

Artigo

Effect of food phenolic compounds on the activity of rat liver CYP2C subfamily enzymes evaluated by a newly validated method of high-performance liquid chromatography

Ramos, C. H.; Rolim, T. S.; Souza, T. P.; Moreira, D. L.;* Paumgarten, F. J. R.; De-Oliveira, A. C. A. X.

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Efeito de compostos fenólicos encontrados em alimentos sobre a atividade de enzimas da subfamília CYP2C do fígado de ratos avaliado por um novo método validado de cromatografia em fase líquida de alta eficiência

Resumo: Neste estudo, avaliamos os efeitos inibitórios de ácidos fenólicos (ácidos cafeico, ferúlico, *p*-cumárico e vanílico) e flavonoides (hesperidina, quercetina e rutina) sobre a atividade de CYP2C12/11 (ortólogos da CYP2C9 humana). Um novo método de cromatografia em fase líquida de alta eficiência (HPLC-DAD) foi desenvolvido e validado para quantificar diclofenaco (DCF, substrato para CYP2C9) e 4'-hidroxiclofenaco (4'-OH-DCF, produto da reação) em microsossomos hepáticos de rato. Os efeitos fenólicos (100 µM) e do fluconazol (controle positivo, 100 µM) sobre a hidroxilação do DCF (marcador para atividade de CYP2C9) foram testados em microsossomos hepáticos de ratos Wistar fêmeas induzidos por tratamento com rifampicina. Quercetina, hesperidina e rutina apresentaram um efeito inibitório modesto (15-21 % de inibição) sobre a atividade catalisada pela CYP2C no fígado de ratos, enquanto os ácidos fenólicos testados não exibiram qualquer efeito. Em conjunto, estes resultados indicaram que, nos níveis encontrados na dieta humana, é improvável que os fenólicos testados afetem o perfil de eliminação de fármacos e outros xenobióticos mediados por enzimas da subfamília CYP2C em ratos.

Palavras-chave: Citocromo P450, CYP2C, farmacocinética, interação medicamentosa, produtos naturais.

Abstract

In this study, we evaluated the inhibitory effects of phenolic acids (caffeic, ferulic, *p*-coumaric and vanillic acids) and flavonoids (hesperidin, quercetin and rutin) on the CYP2C11/12 (rat orthologs of human CYP2C9) activity. A new high performance liquid chromatographic (HPLC-DAD) method was developed and validated to measure diclofenac (DCF, CYP2C9 substrate) and 4'-hydroxy diclofenac (4'-OH-DCF, reaction product) in rat liver microsomes. The effect of phenolic compounds (PCs, 100 µM) and fluconazole (positive control, 100 µM) on the hydroxylation of diclofenac (CYP2C9 activity marker) were tested in rifampicin-induced rat liver microsomes. Quercetin, hesperidin and rutin exhibited a modest (15-21 % inhibition) inhibitory effect on rat liver CYP2C-catalyzed activity, while no effect was noted with the tested phenolic acids. Overall, these findings indicated that, at levels found in the human diet, tested PCs are unlikely to inhibit CYP2C-mediated clearance of drugs and other xenobiotics in the rat.

Keywords: Cytochrome P450; CYP2C; pharmacokinetics; food-drug interaction; plant products.

* Fundação Oswaldo Cruz, Instituto de Tecnologia em Fármacos-ITF, Rua Sizenando Nabuco 100, CEP 21041-000, Manguinhos, Rio de Janeiro-RJ, Brasil.

Fundação Oswaldo Cruz, Laboratório de Toxicologia Ambiental-LTA, Av. Brasil 4036, Manguinhos, CEP 21040-361, Rio de Janeiro-RJ, Brasil.

✉ davyson.moreira@far.fiocruz.br

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Effect of food phenolic compounds on the activity of rat liver CYP2C subfamily enzymes evaluated by a newly validated method of high-performance liquid chromatography

Carlos H. Ramos,^a Thais S. Rolim,^a Thamyris P. de Souza,^a Davyson L. Moreira,^{a,b,*} Francisco J. R. Paumgarten,^a Ana C. A. X. De-Oliveira^a

^a Fundação Oswaldo Cruz, Laboratório de Toxicologia Ambiental-LTA, Av. Brasil 4036, Manguinhos, CEP 21040-361, Rio de Janeiro-RJ, Brasil.

^b Fundação Oswaldo Cruz, Instituto de Tecnologia em Fármacos-ITF, Rua Sizenando Nabuco 100, Manguinhos, CEP 21041-000, Rio de Janeiro, RJ, Brasil.

