JOURNAL OF MEDICINAL FOOD
J Med Food 17 (5) 2014, 543–549

DOI: 10.1089/jmf.2013.0108

Enhancement of Wheat Grain Antioxidant Activity by Solid State Fermentation with *Grifola* spp.

Pablo Postemsky and Néstor Curvetto

Laboratory of Biotechnology of Edible and Medicinal Mushrooms, Center for Renewable Natural Resources of the Semiarid Region (CERZOS), National Scientific and Technical Research Council (CONICET) and National University of the South (UNS), Bahía Blanca, Argentina.

ABSTRACT *Grifola frondosa*, *Grifola gargal*, and *Grifola sordulenta* are edible and medicinal mushrooms with antioxidant properties. To obtain wheat flour (Wf) with a higher antioxidant activity than the one exhibited by regular Wf, solid state fermentation (SSF) of wheat grains with mycelia of those *Grifola* spp. was used to obtain biotransformed wheat grain (BWG) flour. The methanolic extract of control Wf and BWG flour of *G. gargal*, *G. sordulenta*, and *G. frondosa* (*Gf*WG, *Gg*WG, and *Gs*WG, respectively) were studied for their radical scavenging (RS) activity against 2,2-diphenyl-1-picrylhydracyl (DPPH) and their Fe(III) reducing power (RP). The values for RS-EC_{50} decreased in BWG flour, therefore presenting a higher antioxidant activity: *Gg*WG (0.56 mg/mL), *Gf*WG (0.81 mg/mL), and *Gs*WG (5.80 mg/mL) in comparison to Wf (57.60 mg/mL). The antioxidant content for this RS activity in terms of ascorbic acid content (RS-EQ_{AA}) was highest in *Gf*WG, followed by *Gg*WG and *Gs*WG (71.73, 14.46, and 3.02 mg/g, respectively) and lowest in Wf (0.25 mg/g). The RP-EC_{50} values in *Gg*WG, *Gf*WG, and *Gs*WG were low (0.55, 0.64, and 4.20 mg/mL, respectively) with respect to Wf (55.00 mg/mL). Compared with Wf (0.56 mg/g), the RP capacity in terms of ascorbic acid content (RP-EQ_{AA}) was very high in *Gf*WG (193.67 mg/g) followed by *Gg*WG and *Gs*WG (31.42 and 8.74 mg/g, respectively). The high content in gallic acid equivalents was consistent with RS-EQ_{AA} and RP-EQ_{AA} contents. TLC revealed that antioxidant activity in BWG could be related to the presence of phenolic compounds. Thus, a valuable food alternative can easily be obtained with wheat grains, that is, by markedly increasing their antioxidant value through SSF with *Grifola* spp.

KEY WORDS: • ***DPPH-radical scavenging activity*** • ***functional food*** • **Grifola frondosa** • **Grifola gargal** • **Grifola sordulenta** • ***phenols*** • ***radical scavenging*** • ***reducing power*** • ***solid state fermentation*** • ***wheat grains***

INTRODUCTION

OXIDATION IS ESSENTIAL in all living organisms and produces the energy needed to enable their metabolism. However, if an uncontrolled production of free radicals occurs as a result of oxidative reactions, then diseases, aging, and acceleration of degenerative process can develop.[1]

Edible mushrooms possess high nutraceutical value.[2] Among these mushrooms, many studies have shown *Grifola frondosa* to exhibit good antioxidant, antitumoral, and immunomodulatory properties.[3–5]

In the patagonian forests of Argentina and Chile, two macrofungal endemic species grow which are phylogenetically close to *G. frondosa*, *Grifola gargal*, and *Grifola sordulenta*.[6,7] The two latter species have also received attention as a potential source for medicinal metabolites and hence were subjected to studies to reveal their nutraceutical properties.[8,9] Both species are characterized by a special almond-like aroma and flavor, which are present in fruiting bodies, mycelia from liquid cultures, fermented grains, and other fermented substrates.

In a previous work,[10] we used the SMART-eyes *Drosophila melanogaster* bioassay to study the antimutagenic activity of flour made from *G. gargal* biotransformed wheat grains (BWG). Flour made of BWG protects the ommatidia cells from DMBA genotoxic damage.

Fungal solid state fermentation (SSF) of wheat grains exhibiting a higher functional value than normal wheat grains has already been reported. This was found to be the case with *Aspergillus* spp.,[11] *Cordyceps militaris*,[12] and *Grifola frondosa*.[5]

Human consumption of *G. gargal* and *G. sordulenta* mushrooms is very low as they are rare mushrooms that naturally occur in protected forest environments, where mushroom over gathering would negatively impact the forest ecology. Moreover, despite attempts to cultivate them, hitherto the industrial cultivation has not been achieved.

Thus, for the time being, BWG flour by *G. gargal* and *G. sordulenta* is an interesting alternative source for obtaining bioactive metabolites produced by these mushrooms.

Manuscript received 14 September 2012. Revision accepted 6 November 2013.

