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The combined efficacy of neem (*Azardirachta indica*) seed oil and orange (*Citrus sinensis*) peel oil cream as a mosquito repellent

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Abstract

Essential oils obtained from certain plants have repellent potency against insects such as mosquitoes. Most repellents on the market have the synthetic formulation of N, N-diethyl-3-methylbenzamide (DEET) as the main active ingredient. This study is an attempt to contribute to the search for an effective plant-extracted essential oil, Neem and Orange as alternative to DEET.

The oil from *Citrus sinensis* (sweet orange) peels was extracted by steam distillation and that for *Azadirachta indica* (Neem) seed by Soxhlet extraction respectively. Creams were formulated at four concentrations of 5%, 15%, 25% and 30% v/w respectively for Neem, Orange peel and combined Neem-Orange oils, after which laboratory reared mosquitoes were used to assess the efficacy of each of the concentrations. A DEET-based cream of 13% concentration was used as the standard.

Wild mosquito larvae were brought to the laboratory from which the adults female after mating were given a blood meal. Second generation mosquitoes were used for the arm in cage test (AIC).

The combined Neem and Orange-based cream at 30% was as effective as the DEET-based repellent which recorded the highest repellent time, followed by Neem cream and the Orange peel based cream recorded the least repellent time even at 30% concentration. The statistically significant difference at 30% between DEET and Orange peel, Neem, and combined Neem-Orange creams were 0.030, 0 .499 and 0.195, respectively.

This study suggests that the combined Neem and Orange Peel Oil can be used to produce repellent creams due to their synergistic potency and good smell.

Keywords: Natural mosquito repellent; Essential oil; *Anopheles*; *Aedes*

1. Introduction

Mosquitoes are important public health concern especially in developing countries as a result of being vectors to many pathogenic organisms that infect humans[1, 2]. They serve as vectors for the transmission of dengue fever, malaria, filariasis, yellow fever just to mention few[3, 4].

According to the 2021 World Malaria Report, there was an estimated figure of 241 million cases of malaria worldwide in the year 2020 with Africa constituting 95% of the world burden. Additionally, the World Health Organization, Africa

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Region carried 96% burden of the 627 000 total global malaria deaths estimated in 2020 [5]. Malaria is also responsible for a significant maternal deaths in Ghana and Africa as a result of contracting malaria during pregnancy[6]. From the findings of Ameme *et al*, (2014), Malaria accounts for 40% of outpatients cases in Ghana from their cross sectional study. Transmission cycle of human-vector contact can be broken by the use of repellent [8]. Additionally, the application of proper mosquito repellent on clothing and bed nets have yielded massive positive results especially in Africa, conferring protection to the individual using them [9, 10]. Moreover, topical repellent is one of the most effective ways of controlling outdoor biting of mosquitoes[11].

Furthermore, N, N-diethyl-3-methylbenzamide (DEET) based repellents are the standard and most common synthetic products in our markets today. Even though very effective, the repercussions of their continuous use cannot be overlooked. The fear of the side effects of DEET by people is gradually giving market to the natural repellents [12]. The findings of Colucci and Müller[2018] showed that DEET showed at least 6 hours of protection against *Aedes aegypti, Anopheles stephensi* and *Culex quinquefasciatus* in a field studies in Switzerland at 15% [14–16]. Additionally, increasing insecticide resistance has posed a major obstacle to mosquito control programmes and this calls for the development of new repellents[12, 17].

The use of botanicals and derivatives as insect repellent particularly against mosquitoes is a feasible option to achieve protection against the mosquitoes, and thus, reducing the risk of acquiring mosquito borne diseases[16, 18, 19]. Among the various natural organic chemicals, products from the neem tree, *Azadirachta indica*, like neem seed oil have long been established as a potential mosquito repellent[20]. Azadirachtin, a complex tetra-terpenoid limonoid compound in neem seeds, is the major component responsible for the repellent action in insects [21]. Moreover, repellents from essential oils are environmentally friendly, biodegradable and not harmful to non-target organism as compared to the synthetic DEET[22].

