Tropical Biomedicine 41(3): 283-289 (2024)
https://doi.org/10.47665/tb.41.3.008

RESEARCH ARTICLE

Study on the neurotoxicity of the temephos impregnated kenaf cellulose nanofiber (KCNF+T) aerosols against the female *Aedes aegypti* mosquitoes

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ARTICLE HISTORY

Received: 26 November 2023
Revised: 12 March 2024
Accepted: 13 March 2024
Published: 30 September 2024

ABSTRACT

The study has generated visible aerosols with the diameter of 11 to 35 µm from the kenaf cellulose nanofiber (KCNF) impregnated with the temephos (KCNF+T) in water suspension. The study aimed to determine whether the KCNF+T aerosols are capable to elicit neurotoxicity in the adult mosquitoes via the respiratory exposure route by observing their behavioural response and measuring its body acetylcholine esterase (AChE) activity. Adult *Aedes aegypti* mosquitoes were assigned to one negative control group and three treatment groups namely, distilled water (vehicle control), KCNF and KCNF+T. The study adopted the MS1911 whereby the aerosols generated are released into the insecticide bioassay glass chamber (IBGC) separately to achieve four different aerosols concentrations of 6.4 ml/m^3, 12.8 ml/m^3, 19.2 ml/m^3, and 25.6 ml/m^3. Then the 20 sugar-fed mosquitoes were released free-flying into each IBGC to observe its behavioural response (knockdown) at 30, 60, 120, 180, 240, 300 minutes intervals and 24th hour mortality. Results showed that only mosquitoes exposed to KCNF+T aerosols exhibited persistent knockdown. There was significant difference ($p<0.05$) of cumulative mosquito knockdown between the four KCNF+T aerosols concentrations with mean percentages of 89.5%, 96.2%, 98.7% and 100.0%, respectively. There was also significant difference ($p < 0.05$) between observation intervals with the cumulative knockdown of 84.8%, 92.8%, 99.0%, 100.0%, 100.0%, 100.0% compared with the KCNF aerosols. Further, it was distinctive that only KCNF+T is capable to cause the female mosquitoes moribund/mortality at the 24th hour with 90% at the lowest aerosol concentration of 6.4 ml/m^3. The neuroenzyme assay on the mosquito that died from the KCNF+T exposure verified to have reduced AChE enzyme activity. The behavioural response and reduction of the AChE activity strongly suggests the temephos from the KCNF+T aerosols has been released into the mosquito body causing the neurotoxicity but KCNF alone is not neurotoxic.

Keywords: Cellulose nanofiber; temephos; neurotoxicity; mosquito; good health.

INTRODUCTION

Cellulose nanofiber (CNF) is a light solid substance obtained from plant matter which comprises nano-sized cellulose fibrils; 1 to 100 nm in range (Charreau *et al*., 2013). The lateral dimensions of nanocellulose range from 5 to 20 nm, and the longitudinal dimension ranges from 20 nm to several thousand nanometres (Soutter, 2012). They are flexible, has high mechanical strength and high strength-to-weight ratio (Dufresne, 2013). CNF has been studied for its potential use as carrier of active chemicals for pharmaceuticals and pesticides due its favourable physicochemical properties (Wong *et al*., 2019; Md Abu *et al*., 2020). Kenaf (*Hibiscus cannabinus*) is commercially planted in Malaysia to replace tobacco farming. The plant has been identified to be a sustainable renewable source of cellulose (Abdul Khalil *et al*., 2010). The research by Tuerxun *et al*. (2019) at the NANOCAT of University Malaya has established a process to produce CNF using the kenaf bass (KCNF). While in a separate study at AMDI-USM, Pengiran *et al*. (2021a) has successfully impregnated KCNF with the temephos insecticide (KCNF+T).

