SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

# *In vitro* antioxidant activity, cytotoxicity and oxidative transformation of flavonoid derivatives

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# *In vitro* antioxidant activity, cytotoxicity and oxidative transformation of flavonoid derivatives

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The Examination takes place at the Department of Pharmacology, Faculty of Pharmacy, University of Debrecen; 11:00 A.M. May 6, 2019.

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The PhD Defense takes place at the Lecture Hall of Building A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen; 1:00 P.M. May 6, 2019.

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## 1. Introduction and Objectives

Cardiovascular disease (CVD), the group of disorders of the heart and circulatory system is the leading cause of death by being responsible for the 45% of all deaths, killing 3.95 million people a year just in Europe. The two most common types of the CVDs are ischaemic heart disease (IHD) and stroke. Hungary has the 4th highest mortality caused by IHD in the EU; in 2014, 32000 people died by IHD and 31000 more by other CVDs including stroke. Oxidative stress is one of the major factors contributing in the development of several cardiovascular diseases including the cardiac ischemic reperfusion injury. During the oxidative stress, there is imbalance in the equilibrium between the pro-oxidants and antioxidants in favour of the former. The excessive formation of free-radicals can lead to the damage of bio-macromolecules. Antioxidant compounds, when they are present at lower concentration than the oxidizable substrate, prevent or delay its oxidation, and thus counterbalance the oxidative stress and its damaging effect on biomolecules. There is a growing need for exogenous antioxidants reset the balance in the redox state to avoid further functional damage of biological macromolecules. The drug research and development makes the pharmaceutical industry one of the most costly industrial sectors of the modern world. One of the main reasons is the compound failure; new chemical entity which will never attain regulatory success due to failure during the development. To be a successful drug candidate during the research and development, the stability and toxicity must also be evaluated in the early stages of the process to prevent cost-increasing compound failures. Metabolism is one of the most important factors affecting the toxicity of a compound; to avoid compound failures the metabolic fate of the compound in the body must be evaluated. These investigations related to the drug metabolites can be achieved with different *in vivo* and *in vitro* metabolism modelling techniques or biomimetic systems.

The main objective of Study I was to evaluate the *in vitro* antioxidant activity, the cytotoxicity and the oxidative transformation of nine flavonoid derivatives, which were chosen from the molecule bank of the University of Debrecen. The antioxidant activity was measured using different assays (ABTS, FRAP and ORAC), the cytotoxicity and the effects of against $H_2O_2$-induced cell death on H9c2 cardiomyoblasts were determined by MTT assay. The oxidative transformation of the selected flavonoids based on their biological activity was investigated with biomimetic model systems, such as chemical Fenton reaction, synthetic metalloporphyrin system and on-line EC/LC/MS.

The aim of Study II was to design, synthetize and evaluate the biological activity of new flavonol derivatives.

Furthermore, the additional objective was to investigate the possible involvement of the phenyl-*N*,*N*-dimethlyamino group in the biological activity of compounds with 2-phenyl-1,4-benzopyrone skeleton. The *in vitro* antioxidant activity was measured by different assays (DPPH, ABTS, FRAP and ORAC), the cytotoxicity on H9c2 cardiomyocyte cell line was determined using MTT assay. The oxidative transformation of the selected flavonols based on their biological activity was investigated with chemical Fenton reaction as biomimetic model system.

## 2. Materials and Methods

### 2.1. Materials and Methods for Study I

*2.1.1. ABTS Assay*

The ABTS radical cation (100 μM) was generated via the ABTS/$H_2O_2$/metmyoglobin system using dissolved ABTS-salt in citric acid-sodium citrate buffer (pH 6.0, 50 mM) with 2% ethanol, and then it was mixed with the compounds of interest (25 μM). The absorbance of the reaction mixture was recorded at 730 nm for 2 hours at room temperature with a Helios-α-spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at 0, 5, 15, 30, 60, 90 and 120 min. Each experiment was performed three times.

