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Asian Pacific Journal of Tropical Biomedicine
journal homepage: www.elsevier.com/locate/apjtb

Document heading doi:10.12980/APJTB.4.2014C1264

Efficacy of seed extracts of *Annona squamosa* and *Annona muricata* (Annonaceae) for the control of *Aedes albopictus* and *Culex quinquefasciatus* (Culicidae)

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PEER REVIEW

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Comments

This is a good research work in which authors have demonstrated the insecticide activity of two Annonaceae (*A. squamosa* and *A. muricata*) seed extracts against two mosquito species of health importance. The activity was assessed based on biochemical parameters and *in vivo* tests. *A. squamosa* and *A. muricata* were found to be promising candidate plants for future insecticide use on mosquitoes.
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ABSTRACT

Objective: To evaluate the potential efficacy of seed extracts of *Annona squamosa* and *Annona muricata* used as natural insecticides to control adult and larvae of the vectors *Aedes albopictus* and *Culex quinquefasciatus* under laboratory conditions.
Methods: Aqueous and oil extracts of the two plants were prepared from dried seeds. Preliminary identifications of the chemical components of each seed extracts were performed using micro-reactional and GCP techniques. Larvae and adults of *Aedes albopictus* and *Culex quinquefasciatus* were collected from the breeding sites in coastal and highlands regions of Madagascar. WHO standardized tests of susceptibility for larvae and imaginal stage of mosquitoes were realized to determine mortality and LC_{50} of mosquitoes.
Results: Chemical identifications showed that these extracts contain alkaloids and flavonoids compounds that probably confer their biological insecticidal proprieties. CPG analysis showed also the presence of various fatty acids. On adult mosquitoes, significant insecticidal effects were observed with both aqueous and oil extracts of the two plant seeds compared to mortality induced by deltamethrin, an insecticide used as reference. Extracts of *Annona muricata* induced high mortality rate to both species of mosquito compared to extracts of *Annona squamosa* at all concentrations tested. The LC_{50} of seed extracts ranged from 1% to 5% for adults and 0.5% to 1% for larvae.
Conclusions: The seed extracts of these two plants may be used as mosquito controlling agents and offer a new approach to a less costly, practical and environmentally friendly control of vector borne diseases.

KEYWORDS
Annonaceae, Seed extracts, Biological insecticides, Chikungunya, Rift Valley fever, Vector control

1. Introduction

Since 2006, Madagascar was affected by successive outbreaks of chikungunya and Rift Valley fever[1-3]. The chikungunya virus (CHIKV) was detected in the coastal regions of Madagascar such as Toamasina, Antsiranana

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Foundation Project: Supported by the grants FRB-CD-AOOI-07-012 and CMIRA Coopera 2011 from Region Rhône-Alpes 11MIF-MAVINGUI-10851.

Article history:
Received 27 Nov 2013
Received in revised form 12 Feb, 2nd revised form 19 Mar, 3rd revised form 6 Apr 2014
Accepted 17 May 2014
Available online 27 Aug 2014

and Taolagnaro, whereas the Rift Valley fever virus (RVFV) has emerged in several regions of the highlands, including Anjozorobe, Manjakandrina, Antananarivo, Ambatondrazaka, Amparafaravola, and the Haute Matsiatra region (Fianarantsoa I, II, Ambalavao)[2-5]. *Aedes albopictus* (*Ae. albopictus*) has been identified as the main vector of CHIKV and dengue virus in urban areas, because of its distribution which coincided to the epidemic areas and its abundance during periods of outbreaks[1,6]. *Ae. albopictus* were found ovipositing and breeding in microhabitats located in the vicinity of human habitations, including natural and/or artificial pounds, crevices, tree trunk excavations and other artificial containers such as discarded cans, used tires and abandoned buckets[1,6,7]. The most important vectors for RVFV are *Aedes* and *Culex* mosquitoes[8,9]. *Culex* is widely distributed in urban areas of Madagascar[4]. Transmission is facilitated by the feeding behavior of *Culex* which is both endophilic and exophilic. Larval stages are known to breed in large range of polluted waters with lentic facies[6,7].

As no vaccine and efficient therapeutic treatments are currently available for CHIKV (Alphavirus), dengue virus (Flavivirus) or RVFV (Bunyavirus), various methods have been applied to control populations of vectors but none of them were able to eradicate these vectors in Madagascar. Among the wide range of biological methods, natural enemies such as predators of mosqitoes at different developmental stages have been used. Aquatic larvae of *Culex tigripes* play an important role in regulating populations of vectors by consuming the larvae of other species of mosquitoes in natural pounds[6]. However, it has been demonstrated that the most efficient method that may control the numbers of larvae, remains the environmental sanitation by removing any potential breeding sites of the vectors[1], although its application at a large scale is restricted.