Temephos is a well-known classical organophosphate insecticide which is commonly used as active ingredient in mosquito larvicide product for treatment in potable water that being marketed for decades till present, e.g. Abate®1.1G at the recommended dosing of 1 mg in 9 litre of water (BASF, 2019). It is classified as chemical substance with low human health hazard with category 4 oral acute toxicity and category 3 dermal acute toxicity. Part of the temephos molecular structure mimics the acetylcholine (ACh) that is the natural neurotransmitter present particularly at the insect and human nervous system synapse. Thus, allowing temephos to bind to acetylcholinesterase (AChE), subsequently blocking the breaking down the ACh which disrupts the normal electrical signal transmission between neuron cells, of which leads to neuromuscular

incoordination of the insect at lower dose and human at very high dose. (Kamaldin & Jaal, 2018; Adeyinka *et al*., 2022). A closer look into the transverse section of insect abdomen shows the tracheal system from the spiracle opening are directly tubed to the end reaching the insect's ventral nerve cord (Insectomania, 2023). When compared with human respiratory system, it also shows the lung innervation with presence of the neuroepithelial cells and the intrinsic neurons (De Divirgiliis & Di Giovanni, 2020). These detail illustrations show that not only both human and mosquito have similar ACh neurotransmitter, both organisms also have neurons indirectly embedded very close to their respiratory system. Therefore, the respiratory system of both organisms is vulnerable to the airborne organophosphates via inhalation (Rathnayake & Northrup, 2016; Kamaldin & Jaal, 2018).

In the study by Pengiran *et al*. (2021), KCNF+T in water suspension with concentration of 0.01 mg/L has shown to cause increased toxic effects to the mosquito larvae leading to its better larvicidal effectiveness compared to the temephos alone at the same concentration. Another study found the acute toxic effects KCNF+T to the aquatic invertebrate *Daphnia magna* and *Dania rerio* were above 100 mg/L and classified as non-hazardous to aquatic life based on the GHS classification (Pengiran *et al*., 2021). However, there is a knowledge gap, whether airborne KCNF+T in aerosol could also cause toxicity to the adult mosquitoes by entering the mosquito trachea which is connected to the mosquito ventral nerve cord by the thoracic ganglia in the mosquito abdomen as illustrated by Insectomania (2023). Typical aerosol with the diameter ≤ 100 µm is classified as inhalable particulate that is capable to enter the human lungs alveolar ducts which has larger diameter of 250 to 300 µm but relatively larger than the mosquito trachea with 0.1 to 1 µm in diameter. Although, the aerosol of the KCNF+T has larger diameter (11 to 35 µm) than the mosquito trachea, it should not be overlooked that the KCNF+T is solid in water suspension whereby under low humidity and warmer temperature of the air, the water of the aerosol is likely to evaporate leaving the KCNF+T as suspended particle which some has dimension smaller than 1 µm (Sabri *et al*., 2022). Therefore, the KCNF+T aerosols that has potential to enter the mosquito respiratory system and very likely able to travel deep into the human respiratory tract. This may also reflect the potential hazard posed by KCNF+T aerosols to human via inhalation exposure knowing alveolar ducts in the human respiratory tract are also connected to the intrinsic neuron of the parasympathetic nervous system by the smooth muscle cells in the human lung as illustrated by De Divirgiliis and Di Giovanni (2020**)**.

The objectives of the study are (a) to determine whether adult mosquitoes will display behavioural response reflecting the neurotoxicity i.e. knockdown and mortality upon exposure to aerosols containing KCNF or KNCF+T and (b) to measure whether changes occurs in the acetylcholine concentration which reflects the AChE neuroenzyme activity in the mosquito body after the exposure to the KCNF or KNCF+T.

MATERIALS AND METHODS

Source of the KCNF and KCNF+T

In this study, the KCNF stock solution was obtained from the Nanotechnology and Catalysis Research Centre, Universiti Malaya (NANOCAT) under the supervision of Dr. Leo Bey Fen. Details of the suspension preparation technique have been reported by Tuerxun *et al*. (2019). The technical grade (93%) temephos was provided by Hextar R&D International Sdn. Bhd. Malaysia. The impregnation process of temephos onto KCNF fiber matrix is as reported in Pengiran *et al*. (2021), in this study temephos was impregnated onto KCNF in dry powder resulting KCNF+T nanofiber powder with approx. 1% w/w temephos. Subsequently the powder was suspended in the distilled water and homogenised to produce the KCNF+T suspension containing approximately 2.1% w/v cellulose and 0.02% w/v temephos. The characterization of the cellulose in the KCNF+T suspension was verified as nanofiber through transmission electron microscopy and scanning electron microscopy, its dimensions were approximately 114.9 ± 26.2 nm in length and 6.0 ± 1.6 nm in width.

