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RESEARCH REPORT

The effect of *Artemisia annua* essential oil and one of its main components on the biology and enzymatic and non-enzymatic activities of *Ephestia kuehniella* Zeller (Lepidoptra: Pyralidae)

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Abstract

The flour moth, *Ephestia kuehniella*, is a common storage pest worldwide. One of the most commonly used methods for controlling storage pests is fumigation using synthetic chemical compounds, which have serious negative impacts. In this study, the fumigant toxicity effects of *Artemisia annua* essential oil and 1,8-cineole, two environmentally safe substances, were investigated on larvicidal activity, developmental stages, cellular immunity, and enzymatic activities of *E. kuehniella* larvae. The results of fumigation assays on 3rd instar larvae after 24 and 48 h was estimated for *A. annua* essential oil and 1,8-cineole and lethal concentration (LC) values was calculated. The results indicated higher toxicity of 1,8-cineole over *A. annua* essential oil. Moreover, prolongation of larval developmental duration and a decrease in pupation period were observed at LC_{30}-treated larvae with *A. annua* essential oil as well as 1,8-cineole. A significant reduction of total hemocyte count (THC) and differential hemocyte count (DHC), including the number of plasmatocytes and granulocytes was also observed at LC_{30} and LC_{50} concentrations, 24 and 48 h post-treatment. The antioxidant enzymes including catalase and peroxidase were significantly increased compared to the control. Similarly, the metabolic enzymes including alanine aminotransferase, aspartate aminotransferase, acid phosphatase, alkaline phosphatase, lactate dehydrogenase were enhanced. The evaluation of detoxifying enzymes like glutathione S-transferase using DCNB and CDNB substrates, showed a significant increase compared to the control. Conversely, the activity of acetylcholinesterase was significantly decreased compared to the control. The results are indicative of the potentiality of these natural compounds as alternative to classical control measure against this important storage product pest.

Key Words: flour moth; immunity; antioxidant enzymes; fumigation; eucalyptol

Introduction

Loss of food commodities due to pests during storage is a significant challenge in both developed and developing countries, resulting in substantial financial losses (Demeter *et al*., 2021). The flour moth, *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae), is one of the most destructive pests of grain, particularly affecting flour (Kurtuluş *et al*., 2020), and is found globally (CABI, 2018). The larvae of the *E. kuehniella* not only cause direct damage by feeding on grains but also reduce product quality by contaminating them with excrement and webbing (Jallouli *et al*., 2013).

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Control of stored pests heavily relies on the widespread use of various chemical pesticides (Attia *et al*., 2020). However, prolonged use of chemical pesticides has raised long-term issues on human health and the environmental, primarily due to their slow degradation, residues in agricultural products, and the development of pest resistance (Isman, 2023). Fumigation, using chemical compounds, is one of the most common methods used to control storage pests, despite its serious negative impacts on the environment and human health (Arıkan and Turan, 2020; Boukan *et al*., 2024). Continuous use of phosphine as a common fumigation method for stored products pest control may lead to resistance development (Wakil *et al*., 2021). Therefore, attention has turned to alternative and environmentally friendly methods (Freitas *et al*., 2020).

Plant compounds comprise various proportions of alkaloids, phenols, terpenoids, sesquiterpenoids, monoterpenoids, flavonoids, tannins, and lignins

Table 1 The LC values, fumigation toxicity of *A. annua* essential oil, and the active ingredient 1,8-cineole on 3rd instar larvae of *E. kuehniella* after 24 hours (approximately 95% confidence has been calculated)

Treatment	LC_{10} (95% CL)	LC_{30} (95% CL)	LC_{50} (95% CL)	Slope ± SE	χ^2(df)
Artemisia annua	158.584 (93.164 – 208.711)	268.803 (202.374 – 317.425)	387.402 (330.533 – 444.674)	3.304 ± 0.576	0.887 (4)
1,8-cineole	30.818 (7.549 – 59.173)	94.997 (45.496 – 149.545)	207.167 (129.548 – 346.317)	1.549 ± 0.225	4.2680 (4)

LC: lethal concentration (µL/L air), CL: denotes 95% confidence limit, χ2: chi-square value, df: degrees of freedom

(Després *et al*., 2007; Kumrungsee *et al*., 2014). Among these plant compounds, essential oils are complex mixtures of plant secondary metabolites found in aromatic plants. Essential oils have been highlighted as the first choice for many biological pesticides due to their interference with insect metabolic, biochemical, physiological, and behavioral activities (Bakkali *et al*., 2008; Attia *et al*., 2020). Some essential oils exhibit fumigant, contact, repellent, antifeedant, and digestive toxicities against pests and are recommended for pest control due to their effective insecticidal activity (Asadi *et al*., 2019).

The sweet wormwood, *Artemisia annua* L. (Asteraceae), is native to Asia but has spread to many countries (Feng *et al*., 2020). Commonly called Gandvash in Guilan province (37.1172° N, 49.5280° E), that grows wild around paddy fields. *A. annua* is a medicinal plant highly regarded for its biological properties. Its essential oil inhibits acetylcholinesterase activity, reduces fertility and reproduction, and disrupts detoxifying enzymes, metabolic enzymes, antioxidants, digestive enzymes, and the immune system (Mojarab-Mahboubkar *et al*., 2022). Numerous reports highlight the insecticidal, bactericidal, virucidal, fungicidal, nematicidal, molluscicidal, and acaricidal properties of this genus (Deb and Kumar, 2020; El-Nuby *et al*., 2020; Tao *et al*., 2020; Tercino *et al*., 2020; Yang *et al*., 2020). Monoterpenoids constitute the majority of essential oil compounds (Arokiyaraj *et al*., 2022). The main components of *A. annua* essential oil include (±)-Camphor (29.290%), 1,8-cineole (12.564%), α-Pinene (8.659%), and *Artemisia* ketone (8.482%), as previously reported by us (Mojarab-Mahboubkar *et al*., 2023). The monoterpenoid 1,8-cineole, also known as eucalyptol, is a significant component of *A. annua* essential oil (Oftade *et al*., 2020). The 1,8-cineole is readily biodegradable and exhibits antibacterial, antifungal, anticancer, and anti-inflammatory activities (Cai *et al*., 2020). Several studies report its impact on various biological and physiological indices in insects (Ramezani *et al*., 2020). These include reduced larval development duration in *Spodoptera frugiperda* (J.E. Smith, 1797) under the influence of 1,8-cineole extracted from *Salvia keerlii* Benth essential oil (Zavala-Gómez *et al*., 2021). There are also several reports on its effects including, fumigant toxicity on *Tribolium castaneum* (Herbst) pupae (Liška *et al*., 2011), significant changes in the biology and physiology of elm leaf beetle (*Xanthogaleruca luteola* Müller) larvae (Adibmoradi *et al*., 2018), contact toxicity to adult *T. castaneum* (Wanna and Bozdoğan, 2024), inhibition of cytochrome P450 monooxygenase by 1,8-cineole extracted from *Eucalyptus cinerea* F. Muell. ex Benth in houseflies (*Musca domestica* L.) (Rossi and Palacios, 2015), and reductions in digestive enzymes including, alpha-amylase, chitinase, protease, and lipase in *Tribolium confusum* (Jacquelin du Val) (Tine *et al*., 2023).

