CHAPTER 3

Results

3.1 Plant preparations

3.1.1 Preparation of crude plant extracts

Crude plant extracts were obtained from 33 plant species by using conventional extraction methods, steam distillation, and ethanolic solvent extraction, which demonstrated different yields, color, and appearance (Table 3.1). In essential oil isolations, only nine plant samples, including *Angelica sinensis* rhizome and root, *Homalomena aromatica* rhizome, *Acorus calamus* rhizome, *Cinnamomum verum* bark, *Curcuma zedoaria* rhizome, *Murraya paniculata* leaf, *Limnophila aromatica* whole plant, *Petroselinum crispum* fruit, and *Zingiber cassumunar* rhizome, offered liquid oils, with yields of 0.02, 0.20, 0.22, 0.48, 0.66, 0.88, 1.53, 1.74, and 1.75% (v/w), respectively. All steam distillate oils obtained were fragrant and less dense than water; except for *A. sinensis* oil, which had a pungent odor. None of the remaining plants provided any essential oils. In solvent extractions, it was found that plant-ethanol macerations provided varying quantities of 33 extracted products, with the highest yield acquired from *M. paniculata* leaf (33.71%, w/w) and the lowest from *Tamarindus indica* seed (2.52%, w/w).

Table 3.1 Percentage yield (%Yield), color and appearance of plant products, including essential oils and ethanolic extracts

Plant/Chemical	Plant products			
	Essential oil		Ethanolic extract	
	%Yield	Color & Appearance	%Yield	Color & Appearance
A. aspera	0.0	ND	4.29	Green semisolid
A. calamus	0.22	Colorless liquid	7.80	Dark brown semisolid
H. aromatica	0.20	Colorless liquid	2.73	Brown semisolid
A. marina	0.0	ND	10.14	Brown powder
C. papaya	0.0	ND	5.53	Brown semisolid
C. magna	0.0	ND	2.65	Pale yellow powder
C. viscosa	0.0	ND	5.99	Green semisolid
A. annua	0.0	ND	12.09	Green semisolid
A. lancea	0.0	ND	20.50	Brown semisolid
T. erosus	0.0	ND	19.92	Yellow powder
C. moschata	0.0	ND	4.15	Green semisolid
M. charantia	0.0	ND	6.09	Green semisolid
P. amarus	0.0	ND	7.03	Green semisolid
P. erosus	0.0	ND	19.84	Pale yellow semisolid
C. verum	0.48	Pale yellow liquid	7.07	Red brown powder
T. indica	0.0	ND	2.52	Pale green viscous liquid
A. esculentus	0.0	ND	6.63	Green powder
T. crispa	0.0	ND	5.16	Pale green powder
B. monosperma	0.0	ND	26.23	Pale green powder
D. scandens	0.0	ND	9.57	Pale yellow powder
S. orientale	0.0	ND	21.60	Pale yellow semisolid
P. odoratum	0.0	ND	13.30	Dark green powder
M. paniculata	0.88	Colorless liquid	33.71	Dark green semisolid
L. aromatica	1.53	Light yellow liquid	10.47	Dark green semisolid
S. aculeatissimum	0.0	ND	12.09	Dark green semisolid
S. indicum	0.0	ND	25.23	Green powder
A. sinensis	0.02	Golden yellow liquid	20.21	Brown liquid
P. crispum	1.74	Pale yellow liquid	12.26	Dark green semisolid
C. quadrangularis	0.0	ND	3.87	Green semisolid
C. zedoaria	0.66	Golden yellow liquid	15.91	Golden yellow semisolid
C. xanthorrhiza	0.0	ND	9.79	Brown semisolid
D. bulbifera	0.0	ND	6.66	Brown semisolid
Z. cassumunar	1.75	Pale yellow liquid	13.40	Yellow semisolid

ND not determined as no essential oil was obtained from this plant species

3.1.2 Preparation of active extracts from *A. sinensis*

Four active extracts were derived from macerations of *A. sinensis* with different organic solvents of increasing polarity, in order to isolate the bioactive components with the highest repellency as much as possible. The obtained extractants of *A. sinensis*, including hexane extract (AHE), acetone extract (AAE), methanol extract (AME), and ethanol extract (AEE) showed different percentage yields, appearance and physical characteristics as demonstrated in Table 3.2.

Table 3.2 Percentage yield (%Yield), appearance and physical characteristics of *A. sinensis* products, including AHE, AAE, AME, and AEE

***A. sinensis* products**	**%Yield**	**Appearance & physical characteristics**		
		Appearance	**Color**	**Odor**
AHE	1.79	Liquid	Dark brown	Aromatic
AAE	8.0	Liquid	Brown	Aromatic
AME	44.8	Powder	Pale yellow	Aromatic
AEE	20.06	Liquid	Dark brown	Aromatic

3.1.3 Preparation of AHE-based repellent products

Apperance and physical characteristics of AHE-based repellent products, including AHE- and AHEv-ethanol solution (AHE- and AHEv-ES), AHE- and AHEv-nanoemulsion (AHE- and AHEv-NE), and 10% AHEv-nanoemulsion gel (AHEv-NEG) are demonstrated in Table 3.3.

Table 3.3 Apperance and physical characteristics of AHE-based repellent products

AHE-based repellent products	Appearance & physical characteristics		
	Clarity/Consistency	Color	Odor
AHE-ES			
5% AHE-ES	Clear	Pale yellow	Aromatic
10% AHE-ES	Clear	Pale yellow	Aromatic
15% AHE-ES	Clear	Yellow	Aromatic
20% AHE-ES	Clear	Brown	Aromatic
25% AHE-ES	Clear	Brown	Aromatic
AHEv-ES			
5% AHEv-ES	Clear	Pale yellow	Aromatic
10% AHEv-ES	Clear	Pale yellow	Aromatic
15% AHEv-ES	Clear	Yellow	Aromatic
20% AHEv-ES	Clear	Brown	Aromatic
25% AHEv-ES	Clear	Brown	Aromatic
AHE-NE			
5% AHE-NE	Clear	Pale yellow	Aromatic
10% AHE-NE	Clear	Yellow	Aromatic
15% AHE-NE	Clear	Orange	Aromatic
20% AHE-NE	Clear	Brown	Aromatic
25% AHE-NE	Clear	Brown	Aromatic
AHEv-NE			
5% AHEv-NE	Clear	Pale yellow	Aromatic
10% AHEv-NE	Clear	Yellow	Aromatic
15% AHEv-NE	Clear	Orange	Aromatic
20% AHEv-NE	Clear	Brown	Aromatic
25% AHEv-NE	Clear	Brown	Aromatic
AHEv-NEG			
10% AHEv-NEG	Soft	Pale yellow	Aromatic

3.2 Chemical analysis of *A. sinensis* products

GC/MS analysis of *A. sinensis* products, including AEO, AHE, AAE, AME, and AEE led to the identification of 13 to 15 different constituent compounds, representing 92.05%, 95.17%, 95.68%, 94.93%, and 93.46% of the total content, respectively (Table 3.4). It was found that the compositions in these *A. sinensis* products were almost similar as phthalides or phthalates were principal constituents showing the highest peaks (Figure 3.1). A high percentage of phthalides, including 3-*N*-butylphthalide, butylidenephthalide, and ligustilide were found in AHE (83.33%), AEO (78.44%), AME (42.78%), and AEE (39.03%), whereas AAE (56.47%) showed the highest amount of phthalates such as di-iso-octyl phthalate. Of all the compounds identified, 3-*N*-butylphthalide was characterized as the most abundant in AHE (70.14%), AEO (50.71%), AME (35.25%), and AEE (28.46%), whereas a dominant constituent in AAE was di-iso-octyl phthalate (56.47%).

