Journal of Plant Protection Research eISSN 1899-007X

ORIGINAL ARTICLE

# Phytochemical characterization by HS-SPME-GC-MS and exploration of the antifungal, insecticidal and repellent activity of *Ptychotis verticillata* essential oil

Mohamed Taibi[1, 2]*, Amine Elbouzidi[1, 3], Aimad Allali[4, 5], El Hassania Loukili[2, 3], Mohamed Addi[1], Samiah Hamad Al-Mijalli[6], Reda Bellaouchi[7], Abdeslam Asehraou[7], Ammmar AL-Farga[8]*, Hanae Naceiri Mrabti[9], Mohammed Bourhia[10], Bouchra El Guerrouj[1, 2], Khalid Chaabane[1]

[1] Laboratory for Agricultural Productions Improvement, Biotechnology and Environment (LAPABE), Faculty of Sciences, University of Mohammed Premier, Oujda, Morocco

[2] Center of Oriental Water and Environmental Sciences and Technologies (COSTEE), Mohammed First University, Oujda, Morocco

[3] Euro-Mediterranean University of Fes (UEMF), Fes, Morocco

[4] Laboratory of Plant, Animal, and Agro-Industry Productions, Faculty of Sciences, University of Ibn Tofail, Kenitra, Morocco

[5] High Institute of Nursing Professions and Health Techniques annex Taza, Fez, Morocco

[6] Department of Biology, College of Sciences, Princess Nourah Bint Abdulrahman University, Riyadh, Saudi Arabia

[7] Laboratory of Bioresources, Biotechnology, Ethnopharmacology and Health, Faculty of Sciences, Mo-hammed First University, Boulevard Mohamed VI, Oujda, Morocco

[8] Biochemistry Department College of Science University of Jeddah, Jeddah, Saudi Arabia

[9] Center of Data Science and Sustainable Technologies, INTI International University, Nilai, Malaysia

[10] Department of Chemistry and Biochemistry, Faculty of Medicine and Pharmacy, Ibn Zohr University, Laayoune, Morocco

**Vol. 64, No. 2: 92–105, 2024**

**DOI: 10.24425/jppr.2024.149161**

Received: October 08, 2023
Accepted: December 08, 2023
Online publication: May 15, 2024

*Corresponding address:
amalfarga@uj.edu.sa;
mohamedtaibi9@hotmail.fr

Responsible Editor:
Jolanta Kowalska

## Abstract

The objective of this investigation was to assess the chemical makeup of essential oil derived from *Ptychotis verticillata* (PVEO), and to examine its antifungal, insecticidal, and repellent properties. PVEO was extracted through hydrodistillation, and its volatile constituents were analyzed using Headspace Solid-Phase Microextraction Gas Chromatography-Mass Spectrometry. Qualitative and quantitative evaluation of antifungal activity was carried out using the agar diffusion method and the minimum inhibitory concentration (MIC) test against *Candida glabrata*, *Saccharomyces cerevisiae*, *Aspergillus niger* and *Penicillium digitatum*. We evaluated the repellent potential, as well as the contact and inhalation toxicity of PVEO against *Callosobruchus maculatus*. The results of the study indicated that the essential oil of *P. verticillata* was composed mainly of γ-Terpinen (25.86%), β-Cymene (18.70%) *O*-Cymen-5-ol (16.78) and α-Pinene (12.13%). PVEO showed potent antifungal activity against all strains tested. The results of insecticidal activity of this essential oil were promising in adult *C. maculatus*. At a dose of 20 µl · dm$^{-3}$ of air, EO caused maximum mortality with an $LC_{50}$ value of 5.64 µl · dm$^{-3}$ for the inhalation test and 3.4 µl · dm$^{-3}$ for the contact test. In addition, a significant decrease in the number of eggs laid and adult emergence was observed as EO doses increased, reaching a reduction of around 95% at a dose of 20 µl · dm$^{-3}$ of air. In terms of repellent activity, PVEO also showed encouraging results. It demonstrated an average repellent activity of around 92 ± 10.95%. Furthermore, molecular docking simulations corroborated the *in vitro* results and demonstrated that specific *p*-Menthen-3-one compounds formed more robust hydrogen bonding interactions with the target receptors. These experiments underscore PVEO's effectiveness as a fungicide against the tested fungal strains, demonstrating its role as a bio-insecticide against *C. maculatus* adults, and its potential as an appealing repellent. This suggests that PVEO could serve as a valuable alternative within integrated pest management strategies.

**Keywords:** antifungal activity, essential oil, HSPME-GC-MS, insecticidal activity, *Ptychotis verticillata*

## Introduction

Seed legumes, such as chickpeas, lentils, beans, peas, broad beans and peanuts, play a crucial role in human nutrition and agriculture (Maphosa and Jideani 2017). They are an excellent source of vegetable proteins, fibers, vitamins (such as B, C and E) and minerals (such as iron, zinc, magnesium and potassium) (Maphosa and Jideani 2017). They provide essential nutrients needed for a balanced diet. However, several insect pests adversely affect pulses, such as *Callosobruchus maculatus*, a common pest of peas, beans, lentils and broad beans (Hajam and Kumar 2022). Fungi can also cause crop losses and deterioration in seed quality in the field and storage (Magan *et al.* 2004). Fungal contamination poses significant threats to human health, leading to various adverse consequences. The dangers associated with such contamination include respiratory issues, allergies, infections, and the potential exposure to mycotoxins. Mycotoxins, produced by certain molds, can have severe health implications, ranging from liver damage and immune system suppression to carcinogenic effects (Dinakar *et al.* 2010). Some fungi produce naturally occurring toxic substances called mycotoxins, which may be present in infected legumes. Mycotoxins can cause a variety of health problems, including food poisoning and gastrointestinal disorders, as well as neurotoxic, hepatotoxic, nephrotoxic and carcinogenic effects in the event of prolonged exposure. Exposure to these substances, whether by ingestion or inhalation, has been associated with an increased risk of developing various types of cancer (Adam *et al.* 2017). The severity of mycotoxins depends on the type of mycotoxin, the quantity ingested, and the duration of exposure. Synthetic insecticides and fungicides are commonly used to control insect pests and fungi in agriculture (Roark 1935). These chemicals are designed to eliminate or reduce insect and fungal pathogen populations to protect crops and improve yields.

Medicinal and aromatic plants contain various chemical compounds that give them their biological properties (Bencheikh *et al.* 2022; Taibi *et al.* 2023) They have long been used for their insecticidal and fungicidal properties. Many of the compounds present in these plants can act as control agents against undesirable insects and fungi (Wang *et al.* 2022). Essential oils, the volatile extracts of these aromatic and medicinal plants, contain high concentrations of bioactive chemical compounds. They can have a variety of biological activities. They are known for their natural fungicidal and insecticidal activities and can be used as an alternative to synthetic pesticides. They contain compounds that have insecticidal and repellent effects on various insects (Wang *et al.* 2022). *Ptychotis verticillata*, a plant endemic to eastern Morocco, is known for its chemical composition rich in bioactive molecules. Several studies have shown that the essential oil extracted from this plant is very rich in polyphenols, giving it antifungal and insecticidal activity (Bounouira *et al.* 2022).

In this research, our primary objective was to thoroughly evaluate the antifungal and insecticidal activity of the essential oil extracted from the aerial part of *P. verticillata*. We specifically focused on assessing the effectiveness of this essential oil against *C. maculatus*, a notorious pest known for causing substantial damage to chickpea grains in Morocco. Additionally, our aim was to explore the antifungal properties of PVEO, with a particular emphasis on its ability to combat four fungi strains (*Candida glabrata, Saccharomyces cerevisiae, Aspergillus niger*, and *Penicillium digitatum*) that pose a threat to legume crops.

