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Humoral immune response of *Galleria mellonella* after mono- and co-injection with *Hypericum perforatum* extract and *Candida albicans*

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Galleria mellonella is used as a model organism to study the innate immune response of insects. In this study, the humoral immune response was assessed by examining phenoloxidase activity, fungal burden, and the expression of phenoloxidase and antimicrobial peptide genes at different time point following separate and combined injections of *Hypericum perforatum* extract and a nonlethal dose of *Candida albicans*. The administration of a plant extract at low doses increased phenoloxidase activity, while higher doses had no effect. Similarly, co-injection of a low dose of the extract with the pathogen allowed half of the yeast cells to survive after 24 h. Co-injection of plant extract with the pathogen decreased the phenoloxidase activity at the end of 4 h compared to *C. albicans* mono-injection. The phenoloxidase gene expressions was reduced in all experimental conditions with respect to the control. When plant extracts and the pathogen were administered together, gallerimycin and hemolin gene expressions were considerably higher compared to mono-injections of plant extracts and the pathogen. The results of this study reveal that gene activation and regulatory mechanisms may change for each immune gene, and that recognition and signaling pathways may differ depending on the involved immunoregulator.

Key words: Greater wax moth; insect immunity; gene expression; plant extract; pathogen.

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Insects are a diverse group of animals that can survive under adverse environmental conditions through their excellent immune responses. Although it is well known that insects have an innate immune system quite similar to that of vertebrates, recent studies have shown that they also have an adaptive immune system [1, 2]. Therefore, insects are gaining popularity as *in vivo* models for assessing human disease virulence and therapeutic efficiency [3–5]. The innate immune system of insects consists of cellular and humoral defense mechanisms. The cellular immune response includes the formation of phagocytosis, nodulation, and encapsulation, while the humoral immune response includes the production of effector molecules such as reactive oxygen and nitrogen species (ROS and RNS), complement-like proteins (opsonins), antimicrobial peptides (AMPs), melanin formation as a result of phenoloxidase (PO) pathway activation, and coagulation of hemolymph [4, 6–8]. Melanization is the process of producing melanin, a black pigment, which phenoloxidase (EC 1.14.18.1) catalyzes. Phenoloxidase is synthesized in hemocytes and released into hemolymph as an inactive enzyme, prophenoloxidase, which is activated by serine protein kinases. In insects, the antimicrobial peptides are mainly produced by the fat body, hemocytes, digestive and reproductive systems [3]. Of the 3569 AMPs presented in the Antimicrobial Peptide Database (URL: https://aps.unmc.edu), 367 are insect-origin AMPs (accessed on May 12, 2023). Gallerimycin, galiomycin, and hemolin are the most

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commonly found antimicrobial peptides in insects: The gene expression of these AMPs increases in a time-dependent manner in cases of infection [9–12].

The greater wax moth *Galleria mellonella* (Lepidoptera: Pyralidae) is used as a model organism to study the innate immune response because of its short life cycle (6 weeks at ideal environmental variables), large larval size allowing easy inoculation and collection of enough hemolymph and other organs for further analysis, and no ethical constraints [4, 13–15]. As in other insects, *G. mellonella* has a chitin-containing integument that acts as the first line of defense against pathogens. In addition, some physiological barriers, such as the biochemical content of the intestine, low humidity and lack of nutrients in the trachea also prevent the invasion of a pathogen into the hemolymph [14, 16]. When these anatomical and physiological barriers are passed by the nonself molecules called immunoregulators (fungi, bacteria, parasites, viruses, protozoa, the cell wall components of pathogens, toxins, and other chemicals), they are recognized properly by the sensory systems, and the innate immune response mechanisms are activated. The cellular immune response is triggered for the clearance of nonself molecules by hemocytes depending on immunoregulator features (e.g., amount, type, size, and virulence level), exposure time of larvae to pathogen, and larval growing temperature [17–25]. The humoral response is activated by the recognition of immunoregulator by the appropriate pattern recognition receptors (PRRs) that recognize unique structures or patterns on the immunoregulator (pathogen-associated molecular patterns, PAMPs), or recognize the damaged or wound region in the larval body (damage-associated molecular patterns, DAMPs) [4, 26]. The bacterial (e.g., peptidoglycan, lipoteichoic acids, and lipopolysaccharides) and fungal (e.g., β-1, 3-glucan) cell wall components are examples of well-known PAMPs [27, 28]. The sensing of immunoregulator and its properties (e.g., size and amount) induces activation of relevant signaling pathways (e.g., JAK–STAT pathway), which cause the synthesis of defense molecules (proteins, AMPs, and ROS/RNS), melanization, and hemolymph coagulation [4, 29, 30].

Different types of research have been conducted to investigate the innate immune response of *G. mellonella* after bacterial (e.g., *Klebsiella pneumonia*, *Escherichia coli*, and *Staphylococcus aureus*), fungal (e.g., *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*), and viral (e.g., Bovine herpes simplex virus-1) infections to the larvae in various circumstances [3, 14, 23, 24, 31–37]. Different immune response parameters (such as microbial load, antimicrobial activity, larval mortality, amount and types of hemocytes, the process of microbial clearance, ROS activity, melanization and phenoloxidase activity, expression and synthesis of AMPs, and other defense molecules) were determined in these studies after mono- or co-injection of immunoregulators and/or immune priming. Meanwhile, other studies have been conducted on the effectiveness of certain medicinal plants on larval mortality, which is important for developing natural pesticides for the management of *G. mellonella* [38–41]. Also, the effect of *H. perforatum* on the innate immune response of *G. mellonella* was studied by measuring hemocytes density and phenoloxidase activity [42, 43]. However, no comprehensive research has been conducted related to the effect of medicinal herbs on the innate immune response mechanisms of *G. mellonella*.

