris germanica* L. in the Lotus database [53], calculated the molecular descriptors and then virtually sieved each compound using the best performing QSAR models.

Results and discussions

A total of 300 models (with different machine learning regression algorithms, feature selection methods, and fingerprint or descriptor datasets) were built and evaluated by nested cross-validation. Of these, a total of 21 models performed reasonably well in nested cross-

validation (either R2 ≥ 0.70 or CCC ≥ 0.85), and of these, only six met both performance conditions (R2 ≥ 0.70 and CCC ≥ 0.85) (Table S11.I and Fig. S11.1).

We downloaded all chemical compounds reported to date as identified in the IG species from the Lotus database [53] of natural compounds, yielding a dataset of 129 compounds that were subjected to virtual screening using the selected models. Seven compounds in this dataset were outside the DA of all six models and 12 compounds were in the DA of only one of the six models. 60 (46.5%) compounds were in the DA of all six models and 38 (29.5%) were in the DA of five of the six models. No compound in this data set had a predicted IC50 less than 10 nM (Fig. 11.1) and only two compounds were predicted by the models to have an IC50 less than 100 nM, both of which were stereoisomers of the same acetylated isoflavone backbone.

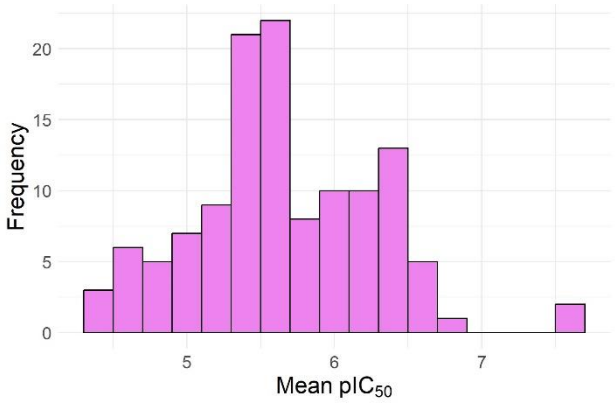
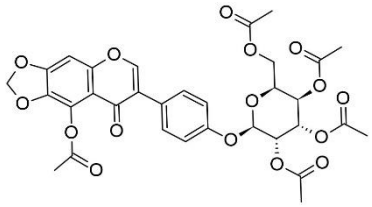
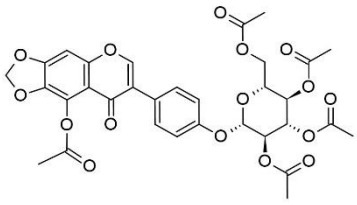


Fig. 11.1. Histogram of predicted pIC50 values for reported chemical compounds in Iris germanica L.



[(2R,3S,4R,5R,6S)-3,4,5-triacetyloxy-6-[4-(9-acetyloxy-8-oxo-[1,3]dioxolo[4,5-g]chromen-7-yl)phenoxy]oxan-2-yl]methyl acetate



[(2S,3S,4R,5S,6R)-3,4,5-triacetyloxy-6-[4-(9-acetyloxy-8-oxo-[1,3]dioxolo[4,5-g]chromen-7-yl)phenoxy]oxan-2-yl]methyl acetate

Fig. 11.2. Two acetylated isoflavonoids from the rhizome of *Iris germanica* L. predicted to be highly active against HMGCoA-reductase.

Conclusions and personal contributions

1. The macroscopic and microscopic examinations of the three analyzed species confirmed their identity, revealed histo-anatomical characters specific to each, and

demonstrated that in all three cases there are no remarkable morphological and anatomical differences between specimens grown in hydroponic and geoponic environments, respectively (Chap. 4).

