Antioxidant and cytotoxic properties of nano and fermented-nano powders of wheat and rice by-products

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Abstract

The use of fermentation and nano technology can promote the absorption efficiency and improve the functionality and the added value of cereal by-products. In this study, nano and fermented-nano powders of wheat and rice by-products were prepared by superfine grinding of raw and solid-state fermented materials. Effects of fermentation and superfine grinding on phytochemical, phenolic acids profile, antioxidant and cytotoxic activity were investigated. The results revealed that phenolic contents of fermented-nano wheat bran (FNWB), fermented-nano wheat germ (FNWG) and fermented-nano rice bran (FNRB) increased by 40.5, 59.2 and 27.9%, respectively compared to their raw samples. The free, conjugated and bound forms of most identified phenolic acids apparently increased. Also, the antioxidant activity of nano and fermented-nano forms significantly increased compared to raw materials. The cytotoxic activity of ultrafine ground samples increased compared to raw materials. NRB extract was the most effective treatment with IC50 value of 4.10 mg/mL. These results indicate that superfine grinding and solid state fermentation altered the rigid structure of wheat and rice by-products and increased their bioactivity through increasing the releasable bioactive molecules.

Introduction

Cereal contains significant levels of dietary antioxidants including phenolic acids, flavonoids, tocopherols and carotenoids (Kumar & Goel, 2019; Przybylska-Balcerek, et al., 2019; Horvat et al., 2020). The most abundant phenolic acids in cereals belong to the chemical class of hydroxycinnamic acids. Ferulic, vanillic, and p-coumaric were major phenolic acids in cereal brans, along with other free phenolic acids including caffeic, chlorogenic, gentisic, syringic, and p-hydroxybenzoic acids (Sun et al., 2001; Adom et al., 2003 and Zhou et al., 2004). Phenolic acids may occur in the free form, but are mostly glycosylated with different sugars, especially glucose (Chandrasekara, 2018). In this concern, Vichapong et al. (2010), Zilic et al. (2012) and Mahmoud et al. (2015) reported that wheat bran, wheat germ and rice bran are a source of phytonutrients with potential health benefits, but the nutritional properties will only be fully exploited if whole-meal products are available. Potentially health beneficial compounds such as phenolics, carotenoids and tocopherols are concentrated in the bran layers and germ.

Qu et al. (2005) and Liu et al. (2012) studied the protective effect of wheat bran against colon and prostate cancer. They found that wheat bran can offer protection against tumor development even when they are consumed with a high-fat diet. Also, Mueller and Voigt (2011) and Saiko et al. (2009) studied the anti-tumor activity of wheat germ and fermented wheat germ extracts. They reported that wheat germ is a multi-substance composition and, besides others, contains agglutinin, 2-methoxy benzoquinone and 2, 6-dimethoxy benzoquinone which are interact with prostate, human pancreatic and colon-cancer cells. Kong et al. (2009) and Takashima et al. (2011) stated that rice bran extracts have prominent in vitro growth inhibition on leukaemia tumour cell and human colorectal adenocarcinoma. Leardkamolkarn et al. (2011) attributed the potential anti-cancer activity of rice bran in human cancer cell line to the apoptotic induction pathway of pro-apoptotic p53, caspase-3, and cyclin proteins. Chung et al. (2009) reported that *Lactobacillus rhamnosus* and *Saccharomyces cerevisiae* fermented rice bran inhibited the melanogenesis

through downregulation of microphthalmia-associated transcription factor, along with reduced cytotoxicity.

The potential of *Saccharomyces cerevisiae, Lactobacillus rhamnosus* and *Rhizopus oryzae* fermentation to improve the health beneficial properties of wheat bran, wheat germ and rice bran were evaluated by Moore et al. (2007), Dordevic et al. (2010), and Prabhu et al. (2014). Fermentation treatments were able to significantly release the phenolic compounds and increase antioxidant properties (Chen et al., 2019). Katina et al. (2012) proposed the main reasons for the improved technological functionality of fermented bran to solubilization of arabinoxylan during fermentation and decreased endogenous xylanase activity. Prabhu et al. (2014) reported that fermented bran can be utilized for enhancement of health properties, giving way to further studies for fermented bran to be incorporated into food.

Also, the possible use of nanotechnology in food becomes the focus of research in many countries (Zhu et al., 2010). Utrafine powders are easier to incorporate into food systems and more available to the body, which would consequently improve the quality of food products and human health (Raghavendra et al., 2004). However, so far the use of this technology in dietary fiber processing remains rather limited, probably due to the toughness and polymer nature of dietary fiber and inadequate equipment support (Zhu et al., 2010). In this concern, ultrafine ball milling was used to decrease the particle size of wheat bran by Hemery et al. (2010, 2011). Also, Rosa et al. (2013) evaluated the potential of using ultrafine grinding and electrostatic separation methods to improve the bioaccessibility of p-coumaric acid, sinapic acid and ferulic acid from wheat bran-rich breads. It was observed that, finer the bran particles in bran-rich breads, the more bioaccessible the phenolic acids. Only the free and conjugated phenolic acids forms were found to be bioaccessible, and the bioaccessibility of sinapic acid was much higher than that of ferulic acid, due to the higher solubility of the former.

The objective of our study was to apply modern techniques including, solid-yeast treatment and superfine grinding, to produce nano and fermented-nano powders. Also, the effects of these methods on phytochemical solubility and antioxidant activity as well as cytotoxic activity of tested materials were investigated.

Material And Methods

Materials

Wheat bran (WB) and wheat germ (WG) were obtained from North Cairo Flour Mills Company, Egypt. Rice bran (RB) was obtained from Rice Research and Training Centre, Sakha, Kafr El-Sheikh, Egypt.

