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# Virtual screening and *in vitro* activity of potential insecticidal agents from natural products

Angie Daniela Torres-Garcia [a], Felipe Victoria-Muñoz [b], Erika Plazas [b], Norberto Sanchez-Cruz [c], Cesar A. Sierra [a,*]

[a] Macromolecules Group, Departamento de Química, Universidad Nacional de Colombia, Sede Bogotá, Bogotá, Colombia
[b] FarmaBioTech Group, Natural and Exact Science Faculty, Salesiana University, Bogotá DC, 111071, Colombia
[c] Computational Chemogenomics Group, Department of Physical Chemistry, Merida Research Unit, Institute of Chemistry, National Autonomous University of Mexico (UNAM), Yucatán, 97302, Mexico

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ABSTRACT

Insecticides research must explore alternative pathways to identify small compounds that satisfy the needs of farmers in both developing and developed countries. Reducing the cost of these inputs is essential to increasing agricultural production and mitigating food insecurity. In this study, a multi-stage computational screening of 407615 natural products were conducted, utilizing filters for volatility (MW $< 350Da$), Protein-Ligand Interaction Fingerprint (PLIF) similarity to a tacrine reference, and a commercial cost threshold of less than $8USD/g$. Out of 16 finalists evaluated *in vitro*, two compounds demonstrated significant inhibitory activity against Acetylcholinesterase from *Electrophorus electricus* (52% and 77% at $250\mu M$, respectively) and *Drosophila melanogaster*. Kinetic analysis of one novel compound, the most potent lead ($K_i = 88.1 \pm 3.1\mu M$), revealed a mixed-type inhibition mechanism. These results validate the identification of affordable, reversible, and volatile natural scaffolds that serve as promising optimizable fragments for the development of sustainable biopesticides.

## 1. Introduction

The search for novel insecticides is a global priority due to the growing limitations of conventional synthetic pesticides. Despite their initial efficacy, many of these compounds exhibit low selectivity toward target organisms, leading to severe environmental toxicity and risks to human health (Bub et al., 2023; Shekhar et al., 2024). This necessity is further exacerbated by the bioaccumulation of these substances within trophic chains and their persistence in ecosystems (Elhamalawy et al., 2024; Gupta and Gupta, 2020). In the context of developing countries, the situation is even more critical, as small-scale farmers with limited access to safer, state of the art technologies often resorts to highly toxic, and obsolete chemicals due to their low acquisition costs (Lee et al., 2025; Rufo et al., 2024).

Within this framework, volatile natural products (VNPs) emerge as a promising alternative. As proposed by (Victoria-Muñoz et al., 2025), the high structural complexity of these compounds may favor greater selectivity, thereby minimizing adverse effects on non-target organisms. Although some volatiles may exhibit acute toxicity, their natural role as semiochemicals, acting as pheromones or kairomones in communication within and between species, suggests an evolved biological potency to interact with specific receptors (Gurr et al., 2023; Pickett and Khan, 2016; Chamberlain et al., 2000). Furthermore, their volatile nature facilitates large-scale application in the agro-industry and reduces residue persistence on crops (Ghirardo and Brilli, 2024; Brilli et al., 2019). Nevertheless, challenges associated with rapid evaporation can be mitigated by developing controlled release formulations or by encapsulation, thereby ensuring their persistence in the field (Chen et al., 2020; Listyaningrum et al., 2026; Zaitoon et al., 2022).

One of the most effective molecular targets for insect control is the enzyme acetylcholinesterase (AChE). Currently, the predominant classes of insecticides acting on this target are organophosphates and carbamates (Sparks et al., 2020; Pang, 2014; Fournier et al., 1992). However, their mechanism of action typically involves binding to the enzyme's catalytic site, which is highly conserved across species, thereby explaining their cross-toxicity in humans and pollinators. Accordingly,

* Correspondence to: Macromolecules Group, Departamento de Química, Universidad Nacional de Colombia, Sede Bogotá, Carrera 30 #45-03, Bogotá 111321, Colombia.
*E-mail addresses:* angtorresga@unal.edu.co (A.D. Torres-Garcia), daniel.victoria@salesiana.edu.co (F. Victoria-Muñoz), erika.plazas@salesiana.edu.co (E. Plazas), norberto.sanchez@iquimica.unam.mx (N. Sanchez-Cruz), casierraa@unal.edu.co (C.A. Sierra).

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recent studies have suggested that this limitation can be overcome by searching for inhibitors that bind to allosteric sites in this target (Nachon et al., 2020). Specifically, it has been demonstrated that a single amino acid variation between *Drosophila melanogaster* and human AChE creates a unique cavity that enables the design of highly insect-selective inhibitors (Nachon et al., 2020; Han et al., 2018).

To optimize the discovery of insect selective AChE inhibitors and reduce research costs, computational tools have assumed a leading role (Sakai and Kaneko, 2025; İnak et al., 2025; Melo and Andrade, 2024). Despite these advances, a gap remains in the scientific literature, as few studies simultaneously integrate criteria of low commercial cost and volatility in the massive screening of hundreds of thousands of molecules. Virtual screening, supported by molecular docking and molecular dynamics, enables the large scale evaluation of the affinities of thousands of ligands before *in vitro* or *in vivo* assays (Hou et al., 2023; Shilla et al., 2026). This approach not only predicts a molecule's binding capacity to a specific target but also allows filtering structures based on their physicochemical properties (Djoumbou-Feunang et al., 2023; İnak et al., 2025).

Under this premise, the present article aims to identify potential insecticides (AChE inhibitors) from a database of natural volatile products, prioritizing those with high commercial availability and low cost. In contrast to traditional covalent and irreversible inhibitors, this research focuses on the search for reversible inhibitors targeting the selective cavity of *D. melanogaster* described by Nachon et al. (2020). To this end, a virtual screening methodology was employed, using tacrine as a comparative standard due to its strategic and specific binding within the insect-selective pocket, which is required for developing insect selective inhibitors, as well as its status as a widely validated reversible inhibitor for studying the architecture of the AChE cavity. Finally, molecules selected using computational filters were validated *in vitro* against the target enzyme and enzymatic extracts from *D. melanogaster* heads, thereby integrating theoretical predictions with experimental evidence.

