ORIGINAL PAPER

ESTIMATION OF PHENOLIC COMPOUNDS AND CONTENT OF TOXIC ELEMENTS FROM MEDICINAL PLANT *LYCOPODIUM CLAVATUM*

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Abstract. *The quality and safety of plant-based products are becoming a major concern worldwide, particularly because of heavy metal contamination. The present study was based on determining the content of toxic elements (Pb, Cd, Cr, Mn, Co, Ni, and Bi) in the medicinal plant Lycopodium clavatum marketed in Romanian shops. Phytochemical analyses were also carried out to determine the total content of phenolic and flavonoid compounds. In addition, the relationship between the phenolic compounds and heavy metals content was determined. The results showed average values of 0.045; 0.035; 1.156; 0.472 and 0.075 mg/kg for Pb, Co, Cu, Cr, and Ni, while for Mn, Cd, and Bi, the concentrations were below the method's detection limit. Data corresponding to correlation coefficients for phenolic compounds and different metals showed significant positive and negative correlations. Although the selected metals pose no threat to the environment at their current level, their accumulation over time in soil and plants can endanger the environment and human health.*

Keywords: *Lycopodium clavatum; phenolic compounds; heavy metals.*

1. INTRODUCTION

The pedicel, called *Lycopodium clavatum*, is a member of the *Lycopodiaceae* family and is a perennial plant. It is found at altitudes above 600m, grows spontaneously on the edge of forests or in coniferous forests, and is a plant that loves soil moisture and deep shade. The flowers bloom from July to September and contain substances that can be used therapeutically: nicotine, phenolic compounds, and minerals [1], while the spores contain phytosterins, fatty acids, and esterified acids. In medicine, the whole aerial part of the plant as well as the spores are used as a diuretic, and anti-inflammatory, in the treatment of gout, rheumatism, alcoholism, cancer [2, 3], Alzheimer's disease [4], as well as for homeopathic remedies.

It has been reported that the main constituents found in *Lycopodium* species are alkaloids. These compounds have beneficial effects in treating cardiovascular and neuromuscular disorders, as well as a positive impact on learning and memory [5].

In parallel with the growing interest in the therapeutic benefits of herbal products, there are major concerns about the safety and toxicity of natural plants and herbal products

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available on the market; their toxicity may be related to contaminants such as pesticides, heavy metals, and microorganisms [6, 7]. In general, geography, soil geochemical characteristics, environmental contaminants and other growth conditions, transport and storage conditions can significantly affect the properties and quality of medicinal plants and products [6].

Among contaminants, the toxicity of heavy metals to human health and the environment has recently attracted considerable attention. Heavy metals tend to accumulate in the food chain. Apart from these zinc, copper, iron, and manganese are essential nutrients; they are important for the physiological and biological functions of the human body. However, an increase in their intake above certain allowable limits can become toxic [8].

To the best of our knowledge, studies on plants of *Lycopodiaceae* family have been carried out on the species *Lycopodium selago* growing in natural areas in the Cluj area. [9, 10], but there is little information available on the safety of consumption of *Lycopodium clavatum* commercialized in Romanian markets.

The present study aims to evaluate the phenolic compounds and heavy metals content in *Lycopodium clavatum* commercialized on the Romanian markets. In addition, the relationship between the phenolic compounds and heavy metals content was also established.

2. MATERIALS AND METHODS

2.1. MATERIALS

Sample preparation

Pedicel *Lycopodium clavatum* was purchased from the local health store, being a plant that grows in natural areas in Romania. For the extraction of polyphenolic compounds, 5 g of dried plant was extracted with 50 ml of solvent (methanol) in an ultrasonic bath at 25°C for 30 min. The extracts were filtered into a 50 mL volumetric flask and diluted with the extraction solvent to the volume.

For the evaluation of the metal content, the dry mineralization method was used; 5 g of the sample was calcined in a muffle furnace at 500°C for 6 hours. The resulting ash was dissolved in 20 mL of concentrated acid mixture (nitric and perchloric acid in a 9:1 ratio for 3 h at 70°C). After dissolution and cooling, the solutions were reconstituted to 20 mL with deionized water, followed by filtration.