Methods

Stabilization of wheat germ and rice bran

Wheat germ and rice bran were stabilized in an air-oven at a temperature of 120 ± 2°C for 1min according to Younas et al. (2011). The stabilized wheat germ and rice bran were ground using Moulinex grinder and

passed through a 40-mesh and packed in polyethylene bags and stored at -30°C until use.

Solid-state yeast fermentation

Yeast strain (*Saccharomyces cerevisiae* FC-620) was obtained from Microbial Chemistry Dept. collection, National Research Centre, Dokki, Cairo, Egypt. The yeast cells were activated, a loopful of the culture was transferred to 250 ml Erlenmeyer flask containing 50 ml broth medium (0.3% yeast extract, 0.3% malt extract, 0.5% peptone and 5% sucrose) and incubated at 30°C for 24h under shaking condition. Solid-state yeast treatments were carried out according to the method of Moore et al. (2007) as follows: 50ml of yeast preparation (1380 cfu/ml) was mixed with 100g sample in a sterile conical flask (1000 ml) to begin the solid-state yeast treatment. Flasks were sealed with cotton seals and incubated at 37°C for 48h. All treated samples were dried at 50 ± 1°C for 16h and stored in polyethylene bags at -30°C for further analysis.

Preparation of nano and fermented-nano materials

The raw and fermented wheat bran, wheat germ and rice bran were ground using 5 ml zirconium oxide balls and zirconium oxide bowl volume 250 ml in a PM 100 Planetary Ball-mill (Retsch, Germany) as previously described by Mohammad et al. (2015). Samples (150 g) were ground at 30 Hz frequency for 60 min at room temperature (25°C).

Preparation of successive extracts

Twenty grams of the raw, nano and fermented-nano materials were extracted using petroleum ether, tetrahydrofuran and methanol in succession using soxhlet apparatus according to the methods of Roopalatha and Nair (2013) with some modifications. Each extract obtained following extraction step was filtered using filter paper Whatman No 1, dried using rotary evaporator and the yield of each extract was recorded. Different extracts were reconstituted in 10 mL dimethylsulfoxide (DMSO) and stored under nitrogen at -30°C till further use.

Determination of total phenolic content

The total phenolic content was determined according to the Folin-Ciocalteu procedure (Zilic et al., 2012). Briefly, the extract (100 µL) was transferred into a test tube and the volume adjusted to 3.5 mL with distilled water and oxidized with the addition of 250 µL of Folin-Ciocalteau reagent. After 5 min, the mixture was neutralized with 1.25 mL of 20% aqueous sodium carbonate (Na_2CO_3) solution. After 40 min, the absorbance was measured at 725 nm against the solvent blank. The total phenolic content was determined by means of a calibration curve prepared with gallic acid, and expressed as milligrams of gallic acid equivalent (mg GAE) per g of sample. Additional dilution was done if the absorbance value measured was over the linear range of the standard curve.

Determination of total flavonoid content

The total flavonoid content was determined according to Zilic et al. (2012) using aluminum chloride ($AlCl_3$) colorimetric assay. Briefly, 300 µL of 5% sodium nitrite ($NaNO_2$) was mixed with 100 µL of extract.

After 6 min, 300 µL of a 10% $AlCl_3$ solution was added and the volume was adjusted to 2.5 mL using distilled water. After 7 min, 1.5 mL of 1 M NaOH was added, and the mixture was centrifuged at 5000 g for 10 min. Absorbance of the supernatant was measured at 510 nm against the solvent blank. The total flavonoid content was determined by means of a calibration curve prepared with catachine, and expressed as milligrams of catechin equivalent (mg CE) per g of sample. Additional dilution was done if the absorbance value measured was over the linear range of the standard curve.

Determination of total carotenoids

Total carotenoids content was determined according to Moore et al. (2005) using the spectrophotometric method at 470 nm. The total carotenoids content was expressed based on β-carotene equivalents (β-carotene; mg/ g sample) using β-carotene standard curve. Additional dilution was done if the absorbance value measured was over the linear range of the standard curve.

Determination of phenolic acids profile

Free, conjugated and bound Phenolic acids of raw, anano and fermented-nano materials were extracted according to Moore et al. (2005) with some modification. Each sample (1g) was transferred to an Erlenmeyer flask, defatted twice with hexane at a 40:1 ratio (v/w), and kept on a mechanical shaker for 1 h. Each time, the mixture was filtered through a filter paper Whatman No. 1, defatted samples were dried in a hood. The defatted sample was then extracted twice with 80% methanol at a 50:1 ratio (v/w) for 1 h. The mixture was filtered through a filter paper Whatman No. 1, and the combined supernatant was concentrated to dryness using a rotary evaporator at 40°C. The residue of each sample, that obtained after rotary evaporating, was re-dissolved in 10 mL acidified water with HCl (pH 2) and partitioned with 30 mL of ethyl ether:ethyl acetate (1:1) in a separating funnel, three times. The organic layers contained free phenolic acids were combined and concentrated to dryness using a rotary evaporator at 40°C and reconstituted in 2 mL methanol. The water phase was neutralized to pH 7 with 2 M sodium hydroxide (NaOH) and dried using a vacuum oven at 50°C overnight. The residue was dissolved in 10 mL of 2 M NaOH and stirred for 4 h at room temperature (25°C). The solution was then acidified to pH 2 with 6M HCl, and extracted three times with ethyl ether and ethyl acetate (1:1). The resulting organic layers contained conjugated phenolic acids were combined and concentrated to dryness using a rotary evaporator at 40°C and reconstituted in 2 mL methanol. The residue, after the methanol extraction, was alkaline hydrolyzed by 40 mL of 2 M NaOH and stirred for 4 h at room temperature (25°C). The solution was then acidified to pH 2 with 6M HCl, and extracted three times with ethyl ether and ethyl acetate (1:1). The resulting organic layers contained bound phenolic acids were combined and concentrated to dryness using a rotary evaporator at 40°C and reconstituted in 2 mL methanol.