Table 3.4 Chemical constituents of *A. sinensis* products, including essential oil (AEO) and solvent extracts (AHE, AAE, AME, and AEE)

Chemical constituent	**%Composition**				
	AEO	**AHE**	**AAE**	**AME**	**AEE**
(E)-Anethole	1.26				
β-Bisabolene	1.33	1.03	0.30		
Butylidenephthalide	25.81	4.85	1.27	3.21	6.64
3-*N*-Butylphthalide	50.71	70.14	20.53	35.25	28.46
β-Chamigrene	0.68				
(+)-Cuparene	1.55				
Cyclohexatriene	0.94				
Diacetone			1.12		
1,3-Dihydro-3,3,7-trimethyl-5-amino-2H-indol-2-one		0.39			
2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one				0.71	
Dihydroxyacetone				2.86	
Di-iso-octyl phthalate		3.53	56.47	7.07	
(E)-*β*-Farnesene	0.20				
β-Funebrene	1.27	0.60			
β-Himachalene	0.51				
5-Hydroxymethylfurfural			1.24	2.59	
Isospathulenol	0.34				
Lepidozene		0.79			
Ligustilide	1.92	8.34	3.34	4.32	3.93
cis,*cis*-Linoleic acid		1.13	5.57	15.66	8.60
Linoleic acid ethyl ester					6.50
Linoleic acid methyl ester		0.52	0.43	8.18	
2-Methoxy-4-vinylphenol	1.55		1.45	1.89	3.16
Mono (2-ethylhexyl) phthalate					10.40
β-Monolinolein					2.33
cis-Ocimene	1.46	1.17			
Oleic acid ethyl ester					1.99
Palmitic acid			2.51	7.43	7.64
Palmitic acid ethyl ester					3.28
Palmitic acid methyl ester				2.34	
Palmitic acid *β*-monoglyceride					0.53
β-Sitosterol		1.80	1.10	2.65	7.63
β-Spathulenol	2.52	0.89			
Stigmasterol			0.36	0.78	2.37
Total identified	**92.05**	**95.17**	**95.68**	**94.93**	**93.46**
No. of identified constituents	**15**	**13**	**13**	**14**	**14**

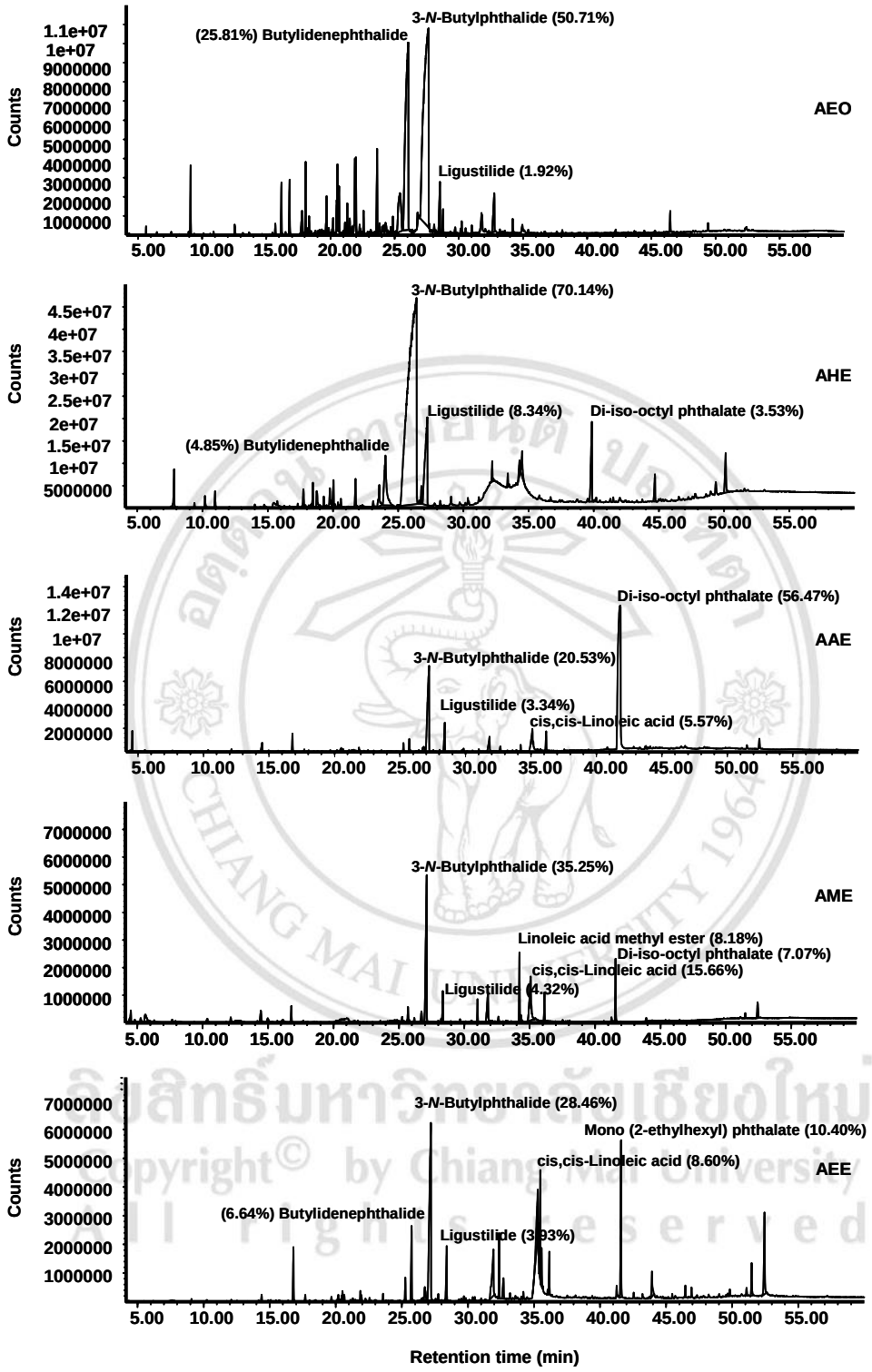

Figure 3.1 GC/MS total ion chromatograms of *A. sinensis* products, including essential oil (AEO) and solvent extracts (AHE, AAE, AME, and AEE)

