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RESEARCH ARTICLE

CHEMICAL CHARACTERIZATION, NUTRACEUTICAL, AND EVALUATION OF THE GEOMICOLOGICAL ACTIVITY OF *RAMARIA FLAVA* WILD AND EDIBLE IN THE STATE OF HIDALGO, MEXICO

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ABSTRACT

The present work describes the chemical and nutraceutical study of *Ramaria flava*, a wild and edible mushroom collected inHidalgo state, Mexico. Reductive power, antioxidant activity, total phenolic compounds content, ß-glucans, phenolic acids (cinnamic, gallic and chlorogenic) in microwave-assisted methanol-water extracts as well as in acidic and alkaline extracts are described. Isolation and identification of sterol as a secondary metabolite of *R. flava*is reported, not previously described for this species in Mexico. The Al, Cd, Cr, Cu, Fe, K, Mg, Mn, Pb concentrations were determinedin extracts as well as the soils of the study area in which the mushroom was collected.The highestvalueswere found for Cu and Fe, in agreement with the literature reporting levels of these metals in the region.An inverse relationship was foundbetween the antioxidant power of the mushroom extracts with the Fe, Cu, Mn and K concentration. The *R. flava* metabolite extraction and chromatographic separation were notreported before on this species in Mexicoand showed up the presence of ergosterol, demonstrated by spectroscopic characterized.

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INTRODUCTION

For some millennia, humanity knew the benefits of mushrooms, the fruiting bodies of the macroscopic fungi. In recent times, China recorded an annual per capita consumption between 20 and 24 kg (Nowacka *et al*., 2014). The most commonly mushroom species produced and consumed internationally are *Agaricus bisporus*, *Pleurotus ostreatus* and Shiitake (*L. edodes*). Mexico stands out as being one of the leading producing countries of aforementioned edible and medicinal mushrooms, representing 90, 9.9 and 0.1% of production respectively;while the annual per capita consumption is 977 g per year (Nava and Martínez, 2003). Thenutrient richness of cultivated speciesdecreases, in contrast to wild species. In Mexico, untamed mushrooms are consumed since precolonial times because it is an adequate source of proteins, carbohydrates, vitamins, fiber, minerals, and fats of low caloric value. The wide range of bioactive components such as phenolic compounds, β-glucans, flavonoids and others are capable of complementing the treatment and preventing chronic diseases such as cardiovascular illnesses, diabetes, hypertension, and cancer (Reis *et al*., 2012). Mexico is a country with a megabiodiversity of wild mushrooms, climates, and soils. Despite, there arescarce studies related to the geomicological interaction affectingtheir mineral content, which can add nutritional value in essential elements such as potassium, magnesium, calciumand increase the antioxidant character of the mushrooms bioactive components.Also, it is essential to prevent health problems due to toxic metals presence such as cadmium, lead and mercury (Gadd, 2007). The species of primary socioeconomic importance in Mexico due to their culinary value are shown in Table 1together with their traditional names.

Ramaria flava develops mainly in conifer and oak forests; it is edible with a pleasant taste. Morphologically, it has a large, thick and fleshy basal part. Its fructification presents numerous thin, cylindrical, fleshy, slightly compact, vertical, pointed ends, in various yellowish or reddish hues, with the darker limbs (Figure 1). This mushroom is commonly known as a

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brush growing in deciduous forests on acid soils, has shown moderate antibiotic activity (Liu *et al*., 2013). This paper reports the chemical and nutraceutical study of the edible wild mushroom *Ramaria flava*, collected in the state of Hidalgo, Mexico. This research aims to describe the reducing power, antioxidant activity, total phenolic compounds content, β-glucans, phenolic acids such as cinnamic, gallic and chlorogenic in extracts obtained by microwave-assisted methanol-water as well as the acid and alkali extracts. The Al, Cd, Cr, Cu, Fe, K, Mg, Mn, Pb concentrations are also determined in extracts as well as in the soils of the study area where the mushroom was collected to know their bioaccumulating capacity based on the metals contents. The bioactive components, the antioxidant properties, and the radical sequestration are determined and analyzed about the present compounds. The study contributes to the chemical knowledge of species.

Table 1. Mexican mushroomsof most socioeconomic importance (Eustaquio *et al*., 2010; Moreno-Fuentes and Hernández-Rico, 2010)

Mushroom name	Traditional name in Mexico	Meaning
Amanita caesarea	Izcalli,yemita	Yellow fungus, yolk
Lyophyllum decastes	Clavito	Small brads
Auricularia delicata	Oreja, trompa	Ear,
Armillaria mellea	Babosito, cazahuate	Sleepy, honey
Agaricus campestris	Champiñón, San Juan	Field Mushroom, Meadow Mushroom
Pleurotus spp.	Blanco patón, avispón, pollitos	Hornet, chicks
Boeltusedulis	Cemita rey, panza	Cep, King Bolete, Belly
Hypomyces lactifluorum	Enchiladas, oreja de puerco	Hot spicy, pig ear
Russula brevipes	Borrego blanco, Iztacnanácatl	White lamb, Short-stemmed Russula
Ramaria flava	Escobetas, coral	Little broom, coral
Collybia Dryophilae	Señoritas, mujercitas	
Amanitarubescens	Mantecoso	Greasy
Cantharellus cibarius	Duraznillo	Little peach

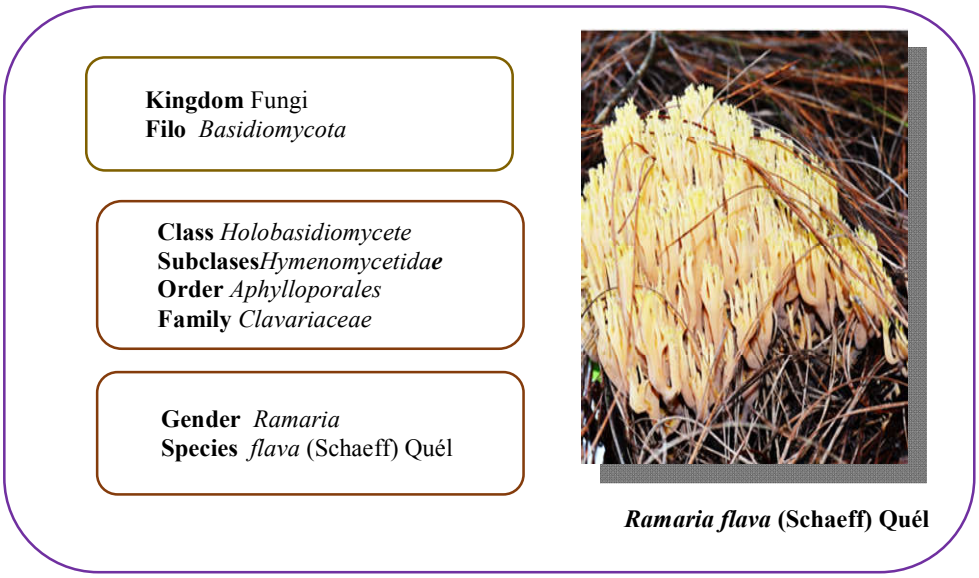

Figure 1. Taxonomic description of *Ramaria flava* (Herrera and Ulloa, 2013)

MATERIALS AND METHODS

Sampling and identification.R. flava and soils samples were collected in the state of Hidalgo, Mexico (Figure 2). The regions were "Los Reyes", Acaxochitlán (latitude 20,152 ° and longitude -98,185 °, 2199 MASL); El Susto (latitude 20.033 ° and longitude -98 .509 °, 2620 MASL) and Paxtepec (latitude 20.033 ° and longitude -98.426 2381 MASL). Sampling was performed during the rainy season of the year 2014 (August-October). The soil samples were obtained at a depth over 25 cm in the sites of amushrooms quest. The species identification was carried out by comparison of the sporocarps according to the identification guides (Læssøe, 2013). *Preparationof mushrooms.*Sampleswere cleaned with a brush and cut into flakes without separating the pile from the stipe. They were dried at 60 °C in a furnace (Riosa EOMI 50.51) until constant weight, then milled and sieved (20 mesh) using a mill (IKA A11 basic), and finally stored in dark plastic cans until analysis. *Extractions for organic analysis*. Itwasaccomplished by the methodologies proposed by Pan *et al*. (2003) and Özyurek *et al*. (2014) with some modifications. Each dry and pulverized sample (200 mg) was extracted with methanol: water mixture (80:20, v/v, 25 mL) using a standard microwave oven (900 W at 10% power). The samples were irradiatedand heat during 7 s (preventing the solvent boiling) and cooled by stirring 30 s in anice bath.The procedure is repeated until 5 min total heating time. The obtained extracts were filtered through filter paper (Whatman No. 4), the filtrate poured into a 25 mL volumetric flaskand filled up to mark with the methanol: water mixture (80:20, v/v). The solutions were stored at 4 °C in amber bottles until the analysis.

