AKADÉMIAI KIADÓ

Acta Veterinaria Hungarica

71 (2023) 1, 16–24

DOI:
10.1556/004.2023.00746

RESEARCH ARTICLE

Effect of hydroxylated and methylated flavonoids on cytochrome P450 activity in porcine intestinal epithelial cells

ZITA KARANCSI†, DÓRA KOVÁCS†*, GYÖRGY CSIKÓ, ORSOLYA PALÓCZ, ÁKOS JERZSELE, PÉTER GÁLFI and ORSOLYA FARKAS

University of Veterinary Medicine, Budapest, Hungary

Received: 18 January 2022 • Accepted: 29 March 2023
Published online: 3 May 2023

ABSTRACT

Cytochrome P450 (CYP) oxidases are among the main metabolizing enzymes that are responsible for the transformation of xenobiotics, including clinically important drugs. Their activity can be influenced by several compounds leading to decreased efficacy or increased toxicity of co-administered medicines. Flavonoids exert various beneficial effects on human and animal health; therefore they are used as food and feed supplements. However, they are also well-known for their CYP modulating potential. Since the amount of CYP enzymes is highest in the liver, interaction studies are mainly conducted in hepatocytes, however, CYP activity in the gastrointestinal tract is also remarkable. In this study, effects of apigenin (API), quercetin (QUE) and their methylated derivatives trimethylapigenin (TM-API), 3-O-methylquercetin (3M-QUE) and 3′,7-di-O-methylquercetin (3′7DM-QUE) on the CYP enzyme activity was examined in IPEC-J2 porcine intestinal epithelial cells. Potential food-drug interactions were studied using flavonoid treatment in combination with inducer and inhibitor compounds. API, TM-API, QUE and 3M-QUE significantly inhibited the CYP3A29 enzyme, while 3′7DM-QUE did not alter its activity. Enzyme inhibition has also been observed in case of some food-drug combinations. Our results support previous findings about CYP modulating effects of flavonoids and highlights the possibility of interactions when flavonoid-containing supplements are consumed during drug treatments.

KEYWORDS

flavonoids, apigenin, quercetin, CYP3A4, CYP3A29, IPEC-J2

INTRODUCTION

Cytochrome P450 (CYP) oxidases are frequently studied Phase I metabolizing enzymes of the human and animal body, that have considerable impact on the expected plasma level of administered drugs and consequently on their safety and efficacy. Investigation of CYP mediated reactions is therefore a necessary part of drug development. Their importance is explained by the fact that in humans, 80% of the whole oxidative metabolism, and 50% of the overall elimination is related to CYP enzymes (Wilkinson, 2005). Highest amount of CYP enzymes can be found in the liver, but the portion in the gastrointestinal (GI) tract is also considerable (Paine et al., 1997) and contributes to the first pass metabolism of clinically important drugs such as midazolam (Paine et al., 1996), cyclosporine (Kolars et al., 1991) and verapamil (Von Richter et al., 2001). Both in the liver and the GI tract, CYP3A is the most frequently occurring enzyme subfamily, constituting 40% of all CYPs in the former and 82% in the latter organ (Paine et al., 2006). In humans, CYP3A4 plays the greatest role, metabolizing more than 50% of administered drugs (Hebert et al., 1992; Paine et al., 1996; Wacher et al., 2001). In pigs, CYP3A29 is the enzyme that is similar to the human CYP3A4 in terms

†Zita Karancsi and Dóra Kovács contributed equally to this paper.

*Corresponding author.
E-mail: kovacs.dora@univet.hu

of expression and activity (Yao et al., 2011). Due to their high prevalence and importance, CYP3A subfamily and CYP3A4 enzyme are the most frequently studied members of the CYP system, however, most investigations focus on hepatic processes despite the importance of GI CYP enzymes in first pass metabolism.

In both organs, CYP enzymes play a role in Phase 1 metabolism of xenobiotics, where the principal aim is to transform substances into hydrophilic compounds, eligible for urinary elimination. However, during CYP-catalysed reactions, biological activity of xenobiotics can also be altered. They might be turned into metabolites with decreased or increased biological effects, or into toxic compounds with various deteriorating impacts on the body. CYP-mediated reactions are also associated with drug-drug and drug-food interactions. If more than one drugs or food components are present in the body, which are transformed by the same CYP enzyme, their competition for the enzyme might result in an alteration in the metabolism and therefore in the expected plasma level of drug(s), leading to possible therapeutic and toxicological consequences. Further interactions can occur if compounds with CYP inducer or inhibitor potential are present (Lin et al., 1998). Inducers and inhibitors can be found among drugs (e.g. barbiturates and dexamethasone - induction, ketoconazole - inhibition) (Lin et al., 1998) and in fruits and vegetables as well (e.g. grapefruit juice - inhibition) (Bailey et al., 1998). Several flavonoids, including quercetin, resveratrol and naringenin have also been shown to modulate CYP enzyme activity (Murray, 2006; Wanwimolruk and Prachayasittikul, 2014).

Flavonoids are the most diverse group of polyphenols: more than 8,000 different flavonoids have already been described (Gonzales et al., 2016). Produced by herbs as secondary plant metabolites, a lot of plant originated nutrients contain flavonoids. The core of their structure contains two aromatic carbon rings connected by a three-carbon unit forming a heterocyclic ring with an oxygen. Based on their chemical structure, flavones, flavonols, flavanones, flavanols (or catechins), isoflavones, anthocyanidins, dihydroflavonols and chalcones can be distinguished (Galvez et al., 2001). Flavonoids possess many beneficial effects on human and animal health such as antioxidant, anti-inflammatory, antibacterial, antiviral, anti-carcinogenic, immunomodulatory, and antithrombotic activity (Scalbert et al., 2005; Kamboh et al., 2015), therefore they are frequently used in food and feed supplements. However, their ability to modulate the activity of certain CYP enzymes must be considered before consumption, especially in case of co-administration of drugs.

