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EVALUATION OF THE EFFECTIVENESS OF *MILLETTIA PACHYLOBA* DRAKE LEAF ETHANOL EXTRACT IN ALLEVIATING OXIDATIVE STRESS INDUCED BY DIAMONDBACK MOTH INFESTATION IN MUSTARD GREENS [*BRASSICA JUNCEA* (L.) CZERN. & COSS.]

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ABSTRACT

The global threat that the diamondback moth (DBM) (*Plutella xylostella*) poses to *Brassica juncea* creates an urgent need to develop effective mitigation strategies. With promising findings, this study explores the potential of plant-derived antioxidants, particularly *Millettia pachyloba*, in mitigating DBM-induced oxidative stress. Greenhouse experiments conducted using *M. pachyloba* leaf ethanol extract (MPEE) at concentrations of 2, 4, 6, 8, and 10 % (w/v) showed the inhibitory effect of MPEE on DBM damage in *B. juncea*. The comprehensive assessment included the effectiveness of MPEE in reducing oxidative stress markers (malondialdehyde, hydrogen peroxide), augmenting metabolites (flavonoids, phenolics), enhancing anti-oxidant compounds (glutathione, ascorbic acid, total protein), and stimulating key enzymes (superoxide dismutase, catalase, and ascorbate peroxidase). The results were compared to controls, in which the plants were treated with water or fipronil. Fipronil and MPEE significantly reduced leaf puncturing and damage, and the impact force increased with MPEE concentration. Both types of protection reduced the content of malondialdehyde and hydrogen peroxide in leaves, stems, and roots. However, a significant increase in the content of flavonoids, phenolic and antioxidant compounds (glutathione, ascorbic acid, and proteins) and enzyme activity (superoxide dismutase, ascorbate peroxidase, and catalase) was observed. The enhancement of metabolites, enzymes, and antioxidants was comparable in plants treated with fipronil and MPEE 10%. This study, with its potential to replace synthetic fipronil with *M. pachyloba* extracts to reduce oxidative stress induced by *Plutella xylostella* on mustard plants, offers a promising solution for protection against this insect.

Key words: *Plutella xylostella*, antioxidants, oxidative stress, stress indicators, enzymes

INTRODUCTION

The Brassicaceae family is considered one of the most economically important plant families. The *Brassica* genus comprises over a hundred species, including oilseed rape (*B. napus* L.), cabbage (*B. oleracea* L.), turnip rapeseed (*B. rapa* L.), and mustard (*B. juncea* L.). These plants are predominantly cultivated for the extraction of oil, vegetables, spices, and as food sources (Chauhan et al. 2022). *B. juncea*, commonly known as mustard greens, stands out as a vegetable widely cultivated throughout the world and is one of the most popular household food crops. However, the production of mustard greens faces significant constraints due to various damaging insect species. These pests include grasshopper (*Melanoplus* sp.), wood-boring beetle (*Agrilus* sp.), leaf-mining fly (*Neoceratitis* spp.), domestic silk moth (*Bombyx mori*), and diamondback moth (*Plutella xylostella* L.). Due to its widespread global distribution, the diamondback moth (DBM) is regarded as the most severe threat among moth species. It is ubiquitous in cruciferous vegetable cultivation areas, exhibiting high migratory capabilities, resistance to natural predators, high reproductive potential, and notably rapid development of insecticide resistance (Legwaila et al. 2014).

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The diamondback moth can potentially induce oxidative stress in plants of the *B. juncea*. Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the biological system's capacity to detoxify these reactive intermediates (Reddy & Guerrero 2000). The manifestations of oxidative stress are closely tied to generating of reactive oxygen species (ROS). ROS can wreak havoc on various biological molecules such as DNA, lipids, proteins, etc., thereby significantly impacting the plant's metabolic processes, limiting growth, and reducing productivity. Oxidative stress in *B. juncea* can lead to diverse physiological and biochemical consequences, affecting the overall health of the plant and its ability to respond to stressors (Javeed et al. 2021). Antioxidants counteract the harmful effects of ROS by either scavenging them or inhibiting their formation. Enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) catalyze the breakdown of ROS into less harmful molecules. Non-enzymatic antioxidants like vitamins C and E, glutathione, and flavonoids directly neutralize ROS or regenerate other antioxidants (Chaudhary et al. 2023). Enzymes are involved in the metabolism and detoxification of ROS. SOD converts superoxide radicals ($O^{2\cdot-}$) into hydrogen peroxide (H_2O_2), which are then further detoxified by CAT or GPx. Peroxidases and thioredoxins play roles in scavenging ROS and regulating redox balance (Jomova et al. 2023). The relationship between antioxidants, enzymes, and ROS is dynamic and balanced. Antioxidants, both enzymatic and non-enzymatic, collaborate to regulate ROS levels and maintain cellular redox homeostasis. When ROS production surpasses the antioxidant defense capacity, oxidative stress occurs, leading to cellular damage and dysfunction (Hong et al. 2024). Therefore, understanding the alterations in antioxidants, enzymes, and ROS induced by DBM in *B. juncea* plants is crucial for devising strategies to mitigate, minimize yield gaps, and harness the full potential of crops (Dawood et al. 2020).

Millettia pachyloba Drake belongs to the Fabaceae family and is a robust climbing plant commonly found in tropical and subtropical regions worldwide. It is particularly prevalent in Indonesia, Malaysia, India, and Vietnam. The plant thrives in natural settings like shrublands and along the edges of evergreen forests, reaching elevations up to 1500 m. Locally, the plant's stem is traditionally utilized for herbal remedies, addressing conditions such as tumors, joint pain, and edema, while the roots serve as a vermifuge (Yan et al. 2019). *M. pachyloba* exhibits an anesthetic effect and induces respiratory distress in fish. It possesses potent insecticidal properties, effectively targeting pests such as termites, cockroaches, weevils, and other insects, making it valuable for use as an insecticide. The *Millettia* genus, includes numerous plants traditionally employed for their diverse pharmacological activities (Jena et al. 2020). The extract of *M. ferruginea* exhibits a plethora of biological activities, encompassing antibacterial, antifungal, larvicidal, anti-tuberculosis, anti-cancer, and antioxidant properties in plants, among others (Yassir et al. 2022). Similarly, the extract from *M. barteri* demonstrates antibacterial activity, antioxidant effects, and the ability to alleviate oxidative stress (Jena et al. 2020). In previous studies, *M. pachyloba* has been linked to cellular toxicity (Yan et al. 2019). However, in-depth research is needed on the antioxidant stress-relieving efficacy of *M. pachyloba* concerning *B. juncea* plants under oxidative stress induced by DMB. Therefore, the current study delves comprehensively into this issue.