*2.1.2. ORAC Assay*

AAPH (37.5 mM) and fluorescein (0.6 μM) stock solutions in phosphate buffer (pH 7.0, 75 mM) were prepared freshly. The investigated compounds was tested in 0.05 mg/mL concentration. Each well contained 65 μL buffer, 20 μL fluorescein, 15 μL sample solution and 100 μL of AAPH solution. The plates were kept at 37 °C. The fluorescence was monitored every 2 min for 1 hour by the FLUOstar OPTIMA (BMG Labtech, Ortenberg, Germany) plate reader at 485 nm excitation and 520 nm emission wavelength. The AUC was calculated using this formula:

$$AUC = 0.5 + (R_2/R_1) + (R_3/R_1) + (R_4/R_1) + \ldots + (R_n/R_1), \quad (1)$$

where $R_1$ is the initial fluorescence at 0 min and $R_n$ is the fluorescence at n min. The net AUC was obtained by subtracting the $AUC_{blank}$ from $AUC_{sample}$. Each measurement was performed in duplicate and repeated four times.

*2.1.3. FRAP Assay*

For the FRAP assay, the following solutions were prepared; acetate buffer (pH 3.6, 300 mM), TPTZ (10 mM) in 40 mM HCl and $FeCl_3$ (20 mM). The FRAP reagent was freshly prepared and consisted of 25 mL of acetate buffer,

2.5 mL of TPTZ and 2.5 mL $FeCl_3$ solution. The reagent was activated by incubation on 37 °C for 15 min. The reaction mixture was prepared by mixing 950 µL FRAP reagent with 50 µL solution of the tested compound (0.05 mg/mL). Following a 15 min incubation period at 37 °C, the absorbance was measured at 593 nm using a Helios-α-spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Trolox standard calibration curve was recorded by measuring the absorbance of samples containing trolox in various concentrations from 0.015 mM to 0.21 mM. Based on this standard curve, the FRAP values were expressed as trolox equivalents (µM/mL). The experiments were run in duplicate and repeated four times.

*2.1.4. MTT Assay*

The cells were dissociated by trituration in DMEM containing 10% FBS, 1% penicillin-streptomycin, and then they were seeded into 96-well plates and cultured for 1 day. The following day, the cells were treated with the compounds of interest (150 µM) in medium. After a 30 min incubation period, 0, 125 or 250 µM $H_2O_2$ was added to the wells. Four hours later, 20 µL MTT solution (5 mg/mL) in PBS was pipetted into the wells and the plates were further incubated for 3 hours at 37 °C in order to allow the mitochondrial uptake. After the removal of the medium, the cells were lysed with 150 µL isopropanol and incubated for 15 min. The absorbance was measured at 570 and 690 nm by a FLUOstar OPTIMA (BMG Labtech, Ortenberg, Germany) plate reader. The experiments were run in four replicate and repeated three times.

*2.1.5. Chemical Fenton System*

400 µL solution of the investigated compound (2.5 mM) was mixed with 50 µL solution of $FeCl_3$ (20 mM), 50 µL EDTA-$Na_2$ (20 mM), 500 µL ascorbic acid (10 mM). The reaction was started by the addition of 1 µL $H_2O_2$ (30%), and the mixture was stirred at room temperature. Samples were drawn at 30 min,

140 min, 18 hours, 71 hours and 261 hours and were immediately injected off-line into an API 2000 Triple Quadrupole mass spectrometer equipped with an ESI source (Applied Biosystems, Waltham, MA, USA) using a syringe pump. The mass spectra were recorded in the range of *m/z* 100–500 in positive-ion mode and were analysed using Analyst 1.5.1. Software (AB SCIEX, Concord, ON, Canada).

*2.1.6. Synthetic Porphyrin System*

50 μL compound of interest (10 mM), 35 μL ACN, 315 μL formic acid (100 mM), 50 μL Fe(III) meso-tetra(4-sulfonatophenyl)porphine chloride (10 mM) and 50 μL $H_2O_2$ (30%) were mixed together and shaken at 37 °C for 30 min at 700 rpm in a ThermoMixer (Eppendorf AG, Hamburg, Germany). Off-line LC-MS analysis (on Kinetex XB-C18 2.6 μm column, using 0.1% formic acid and ACN with 0.1% formic acid as eluents with gradient elution) was performed using a LTQ XL (Thermo Fisher Scientific, Waltham, MA, USA) linear ion trap mass spectrometer equipped with ESI ion source in positive mode.

