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Larvicidal activity and molecular interactions of furoquinoline alkaloids from Araliopsis soyauxii with wild-type and G119S mutant acetylcholinesterase of the malaria vector Anopheles gambiae

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Research Article

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Title: Larvicidal activity and molecular interactions of furoquinoline alkaloids from *Araliopsis soyauxii* with wild-type and G119S mutant acetylcholinesterase of the malaria vector *Anopheles gambiae*

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Running title: Furoquinoline alkaloids against AChE

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Abstract

The use of synthetic insecticides has led to target-site mutations such as G119S in acetylcholinesterase which is a major threat in *Anopheles gambiae* mosquitoes' control. To obtain alternative sources of novel bio-friendly insecticides, our study investigated the larvicidal activity and molecular interactions of four alkaloids isolated from *Araliopsis soyauxii* (Rutaceae) Nkolbisine, Tecleaverdoornine, Kokusaginine, and Flindersiamine against insecticide-susceptible and resistant *An. gambiae* populations.

The alkaloids of the *A. soyauxii* were obtained from stem barks using column chromatography and characterized by NMR spectroscopy. Using WHO larvicidal bioassays protocol, the susceptible Kisumu strain and field collected strains of *An. gambiae* populations characterized as carrying G119S Ace-1 mutation were exposed to the purified alkaloids.

Molecular interactions analysis was conducted via Molecular docking and MM-GBSA analysis.

Nkolbisine was the most potent, with LC_{50} values ranging from 51.63 mg/L to 60.12 mg/L across various strains at 24 h. Tecleaverdoornine was also very active and Kokusaginine and Flindersiamine followed. Our docking results revealed that Nkolbisine and Tecleaverdoornine had a positive binding affinity to wild-type as well as mutant AChE, with docking of scores -8.94 and -7.75 kcal/mol (wild-type) and -9.25 and -7.90 kcal/mol (mutant), respectively. Interactions such as hydrogen bonding with catalytic residues (GLU448, SER283) and π-π stacking were conserved in aromatic residues (TYR493, TRP441). MM-GBSA analysis, showed that Nkolbisine (-46.27 to -29.39 kcal/mol) interacts favorably with both enzymes.

Hence, Nkolbisine and Tecleaverdoornine are eco-friendly potential larvicidal candidates that can be used against both susceptible and resistant populations of *An. gambiae* for larval control in endemic settings.

Keywords: acetylcholinesterase G119S mutation; *Anopheles gambiae*; furoquinoline alkaloids; insecticide resistance; larvicidal activity; molecular docking.

Introduction

Mosquito-borne illnesses continue to be a serious public health concern, especially in sub-Saharan Africa, where *Anopheles gambiae* is the main malaria vector and insecticide resistance is posing a growing threat to vector control initiatives (WHO, 2023). Numerous methods and techniques have been developed in Cameroon to control these vectors. Some

current strategies to eradicate vectors include environmental changes, biological control measures, long-lasting insecticidal nets (LLINs), and indoor residual sprays (IRS) (Antonio-Nkondjio *et al*., 2019). Eliminating mosquitoes at the larval stage by stopping their development is another successful method (Venugopala *et al*., 2020). In addition to the above measures, pyrethroids (PYRs), organochlorides (OCs), organophosphates (OPs), carbamates (CMs), and neonicotinoids are the classes of insecticides recommended by WHO for use in IRS, which has been a significant approach to prevent the disease (WHO, 2016).

Insecticides have long targeted acetylcholine esterase (AChE), a serine hydrolase essential for controlling the neurotransmitter acetylcholine in insects. The bottom and opening portions of this enzyme's deep, narrow active site are referred to as catalytic and peripheral sites, respectively (Han *et al*., 2018). Resistance issues brought on by mosquitoes carrying AChE mutations, such as the G119S Ace-1 mutant, have restricted the use of anticholinesterase insecticides, which confers insusceptibility to organophosphate and carbamate insecticides (Antonio-Nkondjio *et al*., 2006). Also, three main enzyme families including monooxygenases (cytochrome P450s), esterases, and, glutathione S-transferases (GSTs) are associated with metabolic resistance in *Anopheles* mosquitoes, reducing the effectiveness of these insecticides (Wondji *et al*., 2012). This has made vector control tedious and difficult given that the quick development of resistance to carbamates and organophosphates could seriously jeopardise IRS-based control methods in the future (Aïkpon *et al*., 2014; Ngangue-Siewe *et al*., 2022).

Given this situation, there is a need to discover innovative acetylcholinesterase inhibitors having greater selectivity for insect AChE over mammalian AChE, and that remain effective

against insecticide-resistant vector populations. Despite resistance-conferring mutations, because the cholinergic system is a crucial part of the insect nervous system, AChE is still a relevant target in Anopheles mosquitoes and novel inhibitors may overcome existing resistance mechanisms (Rants'o *et al*., 2023). Inhibiting this enzyme disrupts neurotransmission, leading to paralysis and death of the insect (Pang, 2006). Hence, the search for new, eco-friendly alternatives continues (Ranson & Lissenden, 2016). Natural products, particularly plant-derived alkaloids, have shown promise as potential insecticidal agents due to their diverse biological activities and structural complexity (Matsuura & Fett-Neto, 2015). Among the many physiological effects of alkaloids are antibacterial, antimitotic, anti-inflammatory, analgesic, local anaesthetic, hypnotic, psychotropic, and antitumor activity (Kurek, 2019).

Araliopsis soyauxii Engl. (syn. *Vepris soyauxii* (Engl.) Mziray) (Rutaceae) is a medicinal plant from Cameroon, found in the Regions of Adamaoua, Littoral and South-West (Vaquette *et al*., 1976, Nganou *et al*., 2019; Noulala *et al*., 2022). In traditional medicine, it is used to treat gonorrhoea, malaria, and lung and venereal diseases (Nganou *et al*., 2019; Noulala *et al*., 2022). Alkaloids and triterpenes are abundant in the genus *Vepris* (Wabo *et al*., 2005; Noulala *et al*., 2022). Additionally, previous studies on *Araliopsis soyauxii* have demonstrated the presence of pharmacologically active alkaloids with potential insect control properties (Njuabe *et al*., 2022). In this context, phytochemical studies of the stem bark of *Araliopsis soyauxii* led to the isolation and characterization of six known compounds (Lupeol, Nkolbisine, Tecleaverdoornine, Kokusaginine, and Flindersiamine). The present study tests the hypothesis that selected alkaloids from *Araliopsis soyauxii*, specifically

Nkolbisine, Tecleaverdoornine, Kokusaginine, and Flindersiamine act as potential *An. gambiae* AChE inhibitors. We aim to elucidate the molecular interactions underlying their insecticidal activity through *in vivo* larvicidal assays and *in silico* docking analyses.

Materials and Methods

2.1. Plant material

The stem bark of *Araliopsis soyauxii* was collected in July 2018 in Small Babanki, Tubah Subdivision, Mezam Division, in the North-West Region of Cameroon. The plant specimen was identified by Dr Tacham Walter, a botanist from the Faculty of Science at the University of Bamenda, and compared to voucher samples catalogued under registration number 38959/HNC, previously housed at the National Herbarium in Yaoundé, Cameroon. The stem bark was air-dried under shade for one month and then ground into a fine powder.

2.2. General experimental procedures

The isolations were performed using column chromatographic carried out on silica gel (70–230 and 230–400 mesh, Merck, Darmstadt, Germany). Thin layer chromatography was carried out on precoated aluminium (Hartmann, Freiburg, Germany; 0.5 mm layer thickness, predominately silica gel 60, F254); upon which spots were visualized by spraying with ceric sulfate solution followed by heating at 120°C on a hot plate. Melting points were recorded on a Barnstead Electrothermal apparatus (Digital Melting Point IA-90) and are uncorrected. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DRX 500 NMR spectrometer at 500.13 and 125.75 MHz for ^{1}H and ^{13}C, respectively. Tetramethyl silane was

used as the internal reference. Optical rotations were measured using a JASCOP-2000 polarimeter (Japan). All of the solvents and reagents were purified using standard methods.

2.3. Extraction and isolation

The powdered plant material (2 kg) was macerated in 10 L of analytical grade methanol for 72 hours at room temperature in the dark to prevent photodegradation. Solvent evaporation under reduced pressure at 40°C gave 160 g of crude extract. A portion of the extract (80 g) was then subjected to column chromatography (silica gel, n-hexane, n-hexane–EtOAc, and EtOAc, in order of increasing polarity) yielding 250 subfractions. Fraction F5 yielded Lupeol (1, 55.8 mg). Fractions F15-25 yielded Nkolbisine (2, 10.5 mg) and Tecleaverdoornine (3, 11.6 mg). Fraction F40 afforded Kokusaginine (4, 12.2 mg), while fractions F110-113 yielded Flindersiamine (5, 28.5 mg) and β-sitosterol glucopyranoside (6, 8.5 mg).

Among the isolated compounds, the alkaloids Nkolbisine (2), Tecleaverdoornine (3), Kokusaginine (4), and Flindersiamine (5) were selected for larvicidal bioassays and molecular docking studies based on their structural features and previous reports of insecticidal activity. The triterpene Lupeol (1) and β-sitosterol glucopyranoside (6) were not investigated further in this study.

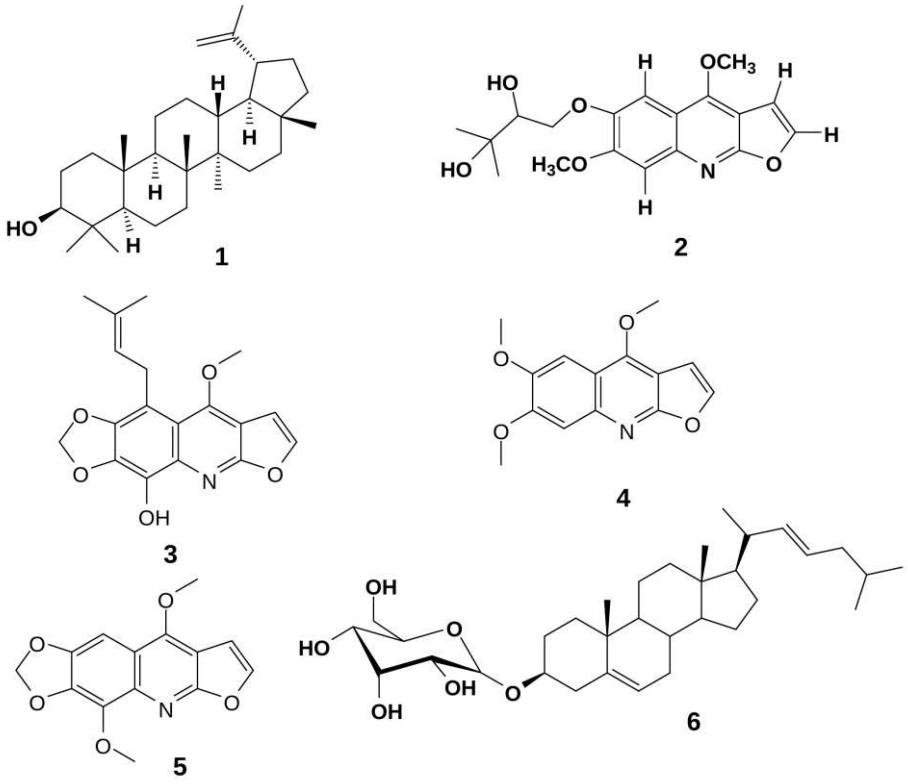


Figure 1: Compounds 1–6 isolated from *Araliopsis soyauxii*

2.4. *Anopheles* strains

Insecticide-sensitive laboratory-reared colonies of *An. gambiae* s.s. (Kisumu strain) maintained at the insectary of the Organization for Coordination of the Fight against Endemic Diseases in Central Africa (OCEAC) and field strains of *Anopheles gambiae* s.l. collected from various larval breeding sites in Mbandjock (Quarters: Mambra and Hausa) and Yaoundé (Quarters: Nouvelle route Bastos and Messa) were used for larvicidal assays. The field strains from Mbandjock and Yaoundé have previously been characterized as harboring the G119S Ace-1 mutation and showing resistance to organophosphates and carbamates (Ngangue-Siewe *et al*., 2022). Based on previous molecular identification of members belonging to