3.3 Repellent bioassays

3.3.1 Laboratory repellent bioassay

3.3.1.1 Preliminary repellent screening

Preliminary laboratory trials for screening repellency against *Aedes aegypti* revealed that all nine essential oils possessed promising repellent potential (0.5 to 7.0 h), whereas only four of thirty three ethanolic extracts demonstrated moderate repellency, with the median complete protection times ranging from 0.5 to 2.5 h (Table 3.5). The most effective repellency in each kind of extracted product, i.e., essential oil and ethanolic extract, was of *A. sinensis*, which recorded the median complete protection times of 7.0 (6.0-7.5) h and 2.5 (2.0-2.5) h, respectively. Apart from *A. sinensis* products, all the other plant extracts exerted moderate repellent activity, with median complete protection times ranging from 0.5-2.0 h. All ethanolic extracts exhibited lower repellent activity than the essential oils of the same plants such as *A. sinensis* and *C. zedoaria*. Furthermore, while the oils of *A. calamus*, *C. verum*, *H. aromatica*, *L. aromatica*, *P. crispum*, *M. paniculata*, and *Z. cassumunar* were effective in repelling *Ae. aegypti* with the median complete protection times of 2.0, 2.0, 1.5, 1.0, 0.5, 0.5, and 0.5 h, respectively; no repellency was observed from the ethanolic extracts of these plants. Therefore, *A. sinensis* that generated the highest repellent activity was selected for further different organic solvents extraction and repellent assessment.

Table 3.5 Repellent activity of plant products, including essential oils and ethanolic extracts, against female *Ae. aegypti*

Plant/Chemical	Thai name	Median complete-protection time (Range, h)*	
		Essential oil	Ethanolic extract
A. aspera	พันงู	ND	0.0
A. calamus	ว่านน้ำ	2.0 (2.0-3.0)	0.0
H. aromatica	เต่าเกียด	1.5 (1.5-1.5)	0.0
A. marina	แสมทะเล	ND	0.0
C. papaya	มะละกอ	ND	0.5 (0.0-0.5)
C. magna	กุ่มน้ำ	ND	0.0
C. viscosa	ผักเสี้ยนผี	ND	0.0
A. annua	โกฐจุฬาลัมพา	ND	0.0
A. lancea	โกฐเขมา	ND	0.0
T. erosus	ดาวเรือง	ND	0.0
C. moschata	ฟักทอง	ND	0.0
M. charantia	มะระขี้นก	ND	0.0
P. amarus	ลูกใต้ใบ	ND	0.0
P. erosus	มันแกว	ND	0.0
C. verum	อบเชยเทศ	2.0 (2.0-2.0)	0.0
T. indica	มะขาม	ND	0.0
A. esculentus	กระเจี๊ยบเขียว	ND	0.0
T. crispa	บอระเพ็ด	ND	0.0
B. monosperma	ทองกวาว	ND	0.0
D. scandens	เถาวัลย์เปรียง	ND	0.0
S. orientale	งาดำ	ND	0.5 (0.0-0.5)
P. odoratum	ผักไผ่	ND	0.0
M. paniculata	แก้ว	0.5 (0.5-0.5)	0.0
L. aromatica	ผักแขยง	1.0 (1.0-1.0)	0.0
S. aculeatissimum	มะเขือขื่น	ND	0.0
S. indicum	มะแว้ง	ND	0.0
A. sinensis	โกฐเชียง	7.0 (6.0-7.5)	2.5 (2.0-2.5)
P. crispum	เทียนป้อม	0.5 (0.0-0.5)	0.0
C. quadrangularis	เพชรสังฆาต	ND	0.0
C. zedoaria	ขมิ้นอ้อย	1.0 (0.5-1.5)	0.5 (0.0-0.5)
C. xanthorrhiza	ว่านชักมดลูก	ND	0.0
D. bulbifera	ว่านสามพันตึง	ND	0.0
Z. cassumunar	ไพล	0.5 (0.0-0.5)	0.0

*There were 2 replicates of each screening test

3.3.1.2 Repellent investigation of active extracts from *A. sinensis*

Four active extractants of *A. sinensis* produced impressive results of repellent activity against *Ae. aegypti* (Table 3.6). Quite marked repellency was observed in *A. sinensis* hexane extract (AHE), proffering the median complete-protection time of 7.5 (6.5-8.5) h, which was greater than that of ethanol extract (AEE: 2.5, 2.0-2.5 h), acetone extract (AAE: 1.75, 0.5-2.5 h), and methanol extract (AME: 0.5, 0-1.0 h). Significant protection afforded by AHE, which was the most effective product, compared favorably with that of its essential oil (AEO: 7.0, 6.0-7.5 h) and DEET (6.25, 5.0-6.5 h). Consequently, AHE was selected for further preparations of AHE-based repellent products.

Table 3.6 Repellent activity of DEET and *A. sinensis* products, including essential oil (AEO) and solvent extracts (AHE, AAE, AME, and AEE) against female *Ae. aegypti*

Repellent samples	**Median complete-protection time (Range, h)***
AEO	7.0 (6.0-7.5)
AHE	7.5 (6.5-8.5)
AAE	1.75 (0.5-2.5)
AME	0.5 (0-1.0)
AEE	2.5 (2.0-2.5)
DEET	6.25 (5.0-6.5)

*There were 4 replicates of each test

3.3.1.3 Repellent investigation of AHE-based repellent products

3.3.1.3.1 Repellency of AHE-ethanol solution

Repellent assessment of ethanolic solutions of AHE and DEET with and without 5% vanillin supplementation demonstrated improved repellency in a dose dependent manner. While 5-25% AHE alone (AHE-ES) provided median complete-protection times of 2.0-6.5 h against *Ae. aegypti*, the addition of 5% vanillin (AHEv-ES) increased AHE repellency, with prolonged median complete-protection times of 4.0-8.5 h (Table 3.7). Accordingly, vanillin also expanded the protection times of 5-25% DEET-ES against *Ae. aegypti* from 2.25-7.25 h to 4.25-8.25 h. Thus, 25% AHEv-ES with a protection time of 8.5 (7.0-10.5) h, which proved to be the best repellent product, was selected for further field study (Field I).