Aerosolization of the KCNF and KCNF+T

Similar to the study by Sabri *et al*. (2022), an ultrasonic atomizer (Brand: CkeyiN, Power: 12W, Frequency: 2.4 MHz, Capacity: 500 ml; Origin: Shenzhen, China) was setup to generate the aerosols (dia. 11 to 35 µm) of KCNF and KCNF+T with discharge rate of 1.0 ± 0.2 ml/min. In this study, the aerosols were released into an insecticide bioassay glass chamber (IBGC) with dimension 70 x 70 x 70 cm equivalent to 0.343 m^3 (DSM, 2006) to achieve four different exposure concentrations i.e. 6.4 ml/m^3, 12.8 ml/m^3, 19.2 ml/m^3 and 25.6 ml/m^3, separately (Figure 1). This IBGC is specially designed and constructed for the testing of household insecticides products against flying insects (DSM, 2006). Initial experimental setup trial found that 25.6 ml/m^3 which takes approx. 25 minutes of aerosolization is the optimum concentration without saturation, which is reflected by non-formation of water droplets on the IBGC inner glass surface. Also considering the practicality to complete 5 hours experiment observation of the 4 IBGC run consecutively as a replicate of a specific concentration within the same day. The aerosols generated consists of water, CNF, and temephos and the estimated amount is presented at the Table 1. After the release of aerosols, the mosquitoes were immediately introduced into the IBGC via small sliding window located in the front panel of the IBGC. The temperature of laboratory was maintained between 24°C to 28°C and 60 to 90% relative humidity, which became the ambient temperature of the experimentation using the IBGC.

Source of the adult mosquito

The *Aedes aegypti* egg (generation F315, strain VCRU) were sourced from an established laboratory colony maintained at 25 to 29°C, 50 to 90% relative humidity and a photoperiod of 12:12 hours in the insectarium of the Vector Control Research Unit, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia. Egg deposited on the filter paper were immersed into a pan of chlorine-free water or distilled water to hatch the eggs. Newly hatched *Aedes* larvae were transferred into polyethylene containers, which contain chlorine-free water (Kamaldin & Jaal, 2018). They were

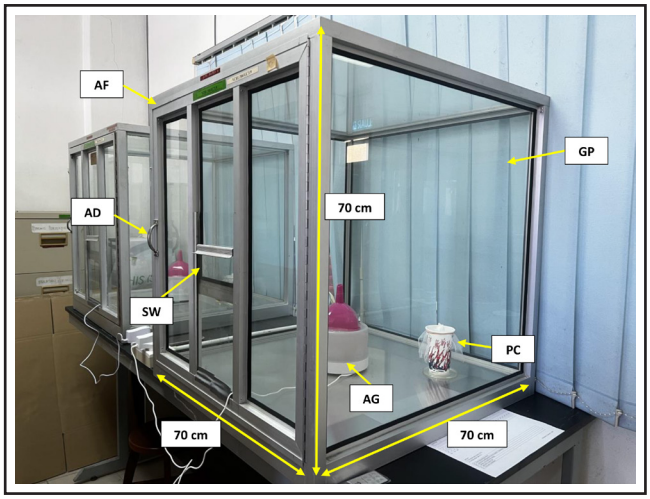

Figure 1. Illustration of the aerosol generator (AG) setup in the insecticide bioassay glass chamber (IBGC); AF : Aluminium frame for structural support; AD : Handle and hinges at front panel as access door; GP : Glass panel for easy observation and cleaning of insecticides residue; SW : Sliding window as access port for experimental purpose i.e. release of mosquitos; AG : Aerosol generator; PC : Paper cup holding cotton wetted with sugar solution.

fed with powdered liver diet *ad libitum* (Asahina, 1964). When the larvae in the container start to moult into 3rd instar larvae or early pupae, they are transferred into a holding cage to collect the adult mosquito upon emerge. Adult mosquitos were fed with 10% sugar solution (Rigby *et al*., 2021).

Neurotoxicity behavioural bioassay

The study adopted the Malaysia national standard method MS1911 – Part 1 (DSM, 2006) for the evaluation of household insecticide products biological efficacy in the IBGC. Live *Aedes aegypti* female adult mosquitoes were separated to four groups each 20 individuals for the different exposure treatments namely (a) distilled water (DW) as vehicle control, (b) KCNF, and (c) KCNF+T. The effect of neurotoxicity was determined by mosquitoes knocked down observed at 30, 60, 120, 180, 240 and 300 minutes after mosquitoes being released into the IBGC. Concurrently, a group of mosquitoes were released into IBGC without any treatment under ambient humidity as negative control, Knockdown is defined as paralysis of an insects where they remain in a state such as to be incapable of coordinating movement and apparently dead (DSM, 2006). Examples of the incapable to coordinate movement are inability to stand in normal posture, abnormal position of wings, lateral recumbency and whole body turn over (Kamaldin *et al*., 2018). The mosquitoes were provided with the cotton wetted with sugar solution placed on a paper cup covered with a cloth mesh for the next 19 hours to observe its 24th hour moribund/mortality, which was recorded on the next day to identify persistent and non-persistent neurotoxicity effect. The experiment was replicated 5 times (DSM, 2006).

