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Nicholus C. Sande

nicholus.sande@gmail.com

University of Zambia

Martin Simuunza

University of Zambia

Kaampwe Muzandu

University of Zambia

Danny Muzata

University of Zambia

Chiluba Zimba

University of Zambia

Busiku Hamainza

National Malaria Elimination Centre

Mulenga Mwenda

PATH

Brenda Mambwe

PATH

Rabecca Ngwira

PMI-VectorLink

Jossy Mweene

National Malaria Elimination Centre

Mutinta Mudenda

National Malaria Elimination Centre

Enala Mwase

University of Zambia

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Insecticidal effectiveness of nicotine from *Nicotiana tabacum* against sub-species of *Anopheles gambiae* sensu lato and *Anopheles funestus* sensu lato (Diptera: Culicidae) from the Luapula Province of Zambia.

Nicholus C. Sande^{1,2*}, Martin Simuunza^{3,4}, Kaampwe Muzandu⁵, Danny Muzata², Chilubaimba⁶, Busiku Hamainza¹, Mulenga Mwenda⁶, Brenda Mambwe⁶, Rabbecca Ngwira⁷, Jossy Mweene¹, Mutinta Mudenda¹, Javan Chanda⁶ and Enala Mwase²

*Correspondence:
Nicholus Chintu Sande
nicholus.sande@gmail.com

Abstract

Background: One of the main challenges affecting malaria control initiatives in sub-Saharan Africa is the swift and widespread emergence of insecticide resistance. Consequently, there is a need to explore alternative insecticides for controlling malaria vectors. This study investigated the effectiveness of tobacco extract on wild *Anopheles* mosquitoes during the dry season (October–November 2021) in Chebele village, Mwense District, Luapula Province, Zambia.

Methods: Wild *Anopheles* larvae were collected using the pipetting and dipping method along the Mwense stream and reared to the F1 progeny. Wild *Anopheles* mosquitoes were identified using morphological taxonomic keys. Specimens belonging to the *Anopheles gambiae* complex and *Anopheles funestus* group were further identified by multiplex Polymerase Chain Reaction (PCR). The solvent extraction method was used to extract tobacco compounds from tobacco leaves.

Filter papers were impregnated with clothianidin (0.132 g/ml; SumiShield® 50WG) in accordance with the World Health Organization (WHO) Standard Operating Procedure for susceptibility testing (tube tests). Clothianidin was used as a positive control, while distilled water served as a negative control. Non-blood-fed, 2- to 3-day-old female wild *Anopheles* mosquitoes were exposed to clothianidin (0.132 g/ml) and tobacco extract at different

concentrations (25% v/v, 33.3% v/v, 50% v/v, 62.5% v/v, 71.43% v/v, and 83.3% v/v) using the World Health Organization (WHO) bottle bioassay. The insectary-reared Kisumu strain (*An. gambiae* sensu stricto) was used as a reference for insecticide susceptibility tests.

Results: Morphological identification of adult mosquitoes collected from 73 households showed that 98.03% were *Anopheles funestus* s.l. (n = 199), 1.48% were *Anopheles gambiae* s.l. (n = 3), and 0.49% were *Culex* species (n = 1). Subsequent PCR analysis of the wild-caught adult mosquitoes revealed that *Anopheles funestus* sensu stricto (s.s.) (n = 93; 46%) and *Anopheles gambiae* s.s. (n = 81; 40%) were the dominant species within the *An. funestus* group and the *An. gambiae* complex, respectively.

Tobacco extract (83.3% v/v) and clothianidin (0.132 g/ml) were found to have mean knockdown rates at 80 minutes of 61.88% (95% CI: 34.03–89.74%) and 85.05% (95% CI: 72.01–98.08%), respectively. Results showed that both clothianidin and tobacco extract elicited 100% mortality in adult wild *Anopheles* mosquitoes and the Kisumu strain after 24 hours. The estimated LC₅₀ for tobacco extract on wild *Anopheles* mosquitoes was estimated to 38.77% v/v (95% CI: 9.99–55.91% v/v), as determined by probit analysis.

Conclusion: The findings of this study suggest that *Nicotiana tabacum* leaf extract may be considered a potential bio-insecticide for controlling *An. gambiae* s.s. and *An. funestus* s.s., which are significant vectors of the malaria pathogen.

Keyword: *Anopheles gambiae*, *Anopheles funestus*, lethal concentration, *Nicotiana tabacum*, vector control, Zambia.

Background

Anopheles mosquitoes are crucial to public health because they transmit diseases that affect humans, such as malaria [38, 35]. The *Anopheles gambiae* and *Anopheles funestus* species complexes are largely responsible for malaria transmission in Africa [12]. Malaria causes approximately 405,000 deaths worldwide each year, with around 90% occurring in Africa [20, 26].

The sub-species of *Anopheles gambiae* sensu lato (s.l.) include *An. gambiae* sensu stricto (s.s.), *An. arabiensis*, *An. merus*, *An. melas*, *An. bwambae*, *An. quadriannulatus*, *An. amharicus*, *An. coluzzii*, and *An. fontenillei* [9, 17]. The *Anopheles funestus* group comprises 11 recognized species and is widely distributed across the African continent [45]. These include *An. funestus*,

An. funestus-like, *An. vaneedeni*, *An. leesoni*, *An. rivulorum*, *An. rivulorum*-like, *An. parensis*, *An. fuscivenosus*, *An. aruni*, *An. brucei*, and *An. confusus* [18].