St. John's wort (*Hypericum perforatum* L.) plant has been used almost all over the world as a traditional herbal medicine. The important biologically active compounds found in *H. perforatum* aerial parts are naphthodianthrones (e.g., hypericin), phloroglucinols (e.g., hyperforin), flavonoids (e.g., quercetin), biflavonoids (e.g., biapigenin), procyanidins (e.g., procyanidin), essential oils (e.g., terpenes), amino acids (e.g., GABA), phenylpropanes (e.g., caffeic acid), and xanthones (e.g., norathyriol) [44]. *H. perforatum* extracts have been used in a variety of applications, including wound healing, depression therapy, fungal infection management, inflammation reduction, treating mycobacterial infections, and as antiviral agents [45–54]. Previously, it was reported that the hydroalcoholic extracts of *H. perforatum* reduced cellular immunity and simultaneously strengthened humoral immunity in mice [55].

The present study was conducted to investigate the immunoregulatory potential of *H. perforatum* extract on *G. mellonella*. After mono-injection of plant extract and co-injection with *C. albicans* into *G. mellonella* larvae, phenoloxidase gene expression and enzyme activity, as well as gene expression of antimicrobial peptides (gallerimycin and hemolin) were measured in a time-dependent manner. The effects of plant extract on *G. mellonella* fungal load were also evaluated.

MATERIALS AND METHODS

Insects and fungi

Galleria mellonella larvae were raised in constant darkness under controlled circumstances at 28.1 °C and 65.5% relative humidity. Two male and four female adult moths were placed in a 1 L glass jar with 2 g of natural blackened honeycomb. When the hatching larvae were seen, they were fed with Bronskill's artificial food (natural blackened honeycomb, wheat bran, honey, water, and

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glycerin) [56]. Last instar larvae of around 160–200 mg in weight were selected for the experiments, which also showed similar motility and color.

Candida albicans (ATCC 10231) culture was prepared in YPD medium (1% Yeast extract, 2% Bacto-peptone, 2% Dextrose) with 120 rpm shaking at 30 °C. The yeast cells were centrifuged at 6000 rpm and washed with sterile distilled water. The yeast pellet was resuspended in sterile distilled water. The density of *C. albicans* was checked by measuring the optical density (OD_{600}) and counting the colony forming units (CFU) after plating a 10-fold serial dilution of the culture on YPD agar. *G. mellonella* larvae were injected with 1.25×10^5 *C. albicans* cells.

Plant sampling and extraction

The aerial parts of *H. perforatum* plant were collected from Alanköy (UTM Latitude: 40.021481 Longitude: 26.772202), Söğütgediği (UTM Latitude: 39.940703, Longitude: 26.766302), and Yaylacık (UTM Latitude: 39.879613, Longitude: 26.738994) regions of Çanakkale province (Turkey) during the flowering period. The plant samples were naturally dried in a dark, ventilated environment before being milled to a fine powder size. The chemical composition of the dried plant material was analyzed using the GC–MS (Thermo ICQ-QD) at ESOGU Central Research Laboratory, and the result was deposited on the Zenodo digital platform with a DOI number (https://doi.org/10.5281/zenodo.8337162). In a soxhlet device, 50 g of plant powder were extracted with 70% ethanol, and the ethanol was then evaporated in the water bath. The dried material was resuspended in sterile distilled water, and the experimental concentrations of 0.001, 0.0025, 0.005, 0.1, 0.5, 1.0, 2.5, 5, 10, 20, 40, 60, 80, and 100 mg/mL were prepared in sterile distilled water. These experimental concentrations were injected into *G. mellonella* larvae, and phenoloxidase activity was measured 24 h after injection. The plant extract doses that caused the highest and lowest enzyme activity were evaluated as high and low doses of the plant extract.

Larval injection

The surfaces of the larvae were sterilized with 70% ethanol before the injection. For the challenge of larvae either with *C. albicans* or plant extracts, a microsyringe (Hamilton, USA) was used to inject 5 μL aliquots of the inoculum into the body cavity of the last proleg. *C. albicans* and plant extract doses (low and high) were injected into larvae alone (mono-injection), and *C. albicans* was injected into larvae with either low or high doses of the plant extract (co-injection). After injection, larvae were reared in the same conditions and incubated in sterile plastic containers. Unless otherwise specified, each experimental group contained n = 16 infected larvae, and each experiment contained three biological replicates. The untreated (naive), needle-injected (null), and sterile distilled water-injected larvae served as the control groups. The larvae or hemolymph were sampled 1, 4, and 24 h post-infection (pi).

Fungal load

To induce fungal infection, five larvae were injected with *C. albicans* (1.25×10^5 yeast cells/larva) or in combination with low and high doses of plant extracts. Hemolymph (approximately 20 μL/larva) was collected and resuspended in PBS buffer. The hemolymph mixture was serially diluted with PBS and plated on YGC (4% Yeast extract glucose chloramphenicol agar) supplemented with ampicillin (100 μg/mL) (Sigma-Aldrich) in addition to chloramphenicol to prevent bacterial growth. The plates were incubated at 30 °C for 2–3 days. The fungal load was calculated as Log CFU/larva.