## 2. Materials and methods

### 2.1. Molecule and target preparation

The COCONUT (Collection of Open Natural Products) database, containing 407615 natural products (Sorokina and Steinbeck, 2019; Chandrasekhar et al. 2025), were utilized (data retrieved in June 2022). For SMILES (Simplified Molecular Input Line Entry System) standardization, the largest fragment of each structure was selected, inorganic molecules were removed, charges were neutralized, and polar groups were protonated according to the predominant ionization state (Bento et al. 2020; Swain, 2019). After removing redundant structures, adding missing hydrogen atoms, and minimizing the structures under the MMFF94 force field. Each molecule with the minimum conformation was finally stored in SDF (Structure Data File) format.

To identify compounds with potential volatility, a molecular weight filter was applied based on the criteria reported by Victoria-Muñoz et al. (2025), using a cutoff value of 350*Da*. Molecules exceeding this threshold were discarded. The crystallographic structure of *D. melanogaster* acetylcholinesterase (*Dm*AChE) was used as the biological target, obtained from the Protein Data Bank (PDB ID: 6XYY) (Nachon et al. 2020). During receptor preparation, cocrystallized ligands, redundant chains, water molecules, and ions were removed. Subsequently, residue protonation states were assigned using PDB2PQR at a simulated physiological pH of 7.4, which accurately defines electrostatic interactions in the active site. These processes were executed using the Python libraries RDKit (v. 2023.3.1) and DeepChem (v. 2.8.0) (Landrum, 2016; Ramsundar et al. 2019).

### 2.2. Molecular docking and PLIF comparison

The docking protocol was validated by redocking the native ligand, using AutoDock Vina (v. 1.2.5). A $10 \times 10 \times 10$ Å grid box was centered on the original ligand's binding site (coordinates: 32.91, 67.71, 9.63). The predicted pose showed high conformational similarity to the experimental crystal structure, achieving an RMSD of 1.12 Å. An exhaustiveness of 16 was configured, and 5 poses per molecule were generated (Trott and Olson, 2010). Results were converted from PDBQT to SDF format to facilitate the generation of Protein-Ligand Interaction Fingerprints (PLIF) using the ProLIF tool (v. 2.0.0) (Bouysset and Fiorucci, 2021). To select the most promising candidates, the cosine similarity was calculated between the Protein-Ligand Interaction Fingerprint (PLIF) of the reference complex (9-N-phenylmethylamino-tacrine) and the average fingerprint derived from the interaction patterns of all generated poses for each ligand. A 75% similarity threshold was established for candidate selection. These calculations were implemented using the Scikit learn library (v. 1.4.1) (Pedregosa et al., 2011).

### 2.3. Accessibility and economic viability filters

Compounds selected in previous phases with interaction patterns similar to those of tacrine were filtered based on commercial availability. An exact SMILES search was performed in the MolPort catalog, selecting only those compounds with a price below 8 USD/g. This criterion ensures that the selected candidates are economically viable for eventual agro-industrial implementation. Fig. 1 schematically summarizes the computational workflow and the reduction of the chemical library after each filter.

### 2.4. General in vitro experimental procedures

Two sources of acetylcholinesterase (AChE) were used for the inhibition assays: commercial *Electrophorus electricus* (*Ee*AChE, E.C. 3.1.1.7, Type VI-S) and a crude extract from *D. melanogaster* pupae (*Dm*AChE). To obtain *Dm*AChE, pupae were homogenized in 0.1 M sodium phosphate buffer (pH 8.0) and centrifuged at 10,000 × g for 5 min at 4 °C. Total protein content in the supernatant was determined by the Bradford method using BSA as a standard, yielding a specific activity of $5.13 \pm 1.22\ U{\cdot}mg^{-1}$ of total protein. The substrate used was acetylthiocholine iodide (ATCI), along with Ellman's reagent (5,5′-dithiobis-2-nitrobenzoic acid, DTNB), which were obtained from Alfa Aesar (USA). Sodium dihydrogen orthophosphate ($NaH_2PO_4{\cdot}2\ H_2O$) and disodium hydrogen phosphate ($Na_2HPO_4{\cdot}2\ H_2O$), as well as all other reagents and solvents, were of analytical grade and were obtained from Merck (Darmstadt, Germany). Absorbance readings were performed on a Varioskan LUX Multimode Microplate Reader.

### 2.5. In vitro enzymatic inhibition screening and mechanistic characterization

Inhibitory activity was assessed using an adaptation of Ellman's colorimetric method by Plazas et al. (2019). Assays were performed in a potassium phosphate buffer (0.1 M, pH 8.0). In 96-well microplates, 50$\mu L$ of the test solutions (final concentration of 250$\mu M$) were pipetted and pre-incubated at 37 °C for 30 min with 50$\mu L$ of the corresponding enzyme (*Ee*AChE or *Dm*AChE at concentrations of $0.03U{\cdot}mL^{-1}$). *Ee*AChE was used as a biological comparison standard due to its extensive validation as a commercial model and its high catalytic activity, which enables precise and reproducible determination of the inhibitory potency of new compounds. The reaction was initiated by adding 100$\mu L$ of a mixture of DTNB (0.5 mM assay concentration) and substrate ATCI (1 mM final concentration). Kinetics were monitored spectrophotometrically at 412 nm for 30 min with readings every minute. Tacrine was