Table 3.7 Repellent activity of the ethanolic solutions of AHE and DEET with and without 5% vanillin supplementation (AHE-ES, AHEv-ES, DEET-ES, and DEETv-ES) against female *Ae. aegypti*

%AHE or %DEET in the ethanolic solutions	Median complete-protection time (Range, h)*			
	AHE-ES	AHEv-ES	DEET-ES	DEETv-ES
5%	2.0 (2.0-3.5)^{aA}	4.0 (3.0-4.5)^{aB}	2.25 (1.5-2.5)^{aA}	4.25 (3.5-6.0)^{aB}
10%	3.0 (2.5-3.0)^{aA}	4.75 (4.5-5.0)^{abB}	3.0 (3.0-4.0)^{bA}	5.0 (4.0-6.5)^{aB}
15%	4.0 (2.5-4.0)^{abA}	5.5 (4.5-6.5)^{bcB}	6.0 (5.0-6.5)^{cB}	7.5 (6.5-8.5)^{bC}
20%	4.75 (4.0-6.0)^{bcA}	7.5 (7.0-7.5)^{dB}	7.0 (6.0-7.0)^{cdB}	8.0 (7.0-8.5)^{bB}
25%	6.50 (6.0-8.0)^{dA}	8.5 (7.0-10.5)^{dA}	7.25 (7.0-8.0)^{dA}	8.25 (8.0-8.5)^{bA}

*There were 4 replicates of each test

*Values followed by different lowercase letters in a column and uppercase letters in a row are significantly different ($P < 0.05$)

3.3.1.3.2 Repellency of AHE-nanoemulsion

Results of laboratory repellent bioassays of AHE-nanoemulsion with and without 5% vanillin supplementation (AHE-NE and AHEv-NE) compared with the ethanolic solutions of AHE and AHEv (AHE-ES and AHEv-ES) are presented in Table 3.8. The repellent test of AHE-ethanolic solutions and AHE-nanoemulsions against *Ae. aegypti* demonstrated improved repellency in a dose dependent manner when vanillin was added. While 5-25% AHE alone (AHE-ES) provided median complete-protection times of 0.5-4.0 h against *Ae. aegypti*, the addition of 5% vanillin (AHEv-ES) increased AHE repellency, with prolonged median complete-protection times of 2.5-6.75 h. Vanillin also expanded the protection times of 5-25% AHE-NE against *Ae. aegypti* from 3.25-5.75 h to 4.0-7.75 h. Consequently, AHEv-NE considered as the best repellent product was subjected for preparation of nanoemulsion gel by formulating low-dose (10%) AHE with various solvents, additives, surfactants, and preservatives. Repellent investigation of 10% AHEv-nanoemulsion gel (10% AHEv-NEG) also was subsequently carried out against three mosquito vectors, including *Ae. aegypti, Anopheles minimus,* and *Culex quinquefasciatus*.

Table 3.8 Repellency of the ethanolic solutions of AHE (AHE-ES and AHEv-ES) and nanoemulsions of AHE (AHE-NE and AHEv-NE) against female *Ae. aegypti*

%AHE-ethanol solutions or %AHE-nanoemulsions	**Median complete-protection time (Range, h)***			
	AHE-ES	**AHEv-ES**	**AHE-NE**	**AHEv-NE**
5%	0.5 (0.5-0.5)	2.5 (2.5-2.5)	3.25 (3.0-3.5)	4.0 (3.5-4.5)
10%	1.25 (1.0-1.5)	4.0 (4.0-4.0)	3.75 (3.0-4.5)	5.25 (5.0-5.5)
15%	2.75 (2.5-3.0)	4.5 (4.5-4.5)	4.75 (4.5-5.0)	5.5 (5.0-6.0)
20%	3.75 (3.5-4.0)	5.75 (5.5-6.0)	5.0 (4.5-5.5)	6.0 (5.5-6.5)
25%	4.0 (4.0-4.0)	6.75 (6.5-7.0)	5.75 (5.5-6.0)	7.75 (7.5-8.0)

*There were 2 replicates of each screening test

3.3.1.3.3 Repellency of 10% AHEv-NEG

According to the results demonstrated in Table 3.9, it appeared that 10% AHEv-NEG was prominently effective in repelling *Ae. aegypti*, *Cx. quinquefasciatus*, and *An. minimus*. The median complete-protection times provided by 10% AHEv-NEG and 10% DEETv-NEG against *Ae. aegypti*, *Cx. quinquefasciatus*, and *An. minimus* were 4.5 (4.0-6.0) h and 7.5 (6.5-9.0) h, 7.75 (6.5-11.5) h and 10.5 (9.5-16.0) h, and 11.0 (9.5-12.0) h and 12.0 (10.0-12.5) h, respectively. The control nanoemulsion gel with 5% vanillin offered no repellency against these mosquito vectors. No skin irritation, rashes, swelling, or other allergic responses were observed during the study period. Consequently, 10% AHEv-NEG and 10% DEETv-NEG were selected for further skin irritation test, biological stability test, and repellent study under field conditions (Field II).

Table 3.9 Repellent activity of 10% AHEv-NEG and 10% DEETv-NEG against female *Ae. aegypti*, *Cx. quinquefasciatus*, and *An. minimus*

Repellent samples	**Median complete-protection time (Range, h)***		
	Ae. aegypti	***Cx. quinquefasciatus***	***An. minimus***
10% AHEv-NEG	4.5 (4.0-6.0)[a]	7.75 (6.5-11.5)[b]	11.0 (9.5-12.0)[c]
10% DEETv-NEG	7.5 (6.5-9.0)[b]	10.5 (9.5-16.0)[cd]	12.0 (10.0-12.5)[d]

*There were 12 replicates of each test

*Values followed by different letters are significantly different ($P < 0.05$)

3.3.2 Field repellent bioassay

3.3.2.1 Field I

In the preliminary field trials, a total of 1,339 adult female mosquitoes, comprising 5 genera, i.e., *Aedes*, *Anopheles*, *Armigeres*, *Culex*, and *Mansonia*, were caught during the surveys. The most predominant mosquitoes were *Culex* and *Armigeres*, followed by *Aedes*, *Anopheles*, and *Mansonia*, which totaled 727 (54.3%), 580 (43.3%), 24 (1.8%), 4 (0.3%), and 4 (0.3%) genera, respectively. Based on these findings, the large and mixed mosquito populations were considered sufficiently abundant for repellency evaluation. During the preliminary trials, sunset occurred at the testing site at around 19.30 h local time, and the mosquitoes gathered around 60 min before and after sunset, with the maximum mean collecting rate of 30.72±13.2 (19.12-19.32 h). After that, the number of mosquitoes decreased gradually, but sufficient numbers were left for testing, with the minimum mean collecting rate of 19.94±9.5 (21.02-21.22 h). Additionally, some mosquito species, particularly *Anopheles* and *Mansonia*, were collected mostly around 30 to 90 min after sunset. This information was then applied to fixing the optimal period for testing and collecting mosquitoes and the period between 18.00 and 21.30 h was deemed to provide the best chance of being bitten.

The results of the Field I applications performed by human-baited techniques against the local mosquito populations are illustrated in Table 3.10 and 3.11, and Figure 3.2. We found that varying species and numbers of mosquitoes were collected from the control volunteers only. Therefore, a highly significant difference between the mean number of mosquitoes collected on the controls and testers treated with 25% AHEv-ES or 25% DEETv-ES was observed at every collecting site (CS); nine 20-min collections at each experiment (Table 3.10). From CS1 to CS4, the mean collecting rates of mosquitoes from the control volunteers increased dramatically. After that, the rates reduced moderately at CS5-CS9 but were still rather high. The maximum mean collecting rate was that of CS4, which was conducted between 19.06 and 19.26 h. These findings were consistent with guidance from the preliminary surveys, in that the mosquito collection period was suitable, due to the populous and mixed mosquitoes collected, which were abundant and available for calculating repellency.