MATERIALS AND METHODS

Experimental design

Design experiments in the laboratory

The experiment was conducted at the Department of Biotechnology, Institute of Biotechnology and Food Technology, Ho Chi Minh City University of Industry, Vietnam. *P. xylostella* was reared according to the method described by Silva et al. (2023) to ensure stability and uniformity throughout the rearing process. Specifically, adult *P. xylostella* specimens were collected from the private *B. juncea* fields. Specimens were housed in plastic cages measuring 60 × 30 cm, each containing a piece of sponge soaked in water to sustain appropriate humidity levels. To stimulate egg-laying, a filter paper disc and a *B. juncea* leaf were placed on the

moist sponge. Adult specimens were supplied with a 10% honey solution via a small hole at the bottom of the cage. The egg-laying leaf was transferred to a glass dish daily until *P. xylostella* hatched. Larvae were reared individually in separate containers until adulthood, with *B. juncea* leaves replaced daily with fresh ones. Pupae were collected in sealed test tubes with gas-permeable caps. Their development was maintained at ambient temperature until adult emergence, after which they were relocated to new housing. The entire life cycle was repeated following the emergence of adult individuals. The population was maintained at an ambient temperature of 22–26°C, with a relative humidity ranging between 55–60% and 75–80%, following a natural light cycle of 12 hours light and 12 hours dark. *P. xylostella* larvae were collected and released at the base of each *B. juncea* plant within the greenhouse model.

Experimental design in the greenhouse

The experiment was conducted at the trial area of Miền Đông Company, located in Tan Hiep Commune, Di An District, Binh Duong Province, Vietnam, in July 2023. *B. juncea* L. var. Southern Giant Curled was selected for testing in a greenhouse environment maintained at a stable temperature of 28 ± 2°C, relative humidity of approximately 65 ± 5%, a 12-hour light/dark cycle, and a CO_2 content ranging from 400 to 600 ppm. To ensure uniformity, in vitro propagated plants were used (Sihovi, Vietnam). The growth substrate was black soil with organic fertilizer Song Gianh BO 60 (Song Gianh Company, Vietnam), mixed in a ratio of 2 : 1 and thoroughly blended.

This experiment followed the method described by Wang et al. (2022) with additional modifications. Plants were grown in plastic pots (15 or 30 cm × 15 cm). The experiment was organized into 3 complete random replications, comprising (2 control treatments + 5 experimental treatments) × 3 replications = 21 pots. The *M. pachyloba* leaf ethanol extract (MPEE) with concentrations 2, 4, 6, 8, and 10% (w/v) denoted as MPEE2, MPEE4, MPEE6, MPEE8, and MPEE10, respectively were used. A commercial product, fipronil 5% SC (100 ppm), was used as a positive control, and water as the negative control. Plants, robust and disease-free with three leaves per plant, were transplanted into the pots, where they underwent a one-week acclimatization period to adapt to the new environmental conditions. Third-instar *P. xylostella* larvae, sourced from controlled laboratory rearing, were then inoculated onto each cabbage plant, with each plant hosting seven DBM larvae. Subsequently, a 50 mL plastic spray bottle was used to apply MPEE, fipronil, and water to the plants, ensuring an even distribution of approximately 20 mL of solution per plant.

Collection plant material

M. pachyloba leaves were collected in May 2023 in Đa Oai Commune, Đa Huoai district, Lam Đong province, Vietnam. The voucher specimen (code MP220523VST) is archived at the Department of Biotechnology, Ho Chi Minh City University of Industry. The collection of plant leaves involved concurrent processes of selecting and eliminating damaged leaves, followed by a thorough cleansing and drying procedure in a shaded environment for three days. Subsequently, the leaves were dried at 60°C until a consistent weight was achieved. Following the drying phase, the leaves were finely ground into a powder, stored in desiccant-containing containers, preserved at room temperature, and shielded from light in preparation for subsequent research phases.

Preparation of *M. pachyloba* leaf ethanol extract

200 g of leaf powder was extracted using 1000 mL ethanol via the Soxhlet extraction method. Subsequently, the extract solution was filtered and dried using a water bath, maintained at a temperature of 45°C. The resulting extract solution, quantified at 62.5 g and designated as MPEE, was then reconstituted in a saline solution (NaCl, 0.9%) for consistent use throughout the research. The MPEE stock solution was diluted to create five different concentrations: 2, 4, 6, 8, and 10% (w/v).

Phytochemical screening and quantification of *M. pachyloba* leaf ethanol extract

Preliminary phytochemical tests for tannins, alkaloids, saponins, cardiac glycosides, terpenoids, flavonoids, steroids, and phenolic compounds in *M. pachyloba* leaves were conducted following the procedures outlined by Bargah and Kushwaha (2017), as shown in Table 1.