Citrus sinensis on the other hand considered in this study has several usefulness. Juice from the fruits is used in the production of different beverages in large quantities. Flavour from the oil of peels are used to season foods and it has repellent effect as well due to the presence of limonene [23–25]. Peels of Citrus fruits (*Citrus sinensis*) has also been proven to possess repellent properties [26]. Exploration of different botanicals against mosquitoes should be continued, thereby reducing the contact of human and mosquitoes as well as the risk of transmission of diseases to humans [27, 28].

This present manuscript elaborates the evaluation of the combined efficacy of neem seed oil and orange peel oil as mosquito repellent against *Aedes aegypti* and *Anopheles gambiae* using DEET as the standard.

2. Material and methods

2.1. Study design

This is an experimental study conducted in the insectary of the Department of Theoretical and Applied Biology at Kwame Nkrumah University of Science and Technology, KNUST Kumasi, Ghana within the period of September 2019 to February 2020.

2.2. Sample collection, extraction, and phytochemical screening

The neem (*Azadirachta indica*) seed oil and sweet orange (*Citrus sinensis*) peel oil were purchased from Agape Moringa Processing in Tamale Northern Region of Ghana. The seed and the peels were collected from Tamale as well. Coloured rind of *Citrus sinensis o*f two oranges were put into Clevenger apparatus and distilled water of 100 cm^3 was added. The flask containing the peels was heated just below 100°C and distillation took place steadily. Distillate of 50 cm^3 was collected in a measuring cylinder and the oil layer is removed with pipette completing the steam distillation method [29].This was done till 200ml of oil was obtained.

Azadirachta indica from Tamale were dried and milled into powder. Oil was extracted from the powder (458.65 g) using Petroleum ether (2.2 L) with the Soxhlet apparatus for 4 days. The extract was concentrated using the rotary evaporator at temperature 45°C and a percentage yield of 43.6% v/w obtained. The oil was then stored in an amber bottle at a cool dry place until ready for use.

$$\text{Yield of extract obtained} = \frac{Volume\ of\ neem\ seed\ oil\ x\ 100\%}{Weight\ of\ plant\ material\ taken}$$

The extracted *Citrus sinensis* and *Azadirachta indica* oils were phytochemically screened according to the method used by [30] and [31] to confirm limonene and azardirachthin respectively which were the active ingredient responsible for the repellent effect before formulating it into a cream.

2.3. Processing of active ingredient into cream

Twenty (20g) of Aqueous Cream was weighed and a calculated measured volume of the active ingredient (neem seed oil, orange peel oil or both) was added to the cream base. The volume of the oil or active ingredient added was determined by the standard formula for preparing aqueous cream which is the quantity to be prepared divided by quantity in the master formula. The mixture was stirred, slightly heated and stirred again with a spatula. The mixture was then homogenized to form the cream which was allowed to cool. Varying concentrations of 5%, 15%, 25% and 30% v/w of the active ingredient (neem seed oil or orange peel oi) was added to the emulsifying ointment. The homogenized cream was allowed to cool or solidify and then packaged into containers. The above concentrations were chosen because Citrus oil contains a compound called bergapten which usually above 30% concentration causes phototoxic skin irritation [32].

2.4. Repellent test by arm in cage test (AIC)

Repellent test was done using the World Health Organisation Pesticide Evaluation Scheme (WHOPES) 2013 guidelines with slight modification [33] thus, larva of *Anopheles gambiae* and *Aedes aegypti* were brought to the laboratory and the second generation of 5 days old were used for the repellent testing.

The twelve (12) volunteers were put into four (4) groups of three (3). Each group of three volunteers was assigned to one concentration of the repellents, thus 5%, 15%, 25% and 30% respectively for orange cream and thereafter Neem only and combined creams.

A confined netted cage on all sides made with wooden bar 20 cm by 20 cm by 20cm. A piece of hollow cloth, big enough to allow for an arm or a glass screen to enter was fixed at one side of the nets. The netted cage was filled with 30 laboratory-reared mated and sorted female mosquitoes of *Anopheles gambiae* and *Aedes aegypti* of 5 days old.

