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Essential Oil-Wax Formulations as an Easy to Use Clothing Impregnating Agent to Prevent Mosquito Bites from the Malaria Vector *Anopheles*

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Abstract: Mosquitoes transmit a wide range of diseases including malaria, a major health problem in Africa. With the lack of an effective vaccine and the emergence of drug resistant parasites against malaria, vector control remains the most effective means to reduce transmission of the disease. Mosquito repellents such as N,N-Diethyl-3methylbenzamide (DEET) have played significant roles in preventing malaria transmission. However, their use is being hampered by the emergence of resistant mosquitoes. Essential oil-based insect repellents have been shown to provide protection against mosquito bites. However, their high volatility makes them unstable and cannot provide protection over a long period of time. In the present study, in order to provide personal protection against mosquito bites, two different behavioral assays were employed to evaluate a cost effective, easy-to-use repellent agent through the impregnation of clothing with essential oils stabilized in waxes. Beeswax and Fjällräven wax were dissolved by heating at 70°C and different active essential oils were added at a final concentration of 2% or 5%. The formulations were used to impregnate clothing by ironing and tested for their repellent effect against laboratory reared *Anopheles stephensi*. The impregnated clothing did not improve the repellent effect after 20 mins of exposure to the mosquitoes. However, the number of mosquitoes with food ingestion after 18-24 h was significantly reduced compared to controls indicating that the formulations prevented mosquitoes from penetrating through the clothing in order to feed. Therefore, it is proposed that impregnating clothing with essential oil-wax formulations can provide protection against mosquito bites and thereby reduce the transmission of malaria.

Keywords: Mosquito, Repellent, Essential Oils, Waxes, Malaria, Transmission

1. Introduction

Mosquitoes transmit a wide range of infectious diseases including malaria, which is a major global health burden, causing more than 216 million clinical infections and an estimated 445.000 deaths annually mainly in Africa [1]. Particularly affected are children under 5 years of age and pregnant women in sub-Saharan Africa. Malaria is caused by blood-borne protozoan parasites of the genus *Plasmodium* and is transmitted from human to human by blood-feeding female *Anopheles* mosquitoes. Efforts to completely eradicate malaria have been hampered by the lack of either an effective vaccine or the rapid emergence of drug resistant parasites against most of the commercial anti-malarial drugs. The most effective measure to prevent malaria infections is to minimize mosquito bites. This has been greatly achieved by the use of insecticide-treated bed nets [2]. However, such prevention measures make use of compounds which are undermined by the spread of insecticide-resistant *Anopheles* in more than 60 countries in Malaria-endemic regions [3, 4]. Also, some of the insecticides being used to impregnate clothing such as pyrethroids and N,N-Diethyl-m-toluamide (DEET) exhibit slight toxicity to humans [5, 6]. Therefore, the need to search for other compounds such as from natural products is required.

Several natural products have been shown to exhibit irritating, repellent or toxic effects on mosquitoes, which could be used to prevent mosquito bites [7]. Among these are essential oils, which are plant products made up of volatile substances found in a variety of species [8]. Some of these essential oils can be easily extracted from plants and exhibit low toxicity against humans but are highly effective against a wide spectra of insects [9]. A major drawback of essential oils is their high volatile nature which makes them unstable resulting in a loss of effectiveness after a short time. Therefore, they cannot be used as mosquito repellents for longer periods, even if they show strong repellent effects on a short time scale. This problem can be solved by using fixing agents to reduce the volatility of the substances and thereby prolong repellent or irritating effects [10, 11]. Another problem hampering the use of essential oils as mosquito repellents is the mode of application. Formulations in ointments, creams or gels that are applied directly on the skin have been described [12]. However, direct application of essential oils on the skin may result in severe side effects. Other studies have generated candle-based essential oil formulations [13–15]. However, these products are able to provide protection against mosquito bites mainly indoors but cannot effectively provide personal protection outdoors.

In this study, using the laboratory strain of the main Asian malaria vector *Anopheles stephensi*, it was evaluated if essential oils with repellent effects stabilized in waxes can be used to impregnate clothing to offer longer personal protection against mosquito bites. The advantage of such waxes as a stabilizing and impregnating agent is their simplicity to use and light weight. For example, they can be used easily during outdoor activities like farming or hiking. An individual simply needs to apply the oil-wax formulation onto the clothing followed by ironing to impregnate it on the clothes. But also camp fires are generally sufficient for the impregnating process as it is known from many products used for outdoor activities. As proof of principle, five different repellent essential oils namely cinnamon, citronella, thyme, lemongrass and cumin [16] embedded in two different type of waxes, beeswax (BW) and a commercially available Fjällräven wax (FRW, a synthetic wax used in the outdoor industry), were tested. The oil-wax formulations were prepared and impregnated on fabrics. Afterwards, these fabrics were investigated for their behavioural effect against laboratory reared *A. stephensi* mosquitoes.

