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Original Article

Toxicity Evaluation of Lycopodiaceae Extracts on Mouse Neuronal Cells and Zebrafish Embryos

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Abstract: Many species in the family Lycopodiaceae possess highly potential medical substances and are widely used as traditional medicines in the treatment of stroke, dementia. Hundreds of Lycopodium alkaloids were described and studied for their biological activities such as anti-inflammatory, antioxidant, immunomodulatory, neuroprotective,… Various members of this family were found in Vietnam and were used as oral traditional medicine for a long time. However, there is a lack of toxicity studies on this subject. In this study, three alkaloid fractions from *Huperzia serrata* (Thunb.) Trevis, *Lycopodium clavatum* L. and *Huperzia squarrosa* (G. Forst.) Trevis were tested in two toxicity models: mouse primary neuronal cell (*in vitro*) and zebrafish (*Danio rerio*) embryos (*in vivo*). The IC_{50} values of *Huperzia serrata* (Thunb.) Trevis, *Lycopodium clavatum* L. and *Huperzia squarrosa* (G. Forst.) Trevis on the mouse primary neuronal cells were 0.839, 1.071 and 0.915 mg/ml and on the zebrafish embryo were 0.180, 0.281 and 0.198 mg/ml, respectively. Notably, three common types of maldevelopment: hemostasis, cardiac sac edema and tail defect were recorded; and the teratogenic index of *Huperzia serrata* (Thunb.) Trevis was 3.418, which indicated high teratogenic effect at embryo developmental stage. These data can be used as a reference in using the alkaloid fractions from these plants in the traditional medicine treatment system, especially when used with high concentration and in treating pregnant women.

Keywords: Lycopodiaceae, alkaloid, toxicity, neuronal cell, zebrafish embryo.

1. Introduction

The family Lycopodiaceae is known as club mosses and has been used as traditional medicine for long time. The alkaloid Huperzine A, extracted from *Huperzia serrata*, [1] is a specific and reversible inhibitor of acetylcholinesterase (AChE). Huperzine A has a high potential for the treatment of *Alzheimer* Disease (AD) [2-5]. Another prominent species is *Lycopodium clavatum* in which many high potency alkaloids have been found, to name a few: Hupezine E, α-Onocerin, α-obscurine, des-N-methyl-α-obscurine,… [6, 7].

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Approximately 276 species have been identified in this family; they are distributed sporadically around the world and differ greatly between regions [8, 9]. A research on 11 species native to Panama has found that most of them differ in AChEI potency with 2 plants: *Huperzia chamaeleon* and *Huperzia reflexa* show high AChEI and antioxidant properties [10]. Chuong et al., have found a new alkaloid: lycosquarosine a in Vietnamese sourced *Huperzia squarrosa* [11]. *Lycopodium japonicum*, a species common in Japan and China, has a unique structure alkaloid group named lycojaponicumins [12]; in 2015 another alkaloid: N-methylhydroxypropyllycodine was discovered [7]. *Lycopodium clavatum* is one of the most widespread species in the family Lycopodiaceae and abundantly found in various areas. As an oral traditional medicine, it was used in the treatment of gastrointestinal, dementia, rheumatic disease, muscle pain. The extracts from *Lycopodium clavatum* showed acetylcholinesterase and antioxidant activities [13]. It could be seen that a lot of potentials from native Lycopodiaceae plants have not yet been discovered, and many researches are being conducted [14, 15].

Despite the rising popularity of Lycopodiaceae as an effective plant base medicine, there are not many researches on the toxicology of its compound on biological model. A recent paper on response of *Toxoplasma gondii* infected mice treated with *Lycopodium clavatum* homeopathic medicine shows that at 200dH concentration, the extract causes "intensified kidney and liver alterations" [16]. Huperzine a has mild adverse effect on the cholinergic system including dizziness, nausea, vomiting, diarrhea, gastrointestinal discomfort, hyper-activity, anorexia, symptoms of gastroenteric headaches and depressed heart rate,… [17].

Over the past twenty years, the zebrafish (*Danio rerio*) model is widely used in biomedical studies. This model has been applied in screening for teratogenicity in developmental toxicity assessment. The standard protocol used for toxicity assessment was proved by the Organization for Economic Co-operation and Development (OECD) as test guideline No. 236. This test allows to calculate the mortality rate of zebrafish as well as to observe the development at embryo stage. Recently, the studies on the zebrafish model have revealed that it is possible to use *Danio rerio* in neurobiological research for designing in vivo models of human diseases such as depression and anxiety, autism [18, 19], obsessive compulsive disorder [20], attention deficit hyperactivity disorder (ADHD) [21, 22], addictions [23-25], or Alzheimer's disease [26, 27].

