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In vitro MOLECULAR IDENTIFICATION AND CHARACTERIZATION OF *Pleurotus* spp. STRAINS IN GUANAJUATO, MEXICO

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ABSTRACT

The consumption of edible fungi has increased worldwide due to its nutritional and functional properties. In the state of Guanajuato, Mexico, the production of these fungi depends on the import of strains from different species. This is due to a lack of isolation and characterization of regional mycogenetic resources such as in the case of the "Maguey mushroom". The aim of this study was to carry out the *in vitro* molecular identification, morphological characterization, and antioxidant biochemistry of a strain of *Pleurotus* sp. in Guanajuato, Mexico, and to compare it to commercial strains. The hypothesis proposed is based on the possibility of isolating a regional strain with adequate mycelial characteristics for its *in vitro* cultivation. A wild strain called UG-01 was collected and isolated from an *Agave mapisaga* plant and its identity was determined by sequencing the region ITS1-5.8S-ITS2. The strain was cultivated *in vitro*, and the mycelium was characterized; an experimental strain and four commercial ones were included as a control. The results were analysed in a totally randomized design. The "Maguey mushroom" strain was identified as *Pleurotus djamor*. Based on the CIE L*a*b system, the coordinates 86.2, -5.6 and -4.1 were obtained, along with 33.1 and 21.7 for Hue and Chroma. The UG-01 strain displayed the highest growth rate with a cottonlike structure and without exudates. In addition, it presented the highest concentration of proline, phenolic compounds, and flavonoids, as well as the lowest remains of 2,2-diphenyl-1-picrylhydrazyl (DPPH). In conclusion, it was possible to cultivate the mycelium of the "Maguey mushroom" *in vitro*, which displayed better morphological and antioxidant biochemistry in comparison with the imported commercial strains.

Keywords: Edible fungi, analysis of images, mycelial growth, antioxidants.

INTRODUCTION

The genus *Pleurotus* represents one of the most important groups of cultivated edible fungi worldwide, since it is second place in terms of production and makes up 19 % of the world production, with 6.46 x 10^6 Mg annually. It is known for being efficient in degrading lignocellulosic waste and for producing edible basidia with highly nutraceutical and organoleptic qualities, as well as vitamins, minerals, and some essential amino acids (Musieba *et al*., 2013).

Basidia of this genus are considered a functional food. They also have antioxidant (Huaping *et al*., 2019), antibiotic, antitumoral (Al-Saffar *et al*., 2020), anti-inflammatory and antimicrobial properties (Bains *et al*., 2020). Active compounds have been isolated from the extracts of this fungus, which are used to treat several chronic degenerative diseases. The species of *Pleurotus* are used in the areas of pharmacology, the food industry, environmental science, and nutrition (Chaurasia *et al*., 2020).

In Mexico, the production of *Pleurotus* at an industrial scale is carried out with foreign strains (León-Avendaño *et al*., 2013). However, there are native genetic resources that make up a broad genetic base with the potential to produce and obtain biologically active secondary metabolites, which can adapt to the environmental conditions of arid regions, such as the species known as the "Maguey mushroom" (*Pleurotus* sp.), which grows in the wild on the necro mass of "agave pulquero" plants.

This type of fungus is appreciated and sold in the central area of the country due to its pleasant taste (Barrales and Mata, 2016). The presence of this fungus has been found in the semi-arid areas of central Mexico, though its distribution may be wider, since the *Agave* genus is typical of the Mexican arid and semi-arid ecosystems, which cover approximately 60 % of Mexico's territory (Montaño *et al*., 2016).

The genus *Pleurotus* represents a wide genetic variability, and therefore the morphological characteristics at *in vitro* cultivation may contribute to a more complete characterization of the organisms. These characteristics can also be used as a parameter of selection to obtain more productive strains (Cardoso *et al*., 2017). The vast majority of studies on the properties of fungi use the fruit-bearing bodies. However, secondary metabolite compounds are also found in the mycelium. The hypothesis presented is based on the possibility of the isolation of a regional strain with adequate mycelial characteristics for its *in vitro* cultivation. The aim of the present study was to perform an *in vitro* molecular identification, morphological characterization, and antioxidant biochemistry of a strain of *Pleurotus* sp. in Guanajuato, Mexico.