Conventional method using chemical insecticides, including organochlorides, pyrethroids mainly the deltamethrin and organophosphates such as malathion and fenthion were still applied as last resort for vector control[9]. These synthetic compounds are not only environmentally polluting but also have concomitant hazardous effects to non-target organisms and to human health[6].

Phenomenon of resistance to the widely used chemical insecticides were found in many vectors of disease, particularly in the case of *Culex quinquefasciatus* (*Cx. quinquefasciatus*)[10-13] causing sudden epidemic spreads of Rift Valley fever and the resurgence of the dengue and chikungunya. Resistance to chemical insecticides in other vectors such as *Ae. albopictus* and *Aedes aegypti* was also confirmed[9-15].

To face the increasing emergence of mosquito resistance to chemical insecticides, a sound option lays on the use of natural products. In this study, seed extracts of *Annona squamosa* (*A. squamosa*) and *Annona muricata* (*A. muricata*) were used to control adult mosquitoes as well as larval stages of the vectors *Ae. albopictus* and *Cx. quinquefasciatus*.

Annonaceae are empirically known to elicit insecticidal activities[12,16]. Plant species in this family contain an array of toxic compounds such as acetogenins, alkaloids, flavonoids that confer to these plants their insecticidal proprieties[12]. Both seeds of *A. squamosa* and *A. muricata* contain a great amount of acetogenins[16]. This special group of chemical compounds is known as mitochondrial complex I inhibitor[12,17,18]. Some alkaloids were found in *A. muricata*. And extracts of *A. muricata* not only affect the mortality rate of pupal and adult stages of mosquitoes but also reduce the reproductive success of surviving adults by decreasing fecundity and egg hatchability[19].

Petroleum ether leaf extracted from leaves of *A. squamosa* were reported to possess an insecticidal and growth regulating activities on three mosquito species namely *Anopheles stephensi*, *Cx. quinquefasciatus* and *Aedes aegypti*[19].

The aim of this study was to evaluate the actual efficacy of seed extracts of *A. squamosa* and *A. squamosa* under laboratory conditions in order to assess their potential uses as natural insecticides to control adult mosquitoes as well as larval stages of the vectors *Ae. albopictus* and *Cx. quinquefasciatus*. A preliminary screening using various micro-reactional techniques and also GC analyses were also performed to identify the range of chemical groups of ingredients in the compositions of the aqueous and oil extracts of the two seeds.

2. Materiels and methods

2.1. Collection of seeds and preparation of plant extracts

A. squamosa and *A. muricata* are commonly planted and harvested in the East coast of Madagascar. Their fruits produce a large quantity of seeds that can be used as insecticides. Fruits of the two species were collected in Manakara (22°8′45.68″ S; 48°0′15.9″ E) at the south east region of Madagascar.

Prior to chemical extraction, seeds were completely dried under a ventilated hood then grinded to powder. A total of 200 g of each grinded seeds of the two plants were then soaked and macerated separately in 1 L of distilled water overnight. Each macerated solution was diluted with distilled water to obtain different lower concentrations (0.5%=1 g/L, 1%=2 g/L, 5%=10 g/L, 10%=20 g/L and 20%=40 g/L). The aqueous extracts were quoted as EAAS for *A. squamosa* and EAAM for *A. muricata*. Similar procedure used to obtain aqueous extracts was adopted to obtain oil extracts by

soaking 200 g of seed powder separately into 1 L of various solvents with different polarity. Three different solvents were used for extractions: ethanol, dichloromethane, and acetone. The oil extracts were quoted EET1 and EET2 for ethanol extracts, DCM1 and DCM2 for dichloromethane extracts of *A. squamosa* and *A. muricata* respectively; and ACE1 for acetone extract of *A. squamosa*.

2.2. Chemical analysis

Preliminary identifications of the chemical ingredients in the composition of the two aqueous extracts from the two seeds were performed using various micro-reactional techniques to detect specific chemical compound families[20] (Table 1). In contrast, oil extracts were injected into Shimazu GCP JC-17A (Version 3 equipped with capillary column: Tracsil TR-wax, 30 m×0.32 m×0.25 m; Injection temperature: 260 °C) for chemical identification of the components of the oil extracts.

2.3. Mosquito collection

Mosquitoes were collected in the coastal regions of Toamasina (18°08′50″ S; 49°23′43″ E; 10 m), Mahajanga (15°45′39.83′′ S; 46°20′9.38″ E; 18 m), and Manakara (22°8′45.68″ S; 48°0′15.9″ E; 20 m) as well as in the highland regions of Antananarivo (18°52′47″ S; 47°34′35″ E; 1245 m), Ambositra (20°54′211″ S; 47°24′159″ E; 1310 m) and Fianarantsoa (21°27′34.05″ S; 47°6′33.75″ E; 1280 m). Adults of *Cx. quinquefasciatus* were collected in human habitations and bedrooms of hospitals early in the morning, whereas *Ae. albopictus* adults were captured with small nets in their natural habitats and shrubs during daytime while flying. Investigations of all potential larval pounds and breeding sites of the two species were also performed and specimens collected as well.

