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Pilot Study on Newly Developed Botanical Larvicides and Repellents against Aedes Mosquitoes in Myanmar

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Abstract

Dengue Haemorrhagic Fever (DHF) is one of the major public health problems in Myanmar. There are no effective vaccine and specific drug for DHF and its containment is totally based on vector Aedes mosquito control. Thus the present study was conducted with the general objective of developing innovative environment-friendly vector control tools mainly focusing on the plant sources. The test plants ? Caesalpinia pulcherrima Linn. And Ervatamia coronaria (Jacq) Stapf. were locally searched in Magway ? central Myanmar, extracted, screened and tested against Ae. Aegypti larvae and adults under the laboratory conditions, and in field trials preceded by animal acute toxicity and skin irritation tests in line with standard procedures and guidelines of WHO and OECD from August through September, 2015. Indepth interviews were undertaken among local residents to evaluate the public acceptance on new control tools. Test plant leaves contained some phytochemicals with larvicidal and repellent properties. LC50 values (95

Index terms— botanical larvicides, repellents, aedes mosquitoes.

Pilot Study on Newly Developed Botanical Larvicides and Repellents against Aedes Mosquitoes in Myanmar Htin Zaw Soe ? , Sein Min ? , Maung Maung Mya ? , Khine Khine Lwin ? , Aye Win Oo ¥ & Myat Khine § Abstract-Dengue Haemorrhagic Fever (DHF) is one of the major public health problems in Myanmar. There are no effective vaccine and specific drug for DHF and its containment is totally based on vector Aedes mosquito control. Thus the present study was conducted with the general objective of developing innovative environmentfriendly vector control tools mainly focusing on the plant sources. The test plants -Caesalpinia pulcherrima Linn. And Ervatamia coronaria (Jacq) Stapf. were locally searched in Magway -central Myanmar, extracted, screened and tested against Ae. Aegypti larvae and adults under the laboratory conditions, and in field trials preceded by animal acute toxicity and skin irritation tests in line with standard procedures and guidelines of WHO and OECD from August through September, 2015. Indepth interviews were undertaken among local residents to evaluate the public acceptance on new control tools. Test plant leaves contained some phytochemicals with larvicidal and repellent properties. LC 50 values (95% FCI) of crude ethyl acetate leaf extract larvicides of C. pulcherrima and E. coronaria against Ae. aegypti larvae were 3.21 (2.95 -3.48) and 4.46 (3.16 -6.05) mg/l respectively. Their repellent ED 50 values (95% FCI) against Ae. aegypti adults were 0.02 (0.01 -0.03) and 0.01 (0.005 -0.02) mg/cm 2 respectively. Their repellent percentage protection (mean ± SD) was 88.4±13.3 (dose, 1.6 mg/cm 2) and 82.1± 6.4 (dose, 0.4 mg/cm 2) at 90 min post application respectively. The results of animal acute toxicity and skin irritation tests using test extract/repellents showed the safe use of new control tools by human. In field trials it was found that larval mortality was 100% in minor water containers treated with C. pulcherrima larvicide (dose, 7.2 -14.4 mg/l) and E. coronaria larvicide (dose, 12.7 -25.4 mg/l) separately in 24 hr. Their repellent percentage protection (mean ± SD) was 98.3±1.4 (dose, 1.6 mg/cm 2) and 97.8± 2.3 (dose, 0.4 mg/cm 2) in 90 min respectively. The local residents were interested in, accepted and demanded the new control tools. In conclusion the present study highlighted that newlarvicides and repellents were found to be very promising to be safely and effectively used to control Aedes mosquitoes.

1 I. Introduction

engue and dengue haemorrhagic fever (DHF) is one of Aedes mosquito-borne diseases. Globally about 2.5 billion people live in more than 100 dengue endemic countries and there are approximately 50 million dengue infections annually. About 500,000 DHF cases required hospitalization each year and case fatality rate is 2.5% 1 . Each year hundreds of thousands of severe cases occur including 20,000 deaths, with 264 disability-adjusted life years (DALYs) per million population lost 2 . Reported cases and deaths in the South-east Asia Region are 232,530 and 2,031 respectively in 2009 1 . In Myanmar average annual reported cases and deaths of DHF were 14,739 and 111 respectively in the last decade (2005-2014). Case fatality rate was under 1%. Up till now there is no reliable effective vaccine and specific treatment for DHF. Thus prevention and control measures are vitally important which are mainly based on vector control methods. The routine vector control methods currently used have several limitations, for example, labour-intensive. Thereforemethods which are locally available, feasible, cheap, ecofriendly and acceptable to the public are urgently needed and to be innovated. In Myanmar botanical larvicides and repellents are rarely studied. The present study was conducted with the general objective of developing innovative environment-friendly vector control tools mainly focusing on the plant sources.

