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Article

Evaluation of Anti-bacterial Activity Induced by *Penicillium mallochii* in the Hemolymph of *Ephestia kuehniella* Zeller (*Lepidoptera: Pyralidae*)

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Abstract: Anti-microbial peptides (AMPs) exhibit anti-bacterial, anti-fungal and anti-parasite activity and are essential effectors for the immune response of insects. Insect hemolymph contains AMPs, which are one of the sources of antibiotics effective on drug-resistant microorganisms. This study was conducted to induce antimicrobial activity in hemolymph by topical application of different doses of *Penicillium mallochii* conidia and its metabolite to *Ephestia kuehniella* larvae. Tetracycline antibiotic disks (TE-10 µg, Sigma), Sulfametaxozole trimethoprim (SXT-25 µg, Sigma), PBS, sterile water, and non-induced hemolymphs of larvae were used as control groups. In total hemolymph induced with metabolite extract, 24-h application was determined to be more effective on test bacteria than 48-h application. The largest zone diameter was observed against *Escherichia coli* (20mm) in hemolymph collected 24 h after metabolite application. Antimicrobial activity was highly increased (24h and 48h) when larvae were induced with *P. mallochii* conidial suspension. The largest zone diameter was observed against *Proteus vulgaris* and *Klebsiella pneumonia* (20 and 24 mm) in hemolymph collected 24 h after conidial suspension application. When larvae were induced with fungus metabolite and conidia, the zone of inhibition was 1.5-2.5-fold larger than that of the control hemolymph, indicating a higher antimicrobial activity after application. In general, this study provides a novel contribution to the knowledge regarding enhancement of antimicrobial activity in response to fungal infections in larvae.

Keywords: Anti-bacterial activity, *Penicillium mallochii*, *Ephestia kuehniella*, Hemolymph, Fungi

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1. Introduction

The class insecta contains far more species than any other class of animals or the entire plant kingdom. Insects constitute approximately 80% of all animal species known, described, and named so far. Insects prosper and evolve in pathogenic and parasitic environments. Accordingly, insects have developed humoral and cellular innate immune mechanisms (Junqueira and Mylonakis, 2019). When a pathogen introduces into an insect hemocoel, it encounters several defensive phenomena known as immune responses. Hemocytes, phagocytosis, encapsulation, and nodule formation play a significant role in the cellular immune responses of the host (Lemaitre and Hoffmann, 2007). The humoral responses of the host are expressed by the synthesis of anti-microbial peptides (AMPs) and lysozymes, the activation of phenol oxidase, and the production of reactive oxygen and nitrogen intermediates and clotting (Browne et al., 2013) (Fig. 1).

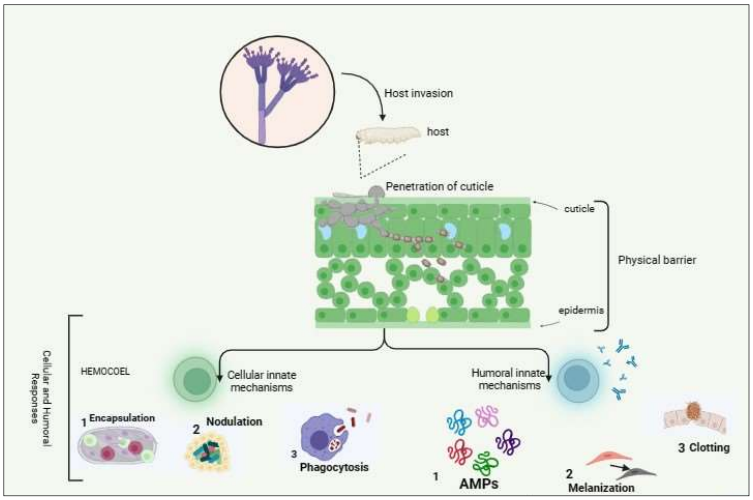

Figure 1. Schematic representation of the insect immune system. After fungal invasion, the spore attaches to the host cuticle and initiates the formation of the appressorium. The cuticle is the first physical barrier. A wide variety of humoral and cellular responses are seen after the cuticle (Created with BioRender.com).

Hemolymph (insect blood) is a clear liquid of yellow-greenish color. Hemolymph constitutes 16-40% of the body weight of insects, this amount varies depending on the insect species and the insect's developmental stages. Hemolymph exists freely in the body cavity and interacts with all internal organs and tissues. Therefore, it plays a critical role in the transport of AMPs to their target site (Ham et al., 2015). After microbial infection, AMPs and lysozyme are first synthesized in the fat body and hemocytes and then released into the hemolymph to destroy pathogens. AMPs play a vital role in host defense and, as they have a broad spectrum, are effective against both Gram (+) and Gram (-) bacteria and fungi (Shafaghat, 2012). With the discovery of natural compounds with anti-microbial properties in invertebrates, especially insects, scientists have started to search for new and effective anti-bacterial compounds. Many insect species have been investigated for AMPs, and defensins, cecropins, and proline-rich peptides and attakines have been found in most insect orders. However, moricin and gloverin have only been described in Lepidoptera (Zahirnia et al., 2023).

