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Bioinspired oxidation in the CYP of isomers orientin and isoorientin using Salen complexes

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Abstract

RATIONALE: Orientin and isoorientin are *C*-glycosidic flavonoids, considered as markers of some plant species as *Passiflora edulis* var. *flavicarpa* Degener and reported in the literature to have pharmacological properties. In order to evaluate and characterize the *in vitro* metabolism of flavonoids, phase I biotransformation reactions were simulated using Salen complexes.

METHODS: These flavonoids were oxidized separately in biomimetic reaction in different proportions, using one oxidant, *m*-chloroperbenzoic acid (*m*-CPBA) or iodozylbenzen (PhIO), and one catalyst, the Jacobsen catalyst or [Mn(3-MeOSalen)Cl]. The [Mn(3-MeOSalen)Cl] was synthesized and characterized by spectrometric techniques. The oxidation potentials of the catalysts were compared. All reactions were monitored and analyzed by UPLC-DAD and HPLC/MS/MS.

RESULTS: The analysis by UPLC-DAD and HPLC/MS/MS showed that isoorientin produces more products than orientin and that the [Mn(3-MeOSalen)CI] produces more products than the Jacobsen catalyst. In addition, the [Mn(3-MeOSalen)CI] catalyst, which has a higher oxidation potential, formed products with an addition of one or two atoms of oxygen, while the Jacobsen catalyst formed compounds with only one added oxygen atom. The products with the addition of one oxygen were mainly epoxides, while those with two added oxygens formed an epoxide in the C-ring and incorporated the other oxygen into the glycosidic moiety.

CONCLUSIONS: The formation of epoxides is common in biomimetic reactions and they may represent a safety risk in medicinal products due to their high reactivity. This study may serve as a basis for subsequent pharmacological and toxicological studies that investigate the presence of these compounds as phase I metabolites, and ensure the safe use of plant products containing orientin as a chemical marker.

Keywords: biomimetic reactions; [Mn(3-MeOSalen)Cl] catalyst; Jacobsen catalyst; orientin; isoorientin.

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1. Introduction

The isoorientin and orientin isomers (**Figure 1 A and B**) are *C*-glycosidic flavonoids of the flavone class and they have been reported in several plant species, including *Trollius chinensis*, *Bambusa vulgaris*, *Cajanus cajan* and *Aspalathus linearis*[1–3]. Like the flavonoids vitexin and isovitexin, isoorientin and orientin are chemical markers of the species *Passiflora edulis* var. *flavicarpa* Degener[4,5], which belongs to the family Passifloraceae and the genus *Passiflora*. This plant species has been reported to have medicinal properties of importance for the treatment of diseases of the central nervous system and for the treatment of insomnia and anxiety[6].

Pharmacological studies for orientin and isoorientin have revealed chemotherapeutic properties against colorectal cancer by anti-inflammatory action, the ability to protect brain lesions by action on oxidative stress, and the reduction of cognitive deficits in Alzheimer's disease mice, together with antiviral, anti-bacterial, cardioprotective, antinociceptive, antidepressant and neuroprotective activities[3,7,8].

Flavonoids are phytochemical constituents which are reported to have health benefits, including anti-inflammatory, antiviral, and anticarcinogenic properties[9,10]. They are in wide popular use, but can be toxic, interacting directly with DNA[11], and mutagenic. It should be noted that medicinal plants have always been used in the treatment of diseases, but their safety has not been fully clarified, leading the Medicines Agency (EMA) to establish community monographs of botanic species[12]. What may be pernicious is the concept that "natural does not harm", leading to self-medication[13], thus evidencing the importance of pharmacokinetic and pharmacodynamic studies.

Due to the need to substantiate the medicinal properties of orientin and isoorientin, studies are necessary to prove their safety and toxicity, including metabolism studies, which involve specific chemical reactions and may cause changes in pharmacological and toxicological activities[14]. Cytochrome P450 (CYP) is a superfamily of enzymes mainly responsible for the biotransformation of drugs, and therefore studies involving CYP are indispensable for the evaluation of drugs that are to be available on the market [15,16], with toxicity and side effects being responsible for the failure of 40% of drug candidates[17].

CYP-bioinspired systems have been used to evaluate *in vitro* metabolism, generating complementary results to *in vivo* metabolism studies and thus reducing the number of animals needed at this stage of development. In this field, the biomimetic model using metalloporphyrins, which are synthetic catalysts that simulate metabolic reactions with the

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addition of oxygen donors, have also been applied to natural products because they have a structure similar to the CYP porphyrin iron core[18–20].

Groves and co-workers studied the use of metalloporphyrins as a bioinspired system, reporting their catalytic activity in the epoxidation of alkenes and hydroxylation of non-reactive alkanes[21]. Subsequently, variation of the binders to the metal gave rise to the generation of novel metalloporphyrins[22]. Although metalloporphyrins are the most suitable catalysts for the CYP enzymatic system, they require high-cost synthesis and purification[20], leading researchers to develop other systems, among them the Jacobsen catalyst (**Figure 1 C**), consisting of Schiff bases coordinated to the metal Mn (III) added to tert-butyl groups and a cyclohexyl substituent. Since its development, the Jacobsen catalyst has been used to catalyze oxidation reactions including those of non-functionalized olefins with enantioselectivity and alcohols[23–25]. In addition, the Jacobsen catalyst has been used in studies with pharmaceuticals and natural products such as carbamazepine[26,27], Monesin A[28], lapachol[29], and grandisin[30].

Although flavonoids are primarily metabolized by phase II reactions[31], it is also necessary to evaluate possible phase I metabolites to monitor efficacy and safety. Thus, biomimetic reactions with the Jacobsen catalyst and Salen catalysts were used to characterize potentially toxic reaction products, and this approach may be complementary to *in vivo* studies that evaluate the toxicity, metabolism and pharmacokinetics of plant extracts containing flavonoids as a chemical marker. This work aimed to evaluate the oxidation reactions of the orientin and isoorientin flavonoids, catalyzed by the Jacobsen catalyst and the synthesized catalyst [Mn(3-MeOSalen)Cl] (**Figure 1 D**), with the two catalysts being able to differentiate the selectivity and formation of the reaction products.

