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Exploiting the bioactive properties of γ -oryzanol from bran of different exotic rice varieties

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The rice industry is one of the most significant food industries since rice is a widely consumed cereal over the world. As a result of this substantial production, the rice industries have a significant amount of side streams, including bran, representing millions of tons of raw materials mainly designated to animal feed. Rice bran is a rich source of γ -oryzanol, a bioactive compound with substantial health benefits. In this perspective, different bran rice samples from distinct germplasm origins (Philippines, Italy and Portugal) were studied for their γ -oryzanol content by HPLC-PDA, cytotoxicity in four human tumour cell lines, hepatotoxicity in a normal cell line and for their antimicrobial effects on different bacterial and fungal strains. The Ballatinao sample presented the strongest activity against all the tumour cell lines, and was also the sample showing the highest amount in γ -oryzanol, suggesting its contribution to the exhibited cytotoxic properties. Regarding the antimicrobial activity, the tested samples were able to inhibit the majority of the bacterial and fungal strains, being the Portuguese Ceres sample the one presenting the highest bacterial inhibition and Maluit and Dinorado samples, the highest fungal inhibition. Overall the results show that rice bran extracts may be considered as potential candidates to be used as antimicrobial agents when incorporated on food matrices.

Introduction

Rice is one of the major staple foods consumed all over the world, being China, India, Indonesia, Bangladesh, Vietnam, Thailand, Myanmar and the Philippines, the countries contributing to the highest rice production, corresponding to 82% of the global rice production and 69% of the global rice consumption¹.

Usually, rice is consumed after dehulling and milling, which leads to a vast amount of side streams with bioactive potential, including husk and rice bran, which are mainly used as animal feed². The percentage of rice side streams is variable, which in the case of bran ranges from 10 to 13%³. Rice bran is considered an important underutilised nutritional resource that could be incorporated into human diets to improve health, particularly in developed nations⁴. Rice bran contains mainly oil and protein⁵, as well as dietary fibre, including cellulose and hemicellulose, which are known for their functional properties⁶. Healthy rice bran benefic

properties have been related to its potential for improving the lipid profile⁷, the muscle strength after resistance training⁸ and for its anti-diabetic properties⁹. Those benefits arise mostly from phytochemicals existing on rice bran lipid fraction, mainly γ -oryzanol, ferulic acid and tocotrienols¹⁰. From these three compounds, γ -oryzanol is the most representative, ranging from 79.2 to 912 mg/100 g on rice bran, depending on the bran origin and other factors such as extraction methods¹¹⁻¹⁶.

The γ -oryzanol is a mixture of ferulate esters of sterols and triterpene alcohol and almost 95% of total γ -oryzanol content is represented by four major compounds: 24-methylenecycloartanyl ferulate, cycloartenyl ferulate, campesteryl ferulate and β -sitosteryl ferulate, by decreasing order of abundance^{17,18}.

The healthy properties of γ -oryzanol have been studied since the early 50's on animals and humans, mainly its effect on lipid metabolism^{19,20}. A recent meta-analysis including randomised clinical trials denoted that rice bran oil intake resulted in the reduction of LDL-cholesterol and total cholesterol, hence reducing cardiovascular disease risk⁷. The cholesterol lowering effect may be related to the triterpene alcohols, a part of γ -oryzanol, that play an important role on inhibiting cholesterol synthesis and absorption²¹. The cytotoxic properties of rice bran, particularly the oil fraction have also been explored in recent years, mainly in cancer cells²² and mice²³. The authors reported an inhibitory growth effect of γ -oryzanol on colon and lung cancer cell lines^{22,23}. Most of the human studies regarding rice bran bioactivity have been focused on the whole

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bran or in the oil fraction and individual compounds especially ferulic acid and γ -oryzanol are considered the main contributors to human health improvement¹⁰. The isolated ferulates of triterpene alcohols and sterols known as γ -oryzanol constituents were tested against TPA-induced inflammation in mice²⁴ and against MCF-7 cells²⁵, revealing anti-inflammatory activity and potential for the chemoprevention of cancer^{24, 25} and synergetic effect between the compounds have also been suggested.

The purpose of this study was to extract and quantify γ -oryzanol of different rice bran varieties from different regions (Philippines, Italy and Portugal). Furthermore, it was also intended to explore its bioactive potential such as the cytotoxicity, antibacterial and antifungal activities.

Materials and methods

Standards and reagents

Sulforhodamine B, trypan blue, trichloroacetic acid (TCA), Tris, ellipticine, Streptomycin ampicillin, *p*-iodonitrotetrazolium chloride (INT) and the HPLC gradient grade solvents (acetonitrile, methanol and isopropanol) were purchased from Sigma (St. Louis, MO, USA). RPMI 1640 medium (HyClone), Hank's balanced salt solution (HBSS) and all the additional culture media components were purchased from Gibco Invitrogen Life Technologies (Paisley, UK). Gamma-oryzanol (CAS number 11042-64-1) was used as standard and purchased from TCI Europe.

The culture media Muller Hinton broth (MHB) and Tryptic Soy Broth (TSB) were obtained from Biomerieux (Marcy l'Etoile, France). Blood agar with 7% sheep blood and Mac Conkey agar plates were purchased from bioMérieux (Marcy l'Etoile, France). Antifungal agents, bifonazole and ketoconazole, were purchased by Srbolek, Belgrade, Serbia and Zorkapharma, Sabac, Serbia, respectively. All other chemicals and solvents were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (TGI PureWater Systems, Greenville, SC, USA).

