Journal of Experimental Biology and Agricultural Sciences, December - 2022; Volume – 10(6) page 1354 – 1365

Journal of Experimental Biology and Agricultural Sciences

http://www.jebas.org

ISSN No. 2320 – 8694

Antifertility Potential of n-Butanol and Ethyl Acetate Extracts of *Penicillium oxalicum* OM282858 in Male Albino Rats as Biological Control Agents

Ayat Taha[1], Shaymaa A. Gouda[2*]

[1]Zoology Department, Faculty of Science, Ain Shams University, Cairo, Egypt
[2]Microbiology Department, Faculty of Science, Ain Shams University, Cairo, Egypt

Received – July 22, 2022; Revision – November 21, 2022; Accepted – November 28, 2022
Available Online – December 31, 2022

DOI: http://dx.doi.org/10.18006/2022.10(6).1354.1365

KEYWORDS

Antifertility

Biological control

Penicillium oxalicum

Testicular toxicity

ABSTRACT

Rodents cause significant damage to many crops, spread diseases, and pose a severe risk to public health. Several synthetic contraceptive agents are available for controlling rodents; however, their use is associated with toxic effects on non-target organisms. *Penicillium oxalicum* has several medical properties, but no reports were available on fertility. This study aimed to assess the antifertility potential of n-butanol and ethyl acetate extracts of *P. oxalicum* in adult male albino rats as biological control agents by lowering the population size of rodent pests. Rats were assigned into three groups (n = 36). The first control group (GI) was injected intraperitoneally with 0.5% dimethyl sulfoxide (DMSO). The second (GII) and third (GIII) groups were injected with a single dose of 200 mg/kg body weight (b.wt.) of n-butanol and ethyl acetate extracts of *P. oxalicum* intraperitoneally, respectively, after dissolving in 0.5% DMSO. Further, *P. oxalicum* was identified morphologically and molecularly and then submitted with accession number OM282858 to the National Center for Biotechnology Information (NCBI) GenBank. The antifertility potential of *P. oxalicum* was evaluated after 24 h (the injection period), 96 h, and 168 h (the recovery periods) of treatments. The effects of the treatments on organ weight, testicular histology, histomorphometry measurements, and sperm characteristics were assessed. Both *P. oxalicum* extracts caused changes in reproductive organ weights, testicular histology, histomorphometry measurements, and spermatogenic arrest accompanied by a significant decrease in the count of epididymal sperm and its motility and an increase in the percentage of sperm abnormalities during the injection and recovery periods. Thus, the results suggest that both *P. oxalicum* extract treatments cause suppression of fertility in adult male rats. Therefore, these outcomes are essential for public health, farming establishments, and vertebrate pest control managers.

* Corresponding author

E-mail: shogouda@sci.asu.edu.eg (Shaymaa A. Gouda)

Peer review under responsibility of Journal of Experimental Biology and Agricultural Sciences.

Production and Hosting by Horizon Publisher India [HPI] (http://www.horizonpublisherindia.in/).

1 Introduction

Rodents are a substantial component of the earth's terrestrial ecosystems due to their economic and pathogenic relevance. However, they cause significant damage to many crops and wreak havoc on native fauna. Furthermore, they are critical vectors for transferring various diseases to humans and domestic and wild animals. As a result, rodents have become a significant concern in pest management and the recovery of endangered rodents in their natural habitats (Tobin and Fall 2004; Abdel-El-Azeem 2008).

Traditional strategies such as poisonous baits, anticoagulant rodenticides, trapping, hunting, and pesticides have been adopted to limit rodent damage or lower rodent populations. These traditional methods are ineffective, and the repetitive use of chemicals endangers human health and causes environmental hazards (Taha and Soliman 2019; Taha 2022). Therefore, pest managers have been directed to search for non-lethal approaches to increase the desire for environmentally friendly control methods at low cost and limit the non-target effects (Aktar et al. 2014; Mahmoud et al. 2018; Taha and Soliman 2019). One of these approaches is biological control using natural materials containing bioactive compounds from plants, algae, animals, microorganisms, and marine biota (Asyura et al. 2017), which is effective against rodent pests, phytopathogens, insects, and weeds.

The *Penicillium* genus is the dominant fungus and is well-known because it produces bioactive secondary metabolites (Zhang et al. 2020). After Fleming experimentally discovered penicillin, the bio-prospecting efforts of thousands of *Penicillium* isolates have been tested for their high biological activities (Zhang et al. 2020; Shankar and Sharma 2022; Weng et al. 2022). *Penicillium oxalicum* is one of the most abundant of all the *Penicillium* species (Currie and Thom 1915) that can produce novel bioactive metabolites (Weng et al. 2022), which are used to control bacteria, fungi, and insects (Kubátová et al. 2019). In the current study, the male antifertility capabilities of *Penicillium oxalicum* n-butanol and ethyl acetate extracts were assessed in adult male rats to integrate both fungal extracts as biological control agents in pest management programs.

2 Materials and methods

2.1 Fungal isolation

In this study, the fungal strain QR20 was previously isolated as a mycoflora isolate from rice rhizospheric soil in Egypt by serial dilution technique according to methods used by Johnson and Curl (1971). Fungal colonies were purified and cultured on potato dextrose agar (PDA) media containing potato slices (200 g/l) and 20.0 g/l for both dextrose and agar (Rotem 1994), then were stored at 4 ℃ in slants and in 15% glycerol at -4℃.

2.2 Fungal morphological identification

The fungal isolate QR20 was identified by observing the macroscopic properties, including colony morphology and color on PDA. The fungal microscopic slides were prepared by the slide culture technique using lactophenol cotton blue stain (Vainio et al. 1998), Conidia and conidiophore were observed on the slides using an optical microscope.

2.3 Molecular identification of the isolated fungus

2.3.1 DNA extraction and polymerase chain reactions (PCRs) protocol

All genomic DNA was extracted and purified from a 7-day-old culture of 100 mg wet fungal isolate QR20 spores using the Quick-DNA™ Miniprep Kit per the manufacturer's protocol. Fungal identifications were based on the internal transcribed spacer (ITS) of their ribosomal DNA (ITS1–5.8S–ITS2). Universal primers: ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used to amplify ribosomal DNA of *Penicillium* spp. (White et al. 1990). The amplification reactions were performed in a 50 µl reaction volume of COSMO PCR Red Master Mix. The program of the thermal cycler used was as follows: initial denaturation at 95 ℃ for 2 minutes, denaturation at 95 ℃ for 15 seconds, annealing at 50 ℃ for 20 seconds, and extension at 72 ℃ for a minute. This program was followed by 35 cycles of denaturation at 95 ℃ for 15 seconds, annealing at 50 ℃ for 20 seconds, and extension at 72 ℃ for a minute. They were followed by a final extension at 72 ℃ for 5 min.

2.3.2 Alignments and phylogeny

The PCR amplicons were sequenced at GATC Company, Germany, using an ABI 3730x1 DNA sequencer. The obtained sequences and other *Penicillium* species sequences from the National Center for Biotechnology Information (NCBI) GenBank nucleotide database were used to carry out the phylogenetic analysis and tree development. The phylogenetic study was performed using MEGA version 11 software (Tamura et al. 2021). The maximum likelihood (ML) method was used to reconstruct the phylogenetic tree, with bootstrap values calculated after a run of 1000 replications using routines included in MEGA software (Felsenstein 1985). The phylogenetic tree was demonstrated by Fig Tree version 1.4.4 (Rambaut 2020).