Address correspondence to: Néstor Curvetto, PhD, Laboratory of Biotechnology of Edible and Medicinal Mushrooms, CERZOS (CONICET-UNS), Camino La Carrindanga Km 7, 8000 Bahía Blanca, Argentina, E-mail: ficurvet@criba.edu.ar

The main objective of the present study was to evaluate the antioxidant activity of methanolic extracts of flour obtained from *G. gargal*, *G. sordulenta*, and *G. frondosa* BWG for both their free radical scavenging (RS) activity against 2,2-diphenyl-1-picrylhydracyl (DPPH) radical and reducing power (RP) activity in Fe(III) to Fe(II). Because phenolics are considered to be important compounds with respect to the antioxidant properties found in mushrooms,[4,5,8,9,13–15] the total phenolic content was also studied. TLC was used as a useful screening tool for revealing the chemical nature of secondary metabolites of *Grifola* spp. exhibiting an antioxidant activity.

MATERIALS AND METHODS

Mushrooms strains

G. gargal Singer (strain: CIEFAP #191) and *G. sordulenta* Mont. (Singer) (strain: CIEFAP #154) were obtained from CIEFAP (Centro de Investigación y Extensión Forestal Andino Patagónico, Argentina). Collection of the *G. gargal* strain was performed in *Nothofagus obliqua* forests (Lanín National Park, Neuquén, Argentina) and the *G. sordulenta* strain in *Nothofagus dombeyi* natural forests (Los Alerces National Park, Chubut, Argentina). The *G. frondosa* strain is of Asiatic origin and was kindly provided by MushWorld Organization (Seoul, Korea).

SSF for obtaining Grifola *spp. BWG*

Grifola spp. BWG were obtained as described elsewhere[16] by using a common method to obtain spawn for mushroom cultivation, that is, SSF is performed after inoculating mushroom mycelium on wheat grains. Briefly, a mixture of 250 g whole wheat grains, 0.6 g $CaCO_3$, and 3.5 g $CaSO_4$, was soaked overnight with 190 mL of water and then sterilized at 1 atm during 90 min. Inoculation was performed using 20-day-old mycelium from 1/4 of a fully colonized Petri dish grown on a MYPA culture medium with addition of sunflower seed hull powder (4g/L).[17] Cultivation was performed during 20 days at 24°C ± 1°C in darkness. The BWG were dried at 60°C in an oven supplied with air circulation. Flour from BWG of *G. frondosa*, *G. gargal*, and *G. sordulenta* was obtained using a Laboratory Sample Mill (Udy Cyclone Corporation, Colorodo, USA). Wheat flour (Wf) to be used in control treatments was obtained by the same procedure, but skipping the mycelium inoculation step.

Extract preparation

Methanolic extractions of control Wf and BWG flour of *G. frondosa*, *G. gargal*, and *G. sordulenta* (*Gf*WG, *Gg*WG and, *Gs*WG, respectively), were done according to Arena *et al.*[18] Three samples of each flour (2–5 g) were dispersed in methanol at a 1/10 mass/volume ratio (g/mL). Each mixture was sonicated for 10 min (Branson Bransonic 220, 50/60 Hz) and then kept 72 h in darkness at 24°C under agitation on an orbital shaker at 0.4 *g*. Each mixture was centrifuged at 1400 *g* for 10 min and the supernatant was separated by decantation and filtration through Whatman #4 filter paper. The extraction yield was gravimetrically obtained. Finally, extracts were pooled in 50-mL plastic tubes, put in a thermal bath at 50°C, and flushed with gaseous nitrogen to obtain an extract concentration of 20–25 mg/mL. Resulting extracts were then kept at −20°C until use.

RS activity

The RS activity was measured according to Arena *et al.*[18] This activity was assayed in the range of 1–20 mg extract dry matter per mL; each sample was mixed with 0.25 mL of methanolic solution containing DPPH radicals (Sigma-Aldrich, St. Louis, MO, USA) to obtain a final concentration of 0.2 mM DPPH in the reaction mixture. Resulting reaction mixtures were allowed to stand at room temperature and darkness for 30 min, and then by using an appropriate mixture as a blank, absorbance readings (λ_{517} nm) were made. The RS activity was obtained using the equation RS (%) = (1 − [ΔAbs sample/ΔAbs blank]) × 100.

For comparison, the RS activities of ascorbic acid (0.5–20 mg/mL; Cicarelli, San Lorenzo, Argentina), butyl hydroxyanisole (BHA 0.25–20 mg/mL; Sigma), and α-tocopherol (0.25–20 mg/mL; Sigma) were also measured. The 50% effective concentration value for this activity (RS-EC_{50} mg/mL) indicates the concentration of the solution with antioxidant activity that causes the extinction of 50% of DPPH radicals; this value was obtained by linear regression analysis. The RS content was also calculated in terms of equivalent activity in ascorbic acid by using the equation RS-EQ_{AA} (mg/g) = [Yield sample (mg/g) × RS-EC_{50} ascorbic acid (mg/mL)]/RS-EC_{50} sample (mg/mL).

