Flavonoids as tyrosinase inhibitors in *in silico* and *in vitro* models: basic framework of SAR using a statistical modelling approach

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**ABSTRACT**

Flavonoids are widely distributed in plants and constitute the most common polyphenolic phytoconstituents in the human diet. In this study, the *in vitro* inhibitory activity of 44 different flavonoids (1–44) against mushroom tyrosinase was studied, and an *in silico* study and type of inhibition for the most active compounds were evaluated too. Tyrosinase inhibitors block melanogenesis and take part in melanin production or distribution leading to pigmentation diseases. The *in vitro* study showed that quercetin was a competitive inhibitor (IC₅₀ = 44.38 ± 0.13 μM) and achieved higher antityrosinase activity than the control inhibitor kojic acid. The *in silico* results highlight the importance of the flavonoid core with a hydroxyl at C7 as a strong contributor of interference with tyrosinase activity. According to the developed statistical model, the activity of molecules depends on hydroxylation at C3 and methylation at C8, C7, and C3 in the benzo-γ-pyran ring of the flavonoids.

1. Introduction

Flavonoids are secondary plant metabolites that can be chemically divided into groups based on their substitutions. Flavonoid moieties can be modified by glycosylation, hydrogenation, hydroxylation, acetylation and methylation, as well as by malonylation and sulfatation. The chemical and biological potentials of flavonoids and their derivatives are connected with the position of diverse substitutions on the molecule and the saturation of double bonds in the structure. Thus, structure-activity relationship (SAR) studies can help predict the biological activities of compounds with related structures and help avoid off-target outcomes due to their toxicity and side effects. The substantial contribution of a treatment is based on selecting the right medicine for a given target. Available theoretical tools (e.g., molecular docking studies) can help predict potential inhibitory activity against enzymes, including tyrosinase, and virtual screening can be used to select compounds that target tyrosinase and to determine expected targets for well-known and newly discovered flavonoids. Tyrosinase is common in mammals, fungi, bacteria, and plants, and it plays a critical role in melanin biosynthesis. Tyrosinase consists of two identical H subunits as a catalytic component and two identical L subunits. The H subunit includes four helices that contain the catalytic binuclear copper-binding site. Each Cu²⁺ cofactor forms coordination bonds with three histidine residues (His61, His85, His94, and His259, His263, His296, respectively) located at the centre of two antiparallel α helix pairs. His85 is covalently bound to Cys83 through a thioether bond, and these two residues are connected to each other via a threonine residue that forms a triad motif that is conserved among tyrosinases and is considered essential for catalytic activity. The histidine ligands of the copper ions are stabilised via interactions with nearby residues such as Phe90 and Phe292 for catalytic activity; thus, interactions with the copper ions as well as their ligands and the nearby residues are required for effective inhibition. In humans, abnormal melanin production or distribution leads to pigmentation diseases, such as overtanning, freckles, age spots, and melanoma. Disorderly melanin production plays a key role in melanic melanoma, and inhibiting tyrosinase activity may reduce melanin content and be a useful process in skin-whitening compounds.