Bare hand without repellent was first introduced into the cage as a negative control followed by aqueous cream base which was used to process the essential oils into repellent creams to see if it has any repellent effect and time recorded. The cream of each concentration of a particular extract was smeared on the arm and the hand was put into the repellent cage with mosquitoes starved for 12 hours. The hand was observed and the time it took for mosquito to land was recorded. This was done by putting the hand in the box every 5 minutes (min) interval after the first 5 min till 30 min. If no mosquito landed after 30 min, the interval time was increased to 15 minutes. Moreover, if no mosquito landed till 6 hours, the six hours was recorded as the complete protection time of the repellent. Also, the conditions of the participants were observed during and after the test for any adverse reactions.

2.5. Statistical analysis

The results were entered into Microsoft Excel and analysed using SPSS. One-Way Analysis of Variance (ANOVA) and independent student t-test were used to compare the Complete Protection Time of the various creams. All tests were performed at a significant level of 0.05.

3. Results

3.1. Repellent results for *Aedes aegypti* mosquitoes

Figure 1 compares the complete protection time (CPT) between neem seed oil cream and orange peel oil cream. At 5% concentration, the CPT for neem seed oil cream was 0.35 ± 0.04 minutes while orange peel oil cream recorded CPT of 0.34 ± 0.05 minutes. The trend did not change when concentrations were increased to 15% and 25%. The CPTs for neem seed and orange peel oil creams were 1.01 ± 0.19 and 0.83 ± 0.07 minutes respectively at 15% concentration and 3.60 ± 0.25 and 1.87 ± 0.23 minutes respectively at 30% concentration. However, at 25% concentration, the CPT of neem seed oil cream (1.53 ± 0.13 minutes) was higher than that orange peel oil cream (1.48 ± 0.07 minutes). Generally, an independent t-test showed that there was no statistically significant difference ($p > 0.05$) between the CPT of neem seed and orange peel oil creams at 5%, 15% and 25% concentrations. However, there was a statistically significant difference ($p = 0.001$) between the CPT of the two products at 30% concentration.

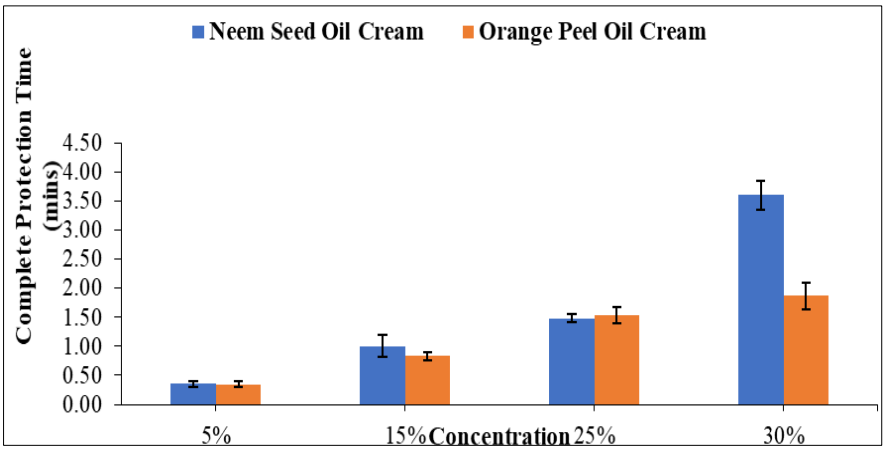

Figure 1 Comparison of Repellent Time of Neem and Orange Creams *(Aedes aegypti)*