HPLC analysis was carried out according to Kim et al. (2006) with slight modifications using an Agilent Technologies 1100 series liquid chromatograph equipped with an auto sampler and a diode-array detector. The analytical column was Agilent Eclipse XDB C18 (150 x4.6 µm; 5 µm) with a C18 guard column. The mobile hase consisted of acetonitrile (solvent A) and 2% acetic acid in water (v/v) (solvent B). The flow rate was kept at 0.8 mL min^{-1} for a total run time of 70 min and the gradient programme was

as follows: 100% B to 85% B in 30 min, 85% B to 50% B in 20 min, 50% B to 0% B in 5 min and 0% B to 100% B in 5 min. There was 10 min of post-run for reconditioning. The injection volume was 10 µL and peaks were monitored simultaneously at 280 and 320 nm for the benzoic acid and cinnamic acid derivatives, respectively. All samples were filtered through a 0.45 µm Acrodisc syringe filter (Gelman Laboratory, MI) before injection. Peaks were identified by congruent retention times and UV spectrum and compared with those of the standards.

Determination of DPPH radical scavenging activity

Free radical scavenging capacity of extracts were determined using the stable $DPPH^{\bullet}$ according to Hwang and Do Thi (2014). The final concentration was 200 µM for $DPPH^{\bullet}$ and the final reaction volume was 3.0 mL. The absorbance was measured at 517 nm against a blank of pure methanol after 60 min of incubation in a dark condition. Percent inhibition of the DPPH free radical was calculated by the following equation:

$$\text{Inhibition (\%)} = 100 \times [(A_{control}-A_{sample})/A_{control}]$$

Where:

$A_{control}$ is the absorbance of the control reaction (containing all reagents except the test compound).

A_{sample} is the absorbance with the test compound.

Extract concentration of sample providing 50% inhibition (IC_{50}) was calculated using linear regression analysis.

Determination of ABTS radical scavenging activity:

The stock solutions of $ABTS^{\bullet+}$ reagent was prepared according to Hwang and Do Thi (2014) by reacting equal quantities of a 7 mM aqueous solution of $ABTS^{\bullet+}$ with 2.45 mM potassium persulfate for 16 h at room temperature (25°C) in the dark. The working solution was then prepared by diluting 1 mL $ABTS^{\bullet+}$ solution with 60 mL of ethanol: water (50:50, v/v) to obtain an absorbance of 1.0 ± 0.02 units at 734 nm using the spectrophotometer. Extracts (50 µL) were allowed to react with 4.95 mL of the $ABTS^{\bullet+}$ solution for 1 h in a dark condition. Then the absorbance was taken at 734 nm using the spectrophotometer. The standard curve was prepared using Trolox. Results were expressed as mM Trolox equivalents (TE)/g sample). Additional dilution was needed if the $ABTS^{\bullet+}$ value measured was over the linear range of the standard.

Ferric reducing activity power (FRAP) assay

The FRAP assay was done according to according to Hwang and Do Thi (2014). The stock solutions included 300 mM acetate buffer [3.1 g sodium acetate ($C_2H_3NaO_2.3H_2O$) and 16 mL glacial acetic acid ($C_2H_4O_2$), pH 3.6], 10 mM TPTZ solution in 40 mM HCl, and 20 mM ferric chloride ($FeCl_3.6H_2O$) solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution, and 2.5

mL $FeCl_3.6H_2O$ solution and then warmed at 37°C before using. Extracts (50 µL) were allowed to react with 3.95 mL of the FRAP solution for 30 min in the dark condition. Readings of the colored product [ferrous tripyridyltriazine complex] were taken at 593 nm. The standard curve was prepared using Trolox and the results were expressed as mM Torlox equivalent (TE/g sample). Additional dilution was needed if the FRAP value measured was over the linear range of the standard curve.

Cytotoxic effect of prepared extracts on human cell line (HCT 116)

Cell viability was assessed by the mitochondrial dependent reduction of yellow MTT to purple formazan in the Bioassay-Cell Culture Laboratory, National Research Centre, Cairo, Egypt, in a sterile area using a Laminar flow cabinet biosafety class II level (Baker, SG403INT, Sanford, ME, USA) according to Mosmann (1983). HCT116 cells were suspended in RPMI 1640 medium. The media were supplemented with 1% antibiotic-antimycotic mixture (10,000U/mL Potassium Penicillin, 10,000 µg/mL Streptomycin Sulfate and 25 µg/mL Amphotericin B), 1% L-glutamine and 10% fetal bovine serum and kept at 37°C under 5% CO_2. DMSO was the vehicle used for dissolution of plant extracts and its final concentration on the cells was less than 0.2%.

Statistical Analysis

All samples were analyzed in triplicates and the results were expressed as means ± standard error, except successive extraction yield and Phenolic acids profile, n = 1. The significant difference between the mean values were determined by using the analysis of variance (ANOVA) and Duncan's multiple range test was conducted at a significance level of $p < 0.05$ using SPSS 11 program.

Results And Discussion

Successive extraction yields of raw, nano and fermented-nano materials

The effect of ultrafine grinding and fermentation of tested materials on the solubility of different types of phytochemical in petroleum ether, tetrahydrofuran and methanol, as well as total yield is illustrated in Fig. 1. The yields of petroleum ether, tetrahydrofuran and methanol extracts were 1.97, 1.07 and 2.74% for WB; 6.85, 1.83 and 15.36% for WG; 2.81, 1.86 and 2.19% for RB, respectively. Similar yields were reported by Oufnac (2006) and Wang et al. (1993). They attributed the higher yield of methanol to that methanol solvent possibly extracts not only lipids and small molecule polar compounds, but also some large molecule polar compounds, such as alcohol soluble proteins and carbohydrates. On the other hand, ultrafine grinding increased the yield for both tested materials and solvents. This could be due to increase the surface area of the produced nano-powders of tested materials. Also, fermentation process increased the solubility of tested materials in all solvents, except FNWG. The low yield values of FNWG, especially in methanol, could be explained by consumption of large portion of micro and macro-nutrients during the growth of yeast cells.