2 II. Materials and Methods

3 a) Test plants

Plant species Caesalpinia pulcherrima Linn. and Ervatamia coronaria **??**Jacq) To find out effective dose (ED) of C. pulcherrimacrude extract against Ae. aegypti female adults, its stock solution (1%) was used with WHO guidelines 6 . Firstly four volunteers including one female from DMR were thoroughly explained about procedure of bioassays and their informed consent was obtained. They were instructed not to use cosmetics/perfumes/ scented soap and not to smoke one day before the bioassays. Those with history of allergy and serious reactions by mosquito bite were excluded. Before the test volunteers' forearm areas from wrist to elbow were measured. Average area of four volunteers was 501.1 $\pm$ 33.5 cm 2 . Next their forearms were thoroughly washed and cleaned with tap water. Secondly the left forearm as control of one volunteer was evenly applied with 1 ml of diluent acetone using a glass rod (30 cm). His hand was protected with a soft plastic glove not to bite the mosquitoes. The diluent was air dried for one min and the forearm was then introduced into a stainless steel cage (30 cm $\times$ 30 cm $\times$30 cm) containing fifty 3-4 day-old, one day-starved, nulliparous female Aedes mosquitoes. The numbers of mosquito landing/ probing on the exposed skin were counted during 30 sec. Thirdly the control forearm was withdrawn and evenly applied with 1 ml of 1% stock solution (extract 0.01 g/ml) as treated forearm and air dried for one min. Afterwards treated forearm was introduced into the same cage and mosquitoes landing/probing were counted during 30 sec. Then additional 1 ml of 1% stock solution was applied on that treated forearm and tested by same procedure till the treated forearm was applied five serial double the concentration doses cumulatively (ie. 0.01, 0.02, 0.04, 0.08 and 0.16 g/ml). Fourthly volunteer's right forearm applied with 1 ml acetone was inserted into the cage again as control. Finally percentage protection (p) was calculated using the formula p = (C -T) /C $\times$ 100 where C is number of mosquitoes landing/probing on control forearm and T is on treated forearm (26 -28C°/RH 70 -79%). The same procedure was performed two replicates per volunteer by four volunteers. ED 50 and ED 90 with 95% FCIwere calculated usingprobit analysis. The same procedure was also conducted for E. coronaria.

ii. For finding percentage protection Percentage protection of C. pulcherrima crude extract against female Aedes mosquitoes was investigated in line with WHO guidelines 6 . Time of the test was between 0800 hr and 1600 hr. Firstly the left forearmas control of one volunteer was evenly applied with 1 ml of diluent acetone. The diluent was air dried for one min and the forearm was then introduced into a stainless steel cage (30 cm $\times$ 30 cm $\times$30 cm) containing fifty 3-4 day-old, one day-starved, nulliparous female Aedesmosquitoes. The numbers of mosquito landing/ probing on the exposed skin were counted during 3 min. Secondly his right forearm was evenly applied with 1 ml of 40% stock solution to get extract 0.4 g/ml (ie. approximately double the dose -0.2 g/ml of its ED 90) and air dried for one min. Afterwards treated forearm was introduced into the same cage and mosquitoes landing/probing were counted during 3 min period. Next the forearm was withdrawn and introduced again into the same cage after 30 min interval. Similar procedure was performed for the period of 150 min. Control forearm was inserted into the same cage every time just before the treated forearm was inserted(25 -28C°/RH 70 -78%). The same procedure was performed two replicates per volunteer by four volunteers. The percentage protection (p) was calculated using the same formula p = (C -T) /C $\times$ 100. Similar procedure was performed for the extract 0.8 g/ml (ie. double the first dose). The same procedures were undertaken for the E. coronaria crude extract at 0.1g/ml (ie. approximately double the dose -0.05 g/ml of its ED 90) and 0.2g/ml (ie. double the first dose). with the polyoxyethylene (20) sorbitanmonolaurate ('Tween' 20) as a vehicle. Each of six control mice was provided with 1 ml 'Tween 20'. Then all mice were watched for 14 days for signs of mortality and toxicity.