The immune system is one of the most crucial and vulnerable systems in insects, responding to plant metabolites through cellular immunity (Afraze and Sendi, 2021). The cellular immunity operates through hemocytes, which eliminate foreign agents via methods such as phagocytosis, nodulation, and encapsulation after their entry into the body (Eleftherianos *et al*., 2021). Two types of hemocytes like, plasmatocytes and granulocytes, are the key cells in cellular immunity and are thus the most effective cells in confronting intruders (Hwang *et al*., 2015). The referenced study provides clear visual documentation of the various cell types present in *E. kuehniella*, these images comprehensively illustrate the cellular diversity, enabling better understanding of the moth's anatomical and physiological characteristics (Ghasemi *et al*., 2013).

Several studies have demonstrated that biochemical processes, including metabolic enzymes, are influenced by plant metabolites (Mojarab-Mahboubkar *et al*., 2022; Tine *et al*., 2023; Mahdaviarab *et al*., 2025). Metabolic enzymes in insects involves two processes: catabolism, which is the breakdown of large molecules into their precursors by specific enzymes, and anabolism, which is the production of larger molecules from their precursors (Klowden, 2013). Another system in insects affected by essential oils and plant extracts is the antioxidant system (Chowański *et al*., 2016; Adesina, 2023). Antioxidants include enzymes like catalases and peroxidases, which form the primary defense line to mitigate oxidative stress caused by secondary metabolites in insects and play a significant

Table 2 The effect of fumigation toxicity of *A. annua* essential oil and 1,8-cineole (LC_{30}) on different life stages duration

Different life stages	Treatment		
	Control	*Artemisia annua*	1,8-cineole
3rd instar larvae (d)	3.20±0.17b	4.13±0.25a	3.66±0.21ab
4th instar larvae (d)	2.66±0.25b	4.46±0.23a	4.20±0.26a
5th instar larvae (d)	2.40±0.16b	4.33±0.31a	2.66±0.12b
Larval period (d)	**8.26±0.58b**	**12.92±0.79a**	**10.52±0.56b**
Prepupation (d)	**4.06±0.24a**	**2.26±0.33a**	**3.60±0.52b**
Pupa (d)	**4.33±0.12a**	**2.00±0.37b**	**1.00±0.33b**
Female longevity (d)	5.20±0.20a	0	0
Male longevity (d)	5.66±0.12a	0	0
Fecundity (eggs/female adult)	**54.93±2.21a**	0	0

The similar letters in each row indicate no significant difference between the means at a 5% significance level using Tukey's test

role in maintaining cellular homeostasis (Adwas *et al*., 2019; Altuntaş *et al*., 2020). Glutathione S-transferase (GST), one of the detoxifying enzymes in insects, catalyzes the conjugation of electrophilic compounds with reduced glutathione, converting reactive molecules into more water-soluble and non-toxic forms that can be easily excreted (Sun *et al*., 2020). GST enzymes are generally crucial in the metabolism of chemical compounds (Guettal *et al*., 2021).

One of the most well-known hypotheses about the mechanism of toxicity of plant essential oils is their ability to inhibit acetylcholinesterase (AChE). Many insecticides, such as organophosphates and carbamates, exhibit their primary toxic effect by inhibiting AChE (Lang *et al*., 2012). AChE is a key enzyme in maintaining neurotransmission in the central nervous system of insects, so its inhibition can cause a wide range of primary and secondary effects (Thany *et al*., 2010). It is hypothesized that components of plant essential oils, including the monoterpenoid 1,8-cineole, are primarily responsible for AChE inhibition (Bajalan *et al*., 2017).

Given the importance and value of environmentally friendly compounds for human health, the flour moth was chosen to examine the fumigant effects of *A. annua* essential oil and one of its natural components (1,8-cineole) on lethality, as well as the irreversible effects of these compounds on the insect's biology and physiology at sub-lethals.

Material and methods

Insect rearing

The eggs of the flour moth (*E. kuehniella*) were obtained from an insectarium (Engineer Frank Mohseni's Plant Protection Production and Services Unit) and incubated under laboratory conditions (temperature 28 ± 1 °C, relative humidity 65 ± 5%, and photoperiod of 16:8 L:D). The hatched larvae were reared on an artificial diet containing whole wheat flour (43 g), yeast (6 g), and glycerin (20 mL) (Lima Filho *et al*., 2001). Newly emerged adult females of *E. kuehniella* were collected from the colony and transferred to a plastic funnel with a mesh screen (18 cm diameter) for oviposition. After seven days, the papers with the deposited eggs were placed in plastic containers (25×12 cm²) with the same artificial diet and kept until larval emergence.