2. Materials and Methods

2.1. Maintenance of *Anopheles stephensi*

The *A. stephensi* colonies used in the study were provided by Dr. Anna Heitmann and Prof. Egbert Tannich from Bernhard-Nocht-Institute for Tropical Medicine, Hamburg Germany. The colonies were maintained under standard insectary conditions at 26 ± 0.5°C, 80 ± 2% humidity and a 12/12 h light/dark cycle in gauze-covered aluminium wire-framed cages [17]. Egg production was induced by blood feeding of adult female mosquitoes once a week with human blood placed at the bottom of a Schott flask containing water at 37°C using parafilm. The blood was purchased from the Department of Transfusion Medicine (University Hospital Aachen, Germany). The University Hospital Aachen Ethics commission approved all work with human blood and the donors remained anonymous. Four days later, the eggs were collected on cotton pads placed inside a plastic bowl containing chlorine-free tap water and 0.1% sea salt solution kept at 26°C. The eggs were carefully transferred into clean plastic bowls containing 0.1% sea salt solution for hatching and emergence of larvae over 2 d. Following emergence of larvae, they were reared at a density of 400-500 larvae per 3 litres of 0.1% sea salt solution in white plastic trays. Larvae were fed on a daily basis with floating dry fodder pellets (Tetra pond food). Any surplus of old food was removed and discarded when necessary. The larvae developed to pupae 6 to 8 d post emergence from eggs and were collected on a daily basis, washed with distilled water and placed in small plastic bowls containing 0.1% sea salt solution. Afterwards, they were transferred to cages for adult mosquito emergence. Adult mosquitoes were fed via a cotton pad soaked with sterile filtered 5% saccharose (Sigma) solution supplemented with 0.05% para-amino benzoic acid (PABA; Sigma).

2.2. Preparation of Essential Oil-Wax Formulations

The essential oils used for embedding in the two different waxes were chosen based on their repellent activity as demonstrated previously [16]. They included cinnamon (*Cinnamomun zeylanicum*), citronella (*Cymbopogon winterianus*), thyme (*Thymus vulgaris*), lemongrass (*Cymbopogon citratus*) and cumin (*Cuminum cyminum*). All essential oils were plant extracts purchased from Sigma-Aldrich. The synthetic repellents DEET and permethrin were used as positive controls while sugar food solution or human blood were used as negative controls, depending on the experiment. As carriers for the essential oils, they were either dissolved in a mixture of silicone oil and ethanol or in two types of waxes, the BW (Carl ROTH) or the FRW (Fjällräven, Sweden). To embed the essential oils in the two waxes, they were cut into small pieces and transferred to Eppendorf tubes and heated at 70°C to melt. The liquid wax was then transferred into a new Eppendorf tube and the desired amount of pure essential oils or the positive controls, DEET and permethrin were added to a final concentration of 2% or 5%. The mixture was vortexed and allowed to cool down. Afterwards, 100 mg of the oil-wax formulation was weighed and impregnated onto a piece of white cotton fabric (3cm x 3cm) using a flat iron heated to 60°C. These fabrics were then tested in feeding assays on mosquitoes for their behavioral response.

2.3. Behavioral Assays

The effect of the different oil-wax formulations was determined using 2 to 7 d old non-blood-fed female *A.*

stephensi mosquitoes which were initially starved for 24 h prior to the experiments. The experiments were conducted between 9 a.m. and 5 p.m. in a dark room to mimic the feeding habit of the mosquitoes. Two setups were used for the feeding experiments, the vertical set up (Figure 1A) and the horizontal set up (Figure 1B). The vertical set up is composed of a white plastic cup of 15 cm in height and 10 cm in diameter in which the top is covered with white non-impregnated mosquito gauze to prevent mosquitoes from escaping (Figure 1A). To insert mosquitoes into the cup, a hole was made at approximately half the height of the cup (7.5 cm) and sealed with a silicone film. For each experiment, 20 female mosquitoes were used for each treatment and five replicates were conducted for each experimental test, resulting in 100 mosquitoes for each oil-wax combination. The mosquitoes were injected into one cup before the impregnated fabric was placed on top in the middle of the gauze. Then a cotton pad containing either a red coloured food solution or blood was placed on top of the fabric thereby functioning as an attractant. The behavioural responses of the mosquitoes were recorded at time points taking into account the number of mosquitoes adhering to the fabric, the number of mosquitoes repelled by the fabric, the number of mosquitoes that took a meal as well as the number of dead mosquitoes. Mosquitoes feeding on the treated fabric were counted as adhering, whereas the mosquitoes sitting on the lower half of the cup were considered as repelled. This set up was first used to test fabrics impregnated with essential oils only at 2% diluted in each of the three carriers (silicone-ethanol, BW or FRW) at the same concentration against the mosquitoes for 20 mins to determine if the activity of the essential oils is still retained, after they have been embedded in one of the carriers.