Phytochemical Analysis

The results of phytochemical analysis (total phenols, total flavonoids and total carotenoids) conducted on successive extracts of tested materials are presented in Table 1. As shown in this table, total phenolic contents in the investigated samples were the highest in WG, 3.00 mg gallic acid equivalent (GAE) per gram sample. Lower total phenolic contents were present in RB 2.65 mg GAE/g sample) and the lowest in WB 1.66 mg GAE/g sample. A similar phenolic content in wheat bran (1.24 mg GAE/g) and rice bran (2.5 mg GAE/g) had been reported by Zhu et al. (2010) and Lai et al. (2009). Ultrafine grinding significantly increased the phenolic contents of NWB and NRB (2.10 and 3.51 mg GAE, respectively) as compared to WB and RB, while this increase in NWG was not significant. Fermentation process did not significantly alter the phenolic content in FNWB or FNRB compared to its nano-forms, while phenolic content of FNWG significantly increased to 4.78 mg GAE/g. Katina et al. (2012) reported that the amounts of total phenolic content did not change in rice bran ferments, while Dordevic et al. (2010) reported that fermentation of wheat bran by both *S. cerevisiae* and *Lactobacillus rhamnosus* increased the phenolic content in wheat extracts. They explained the increase in the total phenolic content by the ability of fungi to degrade lignocellulosic materials due to their highly efficient enzymatic system. Xylanases, in particular, are one type of enzyme missing from *S. cerevisiae* which are important for release of phenolic compounds from cereal matrix (Mathew & Abraham, 2004). This could explain the inability of yeast to release the phenolic compounds from wheat and rice matrix.

Table 1 Phytochemicals of raw, nano and fermented-nano-materials

Sample	Total phenols (mg GAE/g)	Total flavonoids (mg CE/g)	Total carotenoids (mg βCE/g)
WB	1.664^{G}±0.103	0.588^{F}±0.005	1.052^{EF}±0.020
NWB	2.104^{F}±0.032	1.256^{E}±0.011	1.039^{F}±0.006
FNWB	2.338^{F}±0.099	2.038^{CD}±0.029	1.021^{F}±0.002
WG	3.003^{D}±0.030	2.635^{B}±0.036	1.984^{C}±0.127
NWG	3.198^{CD}±0.054	3.071^{AB}±0.068	2.376^{B}±0.107
FNWG	4.780^{A}±0.293	3.539^{A}±0.389	3.577^{A}±0.190
RB	2.649^{E}±0.006	1.206^{E}±0.031	1.262^{E}±0.004
NRB	3.513^{B}±0.067	1.805^{D}±0.006	2.076^{C}±0.009
FNRB	3.389^{CB}±0.072	2.566^{BC}±0.324	1.583^{D}±0.122

- Values in the same column followed by different letters are significantly different ($p < 0.05$)

WB- wheat bran, NWB- nano-wheat bran, FNWB- fermented-nano- wheat bran, WG- wheat germ, NWG- nano-wheat germ, FNWG- fermented-nano-wheat germ, RB- rice bran, NRB- nano-rice bran, FNRB-

fermented-nano-rice bran.

Also, data in Table 1 showed that the total flavonoids content of WG (2.64 mg catachine equivalent (CE)/g) was significantly higher than those of WB and RB (0.59 and 1.21 mg CE/g, respectively). Ultrafine grinding significantly increased the flavonoids content of NWB and NRB to 1.26 and 1.81 mg CE/g, respectively, but its effect on NWG was not significant. Moreover fermentation process significantly increased the flavonoids contents of FNWB and FNRB to 2.04 and 2.57 mg CE/g, respectively. Also, the effect of fermentation process on FNWG flavonoids content was not significant. Similar results were reported by Zilic et al. (2012) for wheat genotypes, El Bedawey et al. (2010) for wheat germ and rice bran. Brewer et al. (2014) compared the flavonoids content of coarse, medium and fine wheat bran from the same wheat cultivar. The order of flavonoid content was determined as: fine > coarse ~ medium. Prabhu et al. (2014) mentioned that fermentation of rice bran by yeast resulted about 14% and 18% increase in flavonoid content after 24 and 48 h of fermentation. This was attributed to the increase in acidic value during fermentation that is liberating bound flavonoid components and making it more bioavailable.

Total carotenoids contents of investigated samples ranged from 1.02 to 3.58 mg β-carotene equivalent (βCE)/g (Table 1). Among the tested raw materials WG had the highest total carotenoids content (1.984 mg βCE/g). There were no significant differences between the carotenoids contents of WB and RB (1.05 and 1.26 mg βCE/g, respectively). Ultrafine grinding significantly increased the carotenoids contents of NWG and NRB to 2.38 and 2.08 mg βCE/g, respectively. This increase in NWB was not significant. Furthermore, fermentation process significantly increased the total carotenoids of FNWG which recorded the highest total carotenoids content (3.58 mg βCE/g) among all tested forms of the investigated materials. Also, the increase in total carotenoids contents of FNWB as a result of fermentation process was not significant. Zilic et al. (2012) found that the total yellow pigments in the brans of bread and durum wheat genotypes ranged from 4.66 to 6.62 mg βCE/kg, and from 8.65 to 12.55 mg βCE/kg, respectively.