To achieve comprehensive insights, we embarked on an in-depth phytochemical characterization of the various components present in PVEO. To facilitate this analysis, we utilized the High-Performance Solid-Phase Microextraction Gas Chromatography-Mass Spectrometry (HS-SPME-GC-MS) technique, which is known for its precision and reliability in identifying volatile compounds.

## Materials and Methods

### Plant origin and hydrodistillation

Samples of P. verticillata were obtained from the provincial market in Oujda, situated in eastern Morocco, in the spring of 2022. The taxonomic identification process was conducted at the Faculty of Sciences, University Mohammed the First, Oujda, Morocco. To extract the essential oil from the plant's aboveground parts, we employed the hydrodistillation technique with a customized Clevenger apparatus.

### HS-SPME-GC-MS analysis

The recent solid-phase microextraction (SPME) technique is an alternative method to traditional approaches to volatile sample preparation. It separates and concentrates volatile fractions by directly sampling the headspace (HS) above the dried powdered plant material using SPME fibers. The extracted gaseous compounds were analyzed using a gas chromatograph (Shimadzu GC-2010) equipped with a fused silica capillary column coupled to a spectrometer detector (GC-MS-QP2010). This technique, called HS-SPME coupled with GC-MS, has proven to

be a simple, solvent-free method for analyzing volatile fractions in plant materials. Constituent identification was performed by comparing mass spectrometry (MS) data with the National Institute of Standards and Technology (NIST147) computer library. LabSolutions software (version 2.5) was used for data collection and processing. To enhance the protocol, we employed the recent SPME technique as an alternative method to traditional approaches for volatile sample preparation. This method facilitates the separation and concentration of volatile fractions by directly sampling the headspace (HS) above the dried powdered plant material using SPME fibers. The extracted gaseous compounds were subsequently analyzed utilizing a gas chromatograph (Shimadzu GC-2010) equipped with a fused silica capillary column coupled to a spectrometer detector (GC-MS-QP2010). This technique, known as HS-SPME coupled with GC-MS, has demonstrated itself as a straightforward, solvent-free method for analyzing volatile fractions in plant materials. Identification of constituents was carried out by comparing mass spectrometry (MS) data with the National Institute of Standards and Technology (NIST147) computer library. LabSolutions software (version 2.5) was employed for data collection and processing. Prior to the application of PVEO on filter paper discs, samples were prepared using the HS-SPME coupled with GC-MS technique to analyze the volatile compounds present in the PVEO itself. Experimental conditions for this analysis were kept constant to ensure the reproducibility of the results.

## Antifungal activity

### Disc diffusion method

Four fungal strains *C. glabrata*, *S. cerevisiae*, *A. niger* and *P. digitatum* were tested for antifungal activity using the agar diffusion method on PDA agar culture medium (Taibi *et al.* 2023). Petri plates containing agar were inoculated with the fungal strains. Sterilized filter paper discs 6 mm in diameter were filled with 20 µl of samples dissolved in DMSO (dimethyl sulf oxide) at a concentration of 20 mg · ml$^{-1}$, each separately. Cycloheximide (1 mg · ml$^{-1}$) was used as a positive control, and DMSO as a negative control. Discs containing the samples, standard antibiotic (cycloheximide) and DMSO were deposited on the agar surface using flamed forceps, then lightly pressed to ensure good contact between the discs and the surface. After 30 minutes of pre-diffusion at room temperature, the Petri dishes were incubated at 25°C for 24 hours. The diameters of the inhibited growth zones were then measured in millimeters (mm) (Loukili *et al.* 2023).

### Determination of the minimum inhibitory concentration (MIC)

In this study, we used the 96-well microplate method to determine the minimum inhibitory concentration (MIC) of yeast using a concentration range from 8% to 0.0015% essential oil. Microplates were incubated at 25°C for 48 hours, after which we added 15 microliters of resazurin solution to each well to assess growth (Taibi *et al.* 2023).

## Insecticidal activity

### Toxicity of PVEO by contact test

To evaluate the insecticidal impact of PVEO, 100 grams of seeds were contaminated with five pairs of insects (both male and female) and placed in a 1-liter glass container with a perforated cover. PVEO was directly applied to the seeds using a pipette, followed by manual shaking for 2 minutes. Various treatments with escalating concentrations (1, 5, 10, 20 l · 100 g$^{-1}$) were administered. After 24 hours, mortality rates were measured, and deceased insects were removed. Egg counts were conducted 12 days after the experiment's commencement, with a routine count of emerging insects performed at the end of the 28-day experimental period.

To adjust the recorded mortality rate, the Abbott formula (1) was applied:

$$Pc = 100 \times \frac{(Po - Pt)}{(100 - Pt)}, \quad (1)$$

where: *Pc* – represents the corrected mortality percentage (%), *Po* – corresponds to the mortality observed in the test group, *Pt* – represents the mortality observed in the control group.

Formula 2 was used to determine the percentage decrease in egg and adult numbers in each PVEO concentration compared to the control:

$$\mathrm{RP} = \frac{(NT - NC)}{(NC \times 100)}, \quad (2)$$

where: RP – represents the percentage, *NC* – corresponds to the number of eggs or insects hatched in the control group, *NT* – represents the number of eggs or insects hatched in the treatment group.

Using these evaluation methods, our aim was to determine the efficacy and suitability of PVEO essential oil as an insecticidal agent against *C. maculatus* by inhalation exposure.

### Toxicity of PVEO tested by inhalation

In this study, we set out to evaluate the efficacy of PVEO by inhalation against *C. maculatus*. To carry out the experiment, we used 1-liter glass jars, to which

we attached small cotton masses suspended by threads inside the lids. Using a micropipette, we precisely applied four different doses of PVEO (1, 5, 15 and 20 µl · dm$^{-3}$) to the cotton. We introduced 10 *C. maculatus* insects (composed of males and females) aged 0–48 h into each jar, ensuring that the closure was perfectly airtight. Each dose was repeated three times, and the results were compared with a control sample (cotton without test solution) (Abdelli *et al.* 2016). To calculate the observed mortality rate, we used the following Abbott formula:

$$Pc = 100 \times \frac{(Po - Pt)}{(100 - Pt)},$$

where: *Pc* – represents the percentage of corrected mortality (%), *Po* – corresponds to the mortality observed in the test group, *Pt* – represents the mortality observed in the control group.

### Repellent activity of PVEO

To evaluate the repellent effect of PVEO on *C. maculatus* adults, we used the preferential surface area method on filter paper as described by McDonald *et al.* 1970. We used 9 cm diameter filter paper discs, which we divided in two to obtain two halves with a surface area of 31.80 cm$^2$ each. On one half, we applied 0.5 ml of different concentrations of PVEO, prepared in acetone, giving doses of 0.016, 0.079, 0.157 and 0.315 µl · cm$^{-2}$ per disk. The other half served as a control, receiving only 0.5 ml of acetone. We then added *C. maculatus* adults to the discs and sealed the Petri dishes with parafilm, leaving them to stand for 30 minutes. We then compared the number of bruchids on the PVEO-treated half of the disc with the untreated half. Each experiment was repeated three times, maintaining environmental conditions consistent with those in which the insects were reared. To calculate the percentage of repellency (RP), we applied the following formula:

$$\mathrm{RP} = \frac{(NC - NT)}{(NC + NT)} \times 100,$$

where: RP – represents the percentage of repellency (%), *NC* – corresponds to the number of insects in the control area, *NT* – corresponds to the number of insects in the treated area.

