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Toxicity of Human Pathogenic Fungi *Candida albicans* and *Aspergillus fumigatus* and their Effect on Development and Hemocytes of the Greater Wax Moth Larvae *Galleria mellonella* (Lepidoptera: Pyralidae)

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ABSTRACT

Toxicity testing on rodents is a prerequisite for using therapeutic agents in humans. However, experiments in mice and rats are expensive and need ethical consideration. Therefore, *Galleria mellonella* has been extensively used as an alternative model to study microbial infections in humans. The current study focuses on the toxicity of human fungal pathogens, *Candida albicans* and *Aspergillus fumigatus* against *G. mellonella* larvae under controlled laboratory conditions. The injection of both fungal species with different doses ($2x10^2$, $2x10^3$, $2x10^4$, $2x10^5$, and $2x10^6$ cells/larva) gave an LD_{50} of $6.8x10^4$ and $4.6x10^4$ for *A. fumigatus* and *C. albicans*, respectively. Larval, pupal, and adult periods were significantly prolonged at $2x10^4$ for both fungal species and afterward, while pupal and adult developmental ratios and adult longevity were significantly reduced, except at the lowest dose ($2x10^4$). Larval treatment with LD_{50} of both fungal species showed a decrease in the concentrations of proteins, lipids, and carbohydrates in larval tissues, and a greater reduction was observed in larvae injected with *C. albicans* compared to *A. fumigatus*. There was a significant decrease in total hemocyte counts (THCs) and hemocyte spreading activity (HSA) after 24 and 48 h of treatment; however, the Variations in plasmatocytes (PLs) and granular cells (GRs) were not statistically significant. In conclusion, this physio-pathological study confirms that changes in some biochemical parameters such as total body proteins, lipids, and carbohydrates as well as cellular immune responses such as changes in hemocyte counts and behavior of *Galleria* larvae are used as indicators of relative toxicity of the tested pathogens in biomedical research.

INTRODUCTION

Candidiasis, caused by the yeast, *C. albicans*, and aspergillosis, caused by the fungus *A. fumigatus* are the most common fungal diseases in humans, including a wide range of mucocutaneous infections and invasive mycoses. These pathogenic fungi produce some effector molecules like enzymes and toxins, facilitating their growth and maintenance in the environment and their invasion of living animals (Brown *et al*., 2012).

Estimating the toxicity of pathogenic microorganisms is essential to reduce the risk of human infection. Toxicity tests are typically carried out using mammals, however, there is a direction to facilitate the usage of mammals in these tests. Insects have a powerful immune system to kill invasive pathogens and ensure host's survival (Dhinau *et al*., 2017). Similarities exist between insects' immune system and mammals' innate immune system (Kavanagh and Reeves, 2004). These similarities include the presence of recognition receptors, through which cellular defense reactions such as phagocytosis, and humoral reactions such as coagulation and the synthesis of immune proteins and enzymes can be initiated (McCloskey *et al*., 2019).

Kavanagh and Reeves (2004) exploited the potential of insects for in vivo toxicity testing of pathogens. Thereafter, many insects such as *Galleria mellonella* (Slater *et al*., 2011), *Drosophila melanogaster* (Ong *et al*., 2015), *Manduca sexta* (Lyons *et al*., 2020), *Bombyx mori* (Montali *et al*., 2020), are now used as models for measuring microbial toxicity or for evaluating the antifungal efficacy.

The wax moth, *G. mellonella* larvae are extensively used and generate results that can be compared to those obtained by mammal models (Thomaz *et al*., 2020). They are widely used for assessing the pathogenicity of bacteria (Nale *et al*., 2016) and fungal pathogens (dos Santos *et al*., 2017). Larvae are inexpensive, easy to handle, and free from ethical restrictions. They can survive at 37 °C, enabling testing of temperature-dependent virulence factors (Rowan *et al*., 2009). Furthermore, using large numbers of larvae can increase the statistical power of studies. Therefore, they can be widely used to investigate the mechanisms of microbial pathogenicity (Tsai *et al*., 2016).