The horizontal set up was used to confirm the results from the vertical set up. It was composed of three chambers arranged in a row separated by two rotatable doors ([16]; Figure 1B). The middle chamber was used to place the mosquitoes to be tested and one end of the two external chambers was covered with a fabric treated with the oil-wax formulation (5%) while the other end was equipped with an untreated control. Both sides were then provided with a cotton pad soaked with food solution and closed with white plastic gauzed lids. Schott flasks containing warm water at 37°-45° C were placed at both ends to serve as additional attractants for the mosquitoes. For each experiment, 20 female mosquitoes were introduced into the middle chamber via a flexible opening. During the experiments, the door of the treated chamber was opened first so that the mosquitoes were exposed to the test substance for 30 sec. Afterwards, the door of the untreated chamber was opened as well and the mosquitoes could now make the choice to move either to the treated or untreated chamber or as well stay in the middle chamber. After 20 mins, both doors were closed, the whole experimental setup was frozen and the number of mosquitoes in each chamber was counted. To analyse whether the oil-wax formulations had a repellent effect, the spatial activity index (SAI) was used as recommended [18] using the formula in equation 1 below:

$$\text{SAI} = \frac{N_U - N_B}{N_U + N_B} \times \frac{N_U + N_B}{N} \qquad (1)$$

N_u correlates to the number of mosquitoes located in the untreated chamber, N_B to the number of mosquitoes in the treated chamber and N indicates the total number of mosquitoes used in the experiment (=20). The SAI gives values between 1 and -1, whereby 1 indicates that all mosquitoes were found in the untreated area (repelled) while -1 indicates that all mosquitoes moved to the treated side (attracted). Zero indicates no response by the mosquitoes.

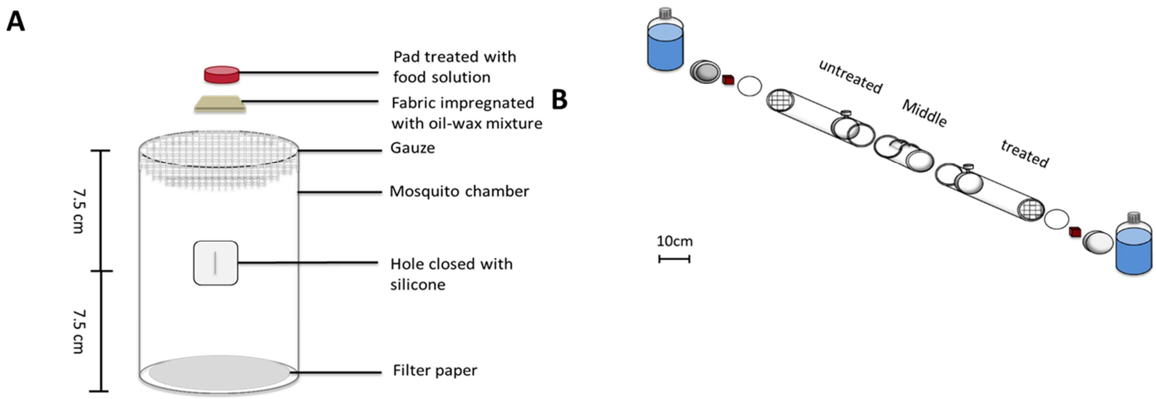

Figure 1. *Experimental set-up A) Schematic showing the vertical experimental set-up. B) Schematic drawing of the horizontal experimental set up.*

2.4. Statistical Analysis

All experiments were performed in five replicates using 20 mosquitoes in each replicate. Statistical analysis was done using GraphPad Prism 5 software (GraphPad Software, La Jolla, USA). The data were tested for Gaussian distribution using the Kolmogorov-Smirnov test. Data that followed Gaussian distribution were afterwards analysed with an ANOVA and the Bonferroni multiple comparison post-test to identify significant differences between the test formulations and the control experiment with food solution or blood, depending on the experimental condition. Also significant differences between the carriers (solvent, BW and FRW) were analysed using the ANOVA and Bonferroni multiple comparison.

3. Results

3.1. Lifespan of *A. stephensi* Mosquitoes Following Starvation

Since food deprived mosquitoes were used in all experiments, it was mandatory to exclude the fact that the mosquitoes were dying due to starvation. Therefore, it was firstly investigated how long the mosquitoes could survive without a food uptake. In this regard, 100 mosquitoes (five replicates each containing twenty mosquitoes) were starved over a period of 120 h and the number of dead mosquitoes was counted after every 24 h. No dead mosquitoes were observed for up to 48 h following starvation, indicating that after a food deprivation for 24 h, the mosquitoes could still be kept for another 24 h without any mortality. Hence, dead mosquitoes during the experiments could be related to the essential oils and not to a lack of food.

3.2. Comparing the Activity of Essential Oils in Solvents and Wax Formulations

To determine if embedding essential oils in waxes and impregnating them onto clothing will retain their activity, the behavioral responses of mosquitoes treated with 2% essential oils dissolved in the solvent carrier (silicone oil and ethanol) to those treated with 2% essential oils embedded in either BW or FRW using the vertical set up were compared. The results show that embedding the essential oils in the waxes reduced the activity of the oils as demonstrated by the 20 mins repellency test (Table 1). In all oil-wax formulations, the percentage of repelled mosquitoes was lower than in cases where the oils diluted in solvents were used. Significantly less mosquitoes were repelled by citronella, cumin, lemongrass ($p<0.05$; ANOVA and Bonferroni's Multiple Comparison Test) and thyme ($p<0.001$; ANOVA and Bonferroni's Multiple Comparison Test) as well as the positive controls DEET ($p<0.01$; ANOVA and Bonferroni's Multiple Comparison Test) and permethrin ($p<0.001$; ANOVA and Bonferroni's Multiple Comparison Test) when they were embedded in the waxes as compared to dissolving them in the solvent carrier.