Rice samples

Seven different varieties of rice (Arabon, Azucena, Ballatinao, Bora, Dinorado, NSICRC9 and Maluit) were selected according to endosperm type and seed-coat colour (Table 1) and delivered by International Rice Research Institute (IRRI)

Table 1 – Germplasm characterisation regarding source country, seed coat colour and endosperm type²⁸.

Variety name	Accession number	Source country	Seed-coat colour	Endosperm Type
Ballatinao	IRGC 44297	Philippines	Purple	Glutinous
Maluit	IRGC 55391	Philippines	Red	Glutinous
Dinorado	IRGC 96108	Philippines	Red	Non-glutinous
Arabon	IRGC 97851	Philippines	White	Non-glutinous
Bora	IRGC 113686	Italy	Beige	Non-glutinous
NSICRC9	IRGC 115128	Philippines	Beige	Non-glutinous
Azucena	IRGC 117264	Philippines	Beige	Non-glutinous
Maçarico	-	Portugal	Beige	Non-glutinous
Ceres	-	Portugal	Beige	Non-glutinous

germplasm bank. Rice varieties were grown in greenhouse facilities and the correspondent seeds were collected during the harvest of 2015. Two Portuguese varieties (Maçarico and Ceres) obtained by INIAV/Cotarroz, Portugal, were also included. Paddy samples were dehusked in a Satake mill (THU, Satake, Taito, Japan). Rice bran samples were obtained by polishing the husked grains in a rice polishing mill (TakaYama TM-05 mini testing mill, Taiwan) and after sieving the seed coat fraction in order to retain the particles between 250 μ m and a 90 μ m. The samples were vacuum sealed until analysis.

Extraction of the bran lipid fraction

The bran lipid fraction containing the γ -oryzanol was extracted with isopropanol (IsOH) solvent according to Massaretto procedure with some modifications²⁶. Briefly, the bran samples (0.8 g) were extracted with 20 mL IsOH, mixed by vortex (Heidoph, Reax 2000 Vortex, Schwabach, Germany) for 2 minutes and the mixture was centrifuged (Sigma, 2K15 Centrifuge with 12139-H rotor, Osterode am Harz, Germany) for 10 minutes at 4500 x g at room temperature. The residues were extracted two more times by the same procedure with 10 mL IsOH. The supernatants were pooled and the IsOH solvent was evaporated to dryness in a rotary vacuum evaporator system (Buchi Rotavapor R-114, Waterbath B-450 and Vacuum system B-169, Flawil, Switzerland) at 40 °C. All extractions were done in duplicate.

Gamma-oryzanol quantification in bran lipid fraction

The γ -oryzanol quantification was performed by reverse phase (RP) high performance liquid chromatography (HPLC) coupled to a photodiode array detector (PDA), according to Rogers et al. with minor modifications²⁷. The lipid residue was dissolved in 5 ml of methanol (MeOH) and filtered through a 0.22 μ m Nylon syringe filter (Filter Lab, Barcelona, Spain). The HPLC system (Alliance Waters 2690, Waters 996 PDA and Waters Millenium 32 software, Milford, Massachusetts, United States) was composed with a C18 column (Spherisorb ODS2 150 mm x 4,6 mm, 5 μ m particle size, Waters) and a C-18 precolumn (Phenomenex AQC18 4mmx3mm) operating in the isocratic mode with a mobile phase of ACN:MeOH, (50:50 v/v), during 30 minutes with a flow rate of 1,2 mL/min and injection volumes of 20 μ L. Solvents were filtered with nylon 0,22 μ m membrane and ultrasound degassed.

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The γ -oryzanol standards (10 to 500 $\mu\text{g}/\text{mL}$) were injected to prepare a calibration curve; the obtained chromatograms at wavelength 325 nm presented 4 peaks whose identification as CAF (cycloartenyl ferulate), 24MCAF (24-methylenecycloartenyl ferulate), CampF (Campesteryl ferulate) and β SF (beta-sitosteryl ferulate) has been confirmed by compounds spectrums at the maximum wavelength and by mass spectrometry. The sum of the areas of the four peaks was calculated and correlated to the standard concentration to obtain the calibration curve with a Pearson coefficient of 0,9993, a slope of 22238 ± 322 , an intercept of -112360 ± 92149 , a detection limit (LOD) of 25 $\mu\text{g}/\text{mL}$ and a quantification limit (LQD) of 75 $\mu\text{g}/\text{mL}$.

All the injections were conducted in duplicate. The γ -oryzanol data were expressed as mg/100 g of bran.