Phenoloxidase activity assay

Phenoloxidase activity was determined at three independent time intervals using hemolymph from 16 larvae. A total of 20 μL of hemolymph from each larva was transferred to sterile cold microcentrifuge tubes including 180 μL of phosphate buffer (0.07541 M $Na_2HPO_4.7H_2O$, 0.02459 M $NaH_2PO_4.H_2O$, pH 7.0). After centrifugation of the hemolymph-buffer mixture at +4 °C, the cell-free supernatant was transferred to a 96-well microplate. The substrate of phenoloxidase, L-DOPA (Sigma-Aldrich), was prepared in phosphate buffer and added to the hemolymph-buffer mixture in the 96-well microplate. Phenoloxidase converted L-DOPA to the black pigmented product, melanin, and the color change of the samples was detected by Multiskan™ GO Microplate Spectrophotometer (ThermoScientific, Finland). Absorbance was measured at 490 nm wavelength for 30 min at 5-min intervals [57]. The total amount of protein was determined using the Bradford method [58]. The results were given as U/mg protein/min.

RNA extraction, reverse transcription, and real-time qPCR

Five larvae from each of group (untreated, null injection, dH_2O injection, *C. albicans* injection, plant extract injection, and co-injection of *C. albicans* with plant extract) were subjected to gene expression quantification at three different time points: 1, 4, and 24 h pi. The larvae were rapidly ground in liquid nitrogen and stored at −80 °C until RNA isolation. Total RNA was isolated from each homogenized sample (approximately 30 mg) using an RNA Purification Kit (Thermo Scientific, K0731), and then the samples were treated with DNase I (Thermo Scientific, EN0525) to remove all DNA from the total RNA. The RNA yield was checked by Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific, Finland) and used to generate the first-strand cDNA by using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, K1622) as recommended by the manufacturer's protocol. Real-time quantitative PCR (RT-qPCR) was performed using a PikoReal™ Real-Time PCR System (Thermo Scientific, Finland) with the RealQ Plus 2x Master Mix Green Without ROX (Ampliqon, A323402, Denmark) according to the manufacturer's instructions.

The primer sequences for gallerimycin, hemolin, phenoloxidase I (PO-I), phenoloxidase II (PO-II), and β-actin genes are given in Table 1. The primer sequences for gallerimycin gene were used as described previously [59]. Other primer sequences were designed using NCBI primer-BLAST tool with *G. mellonella* nucleotide database. The specificity of all primers was checked against the *G. mellonella* genome and confirmed by agarose

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Table 1. Primers used in this study for evaluating gene expressions

Gene	Sequence (5′ to 3′)	NCBI accession number	Reference
Gallerimycin	F: GAAGTCTACAGAATCACACGA R: ATCGAAGACATTGACATCCA	-	[59]
Hemolin	F: ATCACTGTTGGCCCTGATGG R: CCGTGAGGGAGTCGATGAAG	XM_026893524.2	This study
PO-I	F: TGGAAGAGTACGGCGTGATG R: CGGGGTAGTCCAACTGACTG	XM_026902688.2	This study
PO-II	F: TCTTGCCGCCTTCAACTTCT R: CCGGTTCATCGATCCTGTCC	XM_026899262.2	This study
β-actin	F: CCCTGTGCTCACCGA R: ACAGTGTGGGTGACCCCGTC	XM_026904349.2	This study

gel electrophoresis. The reaction sample's final volume contains 20 ng of cDNA, 5 μL of 2× SYBR green, and 10 pmol/μL of each primer. The RT-qPCR conditions were 95 °C for 15 min, 40× (95 °C for 30 s, 52 °C for 30 s, 72 °C for 30 s). Each reaction was performed in triplicate, and the average threshold cycle (Ct) was computed for each sample. The amount of mRNA was normalized to β-actin as the housekeeping gene. Fold differences for each gene were calculated using the comparative CT (−ΔΔCt) method relative to the untreated at 1, 4, and 24 h [60].

Statistics

Unless otherwise noted, all assays were performed in at least triplicate. The obtained data were evaluated with one-way ANOVA and Tukey's HSD test in the SPSS statistical program. Differences were considered statistically significant at $p < 0.05$. All data were expressed as the mean ± SD.

RESULTS AND DISCUSSSION

Effects of *H. perforatum* extract doses on phenoloxidase activity

Hypericum perforatum has been considered a valuable herbal medicinal plant due to its antibacterial, antioxidant, and cytocidal properties. To determine the dose-dependent effect of *H. perforatum* extract on phenoloxidase activity, injection doses were prepared as 0.001, 0.0025, 0.005, 0.1, 0.5, 1.0, 2.5, 5, 10, 20, 40, 60, 80, and 100 mg/mL. Control groups were stated as untreated, sterile distilled water injection and null injection. Phenoloxidase activity was determined by collecting hemolymph 24 h pi. It was observed that there was no significant difference between the phenoloxidase activity of the control groups (untreated, sterile distilled water and null) ($p > 0.05$). Therefore, the results are given as a percent change in phenoloxidase activity relative to untreated (Fig. 1). In addition, a significant change was observed in larvae injected with low (0.001 and 0.0025 mg/mL) and high (20 mg/mL and over) doses of plant extract ($p < 0.05$). Therefore, in subsequent studies, 0.0025 and 20 mg/mL *H. perforatum* extract were determined as low (Hp-L) and high (Hp-H) doses, respectively. It was also observed that the larvae remained alive for 72 h at all *H. perforatum* extract doses (also at 200 mg/mL) used in our study.