154 *Anopheles gambiae* s.l. field strains from Yaoundé were dominated by *Anopheles coluzzi*
155 (>95%) in Yaoundé (Antonio-Nkondjio *et al*., 2014) and those from Mbandjock by
156 *Anopheles gambiae* s.s (Antonio-Nkondjio *et al*., 2013). These field-collected mosquitoes
157 were then maintained in the insectarium of the malaria research laboratory at OCEAC.

158 **2.5. Larvicidal bioassays**

159 The larvicidal activity of these alkaloids was evaluated using the WHO larvicidal bioassay
160 (WHO, 2025). Twenty-five (25) third-instar larvae of each *Anopheles* species were
161 transferred into test cups containing deionized water. All test compounds and the positive
162 control were dissolved in DMSO and diluted with deionized water to achieve final test
163 concentrations ranging from 50 to 300 mg/L. The final DMSO concentration in all test
164 solutions, including controls, was maintained at ≤0.1% (v/v) to avoid solvent-induced
165 mortality. A solvent control (0.1% DMSO in deionized water) was included in all
166 experiments. Propoxur, a known larvicide and anticholinesterase agent, was used as a
167 positive control. The experiments were run in triplicate at 27°C and humidity ≥78%
168 simulating the insectary conditions. Larval mortality was documented at 24 and 48 hours.
169 The larvae were fed with Tetramin® fish food on day 0 and after each mortality assessment
170 to prevent mortality related to starvation. Mortality counts were completed before feeding to
171 avoid disturbing larvae during the counting process. The 50% lethal concentration (LC_{50}) and
172 90% lethal concentration (LC_{90}) values were determined by probit analysis method using
173 SPSS Statistics v27 software.

174 **2.6. *In silico* investigation**

2.6.1. Target proteins preparation

All docking investigations were conducted using the Schrödinger Release 2024-1 docking suite, Maestro version 13.5 (Schrödinger LLC, New York). The *An. gambiae* acetylcholinesterase (AgAChE) wild-type (PDB: 5YDI, resolution: 2.05 Å) and mutant G119S (PDB: 6ARX, resolution: 2.30 Å) crystal structures were obtained from the Protein Data Bank (Han *et al*., 2018; Cheung *et al*., 2018). These structures were selected based on their high resolution and complete coverage of the catalytic domain which confirm the binding site location and enable validation of docking protocols. These conditions were essential to the achievement of correct and valid docking outcomes (Rants'o, *et al.*, 2022).

Preparation of the protein structures was done through GLIDE Protein Preparation Wizard. During this process, water molecules that were found beyond 5 A of the binding site were eliminated (Roberts *et al*., 2008). Also, non-protein heteroatoms were removed and any missing side chains, looped regions, or hydrogen atom were added to bring about the structural integrity. The assignment of proper hydrogen orientations was done using PROPKA by predicting protonation states at pH 7.0, then optimizing it with a physiological pH range of 7.0 ± 2.0. The last step entailed the minimization of the restrained energy, which was done via the OPLS4 force field, to eliminate the strain and correct steric clashes within the molecules (Jorgensen & Tirado-Rives, 1988). Minimization was terminated when the root-mean-square deviation (RMSD) of non-hydrogen atoms converged at 0.3 A.

2.6.2. Receptor grid generation

The 5YDI crystal structure used for docking has two protein chains. Mosquitoes possess two distinct acetylcholinesterase genes, ace-1 and ace-2, which encode the AChE1 and AChE2 isoforms, respectively. Previous studies in insects, including *Drosophila melanogaster*, have identified AChE1 as the major target of organophosphate and carbamate insecticides (Hoffmann *et al*., 1992). In *Anopheles gambiae*, the ace-1 gene, which encodes AChE1, has been directly linked to insecticide resistance through mutation, e.g. G119S (Weill *et al*., 2004). Therefore, only Chain A (representing AChE1) was kept and used for further docking-analyses. The GLIDE grid was constructed using the OPLS4 force field, and the binding site was defined based on key active-site residues: Tyr489, Phe490, Trp441, Gly442, Tyr443, Leu444, Gly445, Ile446, Cys447, Glu448, Pro450, Tyr494, Phe560, Trp592, Met599, and Gly601. These residues enclose the catalytic Ser360, including the classical catalytic triad (Ser360 - Glu486 – His600) essential to enzyme activity (Rants'o *et al*., 2022a).

2.6.3. Ligand preparation

The chemical structures of the chosen ligands such as Nkolbisine, Tecleaverdoornine, Flindersiamine, Kokusaginine, Malathion and, Propoxur were acquired at the PubChem database. These ligands were imported into Maestro 2D Sketcher and then subjected to the Ligand Preparation (LigPrep) workflow. LigPrep was used to convert the 2D inputs into fully prepared 3D structures, including stereoisomer enumeration, prediction of ionization states, tautomer generation, and energy minimization. Using the Epik tool at pH 7.0 ± 2.0, the most likely ionization and tautomeric structures were predicted to ensure physiological relevance (Shelley *et al*., 2007; Greenwood *et al*.,2010). The conformers of the resulting ligand were then minimized in terms of energy with the OPLS4 force field. This produced geometry-

217 optimized 3D structures suitable for high-accuracy molecular docking with GLIDE
218 (Jorgensen and Tirado-Rives, 1988).

219 **2.6.4. Molecular docking**

220 The best poses from the ligands Nkolbisine, Tecleaverdoornine, Flindersiamine,
221 Kokusaginine, Malathion and, Propoxur were docked against both wild-type (5YDI) and
222 mutant (6ARX) *Anopheles gambiae* AChE. Extra Precision (XP) docking tool of GLIDE in
223 Schrodinger suite Ligand Docking panel was utilized to access the receptor grid generated
224 based on the 5YDI structure. Ligands were then imported in the project table and docking
225 was launched. During docking, ligands were considered flexibles and the sampling of barrier
226 to inversion of ring conformations and nitrogen barriers were possible. By default, nonplanar
227 conformations of amide bonds were penalized, and Epik state penalties were applied to the
228 docking scores to account for the energetic cost of adopting ionized or tautomeric states
229 generated during the ligand preparation process (Greenwood *et al.*, 2010). The XP docking
230 protocol was used, as it provides greater scoring accuracy and stricter pose filtering than
231 standard docking. For each ligand, the best-scoring pose was retained, and post-docking
232 minimization was carried out to optimize the geometry of the docked complex.

233 **2.6.5. Molecular dynamics simulations**

234 To assess the stability of the predicted binding modes over time, molecular dynamics (MD)
235 simulations were performed for the two most active alkaloids, Nkolbisine and
236 Tecleaverdoornine, in complex with both wild-type (5YDI) and mutant (6ARX) AgAChE,
237 yielding four simulated systems. Propoxur was also simulated as it served as the reference.

Simulations were carried out in GROMACS version, 2026 using the CHARMM36 force field for the protein and CGenFF for the ligands. Each protein-ligand complex was solvated in a TIP3P water box with a minimum protein-edge distance of 1.0-1.2 nm and neutralized with Na^+/Cl^- ions at a physiological concentration of 0.15 M. Systems were energy-minimized using the steepest-descent algorithm until the maximum force fell below 1000 kJ/mol/nm, followed by sequential NVT and NPT equilibration for 100 ps each at 300 K and 1 bar using the V-rescale thermostat and Parrinello–Rahman barostat. Production MD was run for 100 ns per system, with coordinates saved every 10 ps. Following the simulation, each trajectory was examined using GROMACS software, which calculates trajectories for root mean square deviation (RMSD) and root mean square fluctuation (RMSF). The results were then plotted and viewed using Grace v5.1.25 software

2.6.6. Molecular mechanics simulation

The Gibb's free energy change, ΔG (kcal/mol) was computed using Molecular Mechanics Generalized Born Surface Area (MM-GBSA) (Genheden & Ryde, 2015). The docked poses from XP docking file were imported to the Prime wizard of Schrödinger Maestro's where it was optimized. This was used as input for MM-GBSA rescoring using the VSGB solvation model and the free energy change on binding ΔG, computed by OPLS4 force field. The ΔG binding energy transition was calculated using the following equation:

$$\Delta G\ Bind = \Delta EMM + \Delta GSolv + \Delta GSA$$

The molar Gibbs energy of binding receptor and ligand molecules in solution is shown by ΔG_Bind. The total solvation energies for the receptor and ligand, along with the GBSA

solvation energy for each, are represented by ΔG_Solv. The difference between the minimized energy of receptor–ligand complexes is shown by ΔEMM. The difference between the surface area energies of the complexes is represented by ΔG_SA.

2.7. Data analysis

Excel spreadsheets were used to document the determined mortality rates. In the bioassay, the treated groups' fatalities were adjusted using Abbott's formula if the control group's fatality was between 5% and 20% (Abbott, 1925):

Corrected mortality = (X-Y)/X × 100

Where X = percentage survival in the untreated control and Y = percentage survival in the treated sample.