* davyson.moreira@far.fiocruz.br

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1. Introduction

Phenolic acids and flavonoids are plant phenolic compounds (PCs) present in variable amounts in tubers (sweet potato, burdock root), fruits (peach, apple and pear), vegetables (pumpkin), seeds (corn, beans), spices and herbs (thyme, sage, basil), wine,

olive oil, chocolate, coffee and mate tea.^{1,2} Phenolic acids contain one or more free phenolic hydroxyl and carboxyl groups (mainly C₆-C₁ and C₆-C₃ skeleton) while flavonoids are plant (or fungus) secondary metabolites with a variable polyphenolic structure.³ Based on their flavan nucleus structure (C₆-C₃-C₆ skeleton), flavonoids are classified as flavanones, isoflavanones, flavones,

isoflavones, anthocyanidins, chalcones or flavolignans.^{4,5} There are over 9,000 flavonoids described in the literature, and estimates of daily intake of flavonoids range from 20 mg to 500 mg.⁵ A major contribution to daily intake of flavonoids comes from consumption of dietary supplements and food and beverages such as tea, red wine, apples, onions, tomatoes and others.^{5,6}

Several plant-derived PCs exhibit strong antioxidant activity in *in vitro* and *in vivo* assays. Moreover, experimental evidence suggests that PCs possess a diversity of pharmacological activities of therapeutic interest, such as antibacterial, antiviral, anti-inflammatory, hepatoprotective, immunomodulating and anti-carcinogenic effects.^{2,3,5,6,7,8}

In fact, a number of epidemiology observational studies lend support to the notion that dietary PCs are beneficial to health. Diets containing high amounts of PCs, for instance, have been associated with decreased risks of lung, breast, prostate and colorectal cancers, tumors of other sites and other chronic-degenerative diseases.^{9,10,11,12,13}

Although it is accepted that PCs are, as a rule, healthy food constituents, the hypothesis that some of these compounds might eventually change the clearance of drugs and other xenobiotics deserves to be analysed. This would not be surprising because several edible and medicinal plant

products interfere with drug clearance and by doing so they, at times, dramatically modify therapeutic and adverse effects. A good example along this line is the strong inhibitory effect of grapefruit juice on CYP3A4-mediated drug clearance.¹⁴ Along the same line, CYP3A4- and 2B6- inducing effects of Saint John's wort (*Hypericum perforatum*), and of its constituent hyperforin, also illustrate that some herbs and medicinal plants markedly alter pharmacokinetics and clearance of other medications.¹⁵ Hyperforin is a ligand of pregnane X nuclear receptor (PXR), a ligand-activated transcription factor, the activation of which promotes CYP3A4 transcription.¹⁵

The CYP2C isoforms are the second most abundant CYP enzymes in the liver where enzymes of this subfamily account for the oxidative metabolism of many routinely used drugs, including non-steroidal anti-inflammatory agents such as diclofenac, ibuprofen and piroxicam.^{16,17}

The objective of this study was to evaluate whether plant phenolic acids (caffeic, ferulic, *p*-coumaric and vanillic) and flavonoids (hesperidin, quercetin and rutin) inhibit CYP2C activity in rat liver microsomes. The conversion of diclofenac (DCF, a putative substrate for CYP2C9) into its primary oxidation metabolite (4'-hydroxy diclofenac, 4'-OH-DCF) (Figure 1) by rat liver microsomes was used to study the effects of PCs on CYP2C-catalyzed activity.

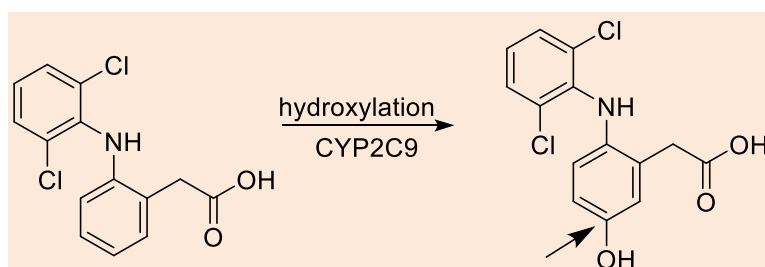


Figure 1. Hydroxylation of diclofenac (DCF) to 4'-hydroxy diclofenac (4'-OH-DCF) by CYP2C9. Arrow indicates the hydroxylation position

2. Experimental

2.1. Inhibition of CYP2C mediated activity in rat liver microsomes

To investigate whether test compounds were capable of inhibiting CYP2C-mediated activity we used an *in vitro* rat liver microsomal fraction (LMF) assay. Aliquots of previously prepared rifampicin (RIF)-induced liver microsomes (300-450 mg/kg bwt/day x 5 days, *po*, female Wistar rats) stored in liquid nitrogen were used.¹⁸ The LMF was prepared essentially as described elsewhere (De-Oliveira *et al* 1997). After thawing the frozen samples of RIF-induced LMF, the microsomal protein concentration was measured by a spectrophotometric method using Coomassie blue dye (Bradford method) and bovine serum albumin as standard.^{19,20} Total-protein concentration in the LMF was expressed as mg of protein per mL. The experimental protocol was approved by the Ethics Committee on Animal Use of FIOCRUZ (2012, P-84/10-7).