Effects of *H. perforatum* extract on fungal load

Detection of changes in the fungal load provides information about the proliferation and survival of the pathogen in the host, as well as the host humoral response [24, 61]. The antifungal activity of *H. perforatum* extracts may cause to change in the fungal load and also host humoral response. Therefore, *G. mellonella* larvae were injected with *C. albicans* alone or together with *H. perforatum* extracts to induce fungal infection. In comparison to the initial fungal load (4.57 Log CFU/larva, 100%), there was no notable alteration in the fungal loads of larvae infected with *C. albicans* alone or co-infected with *H. perforatum* extracts at 1 and 4 h pi (Fig. 2). After 24 h, no yeast cells were observed in the hemolymph of larvae infected with only *C. albicans*. It was reported that no *C. albicans* cells were found in *G. mellonella* hemolymph samples taken 7, 24, 48, and 72 h after *C. albicans* injection [12]. These results indicate that hemocytes start to lyse or phagocytize yeast cells after 4 h (Fig. 2).

However, in larvae co-infected with a low and high dose of plant extract, the fungal load was determined as 2,2 Log CFU/larva (48%) and 0.3 Log CFU/larva (7%), respectively. It was known that there is a positive correlation between fungal infection and hemocyte number. The increase in the fungal load within the larva causes to increase in the amount of hemocytes up to a certain level [24]. The amount of hemocytes in *G. mellonella* larvae varies depending on the microorganism characteristics such as type, size, amount and virulence of a pathogen, and some other factors such as heat stress (e.g., incubation temperature of larva) and

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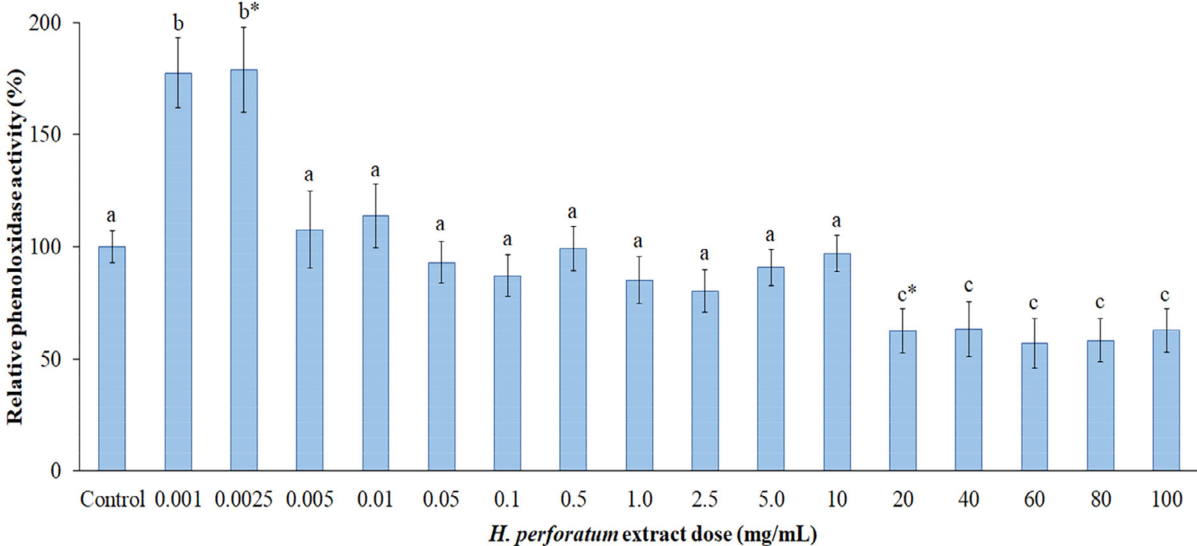

Fig. 1. Percent phenoloxidase activity relative to control (untreated larva). *Indicates the selected low and high dosages of plant extract. According to one-way ANOVA and Tukey's HSD, values followed by different letters are significant ($p < 0.05$) for each treatment (F: 9.116, df: 16/255, p: 0.000).

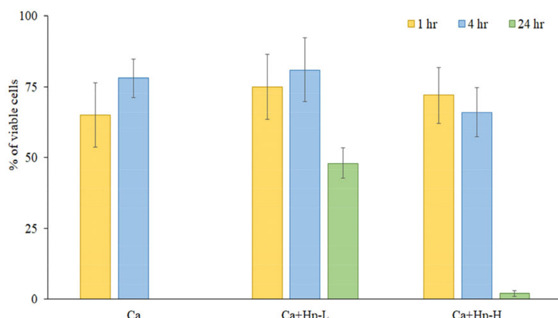

Fig. 2. The fungal load in *G. mellonella* larva after mono- and co-injection of *C. albicans* with low and high doses of *H. perforatum* extracts at three time points: 1, 4, and 24 h pi. Ca: *C. albicans*, Ca+Hp-L: *C. albicans* with low dose of *H. perforatum* extract (0.0025 mg/mL), Ca+Hp-H: *C. albicans* with high dose of *H. perforatum* extract (20 mg/mL). The number of yeast cells injected to *G. mellonella* larva is considered to be the initial fungal load (100%). Values represent the mean ± SD of data from three independent experiments.

physical stress (e.g., shaking larva or UV exposure) [17, 20, 24, 62, 63]. As all these factors were the same in our experimental groups, the survival of yeast cells in the larvae injected with plant extract may be possible by evading the yeast cells from the immune system. The evasion of the pathogen from the host immune system can be done in two ways: by avoiding direct immune detection or by actively interfering with host immune responses [64]. As a result, the molecules in the plant extract might have evaded the host immune system by preventing yeast cells from being recognized, or the immune response could have been delayed due to troubles recognizing different immunoregulators.