Total phenolic content. It was determined spectrophotometrically through the Folin-Ciocalteu (FC) test, based on the ability of phenolic compounds to reduce a mixture of phosphotungstic and phosphomolybdic acids from Mo (VI) to Mo (V) in an alkaline solution to produce a blue color (Huang *et al*., 2015). According to the procedure of Heleno *et al*. (2015)100 µl of methanolic extract were mixed

with the FC reagent (750 µl, 10% v / v) stirred 1 min and rest 5 min.A Na_2CO_3 solution (750 µl, 10% w/v) was added to the mixture, andthe reaction was kept in the dark for 90 min.The absorbance reading was achieved at 725 nm (Genesys 10S VIS Thermo Scientific spectrophotometer). Gallic acid was used to obtain the standard curve (20-600 µ/ml). The total phenolic content was expressed as mg of gallic acid equivalents (GAE) per gram of dried mushroom.

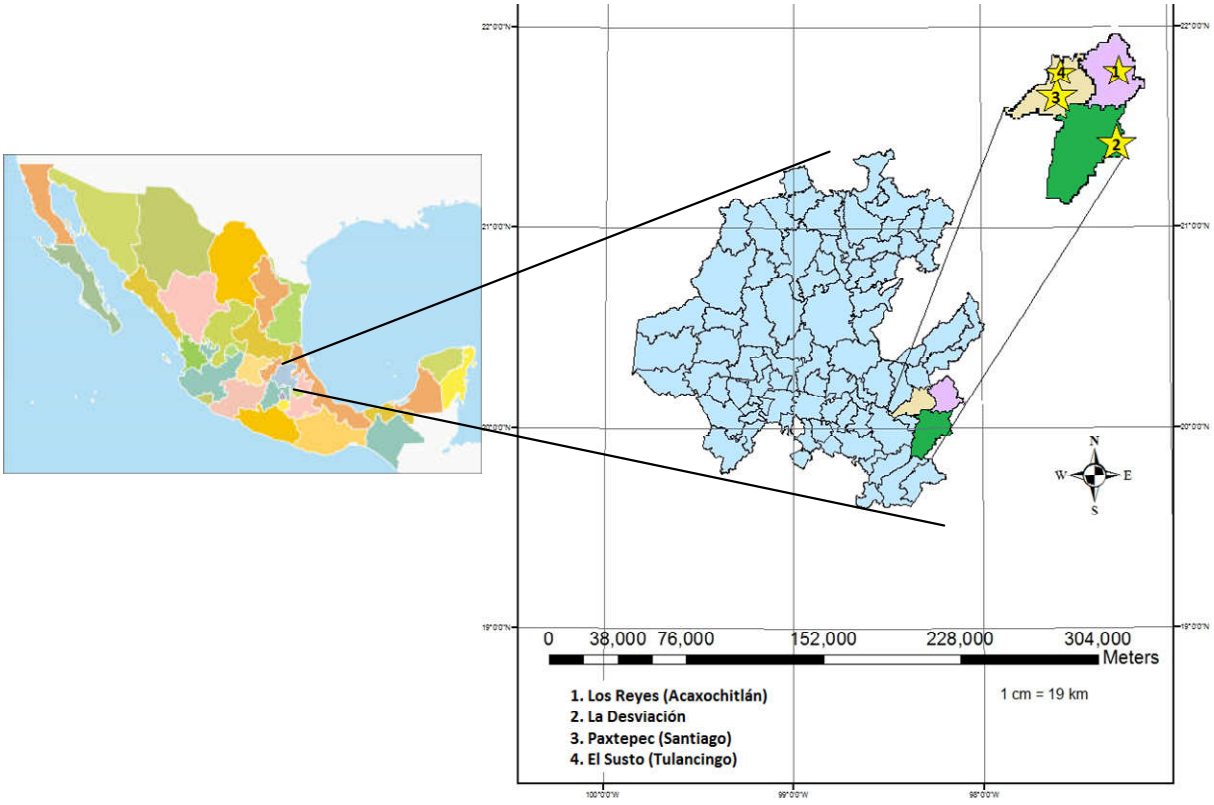

Figure 2. Geographic location of the sampling points

Phenolic acids. The methanolic extracts were filtered through a 0.45 µm nylon disk membrane to performfurther phenolic acid determination by high-performance liquid chromatography by an Agilent 1260 equipped with a diode array detector. The separation was carried out using 5 µl of extract in an Agilent poroshell 120 EC-C18 column (4.6 x 50 mm, 2.7 µm) thermoset at 25 °C. The mobile phase was: (A) 0.1% phosphoric acid in water, (B) acetonitrileHPLC-grade, (C) methanolHPLC-grade. The gradient used was: 95% A, 5% B, flow rate of 1.0 ml/min for 2 min; 50% A, 25% B and 25% C, flow rate at 0.5 ml/min for 8 min. The phenolic acids were quantified by comparing the area of the peaks recorded at 280 nm with the corresponding ones of the calibration curves obtained with the commercial standards of each acid. The results were expressed as µg/g dry arrow.NO ENTENDÍ

Reducing power. It was determined by the procedure reported by Barros *et al*. (2011). The methanolic extract (500 µl) was mixed with a sodium phosphate buffer (500 µl, 200 mM, pH 6.6) and potassium ferricyanide (500 µl, 1% w/v). The mixture was incubated at 50-60 ° C for 20 min, and trichloroacetic acid (500 µl, 10% w/v) was added. An aliquot of the reaction mixture (800 µl) was transferred to vials containing distilled water (800 µl) and ferric chloride (160 µl, 1% w/v). After 90 min, the absorbance (Genesys 10S VIS spectrophotometer, Thermo Scientific) was measuredat 690 nm.The reduction power was obtained as a percentage of the conversion of the orange Fe^{+3} - ferricyanide complex to a Prussian blue color of the Fe^{+2} - ferrocyanide species in the presence of antioxidants (Özen and Türkeful, 2010). Butylated hydroxytoluene (BHT, 1mg/ml) was used as the control by measuring its absorbance in the assay and was assigned 100% of the reducing effect. Aliquots of 60 µl extract were mixed with 540 µl of 2,2-Diphenyl-1-Picrilhidrazilo (DPPH) radical methanolic solution (0.06 mM). The mixture was allowed to stand for 60 min in the dark. Reduction of the DPPH moiety was determined by measuring the absorbance at 515 nm. The radical inhibitory activity (RIA) was calculated as a percentage of the discoloration of the purple solution of the DPPH radical by equation 2:

$$\% \text{ RIA} = ((\text{ADPPH} - \text{Am})/\text{ADPPH}) \times 100$$

where Am and ADPPH are the absorbances of the solution containing the sample and the DPPH solution, respectively (Vaz *et al*., 2011).

Ascorbic acid. Itwas determined by the reported method (Vaz *et al*., 2011). 150 mg of the powdered sample was extracted with 10 ml of metaphosphoric acid (1%, w/v) for 45 min at room temperature with constant stirring and the mixture was filtered through filter paper (Whatman N° 4). The filtrate (0.1 ml) was mixed with 0.9 ml of 2,6-dichloroindophenol (0.00125% w/v) and allowed to stand for 30 min. The absorbance at 515 nm was measuredagainst its target. The ascorbic acid content was calculatedwith the calibration curve of L-ascorbic acid between 0.5 and 2.01 µg/ml and the results expressed as mg of ascorbic acid per g of thedry arrow. *Flavonoids*. The quantification of flavonoids was carried out according to Pereira *et al*. (Pereira *et al*., 2011). For this purpose, 500 µl of methanolic extract was mixed with 150 µl of a sodium nitrite solution (5%) and 2 ml of distilled water;