In this study, we investigated the tyrosinase inhibitory activity of 44 flavonoids, (7-methoxynaringenin (1), 7,4′-dimethoxynaringenin (2), butin (3), isokarin (4), hesperetin (5), hesperetin 7-O-rutinoside (hesperidin) (6), 5′-hydroxyflavone (7), 5′-hydroxy-2′-methoxyflavone (8), 5-hydroxy-2′,6′-dimethoxyflavone (9), zapotin (10), chrysin (11), apigenin (12), apigenin 7-O-glucoside (cosmosin) (13), apigenin 8-C-glucoside (vitekxin) (14), apigenin 8-C-glucosyl-2′-O-glucoside (15), acacetin (16), luteolin (17), luteolin 7-O-glucoside (cyanoside) (18), luteolin 7-O-sambubioside (19), luteolin 6-C-glucoside (isorientin) (20), chrysoseli (21), 5,7,3′-trihydroxy-4′-methoxyflavone-8-C-xylopyranoside-2′-O-glucoside (scleranthoside A) (22), 5,7,3′-trihydroxy-4′-acetoxyflavone-8-C-xylopyranoside-2′-O-glucoside (scleranthoside B) (23), 5,7-dihydroxy-3′-methoxy-4′-acetoxyflavone-8-C-xylopyranoside-2′-O-glucoside (24), 5,7-dihydroxy-3′-methoxy-4′-acetoxyflavone-8-C-xylopyranoside-2′-O-(4′-acetoxy)-glucoside (scleranthoside D) (25),...
kaempferol (26), 8-methoxykaempferol (27), kumatakenin (28), kaempferol 3-O-glucoside (astragalin) (29), kaempferol 3-O-glucoronide (30), kaempferol 3-O-galactoside (hyperoside) (31), kaempferol 3-O-(6’-O-trans-p-coumaryl)-glucoside (tilloside) (32), ermanin (33), icariin (34), quercetin (35), quercetin 3-O-gluconide (miquelanin) (36), quercetin 3-O-rutinoside (rutin) (37), quercetin 3-O-rutinoside-7-O-glucoside (38), 7-O-methoxyquercetin (rhamnetin) (39), 7-O-methoxyquercetin (isorhamnetin) (40), robinetin (41), myricetin (42), daidzein (43), and genistein (44), to find potentially active compounds. First, tyrosinase inhibition and enzyme kinetics were tested in an in vitro model to determine the types of inhibition for the compounds with the highest activity. Second, in an in silico model, docking studies were performed to describe SARs, which were also assessed using a statistical model. Thus, a combination of bioinformatics simulation and biological in vitro studies describe the functional mechanisms of the tested compounds.

2. Materials and methods

2.1. Chemical and reagents

Kojic acid (CAS number: 501-30-4), 3,4-dihydroxy-L-phenylalanine (L-DOPA) (CAS number: 59-92-7; purity ≥98%), and tyrosinase from mushrooms (CAS number: 9002-10-2) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Compounds 5 (CAS number: 69097-99-0), 11 (CAS number: 480-40-0), 12 (CAS number: 520-36-5), 14 (CAS number: 3681-93-4), 20 (CAS number: 4261-42-1), 21 (CAS number: 491-71-4), 26 (CAS number: 520-18-3), 35 (CAS number: 6151-25-3), 39 (CAS number: 90-19-7), 40 (CAS number: 480-19-3), 42 (CAS number: 529-44-2) and 44 (CAS number: 446-72-0) were obtained from Carl Roth (Karlsruhe, Germany), 16 (CAS number: 480-44-4), 34 (CAS number: 489-32-7), and 43 (CAS number: 552-69-0) were obtained from Cayman Chemical (Ann Arbour, MI, USA). Flavonoids 1, 2, 28, 33, 15, 13, 18, 19, 7, 9, 22, 23, 24, 25, 27, 29, and 37 were isolated and identified in the Department of Pharmacognosy at the Medical University of Białystok. Compounds 3 (CAS number: 492-14-8), 4 (CAS number: 1036-49-3), and 10 (CAS number: 14813-19-5) were purchased from Biosynth Carbosynth (London, UK), 6 (CAS number: 520-26-3) and 41 (CAS number: 490-31-3) were purchased from Fluka Analysis (St. Louis, MO, USA), and 30 (CAS number: 22688-78-4) and 36 (CAS number: 22688-79-5) were obtained from Extrasynthese (Genay, France). All isolated compounds were >95% pure as measured by HPLC. Bioassay was performed on BioTek Instruments microplate spectrophotometer EPOCH 2 (Oxfordshire, UK).

2.2. Tyrosinase inhibition assay

The inhibitory effect on mushroom tyrosinase (TYase) was evaluated using a method reported in the literature with some modifications21. This assay was performed in PBS (100 mM, pH = 6.8 in 25°C). Test samples 1–44 (80 μL) were preincubated with TYase solution (40 μL; 250 U/mL) at 25°C for 10 min. Then, L-DOPA (80 μL; 0.19 mg/mL) was added, and after an additional 10 min at 25°C, the absorbance was measured at 492 nm. A blank analysis was performed using PBS instead of a sample, and the positive control was conducted with kojic acid. The inhibitory effect was calculated as follows:

\[ TYaseInh (\%) = \left( \frac{B - C}{C} \right) \times 100 \]

where B and C are the absorbances of the blank and the compounds, respectively. The compound concentration that inhibits 50% of tyrosinase activity (IC50) was calculated. All tests were performed in triplicate.