In many Lepidopteran model insects, fungi have many physiological modes of action including inhibition of insect growth, development, and reproduction. Some biochemical alterations, due to fungal infection, have occurred in different metabolic functions as fungi produce a variety of metabolites including toxins and extracellular enzymes to enable them to colonize and reproduce in the host`s body (Curtis *et al*., 2022). In addition, fungi may also affect the insects' innate immune system which recognizes and eliminates the infection (Trevijano-Contador and Zaragoza, 2014). Therefore, biochemical and immune functions in insects can be used as bioindicators to determine the toxicity of infective fungi.

This work aimed to demonstrate the potential of G. *mellonella* larvae as a simple and inexpensive model for evaluating the toxicity of human pathogenic fungi, *C. albicans*, and *A. fumigatus*. This study presents the first application of *Galleria* larvae for an in vivo LD_{50} study of these fungal species. The effects of fungal injection on various biological parameters including mortality, developmental time, and longevity as biological indicators, some biochemical aspects including protein, lipid, and carbohydrate concentrations as biochemical indicators, and changes in hemocyte counts and behavior as immune indicators were also assessed and compared.

MATERIALS AND METHODS

All chemicals, media, and solvents used in this study were of high purity and

obtained from Sigma-Aldrich, UK unless otherwise stated.

1. The Experimental Insect:

The greater wax moth, *G. mellonella* was reared under controlled laboratory conditions (30 ±2ºC, 65 ±5% RH) using a diet containing pollen and bee wax to sustain their natural habitat in beehives, as previously described by Abo-Kersh and Barakat (2023). The last instar larvae were used in the subsequent experiments.

2. The Tested Fungi:

Human pathogenic fungal suspensions of *C. albicans* and *A. fumigatus*, were prepared as described in the National Committee for Clinical Laboratory Standards, Document M38-A method (NCCLS, 2002) and sub-cultured in Sabouraud-dextrose liquid medium (SAB) at 37°C for 48 h with gentle shaking. Before use, fungal cells were streaked onto the SAB agar solid medium (by addition of 2% agar) for 48 h at 37°C, and green colonies were inoculated into SAB broth for 48 h at 37°C. Fungal cells were washed with sterile phosphate-buffered saline (PBS; 150 mM NaCl, 20 mM phosphate buffer, pH 7.2) to remove non-adherent cells, counted using a hemocytometer (Corning, New York, USA), and adjusted to 10^8 cells/mL.

3. Toxicity Bioassays of Fungi Using *G. mellonella* Larvae:

Assays were carried out according to Curtis *et al*. (2022). 20µL of each fungal suspension (10^4, 10^5, 10^6, 10^7, and 10^8 cells/mL) were injected into the hemocoel of 30 last instar larvae of *G. mellonella*, in three replications, via the last right proleg using a 20-µL syringe (Hamilton, Hamilton Company Reno, NV, USA) with a needle size 26 s. Control groups consisted of 30 untreated larvae and another 30 larvae injected with 20µL of PBS only. Larvae were incubated under the same laboratory conditions and daily observed to determine the percentages of larval and total mortality. Results were analyzed using the probit method to provide the LD_{50} which is used to search for the effects on biochemical and immunological parameters.

4. Biological Studies:

For studying the biological effects of *C. albicans* and *A. fumigatus* against *G. mellonella* larvae, the fungi were injected as previously described. After injection, the alive larvae were transferred individually to clean tubes, kept under the same laboratory conditions, and monitored daily until adult emergence. Emerged adults were also observed daily until their death to record adult longevity. Different biological aspects were recorded as follows; larval and pupal duration and adult emergence time (days) and the ratio of pupation and adult emergence (%) were determined. 30 larvae in three replicates were used for each experimental and control group.

