Flavonoids from *Trollius Europaeus* Flowers and Evaluation of Their Biological Activity

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Abstract

**Objectives:** This paper describes the flavonoid composition of the flowers of *Trollius europaeus* and the method of isolation thereof as well as provides an attempt at investigating the antioxidant activity of the isolated flavonoids and the anti-tyrosinase activity of the extracts from the investigated material.

**Methods:** The compositional data were acquired by combining results of Nuclear Magnetic Resonance (NMR), Ultraviolet spectroscopy (UV), Electrospray ionisation-tandem mass spectrometry (ESI-MS/MS) analyses and those of an analysis of the products of acid hydrolysis of the compounds. The antioxidant activity of the extracts was studied using the 2,2’-diphenyl-1-picrylhydrazyl (DPPH) assay and the tyrosinase inhibitory activity - with the use of mushroom tyrosinase.

**Key finding:** Ten flavonoid derivatives of luteolin and apigenin were isolated from the flowers of *Trollius europaeus* and identified. The investigation into the antioxidant activity revealed that orientin 2”-O-α-arabinopyranoside (4) and orientin 2”-O-β-glucopyranoside (5) had a significant antioxidant effect.

**Conclusion:** The studies conducted led to the development of a method of isolating flavonoid, potentially antioxidant, compounds from *T. europaeus*. They allowed determining which of the investigated flavonoids demonstrated significant antioxidant activity and could be used as natural antioxidants.

**Keywords:** *Trollius europaeus*; Flavonoids; Apigenin; Luteolin; Antioxidants

Introduction

The genus *Trollius* (Ranunculaceae) comprises 31 species of perennial herbs which grow in the northern hemisphere areas with temperate climates. In Europe and western Siberia the species *Trollius europaeus* L. is found. The medicinal part is the whole plant. Various parts of the plant have been used for the treatment of scurvy in folk medicine due to its high content of vitamin C [1]. The previous studies into *T. europaeus* leaves, of which I was a co-author, reported the presence of flavonoids and phenolic acids [2,3]. A more extensive investigation into the flavonoids in *T. europaeus* flowers led to isolation and structural identification of derivatives of luteolin (1-6) and apigenin (7-10). Except for compound 6, which was an O-glycoside, all of them displayed a C-glycoside-like structure. These compounds have been identified and described for the first time in the plant kingdom. Their structures were established on the basis of NMR and MS, UV and comparison with reference samples (standards).

The NMR spectroscopic data included 1H, 13C NMR, COSY, HMQC and HMBC experiments. The antioxidant activity of four of the obtained compounds (4,5,9,10), which had not been investigated before, was subjected to examination with the use of the method with the DPPH radical. This method is based on the reaction of the antioxidant with the oxidant which changes colour from purple to yellow as a result of reduction. The color change was monitored spectrophotometrically by measuring absorbance at the wavelength $\lambda=515$nm. The anti-tyrosinase activity of the extracts from the *T. europaeus* flowers was determined for the first time and was measured by spectrometry, as described by Bendaikha [4]. Tyrosinase is responsible for the process of tyrosine hydroxylation to the laevorotatory form of dopamine (L-DOPA) and the oxidative transformation of L-DOPA into L-Dopaquinone, which spontaneously converts into coloured dopachrome. The tyrosinase activity was monitored by dopachrome concentration at 475nm. Dopachrome is not a direct product of the enzymatic reaction; it results from non-enzymatic reactions of cyclisation and oxidisation of the direct product of this reaction-dopaquinone [5]. A number of studies describing the inhibitory activity of flavonoids and extracts containing flavonoids suggest their activity through formation of copper-flavonoid complexes. This activity depends on the quantity and location of hydroxyl groups [6].
Materials and Methods