Regarding the results demonstrated in Table 3.11, it appeared that 25% AHEv-ES afforded remarkable repellency, which was comparable to that of 25% DEETv-ES. No mosquito bites were observed on the volunteers treated with 25% AHEv-ES or 25% DEETv-ES throughout the testing periods of the field study. Therefore, it should be concluded that 25% AHEv-ES and 25% DEETv-ES produced similarly strong repellency by minimizing bites with a 100% protection against a wide range of field mosquito populations. A total of 5,718 adult female mosquitoes belonging to five genera, i.e., *Aedes*, *Anopheles*, *Armigeres*, *Culex*, and *Mansonia*, were collected during the field trials. Among 13 mosquito species collected, the most prominent were *Cx. quinquefasciatus*, *Ar. subalbatus*, and *Cx. vishnui*, which made up 41.47%, 41.13%, and 10.53%, respectively.

Table 3.10 Number of mosquitoes and mosquito collecting rates (Mean±standard error, SE) captured from human volunteers during field repellent bioassays at Sunpesua subdistrict, Muang district, Chiang Mai province, northern Thailand

Collecting site (CS): Time	Treatment	No. of mosquitoes collected	Mosquito collecting rate (Mean±SE)*
CS 1: 18.00-18.20 h	Control	56	2.0±3.5[a]
	25% AHEv-ES	0	0[b]
	25% DEETv-ES	0	0[b]
CS 2: 18.22-18.42 h	Control	588	21.0±17.8[a]
	25% AHEv-ES	0	0[b]
	25% DEETv-ES	0	0[b]
CS 3: 18.44-19.04 h	Control	787	28.1±22.4[a]
	25% AHEv-ES	0	0[b]
	25% DEETv-ES	0	0[b]
CS 4: 19.06-19.26 h	Control	1,058	37.8±20.8[a]
	25% AHEv-ES	0	0[b]
	25% DEETv-ES	0	0[b]
CS 5: 19.28-19.48 h	Control	672	24.0±8.5[a]
	25% AHEv-ES	0	0[b]
	25% DEETv-ES	0	0[b]
CS 6: 19.50-20.10 h	Control	667	23.8±9.6[a]
	25% AHEv-ES	0	0[b]
	25% DEETv-ES	0	0[b]
CS 7: 20.12-20.32 h	Control	641	22.9±10.1[a]
	25% AHEv-ES	0	0[b]
	25% DEETv-ES	0	0[b]
CS 8: 20.34-20.54 h	Control	650	23.2±13.4[a]
	25% AHEv-ES	0	0[b]
	25% DEETv-ES	0	0[b]
CS 9: 20.56-21.16 h	Control	599	21.4±10.8[a]
	25% AHEv-ES	0	0[b]
	25% DEETv-ES	0	0[b]
Total	Control	5,718	204.2±73.2[a]
	25% AHEv-ES	0	0[b]
	25% DEETv-ES	0	0[b]

*Mean in each collecting site followed by different letters is significantly different ($P < 0.05$)

Table 3.11 Results obtained from field repellent assessment of 25% AHEv-ES and 25% DEETv-ES, undertaken at Sunpesua subdistrict, Muang district, Chiang Mai province, northern Thailand

Mosquito species	**Control**	**25% AHEv-ES**		**25% DEETv-ES**	
	No. of mosquitoes captured (%)	**No. of mosquitoes captured (%)**	**Protection (%)**	**No. of mosquitoes captured (%)**	**Protection (%)**
Genus *Aedes*					
Ae. vexans	123 (2.15)	0(0)	100	0(0)	100
Ae. aegypti	4 (0.07)	0(0)	*ND*	0(0)	*ND*
Ae. albopictus	46 (0.80)	0(0)	100	0(0)	100
Ae. lineatopennis	17 (0.30)	0(0)	100	0(0)	100
Genus *Anopheles*					
An. wejchoochotei	12 (0.21)	0(0)	100	0(0)	100
Genus *Armigeres*					
Ar. subalbatus	2,352 (41.13)	0(0)	100	0(0)	100
Genus *Culex*					
Cx. gelidus	38 (0.66)	0(0)	100	0(0)	100
Cx. vishnui	602 (10.53)	0(0)	100	0(0)	100
Cx. quinquefasciatus	2,371 (41.47)	0(0)	100	0(0)	100
Cx. tritaeniorhynchus	67 (1.17)	0(0)	100	0(0)	100
Genus *Mansonia*					
Mn. indiana	49 (0.86)	0(0)	100	0(0)	100
Mn. uniformis	13 (0.23)	0(0)	100	0(0)	100
Mn. annulifera	24 (0.42)	0(0)	100	0(0)	100
Total	**5,718 (100)**	**0(0)**	**100**	**0(0)**	**100**

ND Not determined, as few specimens of this species were captured

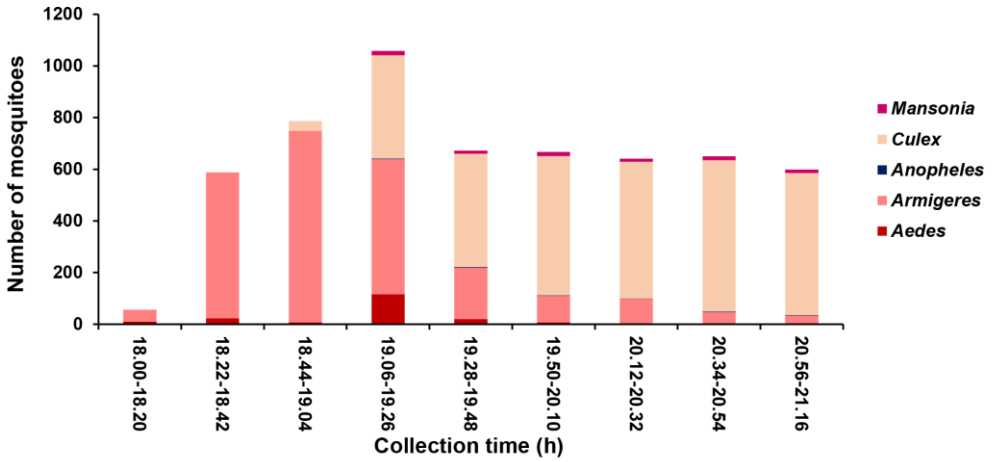

Figure 3.2 Distribution of mosquito species collected during the field repellent bioassays at Sunpesua subdistrict, Muang district, Chiang Mai province, northern Thailand

3.3.2.2 Field II

In the preliminary field trials, a total of 1,510 adult female mosquitoes, comprising 5 genera, i.e., *Aedes*, *Anopheles*, *Armigeres*, *Culex*, and *Mansonia*, were caught during the surveys. The most predominant mosquitoes were *Culex*, *Aedes*, and *Armigeres*, followed by *Mansonia*, *Anopheles*, and unidentified species, which totaled 1,056 (69.9%), 248 (16.4%), 119 (7.9%), 84 (5.6%), 2 (0.1%), and 1 (0.1%), respectively. Based on these findings, the large and mixed mosquito populations were considered sufficiently abundant for repellency evaluation. During the preliminary trials, sunset occurred at the testing site at around 19.30 h local time, and the mosquitoes gathered around 60 min before and after sunset, with the maximum mean collecting rate of 16.4±8.0 (19.34-19.44 h). After that, the number of mosquitoes decreased gradually, but sufficient numbers were left for testing, with the minimum mean collecting rate of 11.5±8.2 (21.02-21.12 h). Additionally, some mosquito species, particularly *Anopheles* and *Mansonia*, were collected mostly around 30 to 120 min after sunset. This information was then applied to fixing the optimal period for testing and collecting mosquitoes and the period between 18.00 and 22.00 h was deemed to provide the best chance of being bitten.

