Preliminary evaluation of phenolic compounds, antioxidant activity and bioactive compounds in some species of basidiomycetes fungi from Paraguay

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Evaluación preliminar de compuestos fenólicos, actividad antioxidante y compuestos bioactivos de algunas especies de hongos basidiomicetes de Paraguay. Se realizó la identificación cualitativa de metabolitos secundarios y se cuantificó el contenido de compuestos fenólicos y el potencial antioxidante de extractos etanólicos de ocho géneros de macrohongos *Amylosporus, Gloeophyllum, Hydnopolyporus, Inonotus, Laccaria, Lentinus, Pisolithus* y *Trametes* por primera vez para el Paraguay. Los análisis químicos cualitativos revelaron la presencia de varios metabolitos secundarios tales como compuestos fenólicos, esteroles y terpenos. La especie *Inonotus splitgerberi* presentó valores de 64,81 ± 2,70 mg GAE.g^{-1} de compuestos fenólicos totales comparables con la especie medicinal *Inonotus obliquus.* También se proporcionan datos noveles para la especie *Amylosporus guaraniticus*.
Palabras claves: hongos, metabolitos secundarios, micoquímica.

Preliminary evaluation of phenolic compounds, antioxidant activity and bioactive compounds in some species of basidiomycetes from Paraguay. The qualitative identification of secondary metabolites was carried out along with the quantification of the content of total phenolic compounds and the antioxidant potential of ethanolic extracts of eight genera of fungi *Amylosporus, Gloeophyllum, Hydnopolyporus*, *Inonotus*, *Laccaria, Lentinus*, *Pisolithus* and *Trametes* for the first time in Paraguay. Qualitative chemical analyses revealed the presence of several secondary metabolites, such as phenolic compounds, sterols and terpenes. The species *Inonotus splitgerberi* showed values of 64.81 ± 2.70 mg GAE. g^{-1} of total phenolic compounds comparable with the medicinal species *Inonotus obliquus*. Novel data for the species *Amylosporus guaraniticus* are also provided.
Key words: fungi, secondary metabolites, mycochemistry.

INTRODUCTION

Secondary metabolites can be considered as natural products originating as response to the stress caused by the ecological niche, the adaptation of an organism to the environment, and are produced by a variety of mushrooms (Arango, 2008; Zaidman *et al.*, 2005; De Silva *et al.*, 2013). These arise from intermediates of primary metabolism, but they can be classified according to their biosynthetic pathway into five main metabolic sources: (1) polysaccharides and pep-

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tidopolysaccharides, (2) the mevalonic acid pathway from acetyl coenzyme A which functions in primary metabolism for the synthesis of sterols, (3) amino acid-derived pathways in the secondary metabolism, (4) the shikimic acid pathway for the biosynthesis of aromatic amino acids, (5) the acetate–malonate pathway from acetyl coenzyme A (Zaidman *et al.*, 2005). Fungi (as well as plants) accumulate a wide variety of secondary metabolites, including phenolic compounds, polyketides, terpenes and steroids (De Silva *et al.*, 2013; Stadler and Hoffmeister, 2015; Sandargo, 2019).

Phenolic compounds contain one or more aromatic rings and one or more hydroxyl groups. They include phenolic acids, flavonoids, hydroxybenzoic acids, lignins, tannins and oxidized polyphenols (Côté, Caillet & Doyon, 2010; D'Archivio, Filesi & Vari, 2010; Sánchez, 2017). **Terpenoids** are fairly common among mushroom metabolites, with sesquiterpenoids, diterpenoids and triterpenoids being the most commonly isolated metabolites from Basidiomycota. Among these, triterpenes are a predominant class of secondary metabolites, especially for the wood-inhabiting polypore species, where they are often present in large quantities in the basidiomata (Sandargo *et al.*, 2019). **Alkaloids** are a large family of more than 15,000 secondary metabolites; they are nitrogenous substances, basic, of natural origin and restricted distribution. Alkaloids have a complex structure and share three characteristics in common: they are soluble in water, contain at least one atom of nitrogen in the molecule, and exhibit biological activity (Ávalos & Pérez, 2009).