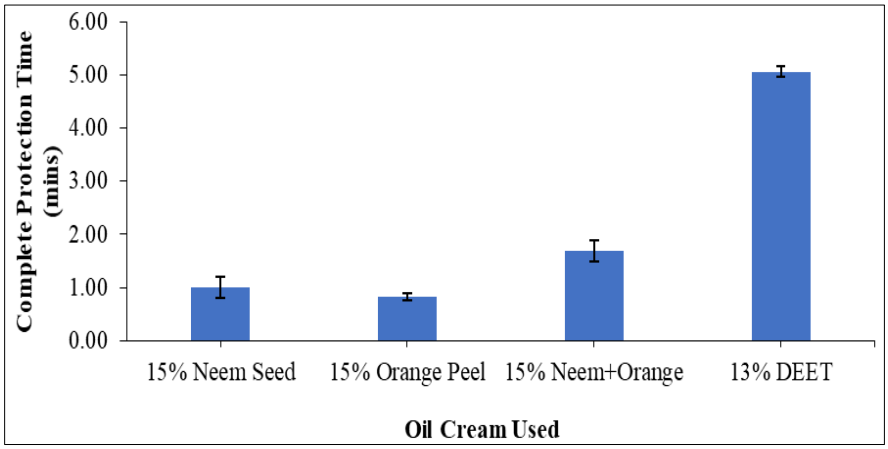

Figure 2 Comparison of natural creams at 15% against DEET *(Aedes aegypti)*

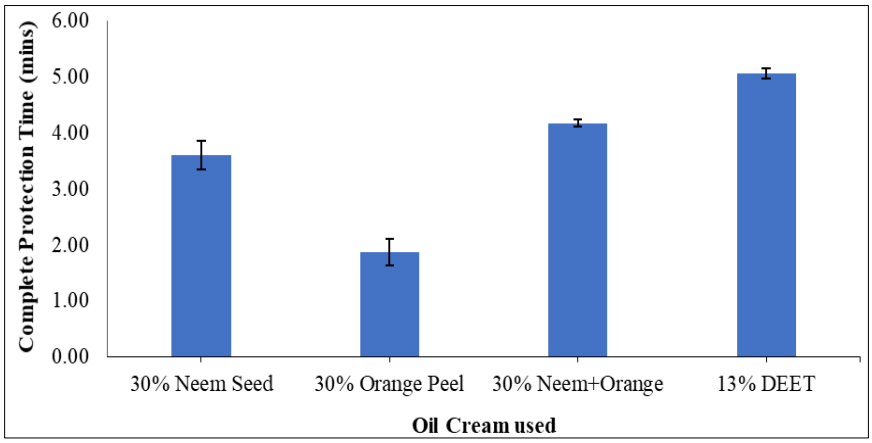

Figure 3 Comparison of natural creams at 30% against DEET *(Aedes aegypti)*

The 13% DEET is standard product on the market and the study compared the CPTs of the prepared products to the standard. The 13% DEET (5.06 ± 0.10 minutes) had a higher CPT compared to the neem seed (1.01 ± 0.19 minutes), orange peel (0.83 ± 0.07 minutes) and combined neem seed and orange peel (1.69 ± 0.20 minutes) oil creams at 15% concentration (Fig 2). There was a statistically significant difference between 15% neem seed vs. 13% DEET ($p < 0.001$); 15% Neem seed vs. 15% combined neem seed and orange peel ($p = 0.002$); 15% orange peel vs. 15% combined neem seed and orange peel ($p = 0.001$); 15% orange peel vs. 13% DEET ($p < 0.001$); and 15% combined neem seed and orange peel vs. 13% DEET ($p < 0.001$).

When the 13% DEET was compared to the CPTs of the prepared products at 30% concentration, the 13% DEET (5.06 ± 0.10 minutes) still had a higher CPT compared to the neem seed (3.60 ± 0.25 minutes), orange peel (1.87 ± 0.23 minutes) and combined neem seed and orange peel (4.17 ± 0.07 minutes) oil creams at 30% concentration (Fig 3). Generally, there was a statistically significant difference ($p < 0.001$) between 13% DEET and all the products at 30% concentration.