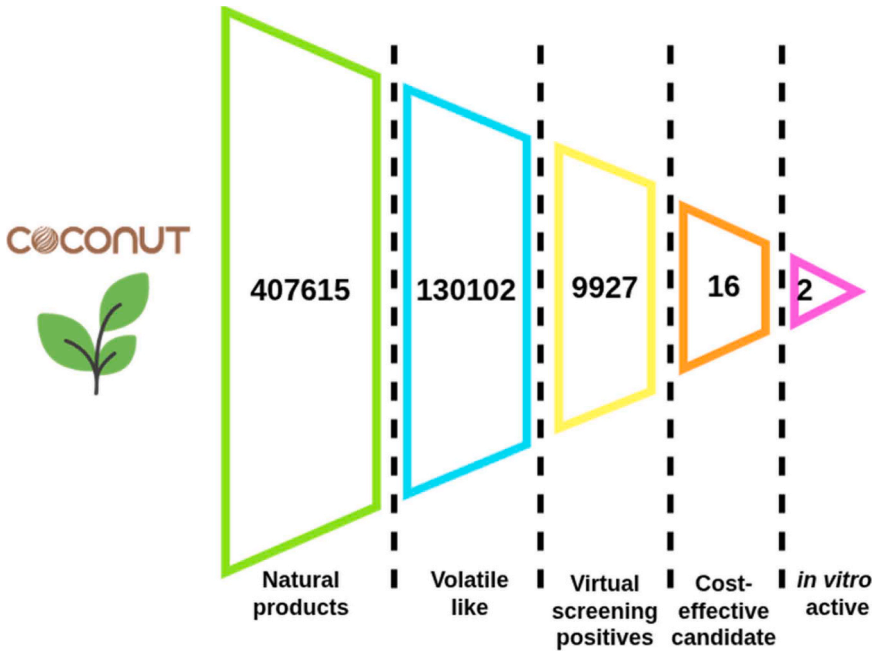



**Fig. 1.** Molecular filtering. Starting with the natural volatiles found in COCONUT, and followed by docking, comparison with reference PLIF (tacrine), accessibility filtering, and ending with in vitro assays.

**Table 1**
Identification, molecular weight, PROLIF-based Tanimoto similarity, and unit price (*USD*/*g*) of the selected compounds. The color gradient (green for molecular weight, red for price) reflects increasing values from light to dark tones.

| ID | Molecular weight (Da) | PROLIF similarity | Price (USD/g) |
|---|---|---|---|
| 1 | 250,4 | 0,85 | 1,2 |
| 2 | 227,6 | 0,86 | 3 |
| 3 | 228,5 | 0,88 | 0,2 |
| 4 | 348,4 | 0,86 | 3,8 |
| 5 | 254,3 | 0,9 | 0,8 |
| 6 | 284,3 | 0,86 | 1,2 |
| 7 | 148,2 | 0,75 | 0,2 |
| 8 | 188,3 | 0,79 | 1,6 |
| 9 | 323,4 | 0,89 | 0,2 |
| 10 | 293,3 | 0,89 | 0,2 |
| 11 | 254,3 | 0,9 | 1,2 |
| 12 | 204,3 | 0,75 | 0,2 |
| 13 | 192,3 | 0,82 | 0,2 |
| 14 | 248,3 | 0,85 | 2 |
| 15 | 266,3 | 0,86 | 6 |
| 16 | 264,3 | 0,85 | 1 |
| Tacrine | 198,3 | 1 | 13 |

used as a positive control (250 $\mu M$ assay concentration), and methanol (2.5%) as a negative control. All experiments were performed in triplicate across three independent sessions.

### 2.6. $IC_{50}$ Determination

The results from the initial screening were used as criteria to select the most promising compounds for $IC_{50}$ (half-maximal inhibitory concentration) determinations. Thus, $IC_{50}$ values were determined for the most potent inhibitors, compounds 4 and 8 (Table 1). For this purpose, inhibitor concentrations ranging from 30 to 500$\mu M$ were used, as previously described. The rate of enzymatic activity was determined from the linear portion of the kinetic reaction, expressed in mAU $min^{-1}$. $IC_{50}$ values were calculated by plotting dose-response curves and fitted using nonlinear regression (log[I] vs. response) in GraphPad Prism 8.0.1. assays were performed in three independent experiments (n = 3), each executed in triplicate.

### 2.7. Enzyme kinetics

Kinetic studies were performed with *Ee*AChE using a methodology similar to that described for the inhibition assay. Compound 8 was evaluated at five concentrations based on its $IC_{50}$ (0.05, 0.1, 0.25, 0.5, and 0.65 × $IC_{50}$). The substrate (ATCI) was used at seven concentrations ranging from 0.1 to 2 mM. Kinetic experiments were performed in duplicate across two independent experiments; all data were analyzed with GraphPad Prism 8.0.1. Constants were calculated from the substrate-velocity curves after fitting the data with the nonlinear regression function for enzyme inhibition kinetics. Furthermore, the inhibition mechanism was determined by fitting the double reciprocal regression equation and plotting Lineweaver-Burk graphs in GraphPad Prism 8.0.1.

## 3. Results and discussion

As shown in Fig. 1, approximately 30% of the molecules reported in the COCONUT database were classified as volatile natural products (VNPs) by meeting the pre-established molecular weight filter (Victoria-Muñoz et al. 2025). To select the appropriate binding site, a comparative analysis of AChE was conducted between *D. melanogaster* (PDB ID: 6XYY) and human (PDB ID: 4EY7) structures (Nachon et al. 2020; Cheung et al. 2012). The binding site of the tacrine derivative revealed a specific open pocket in *D. melanogaster* compared to human AChE, as displayed in Fig. 2(a) and (b). The principal structural difference is the substitution of Asp482 with Tyr449. In human AChE, the hydrogen bond between Tyr449, Trp86, and Trp439 closes the pocket that is open in *D. melanogaster* due to the absence of this interaction, as shown in Fig. 2(c) (Nachon et al. 2020).