MATERIALS AND METHODS

Collection and isolation

A strain of *Pleurotus* sp. was isolated from an adult plant of *Agave mapisaga* species in the Centro Nacional de Agaves (National Agave Center) of the University of Guanajuato in Mexico. The fungus collected was found in the wild in Copal, Irapuato, Guanajuato; located in the coordinates 20° 40 22" N, 101° 20 53" W, at an altitude of 1715 m. The climate is semi-warm and sub-humid, with rains in the summer Acw, and temperatures ranging from 12 to 26 °C, an average annual rainfall of 716 mm and the predominant vegetation is xeric shrubland. A specimen was collected and placed in a previously disinfected container. The carpophore was disinfected with 2 % NaClO for 1 min and was later rinsed using sterile distilled water. The mycelium was isolated vegetatively, cutting 1 cm^2 pieces from the middle of the fruit-bearing body.

For cultivation *in vitro*, a potato-dextrose-agar medium (PDA) was used in Petri dishes with a volume of 95.4 cm^3. The inoculated dishes were incubated at 25 °C in

complete darkness. Several sub-cultivations were performed until pure cultivations were obtained. As a control, the strain IE-837 was included, which had been donated by the Mexican Institute of Ecology (Instituto de Ecología A. C.) (Barrales and Mata, 2016) and four introduced strains which are produced and sold in the area, and which were labelled GR-1, BR-1, BL-1, and BL-2.

Mycelial characterization

From the cultivations *in vitro,* the mycelial growth (MG; cm d^{-1}) was determined by transferring mycelium fragments, 1 cm in diameter, to Petri dishes with freshly prepared PDA medium and incubating at 25 °C. The growth was recorded after 6 d, based on the measurements of two-dimensional distances on the axes (*x,* y) previously traced from the centre of each dish. The Growth Rate (GR; cm d^{-1}) was determined using the formula by Benítez *et al.* (2007). When the mycelium covered the medium in its entirety, digital images were taken under controlled and homogenous conditions, using a box with a white interior and a constant white LED light.

The images were taken using a 13 mega pixel Nikon W150 digital camera, with an HD resolution of 1400 x 720 pixels. The images were taken at a distance of 9.5 cm between the lens and the culture and analysed with a recognition algorithm specifically designed for this study using the software MATLAB R2019a, manufactured by MathWorks®. The color of the samples was determined using the system CIE L*a*b, to then calculate the Hue angle and the Chroma index. Based on the saturation of pixels, the density of the mycelium was established (DEN; g cm^{-3}).

The texture (TEX) was analysed using the visual keys described by Carreño-Ruiz *et al.,* (2013), as well as the presence and/or absence of exudates (EXU). Regarding the biochemical characterization of the mycelium, the concentration of proline (PRO; μg mL^{-1}) was established with the reaction of this amino acid with 2,2-DihydroxyIndeno-1,3-dione, and the spectrophotometric measurement was performed at 517 nm. The concentration of phenolic compounds (PC; μg mL^{-1} of gallic acid) was carried out by spectrophotometry at 750 nm based on the formation of the phosphotungstic-phosphomolybdenum complex. For both PRO and PC, the quantification was carried out with calibration curves ($r^2 > 0.99$).

Antioxidant activity was analysed with 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). Absorbances were determined by their spectrophotometric measurements at 515 and 754 nm, respectively. For both determinations, the results were reported as the percentage of remaining radical.

Molecular identification

Mycelium samples of each strain were planted in a PDA medium. From the recovered mycelium, the genomic DNA was extracted, following the methodology described by Gómez-Luna *et al*. (2012). The integrity of the samples was evaluated by electrophoresis in 1 % agarose gel with a TBE 1X solution, which was stained

with GelRed® (Biotium). The purity as determined by spectrophotometry and the concentrations were normalized at 200 ng μL^{-1}. The region ITS1-5.8S-ITS2 was then amplified by PCR using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990).

Amplifications were performed using the kit MyTaq™ Mix (Bioline), using 50 µL reactions containing 200 ng of DNA, 20 µM of each primer and 50 µL of the Mix 2x. The conditions of the reaction consisted of 30 denaturalization cycles at 95 °C for 5 min, alignment at 51 °C for 30 s and synthesis at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The PCR products were sequenced by capillary technology using the method by Sanger *et al.* (1977). The BigDye® Terminator v. 3.1 kit (Applied Biosystems) and the platform ABI 3730xl (Applied Biosystems) were used.