Table 1. Preliminary phytochemical screening of *M. pachyloba* leaf ethanol extract

Phytoconstituents	Test	Observation
Tannins	2 mL of extract + lead acetate solution	white precipitate
Flavonoids	1 mL of extract + magnesium + a few drops of HCl	bright red color
Terpenoids	2 mL of extract + 2 mL of chloroform + 3 mL of H_2SO_4	brownish-red color
Saponins	5 mL of extract + 2 mL of DW + sodium carbonate and mixed	froth appears
Steroids	1 mL of extract + 1 mL of $CHCl_3$ + a few drops of H_2SO_4	from red with a slight tint of green to coral red
Cardiac glycosides	1 mL of extract + pyridine + $Na_2Fe(CN)_5NO$	colorless mixture

Alkaloid content in MPEE

200 mL of 20% acetic acid solution was added to 5 g of leaf powder, and this mixture was tightly sealed in a 250 mL glass beaker and left undisturbed for 4 h. The resulting solution was filtered, and the volume was reduced to one-fourth by evaporation. Subsequently, concentrated ammonium hydroxide was added dropwise until complete precipitation occured. The entire solution was then allowed to settle, and the precipitate was collected by filtration and weighed. The determination of alkaloid content was made using spectrophotometer at 280 nm. The percentage ratio of total alkaloid content (TAC) in the extracted solution was calculated using the following formula (Senguttuvan et al. 2014):

$$\text{TAC}(\%) = \frac{\text{Weight of dried alkaloid extract}}{\text{Weight of the sample}} \times 100.$$

Total phenolic content

10 mg of gallic acid was placed into dry volumetric flasks, dissolved in methanol, and adjusted to a final volume of 10 mL, resulting in a 1 mg·mL^{-1} concentration. A stock solution of the test substance was prepared by dissolving 10 mg of dry methanol extract in 10 mL of methanol, obtaining a concentration of 1 mg·mL^{-1}. The total phenolic content in the MPEE was quantified using the Folin–Ciocalteu colorimetric method. Gallic acid served as a standard, and results were expressed as gallic acid equivalents (GAE) per gram of sample (Senguttuvan et al. 2014).

Terpenoid content

An aliquot (0.1 g) of the leaf extract from *M. pachyloba*, was individually weighed, mixed with 20 mL of ethanol, and filtered through the Whatman No. 1. A 1 mL portion of the filtrate was withdrawn and mixed with 1 mL of 5% phosphomolybdic acid solution, followed by shaking. To each vial, 1 mL of concentrated H_2SO_4 was added. The mixture was allowed to stand for 30 minutes. Subsequently, 2 mL of ethanol was introduced, and the absorbance was measured at a wavelength of 700 nm. The content of total terpenoid content (TTC) in the extract was calculated using the following formula (Ujah & Onyishi 2023):

$$\text{TTC}\ (\text{mg} \cdot \text{g}^{-1}) = \frac{\text{Absorbance} \times \text{Path length}}{100 \times \text{Weight of sample used}}$$

The impact of diamondback moth on leaf hole formation and leaf damage in *B. juncea*

The disease severity of leaf puncture on *B. juncea* leaves caused by DBM

Ten leaves of similar age and size were gathered and scanned using a flatbed scanner. ImageJ software was employed to precisely quantify leaf area. Subsequently, the total area of damaged leaves and the overall leaf area under each experimental condition were measured. The degree of puncturing (DGP) by diamondback moth (DBM) on each *B. juncea* leaf was computed using the following formula:

$$\text{DGP}\ \% = \frac{\text{TPL}}{\text{TLE}} \times 100$$ (Araya et al. 2023);

where TPL signifies the cumulative area of punctured leaves and TLE represents the total leaf area under each experimental condition.

The disease incidence in the leaf of *B. juncea* caused by diamondback moth

Ten leaves of similar age and size from mustard plants were chosen for inspection. The number of damaged leaves was counted, and the total number under each experimental condition was determined. The leaf damage ratio (LDR) was calculated using the formula:

$$\text{LDR}\ \% = \frac{\text{TLL}}{\text{TLP}} \times 100$$ (Araya et al. 2023);

where TLL is the total count of lost leaves and TLP is the overall leaf count across all plants.

Sample preparation for each repetition of the experiment

To ensure high accuracy and consistency across repetitions, we conducted leaf preparation for each repetition in an experiment, aiming to eliminate or minimize random or undesired factors that could affect the outcome.

Determination of oxidative stress indicators

Malondialdehyde content

The malondialdehyde (MDA) content was assessed using the method outlined by Touzout et al. (2023). The fresh biomass, comprising leaves, stems, and roots of *B. juncea* was initially ground in a 1% trichloroacetic acid (TCA) solution, using a mortar and pestle, with a ratio of 10 mL per gram of fresh weight. The resulting material was centrifuged at 10,000 revolutions per minute for 5 minutes, and 1.0 mL of the supernatant was transferred to a separate test tube. Subsequently, 4.0 mL of 0.5% thiobarbituric acid (TBA) was added to the test tube. The mixture was then heated at 95°C for 30 minutes to facilitate the development of a pink-red chromophore indicative of MDA formation, followed by a cooling phase in an ice bath. The clarified solution was centrifuged at 5,000 rpm for 5 minutes. The absorbance of the resulting solution was measured at 532 nm wavelength, with any non-specific turbidity corrected by subtracting the absorbance value obtained at 600 nm. The blank range was established using 0.5% TBA in 20% TCA. The content of MDA was determined by measuring the absorbance at 532 nm. The MDA content was expressed in micromoles per gram of fresh weight (μmol·g^{-1} FW).

Assay of hydrogen peroxide content

The methodology proposed by Chauhan et al. (2022) was applied. 100 mg of leaves, stems, and root tissues from *B. juncea* were individually homogenized using 0.1% TCA. The resulting mixtures were centrifuged at 12,000 rpm for 15 minutes. A UV-visible spectrophotometer, operating in the ultraviolet range, was employed to measure absorbance at 390 nm after the addition of 0.4 mL of potassium phosphate buffer (10 mM, pH 7.0) and 0.8 mL of potassium iodide (1 M, pH 7.4) to 0.5 mL of the upper supernatant. Following this, a standard curve was constructed for H_2O_2 quantification. The H_2O_2 content was expressed in micromoles per gram of fresh weight (μmol·g^{-1} FW).

Identification of metabolites in mustard plants

Flavonoid content

The flavonoid content was determined following the protocol proposed by Verma et al. (2023). Individual samples of leaves, stems, and roots from *B. juncea*, 500 mg each, were homogenized separately in 3 mL of absolute methanol. Subsequently, the homogenized solutions were centrifuged at 13,000 rpm for 20 minutes. Following this, 1 mL of the supernatant, along with 4 mL of distilled deionized water (DW), 0.3 mL of sodium nitrite ($NaNO_2$), and 0.3 mL of aluminum chloride ($AlCl_3$), was added. The mixture was incubated for 5 minutes, and a pink color developed upon adding 2 mL of sodium hydroxide (NaOH) to 2.4 mL of distilled water. Flavonoid content quantification was accomplished through a standard curve of rutin as the standard, and absorbance was measured at a wavelength of 510 nm. The total flavonoid content was expressed as equivalent to rutin.