Effects of *H. perforatum* and *C. albicans* mono- and co-injection on phenoloxidase activity

Phenoloxidase activity in *G. mellonella* larvae was determined after larvae challenged with nonlethal dose of *C. albicans* (Ca), low and high dose of *H. perforatum* extracts (Hp-L and Hp-H) and nonlethal dose of *C. albicans* with low and high dose of *H. perforatum* extracts (Ca+Hp-L and Ca+Hp-H). The mono- and co-injections of *C. albicans* and the plant extract did not elicit humoral immune responses such as nodulation or melanization in larvae (data not given). No significant difference was detected between enzyme activities measured in the control groups (untreated, sterile distilled water and null) at 1, 4, and 24 h pi. Mono-injection of larvae with *C. albicans* increased the enzyme activity (4- or 5-fold) at 1, 4 and 24 h pi compared to the control (untreated larvae) (Fig. 3). Previously reported that the injection of a nonlethal dose of *C. albicans* (2×10^4 CFU/larva) caused an increase in the phenoloxidase activity at 24 h pi while the injection of a lethal dose (2×10^5 CFU/larva) caused a strong inhibition of enzyme activity [12]. The injection of 1-1.25 × 10^5 CFU/larva has been determined to be a nonlethal dosage, since it does

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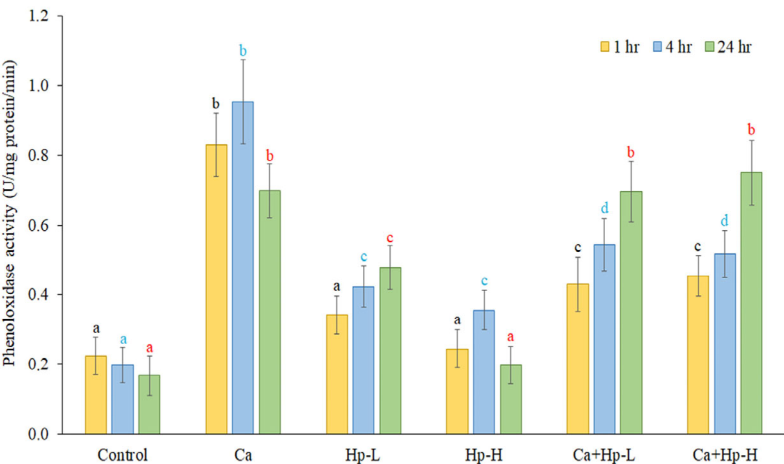

Fig. 3. The phenoloxidase activity in *G. mellonella* larva after mono- and co-injection of *C. albicans* with low and high doses of *H. perforatum* extracts at three time points: 1, 4, and 24 h pi. Control: Untreated larvae, Ca: *C. albicans*, Hp-L: low dose of *H. perforatum* extract (0.0025 mg/mL), Hp-H: high dose of *H. perforatum* extract (20 mg/mL), Ca+Hp-L: *C. albicans* with low dose of *H. perforatum* extract, Ca+Hp-H: *C. albicans* with high dose of *H. perforatum* extract. For each hour, a statistical study was done separately, and a colored lettering system was created within that hour. According to one-way ANOVA and Tukey's HSD, values followed by different letters are significant ($p < 0.05$) for each treatment (for 1 h: F: 4.751, df: 5/54, p: 0.001; for 4 h: F: 7.463, df: 5/54, p: <0.001; for 24 h: F: 6.626, df: 5/54, p: <0.001).

not result in the mortality of *G. mellonella* [65, 66]. Therefore, the increase of phenoloxidase activity was seen in our study as a result of the injection of a nonlethal dose of *C. albicans*, which is consistent with previous research findings [12]. The rapid increase in enzyme activity 1 h after mono- and co-injection of pathogen, and the presence of a high level of the active enzyme at 24 h (even if no viable cells are present) may be due to the prolonged maintenance of active enzyme in a certain level within the system. It was previously observed that the enzyme activity increased at 7 h after the administration of a nonlethal dose of *C. albicans* and continued up to 24 h [12]. The results of this study confirm the outcomes of our inquiry.

The mono injection of a plant extract at a low dose resulted in a twofold increase in enzyme activity at 4 and 24 h pi (Fig. 3). Similarly, when exposed to a high dose of plant extract, the enzyme activity increased twofold at 4 pi but not at 24 h. Co-injection of *C. albicans* and plant extract, on the other hand, resulted in a decrease in enzyme activity at 1 and 4 h pi compared to larvae injected solely with *C. albicans*, although it was still twofold higher than the control. According to these results, the molecules in the plant extract may be slowed down the humoral response by preventing the recognition of the pathogen by the immune system for a while, or by allowing the generation of an immune response against the plant extract first. The existence of viable yeast cells in larvae co-injected with *C. albicans* and plant extract at 24 h pi supports the possibility of a delay in the pathogen recognition process.

It has been previously noted that the immune proteome of co-infected larvae exhibits an altered immune response than that of mono-infected with *C. albicans* or *S. aureus* [66]. In the same way, our results showed that co-infected larvae had a different pattern of phenoloxidase activity than mono-infected larvae. According to our results, the immune response may be regulated depending on magnitude of the challenge and the immunoregulator's pathogenicity. Indeed, previous work has demonstrated that the insect immune system can sense the magnitude of the microbial challenge and provide an "appropriate and proportionate" response to ensure survival while minimizing the use of resources [66, 67]. Also, the link between the magnitude of the immune response and the size of the microbial challenge suggested that the insect immune system may differentiate between a low-level, potentially nonlethal infection and one that threatens survival [68]. Therefore, the immune system might be able to decide which foreign substance is more harmful than the other and generate the first and fast immune response to the most harmful one.