Preparation of A. annua *essential oil and the active compound 1,8-cineole*

The *A. annua* plants were collected in May 2020 from around the rice fields situated in the Faculty of Agricultural Sciences at the University of Guilan. The plant samples (leaves of *A. annua*) were shade dried for one week. After drying, the leaves were ground into a fine powder. A Clevenger type apparatus was used for extraction of essential oil, for this purpose each time 50 g of the dried plant powder were mixed with 750 mL of distilled water and left at room temperature overnight. The distillation process took approximately 2 hours and the process of extraction for obtaining required amount of essential oil was repeated several times. Sodium sulfate was used to remove any remaining water from the essential oil. The active compound 1,8-cineole, with a purity of 99%, was procured from Sigma-Aldrich (Germany). The essential oil was stored in dark vials and maintained at 4 °C in a refrigerator until its utilization in the experimental procedures.

Bioassay of A. annua *essential oil and 1,8-cineole*

The freshly molted 3rd instar larvae (n=10) of the flour moth were placed in small containers of 100 mL volume each time, and the container was covered with mesh lids. These containers were then placed inside a transparent plastic chamber of 8000 mL volume, serving as the fumigation chamber. The chamber's lid was sealed after placing cotton soaked in a blend of 1,8-cineole or essential oil. For control the cotton was placed in the chamber without any

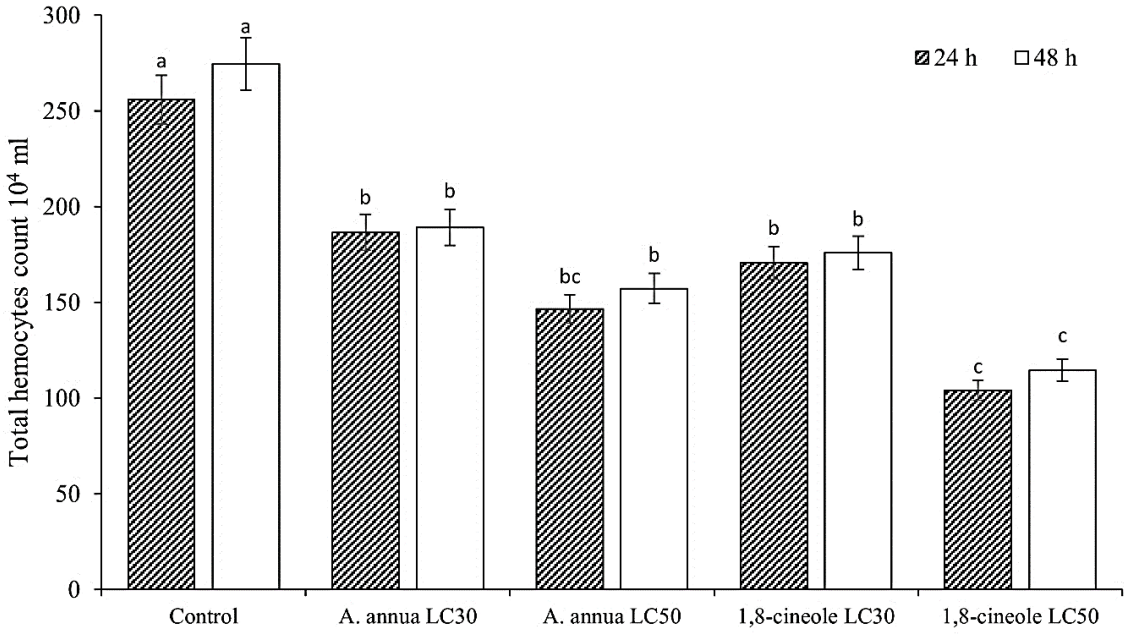

Fig. 1 The Total number of hemocytes in the 3rd instar larvae of flour moth *E. kuehniella* treated with LC_{30} and LC_{50} of *A. annua* essential oil and 1,8-cineole after 24 and 48 hours. Similar letters in each column indicate no significant differences (Mean±SE) (Tukey test, p≤0.05)

treatment (Seyedi *et al*., 2011). In preliminary experiments, concentrations that caused mortality ranging from 10% to 90% of the 3rd instar larvae were used as final concentrations of the essential oil and the 1,8-cineole blend (200, 300, 400, 500, 600, and 700 µL/L of air for the essential oil, and 30, 60, 120, 240, 480, and 960 µL/L of air for the 1,8-Cineole blend). This experiment was evaluated on 10 larvae and repeated 4 times, with mortality recorded after 24 hours (larvae were considered dead if no movement was observed upon stimulation with a fine brush). The experimental containers were kept in an incubator at 25 ± 1 °C, relative humidity of 65 ± 5%, with a photoperiod of 16 hours light and 8 hours dark. LC values (LC_{10}, LC_{30}, and LC_{50}) and 95% confidence intervals were estimated using probit regression analysis with Polo-Plus software (Robertson *et al*., 2017).

Duration of life stages

After determining the lethal and sublethal concentrations on 3rd instar larvae treated with 1,8-cineole and *A. annua* essential oil, the study examined the duration of various life stages (larval, prepupal, pupal, male and female adult), as well as the egg-laying capacity of adult insects at LC_{30} concentration. A control group was also included for comparison.

Immunology

Hemolymph of 3rd instar larvae of the flour moth (collected at 24 and 48 h) treated with LC_{30} and LC_{50} values along with a control group, was collected from the first proleg.

Total Hemocyte Count (THC)

To count the hemocytes, the third instar larvae were soaked in hot water (60 °C for 5 min). The first abdominal proleg was excised with the help of microscissors, and then 5 µL of fresh hemolymph was diluted in 15 µL anticoagulant solution (0.098 M NaOH, 0.186 M NaCl, 0.017 M EDTA, and 0.041 M citric acid, pH 5.4) (Amaral *et al*., 2010) and was added to Neubauer chamber (Mareinfeld CO. Germany), and the number of blood cells in four corners and one central area, each measuring 1 mm^2, was counted. The number of cells was calculated per mL of hemolymph (Khosravi *et al*., 2021).

