Trace Rice

Extraction technologies to optimize bioactive compounds

24th January 2024

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Title: Report of extraction technologies to optimize bioactive compounds (D3.4)

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Executive summary

The objective of this task is to develop extraction methodologies that allow the obtention of bioactive compounds from rice by-products with impact on the human health. If possible, the proposed technologies will be sustainable and efficient. Moreover, the potential bioactive effect on the human health will be assessed by in vitro assays.

1. Introduction

Rice (*Oryza sativa L.*) is the most important staple food crop consumed globally. In terms of production rice is the third most important grain in the world behind wheat and corn. Approximately 757 million tons of rice is harvested worldwide. Rice processing involves milling steps to produce several materials including rice bran, husk and broken rice. By-products recycling is one of many approaches of the circular economy, which is promoted as a sustainable business model with great market potential for the European agriculture and food sector. In this report, we present different approaches to valorise rice by-products by applying diverse extraction methodologies to get bioactive extracts. These extracts will be evaluated to assess their cytotoxicity in human cell lines and further bioactivity namely antioxidant and antiproliferative effect. Additionally, the study incorporates an analysis of the technological and functional properties of different fractions of rice bran.

One of the key health-promoting features of rice oil is its balanced fatty acid composition. It contains a high proportion of monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), particularly oleic acid (C18:1) and linoleic acid (C18:2). These MUFAs and PUFAs contribute to reducing low-density lipoprotein (LDL) cholesterol levels, thereby supporting cardiovascular health[1,2]. Moreover, rice brain oil (RBO) has been studied for its potential anti-inflammatory properties, attributed in part to its composition of bioactive compounds such as γ-oryzanol, tocopherols, and tocotrienols, which collectively exhibit anti-inflammatory effects. γ-oryzanol, one of the prominent components of RBO, has shown the ability to reduce inflammation and antioxidant stress[3,4,5,6]. γ-oryzanol rich extracts as radical scavengers, possess potent anti-inflammatory activities by inhibiting nuclear factor-kB activation, decreasing oxidative stress gene markers[7].

Once rice bran oil is extracted, various interesting components remain in the resulting residue, such as the fibers or proteins of rice bran. Sometimes, due to their characteristics, their applicability is limited. The resulting rice bran, rich in proteins containing essential amino acids like GABA and showing high digestibility, offers numerous health benefits[8]. Furthermore, it has been reported to contain more than 30% fiber, usually not used for human consumption. Nevertheless, several studies

[1] S. Punia et al., "Rice-bran oil: An emerging source of functional oil," Journal of Food Processing and Preservation, vol. 45, no. 4. Blackwell Publishing Ltd, Apr. 01, 2021. doi: 10.1111/jfpp.15318.

[2] R. Singanusong and J. J. Jacoby, "Nutrition and applications of rice bran oil: a mini-overview", doi: 10.16210/j.cnki.1007-7561.2021.05.000.en.

[3] C. Lemus et al., "γ-Oryzanol. An Attractive Bioactive Component from Rice Bran.," in Wheat and Rice in Disease Prevention and Health, Elsevier Inc., 2014, pp. 409–430. doi: 10.1016/B978-0-12-401716-0.00032-5.

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[5] A. Mastinu et al., "Gamma-oryzanol prevents LPS-induced brain inflammation and cognitive impairment in adult mice," Nutrients, vol. 11, no. 4, Apr. 2019, doi: 10.3390/nu11040728.

[6] Maznah Ismail et al., "Gamma-oryzanol rich fraction regulates the expression of antioxidant and oxidative stress related genes in stressed rat's liver," Nutr Metab (Lond), 2010.

[7] P. Klongpityapong et al., "Antioxidant effects of gamma-oryzanol on human prostate cancer cells," Asian Pacific Journal of Cancer Prevention, vol. 14, no. 9, pp. 5421–5425, 2013, doi: 10.7314/APJCP.2013.14.9.5421.

[8] Zheng, Y. et al. (2019). Chapter 11 - Rice Bran Protein: Extraction, Nutraceutical Properties, and Potential Applications. En L.-Z. Cheong & X. Xu (Eds.), Rice Bran and Rice Bran Oil (pp. 271-293). AOCS Press. https://doi.org/10.1016/B978-0-12-812828-2.00011-1

support its capacity to lower blood glucose levels or inhibit lipase activity in the blood[9,10]. The rice bran fiber primarily involves insoluble dietary fiber, composed of cellulose, some hemicellulose, and lignin, with benefits for intestinal flora and transit, increased stool volume, and inhibition of pancreatic lipase[11,12] . Soluble dietary fiber, in lower proportion, has benefits in reducing glycemic response and plasma cholesterol, as well as in immunomodulatory activity and colorectal cancer prevention[12]. An alternative to revalorize and enhance their applicability is to modify their properties through different treatments, such as enzymatic processes.

2. Objectives

- To develop methodologies to extract bioactive compounds from rice and rice by products.
- To evaluate the bioactivity of the obtained extracts
- Using enzymatic treatments to modify the techno-functional properties of rice bran with and without defatting.
- Characterize the different fiber fractions of the treated brans.