2.4 Purification of *Penicillium oxalicum*

To guarantee the purity of *P. oxalicum*, fungal hyphal tips that emerged from previously sub-cultured fungi were taken and sub-

cultured again by putting it on fresh PDA media and incubating for 7 days at 28 ± 2 ℃.

2.5 Inoculum preparation and extraction

Penicillium oxalicum QR20 secondary metabolites extraction was performed according to the procedures of Petit et al. (2009) and Tirumale et al. (2020), with minor modifications for *P. oxalicum* QR20 inoculum preparation. A pure culture of fungus was inoculated on the modified Czapek-Dox broth medium (Dox 1909), which consisted of sucrose as a carbon source (30.0 g/l), KCl (0.5 g/l), $NaNO_3$ (3.0 g/l), K_2PO_4 (1 g/l), $MgSO_4 \cdot 7H_2O$ (0.5 g/l), $Fe(II)SO_4 \cdot 7H_2O$ (0.01 g/l), and pH was adjusted at 7.3 ± 0.2. After fungal inoculation, the culture was incubated at static conditions for two weeks at 28 ± 2ºC. At the end of the incubation period, broth media were first filtered with cheesecloth to remove the mycelial mats and then with Whatman filter paper No. 1 to remove mycelium. The aqueous filtrate was subjected to liquid-liquid extraction with ethyl acetate (EtOAc) solvent three times with an equal volume of culture filtrate (1:1, vol/vol). It was shaken well for 15 min in a separating funnel. The organic layer (EtOAc) was collected, subjected to Na_2SO_4, and concentrated under a vacuum using a rotary evaporator. After evaporation, a crude dried EtOAc extract was obtained. Moreover, the fungal filtrate was extracted with n-butanol (n-BuOH) using the same procedures used for the ethyl acetate extract. Both fungal extracts were prepared to evaluate the antifertility potential.

2.6 Animals

Thirty-six (120–130 g) adult male Wistar albino rats (*Rattus norvegicus*) were obtained from the National Research Center (Cairo, Egypt). Rats were fed *ad libitum* per the standard rodent diet and water intake. The rats were prepared for acclimatization for ten days before the start of the experiment under natural light and dark cycles at 22 ± 2 ℃ and 40- 60% humidity (laboratory conditions). They were humanely handled, and this study protocol was approved by the Ain Shams University Research Ethics Committee (ACUC-FP-ASU RHDIRB2020110301 REC#90).

2.7 Experimental protocol

Rats were randomly assigned into three groups, with twelve rats in each group. Treatments were assigned to each group; the first control group (GI) was administered intraperitoneally with 0.5% dimethyl sulfoxide (DMSO). The second (GII) and third (GIII) groups were injected with a single dose of 200 mg/kg body weight (b.wt.) of n-butanol and ethyl acetate extracts of *P. oxalicum*, respectively (Kaur et al. 2021). Doses were prepared by making a suspension of the fungal extracts, which were dissolved in 0.5% DMSO for intraperitoneal injection.

2.8 Body weight, reproductive organ weights, and sperm characteristics

Rats were weighed, and the obtained weights were recorded at the experiment's beginning and end. Then, the percentage change in body weight was calculated according to the following equation:

Percentage change in body weight = [(recorded end body weight-recorded beginning body weight/ recorded beginning body weight)] x 100

After 24 hours of injection, four rats per group were sacrificed. To assess the reversal or delay effects of antifertility of both fungal extracts, the remaining four rats from each group lasted for 96 and 168 h. They were then sacrificed to test the recovery period.

The anesthetization of rats was carried out with chloroform. First, the male reproductive organs (testes, epididymis, vesicula seminalis, and prostate gland) were autopsied, washed in saline, and then weighed. The organ index weight (relative weights) was recorded as organ weight/body weight × 100. Then, the epididymis was removed and perfused with a modified 2 ml of Tyrode's solution (125 mM NaCl, 2.7 mM KCl, 25 mM $NaHCO_4$, 0.5 mM $MgCl_2$, 1.80 mM $CaCl_2 \cdot 6H_2O$, 05.56 mM glucose, 36 mM $NaH_2PO_4 \cdot 2H_2O$, 100 units of penicillin, and 4 mg/ml BSA) at 34 ℃ to determine sperm parameters and then crumbled by scissors to allow sperm release. The sperm sample was incubated at 38 ℃ for 15 min; then, a hemocytometer was used to count sperm, expressed as 10^6/ml. The same sample was used to evaluate the percentage of sperm motility under a light microscope. A smear of sperm was placed on microscopic slides, and methanol was used to fix sperm and stained with eosin as described by Mahmoud et al. (2018), which was then examined at 1,000x by an objective lens with oil immersion in a light microscope. According to WHO (2000), the sperm were classified as abnormal. The abnormality of sperm was calculated as a percentage of the total spermatozoa count (Taha and Soliman 2019).

2.9 Histopathological evaluation

Before histological analysis under a light microscope, the testis tissue slices were stained with hematoxylin and eosin (H&E) for histopathological examination, as Soliman et al. (2016) described. Testis capsule thickness, epithelial height, and diameter of seminiferous tubules were measured at 400x. The testicular scoring system of Johnsen (1970) was used to assess spermatogenesis in a semi-quantitative manner. The order of maturation was given a degree ranging from 10 to 1 for each sectioned tubule according to the appearance or disappearance of main cell types: complete spermatogenesis (10), spermatozoa present with random spermatogenesis (9), few spermatozoa (8), no spermatozoa but spermatids were present (7), few spermatids were present (6), spermatocytes were only present (5 or 4), presence of

spermatogonia (3), presence of only Sertoli cells (2), and practically empty lumen (1). The mean score was obtained by randomly picking ten seminiferous tubules/rats.

2.10 Statistical analysis

All data values were tabulated and statistically analyzed using one-way variance analysis (ANOVA) and performed by Minitab V17 software. The significant difference was at a p-value ≤ 5%, and the data were expressed as mean ± standard error (SEM).

3 Results

3.1 Morphological and molecular identification of the isolated fungus

The fungal strain (QR20) was morphologically identified according to macromorphology and micromorphology as a member of the genus *Penicillium*. Macroscopic characteristics of fungal growth on PDA media included rapid growth and appearance as white mycelia with a cottony texture that turned bluish-green with a powdery texture due to the production of sexual conidiospores (Figure 1a). Microscopic characteristics of the fungus showed hyphae and long chain conidia with smooth walls (Figure 1b). These results were confirmed by molecular identification.

The molecular identification was carried out using rDNA (ITS1-5.8S-ITS2) region sequencing. In blast similarity analysis, the isolated fungus sequence (ITS-rDNA) revealed a 100% sequence similarity with *P. oxalicum*. The fungal strain was submitted to GenBank with accession number OM282858. Phylogeny was constructed based on rDNA sequences and ITS data of the identified fungal isolate QR20 sequence with the closest relative species sequences obtained from NCBI by Mega11 software. The maximum likelihood (ML) method to study genetic relatedness revealed that the fungal isolate QR20 in accommodate to two taxa (*Penicillium oxalicum* PSF-4 MK720103 and *Penicillium oxalicum* 2-4F MW077049) with 100% bootstrap (BT) support, as shown in Figure 2.