Phenolic acids profiles of wheat and rice by-products

The phenolic acids (gallic, protocatechuic, gentistic, syringic, chlorogenic, caffeic, vanillic, ferulic, sinapic, *p*-coumaric, rosmarinic, trans-cinnamic acids and chyrsin) were investigated in cereal by-products and the concentrations of individual phenolic are shown in Table 2. Phenolic acids profile of WB, WG and RB was nearly similar. Among the tested phenolic acids, only gentistic and chlorogenic acids were not detected in WB and WG while, chlorogenic acid was not detected in RB under the experimental conditions. Ferulic and sinapic acids were the predominant phenolic acids in WB and WG while, ferulic and vanillic acids were the predominant phenolic acids in RB. Most of the ferulic and sinapic acids in WB were bound, with a concentration of 129.51 and 80.15 µg/g, respectively. While, the most of ferulic acid in WG was bound, with a concentration of 105.29 µg/g, but most of sinapic acid was conjugated, with a concentration of 127.48 µg/g. Most of the ferulic and vanillic acids in RB were bound, with a concentration of 147.96 and 56.15 µg/g, respectively.

Ultrafine grinding of raw WB and WG releases detectable free and conjugated amounts of gallic and protocatechuic acids. Also, NRB contained detectable free amounts of sinapic, *p*-coumaric, and rosmarinic acids which were not detected in RB. Moreover, ultrafine grinding of WB, WG and RB apparently increased the free, conjugated and bound forms of all identified phenolic acids except conjugated sinapic acid in WG and RB. This could be due to that ultrafine grinding increased phenolic acids accessibility by increasing the particle surface area of cell walls, and thus increasing the release of intra-cellular contents. Similar results were obtained by Van Craeyveld et al. (2009). They reported that the intensive grinding of wheat bran could partly solubilize the arabinoxylans, possibly contributing to the production of bioaccessible phenolic compounds, i.e. phenolics which are in conjugated or even free forms. While, Rosa et al. (2013) found that the mechanical treatment did not change the phenolic acids structuration state as the conjugated and free forms remained constant among the ground fractions. They mentioned that the conditions of grinding used (frequency and time) probably were not hard enough to break phenolic acids ester link.

Table 2 Phenolic acids profile of raw, nano and fermented-nano materials (µg/g)

Compound	Free			Conjugated			Bound		
	WB	***NWB***	***FNWB***	***WB***	***NWB***	***FNWB***	***WB***	***NWB***	***FNWB***
Gallic	ND	0.81	8.99	ND	6.44	10.96	13.07	2.88	2.03
Protochatchuic	ND	2.40	2.99	ND	0.77	1.20	5.67	10.79	6.22
Caffeic	ND	ND	0.63	ND	0.31	0.81	1.66	2.35	1.23
Syrngic	2.26	2.98	4.75	1.91	7.73	18.72	13.34	16.32	10.17
Vanillic	0.78	2.59	5.61	1.15	1.21	1.63	8.65	14.54	8.06
Ferulic	10.6	9.09	23.74	5.98	11.27	13.03	129.5	136.21	185.03
Sinapic	1.62	1.24	7.30	5.72	18.91	18.19	8015	79.27	57.89
P-Coumaric	ND	ND	10.02	0.41	0.99	0.78	3.19	3.64	7.42
Rosmarinic	0.84	3.36	4.29	0.98	4.04	4.77	23.19	29.04	9.81
Cinnamic	0.55	0.24	0.83	0.15	0.23	0.23	5.51	7.30	5.87
Chyrsin	1.73	1.87	4.42	1.14	1.52	1.54	9.72	10.34	16.61
	WG	***NWG***	***FNWG***	***WG***	***NWG***	***FNWG***	***WG***	***NWG***	***FNWG***
Gallic acid	ND	1.24	16.60	7.59	7.98	12.78	ND	ND	ND
Protochatchuic	ND	0.60	7.38	4.20	2.21	5.49	ND	ND	ND
Caffeic acid	ND	ND	1.21	ND	ND	ND	ND	ND	ND
Syrngic acid	1.58	3.60	9.58	8.99	22.57	10.37	3.14	5.50	2.85
Vanillic acid	0.78	1.94	2.60	3.17	4.40	5.34	12.07	12.68	10.04
Ferulic acid	1.95	6.11	4.77	21.10	28.83	21.54	105.29	107.07	109.81
Sinapic acid	0.47	1.37	7.48	127.48	89.78	89.20	20.70	17.27	40.49
Coumaric acid	0.14	0.13	0.12	0.24	0.52	0.43	1.84	2.93	0.80
Rosmarinic	ND	1.44	6.78	1.88	2.02	1.84	6.28	5.91	3.07
Cinnamic acid	0.16	0.34	0.16	0.15	0.23	0.20	0.44	0.61	0.86
Chyrsin	1.65	4.80	4.42	ND	ND	ND	ND	ND	ND
	RB	***NRB***	***FNRB***	***RB***	***NRB***	***FNRB***	***RB***	***NRB***	***FNRB***
Gallic acid	ND	ND	6.59	6.16	5.58	7.05	ND	ND	ND
Protochatchuic	2.71	13.58	13.31	ND	ND	ND	ND	ND	ND
Gentisic acid	ND	ND	ND	3.59	3.47	8.89	ND	ND	ND

Caffeic acid	0.94	1.06	1.80	ND	ND	ND	ND	1.06	1.26
Syrngic acid	0.94	1.91	2.59	ND	7.87	5.05	5.92	5.92	8.07
Vanillic acid	8.36	10.40	5.16	5.62	6.05	5.41	56.15	32.25	37.99
Ferulic acid	2.82	5.50	14.52	6.71	32.99	20.62	147.9	194.66	251.08
sinapic acid	ND	1.83	2.99	43.23	23.23	26.61	19.39	26.25	38.75
Coumaric acid	ND	4.57	8.40	1.16	1.25	1.94	4.95	6.45	12.58
Rosmarinic	ND	10.17	12.77	3.50	6.47	1.92	15.98	26.71	50.25
Cinnamic acid	1.10	1.11	0.81	0.42	0.31	ND	1.08	1.21	2.19
Chyrsin	ND	ND	ND	11.53	18.92	7.91	5.50	6.56	7.34

WB- wheat bran, NWB- nano-wheat bran, FNWB- fermented-nano- wheat bran, WG- wheat germ, NWG- nano-wheat germ, FNWG- fermented-nano-wheat germ, RB- rice bran, NRB- nano-rice bran, FNRB- fermented-nano-rice bran, ND- not detected.

