

Original Article

Mushrooms to live or die: toxicity of some Basidiomycota using *Artemia franciscana*

Cogumelos para viver ou morrer: toxicidade de alguns Basidiomycota usando *Artemia franciscana*

L. E. Ruiz-González^a , L. Guzmán-Dávalos^b , S. R. Guerrero-Galván^a  and F. Vega-Villasante^{a*} 

^aUniversidad de Guadalajara, Centro Universitario de la Costa, Departamento de Ciencias Biológicas, Laboratorio de Calidad de Agua y Acuicultura Experimental, Puerto Vallarta, Jalisco, Mexico

^bUniversidad de Guadalajara, Centro Universitario de Ciencias Biológicas y Agropecuarias, Departamento de Botánica y Zoología, Zapopan, Jalisco, Mexico

Abstract

Consumption of wild mushrooms has increased in recent years; however, not all of them are edible and there is no precise information on those that may cause poisoning. Therefore, studies to obtain data about their toxicity are needed. For this purpose, we used the brine shrimp *Artemia franciscana*, a crustacean employed in toxicity tests and with wide application in the toxin detection, including mycotoxins. Mushrooms were collected in the state of Jalisco, Mexico, with which aqueous extracts were prepared. Dilutions of the stock solution of each extract were made to final concentrations of 50, 100, 250, 500, and 1000 µg/mL. Potassium dichromate (PD) was used as positive control and artificial seawater as negative control. The median lethal dose (LD50) of extracts on nauplii of *A. franciscana* was calculated. The aqueous extracts obtained from *Amanita amerivirosa*, *A. muscaria*, *Chlorophyllum molybdites*, and *Leucopaxillus amarus* showed a LD50 < 70 µg/mL, similar to PD (LD50 = 37 µg/mL). This is the first indication of the probable toxicity of *Leucopaxillus amarus* in humans. *Cantharellus cibarius* and *Scleroderma texense* caused the lower toxicity to the nauplii. The brine shrimp bioassay was effective in evaluating the toxicity of Basidiomycota. *Scleroderma texense* has been reported to be toxic, but it was not for this crustacean nauplii, and probably not to humans either, as recent literature has reported.

Keywords: LD50, fungi, toxicity test, crustacean.

Resumo

O consumo de cogumelos silvestres aumentou nos últimos anos; porém, nem todas as variedades são comestíveis e não há informações precisas sobre aquelas que podem causar intoxicação. Portanto, estudos para obter dados sobre sua toxicidade são necessários. Para tanto, utilizou-se o artêmia *Artemia franciscana*, crustáceo utilizado em testes de toxicidade e com ampla aplicação na detecção de toxinas, incluindo micotoxinas. Cogumelos foram coletados no estado de Jalisco, México, com os quais foram preparados extratos aquosos. As diluições da solução estoque de cada extrato foram feitas para as concentrações finais de 50, 100, 250, 500 e 1000 µg/mL. Dicromato de potássio (PD) foi usado como controle positivo e água do mar artificial como controle negativo. Calculou-se a dose letal mediana (DL50) dos extratos sobre os náuplios de *A. franciscana*. Os extratos aquosos obtidos de *Amanita amerivirosa*, *A. muscaria*, *Chlorophyllum molybdites* e *Leucopaxillus amarus* apresentaram DL50 < 70 µg/mL, semelhante ao DP (LD50 = 37 µg/mL). Esta é a primeira indicação da provável toxicidade de *L. amarus* em humanos. *Cantharellus cibarius* e *Scleroderma texense* causaram a menor toxicidade aos náuplios. O bioensaio de artêmia foi eficaz na avaliação da toxicidade de Basidiomycota. *Scleroderma texense* foi relatado como tóxico, mas não foi para este náuplio de crustáceo e provavelmente também não para os humanos, como a literatura recente relatou.

Palavras-chave: LD50, fungos, teste de toxicidade, crustáceo.

1. Introduction

Interest in wild mushrooms, mainly Basidiomycota, has increased in recent years, due to their medicinal properties such as antitumor, antioxidant, antibiotic, and antidiabetic activities (Wasser, 2011). Besides, they are considered a healthy and complete food because they are low in fat and calories and contain vitamins, minerals, and essential and non-essential amino acids

(Ribeiro et al., 2008; Sadler, 2003). Not all wild mushrooms are edible, since poisoning may occur by eating them, mostly by accident when edibles are confused with toxic ones (Ruiz-Sánchez et al., 1999). Usually, in Mexico these cases occur in urban and suburban areas of the country, where people collect mushrooms during the summer, without knowing the species (Rodríguez-Maldonado et al., 2008).