3. Development

3.1. Materials

Rice bran and white broken rice from Japonica and Indica varieties were obtained from Ernesto Morgado S.A, Portugal. When received in February 2021, the material was placed into plastic bags and stored at 4°C until additional analyses could be conducted. Throughout the experiments, a specific amount of biomass was milled (< 1 mm) and kept at room temperature in a desiccator (Figure 1).

[9] Qi, J. et al. (2015). Cellulosic fraction of rice bran fibre alters the conformation and inhibits the activity of porcine pancreatic lipase. Journal of Functional Foods, 19, 39-48. https://doi.org/10.1016/j.jff.2015.09.012

[10] Qureshi, A. A. et al., (2002). Effects of stabilized rice bran, its soluble and fiber fractions on blood glucose levels and serum lipid parameters in humans with diabetes mellitus Types I and II. The Journal of Nutritional Biochemistry, 13(3), 175-187. https://doi.org/10.1016/S0955-2863(01)00211-X

[11] Mudgil, D. and Barak, S. (2013). Composition, properties and health benefits of indigestible carbohydrate polymers as dietary fiber: A review. International Journal of Biological Macromolecules, 61, 1–6. https://doi.org/10.1016/j.ijbiomac.2013.06.044

[12] Jia, M. et al., (2019). Structural characteristics and functional properties of soluble dietary fiber from defatted rice bran obtained through Trichoderma viride fermentation. Food Hydrocolloids, 94, 468–474. https://doi.org/10.1016/j.foodhyd.2019.03.047

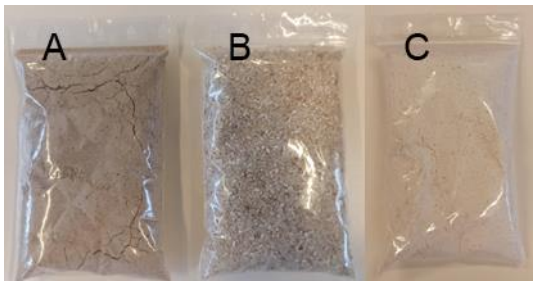

Figure 1. Milled rice bran (A), white broken rice (B) and milled white broken rice (C)

Rice bran for the enzymatic treatments was procured from Arrocería Pons (Valencia, Spain) and defatted (DRB) suspending the RB in hexane with a ratio of 1:3 (w:v). The bran was sieved to obtain a homogenous sample. Enzymes were purchased from Novozymes (Bagsvaerd, Denmark). Depending on their activity, the enzymes were selected as protein-acting enzymes or carbohydrate-acting enzymes.

3.2. Extraction methods

3.2.1. Conventional extractions: Soxhlet

Solid-liquid extraction was performed to characterize the raw material. Based on the experience gained in the research group for other natural matrices, for Soxhlet extraction the rice sample (20 g) was placed into cellulose thimbles (22mm×80mm, Whatman (plugged with glass beads to avoid transfer of sample particles in the distillation flask) and placed in Soxhlet apparatus (Figure 2). Solvent (400 ml), n-hexane or ethanol, was added to the flask and boiled for six hours above their boiling temperature, 69 and 78°C, respectively. The extract was filtered (Qualitative filter paper, 125 mm, from FILTER-LAB) and centrifuged (Eppendorf Centrifuge 5810 R) to remove any remaining solid part of the unsolved raw material. The extract was dried in a rotary evaporator under reduced pressure at 40 °C. Extractions were performed in triplicate.

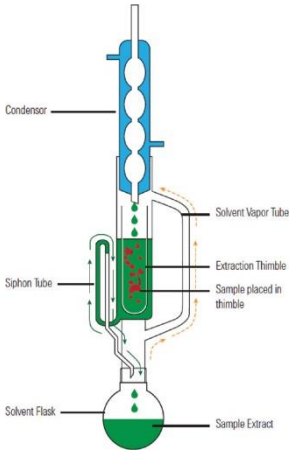

Figure 2. Representation of a Soxhlet apparatus.

3.2.2. Supercritical CO2 extraction

Supercritical CO2 extractions were carried out in a supercritical fluid extraction system (Thar Technology, Pittsburgh, PA, USA, model SFE-500F-2-C50) comprising a 500 mL cylinder extraction cell and two different separators, each of them with 500 mL of capacity, with independent control of temperature and pressure. This apparatus was previously described by Nunes et al[13].

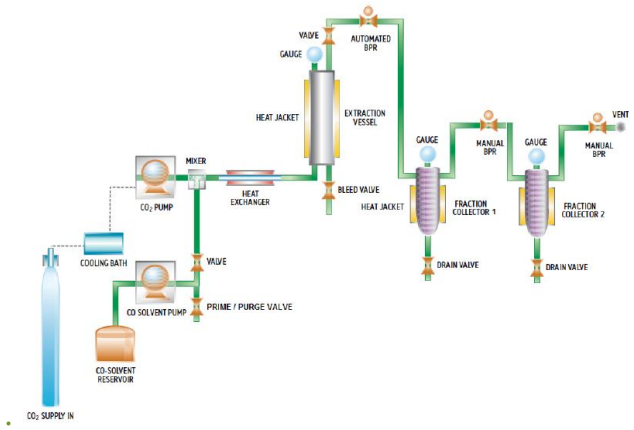

Figure 3. Representation of a supercritical CO2 extractor.