INSERT FIGURE 1

2. Experimental

2.1. Materials

All reagents and solvents were used without further purification. (*S*, *S*)-Jacobsen catalyst ((*S*, *S*)-(+)-*N*,*N*'-Bis(3,5-di-tert-butylsalicylidene)-1,2-cyclohexanediaminomanganese III)), hydrochloric acid (HCl), acetic acid (CH_3COOH), sodium thiosulfate ($Na_2O_3S_2$), ethanol (EtOH), Dimethyl sulfoxide (DMSO), manganese chloride (II) tetrahydrate ($MnCl_2(4H_2O)$), *o*-vanillin PA ($C_8H_8O_3$), silver chloride (AgCl), potassium bromide (KBr), isoorientin standard, orientin standard, sulfuric vanillin and Natural A reagent were purchased from Sigma-Aldrich (St Louis, MO, USA). Methanol (MeOH) was purchased from JT Baker (Phillipsburg, NJ, USA). Ethyl acetate (EtOAc), acetonitrile (ACN), *n*-butanol (BuOH) and *n*-hexane were

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purchased from Merck Millipore (Darmstadt, Germany). Acetone (CH_3COCH_3), formic acid (HCOOH) and trifluoroacetic acid (TFA) were obtained from Proquimios (Rio de Janeiro, Brazil). Sodium hydroxide (NaOH) and chloroform ($CHCl_3$) were obtained from LabSynth (Diadema, Brazil). Sulfuric acid (H_2SO_4), sodium bicarbonate ($NaHCO_3$), potassium iodide (KI) and ethylenediamine ($C_2H_8N_2$) were purchased from Isofar (Duque de Caxias, Brazil). Potassium dichromate ($K_2Cr_2O_7$) was obtained from QEEL-Chemistry (São Paulo, Brazil). Silica gel 60 F254 was purchased from Macherey-Nagel (Düren, Germany) and Sephadex LH-20 from GE Healthcare (Marlborough, MA, USA). The iodozylbenzene oxidant was synthesized by alkaline hydrolysis[32], and its purity determined as 99% by iodometric titration[33].

2.2. Synthesis of the [Mn(3-MeOSalen)Cl] catalyst

The complex was synthesized in 45% yield from the reaction between the Schiff 3-MeOSalen base (409 mg, 1.24 mmol) and manganese (II) chloride tetrahydrate ($MnCl_2$ ($4H_2O$)) (245 mg, 1.24 mmol) in MeOH. The reaction system was stirred and refluxed for two hours, after which time the brown solid obtained was filtered off and kept in a desiccator[34].

Characterization of the [Mn(3-MeOSalen)Cl] and Jacobsen catalysts

The catalysts were characterized by UV-Visible spectroscopy in organic medium in the region of 200 to 900 nm using an Agilent (Santa Clara, CA, USA) 8453 UV-Vis spectrometer, and by IR spectroscopy of the compound dispersed in KBr, in the range 400 to 4000 cm^{-1}, on a Shimadzu (Kyoto, Japan) FTIR-8400S spectrometer using IRsolution software(Shimadzu). To compare the oxidation potential of the catalysts, cyclic voltammetry analysis was performed on a Bioanalytical Systems, Inc. (BASi, West Lafayette, IN, USA) Epsilon potentiostat. The electrochemical cell was composed of a working carbon glass electrode, a platinum electrode as auxiliary, and an Ag/AgCl electrode (silver chloride) as reference. All analyses were performed using a 0.1 mol L^{-1} solution of tetrabutylammonium perchlorate (TBAP) in DMSO as the supporting electrolyte. The scan speed was 100 mV s^{-1}.

2.3. Isolation of orientin and isoorientin

The leaves of *Passiflora edulis flavicarpa* were collected at Coronel Ezequiel, Rio Grande do Norte, Brazil (Latitude: S 06º 23' 44.2"; Longitude: W 36º 10' 27.3"; Altitude: 75 m), carried out under authorization of the Brazilian System of Authorization and Biodiversity (SISBIO) (process number 35016) and SISGEN (process number A925E81). The exsiccate was deposited in the Herbarium of the Federal Rural Semi-Arid University (UFERSA), Mossoró, Rio Grande do Norte, Brazil (ICN 13751.6).

The leaves were oven dried at 40 ºC for 48 hours in a greenhouse with circulating air (400-8D model; Nova Ética, Vargem Granse Paulista, Brazil). The hydroethanolic extract

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(EHF) was prepared in the ratio 1:20 using 70% EtOH by turbo-extraction (150 g:3000 mL, w/v) in an industrial blender (700/650 w, BR2L model, JL Colombo, Itajobi, Brazil) for 5 minutes at 25 ºC[35]. The EHF was filtered and dried on a rotary evaporator at 40 °C. Subsequently the extract was partitioned with solvents of increasing polarity: *n*-hexane, $CHCl_3$, EtOAc and BuOH. The composition of the phases was 200 mL of the extract solubilized in H_2O and 3 x 200 mL for each solvent, except EtOAc where it was 5 × 200 mL. The EHF and the fractions were first analyzed by TLC, using silica gel 60 F254 aluminum chromate plates and mobile phase of EtOAc:CH_3COCH_3 (acetone): CH_3COOH (acetic acid):H_2O (6:2:1:1, v/v/v/v). The developers used were the Natural A reagent of diphenylboryloxyethylamine (0.5%) and vanillin sulfuric methanol, followed by a subsequent UV detection at 254 and 365 nm[36].

The EtOAc fraction (1.2 g) was submitted to column chromatography with compacted silica gel 60 F254 (2 cm x 60 cm, 116 g) as the stationary phase. The mobile phase of the column consisted of 400 mL of $CHCl_3$:EtOH:H_2O in the ratio (4:4:0.5 v/v/v), and the flow rate was 2 mL min^{-1}. All fractions obtained were further purified using another column containing Sephadex LH-20 (30 cm x 2 cm, 100 g) stationary phase The condition for molecular exclusion were a flow rate of 1 mL min^{-1} and a 210 mL gradient mobile phase: EtOH:MeOH (1:1)[36].

The C2-H fraction was selected and purified by HPLC (High Performance Liquid Chromatography) on semipreparative scale in a Thermo Fisher Scientific (Waltham, MA, USA) High-Pressure Liquid Chromatograph, equipped with a diode array detector (DAD), quaternary pump, and automatic injector. A Phenomenex (Torrance, CA, USA) Luna C18 column (250 mm × 10 mm, 5 µm) was used at 25 °C, at a constant flow rate of 2.5 mL min^{-1}. In the DAD, wavelengths ranging from 200 to 500 nm were monitored, but the chromatogram was obtained using a wavelength of 345 nm. The mobile phase consisted of A: 0.05% TFA (trifluoroacetic acid) in H_2O and B: 0.05% TFA in MeOH in an isocratic elution at 18% B[36]. The data were processed using ChromQuest software (Thermo Fisher Scientific)[37,38].

Purity analysis of flavonoids by UHPLC-DAD

A stock solution of each flavonoid was prepared in 8:2 (v/v) ACN:H_2O at a concentration of 0.4 mg mL^{-1}. Its purity was determined by UHPLC-DAD using LCSolution software (Shimadzu), mobile phase A: HCOOH in H_2O (0.1%) and B: HCOOH in ACN (0.1%), a Shimpack (Shimadzu) C18 column (75 mm x 4.6 mm, 2.2 µm), a constant flow rate of 0.3 mL min^{-1}, a column temperature of 25 ° C, and an injection volume of 3 µL. The analytical method employed a gradient starting with 2% B for 3 min (0 to 3 min), followed by 25% B for 22 min (3 to 25 min), then reached 100% B in 3 min (25 to 28 min).