Neurotoxicity AChE neuroenzyme microassay

The study employed method used by Rasli *et al*. (2018) and WHO guidance for entomologists for the monitoring and managing insecticide resistance in *Aedes* mosquito populations (WHO, 2016). Upon checking the 24th hour mortality, the adult mosquitoes found dead or in moribund state after either the three treatments were transferred into microcentrifuge tubes (Eppendorf) and kept refrigerated until the experiment setup is ready for homogenate preparation. While, the mosquitoes that were still alive and active, they were collected with battery-operated aspirator and forced knockdown by cold stress in the refrigerator. The negative control mosquito was used to establish the baseline enzyme activity. Hence, the percentage of difference in enzyme activity equals to the activity shown by the supernatant of the adult mosquito exposed to treatments of DW or KCNF or KCNF+T minus the baseline activity shown by the negative control mosquito.

The potassium phosphate buffer (PBS) was prepared by dissolving 4735 mg of NA_2HPO_4 in 500 ml of distilled water and 4540 mg KH_2PO_4 in 500 ml of distilled water. The buffer solution was adjusted to a pH of 6.8. Then, 0.2 mL of the PBS buffer was pipetted into a 1.5 mL Eppendorf tube containing an individual mosquito, followed by the grinding of the mosquito using a battery-operated mixer. Finally, added in 0.4 mL of PBS buffer, making to a final volume of 0.6 ml of mosquito homogenate. All steps pertaining to the preparation of the mosquito homogenate was performed on ice in order to avoid enzyme degradation. The homogenate was centrifuged at 14,000 rpm at 4°C for 30 min. The pellet was discarded, and the supernatant was used as the enzyme source for the enzyme microassay.

In order to measure enzyme activity in supernatant, a specific substrate was added. This substrate acted upon reaction with the enzyme, leading to a formation of coloured product that can be quantified optically using spectrometry (Varma, 2022). This colour intensity is directly related to the concentration of the enzyme or its activity whereby the colour intensity increases with the increase of enzyme activity (Zarzar *et al*., 2017), which causes less light transmitted through the samples placed in the microplate wells. Thus, the colour optical density (OD) of the sample measured will increase when the colour increases at the appropriate wavelength using a spectrophotometer. The OD of the samples on the microplate wells was determined using an immunoassay reader (Thermo Scientific, Waltham, MA, USA).

The natural substrate for the AChE neuroenzyme is acetylcholine (Trang & Khandhar, 2023). The synthetic substrate for the AChE bioassay is based on Ellman's method (1961) described in Brogdon and Barber (1987), which uses acetylthiocholine iodide (ACTHI) and 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) as substrates. The ACTHI solution was prepared by vortex the mixture of 37.5 mg acetylthiocholine iodide and 5 ml acetone before adding 45 ml of PBS. The DTNB solution was prepared by dissolving 6.5 mg 5,5'-dithiobis (2-nitrobenzoic acid) in 50 ml PBS. The 50 µl aliquot of ACTHI solution and 50 µl aliquot of the DTNB solution were added into each well of the microplate that contains three replicates of 50 µl individual mosquito homogenate. The hydrolysis of acetylthiocholine by AChE yields thiocholine and 5-thio-2-nitrobenzoate anion that reacts to form a dark yellow substance, which is quantified at a wavelength of 410 nm after 30 minutes of incubation at room temperature (Rasli *et al*., 2018). The colour changes represent the natural hydrolysis of acetylthiocholine by the AChE. Low activity of AChE can indicate that the enzyme is inhibited by a substance that prevents it from breaking down acetylthiocholine. High activity of AChE can indicate an increase in the breakdown of acetylthiocholine, which mirrors the decrease in the amount of acetylcholine in the synapse that results decrease in neurotransmission (Rathnayake & Northrup, 2016). The mean and standard deviation of optical density in three replicates were derived from a 10 mosquito's supernatant representing each treatment group and the negative control group.