On the other hand, the concentrations of soluble free and conjugated gallic, syringic, sinapic, *p*-coumaric, and rosmarinic acids of fermented nano-samples showed pronounced increases versus the raw and nano-samples. This indicates that yeast may produce hydrolytic enzymes capable of releasing soluble conjugated or insoluble bound phenolic acids from wheat bran. In contrast, soluble free ferulic and vanillic acids concentrations in FNWG and FNRB, respectively showed decreased values compared to NWG and NRB. This decrease indicates that yeast may be able to convert ferulic and vanillic acids to other compounds through enzymatic reactions. Interestingly, strains of *S. cerevisiae* have been reported to have a variety of phenolic acid biotransformation activities involving ferulic and vanillic acid derivatives (Priefert et al., 2001). This may partially explain the observed changes in soluble free phenolics. Furthermore, results showed that fermentation altered soluble conjugated and insoluble bound concentrations for most detected phenolic acids. Yeast treatment of WB and WG decreased insoluble bound concentrations for all measured phenolic acids versus nano-form, except for ferulic and *p*-coumaric acids. These results suggest that *S. cerevisiae* may have produced enzymes capable of releasing insoluble bound phenolic acids, thereby increasing its soluble free and or soluble conjugated phenolic acid contents. On contrast, fermentation of RB increased insoluble bound concentrations of all measured phenolic acids versus NRB. This could be due to the differences in lignocellulosic materials and phenolic acids profile of wheat and rice cultivars. Moore et al. (2007) and Chen et al. (2019) studied the effect of yeast and fungal fermentation on soluble free, soluble conjugated and insoluble bound phenolic acids of wheat and rice bran, respectively and found similar results.

Antioxidant activity of raw, nano and fermented-nano materials

The extracts of investigated samples were analyzed and compared for their IC_{50} values against $DPPH^{\bullet}$ (Table 3). IC_{50} is the required concentration of sample antioxidants to scavenge 50% DPPH radicals

in the reaction mixtures under the experimental conditions. The IC_{50} values ranged from 1.73 mg for WG to 0.51 mg for NRB, indicating that individual samples may significantly differ in their $DPPH^{\bullet}$ radical scavenging capacities. The scavenging effect against $DPPH^{\bullet}$ radical ranked the samples in the order of rice bran > wheat bran > wheat germ. Scavenging activity of all nano-materials slightly increased compared to raw materials. Also, the scavenging activity of FNWG increased compared to its nano-forms, while the scavenging activity of FNWB and FNRB decreased. This could be due to the ability of yeast to increase extracted phytochemicals. These results were in agreement with those of Moore et al. (2005), Mansour et al. (2013) and Shin et al. (2019). While, Prabhu et al. (2014) depicted that the fermented rice bran extract exhibited about 56% radical scavenging activity with 24 h of fermentation. They attributed this enhancement of scavenging activity to the liberation of bound polyphenolic and flavonoid content by the fermentative action of yeast.

All tested samples exhibited effectual radical cation scavenging activity ranged from 4.61 mM trolox equivalent (TE)/g WB to 8.27 mM TE/g NWB, as seen in Table, 3. There were no significant differences in $ABTS^{\bullet+}$ scavenging potential among WB, WG or RB. Ultrafine grinding significantly increased the scavenging activity of NWB and NRB to 8.27 and 8.08 mM TE/g, respectively. Also, the scavenging activity of FNWB and FNRB were significantly higher than those of WB and RB. On the other hand, neither ultrafine grinding nor fermentation significantly affected the scavenging activity of wheat germ. Moore et al. (2005) found that soft wheat grains had $ABTS^{\bullet+}$ scavenging activities varied from 14.3 to 17.6 µM TE/g. Also, wheat bran had 73.24 % ABTS radical scavenging activity (Shallan et al. 2014). Mahmoud et al. (2015) mentioned that 1µg/ml of wheat germ extract had ability to scavenging 70% from the $ABTS^{\bullet+}$ radicals.

The results of reducing power demonstrate the electron donor properties of tested samples thereby neutralizing free radicals by forming stable products (Table 3). The outcome of the reducing reaction is to terminate the radical chain reactions that may otherwise be very damaging. WB had the lowest reducing power (4.55 mM TE/g). There were no significant differences in reducing power of WG and RB (5.79 and 6.00 mM TE/g, respectively). Ultrafine grinding significantly increased the reducing power of NWB and NRB to 7.60 and 7.40 mM TE/g, respectively. While, the fermentation process only increased the reducing power of FNWG to 6.88 mM TE/g compared to 5.7 mM TE/g for both WG and NWG. Lai et al. (2009) found that the antioxidant activity of the methanolic extract of rice bran was 78% of reducing power. Singh et al. (2012) reported that the reducing power of Wheat bran was 2.532 mM ascorbic acid equivalent (AAE)/g.

