Bioavailability of the ferulic acid-derived phenolic compounds of a rice bran enzymatic extract and their activity against superoxide production†

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Rice bran is an exceptional source of such antioxidant molecules as γ-oryzanol and ferulic acid, but their bioavailability and metabolism within this matrix remain unknown. The aims of this work were to describe the oral bioavailability and metabolic pathways of the ferulic acid-derived phenolic compounds contained in a rice bran enzymatic extract (RBEE), and to determine its effect on NADPH oxidase activity. Wistar rats were administered with RBEE and sacrificed at different times over a period of 24 h to obtain plasma. An additional group was used for collection of urine and faeces over a period of 48 h. The phenolic metabolites were determined by ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS), and plasma pharmacokinetic parameters were calculated. In parallel, aortic rings were incubated in the plasma of rats sacrificed 30 min after RBEE gavage, or in the presence of RBEE, ferulic acid or γ-oryzanol. Endothelin-1-induced superoxide production was recorded by lucigenin-enhanced luminescence. Twenty-five ferulic acid metabolites showing biphasic behaviour were found in the plasma, most of which were found in the urine as well, while in the faeces, colonic metabolism led to simpler phenolic compounds. Superoxide production was abrogated by phenolic compound-enriched plasma and by RBEE and ferulic acid, thus showing the biological potential of RBEE as a nutraceutical ingredient.

1. Introduction

Rice bran (RB), a by-product of rice milling, is an important source of fat, proteins and bioactive molecules with special interest due to its antioxidant and lipid-lowering properties. These bioactive molecules include γ-oryzanol (ORZ) (a mixture of ferulic acid (FA) esters of triterpene alcohols and sterols), tocols (tocopherols and tocotrienols) and unsaturated fatty acids.[1] RB is especially rich in the phenolic compounds ORZ and FA, which have demonstrated hypolipidemic effects (reducing total plasma cholesterol and triglyceride levels, and increasing high-density lipoprotein levels) by mechanisms related to strong antioxidant activity,[2,3] HMG-CoA inhibition[4] and increased cholesterol excretion.[5,6]

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Although RB shows a significant level of natural antioxidants and nutritional proteins,[7] its potential use as a functional food ingredient is limited due to the low water solubility of some of its components, including ORZ. These limitations have been overcome by the development of an enzymatic extraction process, giving rise to the water-soluble rice bran enzymatic extract (RBEE)[8] used in this study. This extraction method preserves the functional properties and improves the solubility of the proteins and antioxidant components of RB,[9,10] providing significant advantages over other RB-derived products, such as rice bran oil.[11] Moreover, the enzymatic treatment also increases the protein concentration and minor functional components, especially ORZ and tocols.

Over recent years, our group has shown that sustained diet supplementation with RBEE is able to improve cardiometabolic markers in obese Zucker rats and prevent the development of atherosclerosis in ApoE−/− mice[12–14] through the reduction of oxidative stress and inflammation markers in the aorta and mesenteric arteries,[13,14] and in adipose tissue.[15–18] Moreover, RBEE counteracted the deterioration of adipose tissue morphology and the expression of genes related to macrophage polarization in high-fat diet-induced obesity.[19] Interest in RBEE is increasing due to its pharmacological activities, revealing the possibility of using it as a nutraceutical ingredient. Therefore, to

explore and determine the mechanisms of action of RBEE bio-active compounds and their role in disease prevention, it is crucial to understand the extent of their absorption and their fate in the organism. Nevertheless, the metabolism and bio-availability of the main phenolic compounds within the rice bran matrix remain unknown, this data being essential for any therapeutic use of the extract in humans.

The main objective of this study was to describe the plasma pharmacokinetics of the main FA-derived metabolites generated after RBEE consumption by pointing out the bioavail-ability of its main metabolites and the metabolic pathways involved. We further evaluated the effects of these plasma FA-derived metabolites on the superoxide production by the NADPH oxidase complex (NADPHox).

2. Material and methods

2.1. Chemicals and reagents

Standards of catechol, *p*-hydroxybenzoic acid, 4-hydroxyphenyl-acetic acid, protocatechuic acid, 3,4-dihydroxyphenylacetic acid, 3-(4-hydroxyphenyl)propionic acid, 3-(2,4-dihydroxyphenyl) propionic acid, caffeic acid, *p*-coumaric acid and FA were purchased from Sigma-Aldrich (St Louis, MO, USA), and vanillic acid from Fluka (Buchs, Switzerland). Methanol (HPLC grade), acetone (HPLC grade), glacial acetic acid and hydrochloric acid (HCl) were provided by Scharlau Chemie (Barcelona, Spain). Acetonitrile (HPLC grade) was from Romyl (Teknokroma, Barcelona, Spain). *ortho*-Phosphoric acid 85% was purchased from Mont Plet & Esteban S.A. (Barcelona, Spain). Water was of Milli-Q quality (Millipore Corp., Bedford, MA, USA).

2.2. Rice bran enzymatic extract (RBEE)

A water-soluble RBEE was prepared as previously described[8] by endoprotease (trypsin- and chymotrypsin like) hydrolysis. The process was conducted in a bioreactor with the pH (pH 8) and temperature (60 °C) controlled. After enzymatic extraction, the nutraceutical composition of the RBEE was characterized as follows: ORZ (8950 ± 850 mg kg^{-1}), phytosterols (3553 ± 66 mg kg^{-1}), free FA (351 ± 5 mg kg^{-1}), tocotrienols (170 ± 15 mg kg^{-1}) and tocopherols (93.4 ± 10 mg kg^{-1}). The ORZ present in the RBEE was composed of different stearyl ferulate esters (such as 2,4-methylene cycloartenyl ferulate, campesteryl ferulate, cycloartenyl ferulate and sitosteryl ferulate).