Data analysis

For the neurotoxicity behavioural bioassay, the observation was reported as a mean percentage of cumulative knockdown at designated time interval up to 300 minutes and the 24th hour moribund/mortality for each of the three different treatments at the four aerosol exposure concentrations and one negative control. For the neurotoxicity based on the AChE neuroenzyme microassay, the results of the optical density of the enzyme activities between the three treatment groups were calculated based on formula modified from the WHO (2005) where OD_{Tx} is the OD of the enzyme activity of a treatment group and OD_{NC} is the OD of the enzyme activity of the negative control group.

Percentage difference of enzyme activity (%)
$= [(OD_{Tx} - OD_{NC}) \div OD_{NC}] \times 100$

The data were analysed using descriptive statistics and Kruskal-Wallis H test, which is also known as nonparametric ANOVA test was performed to determine differences between the four experimental groups at the significance level of $p< 0.05$ using IBM Statistics version 28 software (IBM Corp, 2023).

RESULTS

Neurotoxicity behavioural bioassay

The negative control mosquitoes under the ambient humidity did not show any sign of knockdown throughout the experiment. The findings of the knockdown behavioural observation up to the 300 minutes and 24th hour moribund/mortality of the adult mosquitoes upon release into the IBGC that contained the aerosols of the DW, KCNF and KCNF+T at four different concentrations are shown in Table 1.

Adult mosquitoes that were exposed to DW aerosols were still alive without showing any knockdown behaviour during the 300 minutes of the observation and did not shown sign of moribund/mortality at the 24th hour. The same findings were also observed for mosquitoes exposed to KCNF at the two lower aerosol concentration

Table 1. Mean percentage of cumulative knockdown up to 300 minutes and 24th hours mortality of female *Aedes aeqypti* mosquitoes upon exposure to the four concentrations of the aerosol treatments of the distilled water (DW), kenaf cellulose nanofiber (KCNF) and KCNF impregnated with temephos (KCNF+T) in the insecticide bioassay glass chamber

Aerosol concentration in the IBGC (ml/m^3)	Treatment group*	Composition of whole aerosols			Knockdown** cumulative percentage at the designated interval						Mortality** percentage (Mean ± SD)
		H_2O (ml)	KCNF (mg)	T (mg)	30	60	120	180	240	300	24th hour
6.4	DW	2.2	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	0 ± 0
	KCNF		48	Nil	Nil	Nil	Nil	Nil	Nil	Nil	0 ± 0
	KCNF+T		48	0.5	62 ± 6	79 ± 2	96 ± 4	100 ± 0	100 ± 0	100 ± 0	95 ± 4
12.8	DW	4.4	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	0 ± 0
	KCNF		97	Nil	Nil	Nil	Nil	Nil	Nil	Nil	0 ± 0
	KCNF+T		97	1	85 ± 6	92 ± 5	100 ± 0	100 ± 0	100 ± 0	100 ± 0	98 ± 3
19.2	DW	6.6	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	0 ± 0
	KCNF		145	Nil	12 ± 3	7 ± 3	Nil	Nil	Nil	Nil	0 ± 0
	KCNF+T		145	1.5	92 ± 3	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
25.6	DW	8.8	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	0 ± 0
	KCNF		194	Nil	23 ± 3	13 ± 3	Nil	Nil	Nil	Nil	0 ± 0
	KCNF+T		194	2	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0

Note: # Treatments are: DW (distilled water); KCNF (KCNF 2.2% w/v suspended in DW); KCNF+T (KCNF 2.2% w/v impregnated with 0.02% w/w temephos suspended in DW). ** Knockdown refers to paralysis of an insect where they remain in a state of incapable to coordinate movement and apparently dead (JSM, 2006). *** Mortality of the mosquitoes in knockdown condition at the 24th hour (moribund) (JSM, 2006).

of the 6.4 and 12.8 ml/m^3. Upon exposure to the third higher KCNF concentration of 19.2 ml/m^3, the 13 ± 3% and 7 ± 3% of the mosquitoes started to exhibit the knockdown behaviour at the 30th and 60th minute, respectively. But the mosquitoes recovered from the knockdown condition in the following hours until the 300th minute. When the mosquitoes were exposed to the highest aerosol concentration of 25.6% ml/m^3, the same knockdown behaviour seen at the 30th and 60th minute with an increased number of mosquitoes being knockdown of 23 ± 3% and 13 ± 3%, respectively. Similarly, the mosquitoes were back to normal in the following hour up to the 300th minute. KCNF aerosols of the four concentrations were not capable to cause knockdown permanently and no moribund/mortality at the 24th hour against the mosquitoes.