Oxidative stress can be defined as the state where there is an imbalance between prooxidants and antioxidants, this disturbance results in the increase of oxidative stress and molecular damage of proteins, lipids, carbohydrates and DNA, leading to diseases such as cancer, rheumatoid arthritis, and associated cellular degeneration (Dubost *et al.*, 2007; Lung & Huang, 2012). Antioxidant compounds have the important ability to eliminate free radicals and inhibit the oxidative mechanisms that lead to degenerative diseases; these are distributed in various products from living organisms such as grains, fruits, vegetables, teas, spices, herbs, and fungi (Dubost *et al.*, 2007; Klaus *et al.*, 2011). Numerous wild mushroom species were reported to have antioxidant activity and these properties have been studied extensively (Song and Yen 2002; De Silva *et al.*, 2013). For example, basidiomata of *Fomitopsis pinicola* (Sw.) P. Karst. and *Pleurotus ostreatus* (Jacq.) P. Kumm. show powerful antioxidant capacity related to low molecular weight compounds, in particular those from the phenolic fractions (Ferreira *et al.*, 2009; Heleno *et al.*, 2011; Mohsin *et al.*, 2011; Reis *et al.*, 2011; Akata *et al.*, 2012).

Mushrooms seem to be particularly talented in producing unique terpenoids, for example **pleuromutilin**, a tricyclic diterpenoid, is a naturally occurring antibiotic from the culture of the mushroom *Clitopilus passeckerianus* (Pilát) Singer (Kavanagh, 1947; Stadler and Hoffmeister, 2015; Sandargo *et al.*, 2019). Other examples are **strobilurins**, one of the most successful class of agrochemical fungicides; and **illudins** from *Lampteromyces* Singer and *Omphalotus* Fayod species (Omphalotaceae) which are sesquiterpenes featuring an unusual cyclopropane as source of potential anticancer drugs and agrochemical pesticides (Tanasova and Sturla, 2012).

In recent years, a number of alkaloids have been discovered in Basidiomycota; fungi produce natural alkaloids, the most studied groups are the indoles and isoxazoles, two simple indole alkaloids: psilocin (3- [2 (dimethylamino) ethyl] -4-indolol) and psilocybin ([3- (2-dimethylaminoethyl) - 1H-indol4-yl] dihydrogen phosphate) are present in many mushroom species (Wieczorek *et al.*, 2015; Sandargo, 2019). Other isolated alkaloids are the **Erinacerines** commonly isolated from both the cultures and fruiting bodies of *Hericium erinaceus* (Bull.) Pers. (Sandargo, 2019), and the **Rosellins A** and **B,** red diketopiperazine alkaloids, isolated from the fruiting bodies of the mushroom *Mycena rosella* (Fr.) P. Kumm. (Lohmann *et al.*, 2018), the **Phlebopines A–C** pyrrole alkaloids isolated from fruit bodies of *Phlebopus portentosus* (Berk. & Broome) (Sun *et al.*, 2018; Sandargo, 2019).

Research applied to the chemical profile and biological potential of the native species of macroscopic fungi is underdeveloped in Paraguay. Many novel biologically active compounds have been reported as a result of research on medicinal mushrooms (De Silva *et al.*, 2013, Staniszewska *et al.*, 2017). The search for secondary metabolites of natural products in Paraguay is limited to the Plantae Kingdom, thus restricting the discovery and application of new sources of secondary metabolites with possible biological activities of the Fungi Kingdom, which has been cataloged as the second most diverse group of organisms (2,2 to 3,8 million species) around the world (Hawksworth & Lücking, 2017), exceeding that of terrestrial plants by one order of magnitude (Blackwell, 2011; Dai, 2010), and with immense diversity of species in the different ecoregions of our country. Two records of the mycochemical and biological profile of a species of the genus *Laetiporus* are reported in Paraguay, ergosterol (ergosta-5,7,22-trien-3β-ol) was isolated, crystallized and elucidated for the first time (Vila *et al.*, 2015; Campi, 2017). Herein the chemical profile of nine species of native mushrooms is studied, thus providing novel information for the area of chemistry of natural products with mycological profile in Paraguay.