According to Sitali et al. [43], 98% of malaria cases in Zambia are caused by *Plasmodium falciparum*, 2% by *Plasmodium malariae*, while *Plasmodium vivax* is a rare cause of infection. However, the spatial distribution of *Plasmodium* species in Zambia is not clearly established. Malaria is endemic throughout the country and remains a major public health concern in many areas [37]. The 2021 Malaria Indicator Survey (MIS) found that Luapula Province had the highest prevalence (63.3%) in children under five years of age [36].

For some time, indoor chemical vector control tools have been effective [31]. However, the rapid evolution and spread of insecticide resistance among primary malaria vectors in sub-Saharan Africa have posed a significant threat to vector control efforts. The extensive use of synthetic insecticides has made eco-friendly alternatives a priority [54, 16]. The existence of several bioactive chemicals in botanicals that can inhibit insecticide resistance, alongside their low environmental persistence and lower cost, makes botanical crude extracts advantageous for disease vector control [47].

One of the first known botanical pesticides is nicotine, which was employed to suppress plum bugs from the late eighteenth to the early nineteenth century. Due to its significant toxicity to mammals and the subsequent emergence of more potent synthetic insecticides, its use eventually decreased. Another plant-based pesticide, rotenone, was introduced around 1850 [3]. The usage of botanical insecticides significantly decreased in the 1940s due to the widespread use of synthetic insecticides, especially dichlorodiphenyltrichloroethane (DDT). However, a resurgence of interest in botanical insecticides as potential substitutes was spurred by worries about environmental contamination and the development of insecticide resistance linked to DDT [13].

Nicotine, nornicotine, and anabasine found in tobacco extract are phenolic compounds that act as neurotransmitters, mimicking the action of acetylcholine, similar to organophosphate and carbamate insecticides [28]. Nicotine and nornicotine are the principal alkaloids in *Nicotiana* species with insecticidal properties, although the effectiveness varies depending on the insect species [56]. Nicotine, a key constituent of tobacco, is neurotoxic to insects and causes rapid knockdown effects [1].

The efficacy of nicotine and its derivatives against mosquitoes forms the basis for growing interest in using tobacco crude extracts for mosquito control [21]. Therefore, this study aimed

to determine the potential of *Nicotiana tabacum* leaf extract as a bio-insecticide against malaria vectors.

Materials and Methods

This study was conducted in Mwense District, located in Luapula Province of Zambia (Figure 1), between October and November 2021. Mwense District lies in the northern part of Zambia at coordinates 10°25'0" S and 29°0'0" E [39]. It shares an international boundary with the Democratic Republic of Congo [44]. The district's vegetation cover consists of Miombo forest and grassland, with *Hyparrhenia* as the dominant species, while low-growing grasses and other plant species predominate in waterlogged areas [24]. The average annual temperature in the district ranges from 12.8°C to 35°C, rarely falling below 10.6°C or rising above 37.8°C [14]. Luapula Province receives approximately 719.83 millimetres of rainfall between December and February, and 190.95 millimetres between September and November [51]. The peak of malaria transmission in Mwense District occurs during the rainy season from November to May, with a reported incidence rate of 787 cases per 1,000 persons per year [55]. Previous studies have shown that *Anopheles funestus* s.s. and *Anopheles gambiae* s.s. were the principal malaria vector species in Luapula Province [46, 29]. Mwense District was selected due to its high malaria burden, suitable ecological conditions, and ongoing anthropogenic activities that enhance vector breeding. This study was specifically conducted in Chebele Village, located along the Mwense stream. In this village, anthropogenic activities such as brick making have created deep holes in the soil, which serve as temporary breeding sites for mosquitoes.

Study area

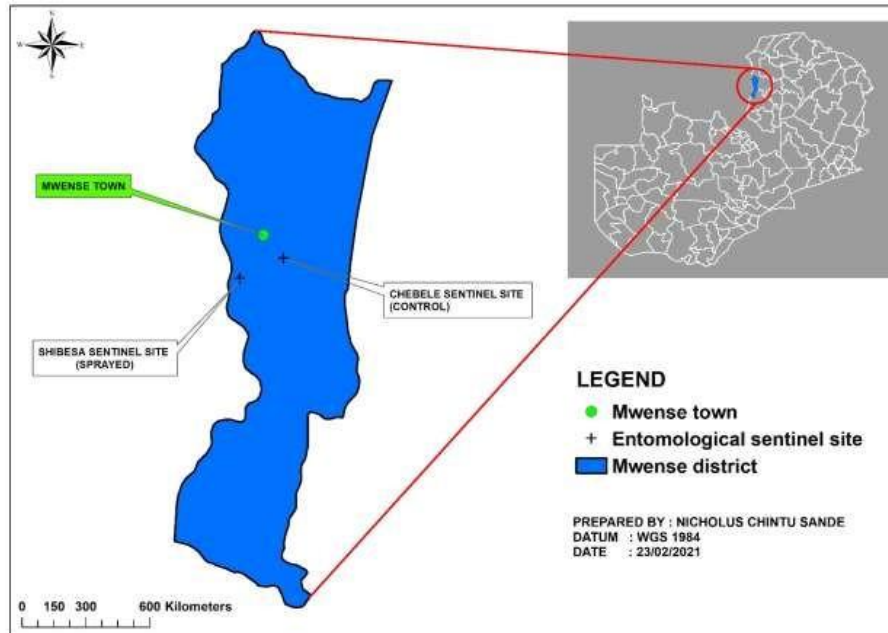


Figure 1: Location of entomological sentinel sites in Mwense district in Luapula Province.