the mixture was stirred for 1 min. After 5 min, 150 µl of aluminum chloride (10%) was added, stirred 1 min and allowed to stand for another 6 min. Finally, 2 ml of sodium hydroxide (4%) and 200 µl of distilled water were added. The mixture was stirredfor 15 min, and the absorbance was measured at 510 nm. Quercetin was used as a calibration standard at concentrations of 1 to 20 µg/ml, and the results were expressed as mg quercetin equivalents (QE) per g dry arrow. *β-glucans.*They were isolated and quantified according to Nitschke *et al*. (2011). 750 mg of mushroom powder was heated with 60 ml of 1 mol/L KOH for 20 min at 60 °C under constant stirring. Subsequently, the suspension was filtered, the solid residue washed with distilled water, and the filtrate was neutralized with 6 mol/L HCl. The filtered volume was poured into a 100 ml volumetric flaskand filled to the mark with distilled water. This extract was called the KOH fraction. The solid residue was suspended with 65 ml of 0.58 mol/L HCl and heated in an oil bath at 100 °C for 1 h.The suspension was filtered, and the solid residue was rinsedwith the HCl solution. The filtratewas neutralized with 6 mol/L NaOH, transferred to a 100 ml volumetric flask and filled to the mark with distilled water. This extract was called HCl-fraction. The solid residue was resuspended with 60 ml of 1 mol/L NaOH and heated at 60 °C for 20 min. The suspension was filtered, the filtrate solid washed with distilled water and the filtrate was neutralized with 6 mol/L HCl, poured into a 100 ml volumetric flask and filled with distilled water to the mark. This portion was called NaOH-fraction. The three fractions were used for the determination of ß-glucans. For the quantification of ß-glucans, 350 µl of each fraction was mixed with 300 µl 0.2 mol/L of citric acid/sodium hydroxide buffer pH 7 and 50 µL of the dye solution (8 mg of red Congo diluted in 10 ml buffer). The absorbances of the mixtures were read at 523 nm using a solution blankprepared with 350 µl of distilled water, 300 µl of buffer and 50 µl of dye. Due to the brown color of the fractions, the measurement required to rule out the background interference at 523 nm. Thus, 350 µl of the sample was mixed with 350 µl of buffer, and the absorbance was measured at 523 nm. The calibration curve was obtained with stock solutions of schizophrenic in the range 225-600 µg/ml. All analyseswere performed in triplicate. The total contents of β-glucans were expressed as mg of β-glucan per g of dried mushroom. *β-carotene and lycopene.* Theywere determined by the reported method (Pereira *et al*., 2012). 500 µl of mushroom extract was shaken with 10 ml of acetone:hexane mixture (4: 6, v/v) for 1 min and the mixture was filtered (Whatman No. 4). Subsequently, the filtrateabsorbance was measured at 453, 505, 645 and 663 nm. The β-carotene and lycopene amounts were calculated by equations 3,4 and expressed as mg / 100 g dry weight of mushroom.

$$\text{ß-carotene (mg/100 mL)} = 0.216(A_{663}) - 0.304(A_{505}) + 0.452(A_{453})$$

$$\text{Lycopene (mg/100 mL)} = -0.0458(A_{663}) + 0.372(A_{505}) - 0.0806(A_{453})$$

Chromatographic separation. A sampleof *Ramaria flava*fresh material (6 kg) was sliced and cut into 2x3 cm pieces, dehydrated in the sun for three days and pulverized to obtain a powder.500 gwas refluxedwith AcOEt for 3 h, filtered and the filtrate was concentrated on a rotary evaporator to obtain the extract for further column chromatographic separation.3.88 g crude extract was eluted with an AcOEt/Hexane (4:1) mixture using a 1.9 x 50 cm chromatographic column packed with silica gel 60 (Merck, 0.063-0.200 mm). A total of 50 fractions (2 mL)were collected at a flow rate of 16 mL/min and concentrated under reduced pressure on a rotary evaporator.A crystalline solid (22 mg) was obtainedfrom fractions 34-47 after the solvent remotion.Thin layer chromatography was performed on silica gel 60 F254 chromatography with UV light developer. *Spectroscopic characterization*. ^{1}H and ^{13}C NMR spectra in $CDCl_3$were obtained at room temperature on a Varian VNMRS 400 spectrometer at 400 and 100 MHz, respectively. The chemical shifts in parts per million (ppm) were calculated from tetramethylsilane (TMS) as a reference, and the coupling constants (J) are given in Hz. Infrared spectra were obtained on a Perkin Elmer Spectrum GX spectrometer in a KBr pelletsand filmsin CsI window.

Soil preparation. The soil samples were dried by sun exposure for 6 h, subsequently sieved (20 mesh) and stored in plastic containers until analysis.

Metal determination. The metal content in mushroom powder and soil samples were carried out by the U.S. EPA methodology. The microwave digestion of 500 mg of each solidwas treated with 5 ml concentrated nitric acid (65%, v/v) in the processing cartridge and heated by a microwave oven (Mars X-5, 1200 W at 100% and 170 °C maximum temperature). The oven was programmed to reach the indicated temperature within 15 min and then, to be cooled.The cartridges contents were pouredinto 50 ml Class A volumetric flaskand filled to the mark with nitric acid (5%, v/v) for nextmeasurement by Atomic Absorption (AA).The flaskswere labeled and storedat 5 °C in suitable plastic containers.The determination was carried out with an Agilent AAS 2200 series AA equipment using air-acetylene and air-nitrous oxide flame.The wavelengths lectures were at 236.7, 228.8, 357.9, 324.7, 372, 404.4, 202.6, 279.5 and 217 nm for Al, Cd, Cr, Cu, Fe, K. Mg, Mn, and Pb, respectively.Multielement standards were used, at a concentration of 0.5 ppm of each element for Cd, Cr, and Pb; K, Cu, and Mn; Al, Fe, and Mg. Eachconcentration determinationwasperformed in triplicate. All assays were performed in triplicate. The results were expressed as themean value ± standard deviation (SD) and analyzed using one-way analysis of variance (ANOVA) followed by the Tukey's HSD test with $\alpha = 0.05$ for results that showed highly significant differences ($p < 0.001$). Statisticswere performedwith SPSS v. 22.0 (IBM Corp., USA).

RESULTS AND DISCUSSION

Total phenolic compounds (TPC). Fungi are sources rich in bioactive compounds with a huge variety of chemical structures. They are well recognizedfor their antitumor, antimicrobial and antioxidant properties; attributed mainly to the presence of phenolic compounds, attributed to secondary metabolites biosynthesized by fungi as anadaptation way to the condition of biotic and abiotic stress (Islam *et al*., 2016). The analysis of the wild species *R. flava* revealed essential contents in total phenolic compounds (Table 1).

Table 1. Contents and composition of total phenolic compounds (TPC) and phenolic acids in *R. flava*

	TPC*	Chlorogenic acid**	Cinnamic acid**	Gallic acid**
R. flava	2.07 ± 0.04	510 ± 8.13 (24.6)	4.9 ± 0.61 (0.24)	n.d.

*mg GAE/g dry mushroom; GAE: gallic acid equivalents. **µg/g dry mushroom. n.d.: Not detected

Previous studies (Palacios *et al*., 2011) reported similar values of TPC for other setae species such as *B. edulis* and *C. cibarius* (5.5 and 2.5 mg of GAE/g of dry mushroom). However, *B. edulis*presents four times higher proportion (8.13 mg of GAE/g of dry mushroom) as itwas recently publishedstudied in Querétarostate, Mexico, (Yahia *et al*., 2017). Because mushrooms are motionless, they require phenolic compounds as a defense strategy against competing organisms and predators as well as for protection against UV light (Heleno *et al*., 2015). The production of the phenolic compounds comes from the shikímico acid route. The concentration variances in the species can be justifiedby the differences in the ecological environment in which the studied mushrooms have been developedabout the need for self-protection according to the surrounding ecological factors which are different in each study region.That is why, the content of bioactive substances, can vary even in fungi of the same species due to differences in the environment where they grew, the maturation stage, fungi freshness and soil conditions such as organic matter content, pH, the presence of mineral salts, microbiota among other factors (Boonsong *et al*., 2016). Also, the differences in the extraction techniques used in reported work generate the observed discrepancies. Hence, these studies allow knowing the phenolic compounds richness of the wild edible species. Among the reported phenolic compounds for edible, inedible and medicinal mushrooms are chlorogenic, gallic and cinnamic acid (Reis *et al*., 2012; Heleno *et al*., 2015; Palacios *et al*., 2011; Taofiq *et al*., 2015). These substances are part of the analyzed total phenolic content and contribute to the antioxidant activity. That is why the analysis of the mentioned compounds in *R. flava* was carried out, and the amounts are shown in Table 1. Gallic acid was not detected. *Chlorogenic acid.*It is present in asignificantamount, 510 µg/g dry mushroom, remarkably higher than in any other studied fungi. Its presencehasbeen reported for all species,ranging from 0.04-1.00 µg/g dry mushroom (Palacios *et al*., 2011; Yahia *et al*., 2017). Its principal health benefitswere reported, such as the obesity reduction in the body due to its triglyceridesand cholesterol lessening action (Cho *et al*., 2013), combined with the powerful antioxidant effect, whose transformation to caffeic acid was demonstrated in vivo and in vitro (Sato *et al*., 2011). The quantification of this phenolic compound in *R flava*hasnot been reported until now. So, thisfindingclaimsitsintake recommendationas part of a diet rich in antioxidants and nutraceutical products. Pancho, esto quizas no sea bueno ponerlo, pues en el articulo de los coreanos hay una tabla con los contenidos del clorogenico en muchos vegetales y son todos del orden de mg/, mucho mayores para unadieta. The presence of chlorogenic acid is chemically justified through the L-phenylalanine content, widely present in fungi.It has akeyinfluenceontaste, and itsproportion constitutes a quality parameter for mushrooms (Sommer *et al*., 2010). Figure 3 illustrates the biosynthesis of chlorogenic acid and shows how chlorogenic acid isrelated to trans-cinnamic acid, acting as a precursor in its biosynthesis. Pancho ver si los metales del suelo actuancon el acido clorogenico y desplazan desplazan el equilibrio hacia su formacion. ese es el aporte importante. kiss en el año 1989 ya reportó muchos complejos de cu(ii).este acido es un glicosido y puede girar para hacer quelatos. fijate que los demas no. tienes mucho cu(ii) en el suelo. quizas no es un poorblema de stress ambiental, sino quimica de coordinacion nada mas. Polyhedron Volume 8, Issue 19, 1989, Pages 2345-2349. The L-phenylalanine 19 undergoes a deamination by means of the enzyme Phenylalanine Ammonium Liasa (PAL) to generate the trans-cinnamic acid 20, which is hydroxylated in para-position by the enzyme cinnamic acid 4-hydroxylase (CAH) to give 4-coumaric acid derivative 21 which in turn is attached to the acetyl coenzyme A by the enzyme 4-coumarate coenzyme A ligase (4CL) and said acetylated product, 22, incorporates the quinic acid 23 to generate the 4- which is converted to chlorogenic acid 25 by means of the enzyme 3-hydroxylase Quitar. solo dejar la figuracon los nombres.