*e-mail: fvillasante@cuc.udg.mx

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There are not many easy ways to detect toxins in mushrooms, being one of them the use of *Artemia franciscana* Kellogg, which has been suggested as a model organism in toxicity tests for some time (Michael et al., 1956; Sorgeloos et al., 1978).

The brine shrimp, *A. franciscana*, is a crustacean of the Artemiidae family, distributed throughout the American continent (Sorgeloos et al., 1986). *Artemia franciscana* has the following advantages that allow its use in this kind of works: low genetic variability, short life cycle, adaptability to different abiotic conditions and nutritional sources, as well as the continuous availability of cysts and organisms at low cost (Nunes et al., 2006).

Brine shrimp cysts and nauplii have great application in the acute toxicity test of plant extracts (Fernández-Calientes et al., 2009; Meyer et al., 1982), cytotoxicity of marine natural products (Carballo et al., 2002), and chemicals such as chlorine dioxide in seawater (Puente et al., 1992). Mostly, the *A. franciscana* nauplii test has been used in mycotoxin toxicity testing and in the detection of other fungal metabolites [e.g., González et al., 2007; Nieto et al., 2008; Ruiz-González et al., 2017; Tan et al., 2011; Vega-Villasante et al., 2013]. On the other hand, the effects of copper, cadmium, zinc (Brix et al., 2006), and some macrofungi were investigated in the hatching success of the *A. franciscana* cysts (Ruiz-González et al., 2017).

The current research reports the toxicity of 23 species of Basidiomycota, collected in Jalisco state, Mexico, using *A. franciscana* nauplii.

2. Materials and Methods

2.1. Mushroom samples

Mushrooms were collected in Jalisco state. Species identification was made through macroscopic and microscopic characteristics (Largent, 1986; Largent et al., 1977), and consulting specialized literature. All specimens used in this study were deposited in the Mycological Collection of the Herbarium of the Instituto de Botánica "Luz María Villarreal de Puga", Universidad de Guadalajara (IBUG). The classification of fungi as edible, inedible, poisonous, and deadly poisonous was obtained from literature.

2.2. Aqueous extract

The extracts were prepared following Vega-Villasante et al. (2013), i.e., 5 g from each dry sample were ground by hand using a mortar and pestle and mixed with 50 mL of artificial sea water. The mixture was heated at 80 °C for 15 min, allowed to cool, and centrifuged at 1500 G for 5 min. The supernatant was stored until further use. Dilutions were performed from each extract's stock solution (100 mg/mL), to final concentrations of 50, 100, 250, 500, and 1000 µg/mL. Potassium dichromate (PD) was used as positive control and artificial seawater as negative control (Nieto et al., 2008; Vega-Villasante et al., 2013).

2.3. *Artemia franciscana* toxicity test

Artemia franciscana INVE®, Salt Lake City, Utah cysts (0.1 g) were hydrated in 50 mL of distilled water for 10 min.

Later, cysts were incubated in a crystal vessel with 1 L of artificial seawater at 40 PSU (practical salinity units), illuminated by a fluorescent light, and air bubbled to the bottom of the vessel to keep all the cysts in continuous motion. After 24 h of incubation, first instar larvae (nauplii) were attracted to one side of the vessel with a light source and collected with a Pasteur pipette (Ocaranza-Joya et al., 2019; Sorgeloos et al., 1978).

For each fungus extract, a total of 60 brine shrimp larvae were distributed in six crystal test tube, in quadruplicate, five tubes containing 5 mL of the different concentrations and one tube only artificial seawater (negative control). The tubes were incubated for 24 h and the numbers of dead brine shrimps were counted. Positive controls were used as a one more test. The LD50 (median lethal dose) was determined for each mushroom extract and positive control.

2.4. Statistical analysis

The LD50 was performed by Probit regression using the IBM® SSPS® Statics software (IBM Corporation, 2023). Standard error of the LD50 for each fungus extract was calculated following the formula indicated by Randhawa (2009). In sequence to evaluate the statistic differences between the LD50 of different aquatic extracts, coefficient of correlation r^2 and T-Student test were executed.