Based on previous analysis, the structures were docked against *Dm*AChE using this binding site (Nachon et al., 2020). The primary selection criterion was the similarity between the Protein- Ligand Interaction Fingerprint (PLIF) calculated using the PROLIF tool for reference and the average fingerprints obtained for each pose of the volatile candidates (Bouysset and Fiorucci, 2021). Following this process, 9927 candidates were selected that exhibited a similarity of over 75% relative to the tacrine derivative. This empirical threshold effectively enriched the dataset to approximately 1% of the total screened candidates, yielding a high-affinity subset with a mean Vina score of −7.49 kcal/mol, thereby ensuring maximum conservation of key binding interactions. Tailoring interaction similarity cutoffs to specific target sites aligns with established interaction fingerprint workflows, as optimal similarity thresholds vary depending on the structural features of the target (Rácz et al. 2018).

Considering that economic constraints represent a key limiting factor in the discovery and development of alternative pesticides, particularly in agroindustrial contexts such as Colombia, where crops like bananas are of major economic importance and widely used pesticides such as chlorpyrifos have been banned, in this study, we incorporated the commercial availability as a selection criterion. Accordingly, screening continued with consideration of the availability of the 9927 candidates. Therefore, this large number of molecules was filtered based on their commercial availability and cost on the MolPort platform (data as of April 2023). Of the 9927 initial candidates, only 517 were available for purchase, with an overall average price of 645$USD/g$. However, as

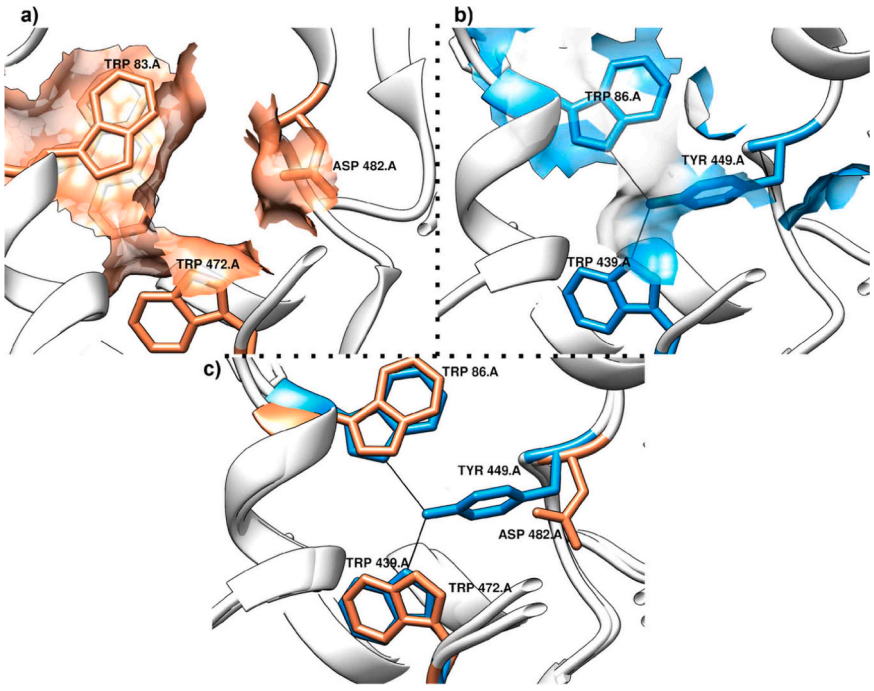



**Fig. 2.** Structural comparison of the binding sites of D. melanogaster and human acetylcholinesterase. Sections (a) and (b) show the binding site surfaces for D. melanogaster and human acetylcholinesterase, respectively. Section (c) displays the structural overlap between the two models.

observed in Table 1, the compounds selected for *in vitro* assays exhibit significantly higher economic viability, with a mean price of $1.8 USD/g$ and a median of $1.2 USD/g$. These final compounds simultaneously meet the criteria of volatility, economic accessibility, and binding profiles similar to those of tacrine (see Table 1 and Fig. 3).

The relevance of this economic approach is evident when analyzing global price trends. According to the U.S. Bureau of Labor Statistics, the cost of pesticides has increased by 38% since 2003, a financial pressure that disproportionately affects developing countries (U.S. Bureau of Labor Statistics, 2026). For instance, Brazil is currently the world's largest importer of these agrochemicals, while Colombia leads in pesticide use per hectare. In this context, the development of low cost alternatives, such as those proposed in this study, is not only a competitive advantage but also an urgent necessity for the region's agricultural sustainability (FAO, 2024).

Additionally, a tacrine derivative (9-N-phenylmethylamino-tacrine) was used as a comparative standard for computational study, providing a strategic advantage in the design of new agrochemicals. Unlike the predominant commercial insecticides such as organophosphates and carbamates, which are often toxic and involve irreversible binding, tacrine allows the search to be directed toward reversible inhibitors with more controlled interaction profiles and potentially lower environmental impact. Fig. 4 illustrates the molecular interactions of compounds 4 and 8 compared to the reference. It is observed that the primary interactions of tacrine consist of $\pi-\pi$ stacking between its aromatic rings and residues Tyr370, Trp83, and His480, in addition to hydrophobic contacts with Tyr370, Trp83, His480, Tyr71, and Gly150. Interestingly, compound 8, despite its lower molecular weight ($188.3 Da$), replicates key $\pi-\pi$ stacking interactions with Tyr370 and Trp83, as well as essential hydrophobic contacts. For its part, compound 4, in addition to reproducing all the reference's hydrophobic contacts, and due to its larger size, incorporates an additional $\pi-\pi$ stacking interaction with Tyr41.