General experimental procedures

The NMR spectra (1H, 13C NMR) were recorded using a Bruker NMR Avance II 400MHz spectrometer; CD3OD or DMSO with TMS as an internal standard. The ESI-MS mass spectra were measured on a Waters/Micromass (Manchester, UK) ZQ mass spectrometer connected with a UV 996 Waters photodiode detector equipped with an electrospray interface operating in a negative and positive ion mode at an optimized sample cone voltage of 30V. The UV spectra were recorded on a UV/VIS Perkin Elmer Lambda 35 spectrophotometer in MeOH, also after addition of the specific reagents (NaOAc/ H3BO3, AlCl3, AlCl3 /HCl, NaOMe, NaOAc), according to Matby et al. [7]. The analytical paper chromatography (PC) was carried out on What man paper chromatography 1 CHR, using the solvent system CH3COOH-H2O (P1, 15:85) and EtOAc-HCOOH-H2O (P2, 10:2:3) upper layer, EtOAc-MeOH-H2O and finally with MeOH (S3) as eluents to yield 8mg of yellow crystalline needles of compound 1. Fraction 43-56 was subjected to column chromatography with cellulose, eluted with H2O, saturated with EtOAc (S1) to give 36 fractions. Fraction 1-14 was purified using Sephadex LH-20 column chromatography, eluted with MeOH (S6) to yield 39 fractions, which were rechromatographed on a column with Sephadex LH-20, eluted by MeOH (S6); next, it was rechromatographed on a column with Sephadex LH-20, eluted with H2O (S6). As a result, yellow crystalline needles of compound 4 (19mg) were obtained.

Fraction F57-F98 was separated by Sephadex LH-20 column chromatography, eluted with MeOH (S6), and the obtained 11 flavonoid fractions. Fraction 1-7 was subjected to Sephadex LH-20CC, eluted with H2O (S6), to give 5mg of compound 2. Fraction 8-11 was purified on columns with Sephadex LH-20, first eluted with MeOH (S6), next with H2O (S6) and finally with MeOH (S6), to provide 36mg of yellow crystalline needles of compound 6. Fraction F99-F143 was applied on a Sephadex LH-20 column. Fractionation was carried out by elution with MeOH (S6) to yield 36 fractions. Fraction 4-27 was re- chromatographed on Sephadex LH-20(H2O saturated with EtOAc-S1) and next rechromatographed on Sephadex LH-20 with H2O(S6) as the eluent, yielding 11mg of compound 3 as yellow crystalline needles.

Fraction F144-F,01 was separated in the same way as fraction F99-F143. As a result of re-chromatography, 16 mg of compound 5 were obtained as yellow crystalline needles (Figure 1).

Acid hydrolysis

The obtained compounds 1-10 were subjected to hydrolysis with 2% HCl at 100oc for 4h. The hydrolysates were extracted with EtOAc and the organic fraction was analyzed by TLC (cellulose) and PC P1 and P2 whereas the water fractions were evaporated and analyzed by TLC (cellulose and silica gel 60) with P4 and aniline phthalate/105oc for detection of sugars. The products of the hydrolysis were identified by comparison with the authentic sample: orientin (luteolin 8-C-glucoside) and isoorientin (luteolin 6-C-glucoside) for 1-5, vitexin (apigenin 8-C-glucoside) and isovitexin (apigenin 6-C-glucoside) for 7-10, apigenin for 6, and adiddationallyxzone for 3, 6, rhamnose for 6,arabinosefor4, 9, glucosefor 5 and galactose for 10.

Spectroscopic data

Compounds 1, 2, 3, 6, 7, 8 were identified by comparison of their spectral data with literature values and confirmed by co- chromatography experiments with authentic standards [3,8-10]. Spectral data of compounds 3, 4, 5, 9, 10 are in (Table 1).
Figure 1: Isolation of flavonoid compounds from *Trollius europaeus* extract.

Table 1: Spectroscopic data of compounds 3, 4, 5, 9, 10.