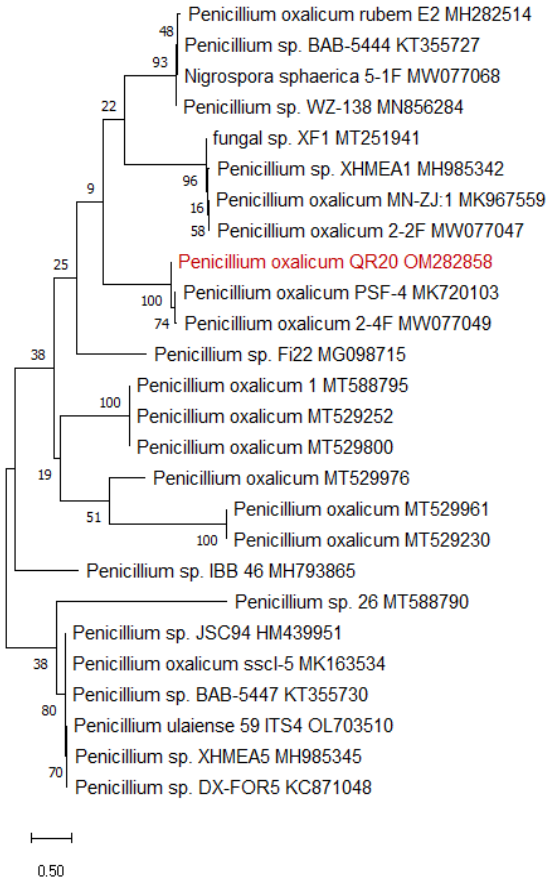

Figure 2 Phylogeny constructed based on ITS sequence dataset of *P. oxalicum* QR20 (OM282858) in red color with other related genes through maximum likelihood analysis. Bootstrap support values (one thousand replicates) are represented at each branch

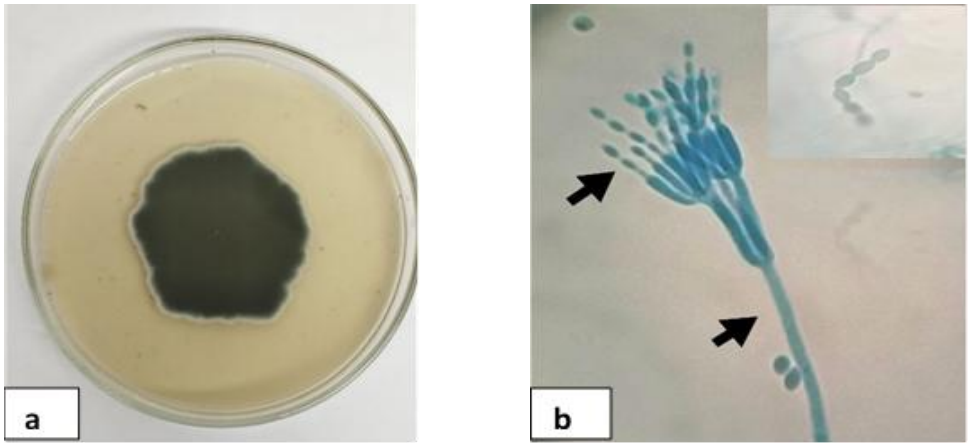

Figure 1 Photograph of *P. oxalicum* isolate (QR20), (a) Colony growth on PDA media after incubation for seven days at 28 ± 2 °C, (b) A microscopic examination showing both conidiophores and conidia (in the inside box) at 400×

3.2 Clinical signs and mortality

There were no toxicity signs in either fungal extract treatment, and all treated rats appeared without abnormalities during both the injection and recovery periods. Additionally, all treated rats had no changes in stool, urine, or eye color. Furthermore, no mortality was recorded in any treated rats during the experiment.

3.3 Effect of *P. oxalicum* extracts on body weight gain and reproductive index organ weights

During the injection period with n-butanol and ethyl acetate extracts, body weight significantly decreased ($p = 0.003$ and 0.018, respectively). On the other hand, there were no significant changes after 168 h in either treatment ($p = 0.189$ and 0.121, respectively) compared to the control rats (Table 1). These results indicated that during the recovery period, body weight could be recovered to the standard (control) range (Table 1).

In GII, there was a significant increase in relative testis weights ($p = 0.044$) and a significant decrease in both relative vesicula seminalis weights ($p = 0.029$) and relative prostate gland weights ($p = 0.008$) after 24 h of treatment (the injection period). Moreover, compared to the untreated rats, relative prostate weights after 96 and 168 hours (the recovery period) showed a highly significant ($p = 0.001$) decrease.

In GIII, there were non-significant changes in testis index weights in treated rats after the injection period ($p = 0.725$) and recovery periods (96 h and 168 h) ($p = 0.387$ and 0.645, respectively; Table 1). There was a significant decrease in vesicula seminalis weight after the injection period of ethylacetate extract ($p = 0.011$). Besides, the relative prostate gland significantly decreased after 96 hours of recovery ($p = 0.008$) compared to the untreated rats (Table 1).

3.4 Effect of *P. oxalicum* extracts on sperm characteristics

During the injection and recovery periods, epididymal sperm count and motility showed a highly significant decrease ($p<0.001$) in comparison to the control rats (Table 2). In the present study, abnormalities found in epididymal sperm were classified as primary abnormalities and identified as the following: (a) abnormalities in the head, which included a compact head, an amorphous head, a headless tail, and a head with a cytoplasmic droplet; (b) abnormalities in the tail, which included a tailless head, a bent tail, and a coiled tail. Total sperm abnormalities demonstrated a significant increase during the injection period of n-butanol extract ($p = 0.021$) and recovery periods of 96 h ($p = 0.019$) and 168 h ($p = 0.013$). Furthermore, in the ethyl acetate extract, total sperm abnormalities revealed a significant increase during the injection ($p = 0.010$) and recovery periods of 96 h ($p = 0.002$) and 168 h ($p = 0.001$) (Table 2).

3.5 Histopathological results

3.5.1 Control group

Untreated rats dissected testes appeared normal in size and color, according to a gross examination. The control group's testicular histology showed a typical histological structure at all phases of spermatogenesis, and the lumen was filled with sperm (Figure 3a and 3b). A thin tunica albuginea entirely encircled the testis (Figure 3a). Testicular tissue contains seminiferous tubules (round or oval) separated by interstitial tissue (Figure 3a). The Sertoli cells are on a thin basal lamina, and a series of spermatogenic cells line each seminiferous tubule (Figure 3b).

