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Allelopathic effects of *Pteridium aquilinum* alcoholic extract on seed germination and seedling growth of *Poa pratensis*

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ABSTRACT

The effects of *P. aquilinum* alcoholic extract on germination, growth and development of *P. pratensis* seedlings were examined. The extract decreased the germination and seedling growth, decreased chlorophyll, caretenoid and protein content depending on the extract concentration. Increasing concentrations decreased all these parameters proportionately simulating the effects of allelopathy in ecosystem. The GCMS analysis of alcoholic extract showed the presence of pterosin B and pterosin F which are inhibitory to the seed germination and development *P. pratensis*.

Key words: Alcoholic extract, allelopathic, extracts, *Pteridium aquilinum, Poa pratensis*, seed germination, seedling growth.

INTRODUCTION

Allelopathy is defined as the beneficial or harmful influence on each other of chemical substances released by plants or microorganisms (5,19). Compounds with allelopathic potential are found in all plant organs [leaves, flowers, fruits, roots, rhizomes, stems and seeds] and in parts which can store these compounds (1,7). Specific inhibitory effects results from the combined action of allelochemicals, interfering in various physiological processes [respiration, photosynthesis, enzyme activity (26,~~2~~6)], water relationships, stomatal opening, hormone levels, availability of minerals cell division, elongation, structure and permeability of cell membranes and cell walls of plant cell (8,11,13) and thereby altering the plant growth (42). Roots are more sensitive to allelochemicals than aerial parts of seedlings. Inhibition of root growth and development may be due to the changes in DNA synthesis in root cells and impaired mitochondrial metabolism (4,14). Soil erosion reduces the vegetation cover, for its control plant species resistant to erosion are sown e.g. *P. aquilinum*, which is poor in nutritional value. These mountain areas are invaded by *P. Aquilinum,* shrubs and woody vegetation.

Most allelopathy studies have focussed on agricultural plants or on the identification and isolation of chemical substances with potential use as herbicides, pesticides etc (4343,33). Ferns produce and release allelopathic chemicals, hence, these

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dominate other species, especially in regeneration after a fire (17,39). Diffusion of chemical compounds with allelopathic potential from the fern inhibits the growth of other plant species (34). The growth of trees and plants is inhibited even after the fern is removed, because the toxins of ferns plants remain active in the soil (20). *Pteridium aquilinum* (bracken, brake or common bracken) is vascular Pterydophyte plant species with allelopathic potential. Its allelochemcials are phenolic compounds [ptaquiloside ($C_{20}H_{30}O_8$), pterosins (sesquiterpenes and norsesquiter-penes) and hydrogen cyanide, etc.]. This fern forms large colonies in various habitats especially in areas where *P. pratensis* (perennial grass specie in pastures, lawns and gardens) is dominant. This study aimed to examine, the effects of *P. aquilinum* extracts on seed germination and development of *P. pratensis* plants.

MATERIALS AND METHODS

All solvents used were of analytical grade and purchased from Merck. While, standard gallic acid, (+)-catechin, (-)-epicatechin, chlorogenic acid, 2N Folin-Ciocalteu phenol reagent and triterpene standards (97% oleanolic acid) were purchased from Sigma-Aldrich. To calculate the retention time of alkanes, C_{12}-C_{60} standard alkane, was from Sigma-Aldrich and C_{21}-C_{40} standard solution was from Fluka.

I. Harvesting and preparation of *P. aquilinum*

P. aquilinum samples were collected in August 2012 from the Cosava, Timis County, Banat, Romania (45°50'53" N, 22°18'43" E, elevation 203 MSL) (9,10). Fresh Plants were cut at 2 cm above ground, air dried at room temperature for 2 weeks and then finely ground. The material was kept in glass containers and stored at - 18°C till extraction. Due to the toxicity of plant material (ptaquiloside and pterosins) protective clothing and gloves were used during the samples collection and processing.

II. Determination of total polyphenol content

(i). Soxhlet extraction of compounds in *P. aquilinum*: Twenty g dried, powdered plant material was placed in a Soxhlet bag and kept in a Soxhlet extractor and extracted with 400 mL absolute ethanol for 6 h. The extract was then cooled, filtered and the solvent was evaporated using a rotary evaporator ROTOFIX 32A. The crude dry extract was weighed and the yield was calculated (10).