Cytotoxicity of bran rice samples

The cytotoxic effects were evaluated in four human tumour cell lines: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma) obtained from DSMZ (Leibniz-Institut DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 10% FBS and 2 mM glutamine, 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin, at 37 °C, in a humidified air incubator containing 5% CO₂. The cytotoxic potential of the rice bran lipid fraction was evaluated by the sulforhodamine B (SRB) assay, according to the procedure described by Abreu et al. For hepatotoxicity evaluation, a cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughterhouse, according to an established procedure and it was designed as PLP2 (porcine liver primary culture)²⁹. The cells cultivation was continued with direct monitoring every two to three days using a phase contrast microscope (Nikon Eclipse 100, Nikon, Tokyo, Japan). Before confluence was reached, cells were subcultured and plated in 96-well plates at a density of 1.0×10^4 cells/well, and cultivated in DMEM medium with 10% FBS, 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin. The rice bran samples were dissolved in DMSO:water, (50:50 v/v), to a stock solution of 8 mg/mL. Cells were treated with different concentrations of the samples (400 – 6.25 $\mu\text{g}/\text{mL}$) and SRB assay was performed as previously described. The results were expressed as GI₅₀ values in $\mu\text{g}/\text{mL}$, the sample concentration that inhibited 50% of the net cell growth. Ellipticine was used as positive control. Two independent experiments were performed for each compound; each one carried out in duplicate. The results are expressed as mean values and standard deviation (SD).

Antimicrobial properties of rice bran samples

Rice bran extracts were dissolved in 30 % ethanol solution and added to Tryptic soy broth (TSB) medium, as described by Sokovic et al.³⁰. The antibacterial activity (MICs and MBCs) was evaluated against three Gram-negative bacteria: *Escherichia coli* (ATCC 25922), resistant *Escherichia coli*, *Pseudomonas aeruginosa* (ATCC 27853), and two Gram-positive bacteria: *Staphylococcus aureus* (ATCC 11632), and MRSA (Methicillin-resistant *Staphylococcus aureus*). The minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations were determined by the microdilution method. Briefly, overnight bacterial cultures were adjusted using the spectrophotometer to a concentration of 10^5 CFU/mL using a spectrophotometer at 625 nm (OD 625). Extracts testing was carried out in different dilutions over the wells containing 100 mL of Tryptic Soy Broth (TSB) and further, 10 μL of the bacterial suspension was added to all the wells. The microplates were incubated for 24 h at 37 °C. The MIC concentrations were achieved following the addition of 40 mL of iodinitrotetrazolium chloride (INT) (0.2 mg mL/1) and incubation at 37 °C for 30 min. The lowest concentration providing a significant inhibition (around 50%) of the bacterial growth in comparison with the positive control was considered as the MIC. Minimal bactericidal concentrations (MBC) were determined by sub-cultivation of 10 mL into microplates containing 100 mL of TSB. The lowest concentration that shows no bacterial growth is considered as the MBC. Standard antibiotics, namely streptomycin and ampicillin, were used as positive controls and 5% DMSO was used as the negative control.

The antifungal activity (MICs and MFCs) was evaluated against four different fungi: *Aspergillus fumigatus* (ATCC 9197), *Aspergillus niger* (ATCC 6275), *Penicillium ochrochloron* (ATCC 9112) and *Penicillium funiculosum* (ATCC 36839)³¹. The bacterial MICs and MFCs were achieved by using the microdilution method. Briefly, the rice extracts were dissolved in 5% solution of DMSO and added to broth malt medium with fungal inoculum. The microplates were incubated for 72 h at 28 °C. The lowest concentrations without observable growth (at the binocular microscope) were defined as MIC. The minimum fungicidal concentrations (MFCs) were determined by serial subcultivation of 2 mL in microtiter plates containing 100 mL of malt broth per well and further incubation for 72 h at 28 °C. The lowest concentration with no observable growth was defined as the MFC, indicating 99.5% killing of the original inoculum. DMSO 5% was used as a negative control, while bifonazole and ketoconazole were used as positive controls.

Statistical analysis

Statistics were performed using IBM SPSS v. 25 (IBM, Armonk, NY, USA). Results were analysed by one-way ANOVA to detect significant differences ($P < 0.05$) between rice varieties and the means were classified by the Duncan comparison test.

Linear relationships between γ -Oryzanol concentration and cytotoxicity activity results were examined by generating Spearman's Correlation Coefficient test ($P < 0.05$).

Results and discussion

Gamma-oryzanol quantification

The mean of total lipid content identified in the bran samples from the nine rice varieties was 13.8 (Table 2). The higher amount of lipids was obtained from the Portuguese variety Maçarico, while Bora revealed the lowest lipid content.

The obtained range of γ -oryzanol variability was between 59.4 ± 0.5 mg and 329.7 ± 5.5 mg per 100 g of bran, being the highest values exhibited by Ballatinao variety with purple pericarp. However, observing the differences between Dinorado and Maluit concentration of γ -oryzanol, the results suggest there is no correlation between the pericarp colour and γ -oryzanol content; these results are in accordance with those found by Sing et al., who analysed 53 varieties of rice, including rice with coloured pericarp, and concluded that γ -oryzanol content is not correlated with pericarp colour³².

The major compounds of γ -oryzanol on the analysed samples are 24-MCAF and CampF, which amount corresponding to 73% of the whole γ -oryzanol compounds. Pokkanta et al. found similar results on 14 samples of rice bran¹⁵. NSICR9 exhibit higher predominance of 24-MCAF and Maçarico and Ceres present higher amounts of CampF when compared with the remaining varieties. The concentrations obtained for γ -oryzanol are within the range found in the literature (79.2 to 912 mg/100 g)^{13, 16, 17, 33-35}. Lower concentrations obtained can be related with the extraction techniques and quantification with different temperatures and solvents, which may affect

not only the lipid yield³⁶ but also the γ -oryzanol content^{11, 12, 16, 37}. Uttama et al. also investigated the relation of γ -oryzanol concentration and different methods of extraction and no significant correlation was obtained³⁷. In fact, for isopropanol selection, we undertake a preliminary study (data not shown) with different type of solvents (isopropanol, ethanol, methanol, acetonitrile, hexane and dichloromethane), temperatures and extraction times for optimization of γ -oryzanol extraction³⁸.