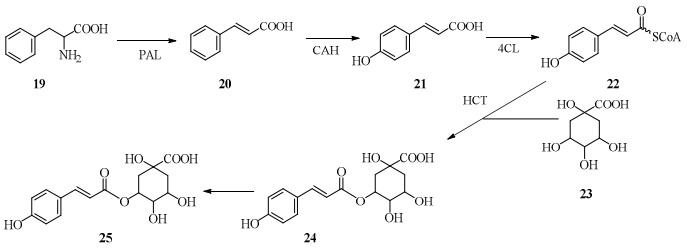

Figure 3. Biosynthesis of chlorogenic acid from L-phenylalanine, Dewick reaction scheme from (Dewick, 2009)

PROPONGO CAMBIAR LA FIGURA POR LA QUE ESTA EN EL ARTICULO DE LOS COREANOS: POR QUÉ? Porque MAS abajo con el análisis del gallic acid, sehabla del ácido shikimic y en la biosíntesis que está aquí no aparece

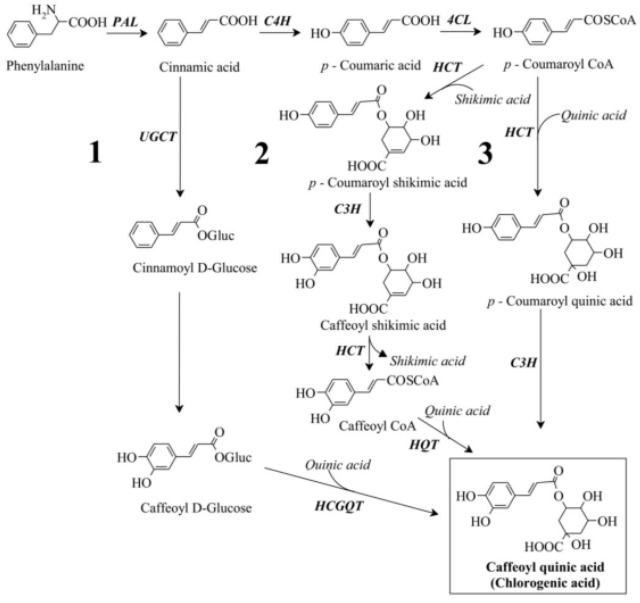

Figure 3. Proposed pathways for chlorogenic acid synthesis in plants.31C(Pham Anh Tuan, Do Yeon Kwon, Sanghyun Lee, Mariadhas Valan Arasu, Naif Abdullah Al-Dhabi, Nam Il Park, and Sang Un Park. Enhancement of Chlorogenic Acid Production in Hairy Roots of Platycodon grandiflorum by Over-Expression of An Arabidopsis thaliana Transcription Factor AtPAP1 *Int. J. Mol. Sci.*2014, *15 15, 14743-14752; doi:10.3390/ijms150814743*

Cinnamic acid. In contrast, cinnamic acid was lesser quantifiedthan chlorogenic acid (4.93 µg/g dry mushroom), and its contribution to TPC was meager. This finding is probably due to the vast extent of the chlorogenic acid biosynthesis, that exhaust the precursor. The reported amount for *B. edulis*in Poland mushrooms was even lower (3.1 µg/g dry mushroom) (Heleno *et al*., 2015). The same species presents three times highercinnamic acid proportion in Portugal (14.2 µg/g dry mushroom) (Taofiq *et al*., 2015), while it was not detected in other studies (Özyurek *et al*., 2014; Heleno *et al*., 2015). *A.*

caesarea presents a low fraction (0.3 µg/g dry mushroom) (Reis *et al*., 2011); about ten times lower than that obtained in this study. These findings support that CFT composition dependson a great extent on the species and the environment.

Gallic acid. In the presentstudy, gallic acid was not detected.The microwave assisted extractive method (methanol/water 80:20, v/v) employed in this paper gave gallic acid reports in high quantities in species where chlorogenic acid is present (*C. Cibarius*, *C. cornucopioides*, *G. lucidum* and *H. marzuolus*) (Palacios *et al*., 2011; Yahia *et al*., 2017; Yildiz *et al*., 2014). Differently, cases are observed in other species, where gallic acid occurs in traces or is undetected (*A. pantherina*, *B. luridus*). Different amounts of gallic acid have been reported in mushrooms with diverse extractivemethods; therefore,a wide range of proportions appears in the literature. Minimal concentrations of*B. edulis* (0.19 µg g dry mushroom) (Özyurek *et al*., 2014); and high amounts (212.96 µg/g of dried mushroom) with simple methanol extraction (Palacios *et al*., 2011). Recently, Islam reported high gallic acid amounts of 231.1 and 18.64 µg/g setae, for *C. cibarius* and *G. lucidum* respectively withmethanol/water/butylated hydroxytoluene extraction (Islam *et al*., 2016). The biosynthesis of gallic acid is shown in Figure 4, coming from shikimic acid, which is, in turn, a precursor of chlorogenic acid (See Figure 3, Path 2). Therefore, its bioavailability is limitedbythe formation of gallic acidin *R. flava*due to the principal chlorogenic acid occurrence (Dewick, 2009). Although the extraction method would influence the results ofamount determination, it is not proven to be such a determining factor to avoidtraces detection.

In this study, the methanol-water extracts of the different mushrooms speciesNOOOOR.flava were tested to calculate the degradation percent of the purple DPPH radical methanolic solution at a single concentration, from the 517 nm absorbance readings vs.the DPPH solution without extract. Esto ya fue explicado en la parte experimental

The extracts of *R. flava* degraded the purple coloration of the DPPH radical in different magnitudes, corresponding to different percentages of DPPH radical inhibition. Table 2 shows the results in percentage (%) inhibition of the DPPH radical for the nine species studied.

Direct relationship between the total content of phenolic compounds and the% inhibition of the DPPH radical. ES UNO SOLO Orhan (Orhan and Üstün, 2011) reported *C. cibarius* total phenolic compound of 31.48 mg GAE/g dry mushroom and 59.87% DPPH inhibition. Likewise, *G. lucidum* has been reported (Orhan and Üstün, 2011) with a phenolic content of 75 mg GAE/g dry mushroom and 74% of the DPPH inhibition. Both species presented higher values than thosereported here for *R flava* (2.07 mg GAE/g dry mushroom and 20.88% DPPH inhibition). With the mentioned reports, thecorrelation between the TPC content and the% DPPH radical inhibition was observed (Figure 5), althoughonly three pairs of values were available. It is due to the ability of phenolic compounds to donate hydrogen atomsdue to the O-H low dissociation enthalpy (102 kcal/mol). Thus, theradicals deactivation by H transfer (Özen and Türkeful, 2010) justifies the low percentage of DPPH radical inhibition.