The enzyme-mediated conversion of DCF into 4'-OH-DCF (catalyzed by CYP2C9 in humans) was used as a marker for CYP2C activity in the rat liver microsomal fraction (CYP2C11/12). To measure the accumulation of reaction product 4-hydroxy-diclofenac (4'-OH-DCF), a high-performance liquid chromatographic method was developed and validated.

2.2. Diclofenac hydroxylation activity

To standardize DCF hydroxylation assay with rat LMF, we evaluated how DCF hydroxylation activity varies over time for different protein concentrations (0.25; 0.5; 1 and 2 mg/ mL). The incubation of reaction mixture containing rat liver microsomal fraction, substrate (DCF), Tris buffer and cofactor solution took place for 10, 20 or 30 min at 37 °C in a shaking water bath (Heto®). 20 mM Tris buffer solution (pH 7.5), 100 µM

DCF and a cofactor solution (0.5 mM β-NADP, 1.24 mM G6P, 0.8 mM MgCl₂ and 0.4 U/ mL G6PD) were added to 1.5 mL eppendorf tubes for a final volume of 250 µL. A reaction mixture containing only the substrate (DCF), Tris buffer and the cofactor solution was pre-incubated for 3 min. The hydroxylation reaction was then initiated by addition of rat LMF into the reaction mixture. After incubation for 10, 20 or 30 min, reaction was stopped by adding acetonitrile and glacial acetic acid (94:6) solution. Tubes with the reaction mixture were subsequently centrifuged at 12,000 x *g* for 4 min to precipitate proteins. The supernatants were then removed to measure the amount of reaction product (4'-hydroxy diclofenac, 4'-OH-DCF) formed. A reaction mixture not containing the microsomal fraction was the negative control (in the absence of the enzyme no 4'-OH-DCF was produced during incubation). Analysis of a set of curves of activity *versus* time for several concentrations of microsomal protein indicated that 1 mg/mL protein and 20 min reaction time were assay conditions ensuring a linear accumulation of reaction product over time. Fluconazole was chosen as positive control for CYP2C inhibition.²¹

2.3. High-Performance Liquid Chromatographic analysis coupled with Diode-Array Detector (HPLC-DAD)

Determinations of DCF and 4'-OH-DCF in the supernatant of rat LMF reaction mixture were performed by using a Shimadzu Nexera® XR, equipped with LC-20AD binary pump, SIL-20AF automatic injector, CTO-20A oven, DAD-UV-VIS SPD- 20MA detector and CBM-20A controller. Chromatograms were visualized through Labsolutions software (Shimadzu®). The analytical method was developed in accordance with BD Bioscience® with a few adaptations. Validation parameters of selectivity, linearity, limits of identification and quantification were determined according to Normative Instruction DOQ-

CGCRE 008 of INMETRO (2008). Different columns and mobile phases were tested to select the most appropriate conditions for 4'-OH-DCF (reaction product) and DCF (substrate) chromatographic analysis. The ultrapure water of mobile phase was from Milli-Q-MiliREP filtration system (Merck), methanol and acetonitrile were from Tedia (Brazil), and acetic acid from Sigma-Aldrich Co.

2.4. Inhibition of CYP2C by phenolic acids and flavonoids

Test compounds (caffeic, *p*-coumaric, ferulic and vanillic acids; rutin, quercetin and hesperidin flavonoids) and positive control (fluconazole) were all dissolved in 20 mM Tris buffer solution (pH 7.5) and added in volumes and concentrations needed to attain a final concentration of 100 μ M in the reaction mixture.

2.5. Statistical analysis

Data were analyzed by ANOVA followed by the Bonferroni *post-hoc* multiple comparison test, when applicable. Differences were considered significant when $p < 0.05$. Statistical analyses and graphics were done using Graphpad Prism5®.

3. Results and Discussion

3.1. Quantification of the reaction product

The chromatographic parameters for separation and quantification of 4'-OH-DCF (reaction product) by HPLC-DAD-UV or UPLC-DAD-UV were determined by using a standard solution in water (25 μ g/mL). Combinations of mobile and stationary phases conditions tested are shown on Table 1. Oven temperature (50 °C) and injection volumes

(ranging from 5 to 20 μ L) were maintained constant in the tests.

The chromatographic condition with the best result in terms of capacity factor (k' 4'-OH-DCF = 5.8; k' DCF = 10.6), signal symmetry (~ 1 for both compounds), retention time, t_R , (4'-OH-DCF 4.89 - 4.95 min; DCF 8.32 - 8.40 min), resolution ($R = 6$) and total analysis time for diclofenac (DCF) separation and 4'-OH-DCF quantification was achieved with UPLC-DAD-UV, using a Supelco Ascentis Express C18 column (75 mm x 2.1 mm i.d. x 2.7 μ m particle size), gradient elution 3 using ultrapure water (Milli-Q-MiliREP) acidified with glacial acetic acid (pH = 3.0), mobile phase B and acetonitrile (ACN) in mobile phase A (acetonitrile (A)/ ultrapure acidified water (B) – initial: 30 (A)/ 70 (B); then 4 min: 50 (A)/ 50 (B); then 6 min: 70 (A)/ 30 (B); then 6-12 min: 70 (A)/ 30 (B); then 12.01: initial condition to re-equilibrate – 5 min), with a flow rate at 0.25 mL/ min, oven temperature 50 °C and injection volume of 1 μ L, for a total analysis time of 17 min (condition 7, Table 1, Figure 2).