Other important factors affecting γ -oryzanol contents may be related to the rice post harvesting treatments, such as drying, storing temperature, packaging type¹⁴ and the sample particle size¹³. On the other hand, the results can be expressed in different bases, such as rice bran oil, refined rice bran oil or rice bran itself, which may result in a difficult comparison.

Cytotoxic activity of bran rice samples

The cytotoxic properties of the of rice bran extracts were tested against four human tumour cell lines (NCI-H460, HeLa, HepG2, and MCF -7) and in a non-tumour primary culture (porcine liver primary cell culture). Lower GI_{50} values indicate a more effective extract ability to reduce cell lines proliferation. From Table 3 it can be observed that the rice bran extract of Ballatinao was the one that presented stronger results for all the tested tumour cell lines by presenting the lowest GI_{50} values ranging from 21.44 to 48.26 $\mu\text{g/mL}$, that may be related to the higher concentration in γ -oryzanol (329.7 mg/100 g) found in this bran rice sample. The Arabon and Bora samples showed activity only in HeLa and MCF7 cell lines, presenting no cytotoxic effects at the maximal tested concentration (400 $\mu\text{g/mL}$) for NCI H460 and HepG2 cell lines. The samples NSICR9 and Azucena presented moderate cytotoxicity activity; however, Azucena sample presented the most promising results for the HeLa and MCF7 tumour cell lines with GI_{50} values of 81.23 and 96.25 $\mu\text{g/mL}$, respectively. After Ballatinao, the Portuguese varieties, Maçarico and Ceres, presented the stronger activity, especially against NCI-460

Table 2 – Total lipid content (%) and quantification of γ -oryzanol in rice bran samples (mg/100g), and percentage of γ -oryzanol components.

Bran rice Variety	Lipid content (%)	γ -Oryzanol mg/100g	CAF (%)	24 MCAF (%)	CampF (%)	β SF (%)
Ballatinao	13.6 ^c \pm 0.5	329.7 ^e \pm 5.5	36.1	35.4	12.4	12.5
Maluit	15.9 ^f \pm 0.0	59.4 ^a \pm 0.5	33.3	35.1	13.7	14.4
Dinorado	14.2 ^{cd} \pm 0.0	281.0 ^f \pm 0.6	37.0	33.3	12.3	13.8
Arabon	15.4 ^{ef} \pm 0.4	219.2 ^{de} \pm 3.7	40.6	36.2	11.4	9.6
Bora	7.4 ^a \pm 0.1	169.1 ^b \pm 0.5	26.3	47.2	8.0	12.6
NSICR9	11.4 ^b \pm 0.1	171.7 ^b \pm 5.0	16.8	68.8	6.9	6.1
Azucena	14.4 ^{cd} \pm 0.5	212.6 ^d \pm 0.3	41.6	33.4	11.0	10.9
Maçarico	17.4 ^g \pm 0.1	194.7 ^c \pm 2.0	28.5	41.2	18.3	6.7
Ceres	14.6 ^{de} \pm 0.2	227.5 ^e \pm 1.9	25.5	41.2	18.2	8.4
Mean	13.8 \pm 1.0	207.2 \pm 25.2	31.7 \pm 7.7	41.3 \pm 10.6	12. \pm 3.7	10.6 \pm 2.9
Range	7.4 – 17.4	59.4 - 329.7	16.8 - 41.6	33.3 - 68.8	6.9 - 18.3	6.1 - 14.4

Values of lipid and γ -Oryzanol are expressed as mean \pm standard deviation ($n = 4$). Different letters in a column show statistically significant differences at $p < 0.05$. CAF (Cycloartenyl ferulate), 24MCAF (24-methylenocycloartenyl ferulate), CampF (campesteryl ferulate), β SF (beta-sitosterol ferulate).

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Table 3. Cytotoxic activity of rice bran extracts on cancer cell lines expressed in GI_{50} values ($\mu\text{g}/\text{mL}$).

	NCI-H460	HeLa	HepG2	MCF-7	PLP2
Ballatinao	21.4 ^a ± 1.8	24.1 ^a ± 1.0	35.3 ^a ± 1.1	48.3 ^a ± 2.4	157.5 ^a ± 8.6
Maluit	254.1 ^d ± 13.0	314.3 ^f ± 18.1	>400 ^c	>400 ^g	>400 ^c
Dinorado	193.2 ^c ± 5.3	82.0 ^b ± 1.6	151.3 ^b ± 5.2	89.3 ^b ± 3.1	238.5 ^c ± 9.6
Arabon	>400 ^e	198.4 ^e ± 3.9	>400 ^c	112.0 ^d ± 6.2	>400 ^c
Bora	>400 ^e	139.3 ^c ± 1.4	>400 ^c	98.3 ^c ± 4.3	>400 ^c
NSICRC9	88.5 ^c ± 3.2	147.1 ^d ± 2.4	188.4 ^b ± 8.1	149.4 ^e ± 5.3	247.4 ^c ± 10.3
Azucena	84.3 ^c ± 2.1	81.2 ^b ± 2.1	127.1 ^b ± 9.1	96.3 ^c ± 3.5	311.3 ^c ± 14.3
Maçarico	74.3 ^b ± 3.3	135.2 ^c ± 2.4	185.0 ^b ± 12.5	225.4 ^f ± 8.44	>400 ^c
Ceres	68.3 ^b ± 2.8	98.5 ^c ± 2.0	111.3 ^a ± 6.4	107.0 ^d ± 3.61	>400 ^c