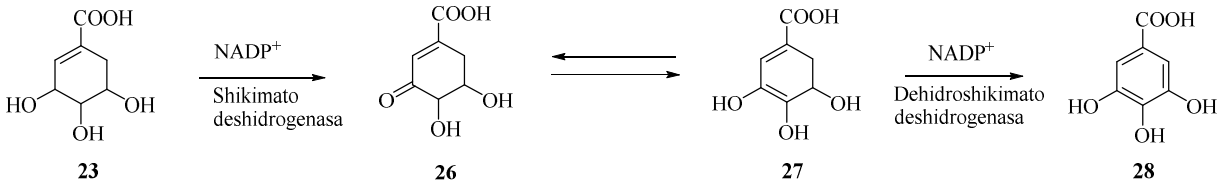

Figure 4. Biosynthesis of gallic acid from shikimic acid (Dewick, 2009)

Table 2. Composition of inhibitory and reductive activity in *R. flava*

	% DPPH radical inhibition	% Fe^{3+} reducing power	Ascorbic acid (mg/g dry arrow)	β-carotenes and lycopenes
R. flava	20.88 ± 3.91	14.73 ± 5.11	3.14 ± 0.04	n.d.

n.d.: Not detected

Wild mushrooms are rich in phenolic compounds (PC) with a unique antioxidant capacity. Discussions have been raised regarding biosynthesis, extraction techniques and sample analysis. The presence of phenolic acids in mushrooms, especiallychlorogenic acid, is attributable to a defense mechanism in which the acid chelates metal ions.Chelationallowsthe bioaccumulating response and prevents the damage to the plants with which they present symbiotic associations using the roots (Gąsecka *et al*., 2017). No entendi la simbiosis con quien?no se entiende Considering the low concentration of gallic acid, and the absence of cinnamicacid, as well as the high Cu content (further discussed here) it is very likely that a quelation effect occurs, affecting the biosynthetic route, showed in Figure 3. Cu chelation by chlorogenic acid has been reported in previous studies (Pham Anh Tuan *et al*., 2014; Tamas Kiss *et al*., 1989)

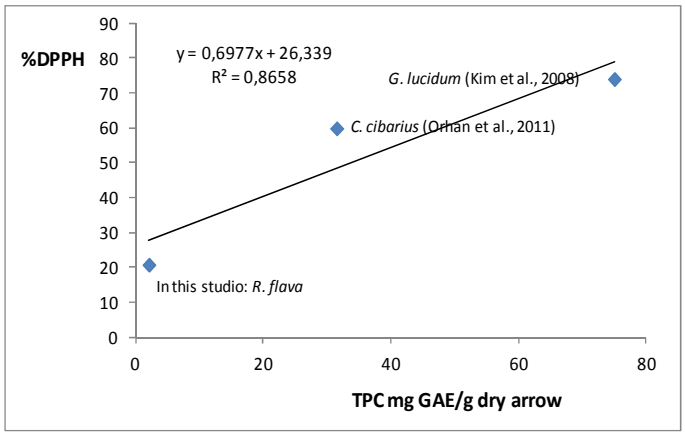

Figure 5. Correlation between% DPPH radical inhibition and TPC content in mushrooms

Free radical scavenging activity. A wide range of different methodologies is described for the evaluation of free radical scavenging activity in the literature (Woldegiorgis *et al*., 2014). Other authors report theEC_{50} parameter, as the TCP concentration (inthe extract) necessary to deactivate 50% of

DPPH radical whichis inversely related to its inactivating activity; thus, the lower EC_{50} valuesget, the more significant radical deactivation by the extract. Recently, EC_{50}has been reportedfor *R. flava* (13 µmolequivalent Trolox/100 g fresh weightmushroom) and other species (Yahia *et al*., 2017). However, further work should be performed at different extract concentrations to obtain better EC_{50} quantitative data to confirm this report, because the way in which authors obtained the test results.Other substances such as ergosterol and some polysaccharides of theβ-glucans family, could bias theradical inhibition and therefore, corrections might be included to avoid the effect of other bioactive molecules in the mushrooms. The weakness of potassium ferricyanide reduction to ferrocyanide correlates with the low TPC content of *R. flava* (Table 2). The reducing effect is directly related to the phenolic compounds present in the mushroom extracts, as it has been demonstrated by (Vaz *et al*., 2011) and the results obtained here are consistent with the content of phenolic compounds. The same phenomenahave also been describedfor *C. cibarius* (Unekwu *et al*., 2014) coincident with a low percentage of DPPH radical inhibition. Also, anotherFe^{3+} reduction dropping effect is associated with the presence of polysaccharides (Tiang *et al*., 2012; Tseng *et al*., 2008; Xiao *et al*., 2012), which are abundant inmushrooms (Barros *et al*., 2008). So, the cause of the reducing effect cannot be limited only to the presence of TPC.

Ascorbic acid. The present study foundthe ascorbic acid in aconcentration of 3.14 ± 0.04 mg/g dry arrowin *R. flava* (table 2). It has been reportedin a low range values, between 0.13 - 0.35 mg/g dry mushroom with depleted TPC content (Barros *et al*., 2008). *A. caesarea* presents theascorbic acid content of 2.08 mg/g dry mushroom (27), close to that determined for *R. flava* in our research. Until now, no other reportsare found in Mexico regarding the ascorbic acid content in wild fungi. In addition to phenolic compounds and tocopherols as defense strategies against the oxidative stress in fungi, ascorbic acid is formedas antioxidant seizing free radical damage. Some authors suggest that the variability in ascorbic acid content in the different fungi species is associated with the genotype and the environmentalconditions where the organisms grew, reaching the antioxidant requirements to face free radicals (Ferreira *et al*., 2009).

β-carotenes and lycopene.Wild fungi are rich in β-carotenes and lycopene, acting as oxygen radicals deactivators as well as lipid peroxidation inhibitors. They reduce the risk of prostate and digestive tract cancer, among other chronic diseases (Robaszkiewicz *et al*., 2010). In this study, the concentrations of ß-carotene and lycopene resulted below the detection limit of the technique. Therefore, no data are reported for this test. It is probably that samples dehydration causes decomposition and fresh sample workupshould be performed to avoid ß-carotene and lycopeneloss.

Flavonoids. It is known that fungi do not have the biosynthetic ability to produce flavonoids. Nevertheless, several papersreport the flavonoids total content in wild mushrooms of different regions (Pereira *et al*., 2012; Woldegiorgis *et al*., 2014; Unekwu *et al*., 2014; Jaworska *et al*., 2014). Some of them provide the quantification methods for some flavonoids such as quercetin, chrysin, andpinocembrin by HPLC (UV/DAD) using a mass detector (Liu *et al*., 2013; Kim *et al*., 2008; Leal *et al*., 2013; Liu *et al*., 2016). Up to date, no report has reliable evidence to explain the origin of flavonoids in fungi. The biosynthetic pathway for the production of these secondary metabolites is knownin plants (Figure 6). This route proceeds through the enzyme PAL (phenylalanine ammonia lyase) biotransformation of the amino acid (Yildiz *et al*., 2014) into cinnamic acid (Dewick, 2009), the leading precursor in the phenylpropanoid route to flavonoids. A second step goes to the hydroxylation of cinnamic acid by cinnamate-4-hydroxylase (C4H) to produce 4-coumaric acid (Gąsecka *et al*., 2017); which is transformed by 4-coumarato-coenzime A ligase (4CL) to the activated 4-coumaroyl-CoA (Woldegiorgis *et al*., 2014). Thechalcone synthase (CHS) is a plant-specific polyketide enzyme that catalyzes the stepwise formationofthree molecules of malonyl-CoA (Orhan and Üstün, 2011) from 4-coumaroyl-CoA (Woldegiorgis *et al*., 2014; Orhan and Üstün, 2011) produces chalcone naringenin (Unekwu *et al*., 2014), the precursor of a large variety of flavonoids. In the last stages of flavonoid biosynthesis, naringenin chalcone is converted to naringenin by chalcone isomerase (CHI), and further flavonoids are formed.

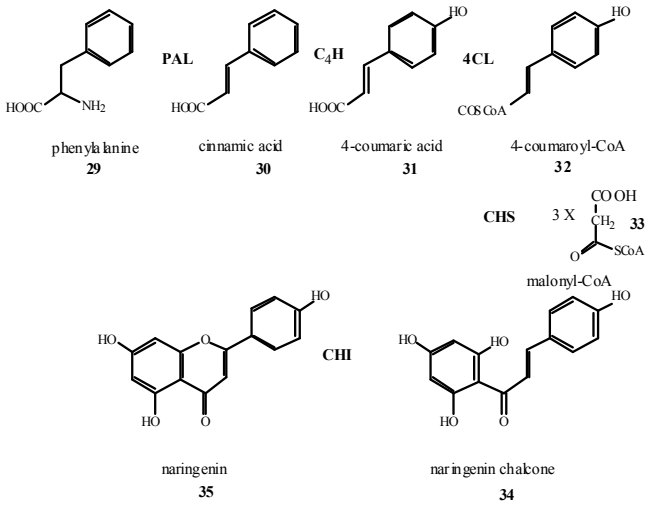

Figure 6. Biosynthesis of flavonoid naringenin (Du *et al*., 2011)