Differential Hemocyte Count (DHC) of the flour moth larvae

For differential hemocyte counting, larvae were acclimated for 5 min in water at 60 °C and dried using filter paper. The first proleg was excised, and a drop of hemolymph was placed on a slide. A smear was prepared using another slide. After air-drying the smear, it was stained with diluted Giemsa stain (1:10 ratio with distilled water) for 25 minutes and then rinsed with distilled water. To distinguish cytoplasmic and nuclear colors, slides were briefly immersed in saturated lithium carbonate solution for 5 seconds and subsequently rinsed with distilled water for several minutes. After air-drying at room temperature, slides were permanently mounted using Canada balsam and dried at 45 °C. Identification of blood cells based on their morphological characteristics was conducted using a Leica light microscope (Rosenberger and Jones, 1960). A total

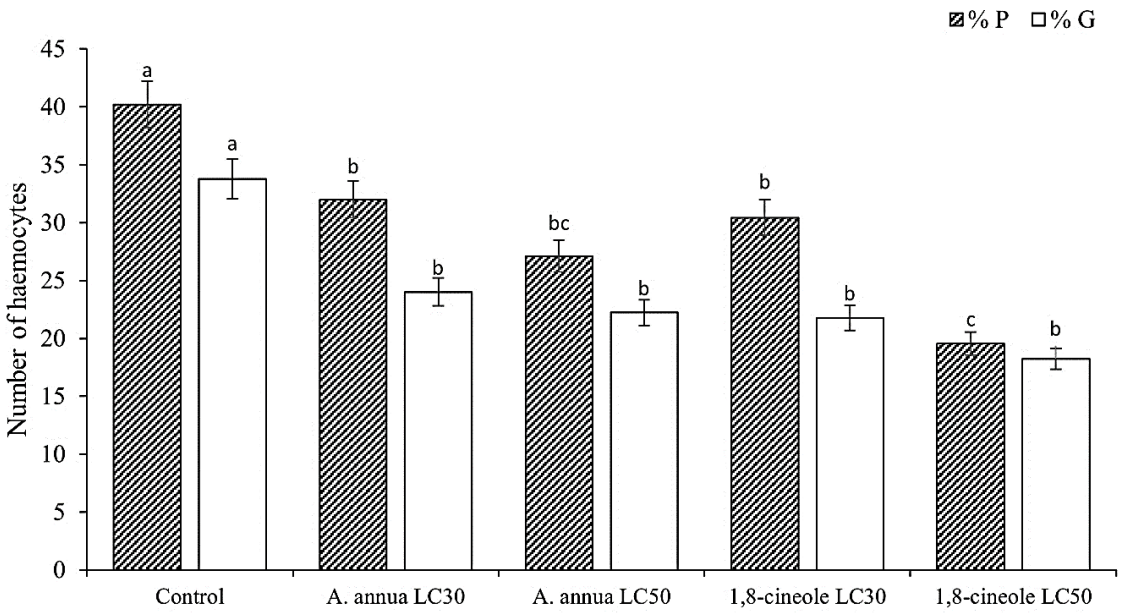

Fig. 2 The number of plasmatocytes and granulocytes of the 3rd instar larvae of flour moth *E. kuehniella* after treatment with LC_{30} and LC_{50} concentrations of *A. annua* essential oil and 1,8-cineole after 24 hours. The similar letters in each column indicate no significant differences (Mean±SE) (Tukey test, p≤0.05)

of 200 cells were randomly counted from the four corners and center of each slide. Total and differential counts of hemocytes were performed in 3 replicates, with 10 larvae in each replicate.

Enzymatic assays

3rd instar larvae of the flour moth were initially treated with LC_{10}, LC_{30}, and LC_{50} values of *A. annua* essential oil and 1,8-Cineole blend via fumigation toxicity. After 24 hours, 5 larvae were randomly selected and homogenized using a manual homogenizer with a 1:1 w/v ratio of phosphate buffer (pH 7). The homogenates were centrifuged at 13000×g for 20 minutes at 4 °C. The supernatants were used for biochemical analysis, with untreated insects included as controls for comparison using the same method. Finally, absorbance readings were taken using a microplate reader (Epoch 2 Microplate reader, BioTek, USA). All enzymatic measurements were performed in three replicates.

Catalase and Peroxidase activity

The catalase (CAT) enzyme activity was measured according to the method of Wang *et al*., (2001). For this purpose, 100 µL of 1% hydrogen peroxide was added to 20 µL of enzyme sample. The reaction mixture was incubated for 10 minutes at 25 °C, and the optical absorbance was finally read at a wavelength of 240 nm.

To measure peroxidase (POX) enzyme activity according to the method of Addy and Goodman, (1963), the reaction mixture included 50 µL of 0.5 mM pyrogallol, 50 µL of 1% hydrogen peroxide, and 20 µL of enzyme sample in 1.0 M phosphate buffer (pH 7). The resulting mixture was incubated for 2 minutes at 25 °C, and the absorbance was read every 30 seconds (5 readings in total) at a wavelength of 431 nm.

Metabolic enzymes activity

Measurement of the activities of two enzymes, Acid Phosphatase (ACP) and Alkaline Phosphatase (ALP), was performed based on the method by Bessey *et al*. (1946) using p-nitrophenyl phosphate as substrate. Initially, 40 µL of Tris buffer (20 mM, pH 5 for ACP and pH 8 for ALP) and 20 µL of substrate were mixed, followed by addition of 20 µL of enzyme sample. Optical absorption at 405 nm was read at one minute interval up to 5 minutes.

Measurement of the activities of two enzymes, Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST), was carried out according to the method by Thomas (1998) using a kit from Parsazmun Co., Karaj-Iran. Firstly, Reagents R1 (Tris buffer pH 7.5, L-Alanine, and lactate dehydrogenase) and R2 (2-Oxoglutarate and NADH) were incubated together for 20 minutes at 25 °C, followed by addition of 20 µL of enzyme sample. Optical absorption at 340 nm was measured.

Measurement of the activity of Lactate Dehydrogenase (LDH) enzyme was performed according to the method by King (1965) using a kit from Parsazmun Co., Karaj-Iran. Initially, Reagents R1 (Phosphate buffer pH 7.5 and Pyruvate) and R2 (Good's buffer pH 9.6 and NADH) were incubated together for 20 minutes at 25 °C, followed by addition of 20 µL of enzyme sample. Optical absorption at 340 nm was measured.

