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Original Article

Antioxidant and antifungal potential of methanol extracts of *Phellinus* spp. from Sonora, Mexico

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ABSTRACT

Background: Among the potential natural sources of bioactive compounds, those of the macroscopic fungi *Phellinus* spp. have been identified by previous researches. Phenolic compounds are among the major antioxidant and antimicrobial contributors due to their bioactive properties.
Aims: The goal of this study was to determine the total phenolic and flavonoid contents, and its relation with the antioxidant and antifungal activity of methanolic extracts of *Phellinus gilvus*, *Phellinus rimosus* and *Phellinus badius*, respectively.
Methods: The collected and identified organisms of *Phellinus* spp. were treated with methanol and the generated aqueous extract was analyzed to quantified total phenolic compounds, total flavonoids, radical scavenging activity against DPPH, trolox equivalent antioxidant capacity, and oxygen absorbance capacity. The antifungal property of the extracts was evaluated against *Alternaria alternata*.
Results: The content of phenolic compounds was of 49.31, 46.51 and 44.7 mg of gallic acid equivalents/g, for *P. gilvus*, *P. rimosus* and *P. badius*, respectively. The total flavonoid content followed the same pattern with values of 30.58, 28, and 26.48 mg of quercetin equivalents/g for *P. gilvus*, *P. rimosus* and *P. badius*, respectively. The variation on the content of phenolic components was reflected on the antioxidant activity of every organism. The antioxidant activity ranked as follows: *P. gilvus* > *P. rimosus* > *P. badius*. The antifungal effect of the different extracts against *A. alternata* showed a significant effect, all of them, inhibiting the growth of this pathogen.
Conclusions: *P. gilvus* showed the best potential to inactivate free radicals, being all the tested fungi effective to inhibit *A. alternata* growth.
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Potencial antioxidante y antifúngico de extractos de metanol de *Phellinus* spp. de Sonora, México

Palabras clave:
Phellinus spp.
Actividad antioxidante
Actividad antimicrobiana
Productos naturales

RESUMEN

Antecedentes: Investigaciones previas han revelado que los hongos de la especie *Phellinus* son una fuente potencial de sustancias bioactivas. Entre ellas, los fenoles son los principales antioxidantes y antimicrobianos que contribuyen a sus propiedades bioactivas.
Objetivos: El objetivo del presente estudio fue determinar el contenido total de fenoles y flavonoides, y su relación con la actividad antioxidante y antifúngica de *Phellinus gilvus*, *Phellinus rimosus* y *Phellinus badius*, respectivamente, extraídos con metanol.
Métodos: Los aislamientos de Phellinus fueron tratados con metanol para la extracción de la fracción acuosa. Mediante análisis espectroscópico del extracto acuoso generado se cuantificó el contenido total de fenoles y flavonoides, la actividad antioxidante frente a 2,2-difenil-1-picrilhidrazil (DPPH), la capacidad antioxidante expresada como equivalentes Trolox® (ET), y la capacidad de absorción del oxígeno reactivo. Las propiedades antifúngicas de los extractos se examinaron frente a *Alternaria alternata*.
Resultados: El contenido de fenoles fue de 49,31, 46,51 y 44,7 mg de equivalentes de ácido gálico/g, para *P. gilvus*, *P rimosus* y *P. badius* respectivamente. El contenido de flavonoides siguió el mismo patrón,

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con valores de 30,58, 28 y 26,48 mg de equivalentes de quercetina/g, para *P. gilvus*, *P. rimosus* y *P. badius*, respectivamente. La variación en el contenido de fenoles se reflejó en la actividad antioxidante de cada hongo. La actividad antioxidante se clasificó del modo siguiente: *P. gilvus* > *P. rimosus* > *P. badius*. El efecto antifúngico de los diferentes extractos frente a *A. alternata* fue significativo, inhibiendo todos ellos el crecimiento de dicho patógeno.

Conclusiones: P. gilvus manifestó la mayor capacidad antioxidante y los extractos de los tres hongos inhibieron el crecimiento de *A. alternata*.

