Kinetic study on the inhibition of xanthine oxidase by acylated derivatives of flavonoids synthesised enzymatically

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ABSTRACT
Studies have reported that flavonoids inhibit xanthine oxidase (XO) activity; however, poor solubility and stability in lipophilic media limit their bioavailability and applications. This study evaluated the kinetic parameters of XO inhibition and partition coefficients of flavonoid esters biosynthesised from hesperidin, naringin, and rutin via enzymatic acylation with hexanoic, octanoic, decanoic, lauric, and oleic acids catalysed by Candida antarctica lipase B (CALB). Quantitative determination by ultra-high performance liquid chromatography–mass spectrometry (UHPLC–MS) showed higher conversion yields (%) for naringin and rutin esters using acyl donors with 8C and 10C. Rutin decanoate had higher partition coefficients (0.95), and naringin octanoate and naringin decanoate showed greater inhibitory effects on XO (IC50 of 110.35 and 117.51 μM, respectively). Kinetic analysis showed significant differences (p < .05) between the flavonoids before and after acylation regarding Km values, whereas the values for Vmax were the same, implying the competitive nature of XO inhibition.

Introduction
Many in vitro and in vivo experiments in animals and humans have reported the biological activity of the flavonoids especially that related to their antioxidant action in the prevention and/or combat of chronic degenerative diseases, and as anti-inflammatory and anti-microbial agents and modulators of the activities of enzymes such as xanthine oxidase (XO)1.

Xanthine oxidase is an enzyme widely distributed in many species ranging from bacteria to human beings and is present in various mammal tissues2. XO catalyses the oxidation of hypoxanthine into xanthine and uric acid during the metabolic processes of purines3, producing superoxide radicals (O2−) and hydrogen peroxide (H2O2)4. Thus, XO is one of the main enzymatic sources of reactive oxygen species (ROS)5. Various inflammatory stimuli regulate XO such as lipopolysaccharides, hypoxia, and cytokines and elevated levels of this enzyme can lead to an excessive formation of EROs and consequently oxidative processes that can result in late XO such as lipopolysaccharides, hypoxia, and cytokines and

Some flavonoids have been described as effective XO inhibitors but the low absorption of flavonoids in vivo is a limiting factor for their bioavailability1,6. The presence of various hydroxyl groups in the flavonoid molecules endows the compound with some degree of polarity and reduces its lipophilicity9,10. Their low solubility in lipophilic systems also limits the applications of flavonoids in the food, pharmaceutical, and cosmetic industries7.

Enzymatic synthesis of acylated derivatives of flavonoids catalysed by lipases has been considered as an effective and promising strategy for improving the liposolubility of these compounds11. The great interest in using enzymatic processes to modify molecules is due to their selectivity, especially of the lipases12 and the lesser number of stages required in comparison with the classical chemical methods13.

Enzymatic acylation of glycosylated flavonoids, catalysed by Candida antarctica lipase B (CALB), is highly regioselective; it proceeds exclusively on the primary alcoholic group of the sugar moiety, and the mild conditions of this reaction do not interfere with the flavonoids structures14. Some authors have investigated the regioselectivity of CALB in the acylation of rutin and isouqueretin by molecular modelling. According to their reports, the aglycon portion of flavonoids is stabilised at the entrance of the enzyme-binding site by hydrogen bonds and hydrophobic interactions, locating its glycoside residue near the centre of the site. Only the primary 6'-OH of the isouqueretin glucose and the secondary 4'-OH of the rutin rhamnose are acylated, as they stabilise close to the catalytic sites15.

Flavonoid selectively acylated with different aliphatic or aromatic acids may not only alter physicochemical properties of these molecules but also improve bioavailability and biological properties compared to the maternal compounds16. These include increased capacity to inhibit microsomal lipoperoxidation of isouqueretin acylated esters17, increased antiproliferative activity of long chain acylated esters of quercetin-3-O-glucoside17, improved antiangiogenic and antitumor properties of silybin18, rutin and naringin esters19, and inhibition of the sarco/endoplasmic reticulum...
with some modifications. The reaction medium consisted 5 ml of acetone, previously treated with 100 mg/ml 4 A molecular sieves activated by being kept for 1 h at 100 °C, 12 h prior to the reaction. The molar mass ratio used between the flavonoid and the fatty acid was 1:5 (0.575 mmol of flavonoid and 2.875 mmol of fatty acid). After the solubilisation of the reagents in the reaction medium, 0.65 g of CALB was added for the experiment. The tests were incubated in sealed flasks in a shaker at 45 °C set at 100 rpm and the aliquots were removed after 12 and 24 h of incubation. Purification of the acylated flavonoid derivatives was based on partition between hexane/water (4:1, v/v) system as previously described. After each rinse the mixture was centrifuged (2800 rpm for 2 min) and the organic phase containing the free fatty acids was discarded. The samples were lyophilized, frozen at −20 °C and forwarded for ultra-high performance liquid chromatography–mass spectrometry (UHPLC-MS) analyses to determine the conversion rates of the acylated derivatives.

