

INHIBITORY EFFECT OF CRUDE ALKALOIDS OF *DATURA STRAMONIUM* SEED AND IT'S FRACTION(S) ON ACETYLCHOLINESTERASE ISOLATED FROM RESISTANT AND SUSCEPTIBLE *ANOPHELES GAMBIAE* MOSQUITO LARVAE

Williams D.G. Chintem^{1,2*}, Mahmoud Suleiman Jada¹, Shehu Sarkiyayi¹ and Abdullahi Usman Wurochekke¹

¹Department of Biochemistry, Faculty of Life Sciences, Modibbo Adama University, Yola, Adamawa State, Nigeria

²Department of Science Laboratory, Taraba State Polytechnic, Jalingo

*Corresponding authors' email: williamschintemdg@gmail.com

Abstract

The increasing resistance of *Anopheles gambiae* to conventional neurotoxic insecticides necessitates the exploration of alternative acetylcholinesterase (AChE) inhibitors with novel modes of action. This study evaluated the inhibitory effects of crude alkaloids and alkaloid fractions from *Datura stramonium* seeds on AChE isolated from susceptible (sAChE) and resistant (rAChE) *Anopheles gambiae* larvae. Crude alkaloids were extracted using acid–base partitioning, while mosquito larvae were field-collected, reared, and biochemically characterized. AChE was isolated from larval homogenates, and enzyme inhibition assays were conducted using the Ellman Assay. Enzyme kinetics (K_m and V_{max}), optimal pH and temperature, and modes of inhibition were also determined. The crude alkaloid extract inhibited AChE in a concentration-dependent manner, with higher sensitivity observed in sAChE ($IC_{50} = 30.3 \mu\text{g/mL}$) compared to rAChE ($IC_{50}=59.6 \mu\text{g/mL}$). Among the alkaloid fractions, DSAF-3 exhibited the strongest inhibitory activity against both strains, achieving up to 89% inhibition at $100 \mu\text{g/mL}$ and the lowest IC_{50} ($22.8 \mu\text{g/mL}$). In contrast, the standard insecticide temephos showed greater potency against rAChE than sAChE, reflecting differential target-site sensitivity. Optimal AChE activity for both strains occurred at pH 7.0–7.5 and 40°C . Kinetic analysis revealed that crude alkaloids and DSAF-3 caused significant reductions in V_{max} (rAChE=0.9802, sAChE=0.4975) and (rAChE=0.7675, sAChE=0.1507) with relatively unchanged K_m values, indicating predominantly non-competitive inhibition, unlike temephos, which exhibited mixed inhibition. These research findings demonstrate that *D. stramonium* seed alkaloids, particularly fraction DSAF-3, effectively inhibit both susceptible and resistant AChE through a resistance-insensitive mechanism, highlighting their potential as lead compounds for the development of novel larvicidal agents in malaria vector control.

Keywords: *Datura stramonium*, Acetylcholinesterase, *Anopheles gambiae* larvae, Alkaloid fractions, Insecticide resistance, Non-competitive inhibition

Introduction

Malaria vector control relies on chemical insecticides that target the nervous systems of *Anopheles* mosquitoes, with acetylcholinesterase (AChE) as a principal biochemical target for organophosphate and carbamate compounds (Cheung et al. 2018). However, the widespread emergence of target-site mutations and multiple metabolic resistance mechanisms in *Anopheles gambiae* populations has compromised the efficacy of these insecticides, prompting renewed

interest in alternative bioactive compounds and modes of action (Fagbohun et al. 2020; Keita et al. 2021). Because AChE remains central to mosquito neurophysiology, plant-derived inhibitors that interact with AChE offer a promising route to novel larvicides that may circumvent existing resistance phenotypes or act synergistically with existing chemistry (Georgiev et al. 2022).