3.2. Repellent results for *Anopheles gambiae* mosquitoes

For *Anopheles* mosquitoes, at 5% concentration, there was no difference between the mean CPTs of neem seed (0.33 ± 0.08 minutes) and orange peel (0.33 ± 0.12 minutes) oil creams. However, at concentrations of 15%, 25% and 30%, the mean CPTs of neem seed (3.43 ± 1.15 minutes, 10.07 ± 0.39 minutes, and 25.38 ± 0.48 minutes respectively) was higher than that of orange peel oil cream (1.83 ± 0.66 minutes, 2.91 ± 1.59 minutes, and 10.01 ± 5.76 minutes respectively) as shown in Fig 4. Statistically, significant difference existed between the two products only at 25% concentration ($p = 0.001$) and 30% concentration ($p = 0.010$).

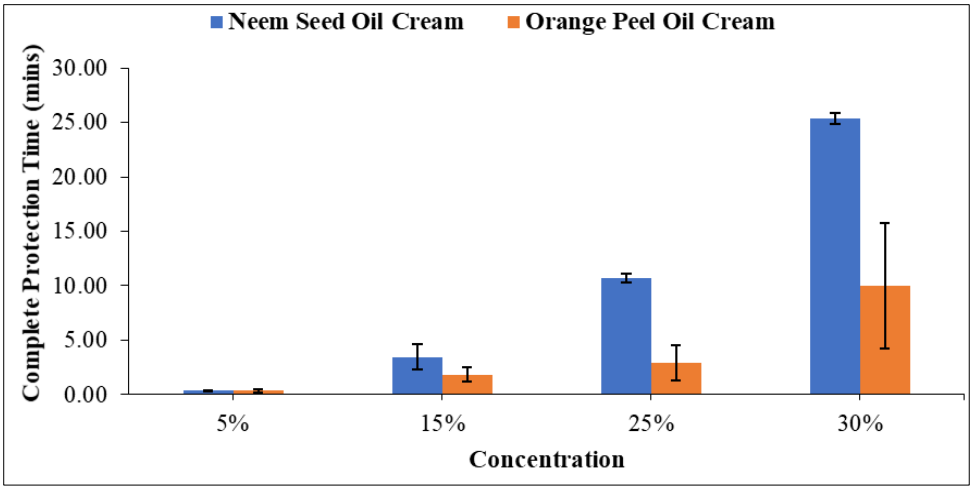

Figure 4 Comparison of neem and orange creams *(Anopheles gambiae)*

The 13% DEET (20.90 ± 0.50 minutes) had a higher CPT compared to the neem seed (3.43 ± 1.15 minutes), orange peel (1.83 ± 0.66 minutes), and the combined neem seed and orange peel (3.75 ± 0.03 minutes) oil creams at 15% concentration (Fig 5). Statistically, significant difference existed between 15% neem seed vs. 13% DEET ($p < 0.001$); 15% orange peel vs. 15% combined neem seed and orange peel ($p = 0.042$); 15% orange peel vs. 13% DEET ($p < 0.001$); and 15% combined neem seed and orange peel vs. 13% DEET ($p < 0.001$).