Phenolic content

The phenolic content was quantified using the method described by Verma et al. (2023). Samples of leaves, stems, and roots of 0.5 g were homogenized in 80% ethanol. The resulting homogenate was centrifuged for 20 minutes at 10,000 rpm. Subsequently, 0.5 mL of Folin–Ciocalteu (FC) reagent and 20% Na_2CO_3 were added to the supernatant. The samples were then assessed at a wavelength of 650 nm, with the phenolic content quantified utilizing gallic acid as the standard.

Quantifying antioxidant compounds

Determination of total glutathione

The content of glutathione (GSH) was assessed following the methodology established by Verma et al. (2023). Leaves, stems and roots specimens of *B. juncea* (1 g each) were ground in 3 mL of tris buffer solution (50 mM, pH 10.0) and subsequently subjected to 20-minute centrifugation at 13,000 rpm. A plant extract consisting of 100 μL, 1 mL of Tris buffer solution (0.2 M, pH 8.2), 50 μL of 0.01 M DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)], and 4 mL of absolute methanol was introduced to the test tube. The mixture underwent a 15-minute incubation at room temperature, followed by another 15-minute centrifugation at 3,000 rpm.

The optical density of the solution was subsequently measured using a spectrophotometer at a wavelength of 412 nm, and the content of glutathione was determined with a reference of mg per 100 mL.

Total ascorbic acid determination (AsA)

The methodology described by Verma et al. (2023) was utilized. Homogenization of 0.5 g of leaves, stems, and root tissues from *B. juncea* was obtained using a 50 mM tris buffer solution with a pH of 10.0, followed by centrifugation for 20 minutes at 13,000 rpm. The plant extracts, comprising 0.5 mL were amended with 100 mg of wood charcoal, 4 mL of distilled deionized water (DDW), and 0.5 mL of 50% TCA and filtrated using Whatman No. 4 filter paper. Incubation occurred at 37°C, 3 h after introducing 0.4 mL of 2,4-dinitrophenylhydrazine (DNPH) solution and 1.6 mL of cold (65%) H_2SO_4 to the mixtures. They were then kept at room temperature for 30 minutes. Results were recorded at an absorbance of 520 nm using a spectrophotometer, with ascorbic acid (1 mg·100 mL^{-1}) serving as the standard solution.

Total protein content

The quantification of the total protein (TPH) content followed the comprehensive methodology outlined by Verma et al. (2023). 500 mg of leaves, stems, and root tissues from *B. juncea* were homogenized with 3 mL of phosphate buffer solution, followed by centrifugation at 10,000 rpm for 10 minutes. Following this, 0.9 mL of distilled water was added to 0.1 mL of the upper supernatant. Subsequently, 5 mL of reagent C was introduced into each tube, forming a mixture of reagents A and B (ammonium iron sulfate in sodium potassium tartrate and sodium carbonate in sodium hydroxide). The reaction mixture stood undisturbed for 10 minutes post-thorough mixing. Then, 0.5 mL of reagent D (FC reagent) was added, stirred, and heated for 30 minutes at room temperature in the dark. The development of a green color indicated the completion of the reaction, and the results were recorded at an absorbance of 660 nm using bovine serum albumin (BSA) as the reference standard.

Measuring antioxidant enzymes

Superoxide dismutase activity

SOD activity was assessed according to the procedure by Wahyuningsih et al. (2020). The reaction mixture comprised 50 mM phosphate buffer solution (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 75 μM nitroblue tetrazolium (NTB), 2 μM riboflavin, and 100 μL of the upper supernatant. The reaction was initiated by placing the tubes under two 15 W fluorescent lights. After 10 minutes, the reaction was halted by removing the tubes from the light source. A dark reaction mixture served as the blank sample. The absorbance of the reaction product was measured at 560 nm using a spectrophotometer. This wavelength was selected for its characteristic property, reflecting the color change associated with NTB reduction and illustrating the level of enzyme activity. This absorbance measurement is an indirect indicator, playing a pivotal role as a significant marker of enzyme efficiency in catalyzing the reaction. This method enables the assessment of enzyme effectiveness during the reaction process by monitoring NTB reduction, thus providing precise insights into the enzyme's ability to facilitate the specific reaction process. SOD activity was expressed in units per milligram of protein.

Ascorbate peroxidase (APx) specific activity

The enzymatic activity of ascorbate peroxidase (APx) was evaluated following the procedure outlined by Verma et al. (2023). Leaf tissues (1 g) from *B. juncea* were homogenized in 3 mL of a 100 mM phosphate buffer solution (pH 7.0) and centrifuged for 20 minutes at 5,000 rpm. Then, 50 μL of the plant extract was introduced into a cuvette containing 2,370 mL of the phosphate buffer solution, along with 0.3 mL of ascorbate (5 mM) and 0.3 mL of H_2O_2 (0.5 mM). The absorbance was recorded at 290 nm using a spectrophotometer. This wavelength is chosen because it corresponds to the absorbance peak of ascorbate, which decreases gradually as it is oxidized by APx in the presence of H_2O_2. By monitoring the decrease in absorbance at 290 nm over time, the rate of ascorbate consumption can be inferred, directly reflecting the enzymatic activity of APx. As APx catalyzes the reduction of H_2O_2 using ascorbate, a decrease in ascorbate content indicates higher APx activity, as it is actively involved in scavenging H_2O_2.

Catalase specific activity

To evaluate the CAT activity, we followed the procedure outlined by Verma et al. (2023). A mixture for extraction was prepared using 1 g of leaf samples from *B. juncea* in 3 mL of phosphate buffer solution (100 mM, pH 7.0) and then centrifuged at 5,000 rpm for 20 minutes. The absorbance was quantified at 240 nm by introducing 50 µL of the plant sample into a solution comprising 300 µL of H_2O_2 (150 mM) and 2,650 mL of 100 mM phosphate buffer solution. The alteration in absorbance at 240 nm reflects the consumption of H_2O_2 by the enzymes present in the plant sample. Enzymes with peroxidase activity catalyze the decomposition of H_2O_2, leading to a reduction in absorbance at 240 nm over time. By tracking the changes in absorbance over time, the rate of H_2O_2 decomposition can be quantified, providing a measure of the activity of CAT in the leaf sample.