2.4. Mosquito rearing

Cx. quinquefasciatus and *Ae. albopictus* caught in the wild were transported to the insectarium of Department of Entomology and were reared following WHO procedures and laboratory rearing method[11,21]. The egg strips and rafts were immersed in natural water which is collected in ponds nearby and placed into plastic bowls (dim: 6 cm diameter). Synchronous hatched larvae were transferred into same

Table1

Micro-reactional techniques used to detect presence of chemical compound groups[20].

Chemical groups	Tests	Reagent	Observations	Indications
Alkaloids	Dragendorff	(NO_2)2BI/IK	Precipitation	Alkaloids
	Hager	Saturated picric acid	Precipitation	
	Marme	I2c.d./IK	Precipitation	
	Mayer	$HgCl_2$/IK	Precipitation	
	Wagner	I2/IK	Precipitation	
Carditonic glycosides	Keller-Killiani test	Few drops of $FeCl_3$+1 mL iced acetic acid+1 mL of H_2SO_4 concentrated	Appearance of red ring color	Rare sugars
	Liberman-Brüchard test	3 drops of anhydride acetic+1 drop H_2SO_4	Crimson, pink, red, violet, aquamarine	Terpenoids, Steroid nucleus
		1-5 mL Kedde regeant+2 to 3 drops of NaOH	pink purple	Lactone insaturated
Anthraquinones	Böntrager test	5 mL of NaOH (1 mol/L)	Pink	Anthraquinones
Cyanides		Heating in a flask intercalated with a picrate sodium filter paper	Shift of color from dark yellow to red brick	Cyanide
Coumarines		Heated for 10 mn and then observed under UV lamp	Fluorescence	Coumarines
Saponines	Foaming test	Vortexed in water	Appearance of 3cm foam layer after 30 mn of resting	Saponines
Flavonoids	Shinoda test	Magnesium ribbon+5 to 6 drops of concentrated HCl	Red	Flavone, flavanol, flavonone, flavanone
Leucoanthocyanins		2 mL HCl (2 mol/L)+2 drops of isopropanol	Red purple	Leucoanthocyanin
Tannins and polyphenols	Formaldehyde	2-3 drops of gelatin 1%	Precipitate	Tannins
		2-3 drops of gelatin- NaCl	Precipitate aquamarine	Tannins
		2-3 drops $FeCl_3$	Precipitate	Polyphenols
	Vanillin-HCl	5-6 drops bromine - water	Precipitate	Tannins Catechin
		3 drops of formaldehyde 40%+6 drops of HCl 10%	Precipitate crimson red	
		1 mL vanillin reageant+1 drop of concentrated HCl+5 mL of lime-water	Precipitate blue gray	Gallic tannin
Cyanogenic glycosides	Colorimetric method	Paper sodium picrate+heating	Brick red color	Cyanide

bowl containing natural water to maintain synchronous development of colonies. Male mosquitoes were fed on 6% sucrose imbibed cotton pad. The females were blood-fed twice a week on laboratory mice. Insectarium temperature was maintained at (25±2) °C, relative humidity at (80±2)% and photoperiod 12 h/12 h.

2.5. Bioassays for adults

WHO standardized method was used to test the susceptibility of *Ae. albopictus* and *Cx. quinquefasciatus* adults to different concentrations of each seed extracts[21]. Bioassays were performed using adapted exposure tubes (WHO tubes, dim: L: 12 cm×4.5 cm diameter) having two adjacent compartments, the exposure compartment and the observation compartment, interconnected by a sliding glass separator that allowed mosquitoes to be grouped within one of the two compartments at a time. Filter papers (0.10 mm thick and 6 cm diameter) were impregnated with 5 mL of aqueous extracts with the respective concentrations of 1%, 5%, 10% and 20% or 1 mL of crude oil extracts, and introduced and walled the exposure tube individually. A number of 25 unfed adult mosquitoes were released into a tube corresponding to a replicate. Four replicates were performed. Time of exposure to the extract was standardized to one hour for each replicate then mosquitoes were transferred into the adjacent observation tube and monitored for 24 h. Controls comprised two different treatments; one using filter paper impregnated with distilled water and another with a filter paper impregnated with deltamethrin 2% taken as the positive reference. During observation, test and control tubes were covered with wooden wall box with aeration aperture and moisten tissues to maintain appropriate humidity. After 24 h, falling or immobile mosquitoes were counted as dead. If mortality rate of mosquitoes of the non-impregnated control tube exceeded 20% (4 mosquitoes) within the 24 h of incubation, the whole batch of test was considered as invalid then repeated.