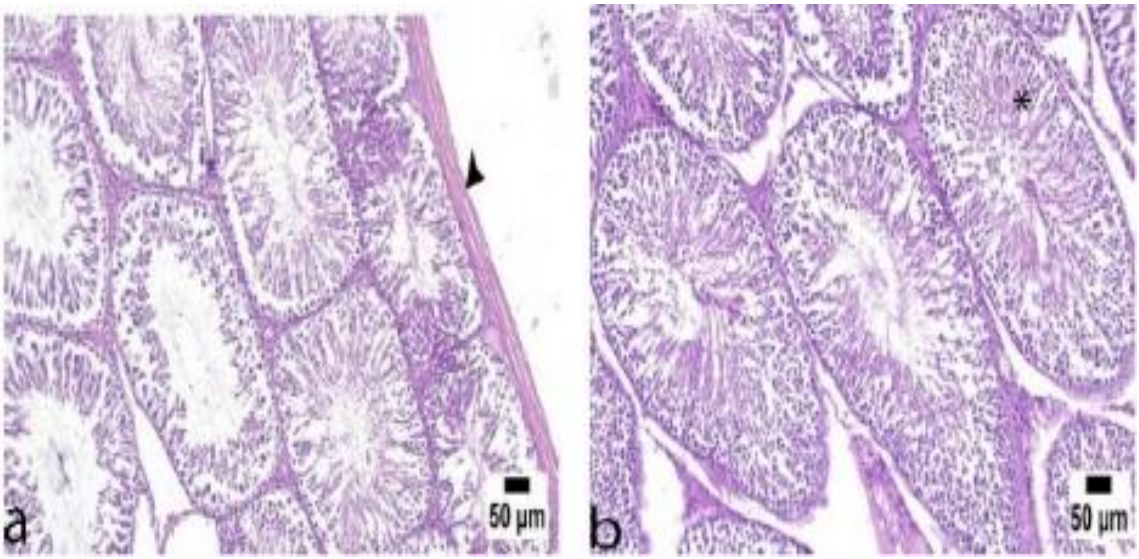

Figure 3 Photomicrographs of testis tissues of albino rats stained by (H&E) of the control group. (a, b) show a thin tunica albuginea layer (arrowheads) seminiferous tubules consisting of stratified germinal epithelium (asterisk)

Table 1 Effect of n-butanol extract (GII) and ethyl acetate extract (GIII) of *P. oxalicum* on body weight, relative reproductive organ weights, stages of spermatogenesis, and testicular morphometry of albino rats.

Treatment		Changes in body weight (%)	Relative reproductive organs weights (%)				Stages of spermatogenesis	Morphometric analysis (µm)		
			Testis	Epididymis	Vesicula seminalis	Prostate gland	Johnsen's score for spermatogenesis	Thickness of tunica albuginea	Diameter of seminiferous tubules	Epithelial height
GI	Control	24.12 ± 6.89	0.685 ± 0.04	0.305 ± 0.088	0.45 ± 0.06	0.307 ± 0.02	9.1 ± 0.27	17.67 ± 0.57	483.16 ± 25.1	107.5 ± 7.82
GII	24 h	-9.070 ± 0.78**	0.79 ± 0.08*	0.235 ± 0.008	0.255 ± 0.014*	0.205 ± 0.0086**	8.0 ± 0.25**	44.27 ± 0.77***	288.5 ± 5.67***	87 ± 1.52**
	96 h	1.28 ± 1.02*	0.735 ± 0.0086	0.265 ± 0.0086	0.265 ± 0.008*	0.145 ± 0.014***	5.6 ± 0.4***	36.60 ± 0.77***	303.5 ± 5.9***	85.5 ± 1.42**
	168h	13.85 ± 0.77	0.771 ± 0.023	0.175 ± 0.025	0.325 ±0.037	0.165 ± 0.002***	6.4 ± 0.22***	32.14 ± 0.57***	287.5 ± 4.78***	74.5 ± 2.5***
GIII	24 h	1.75 ± 1*	0.73 ± 0.115	0.185 ± 0.049	0.205 ± 0.002*	0.277 ± 0.0025	5.9 ± 0.48***	31.069 ± 0.54***	212 ± 11.74***	53 ± 2.09***
	96 h	3.31 ± 3.2*	0.725 ± 0.014	0.285 ± 0.020	0.41 ± 0.017	0.205 ± 0.008**	6.1 ± 0.50***	46.96 ± 1.08***	254.98 ± 8.5***	67.5 ± 1.55***
	168 h	11.1 ± 2.13	0.735 ± 0.095	0.51 ± 0.16	0.47 ± 0.05	0.25 ± 0.017	5.6 ± 0.16***	39.28 ± 0.57***	273.5 ± 8.63***	71.5 ± 1.58***

Values are expressed as means ± SEM; they were significantly different from those of the control group; *p≤0.05; **p≤0.01; ***p≤0.001.

Table 2 Effect of n-butanol extract (GII) and ethyl acetate extract (GIII) of *P. oxalicum* on sperm characteristics in albino rats

Treatments		Epididymal sperm count x 10^6/ml	Sperm motility (%)		Sperm morphology (%)							
			Motile sperm	Immotile sperm	Compact Head sperm	Amorphous head sperm	Headless sperm	Head with cytoplasmic droplet sperm	Tailless sperm	Bent tail sperm	Coiled tail sperm	Total sperm abnormalities (%)
GI	Control	58.5 ± 4.9	70 ± 3	30 ± 2	10 ± 0.7	1.5 ± 0.64	2.5 ±1.04	0	2.75 ± 0.47	0	0	16.75 ± 1.79
GII	24h	7.25 ± 1.10***	0 ± 0***	100***	11.5 ± 1.32	7 ± 1.08**	6 ± 1.08	0.75 ± 0.47	3.5 ± 0.64	0	1.25 ± 0.47*	30 ± 3.85*
	96h	5.5 ± 0.64***	0 ± 0***	100***	10.25 ± 1.30	7.25 ± 0.85**	6 ± 1.08	0.5 ± 0.288	4.25 ± 0.85	0	1 ± 0.40*	29.2 ± 3.47*
	168h	9.5 ± 0.64***	0 ± 0***	100***	10.25 ± 1.6	6.75 ± 0.62***	6.25 ± 1.03*	0.75 ± 0.47	5.25 ± 0.62*	0	1 ± 0.40*	30.2 ± 3.42*
GIII	24h	8 ± 1.4***	0 ± 0***	100***	8.2 ± 1.0	5.7 ± 1.4*	4.75 ± 0.85	0	6.5 ± 0.8**	2 ± 0.40**	0	27.2 ± 2.13**
	96h	7.75 ± 0.85***	4.5 ± 0.5***	95.5 ± 0.5***	11.2 ± 1.3	6.7 ± 0.75**	5.25 ± 1.03	0	7 ± 0.57***	2.25 ± 0.2***	0	32.5 ± 2.5**
	168h	8.75 ± 0.62***	7.5 ± 1.44***	92.5 ± 1.44***	9.5 ± 0.64	7 ± 1.22**	4.75 ± 0.8	0	7.5 ± 0.28***	1.75 ± 0.47**	0	30.5 ± 1.70***

Values are expressed as means ± SEM. They were significantly different from those of the control group. *p≤0.05; **p≤0.01; ***p≤0.001