The results of the repellent effects of the essential oil were interpreted in the classification provided by McDonald *et al.* 1970.

### Molecular docking procedure

Following the methodology outlined in (Kandsi *et al.* 2022), we conducted an *in silico* molecular docking study. In summary, we obtained three-dimensional structures of the phytocompounds identified in PVEO from PubChem in "3D sdf" format and converted them into pdb file format using PyMol. To elucidate the potential insecticidal mechanisms of action of the chemicals identified in PVEO, we gathered information about the target proteins from relevant literature sources (Kilic *et al.* 2021). Subsequently, we accessed the Protein Data Bank (PDB) using the PDB IDs of the target proteins to acquire their crystallographic three-dimensional structures, which were then visualized using the Discovery Studio 4.1 program (Dassault Systems Biovia, San Diego, CA, USA) (Cosconati *et al.* 2010). The results for the docked ligand complexes were expressed in kcal · mol$^{-1}$ values for ΔG binding energies. Furthermore, we created 2D molecular interaction diagrams and investigated protein-ligand binding interactions using Discovery Studio 4.1.

### Statistical analysis

The bioassay experiment followed a randomized design, with three replicates for each treatment. Results were presented as means with their standard error (SE). Statistical analyses were performed using SPSS for Windows R software (Version 21.0). To detect significant differences, data were subjected to a one-way ANOVA. Multiple comparisons were performed using Fisher's minimum significant difference test, setting a significance level $\alpha = 0.05$. To determine the lethal concentrations $LC_{50}$ and $LC_{95}$ and their confidence intervals, we used the probit method, following the recommendations of Finney 1971 (Finney 1971). For the inhalation and contact tests, row values with the same letters (a, b, c, d or e) did not differ significantly (means ± SD, $n = 4$, one-way ANOVA; Tukey's test, $p \leq 0.05$).

## Results

### Phytochemical composition

*Ptychotis verticillata* essential oil was analyzed by solid phase chromatography with mass spectrometry (HS-SPME-GC-MS), an analytical technique used to analyze volatile compounds in samples. It enables the identification and quantification of chemical compounds of interest in complex samples such as essential oils. PVEO is comprised of 13 volatile compounds that contribute to its characteristic aroma and flavor (Fig. 1; Table 1). There are five main compounds in this essential oil. γ-Terpinene, the most abundant compound in this essential oil with a concentration of 25.86%, is a monoterpene naturally present in various aromatic

plants. *p*-Cymene with a concentration of 18.70%, is a monoterpene commonly found in essential oils known for its anti-inflammatory and antioxidant properties. β-cymene may confer specific beneficial properties to PVEO. *O*-Cymen-5-ol, with a concentration of 16.78%, is known for its herbicidal activity (Wang *et al.* 2020). α-Pinene represents 12.13% of the chemical composition of this essential oil. It is a monoterpene found in many plants, notably conifers. α-Pinene is often associated with anti-inflammatory and antiseptic effects. β-Myrcene is also present at a concentration of 6.84%, It is a monoterpene commonly found in the essential oils of various plants (Darshani and Pragadheesh 2023).

## Antifungal activity

The antifungal activity of *P. verticillata* essential oil (PVEO) was tested against four fungal strains: *C. glabrata*, *S. cerevisiae*, *A. niger* and *P. digitatum* (Table 2). The results indicate that PVEO had antifungal activity against *C. glabrata*, with a zone of inhibition of 41.3 mm, suggesting an ability to inhibit the growth of this specific strain. Furthermore, the minimum inhibitory concentration of 0.25% indicates the lowest concentration of PVEO required to inhibit *C. glabrata* growth. These results encourage the potential use of this essential oil as an antifungal agent against this strain. The results also show significant activity against *S. cerevisiae*. The inhibition zone of

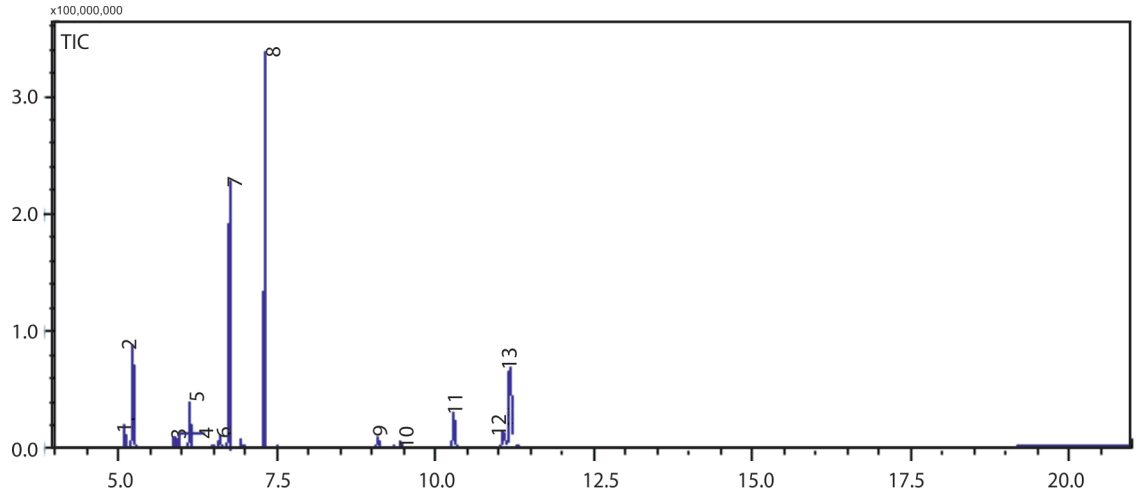


**Fig. 1.** HS-SPME-GC-MS PVEO chromatogram. The peaks in the chromatogram represent the volatile compounds identified and quantified in Table 1

**Table 1.** Phytochemical constituents of *Ptychotis verticillata* (PVEO)

| No. | Compound name | Formula | RI | Mol. Wt. | RT [min] | Peak area [%] |
|---|---|---|---|---|---|---|
| 1 | α-Thujene | $C_{10}H_{16}$ | 926 | 136.23 | 5.092 | 2.43 |
| 2 | α-Pinene | $C_{10}H_{16}$ | 931 | 136.23 | 5.223 | 12.13 |
| 3 | Sabinen | $C_{10}H_{16}$ | 956 | 136.23 | 5.875 | 1.49 |
| 4 | β-Pinene | $C_{10}H_{16}$ | 959 | 136.23 | 5.949 | 2.00 |
| 5 | β-Myrcene | $C_{10}H_{16}$ | 965 | 136.23 | 6.122 | 6.84 |
| 6 | (+)-4-Carene | $C_{10}H_{16}$ | 980 | 136.23 | 6.596 | 2.24 |
| 7 | *p*-Cymene | $C_{10}H_{14}$ | 985 | 134.22 | 6.748 | 18.70 |
| 8 | γ-Terpinene | $C_{10}H_{16}$ | 1002 | 136.23 | 7.306 | 25.86 |
| 9 | D-isomenthone | $C_{10}H_{18}O$ | 1046 | 154.25 | 9.091 | 1.94 |
| 10 | Menthol, trans-1,3,cis-1,4- | $C_{10}H_{20}O$ | 1070 | 156.26 | 9.443 | 1.08 |
| 11 | *p*-Menthen-3-one | $C_{10}H_{16}O$ | 1071 | 152.23 | 10.286 | 4.90 |
| 12 | Carvacrol | $C_{10}H_{14}O$ | 1086 | 150.22 | 11.061 | 3.61 |
| 13 | *o*-Cymen-5-ol | $C_{10}H_{14}O$ | 1088 | 150.22 | 11.173 | 16.78 |

RI – retentions index; RT – retention time

67.5 mm indicates a strong capacity to inhibit the growth of this strain. In addition, the minimum inhibitory concentration of 0.125% suggests a high efficacy of this essential oil at relatively low concentrations. These results indicate the strong potential of this essential oil as an antifungal agent against *S. cerevisiae*. PVEO demonstrates strong antifungal activity against *A. niger*, as evidenced by the substantial 48.1 mm zone of inhibition. Additionally, the low minimum inhibitory concentration of 0.125% suggests that this oil is effective at relatively small doses against *A. niger*. The results suggest that PVEO holds promise as a potential antifungal agent against both the particular strain mentioned and *P. digitatum*. The significant inhibition zone of 46.5 mm and the low minimum inhibitory concentration of 0.125% observed in the analysis of PVEO against *P. digitatum* further reinforce the potential effectiveness of this essential oil as an antifungal treatment for this strain.