| Compounds | Position | 3         | 4         | 5         | 9         | 10        |
|-----------|----------|-----------|-----------|-----------|-----------|-----------|
|           |          | 13CNMR(100MHz) |          |           |           |           |
| 2         | 163.80   | 166.53    | 166.48    | 163.46    | 166.48    |           |
| 3         | 102.50   | 103.65    | 103.70    | 103.89    | 103.74    |           |
| 4         | 181.90   | 184.11    | 184.10    | 181.65    | 184.32    |           |
| 5         | 160.50   | 162.69    | 162.59    | 156.48    | 158.37    |           |
| 6         | 98.13    | 99.47     | 99.54     | 98.68     | 99.46     |           |
| 7         | 162.50   | 164.63    | 164.96    | 161.19    | 162.85    |           |
| 8         | 103.80   | 105.57    | 105.01    | 104.62    | 105.59    |           |
| 9         | 156.20   | 158.50    | 158.35    | 160.53    | 158.37    |           |
| 10        | 103.60   | 104.94    | 105.01    | 104.62    | 105.04    |           |
| 1’        | 122.00   | 124.01    | 123.99    | 121.58    | 123.57    |           |
| 2’        | 114.00   | 114.89    | 114.92    | 128.76    | 128.86    |           |
| 3’        | 145.80   | 147.17    | 147.22    | 115.88    | 117.06    |           |
| 4’        | 149.50   | 150.98    | 151.08    | 160.53    | 162.85    |           |
| 5’        | 115.60   | 116.78    | 116.78    | 115.88    | 117.06    |           |
| 6’        | 119.20   | 120.83    | 120.86    | 128.76    | 130.12    |           |
| 1”        | 71.79    | 73.90     | 73.43     | 71.91     | 75.54     |           |
| 2”        | 81.88    | 81.85     | 82.22     | 78.66     | 82.31     |           |
| 3" | 78.79 | 80.48 | 80.49 | 80.46 | 80.44 |
| 4" | 70.09 | 72.73 | 71.85 | 70.81 | 71.85 |
| 5" | 81.97 | 82.90 | 82.89 | 81.72 | 82.86 |
| 6"a | 60.80 | 63.01 | 63.01 | 61.01 | 62.83 |
| 6"b | 62.65 | 62.65 | 62.65 | 62.65 | 62.65 |
| 2"'-O-Xyl | 2"'-O-Ara | 2"'-O-Glc | 2"'-O-Ara | 2"'-O-Gal |
| 1" | 106.10 | 107.25 | 106.86 | 102.32 | 107.38 |
| 2" | 73.10 | 73.56 | 74.84 | 71.49 | 73.50 |
| 3" | 71.40 | 71.95 | 75.51 | 70.21 | 74.86 |
| 4" | 69.70 | 69.89 | 69.00 | 66.19 | 69.00 |
| 5"a | 66.40 | 66.06 | 75.51 | 63.48 | 73.50 |
| 5"b | 67.40 | 67.40 | 67.40 | 67.40 | 67.40 |
| 6"a | 60.41 | 60.41 | 60.41 | 60.41 | 60.41 |
| 6"b | 60.41 | 60.41 | 60.41 | 60.41 | 60.41 |

$^1$H NMR (400 MHz) ($J$ in Hz)

| 3 | 6.62s | 6.53s | 6.54s | 6.71s | 6.61s |
| 6 | 6.21s | 6.21s | 6.19s | 6.17s | 6.20s |
| 2' | 7.47br | 7.36br | 7.36br | 8.01d8.57 | 7.98d8.78 |
| 3' | 6.87d7.20 | 6.91d8.22 | 6.91d8.30 | 6.88d8.74 | 6.94d8.77 |
| 5' | 6.70bd7.20 | 6.71bd1.90/8.40 | 7.55dd | 6.94d8.77 |
| 6' | 7.50bd7.20 | 7.51dd1.90/8.40 | 7.55dd | 6.94d8.77 |

UV, λnm

| MeOH | 349,268,256 | 345,270,259 | 348,266,256 | 330,270 | 330,272 |
| NaOAc | 387,327,271 | 384,321,280 | 389,328,273 | 351,278; | 386,280 |
| NaOAc/H$_3$BO$_3$ | 369,265 | 372,267 | 374,265 | 336,270 | 334,271 |
| NaOMe | 409,329,273 | 403,336,278,270 | 406,328,273 | 396,329,281 | 395,330,281 |
| $\text{AlCl}_3$ | 421,340,275 | 422,335,304,276 | 423,275 | 382,345,302,277 | 382,342,305,278 |
| $\text{AlCl}_3$/HCl | 386,353,275,260 | 384,354,298,278 | 385,349,276, | 382,341,302,277 | 382,338,303,278 |

ESI-MS
Bioassay

Determination of antioxidant activity

The free radical scavenging capacity was determined in vitro, by using the DPPH (1,1-diphenyl-2-picrylhydrazyl radical) assay [11-13]. The compounds 4, 5, 9, 10 were dissolved in methanol at different concentrations (0.5 - 5.0mg/ml) and mixed with 3.9 ml of 2.2-diphenyl-1-picrylhydrazyl (DPPH) (6.0 x 10-5 mol/L in MeOH). After 30 min of incubation in darkness at room temperature, absorbance (A) was measured at 517nm against a blank.

