Research Article

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Chemical profile and antioxidant activity of *Trollius europaeus* under the influence of feeding aphids

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Abstract: The influence of stress factors on a plant may lead to worse functioning of the plant and the loss of its crop. The effect of *Aulacorthum* solani feeding on *Trollius europaeus* with regard to active compounds in the leaves was investigated in the study. The antioxidant activity of the leaves, the material on which the insects fed, was compared with that of the material that was not infested by the aphids. Stress level was evaluated on the basis of such physiological parameters as chlorophyll fluorescence and photosynthesis activity. An increase of 34.5% in the content of polyphenolic compounds, as compared to control, was demonstrated in the material exposed to the biotic stress caused by aphids. The content of phenolic acids was 28% higher while that of flavonoids rose by 25%. The increase in polyphenolic compounds augmented the antioxidant activity of the material.

Keywords: *Trollius europaeus*, flavonoids, phenolic acids, aphid feeding, chlorophyll fluorescence

1 Introduction

Polyphenols, including flavonoids and phenolic acids, constitute one of the best-studied groups of chemical compounds of a wide range of activity. Their most extensively described biological activity has been their antioxidant activity, which is the focus of a range of medicinal and preventive activity, e.g. against tumours, degenerative diseases, and aging processes. Scientists worldwide have been searching for new and efficient plant sources of polyphenolic compounds. Species of the *Trollius* genus are rich in polyphenolic compounds, particularly flavonoid *C*-glycosides, rarely found in the plant world [1, 2]. They have been commonly utilized in Far East medicine to prevent and treat many diseases, especially infectious ones. Extracts from *T. chinensis* and *T. ledebouri* have been traditionally used to treat upper respiratory tract infections, pharyngitis, tonsillitis, bronchitis, cold with fever, acute tympanitis, aphthae, mouth sores, hemorrhage, gum pain, acute lymphangitis, and acute periostitis [3, 4, 5].

The *T. europaeus* species, which occurs in Europe, may also constitute a source of material rich in polyphenolic compounds and could therefore be used for medicinal purposes. Initial research results suggests that it may have great antioxidant potential suitable for a range of biological and medicinal applications [6, 7].

Previous research identified caffeic, chlorogenic, γ-resorcylic, p-coumaric, vanillic, p-hydroxybenzoic, ferulic, and syringic acid as well as the following flavonoids: isoorientin, orientin, vitexin, vitexin 2”-O-α-arabinopyranoside, vitexin 2”-O-β-galactopyranoside, orientin 2”-O-β-glucopyranoside, orientin 2”-O-α-xyloopyranoside, and 4’-O-α-rhamnopyranosyl (1→2)-β-xylopyranoside of 7-O-methylapigenin [6, 7, 8].

The presence of stress factors affects plant growth and development as well as the size and quality of crops. Two types of stress can be distinguished: abiotic stress connected with environmental factors such as solar radiation, temperature, water availability, soil structure, wind, etc., and biotic stress associated with the presence of pests and pathogens. A natural mechanism of plant defense against stress such as pests is to produce secondary metabolites, which limit negative influence on the development of the plant and protect it from damage and destruction [9].

The aim of this study was to determine the effect of exposure to feeding aphids on the content of flavonoids

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and phenolic acids in the leaves of *T. europaeus* and to compare the antioxidant activity of raw materials growing under conditions with and without the stress factor in the form of feeding aphids.

2 Materials and methods

2.1 Plant material

The plant material to be studied was obtained from three-year-old plants of *Trollius europaeus* grown in natural environment conditions in the Botanical Garden of the Department of Medicinal and Cosmetic Natural Products of Poznan University of Medical Sciences (Haake, 1034). The plants of *T. europaeus* were transferred to pots (one plant per pot) and placed in a greenhouse in controlled conditions with a photoperiod of 16/8h and temperature of 25-30°C. At the beginning of the flowering stage, the 12 most aligned plants were selected. Half of the *T. europaeus* plants were separated by a mosquito net and infested mechanically with *Aulacorthum solani* (five wingless aphid parthenogenetic clones per plant). The aphids were collected from the greenhouse in early spring and then multiplied in controlled conditions. The *Aulacorthum solani* species had been donated by Strażyński at the Institute of Plant Protection in Poznań, Poland. In order to monitor vegetation conditions during the study, the biotically stressed plants as well as the control plants were grown in a controlled greenhouse environment. After 21 days, the leaves were collected and dried under natural conditions at 24-26°C. 15.1 g of dried leaves were obtained from the plants subjected to the biotic stress and 15.6 g from the plants grown without the stress factor.