Alkaloids are a chemically diverse class of plant secondary metabolites known for their potent neuroactive properties, including AChE inhibition and disruption of insect neurotransmission (Sharma et al. 2021). Recent reviews and experimental screening reports show strong AChE-inhibitory activity in alkaloid-rich extracts and isolated alkaloids, and demonstrate measurable insecticidal and larvicidal effects against dipteran pests (Georgiev et al. 2022; Corzo-Gomez 2024). Targeted evaluations of individual alkaloids and crude alkaloid fractions have revealed both immediate toxic effects and sublethal neurophysiological disturbances in insect larvae, supporting the idea that alkaloid extracts could function as AChE-directed bioinsecticides (Corzo-Gomez 2024; Gupta et al. 2024). Thus, to assess the enzyme-inhibiting activity of alkaloid extracts against AChE isolated from resistant and susceptible larvae, it is essential to determine whether *Datura stramonium* alkaloids retain activity against resistant enzyme variants and to elucidate possible inhibition kinetics, as potential alkaloid-based vector control agents.

Materials and Methods

Collection, identification, and preparation of plants

Seeds of *Datura stramonium* were harvested from the study area. The plants were identified at the Herbarium unit of the Department of Plant Science, Modibo Adama University, Yola. The plant seeds were shade-dried for several days, ground to a fine powder and stored in an air-tight container.

Extraction of Alkaloid

Powdered plant material (100g) was wetted with 150 mL of NH_4OH (25%, m/m) and room temperature solvent extraction was performed with 1000 mL of ethyl acetate for 72h. The filtrate was evaporated in a rotary evaporator under reduced pressure at 40°C. The residue dissolved in H_2O and acidified with H_2SO_4 to pH 3-4; extracted with petroleum ether and diethyl ether. Basify to pH 9-10 with NH_4OH (25%, m/m), extracted with chloroform to neutral pH, dried with Na_2SO_4 and concentrated to dryness to obtain crude alkaloid (Khan and Kumar 2019).

Column Chromatography

About 1g of crude alkaloid extract of *D. stramonium* was partially purified by column chromatography, and a total of 12 fractions were collected based on colour separation. Each fraction was allowed to dry and then spotted on a TLC plate. The developed TLC plate was allowed to dry and was viewed under UV radiation at 254 nm (Ejele et al. 2012). The fractions were pooled together into 4 main fractions, each based on the pattern and R_f values. The combined fractions were referred to as *Datura stramonium* Alkaloid DSAF (1-4) and further subjected to Larvicidal bioassay. A further purification step was performed on the bioactive fraction DSAF-3, which was then subjected to Gas Chromatography/Mass Spectrometry.

Rearing of Mosquito Larvae

Anopheles spp. Mosquito larvae were harvested around the Modibo Adama University staff quarters (Lat 9.3378°N, Lon 12.4889°E). The larvae were identified and authenticated by Dr Atinga Atimi and confirmed by Dr Sani Njobdi at the IIDP Laboratory. The larvae were reared in plastic and enamel trays in tap water maintained in a growth chamber at room temperature and fed a diet of brewer's yeast and biscuits (Mamai et al. 2018).

Extraction of crude Acetylcholinesterase enzyme

About 100 death (susceptible and resistant) larvae were frozen at -20 °C, decapitated, and homogenised in 5ml ice-cold 0.1 M sodium phosphate buffer at a pH 8.0, using a tissue homogeniser. The homogenate was centrifuged at 10,000 × g at 4 °C for 20 minutes. The supernatant was then filtered with a 0.22µm filter and used as the AChE preparation Zulkpli et al. 2023).

Enzyme Inhibition Assay

Acetylcholinesterase catalyses the hydrolysis of acetyl-thiocholine (sulfur analogues of their respective natural substrate, acetylcholine). Upon hydrolysis, these substrate analogues produce acetate and thiocholine. The method described by Todd and Gomez (2001). A multichannel pipette was used to transfer 10 µL of the enzyme preparation to each well of a 96-well microtiter plate, then mix with 20 µL of the tested compounds and 150 µL of cold phosphate buffer. The assay microtiter plates were incubated at 25°C for 10 min. The inhibitors (Alkaloid) were dissolved in t several concentrations (3.125, 6.25, 12.5, 25, 50, and 100 µg/ml) and added to test wells in separate rows. Then, 20 µL of 0.4 mM acetylthiocholine iodide (ACThI) and 0.3 mM DTNB were added to the enzyme solution, and the reaction was monitored for 30 min at room temperature. A microplate reader recorded changes in absorbance at 412 nm (Todd and Gomez 2001). The enzyme concentrations used were within the linear range of their toxicity.