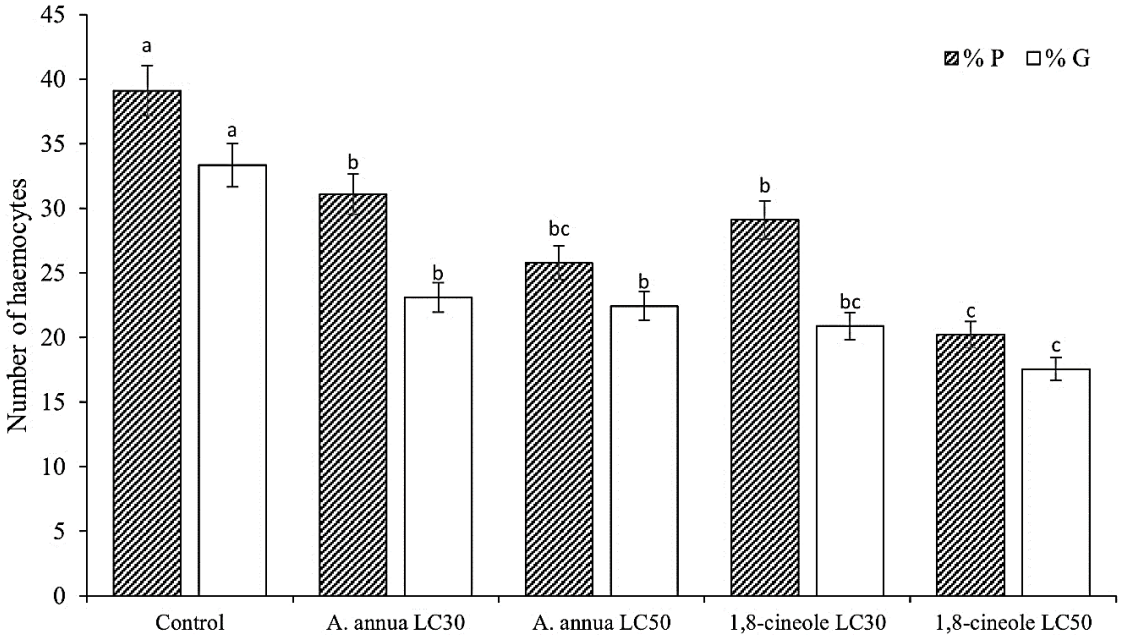

Fig. 3 The number of plasmatocytes and granulocytes of the 3rd instar larvae of flour moth *E. kuehniella* after treatment with LC_{30} and LC_{50} concentrations of *A. annua* essential oil and 1,8-cineole after 48 hours. Similar letters in each column indicate no significant differences (Mean±SE) (Tukey test, p≤0.05)

Glutathione S-Transferase (GST) activity

Measurement of Glutathione S-Transferase (GST) enzyme activity was conducted based on the method by Oppenorth *et al*. (1979) using two substrates, 1-chloro-2,4-dinitrobenzene (20 mM) and 1,2-dichloro-4-nitrobenzene (20 mM). Initially, 50 µL of Universal buffer (20 mM, pH 7), 20 µL of substrate, and 25 µL of reduced glutathione were mixed, followed by addition of 20 µL of enzyme sample. After 5 minutes of incubation at 25 °C, optical absorption at 340 nm was measured.

Acetylcholinesterase (AChE) activity

Acetylcholinesterase (AChE) enzyme activity measurement followed the method of Ellman *et al*. (1961). Initially, 80 µL of sodium phosphate buffer (100 mM, pH 7), 50 µL of acetylthiocholine iodide (10 mM), and 50 µL of 5,5'-dithiobis (2-nitrobenzoic acid) were mixed. After 5 minutes, 20 µL of enzyme sample was added to the mixture and incubated for 30 minutes at 25 °C. Optical absorption at 405 nm was measured.

Data analysis

Probit analysis using POLO-Plus software (Robertson *et al*., 2017) was conducted to assess acute toxicity after 24 hours. The obtained data on mortality, biochemical responses, and life stages were initially normalized using the arcsine square root transformation. Sorting and graphical representation of the data were performed using Excel 2016 software. One-sided analysis of variance (ANOVA) was carried out using Minitab 16 software. Tukey's test was used to compare means at a significance level of 5%.

Results

Toxicity test of A. annua *essential oil and 1,8-cineole*

The toxic effect of the essential oil of *A. annua* and 1,8-cineole against 3rd instar larvae of the flour moth after 24 hours is presented in Table 1. As the concentration of *A. annua* essential oil and 1,8-cineole increased, the mortality rate of the larvae also increased. Additionally, 1,8-cineole exhibited higher toxicity compared to *A. annua* essential oil, reporting LC_{10}, LC_{30}, and LC_{50} values of 30.818, 94.997, and 207.167 µL/L of air, respectively.

The effect of A. annua *essential oil and 1,8-cineole on the life stages of the flour moth*

The results of the impact of sublethal concentration (LC_{30}) of *A. annua* essential oil and 1:8 cineole on the biological stages of the flour moth are presented in Table 2. The sublethal concentration of *A. annua* essential oil on 3rd instar larvae of the flour moth extended the larval period significantly (12.92 ± 0.79) compared to the control (8.26 ± 0.58). However, the sublethal concentration of 1,8-cineole did not show a significant difference in the larval period (10.52 ± 0.56) compared to the control ($F=22.85$, $df=2,44$ $P<0.0001$). The prepupal period in the sublethal concentration of 1,8-cineole (3.60 ± 0.52) was significantly different compared to *A. annua* essential oil (2.26 ± 0.33) and the control (4.06 ± 0.24) ($F=5.88$, $df=2,44$, $P=0.0056$). The shortest pupation period was observed in *A. annua* essential oil (2.00 ± 0.37) and 1,8-cineole (1.00 ± 0.33) compared to the control (4.33 ± 0.12) ($F=35.15$, $df=2,44$, $P<0.0001$). The effect of sublethal concentration of *A. annua* essential oil caused