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2.4. Oxidation reactions

The orientin and isoorientin oxidation reactions were carried out in separate 5-mL penicillin flasks. The experiments were performed using PhIO or *m*-CPBA oxidants, Jacobsen or [Mn(3-MeOSalen)Cl] catalysts, the flavonoid as substrate (stock solution), and ACN: H_2O (9:1, v/v) solution to reach a total reaction volume of 3 mL. The oxidants and catalysts were dissolved in 9:1 ACN:H_2O (v/v) for the reactions, except for PhIO which was dissolved in ACN. All combinations of oxidant, substrate and catalyst were evaluated in catalyst:oxidant:substrate ratios of 1:10:10, 1:20:20, 1:30:30 (0.1mM catalyst:1 mM oxidant:1 mM substrate, 0.1mM catalyst:2 mM oxidant:2 mM substrate and 0.1mM catalyst:3 mM oxidant:3 mM substrate)[28–30]. The control reactions involved the absence of a catalyst. After placing the catalyst:substrate:solvent mixture in the flasks under magnetic stirring at room temperature, the oxidant was added, initiating the reaction. The reactions took up to 24 hours and aliquots of 150 μL were withdrawn after 1, 2, 3, 4.5, and 9 hours, and at the end of the reaction, for quantification of the reaction products by UHPLC-DAD. To remove the catalyst, a partition with 150 μL of *n*-hexane was performed and only the polar phase was analyzed. If necessary for quantification, the samples were diluted with 9:1 ACN:H_2O (v/v). The aliquots at 3, 4.5 and 24 h were analyzed by HPLC/MS and HPLC/MS/MS to characterize the oxidization products.

Analytical curve

In order to calculate the consumption of flavonoids (substrates) in the reactions, the analytical curve was obtained by UHPLC-DAD using the above method by monitoring at a wavelength of 350 nm. The calculation was performed through the mean of the correlation of the mass injected to the area of the chromatographic peak, where each injection volume corresponds to a mass value injection. The injection volumes of the orientin stock solution (0.1 mg mL^{-1}) were 0.1, 0.4, 0.6, 0.8, 1 and 3 μL, corresponding to 0.01, 0.04, 0.06, 0.08, 0.1 and 0.3 μg/μL (0.02, 0.09, 0.13, 0.18, 0.22, 0.67 mM). For isoorientin the injection volumes of the stock solution (0.33 mg mL^{-1}) were 0.3, 0.7, 0.9, 2 and 3 μL, corresponding to 0.099, 0.231, 0.297, 0.660 and 0.990 μg/μL (0.22, 0.52, 0.66, 1.47 and 2.21 mM). .

2.5. Analysis by UHPLC-DAD and HPLC/MS/MS of the oxidation products of orientin

The reactions products were analyzed by UHPLC-DAD, as described above for the analytical curve. To characterize the reaction products, HPLC/MS was used in positive and negative ion mode, followed by HPLC/MS/MS in multiple reaction monitoring (MRM) mode. The HPLC system was a Shimadzu model 20A equipped with a DAD detector, binary pump, oven and automatic injector. The mass spectrometer was a Bruker Daltonics (Bremen,

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Germany) model microTOF II (ESI-TOF/TOF) instrument with DataAnalysis version 4.3 software (Bruker Daltonics). The reactions products were dried and diluted in 600 μL ACN/H_2O solution (9:1) for chromatographic analysis. The chromatographic conditions were Phenomenex Luna C18 column (250 mm × 4.6 mm, 5 μm) at a temperature of 30 °C, 3 μL injection volume and 1 mL min^{-1} flow rate. The mobile phase consisted of ACN (B) and H_2O (A) in negative ion mode and ACN acidified with HCOOH (B, 0.1%) and HCOOH (A, 0.1%) in positive ion mode. The 35-minute gradient chromatographic method started with 8% B, increased to 10% B in 20 minutes, and finally from 10% B to 100% B in 15 minutes. The temperature of the drying gas was 220 °C with a flow rate of 10 L min^{-1} at a pressure of 5.5 bar. The capillary voltage was 3.5 KV. The MS analysis was performed in scanning mode from *m/z* 50 to 600, in automatic MS/MS negative ion mode, and in MRM positive ion mode with the collision energy varying for each ion, with values between 15 and 35 eV.

3. Results and discussion

3.1. Isolation and characterization of orientin and isoorientin

Chemical partition of 3 L of the hydroethanolic extract of the leaves of *Passiflora edulis var. flavicarpa* was performed in order to separate the compounds according to their polarity. The EtOAc fraction obtained a yield of 0.8%, relative to the 1.2 g used in the isolation. A phytochemical screening of the extract and fractions by TLC with vanillin sulfuric acid and Natural A reagent preliminarily identified orientin and isoorientin through their characteristic Rf[39] and this assignment was confirmed by comparison with standards. This fraction was then submitted to column chromatography, where 120 fractions were collected. The C1-I fraction of 144 mg was purified by Sephadex LH-20, after phytochemical screening by TLC, and revealed the presence of orientin and isoorientin. Further column chromatography of this fraction resulted in 65 fractions, with TLC analysis of the C2-H fraction (35 mg) showing the presence of orientin and isoorientin. The C2-H fraction was then purified by semipreparative HPLC, which yielded 16 mg of orientin and 17 mg of isoorientin, with a chromatographic purity of 94.5% and 95.5% respectively.

3.2. Comparison of the UV-Vis and IR spectra of the [Mn(3-MeOsalen)Cl] and Jacobsen catalysts

The electronic spectra of the Jacobsen and [Mn(3-MeOsalen)Cl] catalysts (**Figures 2 A and B**) were obtained in DMSO. Differences were observed in the maximum absorptions of the $\pi \rightarrow \pi^*$ transitions. The Jacobsen catalyst showed three maxima at 293 (8300 L mol^{-1} cm^{-1}), 319 (5450 L mol^{-1} cm^{-1}) and 356 (3320 L mol^{-1} cm^{-1}) nm, while [Mn(3-MeOSalen)Cl]

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exhibited two bands, at 296 (6510 L mol^{-1} cm^{-1}) and 324 (6100 L mol^{-1} cm^{-1}) nm, in a region of higher wavelength. An intra-ligand n→π* band at 415 nm with molar absorptivity around 2200 L mol^{-1} cm^{-1} was found for both catalysts and the d-d band for the metal at a lower wavelength: 622 nm (144 L mol^{-1} cm^{-1}) for the Jacobsen catalyst and 667 (80 L mol^{-1} cm^{-1}) nm for [Mn(3-MeOsalen)Cl]. The Jacobsen catalyst has a stronger receptor effect and consequently needs more energy in the electronic transitions, shifting these to a shorter wavelength[40].