*2.1.7. Electrochemical System*

A thin-layer electrochemical cell (FlexCell, Antec, Zoeterwoude, The Netherlands) with a boron-doped diamond working electrode, a graphite doped teflon counter electrode and a $Pd/H_2$ reference electrode with a homemade potentiostat was used for oxidation. The potential ramp from 0-2500 mV was applied with 10 mV/s scan rate. The solution of the investigated compound in the mixture of ammonium formate (pH 7.4, 10 mM) and ACN was infused through the cell at 10 μL/min flow rate, and then this effluent was immediately analysed with an Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an ESI ion source. The full scan spectra were recorded in positive mode in the range of *m/z* 100–800 with XCalibur 2.1

(Thermo Fisher Scientific, Waltham, MA, USA) software. The three-dimensional mass voltammogram was achieved by plotting mass spectra in against the applied potential using Origin 9.1 (OriginLab, Northhampton, MA, USA) software.

## 2.2. Materials and Methods for Study II

### *2.2.1. General Procedure for the Synthesis of **3a-c***

Benzaldehyde (**2a,b**, 6.3 mmol) suspension in MeOH (5 mL) was added to the mixture of acetophenone (**1a,b,** 1.29 g, 6 mmol) solution (10 mL MeOH) and 50% aq. NaOH (1.26 mL, 24 mmol). After stirring the solution for 1 hour, the reaction mixture was kept at room temperature for 1 day. In order to reach pH 1, 10% HCl solution was added to the flask and the precipitate was filtered off and washed with $H_2O$ (3 × 30 mL) to give **3a-c** with 88-97% yields.

### *2.2.2. General Procedure for the Synthesis of **4a-c***

The chalcone (**3a-c**, 2.7 mmol) was suspended in EtOH (15 mL) and cooled with water-bath, while 8% aq. NaOH (3.9 mL, 8.41 mmol) in order to produce a solution, followed by a dropwise addition of 30% $H_2O_2$ (3.9 mL, 38.2 mmol). The reaction mixture was stirred at room temperature for 2 hours, and then it was poured into ice-water mixture (250 mL). The pH of the solution was set to pH 1 by adding 10% HCl, and it was kept for 1 day to allow sedimentation. The precipitate was filtered off and washed with cc. $NaHCO_3$ solution (2 × 50 mL) and with water (4 × 50 mL) to give **4a-c** with 57-76% yields.

### *2.2.3. General Procedure for the Synthesis of **6a-f***

In a pressure tube the mixture of 3-hydroxyflavone (**4a-c**, 0.265 mmol), KF (46.3 mg, 0.795 mmol), $Pd(OAc)_2$ (3 mg, 0.0133 mmol), XPhos (12.6 mg, 0.0265 mmol) and boronic acid (**5a,b**, 0.53 mmol) in toluene/t-BuOH (6:1, 3.5

mL) were stirred under argon for 4 hours at 100 °C. The solvent was removed using rotary evaporator under reduce pressure, the residue was purified with adsorptive filtration using toluene/EtOAc (2:1) as eluent. Diisopropyl ether was used to wash the crude product, which was then filtered to result the pure product **6a-f** with 63-74% yields.

*2.2.4. ABTS Assay*

The $ABTS^{\bullet+}$ was generated by mixing ABTS solution (7 mM ) with $K_2S_2O_8$ solution (2.45 mM ) and keeping it in the dark for 16 hours. The work solution was prepared freshly by mixing 150 µL of ABTS•+ stock solution with 2.9 mL MeOH. The reaction mixture consisted of 180 µL work solution and 20 µL of the investigated compound or quercetin standard in DMSO (10, 20, 50, 100 and 200 µM). The decrease in $ABTS^{\bullet+}$ concentration was monitored by measuring the absorbance at 737 nm for 2 hours after a short shaking period (10 sec) using a Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Each experiment was run in duplicate and repeated three times. The mean (SD) $IC_{50}$ values were calculated based on the inhibition percentage measured at 120 min.

*2.2.5. DPPH Assay*

In each well, 180 µL freshly made DPPH solution (0.2 mM) in MeOH and 20 µL of the compound of interest or quercetin standard (10, 20, 50, 100 or 200 µM) in DMSO were mixed together. The decrease in DPPH free radical concentration was monitored by measuring the absorbance at 515 nm for 90 min after a short gentle shaking period (10 sec) using a Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Each measurement was performed in duplicate and repeated three times. The mean (SD) $IC_{50}$ values were calculated based on the inhibition percentage measured at 90 min.