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The natural products and herbal medicine industries have become increasingly popular over the past three decades. The recognition of the value of traditional medical systems, particularly those of Asian origin, and the identification of indigenous medicinal plants and fungi with healing power, are factors that have significant influence in the expansion of the natural products industry.[6,24] Furthermore, there is a constant search for new and effective natural drugs, which has been driven by the number of pathogenic organisms reported to have multi-resistance against many of the therapeutic products that are currently available on the market.[21,24]

Nowadays, fungus kingdom has become very attractive as a functional food and as a good source for the development of drugs and nutraceuticals.[6,10] The number of mushrooms species on earth is estimated to be larger than 100,000, suggesting that only 15–20% are known.[6] Even among the known species, the proportion of well-investigated mushrooms, as to their contents in secondary metabolites, and antioxidant and antimicrobial properties, is very low.[7] This fact, together with the knowledge about the great potential of fungi for production of bioactive metabolites (e.g. penicillin from *Penicillium notatum*, ergotamin from *Claviceps purpurea*, ciclosporin from *Tolypocladium inflatum*), in addition to their use in ethnomedicinal, reveals mushrooms as a vast source of bioactive compounds.[6,10]

Among the potential natural sources of bioactive compounds, those of the macroscopic fungi *Phellinus* spp. have been identified in previous studies on the phyla Basidiomycota where they are located.[7] Phenolic compounds are among the major contributors to the bioactive properties of *Phellinus*; however, variation could occur among species and regions of growth.[7] Fractions rich in phenolics have been extracted from *Phellinus baumii* using various solvents like methanol and hot water. These extracts exhibited good inhibition rates, of about 80–90%, by hydroxyl radical scavenging activity, hydrogen peroxide scavenging activity, effect of reducing power on metallic compound formation and antioxidant activity.[18] *Phellinus linteus* extract showed strong anti-angiogenic activity related to its antioxidant activity.[27] Ethyl acetate extract of *Phellinus rimosus* exhibited significant *in vitro* antioxidant activity. However, in all probability, genetically related species share the same "biochemical" composition, implying that they could show the same properties.[7] With this in mind, a greater number of medicinal fungi could emerge, and this would give people more options and consequently reduce the cost of medicinal mushrooms.[6,10,30] Previous review discussed the relevance of testing different medicinal functions of *Phellinus* sensu lato, among these properties, antioxidant and antibiotic properties are the less studied in *Phellinus*.[7] Therefore the study of the bioactive properties of *Phellinus* spp. from different locations is needed. In this context, 11 *Phellinus* species were found in the Ajos-Bavispe natural protected area in Sonora, Mexico, three of them were more commonly collected and studied in the present survey: *Phellinus badius* (Berk. ex Cooke) G., *Phellinus gilvus* (Schwein.) Pat., and *P. rimosus* (Berk.) Pilát. In this context, the objective of the present work was to determine the antioxidant and antifungal activity of methanolic extracts of *P. badius*, *P. gilvus* and *P. rimosus*, respectively, collected in Sonora, Mexico.

Materials and methods

Collection and identification of Phellinus *spp.*

P. badius, *P. gilvus* and *P. rimosus* were collected at the National Forest Reserve and Wildlife Refuge Ajos-Bavispe, located at Sonora, Mexico. Sampling was performed seasonally from fall 2004 to summer 2005. *Phellinus* specimens were collected and conserved following conventional mycological techniques. Taxonomic identification was reached according to Gilbertson and Ryvarden[8] and Larsen & Cobb-Poulle.[14] Three major species were identified as *P. badius*, *P. gilvus* and *P. rimosus*. Ten organisms with an approximate weight of 100 g per organism per species were selected and grouped. Mycelia from each organism of each species were mixed, freeze dried and vacuum stored at −35 °C until extraction.

Preparation of bioactive extracts from Phellinus *spp.*

Methanolic extracts of *Phellinus* spp. were obtained as follows: 1 g of freeze-dried sample was mixed in 15 mL of 80% (v/v) methanol, and homogenized with an Ultra-Turrax T 25 basic (IKA® WERKE, Germany), thereafter the homogenate was sonicated for 15 min and centrifuged at 10,000 rpm at 5 °C for 15 min. The sample was vacuum-filtered through Whatman No. 1 filter paper. This procedure was repeated twice to ensure the maximum extraction of bioactive compounds using 20 mL of methanol. The extracts were collected and made up to final volume of 50 mL. The final concentration of the extract was 0.02 g/mL, which was used for total phenolics, total flavonoids and antioxidant activity determinations.

