Research, Society and Development, v. 11, n. 14, e159111435997, 2022
(CC BY 4.0) | ISSN 2525-3409 | DOI: http://dx.doi.org/10.33448/rsd-v11i14.35997

Microscopic fungi recovered from honey, isolation and pathological lesions by *Penicillium sp* in an experimental model

Fungos microscópicos recuperados de mel, isolamento e lesões patológicas por Penicillium sp em modelo experimental

Hongos microscópicos recuperados de miel, aislamiento y lesiones patológicas por Penicillium sp en un modelo experimental

Received: 09/30/2022 | Revised: 10/17/2022 | Accepted: 10/18/2022 | Published: 10/23/2022

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Abstract

Background: Species of mycotoxin-producing fungi are potentially dangerous to humans and animals. The liver is the best-known organ of action of these substances. The aim of this study was to isolate microscopic fungi from honey and investigate the cytotoxic effect of the extract of *Penicillium* sp. in an experimental model. Methods: Honey samples were cultured in Sabouraud agar. After isolated and identified microscopically, the colonies of the genus Penicillium sp. were transplanted to the Sabouraud dextrose agar culture medium. After its development, they were processed to obtain an extract. Eighteen *Wistar* mice were randomly assigned to experimental (GI) and control (GII) groups. The GI was subjected to an oral inoculation of the extract, while GII received a placebo. Procedures were performed every day for thirty days, after which the liver of each animal was removed for analysis. Results: *Aspergillus* sp. (86.2%), *Geotrichum* sp. (6.89%) and *Penicillium* sp. (6.89%) were isolated. The most frequent species was *Aspergillus niger* (46%). In relation to the cytotoxic effects of the extract of Penicillium sp., the gross findings in the liver of GI suggested mainly congestion. Light microscopy showed that the little hepatic lobules were preserved and there was vascular congestion of sinusoids. Light microscopy of specimens from the experimental group showed that 68.2% were abnormal, whereas 87.5% of the control group were within normal limits.

Conclusions: The results suggest that there was contamination in honey samples. There was a predominance of macroscopic and microscopic changes in the liver of experimental rats, suggesting liver damage by *Penicillium* sp.
Keywords: Microscopic fungi; Honey; *Penicillium* sp.; Liver damage.

Resumo

Introdução: Espécies de fungos produtores de micotoxinas são potencialmente perigosas para humanos e animais. O fígado é o órgão de ação mais conhecido dessas substâncias. O objetivo deste estudo foi isolar fungos microscópicos do mel e investigar o efeito citotóxico do extrato de Penicillium sp. em um modelo experimental. Métodos: Amostras de mel foram cultivadas em ágar Sabouraud. Depois de isoladas e identificadas microscopicamente, as colônias do gênero Penicillium sp. foram transplantados para o meio de cultura Sabouraud dextrose agar. Após o seu desenvolvimento, foram processados para obtenção de um extrato. Dezoito camundongos Wistar foram distribuídos aleatoriamente nos grupos experimental (GI) e controle (GII). O GI foi submetido à inoculação oral do extrato, enquanto o GII recebeu placebo. Os procedimentos foram realizados diariamente durante trinta dias, após os quais o fígado de cada animal foi retirado para análise. Resultados: *Aspergillus* sp. (86,2%), *Geotrichum* sp. (6,89%) e *Penicillium* sp. (6,89%) foram isolados. A espécie mais frequente foi *Aspergillus niger* (46%). Em relação aos efeitos citotóxicos do extrato de *Penicillium* sp., os achados macroscópicos no fígado do GI sugeriram principalmente congestão. A microscopia de luz mostrou que os pequenos lóbulos hepáticos estavam preservados e havia congestão vascular dos sinusóides. A microscopia de luz dos espécimes do grupo experimental mostrou que 68,2% estavam anormais, enquanto 87,5% do grupo controle estavam dentro dos limites normais. Conclusões: Os resultados sugerem que houve contaminação nas amostras de mel. Houve predomínio de alterações macroscópicas e microscópicas no fígado de ratos experimentais, sugerindo lesão hepática por *Penicillium* sp.
Palavras-chave: Fungos microscópicos; Mel; *Penicillium* sp.; Dano hepático.