2.2 Chemicals

Acetone, methanol, ethyl acetate, hydrochloric acid, sodium hydroxide, sodium carbonate, sodium molybdate and sodium nitrite were obtained from POCh (Gliwice, Poland). Caffeic and chlorogenic acids were purchased from Carl Roth GmbH Co, Germany, Folin-Ciocalteu reagent and aluminum chloride from Merck (Darmstadt, Germany), dimethylosulfoxide (DMSO) from Ubichem Ltd. (Hampshire, England), 2,2-diphenyl-1-picrylhydrazyl (DPPH radical) from Sigma-Aldrich, USA, and butylhydroxyanisole (BHA) from Fluka, France. Absorbance was measured with a UV/VIS Lambda 35 spectrophotometer (Perkin-Elmer, USA).

2.3 Determination of total phenolic content

Determination of the total phenolic content in liquid extracts was conducted using the Folin-Ciocalteu reagent [10]. 100 microL of extract was mixed with 0.5 mL of Folin-Ciocalteu reagent and 4.0 mL of water. After 1 minute of incubation, 2.0 mL of a 20% solution of sodium carbonate were added. The mixture was incubated for 30 min. at room temperature. The absorbance was measured at 760 nm with a spectrophotometer (UV/VIS Lambda 35 Elmer-Perkin). Total phenolic content was calculated from the equation of the standard curve of caffeic acid: y=0.093x-0.0287; R^2=0.9503.

2.4 Determination of phenolic acid content

Phenolic acids content was determined by the spectrophotometric method with Arnov's reagent. 0.3 mL of extract was mixed with 6.7 mL of water, 1 mL of hydrochloric acid (18 g/L), 1 mL of the Arnov's reagent (10.0 g of sodium molybdenum, 10.0 g of sodium nitrate in 100.0 mL of water), and 1 mL of sodium hydroxide solution (40 g/L). 1 minute after the addition of the sodium hydroxide solution, the absorbance of the extracts was measured at a wavelength of λ = 490 nm. The total phenolic acids content (TPAC) expressed as a caffeic acid equivalent was calculated from the following formula:

$$\text{TPAC}\ (\%) = A \text{x}\ 500/\text{a}\ \text{x}\ m$$

where A was the absorbance of the test solution, m was the mass of the powdered drug, in grams, and a was the absorbance of caffeic acid, which was determined from the standard curve at a value of 215 [11].

2.5 Determination of total flavonoid content

The total content of flavonoids (TFC) was determined spectrophotometrically according to the boric acid method described in Polish Pharmacopoeia [11]. The stock solution was obtained by boiling 0.5 g of powdered leaves (first extraction 30 min., next 10 min., 60°C) with ethanol (60% (*V/V*), first 40 mL, next 40 mL). 5 mL of the basic solution was evaporated to dryness and the residue was dissolved in 10 mL of a mixture of methanol and glacial acetic acid (1:10): 10 mL of boric acid solution (25 g/L) and oxalic acid solution (20 g/L) in anhydrous acetic acid were then added. Anhydrous acetic acid was poured to a final volume of 25 mL. By the same procedure, replacing

the mixture of boric and oxalic acids with anhydrous formic acid, reference solutions were prepared. After 30 min. of incubation, the absorbance of the tested mixtures was measured at λ = 401 nm. The formula: *X=A* x 0.8/m was used to calculate total flavonoid content. The results were calculated on vitexin, assuming an absorbance typical of vitexin was equal to 628. *A* in the formula is the absorbance of the tested samples and m is the mass of the substance to be examined, in grams.

2.6 DPPH free radical scavenging assay

The antioxidant activity of the methanolic extracts (TAph - the extracts from the leaves of *T. europaeus* on which aphids were present and TE- the extracts from the leaves of *T. europaeus* on which aphids were not present) was determined by using 1,1-diphenyl-2-picrylhydrazyl (DPPH, Sigma-Aldrich, Germany) [12, 13, 14]. A constant temperature of 20°C +/- 1. was maintained during the tests. 0.2 mL of each sample dissolved in water at a concentration of 0.1mg, 0.3mg, 0.5mg, 0.7mg, and 1.0 mg of the youngest fully expanded leaves/100 mL was added to 3.9 mL of the DPPH solution (6.2 x 10^{-3} %). The absorbance was tested at λ = 536 nm after 30 min. of incubation. The scavenging activity was calculated according the formula: DPPH scavenging activity (%) = [A0 – Ai/Ai]x100, (A0 - absorbance of the control, Ai - absorbance of the tested sample). The reference substance was BHA (buthylatedhydroanisole) (100-1000 μg/mL).