*2.2.6. FRAP Assay*

The FRAP working solution was prepared freshly by mixing 10 mL acetate buffer (pH 3.6, 300 mM), 1 mL TPTZ solution (10 mM) in HCl (40 mM) and 1 mL $FeCl_3$ (10 mM) together, followed by 15 min incubation at 37 °C. Solutions (20 µL) of the compound of interest or quercetin standard (10, 20, 50, 100 or 200 µM) were allowed to react with 180 µL FRAP working solution for 30 min in the dark. The absorbance of the coloured ferrous tripyridyltriazine complex was measured at 593 nm by a Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The solution of ferrous sulphate was used as standard and the calculated FRAP values are expressed as mean (SD) µM Ferrous equivalents. Measurements were run in duplicate and repeated three times.

*2.2.7. ORAC Assay*

The oxygen radical absorbance capacity of the derivatives was measured using black 96-well plates. Solutions of fluorescein (50 nM) and AAPH (180 mM) in phosphate buffer (pH 7.0, 75 mM) were prepared freshly for each experiment. The mixture of 20 µL solution of the compound of interest or quercetin standard (2 and 10 µM) in ACN and 160 µL fluorescein (50 nM) was incubated for 15 min at 37 °C. The reaction was initiated by adding rapidly 20 µL AAPH solution (180 mM) to each well. The decrease in the fluorescence of the fluorescein was measured at 485 nm excitation and 520 nm emission wavelength for 2 hours in every 2 minutes using FLUOstar OPTIMA (BMG Labtech, Ortenberg, Germany) plate reader. The AUC was calculated using the following formula:

$$AUC = 0.5 + (A_1/A_0) + (A_2/A_0) + (A_3/A_0) + \ldots + (A_n/A_0), \quad (2)$$

where $A_0$ is the fluorescence at 0 min and $A_n$ is the fluorescence at n min. The ORAC values were calculated by subtracting the blank AUC from the sample

AUC and expressed as mean (SD) AUCnet. Experiments were performed in duplicate and repeated three times.

*2.2.8. MTT Assay*

The cytotoxic evaluation of the investigated compounds cell on cell survival was accomplished by utilizing the MTT assay using H9c2 rat cardiomyocyte cell line (ATCC, CRL-1446, LGC Standards GmbH, Wesel, Germany). The cells were seeded into 96-well plates in 200 μL DMEM media with 10% FBS and supplemented with penicillin and streptomycin, the density was 6000 cells/well. The cells were cultured overnight, followed by the addition of the flavonol derivatives (20 μM). Twelve hours later, 20 μL of MTT solution (5 mg/μL in PBS) was pipetted to the wells, followed by incubation at 37 °C for 4 hours to allow mitochondrial uptake. After the careful removal of the media, 100 μL DMSO was pipetted in order to dissolve the formed formazan crystals. The measurement of absorbance was performed at 540 and 630 nm using a Synergy HT (BioTek, Winoosky, VT, USA) plate reader. The results are given in mean (SD) cell viability percentage. The experiments were carried out in triplicate and repeated two times

*2.2.9. Chemical Fenton System*

The oxidation of the flavonol derivatives was accomplished using chemical Fenton reaction system, which was prepared as written in section 2.1.5.

## 3. Results

### 3.1. Results of Study I

*3.1.1. Antioxidant Properties*

3.1.1.1. ABTS assay

Among the tested derivatives, compound **865** demonstrated the highest scavenging rate followed by **893**. The compound **1019/2** showed results similar to the coumarin standard, while the other investigated compounds have significantly lower scavenging rate.

3.1.1.2. ORAC Assay

During this test, also the compound **865** had the highest ORAC value, but it is noteworthy that all the tested compounds have shown significantly higher capacity than the coumarin standard. Compound **865** was followed by **874**, **890**, **893**, **987/3** and **1010/2**.

3.1.1.3. FRAP Assay

Compound **865** showed significantly increased FRAP activity, while the other tested compounds have slightly increased (**870**, **874**, **991** and **1019/2**) trolox equivalents compared to the coumarin standard or similar (**876**, **890**, **893** and **987/3**).