AMPs are important parts of insect innate immunity and exhibit anti-bacterial, anti-fungal, anti-viral and anti-parasitic activity. The first anti-bacterial activity in insects was determined in bacteria-immunized pupae of giant silk moths *Samia cynthia* and *Hyalophora cecropia* (Boman et al., 1991). Similarly, anti-bacterial activity caused by bacteria was observed in adult flies of *Drosophila melanogaster* (Robertson and Postlethwait, 1986). The first insect AMPs cecropin was purified from the pupa of *H. cecropia* in 1980 (Hultmark et al., 1980). Andra et al., (2001) reported that *Candida albicans* yeast phase growth was inhibited and killed in their study with cecropin A and B from Cecropia silkmoth (*H. cecropia)* as well as porcine cecropin P1. Seraj et al., (2003) isolated and purified an anti-bacterial protein from the hemolymph of the *Periplaneta amreicana*. Latifi et al., (2015) tested the anti-bacterial activity of American cockroach hemolymph against sensitive and resistant nosocomial bacterial species. In the studies on infection and immunity in insects, it has been determined that the insect host defense mechanisms and the innate immune systems of vertebrates are similar in many ways. In addition, their diversity and variable representation in addition to their immune effector functions have made them the focus of invertebrate pathology studies (Haine et al., 2008). Studies with insect AMPs have shown these compounds to be effective in various fields, including pharmaceutical and agricultural fields (Ursic-Bedoya et al., 2011). Storage pests affect stored grains, foods, and fiber crops and reduce crop yields. For the biologic control against agricultural pests that have negative ecological and economic effects to be effective, it is necessary to better understand their immunity and develop new compounds. Insect peptides were identified by the discovery of AMPs induced in response to external stimuli in the hemolymph of insects (Moreno-Garcia et al., 2013).

However, most of these studies have used opportunistic human pathogens. In this case, specific immune strategies developed against natural insect pathogens may not have been discovered (Zahirnia et al., 2023). Fungi are the most common natural insect pathogens and, in this study, an insect-pathogen fungus *Penicillium mallochii* was used to induce hemolymph production of anti-microbial peptides, which have an important role in immune system function. *P. mallochii* Rivera, Urb & Seifert is a rare fungus isolated from caterpillars (*Rothschildia lebeau* and *Citheronia lobesis*) in Costa Rica (Rivera et al., 2012) and *Triplectides* sp. in Brazil (Teixeira et al., 2022). As a result of the entry of a bacterial or fungal pathogen into the insect hemocoel, AMPs are first produced in the cytoplasm and released into the hemolymph. AMPs released into the hemolymph are thought to clear the insect of invasive microorganisms due to their high concentrations and broad activities (Casanova-Torres and Goodrich-Blair, 2013).

This study is based on inhibition zone measurements to demonstrate the change in anti-bacterial activity in non-induced and fungus-induced hemolymph of *E. kuehnialla* larvae. Additionally, this study allows us to obtain new information about fungus-host interactions by inducing the production of high concentrations of AMP in the *E. kuehniella* hemolymph of *P. mallochii*, in contrast to the classical strategies used by the fungus to evade or suppress host immunity.

2. Materials and Methods

2.1. *Rearing of E. kuehniella larvae*

In this study, *E. kuehniella* belonging to the Pyralidae family of the Lepidoptera order, which is an important storage pest, was used as a host. Adult moths were obtained from laboratory colonies at Balikesir University (BAUN), Biology Department, Faculty of Sciences and Arts, Turkey. The moths were reared on a standard artificial diet consisting of 40% wheat flour, 20% corn flour, 20% fine bran, and 20% barley flour. The insects were reared at 25 ± 1 °C, 65 ± 5% RH, and photoperiod: 12:12 h L/D (Kurtuluş et al., 2020). 1500 third-instar larvae were used in these bioassays.

2.2. *P. mallochii culture conditions*

The strain used in this study was identified by molecular methods twice, in Adıyaman University (TR) and Charles River Laboratory (USA) (Genbank number: MG591446). It is stored in Balıkesir University Microbiology Laboratory Mushroom Herbarium with code number CB-16 (Bouhri et al., 2020). Malt Extract Agar (MEA, Oxoid) was used in the preparation of the culture of *P. mallochii*. Three-point inoculation was done on the surface of the media in the Petri dishes (90 mm) then plates were incubated at 28 °C for 7 days. The plates were used for preparation of conidial suspension/metabolite extract. The stock culture was maintained on MEA medium in the deep freeze at –20 ºC.