Total phenolic and flavonoid contents

Concentrations of total phenolic compounds were measured by the methods described by Singleton and Rossi[26] with some modifications. Extracts (50 μL) were mixed with 3 mL of H_2O, and 250 μL of Folin–Ciocalteu's phenol reagent 1 N. After 8 min of equilibration time, 750 μL of Na_2CO_3 (20%) and 950 μL of H_2O were added to the extracts. After incubation for 30 min at room temperature, the absorbance was read at 765 nm with an UV–vis spectrophotometer (Cary, model 50 Bio, Varian, Italy). Concentration of total phenol compounds was calculated using a standard curve of gallic acid equivalents (GAE) and expressed as milligrams per g of dry weight.

Flavonoid content was determined based on the methods described by Zhishen et al.[32] modified as required. Flavonoids were measured in 1 mL of each methanolic extract and it was mixed with 5% $NaNO_2$, 10% $AlCl_3$ and 1 mol/L NaOH and measured spectrophotometrically at 415 nm using quercetin as standard. The results were expressed as mg of quercetin equivalents (QE) per g of dry weight.

Evaluation of antioxidant capacity

DPPH

This assay is based on the measurement of the scavenging ability on antioxidants toward the stable radical DPPH.[9] A 3.9 mL aliquot of a 0.0634 mM of DPPH solution, in methanol was added to 0.1 mL of each extract and shaken vigorously. Tubes were placed at 27 °C for 30 min. A control reaction was prepared as above without extract, and methanol was used for the baseline correction. Changes in the absorbance of samples were measured at 515 nm. Radical scavenging activity was expressed at the inhibition percentage and was calculated using the following equation:

$$\%\text{DPPH radical-scavenging activity} = \frac{\text{control Abs} - \text{sample Abs}}{\text{control Abs}} \times 100.$$

Trolox equivalent antioxidant capacity

This assay is based on the ability of the antioxidants to scavenge the blue-green $ABTS^{+}$ radical compared to the scavenging ability of the water-soluble vitamin E analog trolox.[22] The $ABTS^{+}$ radical cation was generated by the interaction of 5 mL of 7 mM ABTS solution and 88 μL of 0.139 mM $K_2S_2O_8$ solution. After the addition of 2970 μL of $ABTS^{+}$ solution to 30 μL of methanolic extracts (0.2 g/mL) or trolox standards (0 to 20 μM range), the absorbance was monitored exactly 1 and 6 min after the initial mixing. The percentage of absorbance inhibition at 734 nm was calculated and plotted as a function of that obtained for the extracts and the standard reference (trolox). The final TEAC value was calculated by using a regression equation between the trolox concentration and the inhibition percentage and expressed as trolox equivalents (μmol TE) per g of dry weight.

Oxygen radical absorbance capacity

This assay measures the antioxidant ability of different components based on the decline in fluorescence of fluorescein (FL) induced by AAPH, a peroxyl radical generator.[9] The reaction mixture contained 1.65 mL of 0.75 mM phosphate buffer (pH 7), 100 μL of 0.106 μM FL, 150 μL of 0.8 M AAPH, and 100 μL of *Phellinus* methanolic extracts. Phosphate buffer was used as a blank for ORAC. FL, phosphate buffer, and samples were pre-incubated at 37 °C for 15 min. The reaction was started by the addition of AAPH, and the fluorescence was measured and recorded every 5 min until the fluorescence of the last reading declined to less than 5% with respect to the initial reading. One blank and a maximum of 12 samples were analyzed at the same time. The excitation and emission wavelength was set at 484 and 515 nm, respectively. All fluorescence measurements were performed on a Perkin-Elmer LS 55 spectrofluorometer (Norwalk, CT, U.S.A.). Each extract measurement was repeated 4 times. The final ORAC values were calculated by using a regression equation between the trolox concentration and the net area under the FL decay curve. ORAC values were expressed as trolox equivalents (μmol TE) per g dry weight. The area under the curve (AUC) was calculated according to the following equation:

$$\text{AUC} = \left(0.5 + \frac{f_5}{f_0} + \frac{f_{10}}{f_0} + \frac{f_{15}}{f_0} + \frac{f_{20}}{f_0} + \frac{f_{25}}{f_0} + \frac{f_{30}}{f_0} + \cdots + \frac{f_t}{f_0}\right) \times 5$$

where f_0 is the initial fluorescence reading at 0 min and f_t is the fluorescence reading at time *i*.