GI_{50} values correspond to the extract concentration achieving 50% of growth inhibition in human tumour cell lines or in liver primary culture PLP2. Ellipticine GI_{50} values: 1.21 $\mu\text{g}/\text{mL}$ (MCF-7); 1.03 $\mu\text{g}/\text{mL}$ (NCI-H460); 0.91 $\mu\text{g}/\text{mL}$ (HeLa); 1.10 $\mu\text{g}/\text{mL}$ (HepG2) and 2.29 $\mu\text{g}/\text{mL}$ (PLP2). Different letters in a column show statistically significant differences at $p < 0.05$.

tumour cell line (GI_{50} values of 74.29 and 68.27 $\mu\text{g}/\text{mL}$, respectively). Maluit, which showed the lowest concentration in γ -oryzanol (59.4 mg/100 g) was the least active sample, presenting only cytotoxic activity against NCI H460 and HeLa cell lines with the highest GI_{50} values (254.08 and 314.25, respectively).

Dinorado sample showed good activity against HeLa and MCF7 tumour cell lines (GI_{50} values of 82.03 and 89.27 $\mu\text{g}/\text{mL}$, respectively) and moderate activity for the remaining tumour cell lines. For the non-tumour cell line (PLP2) the samples Ballatinao, Dinorado, NSICRC9 and Azucena presented hepatotoxicity, however, the GI_{50} values obtained for this cell line are much higher than those obtained for tumour cell lines, which makes its use effective against the tumour lines studied. A negative correlation have been found between γ -oryzanol concentration and GI_{50} for HeLa, HepG2 and MCF7 (-0.73, -0.76 and -0.73 respectively), which means high concentrations of γ -oryzanol may be effecting on reducing these cells proliferation. This correlation was also reported by Uttama et al who tested a sample of Homali 105 (*Oryza sativa*) rice bran extract against four human cancer cell lines: lung (CORL23), cervical (HeLa), prostate (PC3) and breast (MCF-7) cancer cell lines; and one normal human cell line (MRC5), using sulphorhodamine (SRB) assay³⁷.

To the best of our knowledge, there are no further reports on the literature about the cytotoxic effects of rice bran extracts in the analysed tumour cell lines.

Antibacterial and Antifungal activities of the rice bran samples

The antibacterial activity data expressed as the minimum inhibitory (MIC) and bactericidal concentrations (MBC) are presented in Table 4. In general, the Portuguese variety, Ceres has shown the strongest activity against all the tested bacterial

strains, by revealing the lowest MIC's values. However, the NSICRC9 variety from the Philippines revealed the strongest capacity against *Staphylococcus aureus*, *Escherichia coli*, and for *Pseudomonas aeruginosa* shows null MIC value.

Ballatinao, the extract with the highest content of γ -oryzanol didn't show a significant antibacterial potential compared with the other extracts, which means that γ -oryzanol may not be the only responsible for the antibacterial effect of these extracts. The antifungal activity data expressed as the minimum inhibitory and fungicidal concentrations are presented in Table 5. Maluit bran rice samples revealed the most promising activity against all the tested fungi samples, with the lower MIC values. Maluit extract can be considered more effective against *Penicillium ochrochloron* comparing to antifungal clinical agents. The Portuguese Maçarico and Dinorado from the Philippines also reveal potential activity against *Aspergillus niger*, *Penicillium ochrochloron*, and *Penicillium funiculosum*.

As it happens for the antibacterial activity, the antifungal activity seems to be not related with the content of γ -oryzanol, since the sample that presented the best results is the one that has the lowest amount in this compound.

To the best of our knowledge, there are no reports on the literature regarding the antimicrobial activity of the raw rice bran, in particular from these specific varieties and bacterial strains. However, several antimicrobial activities have been reported in rice bran derived healthy foods. Li et al. evaluated the biofilm inhibitory activities of fermented rice bran broth with effective microorganisms and found growth inhibition of *Escherichia coli* and *Pseudomonas aeruginosa*³⁹.

Ferdes et al. studied the antimicrobial effect of *Monascus purpureus* red rice against some bacterial strains (*Bacillus subtilis*, *Pseudomonas aeruginosa* and *Escherichia coli*) and

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Table 4 - Minimum inhibitory (MIC, mg/mL) and bactericidal concentrations (MBC, mg/mL) of rice bran lipid fraction.