2.3 *Preparation of conidial suspensions*

The conidia were harvested after cultivation on MEA for 10 days at 28 ºC in the dark. Spores were obtained by adding 10 mL of sterile water prepared with 0.01% Tween 20 (Millipore) to Petri dishes (90 mm) and scraping carefully with a glass baguette. While the suspension was being prepared, mycelia and agar pieces were removed by filtering through 4-layer gauze into sterile 50 mL falcon tubes. The conidia number of the suspension was counted on the Thoma slide under the microscope and dilutions were made at the required concentrations and adjusted to $1x10^9$ conidia in 1 mL (Fancelli et al., 2013).

2.4. *Preparation of metabolite extract*

Petri dishes (90 mm) prepared using MEA were used for metabolite production. The fungus was incubated at 28 °C for 14 days for metabolite production. After the incubation period and colony growth were completed, 2 mL of Tween 80 (Sigma-Aldrich) was added to each Petri dish and the spores were scraped away. The agars, the spores of which were removed, were cut into smaller pieces. Small agar pieces (100 g) were transferred to a flask and Ethanol (w/v: 1/2) was added. For the fungal metabolites in the agar to pass into ethanol, it was kept in a shaker (ZHWY-211D) at 160 rpm and 26 °C for 72 h. The resulting solution was passed through 0.45 and 0.22 µm Minisart filters (Supelco). The solvent was evaporated and concentrated with an evaporator (IKA RV 10 basic). The obtained orange-red colored extract was lyophilized with a freeze dryer (CHRIST ALPHA 1–2 LD) (Bouhri et al., 2020). In this part of the study, ethanol extract production was performed in triplicate. The extract was stored in a deep freeze at -20 °C until use.

2.5. *Application of Conidial Suspension Doses and Metabolite Doses*

The extract of *P. mallochii*, weighing 0.5 g, was dissolved in 10 mL of PBS (Phosphate Buffered Salt Solution) to create the main stock solution with a concentration of 50 mg/mL. All stock solutions were stored in a deep freeze at -20 ºC. To prepare 10 mL (10 mg/mL concentration) solution, 2 mL of stock solution was taken, and 8 mL of PBS was added. Therefore, the final solution concentrations were 10 mg/mL. Conidial suspension (1×10^5, 1×10^6, 1×10^7, 1×10^8, and 1×10^9 conidia/mL/PBS and metabolite extract doses (10, 5, 2, 1, 0.5, 0.1 mg/mL/PBS) (in [v/v]) was applied (2 µL) topically to *E. kuehniella* larvae. In each experiment, 20 larvae (25+5 mg) were tested with three replicates for each concentration. Treated and control groups were maintained for 24 and 48 h at 26 ºC, 60% relative humidity, and a 12:12-h L/D photoperiod.

2.6. *Hemolymph collection*

Hemolymph samples were collected from PBS-induced (PBS control), metabolite extract-induced, and conidial suspension-induced larvae at 24 hours and 48 hours after dosing. Control hemolymph samples were also collected from larvae. To collect the hemolymph, the cuticle of the larva on the first proleg was pierced with a fine sterile cutting needle. It was collected by suction with a fine-tipped calibrated glass microcapillary (5µL, Hirschmann). Hemolymphs were added to sterile and cooled Eppendorf containing 1 mg phenylthiourea (Sigma). It was stored at -20 ºC until further analysis to prevent melanization. Three repetitions of twenty larvae were performed at different times for each dose and control group.

2.7 *Anti-bacterial Activity Test*

2.7.1. *Microorganisms*

Five strains of bacteria used were *Bacillus cereus* (BC, ATCC 10876), *Staphylococcus aureus* (SA, ATCC 538), *Klebsiella pneumonia* (KP, ATCC 31488), *Proteus vulgaris* (PV, ATCC 6897) and *Escherichia coli* (EC, ATCC 8739). The stock culture was maintained on a Nutrient agar (NA) medium at 4 ºC in the refrigerator.

2.8. *Disc Diffusion Method*

Disc diffusion technique was applied using Nutrient agar (Merck) to determine the anti-bacterial activity of AMPs induced from hemolymph of *P. mallochii* applied larvae (Saad et al., 2021). An inoculum suspension was prepared according to the 0.5 McFarland standard from the 24-h fresh cultures of the bacteria used in the study. Bacterial colonies for inoculum suspension were prepared in 0.85% w/v NaCl water (El-Saadony et al., 2021). Control groups were made by Tetracycline (TE-10 µg, Sigma) and Sulfamethoxazole trimethoprim (SXT-25 µg, Sigma) antibiotic discs, PBS, sterile

water, conidial suspension, and metabolite doses. The hemolymph samples and control groups were absorbed into sterile blank paper discs (6mm) as 10 µL (Figure 2). The prepared bacterial suspension (500 µL) was spread on the surface of Petri dishes (90 mm). The discs prepared for the control and experimental groups were placed on the Petri surface with the forceps, leaving a 2 cm gap between them. Petri dishes were incubated for 24 h at 37±0.1 °C. When the incubation period was completed, inhibition zones were measured and recorded in mm. The results were compared with standard antibiotic disks and control groups (Marshall and Arenas, 2003). Zone width was calculated by measuring the zone diameter and disc diameter together.