Evaluation of antifungal activity

The antifungal potential of methanolic extracts of *Phellinus* spp. was tested against *Alternaria alternata* using the agar dilution method. For this 0.05, 0.025, 0.0125, 0.00625 g/mL of methanolic extracts of the tested *Phellinus* were added to Petri dishes containing potato dextrose agar, and then inoculated in the center with the fungi. The efficiency of the treatments was evaluated at 5 days of incubation at 25 °C. Controls were pure agar inoculated with the fungi and without exposure to any extract, no effect of the solvent on the fungal growth was observed. Mycelial area was recorded (cm^2) in triplicate by the digital analysis of the picture of every plate using the UTHSCSA ImageTool version 3.0 software.[31]

Statistical analysis

This experiment was based on a completely randomized design with equal replications. Analysis of variance for the treatments was done using Number Cruncher Statistical System version 6.0 software.[11] Mean comparisons of the studied parameters among treatments were done using the least significant difference test at a 5% level ($P < 0.05$).

Results and discussion

Identification of Phellinus *spp.*

Eleven macrofungus *Phellinus* species were found in the Ajos-Bavispe natural protected area, three of them were more commonly collected and studied in the present study. *P. badius* (Berk. ex Cooke) G. Cunn. was found in subtropical scrub and mezquital (plants from the genus *Prosopis*). It has a circumglobal distribution in the tropics and subtropical regions.[8] *P. gilvus* (Schwein.) Pat. was collected in pine-oak wood, oak wood and mezquital (plants from the genus *Prosopis*). It is a common fungus on living and dead hardwoods in many genera, and it is more common to be found on oaks. *P. rimosus* (Berk.) Pilát was observed in pine-oak forest, subtropical scrub and mezquital (plants from the genus *Prosopis*). This macrofungus has been commonly collected in the Sonoran mycobiota.[19]

Total phenolics, flavonoids and antioxidant capacity analysis

Table 1 shows the contents of total phenolic compounds expressed as gallic acid equivalents determined for *P. badius*, *P. gilvus* and *P. rimosus*, respectively. A significant difference ($P < 0.05$) was observed among *P. gilvus* with the highest total phenolic content (49.31 mg GAE/g), followed by *P. rimosus* (46.51 mg GAE/g) and *P. badius* (44.76 mg GAE/g). Similarly, the total flavonoid content of *Phellinus* methanolic extracts showed the same behavior already described for phenolic compounds (Table 1), where *P. gilvus* presented the highest flavonoids content (30.58 mg QE/g), followed by *P. rimosus* (28 mg QE/g) and *P. badius* (26.48 mg QE/g).

Comparison with other kind of macrofungus reported in previous publications showed that *Phellinus* spp. from Sonora contains higher levels of phenolic compounds. The phenolic content of *Leucopaxillus giganteus, Sarcodon imbricatus*, and *Agaricus arvensis* was 6.29, 3.76, and 2.83 mg/g, respectively.[3] However, *Phellinus baumii* samples presented significantly higher levels of phenolic compounds (338 μg/mL).[18] In contrast, other species of fungus such as *Endoptychum arizonycum* and *Disciseda verrucosa* presented lower values of phenolic compounds (9.81 and 16.1 mg/g, respectively; unpublished data). Total phenolic and flavonoid contents

Table 1
Total phenolics, flavonoids and antioxidant capacity of methanolic extracts from *Phellinus* spp.

Antioxidant parameter	*P. badius*	*P. gilvus*	*P. rimosus*
Total phenols (mg GAE/g dw)	44.7638[ca]	49.3062[a]	46.5045[b]
Total flavonoids (mg QE/g dw)	26.4877[c]	30.5831[a]	28.0439[b]
Antioxidant capacity			
DPPH (inhibition%)	77.1980[c]	90.8263[a]	84.0783[b]
TEAC (μmol TE/g)	122.7161[c]	266.3734[a]	212.7126[b]
ORAC (μmol TE/g)	1704.3507[c]	7470.6301[a]	1848.2940[b]

[*] Different superscripts among rows indicate significant differences among means ($P < 0.05$).

of different solvent extracts of *Phellinus igniarius* ranked from 6 to 23 mg/g of extract, and 0.05 to 4.26 mg/g, respectively.[17] Clearly the phenolic content of *P. badius*, *P. rimosus* and *P. gilvus* are higher than those published values in other macrofungus species.