## Insecticidal activity

The main goal of this research was to assess the harmful effects of PVEO on *C. maculatus*, an insect pest. The study exposed adult insects of *C. maculatus* to various doses of the essential oil and monitored them at multiple time intervals (24 hours, 48 hours, 72 hours, and 96 hours). Figure 2A illustrates the outcomes, revealing a notable insecticidal impact of PVEO on the treated adults. As the dose and exposure duration to the essential oil increased, so did the mortality rate. Remarkably, after 96 hours of exposure to a 10 µl · dm$^{-3}$ dose of PVEO, the mortality rate reached 77.50%, highlighting the potent insecticidal action of this oil.

Additionally, the study conducted direct contact tests with *C. maculatus* using different doses of PVEO, and the findings are presented in Figure 2B. The mortality of *C. maculatus* adults increased with higher doses of the essential oil and longer contact times. For instance, the lowest concentration (1 µl · 100 g$^{-1}$) caused 60 ± 2.5% mortality after 96 hours of exposure, while the highest concentration (20 µl · 100 g$^{-1}$) resulted in 97.5 ± 2.5% mortality. Statistical analysis revealed slightly higher $LC_{50}$ (lethal concentration for 50% of the population) and $LC_{95}$ (lethal concentration for 95% of the population) values for the inhalation test (5.64 and 17.62 µl · dm$^{-3}$ of air, respectively) than the contact test (3.40 and 14.66 µl · dm$^{-3}$ of air, respectively) (Table 3). This indicates that PVEO demonstrated significant toxicity against *C. maculatus*, regardless of whether it was applied through inhalation or direct contact, and that higher doses and prolonged exposure enhanced its insecticidal effectiveness.

Despite the significant reduction in *C. maculatus* adult mortality, the essential oil did not wholly prevent female oviposition, as illustrated in Figure 3. At

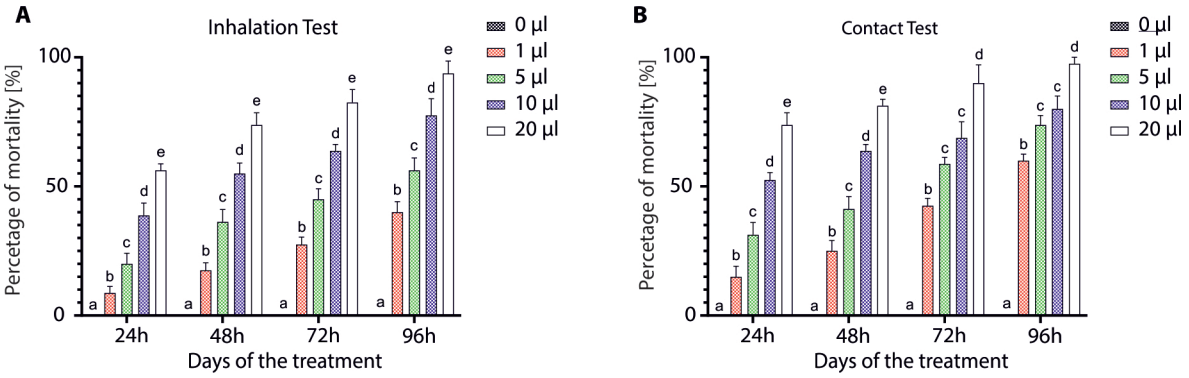


**Fig. 2.** A – percentage of *Callosobruchus maculatus* adult mortality in inhalation; B – contact experiments, presented as mean ± SD mortality values. Row values with the same letters (a, b, c, d or e) did not differ significantly (means ± SD, $n = 3$, one-way ANOVA; Tukey's test, $p \leq 0.05$)

**Table 2.** Evaluation of minimum inhibitory and fungicidal concentrations of *Ptychotis verticillata* (PVEO) against fungal strains

| Fungal strains | PVEO – IZ [mm] | Cycloheximide [1 mg · ml$^{-1}$] IZ [mm] | PVEO – MIC [%] |
|---|---|---|---|
| *Candida glabrata* | 41.3 ± 0.3 | 24 ± 0.5 | 0.25 |
| *Saccharomyces cerevisiae* | 67.5 ± 0.1 | 19 ± 0.23 | 0.125 |
| *Aspergillus niger* | 48.1 ± 0.4 | 20.5 ± 0.3 | 0.125 |
| *Penicillium digitatum* | 46.5 ± 0.1 | 25.5 ± 0.1 | 0.125 |

IZ – inhibition zone; MIC – minimum inhibitory concentration

**Table 3.** $LC_{50}$ and $LC_{95}$ (µl · $dm^{-3}$ of air) responsible for the mortality of adult *C. maculatus* in toxicity tests by contact and inhalation, treated with different doses of *Ptychotis verticillata* (PVEO)

| Test | Exposure duration | df | Slope ± SD | $LC_{50}$ (CI95%) | $LC_{95}$ (CI95%) | Intercept ± SD | *p*-value | $X^2$ |
|---|---|---|---|---|---|---|---|---|
| Inhalation | 24 | 3 | 0.89 ± 0.01 | 16.35 (10.7;39.89) | 34.81 (23.12; 112.07) | −1.46 ± 0.11 | 0.001 | 16.2 |
| | 48 | 3 | 0.1 ± 0.009 | 11.29 (4.46;38.47) | 27.76 (17.48;159.19) | −1.13 ± 0.1 | 0.00 | 27.52 |
| | 72 | 3 | 0.11 ± 0.009 | 8.73 (2.27 ;65.25) | 24.12 (14.47 ;482.72) | −0.93 ± 0.09 | 0.00 | 35.71 |
| | 96 | 3 | 0.14 ± 0.01 | 5.64* | 17.62* | −0.77 ± 0.09 | 0.00 | 49.15 |
| Contact | 24 | 3 | 0.1 ± 0.009 | 11.83 (6.23;27.46) | 27.64 (18.28;90.9) | −1.23 ± 0.1 | 0.00 | 22.39 |
| | 48 | 3 | 0.11 ± 0.009 | 9.19 (0.4;40.15) | 24.47 (14.99;224.52) | −0.99 ± 0.09 | 0.00 | 33.29 |
| | 72 | 3 | 0.11 ± 0.01 | 6.17* | 20.78* | −0.7 ± 0.08 | 0.00 | 53.59 |
| | 96 | 3 | 0.15 + 0.01 | 3.4* | 14.66* | −0.5 ± 0.08 | 0.00 | 66.12 |

$X^2$ – Chi-squared analysis was used to determine the inclination of a line. Slope was determined by probit (p) – constant + Bx; df: degree of freedom; SD – standard deviation; $LC_{50}$ and $LC_{95}$ lethal concentrations (50% and 95% mortality of *C. maculatus* adults) *confidence intervals were too wide; they did not lend themselves to calculation