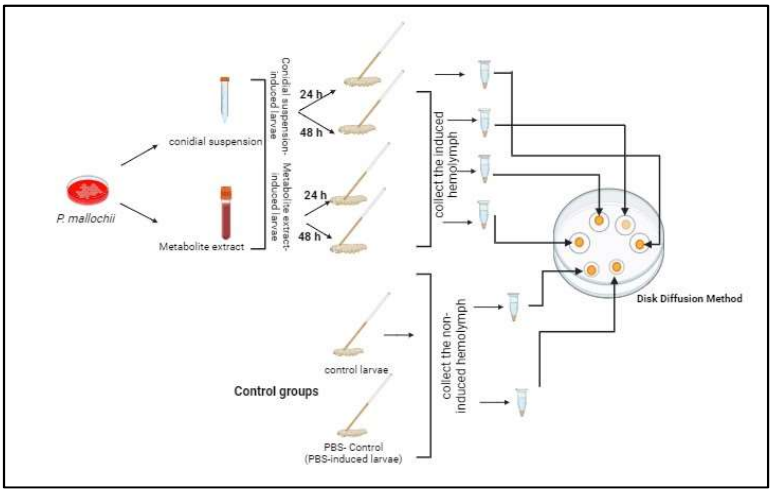

Figure 2. Schematic representation of susceptibility detection of bacteria to *E. kuehniella* hemolymph by disc diffusion method (Created with BioRender.com).

3. Results

For anti-bacterial activities, hemolymph was collected from control groups and treated groups of *E. kuehniella* larvae with the microcapillary tube. The amount of collected hemolymph is shown in Table 1.

Table 1. Amounts of collected hemolymph.

Groups	Collected Hemolymph (µL)	Number of larvae
Control (normal)	224	60
PBS-control/24h	210	60
PBS-control-48h	218,5	60
10mg/mL-24h	187,5	60
10mg/mL-48h	203,7	60
5mg/mL-24h	184	60
5mg/mL-48h	178	60
2mg/mL-24h	206	60
2mg/mL-48h	200,4	60
1mg/mL-24h	174,6	60
1mg/mL-48h	163,9	60
0.5mg/mL-24h	196,5	60
0.5mg/mL-48h	200,5	60
0.1mg/mL-24h	230,6	60
0.1mg/mL-48h	173	60
10^9 conidia/mL-24h	169	60
10^9conidia/mL-48h	196,8	60
10^8 conidia/mL-24h	187	60
10^8 conidia/mL-48h	206,3	60
10^7 conidia/mL-24h	147,9	60
10^7 conidia/mL-48h	198,5	60
10^6 conidia/mL-24h	203,4	60
10^6conidia/mL-48h	201	60
10^5 conidia/mL-24h	203	60
10^5 conidia/mL-48h	163	60

The anti-bacterial activity of *E. kuehniella* hemolymph was performed against *S. aureus, E. coli, K. pneumonia, B. cereus, P. vulgaris*. Anti-bacterial activity results of hemolymph collected from normal larvae (non-induced) and hemolymph collected from larvae induced with PBS for 24 and 48 h were given in Table 2 and Figure 3. Results of control group hemolymphs are presented together with PBS, sterile water, and standard antibiotics (Tetracycline 10µg and Sulfamethoxazole 25µg). A weak anti-bacterial activity was detected against test bacteria in control group hemolymph

(non-induced hemolymph). This activity was not increased by the PBS application. It was observed that hemolymph collected from larvae induced with PBS for 24 h had higher activity compared to 48 h. No anti-bacterial activity was observed for PBS and sterile water disc. The largest inhibition zone diameter (44 mm) was determined against *P. vulgaris* and *K. pneumonia* in Sulfamethoxazole.

Table 2. Anti-bacterial activity of control groups.