3.2.3. Enzymatic methods

Proteases were added at a concentration of 1% relative to the protein content of dry basis of DRB [14] following the optimal conditions of pH (6.1) and temperature (50ºC) for a duration of 120 minutes. Carbohydrate-acting enzymes were applied at a rate of 1% with respect to the fiber content of dry DRB, under the same pH, temperature, and time parameters as the proteases. To inactivate the enzymes, the temperature was raised to 80ºC for 10 minutes.

3.3. Chemical and biological characterization methods

3.3.1. Analysis of γ-oryzanol of SOX and SFE samples

The LC–MS/MS analysis was developed in house and was performed on an SCIEX QTRAP 6500+ (Sciex, USA) system. Chromatographic separation was achieved on a ACQUITY UPLC BEH Shield RP18 Column, 130Å, 1.7 µm, 2.1 mm X 150 mm (Waters cat. 186003376) at 25 °C. For the mobile phase ACN:MeOH, 50:50 (v/v) was used as solvent in an isocratic gradient. The 6500+ QTrap was operated in negative ion mode using electrospray ionization (ESI). Nitrogen was used as the curtain gas (CUR), nebulizing gas (GS1) and drying gas (GS2). All other instrumental parameters were set as follows: CUR at 20 psi, GS1 at 50 psi, GS2 at 35 psi, the drying gas was heated to 650 °C, and

[13] Nunes, A. N. et al.,"Production of a natural red pigment derived from {Opuntia} spp. using a novel high pressure CO2 assisted-process," RSC Adv., vol. 5, no. 101, pp. 83106–83114, 2015, doi: 10.1039/C5RA14998C.

[14] Vallabha, V. S. et al. (2015). Enzymatic process of rice bran: A stabilized functional food with nutraceuticals and nutrients. Journal of Food Science and Technology, 52(12), 8252–8259. https://doi.org/10.1007/s13197-015-1926-9

the ion spray voltage was set at −4300 V. The declustering potential (DP), collision energy (CE), entrance potential (EP), exit potential (CXP) and mass transitions were optimized for each analyte. The MS was operated in multiple reaction monitoring (MRM) mode. Analyst 1.6.3 and MultiQuant 3.0.2 software were used for data acquisition and analyses. The identification of analytes was performed based on their retention times compared to the standard (±2%) and the ratio of the first and second transition abundances (within 90 - 110 % of the value obtained for the standard).

3.3.2. Analysis of fatty acids of SOX and SFE samples

Lipids obtained after extraction of rice oil obtained by Soxhlet and SFE were converted to corresponding FAMEs in a two-step acid-catalyzed method (ISO 5509, 2000). The analysis was performed using a Thermo Scientific TRACE GC Ultra (Thermo Scientific, Milano, Italy) GC-FID. Heptadecanoic acid (1 mg/mL) was used as an internal standard. The separation of sample components was achieved using a J&W DB-23 capillary column (Agilent Technologies, Inc., Santa Clara, CA, USA), 60 m × 0.25 mm internal diameter and 0.25 µm phase thickness.

3.3.3. Proximate composition of samples obtained by enzymatic treatment

Moisture analysis was conducted using the ISO 712:2009 method. Total nitrogen content was determined according to the ISO 16634-2:2016 standard applying 6.25 as a nitrogen to protein conversion factor. The quantification of total dietary fiber (TDF), insoluble dietary fiber (IDF), and soluble dietary fiber (SDF) was conducted using the 37-02 method as outlined by AACC (2000). The determination of neutral detergent fiber (NDF) content followed the procedure proposed by Van Soest et al.[15]. Acid detergent fiber (ADF) and acid detergent lignin (ADL) were analyzed according to the protocols specified in AOAC (973.18). For the ADL analysis, the samples were treated with 72% sulfuric acid for a duration of 3 hours. The hemicellulose fraction was computed as ADF minus NDF, while cellulose was calculated by subtracting ADL from ADF.

3.3.4. Antioxidant activity (ORAC) of SOX-Hex and SFE samples

This assay assessed the ability of the antioxidant species in the sample to inhibit the oxidation of fluorescein catalyzed by peroxyl radicals generated from AAPH. The assay was performed using the method described in Serra et al[16]. Three independent experiments were performed in triplicate.

[15] Van Soest, P. J. et al. (1991). Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition. Journal of Dairy Science, 74(10), 3583–3597. https://doi.org/10.3168/jds.S0022-0302(91)78551-2

[16] A. T. Serra et al., "Identification of bioactive response in traditional cherries from Portugal," Food Chem, vol. 125, no. 2, pp. 318–325, Mar. 2011, doi: 10.1016/j.foodchem.2010.07.088.

3.3.5. Cytotoxicity of SOX-Hex and SFE samples

Cytotoxicity assays were performed using confluent and non-differentiated Caco-2 cells. This cell model is widely used as it shares some characteristics with crypt enterocytes being considered an acceptable intestinal epithelium model. cell viability was expressed in terms of percentage of living cells relative to the control.[17] Three independent experiments were performed in triplicate.