Metal content

A multi-element standard (ICP multi-element standard solution IV, Merck, Germany) was used for the preparation of intermediate solutions to obtain calibration curves. Deionized water (Direct Q UV, Millipore, approximately 18.0MΩ, Analytica Jena Instruments, Germany) was used in the preparation of all solutions. Standard stock solutions (1000 ppm) were diluted to obtain working standard solutions ranging from 1 μg/L to 20 μg/L and stored at 4°C. An acidity of 0.1% nitric acid was maintained in all solutions. A calibration curve was drawn between measured absorbance and concentration (μg/L).

2.2. METHODS

TOTAL PHENOLIC CONTENT

Total phenolic content (TPC) was spectrophotometrically determined by the Folin-Ciocalteau assay, using gallic acid as the standard method [11]. TPC was performed using a Jasco 550 UV -VIS spectrophotometer (Jasco, Germany). Absorbance was measured at 675 nm against the prepared reagent blank. The concentration of total phenolic compounds in the extract was expressed as mg of gallic acid equivalent per kg plant (mg GAE/kg plant). All the experiments were carried out in triplicates, and the average absorbance values obtained at different concentrations of gallic acid were used to plot the calibration curve (R^2=0.9997).

TOTAL FLAVONOID CONTENT

The total flavonoid content (TFC) was determined by $AlCl_3$ method [12]. TFC was performed using a Jasco 550 UV -VIS spectrophotometer (Jasco, Germany). The absorbance of the resulting solution was measured at 510 nm using a UV– VIS spectrophotometer against a reagent blank containing 50% methanol instead of the plant extract. Catechol was used as standard to express total flavonoids contents (mg CT/100g sample). All the samples were analyzed in triplicate, and the total flavonoid content was expressed as catechol equivalents using the linear equation based on the calibration curve (R^2 =0.9974).

METAL CONTENT

Stored samples were used for heavy metal analysis using graphite furnace atomic absorption spectrometry (GF-AAS). Results show the average of three determinations and are expressed in mg/kg dry plant. The optimized operating parameters for GF-AAS, (ContraA 800, Analytic Jena Instruments, Germany) [13] as well as the method performance parameters are listed in Table 1.

Table 1.GF-AAS method performance parameters.

Parameters / Elements	λ [nm]	Pyrolysis Temperature [°C]	Atomization Temperature [°C]	Regression equation	Coefficient of correlation	LOD* [μg/L]	LOQ** [μg/L]
Pb	217	600	1500	y=0.0025x+0.0003	0.9946	1.213	4.448
Cd	228	300	1200	y=0.0190x+0.0172	0.9878	0.904	3.203
Cr	357	1100	2300	y=0.0116x+0.0025	0.9849	2.043	7.198
Mn	279	900	2000	y=0.0432x+0.0273	0.9894	1.704	6.084
Cu	324	900	2000	y=0.0100x+0.0034	0.9864	1.935	6.846
Co	240	1000	2100	y=0.0087x+0.0008	0.9960	1.044	3.871
Ni	232	900	2300	y=0.0080x+0.0067	0.9614	3.286	11.49
Bi	223	550	1900	y=0.0010x+0,0012	0.9927	3.595	12.77

LOD*-Limit of Detection; LOQ**-Limit of Quantification.

3. RESULTS AND DISCUSSION

3.1. RESULTS

The values obtained for the TPC, TFC, and toxic elements content of *Lycopodium clavatum* are presented in Table 2. Table 3 presents the correlations between the evaluated parameters.

Table 2. Descriptive statistics (n = 3).

	TPC [mg/g]	**TFC [mg/g]**	**Pb [mg/kg]**	**Mn [mg/kg]**	**Co [mg/kg]**	**Cu [mg/kg]**	**Cr [mg/kg]**	**Ni [mg/kg]**	**Cd [mg/kg]**	**Bi [mg/kg]**
Minimum	2.156	23.760	0.043	<LOD	0.030	1.520	0.457	0.070	<LOD	<LOD
Maximum	2.209	24.102	0.047	<LOD	0.037	1.601	0.480	0.079	<LOD	<LOD
Median	2.176	23.892	0.045	-	0.034	1.564	0.469	0.075	-	-
Mean	2.194	23.972	0.045	<LOD	0.035	1.560	0.472	0.075	<LOD	<LOD
Standard deviation	0.10	0.67	0.01	-	0.01	0.05	0.02	0.02	-	-
Maximum levels [mg/kg] WHO [20]	-	-	10	2	-	20-150	2	1.5	0.3	-

Table 3. Correlation matrix.