Resumen

Antecedentes: Las especies de hongos productores de micotoxinas son potencialmente peligrosas para humanos y animales. El hígado es el órgano de acción más conocido de estas sustancias. El objetivo de este estudio fue aislar hongos microscópicos de la miel e investigar el efecto citotóxico del extracto de *Penicillium* sp. en un modelo experimental. Métodos: Las muestras de miel se cultivaron en agar Sabouraud. Luego de aisladas e identificadas microscópicamente, las colonias del género Penicillium sp. fueron trasplantados al medio de cultivo agar dextrosa Sabouraud. Luego de su desarrollo, fueron procesados para obtener un extracto. Se asignaron aleatoriamente dieciocho ratones Wistar a grupos experimentales (GI) y de control (GII). El GI se sometió a una inoculación oral del extracto, mientras que el GII recibió un placebo. Los procedimientos se realizaron todos los días durante treinta días, después de lo cual se extrajo el hígado de cada animal para su análisis. Resultados: *Aspergillus* sp. (86,2%), *Geotrichum* sp. (6,89%) y *Penicillium* sp. (6,89%) fueron aislados. La especie más frecuente fue *Aspergillus niger* (46%). En relación con los efectos citotóxicos del extracto de *Penicillium* sp., los hallazgos macroscópicos en el hígado de GI sugirieron principalmente congestión. La microscopía óptica mostró que los pequeños lóbulos hepáticos estaban conservados y había congestión vascular de los sinusoides. La microscopía óptica de las muestras del grupo experimental mostró que el 68,2 % eran anormales, mientras que el 87,5 % del grupo de control estaba dentro de los límites normales. Conclusiones: Los resultados sugieren que hubo contaminación en las muestras de miel. Hubo un predominio de cambios macroscópicos y microscópicos en el hígado de ratas experimentales, lo que sugiere daño hepático por *Penicillium* sp.
Palabras clave: Hongos microscópicos; Miel; *Penicillium* sp.; Daño hepático.

1. Introduction

Fungus are ubiquitous and virtually present in all spaces, i.e., water, soil and air. Although they bring various numerous benefits to nature, they may also eventually harm humans (LACAZ et al., 2002). Honey is a natural product with a complex chemical composition. It is primarily composed of fructose and glucose, but also contains probiotic agents (CHOW, 2002).

The growth of fungus on host animals, also known as mycosis, may be superficial, cutaneous, subcutaneous, systemic or opportunist (Lacaz et al., 2002). Dietary, respiratory or dermal exposure to the toxic metabolites of fungi produce the so-called mycotoxicosis (Bennett & Klich, 2003). These compounds are called mycotoxins, i.e., substances that affect organs and tissues; they may induce cancer, mutagenesis, teratogenesis, immunosuppression, among others (Ferreira et al., 2006). Among the fungi that produce mycotoxins there are the genera *Aspergillus* (aflatoxin, ochratoxin A), *Penicillium* (citrinin, citreoviridin, patulin), *Claviceps* (Ergot Alkaloids), Fusarium (fumonisins, trichothecenes, zearalenone) (Richard, 2007).

In 2006, an outbreak of a clinical syndrome in the southwestern region of Maranhão drew the attention on the importance of fungi in the public health context. After hundreds of reported cases and approximately forty deaths, the conclusion was that the etiology of the unknown clinical syndrome involved the mycotoxin of the *Penicillium* fungus, which contaminated the rice eaten by the population (Lira & Ferreira, 2008).

Among the mycotoxins, the most studied and demonstrably linked to hepatocellular carcinogenesis is aflatoxin, which is produced by *Aspergillus* fungi. As for the fungi of the genus *Penicillium*, although equally abundant in the environment and considered an important source of food contamination, relatively fewer studies were conducted on them to analyze the effects of their toxins on human organs and tissues (Hoeltz et al., 2009).

Because of its eminent role in detoxification and excretion, the liver is a major target organ of toxic substances orally ingested. Lesions are caused by hemorrhagic necrosis, proliferation of bile duct cells and by fatty infiltration of hepatocytes (Espada et al., 1992; Oliveira & Germano, 1997). Moreover, the bleeding observed in the liver of animals has been associated with mycotoxins (Maciel et al., 2007). A chronic ingestion of mycotoxins could lead to neoplastic changes induced by a direct DNA damage, being the liver the most common site (Hoeltz et al., 2009).