INSERT FIGURE 2

The IR bands found for both catalysts (**Figure 2 C**) can be compared with those of the Schiff base Salen, which is characterized mainly by an intense band in the region of 1600 cm^{-1}, assigned to ν_{as}(C=N). The Jacobsen catalyst displayed bands at 1605 cm^{-1}, 1533 cm^{-1} and 1255 cm^{-1}, assigned to ν(C=N), ν(C=C) and the phenol ν(C-O), respectively. [Mn(3-MeOSalen)Cl] had the same bands as the Jacobsen catalyst, with the stretching ν_{as}(C=N) at 1625 cm^{-1} and ν_s (C=N) at 1601 cm^{-1}, ν (C=C) at 1552 cm^{-1}, the phenol ν(C-O) at 1288 cm^{-1} and other stretching bands related to the methoxy group present in the 3-MeOSalen ligand at 1249 cm^{-1} and 1085 cm^{-1} assigned to ν_{as} (C-O-C) and ν_s (C-O-C). As the Jacobsen catalyst is a stronger π receptor, this weakens the C=N bond and ν_{as}(C = N) is observed at a lower wavelength.

3.3. Cyclic Voltammetry of the [Mn(3-MeOsalen)Cl] and Jacobsen catalysts

Cyclic voltammograms of the [Mn(3-MeOSalen)Cl] complexes and the Jacobsen catalyst (**Figure 3**) were obtained in TBAP (tetrabutylammonium perchlorate) solution: 0.1 mol L^{-1} in DMSO. The two catalysts showed similar electrochemical potentials, where the redox couple of the manganese is visible at negative potentials, and the oxidation processes of the respective Salen-derived ligands are visible at high potentials. The data indicate that the [Mn(3-MeOSalen)Cl] catalyst has a higher electrochemical potential. This is demonstrated by the difference of 222 mV between the respective electrochemical potentials; the $Mn^{3+/2+}$ redox couple in the Jacobsen catalyst showed a half potential wave of - 461 mV, whereas in the [Mn(3-MeOSalen)Cl] complex the $E_{1/2}$ value was considerably higher at - 239 mV. However, when reduced, the Salen complex should act as a less potent reducing agent than the Jacobsen catalyst. The difference between the peak potentials for the two complexes indicated that redox processes centered on the metal are almost reversible. The [Mn(3-MeOsalen)Cl] (ΔE = 95 mV) complex was considerably more reversible than the Jacobsen catalyst (ΔE = 176 mV),

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indicating, therefore, a smaller structural change due to the alteration of the oxidation state of the metal[41].

INSERT FIGURE 3

3.4. Optimization of the reaction condition with higher consumption of the substrate

The oxidation reactions were monitored by UHPLC-DAD to evaluate which set of reaction condition gave the highest speed in the consumption of the substrate, calculated through the equation obtained from the analytical curve (available in the supporting information). The reaction was monitored at a wavelength of 280 nm, the region of the strong absorption of the reaction products. The reaction conditions with the oxidant iodozylbenzene in the proportion 1:10:10 showed the highest rate of orientin consumption for both catalysts: in 3 hours of reaction, 69% for the Jacobsen catalyst and 63% for the catalyst [Mn(3-MeOsalen)Cl]. After 4.5 hours of reaction, 71% of the orientin was consumed for the Jacobsen catalyst and 69% for [Mn (3-MeOSalen)Cl]. For isoorientin, the reaction conditions with the oxidant *m*-CPBA in the proportion 1:20:20 showed the highest substrate consumption rate for both catalysts: in 3 hours of reaction, 91 ± 0% for the Jacobsen catalyst and 90 ± 3 % for [Mn(3-MeOSalen)Cl]. The reaction kinetic results for the proportion 1:20:20 are provided in Tables 1 and 2, and the kinetic curves are provided in the supporting information. These data show that, although the [Mn(3-MeOSalen)Cl] catalyst is not yet used in biomimetic studies, it has similar catalytic efficiency to the Jacobsen catalyst that is widely used in biomimetic studies of drugs and drug candidates. In addition, the iodozylbenzene oxidant has only one oxygen atom and it frequently leads to the formation of high-valency reactive intermediates, thus obtaining good results in oxidation reactions, despite their toxicity[24,27]. In the study of the biomimetic oxidation of apigenin-7-O-glycoside[42], the selectivity of different oxidants was evaluated, and PhIO was reported to be the most selective.

INSERT TABLE 1

INSERT TABLE 2

3.5. Characterization of the reaction products by HPLC/MS and HPLC/MS/MS

The flavonoids reaction products were characterized by mass spectrometry using both negative and positive ion mode. For both substrates, the tested oxidants (PhIO and *m*-CPBA) formed the same products; however, there was a divergence in the products when the [Mn(3-MeOSalen)Cl] and Jacobsen catalysts were used, as evidenced by the chromatograms for orientin (**Figure 4**) and isoorientin (**Figure 5**).

INSERT FIGURE 4

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INSERT FIGURE 5

In the positive ion analysis of the orientin reaction products, five peaks were detected for the catalyst [Mn(3-MeOSalen)Cl] and four for the Jacobsen catalyst. Peak 1, present for both catalysts, yields an ion at *m/z* 481 which could correspond to a product with an addition of 32 Da to the orientin molecule, that is, the incorporation of 2 oxygen atoms in the oxidation process. This may represent the formation of an epoxide in ring C and the addition of the other oxygen in the sugar moiety. However, as it was not possible to obtain the MS/MS spectrum of this ion and isolation of the compound was not possible because of the low reaction yield, it is not possible to confirm the structure of oxidation product 1.

Peak 2, present in the chromatograms of both catalysts, yields an ion at *m/z* 465 resulting from the insertion of one oxygen atom into the structure of the orientin, forming an epoxide in ring C (**Figure 6, Table 3**). The proposed reaction mechansim is based on studies carried out previously using the Jacobsen catalyst, in which an epoxide was formed in ring C, as for example in the biomimetic oxidation of apigenin-7-O-glycoside, where among the products formed are apigenin-2,3-epoxy and 3- (hydroxy)-floretine-2,3-epoxy with both compounds epoxidized in the C ring[42]. From the MS/MS data it was possible to confirm the proposed structure based on the formation of the product ion at *m/z* 277. Peak 3 was from orientin (**Figure 6, Table 3**), the substrate of the reaction and the major peak for both catalysts.