The identities of the sequences were determined by comparison in the GenBank data base, with the option BLAST 1.17. Alignments were carried out between the sequences obtained using the software Clustal W 2.1, the phylogenetic tree was created using the software FastTree 2.1.8 and the neighbour-joining method (Saitou and Nei, 1987).

Statistical analysis

Quantitative data (MG, GR, and DEN) were analysed in a totally randomized design with five repetitions performing Tukey mean separation tests ($p \leq 0.05$). For the qualitative variables TEX and EXU, results were analysed in a totally randomized design with 10 repetitions using the Kruskal-Wallis test. All statistical analyses were carried out using the Minitab® 16.2.3 (Minitab, 2010).

RESULTS AND DISCUSSION

Description of the environment

The "Maguey mushroom" was found on the necro mass of *Agave mapisaga* plants from September to December. Barrales and Mata (2016) found *Pleurotus opuntiae* growing on *Agave salmiana* Otto plants in a temperate semi-dry BS1k climate at 2340 m and a temperature of 12-14 °C.

In situ morphological characterization

The specimen collected presented the following morphological characteristics: smooth pileum between 80 and 125 mm, hymenium with creases, insertion of decurrent sheet, wavy margin, eccentric stipe or no stipe, white spores, no volva, or ring. Phylogenetic studies report that *P. agaves, P. opuntiae* and *P. djamor* grow in Mexico (Zervakis *et al.*, 2019). The fungus found in maguey plants has been morphologically classified by some authors as *Pleurotus opuntiae* (Durieu et Lév.) (Camacho *et al.*, 2012).

Molecular identification

PCR products were obtained between 600 and 650 base pairs (bp) with oligonucleotides ITS1-ITS4. The UG-01 strain collected was identified as *Pleurotus djamor* with a maximum identity of 99.3 % (E < 0.0) with the accession number KX573927. The strain

IE-837 (Barrales and Mata, 2016) was identified as *Pleurotus flabellatus*, with a maximum identity of 99.8 % (E < 0.0) with accession number MF459667.1. The commercial strain BL-1 corresponded by 98.7 % (E < 0.0) to *Pleurotus ferulaginis* with accession number MF076894.1, whereas strains BL-2 (99.8 %, E < 0.0, LT627806.1), BR-1 (98.6 %, E < 0.0, KY686275.1) and GR-1 (99.2 %, E < 0.0, LC602510.1) were identified as *Pleurotus ostreatus*. This is one of the most economically important species in the country; it is imported from Europe (Zervakis *et al.*, 2019), Asia and North America.

The species *Pleurotus djamor* presents a pantropical distribution (Salmones, 2017). In Mexico, it has been gathered in tropical and subtropical areas of the south (Chiapas, Tabasco, and Yucatán), centre (Puebla and Morelos), west (Jalisco and Michoacán) and east (Veracruz). It grows on dead trunks and branches of diverse tree and bush species.

The phylogenetic tree shows that the strain UG-01 is found in an independent group, indicating that it is further related to the other strains. It also indicates that commercial strains BL-2 and BL-1 share a common recent ancestor, along with strains BR-1 and GR-1 (Figure 1), and the commercial strains are equally related to the wild strain IE-837.

In vitro morphological characterization

Significant differences were found ($p \leq 0.05$) in luminosity (L), with values ranging from 86.2 to 87.6, with the highest value in strain IE-837. Regarding coordinate a*,