3. Results

3.1. Median lethal dose over *Artemia franciscana* nauplii

The mushrooms species collected for this study and LD50 of each mushroom extracts are shown in Table 1. The extracts of *Amanita amerivirosa*, *A. muscaria*, *Chlorophyllum molybdites*, and *Leucopaxillus amarus* had a LD50 less than 70 µg/mL. Among the mushrooms tested, *Cantharellus cibarius* and *Scleroderma texense* were found with the lowest toxicity over *A. franciscana* (LD50 > 1000 µg/mL), followed by *Ramaria holorbella* (LD50 = 769 µg/mL). *Gymnopilus penetrans*, *Ramaria aurea*, and *Suillus tomentosus* had a LD50 of 341, 341 and 433 µg/mL, respectively. The remaining mushrooms extracts had a LD50 between 92-270 µg/mL.

4. Discussion

The use of brine shrimp in acute toxicity tests is a widely documented topic, which has been used for different purposes, such as in the analysis of natural products (e.g., some plants and mushrooms extracts), in the detection of chemicals, to identify potential medicinal substances, and the safe use of different products (Carballo et al., 2002; Fernández-Calientes et al., 2009; González et al., 2007; Meyer et al., 1982; Moshi et al., 2006; Nieto et al., 2008; Puente et al., 1992; Ruiz-González et al., 2017; Tan et al., 2011; Vega-Villasante et al., 2013).

Meyer et al. (1982) found a toxic effect over brine shrimp if LD50 < 1000 µg/mL. Following this standard, all the mushrooms studied here, except *Cantharellus cibarius* and *Scleroderma texense*, would have some type of toxicity.

Table 1. Median lethal dose (LD50 µg/mL) of aqueous mushroom extracts over *Artemia franciscana* nauplii.

| Control/Species | Sample | LD50 ± SE (µg/mL) | P | Traditional classification |
|---|-------------------------|-------------------|-------|---|
| <i>Amanita muscaria</i> (L.) Lam. | L.E. Ruiz-González 4 | 37 ± 31 | 0.006 | Toxic and hallucinogenic (Guzmán, 1977) |
| Potassium dichromate (PD) | | 37 ± 11 | 0.003 | Positive control |
| <i>Leucopaxillus amarus</i> (Alb. & Schwein.) Kühner | B.A. Arceo-Orozco 460 | 44 ± 5 | 0.058 | Inedible (Guzmán, 1977) |
| <i>Amanita amerivirosa</i> Tulloss, L.V. Kudzma & M. Tulloss | L.E. Ruiz-González 18 | 49 ± 14 | 0.006 | Deadly poisonous (Guzmán, 1977, as <i>A. virosa</i>) |
| <i>Chlorophyllum molybdites</i> (G. Mey.) Massee | L.E. Ruiz-González 24 | 69 ± 19 | 0.001 | Poisonous (Guzmán, 1977) |
| <i>Psathyrella candolleana</i> (Fr.) Maire | L. Guzmán-Dávalos 10717 | 92 ± 29 | 0.002 | Inedible (Guzmán, 1977) Edible (Rubina et al., 2017) |
| <i>Lactarius piperatus</i> (L.) Pers. | L. Guzmán-Dávalos 10740 | 98 ± 49 | 0.006 | Inedible (Guzmán, 1977; Wang et al., 2003) Edible (Das, 2010) |
| <i>Tylopilus violatinctus</i> T.J. Baroni & Both | L. Guzmán-Dávalos 10724 | 167 ± 47 | 0.001 | Unknown |
| <i>Lactarius indigo</i> (Schwein.) Fr. | L. Guzmán-Dávalos 10738 | 171 ± 37 | 0.004 | Edible (Guzmán, 1977) |
| <i>Amanita polypyraxis</i> (Berk. & M.A. Curtis) Sacc. | L.E. Ruiz-González 21 | 175 ± 38 | 0.000 | Poisonous (Guzmán, 1977), as <i>A. alexandri</i>) |
| <i>Amanita jacksonii</i> Pomerl. | L.E. Ruiz-González 5 | 182 ± 163 | 0.001 | Edible (Ruan-Soto, 2018) |
| <i>Hygrophoropsis aurantiaca</i> (Wulfen) Maire | M. Herrera 1397 | 187 ± 72 | 0.000 | Edible (Guzmán, 1977) |
| <i>Lactarius smithii</i> Montoya & Bandala | L. Guzmán-Dávalos 10742 | 190 ± 60 | 0.002 | Unknown |
| <i>Hygrophorus russula</i> (Schaeff. ex Fr.) Kauffman | L.E. Ruiz-González 26 | 195 ± 78 | 0.037 | Edible (Guzmán, 1977) |
| <i>Macrolepiota mastoidea</i> (Fr.) Singer | L.E. Ruiz-González 2 | 221 ± 42 | 0.001 | Edible (Ge et al., 2010) |
| <i>Cortinarius purpureus</i> (Bull.) Bidaud, Moëgne-Loec. & Reumaux | L.E. Ruiz-González 57 | 251 ± 95 | 0.015 | Unknown, suspected toxicity (Errotari, 2023) |
| <i>Pholiota spumosa</i> (Fr.) Singer | L.E. Ruiz-González 55 | 268 ± 44 | 0.002 | Unknown |
| <i>Heimioporus ivoryi</i> (Singer) E. Horak | L.E. Ruiz-González 9 | 270 ± 49 | 0.001 | Unknown |
| <i>Ramaria aurea</i> (Schaeff.) Quél. | L. Guzmán-Dávalos 10851 | 341 ± 61 | 0.001 | Inedible (Guzmán, 1978) |
| <i>Gymnopilus penetrans</i> (Fr.) Murrill | L.E. Ruiz-González 58 | 341 ± 72 | 0.001 | Inedible (Guzmán, 1977) |
| <i>Suillus tomentosus</i> Singer, Snell & E.A. Dick | L.E. Ruiz-González 1 | 433 ± 92 | 0.002 | Edible (Guzmán, 1977) |
| <i>Ramaria holorbella</i> (G.F. Atk.) Corner | L.E. Ruiz-González 56 | 769 ± 355 | 0.000 | Inedible (Guzmán, 1978) |
| <i>Cantharellus cibarius</i> Fr. | M. Herrera 1407 | >1000 | 0.078 | Edible (Guzmán, 1977; Ruan-Soto, 2018) |
| <i>Scleroderma texense</i> Berk. | L.E. Ruiz-González 35 | >1000 | 0.001 | Poisonous (Guzmán, 1977) Edible (Cortés-Pérez et al., 2021) |