There are no reports of the enzymes C4H, 4CL, CHS or CHI in fungi, essential for the biosynthesis of flavonoids in superior plants (Figure 6); and so, it has been stated that fungi are not able to produce flavonoids (Ramírez *et al*., 2016). However, the biosynthetic pathway in the mushrooms has not been studied and would be a considerable contribution.There are reports relating the flavonoid content with the presence of metals in the fungi's fruiting bodies, arising as a defense mechanism through the production of flavonoids capable of sequestering metals, avoiding its toxic effects (Pham Anh Tuan *et al*., 2014). In this study, the flavonoid content in *R flava*was not detected, despite it was reported (Yahia *et al*., 2017) in 4.67 mg eq catechine /g dry arrow for the same species. The difference mg eq. of quercetin (this study) or catechin (21) is irrelevant. Further discussion of the results found in the quantification of some metals in the fruiting body of *R. flava* and its relation to the content of flavonoids is made according to (Gąsecka *et al*., 2017) to distinguish the influence they exert on the total content of these secondary metabolites. *β-D-glucans*. While the plant's cell wall constitutes a vital source of cellulosic dietary fiber, the fungi cell walls contain a mixture of other fibrous components such as chitin, β-D-glucans and mannanes (Cheung *et al*., 2013). The ß-D-glucans have interesting anticancer properties. However, a large number of fungi wild species (edible and non-edible) have not

been investigated searching for β-D-glucans. In Mexico, no reported data provide information regarding the content of β-D-glucans in wild species and in particular, for *R.flava*. This study did not detectβ-D-glucans for this species. Significant differences were found in reported β-D-glucan contents for some mushrooms. Thus, a value of 214.02 mg of ß-glucans/g of dry mushroom for *A. caesarea*was reported in our lab CITA, while Rahar (Rahar *et al*., 2011) reported 0.4 mg of ß-glucans/g of dried mushroom for same species. According to (Rahar *et al*., 2011) the ß-glucans content depends on the habitat, mainly influenced by the altitude and the surroundingvegetation cover. The samples analyzed here were collected at an average altitude of 2620 masl, while those reported by Rahar (Rahar *et al*., 2011) correspond to an altitude of 1,100 masl, this factor is consistent with the low content that these authors also report. Pancho, este articulo es solo de f flava, yo quitara todo este parrafo, Esto Esta aquí, seguramentepor la tesis de Eric, que tenia en la tabla la A cesarea. *Metals.* The metal determination resultsare shown in Table 3. Numerous studies point to fungi as bioaccumulating organisms of metals through an extensive network of mycelium that extends below the ground, for which a variety of different mechanisms have been proposed (Kalač, 2010; Durkan *et al*., 2011). Due to their metals bioaccumulativeability, fungi have been placed as bioindicators of the environmental quality. The BAI (Bioaccumulation Index) defined the proportion of an element concentration in a fruiting body andthe soil. BAI quantifies the fungus ability to accumulate a particular element (Falandysz and Borovička, 2013). This capacityis influenced by biochemical processes to survive and tolerate its metal intake. Among the described mechanisms for metals bioaccumulation in fungal fruiting bodies are: (i) metals cell wall binding; (ii) vacuolar sequestration; (iii) production of chelating metallothioneins, glutathione and plastocyanin; (iv) metals and metalloids toxic effects reducethe oxidative stress through the depleted generation of oxygen reactive species, by means the antioxidant enzymatic system (superoxide dismutases, catalases, glutathione peroxidases)and reactions involving low molecular weight organic substances such as ascorbic acid, tocopherol and phenolic compounds (Pham Anh Tuan *et al*., 2014).

The accumulation of toxic metals is influenced by the fungi symbiosiswith the roots of the trees, acting as attenuators of the metal uptake by the plant, and bioaccumulating them in their fruiting bodies. Other factors affect the metals translocation to the fungus fruiting body such as soil pH, organic matter content and the genotype in species (Gadd, 2007). Another incoming form of toxic metals into the fungal body is through airways (deposition of air contaminated by aerosols, dust, fumes), especially for toxic elements such as Cd, Pb, and Hg. In some cases, the bioaccumulated amount is related to the size of the fruiting body (Michelot *et al*., 1998). The Cd and Pb concentrations found in *R. flava* fruiting body were below the detection limits of the technique (<0.0006 mg/kg,), as well as soils.So, it was not possible to evaluate theirBAI factor. Several Pb and Cd hyperaccumulating mushrooms are described in the literature, such as *Lycoperdon foetidum*, *Abortiporus biennis*, *Amanita strobiliformis* and *Amanita caesarea* (Aloupi *et al*., 2012; Borovička *et al*., 2007; Riganakos *et al*., 2009; Wilkoiazka *et al*., 2011). In (Schweiger *et al*., 2009) report for different species of the genus *Amanita*, high amounts of Cd attributable mainly to the collection of these species in contaminated areas. Regarding the species analyzed here, concentrations for Cd and Pb were not prevention at levels higher than the limit of detection in mushrooms or soil, which can be inferred that the area studied is free of contamination by Cd or Pb.PARA QUE SEGUIR HABLANDO DE ESTO, SI NO HAY ESOS METALES EN EL SUELO?

The Cr concentration in the *R. flava*fruiting body resulted16 ± 0.34 mg/kg dry mushroom, while in the surrounding soil was 5.58 ± 0.48 mg/kg (Table 3), which is a non-contaminated area (limit over 0.5 - 10 mg/kg of soil (Pham Anh Tuan *et al*., 2014). Moreover, the BAI resulted in 0.75,which denotes a Cr high bioaccumulationcapacity, considering that the soil is not classified as contaminatedwith this metal (Kalačk, 2010). Cu was found abundantly in *R. flava*, resulting 1141.4 mg/kg dry arrow, while the soil content was 1141 mg/kg. Then, the study area iscontaminated.Cu values of more than 76 mg/kg (Nikkarinen and Mertanen, 2004) and 12.81-39.7 mg/kg have been reportedfor contaminated soils (Pham Anh Tuan *et al*., 2014). BAI_{Cu} resulted very high (9.88), and so, the R flavais considered a hyperaccumulatingspecies (Table 3). The high origin of Cu in the forest-agricultural zone is unknown. Probably, it might be due to fungicides used for forest areas in Acaxochitlán based on oxychloride and copper sulfate. It is known that fungi are efficient Cu bioaccumulating organisms compared to plants (Firdousi, 2017). The Cu range for wild mushroom species collected at uncontaminated sites was reported between 20 and 100 mg/kg dry matter. Reported fungi with high Cu bioaccumulation capacity (> 200 mg/kg) are *Macrolepiota procera*, *Calvatia utriformis*, *Agaricus bisporus* (Kalačk, 2010). However, the value found herefor *R flava* greatly exceeded the previously reported ranges. The high Cu contamination in the soilprovoked the remarkable bioaccumulationby thefungi in their fruiting bodies, probably through cysteine-rich low molecular weight proteins called metallothioneins (Gadd, 2007; Collin *et al*., 2002) whose Cu cofactor role are involved in oxidation-reduction reactions.It has been proposed that the function of the metallothioneins be to keep low the ionic Cu concentration and to allow the enzymatic activation by the metal as well as in the detoxification there of (Brambila and González, 1993). This finding is likely related to the high chlorogenic acid content reported in this study. The recommended tolerated daily intake (RTDI) for this metal is 30 mg daily (calculated for an adult), andthe ingestionof 300 g fresh mushroom would contribute114%of the Cu RTDI value. Therefore,dailyintake of wild *R flava*cropped in Cu rich soils is recommendedbelow 300 g,and the gastronomic and nutrimental value of the edible mushroomscan be takenan advantage. Fe is an abundant element frequently found in soils. *R. flava*showeda concentration of 471.5 mg/kg dry weight. The very high values in soils indicate that the study area (Acaxochitlán, "Los Reyes")is a Fe contaminated zone, althoughit is surrounded by vast humid forests with some agriculture and no mining activity. Soilshave been previously studied, reporting Fe as iron oxides (goethite) and Al as kaolinite and halloysite (Acevedo *et al*., 2010). The low BAI_{Fe} calculated (0.009) allows to catalog *R. flava*as Fe hypoaccumulator, but the significant content could affect the man. *A. muscaria*collected in Poland contains 67 mg of Fe/kg dry weight (Lipka and Falandysz, 2017). The present study reports a value about seven times higher in the*R. flava* species.Fe is a vital element for almost all living organisms;however, high amounts can be toxic. Fungi can restrict the entry of toxic metal species into their cells through various mechanisms such as 1) reduction of the metal intake and/or increase of its flow, 2) immobilization of the metal through the absorption in the cell wall, extracellular

Table 3. Cr, Cu, Fe, and Mn contents in *R. flava* and soil samples and bioaccumulation index (BAI). In brackets the standard deviation

	Cr*	BAI_{Cr}	Cu*	BAI_{Cu}	Fe*	BAI_{Fe}	Mn*	BAI_{Mn}
Soil	5.58 (0.5)	0.75(0.05)	115 (3)	9.9 (0.4)	53000 42)	0.009(0.001)	193 (2)	0.19 (0.01)
R. flava	4.16 (0.3)		1141 (8)		471 (5)		36 (1)	

*mg/kg dry weight

Table 4. Al, Mg and K contents in *R. flava* and soil samples and bioaccumulation index (BAI). In brackets the standard deviation

	Al*	BAI_{Al}	Mg*	BAI_{Mg}	K*	BAI_{K}
Soil	36476 (13)	0.048 (0.002)	543 (3)	1.06 (0.4)	1053 (6)	7.2 (0.1)
R. flava	1763 (10)		577 (3)		7623 (8)	