3.5.2 n-butanol and ethyl acetate extract of *P. oxalicum* treated groups

During the injection and recovery periods, gross morphological examination of autopsied testes from rat groups demonstrated typical color and size compared with the control group. The testes of both fungal extract-treated groups (GII and GIII) showed highly significant ($p<0.001$) increases in the thickness of tunica albuginea and a decrease in tubule diameter in the injection and recovery periods in comparison to the control group (Table 1 and Figure 4 and 5). In GII, the testicular examination of the injected and recovery periods showed the presence of congested blood vessels, disrupted seminiferous tubules with vacuolated spermatogenic cells, mild interstitial edema, decreased spermatogenesis in the surrounding seminiferous tubules, and wide interstitial spaces (Figure 4). During the injection period of GIII, testicular examination indicated several histopathological alterations, including atrophied tubules characterized by an irregular basement membrane with degenerated seminiferous tubules, the absence of mature sperm, vacuolated spermatogenic cells, congested blood vessels, and wide interstitial spaces (Figure 5a and 5b). During the recovery periods (96 h and 168 h) of GIII, testicular examination revealed numerous vacuolations and exfoliation of spermatogenic cells in seminiferous tubules, wide interstitial spaces, mild interstitial edema, and congested blood vessels (Figure 5c, 5d, 5e, and 5f). In GII, the testicular biopsy (mean of Johnsen's score) was significantly lower ($p = 0.009$) than in the untreated rats during the injection period, while it demonstrated a more significant ($p<0.01$) decrease in testicular biopsy after 96 h and 168 h of exposure to n-butanol extracts (the recovery period). In GIII, the mean of Johnsen's score showed a more significant decrease ($p<0.01$) during the injection and recovery periods (Table 1). The results of Johnsen's scoring in both treatments indicated impaired spermatogenesis (Table 1).

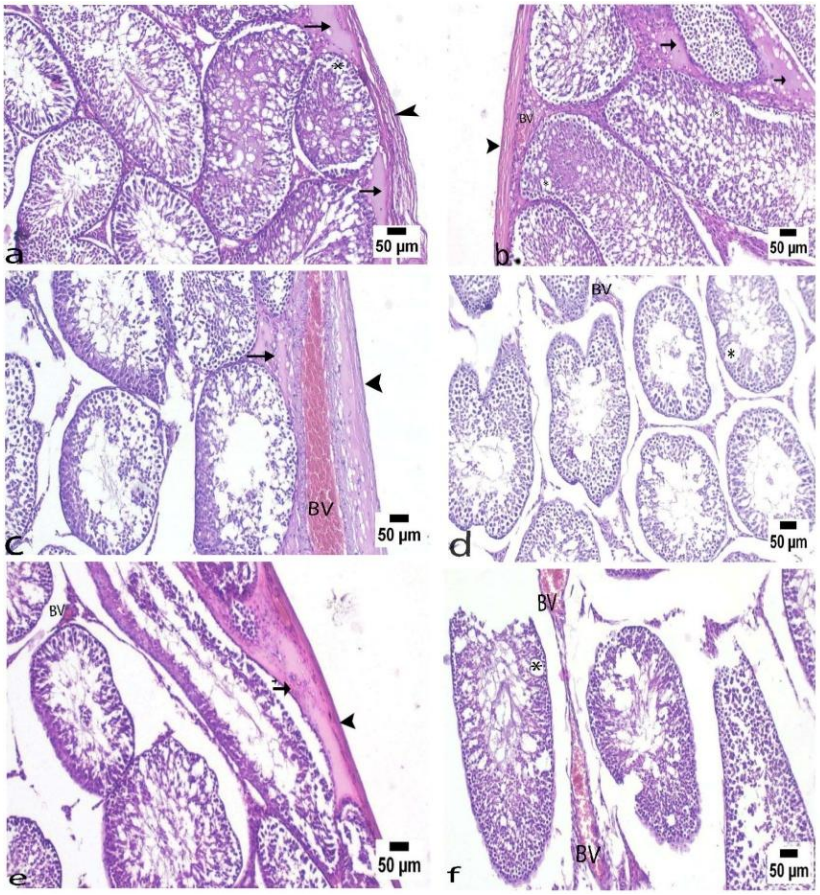

Figure 4 Photomicrographs of testes tissues of albino rats stained by (H&E) treated with n-butanol extract of *P. oxalicum*; (a, b) show thickened tunica albuginea (arrowheads), the presence of congested blood vessels (BV), disrupted seminiferous tubules with vacuolated spermatogenic cells (asterisk), and mild interstitial edema (arrows) after 24 h of treatment (the injection period); (c, d) The recovery period (after 96 h of cessation of treatment) shows thickening in the tunica albuginea (arrowhead), the presence of congested blood vessels (BV), and mild vacuolation of seminiferous tubules (asterisk), and mild interstitial edema (arrow); (e, f) The recovery period (after 168 h of treatment cessation) demonstrates thickening in the tunica albuginea (arrowhead) surrounded by peripheral edema (arrow), a mildly congested blood vessel (BV), and vacuolated spermatogenic cells (asterisk).

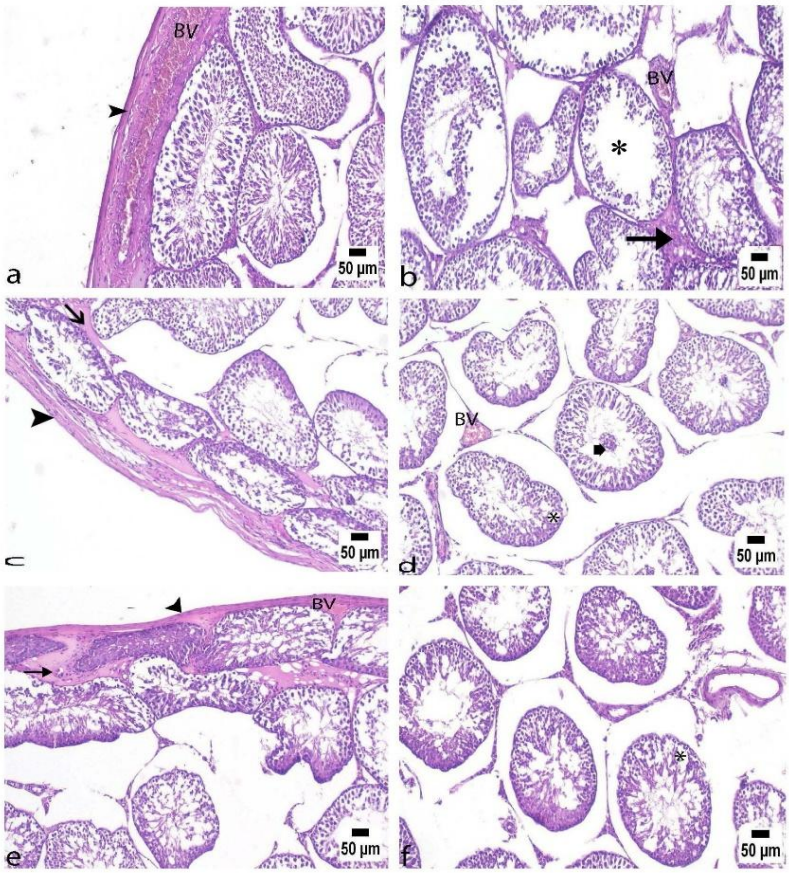

Figure 5 Photomicrographs of testes tissues of albino rats stained by (H&E) treated with ethyl acetate extract of *P. oxalicum*; (a, b) After 24 h of treatment (the injection period), there is a thickening in the tunica albuginea (arrowhead), a presence of congested blood vessels, disrupted seminiferous tubules with vacuolated spermatogenic cells, mild interstitial edema (arrow), empty seminiferous tubule (asterisk), and wide interstitial spaces; (c, d) The recovery period (after 96 h of ceased treatment) shows thickening in tunica albuginea (arrowhead), the presence of mild blood vessels congested (BV), disrupted seminiferous tubules with exfoliated spermatogenic cells (broad arrow), and vacuolated spermatogenic cells (asterisk), and mild interstitial edema (arrow); (e, f) The recovery period (after 168 h of cessation of treatment) shows thickened tunica albuginea (arrowhead), the presence of congested blood vessels (BV), disrupted seminiferous tubules with vacuolated spermatogenic cells (asterisk), and mild interstitial edema (arrow).