Lycopodiaceae was used as traditional medicine in treatment for stroke, dementia for a long time in Vietnam. Lycopodiaceae family has wide variation and growth throughout Vietnam [28, 29], yet there are only a few researches on this topic, mainly focusing on discovering novel alkaloid and confirming chemical properties of found substances [11, 30]. However, the lack of information on the effects of native species on animal model is a major setback. To direct link to traditional medicine, here, we evaluated the toxicity of the extract from *Huperzia serrata* (Thunb.) Trevis, *Lycopodium clavatum* L. and *Huperzia squarrosa* (G. Forst.) Trevis on the model of zebrafish embryo *in vivo* and the model of mouse primary neuronal cell *in vitro*. The results would provide necessary information to create safety profiles for these medicinal plants in Vietnam.

2. Methodology

2.1. Plant Collection

The plants were collected in Vinh Phuc province, Lao Cai province and Lam Dong province in Vietnam from 2018 to 2019. The voucher specimen was kept at the Botanical Museum of Hanoi, VNU University of Science.

2.2. Preparation of Medicinal Plant Extracts

The dried aerial part of *Huperzia serrata* (Thunb.) Trevis, *Lycopodium clavatum* L. and *Huperzia squarrosa* (G. Forst.) Trevis were extracted with ethyl acetate (EtOAc) to get alkaloid fraction as previously described [11]. The plant materials were labeled as AI.1, AI.2 and AI.3 representing for *Huperzia serrata* (Thunb.) Trevis, *Lycopodium clavatum* L) and *Huperzia squarrosa* (G. Forst.) Trevis, respectively. Brieftly, 80 g of plant material was extracted with MeOH and 0.5% NaOH, sonicated for 10 minutes and refluxed three times. The resulting crude extracts (AI.1: 18.39g; AI.2: 18.69 and AI.3: 17.01 g) were dried under reduced pressure and then resuspended in 200 ml of 1N HCl and partitioned with EtOAc (ratio 1:1). The final concentrated of crude alkaloid fractions of AI.1, AI.2 and AI.3 were 6.61 g; 5.19 g and 5.47 g, respectively. The extracts were dissolved in DMSO for further test on the neuronal cell and zebrafish embryo.

2.3. Isolation of Primary Neuronal Cells

The mouse primary cell culture derived from neonatal mouse brain is developed using the previously described methods [31-36] with modification. Briefly, the procedure was as follows:

The wild type Swiss mice were purchased from the National Institute of Hygiene and Epidemiology. The cortex was isolated from postnatal Swiss mice pups (P1-P4), meninges were removed and cleaned with cold PBS, then cut into 1 mm thick slides. The enzyme was digested in 2 ml Trypsin/EDTA 0.25% solution for 25 min at room temperature, then 5 ml cold PBS was added to dilute the solution, and after 5 minutes, the supernatant was discarded. The tenderized brain slides were triturated in 5 ml neuronal medium (PAN-Biotech) and mixed by pipetting. Let the tube rest for 3 - 5 minutes, then transfer the cell suspension into another 15 ml centrifugation tube while carefully keep out the larger tissue pieces, and repeat the trituration 2 more times to optimize the number of harvested cells. Count the cells by haemocytometer then plate them into T25 flask at density of $2.5x10^6$ cells per flask and culture at 37 °C; 5% CO_2. The estimated yield was $7\text{-}8x10^5$ cells per pup. Change the media after the first 24 h, and then every 2 days.

Cell imaging was performed from day 3 in vitro (DIV) onward, all images were taken from Carl Zeiss Axiovert S100 inverted microscope and Optikam B5 at 10x magnification. The images were processed on ImageJ.

2.4. Cellular Toxicity Test

Promega CellTiter-Glo® Luminescent Cell Viability Assay kit was used to evaluate the toxicity of medicinal plant extracts; the procedure followed manufacturer's specification. Briefly, neuronal cells were cultured in 96-well opaque black culture disk (SPL 31496) with 30,000 cells per well. After 24 h, the plant extracts were added with the multiple concentrations. The ranges of extract concentration were 0.4; 0.6; 0.9; 1.0; 1.2; 1.8 (mg/ml) for AI.1; 0.56; 0.8; 0.9; 1.1; 1.2; 1.3; 1.4 (mg/ml) for AI.2, and 0.5; 0.75; 0.9; 1.2; 1.5 (mg/ml) for AI.3. At 48 hours after plating, change the medium with CellTitter-Glo Luminescent solution, then lysis cell completely by Cell Titter shaker at 500 rpm in 10 minutes. Let the disk rest at room temperature for 5 - 10 minutes and then read luminescent signal at 535 nm by a multiplate reader (Berthold Tristar LB 942).