	Control Groups						
	H	H-PBS		PBS	SW	TE	SXT
M.organism		24h	48h				
E.C.	12	9	9	-	-	-	28
S.A.	14	10	8	-	-	32	41
B.C.	9	10	7	-	-	28	-
P.V.	8	7	7	-	-	30	44
K.P.	10	9	7	-	-	37	44

*H: Hemolymph (normal-non induced); H-PBS: Hemolymph (PBS-control); PBS: Phosphate Buffered Salt Solution; SW: Sterile water; TE: Tetracycline; SXT: Sulfamethoxazole trimethoprim; *inhibition zones were measured in mm; (-) not detected.*

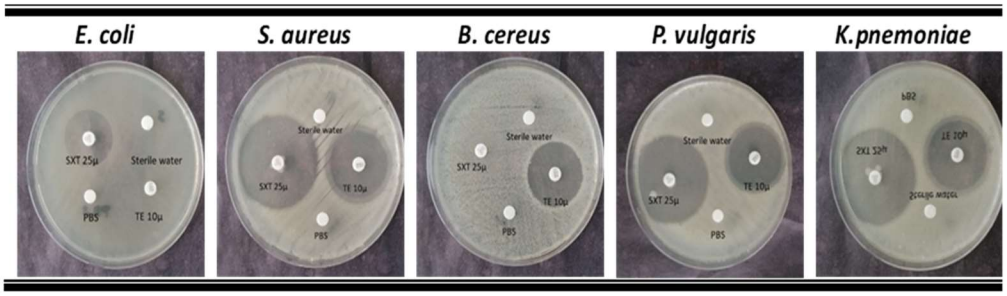

Figure 3. Photograph of inhibition zone of anti-bacterial activity test control groups against different bacteria.

In studies for other control groups, discs were loaded with different concentrations of *P. mallochii* metabolite extract and conidial suspensions. Control groups of metabolite results are shown in Table 3. The highest dose of 10 mg/mL showed anti-bacterial activity on four of the test bacteria. While metabolite doses did not show any effect on *P. vulgaris*, it was determined that it was very effective on *K. pneumonia*. Zone diameters are shown in Figure 4.,

Table 3. Anti-bacterial activity of metabolite extract control groups.

	Control Groups (Metabolite Extract)					
	10mg/mL	5 mg/mL	2 mg/mL	1 mg/mL	0.5 mg/mL	0.1 mg/mL
M.organism						
E.C.	16	13	-	-	-	-
S.A.	16	13	10	9	-	-
B.C.	11	10	9	8	-	-
P.V.	-	-	-	-	-	-
K.P.	20	15	13	10	7	-

**Inhibition zones were measured in mm; (-) not detected.*

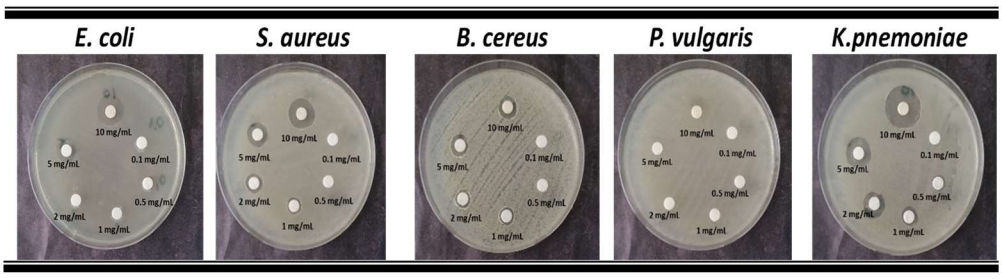

Figure 4. Photograph of inhibition zone of anti-bacterial activity test metabolite control groups against different bacteria.

Control groups of conidial suspension results were shown in Table 4. Conidial suspension doses showed no effect on *B. cereus*, while only the highest doses showed effect on other bacteria. Zone diameters were shown in Figure 5.

Table 4. Anti-bacterial activity of conidial suspension control groups.

	Control Groups (Conidial suspensions)				
M.organism	10^9 conidia/mL	10^8 conidia/mL	10^7 conidia/mL	10^6 conidia/mL	10^5 conidia/mL
E.C.	12	10	9	-	-
S.A.	13	-	-	-	-
B.C.	-	-	-	-	-
P.V.	17	13	-	-	-
K.P.	18	12	-	-	-

**Inhibition zones were measured in mm; (-) not detected.*

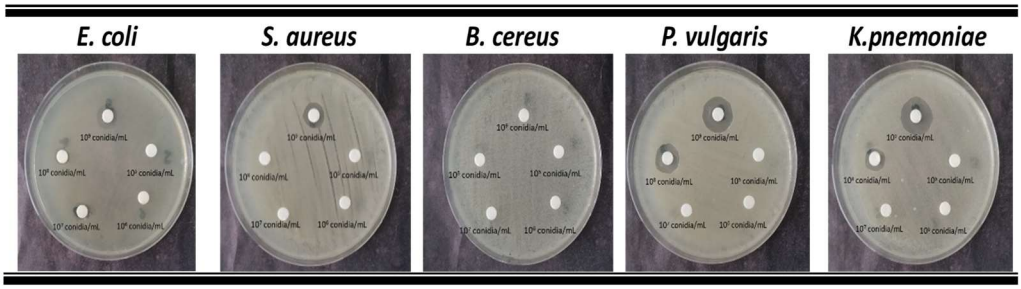

Figure 5. Photograph of inhibition zone of anti-bacterial activity test conidial suspension control groups against different bacteria.