Effects of *H. perforatum* and *C. albicans* mono- and co-injection on phenoloxidase gene expression

The expression of the *G. mellonella* phenoloxidase genes (PO-I and PO-II) in whole larva was

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measured using RT-qPCR at three time points: 1, 4, and 24 h pi. The fold difference for each gene, relative to the control (i.e., $2^{-\Delta\Delta CT} = 1$ for untreated) was calculated using the ΔΔCT method. The expression levels were normalized to the housekeeping gene, β-actin [60].

PO-I and PO-II genes expressions was considerably lower in all experimental groups compared to the control at 1, 4 and 24 h pi, with the exception of PO-I expression in Hp-L (at 1 h pi) (Figs 4 and 5). There was no change in PO-I gene expression in larvae infected with a low dose of plant extract at 1 h pi (Fig. 4).

Despite the increase in phenoloxidase enzyme activity, the decrease in PO-I and PO-II gene expressions indicates that there is no positive correlation between enzyme activity and gene expression. PO-I and PO-II gene expressions were determined using whole larvae while the enzyme activities were determined using hemolymph. Twenty larvae were weighted before and after the hemolymph collection, and it was found that the amount of hemolymph in one larva is approximately 25% of the whole larva (data not given). Hence, the lack of any correlation between enzyme activity and gene expression is a probable consequence.

Phenoloxidase is synthesized as an inactive pro-enzyme, prophenoloxidase (ProPO) in hemocytes, and converted to active phenoloxidase (PO) by prophenoloxidase-activating protein (PAP-1) during melanization. PAP-1 enzyme activity was reported to have increased in *G. mellonella* at 6 h after post-injection with *C. albicans* (5×10^5 CFU/larva) [24]. PO-I gene expression did not change for 12 days in *G. mellonella* larvae feeding with *Nosema apis* and *Nosema ceranae* (10^6 single and mixed *Nosema* spore-containing diet) [69]. Differentially expressed genes involved in the innate immune reaction were determined in hemocytes from untreated and LPS-treated *G. mellonella* larvae and it was found that the PAP-1 gene expressed differentially instead of the phenoloxidase gene [70]. Similarly, the proteomic analysis of co-infected larval hemolymph revealed an increased abundance of prophenoloxidase activating protein rather than prophenoloxidase [66]. Considering the results in previous studies and our results, future research should be focused on determining the PO-I, PO-II, and PAP-1 gene expressions in both hemolymph and whole larvae.

Effects of *H. perforatum* and *C. albicans* mono- and co-injection on gallerimycin and hemolin gene expressions

The antimicrobial peptides, gallerimycin and hemolin, are also found in *G. mellonella* [9, 70]. The expression of gallerimycin and hemolin genes in the whole larva was measured at 1, 4, and 24 h pi.

Gallerimycin gene expression was considerably increased in *G. mellonella* larvae challenged with *C. albicans* after 1 h pi (3-fold higher than control) and continued to increase at 4 h pi (13-fold) but decreased at 24 h pi (Fig. 6). Previously reported that after injection of *C. albicans* (2×10^5 cells/mL) to *G. mellonella*, gallerimycin gene expression increased markedly in whole larvae (36-fold at 1 h, 120-fold at 24 h, and 90-fold at 48 h) [71]. But

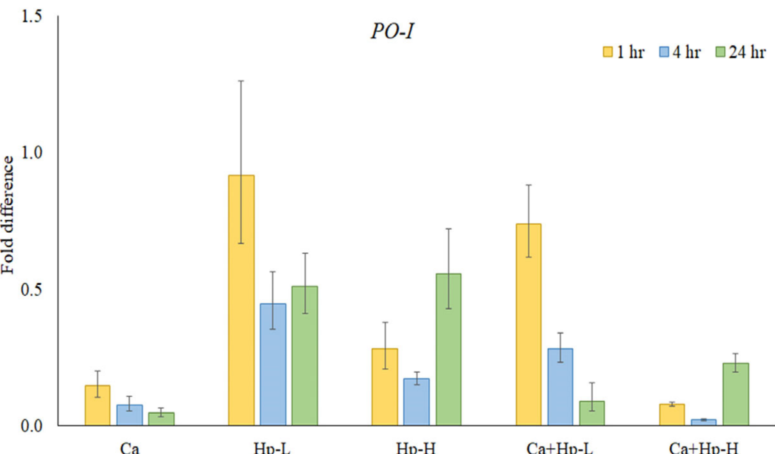

Fig. 4. PO-I gene expression in *G. mellonella* larva after mono- and co-injection of *C. albicans* with *H. perforatum* extracts at three time points: 1, 4, and 24 h pi. Ca: *C. albicans*, Ca+Hp-L: *C. albicans* with low dose of *H. perforatum* extract (0.0025 mg/mL), Ca+Hp-H: *C. albicans* with high dose of *H. perforatum* extract (20 mg/mL). Reference point was the level of gene expression in the control (i.e., $2^{-\Delta\Delta CT} = 1$ for untreated). Values represent the mean ± SD of data from the ΔCt value of the technical triplicates of three independent experiments.