## Materials and methods

### Enzymes and reagents

CALB immobilised in acrylic resin (EC 3.1.1.3, 10,000 U/g) (Novozyme 435), xanthine oxidase from bovine milk, xanthine, allopurinol, hesperidin, naringin, and rutin standards and hexanoic, octanoic, decanoic, lauric, and oleic acids were obtained from the Sigma-Aldrich Chemical Co., St. Louis, MO. All the solvents and reagents were of analytical, spectrometric, or chromatographic quality.

### Enzymatic synthesis of acylated derivatives of flavonoids with different fatty acids

The enzymatic synthesis of acylated derivatives of flavonoids was performed according to a previously described method.

$$k = \frac{C_0}{C_a \times r}$$

where $C_0$ = test compound concentration in octanol, $C_a$ = test compound concentration in aqueous solution, $r$ = ratio of the volumes of the oily and the aqueous phases.
Inhibition of xanthine oxidase (XO) activity by flavonoids and acylated derivatives

Inhibition of XO activity was assessed by measuring the uric acid formed from the xanthine substrate. Solutions of xanthine in various concentrations in a 0.1 M, pH 7.4 phosphate buffer were incubated together with 100 μl of ethanol and the same volume of samples of the solutions with different concentrations (45 and 90 μM in a 0.1 M, pH 7.4 phosphate buffer). The samples were pre-incubated at 37°C for 10 min. The XO solution (0.3 ml, 0.1 U/ml) was added to the reaction mixture and the flasks were incubated at 37°C for 20 min. The enzymatic reaction was interrupted by adding 25 μl of 3.2% HCl. The absorption of the samples was measured using an ELISA reader Epoch, BioTek (Winooski, VT) at 290 nm. The uric acid production was calculated from the differential absorbance with a blank solution without xanthine oxidase. The control was a solution containing xanthine and XO. Allopurinol, a specific XO inhibitor, was used as a positive control. XO inhibitory activity was expressed as the percentage inhibition of XO (XOI, %) by using the following equation:

\[
XOI (\%) = \left( 1 - \frac{Abs_{c}}{Abs_{bc}} \right) \times 100;
\]

where Absc and Absam = absorbance values for the control reaction and for the test samples, respectively.

Measurement of the kinetic constants

The enzymatic kinetics trials were made with those compounds that showed the highest percentage inhibition (XOI) of XO activity. \(V_{\text{max}}\) (maximum reaction rate) and \(K_{m}\) (Michaelis–Menten constant, that is the substrate concentration at 1/2 the maximum reaction rate) were calculated and the type of inhibition kinetics was identified using the Origin 8.6 program and the rectangular hyperbola model. The Michaelis–Menten equation linearized by Lineweaver–Burk was used to determine \(V_{\text{max}}\) and \(K_{m}\) by plotting a graph, that is 1/\(V\) against 1/substrate concentration, and estimated by the intercept and slope respectively. Reaction rate (expressed as uric acid concentration in μmol/min) was obtained from the uric acid standard curve (\(y = 0.024x - 0.1242, R^2 = 0.9786\)). Those concentrations associated to 50% inhibition (IC50) were also calculated.

Statistical analysis

The experiments were performed in triplicate ANOVA one way was used to analyse statistical significance followed by Turkey (Bonferroni) test using Origin Pro 8 (OriginLab, Northampton, MA) statistics software. The results are presented as mean ± SD and statistics were considered significant when the \(p\) values was .05 or less.

Results and discussion

Enzymatic synthesis of the acylated derivatives of flavonoids

Table 1 shows the conversion yields derivatives of naringin, hesperidin, and rutin acylated using hexanoic, octanoic, decanoic, lauric, and oleic acids.

Table 1. Conversion yields (%) of acylated flavonoid derivatives with monocarboxylic acids (hexanoic, octanoic, decanoic, lauric, and oleic acids).

| Flavonoid acylated derivatives | Time (h) | Conversion yields (%) |
|-------------------------------|---------|----------------------|
| Naringin hexanoate            | 12      | 27.30                |
| Naringin octanoate            | 12      | 38.40                |
| Naringin decanoate            | 12      | 28.89                |
| Naringin laurate              | 12      | 9.22                 |
| Naringin oleate               | 12      | 24.32                |
| Hesperidin hexanoate          | 12      | 33.00                |
| Hesperidin octanoate          | 12      | 44.00                |
| Hesperidin decanoate          | 12      | 25.50                |
| Hesperidin laurate            | 12      | 24.60                |
| Hesperidin oleate             | 12      | 32.40                |
| Rutin hexanoate               | 12      | 24.90                |
| Rutin octanoate               | 12      | 20.27                |
| Rutin decanoate               | 12      | 37.95                |
| Rutin laurate                 | 12      | 51.29                |
| Rutin oleate                  | 12      | 34.65                |
|                                | 24      | 28.71                |
|                                | 24      | 9.34                 |
|                                | 24      | 1.53                 |

Kinetiscs of xanthine oxidase (XO) inhibition

Figure 1 displays the percentages of XO inhibition (XOI) obtained for the respective compounds before and after 12 h of enzymatic acylation.