	<i>S. aureus</i> ATCC 11632		MRSA		<i>E. coli</i> ATCC 25922		Resistant <i>E. coli</i>		<i>P. aeruginosa</i> ATCC 27853	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Streptomycin	0.01	0.01	0.10	N/a	0.05	0.10	0.10	0.20	0.03	0.05
Ampicillin	0.01	0.03	N/a	N/a	0.10	0.20	0.20	N/a	0.05	0.10
Ballatinao	1.50	3.00	1.5	3.00	2.00	4.00	0.50	1.00	0.25	0.50
Maluit	4.00	8.00	4.00	8.00	4.00	8.00	4.00	8.00	1.50	3.00
Dinorado	2.00	4.00	2.00	4.00	2.00	4.00	2.00	4.00	1.00	2.00
Arabon	1.99	3.99	1.00	1.99	1.00	1.99	1.00	1.99	0.49	1.00
Bora	2.00	4.00	1.00	1.50	1.00	2.00	1.00	2.00	1.00	1.50
NSICRC9	1.00	2.00	1.00	2.00	1.00	2.00	2.00	4.00	N/a	N/a
Azucena	2.72	5.44	2.72	5.44	2.72	5.44	1.10	2.72	0.68	1.10
Maçarico	2.38	4.76	1.10	2.38	1.10	2.38	1.10	2.38	0.59	1.10
Ceres	1.10	2.12	0.53	1.10	1.10	2.12	1.10	2.12	0.53	1.10

MIC values correspond to the minimal sample concentration that inhibited the bacterial growth. MBC was defined as the lowest concentration with no visible growth, indicating 99.5% killing of the original inoculum.

Alternaria and *Botrytis* genus) and confirmed the bacteriostatic and antifungal activities of the monascidin A pigment⁴⁰. Other study performed by Arpan et al. assessed the antibacterial activity of the rice bran oil concluding that the intake of rice bran oil may help to prevent bacterial pathogenesis by *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The bacterial cultures were supplied with rice bran oil⁴¹. The studies reveal the antibacterial effect and complete inhibition of these microorganisms. More importantly, these authors also described that the presence of

these extracts also inhibited the presence of other contaminants for five consecutive days.

Conclusions

Considering the extension of rice production and their industrial side streams, such as bran, it can be an important raw material for the development of nutraceuticals and functional foods. The bran rice extracts showed cytotoxic effect on tumour cell lines, possibly related to the amount of γ -

Table 5 - Minimum inhibitory (MIC, mg/mL) and fungicidal concentrations (MFC, mg/mL) of rice bran lipid fraction.

	<i>A. fumigatus</i> ATCC 9197		<i>A. niger</i> ATCC 6275		<i>P. ochrochloron</i> ATCC 9112		<i>P. funiculosum</i> ATCC 36839	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Ketoconazole	0.20	0.50	0.20	0.50	1.00	1.50	0.20	0.50
Bifonazole	0.15	0.20	0.15	0.20	0.20	0.25	0.20	0.25
Ballatinao	16.0	>16.00	2.00	4.00	2.00	4.00	1.00	2.00
Maluit	0.50	1.00	1.00	2.00	0.50	1.00	0.50	1.00
Dinorado	1.00	2.00	1.00	2.00	1.00	2.00	1.00	2.00
Arabon	3.99	7.98	1.99	3.99	1.99	3.99	1.99	3.99
Bora	1.00	2.00	2.00	4.00	1.50	3.00	1.50	3.00
NSICRC9	1.00	2.00	1.00	2.00	3.00	6.00	1.50	3.00
Azucena	2.00	4.00	4.00	8.00	2.00	4.00	2.00	4.00
Maçarico	2.38	4.76	1.10	2.38	1.10	2.38	1.10	2.38
Ceres	2.12	4.23	2.12	4.23	2.12	4.23	2.12	4.23

MIC values correspond to the minimal sample concentration that inhibited the bacterial growth. MFC was defined as the lowest concentration with no visible growth, indicating 99.5% killing of the original inoculum.

oryzanol. However, to assure the extension of the γ -oryzanol influence on cytotoxic activity, the synergetic effects with other oil bran bioactives should be accounted.

Regarding the antimicrobial properties, the obtained results show that rice bran lipid extracts can be effective in the inhibition of the studied bacteria and fungi, mainly *Pseudomonas aeruginosa*, for which the extracts reveal lower MIC's and MBC's. Since these pathogenic bacteria and fungus can be easily found on highly manipulated food products, rice bran oil may be explored as a natural additive on processed foods in order to extend their shelf life.

Overall the results show that rice extracts may be considered as potential candidates to be used as antimicrobial agents when incorporated on food matrices, helping to prevent the contamination by mycotoxins. Owing to the numerous health benefits associated with the consumption of rice bran, detailed *in vivo* studies are recommended to create a strong database.

Conflicts of interest

There are no conflicts to declare.