When the number of adhering mosquitoes to the fabric was investigated, a comparable number of mosquitoes was obtained when the oils were diluted in the solvent as a carrier compared to when the essential oils were dissolved in the waxes (Table 1). This indicates that although most of the mosquitoes were sitting on the upper part of the cup, they were not able to adhere to the oil-wax treated fabric to feed within 20 mins of testing.

To determine if the mosquitoes that adhered to the fabrics finally took a meal over a longer period of 18-24 hours indicating that they were not hampered from penetrating the fabric using their proboscis, the abdomen of all mosquitoes were dissected and checked for the presence of the colored food solution within the abdomen. It was observed that all essential oils diluted in the solvent as carrier as well as oil-wax formulations (except thyme in BW) at 2% concentration showed significantly lower numbers of mosquitoes which had a food uptake in comparison to the negative controls ($p<0.0001$; ANOVA and Bonferroni's Multiple Comparison Test). Cinnamon oil in BW and FRW showed the lowest number of mosquitoes which fed (Table 1).

Additionally, the number of dead mosquitoes after 18–24 h following exposure to the different formulations was counted. Exposure to the essential oils in the solvent shows a similar trend for the percentage of dead mosquitoes when compared to the oils embedded in the waxes (Table 1). In most cases, the number of dead mosquitoes was less than 50% except for cinnamon in solvent, BW and FRW (Table 1).

Table 1. *Comparison of the behavior responses of mosquitoes following exposure to 2% essential oils formulations in solvent and waxes. Percentages are indicated as mean ± SD.*

Essential oil	Formulation	20 mins		18-24 h	
		Repelled mosquitoes (%)	Adhering mosquitoes (%)	Mosquitoes with food ingestion (%)	Dead mosquitoes (%)
Cinnamon	Cinnamon + Solvent	74 ± 14.75	0	5 ± 6.12	56 ± 15.17
	Cinnamon + BW	52 ± 5.7	0	1 ± 2.24	60 ± 34.46
	Cinnamon + FRW	54 ± 8.22	0	0	76 ± 26.79
Citronella	Citronella + Solvent	48 ± 16.05	1 ± 2.24	38 ± 13.51	7 ± 4.47
	Citronella +BW	21 ± 13.42	1 ± 2.24	31 ± 22.47	8 ± 6.71
	Citronella+ FRW	20 ± 11.18	0	14 ± 13.42	3 ± 4.47
Cumin	Cumin + Solvent	64 ± 7.42	0	20 ± 9.35	30 ± 11.73
	Cumin + BW	35 ± 9.35	1 ± 2.24	18 ± 10.95	18 ± 15.23
	Cumin + FRW	31 ± 4.18	0	19 ± 18.17	9 ± 8.22
Lemongrass	Lemongrass + Solvent	51 ± 8.22	0	28 ± 8.37	19 ± 11.94
	Lemongrass + BW	29 ± 20.43	0	28 ± 12.55	11 ± 11.40
	Lemongrass + FRW	23 ± 18.23	1 ± 2.24	40 ± 21.51	6 ± 13.43
Thyme	Thyme + Solvent	68 ± 8.37	0	30 ± 14.58	19 ± 4.18
	Thyme +BW	16 ± 4.18	10 ± 7.07	38 ± 20.80	0
	Thyme +FRW	22 ± 10.37	0	23 ± 16.43	10 ± 12.75
DEET	DEET + Solvent	77 ± 13.96	0	0	29 ± 20.43
	DEET + BW	26 ± 8.94	0	2 ± 2.74	26 ± 14.32
	DEET + FRW	28 ± 11.51	0	0	5 ± 11.18
Permethrin	Permethrin + Solvent	100	0	0	100
	Permethrin + BW	52 ± 11.51	2 ± 2.74	13 ± 7.58	23 ± 10.37

Essential oil	Formulation	20 mins		18-24 h	
		Repelled mosquitoes (%)	Adhering mosquitoes (%)	Mosquitoes with food ingestion (%)	Dead mosquitoes (%)
	Permethrin + FRW	48 ± 13.51	4 ± 4.18	2	21 ± 15.57
	BW	3 ± 2.74	42 ± 29.28	63 ± 8.37	1 ± 2.24
	FRW	1 ± 2.24	36 ± 8.94	78 ± 10.37	0
	Food solution	1 ± 2.24	25 ± 12.72	90 ± 9.35	0

3.3. Behavioral Responses of Mosquitoes Using 5% Oil-Wax Formulations

Due to the fact that at a concentration of the essential oils of 2% and an observation period of 20 mins, no strong repellent effect of the essential oil-wax formulations on the behavior of the mosquitoes was observed, the concentration of the essential oils was increased to 5% to investigate if the repellent effect could be increased. The repellency after 20 mins of testing (Table 2) was comparable to the results where the concentration was 2% (Table 1). Interestingly, although a higher concentration of the essential oil did not result in a stronger repellent effect, no mosquito was adhering to the fabric within 20 mins when 5% essential oils were embedded in the two different waxes (Table 2). The effects were significant ($p<0.01$; ANOVA and Bonferroni's Multiple Comparison Test) for all essential oils compared to the negative controls (BW, FRW or Food solution). The number of mosquitoes with food ingestion after 18-24 h was greatly reduced using 5% oil-wax formulations (Table 2) as compared to the situation, where 2% oil-wax formulations were used (Table 1). Cinnamon dissolved in either wax prevented most effectively the mosquitoes from feeding with only DEET showing the same effect. The least efficacy was displayed by citronella dissolved in FRW, where around 15% of the mosquitoes had a food ingestion. Nonetheless, for all essential oils as well as the two positive controls, the number of mosquitoes with food uptake was significantly reduced in comparison to the two negative controls demonstrating the effectiveness of the essential oils. The number of dead mosquitoes was greatly increased with cinnamon embedded in BW killing all mosquitoes (Table 2). Also for the condition with FRW, almost 90% of the mosquitoes did not survive (Table 2).