2.3. Treatment of rats and plasma, urine and faeces collection

Fifty male Wistar rats (body weight 295.8 ± 30.5 g) were purchased from the Central Animal Research Facility of the University of Seville (Espartinas, Seville, Spain). The animals were maintained in groups in an air-conditioned room at 25 °C and 65–70% humidity and with a 12 h light–dark cycle. They were fed a maintenance diet of Harlan (2014, Harlan Laboratories, Madison, USA) and water. All experimental procedures were approved by the University of Seville's (Spain) Committee for Ethical Experimentation (Reference: 12-01-16-002).

At the age of 12 weeks, the rats were randomly assigned to 8 groups ($n = 5$) and starved overnight before oral gavage of the equivalent amount of water-diluted RBEE to 10 g per kg body weight. This dose accounts for a human equivalent dose of 1.6 g per kg body weight (*i.e.* 112 g for a 70 kg person) after applying the 0.162 inter-species rat–human conversion factor.[20] Then, the rats were anesthetized with phenobarbital (i.p. 50 mg kg^{-1}) and blood was extracted by cardiac puncture at the following endpoints: 0 min, 15 min, 30 min, 60 min, 3 h, 6 h, 12 h, 18 h and 24 h. Plasma was obtained by centrifugation ($1500g$, 20 min at 4 °C) and immediately frozen for further determination. An additional group of rats ($n = 5$) was housed in individual metabolic cages and used for the collection of urine (0, 1, 2, 3, 4, 5, 6, 9, 24 and 48 h) and faeces (0, 24, 36 and 48 h). Rats did not have access to food during the course of the study.

2.4. Liquid chromatography analyses (UPLC-ESI-MS/MS)

The phenolic compounds and their generated metabolites were analyzed by AcQuity Ultra-Performance™ liquid chromatography (UPLC) coupled to a triple quadrupole detector (TQD) mass spectrometer (Waters, Milford). The analytical column was an AcQuity BEH C$_{18}$ column (100 mm × 2.1 mm i.d., 1.7 μm,) equipped with a VanGuard™ Pre-Column AcQuity BEH C$_{18}$ (2.1 × 5 mm, 1.7 μm), also from Waters. During the analysis, the column was kept at 30 °C and the flow rate was 0.3 mL min^{-1}. The mobile phase was 0.2% acetic acid (eluent A), and acetonitrile (eluent B). The elution gradient was 0–5 min, 5–10% B; 5–10 min, 10–12.4% B; 10–18 min, 12.4–28% B; 18–23 min, 28–100% B; 23–25.5 min, 100% B isocratic; 25.5–27 min, 100–5% B; and 27–30 min, 5% B isocratic.

Tandem MS analyses were carried out on a triple quadrupole detector (TQD) mass spectrometer (Waters, Milford, MA, USA) equipped with a Z-spray electrospray interface (ESI). Ionization was achieved using the electrospray interface operating in the negative mode [M − H]$^-$ and the data were acquired through selected reaction monitoring (SRM). The ionization source parameters were the ones reported in our previous studies.[20] Two SRM transitions were selected, the most sensitive transition was used for quantification, and a second one for confirmation purposes. The SRM transition for quantification and for identification, as well as the individual cone voltage and collision energy, and the MS^2 fragments for each phenolic compound and metabolite are shown in ESI Table 1S.† The dwell time established for each transition was 30 ms. Data acquisition was carried out with the MassLynx 4.1 software.

In ESI Table 1S† is also shown how each phenolic compound or metabolite has been quantified. Due to the lack of commercial standard phenolic metabolites, these compounds were tentatively quantified by using the calibration curve of their native compound or another phenolic compound with a similar structure, which could partially bias the results obtained.

2.5. Biological sample pre-treatment

Before the chromatographic analyses, the biological samples plasma, urine and faeces were pre-treated in order to extract the phenolic compounds and their generated metabolites and to remove the interference of the biological matrix. There were three replicates of each biological sample.

2.5.1. Plasma samples. The plasma samples were pre-treated by off-line micro-elution solid-phase extraction (µSPE) using OASIS HLB (2 mg, Waters, Milford, MA) micro-cartridges. These were conditioned sequentially with 250 µL of methanol and 250 µL of 0.2% acetic acid. 350 µL of 4% phosphoric acid was added to 350 µL of the plasma sample, and then this solution was loaded into the micro-cartridges. The loaded micro-cartridges were cleaned-up with 200 µL of Milli-Q water and 200 µL of 0.2% acetic acid. Then, the retained phenolic compounds were eluted with 2 × 50 µL of the acetone : Milli-Q water : acetic acid (70 : 29.5 : 0.5, v/v/v) solution. 2.5 µL of the elution solution was injected into the chromatographic system.

2.5.2. Urine samples. The rat urine samples were also pre-treated by off-line µSPE. 100 µL of 4% phosphoric acid was added to 100 µL of the urine sample, and this solution was loaded into the micro-cartridge. The retained phenolic compounds were then eluted with 2 × 50 µL of the acetone : Milli-Q water : acetic acid (70 : 29.5 : 0.5, v/v/v) solution. 2.5 µL of the elution solution was injected into the chromatographic system.