5. Biochemical Studies:

To investigate the changes in protein, lipid, and carbohydrate metabolic products, fungal LD_{50}-treated larvae along with untreated and PBS-treated larvae were collected at 24 and 48 h post-treatment and homogenized in distilled water (1 larva/1 mL) using a glass Teflon tissue grinder (MPW-309 Mechanic-Preczyina, Poland) surrounded with an ice jacket for 3 min. The homogenates were centrifuged (Hettich Zentrifugen, D-7200 Tuttlingen, Germany) at 5000 rpm for 30 min at 5ºC. The supernatant of each group was immediately assayed calorimetrically by using a spectrophotometer (Thermo/Milton Roy Spectronic 601) to determine the total body proteins (TBP) according to Gornall *et al*. (1949), the total lipids content (TBL) according to Van Handel (1985), and the total body carbohydrates (TBC) according to Singh and Sinha (1977).

6. Immunological Studies:

To determine the effects of fungal treatment, at the LD_{50} level, on the total hemocyte count (THC), the differential hemocyte count (DHC), and hemocyte spreading activity (HSA), experiments were conducted on each last instar larva in all experimental and control groups and hemolymph was collected at 24 and 48 h

post-treatment by piercing the larva with a sterile needle on the first hind leg.

6.1 Total and Differential Hemocyte Counts:

To determine the effects of fungi on THCs, the collected hemolymph from control and fungal-treated groups, collected at 24 and 48 h post-treatment, were added in 1: 10 a ratio of ice-cold anticoagulant buffer (17 mM Na_2 EDTA, 98 mM NaOH, 41 mM citric acid, 186 mM NaCl, pH 4.5) into an Eppendorf tube. The cell suspension was loaded to a Neubauer Improved Hemocytometer (Marienfeld, Lauda-Königshofen, Germany) and then examined under an optical microscope (Zeiss, Jena, Germany). Thirty larvae were used for each experiment in three independent replicates.

To count the DHCs, the hemolymph collected at 24 and 48 h post-treatment, was transferred into an Eppendorf tube containing PBS and mixed well. One drop of this diluted hemolymph was smeared onto a microscopic slide and fixed for two min in absolute methyl alcohol. Fixed cells were stained with Giemsa's solution for 20 min, washed several times with distilled water, air-dried, and examined microscopically under oil immersion (1600x) and classified according to Brehélin and Zachary (1986). The proportion of PLs and GRs, which are the center of the immune system in insects, was calculated from a total of 100 hemocytes examined in three replicates of each experiment.

6.2. Hemocyte Spreading Activity:

To observe and record the ability of larval hemocytes to spread on a glass surface, after fungal treatment, which is used as an immune indicator, hemolymph from control and fungal-treated larvae was mixed with PBS (1: 5 v/v) in an Eppendorf tube. 20µL of each fungal suspension was spread on a slide and kept for 30 min at room temperature to allow the hemocytes to attach to the slide. A total of 100 cells per field were examined from each treatment under a light microscope and relative numbers of spreading hemocytes from 10 larvae in three replicates were recorded.

7. Statistical Analyses:

LD_{50} values and biological parameters were carried out among PBS-treated and fungal-treated insects and calculated using Finney's probit analysis method (Finney, 1971), using LDP line software. Other data were expressed as means ± standard deviation (SD) and were normally distributed by one-way analyses of variance followed by the Bonferroni posttest for comparing experimental means. All statistical tests were performed with SPSS version 22.0 software program and results were evaluated as significant when $P<0.05$.

RESULTS

1. Toxicity of Fungi against *G. mellonella* Larvae:

Mortality rates were assessed in two categories larval mortality and total mortality. For both fungal species, larval mortality rates increased from the lowest dose (2×10^3 cells/larva) to the highest dose (2×10^6 cells/larva) in a dose-dependent manner. Similar results were also obtained in total mortality rates. The larvae of *G. mellonella* were found to be more susceptible to *A. fumigatus* ($LD_{50} = 6.8 \times 10^4$ cells/larva), and less susceptible to *C. albicans* ($LD_{50} = 4.6 \times 10^4$ cells/larva). In the control population, the mortality rate did not exceed 5%, and no sign of fungal infection was observed in dead larvae (Table 1).