(ii). Determination of total polyphenol content: To determine the total polyphenol content of alcoholic extract, the extract obtained from Soxhlet extraction of compounds in *P. aquilinum* was diluted in 3 mL water.

Total polyphenol content of the alcoholic extract was determined using the Folin-Ciocalteu (10) and the absorbance was measured at 765 nm using UV-VIS Spectrophotometer [PG Instruments with 5.05 UV WIN software (36)]. Calibration curve (concentration range 20 to 120 µg/mL) obtained using gallic acid was used as standard. Briefly, 10 µL of each standard solution was added to a 10 mL volumetric flask containing 1 ml of Folin-Ciocalteu reagent and 5 ml of 7.5% Na_2CO_3 aqueous solution (10). Deionized water was added to each flask to make volume of 10 mL. After mixing, the solutions were incubated for 1 h (protected from direct light) and the absorbance was measured at 765 nm. By plotting absorption vs concentration, a linear calibration curve was formed. Total polyphenol content in each sample of alcoholic fern extract was expressed as milligrams of gallic acid equivalent (mg GAE) per 100 g of dry weight (g DW). Calculation was made by reference to 100 g of dry weight (g DW). Four replicates for each treatment were used.

(iii). Determination of total triterpene content: The total triterpenes content was determined using perchloric acid and vanillin (staining reagent). The absorbance was measured at 550 nm using UV-VIS Spectrophotometer with UV-WIN 5.05 software. For calibration, 10 mg oleanolic acid was used as standard, dissolved in 50 mL methanol and 0.10, 0.20, 0.30, 0.40, 0.50, 0.60; 0.70 mL of standard solution was transferred to test tubes. 0.3 mL of vanillin in 5% acetic acid and 1 mL of perchloric acid were added to each test tube prior to heating on the water bath for 25 min at 70^oC. The solutions were cooled and 10.0 mL of 5% acetic acid was added to each test-tube. The absorbance of each solution was measured at 550 nm.

Using samples previously prepared, 10 mg of lyophilized fern extract was dissolved in 1 mL of dichloromethane in a test tube. The solvent was then evaporated on water bath and total triterpene content (triterpene constituent in the extract compared to oleanolic acid content, expressed in milligrams) in samples was measured as described previously (10,21). Potential allelopathic compounds were identified by chromatographic analysis. From the original extract, 25, 50, 75, and 100% concentrations were prepared.

III. Germination bioassay

The effects of *P. aquilinum* alcoholic extracts were determined on seeds germination and seedling growth of test specie *P. pratensis*. The distilled water was used as control. Twenty seeds of *P. pratensis* were placed on filter paper in Petri dishes (9 cm dia) lined with 2- filter papers moistened with 5 mL of *P. aquilinum* extract or water as per treatments. The plates were kept in germination room [28^oC ($\pm 2^oC$), 12 h light and 12 h dark]. Germinated seeds were counted at 12 h intervals till 7-days and thereafter, every 24 h till 10 days. The seeds with 2 mm long radicle were considered as germinated. The germination parameters were germination (%) and speed of germination (18). Treatments were replicated 4-times.

IV. Growth bioassay

Twenty *P. pratensis* seeds were germinated in transparent plastic boxes (11·29 x 9.5 cm), lined with filter paper, moistened with 40 mL of extract or water as per treatments. The boxes were covered and placed in transparent plastic bags and kept in climate-controlled room under the same conditions as for the germination test. After 4-days, the length of shoot and primary root was measured with a digital caliper (9).

V. Pigments analysis

To determine the pigment compounds, plant samples were chopped and extracted with acetone in the presence of $CaCO_3$ (10). The pigments were extracted from samples with 80% acetone. Chlorophyll a (Chl a), chlorophyll b (Chl b) and pigments (Xan + Car) were spectrophotometrically estimated based on special absorption coefficients (10, 21). The lack of a significant absorption in the green side of spectrum offered to chlorophylls its characteristic colour, green or blue-greenish.