The free radical activity was calculated by percent inhibition using the following formula:

\[ \% I = \left( \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right) \times 100, \]

where Ablank was the absorbance of the control (containing all reagents except the tested extracts) and Asample was the absorbance of the sample. The results were also expressed using the IC50 parameter, which is defined as the concentration of an antioxidant that causes a 50% DPPH loss of the DPPH radical scavenging activity. The IC50 parameter was obtained by linear regression. BHA was used as the positive control (0.5-5.0mg/ml). The results have been presented in Table 2.

The data obtained in this study were expressed as the mean of six replicates, plus or minus the confidence interval. The significant difference was considered at the p value ≤0.05. The statistical analysis was performed using the Microsoft Excel 2007 software.

Preparation of the Extracts

The dried and powdered flowers of *Trollius europaeus* (5g) were extracted twice with 100ml of methanol-water. (1:1) for 30min using an ultrasonic bath (Elma S 180H, Germany). The extraction was carried out at a temperature of 50oC, 1000W ultrasonic power, and frequency of 37kHz. The extracts were condensed to dry matter. The dry residue was weighed and dissolved in water thus obtaining 2.5% stock solution.

Determination of total flavonoid content (TFC)

The total content of flavonoids (TFC) was determined using the boric acid colorimetric method described in Pharmacopeia Polonica [14]. 5ml of the stock solution were evaporated to dryness and the remains were dissolved in 10ml of a mixture of methanol and glacial acetic acid (1:10), 10ml of the solution of boric acid (25g/L) and oxalic acid (20g/L) in anhydrous acetic acid were added, then anhydrous acetic acid was added to obtain 25ml. The reference was prepared in the same way, but the mixture of boric and oxalic acids were replaced with anhydrous formic acid. The absorbance was measured at λ =401nm, after 30min of incubation. The total flavonoid content was calculated with the following formula: X=Ax 0.8/m, calculated as vitexin, adopting absorbance typical of vitexin equal to 628. The A in the formula is the absorbance of the tested samples, m- the mass of the substance to be examined, in grams.

Tyrosinase inhibitory activity assay

Mushroom tyrosinase, L-dopa used for the bioassay was purchased from Sigma-Aldrich, Chemical Co [14] and hydroquinone from POCH S.A. Poland. The tyrosinase inhibitory activity was measured by spectrophotometry, as described by Bendalidha S et al. [4]. The studies were conducted at a constant temperature of 20oC ±1. The study was conducted for a flavonoid standardized methanol-water extract from the flowers. Five to twenty five ml of the stock solution were condensed to dryness, and then dissolved in 1ml of H2O.

The hundred µL of these concentrations were placed in flasks and then 250µL of 192U/ml of mushroom tyrosinase in phosphate buffer solution (pH 6.8) were added. After pre-incubation at 25 oC for 20 minutes, 200mL of L-dopa (2mM in H2O) were added. The assay mixture was incubated at 25 oC for 10 minutes and the absorbance at 475nm was measured with a spectrophotometer. Hydroquinone, a known tyrosinase inhibitor, was used as a positive control agent. The tyrosinase inhibitory activity was calculated according to the following equation: % inhibition = \[ \frac{(A_{\text{blank}} - A_{\text{test}})}{A_{\text{blank}}} \times 100, \]

where A and B was the absorbance of the enzyme without and with the test material. All the tests were conducted in triplicate. IC50 values were calculated by interpolation of the concentration % inhibition curve by the MS Excel program.

Statistical analyses

The data obtained in this study were expressed as the mean of six replicate determinations plus or minus the confidence interval. The Kruskal-Wallis test was used to assess the significance of the effects of the activity, at p≤0.05. Individual differences between the treatments were identified using the Dunn’s test. The correlation of the samples that provided 50% inhibition (IC50) was obtained by interpolation from a linear regression analysis. The calculation was performed using the STATISTICA 10.0 software (Poland).