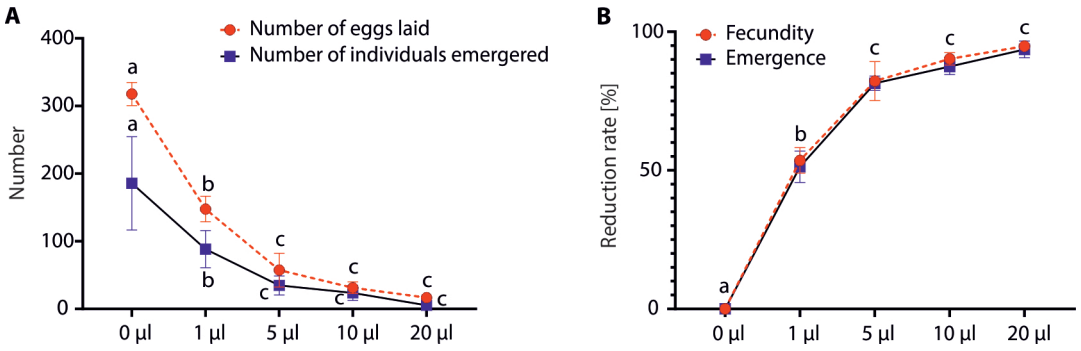


**Fig. 3.** A – fecundity of females (mean values of eggs laid ± SD) and emergence (mean number of individuals emerged ± SD) of *Callosobruchus maculatus* adults after a direct contact toxicity test with different concentrations of PVEO; B – the reduction in fecundity and emergence rates following direct contact with varying doses of PVEO (ranging from 0 to 20 µl). Row values with the same letters (a, b, and c) did not differ significantly (means ± SD, $n = 3$, one-way ANOVA; Tukey's test, $p \leq 0.05$)

a concentration of 10 µl · $dm^{-3}$ PVEO, around 90% of female oviposition was inhibited, and at 20 µl · $dm^{-3}$, almost 95% of oviposition was prevented. The number of eggs laid was inversely proportional to the concentration of essential oil, with the highest concentration resulting in a 94.2% reduction in the number of eggs laid compared to the control group. However, it should be noted that at the highest concentration, a small percentage of eggs still managed to hatch (93.60% reduction in emergence at 20 µl · 100 $g^{-1}$).

## Repellent activity

The findings indicated that the essential oil derived from the plant (PVEO) exhibited a repellent effect at various doses, achieving a maximum repellency rateof 92 ± 10.95% after 120 minutes at a dose of 0.315 µl · $cm^{-2}$. This level of repellency was classified as "Very Repellent (V)" according to (McDonald *et al.* 1970). The study demonstrated the remarkable ability of PVEO to protect legume seeds by significantly reducing the lifespan of adult *C. maculatus* bruchids, even at very low doses. The effectiveness of PVEO was further evidenced by its low $LC_{50}$ value of 3.40 µl · 100 $g^{-1}$ in the contact test, indicating the impact of its bioactive components. The toxicity of PVEO was observed to increase with higher doses, reaching its peak at the highest concentrations used (Table 4.). These results align with the findings of Bounouira *et al.* (2022), who reported repellent activity of this essential oil resulting in repellency rates ranging from 52.5 to 75.5%, depending on the dose. The insecticidal activity of the

**Table 4.** Results of the repellent activity of PVEO against *C. maculatus*. Data are presented as repellency (%) ± SD

| Time [min] | Doses [µl · cm$^{-2}$] | | | |
|---|---|---|---|---|
| | 0.016 | 0.079 | 0.157 | 0.315 |
| 30 | 08 ± 04.47$^{a}$ (I) | 22 ± 08.36$^{bc}$ (II) | 38 ± 04.47$^{de}$ (II) | 80 ± 07.07$^{gh}$ (IV) |
| 60 | 16 ± 05.47$^{ab}$ (I) | 32 ± 08.36$^{cde}$ (II) | 44 ± 05.47$^{ef}$ (III) | 88 ± 04.47$^{h}$ (V) |
| 120 | 26 ± 05.47$^{bcd}$ (II) | 54 ± 08.94$^{f}$ (III) | 68 ± 08.36$^{g}$ (IV) | 92 ± 10.95$^{h}$ (V) |

Each value is a mean ± SD of five repetitions. The means in the same column followed by the same letter(s) are not statistically different ($p > 0.05$) using LSD test. repellency (RP) class: class 0 – RP ≤ 0.1%; class I – 0.1% < RP ≤ 20%; class II – 20% < RP ≤ 40%; class III – 40% < RP ≤ 60%; class IV – 60% < RP ≤ 80%; class V – 80% < RP ≤ 100%

essential oil against *Sitophilus zeamais* also demonstrated significant outcomes based on dose and time (Bounouira *et al.* 2022).

Overall, the presented results clearly establish PVEO as a potent repellent against *C. maculatus*, with the efficacy depending on the dosage (Table 4.). Given its strong insecticidal and repellent activity against various larval stages, PVEO holds promise as an environmentally-friendly natural insecticidal agent for controlling *C. maculatus*.

## Molecular docking

Molecular docking is a widely recognized and extensively employed technique in the field of structure-based drug design (SBDD) (Ferreira *et al.* 2015). Its importance lies in its remarkable ability to predict how small-molecule ligands position themselves within the well-defined binding sites of target proteins with great precision (Ferreira *et al.* 2015). Referred to as molecular docking (MD), this technique has become a fundamental tool in drug discovery, significantly advancing drug development since its inception in the 1980s, coinciding with the development of the initial algorithms supporting it. In this study, we employed molecular docking to investigate how the components found in PVEO may work. We assessed binding affinity values, where a decrease in binding energy typically indicates a stronger compound interaction. These results offer insights into how these molecules interact with a specific target compared to a known inhibitor. To conduct the molecular docking analysis, we followed the procedures outlined in References (Elbouzidi *et al.* 2022; Taibi *et al.* 2023). We used co-crystalized 3D structures of three proteins: acetylcholinesterase (PDB ID: 4EY7), gamma-aminobutyric acid receptor, specifically the GABA(A)R-beta3 homopentamer (PDB ID: 4COF), and ryanodine receptor (PDB ID: 5C30). These protein structures were obtained from the Protein Data Bank (PDB) database.

We represented the docking scores in a table displayed as a heatmap, with a color gradient ranging from red (indicating the lowest energy values, often matching the docking score of the native ligand) to green (representing the highest energy values) (Table 5). Compounds with docking scores equal to or lower than the score of the native ligand were denoted with an asterisk (*).