Previously, health benefits of flavonoids were attributed to their hydrogen-donating antioxidant property, but it has been described that they are also capable of modulating several intracellular signalling pathways (Williams et al., 2004). Flavonoids can have diverse chemical structure resulting in various biological activities. As an example, methylated flavonoids show better intestinal absorption and hepatic metabolic stability, leading to an improved oral bioavailability compared to the corresponding unmethylated substances (Wen et al., 2006), and they can also possess anti-carcinogenic, antibacterial, anti-inflammatory and antioxidant effects (Murakami et al., 2005; Lai et al., 2007; Tang et al., 2007; Farkas et al., 2015). Impact of flavonoids on CYP enzyme activity is also dependent on several factors. It might occur in the form of induction or inhibition and is influenced by the structure and concentration of the substance, as well as the gender of the consumer (Ekstrand et al., 2015). Numerous studies aim to discover the exact structure–activity relationship of flavonoids, but knowledge in this field is still incomplete.

Intestinal cell lines are important tools for modelling the human GI tract when studying toxicity, bioavailability and interaction of drugs, nutrition, and food microbiology (Cencic and Langerholc, 2010). IPEC-J2 is a non-transformed cell line derived from jejunal epithelia of neonatal, unsuckled piglets (Cencic and Langerholc, 2010). Advantages of this cell line include its non-transformed, non-tumorigenic nature, as well as its morphological and functional similarities with *in vivo* properties of the intestinal epithelium (Verchoeckx et al., 2015). IPEC-J2 cells form continuous, polarized monolayers with high transepithelial electrical resistance, develop apical and basolateral parts and are capable of expressing tight junction proteins, synthetizing cytokines, defensins, toll-like receptors and mucins (Schierack et al., 2006; Ayuso et al., 2020). Besides gaining knowledge about the swine GI tract, intestinal cell lines of porcine origin can also be used as models for human intestinal function based on genetical, anatomical and physiological similarities of the two species (Nossol et al., 2015). These cells have stronger resemblance to human conditions than analogous rodent cell lines (e.g. IEC-6, IEC-18) (Brosnahan and Brown, 2012). Considering its above-mentioned qualities, IPEC-J2 cell line is a preferential tool for *in vitro* investigations of both porcine and human intestinal function.

The main goal of our study was to investigate the effects of hydroxylated flavonoids; apigenin (4′,5,7-trihydroxyflavone; API), quercetin (3,3′,4′,5,7-pentahydroxyflavone; QUE) and their methylated analogues; trimethylapigenin (4′,5,7-trimethoxyflavone; TM-API), 3-O-methylquercetin (3′,4′,5,7-tetrahydroxy-3-methoxyflavone; 3M-QUE) and 3′,7-di-O-methylquercetin (rhamnazin, 3,4′,5-trihydroxy-3′,7-dimethoxyflavone; 3′7DM-QUE) on CYP enzyme function in IPEC-J2 porcine intestinal epithelial cell line. The chemical structure of the tested flavonoids can be seen in Fig. 1. Activity of the CYP3A29 enzyme was examined after treatment with the flavonoids alone and in combination with different drugs to model possible food-drug interactions. In pigs, CYP3A29 plays a similar role to the human CYP3A4 enzyme.

MATERIALS AND METHODS

Chemicals and instruments

API, QUE, 3M-QUE, 3′7DM-QUE, phenobarbital, ketoconazole, 4-aminoantipyrine, dimethyl-sulfoxide (DMSO),

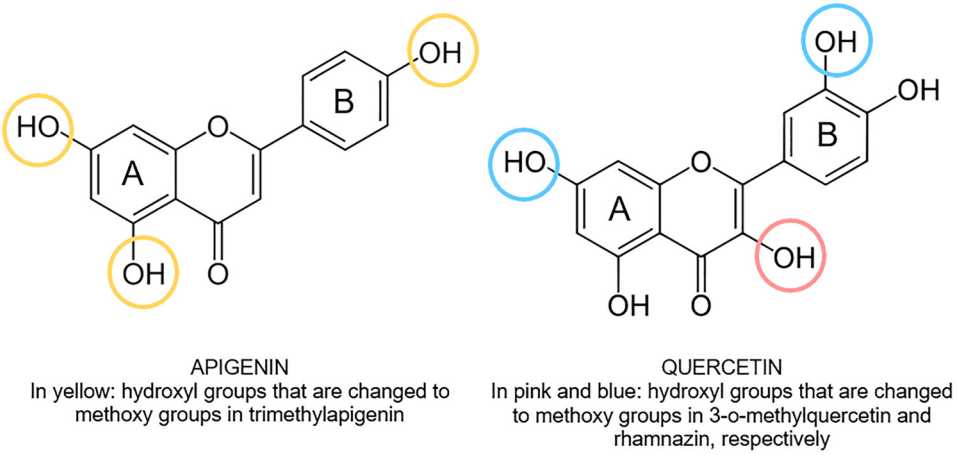

Fig. 1. Chemical structure of the investigated flavonoids

neutral red dye and growth medium of cells were purchased from Sigma–Aldrich (Darmstadt, Germany), while TM-API was obtained from Indofine Chemical Company (Hillsborough, NJ, USA). P450-Glo™ assays were supplied by Promega (Madison, WI, USA). Cell culture plates were ordered from Corning Inc. (Corning, NY, USA). For the measurements, EZ Read 400 Microplate Reader (Biochrom Ltd., Cambridge, UK) and Victor X2 2030 multilabel reader (Perkin-Elmer Inc., Waltham, MA, USA) were used.