3.3.6. *Antiproliferative activity* of SOX-Hex and SFE samples

The capacity of rice bran extracts to inhibit cancer cell proliferation was evaluated in vitro using HT29 cell line, a widely used model for in vitro colorectal cancer studies. Cell viability was expressed in terms of percentage of living cells relative to the control[18,19]. Three independent experiments were performed in triplicate.

3.3.7. Cellular antioxidant activity of SOX-Hex and SFE samples

The formation of intracellular ROS was assessed using a dichloro-dihydro-fluorescein diacetate (DCFH-DA) probe as previously described, with minor modifications[2021]. Results were expressed in terms of percentage of fluorescence intensity relatively to the control (cells treated with DCFH-DA and oxidant (600µM AAPH)). Three independent experiments were performed in triplicate.

4. Results

4.1. Raw material selection

In the first attempt, to obtain rice oil with high concentration of bioactive compounds, the by-products (white Broken Rice and Rice Bran) from two varieties were used (Japonica and Indica). Conventional solid-liquid extraction with n-hexane performed in a Soxhlet apparatus (SOX-Hex) was compared to supercritical fluid extractions (SFE). The extraction conditions for the preliminary tests were based on previous knowledge of the group. SOX was performed for 6h with mass:solvent ratio of 1:20 (g:mL) and SFE at 40ºC, 500 bar, flow rate of 15 g/min (CO2) for 3 h. Results obtained are summarized in Table 1. As expected, as the bran is known for having more lipophilic compounds, the extraction yield (calculated as (g extract/100 g by-product) was higher when using the bran than the white broken rice. Moreover, SOX extraction was shown to be

[17] Rodrigues, L. et al., "Recovery of antioxidant and antiproliferative compounds from watercress using pressurized fluid extraction," RSC Adv, vol. 6, no. 37, pp. 30905–30918, 2016, doi: 10.1039/c5ra28068k.
[18] Melgosa, R. et al., "Supercritical CO2 and subcritical water technologies for the production of bioactive extracts from sardine (Sardina pilchardus) waste," Journal of Supercritical Fluids, vol. 164, Oct. 2020, doi: 10.1016/j.supflu.2020.104943.
[19] Serra, A. T. et al., "Identification of bioactive response in traditional cherries from Portugal," Food Chem, vol. 125, no. 2, pp. 318–325, Mar. 2011, doi: 10.1016/j.foodchem.2010.07.088.
[20] Wang, H. and Joseph, J. A. "Original Contribution quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader," 1999.
[21] Serra, A. T. et al., "Characterization of traditional and exotic apple varieties from Portugal. Part 2 - Antioxidant and antiproliferative activities," J Funct Foods, vol. 2, no. 1, pp. 46–53, Jan. 2010, doi: 10.1016/j.jff.2009.12.005.

slightly more efficient in terms of extraction yield (Table 1). The total fatty acid (FA) content of the rice bran oil obtained by SOX Hex and SFE are compared (Table 1) Oils obtained from Indica variety by SOX and SFE showed a similar total FA content. The oil containing higher amount of FA was obtained from Japonica rice bran by SFE. In general, the most abundant FA in all the samples were palmitic (saturated FA), oleic acid (monosaturated FA, commonly used as excipient and/or as emulsifying) and linoleic acid (essential polyunsaturated omega-6 fatty acid with proven health benefits related to cholesterol and blood pressure).

Table 1. Extraction yield of rice by-products.

		SOX-Hex		SFE	
Variety	**By-product**	**Extraction yield (%)**	**Concentration FA (µg FA/mgextrac)**	**Extraction yield (%)**	**Concentration FA (µg FA/mgextrac)**
Japonica	White Broken Rice	0.8	not determined	0.6	not determined
	Rice Bran	18.0	1097.05	15.5	605.95
Indica	White Broken Rice	1.2	not determined	0.6	not determined
	Rice Bran	13.5	581.08	9.9	510.62

The antioxidant activity of the rice oils was studied by ORAC assay and the results showed a similar ORAC value between SFE and SOX-Hex extracts obtained for each raw material. It is important to note that the ORAC value of rice brain extracts is about two times higher that milled white broken rice samples, indicated that rice brain presented higher content in antioxidants.

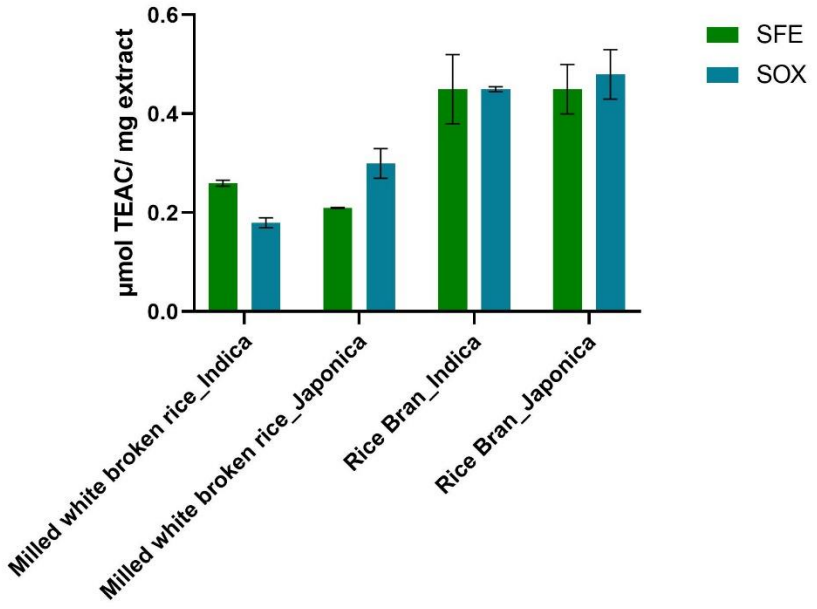

Figure 4. Antioxidant activity of rice oil (ORAC values).