Table 2. *Behavior responses of mosquitoes following exposure to 5% oil-wax formulations and using food solution as attractant. Percentages are indicated as mean ± SD.*

Essential oil	Oil-wax formulation	20 mins		18-24 h	
		Repelled mosquitoes (%)	Adhering mosquitoes (%)	Mosquitoes with food ingestion (%)	Dead mosquitoes (%)
Cinnamon	Cinnamon + BW	62 ± 2.74	0	0	100
	Cinnamon + FRW	56 ± 7.42	0	0	89 ± 8.9
Citronella	Citronella + BW	23 ± 12.55	0	5 ± 6.12	23 ± 23.87
	Citronella + FRW	24 ± 9.62	0	14 ± 5.48	0
Cumin	Cumin + BW	28 ± 7.58	0	3 ± 2.74	77 ± 28.64
	Cumin + FRW	32 ± 10.37	0	3 ± 6.71	78 ± 13.51
Lemongrass	Lemongrass + BW	28 ± 5.70	0	0	91 ± 5.47
	Lemongrass + FRW	30 ± 7.91	0	2 ± 4.47	67 ± 13.04
Thyme	Thyme + BW	19 ± 4.18	0	7 ± 8.37	23 ±7.59
	Thyme + FRW	36 ± 9.61	0	2 ± 2.74	27 ± 17.54
DEET	DEET + BW	38 ± 16.43	0	0	18 ± 10.37
	DEET + FRW	53 ± 8.37	0	0	15 ± 9.35
Permethrin	Permethrin + BW	56 ± 8.37	3 ± 12.94	3 ± 2.74	20 ±11.73
	Permethrin + FRW	54 ± 9.62	0	1 ± 2.23	12 ± 9.08
	BW	3 ± 4.47	31 ± 12.94	78 ± 10.37	1 ± 2.23
	FRW	1 ± 2.24	49 ± 20.43	82 ± 8.37	0
	Food solution	1 ± 2.24	22 ± 4.47	95 ± 3.54	0

3.4. Behavioural Responses of Mosquitoes Using Blood as Attractant

Since female mosquitoes seek blood for their egg production during which the parasites causing malaria are transmitted to humans, it was determined if using blood as an attractant will affect the behavioural responses of the mosquitoes. Again, the vertical feeding assay setup was used, but in this case the pad with the sugar solution was replaced by a small glass feeder containing human blood as an attractant. Here, only the three most promising essential oils were investigated, based on the results of the previous experiments: cinnamon, cumin and lemongrass. DEET was used as a positive control. To perform the experiments over a period of 18-24 h as has been done previously, ten feeders were connected with silicone hoses to a water bath set to 30°C. A pump as commonly used for aquaria allowed for the circulation of warm water through the feeder system and kept the blood constantly at 30°C mimicking the temperature on the surface of the human skin. In this setup, 5% of each oil-wax formulation was prepared and applied to a fabric. The treated fabric was then placed on top of the gauze on the vertical cup containing twenty mosquitoes starved for 24 h. Thereafter, the feeder containing blood at 30°C was placed on top of the fabric to serve as an attractant and fixed with adhesive tape to the cup. The number of mosquitoes adhering and repelled after 20 mins was counted as well as the number of mosquitoes which fed or died after 18-24 h.

The results show that using blood as attractant, the oil-wax formulations displayed a better repellent effect (more than 50% of the mosquitoes being repelled, Table 3) as compared to when a food solution (sugar) was used as attractant (Table 2). Also, no mosquitoes were seen adhering to the test substances within 20 mins of the assay (Table 3). When the mosquitoes were dissected to investigate whether they took a blood meal or died within 18-24 h, a significantly reduced number of mosquitoes was observed which had blood ingestion in all of the oil-wax formulations (Table 3) compared to the controls (p≤0.001; ANOVA and Bonferroni's Multiple Comparison Test). Regarding the percentage of dead mosquitoes after a period of 18-24 h, the mortality dramatically dropped when blood was used as an attractant (Table 3) in comparison to the food solution (Table 2).