Selection of village households and sample size

This study was conducted in collaboration with the Mwense District Health Office and employed a cross-sectional entomological survey using a mixed-methods approach. A Community Health Worker (CHW) assisted with community engagement and supported the sampling of *Anopheles* larvae from identified breeding habitats. Consent was obtained from household heads prior to indoor aspiration of adult mosquitoes using Prokopack aspirators. The number of households included in the study was determined using the sample size formula described by Almeda et al. [5], assuming a margin of error (e) of 0.1. The total number of households (N) in the village was 270. Seventy-three (73) households with open eaves were selected from Chebele village using simple random sampling. All selected houses were geocoded using the Global Positioning System (GPS) Test application. Mosquitoes collected indoors from these households were used for morphological species identification, and were not exposed to insecticides.

The determination of sample size for insecticide susceptibility testing was guided by WHO

Standard Operating Procedures (SOPs) [52], where a minimum of 100 mosquitoes per insecticide is required. These mosquitoes were obtained from field-collected larvae reared to adulthood. A total of 27 WHO bottle bioassays were conducted, with each assay using the recommended 20 to 25 mosquitoes, according to WHO guidelines. This design enabled assessment of wild adult mosquito susceptibility to insecticides during the malaria transmission season.

Collection of *Nicotiana tabacum* and preparation of tobacco extract

Fresh leaves of *Nicotiana tabacum* (2 kg) were sourced from a small-scale tobacco farmer in Nkeyema District, Western Province, Zambia, and transported to the Entomology Laboratory at the field station in Kaoma District for processing. Tobacco crude extract was prepared using a solvent extraction method as described by Al-Dahhan et al. [4]. Briefly, the leaves were airdried until brittle, ground into powder using a mortar and pestle, and sieved through a 1.40 mm mesh. The resulting powder was stored in zip-lock bags at 4°C until use. For extraction, 10 g of tobacco powder were mixed with 5% sodium hydroxide (NaOH) in a 250 ml beaker, stirred for 15 minutes, and filtered using a Büchner funnel. Twenty-five millilitres of petroleum ether were added to the filtrate, agitated for 30 minutes, and left to separate into two layers. The organic layer was dried using Whatman No. 1 filter paper and Magnesium Sulphate Heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$). The final extract was concentrated in a water bath at 80°C to remove residual solvent and stored in glass tubes at 4°C. The presence of nicotine was confirmed by the formation of a red-orange precipitate upon addition of Dragendorff reagent [8]. The nicotine content in *N. tabacum* typically ranges from 0.3% to 5% [10].

Collection of adult wild mosquitoes

Indoor-resting female *Anopheles* mosquitoes were collected from the 73 randomly selected households in Chebele village using a Prokopack aspirator (John W. Hock Company, Florida, USA) between 04:00 and 05:00 hours. The collections targeted endophagic and anthropophilic mosquitoes and were conducted early in the morning as occupants vacated their homes. The Prokopack device, powered by a 12V battery, was used to aspirate mosquitoes from indoor surfaces. Captured mosquitoes were transferred into BugDorm-1 cages (Mega View Science Co., Ltd, Taichung, Taiwan), lined with moist cotton towels to maintain humidity and promote survival. Specimens were temporarily held for 24 hours to monitor for potential knockdown effects due to mechanical aspiration stress. Knocked-down individuals were carefully separated and stored in Eppendorf tubes. All collected mosquitoes were transported to the central field

insectary at Grand Palm Lodge (10°23'7.80"S, 28°40'33.49"E) in Mwense town for further processing.

Laboratory-reared *Anopheles gambiae* s.s mosquitoes

The laboratory strain of *Anopheles gambiae* s.s. (Kisumu strain) that was used as a reference in the experiment was obtained from the National Malaria Elimination Centre (NMEC). This strain had never been exposed to any insecticides and was therefore considered susceptible. The colony was maintained continuously at 25–27°C and 75% relative humidity. Female *An. gambiae* s.s. (Kisumu), aged 2–5 days, were used for the bioassays

Collection of wild *Anopheles* larvae using pipetting and dipping method

Wild *Anopheles* larvae were collected in Mwense district near the banks of Mwense stream (10°23'58.07''S and 28°42'46.77''E) (Figure 2). Dippers, pipettes, and white plastic trays were used to collect the larvae. Dippers were used to scoop wild larvae from breeding sites, followed by the use of pipettes to collect individual larvae from the dippers. The larvae were then transferred into plastic trays, which served as temporary holding containers during sample collection. Additionally, using a pipette, wild larvae were moved from plastic trays into tiny holding breeding cups and then brought to the field laboratory, where they were raised from various instar stages through the pupa stage and ultimately to the adult stage.