IPEC-J2 cell line and culture conditions

IPEC-2 cells were kindly provided by Dr. Jody Gookin (Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC, USA). Cells were propagated on 37 °C, with 5% CO_2, in the 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient (DMEM/F12) until a passage number of approximately 45 for all investigations. In order to provide an optimal environment for cells, DMEM/F12 was supplemented with fetal bovine serum (5%), insulin (5 µg mL^{-1}), transferrin (5 µg mL^{-1}), selenium (5 ng mL^{-1}), epidermal growth factor (5 ng mL^{-1}) and penicillin-streptomycin (1%) for cell culturing (full DMEM/F12). In the working solutions, plain DMEM/F12 (without supplementation) was used. The experiments were performed with the cells seeded on 96-well (neutral red) or 24-well (chemiluminescence) polystyrene cell culture plates, after they had reached a differentiated, confluent monolayer, evaluated by light microscopy.

Cell viability determination by neutral red assay

Before testing CYP enzyme activity, influence of QUE, 3M-QUE and 3′7DM-QUE in different concentrations (25, 50 µM) on the viability of IPEC-J2 cells was examined. Effect of API and TM-API has been investigated previously demonstrating that these could be used safely at the concentration of 25 µM, while higher concentrations caused cell viability decrease in IPEC-J2 cells (Farkas et al., 2015). For preparation of the working solutions, flavonoids were dissolved in the mixture of DMSO and plain cell culture medium. Final concentration of DMSO on the cells was set to 0.1%, which did not alter the measured parameters in control experiments. IPEC-J2 cells were seeded onto 96-well plates and incubated with QUE and its derivatives for 2 h (37 °C, 5% CO_2). Control cells received only plain medium for similar incubation time. At the end of treatments, supernatants were removed, cells were washed with phosphate buffered saline (PBS) and then received full DMEM/F12. The proportion of living cells was determined 24 h later with neutral red uptake assay (Repetto et al., 2008). Absorbance measurement (on 540 nm) was performed with Biochrom EZ Read 400 Microplate Reader. Based on the results of the cell viability assay, it was concluded that QUE and its derivatives could be used safely in the further experimental steps at concentrations of 25 and 50 µM.

CYP activity determination by chemiluminescent method

The effect of 25 µM API and 25 µM TM-API on CYP activity of IPEC-J2 cells was investigated. QUE, 3M-QUE and 3′7DM-QUE were tested at both 25 and 50 µM concentrations as these had been found to be safe in the cell viability determination phase. All flavonoids were dissolved in the mixture of plain medium and 0.1% DMSO (final concentration on cells) for the working solutions. The cells receiving only plain DMEM/F12 served as untreated control. There was no need to include DMSO in the control, as the level of it included in the flavonoid solutions does not interfere with the assay used (as described in the protocol of P450-Glo™ assay, luciferin-IPA substrate is not sensitive to DMSO up to 0.25%). For comparison, well-known CYP inducer and inhibitor compounds have also been applied on some cells. A group of cells was treated with 500 µM phenobarbital (general CYP inducer), while others received 25 µM ketoconazole (CYP1A1 and 3A4 inhibitor) (Palócz et al., 2017). Phenobarbital was also applied in combination with the different flavonoids. 4-aminoantipyrine (1 mM), an analgesic and antipyretic drug was added to other groups of cells alone and in combination with the tested flavonoids for

modelling possible drug-flavonoid interactions. All treatments were applied on cells for 2 h.

For detection of CYP3A29 activity in the untreated and treated cells, P450-Glo™ assay (cell-based, nonlytic) was performed according to the recommendations of the manufacturer. For the assay, luciferin-IPA substrate was used, which is the most sensitive and selective substrate for CYP3A4 (and for CYP3A29). Luminescence in the wells was detected with Victor X2 2030 multilabel reader.

Statistical analysis

For statistical evaluation of the obtained data, R 3.3.2 (2016) software (R Foundation for Statistical Computing, Vienna, Austria) was used. Mean values of the different groups were compared with one-way ANOVA and Tukey post hoc test. Significance was determined if p value was lower than 0.05.

RESULTS

Cell viability

Viability of IPEC-J2 cells after 2 h treatment with QUE and its derivatives at 25 and 50 µM can be seen in Fig. 2. Neutral red uptake assay showed that there was no significant difference in the ratio of viable and dead cells in the treated groups compared to the untreated control. Therefore QUE, 3M-QUE and 3′7DM-QUE could be safely used for further experiments at both tested concentrations. The safe concentration of API and TM-API has been tested previously (Farkas et al., 2015).

CYP3A29 activity after treatment with flavonoids

Activity of the CYP3A29 enzyme after treatment with API, QUE and its derivatives at different concentrations is demonstrated in Fig. 3. Both API and TM-API at 25 µM significantly ($p < 0.05$) inhibited the activity of the enzyme and there was no difference between their actions. Their inhibitory activity was similar to that of ketoconazole. Among QUE and its derivatives, 50 µM QUE and 3M-QUE at both applied concentrations showed significant inhibitory effect ($p < 0.05$). In comparison to each other, their effect did not differ significantly, but their inhibitory activity was milder than that of the ketoconazole. However, 3′7DM-QUE did not alter the enzyme activity regardless of the tested concentration.

CYP3A29 activity after treatment with flavonoids and phenobarbital

Activity of the CYP3A29 enzyme after treatment with flavonoids in combination with phenobarbital is shown in Fig. 4. Phenobarbital alone did not cause significant alteration in the enzyme activity. When flavonoids were combined with phenobarbital, TM-API, QUE and 3M-QUE could still maintain their enzyme inhibitory effect, in contrast to API, which did not influence CYP3A29 activity when applied together with the inducer. Similarly to treatment with 3′7DM-QUE alone, its usage with phenobarbital had no significant effect on the tested enzyme.