***Table 3**. Behavior responses of mosquitoes following exposure to 5% oil-wax formulations and using blood as attractant. Percentages are indicated as mean ± SD.*

Essential oil	Formulations	20 mins		18-24 h	
		Repelled mosquitoes (%)	Adhering mosquitoes (%)	Mosquitoes with food ingestion (%)	Dead mosquitoes (%)
Cinnamon	Cinnamon + BW	80 ± 12.75	0	3 ± 4.47	26 ± 11.94
	Cinnamon + FRW	76 ± 12.94	0	5 ± 8.66	13 ± 8.37
Cumin	Cumin + BW	54 ± 15.17	0	12 ± 13.51	12 ± 2.74
	Cumin + FRW	56 ± 19.49	0	15 ± 9.35	1 ± 2.24
Lemongrass	Lemongrass + BW	46 ± 14.75	0	12 ± 5.70	7 ± 5.70
	Lemongrass + FRW	61 ± 13.42	0	5 ± 7.07	4 ± 4.18
DEET	DEET + BW	57 ± 11.51	0	0	13 ± 7.58
	DEET + FRW	64 ± 11.40	0	0	3 ± 4.47
	BW	1 ± 2.24	55 ± 3.54	93 ± 5.7	4 ± 4.18
	FRW	2 ± 4.47	66 ±14.32	82 ± 2.74	1 ± 2.24
	Blood	1 ± 2.24	63 ± 15.25	90 ± 6.12	2 ± 4.47

3.5. Validation of the Repellent Effect of Mosquitoes to 5% Oil-Wax Formulations Using Horizontal Feeding Assay Set up

In order to confirm the repellent results from the vertical feeding assay setup, a horizontal feeding set up as described elsewhere was utilized [16]. This setup allows for the determination of the spatial repellent effect of the tested formulations through the determination of their spatial activity index (SAI) calculated as described in the materials and methods. The SAI varies from -1 to +1 with a value greater than zero indicating a repellent effect and 1 indicates the highest repellent effect while a value less than zero indicates an attractive effect and -1 the most attractive effect. The setup was used to confirm the repellent effect of the mosquitoes using the 5% of oil-wax formulations. The results show high repellent effects of the formulations similar to those when blood was used as attractant (Figure 2A (BW), B (FRW)). The control samples with only BW or FRW and food solution show attractive effects with negative SAI values. DEET at 5% also showed a slight repellent effect while permethrin at 5% showed a weak attractive effect (Figure 2A, B).

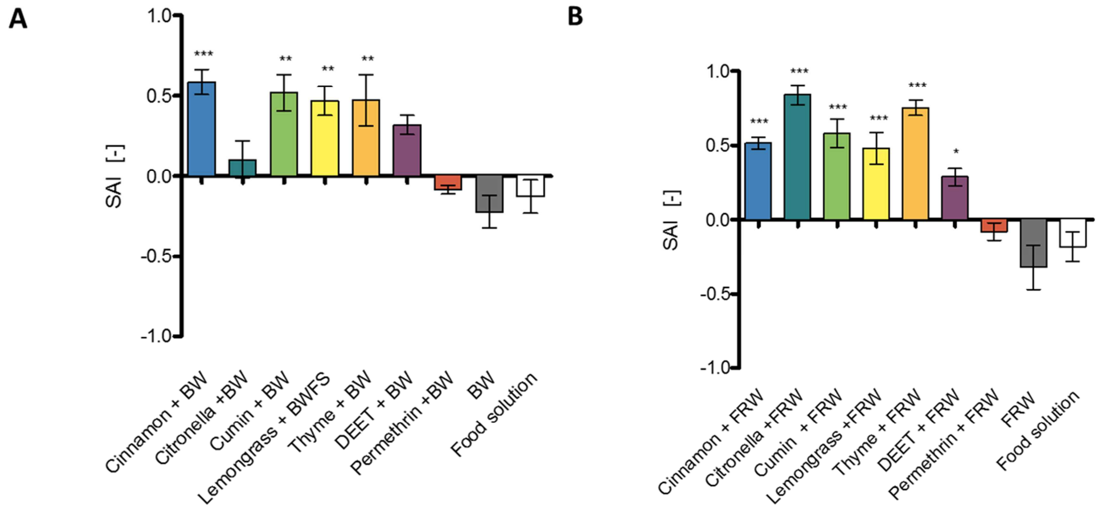

***Figure 2.** Spatial repellent effect of mosquitoes to 5% essential oils embedded in BW and FRW.*

Repellent effect represented by SAI of mosquitoes following exposure for 20 mins to 5% essential oils embedded in BW (A) or FRW (B). "1" indicates that all mosquitoes were repelled while "-1" indicates that all mosquitoes were attracted. These experiments confirm the repellent effect of the essential oils as well as DEET, which are significant for most compounds in comparison to the control condition (food solution; p≤0.05*, p≤0.01**, p≤0.001***; ANOVA and Bonferroni's Multiple Comparison Test). Each experiment was performed in five replicates 20 female mosquitoes each, 2 to 7 d old, 24 h after food deprivation (n=5). Data are represented as mean ± SEM.

3.6. Repellent Effect of Oil-Wax Formulations over Time

To determine if impregnating essential oils in waxes will reduce their volatility and allow them to be active for longer periods, the three most active essential oil formulations (cinnamon, cumin and lemongrass) embedded in both BW and FRW were investigated using blood as attractant for their repellent effect over a period of 10 h. In this experimental series, the number of mosquitoes being either repelled or attracted was counted every hour. In comparison to the previous experiments, this procedure allows for a temporally more precise analysis of the mosquitoes' behaviour over a greater time scale. Furthermore, since most repellents that are commercially available are active between 6 and 10 h, these experiments were designed to compare the repellent effect of the oil-wax formulations with those products.