**Table 5.** Heat-map of the free binding values (binding affinity values are expressed in kcal · mol$^{-1}$) of *Ptychotis verticillata* (PVEO) components

| No | Compounds | Protein IDs [retrieved from Protein Data Bank] | | |
|---|---|---|---|---|
| | | 4EY7 | 4COF | 5C30 |
| | | free binding energy [kcal · mol$^{-1}$] | | |
| - | Native ligand | Galanthamine –7.6 | Securinine –7 | Chlorantraniliprole –8 |
| 1 | α-Thujene | –5.9 | –5.1 | –6.1 |
| 2 | α-Pinene | –5.5 | –5.2 | –5.9 |
| 3 | Sabinene | –6 | –5 | –6.6 |
| 4 | β-Pinene | –5.5 | –5.2 | –6.2 |
| 5 | β-Myrcene | –5.5 | –5 | –5.5 |
| 6 | (+)-4-Carene | –5.9 | –5.1 | –6.6 |
| 7 | *p*-Cymene | –5.8 | –5.1 | –7 |
| 8 | γ-Terpinene | –5.7 | –5 | –6.9 |
| 9 | D-isomenthone | –6 | –5.2 | –6.4 |
| 10 | Menthol, *trans*-1,3, *cis*-1,4- | –5.7 | –5.3 | –6.4 |
| 11 | ***p*-Menthen-3-one** | **–8 *** | **–7.6 *** | **–8 *** |
| 12 | Carvacrol | –6.2 | –5.9 | –7.2 |
| 13 | *o*-Cymen-5-ol | –6 | –5.4 | –6.4 |

4EY7 – acetylcholinesterase protein; 4COF – gamma-aminobutyric acid receptor, the GABA(A)R-beta3 homopentamer; 5C30 – ryanodine receptor

The majority of insecticides function as neurotoxicants, inducing a range of hyperexcitation and paralysis effects in animals. These neurotoxic insecticides predominantly target various neuroreceptors and ion channels. The mechanism of action for many synthetic chemical pesticides, such as organophosphates (OPs), abamectin, and carbamates, primarily revolves around the inhibition of enzymatic processes (Lalah *et al.* 2022). Among the pivotal enzymes targeted in

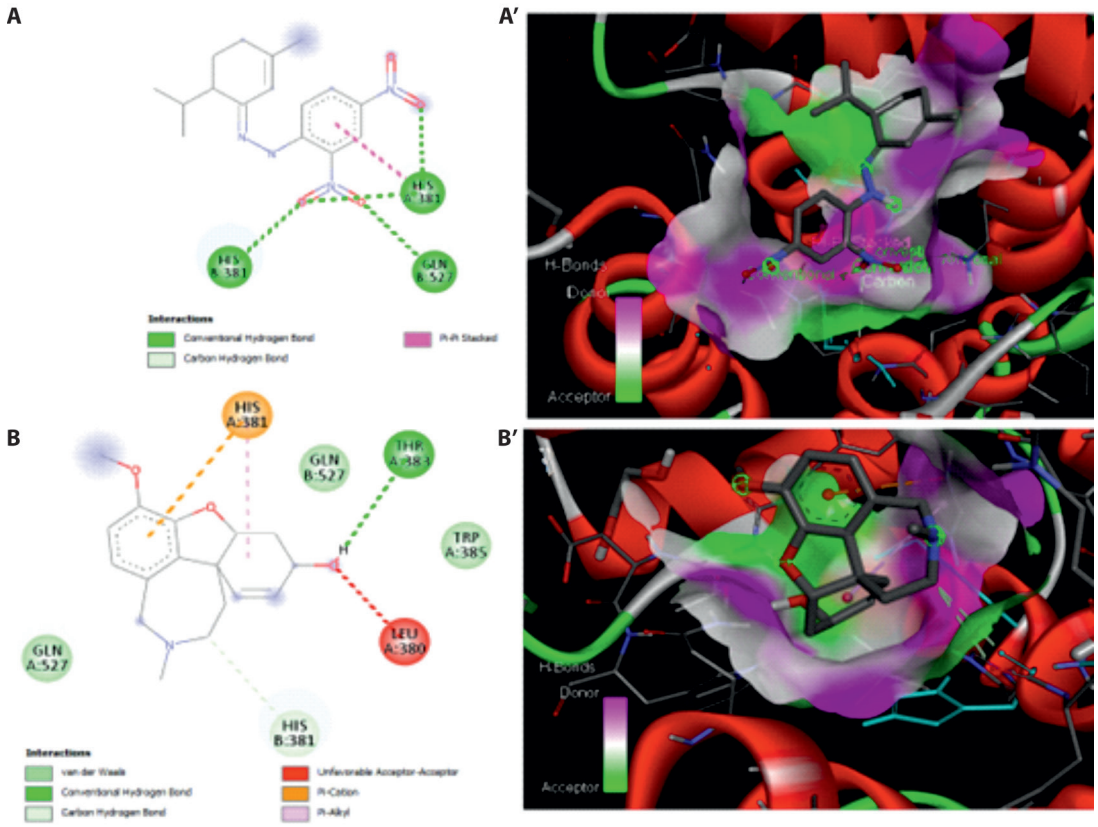


**Fig. 4.** A, A' – two-dimensional and three-dimensional schemes of the interactions of *p*-Menthen-3-one; B, B' – the native ligand (galanthamine) with the target protein 4EY7

this context is acetylcholinesterase (AChE). The inhibition of AChE at cholinergic synapses within the nervous systems of arthropods leads to a reduction in their locomotor activity and the suppression of their reproductive cycles. In the scope of our investigation, a singular ligand demonstrated significant inhibitory potential against AChE, namely *p*-Menthen-3-one, with a notable docking score of –8 kcal · mol$^{-1}$. This score is notably superior to that of the native ligand of the AChE protein, galanthamin, which registered a score of –7.6 kcal · mol$^{-1}$. Notably, *p*-Menthen-3-one established three hydrogen bonds with specific amino acid residues within the active site pocket of AChE, specifically with His A:381, His B:381, and Gln B:527. In contrast, galanthamin formed just one hydrogen bond, linking with Thr A:383 within the active site pocket (Fig. 4.).

Alternatively, another mode of action involves the binding of insecticides to the ionotropic γ-aminonobutyric acid (GABA) primary receptor, thereby inhibiting its interaction with glutamate-dependent chloride channels (GluCls), effectively disrupting synaptic transmission within the arthropod's nervous system. Within the scope of our investigation, it was observed that *p*-Menthen-3-one exhibited strong binding affinity exclusively with the GABA receptor. This interaction suggests its potential to disrupt the functions of this protein, leading to the blockage of synaptic transmission in the nervous system of insects. Notably, *p*-Menthen-3-one established an alkyl bond with the amino acid residue Met E:49 and a carbon-hydrogen bond with Lys A:102 within the active site pocket. This interaction yielded a significant docking score of –7.6 kcal · mol$^{-1}$, as depicted in Figure 5. In contrast, the native ligand, securinine, a well-known GABA receptor antagonist, yielded a docking score of –7 kcal · mol$^{-1}$, as detailed in Table 5.

The Ryanodine receptor (RyR) is a calcium-release channel predominantly situated within the endoplasmic reticulum of cellular structures. Its principal role encompasses the maintenance of calcium homeostasis and the facilitation of muscle contraction, a fundamental process observed in various organisms, including insects. Specifically in insects, the RyR assumes a pivotal role as an indispensable constituent of the excitation-contraction coupling cascade. Herein, it bears the responsibility of liberating calcium ions from the ER into the cytoplasm, thereby instigating the muscular contractile process (Kilic *et al.* 2021). RyR inhibition constitutes a prominent strategy in the endeavor to formulate novel pesticides. The modulation or inhibition of RyR functionality possesses the potential to disrupt the customary operations of insect musculature, resulting in muscular paralysis – an advantageous