Reducing power

RP was determined according to Arena *et al.*[18] One mL of each methanolic extract sample (1–20 mg/mL) was mixed with 2.5 mL of 200 mM sodium phosphate buffer (pH 6.6) and 1 mL of 1% potassium ferricyanide (Sigma); the resulting mixture was incubated at 50°C in darkness for 20 min. After adding 1 mL of 0.1g/mL trichloroacetic acid (Anedra, Buenos Aires, Argentina), the mixture was centrifuged at 1400 *g* for 10 min. A 1-mL aliquot of the upper layer was mixed with 1 mL of deionized water and 0.2 mL 0.1% $FeCl_3$ $6.H_2O$ (Anedra). The absorbance of the final mixture was measured at λ_{700} nm; higher absorbance indicates higher RP. All reference antioxidants (ascorbic acid, BHA, and α-tocopherol) at 10 mg/mL concentration exhibited 100% of RP activity, this RP activity being reached at 1.2 absorbance units. RP was obtained by using the equation RP (%) = (100 − [ΔAbs maximum − ΔAbs sample]/ΔAbs maximum) × 100.

The RP-EC_{50} (mg/mL) reference value corresponded to the extract concentration by which 50% of maximum absorbance was reached, and it was determined by linear regression analysis. The content of compounds in methanolic extracts exhibiting RP was also given in terms of ascorbic acid RP (mg/g RP-EQ_{AA}): RP-EQ_{AA}(mg/g) = Yield sample (mg/g) × RP-EC_{50} ascorbic acid (mg/mL)/RP-EC_{50} sample (mg/mL).

Phenol content

Total phenolic content was determined according to the Folin–Ciocalteu method as described by Arena *et al*.[18] A calibration curve was obtained using 0.01–2 mg/mL gallic acid (Sigma) as standard and the phenol content in flour was expressed in terms of gallic acid equivalents (mg/g EQ_{GA}).

Thin layer chromatography of methanolic extracts

Screening of chemical compounds involved with antioxidant activity was performed using thin layer chromatography (TLC). TLC plates (10 cm × 20 cm) coated with 0.2 mm silicagel (Merck 60 F_{254}) were used. Ten microliter aliquots of solutions containing reference substances or 20 μL methanolic extracts of mushroom BWG flour and Wf were applied as bands of 4 mm width. The solvent system used to run the TLC was chloroform:methanol:water (65:35:10).[19] Compounds exhibiting fluorescence were detected under white light (fluorescent tubes) and ultraviolet (UV) light (λ_{254} nm, λ_{366} nm).

Three chromatographic replicates were run and separated metabolite bands were scored according to their polar character. The first chromatogram plate was sprayed with 2.54 mM DPPH methanol solution.[20] Yellow bands of antioxidant compounds appeared on a violet background at the first 30 min, bands stained after 16 h were also registered. The second chromatogram plate was observed under white light and UV irradiation (λ_{254} nm, λ_{366} nm) before and after the exposure to ammonia vapors. Phenolic compounds were detected under UV (λ_{254} nm, λ_{366} nm) as fluorescent bands and under white light as colored bands, which became more intense in color after exposing them to ammonia vapors.[21,22] Additionally, a freshly prepared developing solution of 1% ferric chloride (Sigma Aldrich, St. Louis, MO, USA) and 1% potassium ferricyanide (Sigma Aldrich) [Cl_3Fe-$K_3Fe(CN)_6$] was sprayed to develop the group of phenolic compounds exhibiting RP.[21] Phenolic compound bands were differentiated by their colors and bands appeared as reported by other authors.[13,23] The third chromatographic plate was sprayed with a solution of 5% anthrone (Sigma Aldrich) made in 15% sulfuric acid in ethanol 96%, followed by 1 min of heating at 120°C[24]; saccharides appeared stained in both red and orange color.

Statistical analysis

Data were subjected to one way-ANOVA, and mean values were then separated using the Tukey multiple range test at $P < .05$, using the Infostat software.[25] To obtain RS-EC_{50} and RP-EC_{50} values at 99% confidence interval, linear regression analysis was done using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

RESULTS AND DISCUSSION

Methanolic extract yield

Methanolic extraction yields of BWG flour were between 2.70% and 19.37% (Table 1). The *Gf*WG extract yield (19.37%) largely exceeded the one obtained with Wf (4.80%). Yields were consistent with the yields reported by Huang *et al*.[5] in a previous work in which a SSF of wheat grains with *G. frondosa* was used and the extraction of the BWG flour was made in ethanol and water. These authors found that a higher extraction yield resulted from the flour coming from highly biodegraded wheat grains.

Table 1. Extraction Yield and Phenolic Content in Methanolic Extracts Obtained from Flour Made of Wheat Grains and from Wheat Grains Biotransformed by *Grifola gargal*, *Grifola sordulenta*, and *Grifola frondosa*

	Methanolic extract yield (%)	EQ_{GA} *(mg/g)*
*Gg*WG	2.70 ± 0.20^{c}	3.28 ± 0.23^{b}
*Gs*WG	5.73 ± 0.25^{b}	1.00 ± 0.04^{c}
*Gf*WG	19.37 ± 0.25^{a}	19.60 ± 0.25^{a}
Wf	4.80 ± 0.60^{b}	0.11 ± 0.01^{d}

Values are expressed as mean ± standard deviation ($n = 3$). Phenolic content values are expressed in terms of equivalents of gallic acid per gram of dry sample (EQ_{GA}).