MATERIALS AND METHODS

Collection and ethanol extraction: Basidiomata were collected in the Departments of Alto Paraguay, Alto Paraná, Central and Cordillera. A sample of each species was deposited in the FACEN Herbarium. The samples were determined taxonomically in the *Laboratorio de Recursos Vegetales- Área de Micología* following the alignments of Robledo & Urcelay (2009) and Wright and Albertó (2002) (Table 1), 20 g of each dried mushroom powder underwent thorough maceration with ethanol 96% for a period of 48 hours, and then every 24 hours until third extractions were completed under periodic agitation. The solution was filtered by gravity and then concentrated with subsequent evaporation of the solvent in a rotary evaporator (Rotav, China). The crude extracts were coded (Table 1) and stored in glass containers at 4 ± 0.5 °C until use (Tiwari *et al.*, 2011 with modifications).

Table 1. Collection of information and codification of each studied species.

Species/Code	Origin	Collection number	Determination
Amylosporus guaraniticus Campi & Robledo /**(AG001)**	Central Department, San Lorenzo city, university campus	M. Campi 014	Campi, Robledo & Maubet
Gloeophyllum striatum (Fr.) Murrill /**(GS002)**	Alto Paraná Department, Bahía Negra city, Estación Biológica 3 Gigantes	M. Campi 149	Campi
Hydnopolyporus fimbriatus (Cooke) D.A. Reid / **(HF003)**	Central Department, San Lorenzo city, university campus	M. Campi 019	Campi
Inonotus splitgerberi (Mont.) Ryvarden **(IS004)**	Cordillera Department, Atyra city, Los Agüero country house	M. Campi 049	Campi & Robledo
Inonotus rickii (Pat.) D.A. Reid /**(IR005)**	Central Department, San Lorenzo city, university campus	M. Campi 270	Campi
Lentinus lindquistii (Singer) B.E. Lechner & Albertó) /**(LL006)**	Central Department, San Lorenzo city, university campus	M. Campi 274	Campi & Maubet
Laccaria fraterna (Sacc.) Pegler /**(LF007)**	Cordillera Department, Piribebuy city	C. Mancuello 001	Campi, Maubet & Niveiro
Pisolithus arhizus (Scop.) Rauschert /**(PA008)**	Central Department, San Lorenzo city, university campus	M. Campi 035	Campi & Maubet
Trametes cubensis (Mont.) Sacc. /**(TC009)**	Alto Paraná Department, Hernandarias city, Refugio Biológico Itabó	M. Campi 007	Campi & Robledo

Qualitative assays of secondary metabolites and reducing sugars: To identify secondary metabolites and reduce sugars of fungal extracts, solutions were prepared and subjected to multiple tests: Dragendorff, Wagner and Mayer for alkaloids; Liebermann-Burchard and Salkowski for triterpenes and steroids and $FeCl_3$ for tannins; Rosenhein for coumarins; and Fehling for reducing sugar (Adebayo, Olok & Olukemi, 2012; Amador *et al.*, 2006; Koolen *et al.*, 2013; Llata, 1994). Each test was performed in triplicate and compared with a positive and negative control sample. The following was used as a criterion to evaluate the results, in relation to the positive control: (+) = faint reaction/coloration, (++) = medium reaction/coloration, (+++) = intense reaction/coloration, (-) = absence.

Total phenolic compounds: The concentration of total phenolics was measured by the method described by Turkoglu *et al.* (2007) with modifications. From the crude extracts, methanolic solutions with a concentration of 1 $mg.mL^{-1}$ were prepared, 2 mL of ddH_2O and 200 µL of the Folin Ciocalteu 2N (analytical grade, Merck) reagent were added to each 1 mL of solution; the mixtures were homogenized and left to stand for 5 minutes, 1.5 mL of 20% sodium carbonate were added, and then the mixtures weres brought to a volume of 10 mL with distilled water. As a prepared blank, 1 mL of methanol was used with the same treatment. The samples were homogenized, and after 1 hour of resting in the dark the absorbance at 760 nm was read in the spectrophotometer. To construct the calibration curve, a gallic acid solution (Sigma-Aldrich®) with concentrations of 0-50 $\mu g.mL^{-1}$ was used, and 200 µL of each concentration was treated in the same way as the sample solutions. The equivalent concentrations of gallic acid of each sample extract were determined from the curve. The results were expressed as the mean of the triplicates of the absorbances in milligrams of gallic acid equivalent per grams of crude extract ($mg.g^{-1}$) ± standard deviation (SD).