4 Discussion

Biological control based on fungi is one of the most promising techniques (Charnley and Collins 2007). The fungal secondary metabolites are significant due to their biological functions and efficiency (Greco et al. 2019; Shankar and Sharma, 2022; Weng et al. 2022). The present study assessed the possible antifertility effects of *P. oxalicum* QR20 extracts (n-butanol and ethyl acetate) on adult male albino rats to be used in integrated pest management programs. The present work relies on the genus *Penicillium*'s ability to produce many different arrays of bioactive metabolites (Zhang et al. 2020).

P. oxalicum is widespread in the soil (Currie and Thom 1915) and a natural source for producing several compounds such as organic acids, toxins, numerous enzymes, and alkaloids (Kubátová et al. 2019). These substances are used in several biocontrol methods, such as antibacterial, antifungal (Lucas et al. 2007; Yang et al. 2008), and insecticidal agents (Santamarina et al. 2002). Therefore, it is recommended as a biocontrol technique against various pests or plant pathogens (Kataria et al. 2018).

The obtained results from macro and microscopic characteristics of the isolated fungus (QR20) revealed that it was *P. oxalicum*, confirmed by the molecular test with 100% identity. The sequence was deposited at GenBank with accession number OM282858. In the molecular characterization, the PCR amplicon amplified with ITS1/ITS4 primers agreed with Umemoto et al. (2009), who revealed *Penicillium* species identification with 100% homology, using sequencing of ITS region, and with Stackebrandt and Goebel (1994) who confirmed that the higher value of identity percentage indicated the higher similarity of DNA sequences. Furthermore,

Gardens and Bruns (1993) reported that investigations of fungal phylogenetics through ITS regions of ribosomal DNA were widely used in genomes, which was attributed to the facilitated examination of these regions by PCR amplification.

The present study showed that n-butanol and ethyl acetate extracts of *P. oxalicum* were injected once intraperitoneally at 200 mg/kg b.wt. and strongly affected the male fertility of albino rats. No clinical signs appeared during the treatments, and all rats were active, healthy, and showed normal behavior. During the injection period, the body weight of both fungal extract-treated rats showed a significant reduction, while it did not indicate any significant changes during the recovery period of 168 h for both treatments compared with the non-treated group. The percentage of change in the body weight of treated rats could be recovered within a week, suggesting that the two fungal extracts had a slight effect on the body weight of treated rats, which was considered a negligible effect on the growth of animals. Body weight fluctuations are a highly sensitive indicator of overall health, and any significant changes in body weight indicate toxic materials (Muharni and Heni 2018; Taha 2022).

Spermatogenesis represents a significant biomarker of chemically-induced male reproductive toxicity (Reddy et al. 2011). The weight of accessory reproductive organs is an endpoint used to estimate the toxic effects of the tested chemical compounds on male fertility (Creasy 2003). In the current study, both fungal extracts generated considerable alterations regarding some reproductive organ weights and caused changes in both the structure and functions of these organs. These might be attributed to androgen insufficiency because androgens are essential for typical male reproductive organ growth (Mahmoud et al. 2018).

In both fungal extract treatments, the count and motility of sperms showed a highly significant ($p<0.001$) decrease during the injection and recovery periods compared to the untreated rats. These results indicated that both fungal extracts had highly destructive effects on the count and motility of epididymal sperm compared to the control rats. The results were consistent with those of other researchers, who reported that sperm count and motility were vital factors and that any changes in sperm count and motility could be used as an indicator for measuring normal fertility by measuring sperm fertilizing capacity in animals (Mahmoud et al. 2018; Taha and Soliman 2019). The reduction in the count and motility of epididymal sperm was attributed to the reduced supply of testosterone in the epididymis (Gong and Han 2006). Changes in sperm characteristics might be attributed to differences in the solvent polarity of n-butanol and ethyl acetate extracts. Different chemical compounds dissolve at different polarities; hence, there are differences in the quantity of both extracts obtained, which lead to different effects (Paini et al. 2014). The activity of the ATPase enzyme in the spermatozoa cell membrane could be affected by alkaloid compounds and the distributing balance of sodium and potassium ions (Hidayati et al. 2018). The morphology of sperm is a vital parameter that reflects their normality and maturity and is directly correlated with male fertility (Memon et al. 1986; Taha and Soliman 2019). Head and mid-piece sperm abnormalities were identified as mainly prime spermatogenesis defects (Schumacher and Moll 2011; Taha and Soliman 2019) and were valuable indicators of testicular degeneration (Bloom 1950; Taha and Soliman 2019). Alterations in sperm parameters were directly associated with the histopathological changes in testicular tissue that caused reproductive dysfunction (De Souza et al. 2010). In this study, both fungal extracts caused numerous testicular histological changes in treated rats during injection and recovery compared to non-treated rats. These results were similar to previous reproductive toxicity publications that showed gonad histology, testis biochemistry, and epididymis histology were used to characterize hazardous compounds that might induce reproduction difficulties in treated animals (Mathur et al. 2010). The increased tunica albuginea thickness resulted from decreasing testicular parenchyma volume (Arenas et al. 1997; Mahmoud et al. 2018; Taha and Soliman 2019). The appearance of numerous mild congestions in the blood vessels of the testes of injected rats was attributed to increasing the production of adenosine, which was consistent with hypoxia and caused vasodilation and expanded blood flow, restoring normal oxygen levels (Huether and McCance 2008; Mahmoud et al. 2018).

The tests on *P. oxalicum* extract-treated rats demonstrated shrinkage in tubular diameter. This was due to the disruption of cell junctions in the Sertoli germ (Mesbah et al. 2008; Taha and Soliman 2019). According to this study, both fungal extract treatments significantly lowered the epithelial height of seminiferous tubules during the injection and recovery periods. These results were accompanied by multiple changes in testicular histology, an increase in tunica albuginea thickness, a decrease in tubular diameter, decreases in the number and motility of epididymal sperm, and an increase in the percentage of sperm abnormalities. Mean score of the testicular biopsy (Johnsen's score) for both fungal extracts during the injection and recovery periods indicated a significant decrease compared to non-treated rats, revealing that both fungal extracts impaired spermatogenesis.