2.5. Zebrafish Maintaining and Husbandry

Zebrafish AB strain was a gift from Dr. Marc Muller, Zebrafish Laboratory at GIGA Institute, University of Liege, Belgium. Fishes are maintained in the Zebrafish Laboratory, VNU University of Science under control conditions: Light cycle 14:10, temperature 27 ± 2 °C, pH: 7 - 8. Mating is conducted in the ratio of 1 male: 2 females to increase the chance of spawning; eggs are collected at the start of light cycle.

2.6. Zebrafish Embryo Toxicity Test

Embryo Zebrafish toxicology test procedure is conducted based on OECD guideline No. 236

on Fish Embryo Acute Toxicity. Briefly, freshly sorted healthy zebrafish embryos at 4 - 8 cells stage (approximately 1 hour after spawn) are placed in a 6-well plate at the density of 20 embryos per well. The dish consists of one vehicle control well filled with E3 and five increasing concentrations (0.06; 0.1; 0.2; 0.6; 2.0 (mg/ml) for AI.1 and AI.2, and 0.05; 0.1; 0.2; 0.5; 2.0 (mg/ml) for AI.3. Experiments extend to 96 h, embryos are observed every 24 h and the number of lethal and malformation (edema - unnatural swelling part, hemostatis - blood clot, skeletal defect,…) individuals are collected. Each test is performed in triplicate, the LC_{50} and EC_{50} values (the concentration that 50% of embryos are perished and develop deformities, respectively) are calculated in Graphpad Prism 8.0.

Mortality (%) = death number/total exposed number × 100

Teratogenicity rate (%) = abnormal number/total surviving number × 100

2.7. Statistical Analysis

Results from at least three independent experiments in each group were statistically analyzed by a Mann- Whitney test to determine significant differences between the experimental groups. A p-value less than 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Isolation of Mouse Primary Neuronal Cells

The primary cells were isolated and cultured in the selected media. As shown in Figure 1, the morphology of neuronal cells could be observed under bright field microscope from 3 days in vitro (DIV). The primary cell culture changed considerably over the course of 22 days. At 3 DIV, many cell types with diverse phenotype could be seen, the variety of cell decrease gradually and generally become homogenous at 22 DIV. On the other hand, the neuronal matrix grew significantly over the course of culturing, clearly visible at 22 DIV.

3.2. Neuronal Cell Toxicity Test

F1 primary neuronal cell was prepared for toxicological test of three medicinal plant extracts: AI.1, AI.2 and AI.3. The effect of three chemicals on neuronal cells are highly similar, cell viability decreases with the increase of concentration. At low concentration (0.4 - 0.5 mg/ml), all three chemicals have little effect on cell viability, however, at concentration higher than 1.5 mg/ml, they all inhibited cell survival. AI.2 proved to be the less adverse effects with the highest IC_{50} at 1.071 mg/ml, while toxicological profile of AI.1 and AI.3 are closely related at 0.839 mg/ml and 0.915 mg/ml, respectively.

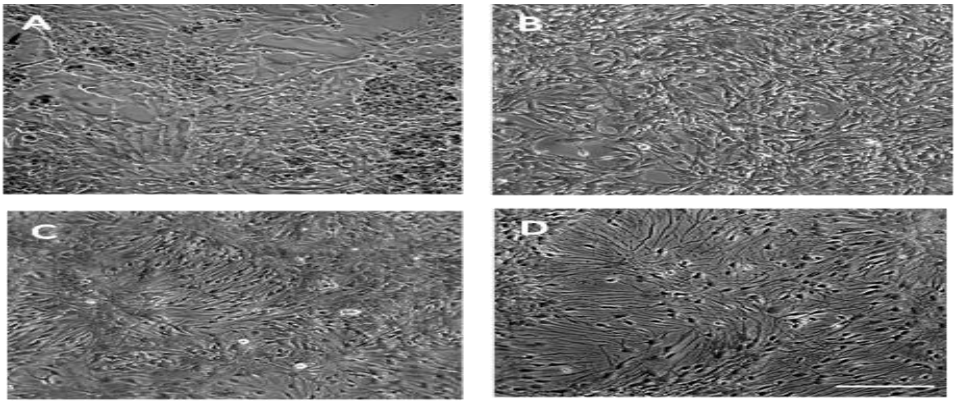


Figure 1. The morphology of primary neuronal cells in continuous culture at 3 (A), 5 (B), 11 (C), 22 (D) days *in vitro* (DIV).
(Cell variety decreased after prolong culture and cell morphology became more homogenous. The neuronal axon could be observed clearly in DIV 22. Scale bar = 100 μm)