In this study we tested the level of anti-bacterial activity in the hemolymph of *E. kuehniella* larvae induced with the different doses of *P. mallochii* conidia and metabolite. Anti-bacterial activity was measured by the disc diffusion method in hemolymph samples obtained in different periods of time after infection. When comparing control group larvae (non-induced and PBS-induced) and experimental group larvae (metabolite and conidia-induced), no difference was seen in larval mortality rates in the first 24 hours and 48 hours after application. It was determined that the inhibition zone of the hemolymph of the fungal metabolite-induced larvae was 1.5-2-fold larger than the inhibition zone of the control larvae hemolymph (non-induced). This indicates increased antimicrobial activity after application. The anti-microbial activity of hemolymph induced by different doses of metabolite extract revealed significant differences. In hemolymph induced with metabolite extract, 24h application was determined to be more effective on test bacteria than 48 h application. The largest zone diameter was observed against *E. coli* (20mm) in hemolymph collected 24 h after metabolite application. The largest zone diameter was observed against *E. coli, P. vulgaris* and *K. pneumonia* (16 mm) in hemolymph collected 48 h after metabolite application. Results are given in Table 5 and Figure 6.

Table 5. Anti-bacterial activity induced by *P. mallochii* metabolite extract in the hemolymph.

	Control Groups (Metabolite Extract)											
	10mg/mL		5 mg/mL		2 mg/mL		1 mg/mL		0.5 mg/mL		0.1 mg/mL	
M.organism	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
E.C.	20	16	15	12	13	10	9	-	7	-	-	-
S.A.	18	11	13	9	9	8	-	-	-	-	-	-
B.C.	10	12	-	-	-	-	-	-	-	-	-	-
P.V.	15	16	11	12	9	-	-	-	-	-	-	-
K.P.	14	16	11	12	8	9	7	-	7	-	-	-

**Inhibition zones were measured in mm; (-) not detected.*

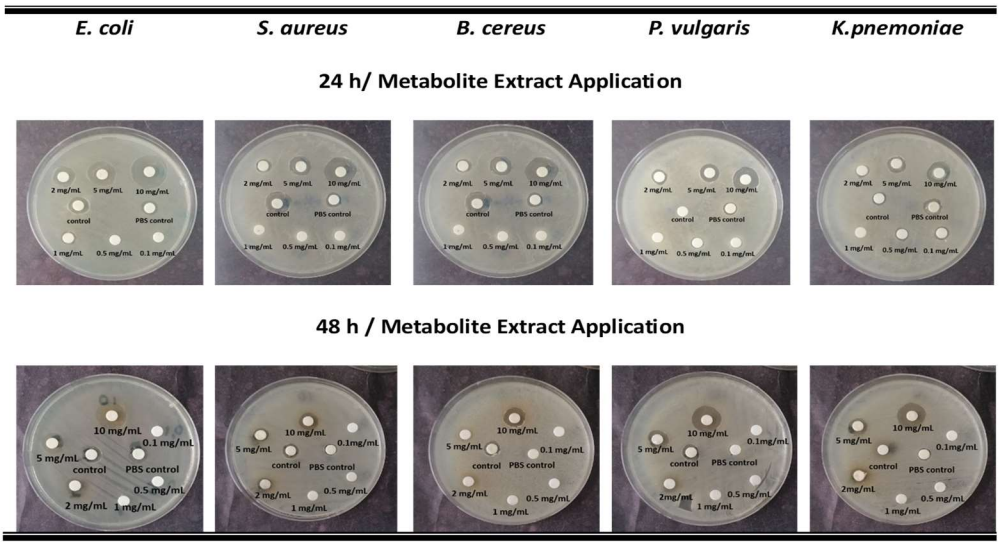

Figure 6. Photograph of inhibition zone of anti-bacterial activity test induced by *P. mallochii* metabolite extract in the hemolymph against different bacteria.

The different conidial suspensions of *P. mallochii* the challenge produced the largest zone of inhibition, with a 2.5- and 2.4-fold increase in size compared with *P. vulgaris* and *K. pnemonia*, respectively. Overall, the anti-microbial activity in larvae hemolymph was increased significantly after the fungus challenge (24h and 48h) (Table 6). Only in *B. cereus* the activity was very low and zone diameter was seen in the collected hemolymph 48 h after the application of the conidial suspension. The largest zone diameter was observed against *P. vulgaris* and *K. pneumonia* (20 and 24 mm) in hemolymph collected 24 h after conidial suspension application. Results are given in Figure 7.

Table 6. Anti-bacterial activity induced by *P. mallochii* conidial suspension in the hemolymph.