The maximum wavelength (λ) obtained by the ultraviolet spectrum for 4'-OH-DCF was 264 - 267 nm and for DCF was 275 - 277 nm. The t_R for 4'-OH-DCF was registered at 4.89 - 4.95 min and for DCF at 8.32 - 8.40 min, demonstrating a complete separation of compounds and, therefore, the method **selectivity**.

The analytical curves obtained for 4'-OH-DCF were done in the concentration range of 3.125 to 50.00 μ g/ mL in three different days. The analytical curves showed a positive linear correlation > 0.99 , therefore, showing excellent **linearity** (r^2 day 1 = 0.9935; r^2 day 2 = 0.9972; r^2 day 3 = 0.9903). Equation 1 was obtained by linear regression and used for quantification of 4'-OH-DCF, and was calculated by the mean and standard deviation of the linear and angular coefficients of the three equations obtained on the three different days:

Equation 1. Concentration μ g/mL of 4'-OH-DCF = (ABS + 1349 \pm 621)/ 7181 \pm 294

Table 1. Analytical parameters for separation of 4'-OH-DCF and DCF and quantification of 4'-OH-DCF by HPLC-DAD-UV or UPLC-DAD-UV

Condition	Mobile Phase (v/v) %	Stationary Phase
1	acetonitrile (50)/ ultrapure acidified water (50)	ACE C18 (250 mm x 4.6 mm i.d. x 5 µm particle size), flow rate at 1 mL/min
2	acetonitrile (70)/ ultrapure acidified water (30)	ACE C18 (250 mm x 4.6 mm i.d. x 5 µm particle size), flow rate at 1 mL/min
3	acetonitrile (75)/ ultrapure acidified water (25)	ACE C18 (250 mm x 4.6 mm i.d. x 5 µm particle size), flow rate at 1 mL/min
4	acetonitrile (78)/ ultrapure acidified water (22)	ACE C18 (250 mm x 4.6 mm i.d. x 5 µm particle size), flow rate at 1 mL/min
5	acetonitrile (70)/ ultrapure acidified water (25)/ methanol (5) - gradient elution 1	ACE C18 (250 mm x 4.6 mm i.d. x 5 µm particle size), flow rate at 1 mL/min
6	kit BD Bioscience (acetonitrile/ ultrapure acidified water (A) methanol (B) – gradient elution 2	ACE C18 (250 mm x 4.6 i.d. mm x 5 µm particle size), flow rate at 1 mL/min
7	acetonitrile/ ultrapure acidified water – gradient elution 3	Supelco Ascentis Express C18 (75 mm x 2.1 mm i.d. x 2.7 µm particle size), flow rate at 0,25 mL/min

Oven temperature at 50 °C, injection volumes from 5 to 20 µL. Gradient elution 1: acetonitrile (A)/ ultrapure acidified water (B)/ methanol (C) – initial: 70 (A)/ 25 (B)/ 5 (C); then 4 min: 50 (A)/ 50 (B); then 6 min: 70 (A)/ 30 (B); then 6 – 12 min: 70 (A)/ 30 (B); then 12.01: initial condition to re-equilibrate – 5 min. Gradient elution 2: acetonitrile 30/ ultrapure acidified water 70 (A)/ methanol 100 (B) – initial: 80 (A)/ 20 (B); then 4 min: 50 (A)/ 50 (B); then 6 min: 50 (A)/ 50 (B); then 6 – 12 min: 60 (A)/ 40 (B); then 12.01: initial condition to re-equilibrate – 5 min. Gradient elution 3: acetonitrile (A)/ ultrapure acidified water (B) – initial: 30 (A)/ 70 (B); then 4 min: 50 (A)/ 50 (B); then 6 min: 70 (A)/ 30 (B); then 6-12 min: 70 (A)/ 30 (B); then 12.01: initial condition to re-equilibrate – 5 min (total run time = 17 min)