2.7 Determination of photosynthesis and fluorescence parameters during aphid feeding

The measurements of the physiological state of the plants were taken 14 and 21 days after the pests were applied. The measurements were conducted under controlled conditions in the greenhouse (photoperiod: 16/8 h, temperature: 25-30°C) on the same day for each of the examined objects, maintaining the order of the replicates. The measurements were always performed on the same, youngest, fully developed leaf because of its high photosynthetic activity and also because of the aphid's preferences (most aphids feed on young shoots and flower buds).

2.7.1 Determination of chlorophyll fluorescence

Chlorophyll fluorescence was measured with the Multi-Mode Chlorophyll Fluorometer (OS5p, OPTI-SCIENCES. INC., Hudson, USA) with PAR Clip (allows for the measurement of Photosynthesis Active Radiation (PAR) and Leaf Temperature along with Yield test). Leaves were dark-adapted (with Dark Clips) for 30 min. prior to the measurement of the maximum fluorescence (Fm), variable fluorescence (Fv), and maximum photochemical efficiency of photosystem II (F_v/F_m). F_v/F_m was calculated through ($Fm-F_0$)/Fm. The yield was measured by means of a light-adapted steady-state photosynthetic test which measures the proportion of the amount of light used in photochemistry in Photosystem II (PS II) to the amount of light absorbed by chlorophyll associated with PSII (Maxwell and Johnson 2000). The plants were light-adapted for about 30 min before the Quantum Yield of Photosynthetic Energy (Y II) and Relative Electron Transport Rate (ETR) of the chlorophyll fluorescence were measured. The equation for the ETR is ETR=(Y(II)) (0.84)(0.50)(PPFD) (OS5p User's Guide). The modulation source used in the tests was red (660 nm). The Modulation Intensity in the Fv/Fm Protocol was set at position 9 and in the Yield Protocol at position 17. The gain setting in the Fv/Fm Protocol was set at position 4 and in the Yield Protocol at 5. The saturation source was a 35W halogen lamp. The Saturation Flash Intensity values were set at Position 30 in the Fv/Fm and 32 in the Yield Protocol. The actinic source was a light source that drove photosynthesis [15].

2.7.2 Determination of photosynthesis and transpiration rate

Photosynthesis Rate (A (μmol CO_2 $m^{-2}s^{-1}$)) and the Transpiration Rate (E (mmol H_2O $m^{-2}s^{-1}$)) of single leaves were measured on the same leaf as chlorophyll fluorescence, with a portable photosynthesis system (LCpro-SD, ADC Bio Scientific Ltd., UK) with a Narrow leaf chamber (area: 5.8 cm^2). The CO_2 concentration (Reference CO_2) in the leaf chamber was kept at 360 vpm, and the leaf chamber temperature (Tch) was maintained at 26 ± 1°C. The flow rate of the air (u) was at 200 $\mu mols^{-1}$. The Reference H_2O and flow (u) stayed at the ambient level. During the measurements, PAR was 400 $\mu molm^{-2}s^{-1}$ and adjusted automatically by a red-blue light-emitting diode (LED) light source (LCP Narrow Lamp, ADC Bio Scientific Ltd., UK). The setting protocols and methods of measurement were selected in accordance with the manufacturer's instructions [15, 16].

2.8 Statistical analyses

The estimated values were calculated as the mean of six replicates, plus or minus the confidence interval. The Kruskal-Wallis test was used to assess the significance of the effects of aphids. The difference was considered significant at a p-value ≤ 0.05. Correlation between the antioxidant capacities and total phenolic contents was analyzed using a simple linear regression, and the coefficient of determination (R^2) was calculated. Statistical analysis was performed using the STATISTICA 10.0 software.