Concentration and antioxidant activity: The concentration and oxidant activity were determined with the DPPH radical absorbance method according to Barros *et al.* (2007) with modifications. Methanolic solutions of 1 $mg.mL^{-1}$ were prepared from the crude extracts, from which 100 µL were taken, 3.9 mL of methanolic solution of the DPPH• (Merck) radical (0.02 mg. mL-1) were added. As a negative control, 100 µL of methanol and 3.9 mL of methanol solution of the DPPH• radical was used. The samples were homogenized and incubated in the dark for 1 hour. The reduction of the reagent was evidenced by the change in coloration from dark violet to light yellow, which was measured with a UV-VIS spectrophotometer (Thermo SCIENTIFIC Genesys 10S Model) at $\lambda = 517$ nm. For the calibration curve, a methanolic solution of 1 $mg.mL^{-1}$ of ascorbic acid was used, from which dilutions of 10 to 100 $\mu g.mL^{-1}$ were prepared; 100 µL were taken from each dilution, which received the same treatment as the samples. From the results of the curve, the equivalent concentration of ascorbic acid was determined. The results were expressed as the mean of the analysis performed in triplicate in milligrams of Ascorbic Acid Equivalent per g of crude extract ($mg.g^{-1}$) ± standard deviation (SD). To calculate the percentage of activity, the following formula was used: % activity = [(absorbance of DPPH - absorbance of the solution) / absorbance of DPPH] × 100.

RESULTS AND DISCUSSION

Mushrooms have become an attractive food and medicinal source. Basidiomycotae produce a wide range of secondary metabolites ranging from structural components with antitumor activity and immunological active to antimicrobial, antifungal, antiviral, cytostatic, enzymes, growth regulators and flavorings (Rathee *et al.*, 2012; De Silva *et al.*, 2013; Stadler and Hoffmeister, 2015; Sandargo, 2019). The mycochemical properties of the basidiomycotae of Paraguay was evaluated.

Qualitative essays

As shown in Table 2, the crude extracts of the basidiomata studied contain second-

dary metabolites such as alkaloids, steroids, coumarins. The presence of reducing sugars in ethanolic extracts was also examined.

Alkaloids: between the tested extracts **LL006** (*Lentinus lindquistii*) showed a red precipitate, which indicates the presence of alkaloids, as confirmed by Hashimoto *et al.* (1972), who described **Eritadenine** for *Lentinula edodes* (Berk.) Singer, an alkaloid with hypoglycemic properties. Another extract that showed a strong positive reaction was LF007 (*Laccaria fraterna*); Matsuda *et al.* (1996) described the alkaloid **Laccarin** for *Laccaria vinaceoavellanea* Hongo, and Spiteller and Schrey (2019) described the **E-Z-proxamidine**, two isomeric alkaloids with a highly unusual core structure for *Laccaria proxima* (Boud.) Pat.; **TC009** (*Trametes cubensis*) showed positive results for alkaloids, previous reports by Bian *et al.* (2017) for the species *Trametes trogii* Berk., reported the presence of **Trametramide** (a pyrimidic alkaloid), qualitative studies revealed the presence of alkaloids in *T. versicolor* (Leliebre-Lara *et al.*, 2015).

The presence of terpenoid compounds - and sterols in the ethanolic extract of all the species studied was checked qualitatively; this coincides with the work of Yokoyama, Natori and Aoshima (1975), who indicated that triterpene carboxylic acids with a lanostane skeleton occur rather widely in Polyporaceae and related higher fungi. The crude extracts that showed the greatest change in coloration were: **IR005** (*Inonotus rickii*), previous studies reported sesquiterpenes as **inonotic acid A** and **B** in the species *I. rickii* and in the close species *I. obliquus*; the presence of **inotodiol, lanosterol, β-sitosterol, romadendrane sesquiterpenoids** named **inonotins** (isolated from the culture) and nostane-type triterpenoids was also established for the genus *Inonotus* (Nomura *et al.*, 2008; Kim *et al.*, 2011; Chen *et al.*, 2014; Isaka *et al.*, 2017). Another positive species was **PA008**; Lobo *et al.* (1983, 1988), Baumert *et al.* (1997), Zamuner *et al.* (2005) and Márquez-Fernandez (2013) described several types of sterols for *Pisolithus tinctorius* (*Pisolithus arhizus*) among them **ergosterol**, **lanosterol** and derivatives, **agnosterol, piscolactone** and the **pisosteral.** Regarding **TC009,** the positive results coincide with those of Leliebre-Lara *et al.* (2015) who obtained positive results for the species *T. versicolor*; with respect to **AG001** the extract indicated the presence of terpenoid compounds and sterols, previous studies.