When the mosquitoes exposed to the KCNF+T, even at the lowest aerosol concentration of 6.4 ml/m^3, marked jump of mosquitoes in the knockdown condition (62 ± 3%) was seen at the first 30 minute, continued to increase steadily to 79 ± 2% and 96 ± 4% at the 60th and 120 minute, respectively. Later, the 100% knockdown persistently achieved from the 180th until the 300th minutes. Then followed with the (95 ± 4 %) moribund/mortality condition was found at the 24th hour. Large hike of mosquitoes' knockdown and mortality was seen observed although the aerosols only contained 0.02%w/v of temephos. The further increase of the KCNF+T aerosol concentration in the IBGC to 12.8 ml/m^3, seems to accelerate whereby 85% and 92% of the mosquitoes were knockdown at the 30th and 60th minute then followed by persistent 100% knockdown up to the 5th hour, ending with 98% moribund/mortality at the 24th hour observation. Meanwhile the exposure of the mosquitoes to the two highest KCNF+T aerosol concentration of 19.2 ml/m^3 and 25.6 ml/m^3 led to 100% knockdown of the mosquitoes from the 60th minute to the 300th minute and 100% mortality, respectively.

The Kruskal-Wallis H test showed that there is a significant difference **(**$p < 0.05$) in percentage cumulative knockdown of the *Aedes aeqypti* mosquitoes among the four KCNF+T aerosols concentrations of 6.4 ml/m^3, 12.8 ml/m^3, 19.2 ml/m^3 and 25.6 ml/m^3 with mean percentages cumulative knockdown of 89.5%, 96.2%, 98.7% and 100.0%, respectively. It is also found that the percentage cumulative of the *Aedes aeqypti* mosquitoes' knockdown upon exposure to KCNF+T aerosols, is significant different ($p < 0.05$) between observation intervals with mean percentages of the cumulative knockdown of 84.8%, 92.8%, 99.0%, 100.0%, 100.0%, 100.0% at the 30th, 60th, 120th, 180th, 240th and 300th minutes, respectively.

For 24th hour moribund/mortality, the result at Table 1 distinctively showed that only aerosols of the KCNF+T which contained 0.02 %w/v of temephos is capable to cause moribund/mortality condition to the female mosquitoes, even at the lowest aerosol concentration of 6.4 ml/m^3 is adequate to caused mortality above 90%.

Neurotoxicity AChE neuroenzyme microassay

Table 2 shows the results of the optical density of the solution in the wells of the microassay plate upon the reaction of the substrate with the solutions of blank and supernatant of the mosquito homogenate. The wells with the blank solution showed consistent low mean OD of 0.09 to 0.14 while solution with supernatant from the negative control mosquito showed relatively higher mean OD of 0.19 due to the presence of the supernatant containing mosquito tissue biochemicals. While for the solution containing supernatant of the DW and KCNF, the formation of the yellow substance from the thiocholine yield by the AChE was apparent with marked increase of the OD reflecting AChE neuroenzyme activity of 189% and 209% higher than the negative control supernatants, respectively. However, for the solution with KCNF+T supernatant, the formation of the thiocholine is suppressed reflected by the decreased OD compared to the negative control resulting 35% reduction in the AChE neuroenzyme enzyme activity.

DISCUSSION

The use of the DW treatment at every replicate of the experiment is essential part of the study. The non-presence of mosquito showing knockdown at all four concentrations of the aerosols as shown at the Table 1 gave assurance the IBGC or the aerosol generator does not have any carry-over of temephos because the chambers were reused and cleaned in according to the test facility standard

Table 2. The optical density of the solution in the wells of the microassay plates of the acetylcholine esterase (AChE) neuroenzyme microassay with the supernatant homogenate of the female *Aedes aeqypti* mosquitoes exposed to the highest concentrations of the 25.6% ml/m^3 aerosol treatments of the distilled water (DW), kenaf cellulose nanofiber (KCNF) and KCNF impregnated with temephos (KCNF+T) in the insecticide bioassay glass chamber