Table 3 The effect of *A. annua* essential oil and 1,8-cineole on the antioxidant enzyme activity in 3rd instar larvae of the flour moth in fumigation toxicity

Concentration	Antioxidant enzyme	
	CAT	POX
Control	0.500±0.005d	0.262±0.008b
Artemisia annua LC_{30}	0.575±0.002b	0.282±0.003b
Artemisia annua LC_{50}	0.664±0.002a	0.382±0.003a
1,8 – cineole LC_{30}	0.541±0.006c	0.277±0.005b
1,8 – cineole LC_{50}	0.576±0.008b	0.364±0.007a
F	111.61	87.96
P	0.0001	0.0001
df	4,14	4,14

The similar letters in each column indicate no significant difference between the means at a 5% significance level using Tukey's test.
CAT: Catalase, POX: Peroxidase

deformities in pupae and eventually led to their mortality. In the treatment with *A. annua* essential oil and 1,8-cineole, pupae did not transform to adult, whereas in the control, the adults hatched from the pupae and exhibited egg-laying behavior (F=120.85, df=2,44, P <0.0001). The number of eggs laid by adults in the control group was 54.93 ± 2.21, while no oviposition was observed in insects treated with the *A. annua* essential oil and 1,8-cineole.

Total Hemocyte Count (THC)

The effect of *A. annua* essential oil and 1,8-cineole on total hemocyte count in 3rd instar larvae of the flour moth 24 and 48 hours after fumigation toxicity at sub-lethal (LC_{30}) and lethal (LC_{50}) concentrations is shown in Figure 1. In larvae treated with LC_{30} and LC_{50} concentrations of *A. annua* essential oil and 1,8-cineole, the lowest total hemocyte count was observed in 1,8-cineole treatment at LC_{50} value. After 24 hours of treatment with *A. annua* essential oil at LC_{30} and LC_{50} concentrations, a significant decrease in total hemocyte count was observed (F = 23.69, df = 4,14, P = 0.0001). After 48 hours, the lowest total hemocyte count was observed in 1,8-cineole treatment at LC_{50} and in *A. annua* essential oil treatments at LC_{30} and LC_{50} (F = 46.71, df = 4,14, P = 0.0001).

Differential Hemocyte Count (DHC)

Plasmatocytes and granulocytes are the most important blood cells in the insect immune system. The percentages of these blood cells after treating 3rd instar larvae of the flour moth with sublethal (LC_{30}) and lethal (LC_{50}) concentrations of *A. annua* essential oil and 1,8-cineole are presented in Figs. 2 and 3, after 24 and 48 h post-treatment. In larvae treated with LC_{30} and LC_{50} concentrations of *A. annua* essential oil and 1,8-cineole, the number of plasmatocytes and granulocytes decreased after 24 and 48 hours. The number of plasmatocytes significantly decreased 24 hours after treating the larvae with LC_{50} concentration of *A. annua* essential oil and LC_{30} concentration of 1,8-cineole (F = 22.61, df = 4,14, P = 0.0001). Additionally, the number of granulocytes in larvae treated with LC_{50} concentration of *A. annua* essential oil and both LC_{30} and LC_{50} concentrations of 1,8-cineole was significantly lower than the control group (F = 7.92, df = 4,14, P = 0.004). The greatest reduction in the number of plasmatocytes was observed 48 hours after treatment with LC_{50} concentration of 1,8-cineole (F = 18.46, df = 4,14, P = 0.0001).

Antioxidant enzymes

The effect of *A. annua* essential oil and 1,8-cineole on antioxidant enzyme activity including CAT and POX in 3rd instar larvae of the flour moth is shown in Table 3. The activity of the enzyme catalase (CAT) significantly increased at LC_{30} and LC_{50} concentrations (F=111.61, df = 4,14, P=0.0001). Similarly, the activity of peroxidase (POX) significantly increased at LC_{30} and LC_{50} concentrations after 24 hours compared to the control (F= 87.96, df= 4,14, P=0.0001).

Metabolic enzymes

The effect of *A. annua* essential oil and 1,8-cineole on metabolic enzymes activity in 3rd instar larvae of the flour moth is shown in Table 4. The activity of metabolic enzymes, including alanine aminotransferase (ALT) (F = 65.91, df = 4,14, P = 0.0001), aspartate aminotransferase (AST) (F = 48.63, df = 4,14, P = 0.0001), alkaline phosphatase (ALP) (F = 24.99, df = 4,14, P = 0.0001), acid phosphatase (ACP) (F = 8.48, df = 4,14, P = 0.0031), and lactate dehydrogenase (LDH) (F = 57.02, df = 4,14, P = 0.0001), significantly increased compared to the control under fumigation toxicity at LC_{30} and LC_{50} concentrations in 3rd instar larvae of *E. kuehniella*.

Table 4 The effect of *A. annua* essential oil and 1,8-cineole on the intermediary enzyme activity in the 3rd instar larvae of the flour moth in fumigation toxicity

Concentration	Intermediary enzymes				
	ALT	AST	ALP	ACP	LDH
Control	0.917±0.004a	0.526±0.009c	0.309±0.001d	0.125±0.009a	0.377±0.003d
Artemisia annua LC_{30}	0.815±0.005bc	0.573±0.005b	0.361±0.011c	0.119±0.008a	0.408±0.005bc
Artemisia annua LC_{50}	0.787±0.013c	0.666±0.012a	0.410±0.011a	0.089±0.0009c	0.490±0.006a
1,8 – cineole LC_{30}	0.905±0.002a	0.586±0.006ab	0.334±0.005dc	0.117±0.0009ba	0.386±0.006cd
1,8 – cineole LC_{50}	0.821±0.000b	0.653±0.004a	0.391±0.005ba	0.090±0.0007bc	0.429±0.007b
F	65.91	48.63	24.99	8.48	57.02
P	0.0001	0.0001	0.0001	0.0031	0.0001
df	4,14	4,14	4,14	4,14	4,14

The similar letters in each column indicate no significant difference between the means at a 5% significance level using Tukey's test
ALT: alanine aminotransferase, AST: aspartate aminotransferase, ALP: alkaline phosphatase, ACP: acid phosphatase, LDH: lactate dehydrogenase

Glutathione S-transferase (GST) activity

The effect of *A. annua* essential oil and 1,8-cineole on the glutathione S-transferase activity in 3rd instar larvae of the flour moth is shown in Table 5. The activity of GST with both CDNB and DCNB substrates in 3rd instar larvae, significantly increased at LC_{30} concentration under the influence of *A. annua* essential oil compared to the control ($F = 87.77$, df = 4,14, $P = 0.0001$) and ($F = 27.43$, df = 4,14, $P = 0.0001$), respectively.