Table 2. Mycochemical profile: (+) faint coloration (++) medium coloration (+++) intense coloration (-) = absent.

Test	AG001	GS002	HF003	IS004	IR005	LL006	LF007	PA008	TC009
ALKALOIDS									
Dragendorff	(+++)(+++)(+++)	(+)(+)(+)	(++)(++)(++)	(++)(++)(++)	(++)(++)(++)	(+++)(+++)(+++)	(+++)(+++)(+++)	(-)(-)(-)	(+++)(+++)(+++)
Wagner	(+++)(+++)(+++)	(+)(+)(+)	(+)(+)(+)	(+)(+)(+)	(+)(+)(+)	(+++)(+++)(+++)	(+++)(+++)(+++)	(-)(-)(-)	(++)(++)(++)
Mayer	(+)(+)(+)	(-)(-)(-)	(-)(-)(-)	(-)(-)(-)	(+)(+)(+)	(+++)(+++)(+++)	(+++)(+++)(+++)	(-)(-)(-)	(++)(++)(++)
TRITERPENES AND STEROIDS									
Liebermann-Burchard	(+++)(+++)(+++)	(+)(+)(+)	(+)(+)(+)	(+++)(+++)(+++)	(+++)(+++)(+++)	(++)(++)(++)	(+)(+)(+)	(+++)(+++)(+++)	(+++)(+++)(+++)
Salkowski	(+)(+)(+)	(+)(+)(+)	(++)(++)(++)	(++)(++)(++)	(++)(++)(++)	(++)(++)(++)	(++)(++)(++)	(+)(+)(+)	(++)(++)(++)
COUMARINS									
Rosenhein	(++)(++)(++)	(+)(+)(+)	(++)(++)(++)	(+++)(+++)(+++)	(++)(++)(++)	(+)(+)(+)	(++)(++)(++)	(+++)(+++)(+++)	(+)(+)(+)
REDUCING SUGARS									
Felhing test	(-)(-)(-)	(-)(-)(-)	(+)(+)(+)	(+++)(+++)(+++)	(+++)(+++)(+++)	(-)(-)(-)	(+)(+)(+)	(+++)(+++)(+++)	(++)(++)(++)

of the genus revealed the presence of terpenoid compounds (Juma *et al.*, 2016); with regard to **GS002**, positive results agree with the work by Cateni *et al.* (2015), who described two triterpene acids for *Gloeophyllum odoratum* (Wulfen) Imazeki, and 10 new ergosteroids, (gloeophyllins A-J) for *Gloeophyllum abietinum* (Bull.) P. Karst (Han *et al.*, 2015).

As for coumarins (Rosenhein and Balje test) ,all the extracts were positive; for the genus *Gloeophyllum*, Oosponol was reported as an isocoumarin for the culture (Rasser *et al.*, 2000). The reducing sugars were analyzed by the Fehling test, the extracts that gave positive with greater intensity were **IR005** and **PA008**, previous studies on *I. obliquus* (Lin *et al.*, 2012) reported the presence of reducing sugars in its ethanolic extracts.

Quantitative studies

The crude extracts show a content of total phenolic compounds with variable ranges (Table 3). The content of total phenolic compounds (TPC) of *Amylosporus guaraniticus* (**AG001**) was 9,30 ± 0,82 mg.g^{-1} of gallic acid equivalents (GAE); these results are higher than those found by Juma *et al.* (2016) for the *Amylosporus* genus, where they reported values corresponding to 2.47 to 3.90 mg. g^{-1} GAE.g^{-1} (TPC). These results constitute novel information because it is the first chemical study ever done since the description of this native species of Paraguay (Robledo & Campi, 2017).

The TPC values of *Gloeophyllum striatum* (**GS002**) were 17.01 ± 1.56 mg GAE.g^{-1}, similar to those found by Sulkowska -Ziaja *et al.* (2012) of 19.88 ± 2.00 mg GAE.g^{-1} (TPC) for the species *Gloeophyllum sepiarium* (Wulfen) P. Karst.