Field study was carried out in the rainy season from 22 June to 7 July 2016 by human-baited-trap, at the same place of preliminary trials; presenting large and varied field mosquitoes populations, which were abundant enough for repellency evaluation. For each nightly collection, the volunteers were exposed to natural field populations of mosquitoes for 240 min, between 18.00 h and 22.00 h, which was indicated in the preliminary study as the suitable period for mosquito collection. Outdoor temperatures during the study periods varied from 26.8 °C-34.1 °C, relative humidity of 61-83%, and 0.00-1.77 m/s for air velocity.

Table 3.12 and 3.13 summarize the repellent results from field application of 10% AHEv-NEG and 10% DEETv-NEG on human volunteers. It was found that 10% AHEv-NEG and 10% DEETv-NEG exerted strong repellency, with 100% and 99.9% protections against all the mosquito species, respectively. There was a highly significant difference between the mean number of field mosquitoes collected on the controls and testers treated with 10% AHEv-NEG or 10% DEETv-NEG in every collecting sites (CS); eleven 20-min exposure sites (Table 3.12). The mean collecting rates of mosquitoes on the control volunteers at CS1, CS2, CS3, CS4, and CS5 were increased dramatically from 15.8±10.3 to 18.3±7.7, 19.7±8.2, 51.5±16.7, and 131.4±64.1, respectively; then decreased, but were still relatively high, to 65.5±25.3, 52.0±14.4, 50.8±16.1, 54.2±26.0, 49.7±20.2, and 39.5±17.3 at CS6, CS7, CS8, CS9, CS10, and CS11, respectively. These results indicated that the maximum mean collecting rate was that of CS5 (19.28-19.48 h) and the crowded mosquitoes were observed between 19.06-22.00 h. This finding likely corresponded to that of the preliminary human-baited-trap trials.

Table 3.12 Number of mosquitoes and mosquito collecting rates (Mean±standard error, SE) captured from human volunteers during field repellent bioassays at Sunpesua subdistrict, Muang district, Chiang Mai province, northern Thailand

Collecting site (CS): Time	Treatment	No. of mosquitoes collected	Mosquito collecting rate (Mean±SE)*
CS 1: 18.00-18.20 h	Control	378	15.8±10.3[a]
	10% AHEv-NEG	0	0[b]
	10% DEETv-NEG	0	0[b]
CS 2: 18.22-18.42 h	Control	439	18.3±7.7[a]
	10% AHEv-NEG	0	0[b]
	10% DEETv-NEG	0	0[b]
CS 3: 18.44-19.04 h	Control	472	19.7±8.2[a]
	10% AHEv-NEG	0	0[b]
	10% DEETv-NEG	1**	0.04±0.14[b]
CS 4: 19.06-19.26 h	Control	1,237	51.5±16.7[a]
	10% AHEv-NEG	0	0[b]
	10% DEETv-NEG	0	0[b]
CS 5: 19.28-19.48 h	Control	3,154	131.4±64.1[a]
	10% AHEv-NEG	0	0[b]
	10% DEETv-NEG	0	0[b]
CS 6: 19.50-20.10 h	Control	1,573	65.5±25.3[a]
	10% AHEv-NEG	0	0[b]
	10% DEETv-NEG	0	0[b]
CS 7: 20.12-20.32 h	Control	1,249	52.0±14.4[a]
	10% AHEv-NEG	0	0[b]
	10% DEETv-NEG	0	0[b]
CS 8: 20.34-20.54 h	Control	1,218	50.8±16.1[a]
	10% AHEv-NEG	0	0[b]
	10% DEETv-NEG	0	0[b]
CS 9: 20.56-21.16 h	Control	1,301	54.2±26.0[a]
	10% AHEv-NEG	0	0[b]
	10% DEETv-NEG	0	0[b]
CS 10: 21.18-21.38 h	Control	1,193	49.7±20.2[a]
	10% AHEv-NEG	0	0[b]
	10% DEETv-NEG	0	0[b]
CS 11: 21.40-22.00 h	Control	947	39.5±17.3[a]
	10% AHEv-NEG	0	0[b]
	10% DEETv-NEG	0	0[b]
Total	Control	13,161	274.2±75.3[a]
	10% AHEv-NEG	0	0[b]
	10% DEETv-NEG	1**	0.02±0.07[b]

*Mean in each collecting site followed by different letters is significantly different ($P < 0.05$)

**_Ar. subalbatus_

Table 3.13 Results obtained from field repellent testing of 10% AHEv-NEG and 10% DEETv-NEG, showing the protection offered against the various species of mosquitoes collected

Mosquito species	Control	10% AHEv-NEG		10% DEETv-NEG	
	No. of mosquitoes captured (%)	No. of mosquitoes captured (%)	Protection (%)	No. of mosquitoes captured (%)	Protection (%)
Genus *Aedes*					
Ae. vexans	2,300 (17.47)	0(0)	100	0(0)	100
Ae. aegypti	37 (0.28)	0(0)	100	0(0)	100
Ae. vittatus	20 (0.16)	0(0)	100	0(0)	100
Ae. gardnerii	23 (0.17)	0(0)	100	0(0)	100
Ae. albopictus	926 (7.03)	0(0)	100	0(0)	100
Ae. lineatopennis	51 (0.39)	0(0)	100	0(0)	100
Genus *Anopheles*					
An. tessellatus	21 (0.16)	0(0)	100	0(0)	100
An. wejchoochotei	18 (0.14)	0(0)	100	0(0)	100
Genus *Armigeres*					
Ar. subalbatus	1,028 (7.81)	0(0)	100	1(0.01)	99.9
Genus *Culex*					
Cx. gelidus	1,423 (10.81)	0(0)	100	0(0)	100
Cx. vishnui	3,179 (24.15)	0(0)	100	0(0)	100
Cx. fuscocephala	2 (0.02)	0(0)	*ND*	0(0)	*ND*
Cx. quinquefasciatus	3,279 (24.91)	0(0)	100	0(0)	100
Cx. bitaeniorhynchus	6 (0.05)	0(0)	*ND*	0(0)	*ND*
Cx. tritaeniorhynchus	250 (1.90)	0(0)	100	0(0)	100
Genus *Mansonia*					
Mn. indiana	524 (3.98)	0(0)	100	0(0)	100
Mn. uniformis	30 (0.23)	0(0)	100	0(0)	100
Mn. annulifera	43 (0.33)	0(0)	100	0(0)	100
Genus *Lutzia*					
Lt. fuscana	1 (0.01)	0(0)	*ND*	0(0)	*ND*
Total	**13,161 (100)**	**0(0)**	**100**	**1(0.01)**	**99.9**