Acetylcholinesterase (AChE) activity

The effect of *A. annua* essential oil and 1,8-cineole on the acetylcholinesterase (AChE) activity in 3rd instar larvae of the flour moth is shown in Table 5. The amount of AChE in 3rd instar larvae significantly decreased at LC_{30} and LC_{50} concentrations when treated with *A. annua* essential oil and 1,8-cineole compared to the control ($F = 54.40$, df = 4,14, $P = 0.0001$).

Discussion

The long-term use of chemical pesticides is not only harmful to the environment but also leads to numerous side effects such as the development of resistance in insects (Pu *et al*., 2020). The secondary plant compounds are being reconsidered as an alternative control method against the chemical control in use due to their rapid biodegradability and reduced resistance development in pests (Razmjou *et al*., 2018; Sharma *et al*., 2023). Essential oils can be extracted from various parts of plants (Regnault-Roger *et al*., 2012). A number of studies have investigated the effects of sweet wormwood essential oil on various insect species, demonstrating its high potential for insect control through various means (Oftadeh *et al*., 2020; Afraze *et al*., 2020; Mojarab-Mahboubkar *et al*., 2022; Mojarab-Mahboubkar *et al*., 2023).

In the present study, the data from the fumigant toxicity of *A. annua* essential oil and 1,8-cineole over a 24-hour period are shown. According to the LC_{10}, LC_{30}, and LC_{50} values, the mortality rate of treated 3rd instar larvae increased with concentration, and 1,8-cineole showed higher toxicity compared to *A. annua* essential oil. It is well established that monoterpenoids, including 1,8-cineole, can penetrate the sensitive tissues of the central nervous system in insects due to their ability to diffuse through insect cuticle and cell membranes (Tak and Isman, 2017). Based on the values obtained in this study, various concentrations of *A. annua* essential oil showed significant fumigant toxicity against *E. kuehniella* larvae. These findings are comparable to the studies on *A. annua* essential oil used against the larvae of the flour moth and the Indian meal moth, representing the main active component, 1,8-cineole. Similarly, in this study, the mortality rate was increased with the increase in essential oil concentration (Bouzeraa *et al*., 2018; Mojarab-Mahboubkar *et al*., 2023). Rosemary essential oil showed high toxicity against the cowpea weevil, which can be attributed to the presence of α-pinene (22.64%), camphor (21.84%), and 1,8-cineole (21.53%) (Krzyżowski *et al*., 2020), which have also been reported in *A. annua* essential oil in our study (Mojarab-Mahboubkar *et al*., 2023).

Molting and metamorphosis are two crucial physiological processes in the life of insects, regulated by molting hormones (20-hydroxyecdysone) and juvenile hormones (Nation, 2008). Plant extracts and essential oils contain phytoecdysteroids or terpenoids, which exhibit hormone-like activity. These factors can interfere with the endocrine system, mimicking the behavior of either juvenile hormones or ecdysteroids thus leading to the formation of defective individuals (Bede and Tobe, 2000). In this study, *A. annua* essential oil prolonged the larval period of the flour moth compared

Table 5 The effect of *A. annua* essential oil and 1,8-cineole on the glutathione S-transferase and acetylcholinesterase activity in the 3rd instar larvae of the flour moth in fumigation toxicity

Concentration	Detoxifying enzyme		
	GST (CDNB)	GST (DCNB)	AChE
Control	0.607±0.003d	0.707±0.010b	0.529±0.016a
Artemisia annua LC_{30}	0.674±0.012bc	0.863±0.012a	0.400±0.005b
Artemisia annua LC_{50}	0.807±0.003a	0.904±0.004a	0.327±0.004c
1,8 – cineole LC_{30}	0.649±0.008c	0.740±0.029b	0.389±0.005b
1,8 – cineole LC_{50}	0.700±0.008b	0.831±0.008a	0.369±0.012bc
F	87.77	27.43	54.40
P	0.0001	0.0001	0.0001
df	4,14	4,14	4,14

The similar letters in each column indicate no significant difference between the means at a 5% significance level using Tukey's test
GST: Glutathione S-transferase, AChE: Acetylcholinesterase
CDNB: 1-chloro2,4-dinitrobenzene, DCNB: 1,2-dichloro-4-nitrobenzene

to the control comparable to the study by Ryan and Byrne, (1988) and Ohadi *et al*., (2024). This prolongation of larval period may be due to an increase in the juvenile hormone levels in the insect's body (Gaur and Kumar, 2020).

The innate immune system in insects is the primary defense mechanism against pathogens and other external factors, playing a crucial role in insect survival (Malagoli *et al*., 2023). Hemocytes, or blood cells, are key components of insect immunity. External factors, such as plant essential oils, can disrupt the immune system through cytotoxicity, affecting both Total Hemocyte Count (THC) and Differential Hemocyte Count (DHC) (Marmaras and Lampropoulou, 2009; Ajamhassani *et al*., 2023). In this study, the THC in *E. kuehniella* larvae is decreased after treatment with the essential oil of *A. annua* and 1,8-cineole. A decrease in mitotic hemocytes, cell damage, cytotoxicity or the inhibitory effects of exogenous compounds (including essential oils) might affect endocrine organs (Ghasemi *et al*., 2014; Ghoneim, 2018; Oftadeh *et al*., 2020; Azizi *et al*., 2024a and b).