Overall, the results obtained showed that the extracts obtained from rice bran of japonica variety presented the highest FA composition and ORAC value. For this reason, this raw material was selected as the most promising biomass for the following tasks. Moreover, SFE was chosen as a plausible method to get bioactive-rich rice oil.

4.2. SFE optimization

In order to maximize the quality of the RBO, a design of experiments (DOE) was planned to obtain high-added value compounds from Japonica rice bran. The extraction procedure was optimized and SFE was evaluated as a green extraction method. The effect of process variables on the extraction yield, γ-oryzanol and FA concentration in the extract was evaluated on a Central Composite Face-Centered Design with two independent variables: temperature and pressure.

The rate (20 g/min) and extraction time (3 h) were kept constant to evaluate the effect of temperature (40 - 80 °C) and pressure (200 - 500 bar) Results obtained regarding extraction yield, total FA and γ-oryzanol concentration are shown in Table 2.

Table 2. Conditions and results of the DOE of the SFE.

				mg/g extract	
Exp.	**T (°C)**	**P (bar)**	**Extraction yield (%)**	**Total FA**	**γ-Oryzanol**
1	40	200	15.1 %	757.64	14.49
2	80	200	14.7 %	772.31	8.94
3	40	500	12.6 %	716.35	24.89
4	80	500	18.2 %	707.41	22.72
5	40	350	13.8%	752.64	20.62
6	80	350	17.6 %	787.22	17.56
7	60	200	15.9 %	723.50	15.4
8	60	500	18.3 %	746.41	18.78
9	60	350	16.8 %	784.49	15.38
10	60	350	15.8 %	782.52	16.75
11	60	350	16.7 %	745.21	19.41
Average Composition	-	-	-	752.34±3.7%	17.72±24.5%

The maximum extraction yield was obtained (18.3%), when the highest pressure and temperature were used: 60/80 °C, 500 bar, and a continuous flow rate of 20 g/min CO2 (Exp. 8). The largest amount of extract was collected at high CO2 density (0.88 g/mL at 500 bar and 80 °C), probably due to the higher lipid solubility in SC-CO2 at higher CO2 density. On the other hand, the lowest value (12.6%) was obtained when the extraction was carried out at the highest pressure but low temperatures (Exp. 3).

In general, total FA extraction was not extremely impacted by the conditions evaluated. The highest values (782.52-787.22 mg/g extract) were achieved at medium pressures (350 bar, Exp. 6, 9 and 10).

Regarding the γ-oryzanol concentration in the extracts, the effect of the pressure is visible when comparing extractions with low and high values used. At the same temperature (80 °C), the SFE performed at 200 bar resulted in an extraction 2.5 times lower (8.94 mg/g extract, Exp. 2)) compared to the ones performed at 500 bar (22.72 mg/g extract, Exp. 4).

The high values for both R2 and Radj2 of these models (Table 3) suggest a close agreement between the experimental data and the theoretical values predicted by the model. Overall, after parameter optimization, the most selective extraction for FA and γ-oryzanol conditions was determined to be 60 °C and high pressure (400 – 500 bar).

Table 3. Model equations for the response surfaces, after SFE optimization, fitted to the values of extraction yield (EY), fatty acids concentration (FA) and γ-oryzanol extracted as a function of temperature (A) and pressure (B) and respective R2 and R_{adj}^2.

Polynomial model equations	**R^2**	**R_{adj}^2**
EY = 3.4 + 0.25A – 0.22A^2 + 0.2AB	0.93	0.88
FA = 776.7 – 26.3B^2	0.63	0.52
γ-oryzanol = 17.6 + 3.75B	0.78	0.72

At 420 bar, 55 °C, with a constant flow rate of 20 g/min flow, the ideal conditions to optimize all responses (extraction yield, FA and γ-oryzanol concentration) was reached. This zone is known as the "sweet spot." However, as the FA concentration in the rice bran oil was comparable for all the conditions, further adjustment was done to promote the extraction of γ-oryzanol rather than FA. The overall concentration of this bioactive was enhanced by increasing the pressure. This optimization resulted in a 17.3 % extraction yield with 784.49 mg FA /g extract and 36.6 mg γ-oryzanol /g extract (Table 4).

SFE performance was compared to conventional solid-liquid extraction using a Soxhlet apparatus with n-Hexane as solvent (SOX-Hex) to extract bioactive compounds from rice bran (Table 4). Similar extraction yields and γ-oryzanol concentrations were obtained for both approaches. However, the total FA content of the extract was higher for the SOX-Hex.