There was a considerable increase in the inhibitory effect on XO activity associated to three of the acylated derivatives, naringin octanoate, naringin decanoate, and hesperidin decanoate (28.80 ± 5.76, 25.61 ± 2.85, and 16.42 ± 3.21, respectively), compared to the untreated flavonoids (14.78 ± 4.80 and 8.24 ± 2.10 for naringin and hesperidin, respectively). Compared to allopurinol, which strongly inhibited XO (89.67%), the derivatives biosynthesised with naringin and hesperidin showed a moderate, but rather promising, activity. For the rutin derivatives, however, the acylation reaction did not enhance the inhibition of XO activity. In the light of those results, the three acylated compounds were selected to assess the kinetic parameters involved in XO inhibition (\(V_{\text{max}}\), \(K_{m}\) and IC50) and to determine their partition coefficients in octanol/water (k).

Two concentrations (45 and 90 μM) of the inhibitors hesperidin decanoate, naringin octanoate, and naringin decanoate and three naringin albeit they efficiently esterified the hesperidin molecule. Earlier studies have reported that the highest CALB-catalysed biosynthesis yields of acylated derivatives of naringin and rutin are obtained using fatty acids with up to 10C12,32. Any further increase of the number of C in the fatty acid chain seems to impede esterification and reduce the efficiency of enzymatic conversion33. According to a recent study, isouceritrin (quercetin-3, O-β-d-glucopyranoside) can be efficiently substituted at 6′-OH by acetate or by C4- to C16-aliphatic acids by CALB. Shorter dicarboxylic acids (C2 to C4) were not substrates for the lipase and did not react at all, while the enzyme has accepted C5- to C12- dicarboxylic acid11.
concentrations of xanthine (250, 500, and 750 μM) were used to determine the kinetic parameters. The reactions were performed in the presence and absence of the inhibitor to obtain the Michaelis–Menten curves and the Lineweaver–Burk double reciprocal plots as shown in Figure 2.

The response of the acylated flavonoids naringin octanoate, naringin decanoate, and hesperidin decanoate in inhibiting XO activity is directly proportional to their concentrations and 90 μM is the concentration with the greatest effect in reducing the reaction rate (uric acid concentration formed/minute). The results were analysed by means of the Lineweaver–Burk double reciprocal plots and the $K_m$ and $V_{max}$ values obtained (Table 2) by graph extrapolation. The $V_{max}/K_m$ ratio was also calculated to determine catalytic efficiency. The double reciprocal graph (B), expressed as $1/V_0$ (y) plotted against $1/[S]$ (x) produces a straight line for which the slope gives the value of $K_m/V_{max}$ the intercept on the $1/V_0$ axis is equal to $1/V_{max}$ and the intercept on the $1/[S]$ axis is equal to $-1/K_m$.

Given that the $V_{max}$ values show no significant alterations and that the $K_m$ (called the apparent $K_m$ and expressed as the [S] in which $V_0 = \frac{1}{2}V_{max}$) were higher in the presence of inhibitors when compared to the control, then it can be supposed that there is a competitive, reversible inhibition reaction mechanism of the untreated and acylated flavonoids. The competitive inhibitors are those most similar to the substrate and they, therefore, occupy the active site. The occupation by the inhibitor thus prevents the substrate from connecting to the active site of the enzyme. Whenever [S] greatly exceeds [I], the probability of an inhibitory molecule connecting to the enzyme is minimized and the reaction will show a normal $V_{max}$ value. That effect on the increase in the apparent $K_m$, together with the absence of any effect on the $V_{max}$ is the diagnosis for competitive inhibition and it is readily revealed in the double reciprocal plots. To compare the inhibitory efficiency of the compounds in regard to XO, the $V_{max}/K_m$ ratios provide better evidence. As seen in the results, the $V_{max}/K_m$ ratios were higher when acylated flavonoid derivatives were used as inhibitors compared to similar untreated flavonoids.

That finding is in agreement with an earlier study that evaluated the inhibitory effect of apigenin, quercetin, myricetin, genistein, and isovitexin on XO activity and reported that they all performed as competitive inhibitors. The presence of sugar units in flavonoid structures also reduces their inhibitory power so that an aglycone form such as quercetin is a more efficient inhibitor than the glycosylated form, rutin.