CYP3A29 activity after treatment with flavonoids and 4-aminoantipyrine

Activity of the CYP3A29 enzyme after treatment with flavonoid and 4-aminoantipyrine combinations is presented in Fig. 5. The analgesic and antipyretic compound, 4-aminoantipyrine slightly inhibited ($p < 0.1$) the enzyme activity, which was further decreased when the drug was applied in combination with API and 50 µM QUE. A weak inhibitory effect could be observed in case of TM-API and

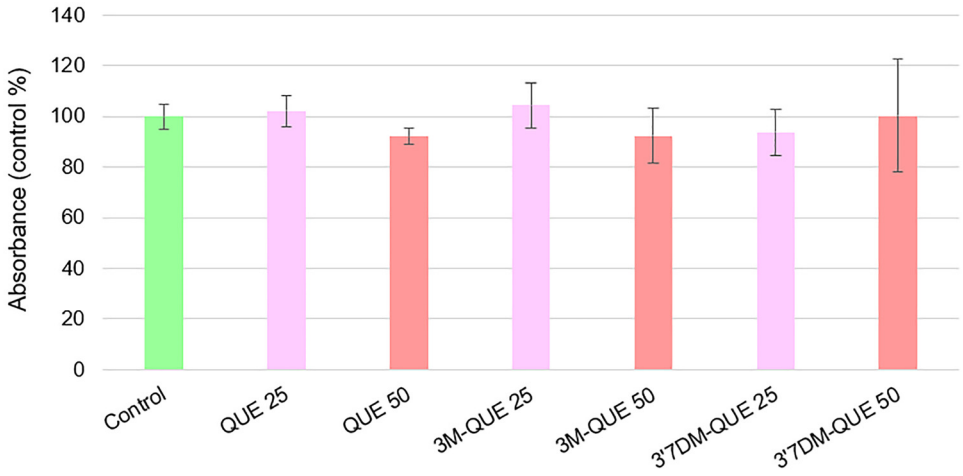

Fig. 2. Viability of IPEC-J2 cells measured by neutral red uptake assay after 2 h treatment with quercetin (QUE), 3-O-methylquercetin (3M-QUE) and 3′7-di-O-methylquercetin (3′7DM-QUE) at different concentrations. Control – treatment with plain medium; QUE 25, 50 – treatment with 25 and 50 µM QUE, respectively; 3M-QUE 25, 50 – treatment with 25 and 50 µM 3M-QUE, respectively; 3′7DM-QUE 25, 50 – treatment with 25 and 50 µM 3′7DM-QUE, respectively. Data are shown as means with standard deviation, and expressed as relative absorbance, considering the mean value of control as 100%, $n = 8$/group

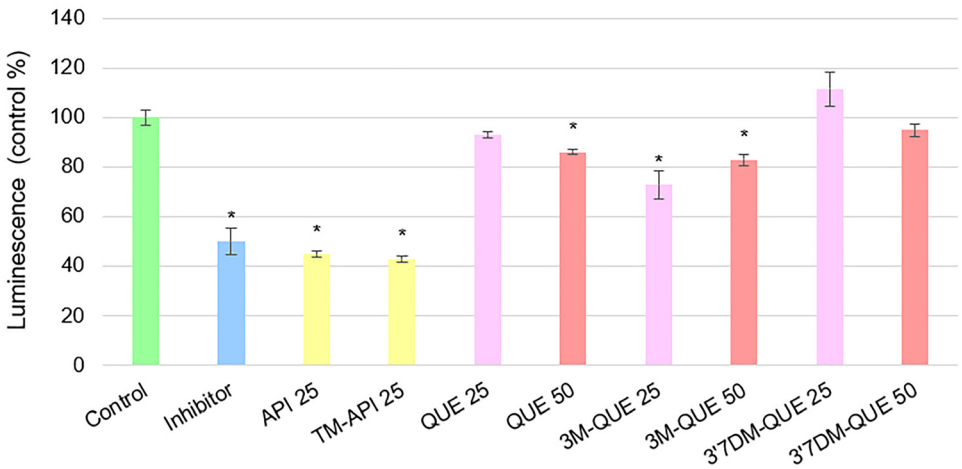

Fig. 3. Activity of CYP3A29 enzyme measured by chemiluminescent method using luciferin-IPA substrate after 2 h of treatment with flavonoids. Control – treatment with plain medium; Inhibitor – treatment with 25 μM ketoconazole; API 25 – treatment with 25 μM apigenin; TM-API 25 – treatment with 25 μM trimethylapigenin; QUE 25, 50 – treatment with 25 and 50 μM quercetin, respectively; 3M-QUE 25, 50 – treatment with 25 and 50 μM 3-O-methylquercetin, respectively; 3′7DM-QUE 25, 50 – treatment with 25 and 50 μM 3′7-di-O-methylquercetin, respectively. Data are shown as means with standard deviation, and expressed as relative luminescence, considering the mean value of control as 100%, $n = 4$/group. Significant difference compared to the untreated control: $^{*}p < 0.05$