	Control Groups (Conidial suspensions)									
M.organism	10^9 conidia/mL		10^8 conidia/mL		10^7 conidia/mL		10^6 conidia/mL		10^5 conidia/mL	
	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
E.C.	16	15	10	11	10	9	9	8	8	-
S.A.	13	9	12	8	9	8	9	7	8	-
B.C.	-	12	-	-	-	-	-	-	-	-
P.V.	20	18	15	11	11	10	10	9	9	8
K.P.	24	26	18	15	15	15	13	13	13	9

**Inhibition zones were measured in mm; (-) not detected.*

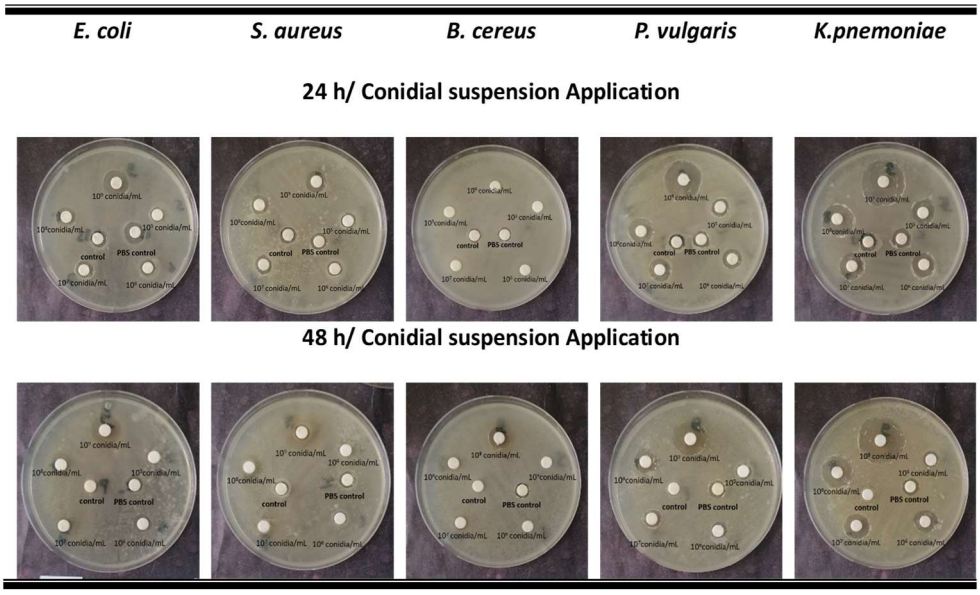

Figure 7. Photograph of inhibition zone of anti-bacterial activity test induced by *P. mallochii* conidial suspension in the hemolymph against different bacteria.

4. Discussion

The need for innovative and effective alternatives such as nanoparticles and peptides are increasing day by day due to multidrug-resistant bacterial strains (El-Saadony et al., 2020; Abdel-Moneim et al., 2021). Resistance mechanisms occur due to unconscious use of antibiotics and this problem has not been solved yet. Determining the pharmacological properties of newly discovered AMPs is seen as a solution to the problem of antibiotic resistance, but studies are needed for the applicability of these peptides (Radwan et al., 2022). Recently, many reviews have been conducted regarding insect AMPs as effective antimicrobial agents against various pathogens (Wojda et al., 2020; Eleftherianos et al., 2021; Manniello et al., 2021). The current research attempts to approve the ability of *E. kuehniella* larvae to induce the anti-microbial peptides in their hemolymph upon application with *P. mallochii* metabolite extract and conidia. Many studies conducted with insects show that bacteria or fungi injected into the hemocoel increase the synthesis of peptides and proteins secreted into the hemolymph (Zhang et al., 2009). After septic body damage and against microbial infection in insects, AMPs are synthesized and released into the hemolymph. The AMPs identified in many insects have been reported to work synergistically against Gram (+) and Gram (-) bacteria. In this study, we tested the level of anti-bacterial activity in the hemolymph of *E. kuehniella* larvae application with the *P. mallochii* metabolite extract and conidia. The anti-bacterial activity of the hemolymph collected at different times (24 and 48 h) after the application was determined by a disc diffusion test. An important analysis of the humoral immune response regarding AMPs is made with the zone of inhibition assays (Morejon and Michel 2023). The anti-microbial activity of insect hemolymph is increased by the secretion of AMPs after microbial challenge (Uvell and Engström, 2007). Our results so far indicate that *P. mallochii* metabolite extract and conidia application to larvae induced anti-microbial activity after 24h. In addition, in studies with other insects such as *Zophobas atratus* and *Bombus terrestris*, it has been reported that the anti-microbial activity of hemolymph reaches its maximum level at 24 and 48 h after microbial challenge (Korner and Schmid-Hempel, 2004). The results show that the larvae of *E. kuehniella* showed that it is more susceptible to conidia than metabolites. Hemolymph of conidia-induced *E. kuehniella* larvae recorded drastic changes in anti-bacterial activities. Differences in the natural environment of infected cells and compounds released by destroyed cells provide information about the disease (Swelum et al., 2020; Yoon et al., 2018). According to the anti-bacterial activity test results, it was determined that normal insect hemolymph showed a very weak anti-bacterial activity against the test bacteria. In non-infected insects, metabolic changes, stressors, and ageing can induce the expression of AMPs (Bland, 2023).