2. Developmental biology and longevity:

Larval duration was significantly elevated at 2×10^4 fungal cells/larva, for both fungal species compared to un-injected and PBS-injected controls. Survival of injected larvae showed insignificant differences among the tested fungi. Fungal treatments also prolonged the pupal period and decreased pupation ratios in a dose-dependent mode compared to controls. Moreover, the adult emergence time of fungal-treated individuals increased above controls; however, a significant increase was observed at all tested fungal

doses except at the lowest one (2×10^2). The longevity of fungal-treated adults was reduced significantly at all doses compared to the control groups. Dose-dependent modes were also detected in adult emergence ratios, that were significant at all applied doses of both fungal species (Table 2).

Table 1. Larval and total mortality of *G. mellonella* larvae treated with different doses of *A. fumigatus* and *C. albicans.*

Larval treatment		% Larval mortality	% Total mortality	χ2	Slope	LD_{50} (confidence limits)
Untreated		0	2.4 ± 0.1 a			
PBS-treated		4.2 ± 0.4 a	4.8 ± 0.2 a			
A. fumigatus (cells/larva)	2×10^2	6.7 ± 0.9 a	14.7 ± 2.9 b	4.199	25.364 ±7.12	6.8×10^4 $8.33 \times 10^3 – 1.84 \times 10^5$
	2×10^3	17.2 ± 2.3 b	21.2 ± 3.1 b			
	2×10^4	65.2 ± 5.6 c	67.5 ± 8.6 c			
	2×10^5	77.5 ± 6.9 d	82.8 ± 8.9 d			
	2×10^6	85.3 ± 9.3 e	93.1 ± 6.8 d			
C. albicans (cells/larva)	2×10^2	6.1 ± 2.8 a	9.5 ± 1.9 a	4.199	25.364 ±7.12	4.6×10^4 $9.87 \times 10^3 – 3.11 \times 10^5$
	2×10^3	15.7 ± 3.2 b	19.7 ± 2.8 b			
	2×10^4	57.5 ± 5.8 c	63.7 ± 6.6 a			
	2×10^5	65.5 ± 7.9 d	74.4 ± 9.1 d			
	2×10^6	79.5 ± 6.9 e	85.8 ± 7.9 d			

Data were expressed as a mean ± SD of 3 independent experiments with 10 larvae per treatment. Larval mortality was applied to probit analysis. (X^2) the total contribution to Chi-square. (X^2 tabulated = 6). (LD_{50}) lethal dose that kills 50% of tested larvae with 95% confidence limits. Groups marked with different letters are significantly different ($P<0.05$, with Bonferroni posttest). Larval mortality ($F = 16.8$, $P<0.01$); and total mortality ($F= 35.3$, $P<0.001$).

Table 2. Developmental parameters of *G. mellonella* following infection of the last instar larvae with different doses of *A. fumigatus* and *C. albicans*