ND Not determined, as few specimens of this species were captured

Regarding the results showed in Table 3.13, it appeared that both repellent products, 10% AHEv-NEG and 10% DEETv-NEG, afforded excellent personal protection against a wide range of mosquito species belonging to six genera, i.e., *Aedes*, *Anopheles*, *Armigeres*, *Culex*, *Mansonia*, and *Lutzia*. However, it was observed that one of volunteers treated with 10% DEETv-NEG was attacked by one *Ar. subalbatus*, whereas no mosquito bite was observed on 10% AHEv-NEG-treated volunteers throughout the testing periods of field study. Therefore, it should be calculated that while 10% DEETv-NEG was effective in reducing bites with 99.9% protection, the protective effect of 10% AHEv-NEG appeared complete (100% protection). Regardingly, it can be concluded that 10% AHEv-NEG offered slightly stronger repellency than the 10% DEETv-NEG against natural populations of mosquitoes. A total of 13,161 adult female mosquitoes comprising 19 species were collected during the field assessments. The predominant mosquitoes collected were *Cx. quinquefasciatus*, *Cx. vishnui*, *Ae. vexans*, *Cx. gelidus*, and *Ar. subalbatus*, which made up 24.91%, 24.15%, 17.47%, 10.81% and 7.81%, respectively.

3.4 Evaluating potential skin irritation from AHE-based repellent products

Table 3.14 displays the results of evaluating skin irritant potential of 25% AHE-ES, 10% AHEv-NEG, 20% SLS, and absolute ethanol, when applied to 30 human volunteers (13 adults males and 17 adults females; age ranged from 22-59 years). The results indicated that none of the 30 human volunteers, who took part in the 4-h patch test, exhibited a positive skin irritant reaction to 25% AHE-ES and 10% AHEv-NEG at any of the assessments. Similar results were also obtained from the application of absolute ethanol, a negative control reference. On the contrary, 21 human volunteers showed positive irritant reaction to a positive control reference, 20% sodium lauryl sulfate (20% SLS), a widely used cosmetic ingredient. At 24 h of SLS-patch removal, 17, 4, and 6 adult volunteers showed positive irritant with slight (+), moderate (++), and severe (+++) reactions, respectively. Additionally, slight, moderate and severe irritation reactions were also observed in 15, 2, and 4 volunteers at 72 h of SLS-patch removal.

Table 3.14 Skin irritant potential of 25% AHE-ES, 10% AHEv-NEG, 20% SLS, and absolute ethanol

Treatment	Number of volunteers	Scoring for skin irritation[a]			
		0	1+	2+	3+
25% AHE-ES	30	30	0	0	0
10% AHEv-NEG	30	30	0	0	0
20% SLS	30	9	15	2	4
Absolute ethanol	30	30	0	0	0

Each site of application on the skin was examined and scored at 24, 48 and 72 h after patch removal. ([a]) number of volunteers whose highest score in these three evaluations was (0) absence of irritation, (+) slight irritation, (++) moderate irritation, (+++) severe irritation

3.5 Testing the physical and biological stability of AHE-based repellent products

3.5.1 Physical and biological stability of AHE (Temperature-time method)

The physical and biological performance of AHE samples, as determined after storage under 4 °C, ambient temperature (AT: 21-35 °C), and 45 °C for 1, 3, and 6 months, showed little difference (Table 3.15). All stored AHE samples exhibited similar characteristics, liquid phases with an aromatic odor, to those of the fresh preparation. However, the color changed from dark brown to very dark brown in samples kept at either ambient temperature for 6 months or 45 °C for 3 and 6 months. These findings indicated relatively changeable appearance depending on the storing conditions of this product.

However, the results obtained from testing these stored AHE samples against *Ae. aegypti* demonstrated that their repellent activity was present for a period of at least 6 months, with varied efficacy. Apart from the AHE samples kept at 4 °C for 1 month, most of the others stored in each condition for 1, 3, and 6 months provided relatively weaker repellency than the fresh sample. Furthermore, a lower repellency was determined from AHE samples with longer storage time. It was plausible that extended storage times as well as fluctuating ambient temperature ranging from 21 to 35 °C, and

a high temperature of 45 °C, partially influenced either the physical or biological stability of AHE materials.

Table 3.15 Appearance, physical property, and repellent activity of the fresh and stored samples of AHE against female *Ae. aegypti*

AHE samples (Temperature/Duration)		**Appearance & physical characteristics**			**Median complete-protection time (Range, h)***
		Phase	**Color**	**Odor**	
Fresh sample		Liquid	Dark brown	Aromatic	6.50 (6.0-8.0)^{ab}
Stored samples					
4 °C	1 month	Liquid	Dark brown	Aromatic	10.0 (8.0-11.0)^{c}
	3 months	Liquid	Dark brown	Aromatic	7.25 (7.0-8.0)^{bd}
	6 months	Liquid	Dark brown	Aromatic	6.0 (4.0-7.0)^{ade}
Ambient temperature	1 month	Liquid	Dark brown	Aromatic	7.25 (6.5-8.5)^{acd}
(21-35 °C)	3 months	Liquid	Dark brown	Aromatic	6.75 (4.0-7.0)^{ade}
	6 months	Liquid	Very dark brown	Aromatic	4.5 (3.0-6.0)^{ef}
45 °C	1 month	Liquid	Dark brown	Aromatic	5.75 (5.0-6.5)^{af}
	3 months	Liquid	Very dark brown	Aromatic	4.5 (4.0-5.5)^{ef}
	6 months	Liquid	Very dark brown	Aromatic	4.5 (4.0-5.0)^{ef}

*Values followed by different letters in a column are significantly different ($P < 0.05$)

3.5.2 Physical and biological stability of 10% AHEv-NEG

3.5.2.1 Temperature-time method

Observation on the stored 10% AHEv-NEG after storage under 4 °C, ambient temperature (AT: 16-30 °C), and 45 °C for 1, 2, 3, and 6 months, revealed little differences in their appearance and physical property. All stored 10% AHEv-NEG samples exhibited similar characteristics, soft and pale yellow in color with an aromatic odor, to those of the fresh preparation. However, changes to yellow and softer were seen in 10% AHEv-NEG samples kept at 45 °C for 1, 2, 3, and 6 months (Table 3.16 and Figure 3.3). Relative repellency (median complete-protection time) against the female *Ae. aegypti* mosquito derived from the stored samples of 10% AHEv-NEG, which kept at conditions that vary in temperature and time storage are shown in Table 3.16. The results demonstrated that 10% AHEv-NEG samples kept at 4 °C and AT for 1, 2, 3, and 6 months yielded equally median protection times of 4.5 (3.5-5.5) h and 4.5 (3.0-5.0) h, respectively which were non-significant and slightly lower than that of the fresh product (4.75: 4.0-5.5 h). Samples of 10% AHEv-NEG stored at 45 °C for 1, 2, 3, and 6 months provided relatively weaker repellency than the fresh sample, with protection times of 3.75 (3.0-5.0) h. It was apparent that the median complete-protection times of 10% AHEv-NEG samples stored at 45 °C for both 2 and 6 months were markedly decreased and manifested a statistically significant difference from that of the fresh sample.