Phenolic compounds are one of the most widely occurring groups of phytochemicals which are of considerable physiological and morphological importance in fungi and plants.[12,20] These compounds play an important role providing protection against pathogens and predators.[20] In humans, phenolic

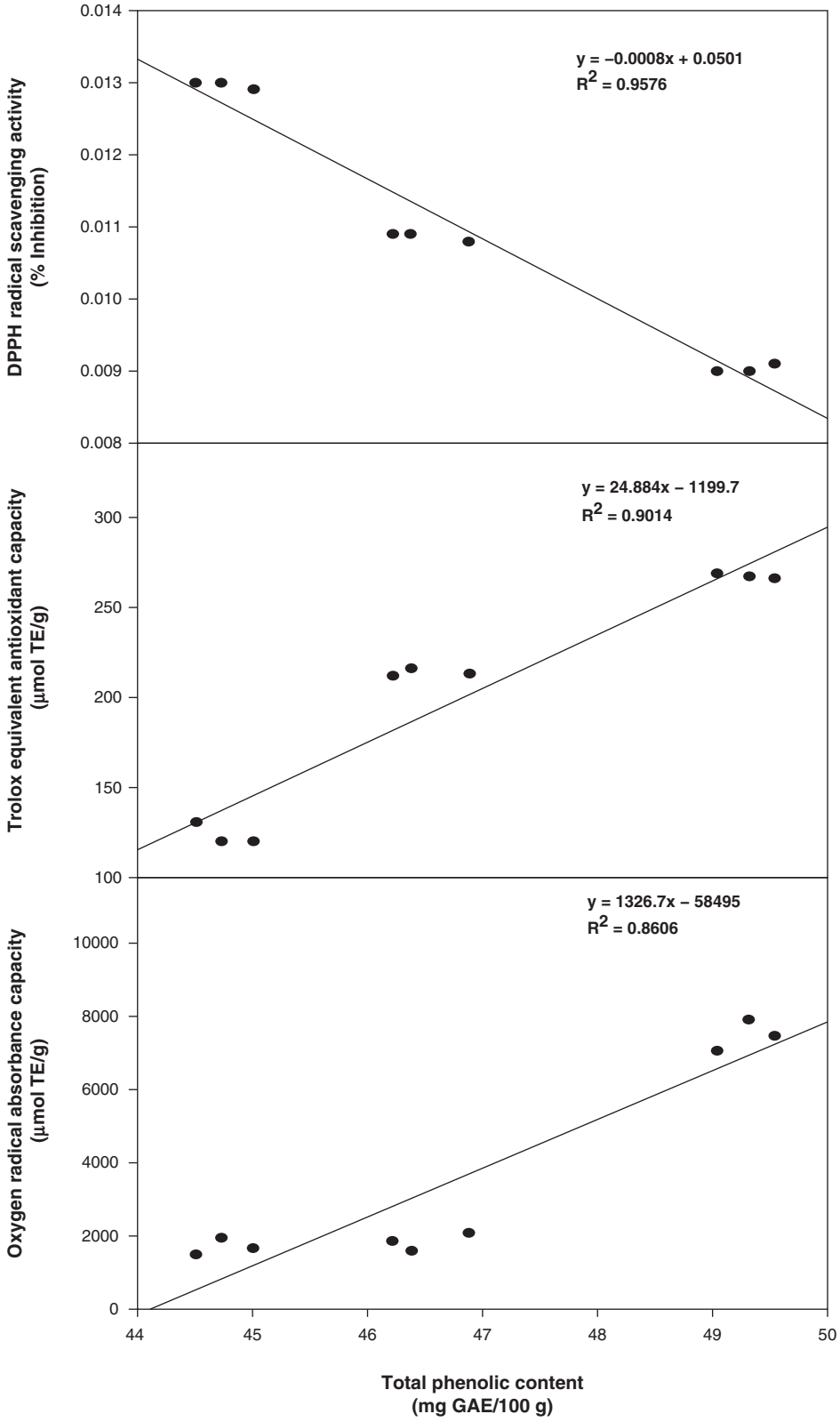

Fig. 1. Correlations of total phenolic compounds and antioxidant capacity values, measured by DPPH, TEAC, and ORAC methods.