2.5.3. Faeces samples. In order to extract the phenolic metabolites, 0.1 g of lyophilized faeces was mixed in 1 mL of methanol : HCl : Milli-Q water (79.9 : 0.1 : 20, v/v/v) and centrifuged (8784g, 5 min at 4 °C) after 15 min of shaking. The supernatant was collected and re-centrifuged under the same conditions. The resulting supernatant was filtered with nylon filters (0.22 µm filter pore size) and 2.5 µL of this solution was injected into the chromatographic system.

2.6. Quality parameters

The instrumental quality parameters, such as linearity, calibration curve, reproducibility, accuracy, detection limit (LOD), quantification limit (LOQ) as well as extraction recovery (%R) and matrix effect (%ME) were determined by spiking blank plasma, urine and faeces with a known concentration of phenolic compounds and extracted according to the extraction procedure described in section 2.5. Blank plasma, urine and faeces were achieved under basal conditions. These parameters were obtained as these are reported in our previous studies.[21] The obtained results are shown in ESI Table 2S.†

2.7. In vitro and ex vivo antioxidant effects of RBEE

Superoxide anion production was measured in isolated aortic rings by lucigenin-enhanced luminescence, as previously described.[22] Two assays were performed. In the first one 5 mm aortic rings were incubated in HEPES-KHS with RBEE (367.6 mg L^{-1}), FA (5.5 µmol L^{-1}) or ORZ (5.5 µmol L^{-1}). The concentration of FA was selected as being that found for the 24 h-bioavailability of FA after RBEE oral gavage, which accounts for the whole FA recovery after a single RBEE dose. That was also the concentration of FA found in 367.6 mg L^{-1} RBEE and 5.5 µmol L^{-1} ORZ. This experiment was planned to study if there were other active molecules in the extract different from FA. Most of the FA biological metabolites are not commercially available. However, owing to their structure some of the metabolites could also exert antioxidant activities. Therefore, in the second assay, we aimed to reproduce the activity of those circulating metabolites present in the rat plasma after the administration of a single dose of RBEE by incubating the aortic rings with plasma samples from rats sacrificed 30 min after oral gavage of (i) 10 g RBEE per kg body weight (RBEE group), which contained 1.56 ± 0.35 µmol L^{-1} FA and different concentrations of its metabolites in plasma 30 min after the RBEE intake (ESI Table 3S†), or (ii) the equivalent volume of water (control group, water) (section 2.3). Diphenyleneiodonium (DPI, 10 µmol L^{-1}), a NADPHox inhibitor, was used as a positive control. Temperature-controlled (37 °C) incubation in the above-described conditions was performed for 2 h in the presence of endothelin-1 (ET-1, 10 nmol L^{-1}). Then, the rings were washed and transferred to tubes containing 500 µL of HEPES-KHS buffer plus lucigenin (5 µmol L^{-1}). Basal relative luminescence units (RLU) were recorded for 3 min. Then, NADPH was added to a final concentration of 100 µmol L^{-1} and RLUs were measured every minute for 5 min in a luminometer (Junior LB 9509, Berthold, Germany). The background and basal luminescence were subtracted, and the RLUs per minute were normalized to the dry weight of the aortic ring tissue.

2.8. Statistical analysis and pharmacokinetic parameters

Statistical analysis was performed using the Graphpad Prism software v5.01 (San Diego, USA). The data were tested for normality (Kolmogorov–Smirnov test) and analysed via one-way analysis of variance (ANOVA) followed by post-hoc Tukey's multiple-difference test.

Values of p lower than 0.05 were regarded as statistically significant. The data are presented as mean values ± standard error of the mean.

The pharmacokinetic (PK) parameters of the main metabolites of RBEE in the plasma were calculated by means of the Microsoft Excel program. The area under the plasma concentration–time curve (AUC) was calculated using the trapezoidal rule method. The maximum plasma concentration (C_{max}) and time to reach C_{max} (t_{max}) were the observed values.

3. Results

Once the quality parameters of the methods were studied and evaluated, these methods were applied for the determination of phenolic compounds and the generated phenolic metabolites in plasma, urine and faeces samples. For the quantification of the phenolic compounds and their generated metabolites, external calibration was used, and a blank of the

biological sample (plasma, urine and faeces) was spiked with a known concentration of phenolic compounds. Under these conditions, the matrix effect was lower than 12%, and then the use of internal standard for the quantification was not considered.

3.1. Determination of ferulic acid-derived phenolic metabolites in plasma samples

After the acute intake of RBEE, different phenolic metabolites were identified in the plasma, urine and faeces. Fig. 1A shows the pharmacokinetic parameters, C_{max} (µM), t_{max} (min), and AUC (µmol L^{-1} min^{-1}), corresponding to the main circulating phenolic metabolites detected in the plasma. A total of 25 phenolic metabolites were identified in the plasma from 0 to 24 h. In ESI Table 3S† the time-course concentrations of all the phenolic metabolites in the plasma generated after the RBEE intake are shown, whose concentration values are significantly different ($p < 0.05$) from the baseline ($t = 0$ h).