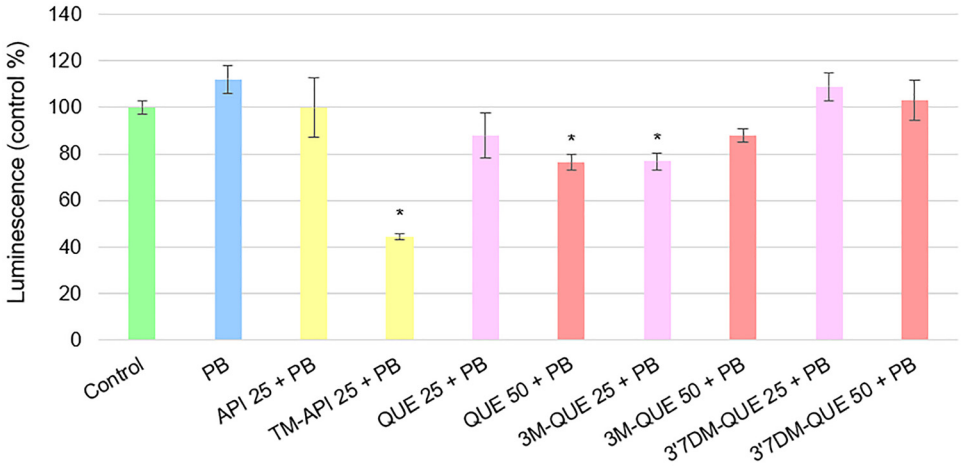

Fig. 4. Activity of CYP3A29 enzyme measured by chemiluminescent method using luciferin-IPA substrate after 2 h of treatment with flavonoids in combination with phenobarbital (PB). Control – treatment with plain medium; PB – treatment with 500 μM PB; API 25 + PB – treatment with 25 μM apigenin + 500 μM PB; TM-API 25 + PB – treatment with 25 μM trimethylapigenin + 500 μM PB; QUE 25, 50 + PB – treatment with 25 and 50 μM quercetin + 500 μM PB, respectively; 3M-QUE 25, 50 + PB – treatment with 25 and 50 μM 3-O-methylquercetin + 500 μM PB, respectively; 3′7DM-QUE 25, 50 + PB – treatment with 25 and 50 μM 3′7-di-O-methylquercetin + 500 μM PB, respectively. Data are shown as means with standard deviation, and expressed as relative luminescence, considering the mean value of control as 100%, $n = 4$/group. Significant difference compared to the untreated control: $^{*}p < 0.05$

4-aminoantipyrine combination ($p < 0.1$), however, no other treatments resulted in significant changes.

DISCUSSION

Flavonoid containing dietary supplements have become popular in recent years due to their versatile health promoting effects (Egert and Rimbach, 2011). However, besides their various beneficial properties, the modulatory effect of flavonoids on CYP enzyme activity has also been described, which can lead to drug-food interactions in case of simultaneous consumption of flavonoids and pharmaceuticals (Murray, 2006; Wanwimolruk and Prachayasittikul, 2014). To better understand these mechanisms and avoid undesired interactions, it is inevitable to discover correlations between the molecular structure of flavonoids and their ability to alter CYP enzyme activity. CYP modulation is among the possible mechanisms behind the anticancer effect of flavonoids, therefore several studies aim to reveal their effect on CYP1A1, 1A2 and 1B1 enzymes that play a role in the bioactivation of carcinogenic agents (Kim et al., 2005).

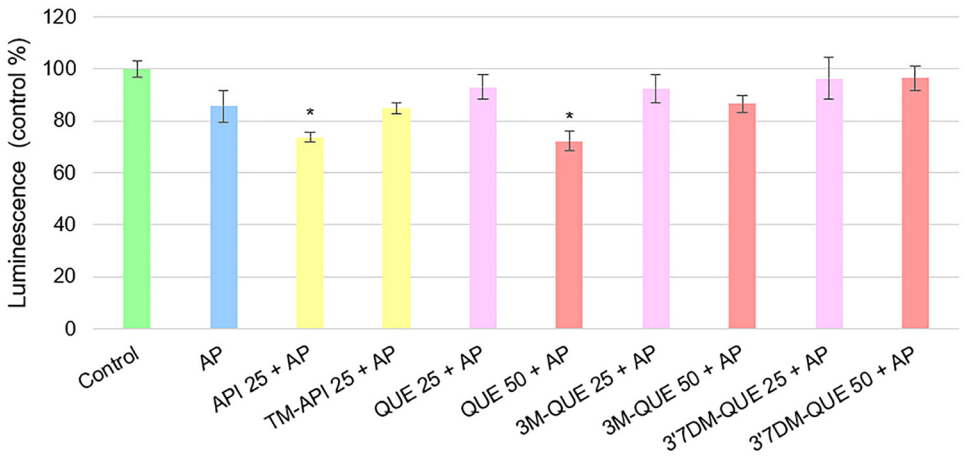

Fig. 5. Activity of CYP3A29 enzyme measured by chemiluminescent method using luciferin-IPA substrate after 2 h of treatment with flavonoids in combination with 4-aminoantipyrine (AP). Control – treatment with plain medium; AP – treatment with 1 mM AP; API 25 + AP – treatment with 25 μM apigenin + 1 mM AP; TM-API 25 + AP - treatment with 25 μM trimethylapigenin + 1 mM AP; QUE 25, 50 + AP – treatment with 25 and 50 μM quercetin + 1 mM AP, respectively; 3M-QUE 25, 50 + AP – treatment with 25 and 50 μM 3-O-methylquercetin + 1 mM AP, respectively; 3′7DM-QUE 25, 50 + AP – treatment with 25 and 50 μM 3′7-di-O-methylquercetin + 1 mM AP, respectively. Data are shown as means with standard deviation, and expressed as relative luminescence, considering the mean value of control as 100%, $n = 4$/group. Significant difference compared to the untreated control: $^{*}p < 0.05$

However, there are fewer studies available about the CYP3A4/CYP3A29 enzyme, which is one of the most important enzymes in drug metabolism.