Results and Discussion

Structural elucidation of flavonoids

The result of this investigation was isolation of flavonoids from the hydromethanolic extract of *T. europaeus* flowers, with the use of column chromatography. The chromatographic separation was monitored on TLC or PC plates under UV 366 nm. The isolated compounds showed brown fluorescence (with Naturstoffreagenz A) changing to orange (1-5) or yellow (6-10). The structures of
the compounds were established on the basis of the results of acid hydrolysis, ultraviolet spectroscopy (UV), mass spectrometry (MS), and nuclear magnetic resonance (\(^1H\) and \(^{13}C\) NMR, \(^1H\)–\(^1H\) COSY, HMBC, HSQC NMR) analyses.

Compounds 1-5 and 7-10 were hydrolysed in a way typical of C-glycosides. Under the conditions of acid hydrolysis, compounds 3-5 and 9-10 first underwent separation of the sugar molecule attached to the O-glycosidic bond (arabinose from 9 and 4, xylose from 3, glucose from 5 and galactose from 10) and, then, interconversion (i.e., Wessely-Moser rearrangement) to a mixture of 6- and 8-isomers (orientin and isoorientin for 1-5, vitexin and isovitexin for 7-10). The Wessely-Moser rearrangement in the same conditions was also observed for compounds 1, 2, 7, 8 which possessed the sugar molecule attached by a C-glycosidic bond [7,15].

For compound 6, the acid hydrolysis was characteristic of di-O-glycosides of flavonoids. The conditions of the hydrolysis first led to the separation of the outer sugar molecule (product-monoglycoside of flavonoid) and, next, of another one until an aglycone were obtained. The results of the chromatographic analysis of the hydrolysis products of compound 6 indicated the presence of two simple sugars corresponding to xylose and rhamnose and an aglycone with standard 7-methylapigenin.

The analysis of the UV spectra see data in Table 1 indicated that compounds 1-5 were flavones with free OH group sat C-3', 4'; 5 and 7, while compounds 7-10 were flavones with free OH groups at C-4', 5 and 7. Compound 6 was a flavone with one free OH group at C-5 [7]. In the ESI-MS spectra of compounds 1-10 protonated and deprotonated molecules of the compounds and their adducts with sodium, potassium and chlorine were observed in the positive and negative ion modes. Based on the results of the ESI-MS analysis, the mass of compounds 1/2=m/z 448, 3=m/z 580, 4 = m/z 580, 5 = m/z 610, 6 = m/z 562, 7/8 = m/z 432, 9 = m/z 564, 10 = m/z 594 was determined. The results indicated that the mass of compounds 1 and 2 corresponded to luteolin substituted with hexose, that of compounds 3 and 4 corresponded to orientin substituted with pentose (C\(_{15}\)H\(_{10}\)O\(_5\)). Compound 7 and 8 indicated to apigenin substituted with hexose, while compound 9 was equivalent to vitexin with bound pentose (C\(_{20}\)H\(_{15}\)O\(_{15}\)) and compound 10- to vitexin substituted with hepxose (C\(_{20}\)H\(_{15}\)O\(_{15}\)). The resonance signals from all the carbons and protons could be assigned by analyzing the \(^1H\)–\(^1H\) COSY and \(^1H\) NMR spectrum compared to the corresponding signal of apigenin, T. ledebouri [9], Deschampsia antarctica[17], and for orientin 2'–O-β-arabinopyranoside isolated from Cannabis sativa, Seteria italic [8].