*3.1.2. Cytotoxic Activity*

Based on the results of the MTT assay none of the investigated compounds showed cytotoxic effect; compounds **893**, **865**, **987/3**, **876**, **1019/2** and **890** augmented the viability when the cells were treated with 125 μM $H_2O_2$, while in case of the treatment with 250 μM $H_2O_2$, all investigated compounds except **874** significantly increased cell viability.

*3.1.3. Oxidative Stability*

3.1.3.1. Chemical Fenton Reaction

Based on the spectral data obtained with off-line ESI-MS, three oxidation products were identified as potential metabolites; **865-$CH_3$** (*m/z* 252.0) with the highest intensity, followed by **865+O** (*m/z* 282.3) and **865-2$CH_3$** (*m/z* 238.0).

3.1.3.2. Synthetic Porphine System

The LC-MS analysis following the oxidation by artificial porphine showed three oxidative products: **865+O** (*m/z* 282.42), **865-2$CH_3$** (*m/z* 238.42) and **865+O-2H** (*m/z* 280.25). The secondary amine (**865-$CH_3$**) peak with *m/z* 252.33 was also present in the control sample; in this case it is considered an impurity of **865**, as there was no evidence to prove otherwise.

3.1.3.3. Electrochemical Oxidation

The oxidation of **865** begins at approximately 1500 mV; its peak (*m/z* 266.1165) intensity has decreased, while the intensity of other peaks has increased. It is noteworthy, that the **865-$CH_3$** (*m/z* 252.1014) was detected even at 0 mV; this can be caused by its oxidation in the ion source, although the intensity increase at 1500 mV confirms **865-$CH_3$** as oxidation product. At higher potential it is oxidized further to **865-2$CH_3$** (*m/z* 238.0858) primary amine. The aromatic hydroxylated product (**865+O** (*m/z* 282.1133)) was also observed along with its dehydrogenated derivative (**865+O-2H** (*m/z* 280.0959)).

## 3.2. Results of Study II

*3.2.1. Chemical Synthesis*

The first step during the synthesis of the flavonols (**6a-f**) was the Claisen-Schmidt condensation (i) of commercially available bromoacetophenones (**1a,b**) and benzaldehydes (**2a,b**) in MeOH using 4 equiv. NaOH, which gave the corresponding **3a-c** chalcones with excellent yields (88-97%). The cyclization

(ii) into the flavonol backbone (**4a-c**) was carried out by adding 14.1 equiv. $H_2O_2$ to the solution of the chalcone (**3a-c**) in EtOH. The bromoflavon derivatives (**4a-c**) were coupled with the appropriate boronic acid (**5a,b**) under argon atmosphere (iii), in the presence of potassium-fluoride, $Pd(OAc)_2$ and XPhos in using toluene/*t*-BuOH (6:1) as solvent, thus providing the compounds of interest with the desired side chains on B ring (**6a-f**) with good yields (63-74%).

*3.2.2. Antioxidant Properties*

3.2.2.1. ABTS Assay

Based on the calculated $IC_{50}$ values compound **6c** was proven to be the most potent scavenger of the ABTS radical cation followed by **6e** and **6a** respectively.

3.2.2.2. DPPH Assay

Investigating the activity against DPPH radical, similarly to the ABTS assay, compound **6c** was shown to have the lowest $IC_{50}$ value, followed by **6a**, **6d** and **6b** respectively.

3.2.2.3. ORAC Assay

In contrast with the two previous assays, in this case at both measured (2 and 10 μM) flavonol concentrations compound **6e** had the highest ORAC value, followed by **6a** and **6b** respectively. Furthermore, the capacity of compound **6a** and **6e** were comparable to the capacity of quercetin standard at 10 μM concentrations, while surprisingly compound **6c** had only the fourth highest ORAC value.

3.2.2.4. FRAP Assay

Compounds **6c** showed the highest FRAP activity at all the tested concentrations followed by **6a** and **6e** respectively.

*3.2.3. Cytotoxic Activity*

The results of the MTT assay using H9c2 cells revealed that compounds **6a**, **6c** and **6e** did not show cytotoxic activity. However, compounds **6b**, **6d** and **6f** significantly decreased the viability of cardiomyoblastoma cells.