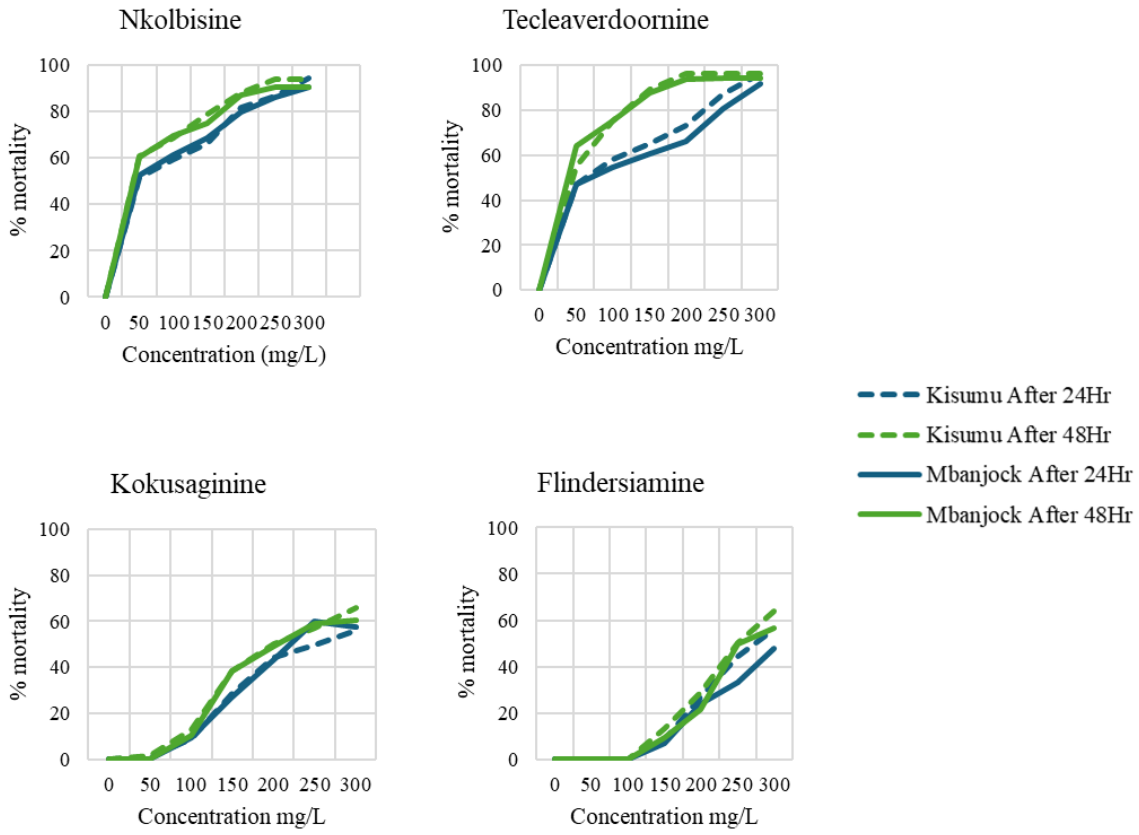
For analysis, data from each replication was combined. IBM SPSS version 27 was used to determine the LC_{50} and LC_{90} values using a log dosage–probit mortality regression line. The LC_{50} readings' mean standard deviation and confidence intervals were calculated.

Results

Larvicidal activity

The comparative larvicidal bioassays using the laboratory-maintained Kisumu strain (insecticide-susceptible) and field-collected strains from Mbandjock, Messa, and NRB (with documented insecticide resistance) revealed strain-dependent variations in susceptibility to the tested alkaloids. This also denoted the larvicidal efficacy of the four extracted alkaloids (Nkolbisine, Tecleaverdoornine, Kokusaginine, and Flindersiamine).

In general, an increase in concentration led to a corresponding rise in larval mortality for all compounds. Mortality was always more intense at 48 hours than at 24 hours, showing an increase in time of action of larvicidal activity. It is important to note that the response curves of certain compounds were different between populations, which indicates that the susceptibility might also differ. Kisumu larvae were more sensitive to the lower concentration of Nkolbisine and Tecleaverdoornine (100-250 mg/L), whereas Mbandjock larvae were less sensitive to the same (Figure 2). Nonetheless, at the maximum concentration examined (300 mg/L), both strains had similar mortality rates and this indicated that though there is a difference in the initial sensitivity, Nkolbisine eventually attains similar efficacy at adequate concentrations across strains. A similar trend of efficacy was also observed with tecleaverdoornine, where the strain exhibited a slightly better LC50 performance at 48 hours in the Mbandjock strain than Kisumu.



291

292 **Figure 2***:* Percentage mortality of insecticide-susceptible *An. gambiae* s.s. (Kisumu strain)
293 and field-collected *An. gambiae* s.l. larvae of Mbandjock (mixture of sibling species with
294 known insecticide resistance) exposed to different concentrations of alkaloid compounds
295 after 24 and 48 hours.

296 As in Figure 2, mortality rose with both concentration and exposure time. In a few cases,
297 however, the Messa and NRB populations showed a slower increase in mortality, particularly
298 with Kokusaginine and Flindersiamine, indicating that they are less susceptible to these
299 substances than Kisumu larvae (Figure 3). This resistance may be attributable to resistance
300 mechanisms selected for by these field populations' prior exposure to conventional
301 insecticides. As with the Mbandjock comparison, Nkolbisine consistently induced high

mortality and had low LC values in both populations. Interestingly, Messa larvae were even slightly more vulnerable than Kisumu larvae, with lower LC50 values observed: the 24 h LC50 of Nkolbisine was 51.63 mg/L in the Messa strain compared to 60.12 mg/L in the Kisumu population, indicating increased baseline sensitivity in the Messa population. Tecleaverdoornine showed a similar pattern, with a smaller 48 h LC90 in the Messa strain than in the Kisumu strain, pointing to a stronger long-term effect in the field strain.

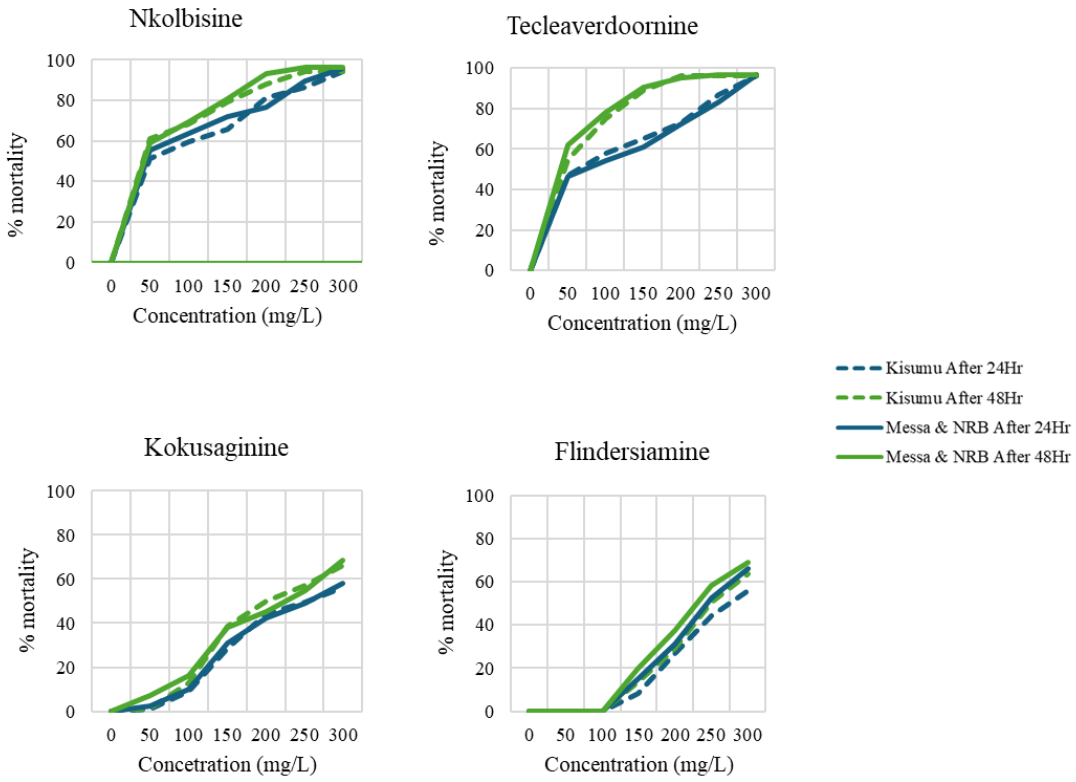
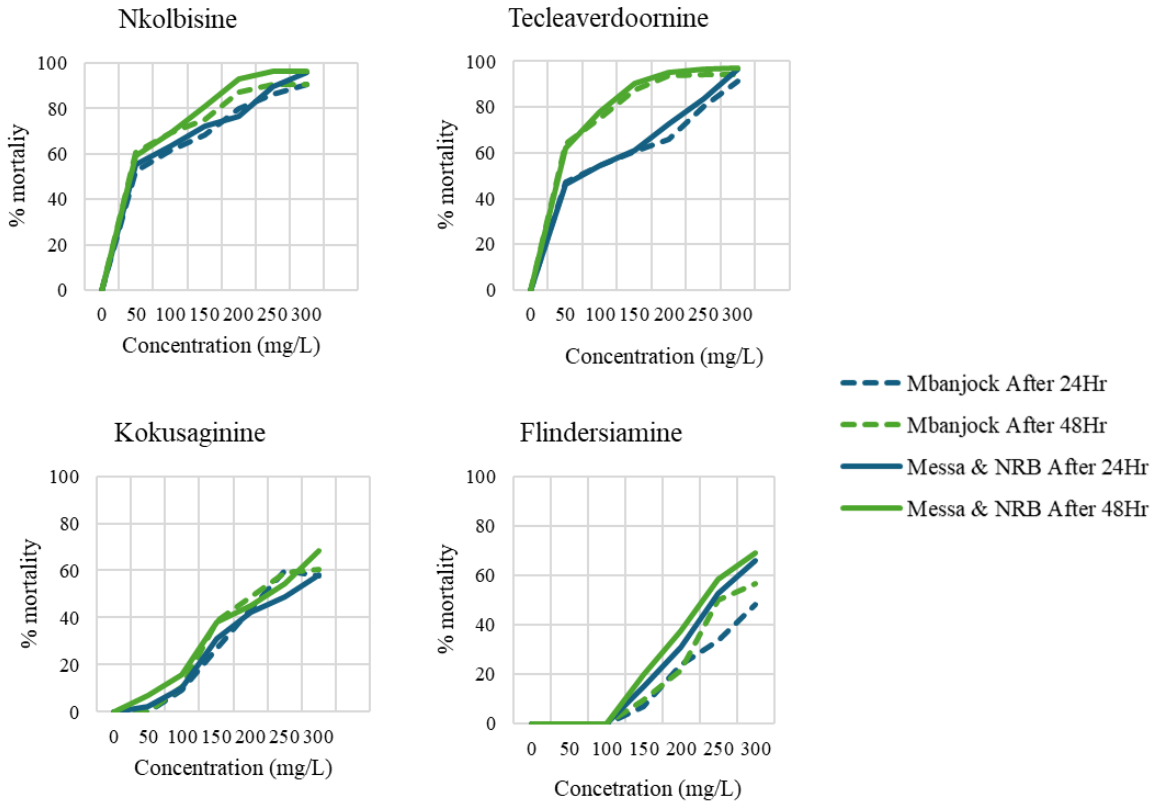


Figure 3: Percentage mortality of *An. gambiae* s.s. larvae (Kisumu strain) and *An. gambiae* s.l. larvae (Messa and NRB strains) upon exposure to alkaloids after 24 and 48 hours.