2.3. Tyrosinase inhibition kinetic analysis

Based on IC50 values, the seven most active compounds (4, 17, 26, 32, 35, 41) were selected for kinetic analysis. The enzyme reaction kinetics of these compounds were measured by constructing Lineweaver-Burk plots of inverse velocities (1/V) to the inverse of substrate concentration (1/S)22,23. Preincubations and measurement times were performed using the same protocol as described above. The enzyme concentration (250 U/mL) was kept constant in the presence of substrate solutions (L-DOPA) between 0.25 and 2 mM in all kinetic studies. The inhibitor concentrations for all test compounds were 0, 25, and 50 μM. The inhibitory types and inhibitory constant (Ki) values were described by 1/V versus inhibitor concentration plots (Dixon plot).

2.4. Molecular docking

The selected flavonoids were sketched in ACD/ChemSketch to build and generate their mol topology format. The compounds were modelled using LigPrep (2021–22, Schrödinger LLC, New York, NY) and MacroModel (2021–22, Schrödinger LLC, New York, NY) according to the OPLS4 forcefield parameters24 and conjugate gradients method. The original configurations of the ligands were conserved, and their ionisation states at pH 7 were modelled. The mushroom tyrosinase structure (PDB ID: 2Y9X5; resolution: 2.78 Å) was downloaded from the RCSB protein data bank (www.rcsb.org)25 and prepared for docking using the Protein Preparation Wizard of Maestro (2021–22, Schrödinger LLC, New York, NY)26. In this process, redundant molecules were removed, H atoms were added, partial charges were assigned, and ionisation, tautomeric states, and H bonds were set. The active site grids were generated using the Receptor Grid Generator panel of Maestro by setting the central coordinates as −0.62, 26.99, and −43.78, and the volume as 27.000 Å³. The ligand was docked to the active site using Glide (2021-2, Schrödinger LLC, New York, NY) in additional precision (XP) mode with 50 runs per ligand, and the results were visually evaluated27. Prior to docking of the ligands, the co-crystallized ligand in the PDB structure, tropolone, was removed and redocked to the active site, and the obtained binding mode was compared with the co-crystallized conformer by calculating the root-mean-square deviation (RMSD) to confirm the precision of the method. The predicted binding mode for tropolone was similar to that of the original conformer (RMSD: 1.33 Å).

2.5. Statistical analysis

Statistical analysis for IC50 calculations was performed using non-linear regression using GraphPad Prism 9 (Trail, GraphPad Software, La Jolla, CA, USA). Data were collected as mean ± SD (n = 3), and the significance of differences was analysed using one-way analysis of variance (ANOVA). Statistical analysis of SARs was performed using Stata/IC 13.1 (StataCorp LP, TX 77845, USA).

3. Results and discussion

3.1. Inhibitory effects of tested compounds on tyrosinase

The effects of different concentrations of selected flavonoids (Figure 1) and kojic acid, a well-known inhibitor of L-DOPA
oxidation by tyrosinase, were studied. The effects, expressed as IC₅₀ values, are shown in Table 1.

Although the rendered activity is accumulative of an entire molecule, a structure-activity relationship was recognised by examining the effect of different substitutions: the presence or absence of hydroxyl groups or their methylation/acetylation/glycosylation in all carbons of the B-ring, as well as in carbons 3, 5, 6, 7, and 8 of the benzo-γ-pyran ring, on the potential inhibitory activity.