*3.2.4. Oxadative Transformation*

Based on the ESI-MS analysis of the reaction mixtures following the chemical Fenton reaction, we were able to detect and identify two oxidation products as potential metabolites: the **6c+O** (*m/z* 434.2) is a product of aromatic hydroxylation, while the most abundant metabolite **6cO-$CH_3$** (*m/z* 404.1) was formed though *O*-demethylation.

## 4. Discussion

### 4.1. Interpretation of Study I Results

The results of the three different assays for compound **865** in Study I are consistent; it was revealed that compound **865**, an *N*,*N*-dimethylamino group containing simple-structured flavonoid has the highest antioxidant activity among the tested nine molecules; **865** demonstrated significantly higher ABTS radical cation scavenging and FRAP activity and it has better oxygen radical absorption capacity. However, the other compounds also showed significantly greater ORAC value than the coumarin standard. Surprisingly, compound **865** has only one of the most significant structural features to be an effective radical scavenger; the conjugation between the 2,3-double bond with a oxo function at position 4, but the other features are not present: OH-functions in positions 3 and 5 and catechol (*o*-dihydroxy) system in the B-ring. The difference in the results obtained by these three methods presumably is caused by their different mechanism; the ABTS and FRAP assays are single electron transfer (SET) based, while the ORAC assay is based on hydrogen atom transfer (HAT) mechanism.

The MTT assay is a first-approach method for measuring cell viability, although it measures the mitochondrial metabolic rate which can be affected by chemical treatments or different conditions, and thus providing results that are not necessarily connected directly to cytotoxicity. However, in this case there are no factors affecting the outcome of the measurement and the results are consistent; the viability of the treated cells in case of compounds **865**, **876**, **893**, **987/3**, **876**, and **1019/2** did not decrease. Similar results can be observed when the cells were treated with $H_2O_2$ besides the flavonoid-derivatives; they prevented the $H_2O_2$-induced cell death of the cardiomyocytes. The antioxidant activity and the lack of cytotoxicity have made the compound **865** a prominent candidate for further *in vivo* testing.

Nevertheless, prior to any *in vivo* experiments, metabolic peculiarities and possible toxicity must be elucidated as early as possible in order to spare time and costs by rapidly identify robust candidates and prevent compound failures. The oxidative metabolism of compound **865** was investigated using three different biomimetic systems; to gather information about the behaviour of the molecule in an oxidative environment, it was oxidized first by chemical Fenton system and the data collection was performed off-line by ESI-MS/MS technique. The Fenton reaction is a biomimetic system used to model phase I metabolic reactions, such as *N*-dealkylation, *N*-oxidation, *O*-dealkylation, *S*-oxidation, dehydrogenation, and different hydroxylation reactions. The chemical Fenton system was able to detect the following potential metabolites of the compound **865**; the *N*-dealkylation yielded the **865-$CH_3$** secondary amine with the highest intensity, and a second *N*-dealkylation afforded **865-2$CH_3$** primary amine. The aromatic hydroxylation of the B ring resulted in the metabolite **865+O**. However, the Fenton system is able to mimic dehydrogenation, the product of this reaction was not detected, probably due to its low concentration being under the limit of detection of the used method.

The synthetic metalloporphine system can mimic *N*-dealkylation, *N*-oxidation, *S*-oxidation, hydroxylation, epoxidation, and dehydrogenation reactions. The off-line LC/MS analysis showed that three reactions of compound **865** were mimicked; the loss of two methyl group via *N*-dealkylation afforded **865-2$CH_3$** primary amine, the aromatic hydroxylation of the B ring yielded **865+O**, and the dehydrogenated derivative of the hydroxylated product **865+O-2H** was also detected. Product secondary amine **865-$CH_3$** was also observed, but it was also present in the control sample and further evidence have not verify its presence as metabolite it is considered as an impurity in this case.

The EC/LC/MS and EC/MS systems have been a useful tool to produce potential metabolites produced by aromatic hydroxylation, dehydrogenation, *S*-, *P*-oxidation, and *N*-dealkylation. This method was used to generate the mass

voltammogram, a graph of the mass spectra plotted against the varying applied potential, which helps to determine the most suitable electrochemical potential at which the highest oxidation conversion rate of the investigated compound can be achieved. For the compound **865**, this potential is 1500 mV. The EC/LC/MS method was able to produce the same potential metabolites as the two other model systems; **865-$CH_3$** secondary amine and the further oxidized **865-2$CH_3$** primary amine, the aromatic hydroxylated product **865+O** and its **865+O-2H** dehydrogenated derivative.