The aim of this study was to isolate microscopic fungi from honey and investigate the cytotoxic effect of the extract of *Penicillium* sp. in an experimental model.

2. Methodology

Honey samples were collected directly from producers in Caxias, Maranhão, between November and December 2008. Producers were identified as P1, P2, P3, P4, being P1 and P2 from urban areas, and P3 and P4 from the countryside. Three honey samples of *M. fasciculata* ("tiúba") and one sample of *A. mellifera* were directly collected at the residence of each producer. They were extracted from beehives under normal conditions of collection. Three aliquots of 5 mL of honey from each producer were stored in sterile test tubes, from which a sufficient volume was withdrawn for the preparation of dilutions in saline solution at 1:1000. Then, 0.1 mL of this solution was used for spreading in two Petri dishes containing Ágar-Sabouraud to promote the growth of fungi. Eight plates were seeded, two for each sample. Two plates containing the same culture medium were opened in the laminar flow cabinet while the sowing was performed as a quality control of the sterile environment.

Plates were kept at room temperature (25°C) and daily monitored for 7-15 days in the Mycology Laboratory of the Nucleum for Basic and Applied Immunology of the Federal University of Maranhão (NIBA - UFMA), where they were observed in a macroscope to identify fungal colonies (CFU). Then, the taxonomic identification of fungi was performed (Lacaz et al., 2002; Guarro & Gené, 1992).

Once isolated and microscopically identified, the colonies of *Penicillium* sp. were transplanted to Sabouraud Agar-dextrose medium and, after their development, five square blocks were cut into approximately 1 cm^2, being deposited in a conical flask containing 250 mL of Sabouraud broth, remaining for 10 days at a temperature of 25°C. Then, the fungal mass was removed, washed 5 times with a 0.15 M saline solution and homogenized in a vial containing glass beads. The lysed cells were separated by centrifugation. The supernatant was used as a crude extract which served as antigens after the filtration procedure and compared to the #4 of the MacFarland scale. The solution obtained, fungal filtrate, was used in *in vivo* tests and stored in a cold room at 4°C until its use.

Eighteen Wistar mice from the animal vivarium of the State University of Maranhão (UEMA), kept at NIBA – UFMA, were used for the procedure. The animals weighted between 20 and 40 grams and had between 40 and 45 days at the time of inoculation. The animals were kept in containers with food and water *ad libitum*, at a constant temperature and with a

daily cleaning of their breeding. Animals were randomized into two study groups: Group I (Experimental Group), comprised of nine animals submitted to an oral inoculation of *Penicillium sp.* fungal extract; and Group II (Control Group), comprised of nine animals submitted to an oral inoculation of placebo (saline 0.9%). The inoculation was performed after 15 days of the conditioning of animals to the environment based on gavage methods. To G1 animals, the fungal extract of *Penicillium sp*. was orally administered at a dose of 20 mg/kg. Regarding G2, it received the saline solution at the same dose. Animals were daily fed in the morning for 30 days. Thirty days after inoculation, the mice were assigned to necropsy for the removal of the liver in the Autopsy Laboratory of the Veterinary Course from UEMA. After opening the peritoneal cavity, the abdominal viscera were displaced and the liver was removed. After a forty-eight hour fixation in formaldehyde, the pieces were subjected to successive washes with alcohol at 70°GL, progressively, until 100°GL. Then, the fragments were subjected to a paraffin embedding technique and 4-5 micrometer-thick cuts (μm) were made with a rotary microtome. The histological cuts from all animals were stained with hematoxylin-eosin (HE) and analyzed by light microscopy (Luna, 1968).

The stained slides in HE were qualitatively evaluated with a description from the histologist, with no prior information of which lamina belonged to which group. The qualitative description sought to assess the viability of hepatocytes, observing the preservation of hepatic lobules, the congestion or dilation of hepatic capillaries sinusoids, the signs of extravasation of red blood cells into the hepatic sinusoids, and hepatocyte integrity. The organs were macroscopically examined taking into consideration criteria such as size and presence of lesions. Slides containing paraffin sections of liver and lungs were stained with hematoxylin and eosin.