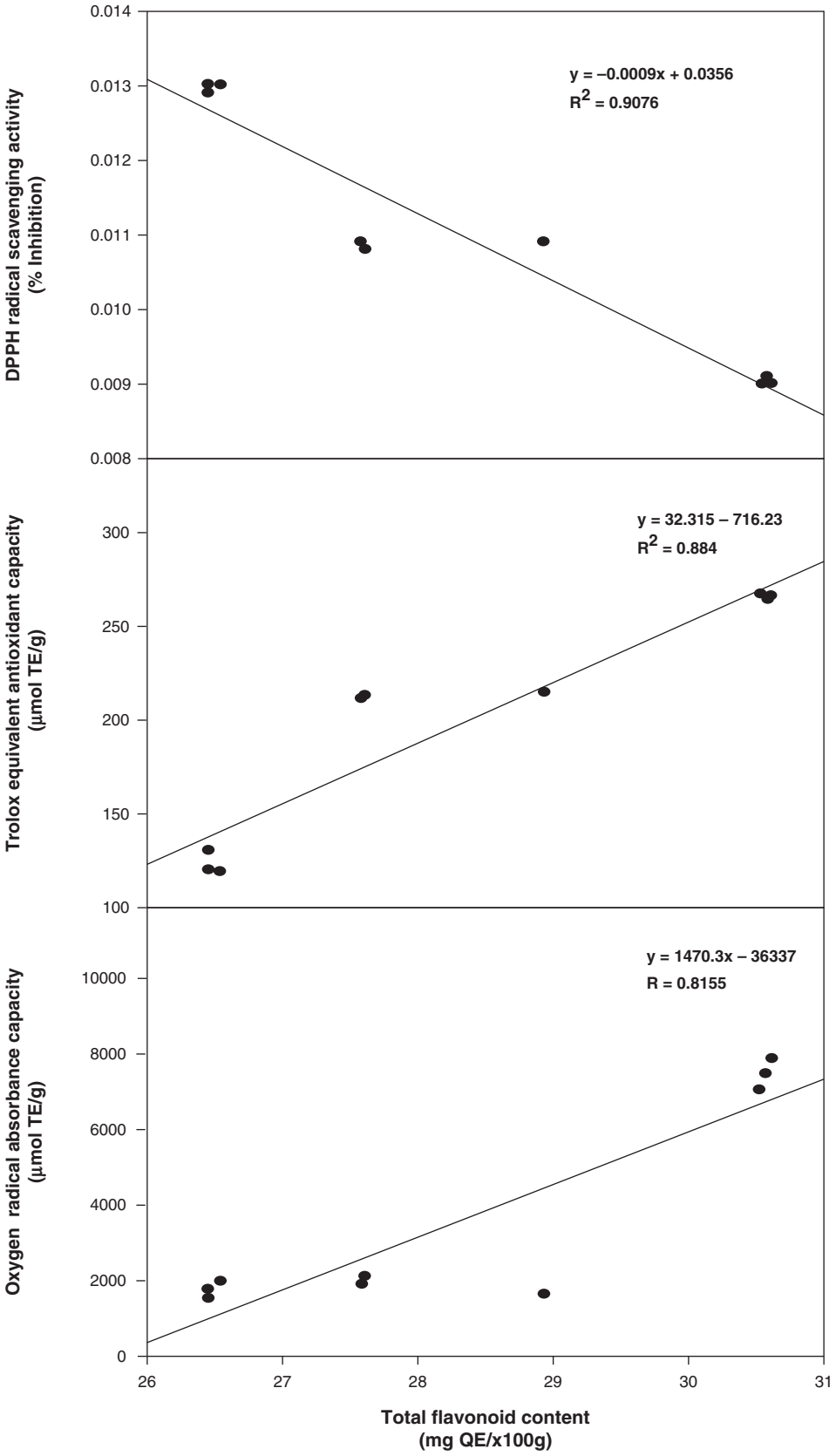

Fig. 2. Correlations of total flavonoid compounds and antioxidant capacity values, measured by DPPH, TEAC, and ORAC methods.