2.6. Bioassays for larvae

Three different concentrations 0.5%, 1% and 2% of each plant aqueous extract were used for bioassays[22]. For each concentration prepared, a batch of two beakers of 100 mL was used. Two controls were run: one blank control with 100 mL of water, and one with 100 mL water supplemented with 1 mL of ethanol that was used as the positive reference. For oil extracts, 1 mL acetone was added to 100 mL of natural breeding pound water prior to adding 1 mL crude oil seed extract of each plant. Twenty-five third instar larvae of *Ae. albopictus* or *Cx. quinquefasciatus* were introduced in each beaker and kept immersed for 24 h.

After incubation time-period, mortality of larvae in each beaker was recorded. Each experiment comprised a minimum number of 4 replicates. Immobile and moribund larvae were counted as dead.

2.7. Statistical analysis

The average values of mortality of mosquitoes were calculated and mortality curves were drawn on log-log graph paper to find out LC_{50} for each extract. Data were analysed using SPSS. 10 (Version, 2010). Mortality rates of different treatments were subjected to ANOVA (α=0.05). When ANOVA results were significant, means were separated using Tukey-Kramer test (α=0.05).

3. Results

3.1. Major families of chemical compounds identified in extracts

Similar families of chemical compounds ranging from flavonoids, leucoanthocyanes, triterpenes, unsaturated sterols, polyphenols and polysaccharides (Table 2) were identified in the aqueous extracts. Alkaloids were present in aqueous extract of *A. muricata* but absent in *A. squamosa* and that was the converse with tannin.

Table 2

Main chemical groups found in aqueous seed extracts of *A. squamosa* and *A. muricata*.

Tests	*A. squamosa*	*A. muricata*
Mayer (Alkaloids)	-	+
Wagner (Alkaloids)	-	-
Dragendorff (Alkaloids)	-	-
Wilstater (Flavones, flavanones, flavanols)	++	-
Modified Wilstater ((Flavones, flavanones)	++	+
Bate-Smith	++	
Anthocyanins	-	
Lieberman Burchard (Triterpenoids)	++	++
Salkowski (Unsaturated sterols)	+	+
Badget-Kedde (laconoid sterols)	-	-
Keller-Killiani (Desoxy-2 sugar)	-	-
Grignard (cyanogenic glycosides)	-	-
Bornsträger (anthraquinones)	-	-
Foam height (saponosides)	-	-
Gelatin 1% (Polyphenols)	++	+
Salted Gelatin (Taninns)	++	-
$FeCl_3$ (condensed hydrolysable tannins)	++	-
Polysaccharides screening	+++	++

-: Not detected, +: Present in small amount, ++: Present in large amount.

Various fatty acids ranging from palmitic acid, oleic acid, linoleic acid were identified in the oil extract of *A. squamosa* (Table 3). In addition to the range of major fatty

acids found in *A. squamosa*, the oil extract of *A. muricata* contained more specific fatty acids including myristic acid, palmitoleic acid, stearic acid, linolenic acid, arachidic acid and others acids with C18, C19 and C20 (Table 3).

3.2. Efficacy of A. squamosa and A. muricata extracts to adult mosquitoes Cx. quinquefasciatus and Ae. albopictus

3.2.1. Aqueous extracts

By using aqueous extracts of *A. squamosa*, numbers of adult mosquitoes *Cx. quinquefasciatus* killed after 24 h were proportional to the concentrations of the extracts: 10±2, 13±2, 18±2 and 23±2 (means±SE) for the concentrations 1%, 5%, 10% and 20%, respectively. When aqueous extracts of *A. muricata* were used, mortalities to adults *Cx. quinquefasciatus* were 18±2, 20±2, 23±2 and 23±2 (means±SE) for the concentrations of 1%, 5%, 10% and 20%, respectively (Table 4). Mortalities of adult mosquitoes using EAAS and EAAM were significantly superior to mortalities observed in the deltamethrin reference tests (14±2) (means±SE) ($F_{(7,111)}$=22.18, n=118, P=0.0001, ANOVA, Tukey-Kramer test). By plotting average values of mortalities on log-log graph, the LC_{50} was found to be at the concentration of 1% for EAAM and 5% for EAAS.

In adults of *Ae. albopictus*, mortalities after 24 h exposure to 1% concentration of EAAS and of EAAM were respectively 18±2 and 24±1 (means±SE) (Table 4). Mortalities of adult mosquitoes treated by EAAM were similar to mortalities observed in the deltamethrin reference tests (23±2) (means±SE), whereas those of *A. squamosa* were lower (18±2) [$F_{(3,36)}$=44.72, n=39, P=0.0001, ANOVA, Tukey-Kramer test]. The LC_{50} was found to be lower than the concentration of 1% for both EAAS and EAAM.