To perform experiments with animals, the project was submitted to the Ethics Committee and Animal Experimentation (EAEC) of the UEMA under protocol no. 18/2009. For statistical analysis of the data obtained on fungal colonies, the arithmetic mean, median and mode were obtained using the Epi Info 2007 program to calculate simple percentages of variables.

3. Results

The plate used as a control did not grow any fungi species. The growth of filamentous fungi was verified in 100% of the Petri plates (n = 8). Table 1 shows the Quantification of Colony Forming Units (CFU) of fungi originated from honeys collected in Caxias, Maranhão, per producer. There was a higher number of CFU in the honey cultivated by producer P2. The genus *Aspergillus* was found in 86.2% of the isolated colonies, followed by the genera *Geotrichum* and *Penicillium*, each one present in 6.89%. Microscopy analysis found in each plate seven species of the genus *Aspergillus*. According to Table 1, the most frequent species was *Aspergillus niger*, found in 46% of colonies.

Table 1 - Percentage of CFU per producer and fungal species isolated from honey in Caxias, Maranhão.

Producer	**No. of CFU**	**(%)**
P1 (Urban area)	10	17.2
P2 (Urban area)	27	46.6
P3 (Countryside)	11	19.0
P4 (Countryside)	10	17.2
Total	58	100

Isolated genus	**Isolated colonies**	
	N	**%**
Aspergillus	50	86.2
Penicillium sp	4	6.89
Geotrichum sp	4	6.89

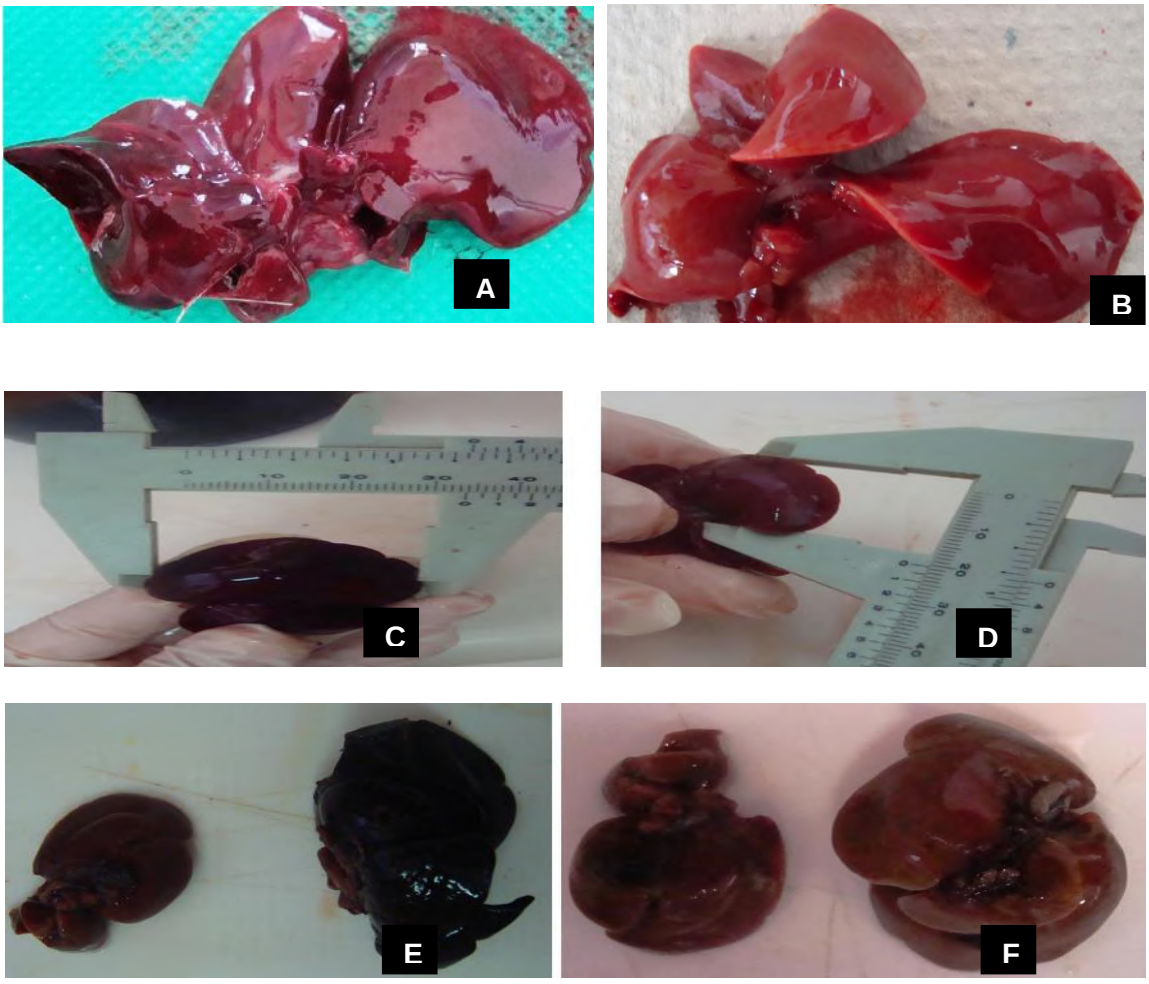
Isolated species of *Aspergillus*	**Isolated Colonies**	
	N	**%**
Aspergillus clavatus	1	2.0
Aspergillus fumigatus	6	12.0
Aspergillus glaucus group	1	2.0
Aspergillus terreus	1	2.0
Aspergillus nidulans	2	4.0
Aspergillus versicolor	2	4.0
Aspergillus flavus	8	16.0
Aspergillus niger	23	46.0
Aspergillus spp	6	12.0
Total	50	100