Dihydroxyphenylacetic acid sulphate was the most abundant metabolite, followed by FA sulphate, (methyl) catechol sulphate, dihydroxyphenylacetic acid, and hydroxybenzoic acid. Dihydroxyphenylacetic acid sulphate could be identified

(A)

Phenolic compound	AUC (µmol/L min^{-1})	C_{max} (µM)	t_{max} (min)
Ferulic acid	336.65 ± 25.80	1.63 ± 0.33	27.00 ± 3.00
Isoferulic acid	17.51 ± 6.51	0.28 ± 0.19	24.00 ± 3.68
Ferulic acid sulphate	3748.03 ± 150.53	13.86 ± 3.46	33.00 ± 7.35
Ferulic acid glucuronide	210.75 ± 8.57	0.74 ± 0.21	36.00 ± 6.00
p-Coumaric acid	395.90 ± 47.73	2.32 ± 0.65	36.00 ± 6.00
Coumaric acid sulphate	946.78 ± 25.66	3.91 ± 0.78	24.00 ± 3.68
Coumaric acid glucuronide	6.80 ± 0.93	0.05 ± 0.02	30.00 ± 0.00
Hydroxyphenylacetic acid	1092.48 ± 64.59	2.10 ± 0.32	21.00 ± 9.93
Hydroxyphenylacetic acid glucuronide	81.49 ± 25.05	1.90 ± 0.43	24.00 ± 3.68
Dihydroxyphenylacetic acid	6186.37 ± 471.22	13.17 ± 2.54	24.00 ± 3.68
Dihydroxyphenylacetic acid sulphate	45657.95 ± 1894.32	160.27 ± 27.16	24.00 ± 3.68
Dihydroxyphenylacetic acid glucuronide	761.14 ± 121.48	2.60 ± 0.70	21.00 ± 3.68
Dihydroxyphenylpropionic acid glucuronide	57.34 ± 5.39	0.11 ± 0.01	33.00 ± 7.35
p-Hydroxybenzoic acid	135.90 ± 15.25	0.80 ± 0.27	27.00 ± 3.00
Hydroxybenzoic acid sulphate	277.60 ± 14.38	0.48 ± 0.07	33.00 ± 7.35
Dihydroferulic acid	56.69 ± 19.30	0.20 ± 0.07	291.00 ± 69.00
Dihydroferulic acid sulphate	596.18 ± 93.62	1.42 ± 0.34	228.00 ± 80.83
Phenylacetic acid	380.45 ± 76.88	1.08 ± 0.22	372.00 ± 191.77
Hydroxyphenylpropionic acid	209.08 ± 27.32	0.80 ± 0.14	228.00 ± 80.83
Hydroxyphenylpropionic acid sulphate	3132.60 ± 177.53	5.22 ± 0.78	294.00 ± 66.00
Catechol sulphate	9473.87 ± 701.10	16.65 ± 1.30	360.00 ± 0.00
Methyl catechol sulphate	8015.91 ± 704.37	13.80 ± 1.40	360.00 ± 0.00
Methyl catechol glucuronide	213.87 ± 48.56	0.45 ± 0.06	294.00 ± 66.00
Hydroxybenzoic acid	6344.40 ± 445.91	13.38 ± 1.31	360.00 ± 0.00
Hippuric acid	764.61 ± 34.25	0.93 ± 0.05	297.00 ± 129.94

(B) Dihydroxyphenylacetic acid sulphate
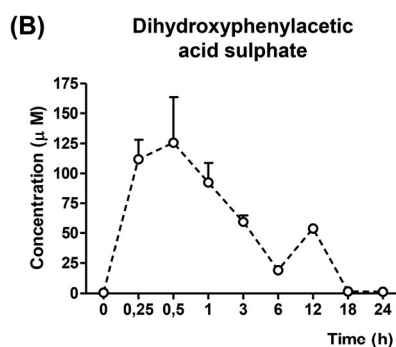
Others: Ferulic acid
Isoferulic acid
Ferulic acid sulphate
Ferulic acid glucuronide
p-Coumaric acid
Coumaric acid sulphate
Coumaric acid glucuronide
Hydroxyphenylacetic acid
Hydroxyphenylacetic acid glucuronide
Dihydroxyphenylacetic acid
Dihydroxyphenylacetic acid glucuronide
Dihydroxyphenylpropionic acid glucuronide
p-Hydroxybenzoic acid
Hydroxybenzoic acid sulphate

(C) Catechol sulphate
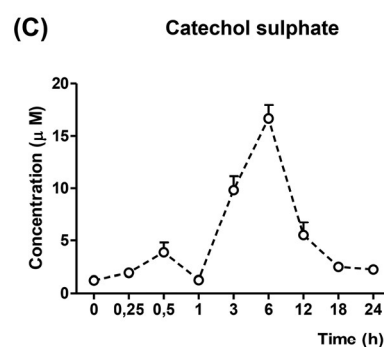
Others: Dihydroferulic acid
Dihydroferulic acid sulphate
Hydroxyphenylpropionic acid
Hydroxyphenylpropionic acid sulphate
Phenylacetic acid
Hydroxybenzoic acid
Methyl catechol sulphate
Methyl catechol glucuronide
Hippuric acid

Fig. 1 Plasma phenolic kinetic parameters expressed as the area under the curve (AUC), maximum peak concentration (C_{max}) and the time at which the C_{max} is observed (t_{max}) (A). Mean concentration (µM) *versus* time of the main phenolic metabolites detected in rat plasma samples after the acute intake of RBEE generated with an early (B) or late (C) t_{max}. The line classifies the metabolites in two groups according to their t_{max}.

by its precursor ion $[M - H]^-$ of m/z 247, and its product ions produced from $[M - H]^-$ fragmentation that were m/z 167, 123 and 108. The product ion m/z 167 was produced by the loss of 80 units, which corresponds to a sulphate moiety. The other fragment ions, m/z 123 and 108 are characteristics of dihydroxyphenylacetic acid. Although this phenolic metabolite and vanillic acid sulphate have the same precursor ion and product ions, these two metabolites could be differenced due to the presence of the product ion m/z 152, which is produced in vanillic acid and not in dihydroxyphenylacetic acid (see ESI Table 1S†).