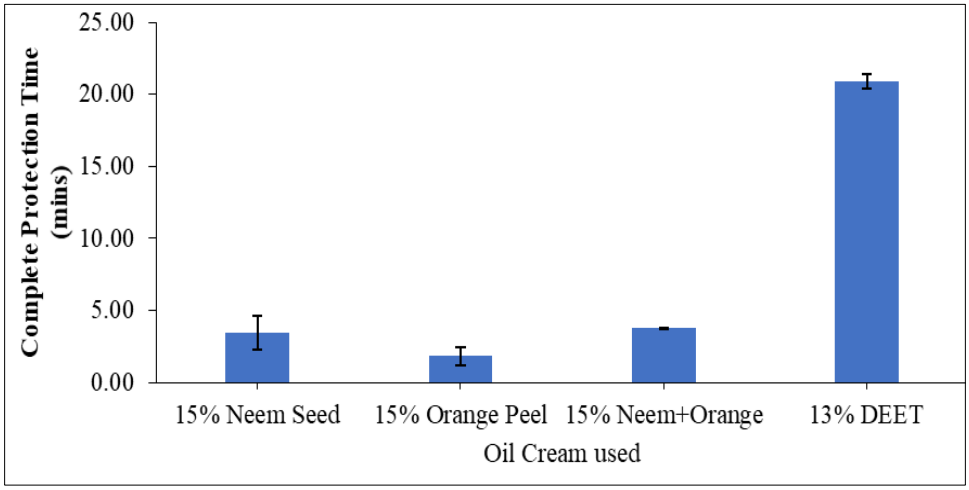

Figure 5 Comparison of natural creams at 15% against DEET *(Anopheles gambiae)*

When concentrations of the products were increased to 30%, the CPTs of 30% neem seed (25.38 ± 0.48 minutes) and 30% combined neem seed and orange peel (27.72 ± 4.75 minutes) were higher than the CPT of 13% DEET (20.90 ± 0.50

minutes) as shown in Fig 6. Statistically significant difference existed only between 30% neem seed vs. 30% orange peel (p = 0.004), 30% orange peel vs. 30% combined neem seed and orange peel (p = 0.002), and 30% orange peel and 13% DEET (p = 0.030).

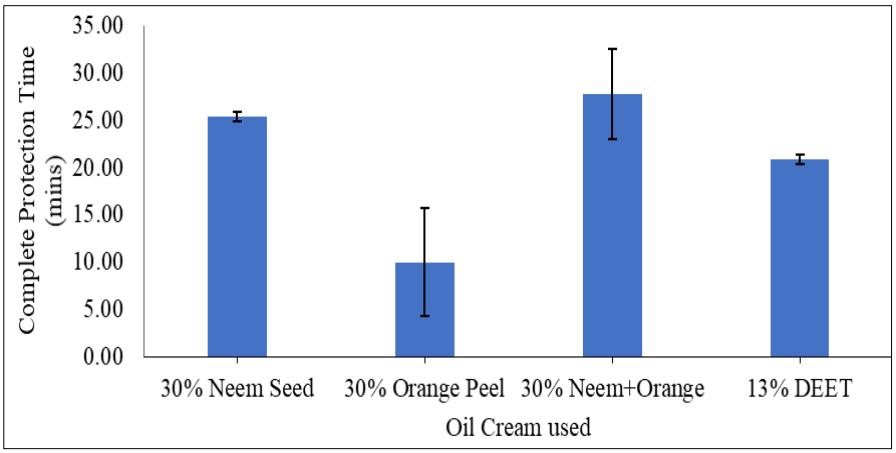

Figure 6 Comparison of natural creams at 30% against DEET (*Anopheles gambiae*)

3.3. Negative Controls

The time recorded for the bare hand and the aqueous cream base used to prepare the repellent creams were the same. Thus, no repellent property in aqueous cream (S1 Table).

Table 1 Comparison of the complete protection time between *Anopheles* and *Aedes* mosquitoes

Product	Complete Protection Time (in minutes)		p – value
	Anopheles	***Aedes***	
5% concentration			
Neem seed	0.33 ± 0.08	0.35 ± 0.04	0.778
Orange peel	0.33 ± 0.12	0.34 ± 0.05	0.897
Neem seed & orange peel	1.27 ± 0.07	0.42 ± 0.08	< 0.001*
15% concentration			
Neem seed	3.43 ± 1.15	1.01 ± 0.19	0.023*
Orange peel	1.83 ± 0.66	0.83 ± 0.07	0.118
Neem seed & orange peel	3.75 ± 0.03	1.69 ± 0.20	< 0.001*
25% concentration			
Neem seed	10.70 ± 0.39	1.48 ± 0.07	< 0.001*
Orange peel	2.91 ± 1.59	1.53 ± 0.13	0.274
Neem seed & orange peel	11.02 ± 0.07	1.88 ± 0.08	0.001*
30% concentration			
Neem seed	25.38 ± 0.48	3.60 ± 0.25	< 0.001*
Orange peel	10.01 ± 5.76	1.87 ± 0.23	0.071
Neem seed & orange peel	27.72 ± 4.75	4.17 ± 0.07	0.013*
Control			
13% DEET	20.90 ± 0.50	5.06 ± 0.10	< 0.001*

Numbers with asterisk (*) indicate the mean difference is significant at the 0.05 level.