Generally, those flavonoids that contain one or more hydroxyl groups are likely to have an inhibitory effect on CYP enzymes, while those compounds that lack hydroxyl groups are proposed to behave as CYP inducers (Ho et al., 2001; Hodek et al., 2002; Quintieri et al., 2008). The presence and number of hydroxyl groups determine the selectivity of flavonoids to the CYP1 enzyme family: those substances, which lack hydroxyl groups on the B-ring (e.g. chrysin) inhibit mainly CYP1A2, while those compounds that have two hydroxyl groups on the same ring (e.g. luteolin) alter mostly the activity of CYP1A1 (Kim et al., 2005). Furthermore, the presence of hydroxyl groups on the A-ring and methoxy groups on the B-ring impact their effect on CYP1B1 enzyme. For example, chrysoeriol, which is a naturally occurring 3′-methoxyflavonoid, has shown to exert a five times greater inhibitory effect on CYP1B1 than on CYP1A1 (Takemura et al., 2010). According to Androutsopoulos et al. (2010), a hydroxyl group in position 3, and the B-ring without substituents increase the inhibitory activity on the CYP1 family. In a human microsomal study on CYP3A enzyme family, it has been shown that those flavonoids, which had no or only one hydroxyl group (e.g. 3-hydroxyflavone, 5-hydroxyflavone, 7-hyroxyflavone) could inhibit CYP3A4 enzyme to a lesser extent, than flavonoids carrying several hydroxyl groups, such as apigenin, chrysin, kaempferol, quercetin, myricetin and diosmetin (Tsujimoto et al., 2009). These results are consistent with the finding of Quintieri et al. (2008), according to which slavigenin, containing only one hydroxyl group, has shown only moderate inhibitory effect on the metabolism of midazolam, while diosmetin and luteolin (possessing more than two hydroxyl groups) have inhibited the metabolism in a concentration-dependent manner. Regarding the position of the substituents, studies have shown that hydroxyl groups at position C 5, 7 and 4′ result in a potent inhibitory effect on CYP3 enzymes, but the presence of a methoxy group in 4′ position reduces the inhibitory effect (Tsujimoto et al., 2009). In contrast with these findings, Androutsopoulos et al. (2010) have concluded, that a methoxy group at position 4′ increases the inhibitory activity on the CYP1 family, while if it is present at position 6 or 7, the inhibition is reduced. However, Wen et al. (2005) have observed remarkable CYP1A1 and CYP1B1 inhibition by 5,7-dimethoxyflavone in Hep G2 tumorous hepatocyte cell line.

Besides the number and position of hydroxyl and methoxy groups, other structural properties might also influence the CYP modulating activity of flavonoids. Small molecules with plane structure have a potent inhibitory effect on the CYP1A2 enzyme. In contrast, flavonones and flavans, which lack the C2-C3 double bond and have a phenyl group on the B-ring, that is almost perpendicular to the molecular surface, inhibit the enzyme to a lesser extent. The same has been experienced in case of CYP3A4: the studied flavones with double bond could inhibit the enzyme to a greater extent, compared to flavonones without double bond (Tsujimoto et al., 2009). Androutsopoulos et al. (2010) have also concluded that the presence of C2-C3 double bond increases the CYP1 inhibitory activity. Based on the observation, that catechins do not alter CYP enzyme activity, it can be stated that a keto group in C4 position is also an important inhibitory factor (Moon et al., 1998).

In our study, inhibition of the CYP3A29 enzyme by different flavonoids was observed after 2 h treatment in a non-transformed, non-tumorigenic porcine intestinal epithelial cell line. API and TM-API could both inhibit CYP3A29, which effect was observed even during co-administration with

4-aminoantipyrine in both cases. There was no significant difference between API and its methylated analogue alone, however, when they were combined with phenobarbital, only TM-API could preserve its inhibitory activity on CYP3A29. QUE and 3M-QUE were also able to significantly inhibit CYP3A29 activity and to show inhibitory effect even in combination with phenobarbital. QUE in 50 µM concentration could further increase inhibition resulting from the 4-aminoantipyrine treatment. API, QUE and 3M-QUE contain hydroxyl groups at position 5, 7 and 4′, which can lead to an enhanced inhibitory activity based on the relevant literature (Tsujimoto et al., 2009; Kim et al., 2000). However, 3′7DM-QUE, that has a methoxy group instead of a hydroxyl group at position 7, did not affect the enzyme activity. In contrast, TM-API, which also has methoxy groups at position 5, 7 and 4′, did show inhibitory effect, therefore, it can be presumed that in case of 3′7DM-QUE, lack of effect was not due to the methoxy group at position 7, which is in line with the findings of Wen et al. (2005). The lack of effect might be correlated with the presence of a methoxy group in position 3′, but this theory needs further research to be proved. In case of CYP1B1 and CYP1A1, the enzyme-modulating effect of 3′,7-dimethoxyflavone has been investigated in a study that focused on the anticancer activity of flavonoids. In this experiment, 3′,7-dimethoxyflavone has been found to significantly inhibit the expression of CYP1A1 and CYP1B1, that were induced by benzo(a)pyrene in human squamous cell carcinoma (SCC-9) cells (Walle and Walle, 2007). The effect of methoxylated flavones on CYP3A4/CYP3A29 is barely known, this area requires further research.

Even though most studies focus on CYP enzymes in the liver, our results underline the importance of studying intestinal metabolism and support the need for further investigations on intestinal cell lines such as the IPEC-J2. Limited information is available about the effect of flavonoids on the metabolism of these cells, although they offer an opportunity to investigate the activity of a main Phase I enzyme, CYP3A4/CYP3A29.

Our results suggest that API, QUE and their methylated derivatives can inhibit the porcine intestinal CYP3A29 enzyme activity. This finding is of greatest importance in the case of concurrent medication and the consumption of flavonoid-containing foods, feeds or supplements, as these may result in interactions that lead to changes in the efficacy or safety of the administered drugs. It is important to note that the assay used in this study is not fully specific to CYP3A29, and the detected enzyme activities may include an overlap between the activity of CYP3A29 and other CYP3A enzymes. Nevertheless, it can be concluded that there is a need to test and consider the effect of flavonoids not only on CYP enzymes in the liver, but also in the GI tract.

ACKNOWLEDGMENTS

We are grateful to Dr. Jody Gookin for providing the IPEC-J2 cell line and to Lubov Nyikolajevna Bakumenko for contributing to the investigations. This research was funded by the European Union and co-financed by the European Social Fund (grant agreement no. EFOP-3.6.3-VEKOP-16-2017-00005, project title: „Strengthening the scientific replacement by supporting the academic workshops and programs of students, developing a mentoring process"). Funding was received from project no. TKP2020-NKA-01 that has been implemented with the support provided from the National Research, Development and Innovation Fund of Hungary, financed under the Tématerületi Kiválósági Program 2020 (2020-4.1.1-TKP2020) funding scheme.