Table 4. Comparison of the results obtained by SFE and Soxhlet extractions.

Exp.	**Extraction yield (%)**	**Total FA (µg FA/mg extract**	**γ-Oryzanol (mg/g extract)**
SFE-opt	17.3	784.5	36.6
SOX-Hex	18.0	1097.1	32.7

FA distribution of SFE-opt and SOX-Hex are shown in Table 5. The rice bran oil extracted by SOX-Hex was found to be richer in total FA and the concentration of PUFA and MUFA were comparable. These FA contribute to reducing low-density lipoprotein (LDL) cholesterol levels, thereby supporting cardiovascular health. The higher differences were seen in the oleic acid (C18:1), and linoleic acid (C18:2) contents, as they appear in a higher amount in the SFE-opt.

Table 5. Fatty Acid profile of rice bran oil obtained by SEF-opt and SOX-Hex.

		µg FA/mg extract	
Fatty Acids		**SFE-opt**	**SOX-Hex**
C14	**Myristic**	2.1	2.2
C16	**Palmitic**	131.7	172.8
C16:1 cis9	**Palmitoleic**	1.4	1.1
C18	**Stearic**	12.5	20.2
C18:1 cis9	**Oleic**	324.1	450.6
C18:2 (9, 12)	**Linoleic**	288.7	413.2
a-C18:3	**alpha Linolenic**	12.5	16.2
C20	**Arachidic**	5.8	8.2
C20:1 cis11	**Gadoleic**	5.8	6.5
C22	**Behenic**	-	6.1
	Total lipids	784.5	1097.1
	MUFA	331.3	458.2
	PUFA	301.1	429.3

Overall, due to the process efficiency and extract composition in terms of γ-Oryzanol and FA, SFE showed to be an alternative method to conventional solid-liquid extraction with organic solvents, to obtain rice bran oil.

4.3. Proximate composition

In order to optimize the revalorization of DRB, an experimental set of different enzymatic treatments was planned. These treatments are specifically designed to modify the fiber fractions within the DRB, with the objective of obtaining a final product with notable improvements in both its functional and technological attributes. The results of the proximate composition and dietary fibre fractions of RB, DRB and different enzymatic treatments are presented in Table 6.

Table 6: Proximate composition of RB with different treatments. RB (rice bran), DRB (deffated rice bran), DRB-Cx (samples treated with carbohydrate-acting enzymes) and DRB-Px (samples treated with protein-acting enzymes)

Proximate composition					
Codes	**Moisture (%)**	**Total Nitrogen (%) db**	**TDF (%) db**	**IDF (%) db**	**SDF (%) db**
RB	11.48 ± 0.04	16.06 ± 0.14	33.52 ± 3.53	30.30 ± 4.53	3.22
DRB	11.97 ± 0.06	20.79 ± 0.43	39.75 ± 0.39	35.82 ± 2.06	3.94
DRB-C1	8.00 ± 0.09	20.74 ± 0.16	39.40 ± 0.64	32.98 ± 0.56	6.43
DRB-C2	9.05 ± 0.04	21.10 ± 0.05	39.74 ± 0.42	33.53 ± 1.62	6.21
DRB-C3	7.59 ± 0.05	20.84 ± 0.22	39.13 ± 1.54	32.69 ± 0.89	6.45
DRB-C4	9.47 ± 0.12	20.71 ± 0.27	42.29 ± 1.02	34.88 ± 0.18	7.41
DRB-P1	9.52 ± 0.33	21.00 ± 0.34	41.36 ± 0.17	34.00 ± 1.18	8.19
DRB-P2	9.04 ± 0.28	21.11 ± 0	41.03 ± 1.65	37.58 ± 0.44	7.16
Composition of TDF (% of db TDF)					
Codes	**IDF (%)**	**Hemicellulose (%)**	**Cellulose (%)**	**Lignin (%)**	**SDF (%)**
RB	82.73 ± 3.56	44.41	27.56 ± 2.93	15.12 ± 0.99	9.61
DRB	90.09 ± 5.18	46.30	27.99 ± 1.02	14.35 ± 1.40	9.91
DRB-C1	83.69 ± 1.42	43.47	20.77 ± 1.98	15.73 ± 2.47	16.32
DRB-C2	84.38 ± 4.07	41.29	26.27 ± 2.83	12.69 ± 0.39	15.63
DRB-C3	83.52 ± 2.27	30.58	27.37 ± 3.72	13.49 ± 0.36	16.48
DRB-C4	82,48 ± 0.42	37.85	23.10 ± 1.90	12.91 ± 2.81	17.52
DRB-P1	79.81 ± 2.75	6.69	21.77 ± 3.74	14.10 ± 2.40	19.80
DRB-P2	82.56 ± 2.60	31.54	26.63 ± 3.36	14.32 ± 0.86	17.45

A substantial increase in protein content in all treatments compared to the control sample RB. DRB-P2 showed the highest increase, reaching 21.11 g protein/100 g RB. Although the treatments had minimal impact on TDF and IDF content, an increasing trend in SDF content was observed compared to RB and DRB. Overall, the fibre fractions showed a similar trend, where IDF was the predominant fraction, followed by hemicellulose and cellulose. The sample DRB-P1 showed significant effects on SDF and increased hydrolysis of hemicellulose and cellulose. However, no decrease in the IDF fraction was observed in any of the samples.