Peak 4, yielding an ion at *m/z* 485, was detected only for the [Mn(3-MeOSalen)Cl] catalyst, (**Figure 6, Table 3**). This represented addition of 36 Da (2 oxygens and 4 hydrogens) to orientin and this could correspond to two molecules of water. The MS/MS spectrum of *m/z* 485 showed the formation of ions at *m/z* 301, 277, 449 and 467, revealing that there was an opening and oxidation of the glycoside ring, in addition to the insertion of a hydroxyl and an H atom across the double bond of ring C.

Peak 5, with an ion at *m/z* 469, was detected only for the [Mn(3-MeOSalen)Cl] catalyst (**Figure 6, Table 3**). This represents a molecule formed by the addition of 20 Da (1 oxygen and 4 hydrogens) to orinentin. Based on what was observed for compound 4 and by analysis of its MS and MS/MS spectrum containing product ions at *m/z* 433, 451 and 415, this peak corresponds to opening of the glycosidic ring of orientin coupled with the addition of an oxygen and a hydrogen atom in ring C.

In the negative ion mode, three peaks were detected for [Mn(3-MeOSalen)Cl] and two for the Jacobssen catalyst. The first peak, detected for both catalysts, shows an unidentified ion at *m/z* 589, which could represent a diglycosylated flavonoid, possibly because the orientin was

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not 100% pure. Peak 3, which was also observed in positive ion mode, is related to the orientin substrate, and peak 4, with an ion at *m/z* 467, was detected only with [Mn(3-MeOSalen)Cl]. **Table 3** shows the spectrometric data of the orientin reaction products identified by HPLC/MS/MS. The proposed fragmentation pathways and MS spectra are given in the supporting information.

INSERT FIGURE 6

INSERT TABLE 3

For isoorientin, seven peaks were detected when the [Mn(3-MeOSalen)Cl] catalyst was used, but only five for the Jacobsen catalyst (**Figure 5**). Peaks 1 and 2 were only observed for the Mn (3 MeOSalen) Cl catalyst and both yielded an ion at *m/z* 481, indicating that the compounds are isomers. As discussed for orientin, these compounds have a molecular mass 32 Da more than that of isoorientin that would result from the addition of two oxygen atoms. The proposed mechanism is that an epoxide was formed in the C ring and the addition of the other oxygen occurred in the sugar moiety, on carbon 5 or 6, as observed for the other identified compounds. However, since it was not possible to obtain the MS/MS spectrum for the compounds and the low reaction yield meant that their isolation was not viable, it is not possible to confirm the structure of these two oxidation products.

Peaks 3 and 4 were observed in the chromatograms of the reactions with both catalysts. Peak 4 yielded an ion at *m/z* 465, associated with the epoxidation of the C-double bond as previously described for orientin and other compounds formed using the Jacobsen catalyst. Peak 3, however, yielded an ion at *m/z* 467, suggesting that it presents a compound whose molecular mass is 18 Da more than that of isoorientin, corresponding to the addition of H_2O across the double bond of ring C. Peak 5 represents the isoorientin substrate, but as it was not detected by UHPLC-DAD, its concentration is below the limit of detection and this illustrates the difficulty in trying to isolate the reaction products for the acquisition of spectroscopic data using other techniques such as ^{1}H and ^{13}C NMR.

Peaks 6 and 7 were detected only for the [Mn(3-MeOSalen)Cl] catalyst. Peak 6 yielded an at *m/z* 469, while peak 7 presented an ion at *m/z* 485, similar to products 4 and 5 observed for orientin. Thus, for the peak 6 compound there was an opening and oxidation of the glycosidic ring, in addition to H_2O insertion across the double bond of the C ring, whereas for compound 7 the same reactions occurred with the addition of another oxygen in the glycosidic portion. The structures of the products and their spectrometric data are shown in **Figure 7** and **Table 4**.

INSERT FIGURE 7

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INSERT TABLE 4

4. Concluding remarks

In order to evaluate the *in vitro* metabolism of the flavonoid orientin and isoorientin, described in the literature as a major secondary metabolites and considered as chemical markers of the species *Passiflora edulis flavicarpa* and other medicinal plants, bioinspired oxidation reactions in CYP were carried out with the Jacobsen and [Mn(3-MeOSalen)Cl] catalysts, and *m*-CPBA and PhIO oxidants. The isoorientin and orientin isomers were revealed to have different reactivities, with isoorientin forming more products than orientin. In addition, the [Mn(3-MeOSalen)CI] catalyst, which has a higher oxidation potential, formed products with addition of one or two oxygens while the Jacobsen catalyst showed only products with one added oxygen. The products were characterized as epoxides which deserve concern due to their high reactivity. Thus, this study may serve as a basis for further pharmacological and toxicological studies that confirm the presence of these putative phase I metabolites and ensure safety in the use of plant products that have orientin and isoorientin as markers, as well as for the synthesis and characterization of new chemical catalysts.

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Table 1. Comparison of orientin consumption in biomimetic reactions using Jacobsen or [Mn(3-MeOSalen)Cl] as catalyst and PhIO or *m*-CPBA as oxidant for reaction ratio 1:20:20.

Time	Jacobsen catalyst		[Mn(3-MeOSalen)Cl]	
(h)	PhIO	*m*-CPBA	PhIO	*m*-CPBA
1	96% ±5	99% ±3	93% ±1	87% ±1
2	94% ±2	95% ±2	88% ±1	83% ±2
3	92% ±2	90% ±1	93% ±0	80% ±2
4,5	81% ±4	83% ±1	85% ±0	77% ±5
9	80% ±2	82% ±4	79% ±3	70% ±0
24	82% ±0	79% ±3	78% ±0	68% ±1

Table 2. Comparison of isoorientin consumption in biomimetic reactions using Jacobsen or [Mn(3-MeOSalen)Cl] as catalyst and PhIO or *m*-CPBA as oxidant for reaction ratio 1:20:20.

Time	Jacobsen catalyst		[Mn(3-MeOSalen)Cl]	
(h)	PhIO	*m*-CPBA	PhIO	*m*-CPBA
1	95% ±0	93% ±1	99% ±1	95% ±6
2	94% ±1	91% ±1	98% ±1	91% ±3
3	94% ±1	91% ±0	96% ±1	90% ±3
4,5	91% ±1	89% ±2	97% ±0	89% ±4
9	91% ±0	86% ±1	95% ±0	85% ±1
24	85% ±0	85% ±1	94% ±1	84% ±1

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Table 3. Mass spectral data of the orientin reaction products identified by HPLC/MS/MS.