Larval treatment		Larval period (Days)	Pupal period (Days)	% Pupation	Adult emergence time (Days)	Adult longevity (Days)	% Adult emergence
Untreated		7.3 ± 0.2 a	6.6 ± o.3 a	100 ±.0.0 a	13.9 ± 0.3 a	12.3 ± 0.7 a	100 ±.0.0 a
PBS-treated		7.1 ± 0.2 a	6.4 ± 0.2 a	99.7 ± 0.5 a	14.3 ± 0.2 a	11.2 ± 0.6 a	99.7 ± 0.5 a
A. fumigatus (cells/larva)	2×10^2	7.2 ± 0.4 a	7.1 ± 0.3 a	91.1 ± 7.7 a	14.5 ± 0.5 a	5.3 ± 0.4 b	53.3 ± 6.6 b
	2×10^3	7.8 ± 0.4 a	7.7 ± 0.5 ab	56.6 ± 1.3 b	22.5 ± 1.0 b	4.6 ± 0.6 b	36.6 ± 3.4 c
	2×10^4	11.6 ± 1.3 b	8.1 ± 0.6 b	48.5 ± 1.1 c	23.0 ± 0.7 b	4.8 ± 0.3 b	25.0 ± 2.3 d
	2×10^5	13.8 ± 0.9 c	9.3 ± 0.7 c	20.3 ± 1.3 d	34.1 ± 2.2 c	3.6 ± 0.6 c	16.6 ± 2.4 e
	2×10^6	14.5 ± 1.1 d	9.9 ± 0.8 c	18.2 ± 1.4 e	33.7 ± 3.4 c	3.1 ± 0.3 c	5.0 ± 0.3 f
C. albicans (cells/larva)	2×10^2	7.0 ± 0.3 a	6.7 ± o.2 a	98.3 ± 0.7 a	14.1 ± 0.2 a	5.3 ± 0.4 b	51.3 ± 5.4 b
	2×10^3	7.4 ± 0.6 a	7.3 ± o.4 a	52.5 ± 1.9 b	20.8 ± 1.3 b	4.6 ± 0.6 b	31.6 ± 3.8 c
	2×10^4	11.2 ± 0.9 b	8.2 ± 0.2 b	42.3 ± 1.8 c	24.1 ± 2.1 c	4.8 ± 0.3 b	24.0 ± 2.8 d
	2×10^5	13.1 ± 1.2 c	9.4 ± 0.7 c	19.3 ± 1.1 d	29.9 ± 2.6 d	3.6 ± 0.6 c	12.1± 2.4 e
	2×10^6	13.7 ± 1.3 d	9.5 ± 0.8 c	12.9 ± 1.8 e	34.8 ± 2.9 e	3.1 ± 0.3 c	4.8 ± 0.32f

Data were expressed as a mean ± SD. Groups marked with different letters are significantly different ($P< 0.05$; with Bonferroni posttest). Larval period ($F =30.2$, $P< 0.001$), pupal period ($F =188$, $P< 0.001$), pupation ratios ($F =188$, $P< 0.001$), adult emergence time ($F= 57. 4$, $P< 0.001$), adult longevity ($F= 27.0$, $P< 0.001$), and adult emergence ratios ($F = 74.7$, $P< 0.001$).

3. Biochemical Assays:

Results in Table (3), expressed the treatment of *G. mellonella* larvae with LD_{50} of both fungal species which revealed a significant reduction in TBP, TBL, and TBC compared to controls. However, the reduction was more in larvae infected with *C. albicans* than with *A. fumigatus* ($p< 0.05$) (212.78 and 227.98 mg/mL for treated and control groups, respectively).

Table 3. Effects of injection of *A. fumigatus* and *C. albicans* with LD_{50} on total body proteins (TBP), total body lipids (TBP), and total body carbohydrates (TBC) (mg/mL body tissues homogenate) of *G. mellonella larvae* after 24 and 48 h post-injection

Larval Treatment	Time after treatment					
	24 h			48 h		
	TBP	TBL	TBC	TBP	TBL	TBC
Untreated control	85.15 ± 8.36 a	212.78 ± 12.72 b	45.15 ± 3.22 a	84.56 ± 7.56 a	210.22 ± 11.38 b	43.26 ± 3.16 a
PBS-treated control	80.55 ± 8.36 a	227.98 ± 18.36 a	42.15 ± 3.51 a	78.12 ± 7.65 a	207.37 ± 16.16 a	42.04 ± 3.12 a
A. fumigatus-treated	32.16 ± 3.76 b	162.37 ± 13.76 b	27.13 ± 3.58 b	30.44 ± 3.76 b	159.30 ± 13.98 b	26.87 ± 2.67 b
C. albicans-treated	30.24 ± 3.46 b	169.56 ± 13.05 b	24.65 ± 4.64 b	30.24 ± 3.46 b	148.66 ± 12.85 b	22.29 ± 4.02 b