2. The differences between the parameters analyzed for hydroponically and geoponically grown specimens are not significant, and any differences that could not be detected due to insufficient statistical power are generally small, so that any undetected differences are unlikely to be practically relevant, at least for the variables of interest analyzed. These make hydroponic cultivation of NE, TO and IG a viable and reasonable alternative to traditional, geoponic cultivation (Chap. 5).
3. We did not, however, obtain in our experiments any evidence of a clear superiority of one cultivation method over the other (but it is possible that further optimization may reveal such superiority, as the literature often - but not always - indicates the superiority of the hydroponic method) (5.4, paragraphs 1-2).
4. In our experiments we did not observe any significant effect of GA3 (on any of the three species), and we assume that this phenomenon is a consequence of the too low concentrations used by us (maximum 0.5 mg/L. (Section 6.3.1, paragraph 1, 6.3.2, paragraph 1, 6.3.3., paragraph 1)).
5. We observed no significant effects of the salicylic acid on leaves, stomatal conductance or leaf mass, underground organs or total dry mass for the species evaluated. At the higher concentration levels tested by us (100 and 150 μM), there tended to be inhibitory effects on leaf number, leaf area and a significant inhibitory effect on stomatal conductance (Section 6.3.1, paragraph 2, 6.3.2, paragraph 2, 6.3.3., paragraph 2).
6. No significant developmental effects were observed for acetylsalicylic acid for the three species evaluated (Section 6.3.1, paragraph 3, 6.3.2, paragraph 3, 6.3.3., paragraph 3).
7. At the concentrations used in the experimental studies (40 mg/L), humic substances had no significant beneficial effects on the development of the three species in hydroponic systems. Limited experimental data suggested that higher levels might be beneficial (in line with the manufacturer's recommendation), but this needs experimental confirmation. (Section 6.3.1, paragraph 4, 6.3.2, paragraph 4, 6.3.3, paragraph 4).
8. All of the three species evaluated tolerate V, Sr and Ba ions very well, with no signs of wilting or other signs of toxicity (Chap. 7).

9. All three species accumulate V, Sr and Ba ions, but not all, and not for all metals evaluated, meet the criteria to qualify as hyperaccumulators (Chap. 7).
10. NE withstands high levels of vanadium while still being unaffected, accumulates relatively large amounts in the underground organs, but the accumulation in the leaves is much less than in the roots. NE concentrated more Sr in the aerial than in subterranean parts and in very large amounts, indicating that it is a hyperaccumulator and highly recommended for phytoremediation, especially since it did not wilt at any of the concentrations tested (Sect. 7.3.1, paragraphs 1-4).
11. TO accumulates large amounts of V in the rhizome, but has the disadvantage that it accumulates little V in the leaves. It also concentrated more Sr in underground than in aboveground parts, but Sr accumulation was in small amounts, much lower than the other species tested (Sect. 7.3.2, paragraphs 1-4).
12. IG showed good tolerance when grown in the presence of vanadium, but accumulated the metal in large amounts only within roots and not in rhizomes or leaves, being resistant to V, but not suitable for phytoremediation (Sect. 7.3.1, paragraphs 1-7).
13. IG accumulated high amounts of strontium in leaves, higher than some hyperaccumulators mentioned in the literature, and is a potentially useful species for phytoremediation of this metal (Sect. 7.3.1, paragraphs 8-9).
14. Although the evaluated species accumulated modest amounts of barium, none of them corresponds to a hyperaccumulator (Sect. 7.3.1, paragraphs. 5-6; par. 5; Sect. 7.3.3., paragraphs. 10-11).
15. The low inhibitory effect of NE leaves grown hydroponically (as well as geoponically) observed in the *Triticum* assay and the low lethality observed in the *Artemia* bioassay suggest that the plant can be safely used for therapeutic purposes (Sect. 8.3.1, paragraphs 1-7; Sect. 8.3.3., paragraphs 1-2).
16. Both tests (*Triticum* and *Artemia*) showed relatively low levels of toxicity of the leaves and subterranean organs of TO, findings consistent with acute toxicity evaluations in rats of extracts obtained from this species (Sects. 8.3.1, paragraphs 8-14; Sects. 8.3.3., paragraphs. 3).
17. All of the toxicity assessments performed by us indicated that extracts obtained from IG, both from leaves and underground organs, have very limited or no acute toxicity. These are in agreement with publicly available data suggesting that IG is a species with low toxicity to humans (but with remarkable toxicity to pets and cattle; the

mechanism of this difference is unclear) (Sec. 8.3.1, paragraphs 15-18; Sec. 8.3.2.; Sec. 8.3.3., paragraphs 4-6).