compounds can exhibit a wide range of physiological properties, such as anti-allergenic, anti-artherogenic, anti-inflammatory, antimicrobial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects.[12,13] The beneficial effects derived from phenolic compounds have been attributed to their antioxidant activity.[12]

Antioxidant capacity analysis

All the macrofungus methanol extracts showed high DPPH radical scavenging activity (Table 1) as expected considering the total phenolic content. The highest antioxidant scavenging effect (90.8%) was showed by the *P. gilvus* extract, followed by *P. rimosus* extract (84%) and *P. badius* extract (77.0%) at a concentration of 0.02 g/mL. The antiradical activity evaluated as the efficient concentration of each extract to inactivate the 50% of the DPPH radical (EC_{50}) was 0.009 g/mL for *P. gilvus*, 0.01 g/mL for *P. rimosus* and 0.013 g/mL for *P. badius*. The EC_{50} values also indicated that the antioxidant activity of the *P. gilvus* extract was higher than that of *P. rimosus*. The antiradical scavenging activity might be attributed to the replacement of hydroxyl groups in the aromatic ring systems of the phenolic compounds as a result of their hydrogen donating ability.[4]

Analysis of ABTS radical scavenging activity of the methanolic extracts of *Phellinus* spp. expressed as trolox equivalent antioxidant capacity (TEAC) (Table 1) showed the same pattern than that of DPPH assay. *P. gilvus* extract presented significantly ($P < 0.05$) higher antioxidant activity (266 μmol TE/g), followed by *P. rimosus* (212 μmol TE/g), and *P. badius* (122 μmol TE/g). The oxygen radical absorbance capacity (ORAC) values confirmed these results (Table 1), being *P. gilvus* (7470 μmol TE/g) the more potent antioxidant extract, followed by *P. rimosus* (1848 μmol TE/g) and *P. badius* (1704.35 μmol TE/g). All the tested extracts were significantly different ($P < 0.05$) among them.

The methanolic extract of *Agrocybe cylindracea* strain B exhibited a DPPH scavenging ability of 93.8% at 0.005 g/mL.[28] Tsai et al.[29] found that scavenging abilities of three ethanolic extracts were 94.9%, 89.2%, and 88.8% at 0.005 g/mL for *Agaricus blazei*, *A. cylindracea*, and *Boletus edulis*, respectively. Furthermore, ethanolic extracts of *Pleurotus citrinopileatus*[15] and *Hypsizgus marmoreus*[16] showed scavenging abilities of 94.9% and 59.7% at 0.005 g/mL, respectively. Our results suggest that the scavenging ability of *P. gilvus* was higher than that of *P. rimosus* and *P. badius*; however, an effect of the solvent extraction method could explain the differences when compared with other studies.

The antioxidant activity of phenolic compounds is due to their ability to scavenge free radicals, donate hydrogen atoms or electron, or chelate metal cations. The structure of phenolic compounds is a key determinant of their radical scavenging and metal chelating activity. The position and number of hydroxyl groups in the phenolics and flavonoids determine the capacity of the molecules to donate an electron and stabilize free radicals. Therefore, antioxidants have the ability to scavenge free radicals and prevent diseases caused by these reactive species. Antioxidants from *Phellinus* sensu lato are composed of hispidin and its derivatives.[13] Also, the catechol moiety is responsible for inhibiting radical generation by chelating metal ions.[13] Therefore, these phenolic compounds could have synergistic effects and be responsible of the high antioxidant capacity of the studied *Phellinus* extracts.

In this study, total antioxidant component contents (phenolic and flavonoids) were positively correlated with the values of antioxidant activity. The correlation coefficients (R^2) of total phenolic compounds versus DPPH, TEAC, and ORAC were 0.9576, 0.9014, and 0.8606, respectively (Fig. 1). However, the correlation coefficients of total flavonoids versus DPPH, TEAC, and ORAC were slightly lower at 0.9214, 0.8840 and 0.8155, respectively (Fig. 2). In addition, an excellent correlation between contents of total phenolics and antioxidant activity was also obtained and pointed out that the antioxidant activity of phenolics was mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators and single oxygen quenchers.[23] Consequently, total phenols in the three methanolic extracts from *Phellinus* spp. were possibly the major components that contribute in higher extent to their antioxidant activities.