### 4.2. Interpretation of Study II Results

The Study II was conducted to further investigate the possible effects of the phenyl-*N*,*N*-dimethylamino group on the antioxidant and cytoprotective activity of flavonoid derivatives. Based on the results of Study I, new flavonol derivatives (**6a-f**) were designed and synthetized with phenyl-*N*,*N*-dimethylamino or methoxymethyl phenyl group connected to the A ring in position 6 or 7. These flavonols have the conjugated system between the 2,3-double bond and the oxo function in position 4, but the formation of catechol moiety on B ring have posed insuperable challenge; the synthesis of the demethylated derivatives requires such forceful conditions, which resulted in the decomposition of the molecules.

The antioxidant activity of the investigated molecules (**6a-f**) was evaluated based on one HAT-based and three SET-based assays. The ABTS $IC_{50}$ value was calculated from the inhibition percentage at 120 min determined in the concentration range of 10–200 μM. As the ABTS abstracts an electron from the antioxidant, it is regenerated to its original form causing a change in the absorbance of the solution at 737 nm, which can be measured by a spectrophotometer. Compound **6c** had the lowest $IC_{50}$ value, and therefore the highest ABTS radical scavenging activity, followed by **6e** and **6a**. These compounds bear with the phenyl-*N*,*N*-dimethylamino group on their A ring,

therefore because the electronegativity difference and the unpaired electrons of thenitrogen atom, their ability to donate an electron during the single electron-transfer to the ABTS radical cation is better than the methoxymethyl group containing derivatives (**6b**, **6d**, and **6f**).

The DPPH assay is based on similar principle as the ABTS assay; i.e., the solution of the stable DPPH radical is deep violet, which loses its colour as the DPPH is reduced by the antioxidant. This change can be detected at 515 nm. The DPPH $IC_{50}$ values were calculated from the inhibition percentage at 90 min obtained in the concentration range of 10–200 μM. Compound **6c** exhibited the lowest DPPH $IC_{50}$ value, followed by **6a**, **6d**, and **6b**. These derivatives (**6a**, **6b**, **6c**, and **6d**) have only two methoxy groups on the B ring, while the other compounds (**6e** and **6f**) have three. *Ortho*- and *para*-substitution pattern of two hydroxyl groups on the B ring in chalcones have much higher antioxidant activity than the *meta*-substitution pattern. The *ortho*- and *para*-dihydroxylated benzene ring systems are much more efficient in delocalizing electrons than the meta- dihydroxylated ones, and thus resulting in more stable chalcone adduct formation during a reaction with free radicals. Furthermore, the presence of three methoxy groups on B ring increases the steric hindrance causing a perturbed planarity leading to decreased ability of hydrogen abstraction, which would be easier in planar geometrical configuration, and hence these compounds (**6e** and **6f**) have lower radical-scavenging ability. The fact, that phenyl-*N*,*N*-dimethylamino group containing **6c** was the most active molecule in both ABTS and DPPH assay correlates well with pervious observations; among the investigated 3-hydroxyflavone derivatives, the 4'-*N*,*N*-dimethyl flavonol exhibited DPPH scavenging potency, comparable with a standard compound quercetin, and it had the highest ABTS cation radical scavenging potency. Based on the results of radical scavenging assays in both Study I and Study II, the phenyl-*N*,*N*-dimethylamino-group could play a key part in the radical scavenger activity of the investigated flavonoid derivatives.

Among the investigated derivatives at all concentrations (10, 20, 50, 100 and 200 μM) compounds **6c**, **6a**, and **6e** exhibited the highest FRAP values, whilst compounds **6d**, **6f**, and **6b** had significantly lower ferric reducing antioxidant potential. However, the fact that each tested derivatives (**6a-f**) showed significantly lower FRAP value compared to the quercetin standard compound is not a surprise, as it is well-known that the methoxylation of flavonoids leads to decreased antioxidant activity compared to the unmethylated derivatives. A previous study showed that the methylated quercetin had a FRAP value 650-times lower than quercetin, but this drastic decrease was not observed in Study II, probably due to the presence of phenyl-*N*,*N*-dimethylamino group at ring A and the free OH group in position 3. The outcome of the FRAP assay also indicates the importance of the phenyl-*N*,*N*-dimethylamino group; all the tested compounds have the free OH group in position 3, however only those compounds had higher ferric reducing antioxidant potential, which have the phenyl-*N*,*N*-dimethylamino group (**6c**, **6e**, and **6a**). The change in the ORAC values was not as significant as in the FRAP results.