Table 3.16 Appearance, physical property, and repellent activity against female *Ae. aegypti* of the fresh and stored products of 10% AHEv-NEG

10% AHEv-NEG products (Temperature/Duration)		**Appearance & physical characteristics**			**Median complete-protection time (Range, h)***
		Consistency	**Color**	**Odor**	
Fresh product		Soft	Pale yellow	Aromatic	4.75 (4.0-5.5)^{ab}
Stored products					
4°C	1 month	Soft	Pale yellow	Aromatic	5.25 (5.0-5.5)^{a}
	2 months	Soft	Pale yellow	Aromatic	4.50 (4.0-5.0)^{ab}
	3 months	Soft	Pale yellow	Aromatic	4.25 (3.5-4.5)^{bcd}
	6 months	Soft	Pale yellow	Aromatic	4.25 (3.5-4.5)^{beg}
Ambient temperature	1 month	Soft	Pale yellow	Aromatic	4.75 (4.5-5.0)^{acefh}
(16-30°C)	2 months	Soft	Pale yellow	Aromatic	4.25 (3.5-4.5)^{bfi}
	3 months	Soft	Pale yellow	Aromatic	3.50 (3.0-4.5)^{bfi}
	6 months	Soft	Pale yellow	Aromatic	4.25 (4.0-4.5)^{bfi}
45°C	1 month	Softer	Yellow	Aromatic	4.50 (3.5-5.0)^{ab}
	2 months	Softer	Yellow	Aromatic	3.50 (3.0-4.0)^{dgij}
	3 months	Softer	Yellow	Aromatic	3.75 (3.5-4.5)^{bhj}
	6 months	Softer	Yellow	Aromatic	3.50 (3.5-4.0)^{dgij}

*Values followed by different letters in a column are significantly different ($P < 0.05$)

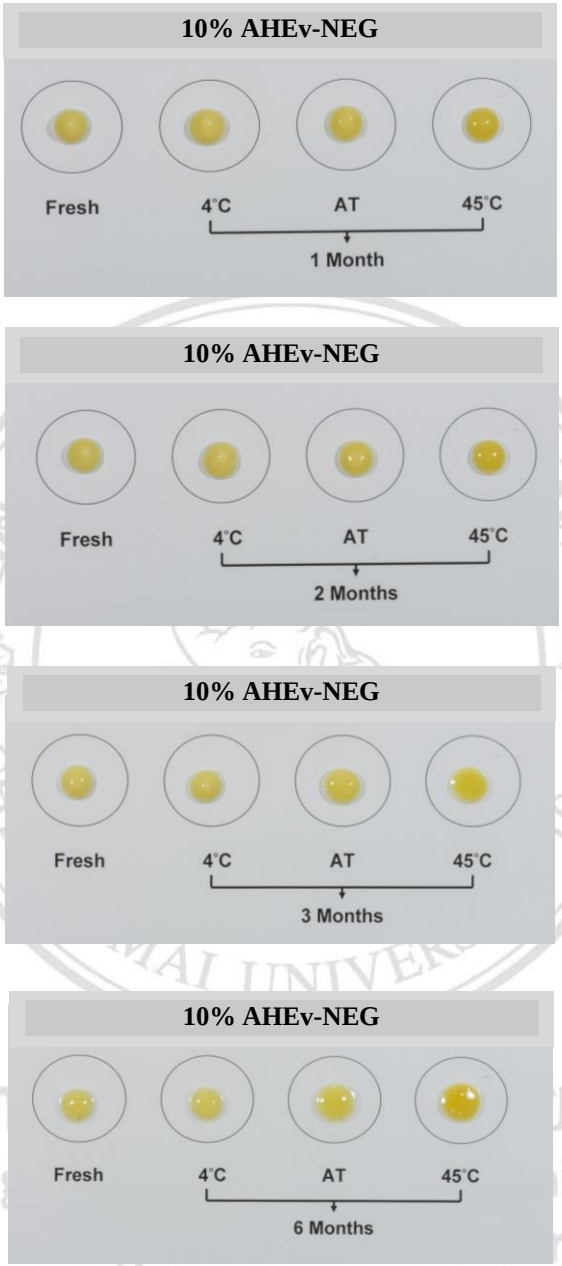

Figure 3.3 Samples of 10% AHEv-NEG after kept at conditions that vary in temperature and time storage compared with fresh preparation

3.5.2.2 Heating and cooling method

For physical observation, the appearance and odor of all stored 10% AHEv-NEG were similar to those of the fresh preparation, with a soft, pale yellow color, and pleasant aromatic odor (Table 3.17 and Figure 3.4). In order to investigate the persistence of repellency, 10% AHEv-NEG was tested after being kept under 2 and 4 cycles of heating and cooling (1 cycle: heated at 45 °C for 48 h and cooled at 4 °C for 48 h). According to the results, it was indicated that 10% AHEv-NEG samples kept under 2 and 4 heating and cooling cycles provided the median complete-protection time of 4.50 (4.0-4.5) h and 3.75 (3.5-4.5) h, respectively, which was slightly lower than that of the fresh preparation, 5.50 (5.0-6.0) h.

Table 3.17 Appearance, physical property, and repellency against *Ae. aegypti* of 10% AHEv-NEG samples after being kept under 0, 2, and 4 cycles of heating and cooling

10% AHEv-NEG:	**Appearance & physical characteristics**			**Median complete-protection time**
Cycles of heating and cooling	**Consistency**	**Color**	**Odor**	**(Range, h)***
0	Soft	Pale yellow	Aromatic	5.50 (5.0-6.0)[a]
2	Soft	Pale yellow	Aromatic	4.50 (4.0-4.5)[b]
4	Soft	Pale yellow	Aromatic	3.75 (3.5-4.5)[b]

*Values followed by different letters in a column are significantly different ($P < 0.05$)

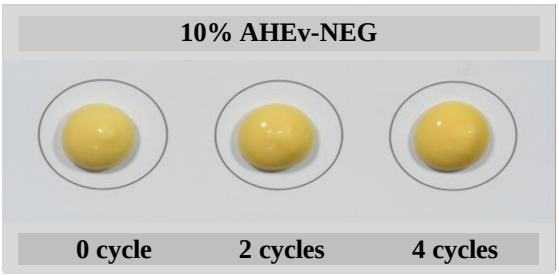

Figure 3.4 Samples of 10% AHEv-NEG after being kept under 0, 2, and 4 cycles of heating and cooling