On the field strains, the general mortality patterns represented were also in agreement with previous observations, with the increased mortality at 48 hours in all the compounds tested.

313 The greatest differences between these two populations in terms of susceptibility occurred
314 with Tecleaverdoornine and Flindersiamine with Mbandjock larvae being most susceptible
315 compared to Messa and NRB, especially at intermediate levels (Figure 4). Although there
316 was similarity in the general patterns of concentration- and time-dependent mortality in the
317 two populations, the extent of response varied with the magnitude to particular compounds.
318 Mbandjock larvae were more responsive than Messa and NRB to Tecleaverdoornine and
319 Flindersiamine at intermediate concentrations (Figure 4), which indicated that there were
320 specific differences in the susceptibility of these field populations to these compounds. Messa
321 larvae were a little more susceptible to Nkolbisine at 24 hours, and the LC50 was 51.63 mg/L
322 versus 57.84 mg/L at Mbandjock. Tecleaverdoornine was also more effective in Mesa after
323 48 hours, indicating that the long-acting effect of this compound would be more useful in
324 some ecological settings.



325

326 **Figure 4**: Percentage mortality of *An. gambiae* s.l. larvae from Mbandjock and *An.*

327 *gambiae* s.l. larvae from Messa and NRB upon exposure to alkaloids after 24 and 48 hours.

328 **Molecular docking analysis.**

329 Following Extra Precision (XP) docking both protein targets (*An. gambiae*

330 acetylcholinesterase (PDB: 5YDI)) and its mutant (PDB: 6ARX)) against the four ligands

331 tested we were able to get the following results.

332 When docked against wild-type AgAChE (5YDI) it was observed that all the four alkaloids

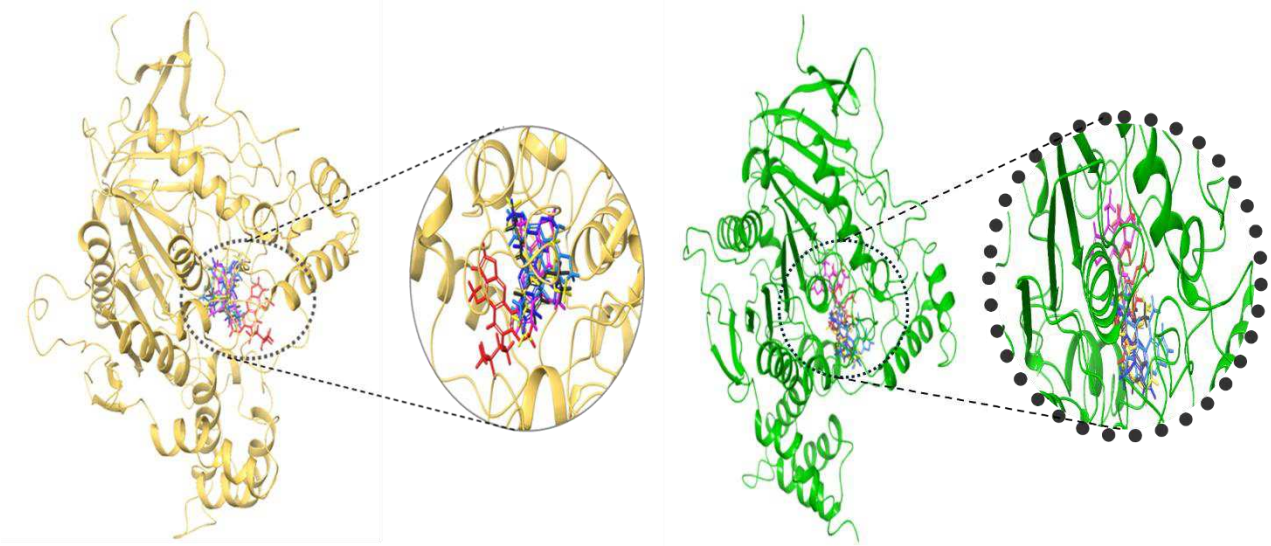
333 were bound in the active site gorge observed in Figure 5 (in gold). Nkolbisine was most

334 predicted to bind with a docking score of -8.94 kcal/mol falling within the range of predicted

335 binding of the protocols of GLIDE XP (Warren *et al*., 2006; Roberts & Mancera, 2008).
336 Tecleaverdoornine was also found to bind well (-7.75 kcal/mol) whereas Flindersimamine (-
337 6.87 kcal/mol) and Kokusaginine (-6.09 kcal/mol) showed intermediate binding scores
338 (Table 1). Malathion (-5.89 kcal/mol) and propoxur (-5.62 kcal/mol) conventional
339 insecticides yielded low docking scores and it can be argued that the binding qualities of the
340 alkaloids especially the Nkolbisine and Tecleaverdoornine are favorable. Nevertheless,
341 propoxur, a well-known AChE inhibitor has only relatively low-scores, suggesting that our
342 non-covalent docking protocol might be underestimating the interactions with carbamates,
343 and the comparative ranking of compounds is a stronger guide than absolute score cut-offs.

344 The presence of all the alkaloids in the active site in the mutant AgAChE (6ARX) indicated
345 possible interactions with the mutant enzyme (Figure 5 in green). Nkolbisine once more had
346 the highest docking score (-9.25 kcal/mol), which is why it was classified as a strong
347 predicted binder (Table 2). Tecleaverdoornine (-7.90 kcal/mol) also maintained a high
348 binding score and Kokusaginine (-5.70 kcal/mol) and Flindersiamine (-5.42 kcal/mol) scored
349 moderately and weakly, respectively. Interestingly, the ranking by the binding energies was
350 marginally different as the Flindersiamine (-31.86 kcal/mol) exhibited more positive
351 energetics than Kokusaginine (-29.39 kcal/mol) but had a lower docking score (Table 1). This
352 difference demonstrates the relevance of both measures, where MM-GBSA calculations take
353 into the account the effects of solvation and strain what the docking scores do not represent
354 (Shelley *et al*., 2007). The conventional insecticides propoxur (-4.07 kcal/mol) and malathion
355 (-5.12 kcal/mol) produced notably lower docking scores than the alkaloids, though this may
356 partly reflect the limitations of non-covalent docking for compounds with partially covalent

357 mechanisms. The binding energies followed a similar trend, in both target proteins with
358 Nkolbisine showing the most favorable value (-46.27 kcal/mol), followed by
359 Tecleaverdoornine (-37.84 kcal/mol), Kokusaginine (-31.43 kcal/mol), and Flindersiamine (-
360 31.38 kcal/mol).


361

362 **Figure 5:** Modelled *An. gambiae* acetylcholine esterase wild-type: 5YDI (in gold) and
363 mutant: 6ARX (in green) with alkaloids

364 **Table 1:** Docking scores and binding energies of alkaloids and synthetic inhibitors against
365 wild-type AgAChE (5YDI) and mutant-type (6ARX)

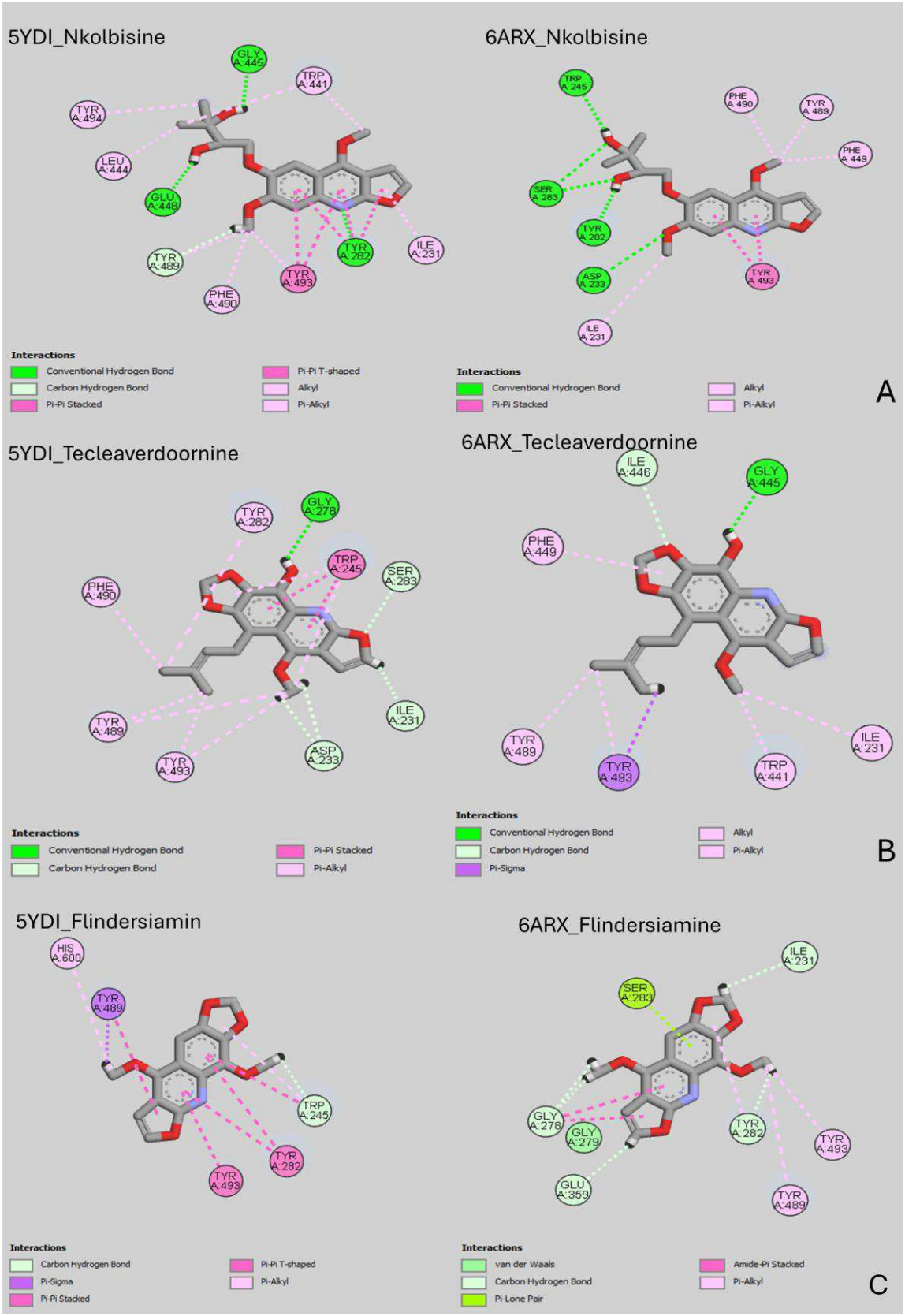
		AgAChE wild-type		**AgAChE mutant**	
Ligand	**Compound ranking based on binding energy**	**Docking score (kcal/mol)**	**Binding energy (kcal/mol)**	**Docking score (kcal/mol)**	**Binding energy (kcal/mol)**
Propoxur	synthetic inhibitor	-5.62	-29.94	-4.07	-29.79