*mg/kg dry weight

precipitation of neoformed secondary minerals (e.g., oxalates) and 3) extracellular sequestration by exopolysaccharides and other extracellular metabolites. Fungi that are metal tolerant may survive because of their intracellular chelation abilities by the formation of metallothioneins and phytochelatins, and thereby achieve sequestration and immobilization of the metal in the vacuoles. Fungal vacuoles play an essential role in the concentration regulation of metal ions and the detoxification of potentially toxic metals bysequesteringmetals, principally Mn^{2+} and $Fe^{2+}$and monovalent cations like K^{+} (Gadd, 2007). When comparing the amount of Fe concerning the TPC, an inverse relationship is observed. Thus species-rich in total phenolic compounds are low in Fe, and conversely, Fe-rich mushrooms are low in TPC contents. On the other hand, the content of ß-glucans is also affected inversely by the presence of Fe in mushrooms. Thus, in *A. caesarea* Fe is abundant and the β-glucan content is small in comparison to *S. calvatus*, in which case the β-glucan content has been higher but is low in Fe content. In (Kostic *et al*., 2013) have demonstrated the inverse relationship between high Fe contents and low phenolic compounds in plants while also shows (Gursoy *et al*., 2009) an inverse relationship between the amount of total phenolic compounds in *Morchella* samples concerning the Fe concentration. Esto es de todos los hongos de la tesis, no del articulo. Mn is useful for plants as a structural component of chloroplasts and in oxidation-reduction reactions.It is essential for biogeochemical processes in white rot fungi, as a cofactor of the lignin-degrading enzyme (Richardson, 2017). In the present research, *R flav*apresent Mnin moderate content (36.2 mg/kg) despite thehigh concentration of 193.0 mg/kg in thesoil,resulting in a low BAI_{Mn} for *R. flava*and is classified as hypoaccumulator of this metal. Species with high Mn content showed low total TPC contents, as well as β-glucans. Accordingly, it is possible to infer that the presence of this metal attenuates the existence of phenolic compounds and β-glucans. For *A. muscaria*, 24 mg/kg has been reported (Lipka and Falandysz, 2017). Mn in moderate amounts helps the human body in the proper function of vitamins B1 and E, the production of the hormone thyroxine, as well as the production of acetylcholine, a powerful neurotransmitter. Concerning the RTDI of the Mn, the *R. flava* species contributes 36%. Al is a metal present in the earth's crust with an abundance of 80,000 mg/kg in soils and papers have reported large range contents for several fungi speciesbetween 1500 - 50 mg/kg in fruiting bodies (Durkan *et al*., 2011). In this work, the Al in *R. flava*was found at a concentration of 1762.9 mg/kg in dry weight (Table 4), while soil concentrations were found at 36476.2 mg/kg at two different points in Los Reyes, Acaxochitlán. The high Al content present in soils is notable as it comes from mineralogical sources (Acevedo *et al*., 2010). The high presence of Al and Fe in the studied areas comes from the geological formation of Atotonilco el Grande, constituted by basaltic rocks of the tertiary period, which through the passage of time have undergone processes of aggressive weathering given by the presence of clay and oxides of iron and aluminum (Acevedo *et al*., 2010). On the other hand, the low BAI_{Al} calculated value allowsbeing classified as hypoaccumulatory. Al does not play any specific role in fungi that facilitated bioaccumulation. Notwithstanding the Al-hypoaccumulatory character of *R flava*, the high content of this element in edible mushrooms stands out.

The RTDI for this metal is 0.75 mg/kg bw/d (55 mg/d calculated for an adult) (INCHEM, 2016), andthe ingestion of 300 g fresh mushroom (30 g dry mushroom) would contribute56 %of the AlRTDI value. Therefore, dailyintake of wild *R flava*cropped in Al-rich soils is safe. The soils analyzed presented homogeneous Mg contents (543.09 ± 3.16 mg/kg dry soil) and *R. Flava* presents 577 ±3 mg/kg dry mushroom (Table 4). A total content of 1250 mg/kg for this species has been reportedrecently, more than twice Mg as described here (Falandysz and Jarzyńska, 2017); and years ago, a similar but somewhat higher report of 702.5 mg/kg (Kishner *et al*., 1982). The calculated BAI_{Mg} was 1.06, which shows the moderate Mg-hyperaccumulating nature of *R. flava*. The hyperaccumulating capacity of the mushrooms is notorious for the high content of fruiting bodies concerning the soils. The Mg uptake and bioaccumulation occurby the organic acids release (succinic and oxalic) that solubilize the calcium and magnesium carbonate present in limestone and dolomite soil, allowing their absorptionas salts through the mycelial networks (Gadd, 2007). This bioaccumulating capacity is linked to the essential metabolic functions vital to the growth of the fungus as it functions as an essential element. All the mushrooms supply moderate Mg amounts to the Daily Intake.Considering the recommended RTDI for Mg, a fresh portion of 300g (30g dry *R. flava*) contributes by 4%. Although mushrooms are not sourced rich in Mg, they are considered valuable promoters of health because oftheir antioxidant properties and other essential minerals.

Among the metals studied, K was the most abundant. The concentration found in *R. flava* was 7632.2 mg/kg in dry weight. For fungi, values of 3,000 mg/kg K have been reported, as it is the case of *S. granulatu* (Gadd, 2007). However, this studyfound that *R. flava* has twice the amount reported. According to WHO, the intake for an adult should be 3600 mg/day, and 300 g of this mushroom can provide 7% of the total RTDI. In contrast to high values obtained here, a low content of 35.250 mg/kg was recently published (69) for *A. muscaria*. For K, the inverse relationship between concentration in fruiting bodies concerning the total content of

phenolic compounds, β-glucans and the reducing and inhibiting effect of the DPPH radical is quite impressive. It is widely known that fungi bioaccumulate K, and the highest BAI_k values have been reported for the genus Amanita (57.21), which hadbeen attributed to mineral solubilization by the release of carboxylic acids from the mycelium (Falandysz and Jarzyńska, 2017). It has been discussed the observed relationship between the concentration of some metals such as Fe, Mn, Al and K with the antioxidant properties in species studied. We found an inverse relationship and some proportional relationship. NO SE HA MOSTRADO NINGUNA GRAFICA ES UN SOLO HONGO. *Extract characterization. R. flava* is an abundant species and allowed enoughextract to carry out the chromatographic separation and purification of substances. A significantmetabolite was isolated with AcOEt by column chromatographyfrom the fractions 34-47 and further purified.A crystalline solid was isolated and characterized. The FTIR spectrumis shown in Figure 7. The main bands are assigned, such as 3432 cm^{-1} broadband corresponding to the -OH stretching,3045 and 2954 cm^{-1} absorptions of the aromatic and aliphatic CH stretching,the1630 cm^{-1} band of C=C stretching,some CH bending in the range 1400 to 1500 cm^{-1} and the C-O stretching at 1039 cm^{-1}. The spectrum is consistent with the ergosterol IR reported in the literature (Kishner *et al*., 1982).

The ^{1}H NMR spectrum in $CDCl_3$at r.t.of a sample of 22 mg crystalline solid isolated from column chromatography hexane:AcOEt (4:1) fractions 34-47yielded the following signals: two CH_3groups at δ 0.56 (H-18 3H, s) and δ 0.88 ppm (H-19, 3H, s)and four CH_3 at δ 0.76 (H-26, H-27); 0.85 (H-21) and 0.97(H-28) (3H, d, J = 6.6 Hz, each).CH groups at δ 2.21 (H-4b,1H, t, J = 12.9 Hz), 2.40 (H4a,1H, ddd, J = 14.2, 4.2 and 2.6 Hz) and 3.57 (H3, 1H, m).Vinylic protons signals are at δ 5.13 (H-22, H-23, 2H, m); 5.50 (H-6,1H, m) and 5.31 (H-7,1H, m) (Figure 8). The assignments were compared with the 1H NMR (Table 5) and 13C (Table 6) spectroscopic data reported by (71),concluding the identity of the isolated compound as ergosterol, a secondary metabolite present in the Ramaria flava from Acaxochitlán, Hidalgo, Mexico.

The ^{13}C NMR spectrum in $CDCl_3$confirm the ergosterol signals, assigned to the following carbons: six primary CH_3groups at δ 12.1, 16.3, 17.6, 19.7 and 21.1 ppm for carbons C-18, C-19, C-28, C-26, C-27, and C-21, respectively. The quaternary carbons signals observed at δ 141.1, 139.8, corresponding to the C-5, C-8, and the alkene CH atoms at δ 135.6, 132.0, 119.6 and 116.3 assigned to C-22, C-23, C-6, and C-7 respectively (Figure 9).