Some flavonoids (4, 17, 35) exhibited IC₅₀ values that were comparable to the positive control, kojic acid (IC₅₀ = 49.48 ± 0.23 μM). Compound 4 (IC₅₀ = 58.43 ± 0.38 μM), tested in this study for the first time, was one of the most effective inhibitors of tyrosinase, along with compound 17 (IC₅₀ = 58.88 ± 0.78 μM) and compound 35 (IC₅₀ = 44.38 ± 0.13 μM). The distinction in position C3 in 35 and 36 (IC₅₀ = 220.10 ± 1.14 μM) or 37 (IC₅₀ = 141.67 ± 1.30 μM) is only a difference in the glycosylation of a hydroxyl group. However, this small difference in their structure causes immense differences in their inhibitory potentials. It is presumed that substitutions at C3 led to steric hindrances that prevent molecules from binding to enzymes. However, the most typical locations for C-glycosyl radicals are the C6 and C8 positions at the A-ring. It seems that aglycone 4 (IC₅₀ = 58.43 ± 0.38 μM) is a more effective tyrosinase inhibitor than its glycoside: 20 (IC₅₀ = 132.55 ± 2.32 μM). The exact location of the glycosidic residue (carbon 6 or 8) does not affect activity. Also, disaccharides linked with the flavonoid core in C8 (22, 23, 24, 25) exhibit weak inhibitory potential with IC₅₀ values that are higher than those of the maximum tested concentration. When the antityrosinase activity of methylated 26, 27, and 28 molecules with IC₅₀ values 65.11 ± 1.09 μM, 290.46 ± 1.19 μM, and >500 μM, respectively, was compared, the presence of methyl groups likely prevents appropriate interactions with the enzyme active site. The same SAR pattern was observed with compounds 4 (without methylation), 1 (one methyl group at C7), and 2 (two methyl groups at C7, C4'), with IC₅₀ values 58.43 ± 0.38 μM, 441.92 ± 1.91 μM, and >500 μM, respectively. The links of the B-ring in the C-ring allow flavone and isoflavone activity to be compared. Isoflavones (3-B-ring) with IC₅₀ values >500 μM (43 and 44) exhibit no inhibitory potential. Additionally, the confrontation of the activity of 35 (IC₅₀ = 44.38 ± 0.13 μM) with 42 (IC₅₀ = 100.33 ± 1.86 μM) leads to the conclusion that the additional hydroxyl group in C5' reduces phenol inhibitory potency. It was not possible to determine the IC₅₀ values of flavonoids 2, 21, 22, 23, 25, 28, 40, 43, and 44 up to 500 μM, which was the highest tested concentration. Thus, based on the in vitro study, molecules containing more unsubstituted hydroxyl groups in C3, C5, and C7, as well as in C3' and C4', generally achieve highly potent activity against tyrosinase, this finding is consistent with previous

Figure 1. Molecular structures of the tested compounds.
8. Similar conclusions of SAR were presented using inhibitors of xanthine oxidase and elastase and scavengers of superoxide radicals. 29,30

### 3.2. Kinetic analysis of tyrosinase inhibition

Kinetic analysis of compound-induced inhibition was performed to determine the type of inhibition of the most active constituents (i.e., IC50 values below 100 μM). Lineweaver-Burk plots (Figure 2) in double-reciprocal form and Dixon plots (Figure 3) were used to determine the inhibition types and evaluate the dissociation constants for the inhibitors (K) (Table 2). The inhibitory type of isookanin (4) and robinetin (41) was tested for the first time in this study.

As shown in Figure 2(A,B,F), the straight lines intersected at the same point on the x-axis, implying that the Km value remained constant, while the maximum reaction rate (Vmax) decreased with increasing concentrations of tested inhibitors (0, 25, and 50 μM).

Thus, isookanin (4), luteolin (17) and robinetin (41) likely caused non-competitive tyrosinase inhibition. Compounds 4, 17 and 41 produce allosteric regulation, which is a specific type of enzyme inhibition that is characterised by an inhibitor binding to an allosteric site, resulting in decreased enzyme efficacy. The luteolin type of inhibition was consistent with previous studies. The inhibition constant K was obtained from Dixon plots (Figure 3(A,B,F)) as 18.64 ± 0.53 μM, 11.32 ± 0.77 μM and 27.42 ± 0.62 μM for compounds 4, 17, and 41, respectively.

In contrast, kaempferol (26), tiliroside (32) and quercetin (35) exhibit tyrosinase inhibition that are similar11,32,33. As shown in Figure 2(C,D,E), all straight lines crossed at the same point on the y-axis, which indicated that Vmax remained constant. As the x-axis intercept increased with increasing inhibitor concentrations, Km increased because a higher concentration of substrate is required to overcome the inhibitory effects of a competitor. Thus, compounds 26, 32, 35 can bind to the active site and prevent binding to the real substrate. Based on Dixon plots (Figure 3(C–E)), K for