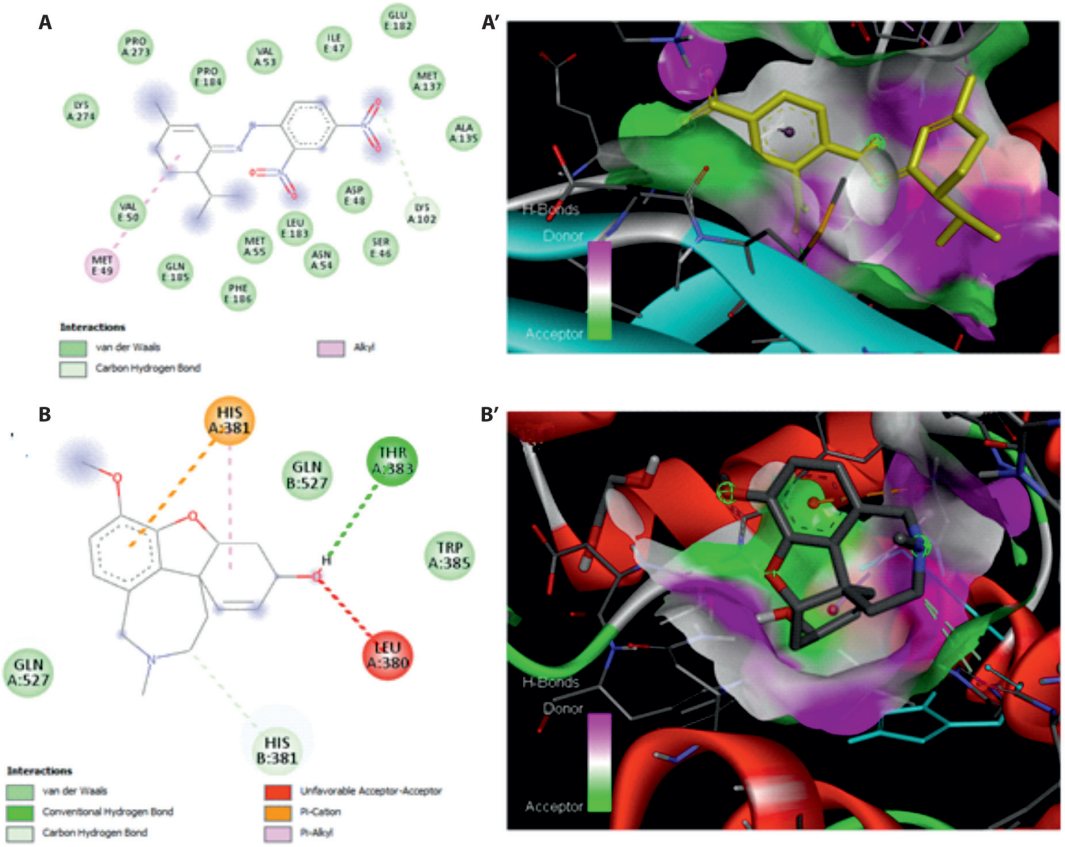


**Fig. 5.** A, A' – two-dimensional and three-dimensional schemes of the interactions of *p*-Menthen-3-one; B, B' – the potent ligand (securinine) with the target protein 4COF

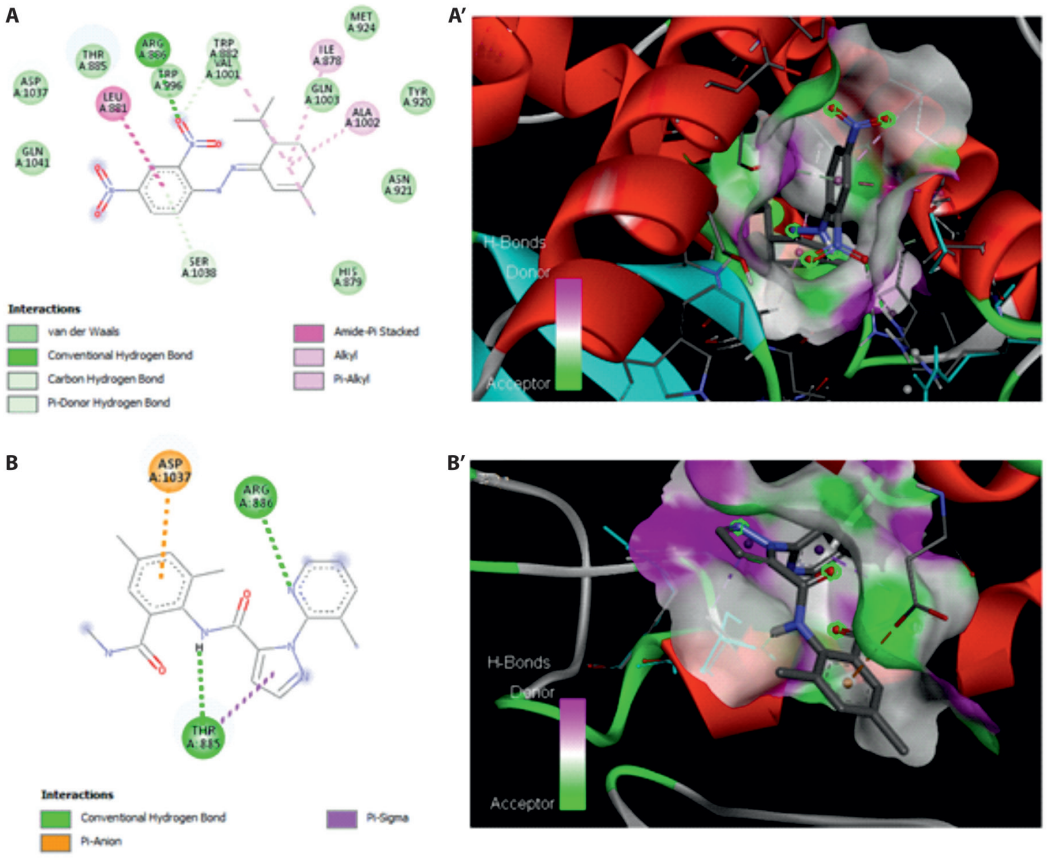


**Fig. 6.** A, A' – two-dimensional and three-dimensional schemes of the interactions of *p*-Menthen-3-one; B, B' – the potent ligand (chlorantraniliprole) with the target protein 5C30

outcome in the context of insect pest management. Moreover, it is noteworthy that insects can develop resistance to conventional insecticides with diverse modes of action over time. By targeting the RyR, we introduced an alternative mechanistic approach that may serve to decelerate the emergence of resistance within pest populations (Sun and Xu 2019). In our study, we identified *p*-Menthen-3-one as a strong inhibitor of the RyR, forming a hydrogen bond with Arg A:886. Similarly, chloroantraniliprole, categorized as a ryanodine receptor modulator, engaged in two hydrogen bonds (Fig. 6). It is noteworthy that both *p*-Menthen-3-one and chloroantraniliprole demonstrated identical ree binding energies (–8 kcal · mol$^{-1}$, as depicted in Table 5). This suggests that both of these compounds may yield the same beneficial effect within this specific context.

## Discussion

Analysis of *P. verticillata* essential oil revealed a compound of 13 volatile compounds that contribute to its distinctive aroma, the main ones being **γ**-terpinene (25.86%), *p*-cymene (18.70%), *O*-cymen-5-ol (16.78%), α-pinene (12.13%), and β-myrcene (6.84%). These terpenic compounds have various properties, including anti-inflammatory, antioxidant, and herbicidal. The presence of β-cymene may confer specific beneficial properties to PVEO. *O*-Cymen-5-ol, with a concentration of 16.78%. **α**-Pinene represents 12.13% of the chemical composition of this essential oil. It is a monoterpene found in many plants, notably conifers. α-Pinene is often associated with anti-inflammatory and antiseptic effects (Shafaroodi *et al.* 2021), and β-Myrcene, present at a concentration of 6.84%, is a monoterpene commonly found in the essential oils of various plants (Darshani and Pragadheesh 2023). These results differ from those reported by (Taibi *et al.* 2023). These researchers characterized the chemical composition of the volatile part of PVEO using the GC-MS technique. They identified a total of 24 components, the three main ones being *D*-limonene (22.10%), **γ**-terpinene (9.78%), and β-cymene (9.35%). These bioactive molecules may contribute to several biological activities of this essential oil.