The circulating phenolic metabolites showed two different kinetics of absorption according to their time of appearance in the plasma (see t_{max} in Fig. 1A). The two groups showed biphasic behaviour (from 0 to 24 h) with two maximum absorption peaks but different t_{max} (Fig. 1B and C). In the first group, represented by dihydroxyphenylacetic acid sulphate, the maximum absorption occurs 30 min after ingestion of the RBEE extract. Then, their concentrations decreased until 6 h, and a second absorption peak was observed at a lower concentration at 12 h (Fig. 1B). The second group of phenolic compounds detected in the plasma, represented by catechol sulphate, showed the first absorption peak 30 min after ingestion of the RBEE extract and the second peak at 6 h (Fig. 1C), reaching a higher plasma concentration compared with the

Fig. 2 Urine molar excretion (µmol) of the main phenolic metabolites observed after the acute intake of RBBE at different time intervals.

first group of compounds. Assuming that any increase observed after RBEE administration comes from FA or ORZ-derived FA, the total phenolic recovery was 18.8% of the original dose administered over the 24 h period studied.

3.2. Determination of ferulic acid-derived phenolic metabolites in urine samples

The main circulating phenolic metabolites identified in the plasma samples were also found in the urine samples from 0 to 48 h. Fig. 2 shows the main phenolic compounds excreted in the urine (µmol) at different time intervals after ingestion of the RBEE extract: from 0–4 h, 4–9 h, 9–24 h, and 24–48 h. The main phenolic metabolites excreted were FA sulphate, hydroxyphenylpropionic acid and its sulphate form, dihydroxyphenylacetic acid sulphate, and (methyl) catechol sulphate. As can be seen in this figure, the concentration of the phenolic metabolites was increased with the time after the RBEE intake. The highest urine excretion was observed between 9 and 24 h and between 24 and 48 h, in comparison with 0–9 h after the RBEE intake.

3.3. Determination of ferulic acid-derived phenolic metabolites in faeces samples

Regarding the analysis of the faeces, different catabolic metabolites were determined from 0 to 48 h after the RBEE intake. Table 1 shows the phenolic metabolites generated after the RBEE intake, whose concentration is significantly different ($p < 0.05$) from the baseline ($t = 0$ h). All the phenolic metabolites detected, with the exception of dihydroxyphenylacetic acid sulphate, were unconjugated simple aromatic acids. Hydroxyphenylpropionic acid, followed by dihydroxyphenylacetic acid and dihydroferulic acid, were the main catabolic metabolites (end products) quantified in the faeces (Table 1).

3.4. RBEE phenolic metabolites attenuate superoxide production

Superoxide production significantly increased after ET-1 incubation ($p < 0.001$) in both the HEPES-KHS- and plasma-incubated rings (Fig. 3). The incubation with RBEE and FA prevented ET-1-induced superoxide production ($p < 0.05$) (Fig. 3A). OZR reduced luminescence reaching values similar
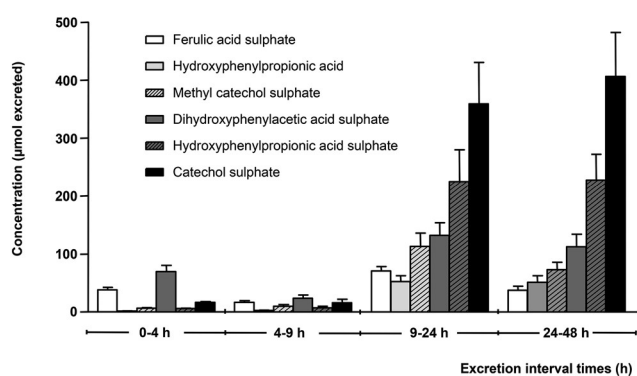
Table 1 Concentration of phenolic metabolites detected in rat faeces (µmol g^{-1}) after the acute intake of rice bran enzymatic extract

Phenolic metabolite (µmol g^{-1} faeces)	0 h	24 h	36 h	48 h
Ferulic acid	3.37 ± 1.39[a]	3.75 ± 1.60[a]	5.08 ± 2.05[a]	5.60 ± 1.76[a]
Dihydroferulic acid	3.86 ± 1.96[b]	3.88 ± 1.61[b]	5.71 ± 2.20[ab]	8.05 ± 2.42[a]
Ferulic acid sulphate	0.01 ± 0.02[b]	0.03 ± 0.01[b]	0.31 ± 0.04[a]	0.01 ± 0.02[b]
Caffeic acid	0.05 ± 0.03[a]	0.09 ± 0.03[a]	0.09 ± 0.03[a]	0.09 ± 0.02[a]
Dihydroxyphenylacetic acid	6.80 ± 1.13[b]	11.67 ± 2.75[a]	8.93 ± 2.35[b]	7.60 ± 2.61[b]
Dihydroxyphenylacetic acid sulphate	n.d.[b]	0.20 ± 0.15[b]	3.61 ± 0.20[a]	0.10 ± 0.02[b]
Hydroxyphenylpropionic acid	24.67 ± 13.14[b]	77.45 ± 19.70[a]	56.25 ± 13.95[b]	66.85 ± 23.42[a]
Protocatechuic acid	n.d.[b]	0.72 ± 0.27[a]	0.48 ± 0.22[a]	0.46 ± 0.28[a]
Hydroxybenzoic acid	0.90 ± 0.30[b]	3.09 ± 1.05[a]	2.81 ± 1.36[a]	3.18 ± 0.58[a]
Hydroxybenzoic acid sulphate	0.12 ± 0.09[b]	0.10 ± 0.08[b]	0.78 ± 0.13[a]	n.d.[b]