$$\% \text{ inhibition} = \left[\frac{A_{\text{conc.}} - A_{\text{sample}}}{A_{\text{conc.}}} \right] \times 100$$

Determination of Enzyme kinetics (K_m and $V_{\max}$) and Mode of Inhibition

Lineweaver-Burk plot analysis was performed to determine the mode of enzyme inhibition and enzyme kinetics of the Alkaloid compound on AChE (Resistant and susceptible larvae). The enzyme inhibition kinetics was carried out in the absence and presence of *D. Stramonium* Alkaloid/fractions (ug/ml) at various concentrations of substrates (3.125, 6.25, 12.5, 25, 50 and 100mM). Kinetic parameters, including Michaelis-Menten (K_m) constant and maximum velocity ($V_{\max}$), were derived from the Lineweaver-Burk plot, while the inhibition constant (K_i) was derived from the Dixon plot (Khan and Kumar 2019).

Results and discussions

Inhibitory effect of the alkaloids on Acetylcholinesterase isolated from Resistant and Susceptible *Anopheles gambiae* Mosquito Larvae.

Table 1 presents the enzyme inhibitory effect of the crude alkaloid extract and the standard insecticide, Temephos (Abate), on acetylcholinesterase (AChE) isolated from both susceptible and resistant *Anopheles gambiae* mosquito larvae. The results indicate that both the alkaloid extract and Temephos inhibited acetylcholinesterase activity in a concentration-dependent manner. The alkaloid extract inhibited growth progressively with increasing concentration, ranging from 13.2% to 84.5% in the susceptible strain and 8.0% to 76.0% in the resistant strain. The inhibitory activity of the alkaloid extract was higher in susceptible larvae than in resistant larvae across all concentrations. The IC_{50} value of the alkaloid extract (30.3µg/ml) further supports this observation for the susceptible strain, compared to 59.6 µg/ml for the resistant strain. The standard insecticide Temephos consistently exhibited high inhibition, ranging from 48.8% to 99.4% across concentrations. The corresponding IC_{50} values of 52.1µg/ml (susceptible) and 19.1 µg/ml (resistant) reveal that Temephos inhibited acetylcholinesterase in resistant larvae more efficiently than in the susceptible strain.

Table 1: Inhibitory effect of the alkaloids on Acetylcholinesterase isolated from Resistant and Susceptible *Anopheles gambiae* Mosquito Larvae

Inhibition (%)	
Alkaloid	Temephos

Conc. (µg/ml)	sAChE	rAChE	sAChE	rAChE
3.125	13.2	8.0	0.0	48.8
6.25	32.5	15.6	0.0	50.0
12.5	46.7	18.0	0.0	98.0
25	64.3	19.9	15.2	93.2
50	68.1	53.0	56.1	98.8
100	84.5	76.0	77.5	99.4
IC ₅₀	30.3	59.6	52.1	19.1

sAChE=Acetylcholinesterase from susceptible *Anopheles gambiae* Larvae, rAChE=Acetylcholinesterase from resistant *Anopheles gambiae* Larvae

Inhibitory effect of the *D. stramonium* alkaloid fractions (DSAF) on Acetylcholinesterase isolated from Resistant and Susceptible *Anopheles gambiae* Mosquito Larvae.

Table 2 presents the inhibitory activity of four *Datura stramonium* alkaloid fractions (DSAF-1 to DSAF-4) on acetylcholinesterase (AChE) isolated from susceptible and resistant mosquito larvae, compared with temephos. The results show that among the fractions, DSAF-3 produced the highest inhibition (about 20%) in both strains, while DSAF-4 exhibited the lowest activity (3.0%) at the initial concentration. At 12.5 µg/ml, DSAF-3 showed the strongest inhibition (49.0% in both strains), followed by DSAF-1 and DSAF-2. At the same time, DSAF-4 and Temephos showed a sharp increase in resistant larvae (98.0%) but had no effect on susceptible larvae. At higher concentrations (25–100 µg/ml), the fractions maintained the same performance trend. DSAF-3 consistently produced the highest AChE inhibition, reaching 80% in both susceptible and resistant larvae at 50 µg/ml, and 89% at 100 µg/ml. DSAF-3 had the lowest IC₅₀ (22.8 µg/ml) in both susceptible and resistant larvae, indicating it is the most potent inhibitor among the plant fractions. Interestingly, temephos exhibited a strong inhibitory effect in resistant larvae (IC₅₀ = 19.1 µg/ml) but much lower potency in susceptible larvae (IC₅₀ = 52.1 µg/ml), suggesting differential sensitivity.