Results were expressed in $\mu g g^{-1}$fw.

$$Chl\ a = (13.95 \cdot A_{665}) - (6.88 \cdot A_{649});$$
$$Chl\ b = (24.96 \cdot A_{649}) - (7.32 \cdot A_{665});$$
$$Pigments\ (Xan + Car) = [(1000 \cdot A_{470}) - (2.05 \cdot Chl\ a) - (114.8 \cdot Chl\ b)]/245.$$

To determine the total protein/nitrogen in samples was estimated by the Kjeldahl method. These estimation methods were from the Romanian Pharmacopeia and the techniques corresponded to international standards (2,24,35).

VI. GC-MS analysis

The GC-MS analyses the extract obtained from Soxhlet extraction of compounds in *P. aquilinum* were done on a gas chromatograph coupled with a mass spectrometer Agilent type as detection system (mass detector). The mobile phase used was helium with a flow rate of 1 mL/min. Initial measurements were done on non-polar column (DB 5 MS, 30 m, 0.25 mm and 0.25 µm film thickness deposited on the walls of the column). MS was equipped with ion-trap, capable of recording sequential ionization spectra until MS11. Data acquired during recording was processed with specialized software, Dissect For Data Analysis 3.4. Electron impact ionization was applied using beam energy of 70 eV. All determinations respected ethics protocols/ research ethics committees (29,30).

RESULTS AND DISCUSSION

Identification of pterosin F and B

The chromatogram of *P. aquilinum* extract showing the chemical composition is given in Figure 1. Although Ptaquiloside derived from the fern are found in large quantities, but this compound is difficult to quantify due to its instability.

Pterosin B, a final product of ptaquiloside decomposition, is used to quantify the ptaquiloside. The results were confirmed from other authors GC-MS analysis of pterosin B (28). Along with pterosin B, pterosin F ($C_{14}H_{17}OCl$) was also detected in *P. aquilinum* extract. Pterosin F has a structure similar to pterosin B, except that hydroxyl group at the side chain is replaced by chlorine. Pterosin F appears in the chromatogram at 20.57 min, identification was achieved by GC-MS. Characteristic ions were identified at m/z 187, 201 and 236. By the decomposition of the benzyl side chain, for pterosin B the m/z 187 ion is formed. The loss of chlorine from the side chain causes the formation of m/z 201 ion. The m/z 236 molecular ion, characteristic to m/z 236 and 238 rate reflects the relative abundance of chlorine isotopes (^{35}Cl: ^{37}Cl = 3:01). The presence of pterosin F extract indicated that the substance exists in the leaves of *P. aquilinum* before extraction. In previous studies, pterosin F was found in methanol extract of young *P. aquilinum* leaves (3). It is not known whether pterosin F is synthesized or it is a product of a metabolic precursor activated in leaves of *P. aquilinum*.

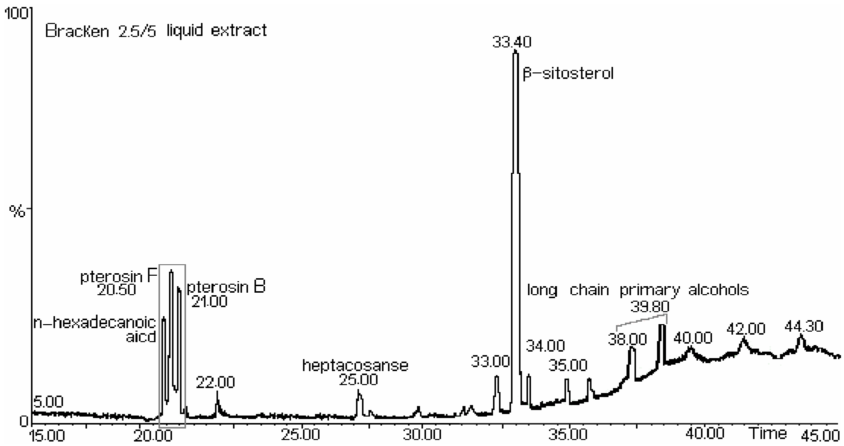

Figure 1. Chromatogram of *P. aquilinum* extract

Several studies suggests that pterosin B is formed from decomposition of ptaquiloside in the presence of light (28). It is possible that ptaquiloside is a source for pterosin B in leaves. Another compound indanone-type of compounds, pterosin A, was isolated from fern and we took into consideration that its precursor is also ptaquiloside, named dennstoside (29). For pterosin F, precursor and the biosynthetic mechanism are unknown.