3.2.2. Oil extracts

Mortalities of *Cx. quinquefasciatus* adults after 24 h were 23±2, 22±3, 19±3, 22±3 and 21±4 (means±SE), respectively for DCM1, DCM2, EET1, EET2 and EAC1 (Table 5). Mortalities of

Table 3

Fatty acid groups for *A. muricata* and *A. squamosa*.

N°	*A. muricata*		*A. squamosa*	
	Fatty acids	Relative concentration of the analyzed sample (%)	Fatty acids	Relative concentration of the analyzed sample (%)
1	Myristic acid	0.07		
2	Palmitic acid 16:0	20.93	Palmitic acid	18.37
3	Palmitoleic acid 16:1w7	2.29		
4	Stearic acid 18:0	3.90		
5	Oleic acid C18:1w9	42.97	Oleic acid C18:1w8	4.06
6	Linoleic acid c18:2w6	29.29	Linoleic acid	38.32
7	C18:3(n-3) or C19:1(n-8)	0.88	Polyinsaturated C18	35.30
8	Linoleic acid C18:3w3	0.20	Linoleic acid c18:3w3	2.51
9			Polyinsaturated C19	0.81
10	Arachidic acid	0.53		
11	C20:1(n-7)	0.22		
12	C20:4(n-6)	0.52		
13	C20:3(n-6)	0.17		
14			C21:0	0.06

Table 4

Mortality of adult mosquitoes induced by aqueous seed extracts of *A. squamosa* and *A. muricata*.

Treatments	EAAS 1%	EAAM 1%	EAAS 5%	EAAM 5%	EAAS 10%	EAAM 10%	EAAS 20%	EAAM 20%	Control	Deltamethrine 2%
Average mortality of *Cx. quinquefasciatus* (means±SE)	10±2^{c}	18±2^{ab}	13±2^{c}	20±2^{ab}	18±2^{ab}	23±2^{a}	23±2^{a}	23±2^{a}	1±2^{d}	14±2^{bc}
Number of individuals tested	550	350	200	200	300	200	200	200	475	200
Average mortality of *Ae. albopictus* (means±SE)	18±2^{a}	24±1^{a}	ND	ND	ND	ND	ND	ND	1±1^{b}	23±2^{a}
Number of individuals tested	300	200	ND	ND	ND	ND	ND	ND	200	300

Different letters indicate significant means (P=0.05, ANOVA, Tukey-Kramer test). ND: not determined.

Table 5

Mortality of adult mosquitoes to oil seed extracts of *A. squamosa* and *A. muricata*.

Treatments	DCM1 crude 1 mL/pap	DCM2 crude 1 mL/pap	EET1 Crude 1 mL/pap	EET2 Crude 1 mL/pap	EAC1 Crude 1 mL/pap	Control	Deltamethrine 2%
Average mortality of *Cx. quinquefasciatus* (means±SE)	23±2^{a}	22±3^{a}	19±3^{ab}	22±3^{a}	21±4^{ab}	1±3^{c}	14±2^{b}
Number of individuals tested	200	100	100	100	50	100	200
Average mortality of *Ae. albopictus* (means±SE)	24±1^{a}	25±1^{a}	24±1^{a}	ND	ND	1±1^{b}	23±2^{a}
Number of individuals tested	200	100	25	ND	ND	200	200

Different letters indicate significant means (P=0.05, ANOVA, Tukey-Kramer test). ND: not determined.

Table 6

Mortality of mosquito third instar larvae to different concentration of aqueous seed extracts of *A. squamosa* and *A. muricata*.

Treatments	EAAS 0.5%	EAAM 0.5%	EAAS 1%	EAAM 1%	EAAS 2%	EAAM 2%	Ethanol	Control
Average mortality of *Cx. quinquefasciatus* (means±SE)	8 ± 2^{e}	12 ± 1^{d}	19 ± 1^{bc}	18 ± 1^{c}	22 ± 1^{b}	24 ± 1^{a}	1 ± 1^{f}	1 ± 1^{f}
Number of individuals tested	100	250	500	300	200	200	250	200
Average mortality of *Ae. albopictus* (means±SE)	15 ± 1^{c}	20 ± 2^{b}	22 ± 1^{b}	24 ± 1^{a}	24 ± 1^{a}	ND	1 ± 1^{d}	1 ± 1^{d}
Number of individuals tested	150	100	350	100	250	ND	200	200

Different letters indicate significant means (P=0.05, ANOVA, Tukey-Kramer test). ND: not determined.

Table 7

Mortality of mosquito third instar larvae to different concentration of oil seed extracts of *A. squamosa* and *A. muricata*.