4.4. Bioactivity evaluation of SOX and SFE samples

4.4.1. Antioxidant activity: chemical and cell based assays

A variety of techniques have been developed to evaluate the effectiveness of dietary antioxidants, whether they are present as pure substances or in food extracts. These techniques focus on several processes of the antioxidant defense system, such as the removal of lipid peroxyl radicals, the suppression of lipid peroxidation, and the chelation of metal ions. The oxygen radical absorbance capacity (ORAC) assay is one of the in vitro techniques frequently used to gauge the antioxidant capacity of food components.

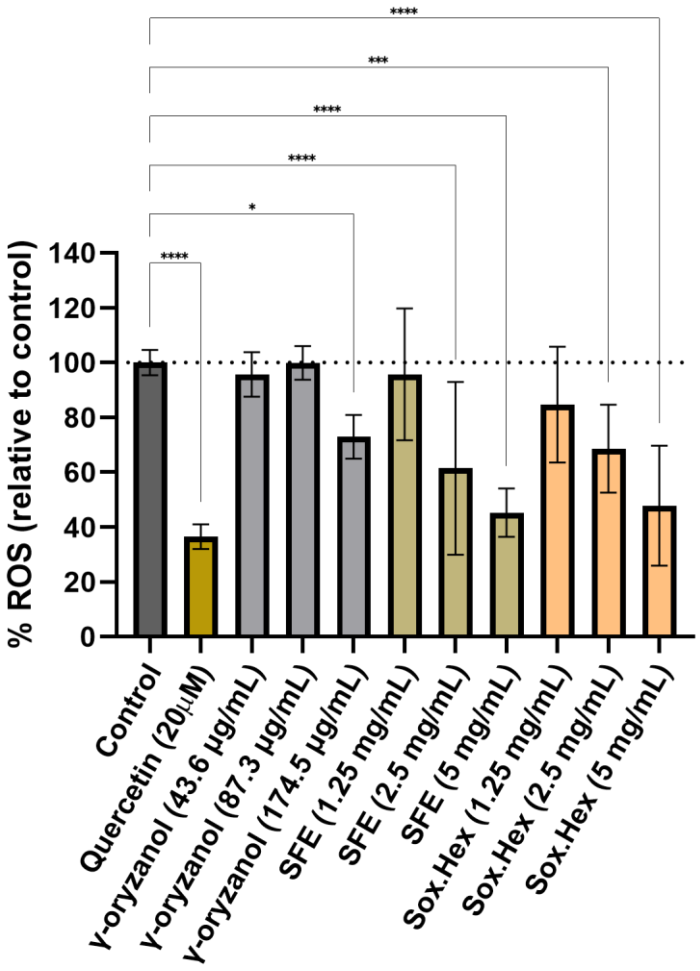

*Figure 5. Cellular antioxidant capacity, expressed as % of ROS inhibition relative to the control (cells incubated with chemical stressor) AAPH, of each extract and γ-oryzanol. The results are expressed as mean ROS percentage relative to the control ± SD. The symbol * indicates significance relative to the control; * p-value ≤ 0.05, ** p-value ≤ 0.01, *** p-value ≤ 0.001, **** p-value ≤ 0.0001*

In this project the SFE and SOX-Hex extracts were evaluated in terms of their antioxidant capacity using a chemical assay, namely ORAC, and a cell-based assay (CAA) using a Caco-2 cell line. This cell model mimics the human intestinal epithelium, as it shares some characteristics with crypt enterocytes, being considered a valid intestinal model and has been widely implemented to assess the effect of chemical and food compounds on intestinal function[17,22]. CAA assay allowed to better understand the bioactivity, namely antioxidant effect of extracts, because some processes related to the uptake, distribution or metabolism of bioactive compounds are better addressed than in chemical techniques[23].

[22] R. Valério, R. et al., "Combined hydrothermal pre-treatment and enzymatic hydrolysis of corn fibre: Production of ferulic acid extracts and assessment of their antioxidant and antiproliferative properties," Ind Crops Prod, vol. 170, Oct. 2021, doi: 10.1016/j.indcrop.2021.113731.

[23] K. L. Wolfe and H. L. Rui, "Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements," *J Agric Food Chem*, vol. 55, no. 22, pp. 8896–8907, Oct. 2007, doi: 10.1021/jf0715166.

Results showed that both extracts presented a similar ORAC value - 480 ± 50 µmol TEAC/g and 450 ± 48 µmol TEAC/g, for SOX-Hex and SFE respectively. For CAA, non-toxic concentrations of the extracts (1.25; 2.5 and 5 mg/mL) were selected for this assay. The cellular antioxidant activity was estimated by evaluating the capacity of each sample to scavenge intracellular ROS, generated by AAPH. In general, both extracts inhibited ROS formation and showed a similar antioxidant profile with an observed dose-dependent effect (Figure 5). When compared with γ-oryzanol (at the same concentration range present in the extracts- 43.6-174.5ug/mL) (Figure 5), all samples presented higher antioxidant capacity. This result suggest that other compounds present in the extracts may have antioxidant effect at a cellular level.