To experimentally validate the computational results, 16 molecules

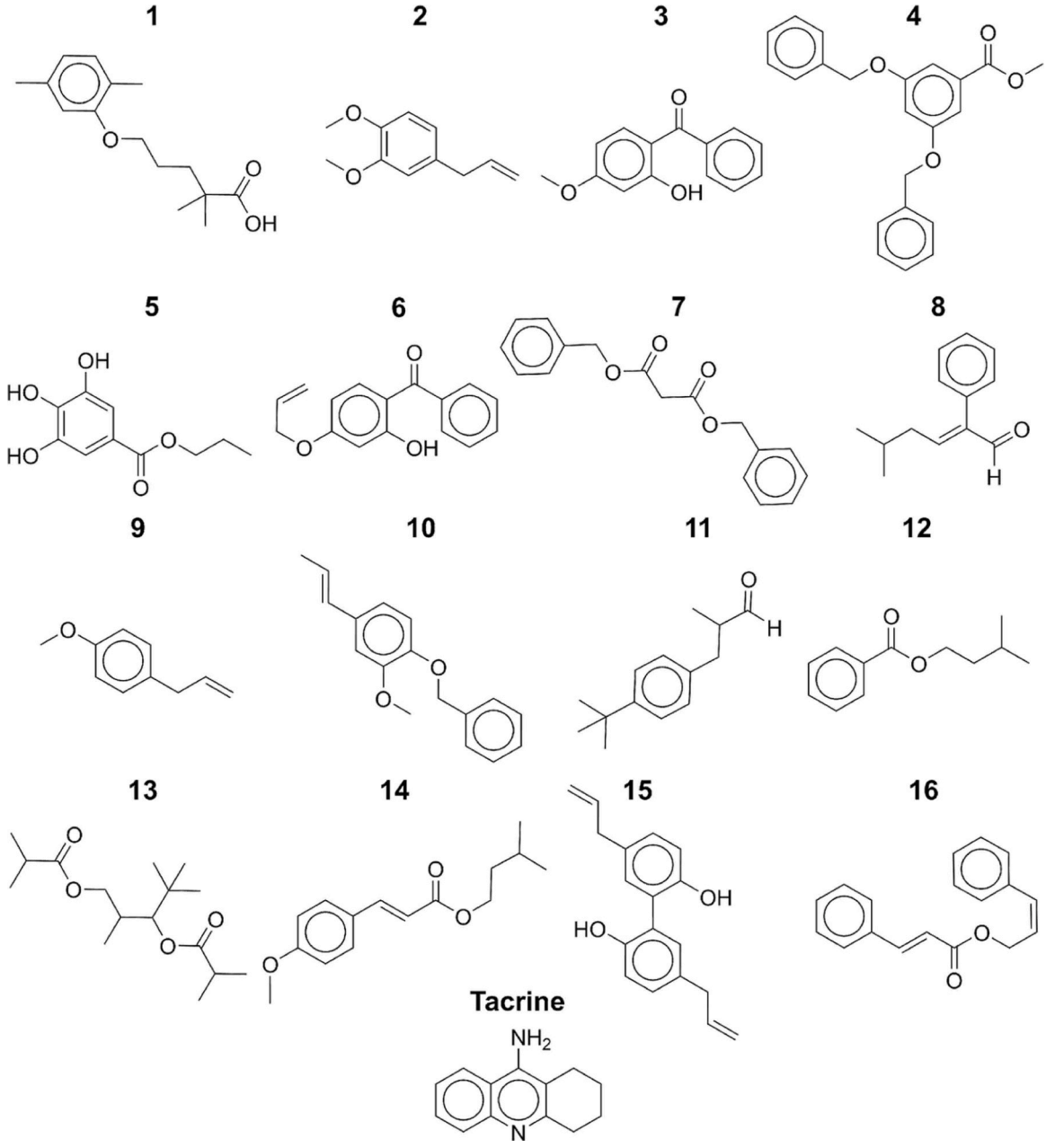



**Fig. 3.** Chemical structures of selected compounds. These molecules were further evaluated in the biological activity steps described below.