Table 2: Inhibitory effect of the *D. stramonium* alkaloid fractions (DSAF) on Acetylcholinesterase isolated from Resistant and Susceptible *Anopheles gambiae* Mosquito Larvae

Conc (µg/ml)	DSAF-1		DSAF-2		DSAF-3		DSAF-4		Temephos	
	sAChE	rAChE	sAChE	rAChE	sAChE	rAChE	sAChE	rAChE	sAChE	rAChE
3.125	10.7	11.0	5.9	5.8	20.1	20.0	3.0	3.0	0.0	48.8
6.25	20.0	20.0	11.1	11.1	33.4	33.3	5.9	5.8	0.0	50.0
12.5	32.3	32.4	19.3	19.3	49.0	49.0	10.8	10.7	0.0	98.0
25	50.0	50.0	33.4	33.4	66.7	66.7	20.0	19.9	15.2	93.2
50	66.6	66.7	50.0	50.1	80.0	80.1	33.6	33.4	56.1	98.8
100	80.0	80.0	66.7	66.7	88.9	89.0	50.3	50.0	77.5	99.4
IC ₅₀	42.8	42.7	63.7	63.6	22.8	22.8	93.3	94.0	52.1	19.1

DSAF=Datura stramonium Alkaloid fraction, sAChE=Acetylcholinesterase from susceptible *Anopheles gambiae* Larvae, rAChE= Acetylcholinesterase from resistant *Anopheles gambiae* Larvae

Effect of Temperature and pH on Resistant and Susceptible Acetylcholinesterase Enzyme after exposure to alkaloid extract

Figure 1 shows the effect of pH and temperature on the activity of resistant and susceptible Acetylcholinesterase (AChE) enzymes after exposure to the *Datura stramonium* alkaloid extract.

Effect of pH on enzyme activity for both resistant and susceptible AChE increased steadily as the pH rose from 5.0 to 7.5. At pH 5.0, resistant and susceptible enzymes showed low activity, 31% and 35%, respectively. Activity increased sharply between pH 6.0 and 7.5, with the resistant enzyme reaching 100% at pH 7.5 and the susceptible enzyme achieving 100% as well. Beyond pH 7.5, enzyme activity began to decline; the optimal AChE activity occurs at pH 7.0–7.5.

Effect of Temperature: The rAChE activity rose from 68% at 20°C to a peak of 92% at 40°C, while the susceptible enzyme activity ranged from 65% to 81%. However, enzyme activity declined sharply at higher temperatures, dropping to 80% and 74% at 45°C, 60% and 54% at 50°C, 37% and 32% at 55°C, and only 11% and 10% at 60°C for resistant and susceptible enzymes, respectively. The enzyme performs best at pH 7.5 and 40°C.

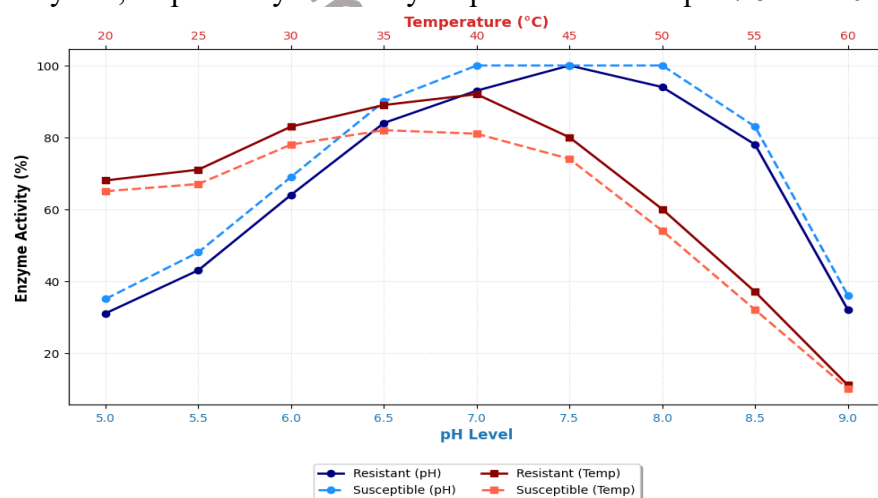


Figure 1: Effect of Temperature and pH on Resistant and Susceptible Acetylcholinesterase Enzyme after exposure to alkaloid extract