These data are in according to other studies found in the literature that evaluated the cellular antioxidant capacity of rice-based extracts. Tyagi, A. et al. demonstrated that ethanolic rice extracts show a dose-dependent cellular antioxidant activity with increasing concentrations of extracts from 0.5 mg/mL to 5 mg/mL in Caco-2 cell model[24].

4.4.2. Antiproliferative effect

The antiproliferative effect of the samples was evaluated using a human colorectal adenocarcinoma cell line (HT29). This cell line was subjected to treatment with non-cytotoxic concentrations of extracts, previously evaluated in Caco-2 cell model (data not shown) for 24 h. Both extracts SFE and SOX inhibited the proliferation of HT29 in a dose-dependent manner (Figure 6) and the SFE extract induced a higher antiproliferative effect. This effect could be explained by the presence of other bioactive compounds rather than oryzanol, since, for the same concentration range the standard compound alone did not show antiproliferative effect (data not shown). The antiproliferative effect of rice bran was already reported by other authors. Ghasemzadeh, A. et al. studied the antiproliferative properties of black, red, and brown rice bran in breast cancer cell lines (MCF-7 and MDA-MB-231) and obtained EC50 values ranging from 119 to 382 mg/mL[25]. In *in vivo* studies, dietary black and brown rice brans (rich in phenolic and flavonoid compounds) seemed to promote the induction of natural killer (NK) cells and macrophages activity, together with the inhibition of angiogenesis, contributing to tumor regression, in mice[26] . Our results suggest that the extracts developed in this work, mainly the rice bran

[24] Tyagi, A. et al., "Phytochemical profiling and cellular antioxidant efficacy of different rice varieties in colorectal adenocarcinoma cells exposed to oxidative stress," PLoS One, vol. 17, no. 6 June, Jun. 2022, doi: 10.1371/journal.pone.0269403.

[25] Ghasemzadeh, A. et al., "Phytochemical constituents, antioxidant activity, and antiproliferative properties of black, red, and brown rice bran," Chem Cent J, vol. 12, no. 1, Feb. 2018, doi: 10.1186/s13065-018-0382-9.

[26] Choi, S. P. et al., "Antitumor effects of dietary black and brown rice brans in tumor-bearing mice: Relationship to composition," Mol Nutr Food Res, vol. 57, no. 3, pp. 390–400, Mar. 2013, doi: 10.1002/mnfr.201200515.

extract developed by the optimized SFE, may have antiproliferative activity in colorectal cancer cells. However, more studies are needed to identify the compounds responsible for this effect in SFE extracts as well as to understand the mechanism of action of the antiproliferative effect.

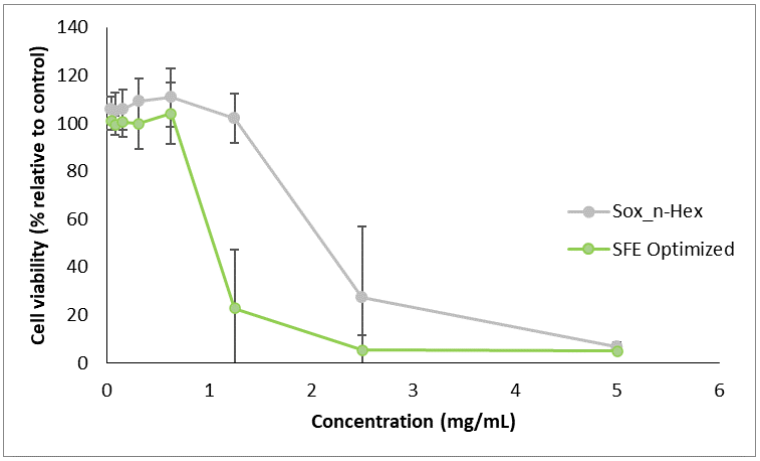

Figure 6. Antiproliferative effect of rice bran extracts in HT29 cell line.

5. Conclusions:

- ✓ Rice bran is a promising source to extract bioactive rich rice oil, richer in lipophilic compounds that the white broken rice (13.5-18.0 mg extract/100 mg biomass vs 0.8-1.2 mg extract/100 mg biomass).
- ✓ SFE at the optimized conditions (500 bar and 62 °C) resulted in a 17.3 % extraction yield and a concentration of 784.5 µg FA/mg extract and 36.6 mg γ-Oryzanol /g extract). These results are comparable to the obtained by conventional SOX extraction using hexane (18 % exaction yield, 1097.1 µg FA/mg extract and 32.7 mg γ-Oryzanol /g extract). This shows that SFE is a sustainable method that can be used as an alternative to conventional solid-liquid extraction with organic solvents, to obtain rice bran oil.
- ✓ The oil obtained was bioactive-rich containing 784.49 mg FA /g and 36.6 mg γ-oryzanol /g and presented bioactive properties including antioxidant effect and higher antiproliferative capacity than a conventional extract obtained by Sohxlet.
- ✓ Enzymatic treatment (using different enzymes) of DRB for 120 minutes increases the soluble fiber content by 5.72 to 9.89% in all of cases.

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