Decreased number of plasmatocytes and granulocytes in *E. kuehniella* larvae after treatment with *A. annua* essential oil and 1,8-cineole is consistent with the use of castor and camphor essential oils against *Spodoptera litura*. reduced. The toxicity of essential oils is attributed to the presence of monoterpenoids which affect insect tissues, inhibit cellular respiration, and disrupt cell membrane permeability (Ali and Ibrahim, 2018; Diksha *et al*., 2023). The reduction in THC and DHC may thus may result from the cytotoxic effects of the essential oils on hemocytes or their inhibitory effects on hematopoietic organs or prohemocytes (Ghasemi *et al*., 2014).

Antioxidant enzymes, such as catalases and peroxidases, play an important role in maintaining cellular homeostasis (Dubovskiy *et al*., 2008; Adwas *et al*., 2019). The results of this study showed that the essential oil of *A. annua* and 1,8-cineole significantly increased the activity of catalase and peroxidase in treated larvae compared to the control. This increase in enzyme activity may indicate an adaptive response to stress induced by the applied compounds to eliminate the negative effects of reactive oxygen species (ROS). A significant increase in the activity of catalase and peroxidase enzymes compared to the control was observed in *G. pyloalis* larvae treated with an aqueous extract of *A. annua* (Afraze and Sendi, 2021). Another study found that this essential oil increased the activities of CAT and POX in adults of *Sitophilus oryzae* (Mojarab-Mahboubkar *et al*., 2023). Overall, this study suggests that high levels of oxidative stress are activated by plant metabolites, and the increase in antioxidant activity may be due to an increase in the production of reactive oxygen species and hydrogen peroxide as a result of oxidative stress.

Acid phosphatase (ACP) and alkaline phosphatase (ALP) are important enzymes participating in dephosphorylation (Senthil-Nathan, 2013). The results of this study showed that the levels of these metabolic enzymes in third-instar larvae treated with *A. annua* essential oil and 1,8-cineole significantly increased after 24 hours compared to the control. This aligns with the findings of Hasheminia *et al*. (2011) in *Pieris rapae* L. and (Mojarab-Mahboubkar and Jalali Sendi, 2016) in fall armyworm larvae when exposed to *A. annua* extracts or essential oil respectively.

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are two enzymes involved in various biochemical reactions in insect hemolymph and fat bodies, allowing them to adapt to oxidative stress (Klowden *et al*., 2013). In a study on rice weevil (*S. oryzae*) increased activity of these enzymes were observed under the influence of *Cinnamomum camphora*, *Mentha piperita* L., and *Jasminum*

officinale L. (Draz *et al*., 2016). Increased metabolic enzymes activity indicates detoxification processes (Mojarab-Mahboubkar and Jalali Sendi, 2016). Lactate dehydrogenase (LDH) is a crucial glycolytic enzyme involved in carbohydrate metabolism. In the present study, LDH levels in flour moth larvae was significantly increased in all treatments. Increased LDH activity is due to the oxidative effect of plant essential oils (Sugeçti and Büyükgüzel, 2018), indicating the negative effects of toxic substances on tissues and cell death (Shekari *et al*., 2008).

The glutathione S-transferases (GSTs), belong to the phase II detoxification system against the toxic effects of plant metabolites on tissue structure and function, playing a crucial role in protecting cells from oxidative damage. The plant metabolites may have differential roles in inhibiting or activating GST (Ramsey *et al*., 2010; Alias, 2016; Nemati *et al*., 2024; 2025). The increased GST activity in flour moth larvae may result from oxidative stress caused by plant compounds, leading to the inactivation of lipid peroxidation products accumulated due to oxidative stress (Khoobdel *et al*., 2022). Another study also reported that *A. annua* essential oil increased GST activity in *S. oryzae* (Mojarab-Mahboubkar *et al*., 2023), possibly due to the binding of GSTs' -SH groups to plant compounds (Chaudhari *et al*., 2021). Previous findings confirm that GSTs play a crucial role in detoxifying enzymes induced by secondary metabolite regulation of lectin extracted from azadirachtin in adult *S. oryzae* and *S. granarius* (Guettal *et al*., 2021).

Acetylcholinesterase is considered a key enzyme in the insect nervous system, catalyzing the hydrolysis of acetylcholine into acetate and choline in the synaptic space. Numerous studies have shown that plant essential oils contain many terpenoid compounds that can disrupt acetylcholinesterase activity in insects (Jankowska *et al*., 2017). The results of this study indicated that AChE activity in third-instar larvae was significantly decreased 24 hours after treatment with *A. annua* essential oil and 1,8-cineole compared to the control. The compound 1,8-cineole inhibited AChE activity in the red flour beetle *T. castaneum*, consistent with our findings (Kim *et al*., 2013). Plant essential oils demonstrated varying inhibitory properties on AChE, which may be attributed to differences in lipophilicity and volatility of the oils (Lee *et al*., 2001). Research on different *Artemisia* species shows that *A. brachyloba* Franch. essential oil and its main compounds (terpineol and davanone) inhibited AChE activity in *T. castaneum* (Hu *et al*., 2019). The main compounds of essential oil, including the monoterpenoids significantly reduced AChE activity in *S. granaries, S. oryzae Planococcus lilacinus* and *Diaphorina citri* (Kim *et al*., 2013; Rizvi *et al*., 2018; Arokiyaraj *et al*., 2022; Nenaah *et al*., 2023).

Conclusion

The results of the present study indicated that *A. annua* essential oil and its active compound 1,8-cineole have the potential to be considered as a natural choice for future pest control, particularly in areas where the use of chemical pesticides is restricted. Since these chemical metabolites degrade rapidly and do not persist in the ecosystem, their use is justified. Given that *A. annua* essential oil and 1,8-cineole have shown almost irreversible changes in all physiological systems of insects, developing a formulation that can maximize efficacy with minimal side effects will be of greater interest. Moreover, 1,8-cineole can be relatively inexpensive to extract and purify from natural sources, further supporting its value as a cost-effective and environmentally sustainable option for integrated pest management programs.

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