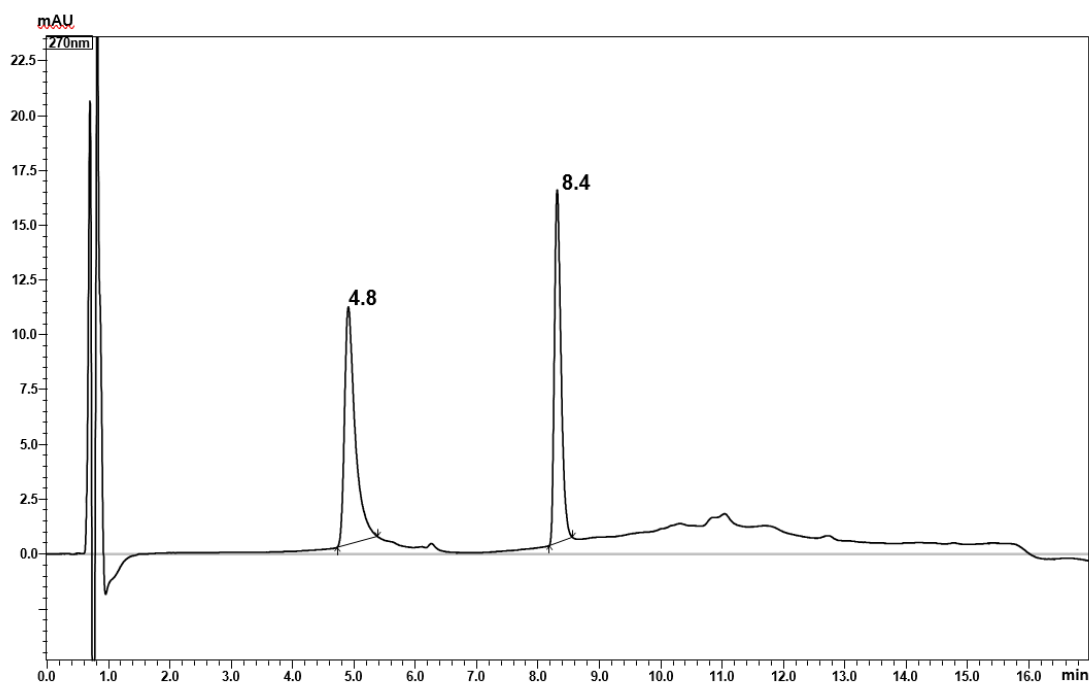


Figure 2. Chromatogram (270 nm) of a mixture of 4'-OH-DCF (water solution, 25 µg/mL) and DCF (water solution, 25 µg/mL): 4'-OH-DCF at retention time (tR) 4.8 min and DCF at tR 8.4 min

The **limit of detection (LOD)** and the **limit of quantification (LOQ)** were determined by successive dilutions as 100 for 4'-OH-DCF and 150 ng/mL for DCF.

Zi *et al.*²² used a Agilent Technologies C18 column (250 mm x 4.6 mm i.d. x 5 µm particle size) with a mobile phase consisting of ultrapure water acidified with perchloric acid with 20 % of acetonitrile and methanol (70:30 v/v), flow rate at 0.8 mL/ min, oven temperature at 40°C, and ultraviolet absorption spectrum at 280 nm. The tR were recorded at 14.5 and 18.5 min for 4'-OH-DCF and DCF, respectively. Another study conducted by Kimura *et al.*²³ used a YMC-Pack ODS a-302 (150 mm x 4.6 mm i.d.) column, flow rate at 1.0 mL/ min, oven temperature at 40 °C, and a gradient elution using in the mobile phase (A) water, acetonitrile and formic acid (69:30:1) and in the mobile phase (B) methanol; with ultraviolet detection at 280 nm for DCF and a total analysis time of 20 min. Our developed and validated method showed

excellent selectivity, signal symmetry, resolution for DCF and 4'-OH-DCF separation in total analysis time of 17 min (shorter analysis time), linearity, non-use of methanol in the mobile phase and, mainly, lower mobile phase consumption (about 4 mL/ run). Thus, the method developed and validated in this study has clear advantages over 4'-OH-DCF analysis methods published in the literature.

3.2. Diclofenac hydroxylation activity assay

DCF hydroxylation activity assay was standardized by varying concentrations of non-induced female rat LMF (0.25, 0.5, 1 and 2 mg/mL) and reaction times (10, 20 or 30 min). As shown in Figure 3, good linearity of product accumulation over time was obtained for 1 or 2 mg/ mL of protein and 20 min of reaction time.

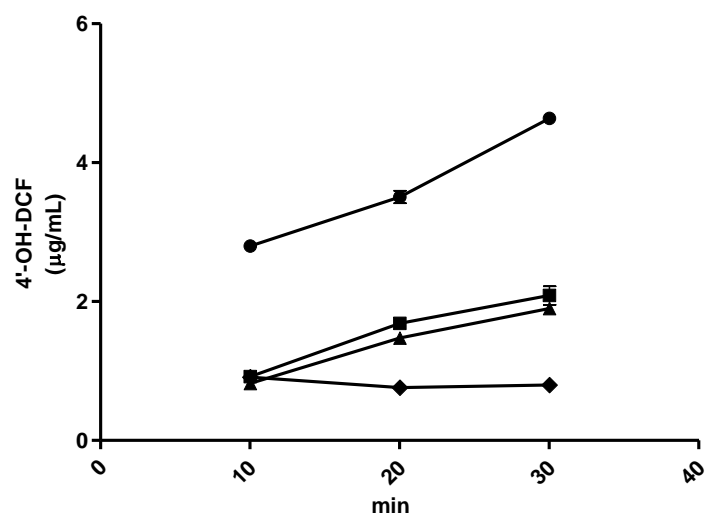


Figure 3. DCF hydroxylation assay: Accumulation of 4'-OH-DCF ($\mu\text{g}/\text{mL}$) over time (10, 20 or 30 min) for microsomal protein concentrations of 0.25 (\blacklozenge); 0.5 (\blacktriangle); 1.0 (\blacksquare) or 2.0 (\bullet) mg/mL . Liver microsomal fraction (LMF) was from untreated female Wistar rats