### Table 1. Compounds 1–44 measured for anti-tyrosinase activity and their respective IC50 values.

| Compounds | B-ring | Benzo-γ-pyran ring |
|-----------|--------|------------------|
| 1         | H H OH | H H H H H H OMe |
| 2         | H H OMe| H H H H H OMe |
| 3         | H OH   | H H H H OH H OH |
| 4         | H OH   | H H H H OH OH H |
| 5         | H OH   | H H H H O Me H OMe |
| 6         | H OH   | H H H H OH O Me |
| 7         | H OH   | H H H H OH H H |
| 8         | + O Me | H H H H OH H H |
| 9         | + O Me | H H H H O Me H |
| 10        | + O Me| H H H H O Me H |
| 11        | + H H H| H H H H OH H OH |
| 12        | + H H H| H H H H OH OH H |
| 13        | + H H H| H H H H OH O Me |
| 14        | + H H O| H H H H OH OH H |
| 15        | + H H O| H H H H OH Glc |
| 16        | + H H O| H H H H OH (6′-p-Co)-Glc |
| 17        | - O Me| H H H H OH OH |
| 18        | - O Me| H H H H OH O Me |
| 19        | - O Me| H H H H OH O Me |
| 20        | - O Me| H H H H OH Glc |
| 21        | - O Me| H H H H OH Glc |
| 22        | - O Me| H H H H OH O Me |
| 23        | - O Me| H H H H OH O Me |
| 24        | - O Me| H H H H OH O Me |
| 25        | - O Me| H H H H OH O Me |
| 26        | - O Me| H H H H OH O Me |
| 27        | - O Me| H H H H OH O Me |
| 28        | - O Me| H H H H OH O Me |
| 29        | + O Me| H H H H OH O Me |
| 30        | + O Me| H H H H OH O Me |
| 31        | + O Me| H H H H OH O Me |
| 32        | + O Me| H H H H OH O Me |
| 33        | + O Me| H H H H OH O Me |
| 34        | + O Me| H H H H OH O Me |
| 35        | + O Me| H H H H OH O Me |
| 36        | + O Me| H H H H OH O Me |
| 37        | + O Me| H H H H OH O Me |
| 38        | + O Me| H H H H OH O Me |
| 39        | + O Me| H H H H OH O Me |
| 40        | + O Me| H H H H OH O Me |
| 41        | + O Me| H H H H OH O Me |
| 42        | + O Me| H H H H OH O Me |
| 43        | + O Me| H H H H OH O Me |

Abbreviations: π C2-C3 +/− present or absence of double bond; Me: methyl; Bu: butenyl; Ac: acetyl; glc: glucose; gluc: glucuronide; Co: para-coumaroyl; man: mannoside; rut: rutinoside; xyl: xylose.
compounds 26, 32, 35 were calculated to be $40.20 \pm 0.46 \, \text{mM}$, $9.83 \pm 0.57 \, \text{mM}$, and $8.21 \pm 0.86 \, \text{mM}$, respectively. The inhibition constant for quercetin (35) was the lowest, suggesting that the inhibitory effect of this compound is leading among all tested flavonoid structures. This result is consistent with the tyrosinase inhibitory activity test $(IC_{50} = 44.38 \pm 0.13 \, \text{mM})$.

3.3. Molecular docking studies

Active compounds were generally predicted to show high affinity to the active site, as represented by their docking scores (Table 3). Compounds 26 and 41 were particularly noteworthy based on their docking scores, which were based on a set of profitable interactions with the enzyme and its cofactors.

Isookanin’s (4) binding mode lacked interactions with Cu$^{2+}$ and its ligands (Figure 4(A)), which resulted in a moderate docking score. This result was most likely due to the lack of aromaticity (i.e., a double bond between C2 and C3) of its flavonoid core, which, in the case of other compounds, was able to reach deeper into the copper zone and make $\pi$-stacks with the key histidine residues. The lack of aromaticity in isookanin’s flavonoid core also cancelled out the compound’s planarity and led to bending, which may have made it sterically difficult to approach the copper ions.

Conversely, compounds 17, 26, 32, 35, and 41 were found to be suitable chelators of Cu$^{2+}$ due to the common flavonoid core and the ionisable OH substituent at the 7th position (Figure 4(B–F)). The ionised hydroxyl was effective even for double Cu$^{2+}$ engagement in the case of 26, 32 and 41, which were the best scoring compounds (see Table 3). Aromaticity of the flavonoid core enabled $\pi$ stacking with the histidine residues serving as
copper ligands, as well as His244. The hydroxyl groups on ring B of 17 and 35 formed H bonds with Glu322. The hydroxyphenyl attached to the glucopyranosyl moiety of 32 made additional π stacking with His85. Ring B of 17, 26, 35, and 41 was observed to face active site entry and disposed to solvent molecules (Figure 5). With three hydroxyls on ring B, 41 was the most advantageous compound in this situation, which contributed to its docking score.