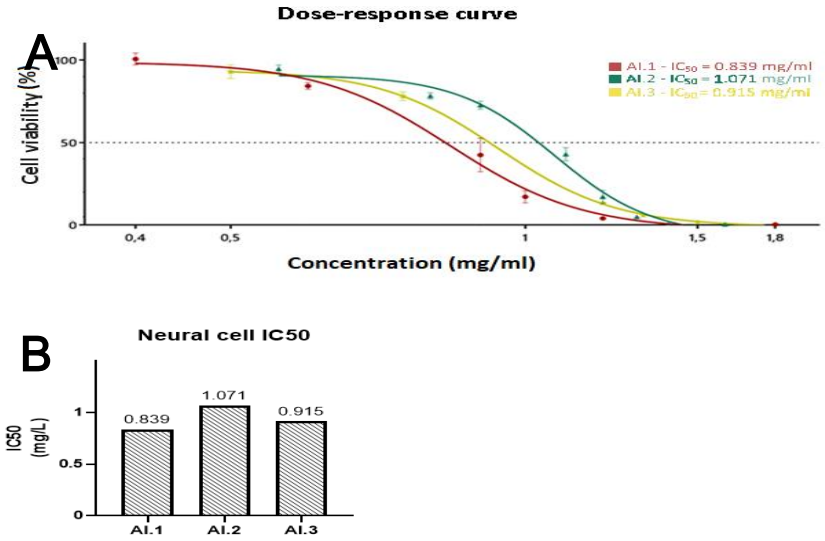
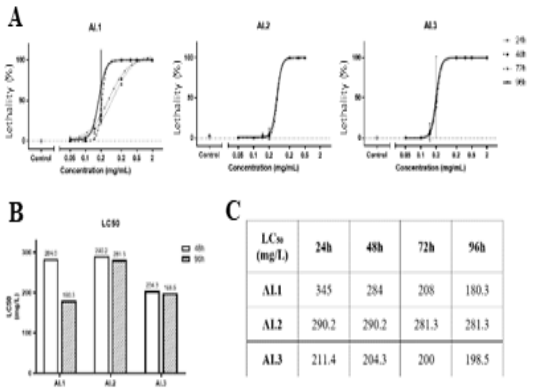


Figure 2. Primary neuronal cell toxicological dose response curve and IC_{50}.
(Bar graphs show tightly correlated toxicology profiles of three alkaloid fractions from AI.1, AI.2, AI.3).

3.3. Zebrafish Embryo Toxicity Test

To investigate the effect of three alkaloid fractions from *Huperzia serrata* (Thunb.) Trevis, *Lycopodium clavatum* L. and *Huperzia squarrosa* (G. Forst.) Trevis, we tested *in vivo* using the model of zebrafish embryo. The toxicity of these plant extracts on zebrafish embryos is shown in Figure 3. At the lower concentration (less than 0.1 mg/ml) all of these extracts did not affect the survival ability of zebrafish embryo until 96 h. But it was shown that the adverse effect was dose dependent and could be observed at early development stage proved by 100% lethal embryo in the highest concentration at 24 h (2 mg/ml). The exposure length is not important in AI.2 and AI.3, and there are clear overlaps in the illustration line graph of 4 endpoints (24 h - 96 h), which represents the lack of change in toxicity. In contrast, the toxicity of AI.1 increased daily and peaked at the last endpoint of 96 hours after fertilization (hpf). Relative lethal effect of these extracts can be seen by their LC_{50} at 96 h. AI.1 is the most potent substance with LC_{50} at 96 h of 0.180 mg/ml; AI.3 has similar LC_{50} of 0.198 mg/ml, while AI.2 is the least toxic with LC_{50} of 0.281mg/ml.



LC_{50} (mg/L)	24h	48h	72h	96h
AI.1	345	284	208	180.3
AI.2	290.2	290.2	281.3	281.3
AI.3	211.4	204.3	200	198.5

Figure 3. Line graph illustration of the curve fit of three extracts of AI.1, AI.2 and AI.3.
(Bar graphs show the comparison of three extracts toxicity on zebrafish embryos.
The Error Bars show SD).

3.4. Zebrafish Teratogenic Effect

To further study the effect of three alkaloid fractions, we observed the teratogenic of zebrafish embryos under extracts treatment. To observe the malformation, we used the concentration range of 0.05; 0.1; 0.2 and 0.5 mg/ml crude extracts and observed every 24 h. All three natural extracts could cause development retard in zebrafish larvae as shown in Figure 4.