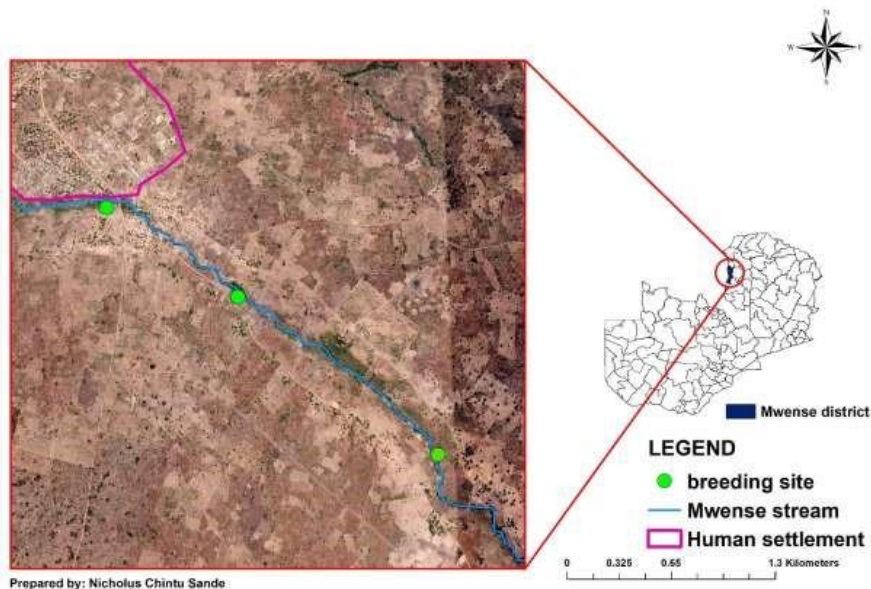


Figure 2: Location of breeding sites of wild *Anopheles* mosquitoes in Chebele study site in Mwense district of Luapula Province in Zambia.

Rearing of wild *Anopheles* larvae to the adult stage

Wild *Anopheles* larvae were reared to the F₁ generation under field conditions at the central field insectary located at Grand Palm Lodge in Mwense Town. Larvae were housed in plastic trays containing water with organic material and were fed twice daily, in the morning and evening, using a pinch of powdered Tetramin flakes fish feed. Feeding was done carefully to avoid overfeeding due to the presence of organic substances in the water. Upon pupation, larvae were collected using pipettes, transferred into plastic cups containing distilled water, and placed inside adult rearing cages to allow adult emergence. Emerged adults were maintained on a 10% glucose solution. Rearing was conducted under ambient field conditions, with temperatures maintained at $30 \pm 2^\circ\text{C}$ and relative humidity between 50–60%.

Preparation of insecticide-treated materials and bioassay procedures for Clothianidin (SumiShield® 50WG) and Tobacco Extract

Standardized procedures developed and optimized by PMI-AIRS were followed to prepare insecticide-treated filter papers for clothianidin (SumiShield® 50WG) [42]. Whatman® No. 1 filter papers (12 cm × 15 cm) were treated with 2 ml of a 0.132 g/ml suspension, prepared by dissolving 2.64 g of SumiShield® 50WG (50% clothianidin active ingredient) in 20 ml of distilled water. The mixture was evenly distributed onto filter papers placed on a nail bed to ensure uniform absorption. Papers were dried overnight and stored in zip-lock plastic bags at 4°C. Control papers were treated with 2 ml of distilled water.

For the plant-based insecticide, various concentrations of *Nicotiana tabacum* (25%, 33.3%, 50%, 62.5%, 71.43%, and 83.3% v/v) were prepared using distilled water. Due to its volatility, 2 ml of each concentration was absorbed onto circular cotton wool pads (Premier MPC Ltd., Lusaka, Zambia). Pads treated with 2 ml of distilled water served as the negative control.

Susceptibility bioassays were conducted on non-blood-fed wild *Anopheles* mosquitoes aged 2 to 3 days, following WHO guidelines [50, 52]. A total of 142 wild *Anopheles* mosquitoes were tested against clothianidin (0.132 mg/ml) across six replicates, with one replicate (n = 25) serving as a control. For tobacco extract, a total of 370 wild *Anopheles* mosquitoes from Chebele were tested at six different concentrations (25% to 83.3% v/v) in 15 replicates. In addition, 104 insectary-reared Kisumu strain mosquitoes were tested with tobacco extract (83.3% v/v) across four replicates, plus one control replicate.

Mosquitoes were exposed for 80 minutes and transferred to holding tubes with 10% sugarsoaked cotton wool. Knockdown was recorded at intervals (10–80 min), and mortality assessed after 24 hours. Bioassays were conducted under controlled conditions (25°C ± 2°C, 75% ± 10% RH) per WHO guidelines, with actual room temperature and humidity recorded.

Both susceptible and resistant wild mosquitoes were preserved in 1.5 ml Eppendorf tubes containing silica gel and transported at room temperature to the National Malaria Elimination Centre (NMEC) in Lusaka for molecular analysis.