Statistical analysis

The statistical analysis involved an initial analysis of variance (ANOVA) followed by a subsequent post hoc multiple range test (DMRT). The results are presented as means ± standard deviation (SD) for each group of samples. Significance was determined at p-values < 0.05, and the statistical computations were executed using Statgraphics Centurion XIX software (Nascimbeni et al. 2010).

RESULTS

Examination and quantification of phytochemicals in the *M. pachyloba* leaf ethanol extract

The analysis of the phytochemical composition of the MPEE revealed the presence of various compounds: alkaloids, tannins, saponins, polyphenols, terpenoids, and flavonoids, while cardiac glycosides and steroids were notably absent. Particularly noteworthy were the contents of total flavonoids at 72.37 ± 4.64 mg GAE·g^{-1}, total alkaloids at 1.95 ± 0.06%, and total terpenoids at 1.61 ± 0.04 mg·g^{-1}.

The impact of diamondback moth (DBM) on leaf hole formation and leaf damage in *B. juncea*

The analysis of the efficacy of MPEE in controlling the degree of puncture (DGP) and leaf damage rate (LDR) in *B. juncea* plants damaged by DBM are presented in Figure 1. The results demonstrated that DBM inflicted the highest damage on *B. juncea* plants in the control group, with a DGP of 92.3% and an LDR of 88.4%. The lowest damages were found in the Fipronil treatment group (DGP 23.8% and LDR 16.7%). However, a significant damage reduction was observed in the groups treated with MPEE, specifically at MPEE10, where the DGP and LDR correspondingly decreased by 15% and 16% with efficacy comparable to Fipronil.

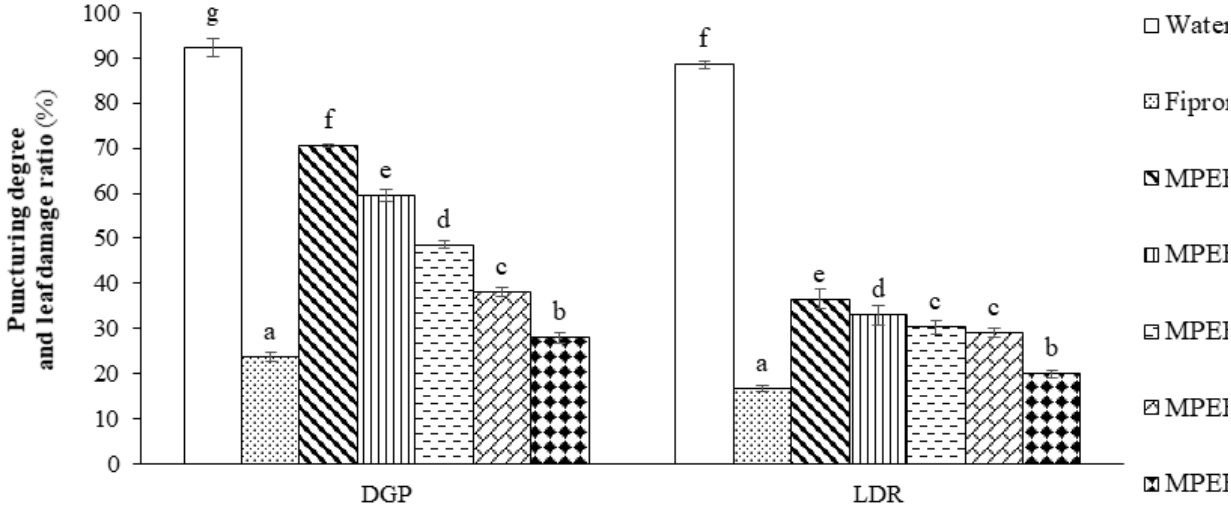

Figure 1. Efficacy of *M. pachyloba* leaf ethanol extract leaves on the degree of puncturing (DGP) and leaf damage ratio (LDR) in *B. juncea* plants induced by the diamondback moth

Results are expressed as mean ± SD; Values marked with the same letter in the same measurement period do not differ significantly at < 0.05, according to the post hoc multiple-range test; MPEE2, 4, 6, 8, and 10 indicate the *M. pachyloba* leaf ethanol extract at concentrations of 2, 4, 6, 8, and 10% (w/v), respectively

Oxidative stress indicators

When mustard plants were subjected to the phenomenon of oxidative stress escalation was conspicuously observed in mustard plants when subjected to the impact of DBM. This occurrence is frequently accompanied by a notable increase in the contents of compounds such as malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) (Sies et al. 2017). The leaves, stems and roots of untreated plants exhibit the highest levels of MDA and H_2O_2. Plant parts treated with fipronil and 10% MPEE reduced significantly by (31–35%) MDA and H_2O_2 contents (Table 2).

Flavonoids and phenolics

The contents of flavonoids and phenolics observed in the leaves, stems, and roots of mustard plants affected by DBM and not treated with MPEE, indicated pronounced oxidative stress (Table 3). However, the treatment with fipronil and MPEE significantly increased the content of above metabolites by 31–33%. Their highest content was in the extracts of plants treated with fipronil and 10% MPEE.

Antioxidant compounds

Upon exposure to oxidative stress induced by DBM, the contents of antioxidant compounds in the leaves, stems, and roots were significantly lower than in in plants treated with Fipronil and MPEE (Table 4). Treatment with Fipronil and 10% MPEE caused an increase of GSH and AsA in leaves, stems and roots compared with untreated control. These increases were about 45% at the highest MPEE concentration, 10%, and were equal to results obtained after Fipronil treatment. Similar results were obtained for the TPH content (Fig. 2). Following treatment with MPEE, the contents of GSH, AsA, and TPH increased significantly.