Regarding *Hydnopolyporus fimbriatus* (**HF003**), values of 6.66 ± 0.79 mg GAE.g^{-1} (TPC) were determined, for the TPCs the reported range is lower than those found by Graça *et al.* (2016) where they obtained a concentration of 25.85 mg GAE.g^{-1} (TPC), it is worth mentioning that they analyzed extracts from the mycelial tissue and not from the basidiomata.

Inonotus rickii (**IR005**) presented values of 3.70 ± 0.48 mg GAE.g^{-1} (TPC), well below the results found by Zhang *et al.*, (2015) which cited values of 55.94 mg GAE. g^{-1} (TPC) for *Inonotus obliquus* (Fr.) Pilát, considered a medicinal mushroom; *Inonotus splitgerberi* (**IS004**), on the other hand, showed values of 64, 81 ± 2.70 mg GAE.g^{-1} (TPC), comparable to the medicinal species.

Regarding *Lentinus lindquistii* (**LL006**), values of 15.11 ± 1.12 mg GAE.g^{-1}(TPC) were obtained, reports of species of the same genus cited by Reis *et al.*, (2011) for *Lentinus tigrinus* (Bull.) Fr. reported 17.3 mg GAE.g^{-1} (TPC) similar to those obtained with **LL006**. These results provide important information to be classified as an edible fungus.

Laccaria fraterna (**LF007**) yielded results of 6.76 ± 0.30 mg GAE.g^{-1} (TPC); in terms of total phenolic compounds, the results obtained are greater than those reported by Heleno *et al.* (2010), who reported 2.85 mg.g^{-1} GAE.g^{-1} for *Lacaria amethystina* Cooke and 1.59 mg GAE.g^{-1} for *Laccaria laccata* (Scop.) Cooke., meanwhile Liu *et al.* (2012) reported results of 9.8 mg GAE.g^{-1} (TPC) for *Lacaria amethystina*.

Table 3. Values of total phenolic compounds, DPPH and percentage of antioxidant activity

Species	Phenolic compounds (mg GAE.g^{-1})	Antioxidant concentration (mg.g^{-1} AAE)	% Activity
AG001	9.30 ± 0.82	7.64 ± 0.87	3.88
GS002	17.01 ± 1.56	10.00 ± 1.00	7.02
HF003	6.66 ± 0.79	4.95 ± 1.69	4.80
IS004	64.81 ± 2.70	94.12 ± 0.87	12.4
IR005	3.70 ± 0.48	7.27 ± 0.49	7.20
LL006	15.11 ± 1.12	9.51 ± 0.35	6.65
LF007	6.76 ± 0.30	5.37 ± 0.37	6.09
PA008	64.45 ± 3.88	36.66 ± 0.51	9.13
TC009	8.98 ± 0.89	5.94 ± 1.14	4.80

*GAE: Gallic Acid Equivalents QE: quercetin equivalents AAE: Ascorbic acid equivalent.

With respect to *Pisolithus arhizus* (**PA008**), 64.45 ± 3.88 mg GAE.g^{-1} (TPC) were obtained; Reis *et al.*(2011) reported concentrations of 298 mg GAE.g^{-1} (TPC), much higher than those found in **PA008**, however to Khadhri, Aouadhi & Aschi-Smiti (2017) found values of 16.0 ± 0.3 mg GAE.g^{-1} for *Pisolithus albus*.

Trametes cubensis (**TC009**) presented total phenolic compound values of 8.98 ± 0.89mg.g^{-1} GAE.g^{-1} (TPC), these represent results similar to those found by Orhan & Üstün (2011) for *Trametes versicolor* (L.) Lloyd where they reported values of 9.58 mg.g^{-1} GAE.g^{-1}; however, Matijašević *et al.* (2016), found higher values for the same species: 25.8 mg.g^{-1} GAE.g^{-1} (TPC).

It is worth mentioning that the species with the highest GAE content is *Inonotus splitgerberi* (**IS004**), with values of 64.81 ± 2.70 mg GAE.g^{-1}, this could be due to the fact that the Hymenochataceae group which includes *Phellinus* and *Inonotus* species were shown to produce phenolic compounds such as **phelligridins** and **inonoblins** (Lee *et al.*, 2007; De Silva *et al.*, 2014).