The results of PVEO's antifungal activity evaluation showed strong antifungal activity against *C. glabrata*, with an inhibition zone of 41.3 mm and a minimum inhibitory concentration of 0.25%. It also showed significant activity against *S. cerevisiae*, with an inhibition zone of 67.5 mm and a minimum inhibitory concentration of 0.125%. In addition, PVEO demonstrated strong antifungal activity against *A. niger*, with an inhibition zone of 48.1 mm and a low minimum inhibitory concentration of 0.125%. These results suggest the strong potential of PVEO as an antifungal agent against these strains. In addition, its efficacy against *P. digitatum*, with an inhibition zone of 46.5 mm and a minimum inhibitory concentration of 0.125%, reinforces its potential as an antifungal treatment. The presence of high levels of β-cymene in this oil may be a significant factor contributing to its antifungal activity. Previous studies have demonstrated that β-cymene, a monoterpene, possesses antifungal properties against various fungal strains (Loukili *et al.* 2023). Additionally, the main compound, *O*-Cymen-5-ol (also known as 4-Isopropyl-3-methylphenol), which is present in the oil, exhibits inhibitory activity against several fungal strains, including *A. niger* (Kim *et al.* 2016). The robust antifungal activity of this oil may be attributed to various factors, including the synergistic action of its chemical compounds against fungal strains. This suggests that the combined effect of the individual molecules present in the oil can result in a more potent antifungal effect than their separate action.

Regarding the insecticidal activity, adult specimens of *C. maculatus* were subjected to various concentrations of the oil, revealing a noteworthy escalation in mortality rates with higher doses and prolonged exposure, culminating in a mortality rate of 77.50% at 10 µl · l$^{-1}$ after 96 hours. Direct contact tests further corroborated the heightened mortality associated with increased doses. Statistical scrutiny unveiled slightly elevated $LC_{50}$ and $LC_{95}$ values for the inhalation test compared to the contact test, signifying substantial oil toxicity independent of the application mode. Notwithstanding the substantial reduction in mortality, the oil failed to completely impede oviposition in females, demonstrating a 90% inhibition at 10 µl · dm$^{-3}$ and 95% at 20 µl · dm$^{-3}$, accompanied by a proportionate decline in the number of laid eggs. Nevertheless, at the higher concentration, a modest percentage of eggs successfully hatched, underscoring the intricate impact of the oil on the reproductive cycle of *C. maculatus*. The results of this study corroborate previous research suggesting that essential oils containing bioactive molecules similar to those present in PVEO essential oil exhibit potent insecticidal activity. As a result, this essential oil appears promising as a natural insecticide.

The plant's essential oil (PVEO) showed a powerful repellent effect, reaching a maximum rate of 92 ± 10.95% after 120 minutes at a dose of 0.315 µl · cm$^{-2}$. Classified as "Very repellent (V)" according to McDonald *et al.* (1970), it demonstrated a remarkable ability to protect legume seeds by reducing the life span of adult *C. maculatus* bruchids, even at low doses. PVEO confirmed its efficacy with a low LC50 of 3.40 µl · 100 g$^{-1}$ in the contact test, showing the impact of its bioactive

components. PVEO toxicity increased with higher doses, aligning the results with those of Bounouira *et al.* (2022). Overall, PVEO emerges as a powerful and environmentally friendly repellent for the control of *C. maculatus*, thanks to its marked insecticidal activity against different larval stages. Several studies have highlighted the beneficial effects of essential oils in reducing the longevity of stored grain pests, notably *C. maculatus*. These essential oils, rich in volatile compounds, notably monoterpenes, act as insecticides by disrupting the growth of insects at different life stages (Aimad *et al.* 2022). Studies have shown that certain terpene compounds, particularly oxidized monoterpenes such as carvacrol, are widely associated with their efficacy in acute toxicity in insects, probably due to their lipophilic properties that promote better penetration of the insect cuticle (Ikbal and Pavela 2019). Numerous studies have also highlighted the neurotoxic actions of monoterpene-rich essential oils in insects. Mixtures of monoterpenes act as neurotoxic agents targeting different parts of the insect nervous system, causing paralysis followed by death. Another study showed that **γ**-terpinene, a major terpene compound of PVEO, showed potential insecticidal properties against various insect pests, such as *S. littoralis* (Abbassy *et al.* 2009). These results suggest that the insecticidal and repellent activity of the essential oils studied may be due to their monoterpene-rich chemical composition and the predominant presence of γ-terpinene. Therefore, the phytochemical profiles of essential oils play a crucial role in their efficacy as insecticides and their potential for pest control in stored cereal crops. This indicates that PVEO is of great interest as a natural insecticide to control stored grain pests and is often considered a safer alternative to synthetic chemical insecticides for the environment and human health.

## Conclusions

Using natural aromatic products derived from medicinal plants represents a promising strategy for controlling fungi and insects, offering several advantages over current synthetic products. In this study, we carried out an in-depth phytochemical characterization of PVEO essential oil and examined its antifungal, insecticidal and repellent activities. The abundance of γ-terpinene and monoterpenes in this essential oil was identified as the primary contributor to its biological activities. The results suggest that this plant could serve as a promising source of natural agents, offering multiple beneficial applications in health, agriculture and food. Molecular docking corroborated the in vitro results and demonstrated that specifically p-Menthen-3-one is the compound responsible of the observed insecticidal effects. These experiments underscored PVEO's effectiveness as a fungicide against the tested fungal strains, its role as a bio-insecticide against *C. maculatus* adults, and its potential as an appealing repellent. This suggests that PVEO could serve as a valuable alternative within integrated pest management strategies. However, despite these advantages, it is essential to note that using essential oils as natural insecticides requires ongoing research to optimize their efficacy, determine appropriate doses, assess their persistence in the environment and understand their overall impact on insect populations and the ecosystem. Further investigations are imperative to comprehensively evaluate the efficacy of essential oils (EOs), particularly PVEO, against an extensive spectrum of insect pests. While the current study highlights promising results in terms of antifungal and insecticidal properties, a more expansive exploration across various pest species would provide valuable insights into the broad-spectrum applicability of these bio-fungicidal agents. In addition to the assessment of insect pests, future studies should focus on isolating and characterizing the active constituents within these EOs. The identification of specific compounds responsible for the observed antifungal and insecticidal effects is crucial for the development of targeted biopesticides.

**Table of abbreviations**

| Acronyme | |
|---|---|
| PVEO | *Ptychotis verticillata* essential oil |
| EO | Essential oil |
| HS-SPME-GC-MS | High-Performance Solid-Phase Microextraction Gas Chromatography-Mass Spectrometry |
| SPME | Solid-phase microextraction |
| HS | Headspace |
| MS | Mass spectrometry |
| GC | Gas chromatography |
| NIST147 | National Institute of Standards and Technology |
| PDA | Potato dextrose agar |
| DMSO | Dimethyl sulfoxide |
| MIC | Minimum inhibitory concentration |
| RP | Repellency percentage |
| PDB | Protein data bank |
| SE | Standard error |
| $LC_{50}$ | Lethal concentration 50 |
| $LC_{95}$ | Lethal concentration 95 |
| MD | Molecular docking |
| AChE | Acetylcholinesterase |
| RyR | Ryanodine receptor |

## Acknowledgements

The authors express their gratitude to the Moroccan Ministry of Higher Education, Scientific Research and Innovation, and the European Union for supporting this work under the PRIMA Programme (OLI4FOOD, ID No 1845), as well as for the support provided by the researcher support project (PNURSP2024R158) of the Princess Nourah Bint Abdulrahman University in Riyadh, Saudi Arabia.

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