Enzyme Kinetics (K_m and V_{max}) and Mode of Inhibition of Crude Acetylcholinesterase Isolated from *Anopheles gambiae*

The effects of the tested inhibitors on the enzyme kinetics of crude acetylcholinesterase (AChE) isolated from *Anopheles gambiae* larvae are presented in Table 3, showing the maximum reaction velocity (V_{max}) and the Michaelis-Menten constant (K_m) for the resistant and susceptible strains. In Table 3, for Temephos, the susceptible larvae exhibited a considerably higher V_{max} (1.455) than the resistant strain (0.7246). While the K_m value for the resistant strain (488.03) was markedly elevated relative to the susceptible strain (32.050), thus reducing the affinity of the resistant AChE for the substrate when exposed to Temephos.

When treated with the alkaloid-rich extract, the resistant AChE sample showed a slightly higher V_{max} (0.980) than the susceptible strain (0.498). However, K_m values for both strains were low and relatively similar (13.139 and 14.199, respectively).

The DSAE-F3 fraction produced a V_{max} of 0.765 in the resistant strain and 0.151 in the susceptible strain. In both cases, K_m values were close (25.776 and 25.054, respectively), implying similar inhibitor-enzyme interactions across the two strains. The significantly lower V_{max} observed in the susceptible strain suggests a stronger inhibitory effect of DSAE-F3 on the sAChE compared to the rAChE.

The kinetic assessment of Acetylcholinesterase (AChE) inhibition in *Anopheles gambiae* in Figure 2 shows that Temephos produced a mixed mode of inhibition with a strong competitive component. In contrast, the alkaloid extract and the purified fraction DSAE-F3 demonstrated

non-competitive inhibition. The natural extracts predominantly exhibited non-competitive inhibition, whereas Temephos displayed mixed inhibition behaviour. Thus indicating that alkaloid extract and the purified fraction DSAE-F3 do not bind primarily to the enzyme's active site where acetylcholine is hydrolyzed. Instead, they likely interact with allosteric sites on the enzyme, causing conformational changes that reduce catalytic activity regardless of whether the substrate is bound. On the other hand the mechanism differs from that of Temephos, which showed mixed inhibition with a strong competitive component, indicating interaction near the active site.

Table 3 Enzyme kinetics (K_m and V_{max}) and mode of inhibition of Acetylcholinesterase isolated from *Anopheles gambiae*

Tested inhibitor	Enzyme strain	V_{max}	K_m
Temephos	Resistant	0.7246	488.030
	Susceptible	1.4550	32.0500
Alkaloid Extract	Resistant	0.9802	13.1396
	Susceptible	0.4975	14.1993
DSAE-F3	Resistant	0.7649	25.7764
	Susceptible	0.1507	25.0540