Malathion	synthetic inhibitor	-5.89	-30.12	-5.12	-30.45
Nkolbisine	1	-8.94	-46.27	-9.25	-39.85
Tecleaverdoornine	2	-7.75	-37.84	-7.90	-33.63
Flindersiamine	3	-6.87	-31.38	-5.70	-29.39
Kokusaginine	4	-6.09	-31.43	-5.42	-31.86

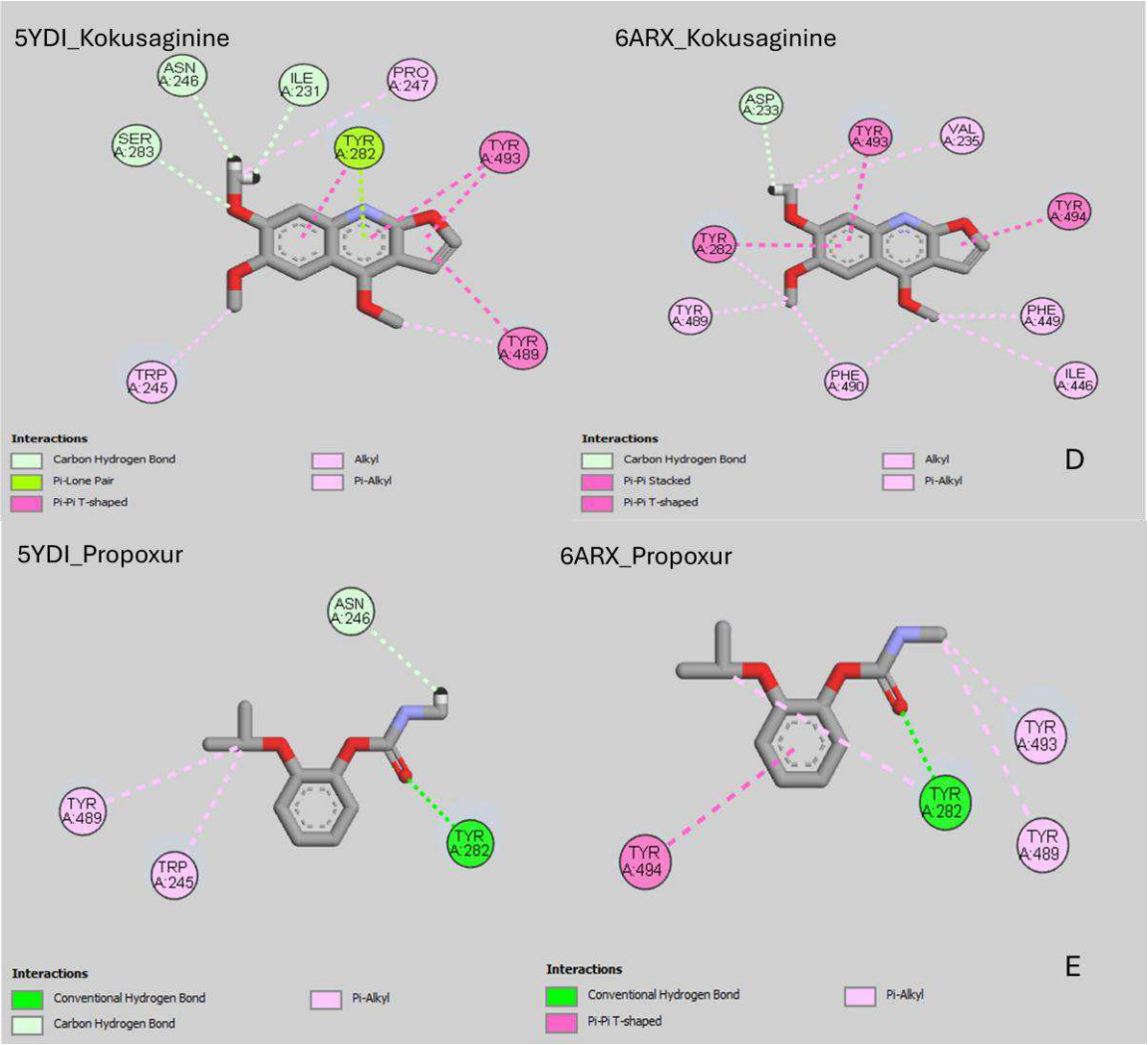
Docking analysis of alkaloids binding in AgAChE wild-type (5YDI) and mutant (6ARX)

While Nkolbisine retains important binding properties in both enzyme forms, a comparative analysis of the docking interactions shows significant variations in residue involvement and interaction geometry. In the wild-type 5YDI, the ligand is deeply embedded within the binding gorge, stabilized by a wide range of hydrogen bonds, π-π stacking interactions, and van der Waals forces. In contrast, the binding in mutant 6ARX shows a shift in interaction profile: while hydrophobic interactions are more peripheral and less extensive than in the wild-type, polar contacts with catalytically relevant residues (SER283, ASP233) become more prominent (Figure 6A). Such alteration of binding mode is probably due to structural changes caused by the G119S mutation, which changes the architecture of the pockets. An anchoring role is conserved as both systems retain interactions with TYR493. The interaction between tecleaverdoornine and wild-type (5YDI) and mutant (6ARX) acetylcholinesterase show significant differences in the level of interaction and depth of interaction with the residue (Figure 6B). The ligand in 5YDI is relatively superficial, with most of the interactions with outer residues being π-alkyl and van der Waals. In comparison, the 6ARX complex

382 exhibits a more favorable orientation in terms of functionality, with direct interaction with
383 catalytically relevant residues and a large π-π stacking in the active site gorge. These
384 observations indicate that it could be more probable that the mutation in 6ARX may increase
385 the binding of Tecleaverdoornine through deeper insertion into the active site and formation
386 of stronger interaction networks. Flindersiamine binding profile of the wild-type and mutant
387 AgAChE is different. In 5YDI, a π-π interaction with limited polar access holds the ligand in
388 place, indicating a superficial mode of binding. On the other hand Flindersiamine in the
389 6ARX mutant is stabilized by a denser hydrogen bonding network and enhanced interactions
390 with residues around the catalytic core resulting in a deeper buried and more stabilized pose
391 (Figure 6C). Figure 6D indicates key differences in the pairs of Kokusaginine binding
392 interactions of wild-type (5YDI) and mutant (6ARX) AgAChE. Substantial modifications of
393 the composition and type of interaction suggest the existence of functional divergence despite
394 the presence of conserved aromatic residues, such as TYR282, TYR489, and TYR493, that
395 are used to form π-π interactions in both enzymes. In 5YDI, polar stabilization of
396 Kokusaginine is observed by three carbon-hydrogen bonds (SER283, ASN246, and ILE231)
397 but in 6ARX, these bonds are missing. New residues such as ILE446, VAL235, PHE449 and
398 PHE490 mediate alkyl and π-alkyl interactions, and therefore, 6ARX provides a more
399 hydrophobic binding pocket. The anchoring role of propoxur towards TYR 282 of wild and
400 mutant AgAChE is confirmed by the conserved hydrogen bond that propoxur displays with
401 that site. Nevertheless, 5YDI possesses a reduced number of hydrophobic interactions with
402 TYR489 and TRP245 being the predominant π-alkyl contacts (Figure 6E). However, 6ARX
403 exhibits greater aromatic stabilization by π-π stacking with TYR494 and more compact π-
404 alkyl contacts.


5YDI_Nkolbisine
GLY A:445
TRP A:441
TYR A:494
LEU A:444
GLU A:448
TYR A:489
TYR A:282
ILE A:231
TYR A:493
PHE A:490
Interactions
Conventional Hydrogen Bond
Carbon Hydrogen Bond
Pi-Pi Stacked
Pi-Pi T-shaped
Alkyl
Pi-Alkyl
6ARX_Nkolbisine
TRP A:245
PHE A:490
TYR A:489
PHE A:449
SER A:283
TYR A:282
ASP A:233
TYR A:493
ILE A:231
Interactions
Conventional Hydrogen Bond
Pi-Pi Stacked
Alkyl
Pi-Alkyl
A
5YDI_Tecleaverdoornine
TYR A:282
GLY A:278
TRP A:245
SER A:283
PHE A:490
ILE A:231
TYR A:489
ASP A:233
TYR A:493
Interactions
Conventional Hydrogen Bond
Carbon Hydrogen Bond
Pi-Pi Stacked
Pi-Alkyl
6ARX_Tecleaverdoornine
ILE A:446
GLY A:445
PHE A:449
TYR A:489
TYR A:493
TRP A:441
ILE A:231
Interactions
Conventional Hydrogen Bond
Carbon Hydrogen Bond
Pi-Sigma
Alkyl
Pi-Alkyl
B
5YDI_Flindersiamin
HIS A:600
TYR A:489
TRP A:245
TYR A:282
TYR A:493
Interactions
Carbon Hydrogen Bond
Pi-Sigma
Pi-Pi Stacked
Pi-Pi T-shaped
Pi-Alkyl
6ARX_Flindersiamine
ILE A:231
SER A:283
GLY A:278
GLY A:279
TYR A:282
TYR A:493
GLU A:359
TYR A:489
Interactions
van der Waals
Carbon Hydrogen Bond
Pi-Lone Pair
Amide-Pi Stacked
Pi-Alkyl
C

405



406

407 **Figure 6:** Comparative analysis of 3D ligand interactions between AgAChE wild-type

408 (5YDI) and mutant (6ARX) active sites for Nkolbisine (A), Tecleaverdoornine (B),