In the HMBC spectrum of compound 4 correlations observed among H-2' and C-1'' confirmed the connection 1→2 between the sugar molecules. The anomic proton of glucose was correlated with C-3' and C-9 of the aglycone. H-3 showed cross peaks with C-10, C-1', C-4' and H-8 showed HMBC correlation with C-6, C-9 and C-10, as well as H-6 to C-5, C-8 and C-10, confirming the positions of these hydrogens. In the HMBC spectrum of 5, the long-range correlations between H-1' of glucose and C-7, C-9, C-10 indicated that glucose was attached to the C-8 position of the aglycone. Correlations between C-2'and H-1'' suggested 1→2 interglycosidic linkage. The long-range correlations from H-6 to C-5, C-8, C-10, as well as between H-3 and C-8, C-10, C-1', C-4', confirmed the positions of these hydrogens. The hydrogens of ring B correlated in the following manner: between H-2' and C-2, C-4', also between H-5' and C-1', C-3', C-4', and from H-6' to C-2', C-3', C-4'. The \(^1H\) NMR spectrum of compound 9 and 10, in the region of the aglycone, revealed signals typical of a pigenin: singlets from H-6 at 8.62 ppm for compound 9 and at 8.617 ppm for compound 10, from H-3 at 8.661 ppm for compound 9 and at 8.617 ppm for compound 10, and doublets from H-2' and H-6' at 8.798 ppm and 88.1 ppm, respectively, whereas for H-3' and H-5' at 8.694 ppm and 5.88 ppm, respectively. The doublets were characterized by coupling constants about 9.0 Hz.

Additionally, no signal of proton H-8, which should have appeared at 8.39 to 8.56 ppm, and the signal shift at C-8 in the 13C NMR spectrum compared to the corresponding signal of apigenin, may suggest that compound 9 and 10 had a substituent at C-8. In the region of the anomic proton signals doublets at \(\delta 4.15\) ppm for compound 9 and \(\delta 4.82\) ppm for compound 8 with the coupling constants about 10.0, indicated \(\beta\) configuration. In the 13C NMR spectrum, in the range of the signals of the anomeric carbon sugar molecules, compared with luteolin, indicating that sugar linked at C-8 by a C-glycosidic bond. In the region of anomic sugar protons there were doublets of the sugar molecule in the range of 85.03 ppm to 85.14 ppm (d, H-1', J=10.0 Hz) attached directly to the aglycone, and from the outside of the sugar molecule at 84.78 ppm (d, H-1'', J=9.8), 84.17 ppm (d, H-1'', J=6.43 Hz) and 84.18 ppm (d, H-1'', J=7.66 Hz), respectively. The constant coupling signals from the anomeric protons of the sugars showed that they occurred in the \(\beta\) configuration. The correlation occurring between the protons and carbons was determined on the basis of 1H-13C HSQC. The anomic carbon of glucose, which constituted the internal sugar molecule, disclosed in the range of \(\delta 71.79\) to 73.90 ppm, whereas the one of the sugar attached to glucose in the range 610.10 to 107.25 ppm. It was observed that the C-2' resonance signal of glucose was shifted downfield by about 10 ppm, while the signals of the adjacent carbons were shifted up field, which suggested substitution of C-2' sugar molecule with a C-glycoside bond (Table 1). [15,16] The values of the signals and their shifts for the investigated compounds were comparable to the results of the identification analyses described for orientin 2'–O-β-arabinopyranoside isolated from T. ledebouri [9], Deschampsia antarctica[17], and for orientin 2'–O-β-glucopyranoside isolated from Cannabis sativa, Seteria italic [8].

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The data indicated that the presence of hydroxyl groups at the aglycone could enhance the antioxidant activity of the flavonoids, as orientin showed greater scavenging activity than vitexin. The results of previous studies suggested stronger antioxidant activity of orientin 2'-O-xyloside from *Setaria viridis* (Gramineae) than that of vitexin 2'-O-glucoside, while vitexin 2'-O-xyloside was inactive [22]. Orientin 2'-O- galactoside (OGA), separated from *T. chinensis* flowers, had been previously isolated and identified in the extract from the leaves of *T. europaeus*. This spectroscopic data (*'H and 13C NMR and ESI-MS, UV-spectrophotometric analysis*) were in good agreement with those reported in the literature [6, 10].

4'-O-α-rhamnopyranosyl (1→2)-β-xylopyranoside of 7-O-methylapigenin (6), identified in the flowers, had been previously isolated from and identified in the extract from the leaves of *E. officinalis*. This spectroscopic data (*'H and 13C NMR and ESI-MS, UV-spectrophotometric analysis*) were in good agreement with those reported in my first study [3].