When a bacterial or fungal pathogen invades the hemocoel, it can be detected by pattern recognition receptors. Accordingly, the regulation of AMPs begins in the fat body and hemolymph (Boderick et al., 2009). Gram (+) bacteria and fungi activate the Toll pathway, which triggers the production of AMPs in the insect. In our study, the application of different doses of conidia and metabolite on the larvae of *E. kuehniella* induced the appearance in their hemolymph of potent anti-bacterial activity. For metabolite-induced hemolymph, the best inhibition zone with the Gram (+) bacteria *S. aureus* was (18 and 11 mm /24 and 48 h) with *B. cereus* was (10 and 12 mm /24 and 48h). Also, the inhibition zone with Gram (-) bacteria *E. coli* was (20 and 16 mm/24 and 48h) with *P. vulgaris* (15 and 16 mm/24 and 48 h) with *K. pneumonia* was (14 and 16 mm /24 and 48 h). Data obtained from metabolite-induced hemolymph showed that AMPs produced by the insect defense mechanism were effective in both bacterial groups. For the anti-bacterial activity of conidia-derived hemolymph, the best inhibition zone (13 and 9 mm /24 and 48 h) with the Gram (+) bacteria *S. aureus* was determined. While no zone formation was observed in other bacteria *B. cereus* in 24 h, it was observed that zone formation increased in 48 h. The authors suggest that the replication rate of the bacteria tested may be responsible for these observations and that in vitro assays of the rapidly replicating strains outstripped the anti-microbial activity of the hemolymph, thus inhibiting zone formation (Haine et al., 2008; League et al., 2017). The inhibition zone with Gram (-) bacteria *E. coli* was (16 and 15 mm/24 and 48h) with *P. vulgaris* was (20 and 18 mm/24 and 48 h) *K. pneumonia* was (24 and 26 mm /24 and 48 h). The data obtained from the results of the anti-bacterial activity of hemolymph induced by conidia showed that AMPs produced by the insect defense mechanism were more effective on Gram (-) bacteria. Pathogenic bacteria sensitive to AMPs include *K. pneumoniae, E. coli*, *Citrobacter freundii, Francisella tularensis*, *Streptococcus sanguinis*, *S. aureus*, and *Bacillus coagulans* (Ursic-Bedoya et al., 2011), and in our study, AMPs induced by *P. mallochii* were found to be sensitive to *E. coli, K. pneumonia* and *S. aureus*.

The anti-microbial activity of hemolymph, which we detected in the inhibition zone analysis, was obtained by triggering the fungus species *P. mallochii* used in the study. This supports the literature on humoral innate immune responses that provide broad defence against different microbial challenges (Coggins et al., 2012; Rhodes et al., 2018). Insect hosts have developed various control mechanisms to monitor and maintain the balance between non-self-signals (microbial compounds) and danger signals (molecules released by host cells upon damage). Thus, the defense mechanisms of insects allow beneficial microbes to be recognized and maintain their communities, while when it recognizes pathogenic microbes that cause cell damage, it responds to eliminate them. (Lazzaro and Rolff, 2011). In addition to distinguishing symbionts from pathogens, there are immune responses triggered by the presence of pathogens in the body cavity. These responses are genus specific as they allow the expression of different AMPs for different pathogen strains (Lu and St. Leger, 2016). It is thought that the conidia and metabolites of *P. mallochii* used in the study trigger different anti-microbial peptide synthesis in the hemolymph.

5. Conclusions

The discovery of natural antibiotic compounds from invertebrates or animals interacting with environmental wastes and pollutants has been a popular field of study. Studies on the anti-microbial activity of hemolymph and insect AMPs provide new perspectives on the interactions between microorganisms and their hosts and contribute to the formation of new models for pathogenesis. Therefore, further identification of potential compounds with biological activity, in vivo and clinical testing, and synthesis of new drug precursors are of great importance. In conclusion, this study contributed to the

regulation of anti-microbial activity in response to microbial infections in larvae and the availability of natural antibiotic substances that can be obtained from insects.

Conflicts of Interests

No conflict of interest is declared by the authors.

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Statement contribution of the authors

Conception/Design of study: P.G., T.A., A.E.; Data Acquisition: P.G., T.A., A.E.; Data Analysis/Interpretation: P.G., T.A., A.E.; Drafting Manuscript: P.G.; Critical Revision of Manuscript: T.A., E.R.; Final Approval and Accountability: P.G., T.A., A.E.; Technical or Material Support: T.A., A.E.; Supervision: T.A., A.E. All authors have read and agreed to the published version of the manuscript.

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