[abcd]Means with different letters within columns are significantly different (Tukey, $\alpha = 0.05$).

*Gg*WG, *Gs*WG, and *Gf*WG: wheat grains biotransformed by *G. gargal*, *G. sordulenta*, and *G. frondosa*, respectively; Wf, wheat flour.

RS activity

Ascorbic acid and α-tocopherol are vitamins required daily; they play an important role as antioxidants.[26,27] BHA is an artificial phenolic compound used in the food industry to prevent fat oxidation.[28] These compounds show a high antioxidant activity (85–95% RS activity at 1 mg/mL, Fig. 1A), hence, they were included for comparison purposes. The RS activity in control Wf indicates the basal antioxidant activity of wheat grains, and reached 13% of the maximum RS at the highest methanolic extract concentration of 20 mg/mL (Fig. 1A). Interestingly, an increase in the RS antioxidant activity occurred in all the BWG flour, reaching the following maximum values of 85% at 1 mg/mL *Gg*WG, 62% at 1 mg/mL *Gf*WG, and 90% at 10 mg/mL *Gs*WG.

The EC_{50} value of antioxidant activity shows the concentration at which 50% of maximum antioxidant activity is reached; a higher antioxidant activity occurs at lower EC_{50} values.[18] Thus, this EC_{50} value is used as a reference to score the antioxidant activity of different compounds. Considering the high RS-EC_{50} value of 57.60 mg/mL found in Wf, a low value of 5.80 mg/mL was obtained in *Gs*WG (Table 2) and very low values of 0.56 and 0.81 mg/mL were found in *Gg*WG and *Gf*WG, respectively.

The RS-EC_{50} in fruiting bodies of *G. frondosa* and others mushrooms with good antioxidant activity ranged from 2 to 10 mg/mL of methanolic extract concentration.[14,13] However, in the case of *Gg*WG and *Gf*WG, the RS-EC_{50} values are closer to those found in the range of 0.5–2.0 mg/mL, considered to possess a high antioxidant activity, for example, as was the case for *Auricularia* spp. and *Tremella* spp.[29]

Because of the low content of mycelium occurring in BWG (4 mg/g, dry basis),[10] the above-mentioned results

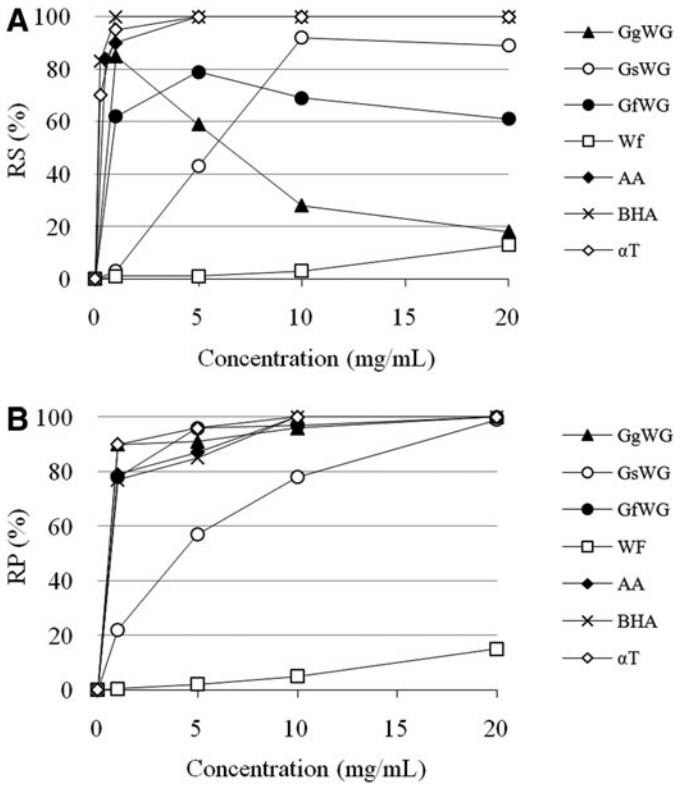

FIG. 1. Antioxidant activity of methanolic extracts. Different extract concentrations in methanol (mg/mL) were evaluated in **(A)** radical scavenging activity against DPPH (RS), and **(B)** Fe(III) reducing power (RP) activity. Ascorbic acid (AA), butylhydroxianisole (BHA), and α-tocopherol (αT) were used as controls. Values are the average of three determinations. Wheat flour (Wf): extracts from flour of wheat grains; *Gg*WG, *Gs*WG, and *Gf*WG: extracts from flour of wheat grains biotransformed by either *Grifola gargal*, *G. sordulenta*, or *G. frondosa*, respectively; DPPH, 2,2-diphenyl-1-picrylhydracyl.

indicate that the antioxidant activity should come from an accumulation of highly active antioxidant metabolites, which in these BWG allow the building of a high antioxidant activity, even to the level found in mushrooms exhibiting the best antioxidant activity.