4. Discussion

Previous study [34] indicated that 2% neem oil mixed in coconut oil was sufficient to provide 12h protection from the bites of *anopheline* mosquitoes. In another study, the combination of the neem oil with kerosene oil showed protection against mosquitoes [35]. In 2004, a commercial product called Bite Blocker which was made up of glycerine, lecithin, oils of coconut, geranium and 2% soyabean showed 7.2 hours protection against *Aedes albopictus*[36].

Moreover, a laboratory study of *Citrus sinensis* oil at 0.02 mg/cm against *Anoheles dirus* provided 40% protection. A recent study from Ethiopia has shown about 20% neem oil combination provide 3-hour protection against *Anopheles arabiensis* in a field study [8]. Application of neem oil in water, (5 g/l; 0.3% Azadiractin in 1 litre containing the emulsifier Sorbitan, tri-9-octadecenoate, poly(oxy-1,2-ethanediyl) was effective in reducing the abundance and oviposition of the mosquito *Aedes albopictus* [37]. However, a systemic review in 2018 demonstrated that *Ligusticum sinense* extract was able to give 11 hours of protection against Anopheles mosquitoes[38].

From Figs 1 and 4, an increase in concentration corresponded with an increase in complete protection time (CPT) thus, 5%, 15%, 25% to 30% in that order. This trend was not different from an earlier research conducted on neem seed oil as mosquito repellent and repellent action of orange peels oil on mosquitoes [26, 35]. Generally, there was a statistically significant difference ($p < 0.001$) between the various concentrations of Neem seed and Citrus oil creams. However, a post hoc test showed no significant difference ($p = 0.079$) between orange peel oil creams at 25% and 30% concentrations for *Aedes aegypti* and 5% vs. 30% concentrations ($p = 0.018$), and 15% vs. 30% concentrations ($p = 0.042$) for *Anopheles gambiae*.

Furthermore, the high volatility of citrus could be responsible for almost an equal protection time between Neem and Orange oil Creams at lower concentrations but drastically reduced at higher concentrations, especially at 30% [39]. Interestingly, the combined neem seed and orange peel oil cream did better than the individual creams (Figs 2, 3, 5, 6 and Table 1). The results indicated synergy between orange and neem oils because the formulation was 50:50 v/v. For instance, the CPT of *Anopheles gambiae* at 30% for the combined cream gave (27.72 ± 4.75), this constituted 15% neem seed oil and 15% orange peel oil which would have given (3.43 ± 1.15) and (1.83 ± 0.66) CPT individually (Table 1). The synergistic repellent effect of combined essential oils observed confirmed recent findings by Noosidum *et al*, [2014] and Feroz, [2020].

Repellent test involving skin application of Citrus peel oil at 20% and other essential oil produced irritancy in previous studies [26, 40]. On the contrary, there was no record of any adverse reaction by all the participants during and after this study probably due to incorporation of the oil into cream.

Also, DEET had excellent protection against *Aedes aegypti* compared to the natural creams at all concentrations. However, *Anopheles gambiae* at 30% of neem only, and combined neem-orange creams (Figs 3 and 6) compared with DEET gave a CPT mean difference of 4.48 and 6.82 respectively without significant difference between them. This is an important indication that Combined Neem and Orange peel oil cream can be used as an alternate effective mosquito repellent in Malaria endemic regions.

Finally, it is evident from the results that the CPTs of *Aedes aegypti* were much lower than that of *Anopheles gambiae*, similar to research conducted in 2018 to evaluate CPTs of both Arm In-Cage Test (AIC) and field for two standard mosquito repellents in Switzerland. For the AIC, the CPTs for *Aedes aegypti* and *Anopheles gambiae* were 0.5 and 2 hours respectively for DEET [13]. However, the results here for DEET (Table 1) are much lower than that recorded in their work probably due to lower concentration used or environmental changes. This could also be due to continuous reduction in repellence of DEET to *Aedes aegypti* upon previous exposure conferring genetic resistance [42]. As well as an indication of resistance of mosquitoes to repellents especially the standard DEET on the market.