For each fungal sample, specimen data are name collector and collection number. All specimens were stored in IBUG Herbarium of Universidad de Guadalajara, Jalisco Mexico. SE = standard error; P = test significance.

Among the mushrooms with higher toxicity, *Amanita muscaria* causes nervous and muscarine poisonings due to the presence of ibotenic acid and muscarine, respectively (Michelot and Melendez-Howell, 2003). *Amanita amerivirosa* provokes a destructive cell poisoning, mortal in 90% of the cases (Ruiz-Sánchez et al., 1999, as *Amanita virosa*), due to the presence of α -amanitin, phalloidin, phalloidin, and phalisacin (Rittgen et al., 2008). *Chlorophyllum molybdites* is a very common mushroom growing in grasslands and gardens in Mexico (Guzmán, 1978), associated to a gastrointestinal poisoning (Peréz-Silva and Herrera, 1986). This species was studied by Yamada et al. (2012), who identified and characterized a toxic metalloendopeptidase called molybdophyllysin.

On the other hand, *Leucopaxillus amarus* showed a LD50 close to the positive control (PD) and previous species. However, the toxicity of *L. amarus* is unknown, except for the reference of Guzmán (1977), who mentioned that *L. amarus* is not edible due to its bitter taste. Previously, Ruiz-González et al. (2017) tested aqueous extracts of *A. amerivirosa* (as *A. virosa*), *A. muscaria*, and *L. amarus* on the hatching of *Artemia franciscana*, documenting the toxicity and hatch inhibition effects on the cyst. Our results confirmed that *A. franciscana* was sensitive to some substances of these mushrooms species. Therefore, these mushrooms contain metabolites toxic to *A. franciscana* and in particular, *L. amarus* could be considered toxic to humans.