It was observed that using both BW and FRW, the repellent effect of the essential oils reduced over time, as compared to DEET which remained stable (Figure 3A, B). Interestingly, the repellent effect of DEET increased over time in both conditions: at the beginning, about 60% of the mosquitoes were in the lower half of the experimental setup while after 10 hours; about 90% of the mosquitoes were observed close to the bottom in the behavioural setup. A closer analysis for BW (Figure 3A) revealed that all essential oils as well as DEET were statistically more efficient than the negative control (blood; $p \leq 0.05$; two-way-ANOVA). In comparison to DEET, the effect of cinnamon significantly increased after 1 hour and decreased significantly after 7 hours, for cumin after 2 hours and for lemongrass after three hours ($p \leq 0.05$; two-way-ANOVA). Thus, cumin and lemongrass rapidly lost their repellent effect in comparison to the positive control. In the condition with FRW (Figure 3B), the results were similar if one compares the effect with the negative control (blood). For cumin, the repellent effect did not differ significantly after 7 and 10 hours, respectively ($p > 0.05$; two-way-ANOVA). A comparison with DEET also reveals similar results: the repellent effect of cumin (after 1 hour) and lemongrass (after 2 hours) decreases significantly faster than compared to DEET, whereby cinnamon has a comparable averse effect up to six hours, before the efficacy also decreases significantly ($p \leq 0.05$; two-way-ANOVA). In summary, DEET displayed the most effective repellent effect over the entire time period. Nonetheless, cinnamon in BW and FRW was still able to repel more than 50% of the mosquitoes after 10 h indicating a considerable repellent effect.

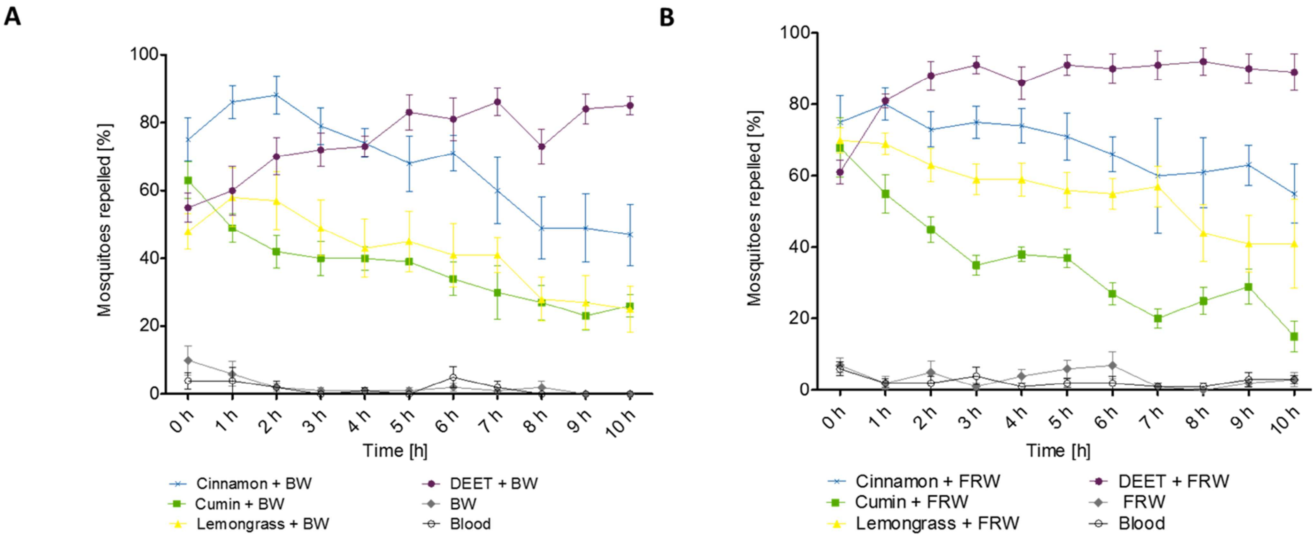

Figure 3. *Repellent effect of fabrics impregnated with essential oils embedded in BW and FRW over time.*

The repellent effect of impregnated fabrics with essential oils embedded in BW (A) and FRW (B) was determined using the vertical setup and human blood at 30°C as attractant. The number of repelled mosquitoes were counted every hour over a period of 10 h. Data are represented as mean ± SEM. In both cases, the repellent effects of the essential oils decrease over time, while DEET remained stable. For both BW (A) and FRW, the repellent effects of the essential oils and DEET were stronger than those of the negative control.

4. Discussion

Essential oils have been shown to provide sufficient protection against mosquito bites. However, their high volatility as well as their mode of application exhibit a significant draw back for their use as mosquito repellents. It is therefore necessary to improve both the stability of essential oils as well as a practicable and effective mode of application before they could be effectively used as mosquito repellents. This study aimed not only to reduce the volatility of known repellent essential oils by embedding them in waxes, but also to provide a simple application by impregnating the formulations on clothing so as to provide outdoor personal protection against mosquito bites. It was hypothesized that these waxes will preserve the activity of the essential oil leading to a gradual release of the active components and therefore retain their activity over a longer period of time. Comparable approaches have for example been conducted with gelatine-gum Arabic microcapsules that preserved the repellent effect for about one month [19]. Binding essential oils to waxes might enable an easy to use application since the stable oil-wax formulations can easily be impregnated on clothing by ironing or even camping fires. Therefore, such applications will be ideal in economically weaker countries where malaria is endemic.