Data were expressed as a mean ± SD of 3 independent experiments with 10 larvae per treatment. Groups marked with different letters are significantly different ($P< 0.05$; with Bonferroni posttest). The concentrations of all biochemical parameters assayed were expressed as mg/mL. TBP at 24 h ($F = 13.4$, $P< 0.001$), and 48 h ($F = 22.7$, $P< 0.001$). TBL at 24 h ($F =117.1$, $P< 0.0001$), and 48 h ($F = 242.2$, $P< 0.0001$). TBC at 24 h ($F = 11.49$, $P< 0.001$; and at 48 h ($F = 15.4$, $P< 0.001$).

4. Hemocyte Counts and Spreading Behavior:

In this study, THC in the hemolymph samples of untreated *G. mellonella* larvae were 3.42 and 3.81 $x10^4$ cells/uL at 24 and 48 h, respectively (Table 4). Fungal treatment caused a remarkable decrease in THC as compared to controls at 24 and 48 h. The minimum count was detected at 48 h after *C. albicans* treatment.

In addition, PLs comprised 37.52% and 35.28% of the total hemocyte population of untreated *G. mellonella* larvae at 24 and 48 h, respectively, while GRs compressed 32.80 % and 34.11% at the same times (Table 4). Fungal treatment caused alterations in both PLs and GRs ratios at 24 and 48 h after treatment, however the changes were insignificant ($P> 0.05$).

The ratio of hemocyte spreading was 38.6% and 35.8% in untreated *G. mellonella* larvae at 24 and 48 h periods, respectively. The percentage of hemocytes from fungal-treated larvae exhibiting spreading activity decreased as compared to control groups at 24 and 48 h after treatment. The minimum count was detected at 48 h after *C. albicans* treatment. This decrease was more pronounced in the hemocytes of *C. albicans*-treated larvae (Table 4).

T**able 4.** Effect of injection of *A. fumigatus* and *C. albicans* LD_{50} on total hemocyte count ($THCx10^4$ cells/uL), differential hemocyte count (DHC, %), and hemocyte spreading activity (HSA%) of *G. mellonella* larvae after 24 and 48 h post-injection

Larval Treatment	Time after treatment							
	24 h				48 h			
	THC (10^4 cells/uL)	PLs %	GRs %	HSA %	THC (10^4 cells/uL)	PLs %	GRs %	HAS %
Untreated control	3.42 ± 0.11 a	37.52 ± 4.17 a	32.80 ± 3.72 a	38.6 ± 3.2 a	.81 ± 0.14 a	35.28 ± 3.58 a	34.11 ± 3.16 a	35.8 ± 2.4 a
PBS-treated control	3.65 ± 0.23 a	41.18 ± 4.36 a	32.15 ± 3.61 a	24.7 ± 3.6 b	3.55 ± 0.16 a	35.99 ± 3.26 a	32.14 ± 3.02 a	21.5 ± 3.3 b
A. fumigatus-treated	3.09 ± 0.26 b	42.37 ± 2.76 a	30.13 ±3.48 a	21.5 ± 3.2 b	3.06 ± 0 .12 b	37.37 ± 3.66 a	31.67 ± 3.17 a	28.3 ± 2.2 c
C. albicans-treated	3.04 ± 0.46 b	41.56 ± 3.05 a	29.65 ±4.54 a	22.7 ± 4.24 b	2.9 4 ± 0.15 b	37.86 ± 3.25 a	30.69 ± 3.34 a	18.9 ± 2.5 d

Data were expressed as a mean ± SD of 3 independent experiments with 10 larvae per treatment. Groups marked with different letters are significantly different ($P< 0.05$; with Bonferroni posttest). THC at 24 h ($F = 14.5$, $P< 0.001$), and 48 h ($F = 25.8$, $P< 0.001$). PLs at 24 h ($F =17.1$, $P< 0.32$), and 48 h ($F = 24.2$, $P< 0.125$). GRs at 24 h ($F = 12.9$, $P< 0.526$), and GRs at 48 h ($F = 21.9$, $P< 0.21$). HAS at 24 h ($F = 7.89$, $P< 0.001$; and at 48 h ($F = 10.4$, $P< 0.001$).