Antioxidant enzymes

Antioxidant enzymes, SOD, CAT, and APx play a crucial role in the plant's defense mechanism against oxidative stress induced by DBM. Figure 3 illustrates the increase in the content of these enzymes in the leaves of mustard plants protected by fipronil and MPEE. The activity values caused by fipronil and MPEE 10% were equally higher by 40–50% compared with water control.

Table 2. Evaluation of the effectiveness of *M. pachyloba* leaf ethanol extract on malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) contents in leaves, stems, and roots of *B. juncea* plants in alleviating diamondback moth-induced oxidative stress

Treatments	MDA (μmol·g^{-1} FW)			H_2O_2 (μmol·g^{-1} FW)		
	leaves	stems	roots	leaves	stems	roots
Water	11.5 ± 1.1^{d}	10.2 ± 1.2^{e}	9.9 ± 0.3^{e}	11.9 ± 1.1^{d}	10.6 ± 0.6^{f}	10.6 ± 0.6^{e}
Fipronil	7.6 ± 0.3^{a}	6.8 ± 0.4^{a}	6.6 ± 0.3^{a}	7.9 ± 0.2^{a}	7.1 ± 0.3^{a}	6.9 ± 0.3^{a}
MPEE2	9.6 ± 0.3^{c}	8.5 ± 0.3^{d}	8.2 ± 0.2^{d}	8.0 ± 0.4^{c}	8.1 ± 0.2^{e}	9.1 ± 0.2^{d}
MPEE4	9.2 ± 0.2^{bc}	8.2 ± 0.3^{cd}	7.9 ± 0.5^{cd}	9.9 ± 0.4^{bc}	8.8 ± 0.2^{de}	8.7 ± 0.2^{cd}
MPEE6	8.8 ± 0.4^{bc}	7.8 ± 0.2^{bcd}	7.6 ± 0.2^{bc}	9.5 ± 0.4^{bc}	8.5 ± 0.3^{cd}	8.4 ± 0.4^{c}
MPEE8	8.4 ± 0.3^{ab}	7.5 ± 0.3^{abc}	7.3 ± 0.3^{b}	8.7 ± 0.4^{ab}	7.8 ± 0.3^{bc}	7.7 ± 0.3^{b}
MPEE10	7.9 ± 0.3^{a}	7.0 ± 0.3^{ab}	6.7 ± 0.2^{a}	8.2 ± 0.4^{a}	7.3 ± 0.3^{ab}	7.2 ± 0.4^{ab}

Results are expressed as mean ± SD; the letters (a, b, c, d, e, and f) indicate differences between treatments ($p < 0.05$); MPEE2, 4, 6, 8, and 10 indicate *M. pachyloba* leaf ethanol extract at concentrations of 2, 4, 6, 8, and 10% (w/v), respectively

Table 3. Evaluate the impact of the *M. pachyloba* leaf ethanol extract on the levels of flavonoids and phenolics in the leaves, stems, and roots of *B. juncea* plants to alleviate oxidative stress caused by the Diamondback moth

Treatments	Total flavonoid content (mg RE·g^{-1})			Total phenolic content (mg GAE·g^{-1})		
	leaves	stems	roots	leaves	stems	roots
Water	53.4 ± 2.4^{a}	47.7 ± 2.1^{a}	46.5 ± 3.3^{a}	59.2 ± 3.1^{a}	52.9 ± 2.5^{a}	51.5 ± 2.7^{a}
Fipronil	80.1 ± 3.3^{e}	71.5 ± 2.1^{e}	69.7 ± 3.1^{e}	88.8 ± 2.4^{e}	79.3 ± 4.2^{f}	77.2 ± 3.1^{e}
MPEE2	64.2 ± 2.1^{b}	57.2 ± 2.1^{b}	55.8 ± 2.3^{b}	71.1 ± 2.8^{b}	63.4 ± 2.0^{b}	61.8 ± 2.6^{b}
MPEE4	66.8 ± 2.4^{bc}	59.6 ± 2.8^{bc}	60.6 ± 3.2^{bc}	74.1 ± 2.7^{bc}	66.1 ± 3.3^{bc}	64.3 ± 2.2^{bc}
MPEE6	69.7 ± 3.7^{cd}	62.2 ± 2.1^{cd}	63.4 ± 2.2^{cd}	77.2 ± 2.4^{cd}	68.9 ± 3.2^{cd}	67.1 ± 2.5^{cd}
MPEE8	72.8 ± 2.1^{d}	64.9 ± 2.2^{d}	67.7 ± 2.1^{de}	80.7 ± 2.0^{d}	72.1 ± 2.0^{de}	70.2 ± 3.3^{de}
MPEE10	77.8 ± 2.1^{e}	69.4± 2.8^{e}	68.1 ± 3.3^{de}	86.2 ± 1.1^{e}	76.9 ± 2.2^{ef}	74.9 ± 2.5^{ef}

Note: see Table 2

Table 4. Effect of *M. pachyloba* leaf ethanol extract on the contents of glutathione (GSH) and ascorbic acid (AsA) in the leaves, stems, and roots of *B. juncea* plants to alleviate oxidative stress induced by the diamondback moth

Treatments	GSH (μg·g^{-1} FW)			AsA (μg·g^{-1} FW)		
	leaves	stems	roots	leaves	stems	roots
Water	4.7 ± 0.2^{a}	4.6 ± 0.2^{a}	4.4 ± 0.2^{a}	3.9 ± 0.2^{a}	3.8 ± 0.2^{a}	3.8 ± 0.2^{a}
Fipronil	7.0 ± 0.2^{e}	6.9 ± 0.2^{d}	6.7 ± 0.2^{e}	5.9 ± 0.1^{e}	5.7 ± 0.2^{e}	5.7 ± 0.2^{e}
MPEE2	5.6 ± 0.2^{b}	5.5 ± 0.2^{b}	5.3 ± 0.2^{b}	4.7 ± 0.2^{b}	4.6 ± 0.2^{b}	4.6 ± 0.2^{b}
MPEE4	5.8 ± 0.1^{bc}	5.7 ± 0.2^{b}	5.6 ± 0.2^{bc}	4.9 ± 0.2^{bc}	4.8 ± 0.2^{bc}	4.8 ± 0.2^{bc}
MPEE6	6.1 ± 0.21^{cd}	7.0 ± 0.2^{b}	5.8 ± 0.2^{cd}	5.1 ± 0.2^{cd}	5.0 ± 0.2^{cd}	5.0 ± 0.2^{cd}
MPEE8	6.4 ± 0.2^{d}	6.2 ± 0.2^{c}	6.1 ± 0.2^{d}	5.3 ± 0.2^{d}	5.2 ± 0.2^{d}	5.2 ± 0.2^{d}
MPEE10	6.8 ± 0.2^{e}	6.6 ± 0.2^{d}	6.5 ± 0.2^{e}	5.7 ± 0.2^{e}	5.6 ± 0.2^{e}	5.5 ± 0.2^{e}