Compound number	Compound name	$[M-H]^-$	$[M+H]^+$	MS/MS in positive mode
2	1-(3,4-dihydroxyphenyl)-4,6-dihydroxy-3-(3,4,5-trihydroxy-6-(hydroxymethyl) tetrahydro-2H-pyran-2-yl)-1aH-oxirene[2,3- b]chromen-7 (7a H)-one	-	465.1096	266.8836; 276.4811; 281.5347; 293.0095; 322.8963; 424.6973; 429.3210; 443.0203.
3	Orientin	447.0950	449.1070	252.0858; 296.1186; 449.1070
4	2-(3,4-dihydroxyphenyl)-8-(1,2,3,4,5,6-hexahydroxyhexyl)-3,5,7-trihydroxychroman-4-one	-	485.1075	277.1119; 301.2198; 365.0528; 403.0891; 431.0259; 449.0883; 467, 0969.
5	2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-8-(2,3,4,5,6-pentahydroxyhexyl)chroman-4-one	467.116	469.1193	319.0711; 349.0741; 373.0565; 415.0817; 433.0836; 451.1005.

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Table 4. Mass spectral data of the isoorientin reaction products identified by HPLC/MS.

Compound number	Compound name	$[M+H]^+$
3	1a-(3,4-dihydroxyphenyl)-4,6-dihydroxy-5-(3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)-1aH-oxireno[2,3-b]chromen-7(7aH)-one	465.1021
4	2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-6-(3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)chroman-4-one	467.0738
5	Isoorientin	449.1079
6	2-(3,4- dihydroxyphenyl)-3,5,7-trihydroxy-6-(1,2,3,4,6-pentahydroxyhexyl)chroman-4-one	469.1235
7	1-(2-(3,4-dihydroxyphenyl)-3,4,5,7-tetrahydroxychroman-6-yl)hexan-1,2,3,4,5,6-hexaol	485.1193

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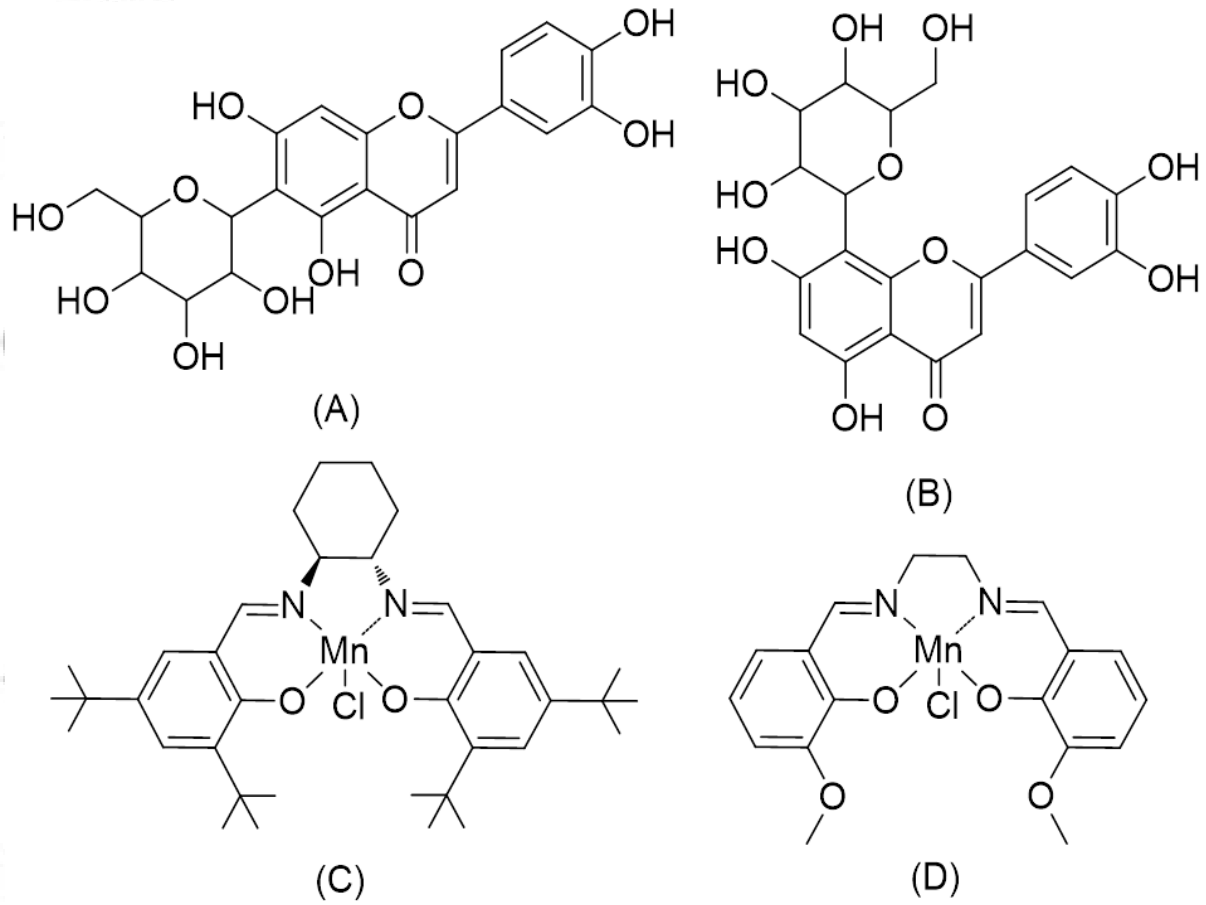

Figure 1. Flavonoid isoorientin (A), flavonoid orientin (B). Jacobsen Catalyst (C) and [Mn (3MeOSalen) Cl] (D).