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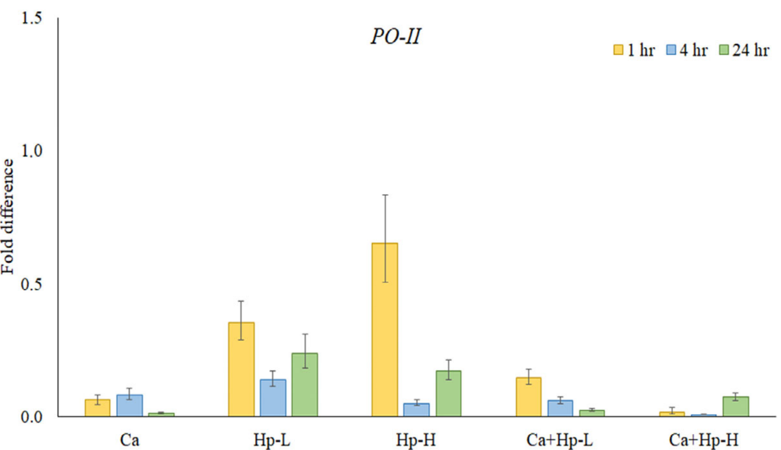

Fig. 5. PO-II gene expression in *G. mellonella* larva after mono- and co-injection of *C. albicans* with *H. perforatum* extracts at three time points: 1, 4, and 24 h pi. Ca: *C. albicans*, Ca+Hp-L: *C. albicans* with low dose of *H. perforatum* extract (0.0025 mg/mL), Ca+Hp-H: *C. albicans* with high dose of *H. perforatum* extract (20 mg/mL). Reference point was the level of gene expression in the control (i.e., $2^{-\Delta\Delta CT} = 1$ for untreated). Values represent the mean ± SD of data from the ΔCt value of the technical triplicates of three independent experiments.

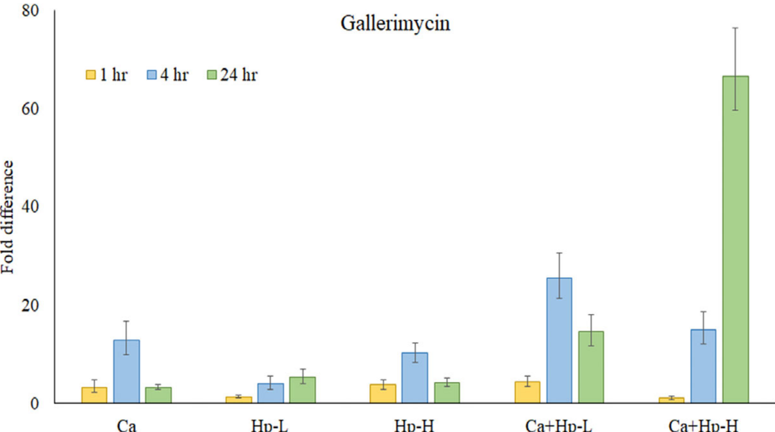

Fig. 6. Gallerimycin gene expression in *G. mellonella* larva after mono- and co-injection of *C. albicans* with *H. perforatum* extracts at three time points: 1, 4, and 24 h pi. Ca: *C. albicans*, Ca+Hp-L: *C. albicans* with low dose of *H. perforatum* extract (0.0025 mg/mL), Ca+Hp-H: *C. albicans* with high dose of *H. perforatum* extract (20 mg/mL). Reference point was the level of gene expression in the control (i.e., $2^{-\Delta\Delta CT} = 1$ for untreated). Values represent the mean ± SD of data from the ΔCt value of the technical triplicates of three independent experiments.

in another study, the administration of the low (2×10^4 CFU/larva) and high (2×10^5 CFU/larva) dose of *C. albicans* to *G. mellonella* caused 5- and 25-fold increase in gallerimycin gene expression in fat body at 24 h pi, respectively [12]. Although the *C. albicans* dose applied in our study was close to the lethal dose, the mRNA level of gallerimycin decreased at the end of 24 h pi (4-fold less mRNA observed than at 4 h pi). Meanwhile, the fungal load was around 65% at 4 h pi, and no *C. albicans* cells were detected at the end of 24 h pi (Fig. 2). So, the decrease in the pathogen level may have caused the repression of gallerimycin gene expression.

When the high concentration of plant extract was injected into *G. mellonella* larvae, the gallerimycin gene expression pattern was similar to *C. albicans* injection at 1, 4, and 24 h pi. The size of the molecules in the injected plant extract is very tiny, although their abundance is significant (The chemical composition of the plant extract is accessible at https://zenodo.org/record/8337162). Conversely, the injected yeast cells have a larger size but are present in a comparatively smaller quantity when compared

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to the plant extract. Therefore, it may be meaningful to obtain similar gene expression profiles between the mono-injection of *C. albicans* and the high concentration of plant extract. However, gallerimycin gene expression in larvae injected with a low concentration of plant extract was not substantially different from the control at 1 h pi. The gene expression increased 4-fold at 4 h pi and maintained its level up to 24 h pi. When the injected plant extract concentration was decreased, gallerimycin gene activation was delayed and the expression level decreased.

The co-injection of a low concentration of plant extract with the pathogen began to induce gallerimycin gene expression at 1 h pi (4-fold) and continued to increase up to 4 h pi (26-fold), but began to decrease at 24 h pi (15-fold) (Fig. 6). Gallerimycin gene expression was the same as the control level in co-injected larva with high plant extract for the first hour, but it increased up to 15-fold after 4 h, and continued to increase (67-fold) by the end of 24 h pi. Gallerimycin gene expression following co-injection with a low plant extract was observed to be two and five times greater than *C. albicans* mono-injection at 4 and 24 h pi, respectively. Gallerimycin expression after co-injection with a high plant extract was shown to be similar to *C. albicans* mono-injection at 4 h pi, but 22 times higher at 24 h pi. The expression of gallerimycin was shown to be maximum at 4 and 24 h when the plant extract dose was low and high, respectively. This shows that when different immunoregulators (e.g., *C. albicans* and plant extract) enter the organism in high amounts, the immunoregulator detection/recognition process of the immune system slows down and the immune response takes longer to occur. The fungal load was extremely low at 24 h pi after a high dose of plant extract injection (Fig. 2). Despite the presence of a negligible amount of viable pathogens in the system, the increased gene expression may be due to the extension or shifting of immune response generation as a result of delay in the immunoregulator detection/recognition process. In spite of the presence of around 50% viable cells in the system, gene expression was shown to be reduced 24 h after injection of a low plant extract concentration. In this situation, once the immune system has characterized the type and amount of the immunoregulator, a signal is generated within 4 h, taking into consideration the initial properties of the immunoregulator, and this signal is long lasting until the next signal is generated.