Figure 4 shows the relationship between the concentration of microsomal protein (1 or 2 mg/mL) of non-induced and RIF-induced LMFs (from female Wistar rats) and the accumulation of 4'-OH-DCF for a reaction time of 20 min. As expected, results demonstrated that the highest concentration of microsomal protein yielded a greater amount of reaction product (4'-OH-DCF). Moreover, results also confirmed that for identical microsomal

protein concentrations and reaction time (20 min), RIF-induced rat LMF yielded an amount of 4'-OH-DCF greater than the amount formed by untreated (non-induced) rat LMF. In other words, RIF treatment did in fact increase the activity (or relative abundance) of CYP enzymes that catalyze the conversion of DCF into its primary oxidation metabolite (4'-OH-DCF) in the rat liver.

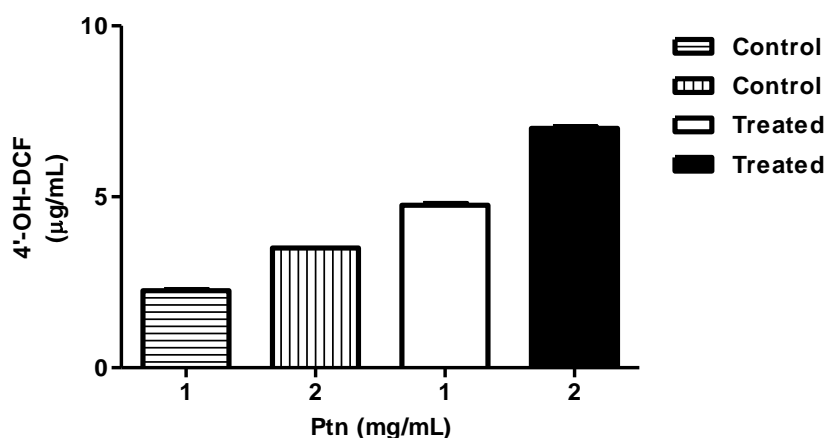


Figure 4. DCF hydroxylation assay. Formation of reaction product (4'-OH-DCF; $\mu\text{g}/\text{mL}$) as function of concentration of microsomal protein (1 and 2 mg/mL) for both non-induced (Control) and RIF-induced liver microsomal fractions (Treated) from female Wistar rats. Reaction time was 20 min

Based on the foregoing results, DCF hydroxylation assay to test PCs was conducted with RIF-induced rat LMF with microsomal protein concentration and reaction duration of 1 mg/mL and 20 min, respectively.

3.3. Inhibition of DCF hydroxylation (CYP2C) by phenolic acids and flavonoids

The four phenolic acids (caffeic **1**, ferulic **2**, *p*-coumaric **3** and vanillic **4**, Figure 5), tested at a concentration as high as 100 μ M in the assay medium, did not decrease DCF hydroxylation activity whereas fluconazole (100 μ M), a putative CYP2C9 inhibiting drug²¹, caused a nearly 24 % reduction in the hydroxylation activity (Table 2, Figure 6). The flavonoids hesperidin (**5**) and quercetin (**6**), also tested at 100 μ M, caused only a slight to moderate (15-21 %) inhibition of DCF-hydroxylation activity. Rutin (100 μ M), or quercetin 3-*O*-rutinoside (**7**) (Figure 7), decreased by approximately 21 % the DCF hydroxylation activity while fluconazole 100 μ M reduced it by 27 % or so (Table 3, Figure 8).

Martignoni *et al.*²¹ reported that rat tissues express several CYP2C family isoforms, including CYP2C6, CYP2C7, CYP2C11, CYP2C12, CYP2C13 and CYP2C22. The expression of a particular CYP2C isoform, however, varies with rat gender and age,

being generally higher in adult animals. CYP2C12 is highly expressed in the liver of female adult rats, whereas CYP2C11 is the predominant isoform in male rats, comprising up to 50 % of the total CYP content of the hepatic tissue. Humans express CYP2C8, CYP2C9 and CYP2C19 isoforms the expression of which is inducible by ligands known to activate PXR nuclear receptor, such as rifampicin and dexamethasone, or Constitutive Androstane Receptor (CAR), a nuclear factor typically activated by phenobarbital.²⁴ Therefore, it is fair to think that treatment with rifampicin (RIF) enhanced expression of CYP2C12 in the female rat liver. RIF, however, is also a classical inducer of CYP3A enzymes.²⁵