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References

1. Grain: World Markets and Trade, USDA, <https://www.fas.usda.gov/data/grain-world-markets-and-trade>, (accessed November 2018)
2. N. M. Esa, T. B. Ling and L. S. Peng, By-products of rice processing: An overview of health benefits and applications, *J Rice Res*, 2013, **1**, 1.
3. D. Mohapatra and S. Bal, Effect of degree of milling on specific energy consumption, optical measurements and cooking quality of rice, *J Food Eng*, 2007, **80**, 119-125.
4. E. C. Borresen and E. P. Ryan, in *Wheat and Rice in Disease Prevention and Health*, eds. R. R. Watson, V. R. Preedy and S. Zibadi, Academic Press, San Diego, 2014, 301-310.
5. E. Revilla, C. S. Maria, E. Miramontes, J. Bautista, A. García-Martínez, O. Cremades, R. Cert and J. Parrado, Nutraceutical composition, antioxidant activity and hypocholesterolemic effect of a water-soluble enzymatic extract from rice bran, *Food Res Int*, 2009, **42**, 387-393.
6. S. Qiu, M. P. Yadav and L. Yin, Characterization and functionalities study of hemicellulose and cellulose components isolated from sorghum bran, bagasse and biomass, *Food Chem*, 2017, **230**, 225-233.
7. A. Bumrungpert, R. Chongsuwat, C. Phosat and A. Butacnum, Rice Bran Oil Containing Gamma-Oryzanol Improves Lipid Profiles and Antioxidant Status in Hyperlipidemic Subjects: A Randomized Double-Blind Controlled Trial, *J Altern Complement Med*, 2018, **0**, 1-6.
8. S. Eslami, N. M. Esa, S. M. Marandi, G. Ghasemi and S. Eslami, Effects of gamma oryzanol supplementation on anthropometric measurements & muscular strength in healthy males following chronic resistance training, *Indian J Med Res*, 2014, **139**, 857-863.
9. B. S. Sivamaruthi, P. Kesika and C. Chaiyasut, A comprehensive review on anti-diabetic property of rice bran, *Asian Pac J Trop Biomed*, 2018, **8**, 79-84.
10. C. Perez-Tenero, M. A. de Sotomayor and M. D. Herrera, Contribution of ferulic acid, gamma-oryzanol and tocotrienols to the cardiometabolic protective effects of rice bran, *J Funct Foods*, 2017, **32**, 58-71.
11. M. Peanparkdee, R. Yamauchi and S. Iwamoto, Characterization of Antioxidants Extracted from Thai Riceberry Bran Using Ultrasonic-Assisted and Conventional Solvent Extraction Methods, *Food Bioprocess Tech*, 2018, **11**, 713-722.
12. J. Kamimura, K. K. Aracava and C. E. C. Rodrigues, Experimental data and modeling of rice bran oil extraction kinetics using ethanol as solvent, *Separation Sci Tech*, 2017, **52**, 1921-1928.
13. K. C. Massarolo, A. C. Ribeiro, E. B. Furlong and L. A. D. Soares, Effect of particle size of rice bran on gamma-oryzanol content and compounds, *Journal of Cereal Science*, 2017, **75**, 54-60.
14. O. Norkaew, P. Boontakham, K. Dumri, A. N. L. Noenplab, P. Sookwong and S. Mahatheeranont, Effect of post-harvest treatment on bioactive phytochemicals of Thai black rice, *Food Chem*, 2017, **217**, 98-105.
15. P. Pokkanta, P. Sookwong, M. Tanang, S. Setchaiyan, P. Boontakham and S. Mahatheeranont, Simultaneous determination of tocopherols, gamma-oryzanols, phytosterols, squalene, cholecalciferol and phylloquinone in rice bran and vegetable oil samples, *Food Chem*, 2019, **271**, 630-638.
16. P. Wanyo, N. Kaewseejan, N. Meeso and S. Siriamornpun, Bioactive compounds and antioxidant properties of different solvent extracts derived from Thai rice by-products, *Appl Biol Chem*, 2016, **59**, 373-384.
17. P. Goufo and H. Trindade, Rice antioxidants: phenolic acids, flavonoids, anthocyanins, proanthocyanidins, tocopherols, tocotrienols, gamma-oryzanol, and phytic acid, *Food Sci Nutr*, 2014, **2**, 75-104.
18. S. Yu, Z. T. Nehus, T. M. Badger and N. Fang, Quantification of Vitamin E and γ -Oryzanol Components in Rice Germ and Bran, *J Agr Food Chem*, 2007, **55**, 7308-7313.