*CFU - Colony Forming Unit *P = Producer. Source: Authors.

The main macroscopic changes in livers of the GI were increased organ size (hepatomegaly), dark-red color and thickened edges suggesting congestion. The edges were blunt, with a smooth surface, without nodules (Figure 1A). The GII had well-preserved hepatic lobules, with smooth surfaces without nodules, thin edges and normal size. No hepatic congestion or fatty infiltration was noted. (Figure 1B).

After the macroscopic inspection of the liver, we proceeded to the verification of one of the liver lobes of animals using a caliper. The right lobe was chosen for the microscopic examination because of its size, which provides a better flexibility in the preparation of the histological slides (Figures 1C and 1D). The macroscopic comparison of livers from the control group with livers of the experimental group showed a significant difference in the size of organs, as shown in Figures 1E and 1F. The experimental group had a higher average size of the right lobe, 30.5 cm length and 20.3 cm wide, suggesting hepatomegaly.

Figure 1 – A – Animal liver from the experimental group showing hepatomegaly and hepatic congestion. **B** - Animal liver from the control group without macroscopic changes. **C and D –** verification with a caliper of length x width (in cm) sizes of the right lobe of the liver. **E and F –** size difference between the liver removed from an animal of the control group (E) and the liver form the experimental group (F).

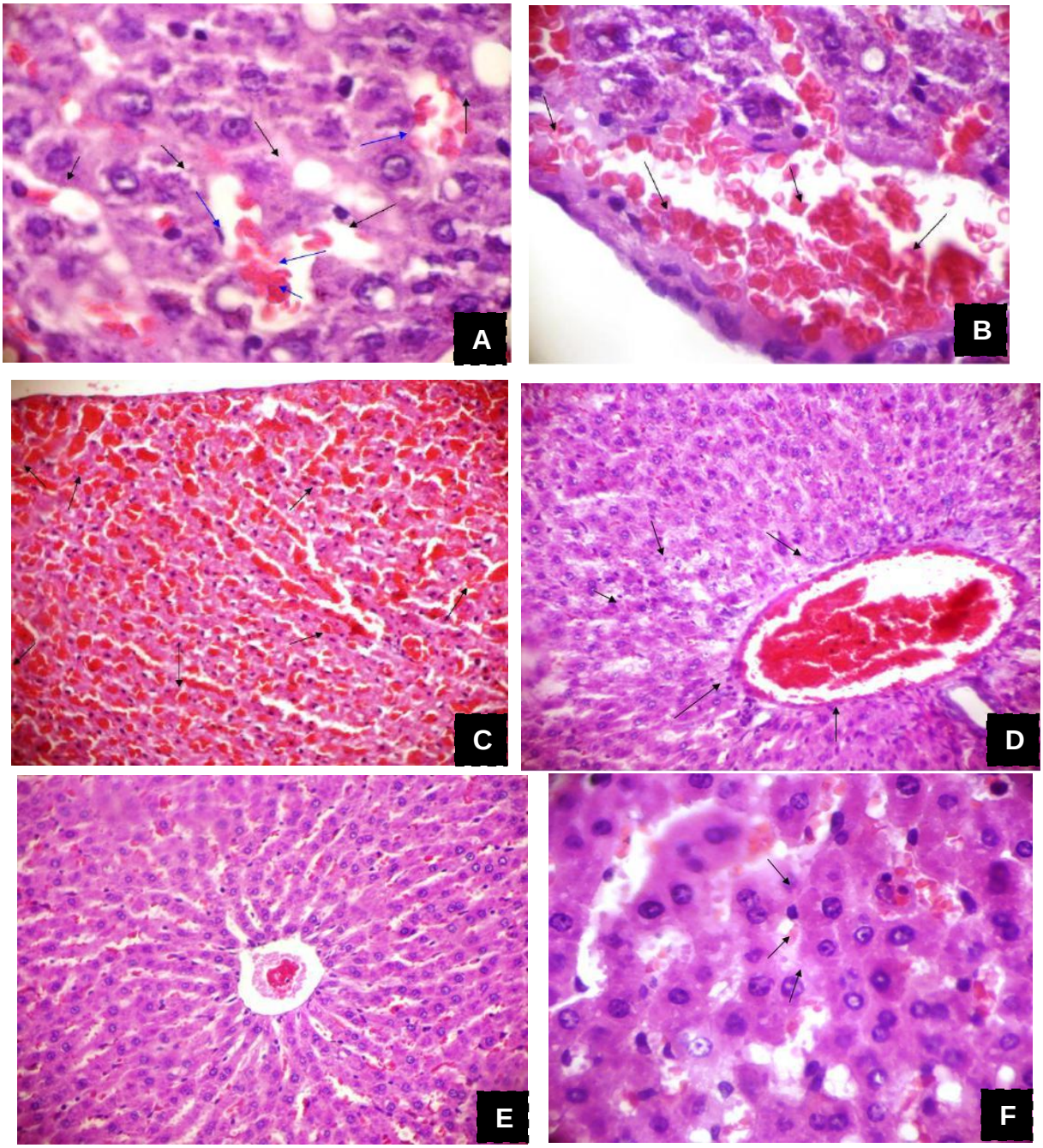


Source: Authors.

Upon microscopic examination, the hepatic lobules showed signs of degeneration. A vascular congestion of sinusoids and extravasation of some red blood cells between hepatocytes were noted. The right lobe of the liver showed a mild pericapsular hemorrhage and a moderate sinusoidal congestion (Figure 2A and 2B). The most important change evidenced in experimental animals was a sinusoidal dilatation (28.6%), followed by liver congestion and pericapsular hemorrhage (14.3%) (Figure 2C and 2D).