Treatments	DCM1 1%	DCM2 1%	EET1 1%	EET2 1%	Ethanol 1%	Control
Average mortality of *Cx. quinquefasciatus* (means±SE)	17 ± 1^{b}	24 ± 1^{a}	5 ± 2^{c}	13 ± 2^{b}	1 ± 1^{e}	1 ± 1^{e}
Number of individuals tested	200	200	100	100	250	200
Average mortality of *Ae. albopictus* (means±SE)	23 ± 2^{a}	24 ± 1^{a}	ND	ND	1 ± 1^{d}	2 ± 1^{e}
Number of individuals tested	100	200	ND	ND	100	100

Different letters indicate significant means (P=0.05, ANOVA, Tukey-Kramer test). ND: not determined.

mosquitoes using oil extracts of *A. squamosa* (DCM1, EET1 and EAC1) and *A. muricata* (DCM2 and EET2) were significantly superior to mortalities observed in deltamethrin reference tests (14±2) (means±SE) [$F_{(5,44)}$=5.89, n=49, P=0.0003, ANOVA, Tukey-Kramer test].

For *Ae. albopictus* adults, mortalities after 24 h were 24±1, 25±1, 24±1 (means±SE) respectively to DCM1, DCM2 and EET1 (Table 5). There were no difference in mortalities between extracts of *A. squamosa* (DCM1, EET1) and *A. muricata* (DCM2) to that induced by deltamethrin (23±2) ($F_{(4,21)}$=56.33, n=25; P=0.0001, ANOVA, Tukey-Kramer test).

3.3. Efficacy of A. squamosa and A. muricata extracts to third instar nymph mosquitoes Cx. quinquefasciatus and Ae. albopictus

3.3.1. Aqueous extracts

For the three concentrations 0.5%, 1% and 2%, the mortalities of third instar larvae of *Cx. quinquefasciatus* after 24 h were respectively 8±2, 19±1 and 22±1 (means±SE) for the aqueous extracts of *A. squamosa* (EAAS), and 12±1,18±1 and 24±1 (means±SE) for the aqueous extracts of *A. muricata* (EAAM), respectively (Table 6). Mortalities of larvae using EAAS and EAAM were significantly superior to mortalities observed in the ethanol and control reference tests (both 1±1) (means±SE) [($F_{(7, 88)}$=320.05, n= 95, P=0.0001, ANOVA, Tukey-Kramer test]. By plotting average values on log-log graph, the LC_{50} was found to be 0.5% for EAAM and 1% for EAAS.

For the two concentrations 0.5% and 1% after 24 h of exposure, third instar larvae of *Ae. albopictus* exhibited mortalities of 15 ± 1^{c} and 22 ± 1^{b} (means±SE) for the aqueous extracts of *A. squamosa* (EAAS), and 20±2 and 24±1 (means±SE) for the aqueous extracts of *A. muricata* (EAAM), respectively (Table 6). Mortalities of larvae using EAAS and EAAM were significantly higher than mortalities observed in the ethanol and control reference tests (both 1±1) (means±SE) [$F_{(6,47)}$=284.50, n=54, P=0.0001, ANOVA, Tukey-Kramer test]. The LC_{50} was found to be lower than 0.5% for both EAAS and EAAM.

3.3.2. Oil extracts

Mortalities of third instar larvae of *Cx. quinquefasciatus* after 24 h were respectively 17±1, and 5 ± 2^{c} (means±SE) for the oil extracts DCM1 and EET1, and 24±1 and 13±2 (means±SE) for the oil extracts DCM2 and EET2 respectively (Table 7). Mortalities of mosquitoes under oil extracts of *A. muricata* (DCM2 and EET2) were significantly higher than mortalities observed in the ethanol and the control reference tests (both 1±1) (means±SE) whereas oil extracts of *A. squamosa* (DCM1 and EET1) induced a mild mortality to third instar larvae of *Cx. quinquefasciatus* ($F_{(7,48)}$=58.97, n=55, P=0.0001, ANOVA test, Tukey-Kramer test). Mortalities of third instar larvae of *Ae. albopictus* by using oil extracts of *A. squamosa* (DCM1) and *A. muricata* (DCM2) were significantly higher than mortalities observed in the ethanol and the control reference tests (1±1 and 2±1 respectively) (means±SE) [$F_{(5,24)}$=650.73, n=30, P=0.0001, ANOVA, Tukey-Kramer test].

Overall, the results showed that *Ae. albopictus* populations were more sensitive to the seed extracts than populations of *Cx. quinquefasciatus*.