409 Flindersiamine (C), Kokusaginine (D), and Propoxur (E)

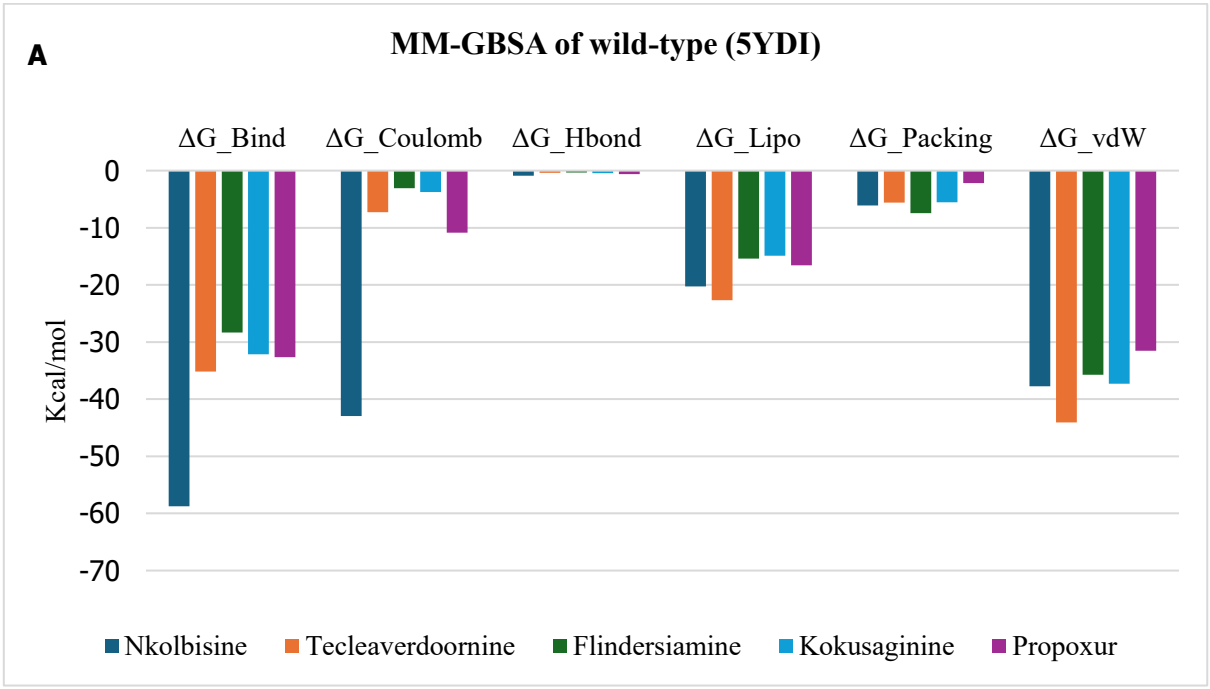
410 **MM-GBSA binding analysis**

411 When MM-GBSA calculations were done, it was shown that all the ligands exhibit

412 thermodynamically favorable binding to the wild-type (5YDI) and mutant (6ARX) proteins,

413 but binding affinity significantly decreases on mutation. The wild-type protein had total

414 binding free energies (ΔG_bind) in the range of - 30 to - 60 kcal/mol with Nkolbisine and
415 having the highest affinity. These favorable values were mainly contributed by significant
416 van der Waals (ΔG_vdW= -35 to -45 kcal/mol) and lipophilic (ΔG_Lipo = -15 to -25
417 kcal/mol) interactions, the optimum hydrophobic complementarity in the native binding
418 pocket (Figure 7A). By contrast, the mutant 6ARX system exhibited relatively less binding,
419 and ΔG_bind values decreased to some level of about -35 to -50 kcal/mol throughout the
420 ligand set. This reduction was mainly related to attenuation of van der Waals (ΔG_vdW = -
421 30 to - 40 kcal/mol) and lipophilic contributions (ΔG_Lipo = -10 to 20 kcal/mol) (Figure
422 7B), which suggests that the mutation disrupted the efficient hydrophobic packing. The
423 electrostatic (ΔG_Coulomb = -10 to -15 kcal/mol) and hydrogen bonding contributions
424 (ΔG_Hbond = 0 to -2 kcal/mol) were insignificant and comparable in both types of proteins
425 indicating that they might not play a significant role in the modulation of binding by the
426 mutation. All of this suggests that the mutation in 6ARX disrupts the binding of ligands in
427 part by destabilizing hydrophobic and dispersive contacts that contribute to high-affinity
428 binding in the wild-type protein.



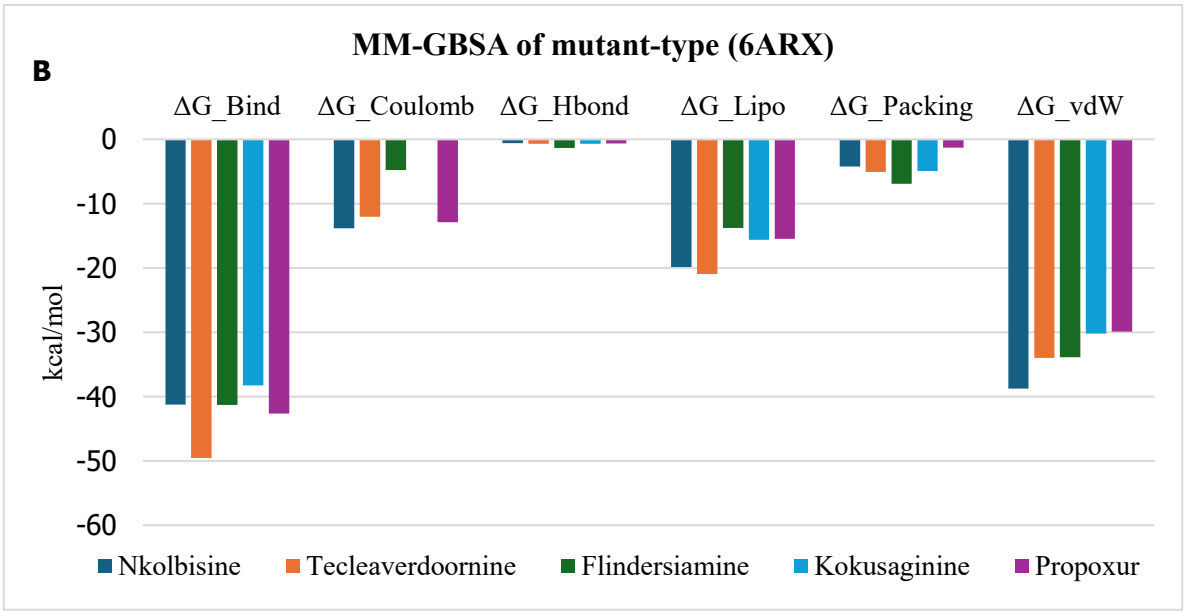


Figure 7: MM/GBSA profile for selected ligands with **(A)** *An. gambiae* AChE1 wild-type (5YDI) and **(B)** mutant (6ARX) protein. ΔG_Bind = binding energy, ΔG_Coulomb = Coulomb energy, ΔG_Hbond = hydrogen-bonding correction, ΔG_Lipo = lipophilic energy, ΔG_Packing = Pi-Pi packing correction, ΔG_vdW = Van der Waals energy.

Molecular Dynamics simulation

Nkolbisine, Tecleaverdoornine and Propoxur complexes were simulated in both the wild-type and mutant backgrounds to provide a dynamics-based reference alongside the docking and MM-GBSA propoxur control, giving six simulated systems in total (5YDI-Nkolbisine, 5YDI-Tecleaverdoornine, 5YDI-propoxur, 6ARX-Nkolbisine, 6ARX-Tecleaverdoornine, 6ARX-propoxur).

Protein backbone RMSD trajectories revealed a consistent stabilising effect of the G119S mutation across all three ligand-bound systems (Figure 8A, B). In the wild-type enzyme (5YDI), mean backbone RMSD was similar for the Nkolbisine- and Tecleaverdoornine-bound complexes (2.35 Å and 2.30 Å, respectively) and higher than for the propoxur-bound reference complex (1.73 Å), indicating that both alkaloids induce a comparable, moderate degree of global backbone flexibility relative to the synthetic inhibitor. In the mutant enzyme (6ARX), this ligand-dependent difference largely disappeared: mean backbone RMSD converged to 1.48–1.57 Å across all three ligands and was uniformly lower than the corresponding wild-type value for every ligand (Nkolbisine: 1.57 vs 2.35 Å; Tecleaverdoornine: 1.56 vs 2.30 Å; propoxur: 1.48 vs 1.73 Å). This pattern is consistent with a rigidifying effect of the G119S substitution on the AgAChE backbone that is largely independent of the identity of the bound ligand.

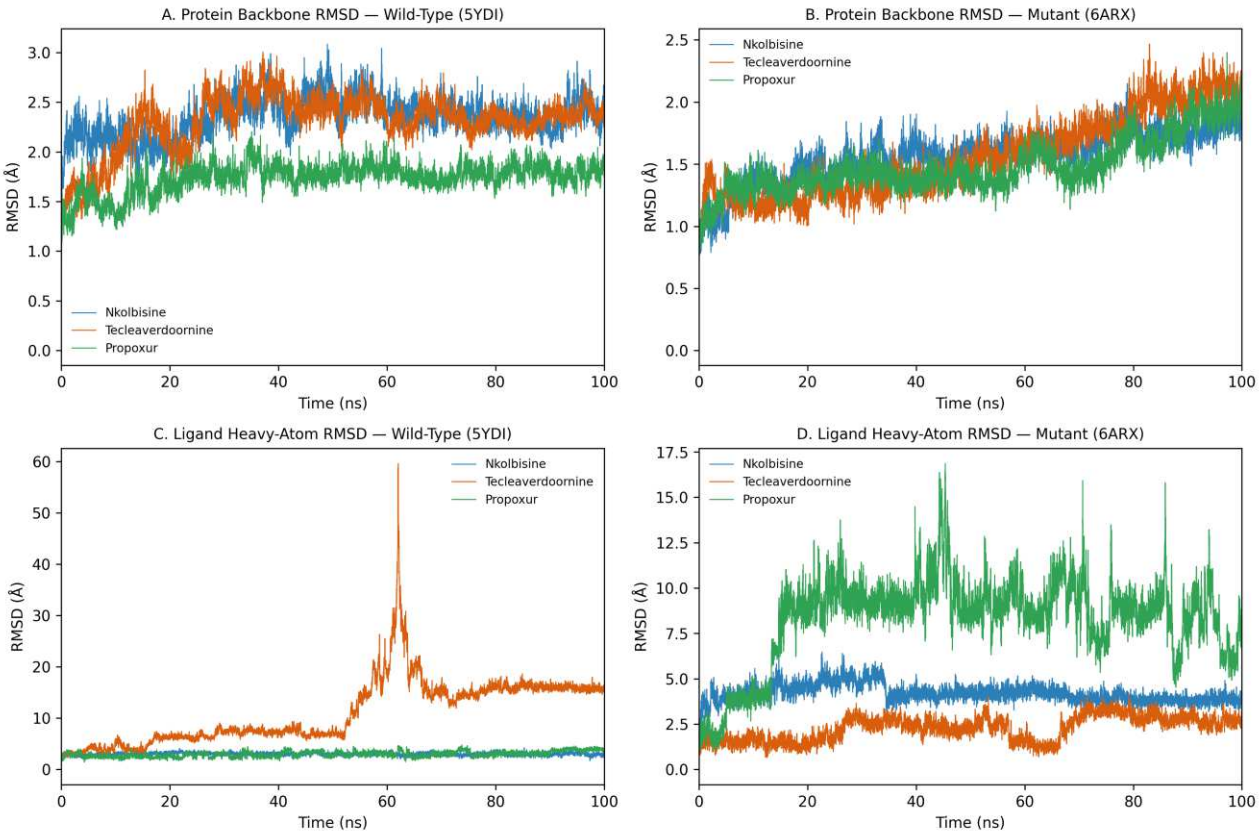


Figure 8: Molecular dynamics trajectory analysis across wild-type (5YDI) and mutant (6ARX) AgAChE complexes over 100 ns. (A) Protein backbone RMSD, wild-type. (B) Protein backbone RMSD, mutant. (C) Ligand heavy-atom RMSD (fit to protein backbone), wild-type. (D) Ligand heavy-atom RMSD, mutant.