3 Results and discussion

The aim of the study was to determine the effect of aphid feeding on the content of polyphenolic compounds, including flavonoids and phenolic acids, on the antioxidant activity of extracts prepared from the youngest, fully expanded leaves obtained from plants exposed to aphid feeding compared to control. The level of stress was evaluated on the basis of such physiological parameters as chlorophyll fluorescence and photosynthetic activity. As a result of aphid feeding, a decrease in Fm value by 8.7% was observed after 14 days and by 24.8% after 21 days, in comparison to control. The decline in Fm value showed that the examined photosynthesizing plants were under the influence of stress, which causes some electron acceptors in PSII not to be fully reduced. The Fv value of the infested plants dropped by 7.5% after 14 days and by 30.31% after 21 days, in comparison to control, which indicated weak PSII activity and excitation energy dispersal in the form of heat [16, 17]. Furthermore, no differences in the value of the F_v/F_m parameter were observed in either sample after 14 days. A significant difference was found in the case of the plants subjected to the biotic stress factor after 21 days of the study. The decrease in the Fv/Fm value by 7.3% in the plants subjected to the biotic stress showed impaired function of PSII. A decline in the ETR parameter by 4.4% was also observed after 14 days and by 15.6% after 21 days (Table 1). The study of the photosynthetic activity included the determination of the Photosynthesis Rate (A (μmol CO_2 $m^{-2}s^{-1}$)) and Transpiration Rate (E (mmol H_2O $m^{-2}s^{-1}$)). In the plants subjected to aphid feeding, CO_2 assimilation was reduced by 0.4 μmol$m^{-2}s^{-1}$ and 0.1 μmol$m^{-2}s^{-1}$ after 14 and 21 days, respectively. The transpiration value increased by 0.4 mmol H_2O $m^{-2}s^{-1}$ after 14 days. No significant changes in transpiration were observed after 21 days, as compared to control (Table 1).

Table 1. Chlorophyll fluorescence and gas exchange measurements of control plants and greenhouse aphid-stricken plants in light conditions after 14 and 21 days of exposure.

There is a myriad of studies confirming differences in chlorophyll fluorescence and photosynthesis measurement parameters between aphid resistant and susceptible species. Chlorophyll fluorescence parameters, as well as the photosynthetic capacity in both cultivars,

Table 1. Chlorophyll fluorescence and gas exchange measurements of control plants and greenhouse aphid-stricken plants in light conditions after 14 and 21 days of exposure

Plant physiological parameters	14 days of biotic stress		21 days of biotic stress	
	TE	TAph	TE	TAph
chlorophyll fluorescence after adaptation to darkness				
Fo	467.75 b	541.00 a	544.00 ns	550.88 ns
Fm	2654.50 a	2422.81 b	2888.25 a	2173.00 b
Fv	2113.50 a	1955.06 b	2337.38 b	1629.00 a
Fv/m	0.81 ns	0.80 ns	0.809 b	0.75 a
chlorophyll fluorescence in light conditions				
Y	0.73 a	0.70 b	0.76 a	0.67 b
ETR	30.73 a	29.38 b	31.96 a	26.99 b
gas exchange				
A (μmol CO_2 $m^{-2}s^{-1}$)	6.83 ns	6.39 ns	6.97 ns	6.90 ns
E (mmol H_2O $m^{-2}s^{-1}$)	2.50 b	2.88 a	2.68 ns	2.67 ns

Y- quantum field of photochemical reaction in PSII; ETR- rate of electron transport through photosystems; Fo- initial fluorescence; Fm- maximum fluorescence; Fv- variable fluorescence; Fv/m – maximum photochemical efficiency PSII; A- CO_2 assimilation; E- transpiration. Statistical notations following mean values in columns: a, b - statistically significant differences, ns- statistically insignificant differences.

are often very similar between aphid-infested and control plants; differences are significant but small. Aphid feeding negatively impacts photosynthesis and chlorophyll fluorescence parameters, but this effect is greater in susceptible plants [18, 19]. Moreover, research proves that photosynthetic adjustments can significantly contribute to plant tolerance resulting from pest injury [20], which confirms the synthesis of phenolic compounds by the plant exposure to aphid feeding.

The determination of the content of total polyphenols was performed by means of the Folin-Ciocalteu reagent method. All results were analyzed statistically and presented in Table 2.

Table 2. The content of phenolic acids (TPAC), flavonoids (TFC) and total phenolic compounds (TPC) and the ratio of TPAC, TFC to TPC in the extracts from the leaves of *T. europaeus* on which aphids were present (TAph) and were not present (TE), n = 6

The results showed that the content of total polyphenols (TPC) was 34.5% higher in extracts from plants leaves on which the aphids fed in comparison with leaves obtained from plants not infested with aphids (TE). Similar results were obtained when determining the content of phenolic acids (TPAC) by the Arnov's reagent method described in Polish Pharmacopoeia X. The leaves from plants infested with the aphids was characterized by a 28% higher phenolic acid content compared to control. The content of the phenolic acids in the TAph extract was 0.32%, while that of the TE extract was 0.25%. The content of the total flavonoids (TFC) calculated as vitexin was 0.25% in the TAph extract and was thus 25% greater than that of the control (TFC = 0.20%).