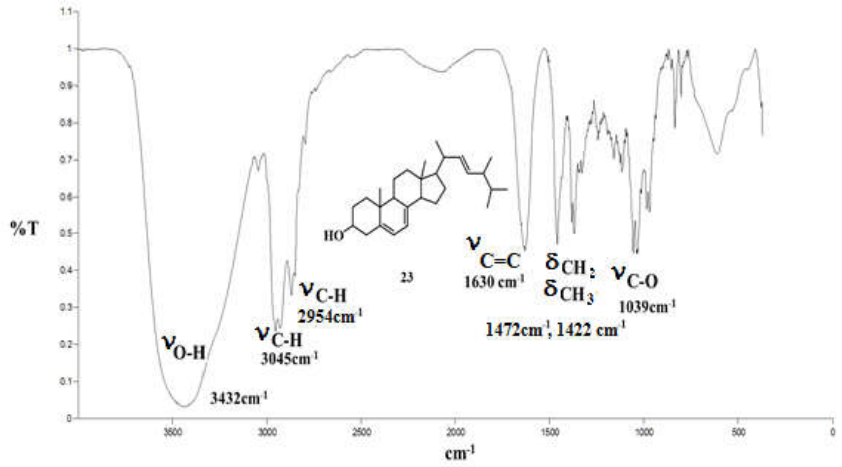

Figure 7. IR spectrum of the secondary metabolite isolated from fractions 34-47. Pancho hay que aumentar la resolucion de esta imagen.le arreglé las asignaciones

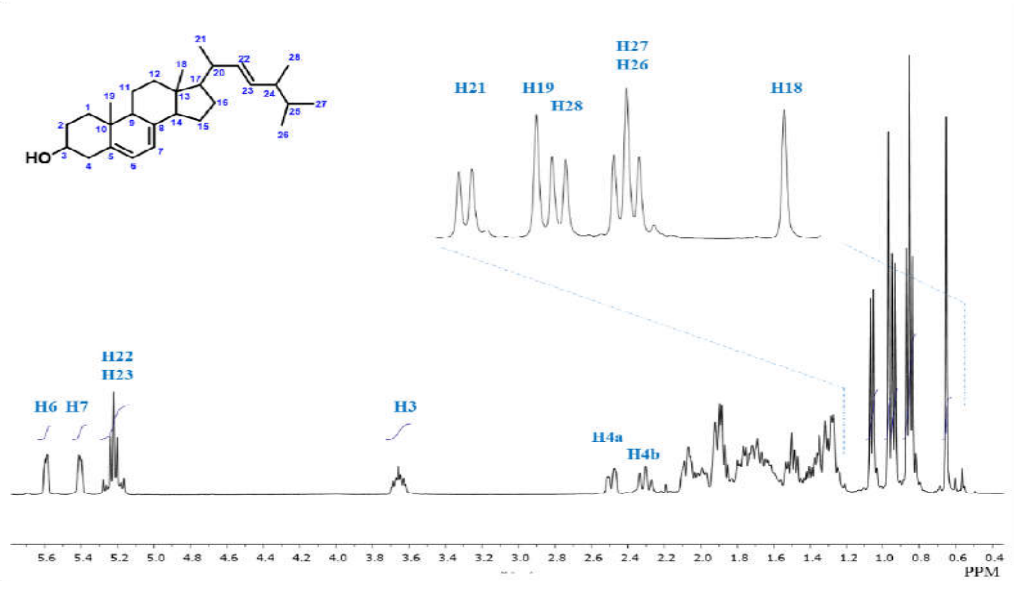

Figure 8. ^{1}H-NMR spectrum ($CDCl_3$) of theisolated metaboliteat 400 MHz. Identified as ergosterol

Table 6. ^{13}C NMR chemical shifts ($CDCl_3$) for ergosterol (Carmen and Bacigalupo, 2012) and the isolated metabolite

Signal	(δ)ppm[A]	(δ)ppm[B]	Fragment Type
C-1	38.4	38.3	CH_2
C-2	32	31.9	CH_2
C-3	70.5	70.4	CH
C-4	40.8	40.7	CH_2
C-5	141.1	141.3	C
C-6	119.6	119.6	CH
C-7	116.3	116.3	CH
C-8	139.8	139.7	C
C-9	46.3	46.2	CH
C-10	37	37	C
C-11	21.1	21.1	CH_2
C-12	39.1	39.1	CH_2
C-13	42.8	42.8	C
C-14	54.6	54.5	CH
C-15	23	23	CH_2
C-16	28.3	28.2	CH_2
C-17	55.8	55.7	CH
C-18	12.1	12	CH_3
C-19	16.3	16.2	CH_3
C-20	40.4	40.4	CH
C-21	21.1	21.1	CH_3
C-22	135.6	135.5	CH
C-23	132	131.9	CH
C-24	42.8	42.8	CH
C-25	33.1	33	CH
C-26	19.6	19.6	CH_3
C-27	20	19.9	CH_3
C-28	17.6	17.6	CH_3

a: Reported (71).b: This work.^{13}C NMR spectrum at 100 MHz $CDCl_3$

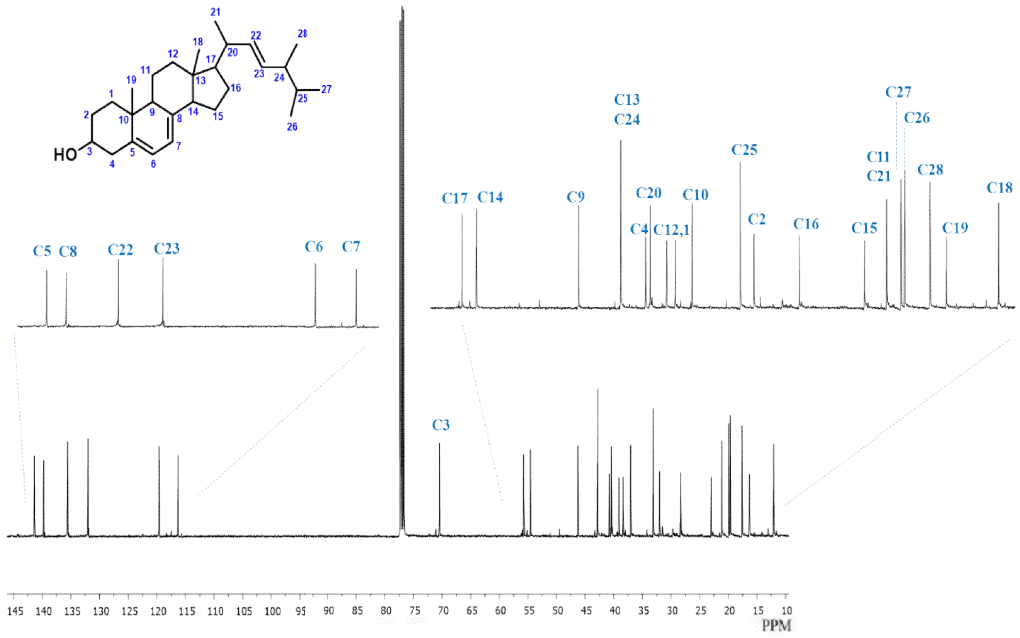

Figure 9. ^{13}C NMR spectrum of (23) in 100 MHz $CDCl_3$

Table. ^{1}H NMR chemical shifts ($CDCl_3$) for ergosterol (71)and the isolated metabolite

Signal	(δ) ppm[a]	I[a]	M[a]		*J* (Hz)[a]		(δ) ppm[b]	I[b]	M[b]		*J* (Hz)[b]	
H-3a	3.57	1H	tt	11.2	4.2		3.63	1H	m			
H-4a	2.40	1H	ddd	14.3	4.2	2.7	2.47	1H	ddd	14.2	4.5	2.2
H-4b	2.21	1H	t	12.9			2.27	1H	t	11.9		
H-6	5.50	1H	d	3.7			5.57	1H	m			
H-7	5.31	1H	dd	2.7	2.7	2.7	5.37	1H	m			
H-18	0.56	3H	s				0.63	3H				
H-19	0.88	3H	d	-			0.94	3H	s			
H-21	0.85	3H	d	6.6			1.03	3H	d	6.6		
H-22 H-23	5.13	2H	m				5.19	2H	m			
H-26 H-27	0.76	6H	d	6.4			0.81	3H	d	6.6		
H-28	0.97	3H	d	6.6			0.92	3H	d	6.8		

a: Reported (71).b: This work.^{1}H NMR spectrum at 400 MHz $CDCl_3$.I = Integral, M = Multiplicity.

Conclusion

The chemical and nutraceutical characteristics of the wild and edible species *Ramaria flava*are reported, concerning their antioxidant activities,total phenolic compounds, % DPPH radical inhibition, the% of Fe^{3+}reducing theeffect, ascorbic acid and total flavonoids contents. A study was carried out about the presence of essential, non-essential and toxic metals in the mushroom fruitbody related tothe quality of the soil where they were collected.The metal Bioaccumulation Indiceswere calculated and reported in this paper. The soils quality in the study area where *R. flava* samples were collected was determined by chemical analyses, and high levels of Cu and Fe were observed, coincident with previous literature reports that classified the area as contaminated. A relation between the unexpected low content of gallic acid together with the high chlorogenic acid concentration and the high value of BAI_{Cu} determined for R flava is proposed, based on the reported data.

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