Table 3 Antioxidant activity of raw, nano and fermented-nano-materials

Sample	DPPH IC_{50} (mg/mL)	ABTS (mM TE/g)	FRAP (mM TE/g)
WB	1.682	4.613 ^{C} ±0.202	4.556 ^{D} ±0.197
NWB	1.080	8.269 ^{A} ±0.360	7.602 ^{A} ±0.248
FNWB	1.176	6.128 ^{B} ±0.365	5.788 ^{C} ±0.039
WG	1.730	6.311 ^{B} ±0.582	5.797 ^{C} ±0.126
NWG	1.432	6.343 ^{B} ±0.052	5.736 ^{C} ±0.030
FNWG	1.400	6.500 ^{B} ±0.409	6.886 ^{B} ±0.047
RB	1.331	6.429 ^{B} ±0.170	6.002 ^{C} ±0.098
NRB	0.505	8.082 ^{A} ±0.118	7.403 ^{A} ±0.178
FNRB	0.89	6.839 ^{B} ±0.096	7.354 ^{A} ±0.126

-Values in the same row followed by different letters are significantly different ($p < 0.05$)

WB- wheat bran, NWB- nano-wheat bran, FNWB- fermented-nano- wheat bran, WG- wheat germ, NWG- nano-wheat germ, FNWG- fermented-nano-wheat germ, RB- rice bran, NRB- nano-rice bran, FNRB- fermented-nano-rice bran.

Cytotoxic activity of raw, nano and fermented-nano materials

The effect of successive extracts of tested samples on proliferation of human colon cancer cell line HT-116 was investigated using MTT assay at 4 concentrations (10, 7.5, 5 and 2.5 mg/ml) and IC_{50} and IC_{90} were calculated using the probit analysis as shown in Table 4. Among the tested raw materials only RB extract was effective against cancer cell proliferation with IC_{50} values of 6.47. Cytotoxic activity of WB and WG successive extracts showed a dramatic inhibition drop against cancer cell growth from 63.8 and 82.6% at 10 mg/ml, respectively to 0% at 5 mg/ml. The anticancer activity of ultrafine ground samples increased compared to raw materials. Also, NRB extract was the most effective treatment with IC_{50} value of 4.10 mg/ml followed by 7.77 mg/ml for NWG and 14.30 mg/ml for NWB. Also, the extracts of FNWB and FNWG showed lower IC_{50} values compared to the extracts of raw and nano forms which indicate that fermentation process increased the anticancer activity of these materials. In this concern, some identified phenolic acids including *p*-coumaric, ferulic, and sinapinic acids have been previously shown to inhibit the growth of some cancer cell lines (Jaganathan, 2013; Peng et al., 2013). The antiproliferative activities of *p*-coumaric, ferulic, and sinapinic acids against HeLa, HCT116, and HT29 cancer cell lines were examined by Senawong et al. (2014). The MTT assay showed that ferulic, sinapinic and *p*-coumaric acids could inhibit the growth of tumor cells at millimolar concentrations. *p*-Coumaric acid exhibited the greatest

anticancer activity against all tested cancer cell lines. Moreover, rice bran fermented products were found to arrest the cancer cell cycle, promote cancer cell apoptosis and enhance the chemo-preventive effects (Yu et al., 2019).

Table 4 Cytotoxic activity of raw, nano and nano-fermented materials

Sample	LC_{50} (mg/ml)	LC_{90} (mg/ml)	Remarks (at 10 mg/ml)
WB	------	------	63.8%
NWB	8.90	14.30	72%
FNWB	5.96	8.14	100%
WG	------	------	82.6%
NWG	5.39	7.77	100%
FNWG	3.08	5.11	100%
RB	6.47	11.11	76.3%
NRB	2.63	4.10	100%
FNRB	1.62	4.23	100%
DMSO	------	------	1%
Negative control	------	------	0 %

WB- wheat bran, NWB- nano-wheat bran, FNWB- fermented-nano- wheat bran, WG- wheat germ, NWG- nano-wheat germ, FNWG- fermented-nano-wheat germ, RB- rice bran, NRB- nano-rice bran, FNRB- fermented-nano-rice bran, DMSO- dimethylsulphoxide, ------- = 0 inhibition at concentration lower than 5mg/ml.

Correlation between antioxidants, antioxidant activity and cytotoxic activity of raw, nano and fermented-nano materials

Data in Table 5 showed high correlation between the techniques used for determining antioxidant activity. High negative correlations among IC_{50} determined based on DPPH assay and both ABTS and FRAP assays were found ($r = -0.821$ and 0.825, respectively, $p < 0.01$). Also, correlations among ABTS and FRAP assays were positively high ($r = 0.997$, $p < 0.01$). Connor et al. (2002) found high correlation among ORAC, FRAP, and methyl linoleate oxidation assays in blueberries. Awika et al. (2003) also found high correlation between ORAC, ABTS, and DPPH in sorghum and its products. Moreover, DPPH, ABTS and FRAP were highly correlated with both total phenols ($r = -0.836$, 0.998 and 0.992, respectively, $p < 0.01$) and total flavonoids ($r = -0.808$, 0.992 and 0.995, respectively, $p < 0.01$) of the tested materials (Table 5). Whereas, the correlation between antioxidant activity assay methods and total carotenoids was not significant (r =

-0.441, 0.238 and 0.286, respectively). Both total phenols and total flavonoids showed high correlation with antioxidant activity as determined by all assays, which indicates that they are important contributors to antioxidant activity in tested extracts. Gil et al. (2002) found high correlation ($r = 0.9$, $P < 0.05$) between antioxidant activities determined by DPPH or FRAP assays and total phenols.

On the other hand, negative correlation between cytotoxic activity (IC_{50} value) and both total phenols and total flavonoids ($r = -0.527$ and -0.555, respectively, $P < 0.05$), while the correlation between cytotoxic activity and total carotenoids was not significant (r -0.028). Also, there was negative correlation between cytotoxic activity and both ABTS and FRAP ($r = -0.534$ and 0.539, respectively, $P < 0.05$). The highest positive correlation was found between cytotoxic activity and DPPH ($r = 0.648$, $P < 0.01$). For this reason, phytochemicals could contribute, at least in part, induced cytotoxic effect in the tested cell through its antioxidant activity.