Treatment	Well sample	No of mosquito homogenate	No of microassay plate well	Optical density* Mean ± SD	Optical density* Mean ± SD (minus blank)	Percentage difference of enzyme activity** (%)
Negative control	Blank	0	3	0.09 ± 0.00	0.10 ± 0.08	Not Applicable
	With supernatant	10	30	0.19 ± 0.08		
DW	Blank	0	3	0.09 ± 0.00	0.29 ± 0.11	+ 189
	With supernatant	10	30	0.38 ± 0.11		
KCNF	Blank	0	3	0.14 ± 0.04	0.31 ± 0.24	+ 209
	With supernatant	10	30	0.45 ± 0.23		
KCNF+T	Blank	0	3	0.09 ± 0.02	0.07 ± 0.04	– 35
	With supernatant	10	30	0.15 ± 0.03		

Note: * Blank and supernatant optical density (OD) for AChE activity were measured using the Thermo-Scientific immunoassay reader at the wavelength of 410 nm after 30 minutes (WHO, 1998). ** The percentage difference of the enzyme activity was calculated using the equation, [(OD treatment group) – (OD negative control) ÷ OD negative control × 100%].

procedures. It has been an interesting observation whereby the study found that aerosolised 2.2% w/v KCNF suspended in the distilled water at the concentration of 19.2 and 25.6 ml/m^3 was capable to cause the 7 to 23% of free flying adult mosquitoes to exhibit short term knockdown behaviour within the first 60 minutes. However, it is suspected the knockdown behaviour was not effects induced by neurotoxicity. The observation seems to suggest that knockdown was due to the presence of KCNF particulate matter. There is possibility if the composition was higher than 2.2% w/v, it may prolong the knockdown effect. This suggestion is based on the report by Wang *et al*. (2023) that found particulate matter from the air pollution adversely affected the antennae functionality of the houseflies (*Musca domestica*) causing detrimental behavioural change. The behaviour exhibited by the mosquitoes namely antenna rubbing, abnormal standing position, loss of coordination and lateral recumbency further reaffirms this suggestion. Mosquito antennae are equipped with sensory structure, including tiny hairs called sensilla, which detect chemical cues from environment (Zwiebel & Takken, 2004). Hence by rubbing their antenna, the mosquitoes able to remove debris, dust, particle that interfere with their ability to detect odours or other sensory information (Boroczky *et al*., 2013), which explains the reversible knockdown on the 2nd hour onwards. While the variation of the neuroenzyme activities seen between the DW and KCNF treatment could be due to the typical population variation of the detoxification enzymes quantity between individual mosquitoes (Wu *et al*., 2022). Study done by Leong *et al*. (2019) regarding enzymatic and molecular characterization of insecticide resistance mechanisms in field populations of *Ae. aegypti* from Selangor, Malaysia found that the mosquito showed various levels of resistance against organochlorine and pyrethroids. Although, they exhibited susceptibility against malathion (organophosphates) and propoxur (carbamate). This suggest that certain mosquito strains may possess genetically lower level of metabolic detoxifying enzyme.

The results also seem to suggest that KCNF despite of it nanosize, which is smaller than the spiracle and trachea diameter, it doesn't seem to cause acute toxic effects to the mosquito respiratory system by clogging it. It is either the KCNF was unable to enter the mosquito's respiratory tract due to the nature of the tracheal system or it was able to travel in and out of the tracheal system due to its nanosize as respirable particles (Insectomania, 2023). The trachea of a mosquito is connected to the external environment through pairs of openings called spiracles. Air enters the system through spiracles, filling the tracheae with air. A short trachea connects to longitudinal tracheal trunks, which is the main tubes of the respiratory system. From these trunks, smaller tracheae branch out to supply air to the various tissues like muscles, nervous tissues, and the gut (North Carolina State University, 2015). The tracheoles of mosquito, originating from cells called tracheoblasts derived from epidermal cells that line the trachea, have a diameter of approximately 1 µm near their origin and gradually narrow down to around 0.1 µm as they extend distally (North Dakota State University, 2023). Thus, theoretically, any particles presence in the air will be taken in through the mosquito respiratory system, allowing for contact with the inhaled substance, such as particles of the KCNF and KCNF+T. A study by Di Novo *et al*. (2021) demonstrated that 1 µL water droplet takes less than 30 minutes to evaporate at the temperature of 20°C and 60% RH, the time becomes faster when the environmental relative humidity reduces and temperature increases. The neurotoxic effects observed in mosquitoes are unlikely to be a result of direct exposure to temephos on their external body, as mosquitoes have a hydrophobic layer of cuticle with scales covering their body (Balabanidou *et al*., 2018). The outer covering of a mosquito exoskeleton is covered with microscopic scale and setae that make it hydrophobic (Bello *et al*., 2023). Thus, the entry of the KCNF and KCNF+T via the mosquito respiratory system is still likely to occur.