Morphological identification of *Anopheles* mosquitoes

All wild *Anopheles* mosquitoes obtained from larval and indoor adult collections were initially identified morphologically in the field using the Coetzee identification key [17]. Morphological

identification was used to classify specimens to species complex or group level (*An. funestus* s.l. and *An. gambiae* s.l.).

Determination of mosquito abdominal (feeding) status

The feeding status of adult female mosquitoes was determined during morphological identification based on visual examination of the abdomen using standard entomological criteria. Mosquitoes were classified as unfed, blood-fed, half-gravid, or gravid according to abdominal distension and coloration [50].

Molecular identification of *Anopheles* species

Only mosquitoes collected as larvae were subsequently subjected to molecular analysis. Specimens that were phenotypically classified as susceptible or resistant to the two insecticides were individually labelled and preserved in 1.5 ml Eppendorf tubes containing silica gel and cotton wool. Samples were stored at room temperature and transported to the National Malaria Elimination Centre (NMEC) laboratory in Lusaka for molecular analysis.

Genomic DNA was extracted from mosquito body parts (legs, wings, and abdomen) of morphologically identified *An. funestus* s.l. and *An. gambiae* s.l. specimens [30] using the Tween–Chelex extraction method [47]. Extracted DNA was used for PCR-based species identification. The PCR primers used as species identifiers within *An. gambiae* sensu lato and *An. funestus* sensu lato are shown in table 1.

Table 1: Sequences of primers used for species identification of *Anopheles gambiae* s.l and *Anopheles funestus* s.l

Species Group	Primer Name	Sequence (5'–3')	Direction	Reference
<i>An. gambiae</i> s.l	UN	GTG TGC CCC TTC CTC GAT GT	Forward	[30]
	GA	CTG GTT TGG TCG GCA CGT TT	Reverse	[30]
	MR	TGA CCA ACC CAC TCC CTT GA	Reverse	[30]
	AR	AAG TGT CCT TCT CCA TCC TA	Reverse	[30]
	QD	CAG ACC AAG ATG GTT AGT AT	Reverse	[30]
<i>An. funestus</i> s.l	UV	TGT GAA CTG CAG ACA T	Forward	[32]
	FUN	GCA TCG ATG GGT TAA TCA TG	Reverse	[32]
	VAN	TGT CGA CTT GGT AGC CGA AC	Reverse	[32]
	RIV	CAA GCC GTT CGA CCC TGA TT	Reverse	[32]

Species Group	Primer Name	Sequence (5'–3')	Direction	Reference
	PAR	TGC GGT CCC AAG CTA GGT TC	Reverse	[32]
	RIV-LIKE	CCG CCT CCC GTG GAG TGG GGG	Reverse	[32]
	LEES	TAC ACG GGC GCC ATG TAG TT	Reverse	[32]

Sub-species of *An. funestus* sensu lato and *An. gambiae* sensu lato. were identified using a multiplex Polymerase Chain Reaction (PCR) assay, with expected amplicon sizes of 505 bp for *An. funestus* s.s. and 390 bp for *An. gambiae* s.s. PCR assays for *Anopheles funestus* s.l. and *Anopheles gambiae* s.l. were performed as previously described [11, 23, 32, 49]. Briefly, the *An. funestus* s.l. assay targets species-specific single nucleotide polymorphisms (SNPs) within the internal transcribed spacer 2 (ITS2) region, while the *An. gambiae* s.l. assay targets the intergenic spacer (IGS) region. Each PCR run included a positive control (DNA from morphologically confirmed reference species) and a negative control (PCR-grade water)

Gel electrophoresis of the PCR product

The PCR products were electrophoresed on a 1.5% agarose gel prepared with Tris-Borate/EDTA (TBE) buffer containing Tris-Base (89 mM), Boric Acid (89 mM), and EDTA (2 mM), and prestained with 4 µl of Midori Green dye. For each sample, 8 µl of PCR product was mixed with 2 µl of loading dye and loaded into individual wells. A 100 bp DNA ladder was included as a molecular size marker. The gel was run and visualized under ultraviolet (UV) light using a gel documentation system, and amplicon sizes were determined by comparison with the DNA ladder. Amplicon sizes were determined relative to a 100 bp ladder.

Statistical analysis

Data were entered in the Microsoft excel® (Microsoft Corporation, Redmond, WA, 2010) software and later transferred to SPSS statistics 23.0 version (IBM, USA) for analysis. Efficacy of insecticides against wild *Anopheles* were calculated as percentage mortality following WHO standards to determine phenotypic resistance frequency with 98% - 100% mortality signifying susceptibility; 90% - 97% mortality signifying possible resistance and mortality less than 90% signifying resistance [52]. Data from the WHO bottle bioassays were further analyzed using Probit regression to estimate the lethal concentration required to kill 50% of the test population (LC₅₀), along with the corresponding 95% confidence intervals. The concentrations of the insecticides were log transformed during the analysis and exponentiated back to their original scale during

interpretation. A non-significant Chi-square goodness of fit test was used to determine that the model fitted the data. All statistics were considered significant at $p \leq 0.050$.