DISCUSSION

Given the interest in developing other non-mammalian models for studying microbial pathogens, to evade the ethical implications of animal testing, here the toxicity of the disease-causing fungi; *C. albicans* and *A. fumigatus* to *G. mellonella* larvae was compared to exploring the potential of this model insect. Because the insect immune system shares many features with human innate defense, insect models may be used to evaluate the toxicity of different agents, including bacterial and fungal pathogens, and the results showed complete agreement with the results of mammals (Kavanagh and Reeves, 2004).

The present study shed light on how fungal infection in humans develops as it does in larvae during the development of infection. The most common and accurate method of infection is by larval injection. This allows precise doses of fungi to be administered; thus, the LD_{50} can be calculated precisely. In the case of treatment of *G. mellonella* larvae via intra-hemocoel injection, the resulting LD_{50} values showed a close correlation with those found in mice (Maguire *et al*., 2016). However, our results concerning the administration of human fungal pathogens, *A. fumigatus*, and *C. albicans* to the larvae of *G. mellonella* by injection showed some differentiation based on comparative toxicity. These differences may be explained by the wide range of virulent factors or perhaps by the high expression of these factors (Cotter *et al*., 2000), or by differences in the growth rates of fungi and the production of tissue-damaging extracellular enzymes (Rossoni *et al*., 2013), however, Ramos *et al*. (2017) and Silva *et al*. (2018) cannot exclude this hypothesis. Larval mortality is due to large numbers of fungal cells in the insect's hemolymph (Dunphy and Thurston, 1990). These results agree with the results of Reeves *et al*. (2004) and Slater *et al*. (2011) who worked on *A. fumigatus*, and those of Cotter *et al*. (2000) and Brennan *et al*. (2002) who worked on *C. albicans* showed a strong correlation with results obtained using rats.

Different biological effects were observed due to fungal treatment in the last larval instar of *G. mellonella* by intra-hemocoel injection, and this is evident by a

dose-dependent increase in larval mortality, survived larval, pupal and adult emergence time prolongation and inhibition of pupal and adult emergence ratios. No differences among the tested fungi, concerning their effect on insect development. This is due to the possession of these fungi the same modes of action in insects. Delayed adult emergence due to fungal treatment has been documented earlier in numerous lepidopteran species; *Agrotis ipsilon* (Abd El-Aziz and Nofal, 1998), *Spodoptera littoralis* (Meshrif *et al*., 2007), and other insects such as the horn fly, *Haematobia. irritans* (Angel-Sahagun *et al*., 2005). These adverse biological consequences in growth and developmental processes which lead to reduced pupation and failure of adult emergence may be explained by unbalanced secretion of the juvenile hormone (Shalaby *et al*., 1987; Abd El-Aziz and Nofal, 1998).

Reductions in different metabolic products (TBP, TBL, and TBC) were observed in our study following fungal injection into *Galleria* larvae, this reduction was greatly remarkable in larvae treated with *C. albicans* than with *A. fumigatus*. The data obtained here confirms the possibility of distinguishing between different levels of pathogenicity within the different fungal groups using the Galleria model. Similar results were observed in *Spodoptera littoralis* treated with entomophilous nematodes (Ghally *et al*., 1988).

Owuama (2001) stated that the lowest level in total proteins, lipids, and carbohydrates may be because of pathogens released into the insect hemocoel to produce virulent factors that suppress host immune responses. These factors include toxins and hydrolyzing enzymes. Other potential virulence factors include a complex array of extracellular enzymes, like lecithinases, and phosphatases.