5. Conclusion

The observation from this study has shown clearly that DEET-based repellent is more effective against *Aedes aegypti* than the plant products. However, Combined Neem Seed and Orange Peel Oil Cream at 30% is as effective as DEET and therefore can be used as an effective mosquito repellent in Malaria endemic regions. There was generally reduced complete protection time compared to previous studies for the standard, DEET and the plant repellents.

Compliance with ethical standards

Acknowledgments

I say a big thank you to Mr. Akuamoah Yaw and Patrick Obuam for their assistance for the field work and Esther Naanbir (Agape Moringa Processing) for the extraction of the essential oils. And, the Department of Theoretical and Applied Biology, KNUST for giving us the insectary for this research.

Disclosure of conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Statement of ethical approval

Ethical clearance with reference CHRPE/AP/417/19 was obtained from the Committee on Human Research Publication and Ethics Kwame Nkrumah University of Science and Technology and Komfo Anokye Teaching Hospital on the 3rd of July 2019. Also, participant information and consent forms were given to volunteers with the explanation of the purpose of the research. The twelve volunteers who consented were enrolled into the research for the repellent test.

Supporting information

Table S1 Raw results for *anopheles gambiae*

***Anopheles gambiae* mosquito**							
Concentration	**Neg. Ctrl barehand (sec.)**	**Replicates (in sec.)**				**CPT (in sec.)**	**CPT (in min.)**
		1	2	3	Average	Avg. – Control	
Complete Protection Time (CPT) for Neem Seed Oil Cream							
5%	10	35	30	25	30	20	0.33
15%	5	145	205	283	211	206	3.43
25%	4	640	672	626	646	642	10.70
30%	6	1503	1524	1560	1529	1523	25.38
Complete Protection Time for Orange Peel Oil Cream							
5%	9	22	29	36	29	20	0.33
15%	5	89	95	160	115	110	1.83
25%	3	145	102	285	177	174	2.91
30%	5	647	241	928	605	600	10.01
Complete Protection Time for Orange Peel Oil and Neem Seed Oil Cream							
5%	6	86	82	78	82	76	1.27
15%	5	232	228	230	230	225	3.75
25%	3	668	664	660	664	661	11.02
30%	6	1830	1340	1838	1669	1663	27.72
Complete Protection Time for Aqueous Cream (Negative control) and Positive Control (DEET)							
13% DEET	6	1290	1260	1230	1260	1254	20.90
Aqueous Cream	5	5	6	6	6	1	0.01

Note: *There is no mean difference between the Negative controls of bare hands (without repellent) and Aqueous Cream Base used to prepare the repellent.

Table S2 Raw results for *Aedes aegypti* mosquitoes

***Aedes aegypti* mosquito**					
Concentration	**Replicates (in seconds)**				**CPT (in min.)**
	1	2	3	Average	
Complete Protection Time (CPT) for Neem Seed Oil Cream					
5%	19	20	24	21	0.35
15%	60	49	72	60	1.01
25%	89	93	85	89	1.48
30%	231	201	216	216	3.60
Complete Protection Time for Orange Peel Oil Cream					
5%	20	18	24	21	0.34
15%	50	46	54	50	0.83
25%	92	84	100	92	1.53
30%	126	98	112	112	1.87
Complete Protection Time for Orange Peel Oil and Neem Seed Oil Cream					
5%	25	20	30	25	0.42
15%	100	114	90	101.3	1.69
25%	118	108	112	112.7	1.88
30%	250	254	246	250.0	4.17
Complete Protection Time for Aqueous Cream Base (Negative Control) and Positive Control (DEET)					
13% DEET	300	310	300	303	5.06
Aqueous Cream	3	1	2	2.0	0.03

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