Kimura *et al.*²³ tested 60 polyphenols (at 100 μ M) for inhibitory effects on the 6 β -hydroxylation of testosterone by recombinant human CYP3A4 (rhCYP3A4) and hydroxylation of diclofenac (DCF) by recombinant human CYP2C9 (rhCYP2C9). The authors found that several coumarins, flavones and flavonoids strongly inhibited rhCYP3A4 and rhCYP2C9 (>80 %) whereas inhibitory effects of flavanones, chalcones and isoflavones on these enzymes are weaker (<50 %).²³ According to Kimura *et al.*, glycosylated flavonoids are weaker inhibitors of both enzymes (<55 %), while anthocyanidins and monoterpenes have a weak effect on rhCYP2C9.²³

Table 2. Activities (Mean \pm SE) of diclofenac hydroxylation (4'-OH-DCF; ng/mg ptn/min) in the absence (control) and in the presence (100 μ M) of caffeic, ferulic, *p*-coumaric and vanillic acids, as well as in the presence of the positive control fluconazole (100 μ M). Percentage of inhibition (%) is shown in brackets

control	caffeic	ferulic	<i>p</i> -cumaric	vanilic	fluconazole
Phenolic acid					
12.4 \pm 0.3	12.3 \pm 0.3	11.6 \pm 0.2	12.7 \pm 0.2	11.6 \pm 0.3	9.4 \pm 0.2
(100 %)	(0.8 %)	(0.6 %)	(0 %)	(0.6 %)	(24.2 %)

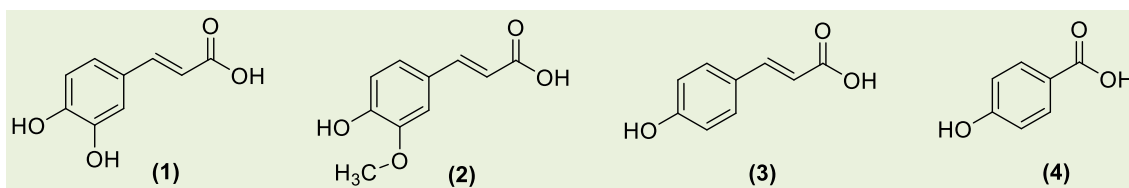


Figure 5. phenolic acids structures: 1 – caffeic; 2 – ferulic; 3 - *p*-coumaric and 4 – vanillic

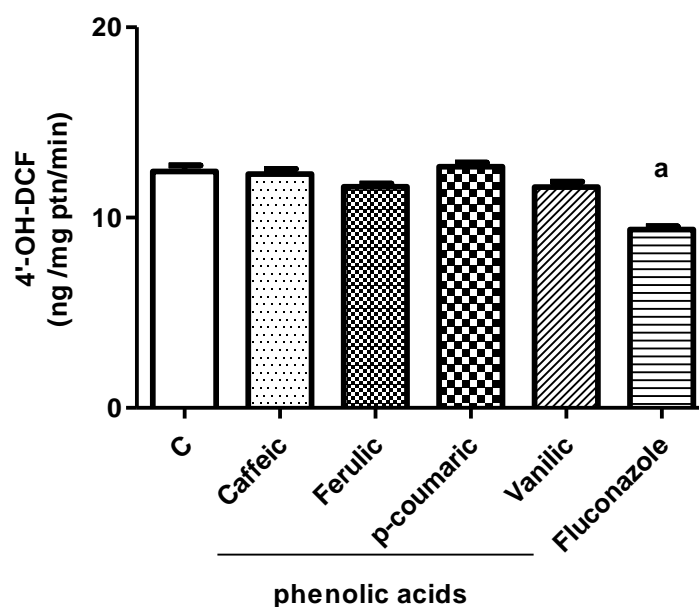


Figure 6. Absence of inhibitory effects of phenolic acids (caffeic, ferulic, *p*-coumaric and vanillic acids) on diclofenac (DCF) hydroxylation activity (4'-OH-DCF ng/ mg protein/ min). Rifampicin-induced liver microsomes from female Wistar rats. Histogram bar heights are Mean \pm SEM. ANOVA followed by the Bonferroni's multiple comparison test: a: \neq from control ($p < 0.05$)

Table 3. Activities (Mean \pm SE) of diclofenac hydroxylation (4'-OH-DCF ng/mg ptn/min) in the absence (control) and in the presence (100 μ M) of the flavonoids hesperidin, quercetin and rutin, as well as in the presence of the positive control fluconazole (100 μ M). Percentage of inhibition (%) is shown in brackets

control	hesperidin	quercetin	rutin	fluconazole
13.0 \pm 0.4	10.7 \pm 0.3	11.0 \pm 0.07	10.3 \pm 0.07	9.0 \pm 0.2
(100 %)	(15 %)	(18 %)	(21 %)	(27 %)