Conclusions

The current study concluded that n-butanol and ethyl acetate extracts of *P. oxalicum* have significant antifertility effects due to the inhibition of spermatogenesis. Further studies are required to clarify the exact mechanism of the antifertility effect and the separation and identification of the active ingredients responsible for the antifertility. To the best of our knowledge, this is the first research that evaluated the potential of *P. oxalicum* extracts as antifertility agents.

Acknowledgment

The authors gratefully acknowledge Prof. Naziha Mohamed Hassanein, Department of Microbiology, Faculty of Science, Ain Shams University for her assistance on the fungal isolation.

Author contributions

All author conceived, designed the study, and contributed to data analysis and manuscript preparation. All authors read and approved the manuscript.

Conflict of interest

No conflicts of interest. Alone, authors are responsible about the content and writing of paper.

References

Abdel- Azeem, M. I. (2008). Ecological studies on some commensal rodent species and their ectoparasites in different habitats at Sharkia governorate. *M. Sc. Thesis, Faculty of Agriculture Sciences, Suez Canal University, Egypt.* 193.

Aktar, M. T., Hossain, K. S., & Bashar, M. A. (2014). Antagonistic potential of rhizosphere fungi against leaf spot and fruit rot pathogens of brinjal. *Bangladesh Journal of Botany, 43*(2), 213-217. https://doi.org/10.3329/bjb.v43i2.21675

Arenas, M. I., Bethencourt, F. R., De Miguel, M. P., Fraile, B., Romo, E., & Paniagua, R. (1997). Immunocytochemical and quantitative study of actin, desmin and vimentin in the peritubular cells of the testes from elderly men. *Reproduction*, *110*(1), 183–193. https://doi.org/10.1530/jrf.0.1100183

Asyura, C., Hasan, A., Hasim, Julistiono, H., Husnawati, Bermawie, N., & Riyanti, E. (2017). Effectiveness of ethyl acetate extract of endophytic fungi in soursop leaves towards the growth of mammary tumor induced by 7,12-dimethylbenz(α)anthracene in female rats. *Annual Research & Review in Biology, 18*(5), 1–8. https://doi.org/10.9734/arrb/2017/34656

Bloom, E. (1950). Fertility in male animals. *Journal of Fertility and Sterility, 1*, 223-224.

Charnley, A.K., & Collins, S.A. (2007). Entomopathogenic fungi and their role in pest control. In: C.P. Kubicek, & I.S. Druzhinina, (Eds.), The Mycota IV: Environmental and Microbial Relationships, 2nd Edition, Springer-Verlag, Berlin, pp. 159-187.

Creasy, D. M. (2003). Evaluation of testicular toxicology: A synopsis and discussion of the recommendations proposed by the society of toxicologic pathology. *Birth Defects Research Part B: Developmental and Reproductive Toxicology*, *68*(5), 408–415. https://doi.org/10.1002/bdrb.10041

Currie, J. N., & Thom, C. (1915). An oxalic acid producing *Penicillium*. *The Journal of Biological Chemistry*, *22*, 287–293. http://dx.doi.org/10.1016/S0021-9258(18)87646-3

De Souza Predes, F., Diamante, M. A. S., & Dolder, H. (2010). Testis response to low doses of cadmium in Wistar rats. *International Journal of Experimental Pathology*, *91*(2), 125–131. https://doi.org/10.1111/j.1365-2613.2009.00692.x

Dox, A. W. (1909). The intracellular enzymes of lower fungi, Especially those of *Penicillium camemberti*. *Journal of Biological Chemistry*, *6*(5), 461–467. https://doi.org/10.1016/s0021-9258(18)91606-6

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, *39*(4), 783–791. https://doi.org/10.1111/j.1558-5646.1985.tb00420.x

Gardes, M., & Bruns, T. D. (1993). ITS primers with enhanced specificity for basidiomycetes application to the identification of mycorrhizae and rusts. *Molecular Ecology, 2*, 113-118. http://dx.doi.org/10.1111/j.1365-294X.1993.tb00005.x

Gong, Y., & Han, X. D. (2006). Effect of nonylphenol on steroidogenesis of rat leydig cells. *Journal of Environmental Science and Health, Part B: Pesticides, Food Contaminants, and Agricultural Wastes*, *41*(5), 705–715. https://doi.org/10.1080/03601230600701866

Greco, C., Keller, N. P., & Rokas, A. (2019). Unearthing fungal chemodiversity and prospects for drug discovery. *Current Opinion in Microbiology*, *51*, 22–29. https://doi.org/10.1016/j.mib.2019.03.002

Hidayati, N. L. D., Tita, N., Nur, R., Yuliana, D., & Yani, S. (2018). Antifertility activities of ethanol extract of sugar apple leaves (*Annona squamosa* L.) in the reproductive system: spermatogenesis and sperm quality study. *Pharmaciana, 8*(2), 339-348.

Huether, S. E., & McCance, K. L. (2008). *Understanding Pathophysiology*, (3rd ed.). St. Louis, Mo. Mosby/Elsevier, pp. 488-501.

Johnsen, S. G. (1970). The stage of spermatogenesis involved in the testicular-hypophyseal feed-back mechanism in man. *Acta Endocrinologica*, *64*(2), 193–210. https://doi.org/10.1530/acta.0.0640193

Johnson, L. F., & Curl, E. A. (1971). Methods for research on the ecology of soil-borne plant pathogens. Burgess International Group Incorporated

Kataria, S. K., Singh, P., Pandove, G., Kalia, A., & Chandi, R. S. (2018). *Penicillum oxalicum* spg1: A novel entomopathogenic fungus isolated from mummified *Bemisia tabaci* (Gennadius) of cotton. *Journal of Applied and Natural Science, 10*(1), 138-143. http://dx.doi.org/10.31018/jans.v10i1.1593

Kaur, M., Chadha, P., Kaur, S., & Kaur, A. (2021). *Aspergillus flavus* induced oxidative stress and immunosuppressive activity in *Spodoptera litura* as well as safety for mammals. *BMC Microbiology*, *21*(1), 180. https://doi.org/10.1186/s12866-021-02249-4

Kubátová, A., Hujslová, M., Frisvad, J. C., Chudíková, M., & Kolaík, M. (2019). Taxonomic revision of the biotechnologically important species, *Penicillium oxalicum* with description of two new species from acidic and saline soils. *Mycological Progress*, *18*(1-2), 215-228. https://doi.org/10.1007/s11557-018-1420-7

Lucas, E. M., Castro, M. C., & Takahashi, J. A. (2007). Antimicrobial properties of sclerotiorin, isochromophilone VI and pencolide, metabolites from a Brazilian cerrado isolate of *Penicillium sclerotiorum* Van Beyma. *Brazilian Journal of Microbiology*, *38*(4), 785-789.http://dx.doi.org/10.1590/S1517-83822007000400036

Mahmoud, Y., Taha, A., & Soliman, S. (2018). 3-Monochloropropane-1,2-diol (alpha-chlorohydrin) disrupts spermatogenesis and causes spermatotoxicity in males of the Egyptian fruit-bat (*Rousettus aegyptiacus*). *Biotechnic & Histochemistry*, *93*(4), 293–300. https://doi.org/10.1080/10520295.2018.1437471