Hemolin gene expression was insensitive to the pathogen at 1 h pi, but it was induced after 4 h (6-fold) and remained at the same level until 24 h pi. (Fig. 7). However, after mono-injection of the plant extract (low and high doses), the hemolin gene expression was found to be 4-fold induced at 1 h pi. The gene expression level determined at 4 and 24 h after mono-injection of the plant extract was similar to that of the pathogen mono-injection level. This finding implies that, like *C. albicans*, the compounds in plant extract can trigger the expression of hemolin and gallerimycin genes. Co-injection of a low dosage of plant extract increased hemolin gene expression 14-fold at 1 and 4 h pi, but reduced it to 6 fold at the end of 24 h pi. In contrast, after co-injection of a high dose of plant extract, hemolin expression

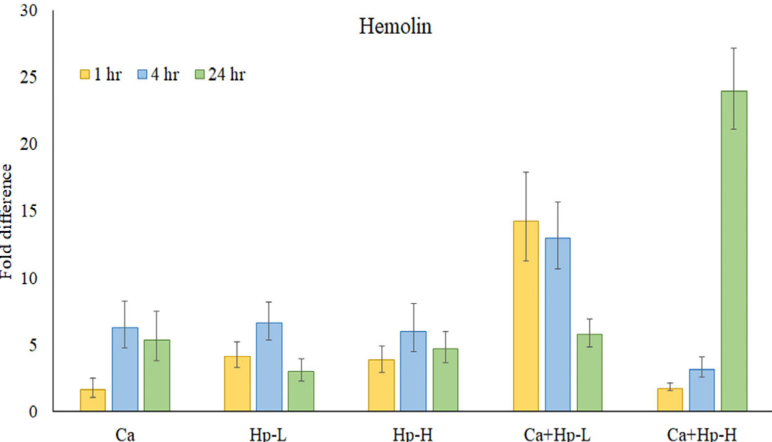

Fig. 7. Hemolin gene expression in *G. mellonella* larva after mono- and co-injection of *C. albicans* with *H. perforatum* extracts at three time points: 1, 4, and 24 h pi. Ca: *C. albicans*, Ca+Hp-L: *C. albicans* with low dose of *H. perforatum* extract (0.0025 mg/mL), Ca+Hp-H: *C. albicans* with high dose of *H. perforatum* extract (20 mg/mL). Reference point was the level of gene expression in the control (i.e., $2^{-\Delta\Delta CT} = 1$ for untreated). Values represent the mean ± SD of data from the ΔCt value of the technical triplicates of three independent experiments.

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increased 2- and 3-fold at 1 and 4 h pi, respectively. Furthermore, at 24 h pi, the level of hemolin expression increased 24-fold.

The co-injection of a low concentration of plant extract with the pathogen induced the hemolin expression 14-fold which was nearly 4-fold greater than gallerimycin expression at 1 h pi. On the other hand, co-injection of a high concentration of plant extract induced the hemolin expression 6-fold which was nearly 5-fold lower than gallerimycin expression at 4 h pi. Generally, the hemolin gene expression was lower than gallerimycin gene expression. These results suggest that, even when similar immunoregulators are introduced into the host, the regulation of antimicrobial peptide gene expression occurs in a complicated and distinct manner, revealing a stringent and tightly controlled system.

The activation of gallerimycin and hemolin gene expressions reached the maximum level at the end of 24 h after co-injection of a high concentration of plant extract. As in the gallerimycin, when the amount and type of immunoregulators in the host increase, the immune response to these immunoregulators is delayed due to the interference of recognition and signaling systems.

Overall, co-infection of the pathogen and the plant extract resulted in increased gallerimycin and hemolin gene expression relative to mono-infections. It has been reported that the synthesis of antimicrobial peptides involved in the formation of humoral immunity is related to the virulence level rather than the amount of pathogen, and the expression of AMP increases as the pathogenicity increases [71]. In this study, it was shown that the gallerimycin and hemolin gene expressions (in the Ca+Hp-L and Ca+Hp-H) increased when the amount of immunoregulator (e.g., plant extract) increased. Hence, with considerations of size, amount, and virulence capacity of the immunoregulator, the processes related to the recognition of the causative agent(s) or pathogen, and the subsequent signaling mechanisms are of crucial significance.

CONCLUSION

The immunoregulatory potential of *H. perforatum* extract on the humoral immune response of *G. mellonella* was investigated using different parameters. The fungal load, phenoloxidase activity, and the expressions of phenoloxidase, gallerimycin and hemolin encoding genes were determined in a time-dependent manner after mono- and co-injections of larva with *H. perforatum* extract and *C. albicans*. The injection of *H. perforatum* plant extract to *G. mellonella* larva promotes humoral immunity and keeps the immune system ready to defend against potential invaders. Hemolin and gallerimycin, two key defensins in *G. mellonella*, were found to be notably expressed after co-injection of plant extract with the pathogen. As a result, *H. perforatum* stimulates humoral immunity in living organisms by enhancing immune gene expressions. In addition, the insect immune system is able to recognize and discriminate the type and composition of the immunoregulator, as well as organize the appropriate humoral immune response, which can be adjusted depending on the severity and intensity of the immunoregulator.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Hypericum GC–MS at https://zenodo.org/record/8337162.

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