High RS-EC_{50} values were also obtained in the ethanolic and aqueous extracts from *G. frondosa* fermented wheat grains (2.76 and 7.34 mg/mL, respectively),[5] showing that the antioxidant capacity depends on the solvent polarity, thus, the methanolic and ethanolic extracts being more active.

As an alternative way to show the antioxidant content present in BWG flour, the ascorbic acid effective concentration value RS-EC_{50} was also obtained (see RS activity in Materials and Methods section). In our study, for ascorbic acid, an RS-EC_{50} value of 0.30 mg/mL was obtained (Table 2, Fig. 1A), which was found to be either lower (0.07 mg/mL)[15] or higher (3.0 mg/mL)[4,30] than other reported values.

SSF of wheat grains increased the content in RS-EQ_{AA} from 0.25 mg/g in Wf to 71.73, 14.46, and 3.02 mg/g in *Gf*WG, *Gg*WG, and *Gs*WG, respectively. Furthermore, the RS-EQ_{AA} content in *Gf*WG was fivefold higher than in *G. frondosa* fruiting bodies, which was ca. 13 mg/g. The latter value was obtained using the equation for RS-EQ_{AA} (mg/g) (see RS activity in Materials and Methods section) with the reported values of extraction yield and RS-EC_{50} reported by Mau *et al*.[4]

In addition, a high accumulation of pigmented metabolites in *Gg*WG and *Gf*WG was also evident over 5 mg/mL concentrations. This fact produced a masking effect in the analysis of RS antioxidant response, which was not previously reported in similar studies.[4,5,14,15,29]

Reducing power

The RP activity on Fe(III) ions indicates the electron donor capacity exhibited by antioxidant compounds.[13] In Figure 1B, a high RP activity of *Gg*WG and *Gf*WG is shown, while the RP activity of *Gs*WG was slightly lower, and reached 100% of RP activity at 20 mg/mL. In fact, the RP-EC_{50} values obtained in *Gg*WG and *Gf*WG were as low

Table 2. DPPH Radical Scavenging and Fe(III) Reducing Power Activities in Methanolic Extracts Obtained from Flour of Wheat Grains and from Wheat Grains Biotransformed by *Grifola gargal*, *Grifola sordulenta*, and *Grifola frondosa*

	DPPH radical scavenging		*Fe(III) RP*	
	RS-EC_{50} (mg/mL)	*RS-EQ_{AA} (mg/g)*	*RP-EC_{50} (mg/mL)*	*RP-EQ_{AA} (mg/g)*
*Gg*WG	0.56 (0.50–0.62)	14.46 ± 1.07^{b}	0.55 (0.50–0.60)	31.42 ± 2.33^{b}
*Gs*WG	5.80 (5.45–6.15)	3.02 ± 0.13^{c}	4.20 (3.52–4.88)	8.74 ± 0.38^{c}
*Gf*WG	0.81 (0.72–0.90)	71.73 ± 0.93^{a}	0.64 (0.42–086)	193.67 ± 2.52^{a}
Wf	57.60 (38.6–76.6)	0.25 ± 0.03^{d}	55.00 (42.40–67.60)	0.56 ± 0.07^{c}
Ascorbic acid	0.30 (0.29–0.31)	—	0.64 (0.63–0.65)	—
α-tocopherol	0.18 (0.17–0.19)	—	0.66 (0.59–0.73)	—
BHA	0.15 (0.14–0.16)	—	0.56 (0.52–0.60)	—

Values are expressed as mean (99% confidence interval) or as mean ± standard deviation ($n = 3$). Ascorbic acid (AA), α-tocopherol, and butylhydroxianisole (BHA) were used as controls.

[abcd]Means with different letters within columns are significantly different (Tukey, $\alpha = 0.05$).

DPPH, 2,2-diphenyl-1-picrylhydracyl; RP, reducing power; RS, radical scavenging.

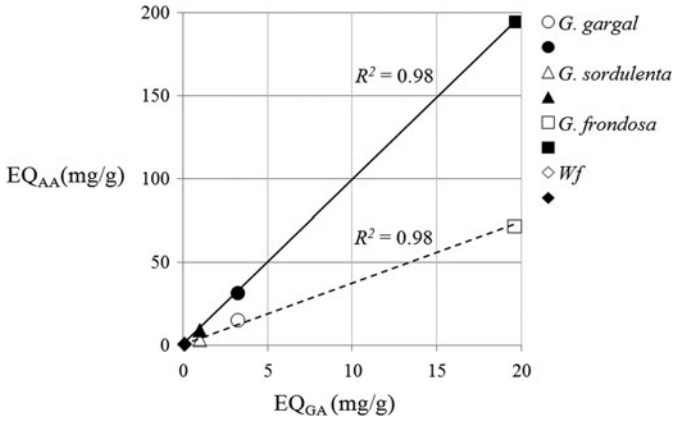

FIG. 2. Linear regression between phenolic content (EQ_{GA}) and antioxidant content considered either as RS-EQ_{AA} or RP-EQ_{AA}. Open symbols and dashed line are for RS-EQ_{AA} determined for DPPH extinction, filled symbols and solid line are for RP-EQ_{AA} obtained in the analysis of Fe(III) reducing power.