Although the aqueous mushroom extracts were toxic over *A. franciscana* nauplii, like PD, they showed very low toxicity compared to some pure mycotoxins. This is the case of ochratoxin A with a LD50 of 10.1 µg/mL, gliotoxin with 3.5 µg/mL, GI aflatoxin with 1.3 µg/mL, sterigmatocystin with 0.54 µg/mL, and diacetoxyscirpenol with 0.47 µg/mL (Harwig and Scott, 1971). Furthermore, the mushroom extracts were less toxic than some biocidal substances such as Tolcide® with a LD50 of 0.26 mg/L, dodecyl ethyl ammonium bromide (DEAB) with 0.48 mg/L, and Oxone® with 11.58 mg/L, but more toxic than 2-bromo-2-nitro-1,3 propanediol (BNP) with a LD50 of 167.61 mg/L (Bartolomé-Camacho and Sánchez-Fortún Rodríguez, 2007).

In the case of *Psathyrella candolleana* (LD50 = 92 µg/mL) and *Lactarius piperatus* (LD50 = 98 µg/mL), they had a LD50 similar to that of the alcoholic extract of *Paxillus involutus* (Batsch) Fr., which was considered unsafe for humans by Nieto et al. (2008). However, *Psathyrella candolleana* was previously classified as inedible or as edible (Guzmán, 1977; Rubina et al., 2017). Guzmán (1977) indicated *Lactarius piperatus* as inedible due to its spicy flavor, Wang et al. (2003) classified it as inedible as well, and Das (2010) mentioned that it is an edible and medicinal fungus. Thus, further studies are required to confirm the presence of toxic metabolites in the basidiomes of these species.

Regarding the edible species (*Amanita jacksonii*, *Hygrophoropsis aurantiaca*, *Hygrophorus russula*, *Lactarius indigo*, *Macrolepiota mastoidea*, and *Suillus tomentosus*) and species with unknown properties (*Heimioporus ivory*, *Lactarius smithii*, *Pholiota spumosa*, and *Tylopilus violatinctus*), all showed some toxic activity over *Artemia franciscana* nauplii (LD < 500 µg/mL), without previous poisoning records in humans. Sasidharan et al. (2011) tested the alcoholic extract of the medicinal mushroom *Ganoderma boninense* Pat. in the brine shrimp, with a LD50 = 640 µg/mL, for which they concluded that *G. boninense* was “relatively safe” and could be used in natural product-based medicine. On the other hand, Wong et al. (2013) studied the toxicity and antibiotic potential of *Auricularia polytricha* (Mont.) Sacc. [= *A. nigricans* (Sw.) Birkebak, Looney & Sánchez-García], resulting with a LD50 = 115.8 µg/mL on *Artemia franciscana* and a positive antibiotic activity over *Staphylococcus aureus* Rosenbach (Gram-positive) and *Pseudomonas aeruginosa* Schroeter (Gram-negative). Furthermore, there are evidences of the presence of antibiotic and cytotoxic compounds in some mushrooms such as *L. indigo* and *P. spumosa* (Ochoa-Zarzosa et al., 2011; Russo et al., 2007). According to Moshi et al. (2006), the brine shrimp test also can be used to identify potential anticancer substances. Therefore, some edible mushroom toxicity in this study could be explained by the presence of bioactive compounds with potential anticancer, antibiotic, or antioxidant properties (Briuela et al., 1998; Wasser, 2011).

The toxicity of *Amanita polypyramis*, *Cortinarius purpureus*, *Gymnopilus penetrans*, and *Ramaria aurea* was confirmed with regard to the literature reports. *Amanita polypyramis* was associated to gastrointestinal poisoning (as *A. alexandri*, Guzmán 1977); *Cortinarius purpureus* was recorded with suspected or unknown toxicity (Errotari), and *Gymnopilus penetrans* was considered as inedible (Guzmán 1977). The LD50 of *A. polypyramis* was similar to that recorded for

Psilocybe cubensis (Earle) Singer by Vega-Villasante et al. (2013), with a LD of 135 µg/mL, which made the authors consider *P. cubensis* unsafe for humans. Moreover, *P. cubensis* causes psychodysleptics effects (Schultes and Hofmann, 2000) and adverse effects like anxiety, ataxia, confusion, hypotension, hypertension, paresthesia, dysphoria, muscle spasms, mydriasis, nausea, tachycardia, unconsciousness, among others (Asselborn et al., 2000; Berger and Guss, 2005; Beug et al., 2006).