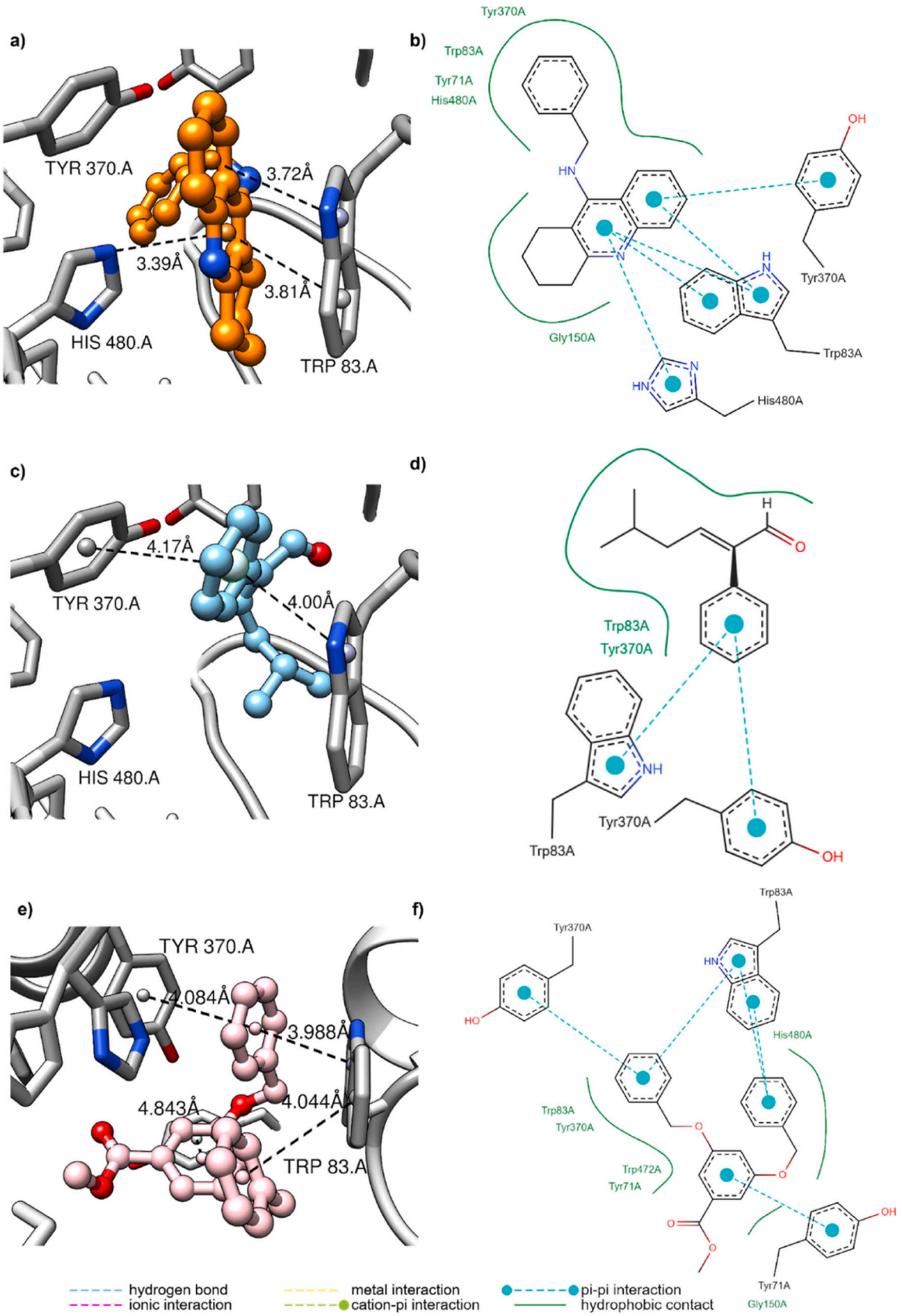



**Fig. 4.** Detailed binding mode and key molecular interactions within the DmAChE catalytic site. The ligands are color coded as follows: a) and b). 9-N-phenylmethylamino-tacrine (orange); c) and d). compound 8 (blue); e) and f). compound 4 (pink). Sections (a, c, e) show the three-dimensional (3D) docking poses, where black dashed lines represent π −π stacking interactions between the ligands and key residues. Sections (b, d, f) provide two-dimensional (2D) interaction maps; in these diagrams, light blue dashed lines indicate π −π stacking, while solid green lines delineate hydrophobic contacts. The specific interactions for each compound are displayed as follows: tacrine derivative (a, b), compound 8 (c, d), and compound 4 (e, f).

were tested in vitro against *Ee*AChE (Fig. 5). As expected by the molecular docking experiments, molecules 4 and 8 stood out for their activity, showing 52% and 77% inhibition at 250$\mu M$, respectively. These results were further corroborated by assays against *D. melanogaster* protein homogenate, in which these same compounds exhibited 16% and 46% inhibition, respectively, following a similar trend to that observed with *Ee*AChE. These findings correlate closely with the *in silico* predictions, as both structures replicate approximately 80% of the key interactions formed by tacrine in the binding site. The variations observed in inhibition levels between the two biological models could be attributed to the nature of the samples: while *Ee*AChE is a purified enzyme, the *D. melanogaster* homogenate constitutes a complex enzymatic cocktail that may influence compound bioavailability or assay kinetics. Consequently, further investigations using purified *Dm*AChE fractions are necessary to elucidate the precise inhibitory mechanisms and eliminate potential matrix effects.

Conversely, the remaining 14 compounds failed to reach the 20% inhibition threshold established for this screening. Although this experimental success rate is consistent with the 10% benchmark commonly observed in computational screening, integrating another tool could further optimize these filters to maximize the yield of active compounds (Zare et al., 2024; Yang et al., 2011). Nevertheless, this methodology could be effective for selecting small, lipophilic compounds ($MW < 350Da$). This is likely due to its ability to account for the entropic gain associated with the release of ordered water molecules from the enzymatic cavity, a phenomenon that often leads to an underestimation of binding energy in conventional docking simulations. This advantage is supported by the favorable docking scores obtained for our compounds (Compound 4: $-7.788kcal/mol$; Compound 8: $-6.773kcal/mol$), which, while lower than the reference value ($-10.125kcal/mol$), still indicate a strong binding affinity suitable for experimental testing (see Table S1 in Supporting Information) (Ramírez and Caballero, 2018; Xu et al. 2022). The identification of these two candidates validates the workflow's efficiency for the massive screening of natural chemical libraries. Furthermore, the fact that these compounds mimic the profile of tacrine reinforces the feasibility of seeking reversible inhibitors with controlled interaction profiles, which represent a strategic and potentially less harmful alternative to irreversibly binding organophosphate insecticides (Rahman et al. 2021; Araújo et al. 2023; Welling and de Vries, 1985).

For the compounds that exhibited greater than 50% inhibitory activity in the initial screening, the half-maximal inhibitory concentration

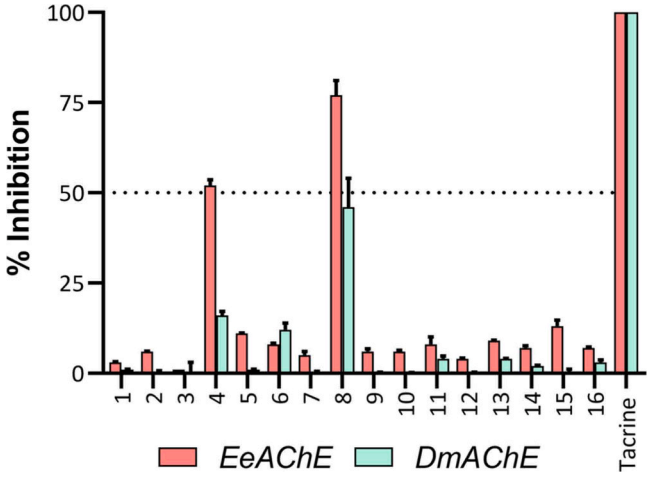



**Fig. 5.** Inhibitory activity of compounds 1–16 (250μM) against EeAChE (pink) and DmAChE homogenate (light blue). The black dotted line indicates the ≥ 50% inhibition threshold used to select compounds for $IC_{50}$ analysis. Tacrine was used as a positive control. Results are expressed as a percentage of inhibition ± SEM (n = 3).