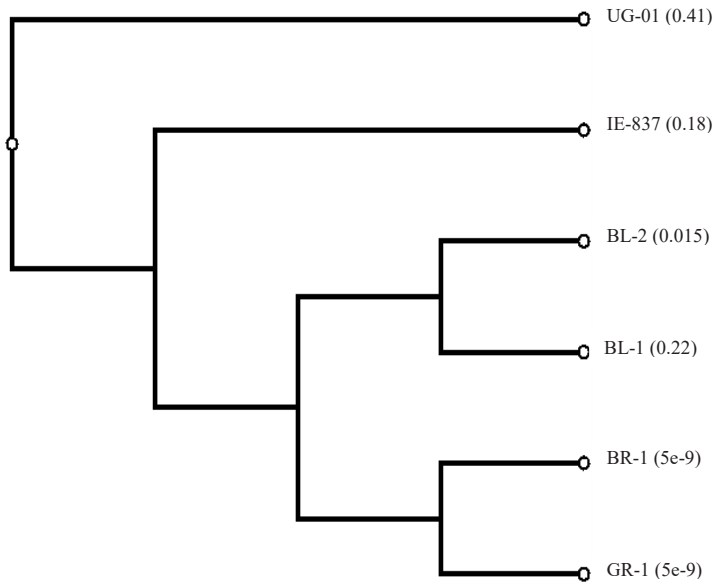

Figure 1. Phylogenetic tree of *Pleurotus* spp. strains. IE-837: donated strain, *Pleurotus flabellatus*, UG-01: gathered strain, *Pleurotus djamor*, BL-2: commercial strain, *Pleurotus ostreatus*, BL-1: commercial strain, *Pleurotus ferulaginis*, BR-1: commercial strain, *Pleurotus ostreatus*, GR-1: commercial strain *Pleurotus ostreatus*.

significant differences were found ($p \leq 0.01$). Negative values indicate green tones, with strains BL-1 and BR-1 being those that were located in this space (Table 1).

In coordinate b*, significant differences were identified ($p \leq 0.05$); the negative values for b* were coloured blue, and the strain that presented pigments of this colour was GR-1, with a value of -4.6. In the case of the Hue index, significant differences were identified ($p \leq 0.01$) with values between 33.1 and 38.5. In the case of the Hue index, values from 0 to 90 indicate a tendency that gradually goes from red to a combination with yellow until the latter is fully defined. Colour is related to the presence of some secondary metabolites; phenolic compounds display colours such as red, pink, blue or purple, whereas some flavonoids display colours in the yellowish-white tones (Gülçin, 2012).

So far, most studies have used visual keys or references to determine the colour by comparison. According to this method, the species *Pleurotus djamor* presents a white or yellowish mycelium (León-Avendaño *et al.,* 2013).

The analysis of images of mycelia helped differentiate variations in the colour that would correspond to an equally different biochemical composition (Figure 2).

Regarding MG, significant differences were found between treatments ($p \leq 0.01$). Strain UG-01 covered 100 % of the area of the Petri dish after 6 d. In general terms, the commercial strains had a similar growth, whereas IE-837 covered 55 % in the same period, displaying slower growth. This difference may be due to this strain coming from an area 2345 m, in which the average temperature is 12-14 °C. Ahmad *et al*. (2015) reported that *Pleurotus djamor* grew in 5.25 d. The difference in mycelial growth relates to the type of enzymes present in each species and their ability to make use of energy sources**.**

Table 1. Morphological characteristics evaluated in the mycelia of the *Pleurotus* spp. strains.

Variables	UG-01	IE-837	BL-1	BL-2	BR-1	GR-1
Quantitative						
L	86.2 b	87.6 a	86.9 ab	87.0 ab	87.2 ab	86.8 ab
a	-5.6 bc	-5.0 ab	-5.7 c	-5.2 a	-5.7 c	-5.4 abc
b	-4.1 ab	-4.1 ab	-3.9 a	-4.2 ab	-4.5 ab	-4.6 b
Chroma	21.7 a	22.3 a	23.9 a	20.3 a	21.2 a	22.4 a
Hue	33.1 b	37.5 ab	36.5 ab	36.8 ab	34.0 ab	38.5 a
CM	4.0 a	2.2 d	3.8 ab	3.3 c	3.9 ab	3.7 b
TC	0.9 d	0.3 a	0.7 c	0.4 b	0.6 c	0.6 c
DEN	0.988 ab	0.986 ab	0.985 b	0.989 ab	0.989 ab	0.990 a
Qualitative						
TEX	Cottonlike	Cottonlike	Zoned	Zoned	Zoned	Zoned
EXU	Absent	Absent	Absent	Absent	Absent	Absent

Variables: L: Luminosity, a: red-green coordinates, b: yellow-blue coordinates, Chroma: saturation, Hue: tone, MG: mycelial growth (cm d^{-1}), DEN: density (g cm^{-3}), GR: growth rate (cm d^{-1}), TEX: texture, EXU: exudates. Means with different letters in each row indicate significant differences among evaluated mycelia (Tukey; $p \leq 0.05$).