Germination bioassay

Regardless of concentration used, *P. aquilinum* extracts did influence the germination (%) of *P. pratensis* seeds at any test concentrations 100 the alcoholic extract significantly decreased the germination (%). Regarding the speed of germination, all extracts delayed the germination (%) except control (Table 1 and Figure 2).

Table 1. Inhibitory allelopathic effects of *P. aquilinum* extract on growth of *P. pratensis* seedlings

Extract Conc. (%)	Germination (%)	Seedling height (cm) at 17 d		
		Root length	Shoot length	RL/SL
Control	94.45	6.02	9.50	0.63
25	84.15	5.78	8.05	0.72
50	77.01	4.71	7.80	0.60
75	67.87	3.85	7.17	0.54
100	64.28	3.07	6.02	0.51

Mean ± (SE) values followed by the same letters within each column are not significantly different at 0.05.

The changes in patterns of germination may be caused by changes in cell membranes permeability, transcription and translation of RNA integrity of second messengers, respiration (44), enzyme and receptor conformation or a combination of these changes (37). Compounds with allelochemical from alcoholics extract presents potential inhibits the germination of seed by preventing the stimulation of α-amylase synthesis, which mobilizes the stored reserves and maintain the seeds respiratory activity.

All allelochemicals may increase the total lipid content in cotyledons (24), due to reduced mobilization of reserves during the germination. In most cases, allelopathic effect is not observed in final germination (%), but is seen in the rate of germination (32), which indicates the allelopathic potential of tested extract. Fern alcoholic extracts changed the seed germination process in *P. pratensis*, demonstrating that these changes may also occur in nature. The extract with the highest concentration caused maximum inhibition in germination and the inhibitory effects depended on the concentration used.

Growth bioassay

The growth was statistically stimulated when *P. pratensis* seedlings were cultivated in presence of *P. aquilinum* alcoholic extracts at 50, 75 and 100 % concentrations compared to control (Table 1 and Figure 2). These results are similar to the results of germination bioassay. The 100 *P. aquilinum* extracts were most inhibitory to seedlings growth. Root exudates of *P. aquilinum* are main sources of allelochemicals released into the soil (27, 27). These compounds are stored in the root cells for subsequent release. *P. aquilinum* root exudates released in the soil, could be the reason for the inhibitory effects on the development of other species.

This probably indicates interferences between the *P. aquilinum* allelochemicals and phytohormones for growth. Perhaps these effects are related to the metabolism, concentration and sensitivity to various plant hormones, but are also influenced by the environmental conditions (22). Ptaquiloside in alkaline pH between 8 and 11, loses one molecule of D-glucose, and in aqueous solutions at acidic pH loses D-glucose, converting into pterosin B. For example, depending on the soil pH, ptaquiloside may decompose as per the following reaction scheme.

In this study, fern alcoholic extracts reduced the primary root growth of *P. pratensis*. The various alcoholic extracts of *P. aquilinum* influenced the germination and seedling growth of *P. pratensis*. The inhibition in germination and growth depended on the concentration used with complete suppression of germination at 100 % concentration (23, 27).

The concentration of chlorophyll a, chlorophyll b, total chlorophyll, caroteniods and proteins of *P. pratensis* decreased proportionally to the increasing alcoholic extract concentration of *P. aquilinum* (Table 2, Figure 3).

Table 2. Allelopathic effect of extracts on pigments, protein content in leaves of *P. pratensis*

Extract Conc (%)	Pigment (mg g^{-1} FW) Chl a	Chl b	Total Chl	Caroteniods	Protein (mg g^{-1} FW)
Control	5.25	0.50	5.74	2.07	28.35
25	3.54	0.39	3.93	1.00	24.68
50	2.82	0.51	3.33	0.73	23.10
75	2.64	0.50	3.14	0.72	18.40
100	2.34	0.46	2.80	0.65	16.40

Chl: Chlorophyll Mean±(SE) values followed by the same letters within each column are not significantly different at 0.05 (ANOVA test n = 4).