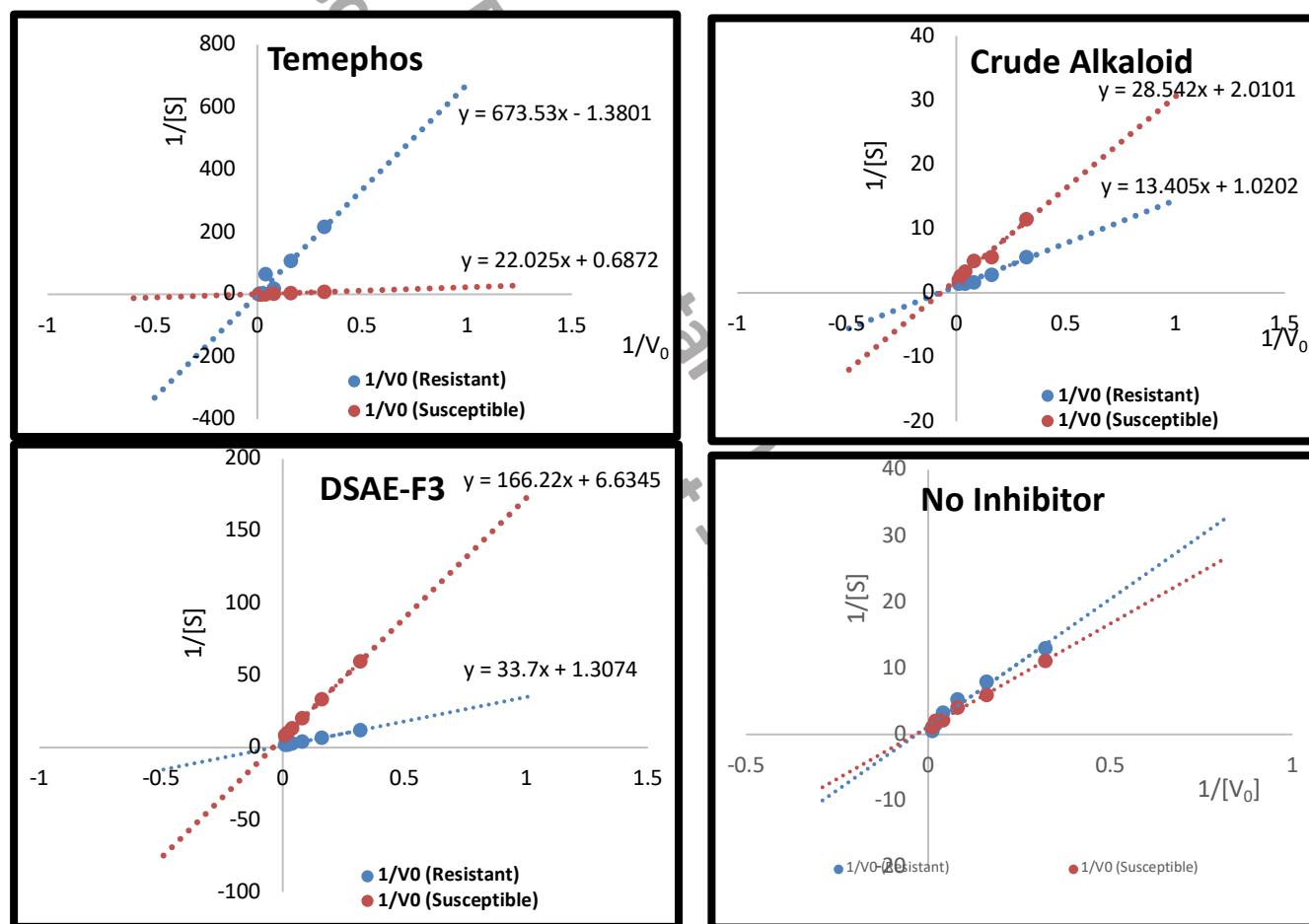


Figure 2: Lineweaver-Burk plot of Temephos, Crude Alkaloid and DSAE-F3 against AChE from Resistant and susceptible *Anopheles gambiae* larvae.

DISCUSSION

Inhibitory effect of the alkaloids on Acetylcholinesterase isolated from *Anopheles gambiae* Resistant and Susceptible Mosquito Larvae

The present study demonstrates that acetylcholinesterase (AChE) activity in *Anopheles gambiae* larvae was effectively inhibited by *Datura stramonium* alkaloid extracts and their purified fraction (DSAE-F3). Kinetic analysis revealed that inhibition by both the crude alkaloid extract and DSAE-F3 was predominantly non-competitive, indicating that enzyme activity was reduced through a mechanism independent of substrate competition at the catalytic active site. AChE inhibition disrupts synaptic transmission and induces neuromuscular paralysis, a mechanism known for many botanical and synthetic insecticides (Hemingway and Ranson 2017). The crude alkaloid extract produced higher inhibitory effects in susceptible larvae (13.2–84.5%) than in resistant larvae (8.0–76.0%), as reflected in the IC₅₀ values. The consistency of IC₅₀ values across susceptible and resistant strains further reinforces the hypothesis that DSAF-3 acts through a conserved binding site unaffected by resistance-associated biochemical changes. This pattern suggests reduced enzyme sensitivity in resistant populations, a phenomenon that resistance often results from AChE insensitivity or conformational changes that reduce inhibitor binding (Fournier 2018; Widiastuti et al. 2020). The inhibitory effects of *D. stramonium* alkaloid fractions were interesting: DSAF-3 exhibited similar inhibitory profiles in both susceptible and resistant larvae; temephos again showed disproportionately stronger inhibition in resistant larvae. This contrasts with previous reports that resistant *Anopheles* strains often exhibit lowered sensitivity to organophosphates due to ace-1 mutations (Djogbenou et al. 2018). The current results may reflect alternative resistance pathways in the studied population, such as metabolic resistance or mixed-function oxidase induction, rather than true AChE insensitivity. The plant seed of *D. stramonium* contains potent neuroactive alkaloids capable of inhibiting AChE, while their effectiveness differs between susceptible and resistant larval strains. The increased potency of DSAF-3 confirms that fractionation enhances bioactivity, highlighting its promise as a botanical larvicide candidate for vector management and control.