The control group showed no cell lesions, prevailing integral cells with no evidence of atypical mitoses and cellular atypia. Well-preserved hepatic lobules were observed, with intact hepatocytes. In this group, the absence of vascular congestion of hepatic sinusoids and rare extravasation of red blood cells into sinusoidal spaces were also noted. Fatty infiltrations in the hepatocytes were not observed. The nuclei showed no alterations, as illustrated in figures 2E and 2F. The control group had a 87.5% normality in the microscopic findings.

Figure 2 A - Photomicrograph of the liver tissue from an animal of the experimental group showing a moderate dilatation of sinusoids (black arrows) with the presence of red blood cells within them (blue arrows). HE, 400x. **B** - Photomicrograph of the liver tissue of an animal from the experimental group with pericapsular hemorrhage with red blood cells close to the capsule that covers the liver, marked by black arrows. HE, 400x. **C** - Photomicrograph of the liver tissue of an animal from the experimental group with hepatic congestion with red blood cells present in all the tissue between hepatocytes, indicated by black arrows. HE, 100x. **D** - Photomicrograph of the liver tissue from an animal of the experimental group showing the central vein preserving the integrity of hepatic lobules and hepatocytes (black arrows), the absence of dilation of sinusoids and hepatic congestion. HE, 100x. **E** - Photomicrograph of the liver tissue of an animal from the control group showing hepatocytes and nucleus without changes in morphology, absence of dilation of sinusoids, no evidence of cancerous changes such as atypical mitoses and cellular atypia. HE, 100x. **F** - Photomicrograph of the liver tissue of an animal from the control group showing hepatocytes and nucleus without changes in morphology (black arrows), the absence of dilation of sinusoids and hepatic congestion. HE, 100x.