RESULTS Mosquito species composition from households

A total of 203 indoor resting biting mosquitoes were collected in 73 selected houses in Chebele village in Mwense district using Prokopack Aspirator, of which 86.70% (n=176) were morphologically identified as fed female of *An. funestus* s.l Giles, 8.87% (n=18) as unfed female *An. funestus* s.l Giles, 2.46% (n=5) as unfed male *An. funestus* s.l Giles, 0.49% (n=1) as fed female *An. gambiae* s.l Giles, 0.99% (n=2) as unfed female *An. gambiae* s.l Giles and 0.49% (n=1) as *Culex* species.

Species identification

Multiplex Polymerase chain reaction (PCR) was performed on a random subsample (n=202) of only wild female *An. funestus* s.l and wild female *An. gambiae* s.l that were exposed to (clothianidin) SumiShield® 50WG and tobacco extract. Molecular analysis confirmed that 40% were *An. gambiae* s.s, while 14% failed to amplify. About 46% of the samples were confirmed to be *An. funestus* s.s.

Bioassay of Tobacco Extract and Clothianidin Against *Anopheles* Mosquitoes

A total of 512 mosquitoes were exposed to varying concentrations of tobacco extract and clothianidin in bioassays to evaluate knockdown and mortality rates. Tobacco extract showed a concentration-dependent effect on wild *Anopheles* mosquitoes from Chebele. Mortality at 24 hours ranged from 66.67% at 25% v/v to 100% at 83.3% v/v (Table 2). Resistance was observed at concentrations below 83.3% v/v, with full susceptibility (100% mortality) noted only at the highest concentration (83.3% v/v). Knockdown increased progressively across time intervals, with 99.41% knockdown at 80 minutes at 83.3% v/v (Table 2).

Mosquitoes exposed to clothianidin (0.132 mg/ml) showed 100% mortality within 24 hours, indicating complete susceptibility. The Kisumu strain and wild *Anopheles* exposed to distilled water showed 0% mortality, confirming the validity of the controls. The Kisumu strain exposed

to tobacco extract (83.3% v/v) exhibited 100% mortality, confirming susceptibility and supporting the bio-efficacy of the extract at this concentration. The percentages of mosquitoes classified as susceptible or resistant are presented in Figure 3.

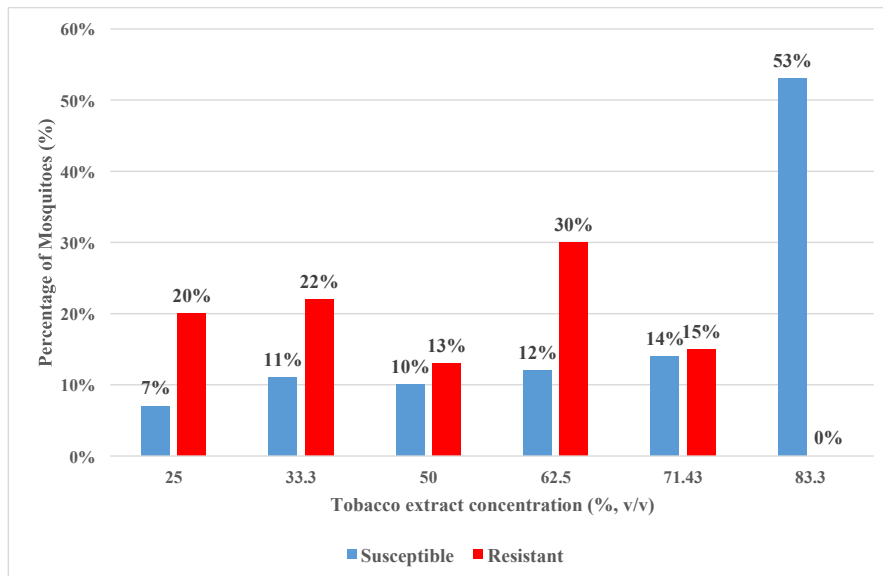


Figure 3: Susceptibility and resistance of mosquitoes exposed to varying concentrations of tobacco extract in Mwense District, Luapula Province, Zambia.

Table 1: Cumulative percentage mortality and knockdown percentage of female wild *Anopheles* mosquitoes and Kisumu susceptible strain populations in response to clothianidin and Tobacco extract concentrations.

Site/Population	Insecticide	Sample size (N ^a)	%Knock down at 10 min	%Knock down at 15 min	%Knock down at 20 min	%Knock down at 30 min	%Knock down at 40 min	%Knock down at 50 min	%Knock down at 60 min	%Knock down at 80 min	%Mortality	
											24 hours	Status
^b Chebele	Tobacco extract (25% v/v)	33	24.24	33.33	27.3	63.64	60.6	78.8	66.67	75.76	66.67	R
Wild <i>Anopheles</i>												
^b Chebele	Tobacco extract (33.33% v/v)	46	13.04	21.7	30.4	56.52	69.6	73.9	73.91	84.78	73.91	R
Wild <i>Anopheles</i>												
^b Chebele	Tobacco extract (50% v/v)	37	27.03	27.03	37.8	40.54	32.4	48.5	48.65	72.97	81.08	R
Wild <i>Anopheles</i>												
^b Chebele	Tobacco extract (62.5% v/v)	54	9.26	9.26	16.7	27.78	33.3	44.4	57.41	79.63	70.73	R
Wild <i>Anopheles</i>												
^b Chebele	Tobacco extract (71.43% v/v)	32	12.5	31.3	34.4	59.38	71.9	78.1	90.63	93.75	75.00	R
Wild <i>Anopheles</i>												