($IC_{50}$) was determined against both enzymes, as detailed in Table 2 and Figure S1. The $IC_{50}$ curves were calculated using non-linear regression models, and the reported values represent the mean of three independent assays performed in triplicate. Both compounds showed $IC_{50}$ values higher than those of the reference standard (tacrine). However, due to its low molecular weight, compound 8 can be classified as an "optimizable fragment" (Xu and Kang, 2025; AlKharboush et al. 2025). This technical characteristic enables future structural modifications to enhance enzymatic affinity, emulating optimization strategies observed in synthetic quinoline derivatives, where specific functional group substitutions drastically increase potency.

Additionally, compound 8 offers a competitive advantage in terms of biosafety, as its previous use as a flavoring agent in various industrial applications suggests a favorable toxicological profile for humans and non-target organisms (Younes et al., 2022; Rychen et al., 2017).

Due to limited aqueous solubility, compound 4 precipitated at elevated doses, precluding the generation of a valid $IC_{50}$ curve in *Dm*AChE and subsequent $K_i$ kinetic modeling. Nevertheless, Compound 4, despite its bulkier architecture, features a molecular scaffold that favors multiple hydrophobic contacts and an additional $\pi-\pi$ stacking interaction with Tyr41. This structural complexity is comparable to that reported in studies of complex natural alkaloids and synthetic heterocyclic derivatives, which achieve robust inhibition by interacting with multiple sites within the enzymatic cavity (Plazas et al. 2019; Sirinzade et al. 2026). Consequently, compound 4 not only emerges as a promising candidate for pest control. Its multi-target potential is supported by the rigidity and planarity inherent to aromatic systems, which enhance molecular interactions such as $\pi-\pi$ stacking and hydrophobic binding, key features frequently associated with multitarget activity in polypharmacological design (Kabir and Muth, 2022; Yasuda, 2023; Guo et al. 2024). Furthermore, the rigid nature of these aromatic frameworks minimizes the entropic penalty upon binding, allowing the scaffold to maintain high affinity across structurally diverse enzymatic targets (Skoreński and Sieńczyk, 2021; Welsch et al. 2010; Barreiro, 2015).

Complementing the $IC_{50}$ values, the assays conducted in *D. melanogaster* head homogenates (*Dm*AChE) enhanced the robustness of our findings, as they represent a biologically relevant system that more closely approximates insect-specific enzyme inhibition within a complex matrix. As illustrated in Fig. 5, compounds 4 and 8 were the most effective inhibitors within this homogenate, maintaining the activity trend observed with the purified enzyme. The proportional decrease in inhibition levels when moving from the pure enzyme to the homogenate is an expected phenomenon, likely due to the presence of multiple isoforms and other non-target esterases that may sequester the ligand or compete for the active site (Melanson et al. 1985; Toutant et al. 1988).

Kinetic studies to determine the mechanism of inhibition of compound 8 were performed against *Ee*AChE; the results are graphically represented in a Lineweaver–Burk double reciprocal plot (Fig. 6). Analysis of the slopes and intercepts revealed a decrease in the maximum velocity ($V_{max}$) accompanied by an increase in the Michaelis Menten constant ($K_m$) as shown in Fig. 6. This behavior is characteristic of a mixed type inhibition mechanism. The intersection of the lines in

**Table 2**
*Inhibitory activities* ($IC_{50}$) of selected compounds against EeAChE and DmAChE.

| Compound | *Ee*AChE[a] ($IC_{50}$ $\mu$M) | *Dm*AChE[b] ($IC_{50}$ $\mu$M) |
|---|---|---|
| 4 | 189.10 ± 5.10 | n.d |
| 8 | 182.00 ± 12.70 | 167.10 ± 5.00 |
| Tacrine | 0.20 ± 0.03 | n.d |

$IC_{50}$ values were calculated using non-linear regression (GraphPad Prism 8.0.1). Data are presented as mean ± SEM of three independent experiments performed at least in duplicate. n.d.: not determined. [a] *Electrophorus electricus* AChE. [b] *Drosophila melanogaster* AChE.