n.d.: not detected. Values in a row without a common superscript letter differ significantly ($p < 0.05$).
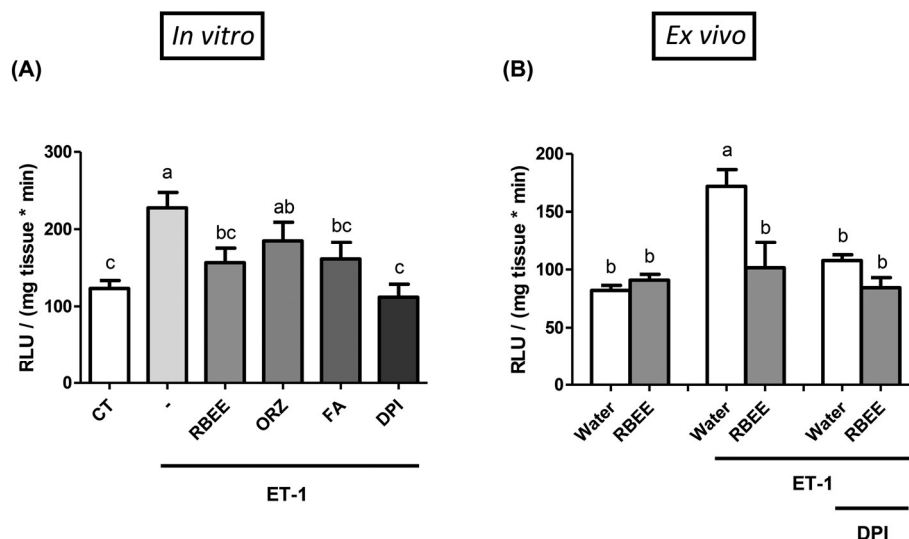
Fig. 3 Superoxide production is reduced by RBEE phenolic metabolites. Superoxide production of ET-1 exposed aortic rings incubated in HEPES–KHS buffer in the presence or absence of RBEE (367.6 mg L^{-1}), ORZ (5.5 µmol L^{-1}), FA (5.5 µmol L^{-1}) or DPI (A). Superoxide production of aortic rings incubated in rat plasma after oral gavage (30 min) of rice bran enzymatic extract (RBEE) or water (CT) in the presence or absence of endothelin-1 (ET-1, 10 nmol L^{-1}) or diphenyleneiodonium (DPI, 10 µmol L^{-1}) (B). Values were mean ± SEM (n = 5). Columns with different letters differ significantly (one-way ANOVA followed by Tukey).

to those found in RBEE- and FA-incubated rings (Fig. 3A). Moreover, the phenolic compound-enriched plasma (30 min after RBEE intake) completely inhibited the ET-1-induced NADPHox activation (p < 0.001) (Fig. 3B).

4. Discussion

FA is a ubiquitous phenolic molecule found in the cell walls of the seeds and leaves both in its free form and conjugated to polysaccharides, glycoproteins, polyamines and fatty acids. Rice bran is rich in FA, found as a part of the γ-oryzanol molecule. Both molecules are known for their antioxidant activities. However, their bioavailability and metabolic pathways within the rice bran matrix remain unknown. Therefore, we aimed to evaluate the bioavailability and the metabolic routes of the FA-derived compounds after oral consumption of a rice bran enzymatic extract (RBEE) and to study their antioxidant activity on the NADPHox complex at the concentrations found in the plasma.

After RBEE consumption, a good number of phenolic compounds derived from FA were identified in plasma, urine and faeces. Free FA (351 ± 5 mg kg^{-1} RBEE) is present in the RBEE used in the present study, the ORZ (FA conjugated form) being an additional source (8950 ± 850 mg kg^{-1} RBEE). The study of ORZ absorption has been gathering interest since the early 1980s. Fujiwara *et al.* reported the metabolism and oral absorption of ORZ, choosing a ^{14}C-labelled located in the FA to motorize ORZ.[23,24] However, the study of the bioavailability of the whole ORZ molecule has been addressed without success to date.[25] In contrast, the FA moiety has been identified in plasma after the ORZ intake.[24,26] The low or null ORZ

absorption could be related to the high molecular weight, or to its low solubility in water, but more importantly, it could be the consequence of the esterase activity in the small and large intestine leading to the ORZ cleavage and formation of FA which can remain in faeces or be absorbed.[27,28] While FA recovery from grain samples ranged from 0.4% to 5% in previous animal and human studies,[29–31] FA recovery after RBEE consumption accounted for 18.8% of the dose administered. This increase compared to previous studies could be due to the enzymatic treatment of rice bran, which reduces the protein size allowing interactions with hydrophobic components such as ORZ, making them soluble and improving its solubility.[8]