	TPC	**TFC**	**Pb**	**Co**	**Cu**	**Cr**	**Ni**
TPC	1	0.34	0.52	0.48	0.12	0.44	-0.24
TFC		1	0.58	-0.03	-0.35	-0.34	-0.33
Pb			1	-0.52	-0.16	-0.23	-0.06
Co				1	-0.27	0.03	-0.42
Cu					1	0.36	0.50
Cr						1	0.57
Ni							1

3.2. DISCUSSION

Phenolic compounds are important constituents of plants with redox properties, thus conferring antioxidant activity, as the hydroxyl groups in these compounds are responsible for facilitating free radical scavenging [14].

Flavonoids are among the major groups of phenolic compounds with a broad spectrum of chemical and biological activities, especially radical scavenging and antimicrobial activities, antibacterial, anticancer, and antiallergic activities [15, 16]. In this study, the values obtained for the total polyphenolic acids and flavonoids content of *Lycopodium clavatum* are in agreement with literature data on the presence of these compounds in *Lycopodium* species [17-19]; TPC was on average 2.194 mg GAE/g DM, while TFC 23.972 mg CT/g DM. (Table 2).

Normally, plants tend to absorb mineral elements (Cu, Fe, Mn, Ni, and Zn) in low concentrations as trace elements for their biochemistry and physiology, but in high concentrations, these elements have toxic effects on both plant metabolism and plant consumers [21]. Long-term exposure of plants to toxic metals results in enzyme inactivation, reduced photosynthesis, and reduced carbohydrate and nutrient uptake, thus leading to visible symptoms such as wilting, growth retardation, and reduced biomass production.

Toxic metals are usually persistent and therefore accumulate in soil and plants [22]. In addition to natural origins, they can enter the soil from various man-made sources, such as the use of mineral fertilizers, and pollution from industrial and urban areas. Numerous studies have shown that the presence of toxic elements in plants can also be a consequence of factors such as soil type and texture, the chemical form in which the elements are present in soil and plants, and the origin of the plants [23]. Acidic soils, in particular, increase the mobility of metals and their uptake by plants, especially Pb [23]. Therefore, levels of accumulated toxic metal concentrations in plants may be an indication of soil quality, but also of human exposure to them. Some external mechanisms that limit metal uptake by roots may help plants to tolerate a certain amount of toxic metal in the soil; for example, the formation of non-toxic metal-ligand chelates in the rhizosphere, involving organic acids and substances emanating from the roots, may reduce the uptake process [24].

In this regard, the World Health Organization (2007) [20] has established national limits for heavy metals in plants (Table 2). Comparing the data on the content of toxic elements in the *Lycopodium clavatum* plant with the reference values (Table 2) it can be seen that these were within safe limits for some of them (Pb, Co, Ni, Cr, Cu), while for others (Cd, Mn, Bi) the concentrations were below the detection limit of the method used.

Lead is known to be the most common and stable toxic element in nature, being toxic to plants and living organisms [25]. Its accumulation by plants has detrimental effects, on morphological, physiological, and biochemical functions. When Pb is taken up by the plant, toxicity is given by alteration of cell membrane permeability, by reaction with metabolically active enzyme groups, or by substitution of essential ions [26].

The Pb uptake process depends on the soil pH, so at higher pH this metal is immobilized. It is also known that due to the low transfer factor, Pb is not taken up by plants below a concentration of 300 μg/g in soil, and if it is taken up, it would accumulate more in leaves than in fruits [27-29].

Fertilizer use, fuel combustion, and sewage sludge are major sources leading to contamination with this element. The mean value of Pb content in *Lycopodium clavatum* species was 0.045 mg/kg, which is much lower than the standard value of 10 mg/kg in medicinal plants [20]. As with other metals, Co contamination of soils is largely due to mining, sewage sludge spreading, and fertilizer use, which can pose a risk to the environment [30]. Information in the literature on Co toxicity and risk to plants is limited, but some studies have shown that when administered in high doses, Co becomes toxic to plants [31]. It has been reported that the uptake and distribution of Co in plants depends on the chemical species, and is controlled by different mechanisms; Co^{2+} species involve an active transport process across cell membranes, but a limited distribution from roots to shoots. Its distribution is assumed to involve complexation reactions with certain organic ligands [30]. The results in *Table 2* show values between 0.030 and 0.037 mg/kg in *Lycopodium clavatum* being much lower than the critical levels reported in medicinal plants, between 30 and 40 mg/kg [32].