A shown at Table 1, the results suggest the impregnation of temephos onto KCNF as little as 0.02% w/w (KCNF+T) and dispersed as water aerosols into the air of the IBGC at the lowest concentration of 6.4 ml/m^3, is potent enough to knockdown more than 15 out of 20 mosquitoes within the first hour. The use of temephos as the active ingredient for the control of mosquito larvae is widely known but it is rarely highlighted of its significant use for mosquito adulticide activities. Its aerosol concentration 19.2 ml/m^3 substantial performance in causing 100% knockdown consistently within the first hour of exposure and 100 % mortality at the 24th hour as seen in the study was not anticipated. The KCNF seem to act as synergistic agent to enhance toxic effect of the temephos by facilitating temephos delivery into mosquito body (Chai *et al*., 2020; Adhikari & Khanikor, 2021; Pengiran *et al*., 2021; Demirak & Canpolat, 2022).

The AChE neuroenzyme microassay results as shown at Table 2, evidence that the knockdown behaviour seen in the first 5 hours and the subsequent moribund/ mortality experienced by the adult mosquitoes are due to the intoxication cause by the inhibition of the ACh hydrolysis by a xenobiotic agent, in this case is the temephos from the KCNF+T. Once the temephos is inside the mosquito body, they undergo a process called bioactivation, where they are converted into a more reactive compound. The

activated temephos bind irreversibly to the active site of AChE, which is present in the nerve synapse and neuromuscular junctions. This binding prevents AChE from functioning properly, leading to an accumulation of ACh in the synaptic cleft (Araujo *et al*., 2023). This causes an overstimulation of cholinergic receptors, leading to excessive nerve impulse and prolonged stimulation of the affected tissue, which led the mosquitoes to knockdown as shown at Table 1. (Rathnayake & Northrup, 2016; Trang & Khandar, 2023). As a result of the inhibition of AChE, its activity was also reduced. This explains the low level of AChE neuroenzyme activity in supernatants of mosquito of the KCNF+T treatment group as recorded at the Table 2 compared to the other DW and KCNF treatment groups. Thus, the results of the microassay at Table 2 reaffirms the suggestion that the temephos was release from the KCNF+T nanofibers. It is consistent with findings of Pengiran *et al*. (2021), whereby the temephos was released from the KCNF+T in the water suspension that caused intoxication to the mosquito larvae.

In summary, the results of the neurotoxicity behavioural study using the IBGC and the AChE neuroenzyme microassay provided meaningful information that the adult mosquitoes displayed behavioural responses reflecting the neurotoxicity, i.e. knockdown and mortality upon exposure to aerosols containing KCNF or KNCF+T. This is possible due to the capability of KCNF to hold and disperse temephos indoors via aqueous aerosol generation and the nanosize of KCNF may facilitate the delivery of the temephos to the nervous system target site within the mosquito body e.g. through the respiratory system. Changes in the acetylcholine concentration reflected the AChE neuroenzyme activity in the mosquito body after exposure to the KCNF or KNCF+T. The KCNF+T concentration can be built up and sustained airborne to a specific concentration, it is capable of inducing neurotoxicity effects on free-flying adult mosquitoes (Britannica, 2023; Ferng, 2023). However, the authors believe further studies on the KCNF and KCNF+T are needed to ascertain the pathway of the temephos entry into the mosquito body.

ACKNOWLEDGEMENT

The authors express their heartiest appreciation to the Hextar R&D International Sdn. Bhd. for the supply of temephos, Dr. Rahmatullah Holding Sdn. Bhd. for the supply of raw kenaf bast, Vector Control Research Unit, Universiti Sains Malaysia for the supply of larvae and laboratory facilities, Medical Entomology Unit, Infectious Disease Research Centre, Institute for Medical Research, National Institute of Health, Ministry of Health, Selangor, Malaysia for the technical guidance and facility in conducting the neuroenzyme assay. The Ministry of Higher Learning, Malaysia and Universiti Teknologi MARA are also acknowledged for the doctorate study scholarship. This work has been funded by the UIAA Intellectual Advisory.

Conflict of Interest

The authors declare there is no conflict of interest.

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