4. Discussion

Chemical screening and GC analyses results showed a range of potent and bioactive compound groups such as flavonoids, and fatty acids in both *A. muricata* and *A. squamosa* aqueous and oil seed extracts. Chemical screening was in agreement with the results obtained by different authors who have already identified a broad range of alkaloids, flavonoids and acetogenins compounds produced in seeds of *A. squamosa*[16,17,19] and *A.*

muricata[16,23] suggesting their probable role in insecticidal activities[23]. Alkaloid compounds including nicotine found in tobacco, have been demonstrated to inhibit the active site of acetylcholine in many organisms[10,19,24]. These alkaloids were only found in *A. muricata* aqueous extracts. Acetogenins were discovered in 1982 by Jolad *et al*., they are specific of Annonaceae[17,23]. These compounds are derivatives of 32 to 37 carbons long chain fatty acids[12]. The major acetogenins in seeds are respectively annonacin in *A. muricata*[23] and squamocin in *A. squamosa*[17]. Although typical acetogenins were not detected with the two methods used, an in-depth identification of the factual nature of the active ingredients belonging to all the chemical families using GC-MS will enable further determination of their precise roles in the insecticidal activities of the extracts.

Numerous types of toxicity result from physical proprieties of fatty acids: toxicity by inhalation due to volatile organic compounds, toxicity by contact due to aggregation and formation of thin film at the surface of water which does not allow respiration of aquatic insects, and by penetration due to the amphibolic propriety of certain molecules; Oleic acid C18 and undecylenic acid C11 possess direct insecticidal activities or may enhance the toxicity of other toxic compounds[25].

Currently, plant-based chemical control may broaden up gradually the existing arsenal of methods in vector control. Many plants are used as biocides in different parts of the world, naming *Piper nigrum* (black pepper), *Nicotiana tabacum* (tobacco), *Melia azedarach*, *Derris*, *Lonchocarpus* and *Tephrosia* and *Azadirachta indica* (neem) [11,12,24,26]. The latter plant is the most widely used biocide as its insecticidal properties have been known for over 300 years and was applied as a foliar spray and vapor to control insects, particularly in greenhouses and other protected crop fields.

In comparison to synthetic deltamethrin, a compound usually taken as standard reference by WHO in susceptibility tests for mosquitoes, a higher insecticidal effect on both adult and larvae of *Ae. albopictus* and *Cx. quinquefasciatus* was observed when using the aqueous and oil seed extracts of *A. squamosa* and *A. muricata*. This higher efficacy opens the possibility to exploit these extracts as biological insecticides to control the two tested mosquito species, and extendedly likely to other mosquito vector taxa. Results of bioassays likely were in concordance with those obtained on larva of *Cx. quinquefasciatus*. Thus, they confirm the insecticidal activities of *A. muricata*[16] and *A. squamosa*[12,16,19]. Strikingly, the present study also brought up additional evidence and support concerning the prevalence of resistance to deltamethrin, the insecticide reference advocated by WHO[10,12].

It is also found that the aqueous extracts of *A. muricata* elicited higher adulticidal activities in *Ae. albopictus* than those of *A. squamosa* at the same concentration. Similar trend was observed when using both aqueous and oil extracts on the larvae of *Cx. quinquefasciatus*. This difference of efficacy between the two species is probably due to the alkaloids present in *A. muricata* which are not found in *A. squamosa*[16]. Aqueous extracts were all efficient at a very low dose (0.5%) and with a proportional increase of the lethal activity following the gradual increase of extract concentrations. At the highest dose (2%), all mosquitoes tested were killed during the tests. Although extracts obtained from all organic solvent elicited adulticidal activities, dichloromethane extracts of both seed plants were significantly potent (DCM1, DCM2) compared to ethanolic extracts, in particular for larval stages. In contrast, only ethanolic extracts (EET2) of *A. muricata* elicited adulticidal activities against *Cx. quinquefasciatus*, with regard to oil extracts.

For all extracts and at all concentrations tested, the populations of *Ae. albopictus* caught from five regions of Madagascar were more susceptible than those of *Cx. quinquefasciatus*. This higher susceptibility might be due to the intrinsic natural ecology and behavior of *Ae. albopictus* larvae that usually breed into relatively clean and less polluted water. This behavior reduced at its minimum their contact to organic compounds into highly polluted waters, which in turn might be among the mechanisms that prevented populations of the species to remain susceptible. In contrast, larvae of *Cx. quinquefasciatus* are known to breed into polluted water containing a broad variety of organic compounds namely alkaloids and/or derivative compounds. The repeated contacts of *Cx. quinquefasciatus* larvae to these compounds might have conferred to these larvae and consequently to adults, a range of adaptations to various chemicals hence their relative moderate sensitivity to these plants extracts.