In contrast to this relatively uniform protein-level response, ligand positional stability diverged sharply between compounds and, strikingly, reversed direction between the two enzyme forms (Figure 8C, D). In the wild-type complex, Nkolbisine remained tightly bound throughout the simulation (mean RMSD 2.99 Å), closely matching the stability of the propoxur reference (2.98 Å). Tecleaverdoornine, however, underwent a clear dissociation event: its RMSD climbed steadily from a bound configuration (<1 Å) to a displaced

464 configuration of approximately 15–16 Å by around 60 ns, and remained at this new position
465 (SD 0.55 Å over the final 20 ns) for the remainder of the simulation, indicating loss of the
466 docked binding pose in the wild-type enzyme.

467 Remarkably, this relationship inverted in the mutant enzyme. Tecleaverdoornine became the
468 most positionally stable of the three ligands in the 6ARX complex (mean RMSD 2.27 Å, SD
469 0.30 Å over the final 20 ns) more stable, by this measure, than propoxur was even in its own
470 wild-type complex. Nkolbisine showed intermediate mobility (4.18 Å). Propoxur, by
471 contrast, showed the largest and most variable displacement of the three ligands in the mutant
472 complex (mean RMSD 8.25 Å, SD 1.57 Å over the final 20 ns, with transient excursions to
473 approximately 17 Å), consistent with sustained repositioning within, or partial escape from,
474 the binding gorge rather than a single discrete dissociation event.

475 These trajectory-based findings complement and extend the static docking and MM-GBSA
476 results reported above. While XP docking predicted favourable and largely comparable
477 binding scores for Nkolbisine in both wild-type and mutant AgAChE (−8.94 and −9.25
478 kcal/mol, respectively), the MD trajectories indicate that a single docking score does not fully
479 capture ligand behaviour over time: Nkolbisine's binding pose is essentially unperturbed in
480 the wild-type enzyme but becomes measurably more mobile in the mutant, despite a
481 nominally improved docking score. Tecleaverdoornine shows the opposite, and more
482 dramatic, discrepancy a favourable wild-type docking score (−7.75 kcal/mol) belies an
483 underlying instability that manifests as full pocket dissociation over the 100 ns trajectory,
484 while its mutant-bound pose (docking score −7.90 kcal/mol) is in fact the most stable of all
485 six simulated systems. This divergence between single-pose docking/MM-GBSA metrics and

time-resolved MD stability underscores the value of dynamics-based validation: a favourable docking score alone does not guarantee a persistent binding mode, and the loss of a bound pose over an extended trajectory would not have been captured by static evaluation.

The behaviour of propoxur across the two protein backgrounds is particularly noteworthy in the context of the G119S resistance mutation. As the positive control and a well-characterised carbamate AChE inhibitor, propoxur was expected to serve as a stable reference across both systems; however, it showed the least stable ligand-level behaviour of any compound in the mutant complex, despite the mutant protein backbone itself being more rigid than the wild-type. This suggests that the local, ligand-specific consequences of the G119S substitution on pocket geometry are not simply captured by whole-protein rigidification, and that the mutation's effect on ligand retention is compound-specific rather than uniform. Combined with Tecleaverdoornine's improved retention in the same mutant background, these results are consistent with the possibility that the G119S mutation reshapes the AgAChE binding pocket in a manner that differentially favours retention of the furoquinoline alkaloid scaffold over the carbamate scaffold, a finding with direct relevance to the compounds' potential utility against resistant vector populations, and one that merits confirmation through contact-persistence and pocket-occupancy analysis.

Per-residue RMSF analysis of the wild-type complexes showed that Ser360, the nucleophilic residue of the catalytic triad, consistently occupied a local flexibility minimum below the whole-protein mean RMSF in all three wild-type systems (0.43–0.74 Å), supporting conservation of catalytic-site geometry regardless of the bound ligand. The remaining triad residues showed more ligand-dependent behaviour: in the propoxur complex, both showed

low RMSF consistent with a fully stabilised triad, whereas in the Tecleaverdoornine complex both exceeded the elevated protein-wide mean, consistent with loss of stabilising ligand contacts accompanying pocket dissociation. Residue-resolved RMSF for the mutant (6ARX) complexes is part of ongoing analysis and will be incorporated in a subsequent revision; the wild-type comparison nonetheless indicates that triad-level flexibility is not uniformly protected across ligands, and that Ser360 specifically is the most consistently shielded of the three catalytic residues.

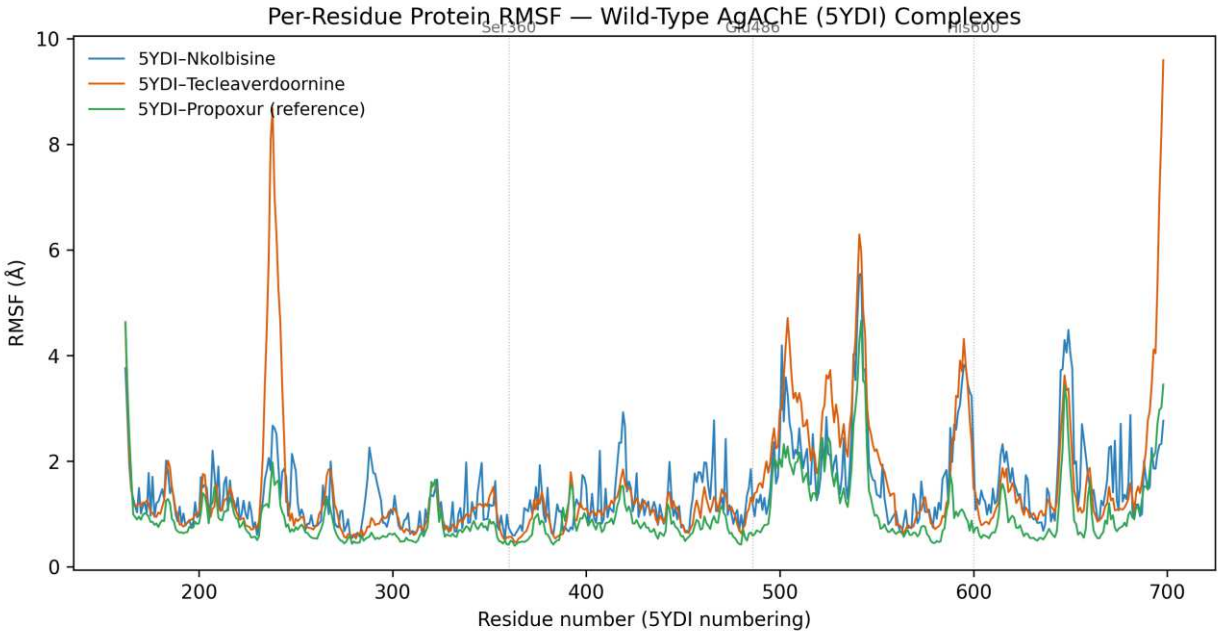


Figure 9: Per-residue protein RMSF for the wild-type (5YDI) Nkolbisine, Tecleaverdoornine, and propoxur complexes over 100 ns, with catalytic triad positions (Ser360, Glu486, His600) indicated. His600 is unresolved in the Nkolbisine complex topology (see note below).

Discussion

The time- and concentration-dependent increase in mortality observed for Nkolbisine, Tecleaverdoornine, Kokusaginine and Flindersiamine indicates a dose- and time-dependent larvicidal effect. The progressive efficacy of natural-product larvicides is often attributed to metabolic inhibition, delayed cellular disruption, or the accumulation of oxidative stress in larvae, consistent with this pharmacodynamic trend (Ali and Venugopalan, 2021). In a similar case, the findings in Cameroon conducted with crude extracts of seeds of *Balanites aegyptiaca* against the Anopheles larvae revealed gradual rise of mortality following a period of 24-48 hours and pronounced reduction in LC_{50} with time (Yonki *et al.*, 2023). Although it is complicated to make direct comparison with pure compound activity because of multiple constituents of crude extracts, the time-dependent pattern of mortality coincides with our observation. A previous study revealed that chelerythrine is an alkaloid of *Zanthoxylum lemairei* that had an LC_{50} of more than 125 mg/L in 24 hours against *Anopheles gambiae* larvae (Talontsi *et al.*, 2011). In comparison, Nkolbisine exhibited low LC_{50} values (51-57 mg/L) in our experiment, which means that Nkolbisine may have a higher larvicidal activity. Nonetheless, direct comparison is to be understood with some degree of caution because of the potential dissimilarities in experimental circumstances, strains of larval and compound purity across studies. Evodiamine and rutaecarpine, indole alkaloid of Evodia rutaecarpa, exhibited LC_{50} of 12.5-28.7 mg/L, at 24 hours, against *Aedes albopictus* (Liu *et al.*, 2012). Although these values indicate greater potency than Nkolbisine, these values cannot be directly compared to *Aedes* and Anopheles mosquitoes due to the possibility of intrinsic differences to alkaloid susceptibility. On the other side, Flindersiamine from *Vepris uguenensis* (Rutaceae), found in Kenya has been found to be a synergist to pyrethrins on housefly *Musca domestica* control (Langat, 2011). The existing evidence indicates a

544 marginally greater tolerance in the Messa and Mbandjock strains, with Flindersiamine
545 causing a slower increase in mortality, which probably indicates a variation in detoxification
546 enzymes or membrane permeability. Ricinine is an alkaloid of *Ricinus communis* which has
547 great insecticidal activity against mosquitoes and storage pests in Central Africa. LC_{50} was
548 found to be 15.5 mg/L in methanol seed extracts against *Aedes aegypti* and 9.4 mg/L in
549 against *Anopheles culicifacies* after 24 hours (Wachira *et al.*, 2014). In another experiment,
550 methanol extracts had LC_{50} of 420 mg/L against the *Anopheles gambiae* larvae (Yonki *et al.*,
551 2023). This makes Nkolbisine and Tecleaverdoornine superior options, as they have lower
552 LC thresholds and can be used in the field.
553 Kisumu larvae were also more likely to die in concentrations of 100 to 250 mg/L of
554 Nkolbisine and Tecleaverdoornine, this likely explains the reduced level of baseline
555 tolerance, which may be the result of laboratory breeding which are not exposed to
556 evolutionary stress with xenobiotics or insecticide (Talipouo *et al.*, 2023). This observation
557 is supported by the studies on the Anopheles larvae of the various ecological regions in
558 Cameroon (Talipouo *et al.*, 2023). Likewise, it has been found that urban agriculture, contrary
559 to pollution, contributes significantly to the insecticide resistance development (Antonio-
560 Nkondjio *et al*., 2011) presumably because of potential differences in enzyme-based
561 detoxification. This lowered susceptibility in some of the field populations is related to larger
562 scale reasons of variation in the Cameroon mosquito populations, in which we have found a
563 variation of responses to a number of control agents such as bacterial larvicides such as
564 *Bacillus thuringiensis israelensis* (Bti) (Edmond *et al.*, 2021; Talipouo *et al.*, 2023).
565 Although the underlying mechanisms are probably different (Bti resistance is associated with
566 gut receptor alterations, whereas alkaloid tolerance is possibly a result of metabolic

567 detoxification), these findings highlight the need to measure the efficacy of natural products
568 with different field populations with different histories of exposure. Nevertheless, in spite of
569 this resistance pattern, Nkolbisine has demonstrated high activity with the LC_{50} being 51.63
570 mg/L in Messa larvae being less than the 60.12 mg/L in Kisumu. This implies that Nkolbisine
571 can have a more extensive range or be more active of action, less reactive to the metabolic
572 detoxification pathways. Earlier studies of alkaloids such as berberine and evodiamine give
573 some evidence of such broad-spectrum effects, and they are effective against resistant
574 mosquito strains (Wu *et al.*, 2021).