In order to achieve our goal, essential oils previously reported to display high repellent activity were used as proof of principle [16]. The oils were embedded in BW and FRW and their effects were determined through behavioral experiments using laboratory reared female *A. stephensi* mosquitoes. Comparing the repellent activity of 2% of the essential oils to that of the oil- wax formulations after 20 mins showed that the unembedded oils displayed a higher repellent effect as compared to when they were embedded in the different waxes. The reduced activity of the oil-wax formulations is probably due to the fact that during the embedding process the waxes were heated, which may have resulted in higher evaporation of the active ingredients of the oils. Nonetheless, this reduced effect might also be accompanied by a lowering of the vapor pressure when adding the essential oils to the waxes. Thus, less active molecules of the essential oils are volatile and can reach the sensory organs of the mosquitoes. Interestingly, the adhering effect of the mosquitoes seems similar when the oils were used alone and when they were embedded in the waxes indicating that although most of the active ingredients of the oils evaporated during the embedding process, the small amount trapped in the waxes could be sufficiently high to hinder the mosquitoes from adhering to it. This was further justified by the fact that the number of mosquitoes which took a meal after 18-24 h was comparable in both treatments.

To determine if the repellent effect could be enhanced, the concentration of the oil-wax formulations was increased from 2% to 5%. It was observed that except for cinnamon in both BW and FRW, which showed a repellent effect greater than 50%, the other formulations still had very low repellent effects on the mosquitoes. However, no mosquito adhered to the formulations after 20 mins and the number of mosquitoes that did feed was dramatically decreased compared to the condition with 2% of the essential oils. Furthermore, a higher mortality after 18-24 h in the test condition with 5% of the essential oils indicates a gradual release of the active ingredients from the oils following embedding. When the attractant was changed from sugar food solution to blood at 30°C, the repellent effect of the essential oils increased indicating that the heated blood causes a higher as well as a faster evaporation of the active ingredients due to an increase of the vapor pressure. This suggests that once the formulations are applied to clothes and worn, the body temperature from the skin will help to increase the release of the active ingredients of the oils which is sufficiently enough to displace a considerable repellent effect. On the other hand, this might result in a much faster decay of the repellent effect over time as might be seen in less dead mosquitoes after 18-24 h compared to the conditions where sugar solution was used as an attractant.

To determine if the embedded oils could display repellent effects over a longer period of time, the most active essential oil-wax formulations at 5% were tested for 10 h and the repellent effects were compared with those of 5% DEET. The results show that while the repellent effect of DEET remained stable, the effects of the oil-wax formulations decreased gradually over time. However, cinnamon in FRW showed a very slow loss of its repellent activity with more than 50% of mosquitoes repelled after 10 h indicating that further improvement of the formulation may result it a potential mosquito repellent. Compared to other approaches, the repellent effect of around 60% for cinnamon is promising. For example, microencapsulated essential oils tested with *Culex quinquefasciatus* for 8 h showed a repellency of around 55 - 67% when applied in the same concentration [6]. However, in the same study, the effect of DEET also decreased dramatically within this period and repelled only 77.05% of the mosquitoes. This is much less than observed in the results of this study. The efficacy of a novel insect repellent – ethyl anthranilate – decreased even faster over time when tested at the same concentration of 5% with *A. stephensi* mosquitoes [20]. After 4 h, only 13.3 ± 9.8% of the mosquitoes were repelled. At this time point, about 40% of the mosquitoes were repelled in the experiments in the present study when cumin and lemongrass were applied. For cinnamon, more than 75% of the mosquitoes did not come in contact with the food solution. Moreover, the long-lasting effectiveness of essential oils such as cinnamon and lemongrass is supported by a study of Amer and Mehlhorn [21], which also demonstrated a strong repellent effect of 100% over 8 h of these oils for *A. stephensi* mosquitoes, although their test concentration was 20% instead of 5%.

As has been shown, several other different stabilizing agents have been tested with the aim to stabilize essential oils but none of the formulations displayed a comparable efficacy as DEET. Microencapsulation of essential oils in lotion formulations showed a greatly improved repellent effect of the essential oils but it was not comparable to that of DEET [6]. Also, encapsulated citronella oil in nanoemulsion led to an increase in retention of the essential oil and a slow release of the active ingredient of the oil thereby resulting in a sustained mosquito protection time [22]. However, the activity could not be compared with that of DEET as well. The same holds true for the experiments in this study.

5. Conclusion

To conclude, embedding essential oils in waxes and impregnating the formulation to clothing did not improve the repellent effect against *Anopheles* mosquitoes after 20 mins. However, most of the mosquitoes were still not able to feed after 18-24 h of exposure to the essential oils indicating a slow release of the active ingredients of the essential oils after embedding in waxes. Therefore it is recommended to improve the wax embedding process in order to provide a longer lasting effect of the essential oils. Additionally, higher concentrations of the essential oils or even blends of several mixed essential oils might prolong the repellent effects of such products which might be used in malaria-endemic countries.

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Conflicts of Interest

The authors declare that they have no competing interests.

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