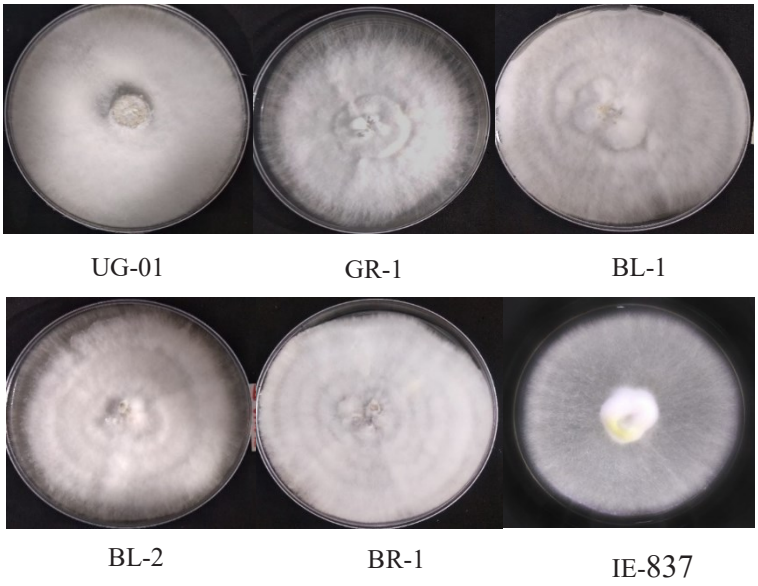

Figure 2. Colour of the different strains of the genus *Pleurotus* determined by an image analysis system with the coordinates L*a*b and the index C and Hue, isolated and stored in a PDA culture.

Significant difference ($p \leq 0.01$) was found in the GR, with a daily growth interval of 0.3 to 0.9 cm d^{-1}. Strain UG-01 surpassed the growth of commercial strains (GR-1, BR-1, BL-1, BL-2); the lowest GR was displayed by IE-837 (0.3) (Table 1). Aguilar-Pumahuillca *et al*. (2019) reported a growth rate over 1.09 – 1.14 cm d^{-1} for *P. djamor* in a PDA culture.

It is important to consider that a faster mycelial growth in the culture medium favours the reduction of cultivation cycles in the production process and the reduction in the capture of contaminants. The strain gathered has the potential to be cultivated *in vitro*, since it responds positively to temperature, making this a very important factor. The cultivation of *Pleurotus* in the state of Guanajuato has had limited development since imported commercial strains are used, which grow and bear fruit at temperate temperatures..

The evaluated strains presented significant differences ($p \leq 0.01$) in DEN, with strain GR-1 presenting the highest density, with a value of 0.989 g cm^{-3}. Strain UG- 01 presented a regular density with the presence of a creeping, scarce and homogenously distributed mycelium. Under favourable conditions, fungi increase the branching of their hyphae and therefore the amount of biomass, increasing the efficiency of the supply of nutrients by increasing their surface area. High densities are distinguished by the presence of an aerial, abundant and homogenously distributed mycelium.

León-Avendaño *et al.* (2013) report that *Pleurotus djamor* strains present a high density, which coincides with the results of this study. Regarding TEXm statistical differences

($p \leq 0.01$) were identified. The commercial strains GR-1, BL-1, BR-1, and BL-2 developed a zoned texture, which may be a chemotrophic response induced by the gradient of nutrients in the medium and the incubation temperature. Meanwhile, wild strains IE-837 and UG-01 presented a cottonlike texture. In turn, Acosta-Urdapilleta *et al.* (2016) mentioned that the *Pleurotus* spp. strains may present a cottonlike, velvety or woolly texture. Regarding the presence of exudates (EXU), no significant differences were found ($p > 0.05$).

The species *Pleurotus djamor* and *Pleurotus albidus* present light brown or amber-coloured exudates in a PDA medium, which is related to the secondary metabolism and the synthesis of diverse chemical compounds as a natural survival response (Carreño *et al.*, 2013).