Note: see Table 2

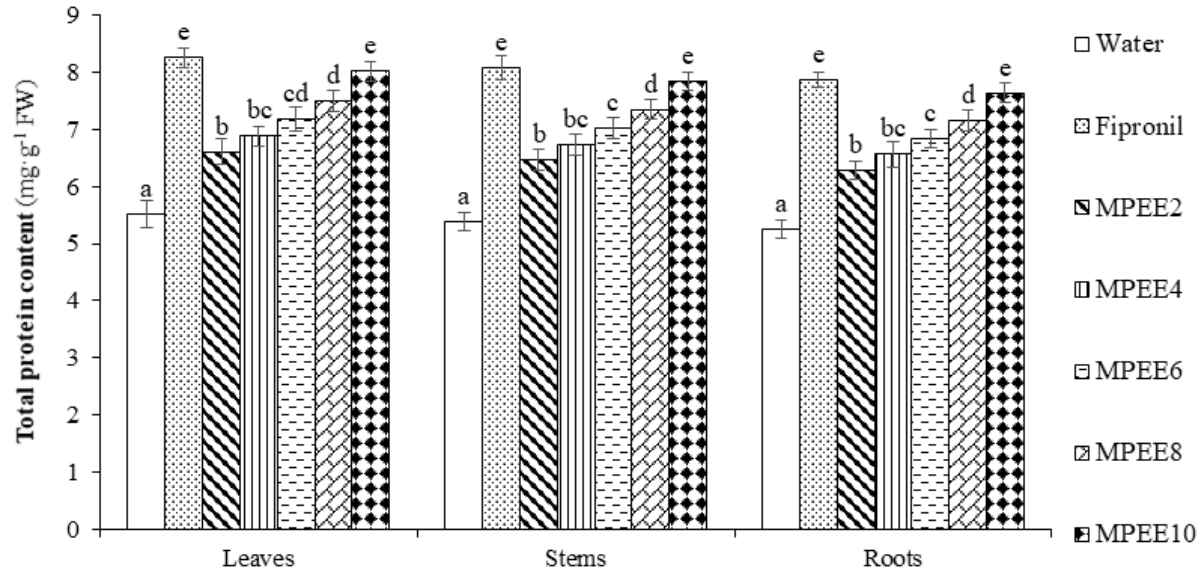

Figure 2. The impact of *M. pachyloba* leaf ethanol extract on the total protein content in the leaves, stems, and roots of stressed *B. juncea* plants caused by the diamondback moth
Note: see Figure 1

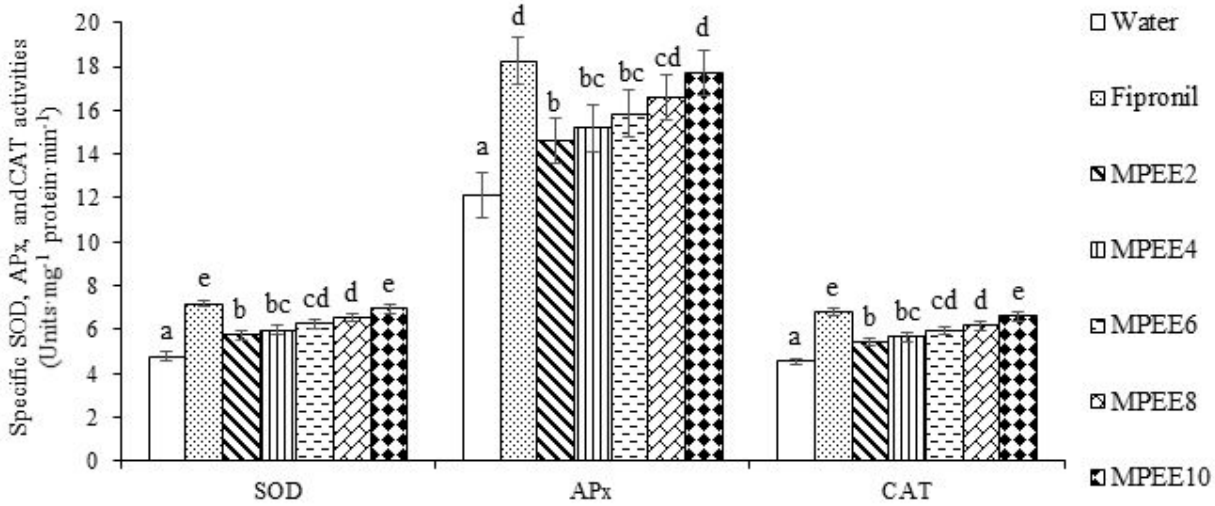

Figure 3. The impact of the *M. pachyloba* leaf ethanol extract on the activities of catalase (CAT), ascorbate peroxidase (APx), and superoxide dismutase (SOD) in stressed *B. juncea* plant leaves, stems, and roots induced by the diamondback moth
Note: see Figure 1

DISCUSSION

DBM and other caterpillars can induce oxidative stress in crops (Kihata & Shikano 2022) by triggering oxidative reactions such as superoxide (O^{2-}), hydrogen peroxide (H_2O_2), and hydroxyl radicals (^OH), leading to cellular and tissue damage in plants (Savchenko & Tikhonov 2021). Plants defend themselves by activating endogenous antioxidant systems, including enzymes like SOD, CAT, and aAPx. These enzymes and antioxidants can also be produced to mitigate oxidative damage (Chauhan et al. 2022). MPEE shows potential in alleviating oxidative stress, as evidenced by various supportive biological and chemical properties. MPEE significantly reduced puncture and leaf damage rates in *B. juncea*, decreased MDA and H_2O_2 contents, reversed DBM-induced oxidative stress, and significantly increased flavonoid, phenolic, GSH, AsA, TPH, SOD, APx, and CAT levels upon treatment. Importantly, MPEE treatment at concentrations of 8% and 10% (w/v) demonstrates efficacy comparable to fipronil.