575 The current docking analysis sheds light on the interaction of natural alkaloids with wild-
576 type (5YDI) and mutant (6ARX) *Anopheles gambiae* acetylcholinesterase (AgAChE), with
577 the implication of an insecticide resistance mechanism and rational inhibitor design.
578 Acetylcholinesterase is still the key target of insecticides, especially carbamates,
579 organophosphates including propoxur and malathion, which act by inhibiting the catalytic
580 activity of the enzyme (Rao *et al*., 2021). The in-silico comparisons that we have conducted
581 show that the G119S mutation that is commonly identified with the development of
582 organophosphate and carbamate resistance produces significant changes in the ligand-
583 binding profile of AgAChE. Nonetheless, some of the major aromatic residues especially
584 TYR493 were retained in both wild-type and mutant enzymes. This tyrosine belongs to the
585 aromatic gorge in the lining that directs the substrate into the active site and provides the
586 inhibitors with stacking interactions. Its conservation implies that even with the resistance-
587 conferring mutations, the basic structure of the gorge is not lost to provide the chances of
588 new kinds of inhibitors, binding the conserved residues. This is in line with previous

589 observations of Rao et al. (Rao *et al*., 2021), who found conserved aromatic residues to be
590 structural anchors in the active-site gorge of AChE in mosquito species. The docking scores
591 below -7.0 kcal/mol tend to be strongly bound whereas a score above -9.0 kcal/mol indicates
592 similar potency as optimized synthetic inhibitors (Warren *et al*., 2006; Roberts & Mancera,
593 2008). According to these standards, Nkolbisine (wild-type: -8.94; mutant: -9.25) and
594 Tecleaverdoornine (wild-type: -7.75; mutant: -7.90) can be classified as good predicted
595 binders in all forms of the enzyme. The slightly weaker scores of Flindersiamine and
596 Kokusaginine (-5.4 to -6.9) indicate weaker yet possibly still relevant binding especially in
597 view of the fact that the standard insecticides such as propoxur gave out scores within a
598 comparable range or even less in our protocol.

599 The MM-GBSA binding energies (-30 to -49 kcal/mol) are in agreement with values found
600 in other enzyme-inhibitor systems when entropic contributions have been neglected
601 (Genheden & Ryde, 2015). The agglomeration of more favorable energetics of Nkolbisine (-
602 46.3 kcal/mol wild -type; -39.9 kcal/mol mutant) compared to other alkaloids implies that it
603 forms the most stable complex with AgAChE. These absolute values however are to be taken
604 with care because they are not directly proportional to inhibition constants unless tested
605 experimentally. In this study, Nkolbisine and Tecleaverdoornine were consistently ranked
606 higher than Flindersiamine and Kokusaginine by both scoring methods and in both enzyme
607 forms, making this comparative ranking a better guide to prioritising compounds for further
608 investigation than the absolute score values. A notable limitation of our docking protocol is
609 that propoxur, a carbamate insecticide well documented to inhibit AChE, received relatively
610 low scores. Carbamates and organophosphates such as propoxur and malathion interact with

611 their targets in part by forming covalent or partially covalent interactions with the catalytic
612 serine, which are not effectively trapped by conventional non-covalent docking protocols
613 (Rants'o *et al*., 2022a). This is probably the reason why they scored lower than the alkaloids
614 in our experiment and also indicates that our protocol is underestimating the activity of these
615 traditional insecticides. The positive results with the alkaloids, therefore, ought to be viewed
616 as indicative of the presence of strong non-covalent binding properties, but that their true
617 inhibitory effect must be experimentally validated by carrying out an enzyme inhibition
618 study. Interestingly, there was a variation in the binding behavior of some compounds to the
619 mutant enzyme. Nkolbisine had great binding in the mutant (docking score -9.25 vs. -8.94
620 kcal/mol in wild-type), but with weaker binding energy (-39.85 vs. -46.27 kcal/mol).
621 Tecleaverdoornine was a little better docking (-7.90 vs. -7.75 kcal/mol) but still not as good
622 binding energy (-33.63 vs. -37.84 kcal/mol). These conflicting findings suggest that the
623 G119S mutation does not uniformly alter the binding landscape but instead modulates affinity
624 in a compound-specific manner, favouring alternative binding modes or chemotypes for some
625 ligands. This is consistent with the increased binding observed with donepezil analogues in
626 AChEs (van Greunen *et al.*, 2017), where such interactions were observed in the immediate
627 vicinity of the catalytic triad.

628 Docking and MM-GBSA rank Nkolbisine as the strongest predicted binder in both enzyme
629 forms, yet the MD trajectories show that positional stability does not track this ranking in a
630 simple way: Nkolbisine's bound pose is essentially undisturbed in the wild-type enzyme but
631 becomes measurably more mobile in the mutant, while Tecleaverdoornine fully dissociates
632 from the wild-type pocket over the trajectory yet becomes the most positionally stable ligand

of all six simulated systems once bound to the mutant. This is consistent with the broader observation that docking scores and short-timescale rescoring methods, including MM-GBSA on a single minimized pose, can diverge from the behaviour of a complex once conformational sampling and solvent dynamics are allowed to act over tens of nanoseconds (Plewczynski et al., 2011). The practical implication for this study is that Tecleaverdoornine's apparent underperformance relative to Nkolbisine in the docking and MM-GBSA analysis should not be read as a stable ranking across all conditions: its behaviour is highly enzyme-form-dependent, and in the mutant background specifically it outperforms every other ligand tested, including the synthetic reference propoxur.

As the positive control, propoxur remained fully stable in the wild-type pocket but showed the largest and most variable ligand-level displacement of any compound in the mutant complex, despite the mutant protein backbone itself being globally more rigid. Taken together with Tecleaverdoornine's improved retention in the same mutant background, this pattern is compatible with the hypothesis that the G119S substitution reshapes the AgAChE binding pocket in a manner that is not uniformly unfavourable to ligand binding, but instead redistributes which chemotypes are accommodated favouring the furoquinoline alkaloid scaffold at the possible expense of the carbamate scaffold.

The RMSF analysis of the wild-type complexes offers a partial structural explanation for these differences. Ser360 occupied a consistent local flexibility minimum across all three wild-type systems regardless of the bound ligand, indicating that the nucleophilic residue of the catalytic triad is protected even when the rest of the complex is not. Glu486 and His600, by contrast, remained near their respective protein-wide means in the Tecleaverdoornine

complex rather than showing the same protection, consistent with a loss of stabilising ligand contacts in the run-up to the dissociation event captured in the ligand RMSD trace. This localized loss of triad protection, rather than a uniform loosening of the whole binding gorge, may be a more sensitive early indicator of an unstable pose than global protein RMSD alone, and could be explored as a diagnostic metric in future screening of candidate alkaloids against both enzyme forms.

Flindersiamine gave a good example of the divergence of wild-type and mutant AChEs in their recognition of alkaloids. Flindersiamine displayed well-defined, selective interactions with residues through T-shaped π-π stacking and π-sigma bonds with TYR282, TYR489, and TRP245 in 5YDI, forming a stable interaction profile. Such interactions are consistent with the importance of preserved aromatic residues in high-affinity ligand recognition (Pang, 2006; Rants'o *et al.*, 2022b). Conversely, the mutant enzyme had lost or disturbed these aromatic interactions to form weaker and less specific interactions of van der Waals forces and carbon-hydrogen bonding. Such a change in anchoring to hydrophobic binding in the mutant corresponds with structural reports that the G119S mutation changes active site structure by introducing a serine hydroxyl with the potential to form new hydrogen bonds and with the potential to subvert existing contacts (Cheung *et al*., 2018). This trend was further brought out by the docking of Kokusaginine. Strong aromatic and polar interaction in wild-type AgAChE facilitated good orientation and selectivity in binding with the contribution of TYR282, ILE231, and ASN246. In the mutant form, however, these polar residues were substituted by hydrophobic residues including PHE449 and VAL235 resulting in ligand retention that is not precisely oriented, which was also found in previous studies of

resistance (Balachandran *et al.*, 2021; Rants'o *et al.,* 2023). The change of TRP245 to other residual ones such as TYR494 in the mutant is an indication of an adaptive process of wide-spectrum retention at the cost of catalytic positioning. Taken together, these results demonstrate how mutations that confer resistance, including G119S, reshape the electrostatic and structural landscape of AgAChE, resulting in changes in ligand affinity and selectivity. The four furoquinoline alkaloids examined here, Nkolbisine, Tecleaverdoornine, Kokusaginine, and Flindersiamine, showed differing sensitivity to these variants, indicating that this scaffold offers scope for further optimization against resistant AChE variants. Their H-bonds, π-stacking, and hydrophobic contacts resemble those of FDA-approved AChE inhibitors (Zhu & Hu, 2022) and make them a promising candidate to structure-based design. Future compounds, however, will possibly be required to include water-mediated bonding or flexible groups to be more reactive to the dynamic binding sites of resistant AChEs.

Conclusion

In our study, we have found Nkolbisine and Tecleaverdoornine as effective botanical larvicides with high activity to mosquito larvae and Kokusaginine and Flindersiamine with characteristic binding modes that indicates future structural optimization. Molecular docking established that these furoquinoline alkaloids retain positive correlations with both wild-type and G119S mutant acetylcholinesterase in *Anopheles gambiae* implying that they might be effective even when dealing with resistant mosquito populations. Remarkably, Nkolbisine and Tecleaverdoornine exhibited the greatest predicted binding affinities to both forms of enzymes. It can be concluded that the future of using these plant alkaloids in replacing synthetic insecticides in mosquito management is bright due to the combination of larvicidal

699 assays and molecular docking. They need to be further confirmed by enzyme inhibition
700 studies and field tests to confirm their practical use.

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712 bioassays with mosquito larvae are not subject to institutional or national ethics review under
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715 **Consent for publication:** Not applicable.

716 **Availability of data and materials:** The datasets generated and/or analysed during the
717 current study, including raw larvicidal bioassay data, molecular docking output, and
718 molecular dynamics trajectory files, are available from the corresponding author on

reasonable request. The crystal structures used (PDB: 5YDI, 6ARX) are publicly available in the Protein Data Bank (https://www.rcsb.org).

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