Source: Authors.

4. Discussion

Until now, honey was believed to be a contamination-free product due to its antifungal and bacteriostatic action. However, studies with laboratory collections and analysis found the presence of these pathogenic microorganisms in honey, making it often unsuitable for consumption. Although the main foods susceptible to contamination by mycotoxins are cereals, numerous national and international scientific investigations have been ascertaining the possibility of microbial growth in honey, for example, filamentous fungi producing mycotoxins (Gelbi et al, 1990; Matuella & Torres, 2000; Martins et al, 2003; Abreu et al., 2005; Oliveir et al., 2005; Rios et al., 1992; Douthat et al., 2006).

The fungal growth observed in 100% of the samples analyzed in the present study confirms these studies. This result may reflect how samples were obtained. They were all directly collected by the producers, possibly using inappropriate methods. However, it may be also be due to environmental contamination. An indicator of the probable contamination of the environment is that the sample with the highest percentage of CFU (46.6%) came from the urban area. It is assumed that urban areas are more abundant in environmental pollutants.

Regarding the fungal species found, the present study supports the literature. In most studies, *Aspergillus* spp. is the most commonly found fungus (Gelbi et al, 1990; Matuella & Torres, 2000; Martins et al, 2003). The genus *Penicillium* was chosen as the object of this research due to the relatively few researches on the occurrence of this fungus in honey and due to an intention to study the organic consequences of its mycotoxins *in vivo*.

Penicillium fungi present mycotoxins known in literature. When chronically ingested, they potentially cause organic damage, mostly in the kidney and liver, which can be identified in histopathologic studies. Depending on growth conditions, various mycotoxins can be produced by different species of *Penicillium* spp, e.g., citrinin (CIT), ochratoxin A (OTA) and Patulin (PAT) (El-Arab et al., 2006).

A research conducted in Rio de Janeiro found that a group inoculated only with aflatoxins showed liver lesions consistent with aflatoxicosis, with hyperplasia in the bile ducts, periportal infiltration of inflammatory cells, coagulation necrosis, vacuolation of hepatocytes, and apoptosis and megalocytosis areas. In control animals, no lesions were observed in the liver tissue (Cardoso et al., 2008).

The main changes observed in the liver of GI were hepatomegaly and congestion. The edges were blunt, with smooth surfaces without nodules. The GII showed well-preserved liver lobes, with smooth surfaces without nodules. In our study, GI had little-preserved hepatic lobules in microscopy. Vascular congestion of sinusoids and the extravasation of some erythrocytes between hepatocytes were observed. As for GII, it had no cellular lesions, prevailing intact cells, without evidence of atypical mitoses and cellular atypia. According to studies conducted by the AFIP, Armed Forces Institute of Pathology (1994), the damages most commonly found in livers subjected to the consumption of mycotoxins are predominantly perivascular focal necrosis, dilation and congestion of hepatic sinusoids and central veins, necrotic areas of the liver parenchyma infiltrated by neutrophils and interstitial liver fibrosis.

The occurrence of some species of fungi in food, contributing to the loss of product quality and damage to human health, has served as a warning to the risk of contamination with mycotoxins (Silva et al., 2007). According to El-Arab et al. (2006), fungi of the genus *Penicillium* has mycotoxins already known in the literature, which, when chronically ingested, cause potentially organic, renal and especially liver damage that can be identified on histopathology.

According to Toledo et al. (2006), the association between diseases and the toxic metabolic products of these microorganisms is an extremely important issue for both the food industry and public health.

5. Conclusion

The research results suggest that there was an environmental contamination in the collected honey samples due to the isolation of filamentous fungi. From the isolation of fungi, the extract of *Penicillium* sp. inoculated into the experimental model showed the occurrence of macroscopic and microscopic liver changes with an acute toxicity.

Further studies need to be performed to evaluate the molecular pathways of *Pencillium*-induced liver toxicity.

Acknowledgments

To FAPEMA (Research Support Foundation of Maranhão State), DECIT/SCTIE/MS, for the financial support, and to PIBIC CNPQ/FAPEMA/UFMA.

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