Cantharellus cibarius, *Scleroderma texense*, and *Ramaria holorubella* were the species that showed a high LD50 and therefore the lowest toxicity (LD50 = > 1000 µg/mL in the first two and 769 µg/mL in the last one). Of these species, *Cantharellus cibarius* is a very well-known and appreciate edible mushroom (Das, 2010; Guzmán, 1977; Ruan-Soto, 2018). No information was found on the properties of *R. aurea* and *R. holorubella*, except for the reference by Guzmán (1978) for *Ramaria* spp., in which he mentioned that mushrooms of this genus with bitter taste can be considered unsafe to eat. In this work, both *Ramaria* species studied had a bitter taste and low toxicity over *Artemia franciscana*. LD50 lower than 1000 µg/mL may be due to the presence of low concentration of bioactive compounds, according to Meyer et al. (1982) and Olajuyigbe and Afolayan (2012).

In the case of *Scleroderma*, there is confusing information in the literature about its toxicity. *Scleroderma texense* causes gastrointestinal poisoning following Guzmán (1977), Guzmán et al. (1985), Guzmán-Dávalos and Guzmán (1985) and others (e.g., Chanona-Gómez, 2014). However, it is an edible mushroom together with *S. citrinum* in Nepal according to Boa (2005), along with other species in other countries, such as *S. citrinum* in Bulgaria, *S. bovista* and *S. polyrhizum* in Hong Kong, *S. radicans* and *S. verrucosum* in India, *Scleroderma* sp. in Indonesia, *S. citrinum* in Kyrgyzstan, and *S. aurantiacum* in Ukraine (Boa, 2005). Other species of *Scleroderma* have been reported to provoke intoxications, specifically *S. albidum* caused muscarinic effects in a 66 year-old Japanese man (Sato et al., 2019). Beug (2016, 2021) presented in the reports of the Toxicology Committee of the NAMA (North American Mycological Association), several cases in which *Scleroderma* basidiomata were ingested because they were mistaken for puffballs (such as *Lycoperdon*) or truffles, causing diarrhea and vomiting. Recently, Cortés-Pérez et al. (2021) recorded *S. texense* as edible in Oaxaca. *Scleroderma citrinum* has been shown to contain sclerocitrin; however, its biological effect is unclear (Winner et al., 2004).

Scleroderma texense, along with *Cantharellus cibarius*, showed the lowest mortality on *A. franciscana* nauplii (LD50 > 1000 µg/mL). Nieto et al. (2008) reported LD50 > 1000 µg/mL for *Pleurotus ostreatus* (Jacq.) P. Kumm. and *P. pulmonarius* (Fr.) Quél. alcoholic extracts, species considered safe for human consumption. Harwig and Scott (1971) tested pure mycotoxins over *A. franciscana* nauplii; these authors found that nauplii were insensitive for griseofulvin, luteoskyrin, oxalic acid, and 8-nitropropionic acid. Also, Ruiz-González et al. (2017) evaluated the effect of *S. texense* aqueous extract, reporting a hatching inhibition close to 80%, but with a low mortality over *A. franciscana* cysts (LD50 > 1000 µg/mL), similar to this research.

Thus, the results obtained with *S. texense* can be explained in two ways: 1) Taking into consideration that *A. franciscana* is not sensitive to some mycotoxins, it could be possible that is also insensitive to *S. texense*'s compounds, explaining the low mortality recorded. 2) On the other hand, it is possible that this mushroom is not poisonous either to *A. franciscana* nauplii and to humans, option confirmed by its use as edible in some countries, among them in Mexico.

The brine shrimp test can be a useful tool in the search for natural products due to its high application in the detection of compounds present in low concentrations. Moreover, this test has a high reproducibility and the target substances can be evaluated in a large population of nauplii. The toxicity of *Amanita amerivirosa*, *A. muscaria*, *A. polypyraxis*, and *Chlorophyllum molybdites* for *Artemia franciscana* was established, confirming their toxicity for humans. The properties of *Lactarius piperatus* and *Leucopaxillus amarus* were unknown; in this investigation these mushrooms were found as toxic. The non-toxicity of *Cantharellus cibarius* was demonstrated. Some mushrooms showed a moderate toxicity on *A. franciscana*, but probably the reason was that they contain bioactive compounds in low concentration with medicinal potential. Further studies on the presence, characterization, isolation, and medicinal potential of the bioactive compounds in these Basidiomycota are needed.

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