The phenolic compounds detected in rat plasma in the present study followed biphasic behaviour with two different profiles, following an early or late C_{max} peak (Fig. 1). The group with an early t_{max} followed by a second peak of lower magnitude could be explained by gastro-intestinal absorption followed by the re-absorption through the enterohepatic re-cycling circulation (Fig. 1B). On the other hand, the late t_{max} group could be assigned to molecules that suffer from a higher colonic microbial metabolism, which would also explain the lower plasma concentration (Fig. 1C), in comparison with the first group. The results obtained were in agreement with the studies reported in the literature for the analysis of the bioavailability of hydroxycinnamic acids after the consumption of coffee[32–34] and cereals.[35] In these studies, the metabolites identified could also be classified into two groups: in one group, the metabolites absorbed in the small intestine, which were mainly phase-II metabolites (sulphated and glucuronidated), such as caffeic and FA conjugates; and in the second group, the phenolic compounds absorbed in the large

intestine (colonic origin), such as dihydrocaffeic and dihydroferulic acids. As in our study, FA in its sulphate and glucuronide forms was also reported in the plasma samples (0 to 6 h) after the consumption of cereals,[35–37] while the generation of dihydroferulic acid from FA has been reported to occur in the colon[35,38] as a consequence of the microflora of the large intestine by mutant strains of *Pseudomonas fluorescens*.[38]

The study of FA-derived phenolic metabolites in urine and faeces was aimed at identifying the RBEE metabolic pathways. Most of the metabolites found in plasma were also identified in urine, indicating a fast metabolization rate. As in our study, hydroxyphenylpropionic acid sulphate was identified in urine samples after a sustained daily intake for 3 weeks of olive oil enriched with its phenolic compounds plus complementary phenolics from thyme (which is rich in rosmarinic acid),[39] and also after the consumption of cereals.[35] Similarly, FA sulphate was the main metabolite excreted in urine after the oral administration of γ-oryzanol-^{14}C in rats.[23]

The metabolite concentration in faeces increases sensibly but not to a great extent for most of the metabolites. This fact could be due to the presence of phenolic compounds in the chow, but also because the absorption from the RBEE matrix was very effective, as suggested by the fast increase in the plasma levels shortly after consumption, followed by rapid metabolization and increased urine excretion between 9 and 48 h. All the metabolites detected were simple phenolic acids with the exception of dihydroxyphenylacetic acid sulphate. These phenolic compounds could be formed from phase-II metabolites secreted *via* the biliary route into the duodenum, where they are subjected to the action of bacterial enzymes, especially β-glucuronidase, in the distal segments of the intestine, after which they may be reabsorbed. This enterohepatic recycling may lead to a longer presence of phenolic metabolites in the body, explaining the maximum plasma concentration observed at 6 or 12 h after the RBEE intake (Fig. 1C). Similarly, hydroxyphenylpropionic and dihydroferulic acids were also reported to be the main metabolites formed after the consumption of cereals,[35–37] and after the *in vitro* colonic fermentation of coffee with human faecal inocula.[40] Additionally, hydroxyphenylpropionic acid was also reported to be the main
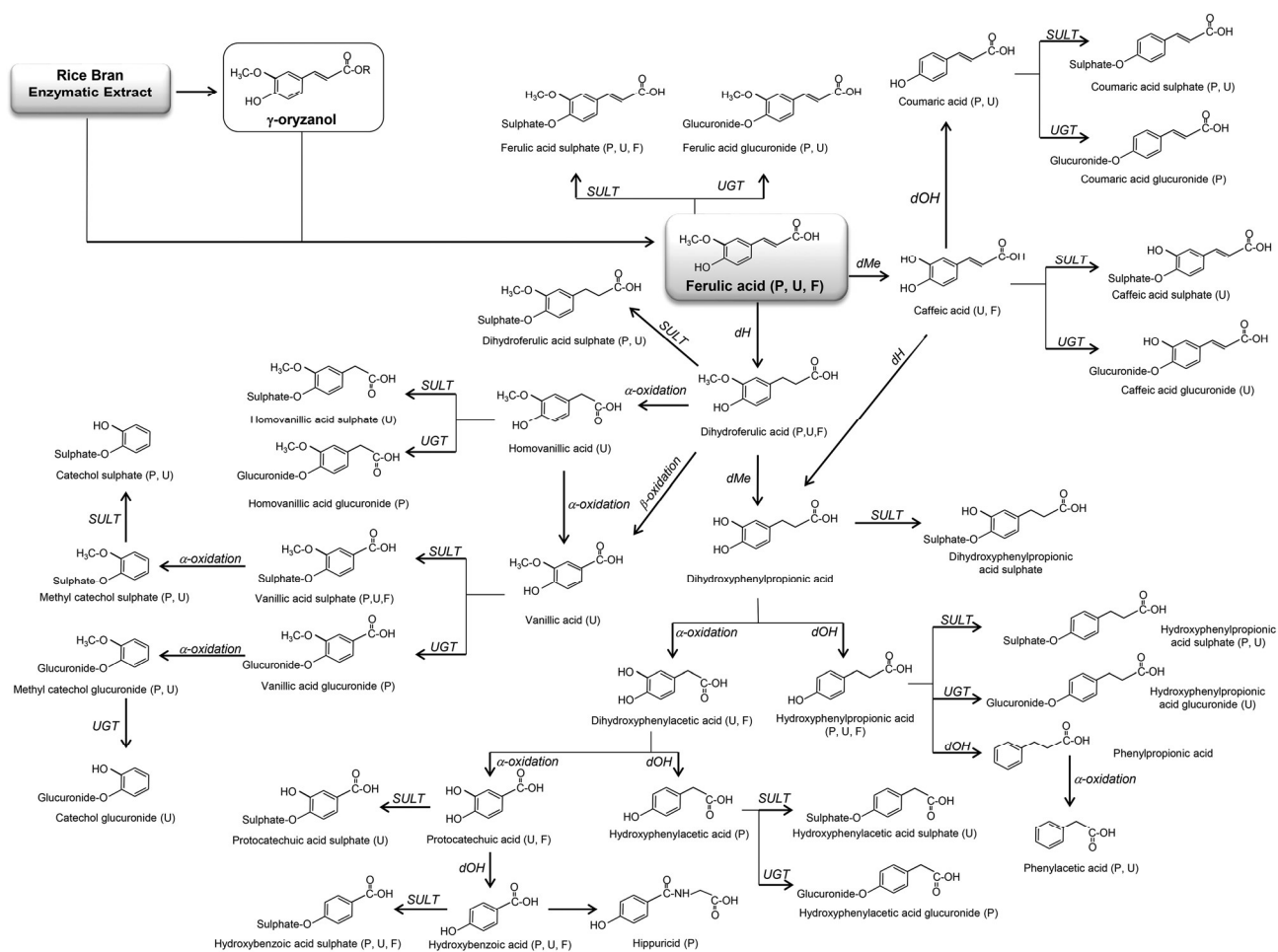