It's well known that fungi produce a variety of molecules including toxins and hydrolytic enzymes enabling them to colonize and reproduce in the insect host. Protease represents the most important enzyme produced that plays a role in insect cell damage because its activity leads to the breakdown of body proteins (Bowen *et al*., 2003). Alkaline metalloproteases play a role in the conversion of dead tissues into a nutritious soap ideal for microbial development (Bowen *et al*., 2003; Joyce *et al*., 2006; Crawford *et al.,* 2010).

The observed reduction in the TBL of *G. mellonella* larvae may result from the secretion of lipases. Fungi secrete several enzymes, including hemolysins, lipases, and proteases, contributing to their virulence or feeding upon the host's fat body (Richards and Blair, 2010). In addition, the decrease in TBC may result from the fungus feeding to gain the energy needed for its reproduction.

This study also demonstrated that fungi interact with the *Galleria* immune system which led to a decrease in THC at 24 and 48 h post-treatment. Similar results were observed by several authors (Bergin *et al*., 2003; Browne *et al*., 2015; Sheehan and Kavanagh, 2018). Many authors explained the decline in THC due to chemical or microbial treatment, it may be due to the aggregation and clumping of hemocytes, the main components of the immune system (Smith *et al*., 2022), or due to the inhibitory effects on endocrine glands, especially inhibiting the secretion of ecdysteroids from the prothoracic gland, that lead to the inhibition of hematopoietic function in insects Nunes *et al*. (2021), or may be due to the decline in mitotic division of prohemocytes (Rajak *et al*., 2015), however, this hypothesis needs further study to confirm it. It was recently reported by Nunes *et al*. (2021) that cellular defense in insects is influenced by hemolymph hormones. In addition to what was mentioned above, the decrease in THC may also be linked to the apoptotic death of hemocytes (Zhao *et al*., 2019).

Despite the decrease in the THC reported in this study, no significant change in the ratio of PLs and GRs was observed. Several studies reported significant changes

in DHCs caused by chemical and microbial treatment in various insects (Dorrah *et al*., 2019). Nunes *et al*. (2021) also suggested that the DHCs in insects are influenced by the circulating hormones in the hemolymph (e.g., ecdysone). In addition, the variation in the numbers of PLs and GRs may be due to the conversion of some hemocyte types into other types needed for cellular immune functions (e.g., phagocytosis), combating against foreign invaders or apoptotic agents (Dorrah *et al*., 2019). However, the decrease in HAS observed here appears to prevent this cell conversion. Hemocyte spreading activity is also an immune indicator in insects that occurs before cellular immune responses (phagocytosis, nodule formation, and encapsulation) as it allows PLs and GRs to adhere to foreign materials (Lavine and Strand, 2002). Here we detected significant reductions in the spreading activity of hemocytes from fungal-treated larvae at 24 and 48 h after treatment. However, this effect showed no significant difference between the tested fungi. These results agree with those demonstrated in hemocytes of *G. mellonella* on exposure to other fungal species (Torres *et al*., 2020), and other botanicals (Zibaee *et al*., 2012). Based on studies conducted by Sun *et al*. (2018) and Liu *et al*. (2019), toxic materials limit cell adhesion ability by interfering with the genes responsible for hormone biosynthesis. Considering the relationship between hormones and the behavior of hemocytes stated by Nunes *et al*. (2021), the decreased HSA reported in this study may be a result of fungal infection causing hormonal imbalance associated with immune regulation

Conclusion

After conducting this pathophysiological study, we can conclude that *G. mellonella* is a good model for examining the toxicity of human pathogenic fungi. By intra-hemocoel injection, the larvae give an indication of fungal doses and potential effects similar to what occurs in mammalian models without having to use large numbers of them in preliminary assays, which emphasizes the need for measures to identify and limit these effects.

Declarations:

Ethical Approval: Not applicable.

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