Compounds **6e**, **6a**, and **6b** exhibited the highest oxygen radical absorption capacity, which was comparable to the capacity of the quercetin at a 10 μM concentration. Strikingly, the compound **6c** had only the fourth highest ORAC value at both concentrations. These results are consistent with the findings of others; in case of methylated polyphenols the change in the ORAC values was not as significant as in the FRAP results compared to the unmethylated derivatives.

To test the cytotoxic effect of the flavonol derivatives, H9c2 cells were treated with 20 μM flavonol solutions for 12 h. The results of the MTT assay showed no cytotoxic activity in case of the phenyl-*N*,*N*-dimethylamino functional group containing molecules (**6a**, **6c** and **6e**); the cell viability percentages were comparable to the quercetin standard compound and untreated control as well. However, the compounds with methoxymethyl groups on A ring

(**6b**, **6d**, and **6f**) significantly decreased the cell viability. This negative effect of the treatment was the most significant in case of the compound **6d**; compared to the control the cell viability was decreased to 46%.

The possible oxidative transformation route and the formation of potential metabolites of compound **6c** were investigated due to its aforementioned antioxidant and non-cytotoxic characteristics. The chemical Fenton system was chosen as biomimetic model system of phase I metabolic reactions. The generated oxidative products of compound **6c** were detected and identified with ESI-MS and confirmed also by LC/ESI-MS analysis; based on the peak intensity the most abundant product were the *O*-demethylated **6cO-CH$_3$** derivative, and the atomatic hydroxylation on the B ring afforded the **6c+OH** potential metabolite. As in Study I, the place of these reactions was determined based on the retro Diels-Alder rearrangement fragmentation pattern characteristic to the 3-hydroxyflavone structure. Additional investigative methods, such as electrochemical oxidation and synthetic porphyrin systems could generate other metabolites; secondary **6cN-CH$_3$** and primary amine **6cN-2CH$_3$** after *N*-dealkylation. Although, the exact positions of these transformations cannot be determined due to the limitations of the analytical methods used.

## 5. Summary

In Study I, nine flavonoids were tested for their possible antioxidant and cytotoxic activities. As the result of this investigation the significant antioxidant potency, cytoprotective activity, and the metabolic stability of 4-*N*,*N*-dimethylamino-flavon (compound **865**) were observed.

Based on this outcome, six new flavonols were designed and synthetized for Study II, in which we investigated the effect of phenyl-*N*,*N*-dimethylamino group on the antioxidant activity. The *N*,*N*-dimethylamino group containing flavonol derivatives (**6a**, **6c** and **6e**) showed increased activity during different antioxidant assays, and the also exhibited cytoprotective effect on H9c2 cardiomyoblast cells.

The results of both Study I and Study II suggest that the phenyl-*N*,*N*-dimethylamino group has a crucial role in the antioxidant potency and the cytotoxicity of flavonoid derivatives. The oxidative stability of **865** and **6c** also makes them an ideal candidate for the investigation of oxidative stress related diseases in suitable animal models.

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## List of publications related to the dissertation

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2. Csépányi, E., **Szabados-Fürjesi, P.**, Kiss-Szikszai, A., Frensemeier, L. M., Karst, U., Lekli, I., Haines, D. D., Tósaki, Á., Bak, I.: Antioxidant Properties and Oxidative Transformation of Different Chromone Derivatives.
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# List of other publications

3. Csépányi, E., Czompa, A., **Szabados-Fürjesi, P.**, Lekli, I., Balla, J., Balla, G., Tósaki, Á., Bak, I.: The Effects of Long-Term, Low- and High-Dose Beta-Carotene Treatment in Zucker Diabetic Fatty Rats: the Role of HO-1.
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