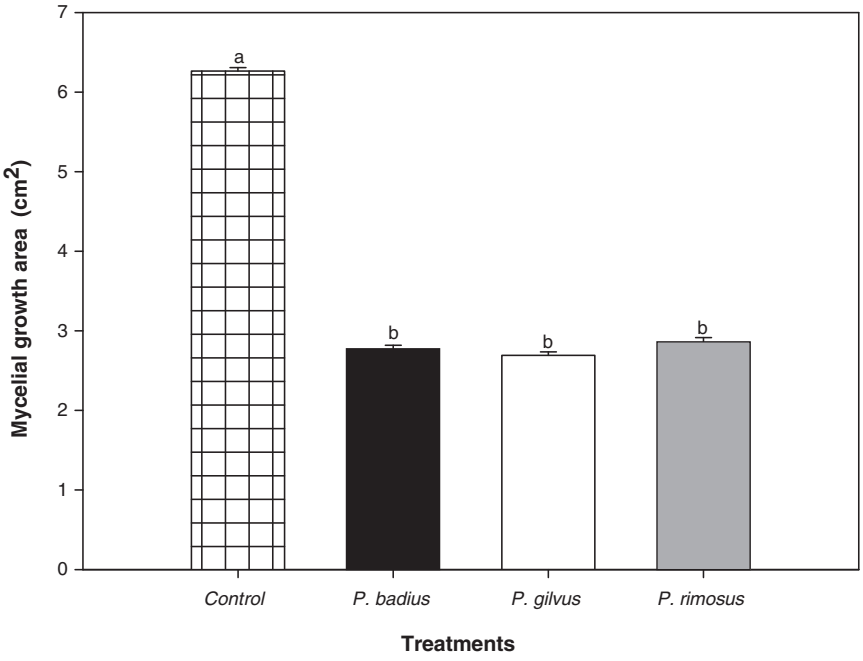

Fig. 3. Antifungal activity of methanolic extracts from *Phellinus* spp. against *Alternaria alternata*. Different letters among bars indicate significant differences among means ($P < 0.05$).

Antifungal activity

The antifungal potential of methanolic extracts of *P. badius*, *P. gilvus* and *P. rimosus* was determined (Fig. 3). Their antifungal potential was tested against *A. alternata*, a mold highly related to plant food spoilage. The three tested macrofungus extracts were effective to inhibit the growth of *A. alternata*, however, no significant differences among them were observed. The antifungal activity of all the extracts did not show a concentration-dependent behavior and no significant ($P > 0.05$) effect of concentration was observed.

The antimicrobial activities of a variety of naturally occurring phenolic compounds from fungi have been studied.[2] These compounds can play an important role in tissue's protection against pathogenic agents, penetrating the cell membrane of microorganisms, causing lysis.[12,13] Phenolic compounds from spices such as gingeron, zingerone, and capsaicin have been found to inhibit the germination of bacterial spores.[5] Polyphenols contained in green tea (*Camellia sinensis*) are effective against *Vibrio cholerae* O1, *Streptococcus mutans*, and *Shigella*.[25] The antimicrobial activity of other *Phellinus* extracts has not been widely evaluated, only two reports from antibiotic activity of *Phellinus fastuosus* and *Phellinus ribis* were found in the literature.[7] In this context, the mixture of phytochemical constituents in methanolic extracts of *Phellinus* spp. can be responsible for their antioxidant and antifungal activity.[1]

Conclusions

Methanolic extracts from mycelia of macrofungus *P. gilvus*, *P. rimosus* and *P. badius* isolated from Sonora, Mexico, revealed considerable antioxidant and antifungal properties as evidenced by their high total phenolics and flavonoid contents as well as their antioxidant capacity values. However, *P. gilvus* exhibited the highest antioxidant potential. The presence of *Phellinus* methanolic extracts was effective in the growth inhibition of *A. alternata*.

Conflict of interest

The authors have no conflict of interest to declare.

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