![Figure 3. Dixon plots for inhibition of tyrosinase in the presence of compounds: 4 (A), 17 (B), 26 (C), 32 (D), 35 (E), and 41 (F). The concentrations of the compounds were 0.00, 25, and 50 μM. The substrate L-DOPA concentrations were 0.25, 0.50, 1, and 2 mM.](image-url)

### Table 2. Kinetic analysis of active compounds on tyrosinase.

| Compound | Type of inhibition | $K_i$ (μM) |
|----------|-------------------|------------|
| 4        | non-competitive   | 18.64 ± 0.53 |
| 17       | non-competitive   | 11.32 ± 0.77 |
| 26       | competitive       | 40.20 ± 0.46 |
| 32       | competitive       | 9.83 ± 0.57 |
| 35       | competitive       | 8.21 ± 0.86 |
| 41       | non-competitive   | 27.42 ± 0.62 |

*All data are represented as $K_i$ values with standard deviation from triplicate measurements.

### Table 3. Docking scores of the active flavonoids.

| Compound | Docking score (kcal/mol) |
|----------|--------------------------|
| 4        | −6.6                     |
| 17       | −7.9                     |
| 26       | −9.1                     |
| 32       | −8.5                     |
| 35       | −7.9                     |
| 41       | −10.3                    |
These results highlight the importance of the flavonoid core with a free hydroxyl at C-7 as a key component for copper chelation and for interactions with the key residues of the tyrosinase active site. The exceptional binding mode of 4 in the catalytic site with respect to its activity can be explained by more effective binding to a possible allosteric site.

3.4. Statistical modelling approach

To create a statistical model, 26 attributes (Table 4) that were consistent with all tested flavonoid compounds and their variables (IC50 values) were identified.

Table 5 shows the multivariate linear regression model for the ln IC50. Statistical analysis explained more than 50% of the variability of the IC50 variable (adjusted R² = 50.88%) with p = 0.0002. All 11 independent variables in this approach yield p < 0.2, while five of them (A4, A10, A18, A21 and A22) have a statistically significant influence on the dependent variable (IC50).

Based on these results, the presence of methoxy substitution at C3’ (A4) increases the mean IC50 value by approximately 4.15 times, and increasing this value (by approximately 2.47 times) relates to methoxy substitution at C7 (A18). Methylation at C8 (A22) caused an increase in the IC50 value, as shown by lnIC50 (1.452888), which indicates that, in this case, the mean IC50 increases by approximately 4.28 times. Conversely, the presence of hydroxy substitution at C3 (A10) and hydroxy substitution at C8 (A21) reduced the mean IC50 by approximately 2.83 and 5.11 times, respectively. Overall, statistical modelling suggests that flavonoids with higher antityrosinase activity possess an OH group at C3 and at C8, while methylation of the hydroxyl group at carbon 3 and at carbon 8 notably reduces the inhibitory activity of the molecules.

4. Conclusions

Results from the literature that describe the effect of flavonoids on mushroom tyrosinase activity are dispersed and sometimes contradictory. This dissidence may relate to various experimental conditions (e.g., concentration of enzyme or substrate L-DOPA, wavelength), which led to different ranges of reported inhibitory activity. Furthermore, generally few flavonoids were included in such investigations, which yields restricted structure-activity relationships information. The statistical model developed in this study provides accurate SAR data in a comprehensive format.
Many flavonoids were tested under the same conditions to obtain values that are as close to those in the real world as possible. In summary, a comparison of IC50 values, kinetic reactions, molecular docking scores and statistical data allowed us to identify the characteristics of flavonoid structures that facilitate tyrosinase inhibition: the presence of hydroxyls at C3 and C7, O- and C-glycosylation, methylation and acetylation of OH groups. As in many other cases reported in the literature, the natural products represent a gold mine for interesting biological activities which can be translated to potential biomedical applications.