Nickel is considered a vital element for plants at concentrations of 0.01 to 5 μg [33]. Ni uptake by the plant occurs through active transport and passive diffusion [34]. Studies on medicinal plants have shown that at concentrations above 10 mg/kg, this metal has negative effects on photosynthesis, and mineral nutrient uptake [35-37]. The average value of Ni in *Lycopodium clavatum* was 0.075 mg/kg, well below the WHO recommended level [20].

Copper is an essential element for various metabolic processes, its content in many plant species varies between 20 and 30 mg/kg. Because it is required only in trace amounts, Cu becomes toxic at high concentrations [38]. The average value of Cu in *Lycopodium clavatum* was 1.560 mg/kg, which is within the limits established in medicinal plants.

Cr is considered a non-essential metal for plants, and its toxicity on plant growth and development has been reported in several studies [39]. The accumulation and diffusion of Cr in plant tissues depends on its ionic state; it accumulates mainly in roots in its trivalent form, Cr(III), and is translocated to shoots in the Cr(VI) form, following oxidation-reduction processes [36]. As with other toxic metals, Cr reduces the uptake of many essential elements, (Fe, Mg, Mn, Ca, P, and K) with negative effects on plant growth [40]. The average value of chromium in the analyzed plants was 0.472 mg/kg.

It can be concluded that Pb, Cr, Cu, and Co were present within tolerable limits in *Lycopodium clavatum* species, while other elements were below the detection limit of the method used. Although the selected metals pose no threat to the environment at their current level, their accumulation over time in soil and plants can endanger the environment and human health.

Table 3 shows the correlation coefficients for phenolic compounds and metal concentrations in *Lycopodium clavatum*. The r values were found to be significant at ±0.22 ($p<0.05$), showing positive and negative correlations between the parameters evaluated.

In the case of phenolic compounds, a significant positive correlation was observed between TPC and TFC with an r=0.34 value; this suggests cooperation between these constituents in the metabolic processes occurring in the plant [41-44].

Regarding the relationship between metals, a significant positive correlation was observed between Cr and Ni at a value of r=0.57, showing the existence of a similar source origin. The next strongest positive correlation includes the Cu-Ni pair with r = 0.50. Similarly, significant negative correlations of Pb and Ni with Co were observed, with values of r=-0.52 and -0.042, indicating an increase in the concentration of one metal with a decrease in the concentration of other metals.

Data corresponding to correlation coefficients for phenolic compounds and different metals also show significant positive/negative correlations; Pb showed positive, significant correlations with TPC and TFC (r=0.52, respectively r=0.58); another significant correlation was observed between TPC and Co and Cr elements, respectively, of r=0.48 and r=0.44, indicating that these minerals coordinate with phenolic compounds and determine their antioxidant action in the plant. On the other hand, Cu might not affect the antioxidant properties of phenolic compounds, as insignificant correlations were observed between this element and flavonoid content.

Research on the effect of different concentrations of heavy metals on plant growth shows that at low concentrations, metals stimulate the production of secondary metabolites, while at higher concentrations, they inhibit the synthesis of secondary metabolites. Also, a high concentration of heavy metals causes a decrease in the accumulation of phenolic compounds, due to the inability of the plant to synthesize new phenolic compounds and flavonoids [45, 46].

In the study, significant positive and negative correlations were observed between phenolic compounds and toxic elements. In general, phenolic agents are characterized by at least one aromatic ring (C6) possessing one or more hydroxyl groups capable of binding to metal ions. Studies have shown that the roots of many plants produce significant amounts of secondary metabolites (phenolic compounds); these compounds can inactivate metals by complexation, thus protecting plants from the toxic action of metals [47].

4. CONCLUSIONS

The study showed: (i) the presence of secondary metabolites (polyphenolic compounds) in the plant *Lycopodium clavatum*; (ii) the concentrations of Pb, Co, Ni, Cr were within the permissible limits, while Mn, Cd and Bi were below the detection limit; (iii) the data corresponding to the correlation coefficients for phenolic compounds and different metals showed significant positive/negative correlations; (iv) Medicinal plants grown in their natural areas can accumulate toxic metals from the soil, and their use for the preparation of medicinal teas and extracts requires continuous analysis, and the results should be shared with local users and managers.

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