Regarding the biochemical characterization of the mycelium, significant differences were identified between strains ($p \leq 0.01$). Strain UG-01 displayed the highest levels of proline (57 μg mL^{-1}), since it may have developed adaptations that help it survive in minimal water conditions and continue the biological cycle. Water limitations are typical of the state of Guanajuato, where the strain UG-01 was isolated. The accumulation of proline acts as an osmotic agent to protect the mycelium from drying. Differences in PC were also significant among strains ($p \leq 0.01$). Strain UG-01 displayed the highest value (362.0 μg mL^{-1} of gallic acid). Strains BL-2, GR-1, and BR-1, identified as *P. ostreatus*, presented statistically different values, which may be due to the different isolations of one same species being able to produce significantly different secondary metabolites, leading to the individuality of the species and that depend on their genetic characteristics.

Ferrer-Romero *et al.* (2019) reported that the *P. ostreatus* mycelium presents diversity in its phenolic compounds, which vary according to the genetic factors and the conditions of cultivation. Vamanu (2014) reported a concentration of 35.4 mg g^{-1} of gallic acid in the mycelium of *P. ostreatus*. Likewise, significant differences ($p \leq 0.01$) were found in FLA with values ranging from 30 to 69 mg mL^{-1} (Table 2). In *P. ostreatus* mycelia, total values of 28.0 (Vamanu, 2014) and 4.4 mg EQ g^{-1} (Ćilerdžić *et al.*, 2015) have been reported in an ethanolic extract. The fungi contain flavonoids such as naringenin, rutin,

Table 2. Antioxidant biochemical variables evaluated in the mycelia of *Pleurotus* spp. strains.

Variable	UG-01	IE-837	BL-1	BL-2	BR-1	GR-1
PR	57.0 a	54.0 c	56.0 b	56.0 b	56.0 b	56.0 b
CF	362.0 a	94.0 e	246.0 d	342.0 ab	327.0 bc	305.0 c
FLA	69.0 a	30.0 d	35.0 d	64.0 a	57.0 b	48.0 c
DPPH	20.8 e	78.6 a	62.4 b	23.3 e	30.4 d	43.2 c
ABTS	28.5 cd	83.7 a	48.3 b	23.3 d	26.5 d	43.8 bc

Concentration of proline (PR, μg mL^{-1}), concentration of phenolic compounds (PC, μg mL^{-1} of gallic acid), concentration of flavonoids (FLA, mg mL^{-1}), antioxidant activity based on the remaining DPPH and ABTS (DPPH, ABRS, %). Means with different letters in each row are statistically different (Tukey; $p \leq 0.05$).

morin and resveratrol. Finally, the amount and variety of compounds also depends on the fungus species.

Significant differences ($p \leq 0.01$) were found in the antioxidant capacity using both 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2´-azino-bis(3-ethylbenzothiazolin)-6-sulfonic acid (ABTS). Absorbances were determined as. In the former case, strains UG-01 (20.8 %) and BL-2 (23.3 %) displayed the lowest remaining radical percentages, whereas strain IE-832 presented the highest percentage (78.6 %), indicating the lowest antioxidant activity rate. Vamanu (2012) indicated that the cleansing activity of DPPH at a maximum concentration of 20 mg mL^{-1} fluctuated between 58.8 and 89.9 % with an ethanolic extract of *Pleurotus ostreatus* mycelia. Oropeza-Guerrero *et al.* (2018) reported a mean maximum inhibiting concentration of 10.76 ± 0.27 mg mL^{-1} in the reduction of DPPH, in the mycelium of *Pleurotus djamor* var. Roseus. Islas-Santillán *et al.* (2017) indicated a positive correlation between the concentration of polyphenols and the antioxidant activity in the genus *Ganoderma.*

Regarding ABTS, the strains that displayed the highest antioxidant activity were BL-2, BR-1 and UG-01, with values of 23.3, 26.5 and 28.5 %, respectively (Table 2). The neutralizing activity of DPPH and ABTS is linked in 62.4 % to the total content of phenolic compounds (Rivas-Mena *et al.*, 2015). Vamanu (2012) concluded that the *Pleurotus* mycelium extracts can be used as a rich source of antioxidants in pharmaceutical products. This is due to an important group of antioxidant compounds found in this fungus reacting by the transfer of hydrogen radicals and the transfer of electrons.

CONCLUSIONS

The UG-01 strain collected from the "Maguey mushroom" was identified as *Pleurotus djamor*. In the *in vitro* cultivation, this strain displayed a higher mycelial growth and antioxidant capacity than the imported strains, the mycelia of which are used in the commercial production.

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