Mushrooms do not contain flavonoids

Although several authors confirm the presence of "total flavonoids" in fungal extracts, (until October 2015 a total of 136

scientific reports were published descrybing the presence of flavonoids for fungi (Gil-Ramírez *et al.*, 2016)) others sustain the absence of this metabolite within the Fungi Kingdom (Ruíz-Rodríguez *et al.*, 2009; Gil-Ramírez *et al.*, 2016).

Most of the authors used an unspecific colorimetric method developed to determine them in plants or plant products; the colorimetric method used is aluminum chloride ($AlCl_3$) as a selective reaction agent with certain flavonoids depending on the reaction medium used (Christ & Müller, 1960), this technique is nonspecific for fungi, since they contain chlorogenic acid, hydroxycinnamic acid, o-diphenols and molecules that include catechol residues as pigments; all of these included ergosterol, and can give false positives in the $AlCl_3$ colorimetric assays (Gil-Ramírez *et al.*, 2016).

In addition to these possible false positives, no coding sequences for chalcone synthase or chalcone isomerase (key enzymes involved in the biosynthetic pathway of flavonoids) have been identified for fungi, this would imply the impossibility of transforming phenolic compounds to flavonoids due to the absence of the transformation catalysts (Gil-Ramírez *et al.*, 2016). However, several authors confirmed the presence of flavonoids such as: quercetin, luteolin, myricetin, naringenin, naringin, hesperetin, rutin, morin, kaempferol, chrysin and derivatives, genistein, apigenin or catechin (Mattila *et al.*, 2001; Ribeiro *et al.*, 2006; Ribeiro *et al.*, 2007; Turkoglu *et al.*, 2007; Kim *et al.*, 2008; Barros *et al.*, 2009; Palacios *et al.*, 2011; Gąsecka *et al.*, 2017). Notably, these studies rarely used state-of-the-art analytical chemistry including analytical and preparative HPLC, high resolution mass spectrometry and NMR spectroscopy (De Silva *et al.*, 2013).

The lack of coding sequences for enzymes involved in the flavonoid biosynthetic pathway in mushrooms is determinant to confirm the absence of production of flavonoids in the Fungi Kingdom, the use of nonspecific techniques developed in plants is the first error committed over time. Today we know that fungi are organisms with a metabolism independent from plants and therefore we must establish specific tests taking into account the metabolites produced by them.

With respect to the DPPH assay and the percentage of antioxidant activity (Table 2), the extracts **AG001**, **GS002**, **HF003**, **IR005**, **LL006**, **LF007** and **TC009** showed positive correlation between the concentrations of phenolic compounds and the antioxidant activity: low concentrations of these compounds (Table 1) indicated moderate to low antioxidant activity (Table 2), consistent with Olennikov (2011) and Keleş *et al.* (2011) who explain that these are the main antioxidant compounds of the secondary metabolism of fungi, mainly emphasizing phenolic compounds.

However, **PA008** extract showed moderate concentrations of total phenolic compounds (Table 1), without expressing efficient antioxidant capacity (Table 2) (9.13% and DPPH 36.66 ± 0.51 $mg.g^{-1}$), these results are reflected in the work of Reis *et al.* (2011), where they reported high concentrations of total phenolic compounds but specify low antioxidant power, this could be understood as a contradiction to the assertions that total phenolic compounds are the main metabolites contributors in the antioxidant action of fungal extracts (Keleş *et al.*, 2011).

CONCLUSIONS

Regarding the crude extracts analyzed, the species *Inonotus splitgerberi* **IS004**, *Inonotus rickii* **IR005** and *Pisolithus arhizus* **PA008** presented a good chemical profile when testing positive bioactive secondary metabolites such as phenolic compounds, reducing sugars and alkaloids, among others; as well as a high percentage of antioxidant activity. **IS004** showed a chemical profile similar to the *Inonotus obliquus* species considered medicinal, used as an antitumor and immunomodulatory agent. These preliminary results confirm the presence of metabolites with possible biological properties and mark the beginning of the chemical study within the Fungi Kingdom in Paraguay. Future studies of isolation and elucidation of metabolites through analytical and preparative HPLC, high resolution mass spectrometry, and NMR spectroscopy of those extracts that showed a good chemical profile are necessary.

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