Mathur, N., Jain, G. C., & Pandey, G. (2010). Effect of tecoma stans leaves on the reproductive system of male albino rats. *International Journal of Pharmacology*, *6*(2), 152–156. https://doi.org/10.3923/ijp.2010.152.156

Memon, M. A., Bretzlaff, K. N., & Ott, R. S. (1986). Comparison of semen collection techniques in goats. *Theriogenology*, *26*(6), 823–827. https://doi.org/10.1016/0093-691x(86)90011-7

Mesbah, S. F., Shokri, S., Karbalay-Doust, S., & Mirkhani, H. (2008). Effects of nandrolone decanoate on ultrastructure of testis in male adult rats. *Iranian Journal of Medical Sciences*, *33*, 94-100. https://doi.org/10.1016%2Fj.sjbs.2020.09.039

Muharni, M., & Heni, Y. (2018). A subchronic toxicity test of ethyl acetate extract from endophytic fungus *Penicillium* sp. of kunyitputih (*Curcuma zedoaria*) against Swiss albino mice. *Journal of Chinese Pharmaceutical Sciences, 27*(2),123–130. https://doi.org/10.5246/jcps.2018.02.014

Paini, S. W., Tarsisius, D. W. B., Fenny, A. K., & Evelyn, L. W. (2014). Difference of solvent polarity to phytochemical content and antioxidant activity of *Pluchea indicia* less leaves extracts. *International Journal of Pharmacognosy and Phytochemical Research, 6*(4), 850–855.

Petit, P., Lucas, E. M. F., Abreu, L. M., Pfenning, L. H., & Takahashi, J. A. (2009). Novel antimicrobial secondary metabolites from a *Penicillium* sp. isolated from Brazilian cerrado soil. *Electronic Journal of Biotechnology*, *12*(4), 1-9. https://doi.org/ 10.2225/vol12-issue4-fulltext-9

Rambaut, A. (2020). Figtree, version 1.4.4; Available online: http//tree.bio.ed.ac.uk/software/figtree/.

Reddy, P. S., Rani, G. P., Sainath, S. B., Meena, R., & Supriya, CH. (2011). Protective effects of N-acetylcysteine against arsenic-induced oxidative stress and reprotoxicity in male mice. *Journal of Trace Elements in Medicine and Biology*, *25*(4), 247–253. https://doi.org/10.1016/j.jtemb.2011.08.145

Rotem, J. (1994). *The Genus Alternaria. Biology, Epidemiology and Pathogenicity*. APS Press, Saint Paul.

Santamarina, M. P., Roselló, J., Llacer, R., & Sanchis, V. (2002). Antagonistic activity of *Penicillium oxalicum* Currie and Thom, *Penicillium decumbens* Thom and *Trichoderma harzianum* Rifai isolates against fungi, bacteria and insects in vitro. *Revista Iberoamericana de Micologia*, *19*, 99-103.

Schumacher, J., & Moll, H. D. (2011). Collection of semen. In J. Schumacher, and H. D. Moll, (Eds.) *A Manual of Equine Diagnostic Procedures*. Teton New Media, Jackson, WY, International Veterinary Information Service, USA, No. A5420.1011.

Shankar, A., & Sharma, K. K. (2022). Fungal secondary metabolites in food and pharmaceuticals in the era of multi-omics. *Applied Microbiology and Biotechnology*, *106*(9-10), 3465–3488. https://doi.org/10.1007/s00253-022-11945-8

Soliman, S., Mahmoud, Y. M., & Taha, A. (2016). Evaluating the efficacy of the male chemosterilantalpha-chlorohydrin on three Egyptian wild rodent pests under laboratory conditions. *Egyptian Journal of Zoology*, *66*, 71-84. http://dx.doi.org/10.12816/0034709

Stackebrandt, E., & Goebel, B. (1994). Taxonomic Note: A Place for DNA-DNA Reassociation and 16S rRNA Sequence Analysis in the Present Species Definition in Bacteriology. *International Journal of Systematic Bacteriology*, *44*, 846-849. http://dx.doi.org/10.1099/00207713-44-4-846

Taha, A. (2022). Assessment of non-target toxicity of profenofos insecticide on the aquatic bird; the white egret, *Egretta alba*. *Egyptian Journal of Aquatic Biology and Fisheries*, *26*(2), 263 – 276. http://dx.doi.org/10.21608/ejabf.2022.228725

Taha, A., & Soliman, S. (2019). Effect of α-chlorohydrin water-bait on the fertility of captive males of the Egyptian fruit-bat (*Rousettus aegyptiacus*) and the proper time for controlling its free-ranging populations in Egypt. *Egyptian Journal of Aquatic Biology and Fisheries, 23*(4), 27-237. http://dx.doi.org/10.21608/ejabf.2019.53599.

Tamura, K., Stecher, G., & Kumar, S. (2021). MEGA 11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology and Evolution, 38* (*7*), 3022–3027. https://doi.org/10.1093/molbev/msab120

Tirumale, S., Wani, N., & Khanday, W. (2020). Phytochemical analysis and evaluation of antibacterial activity of different extracts of soil-isolated fungus *Chaetomium cupreum*. *Journal of Natural Science, Biology and Medicine*, *11*(1), 72. https://doi.org/10.4103/jnsbm.jnsbm_150_19k

Tobin, M.E., & Fall, M. W. (2004). *Pest control: rodents. USDA National Wildlife Research Center - Staff Publications, pp*. 67.

Umemoto, S., Odake, Y., Takeuchi, T., Yoshida, S., Tsushima, S., & Koitabashi, M. (2009). Blue mold of tomato caused by *Penicillium oxalicum* in Japan. *Journal of General Plant Pathology, 75*, 399-400. http://dx.doi.org/10.1007/s10327-009-0180-2.

Vainio, E. J., Ktwhiteorhonen, K., & Hantula, J. (1998). Genetic variation in Phlebiopsis gigantea as detected with random amplified microsatellite (RAMS) markers. *Mycological Research*, *102*(2), 187–192. https://doi.org/10.1017/s0953756297004577

Weng, W., Li, R., Zhang, Y., Pan, X., Jiang, S., et al. (2022). Polyketides isolated from an endophyte *Penicillium oxalicum* 2021CDF-3 inhibit pancreatictumor growth. *Frontiers in Microbiology*, *13*, 1033823. https://doi.org/10.3389/fmicb.2022.1033823

White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols*, 315–322. https://doi.org/10.1016/b978-0-12-372180-8.50042-flrpet

World Health Organization (2000). Laboratory manual: for the examination of human semen and sperm-cervical mucus interaction, 4thedn. Cambridge, Cambridge University Press.

Yang, L., Xie, J., Jiang, D., Fu, Y., Li, G., & Lin, F. (2008). Antifungal substances produced by *Penicillium oxalicum* strain PY-1—potential antibiotics against plant pathogenic fungi. *World Journal of Microbiology & Biotechnology*, *24*, 909–915. http://dx.doi.org/10.1007/s11274-007-9626-x.

Zhang, P., Wei, Q., Yuan, X., & Xu, K. (2020). Newly reported alkaloids produced by marine-derived *Penicillium* species (covering 2014–2018). *Bioorganic Chemistry*, *99*, 103840. https://doi.org/10.1016/j.bioorg.2020.103