18. The most abundant content of polyphenols, flavonoids and the highest antioxidant capacity were recorded in the IG species, while the lowest values of the same variables were recorded in the TO species (IG>NE> TO) (Sect. 9.3).
19. In general, the values for polyphenols, flavonoids and antioxidant capacity, were higher for underground organs than for leaves, except for extracts obtained from NE grown in the hydroponic medium (Sect. 9.3).
20. The polyphenol content, flavonoid content, and antioxidant capacity were higher for specimens grown geoponically than hydroponically, except for leaves of hydroponically grown NE, in which flavonoid content and antioxidant capacity were higher (Sect. 9.3).
21. Our results suggest that the hydroponic culture would not be advisable for TO, at least with the cultivation parameters used by us (Sect. 9.3).
22. Optimization studies are needed that may result in more abundant contents of polyphenols and flavonoids in all organs of the three plant species investigated (Sect. 9.4).
23. The acute toxicity assessment in accordance with the OECD guideline 420, adopted in July 1992, revealed that no lethal effect occurred following administration of the tested extracts at a dose of 2000 mg/kg bw administered p.o. Therefore, according to the cited Guideline the extracts NERG, NERH, NEFG, NEFH, IGRG, IGRH, IGFG, IGFG, IGFH, TORG, TORH, TOFG and TOFH were classified as category 5 (LD50 > 5000 mg/kg, p.o.), according to the data included in the Global Harmonized System (GHS) - ENV/JM/MONO(2001)6, 2001] (Section 10.1).
24. We evaluated the anxiolytic effect for extracts obtained from rhizomes and leaves of *Nephrolepsis exaltata* grown in hydroponic and geoponic media using the plus maze test. The anxiolytic action expressed by increased time spent in the open arms was not evidenced for any of the test substances. Likewise, the number of animal entries into the open arms of the maze did not change statistically significant for any of the test substances (Sect. 10.2, paragraphs. 24-29).
25. A statistically significant anxiogenic effect, compared to the control group, was shown after 7 days of treatment for the groups treated with *Nephrolepsis exaltata* extract from rhizomes grown in hydroponic and geoponic environments and for the

leaf extract grown in the hydroponic environment. The same effect is also evident for caffeine, the reference anxiogenic substance (Sect. 10.2, paragraphs 24-29).

26. The tested extracts significantly modified neither the time spent in the central zone nor the anxiety index, and it can be concluded that none of the four extracts showed anxiolytic activity (Sect. 10.2, paragraphs. 24-29).
27. The evaluation of the motor activity of animals treated with the test extracts demonstrated decreased vertical and horizontal motor activity for animals treated with extracts obtained from leaves and rhizomes of *Nephrolepsis exaltata* grown in hydroponic and geoponic environments. It can be concluded that extracts obtained from the leaves of *Nephrolepsis exaltata* grown in hydroponic and geoponic environments have a depressant effect on the CNS, quantified by a significant decrease in horizontal and vertical motor activity in treated animals (Sect. 10.2, paragraphs 30-34).
28. The evaluation of the coordinated motor activity in the rotarod test for the extracts obtained from the rhizomes and leaves of *Nephrolepsis exaltata* grown in hydroponic and geoponic environments demonstrated stimulation of motor performance and increased rotational speed at which coordination is maintained for the extract obtained from rhizome of *Nephrolepsis exaltata* grown in hydroponic environments. The same effect was obtained on the endurance time parameters of the rotational speed at which coordination is maintained after administration of 7 consecutive doses (Sect. 10.2, paragraphs 35-37).
29. A series of adequately validated QSAR models with good performance have been developed and used for virtual screening of chemical compounds in IG, and compounds with moderate HMG-CoA reductase inhibitory activity have been identified that could explain the hypocholesterolemic activity of rhizome extracts. Among these, the most potent are two stereoisomers of an acetylated isoflavone and a sesquiterpene derivative (4-methyl-2-[(1S,5R)-2,5,6,6-tetramethylcyclohex-2-en-1-yl]furan (Chap. 11).

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List of published scientific papers

Articles published in specialized journals:

- **Popovici** PC, Ancuceanu VR, Olaru OT, Stoicescu CS, Dinu M,

Toxicity Assessment of *Nephrolepis exaltata* (L.) Schott, Fam. *Nephrolepidaceae,* Acta Biologica Marisensis, 2018, 1(1):27-36

DOI: 10.2478/abmj-2018-0004

Chapter 4, pages 27-29, 36; Chapter 8, pages 79-81.

- **Popovici** PC, Ancuceanu VR, Dinu M, Microscopic characterization and toxicological assessment of *Iris germanica* L. cultivated under hydroponic and geoponic conditions, Farmacia, 2022, 70(5): 861

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Chapter 4, pages 32-36; Chapter 8, pages 84-90.