The XO (%) as a function of flavonoid concentration and the IC$_{50}$ values are displayed in Table 3 and Figure 3. For each equation corresponding to the straight lines of the graph, calculations were made to obtain the value of x when y = 50 (IC$_{50}$). It can be seen that the acylation reaction led to a reduction in the IC$_{50}$ for the three acylated derivatives. Naringin octanoate showed the greatest inhibitory effect on XO activity (IC$_{50}$ = 110.35 μM), followed by naringin decanoate (IC$_{50}$ = 171.51 μM) and hesperidin decanoate (IC$_{50}$ = 198.96 μM), whereas the standard inhibitor of xanthine oxidase (allopurinol) had an IC$_{50}$ value of 14.67 μM.

Higher inhibition activities were reported for isoquercitrin oleate (C18:1), which presents an unsaturation in the acyl group, when compared to its saturated analogue (IC$_{50}$ values of 27 and 61 μM, respectively). The authors observed an increase in the IC$_{50}$ values of saturated esters with the decrease in the carbon chain length (from 61 to 144 μM for isoquercitrin stearate and butyrate, respectively).

Based on the structure of flavonoids, a method for predicting IC$_{50}$ values of XO by calculating the contribution of each hydroxyl moiety towards inhibition of this enzyme was described. The group with the strongest negative contribution to inhibition of XO is the 2'-hydroxyl moiety as can be concluded from comparing kaempferol with morin in which the IC$_{50}$ increases from 2.5 to 40 μM, respectively. The flavones (like quercetin, kaempferol, and morin) are capable of inhibiting the activity of this enzyme even when they are only present in low concentrations.

Hydroxyl groups at C-2 and C-7 and the double bond between C-2 and C-3 were described as essential for high inhibitory activity against XO. The planar structure of flavones and flavonols and the C2 = C3 double bonds of flavonoids were considered advantageous for XO inhibition. Unfortunately those authors did not assess hesperidin, naringin, or any other acylated flavonoid derivative.
Partition coefficients of acylated derivatives of flavonoids in 1-octanol/water

Partition coefficient in 1-octanol/water ($k$) was measured for all prepared compounds as a basic empirical determination of hydro- or lipophilicity (Table 4). This value is a parameter that relates single-solute partitions between polar (water) and nonpolar (octanol)
phases, which determines in vitro solubility in appropriate pharmaceutical and cosmetic preparations.

Among the flavonoids and their acylated derivatives that have been analysed, the aglycone form, quercetin, is the one that presents the highest partition coefficient and therefore the highest degree of lipophilicity (1.46). That high partition coefficient of quercetin in an octanol/water system when compared to its glycosylated form has already been reported\(^39,40\). The derivatives biosynthesised with decanoic acid had partition coefficients of 0.95, 0.83, and 0.86 for rutin decanoate, hesperidin decanoate, and naringin decanoate, respectively. Those values were higher than the coefficients obtained for the untreated flavonoids, 0.48, 0.42, and 0.37 for rutin, hesperidin, and naringin, respectively. The same K values were obtained for the naringin derivatives whether they were acylated with 8C chains or 10C chains.

The variety of substituents on the flavonoid molecules largely influence their physicochemical properties such as dipole moment or hydrophobicity, and thus determine the partitioning into lipid membranes\(^41\). Flavonoid acylated derivatives are expected to exhibit a higher affinity for phospholipidic membranes and so to be transferred into cells\(^28\). The result indicated that the degree of lipophilicity played a major role in improving enzyme inhibitory activity. These results are in alignment with reports in the literature which indicate that increasing the lipophilicity of the flavonoid molecule enhances its inhibitory effect on XO, as has been described for isoquercitrin\(^27\), insofar as it increases the accessibility of the compound to the active site of the enzyme\(^26\).

### Conclusion

Flavonoid acylation, as has been described, provides a useful tool for flavonoid ester formation with improved characteristics. The results suggest that selective enzymatic synthesis of acylated flavanone derivatives catalysed by CALB lipase may represent a new approach to the production of XO competitive inhibitors with greater lipophilicity. The most potent XO inhibition was observed in naringin derivatives with octanoic and decanoic acids (naringin octanoate and naringin decanoate). The results provide the basis of the kinetics of the interaction mechanisms of acylated flavonoid derivatives with XO that may lead to the development of potential new drugs for XO inhibition. This approach also enables the use of flavonoid fatty acid esters in oil-based systems. Thus, these acylated derivatives are promising candidates to be used in pharmaceutical, cosmetic, and nutritional preparations for preventive and/or therapeutic purposes.

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### Disclosure statement

The authors declare no conflict of interests.

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