as the reference antioxidant compounds: 0.55–0.64 mg/mL (Table 2). However, RP activity of wheat grains without SSF barely reached 15% at the higher methanolic extract concentration, that is, 20 mg/mL. Otherwise, the RP-EC_{50} obtained in *Gs*WG (4.20 mg/mL) was comparable to the one obtained in other mushrooms, considered to have good antioxidant RP activity, like *G. frondosa*, *Agaricus blazei*, and *Hericium erinaceus*.[4,13,31] With regard to *Gf*WG, no differences were found between methanolic extracts and the reported antioxidant values of ethanolic or aqueous extracts (RP-EC_{50}: 2.0 and 1.1 mg/mL, respectively),[5] and the observed difference between RS and RP in the analyzed extracts was attributed to the presence of different antioxidant compounds because of the differences in the polarity properties of the extractants used.

The RP-EC_{50} obtained with ascorbic acid was quite similar to the values reported elsewhere.[4] SSF of wheat grains produced a noticeable increase in the antioxidant activity. RP expressed as RP-EQ_{AA} in *Gf*WG, *Gg*WG, and *Gs*WG was 344, 56, and 16 times higher, respectively, than the basal activity obtained in Wf (Table 2).

Thus, RS and RP analysis revealed that common wheat grains can be transformed to a functional food by mushroom SSF. This alternative source of metabolites can be useful in the case of mushrooms growing in protected areas and with rare species difficult to cultivate, as is the case of *G. gargal* and *G. sordulenta*. In addition, flour obtained using these species has an almond-like flavor and aroma.

Antioxidants present in BWG by Grifola *spp.*

In fungi, antioxidant activity is generally attributed to the presence of phenolic compounds rather than to polysaccharides.[32–36] The phenolic content, expressed in terms of gallic acid equivalents (EQ_{GA}), was found to be in the order *Gf*WG > *Gg*WG > *Gs*WG > Wf (Table 1).

The high phenolic content in *Gf*WG (19.60 mg/g) was comparable to the content reported for aqueous and ethanolic extracts of BWG (13.35 and 23.01 mg/g, respectively)[5] and to phenolic contents found in *G. frondosa* fruiting bodies (12.3 mg/g).[4] The phenolic content in *Gg*WG (3.28 mg/g) was also similar to the content found in fruiting bodies of *G. gargal* (3–5 mg/g)[8,9] (considering 85% relative humidity).

Table 3. Thin Layer Chromatography Analysis of Methanolic Extracts

Sample	*Rf*	*RS activity*[a]	λ_{254}	λ_{366}	*Ammonia*[b]	*Phenol*	*Saccharides*
Wf							
Polar	0.00–0.20	*Low*	−	−	−	+	+
Nonpolar	0.95–1.00	*Moderate*	+	+	−	−	+
*Gg*WG							
Polar I	0.00–0.20	*High*	+	+	+	+	−
Polar II	0.20–0.40	*High*	+	+	+	+	−
Polar III	0.40–0.60	*Moderate*	+	−	+	+	−
Intermediate polar	0.60–0.80	*Low*	+	−	+	+	−
Nonpolar	0.95–1.00	*High*	+	+	+	−	−
*Gs*WG							
Polar	0.00–0.50	*Moderate*	+	−	−	+	−
Intermediate polar	0.60–0.80	*Low*	+	+	−	−	+
Nonpolar	0.95–1.00	*High*	+	+[c]	−	+	+
*Gf*WG							
Polar I	0.00–0.20	*High*	+	+[c]	+	−	−
Polar II	0.20–0.40	*High*	+	+	+	+	−
Intermediate polar	0.60–0.90	*Low*	+	+	+	−	−
Nonpolar	0.95–1.00	*High*	+	+	−	+	+

Bands with radical scavenging (RS) activity reaction (high, moderate, low) are shown with their characteristics (+, presence; −, absence) under ultraviolet light (λ_{254} and λ_{366}) and under white light irradiation following exposure to ammonia vapors, 1% ferric chloride and 1% potassium ferricyanide (phenols) and to anthrone sulfuric acid reagent (saccharides).

[a]DPPH radical extinction observed under white light. Antioxidant activity: high (intense in 30 min); moderate (intense in 16 h); and low (faint in 16 h).

[b]Color darkening observed under white light following ammonia vapor exposure was related to phenolics.

[c]Bright blue at λ_{366}, which increases after ammonia exposure was related to flavonoids.

In addition, it was reported that 5–10% of the phenolics present in *G. gargal* fruiting bodies are flavonoids.[9]

Despite the low content in mycelia present in BWG (~0.4%, determined by glucosamine content),[10] it is interesting that the phenolic contents of both *Gf*WG and *Gg*WG were found to be similar to that found in fruiting bodies.

The correlations between phenolic content (EQ_{GA}) versus antioxidant content (RS-EQ_{AA} and RP-EQ_{AA}; Fig. 2) show that phenolics play a marked role in the antioxidant activity exhibited by these mushrooms.