19. M. Shinomiya, N. Morisaki, N. Matsuoka, S. Izumi, Y. Saito, A. Kumagai, K. Mitani and S. Morita, effects of gamma-oryzanol on lipid-metabolism in rats fed high-cholesterol diet, *Tohoku J Exp Med*, 1983, **141**, 191-197.
20. K. Sakamoto, T. Tabata, K. Shirasaki, T. Inagaki and S. Nakayama, effects of gamma-oryzanol and cycloartenol ferulic acid ester on cholesterol diet induced hyperlipidemia in rats, *Jpn J Pharmacol*, 1987, **45**, 559-565.
21. N. R. Jolfaie, M. H. Rouhani, P. J. Surkan, F. Siassi and L. Azadbakht, Rice Bran Oil Decreases Total and LDL Cholesterol in Humans: A Systematic Review and Meta-Analysis of Randomized Controlled Clinical Trials, *Horm Metab Res*, 2016, **48**, 417-426.
22. S. Doello, Z. Liang, I. K. Cho, J. B. Kim and Q. X. Li, Cytotoxic Effects of 24-Methylenecycloartanyl Ferulate on A549 Non-small Cell Lung Cancer Cells through MYBBP1A Up-Regulation and AKT and Aurora B Kinase Inhibition, *J Agr Food Chem*, 2018, **66**, 3726-3733.
23. S. P. Kim, M. Y. Kang, S. H. Nam and M. Friedman, Dietary rice bran component γ -oryzanol inhibits tumor growth in tumor-bearing mice, *Mol Nutr Food Res*, 2012, **56**, 935-944.
24. T. Akihisa, K. Yasukawa, M. Yamaura, M. Ukiya, Y. Kimura, N. Shimizu and K. Arai, Triterpene alcohol and sterol ferulates from rice bran and their anti-inflammatory effects, *J Agr Food Chem*, 2000, **48**, 2313-2319.
25. H. F. Luo, Q. Li, S. Yu, T. M. Badger and N. Fang, Cytotoxic hydroxylated triterpene alcohol ferulates from rice bran, *J Nat Prod*, 2005, **68**, 94-97.
26. I. L. Massaretto, PhD thesis, Características químicas e nutricionais de arroz-preto, vermelho e selvagem e comparação por análise estatística multivariada, Universidade de São Paulo, 2013.
27. E. J. Rogers, S. M. Rice, R. J. Nicolosi, D. R. Carpenter, C. A. McClelland and L. J. Romanczyk, Identification and quantitation of γ -oryzanol components and simultaneous assessment of tocopherols in rice bran oil, *J Am Oil Chem Soc*, 1993, **70**, 301-307.
28. The international Rice Genebank collection information system, <http://www.irgicis.irri.org:81/grc/SearchData.html>, (Accessed September 2018)
29. R. M. Abreu, I. C. Ferreira, R. C. Calhelha, R. T. Lima, M. H. Vasconcelos, F. Adegas, R. Chaves and M. J. Queiroz, Anti-hepatocellular carcinoma activity using human HepG2 cells and hepatotoxicity of 6-substituted methyl 3-aminothieno[3,2-b]pyridine-2-carboxylate derivatives: in vitro evaluation, cell cycle analysis and QSAR studies, *Eur J Med Chem*, 2011, **46**, 5800-5806.
30. M. Sokovic, J. Glamoclija, P. D. Marin, D. Brkic and L. J. van Griensven, Antibacterial effects of the essential oils of commonly consumed medicinal herbs using an in vitro model, *Molecules*, 2010, **15**, 7532-7546.
31. M. Kostic, M. Smiljkovic, J. Petrovic, J. Glamoclija, L. Barros, I. Ferreira, A. Ciric and M. Sokovic, Chemical, nutritive composition and a wide range of bioactive properties of honey mushroom *Armillaria mellea* (Vahl: Fr.) Kummer, *Food Funct*, 2017, **8**, 3239-3249.
32. S. X. Sing, H. H. Lee, S. C. Wong, C. F. J. Bong and P. H. Yiu, Ferulic acid, gamma oryzanol and GABA content in whole grain rice and their variation with bran colour, *Emir J Food Agr*, 2015, **27**, 706-711.
33. V. Ziegler, C. D. Ferreira, M. M. C. Cardozo, M. de Oliveira and M. C. Elias, Pigmented rice oil: Changes in oxidative stability and bioactive compounds during storage of whole grains, *J Food Process Pres*, 2017, **41**, 7.
34. M. Irakli, F. Kleisiaris, A. Mygdalia and D. Katsantonis, Stabilization of rice bran and its effect on bioactive compounds content, antioxidant activity and storage stability during infrared radiation heating, *J Cereal Sci*, 2018, **80**, 135-142.
35. T. D. Jung, G. H. Shin, J. M. Kim, S. I. Choi, J. H. Lee, S. J. Lee, S. J. Park, K. S. Woo, S. K. Oh and O. H. Lee, Comparative Analysis of gamma-Oryzanol, beta-Glucan, Total Phenolic Content and Antioxidant Activity in Fermented Rice Bran of Different Varieties, *Nutrients*, 2017, **9**, 571.
36. N. S. M. Daud, D. N. A. Zaidel, K. S. Lai, N. Khairuddin, Y. M. M. Jusoh and Muhamad, II, Crude Oil Yield and Properties of Rice Bran Oil from Different Varieties as Affected by Extraction Conditions Using Soxhlet Method, *Arab J Sci Eng*, 2018, **43**, 6237-6244.
37. S. Uttama, I. Sakkapdeejaroen and A. Itharat, Correlation of cytotoxicity, antioxidant activities, gamma-Oryzanol content and extraction methods of Hom Mali 105 rice bran (*Oryza sativa*), *Planta Med*, 2010, **76**, 136.
38. M. H. Chen and C. J. Bergman, A rapid procedure for analysing rice bran tocopherol, tocotrienol and γ -oryzanol contents, *J Food Compos Anal*, 2005, **18**, 139-151.
39. Z. Li, J. Lee and M. H. Cho, Antioxidant, antibacterial, tyrosinase inhibitory, and biofilm inhibitory activities of fermented rice bran broth with effective microorganisms, *Biotechnol Bioprocess Eng*, 2010, **15**, 139-144.
40. M. Ferdes, C. Ungureanu, N. Radu and A. A. Chirvase, Antimicrobial effect of *Monascus purpureus* red rice against some bacterial and fungal strains, *New Biotechnol*, 2009, **25**, 194-194.
41. D. Arpan, J. Praveen and S. Ajay, Antibacterial activity of rice bran oil, *Recent Res Sci Technol*, 2013, **5**, 18-19.

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