Effect of Enzyme Kinetics (K_m and V_{max}) and Mode of Inhibition of Crude Acetylcholinesterase Isolated from *Anopheles gambiae*

The kinetic behaviour of crude AChE from *Anopheles gambiae* demonstrated distinct inhibition patterns across the tested compounds. The alkaloid extract and its fraction DSAF3 both exhibited non-competitive inhibition, characterized by reduced V_{max} with minimal change in K_m . This is consistent with recent work demonstrating that plant-derived compounds, including alkaloids and essential oils, frequently inhibit AChE by interacting with allosteric or peripheral sites rather than the catalytic centre, thereby reducing catalytic efficiency without significantly altering substrate affinity (Rants'o et al. 2022; Hillary 2024). The markedly lower V_{max} in the susceptible strain exposed to DSAF3 indicates greater inhibition, a phenomenon also reported for purified fractions that exhibit greater potency than crude extracts (Okumu et al. 2024).

The non-competitive inhibition observed for the alkaloid extract and DSAE-F3 indicates that these compounds act through allosteric modulation of AChE, enabling them to suppress enzyme activity in both susceptible and resistant strains of *Anopheles gambiae*. The results suggest that while resistance-associated structural changes strongly influence Temephos inhibition in AChE, the alkaloid extract and DSAF3 act through mechanisms that circumvent active-site modifications. This supports the growing advocacy for botanical-based insecticides as promising alternatives for managing resistant mosquito populations (Hillary 2024; Rants'o et al. 2022). These findings suggest that *D. stramonium* alkaloids, particularly the DSAF-3 fraction, inhibit AChE through a reversible, allosteric, and resistance-independent mechanism. This distinct mode of action compared to Temephos highlights their potential as alternative bioinsecticidal agents for managing insecticide-resistant *Anopheles gambiae* populations.

Conclusions

This study demonstrates that alkaloids derived from *Datura stramonium* seeds possess substantial inhibitory activity against acetylcholinesterase (AChE) isolated from both susceptible and insecticide-resistant *Anopheles gambiae* larvae, underscoring their potential relevance in malaria vector control. The crude alkaloid extract exhibited clear concentration-dependent AChE inhibition in both strains, with greater potency against the susceptible enzyme but sustained activity against the resistant variant, as reflected by comparatively moderate IC₅₀ values. The fractionation of the crude alkaloid enhanced bioactivity, with DSAF-3 as the most potent fraction, achieving high levels of AChE inhibition in both resistant and susceptible larvae and displaying identical IC₅₀ values across strains. This indicates a reduced sensitivity of DSAF-3 to resistance-associated target-site modifications. Biochemical characterisation showed that optimal AChE activity occurred at pH 7.0–7.5 and 40°C. Enzyme kinetic analyses further revealed mechanistic distinctions between the plant-derived inhibitors and the conventional organophosphate temephos. While temephos exhibited mixed inhibition with a strong competitive component and reduced substrate affinity in resistant AChE, the crude alkaloid extract and DSAF-3 displayed non-competitive inhibition, suggesting that *D. stramonium* alkaloids interact with AChE at sites distinct from the classical organophosphate binding region, thereby bypassing established resistance mechanisms. The findings provide strong biochemical evidence that *D. stramonium* seed alkaloids, particularly fraction DSAF-3, represent promising lead candidates for the development of novel, plant-based larvicides or synergistic agents targeting resistant *Anopheles* populations.

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