Table 3 shows the TLC of methanolic extracts from flour made from wheat grains biotransformed by *Grifola* spp. The Wf showed a weak and moderate antioxidant activity, which appeared in one polar and one nonpolar band, respectively (Table 3), possibly related to phenolics, tocopherols, and carotenoids known to be the major DPPH radical scavenger compounds present in wheat grains.[37,38]

The *Gg*WG extract showed one nonpolar and two polar bands, with high antioxidant activity, with migrating and staining features of phenolic compounds. The polar and intermediately polar compounds appeared as a continuum from the origin to an Rf of 0.80, being the highest antioxidant bands located in the chromatogram position corresponding to the most polar compounds (Rf: 0.00 to 0.40, Table 3).

In *Gs*WG, a moderately antioxidant polar band and a highly antioxidant nonpolar band were found. Both of them show features consistent with phenolics and saccharides.

In the case of *Gf*WG, two polar bands with high antioxidant activity exhibited characteristics of phenolic compounds. A similar continuum as was described above for *Gg*WG extracts was also observed. The third band with a high antioxidant activity was nonpolar and presented both phenolic and saccharide characteristics.

This particular analysis also showed the presence and low activity of antioxidants in Wf in comparison with the high antioxidant activity and the presence of numerous compound bands found in methanolic extracts of BWG flour obtained with SSF process and *Grifola* spp. mycelia.

In conclusion, the three *Grifola* spp. were able to biotransform wheat grains and during this process, a high accumulation of antioxidant metabolites resulted. This good antioxidant activity shows the nutraceutical value of BWG flour and the nutraceutical possibilities of *Grifola* spp. hydroalcoholic extracts, that is, by either adding value to a food that could be prepared by using the flour obtained with mushroom BWG, or even to obtain an extract of bioactive compounds by preparing a hydro-alcoholic liqueur. The almond flavor and aroma are also interesting features to be profited from these special wheat/mushrooms flour. This innovative approach could even be done with other cereal/medicinal mushroom combinations, thus contributing to the food industry to provide better and healthier foods.

ACKNOWLEDGMENTS

We thank Silvia Delmastro and Ricardo Devalis for their expert technician help. This research was supported by grants from the CONICET and Universidad Nacional del Sur, Argentina.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

REFERENCES

1. Halliwell B: Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. *Plant Physiol* 2006;141: 312–322.
2. Cheung PCK: The nutritional and health benefits of mushrooms. *Nutr Bull* 2010;35:292–299.
3. Illana-Esteban C: El hongo maitake (*Grifola frondosa*) y su potencial terapéutico. *Rev Iberoam Micol* 2008;25:141–144.
4. Mau J, Chang C, Huang S, Chen C: Antioxidant properties of methanolic extracts from *Grifola frondosa*, *Morchella esculenta* and *Termitomyces albuminosus* mycelia. *Food Chem* 2004;87: 111–118.
5. Huang SL, Tsai SY, Lin SY, Liang CH, Lian PY, Mau JL: Preparation of culinary-medicinal Maitake mushroom, *Grifola frondosa* (Dicks.: Fr.) S.F. Gray (*Aphyllophoromycetideae*)-fermented wheat and its antioxidant properties. *Int J Med Mushrooms* 2011;13:61–71.
6. Shen Q, Geiser DM, Royse DJ: Molecular phylogenetic analysis of *Grifola frondosa* (maitake) reveals a species partition separating eastern North American and Asian isolates. *Mycologia* 2002;94:472–482.
7. Rajchenberg M: The genus *Grifola* (*Aphyllophorales, Basidiomycota*) in Argentina revisited. *Bol Soc Argent Bot* 2002;37:19–27.
8. Brujin J, Loyola C, Aqueveque P, Cañumir J, Cortéz M, France A: Influence of heat treatment on the antioxidant properties of *Grifola gargal* hydro-alcoholic extracts. *Micol Apl Int* 2008;20: 27–34.
9. Brujin J, Loyola C, Aqueveque P, Cañumir J, Cortéz M, France A: Antioxidant properties of extracts obtained from *Grifola gargal* mushrooms. *Micol Apl Int* 2009;21:11–18.
10. Postemsky PD, Palermo AM, Curvetto NR: Protective effects of new medicinal mushroom, *Grifola gargal* Singer (higher basidiomycetes), on induced DNA damage in somatic cells of *Drosophila melanogaster. Int J Med Mushrooms* 2011;13:583–594.
11. Bhanja T, Kumari A, Banerjee R: Enrichment of phenolics and free radical scavenging property of wheat koji prepared with two filamentous fungi. *Bioresour Technol* 2009;100:2861–2866.
12. Das S, Masuda M, Sakurai A, Sakakibara M: Medicinal uses of the mushroom *Cordyceps militaris*: current state and prospects. *Fitoterapia* 2010;81:961–968.
13. Soares AA, Marques de Souza CG, Daniel FM, Pezente Ferrari G, Gomes da Costa SM, Peralta RM: Antioxidant activity and total phenolic content of *Agaricus brasiliensis* (*Agaricus blazei* Murril) in two stages of maturity. *Food Chem* 2009;112:775–781.
14. Yang JH, Lin HC, Mau JL: Antioxidant properties of several commercial mushrooms. *Food Chem* 2002;77:229–235.
15. Liu X, Dong M, Chen X, Jiang M, Lv X, Yan G: Antioxidant activity and phenolics of an endophytic *Xylaria* sp. from *Ginkgo biloba. Food Chem* 2007;105:548–554.
16. Curvetto N, González Matute R, Figlas D, Delmastro S: Cultivation of oyster mushrooms on sunflower seed hull substrate. In: *Mushroom Growers' Handbook 1: Oyster Mushroom Cultivation*. (MushWorld, eds.) Seoul, Korea, 2004, pp. 101–106.