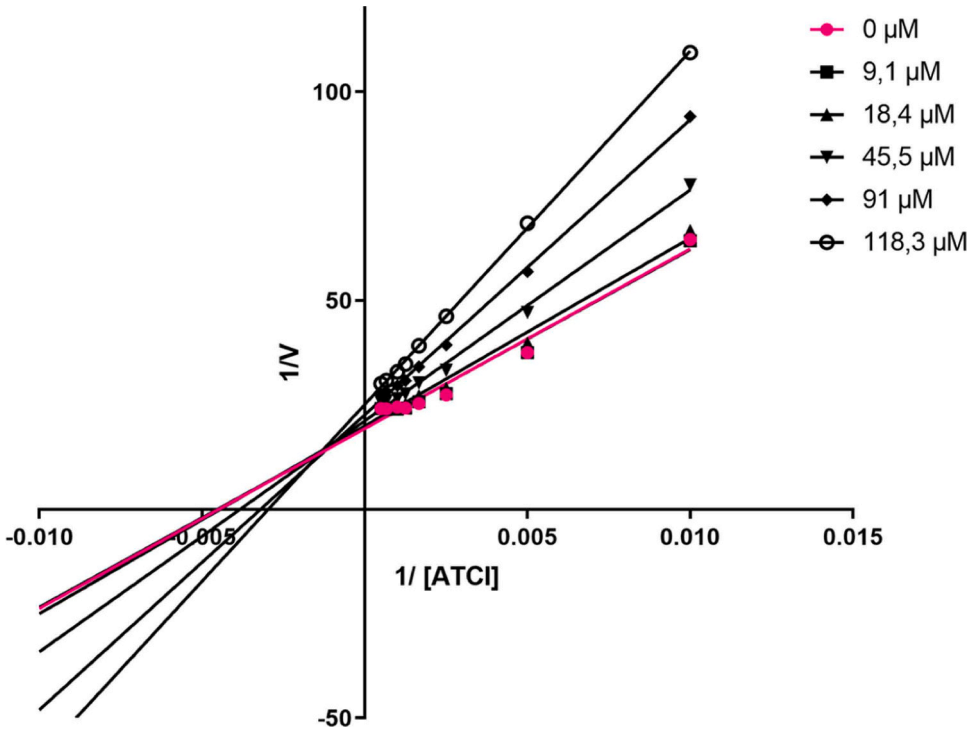



**Fig. 6.** Lineweaver–Burk plots for the inhibition of EeAChE by compound 8. The enzyme activity was measured at five inhibitor concentrations (0–118.3 µM, based on its $IC_{50}$) and seven substrate concentrations (ATCI) ranging from 0.1 to 2 mM.

the second quadrant indicates that the inhibitor possesses a higher affinity for the free enzyme than for the enzyme-substrate complex (Plazas et al. 2019). This preferential competition suggests that the compound interacts with both the active site and a peripheral anionic site (allosteric site) of AChE, consistent with a design oriented toward the target's selective cavity.

Finally, the inhibition constant ($K_i$) was determined by fitting the experimental data to a non-linear mixed-inhibition model. Compound 8 was found to have a $K_i$ of 88.1 ± 3.1 $\mu$M. Comparing this value with the $K_i$ of tacrine (0.19 ± 0.02 $\mu$M) reported by Sirinzade et al. (2026), it is evident that while the natural product's potency is lower, its reversible mechanism and nature as an optimizable fragment position it as a safe and promising scaffold. Furthermore, when compared with other volatile natural products recognized for their anticholinesterase and insecticidal properties, compound 8 shows competitive potency. Its $K_i$ (88.1 $\mu$M) is comparable or superior to those reported for common monoterpenes and phenylpropanoids, such as $\alpha$-pinene (81.7 $\mu$M), (+)-limonene (225.9 $\mu$M), (+)-sabinene (176.5 $\mu$M), and trans-anethole (134.7 $\mu$M) (Ahmed et al. 2013; Gajendra et al. 2024).

Regarding its structural features, compound 8 retains an aromatic framework similar to that of trans-anethole and a molecular size comparable to that of $\alpha$-pinene, (+)-limonene, and (+)-sabinene, all of which are common active ingredients in commercial biopesticides. These structural similarities suggest that compound 8 could be integrated into multi-component formulations, potentially broadening the insecticidal spectrum by synergy effects and reducing the likelihood of resistance development.

## 4. Conclusions

The implementation of an integrated computational methodology allowed for the identification of novel acetylcholinesterase (AChE) inhibitors from natural sources for *Electrophorus electricus* and *Drosophila melanogaster*. In this process, prioritization criteria based on volatility and cost were decisive for the success of the virtual screening. Through molecular docking and Protein-Ligand Interaction Fingerprint (PLIF) analysis, 16 candidates were selected from a natural product database of over 400000 compounds. All selected candidates met the requirements for potential volatility and a commercial cost of less than 8 *USD*/*g*, demonstrating the utility and efficiency of bioinformatic tools in narrowing the chemical search space.

Nevertheless, the experimental success rate indicates that selection filters must be refined, possibly by incorporating complementary techniques, such as molecular dynamics, since only 2 of the 16 candidates exhibited inhibition greater than 50%. Despite this, the economic analysis validated that compounds 4 and 8 have significantly lower prices than the reference (tacrine) and are readily available; specifically, compound 8 stands out for its proven safety profile for human consumption, which facilitates its potential adoption in the agro-industry.

Regarding biological activity, although the $IC_{50}$ values are higher than those of the reference, compound 8 represents a promising structural *"fragment"* due to its simple single-ring architecture. This allows for the projection of future optimizations aimed at improving its enzymatic affinity and potency through side-chain modifications. On the other hand, compound 4 requires further in-depth study to determine its viability as an insecticide, including evaluation of critical parameters such as photodegradation stability, environmental persistence, and its ability to penetrate the insect cuticle upon exposure via inhalation or contact. Finally, it is imperative to conduct *in vivo* activity assays to corroborate the biological efficacy of these compounds. Such studies should encompass both laboratory strains and pest species under field conditions, with the aim of consolidating these volatile natural products as sustainable, safe, and economically viable alternatives to traditional synthetic insecticides.

Finally, it is imperative to conduct *in vivo* activity assays to corroborate the biological efficacy of these compounds, encompassing both laboratory strains and pest species under field conditions. Beyond such testing, the translation of compound 8 into a commercial biopesticide will depend on controlled-release formulation strategies that mitigate its volatility and extend its persistence in the field. Its established status as a flavoring agent could additionally facilitate regulatory approval within risk-based biopesticide frameworks, an advantage reinforced by its

predicted mammalian safety margin (rat oral $LD_{50} \approx 4100$ mg/kg, GHS category 5; Table S1), which is consistent with the low-toxicity profile expected of a biochemical active substance. Together with its low cost and commercial availability, these attributes position compound 8 as an economically viable, optimizable scaffold, and support the consolidation of volatile natural products as sustainable, safe and economically viable alternatives to traditional synthetic insecticides.

## CRediT authorship contribution statement

**Angie Daniela Torres-Garcia:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Felipe Victoria-Muñoz:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Erika Plazas:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Norberto Sanchez-Cruz:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Cesar A. Sierra:** Writing – review & editing, Methodology, Investigation, Funding acquisition, Formal analysis.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.compbiolchem.2026.109377.

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