Commented [U1]:

^b Chebele	Tobacco extract (83.3 % v/v)	142	4.93	26.76	43.66	69.01	78.87	83.1	88.73	100.00	100.00	S
<i>Anopheles</i> Wild												
^b Chebele	Clothianidin (0.132 g/ml)	116	14.66	50.00	64.66	87.93	94.83	96.55	95.69	98.28	100.00	S
<i>Anopheles</i> Kisumu Strain												
^c Chainama	Tobacco extract (83.3% v/v)	104	98.08	98.08	98.08	98.08	98.08	98.08	98.08	98.08	100.00	S
Kisumu strain												
^c Chainama	Clothianidin (0.132 g/ml)	116	14.66	50.00	64.66	87.93	94.83	96.55	95.69	98.28	100.00	S
Kisumu Strain												
[*] Chebele												
Wild <i>Anopheles</i>	Distilled water	25	0	0	0	0	0	0	0	0	0	-
[*] Chainama												
Kisumu strain	Distilled water	25	0	0	0	0	0	0	0	0	0	-

^aTotal number of mosquitoes exposed to each insecticide, ^b Source of wild mosquitoes, ^cSource of Kisumu strain, ^{*}Negative control, R= Resistance, S=Susceptible

Probit model estimation

The Chi-square goodness of fit test was non-significant ($p = 0.459$), indicating that the model fitted the data. The analysis showed that mosquito mortality was significantly associated with the concentration of the tobacco extract (Estimate = 1.178, (95% CI = 0.220–2.135), $p = 0.016$), The LC_{50} was estimated to be 38.77% v/v (95% CI: 9.99 – 55.91% v/v).

DISCUSSION

This study aimed to evaluate the insecticidal effectiveness of tobacco (*Nicotiana tabacum*) leaf extract against wild sub-species of *Anopheles gambiae* sensu lato (s.l.) under field conditions in Chebele, Mwense District, Luapula Province, Zambia. Specifically, the study sought to identify *Anopheles gambiae* sensu stricto (s.s.) and *Anopheles funestus* s.s. within their respective species complexes and determine the lethal concentrations of tobacco extract required to knock down these malaria vectors. Determining effective concentrations of plant-based insecticides is essential for understanding vector susceptibility and developing alternative tools for monitoring and managing insecticide resistance. With resistance to conventional insecticides threatening the sustainability of malaria control programmes [14], the exploration of botanicals such as tobacco extract offers a promising, locally accessible alternative for vector management in endemic areas. The use of synthetic insecticides in mosquito control has been restricted due to their high cost, adverse effects on environmental quality, non-biodegradable nature, harmful impacts on human and animal health, and the development of resistance [5]. One potential way to overcome these challenges is to use plant-derived insecticides as substitutes for synthetic ones [25].

This study identified *Anopheles funestus* s.s. and *Anopheles gambiae* s.s. as the only confirmed members of the *An. funestus* and *An. gambiae* species complexes in Chebele village, based on PCR analysis of 202 samples. However, 14% of the samples failed to amplify, as such the presence of other sibling species cannot be ruled out. Furthermore, the larval collection results indicated that *An. funestus* s.l. was the predominant *Anopheles* species compared to *An. gambiae* s.l. in Chebele village during the dry season (October–November 2021). This finding aligns with a previous study, which reported that *An. funestus* s.l. was the dominant malaria vector over *An. gambiae* s.l., with an overall proportion of 87.6% from August 2018 to June 2019 in Mwense District [42].

The study's findings showed that clothianidin (SumiShield® 50WG) and tobacco extract had a knockdown effect on wild *An. funestus* s.s. and *An. gambiae* s.s. Under field conditions, both compounds exhibited distinct mean knockdown percentages after 80 minutes of exposure, with tobacco extract demonstrating a greater knockdown effect. One hundred percent (100%) mortality was observed 24 hours post-exposure to both clothianidin (SumiShield® 50WG) and tobacco extract against wild *An. funestus* s.s. and *An. gambiae* s.s. Similar studies by Oxborough et al. [40] and Ileke et al. [27] also reported 100% mortality at 24 hours following exposure to clothianidin and tobacco extract. However, a study conducted in Northern Tanzania found that clothianidin at a concentration of 2% produced 80% mortality against a wild, resistant population of *Anopheles arabiensis* [33]. In contrast, in Benin (West Africa), a WHO cone bioassay assessing the effectiveness of clothianidin against wild *An. gambiae* s.l. recorded a 91.7% mortality rate at 120 hours post-exposure, and the mortality rate continued to increase over time [2]. This study confirms that clothianidin (SumiShield® 50WG) is effective in inducing high mortality in *An. funestus* s.s. and *An. gambiae* s.s., despite its slow knockdown effect. These findings are consistent with those reported by Oxborough et al. [40], which also reported delayed knockdown following clothianidin exposure. Furthermore, the present study demonstrates the effectiveness of *Nicotiana tabacum* (tobacco) leaf extract in inducing concentration-dependent mortality in wild adult *An. gambiae* s.s. and *An. funestus* s.s. These findings indicate its potential utility as a botanical insecticidal agent against these malaria vector species.