Table 5 Correlation coefficient of antioxidants, antioxidant activity and cytotoxic activity of raw, nano and fermented-nano materials

Trait	TPH	TF	TC	DPPH (IC_{50})	ABTS	FRAP
TF	0.986**					
TC	0.264^{ns}	0.27^{ns}				
DPPH (IC_{50})	-0.836**	-0.808**	-0.441^{ns}			
ABTS	0.998**	0.992**	0.238^{ns}	-0.821**		
FRAP	0.992**	0.995**	0.286^{ns}	-0.825**	0.997**	
CA (IC_{50})	-0.527*	-0.555*	-0.028^{ns}	0.648**	-0.534*	-0.539*

TPH = total phenols, TF = total flavonoids, TC = total carotenoids and CA = cytotoxic activity. ^{ns} = non significant, * = Correlation is significant at $p < 0.05$ and ** = Correlation is significant at $p < 0.01$.

Conclusion

This study demonstrates that cereal by-products including wheat bran, wheat germ and rice bran are good sources of dietary antioxidants. High energy ball milling and solid state fermentation are promising techniques in food processing. These techniques were able to produce nano and fermented-nano powders from cereal by-products with higher antioxidant and cytotoxic activity through reducing the particle sizes and alleviate the phytochemicals.

Declarations

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Data Availability: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

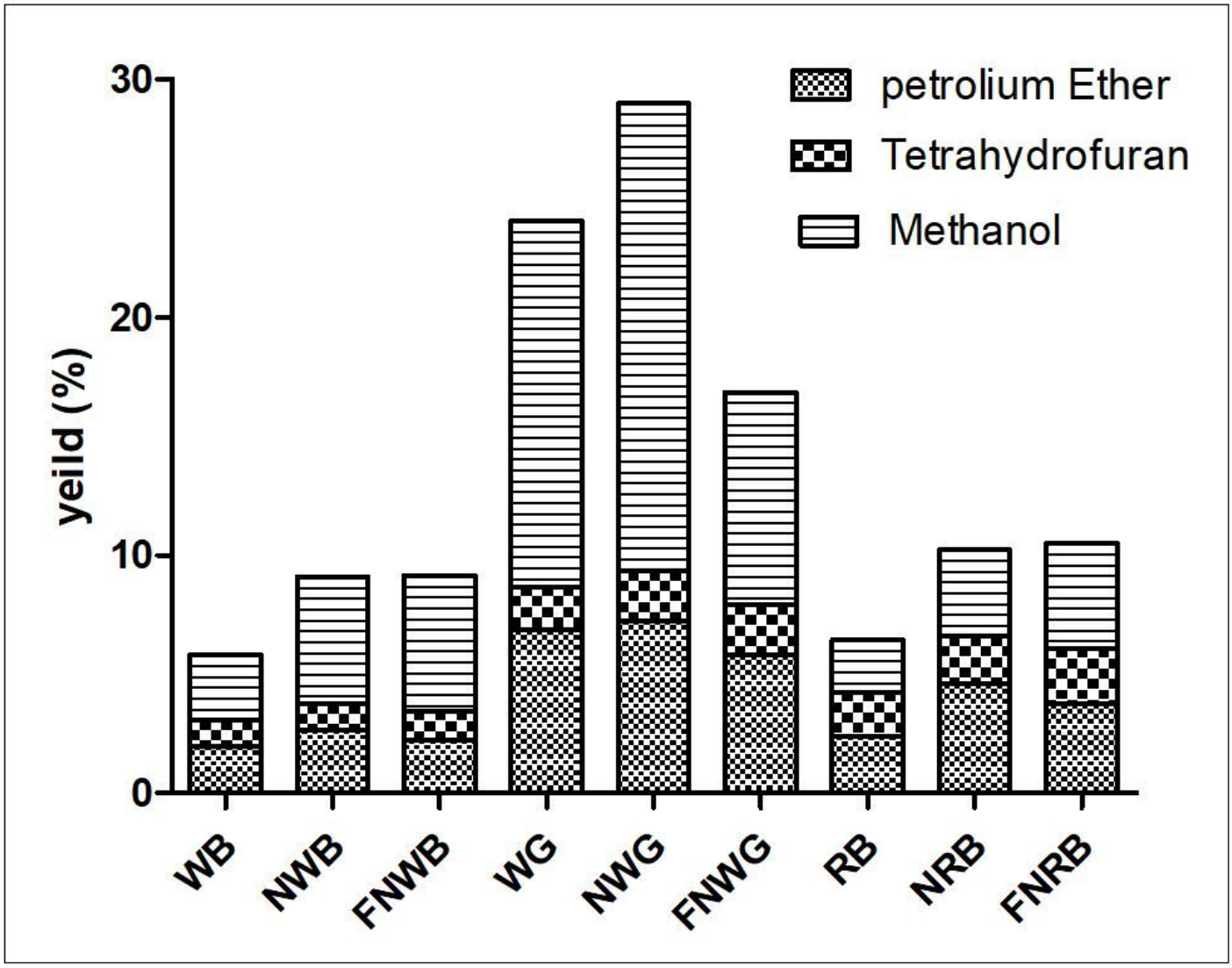

Figure 1

Successive extraction yield of raw, nano and fermented-nano materials

WB- wheat bran, NWB- nano-wheat bran, FNWB- fermented-nano- wheat bran, WG- wheat germ, NWG- nano-wheat germ, FNWG- fermented-nano-wheat germ, RB- rice bran, NRB- nano-rice bran, FNRB- fermented-nano-rice bran.