**Table 2:** DPPH radical scavenging activity (%) of compounds 4, 5, 9, 10 and BHA.

| Concentration (mg/ml) | 4       | 5       | 9       | 10      | BHA     |
|-----------------------|---------|---------|---------|---------|---------|
| 0.5                   | 39.40±0.76 | 39.40±0.76 | 4.39±0.76 | 2.23±0.1 | 34.37±0.13 |
| 1                     | 50.72±1.33 | 30.69±0.69 | 4.46±0.28 | 2.28±0.17 | 58.39±0.40 |
| 2                     | 74.38±1.10 | 32.80±0.46 | 4.61±0.34 | 2.46±0.17 | 74.06±0.46 |
| 4                     | 84.20±0.29 | 63.78±0.79 | 4.86±0.80 | 2.48±0.41 | 77.73±0.28 |
| 5                     | 87.72±0.35 | 71.71±0.81 | 5.01±1.43 | 2.54±0.65 | 82.34±0.38 |
| IC50 (mg/ml)           | 1.67     | 3.41    | 142.9   | 57.65   | 1.67    |

The data indicated that the presence of hydroxyl groups at the aglycone could enhance the antioxidant activity of the flavonoids, as orientin showed greater scavenging activity than vitexin. The results of previous studies suggested stronger antioxidant activity of orientin 2'-O-xyloside from *Setaria viridis* (Gramineae) than that of vitexin 2'-O-glucoside, while vitexin 2'-O-xyloside was inactive [22]. Orientin 2'-O- galactoside (OGA), separated from *T. chinensis* flowers, showed greatest scavenging activity against...
DPPH (IC50 = 23.9 μg/ml) and was followed by orientin and vitexin which demonstrated moderate scavenging activity against DPPH (112.4 μg/ml and 217.9 μg/ml, respectively). It was observed that the tested compounds showed weak or no scavenging activity against ABTS. By comparison with orientin, OGA showed particularly strong scavenging activity in this case, which might be ascribed to its improved polarity and solubility by an additional galactoside moiety [23, 24].

Table 3: Inhibitory effects on mushroom tyrosinase of methanol-water extracts from flowers of *T. europaeus*(TK) and hydroquinone (HQ).

| TK concentration [mg/mL] | TK inhibition [%] | HQ inhibition [%] | IC50 [mg/mL] |
|--------------------------|-------------------|-------------------|-------------|
| 25.00                    | 36.92±0.26        | 18.46±0.14        | 478.6       |
| 50.00                    | 48.7±0.62         | 34.70±0.58        | 98.57±1.56  |
| 75.00                    | 62.05±0.88        | 40.19±1.18        | 50.00       |
| 100.00                   | 96.67±1.32        | 52.34±0.40        | 23.84       |
| 125.00                   | 98.57±1.56        | 60.12±0.54        | 20.00       |

**Inhibitory effect of *Trollius* flowers extract (TK) on mushroom tyrosinase**

Tyrosinase is a glycosylated multi-copper mono oxygenase enzyme. It is responsible for the pigmentation of eyes, hair, and skin. It contributes to undesired browning of fruits and vegetables. The changes in pigmentation in mammal organisms (hypos-/hyperpigmentation), as well as fruit and vegetable browning, may be connected with disorders of tyrosinase activity. This has prompted scientists to search for new, safe inhibitors of the enzyme for use in foods and cosmetics (Table 3).

The antityrosinase activity was determined for a methanol-water extract from the flowers of *T. europaeus* (TK) standardised for the content of flavonoids calculated as vitexin. The total content of flavonoids in the investigated extract was 0.4±0.03%. The determination of tyrosinase inhibition was performed with the use of the mushroom tyrosinase assay for different concentrations of the TK extract corresponding to 125.00 to 250 mg/ml of the raw material. The IC50 of the TK extract was 478.6 mg/ml, whereas for hydroquinone HQ used as the positive control it was 23.84 mg/ml. The extract from the flowers of *T. europaeus* containing 0.4% of flavonoids also seems a potentially good factor of antityrosinase. This ability may be related to the structural similarity of polyphenols to L-DOPA and tyrosine, which are natural substrates for tyrosinase [25].

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