According to the results from the probit analysis, the LC_{50} was estimated at 38.77% v/v (95% CI: 9.99 – 55.91% v/v), which was effective in exterminating 50% of the *An. funestus* s.s. and *An. gambiae* s.s. populations under field conditions. Similarly, in Nigeria, a crude extract of *Nicotiana tabacum* showed comparable effectiveness against adult *An. gambiae* [27]. According to Ileke et al. [27], tobacco extracts with higher LC_{50} values are generally considered moderately effective, biodegradable, environmentally benign, and exhibit minimal non-target toxicity. Nonetheless, lower concentrations of tobacco extract (25%, 33%, 50%, 62.5%, and 71.43% v/v) produced knockdown effects on wild female and male *Anopheles* mosquitoes, although possible resistance was observed across all these concentrations.

This study provides important baseline evidence on the susceptibility of wild *Anopheles* populations to clothianidin (SumiShield® 50WG) and *Nicotiana tabacum* leaf extract in Chebele village. Larval sampling was conducted during the dry season (October–November 2021), which influenced species composition and resulted in a predominance of *An. funestus* s.l. in the bioassays. Consequently, the findings primarily reflect responses of this vector species under dry season conditions.

These results highlight the need for longitudinal investigations encompassing both dry and rainy seasons to capture seasonal variation in vector abundance and insecticide susceptibility, particularly for *An. gambiae* s.l. Future studies incorporating larger sample sizes and expanded molecular identification will strengthen species-specific inferences and support the development of alternative and integrated vector control strategies, including the use of botanical insecticides alongside existing interventions. Although most samples appeared morphologically to be *An. funestus* s.s. and *An. gambiae* s.s., a few did not amplify, and thus it cannot be confidently concluded that all individuals in the *funestus* and *gambiae* groups belonged to their respective species. Therefore, further studies are needed to assess insecticide susceptibility status and confirm species identity both morphologically and molecularly for primary and secondary malaria vectors in the study area. This information will help guide the Technical Advisory Committee (TAC) on Insecticide Resistance Management (IRM) under the National Malaria Elimination Programme (NMEP) in Zambia.

Conclusion

The results of this study’s insecticide susceptibility tests demonstrated that leaf extract from *N. tabacum* led to considerable knock down effect in adult population of wild *Anopheles* mosquitoes (*An. gambiae* s.l and *An. funestus* s.l). However, the knock down effects for clothianidin and tobacco extract were different, and tobacco extract was found to have better knock down effects on wild adult *Anopheles* mosquitoes. Therefore, *N. tabacum* leaf extract may be considered as a potential bio-insecticide for the control of *An. gambiae* s.s and *An. funestus* s.s, significant vectors of malaria pathogen.

Abbreviations

An	<i>Anopheles</i>
PCR	Polymerase Chain Reaction
WHO	World Health Organization
MIS	Malaria Indicator Survey
DDT	Dichlorodiphenyltrichloroethane
CHW	Community Health Worker
SOP	Standard Operating Procedure

LC ₅₀	Lethal concentration
NMEC	National Malaria Elimination Centre
DNA	Deoxyribonucleic acid
IRS	Indoor Residual Spraying
LLINs	Long Lasting Insecticide Nets

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Author contributions

NCS, MS, KM and ETM conceived and designed the study. NCS, JM and ETM carried out the field research and conducted field insecticide susceptibility tests. RN reared and provided the Kisumu insectary susceptible species. NCS, DM and CZ morphologically identified and sorted the mosquitoes for molecular analysis. NCS and BM performed the molecular analysis, NCS and MS performed data analysis, NCS wrote the initial draft of the manuscript, which was revised by ETM, MS, JC, KM, BH and MM. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations Ethics approval and consent to participate

The study protocol and informed consent forms were reviewed and approved by the University of

Zambia Biomedical Research and Ethics Committee (UNZABREC/Approval number: Ref. 18342021) and the National Research Health Authority (NHRA/Approval number: Ref. NHRA000007/15/10/2021). Written permission was obtained from the Ministry of Health through the National Malaria Elimination Centre (NMEC) and Mwense District Medical Office. Local and traditional leadership were also informed about the purpose of the study. Participation in the study was voluntary, and informed consent was obtained from household's heads and every participant above the age of 18 years. Verbal consent was obtained from household's heads before mosquito collections.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Author details

¹National Malaria Elimination Centre, Lusaka, Zambia.

²Department of Paraclinical Studies, University of Zambia, School of Veterinary Medicine, Lusaka, Zambia.,

³Africa Centre of Excellence for Infectious Diseases of Humans and Animals, University of Zambia, Lusaka, Zambia,

⁴Department of Disease Control, School of Veterinary Medicine, University of Zambia, Lusaka.

⁵Department of Biomedical Sciences, School of Veterinary Medicine, University of Zambia, Lusaka

⁶PATH, Lusaka, Zambia.

⁷PMI-VectorLink, Lusaka, Zambia.

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