The diamondback moth is notorious for its ability to create nests on plant leaves and consume them, resulting in leaf area loss, reduced crop quality, and overall yield impact. DBM larvae typically attack plant leaves by feeding on leaf tissue, causing punctures and surface damage (Chi et al. 2024). MPEE, containing various components such as alkaloids, tannins, saponins, polyphenols, terpenoids, and flavonoids, has been demonstrated to effectively eradicate DBM and hinder their developmental process. Mainly, within the MPEE10 treatment group, a significant reduction in DGP and LDR in *B. juncea* plants has been observed, with efficacy equivalent to fipronil. At a concentration of 10% (w/v), MPEE harbors higher contents of bioactive compounds, possessing more potent insecticidal and antioxidative properties. MPEE at 10% (w/v) is considered an optimal dosage to achieve maximum efficacy in combating DBM-induced damage while maintaining a balance between providing sufficient bioactive compounds for insecticidal and antioxidative effects and avoiding potential negative impacts on plant health.

By consuming plant leaves, the diamondback moth induces punctures and holes, thus reducing leaf area and quality. Throughout this process, plant cells experience oxidative stress due to the production of free radicals, leading to cellular damage and reduced antioxidant capacity, resulting in oxidative stress effects (Chauhan et al. 2022). Oxidative stress in DBM-infested plants is often accompanied by elevated levels of MDA and H_2O_2. Increased MDA levels indicate cellular damage (Nhung & Quoc 2024). Antioxidants such as polyphenols, terpenoids, and flavonoids in MPEE can inhibit ROS production, reduce lipid peroxidation, and decrease MDA and H_2O_2 production in *B. juncea* plants. The efficacy of MPEE in reducing oxidative stress and controlling DBM increases with concentration, inhibiting the activity of oxidative agents and reducing ROS production in plants, thereby minimizing cellular damage and oxidative stress. Selecting the appropriate concentration of MPEE is crucial for achieving maximum effectiveness. Our experiment identified a concentration of 10% (w/v) MPEE as the optimal level to achieve results equivalent to fipronil.

Studies have identified that flavonoids and phenolics, two types of natural antioxidants, increase significantly in proportion to the concentration of MPEE. These compounds protect crops from oxidative stress (Kiani et al. 2021). When using MPEE, these compounds are supplied from plant extracts, reinforcing the plant's antioxidant system and minimizing damage from oxidative stress. In our case, treatment with MPEE led to a significant increase in flavonoid and phenolic content corresponding to the increase in extract concentration, helping to balance the chemical environment within the crop and reduce oxidative stress, thereby protecting the crop from DBM infestation. The effectiveness of MPEE depends on the amount of synergistic compounds in the extract. As the concentration of MPEE increases, the levels of bioactive compounds such as polyphenols, terpenoids, and flavonoids also increase, thereby providing a more considerable amount of antioxidants and other compounds capable of inhibiting the development of DBM.

In coping with the oxidative stress induced by DBM, antioxidant compounds such as GSH, AsA, TPH, SOD, CAT, and APx play pivotal roles in the plant's defense mechanism. They help balance antioxidants and free radicals, prevent oxidative stress, and protect cells from ROS-induced damage (Rajput et al. 2021). In the context of DBM-infested mustard plants, MPEE is a crucial source of antioxidant compounds, positively influencing the plant's biochemical mechanisms, aiding in recovery, and sustaining antioxidant levels. MPEE stimulates the plant's endogenous mechanisms, enhancing the synthesis of GSH, AsA, and TPH. The antioxidant components in MPEE provide essential raw materials and stimulate

enzymes and associated metabolic processes, bolstering antioxidant production. Compounds within MPEE have been demonstrated to protect plant cells from oxidative stress-induced damage, contributing to the maintenance of enzyme activity and antioxidant production mechanisms (Qamer et al. 2021). The results of this study indicate that the use of MPEE led to a significant increase in antioxidant compounds, with this increase directly proportional to the concentration of MPEE. Notably, the level of antioxidants in the MPEE-treated group at a concentration of 10% (MPEE10) was nearly equivalent to that observed in the group treated with Fipronil. The efficacy of this extract depends on the amount of active components present. As the concentration of MPEE increases, the levels of bioactive compounds such as polyphenols, terpenoids, and flavonoids also increase, thereby providing a greater quantity of antioxidants and other compounds capable of inhibiting the development of DBM. Therefore, selecting the appropriate concentration of MPEE is crucial for achieving optimal effectiveness in controlling DBM and reducing oxidative stress in crops.

CONCLUSIONS

The practical implications of the research findings on *Millettia pachyloba* ethanol extract are significant. The extract has shown potential in mitigating oxidative stress induced by the diamondback moth on *Brassica juncea* plants, significantly decreasing the contents of malondialdehyde and hydrogen peroxide in plant tissues affected by diamondback moth. It also can reverse the decline in flavonoid and phenolic levels, increase the contents of antioxidants glutathione, ascorbic acid, and proteins in the whole plant of *B. juncea* plants, and stimulate the increase in the activity of antioxidant enzymes in plant leaves. As a result, it markedly reduces leaf perforation and damage. The efficacy of *M. pachyloba* leaf ethanol extract therapy at a concentration of 10% (w/v) is nearly equivalent to that of Fipronil treatment. These findings highlight the promising potential of *M. pachyloba* leaf ethanol extract as effective practical agricultural management approach, offering a valuable strategy for alleviating oxidative stress induced by by diamondback moth and protecting *B. juncea* against diamondback moth. This research provides a practical solution for agricultural researchers, plant biologists, and professionals in agricultural management.

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