REFERENCES

Androutsopoulos, V. P., Papakyriako, A., Vourloumis, D., Tsatsakis, A. M. and Spandidos, D. A. (2010): Dietary flavonoids in cancer therapy and prevention: substrates and inhibitors of cytochrome P450 CYP1 enzymes. Pharmacol. Ther. **126**(1), 9–20.

Ayuso, M., Van Cruchten, S. and Van Ginneken, C. (2020): A medium-throughput system for *in vitro* oxidative stress assessment in IPEC-J2 cells. Int. J. Mol. Sci. **21**, 7263.

Bailey, D. G., Malcolm, J., Arnold, O. and Spence, J. D. (1998): Grapefruit juice–drug interactions. Br. J. Clin. Pharmacol. **46**(2), 101–110.

Brosnahan, A. J. and Brown, D. R. (2012): Porcine IPEC-J2 intestinal epithelial cells in microbiological investigations. Vet. Microbiol. **156**, 229–237.

Cencic, A. and Langerholc, T. (2010): Functional cell models of the gut and their applications in food microbiology — a review. Int. J. Food Microbiol. **141**, S4–S14.

Egert, S. and Rimbach, G. (2011): Which sources of flavonoids: complex diets or dietary supplements? Adv. Nutr. **2**(1), 8–14.

Ekstrand, B., Rasmussen, M. K., Woll, F., Zlabek, V. and Zamaratskaia, G. (2015): *In vitro* gender-dependent inhibition of porcine cytochrome P450 activity by selected flavonoids and phenolic acids. BioMed Res. Int. **2015**, 387918.

Farkas, O., Palócz, O., Pászti-Gere, E. and Gálfi, P. (2015): Polymethoxyflavone apigenin-trimethylether suppresses LPS-induced inflammatory response in nontransformed porcine intestinal cell line IPEC-J2. Oxid. Med. Cell. Longev. **2015**, 673847.

Galvez, J., Sanchez De Medina, F., Jimenez, J. and Zarzuelo, A. (2001): Effects of flavonoids on gastrointestinal disorders. Stud. Nat. Prod. Chem. **25**, 607–649.

Gonzales, G. B., Van Camp, J., Smagghe, G., Raes, K. and Mackie, A. (2016): Flavonoid–gastrointestinal mucus interaction and its potential role in regulating flavonoid bioavailability and mucosal biophysical properties. Food Res. Int. **88**, 342–347.

Hebert, M., Roberts, J., Prueksaritanont, T. and Benet, L. (1992): Bioavailability of cyclosporine with concomitant rifampin administration is markedly less than predicted by hepatic enzyme induction. Clin. Pharmacol. Ther. **52**(5), 453–457.

Ho, P. C., Saville, D. J. and Wanwimolruk, S. (2001): Inhibition of human CYP3A4 activity by grapefruit flavonoids, furanocoumarins and related compounds. J. Pharm. Sci. **4**(3), 217–227.

Hodek, P., Trefil, P. and Stiborov, A. M. (2002): Flavonoids-potent and versatile biologically active compounds interacting with cytochromes P450. Chem. Biol. Interact. **139**(1), 1–21.

Kamboh, A. A., Arain, M. A., Mughal, M. J., Zaman, A., Arain, Z. M. and Soomro, A. H. (2015): Flavonoids: health promoting phytochemicals for animal production–a review. J. Anim. Health Prod. **3**(1), 6–13.

Kim, H. J., Chun, H. S. and Yang, R. (2000): Inhibition of benzo[a] pyrene-induced cytotoxicity and cytochrome P450 1A activity by dietary flavonoids in human liver cell model: structure-activity relationship. Biotechnol. Lett. **22**, 1941–1946.

Kim, H. J., Lee, S. B., Park, S. K., Kim, H. W., Park, Y. I. and Dong, M. S. (2005): Effects of hydroxyl group numbers on the B-ring of 5,7-dihydroxyflavones on the differential inhibition of human CYP 1A and CYPIB1 enzymes. Arch Pharm. Res. (Seoul) **28**(10), 1114–1121.

Kolars, J. C., Awni, W. M., Merion, R. M. and Watkins, P. B. (1991): First-pass metabolism of cyclosporin by the gut. Lancet **338**(8781), 1488–1490.

Lai, C. S., Li, S., Chai, C. Y., Lo, C. Y., Ho, C. T., Wang, Y. J. and Pan, M. H. (2007): Inhibitory effect of citrus 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone on 12-O-tetradecanoylphorbol 13-acetate-induced skin inflammation and tumor promotion in mice. Carcinogenesis **28**(12), 2581–2588.

Lin, J. H. and Lu, A. Y. H. (1998): Inhibition and induction of cytochrome P450 and the clinical implications. Clin. Pharmacokinet. **35**(5), 361–390.

Moon, J. Y., Lee, D. W. and Park, K. H. (1998): Inhibition of 7-ethoxycoumarin O-deethylase activity in rat liver microsomes by naturally occurring flavonoids: structure–activity relationships. Xenobiotica **28**(2), 117–126.

Murakami, A., Shigemori, T. and Ohigashi, H. (2005): Zingiberaceous and citrus constituents, 1'-acetoxychavicol acetate, zerumbone, auraptene, and nobiletin, suppress lipopolysaccharide-induced cyclooxygenase-2 expression in RAW264.7 murine macrophages through different modes of action. J. Nutr. **135**, 2987S–2992S.

Murray, M. (2006): Altered CYP expression and function in response to dietary factors: potential roles in disease pathogenesis. Curr. Drug. Metab. **7**(1), 67–81.

Nossol, C., Barta-Böszörményi, A., Kahlert, S., Zuschratter, W., Faber-Zuschratter, H., Reinhardt, N., Ponsuksili, S., Wimmers, K., Diesing, A. K. and Rothkötter, H. J. (2015): Comparing two intestinal porcine epithelial cell lines (IPECs): morphological differentiation, function and metabolism. PLoS One **10**(7), e0132323.