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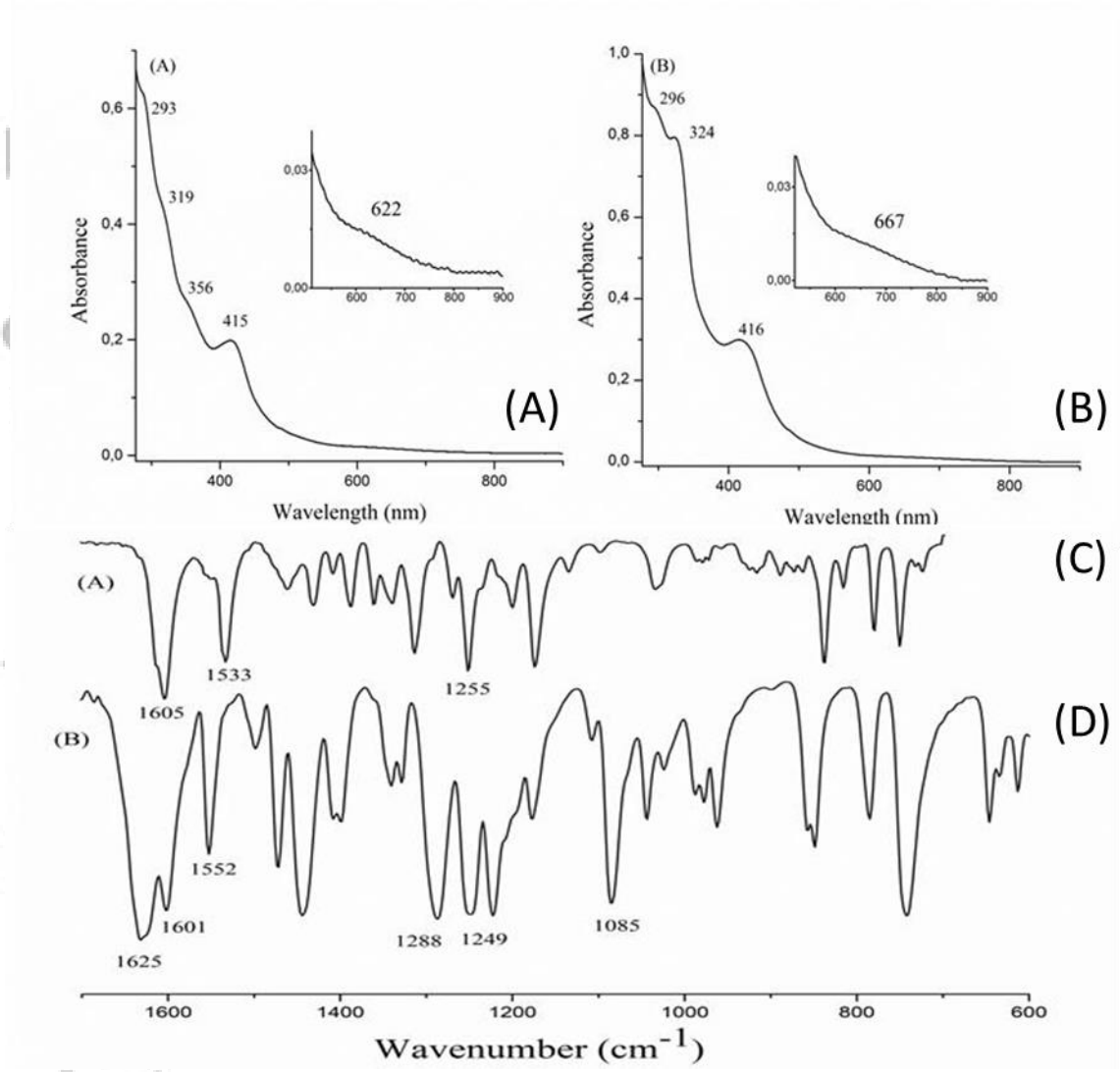

Figure 2. Electronic spectra of the Jacobsen catalyst (A) and [Mn (3-MeOSalen)Cl] (B). Infrared Spectra of Jacobsen catalyst (C) and [Mn(3-MeOSalen)Cl] (D) in region from 1700 to 600 cm^{-1} (C).

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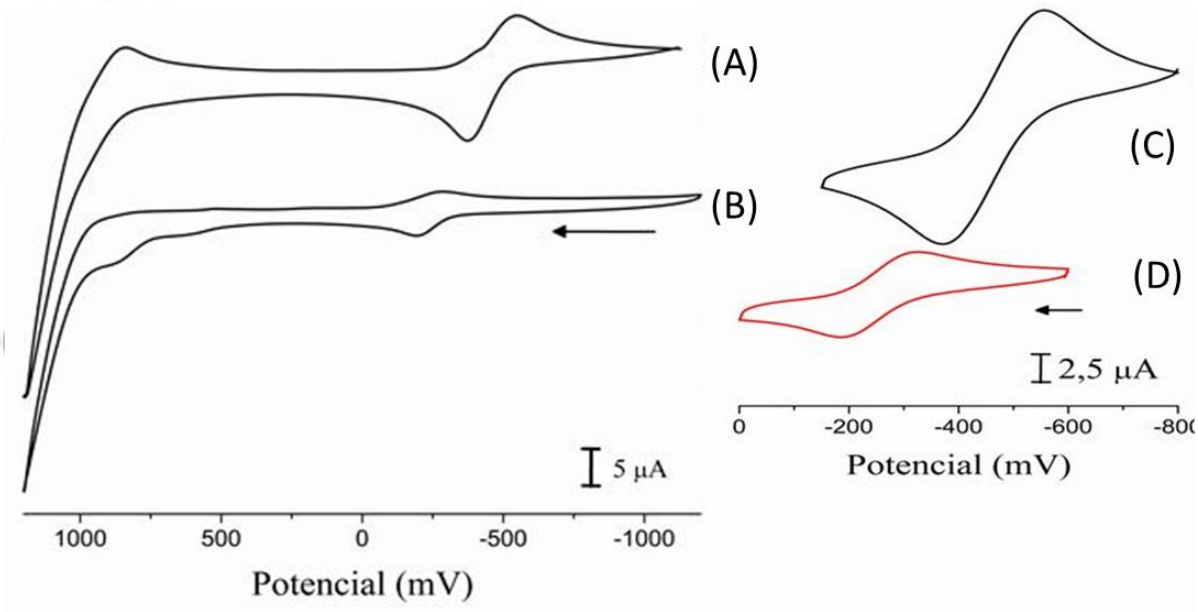

Figure 3. Cyclic voltammogram in 0.1 mol L^{-1} of TBAP in DMSO from -1200 to 1200 for Jacobsen Catalyst (A) and [Mn(3-MeOSalen)Cl] (B); also in the region of 0 to -600 mV for Jacobsen Catalyst (C), and [Mn (3-MeOSalen)Cl] (D).

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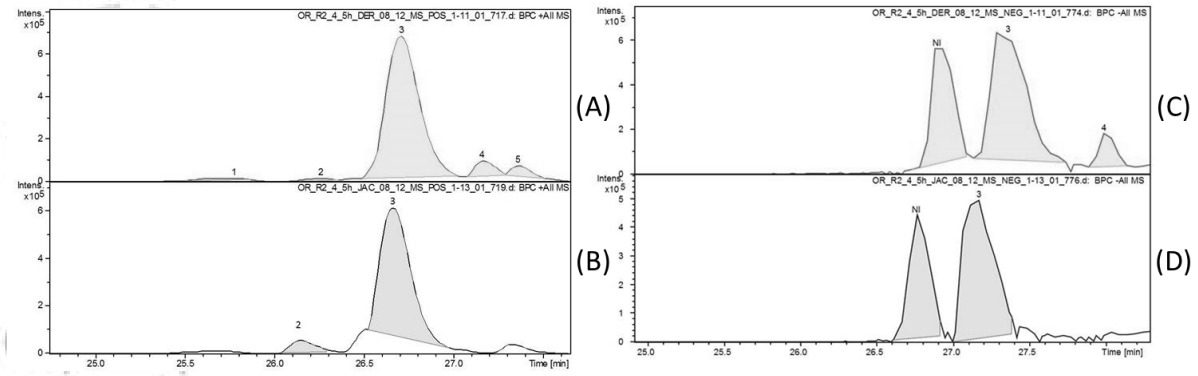

Figure 4. HPLC/MS chromatograms in positive ion mode for the best orientin consuming conditions using [Mn(3-MeOSalen)Cl] catalyst (A) and Jacobsen catalyst (B), and HPLC/MS chromatograms in negative ion mode for the best orientin consuming conditions using [Mn(3-MeOSalen)Cl] catalyst (C) and Jacobsen catalyst (D). NI: not identified compound.