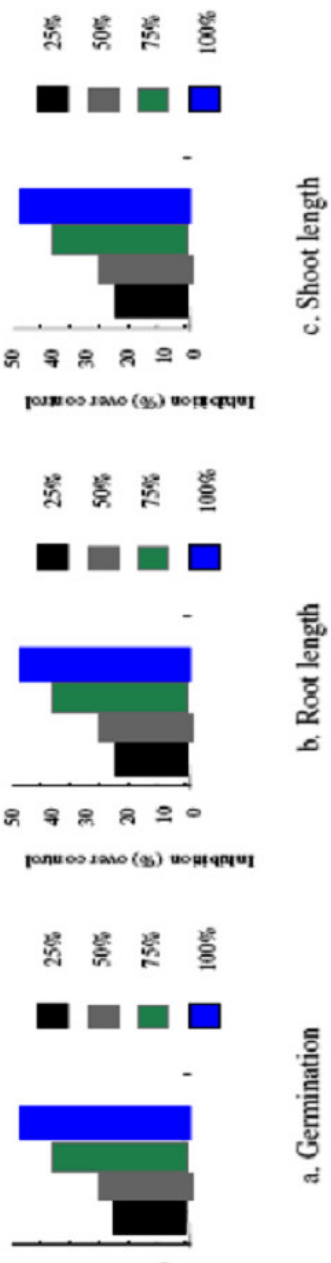

Figure 2. **Inhibitory** effects of *P. aquilinum* extract on seed germination and seedling growth of *P. protensis.*

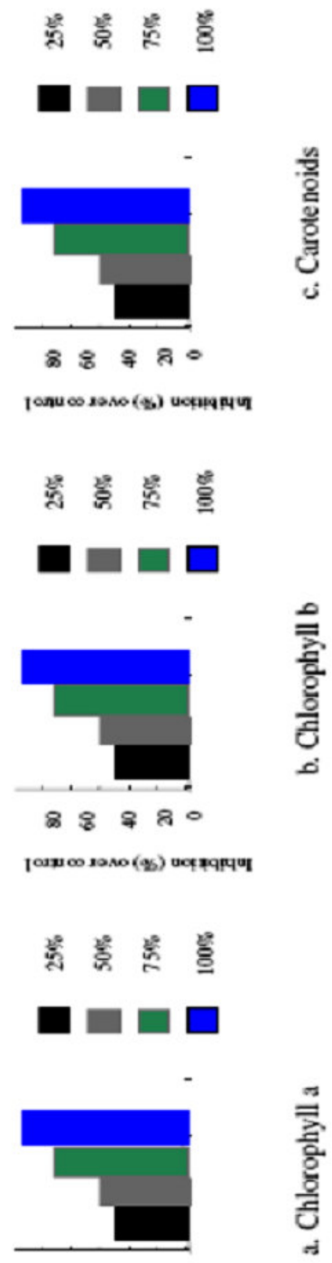

Figure 3. **Inhibitory** effects of *P. aquilinum* extract on **Chlorophyll** a, Chlorophyll b and Carotenoids in *P. protensis.*

It was found how changes in chemical composition of *P. pratensis* plants can be quantitatively attributed to different sources of variation of fern alcoholic extract concentration (165,16).

Future allelopathy research should focus on: (i). determining the role of allelopathy in nature and in agro-ecosystem, (ii). screening of compounds with allelopathic potential in plants or microorganisms (iii). development of new isolation methods of allelochemicals in plants and soils, (iv). to study the mode of action of allelopathic substances, (v). enhancement of allelopathic properties in crop plants, (vi). use of biotechnology to transfer the potential allelopathic chemical products from one plant to another and (vii). development of practical methods to use allelopathy in crop production.

CONCLUSIONS

The concentration of alcoholic extract of *P. aquilinum* decreased the germination, root length and length of seedlings. All higher concentrations of alcoholic extract of *P. aquilinum* decreased the quantity of plant pigments (chlorophyll a, chlorophyll b and carotene). The GCMS analysis of alcoholic extract showed the presence of pterosin B and pterosin F in *P. aquilinum* were inhibitory to seed germination and development *P. pratensis*.

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