Antioxidant activity was defined by the IC_{50} which measures the antioxidant concentration that causes a decrease in the initial concentration of radicals by 50%. The IC_{50} was 0.79% for the TAph extract and 9.2% lower than the IC_{50} for the TE extract (IC_{50}=0.87), whereas the IC_{50} amounted to 0.27 mg/mL for BHA. These studies have confirmed a correlation between the content of the polyphenols in the investigated extracts and their DPPH free radical scavenging activity (Table 3).

Table 3. DPPH radical scavenging activity (%) of methanol extract from the leaves of *Trollius europaeus* on which aphids were present (TAph) and on which they were not present (TE) and of BHA

Antioxidant activity of chemical compounds, extracts and substances obtained from plants is widely used to treat a number of disorders, from neurodegenerative ones to ordinary colds. There is a range of studies confirming the influence of polyphenolic compounds, including derivatives of orientin and vitexin, found in species of the *Trollius* genus, on their antiviral, antibacterial, anti-inflammatory, antioxidant, and radioprotective activity [3, 4] and protective effects against pharyngitis, tonsillitis, bronchitis, cold with fever, acute tympanitis, aphthae, mouth sore, hemorrhage, gum pain, acute lymphangitis, and acute periostitis. Moreover, this work suggests the possibility of obtaining raw material for medicinal purposes from plantations run by a modified method with the use of controlled biotic stress conditions. Fifteen grams

Table 2. The content of phenolic acids (TPAC), flavonoids (TFC) and total phenolic compounds (TPC) and the ratio of TPAC, TFC to TPC in the extracts from the leaves of *T. europaeus* on which aphids were present (TAph) and were not present (TE), n = 6

Content [%]	**Extract**		**TAph/TE [%]**
	TAph	**TE**	
TPAC (%CA)	0.32 ± 0.01	0.25 ± 0.01	28.0
TFC (%V)	0.25 ± 0.03	0.20 ± 0.04	25.0
TPC (%CA)	3.86 ± 0.02	2.87 ± 0.02	34.5
Ratio [%]			
TPAC / TPC	8.3	8.7	
TFC / TPC	6.5	7.0	
(TPAC + TFC) / TPC	14.8	15.7	

CA- caffeic acid, V- vitexin, TAph/TE - Increase in the content of the compounds in TAph compared to TE [%], TAph- the methanol extract from the leaves of *Trollius europaeus* on which aphids were present, TE- the methanol extract from the leaves of *Trollius europaeus* on which aphids were not present.

Table 3. DPPH radical scavenging activity (%) of methanol extract from the leaves of *Trollius europaeus* on which aphids were present (TAph) and on which they were not present (TE) and of BHA

Concentration (mg/ml)	DPPH radical scavenging activity (%)		
	methanol extract		
	TAph	TE	BHA
0.1	7.62 ± 0.30	6.84 ± 0.22	32.16 ± 0.21
0.3	19.41 ± 0.94	17.02 ± 0.31	56.27 ± 0.11
0.5	29.56 ± 0.65	26.76 ± 0.60	71.14 ± 0.53
0.7	47.85 ± 0.81	43.23 ± 0.82	74.27 ± 0.66
1.0	62.02 ± 0.64	56.20 ± 0.78	80.62 ± 0.14
IC50 (mg/ml)	**0.79**	**0.87**	**0.27**

DPPH- 2,2-diphenyl-1-picrylhydrazyl, TAph- the methanol extract from the leaves of *Trollius europaeus* on which aphids were present, TE- the methanol extract from the leaves of *Trollius europaeus* on which aphids were not present, BHA- 2-tert-Butyl-4-hydroxyanisole, IC_{50}- the half maximal inhibitory concentration of extracts, DPPH radical scavenging activity (%) = [A0 – Ai/Ai]x100, (A0 - absorbance of the control, Ai - absorbance of the tested sample).

of dry leaf matter was obtained from the experimental plantation, both from the plants subjected to the aphid feeding and those grown without the pest stress factor. The introduction of controlled biotic stress did not cause significant losses in the crop. Removal of the pest did not require any chemical agents since *Aulacorthum solani* is a greenhouse pest. The aphids were removed by a change of environment from greenhouse to natural conditions.

4 Conclusion

Induced aphid feeding did not significantly affect photosynthetic activity, which implies that it will not limit plant growth, development, or yield. It may, however, cause an increase in the content of medicinally active compounds. Defense mechanisms of plants result in an increased production of active compounds which determine the medicinal activity of plant material. This, in turn, proposes the possibility of reducing the applied doses of herbal material thanks to a greater content of active substances in extracts obtained from the plants used to produce medicinal preparations. Controlled induction of stress while maintaining spatial isolation and diligence in methodology could be an alternative to traditional methods of obtaining medicinal materials.

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