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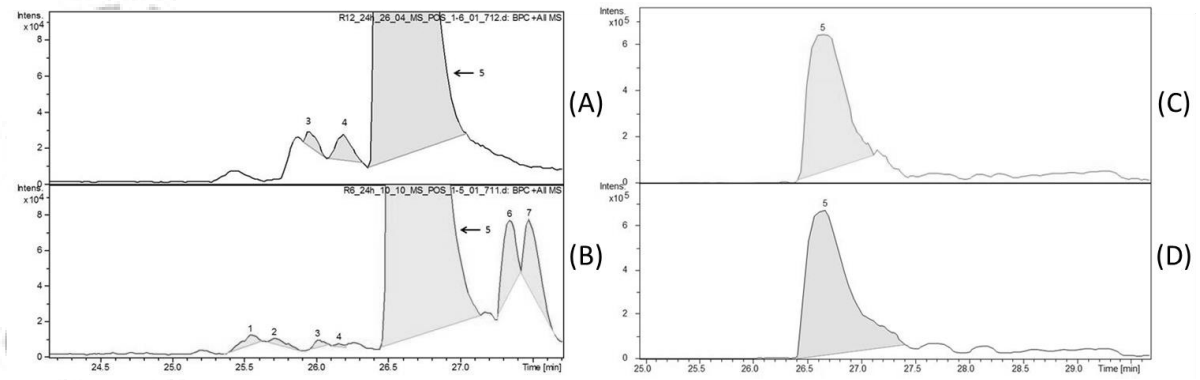

Figure 5. HPLC/MS chromatograms in positive ion mode for the best isoorientin consuming conditions using Jacobsen catalyst (A) and [Mn(3-MeOSalen)Cl] catalyst (B), and HPLC/MS chromatograms obtained in negative ion mode for the best isoorientin consuming conditions using [Mn(3-MeOSalen)Cl] catalyst (C) and Jacobsen catalyst (D).

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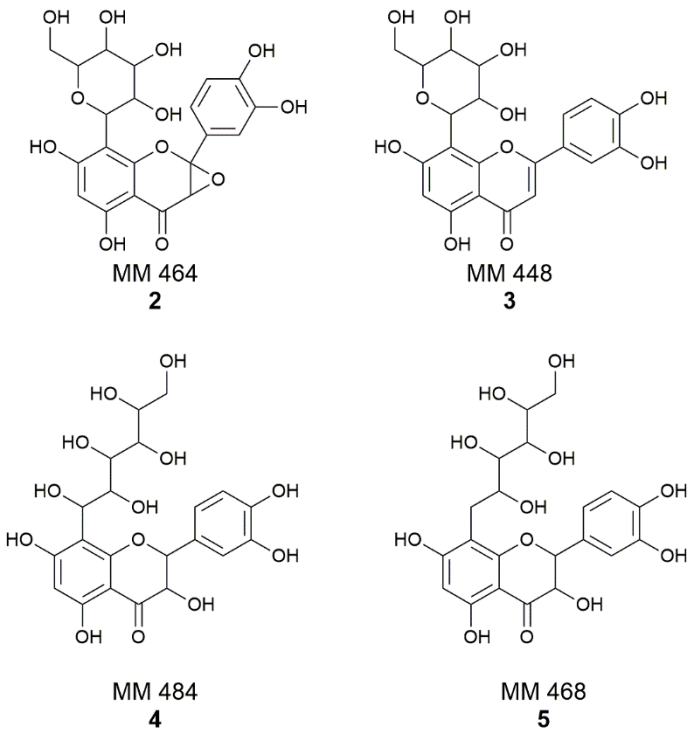

Figure 6. Structures of the orientin reaction products.

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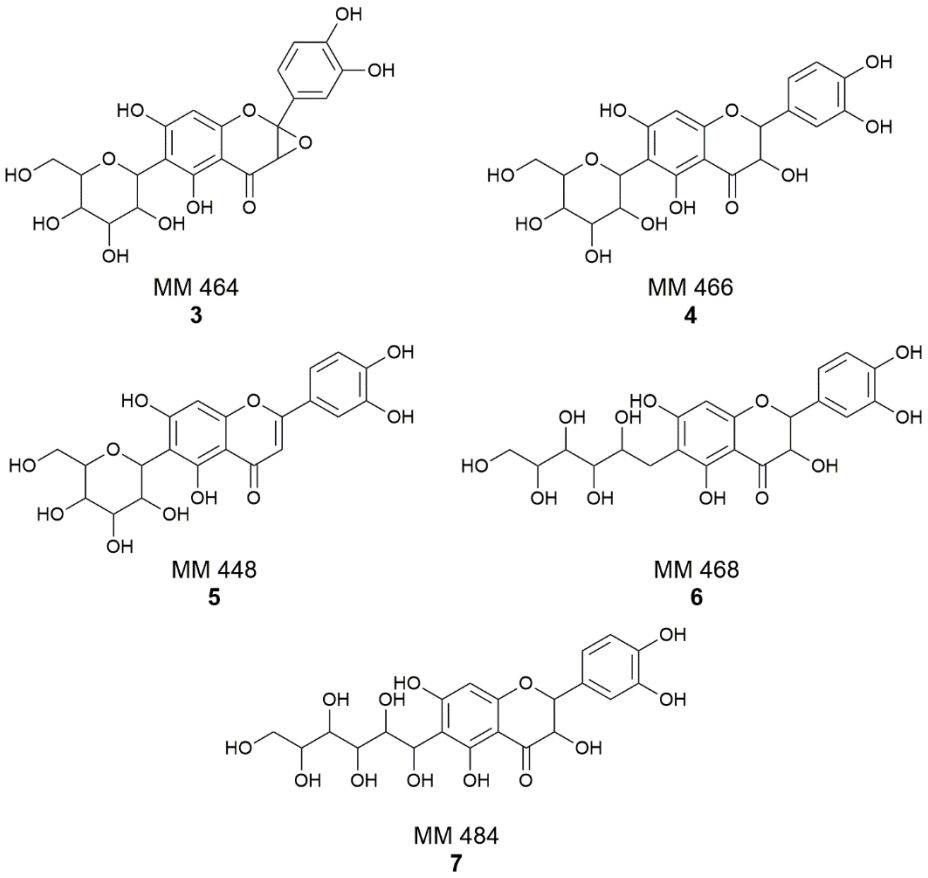

Figure 7. Structures of the isoorientin reaction products.

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