**Table 4.** Attributes definitions used to for the statistical analysis.

| Number | Definition                                                                 |
|--------|---------------------------------------------------------------------------|
| A1     | presence or absence of double bond between C2 and C3                      |
| A2     | OMe substitution at C2’                                                   |
| A3     | OH substitution at C3’                                                    |
| A4     | OMe substitution at C3’                                                   |
| A5     | OH substitution at C4’                                                    |
| A6     | OMe substitution at C4’                                                   |
| A7     | OAc substitution at C4’                                                   |
| A8     | OH substitution at C5’                                                    |
| A9     | OMe substitution at C6’                                                   |
| A10    | OH substitution at C3’                                                    |
| A11    | OMe substitution at C3                                                    |
| A12    | O-gluc/O-gluc/O-rut/6-deoxy-a-L-man substitution at C3                    |
| A13    | O-(6’”p-Co)-glc substitution at C3                                        |
| A14    | OH substitution at C5                                                    |
| A15    | OMe substitution at C5                                                    |
| A16    | glc substitution at C6                                                    |
| A17    | OH substitution at C7                                                    |
| A18    | OMe substitution at C7                                                    |
| A19    | O-rut/O-gluc substitution at C7                                           |
| A20    | O-xyr-gluc substitution at C7                                             |
| A21    | OH substitution at C8                                                    |
| A22    | OMe substitution at C8                                                    |
| A23    | glc substitution at C8                                                    |
| A24    | 3-Me-2-Bu substitution at C8                                              |
| A25    | glc-O-gluc/xyr-O-gluc substitution at C8                                  |
| A26    | (4”acetoxy-xyr)-O-gluc substitution at C8                                 |

Abbreviations: Me: methyl; B: butenyl; Ac: acetyl; glc: glucose; gluc: glucuronide; p-Co: para-coumaroyl; man: mannose; rut: rutinose; xyl: xylose.

**Table 5.** The multivariate linear regression model for the ln IC50

| Attribute | Coefficient | $e^{\text{Coefficient}}$ | p Value | [95% Conf. Interval] |
|-----------|-------------|--------------------------|---------|---------------------|
| A4        | 1.423116    | 4.15003                  | <0.001  | 0.7909708 2.055261  |
| A5        | 0.3649103   | 1.44038                  | 0.126   | -0.1077861 0.8376067|
| A6        | 0.4055184   | 1.50008                  | 0.148   | -0.1492667 0.9627536|
| A10       | -1.041826   | 0.35281                  | <0.001  | -1.521979 -0.5616735|
| A13       | -0.8969235  | 0.40782                  | 0.088   | -1.935625 0.1417784|
| A14       | -0.4376985  | 0.64552                  | 0.167   | -1.068768 0.1933707|
| A15       | -1.317022   | 0.26793                  | 0.063   | -2.71015 0.0761047 |
| A18       | 0.9049781   | 2.47188                  | 0.002   | 0.3562236 1.453733  |
| A21       | -1.630273   | 0.19588                  | 0.008   | -2.805552 -0.4549943|
| A22       | 1.452888    | 4.27544                  | 0.012   | 0.3464813 2.559294  |
| A25       | 0.59333549  | 1.73908                  | 0.056   | -0.0138585 1.120568 |

Adjusted $R^2 = 0.5088$.

Many flavonoids were tested under the same conditions to obtain values that are as close to those in the real world as possible. In summary, a comparison of IC50 values, kinetic reactions, molecular docking scores and statistical data allowed us to identify the characteristics of flavonoid structures that facilitate tyrosinase inhibition: the presence of hydroxyls at C3 and C7, O- and C-glycosylation, methylation and acetylation of OH groups. As in many other cases reported in the literature, the natural products represent a gold mine for interesting biological activities which can be translated to potential biomedical applications.

**Acknowledgement**

The authors express thanks to J.W. Strawa and A.M. Juszczak from the Medical University of Białystok for isolation of compounds to in vitro studies.
Author contributions

Study design: KJ, and M.T.; Biological evaluation: K.J.; Data Interpretation: K.J.; Computational studies: S.S., and D.S.; Statistical analysis: R.M., and K.J.; Supervised experiments: M.T., and D.S.; Wrote the original paper: K.J., S.S., and R.M.; Manuscript formal analysis: M.T., and C.T.S.

Disclosure statement

No potential conflict of interest was reported by the authors. CT Supuran is Editor-in-Chief of Journal of Enzyme Inhibition and Medicinal Chemistry and he was not involved in the assessment, peer review or decision making process of this paper. The authors have no relevant affiliations of financial involvement with any organisation or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Funding

The author(s) reported there is no funding associated with the work featured in this article.

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