Fig. 4 Metabolic pathways proposed for the phenolic metabolites generated from ferulic acid from the rice bran enzymatic extract. dH, dehydrogenation (reductase); dOH, dehydroxylation; dMe, demethylation; α-oxidation, one decarboxylation; SULT, sulphotransferase; and UGT, glucuronosyl-transferase.

catabolic metabolite when different sources of caffeic acid, such as rosmarinic acid,[41] caffeic acid,[42] chlorogenic acid[42,43] and caftaric acid,[42] were incubated with human faecal samples *in vitro*. Another study by Pereira-Caro *et al.* showed that after the *in vitro* colonic fermentation of FA, methoxy hydroxy-phenylpropionic and hydroxyphenylpropionic acids were the main catabolites formed.[44] Nevertheless, the methoxy form of hydroxyphenylpropionic acid was not detected in faeces in our study.

In order to understand the generation of the phenolic metabolites identified in the different biological samples (plasma, urine and faeces) in the present study, different metabolic pathways have been proposed (Fig. 4). After the RBEE intake, the phenolic metabolites generated were mainly sulphated and glucuronided conjugates formed through the action of sulphotransferases (SULFs) and uridine-5′-diphosphate glucuronosyl-transferases (UGT), respectively. Nevertheless, sulphation was the main phase-II metabolic transformation. On the other hand, part of the phenolic metabolites were not absorbed in the upper gastrointestinal tract and reached the colon, where they could be extensively metabolized by microflora enzymes resulting in a further modification of their chemical structure. Decarboxylation (α-oxidation) and dehydroxylation were the main reactions that may be carried out. The microbial catabolites generated could then undergo phase-II metabolism locally and/or be absorbed and reach the liver, where they would be subjected to enzymatic metabolism before re-entering the systemic blood circulation and finally be excreted in urine.

As discussed above, FA is released from ORZ during digestion, and is absorbed and found in the plasma. Here, we showed that the NADPHox inhibitory activity exerted by RBEE is due to the release of FA from the ORZ molecule since the antioxidant activity was maximized when the aortic rings were incubated with FA (Fig. 3A). This result confirmed the hypothesis that the main antioxidant molecule in RBEE is FA. However, FA is rapidly metabolized giving rise to multiple metabolites that maintained the inhibitory activity upon NADPHox (Fig. 3B). Most of the FA metabolites identified in plasma are not commercially available, which limited the study of the individual effects of all of the metabolites generated. The *ex vivo* study with the phenolic compound-enriched plasma allowed an assessment of their potential as antioxidant compounds. Even though the FA concentration at the t_{max} (30 min) was lower than the dose used in the *in vitro* experiment, the FA metabolite-enriched plasma was able to completely inhibit NADPHox activity, which indicates that some of the FA metabolites present in plasma at 30 min (t_{max}) are active as well and point out potential synergistic effects. These results allow us to directly relate the antioxidant activity as responsible for the protective effects found in other studies of chronic treatments with RBEE. We previously found the down-regulation of NADPHox subunits in the aorta and small mesenteric arteries of ApoE−/− mice[13,14] and Zucker rats.[16,17] Therefore we hypothesize that it is the FA metabolite enriched-plasma of treated animals which is able to prevent the deleter-

ious effects of the activation of NADPH oxidase in the arteries of these animals, confirming the biological activity of RBEE as a source of FA.

5. Conclusions

In conclusion, in this work, the oral bioavailability and metabolic pathways of FA-derived metabolites from a rice bran enzymatic extract (RBEE) are described for the first time. Based on the analysis of plasma after the RBEE intake, the metabolism of FA was characterized by its fast biphasic absorption and metabolization. Two groups of FA metabolites were identified according to the plasma pharmacokinetic parameters (C_{max} and t_{max}), which were governed by enterohepatic recirculation and colonic bacterial metabolization processes. The potential interest of RBEE as a nutraceutical ingredient in the prevention of oxidative stress-related processes arises as a result of its phenolic compound being absorbed and it showing the prevention of superoxide production *in vitro*. A lower but sustained dose could exert the same effects as those found here, but this point should be further studied as well as the metabolite characterization in the main organs involved in polyphenol metabolism or the organs affected by a certain disease that could be prevented by the action of polyphenols.

Abbreviations

AUC	Concentration–time curve;
C_{max}	Maximum plasma concentration
DPI	Diphenyleneiodonium
ET-1	Endothelin-1
FA	Ferulic acid
NADPHox	NADPH oxidase
RBEE	Rice bran enzymatic extract
RLU	Relative luminescence units
t_{max}	Time to reach C_{max}
γ-Oryzanol	ORZ

Conflict of interest

All authors declare no conflict of interest concerning the content of the manuscript.

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