17. Postemsky PD, González Matute R, Figlas D, Curvetto N: Optimizing *Grifola sordulenta* and *Grifola gargal* growth in agar and liquid nutrient media. *Micol Apl Int* 2006;18:7–12.
18. Arena ME, Postemsky PD, Curvetto NR: Accumulation patterns of phenolic compounds during fruit growth and ripening of *Berberis buxifolia*, a native Patagonian species. *NZ J Bot* 2012; 50:15–28.
19. Farombi E, Britton G, Emerole G: Evaluation of the antioxidant activity and partial characterisation of extracts from browned yam flour diet. *Food Res Int* 2000;33:493–499.
20. Gu L, Wu T, Wang Z: TLC bioautography-guided isolation of antioxidants from fruit of *Perilla frutescens* var. Acuta. *LWT Food Sci Technol* 2009;42:131–136.
21. Hammerschmidt R, Nicholson R: Resistance of maize to anthracnose: changes in host phenols and pigments. *Phytopathology* 1977;67:251–258.
22. Toledo Castro S, González Lavaut J, Echemendía O, García González N: Evaluación fitoquímica y farmacológica de frutos de *Erythroxylum minutifolium* Griseb. (*Erythroxylaceae*). *Rev Cubana Plant Med* 2008;13:1028–4796 (Online).
23. Wanasundara U, Amarowicz R, Shahidit F: Partial characterization of natural antioxidants in canola meal. *Food Res Int* 1995;28:525–530.
24. Helbert J, Brown K: Color reaction of anthrone with monosaccharide mixtures and oligo and polysaccharides containing hexuronic acids. *Anal Chem* 1957;29:464–1466.
25. Di Rienzo J, Casanoves F, Balzarini M, González L, Tablada M, Robledo CW: Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina, 2010. www.infostat.com.ar (accessed January 2010).
26. Khalid N, Kobayashi I, Neves M, Uemura K, Nakajima M: Preparation and characterization of water-in-oil emulsions loaded with high concentration of L-ascorbic acid. *LWT Food Sci Technol* 2013;51:448–454.
27. Frank J: Beyond vitamin E supplementation: an alternative strategy to improve vitamin E status. *J Plant Physiol* 2005;162:834–843.
28. Dolatabadi J, Kashanianb S: A review on DNA interaction with synthetic phenolic food additives. *Food Res Int* 2010;43:1223–1230.
29. Mau JL, Chao GR, Wu KT: Antioxidant properties of methanolic extracts from several ear mushrooms. *J Agr Food Chem* 2001; 49:5461–5467.
30. Sharma O, Bhat T: DPPH antioxidant assay revisited. *Food Chem* 2009;113:1202–1205.
31. Mau JL, Lin HC, Song SF: Antioxidant properties of several specialty mushrooms. *Food Res Int* 2002;35:519–526.
32. Tseng YH, Yang JH, Mau JL: Antioxidant properties of polysaccharides from *Ganoderma tsugae*. *Food Chem* 2008;107:732–738.
33. Barros L, Baptista P, Ferreira I: Effect of *Lactarius piperatus* fruiting body maturity stage on antioxidant activity measured by several biochemical assays. *Food Chem Toxicol* 2007a;45:1731–1737.
34. Heleno S, Barros L, Sousa MJ, Martins A, Ferreira I: Tocopherols composition of Portuguese wild mushrooms with antioxidant capacity. *Food Chem* 2010;119:1443–1450.
35. Liu X, Zhou B, Lin R, Jia L, Deng P, Fan K, Wang G, Wang L, Zhang J: Extraction and antioxidant activities of intracellular polysaccharide from *Pleurotus* sp. mycelium. *Int J Biol Macromol* 2010;47:116–119.
36. Liu W, Wang H, Pang X, Yao W, Gao X: Characterization and antioxidant activity of two low-molecular-weight polysaccharides purified from the fruiting bodies of *Ganoderma lucidum. Int J Biol Macromol* 2010;46:451–457.
37. Chen Y, Dunford NT, Goad C: Phytochemical composition of extracts from wheat grain fractions obtained by tangential abrasive dehulling. *LWT Food Sci Technol* 2013;54:353–359.
38. Mateo Anson N, van den Berg R, Havenaar R, Bast A, Haenen GRMM: Ferulic acid from aleurone determines the antioxidant potency of wheat grain (*Triticum aestivum* L.). *J Agric Food Chem* 2008;56:5589–5594.