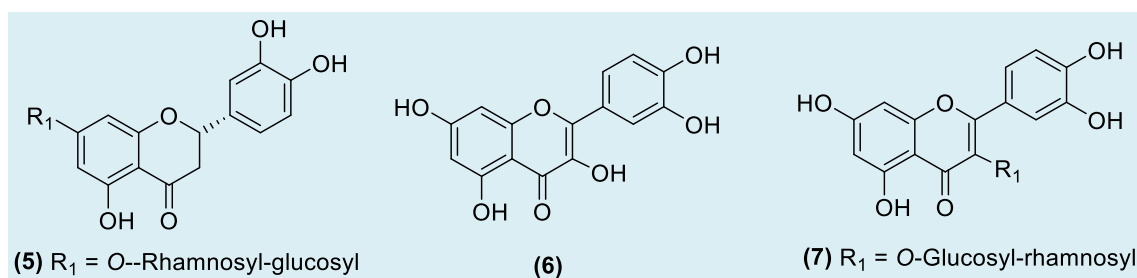


Figure 7. Flavonoids structures: 5 – hesperidin; 6 – quercetin; 7 – rutin

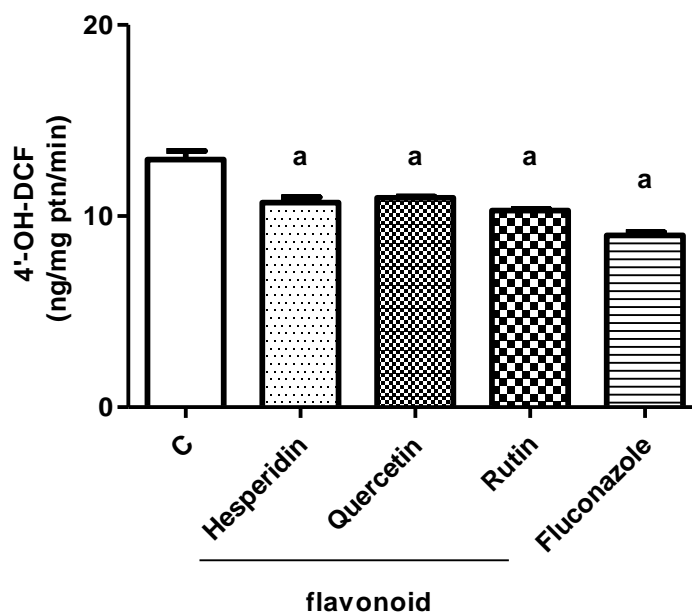


Figure 8. Inhibition of diclofenac (DCF) hydroxylation activity (4'-OH-DCF ng/ mg protein/ min) by flavonoids hesperidin, quercetin and rutin. Rifampicin-induced liver microsomes from female Wistar rats. Histogram bar heights are Mean \pm SEM. ANOVA followed by the Bonferroni's multiple comparison test: a: \neq from control ($p < 0.05$)

Our results regarding the inhibition of DCF hydroxylation by rutin in the rat LMF (ca 21 % inhibition) were similar to those obtained by Kimura *et al.*²³ for the inhibition of rhCYP2C9 by this glycosylated flavonoid (ca 25 % inhibition). Quercetin 100 μ M, however, caused a 100 % inhibition of DCF hydroxylation catalyzed by rhCYP2C9 and a weak inhibition of DCF hydroxylation mediated by the RIF-induced rat LMF. The reasons for this apparent discrepancy between inhibitory effects of quercetin on rat LMF (a complex mixture of CYP2C12 and many other CYP forms) and on isolated human CYP2C9 enzyme are not entirely clear. Quercetin seemed to be an inhibitor of (isolated) human CYP2C9 with an IC_{50} as low as

26.1 μ M.²³ Further studies are necessary to clarify this question, including investigation of the inhibitory effects of quercetin on the hydroxylation of DCF by human liver microsomal fractions and a clarification of which rat liver CYP forms are substrates for quercetin and DCF.

4. Conclusion

A new UPLC-DAD-UV method to quantify the hydroxylation product of diclofenac (4'-hydroxydiclofenac) was developed and validated. This new chromatographic method compares favorably with previously published

methods in various aspects such as to use a methanol free mobile phase, to be less time-consuming and to require the use of smaller volumes of solvent. Moreover, the analytical method proved to be suitable to quantify the product of the DCF hydroxylation (diclofenac conversion to 4'-hydroxydiclofenac) in the liver microsomal fraction.

Overall, results showed that the tested PCs, at a concentration as high as 100 µM, did not inhibit at all or have only a weak to modest inhibitory effect on the DCF hydroxylation by rat liver enzymes. These findings indicated that, at levels found in the human diet, caffeic, ferulic, *p*-coumaric and vanillic acids, as well as hesperidin and quercetin do not interfere with the CYP2C-mediated clearance of drugs and xenobiotics in the rat. The modest inhibition of DCF hydroxylation by quercetin, hesperidin and rutin (100 µM) suggests that at concentrations commonly found in food and medicinal plants these flavonoids are unlikely to alter the pharmacokinetics of DCF and related drugs in rats.

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