However, AI.1 is the only substance that has considerable malformation effect on larvae even in the lowest concentration (0.05 mg/ml). As shown in Figure 4I, malformations only occurred sporadically in AI.2 and AI.3 at sub-lethal doses, while teratogenic phenotypes could be observed in most concentrations of AI.1. Three common types of maldevelopment hemostasis, cardiac sac edema and tail defect are recorded. Zebrafish embryos could develop all three types of deformities simultaneously in high concentrations of AI.1, AI.2, AI.3 extracts. These results show that alkaloid fractions from *Huperzia serrata* (Thunb.) Trevis, *Lycopodium clavatum* L. and *Huperzia squarrosa* (G. Forst.) Trevis could have adverse effects on vascular development and angiogenesis as well as skeletal formation process.

Teratogenic index (TI), which was calculated as ratio of LC_{50}/EC_{50}, could only be found in AI.1 for teratogenic effect was not significant in AI.2 and AI.3, so no EC_{50} of two substances could be found. TI value of AI.1 is 3.418, much greater than the others, which shows that AI.1 has high teratogenic effect over embryotoxicity.

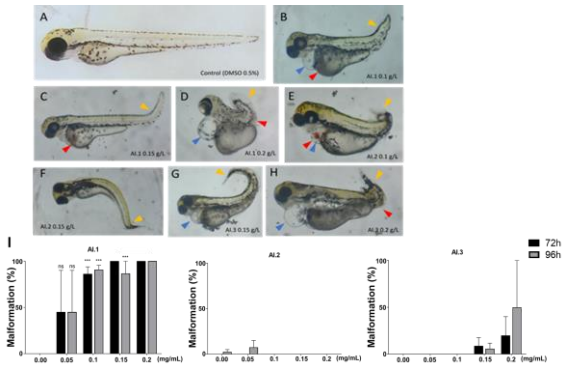


Figure 4. Zebrafish larvae show varied development deformities after their exposure to alkaloid fractions.
(A. Control; B, C, D. AI.1 0.1 - 0.15 - 0.2 g/L; E, F. AI.2 0.1 - 0.2 g/L; G, H. AI.3 0.1 - 0.2 g/L. I. Bar graphs illustrate the tetratogenic effect of three extracts. Red arrow denotes hemostatis; blue arrow points to edema area, and yellow arrow shows tail defect. The error bars show SD; ns: none significant; ***: significant differences with p value < 0.01).

These data for the first time provide a scientific proof for the safe concentration of alkaloid fractions from *Huperzia serrata* (Thunb.) Trevis, *Lycopodium clavatum* L. and *Huperzia squarrosa* (G. Forst.) Trevis in Vietnam. In the *in vitro* test, all of these extracts completely inhibited primary neuronal cells survival at 1.5 mg/ml. The malformation *Huperzia serrata* (Thunb.) Trevis extract was observed: hemostasis, cardiac sac edema and tail defect at embryo developmental stage. Some commercial brands using extracts of Lycopodiaceae (Lohha Tri Nao, Kinh Vuong Nao Bo, Cao Thong Dat) in treatment of neuroprotection and dementia. Brand Kinh Vuong Nao Bo and Cao Thong Dat used 65 and 60 mg extract of *Huperzia serrata* (Thunb.) Trevis per tablet. Based on our data, these products should be considered when used at high concentration and in the case of pregnant women.

4. Conclusion

In this study, we tested the toxicity of alkaloid extracts from *Huperzia serrata* (Thunb.) Trevis, *Lycopodium clavatum* L. and *Huperzia squarrosa* (G. Forst.) Trevis collected in Vietnam in two models of primary neuronal cells and zebrafish embryo. Our results showed that the cell toxicity had IC_{50} values of *Huperzia serrata* (Thunb.) Trevis, *Lycopodium clavatum* L. and *Huperzia squarrosa* (G. Forst.) Trevis as 0.839; 1.071 and 0.915 mg/ml, respectively. On the other hand, the LC_{50} values of these extracts on zebrafish embryo at 96 h were 0.180 mg/ml (*Huperzia serrata* (Thunb.) Trevis); 0.281 mg/ml (*Lycopodium clavatum* L.) and 0.198 mg/ml (*Huperzia squarrosa* (G. Forst.) Trevis. Notedly, the malformation was observed at low concentration (0.05 mg/L) of *Huperzia serrata* (Thunb.) Trevis extract. The teratogenic index (TI) of this species was 3.418, indicating high teratogenic effect at embryo developmental stage. These data can be used as a reference in using the extracts of these plants in the traditional medicine treatment system.

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