Nevertheless, the use of plant-based pesticides presents a number of challenges and advantages[9,26] as follows: (i) the active ingredients of many plant-based pesticides are not known, but are essential for the development of specifications. (ii) The active ingredients can vary both in composition and concentration in the same plant species, in different clones, in different parts of the plants, at different stages of plant growth and under different climatic and soil conditions. (iii) The activity may not be due to a single ingredient but to a mixture of compounds, which may act synergistically. (iv) The development of resistance to pesticides containing a mixture of active ingredients may occur less readily. (v) Adequate toxicological and eco-toxicological data are not available for many plant-based pesticides. (vi) Analytical standards of the active ingredients may not be easily obtainable.

As the two tested plants have a wide geographical distribution in eastern region of Madagascar where malaria

and other virus-borne vectors are endemic[1], integrating and promoting their use as an alternative plant-based method in vector control could be advocated, especially in the case of Madagascar. If used properly at an efficient dose, these extracts of *Annona* were revealed to be a low cost and powerful environmentally friendly tool by avoiding accumulation and negative impacts on non-target entomofauna. The possibility of newly synthesized bioactive molecules forming all extracts and that from the different organic solvent used thus remains pivotal in the design of efficient and practical plant extract-based methods in vector control.

The aqueous and oil extracts of *A. squamosa* and *A. muricata* both showed insecticidal properties though at varying levels. Extracts of *A. muricata* displayed higher insecticidal potential compared to extracts of *A. squamosa*. Besides, *Ae. albopictus* was more susceptible than *Cx. quinquefasciatus* to all extracts. Lethal concentrations (LC_{50}) of all extracts of the two plants tested ranged from less than 1% for adult mosquitoes and 0.5% for larvae of *Ae. albopictus*, and 1% to 5% for adult mosquitoes and 0.5% to 1% for larvae of *Cx. quinquefasciatus*. Dichloromethane extracts of *A. muricata* showed high lethal effect to larval and adult stages of *Ae. albopictus* and *Cx. quinquefasciatus* compared to deltamethrin which is an OMS insecticide reference product. Hence, this extract might already be considered having high insecticidal potential as an alternative agent in the control of mosquitoes though further studies on identification and chemical characterization of the active ingredients of these two plants are still needed for its complete integration as mosquito controlling agents.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

We are grateful to the Ministry of Water and Forestry and to Madagascar National Parks (formerly ANGAP) for fully authorizing collection of wild mosquitoes within parks. We thank Rahanitriniaina Sahondra and Rajaonera Tahina Ernest for assistance and cooperation, Rajaonarivo Solofoniaina for providing all the plant extracts, and Brown Rakotoarisoa and Ratsimbazafy Jonah for helpful suggestions on the manuscript. This work was funded by the grants FRB-CD-AOOI-07-012 and CMIRA Coopera 2011 from Region Rhône-Alpes 11MIF-MAVINGUI-10851, and was carried out within the frameworks of GDRI "Biodiversité et Développement Durable à Madagascar".

Comments

Background

Ae. albopictus and *Cx. quinquefasciatus* are mosquito vectors of several human diseases all over the world. Among them chikungunya and Rift Valley fever diseases have been recently reported for severe outbreaks in several countries, such as the islands of the south west part of the Indian Ocean. Most of the methods used today to eradicate those vector populations are not efficient or too costly for the country. Therefore there is need of new molecules to improve those control methods.

Research frontiers

The present research work depicts insecticide activity of aqueous and oil extracts of *A. squamosa* and *A. muricata* (Annonaceae) seeds against 2 mosquito species of health importance: *Ae. albopictus* and *Cx. quinquefasciatus* by identifications of the chemical components of each seed extracts and *in vivo* tests of susceptibility for larvae and imaginal stage of mosquitoes (determining mortality and CL 50 of mosquitoes).

Related reports

Annonaceae are empirically known to elicit insecticidal activities. Plant species in this family contain an array of toxic compounds such as acetogenins, alkaloids, flavonoids that confer to these plants their insecticidal proprieties. The folklore medicine has evidence of effectiveness of those extracts as insecticides.

Innovations and breakthroughs

Annonaceae are empirically known to elicit insecticidal activities however in the present study, authors have looked for their different chemical compounds and demonstrated the insecticide activity of *A. squamosa* and *A. muricata* seeds against 2 mosquito species of health importance.

Applications

This scientific study is, as mentioned by author, a preliminary work, screening 2 plant species for their insecticide activity. Nevertheless their results even preliminary are supporting and suggesting these seeds extract for future insecticide use.

Peer review

This is a good research work in which authors have demonstrated the insecticide activity of two Annonaceae (*A. squamosa* and *A. muricata*) seed extracts against 2 mosquito species of health importance. The activity was assessed based on biochemical parameters and *in vivo* tests. *A. squamosa* and *A. muricata* were found to be promising candidate plants for future insecticide use on mosquitoes.

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