Paine, M. F., Hart, H. L., Ludington, S. S., Haining, R. L., Rettie, A. E. and Zeldin, D. C. (2006): The human intestinal cytochrome P450 „pie". Drug Metab. Dispos. **34**(5), 880–886.

Paine, M. F., Khalighi, M., Fisher, J. M., Shen, D. D., Kunze, K. L., Marsh, C. L., Perkins, J. D. and Thummel, K. E. (1997): Characterization of interintestinal and intraintestinal variations in human CYP3A dependent metabolism. J. Pharmacol. Exp. Ther. **283**, 1552–1562.

Paine, M. F., Shen, D. D., Kunze, K. L., Perkins, J. D., Marsh, C. L., Mcvicar, J. P., Barr, D. M., Gillies, B. S. and Thummel, K. E. (1996): First-pass metabolism of midazolam by the human intestine. Clin. Pharmacol. Ther. **60**(1), 14–24.

Palócz, O., Farkas, O., Clayton, P. and Csikó, Gy. (2017): Changes in cytochrome P450 gene expression and enzyme activity induced by xenobiotics in rabbits *in vivo* and *in vitro*. World Rabbit Sci. **25**, 173–180.

Quintieri, L., Palatini, P., Nassi, A., Ruzza, P. and Floreani, M. (2008): Flavonoids diosmetin and luteolin inhibit midazolam metabolism by human liver microsomes and recombinant CYP3A4 and CYP3A5 enzymes. Biochem. Pharmacol. **75**(6), 1426–1437.

Repetto, G., del Peso, A. and Zurita, J. L. (2008): Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nat. Protoc. **3**, 1125–1131.

Scalbert, A., Manach, C., Morand, C., Remesy, C. and Jimenez, L. (2005): Dietary polyphenols and the prevention of diseases. Crit. Rev. Food Sci. Nutr. **45**(4), 287–306.

Schierack, P., Nordhoff, M., Pollmann, M., Weyrauch, K. D., Amasheh, S., Lodemann, U., Jores, J., Tachu, B., Kleta, S., Blikslager, A., Tedin, K. and Wieler, L. H. (2006): Characterization of a porcine intestinal epithelial cell line for *in vitro* studies of microbial pathogenesis in swine. Histochem. Cell Biol. **125**, 293–305.

Takemura, H., Uchiyama, H., Ohura, T., Sakakibara, H., Kuruto, R., Amagai, T. and Shimoi, K. (2010): A methoxyflavonoid, chrysoeriol, selectively inhibits the formation of a carcinogenic estrogen metabolite in MCF-7 breast cancer cells. J. Steroid Biochem. Mol. Biol. **118**(1–2), 70–76.

Tang, M., Ogawa, K., Asamoto, M., Hokaiwado, N., Seeni, A., Suzuki, S., Takahashi, S., Tanaka, T., Ichikawa, K. and Shirai, T. (2007): Protective effects of citrus nobiletin and auraptene in transgenic rats developing adenocarcinoma of prostate (TRAP) and human prostate carcinoma cell. Cancer Sci. **98**(4), 471–477.

Tsujimoto, M., Horie, M., Honda, H., Takara, K. and Nishiguchi, K. (2009): The structure–activity correlation on the inhibitory effects of flavonoids on cytochrome P450 3A activity. Biol. Pharm. Bull. **32**(4), 671–676.

Verhoeckx, K., Cotter, P., López-Expósito, I., Kleiveland, C., Lea, T., Mackie, A., Requena, T., Swiatecka, D. and Wichers, H. (2015): The Impact of Food Bioactives on Health: *in Vitro* and *Ex Vivo* Models. Springer International Publishing: Switzerland, 2015, 125–129.

Von Richter, O., Greiner, B., Fromm, M. F., Fraser, R., Omari, T., Barclay, M. L., Dent, J., Somogyi, A. A. and Eichelbaum, M. (2001): Determination of *in vivo* absorption, metabolism and transport of drugs by the human intestinal wall and liver with a novel perfusion technique. Clin. Pharmacol. Ther. **70**(3), 217–227.

Wacher, V., Salphati, L. and Benet, L. (2001): Active secretion and enterocytic drug metabolism barriers to drug absorption. Adv. Drug Deliv. Rev. **46**(1–3), 89–102.

Walle, T. and Walle, U. K. (2007): Novel methoxylated flavone inhibitors of cytochrome P450 1B1 in SCC-9 human oral cancer cells. J. Pharm. Pharmacol. **59**(6), 857–862.

Wanwimolruk, S. and Prachayasittikul, V. (2014): Cytochrome P450 enzyme mediated herbal drug interactions (Part 1). EXCLI J. **13**, 347–391.

Wen, X. and Walle, T. (2006): Methylated flavonoids have greatly improved intestinal absorption and metabolic stability. Drug Metab. Dispos. **34**(10), 1786–1792.

Wen, X., Walle, U. K. and Walle, T. (2005): 5,7-Dimethoxyflavone downregulates CYP1A1 expression and benzo[a]pyrene-induced DNA binding in Hep G2 cells. Carcinogenesis **26**(4), 803−809.

Wilkinson, G. R. (2005): Drug metabolism and variability among patients in drug response. N. Engl. J. Med. **352**, 2211–2221.

Williams, R. J., Spencer, J. P. E. and Rice-Evans, C. (2004): Flavonoids: antioxidants or signalling molecules? Free Radic. Biol. Med. **36**(7), 838–849.

Yao, M., Dai, M., Liu, Z., Huang, L., Chen, D., Wang, Y., Peng, D., Wang, X., Liu, Z. and Yuan, Z. (2011): Comparison of the substrate kinetics of pig CYP3A29 with pig liver microsomes and human CYP3A4. Biosci. Rep. **31**, 211–220.