Similar procedure was conducted for E.coronaria. For skin irritation tests,lab-reared 4 month old female guinea pigs Caviaporcellus were used. Hairs on the area (4 cm $\times$ 4 cm) of the backside of each of three healthy guinea pigs were removed by shaving. The resultant bare areas were applied with C.pulcherrima crude extract prepared with acetone at 1.6 mg/cm 2 (ie. approximately four times its ED 90). One control guinea pig was applied with 1 ml of acetone. Next they were monitored whether they developed skin reactions in 72 hr (25 -26C°/RH 72

-74%). Similar procedure was performed with E.coronaria at 0.4 mg/cm 2 (ie. approximately four times its ED 90).

4 g) Field trials

Study area and period: Trials were conducted in Ward Aungmingala purposively selected inMagway Townshipin August -September, 2015 because of its highest proportion (35.7%) of DHF cases (10 cases) in 2015. House Index, Container Index and Breteau Index of the ward in July 2015 were 18%, 23% and 82 respectively.

Larval survey and introduction of test larvicides: Larval survey was conducted at 50 randomly selected houses.Next out of 48 randomly chosen Ae.aegypti larva-positive minor water containers (flower vases and spiritual bowls) in and around the surveyed premises, 24 containers were marked and treated with C. pulcherrimalarvicide at 7.2 -14.4 mg/l (ie. its LC 90 to twofold dose) and remaining 24 containers with E. coronarialarvicide at 12.7 -25.4 mg/l (ie. its LC 90 to twofold dose). All treated containers were checked for larval mortality at 24 hr.

Percentage protection of test repellents: Field trials were conducted for two days using the methods by Choochote W et al for percentage protection 8 with the help of four well-trained male volunteers from Medical Entomology Section of Health and Disease Control Unit, Nay Pyi Taw during 0800 -1600 hr. Each volunteer had to sit indoors and catch/count Aedes mosquitoes in nine assigned houses at least 10 metres apart from each other. Using mosquito coils, burning thrash and smoking in and around the premises by householders were not allowed. Firstlyvolunteer's legs were thoroughly washed and cleaned with tap water and right leg was treated with C. pulcherrima crude extract (dose:1.6 mg/cm 2). Control left leg was treated with acetone 1 ml. Areas of both legs above knees and below ankles were covered with short trousers and socks respectively to prevent the mosquito bites. The volunteer had to sit indoors and count/catch mosquitoes landing/probing on exposed areas of both legs within 10 min with mouth aspirators. The mosquitoes caught were kept in a paper cup for species identification and calculation of landing/ biting rate. After 10 min at first house volunteer moved to his second assigned house and took the similar functions for 10 min. This procedure was completed in 2 hr. Volunteers performed their second replicate in different houses. Then percentage protection within 90 min exposure was calculated using p = (C -T) /C × 100. Next day the same procedure for two replicates was carried out for E. coronaria(dose: 0.4 mg/cm 2) in different houses (28 -34 C°/RH 48 -72%).

5 h) Indepth interviews

Ten local residents were recruited at Day 7 of field trials and Principal Investigator (PI) disseminated field trial results. Next indepth interviews were performed by PI himself. Their opinions on results of new larvicides and repellents in their ward were mainly elicited and their actual wordings were recorded, transcribed and translated into English. i) Statistical analysis LC 50 , LC 90 ,ED 50 and ED 90 were calculated by probit analysis using SPSS version 16.0. Chi-squared test was used to find out homogeneity of test mosquitoes and paired t test to find out significant difference between landing/biting rates at significance level 0.05.

6 j) Ethical considerations

Research proposal was submitted to Ethical Review Committee of University of Community Health, Magway and ethical clearance was obtained. Informed consent from study volunteers in laboratory and field trials was also received.

7 III. Results

8 a) Laboratory results

Preliminary phytochemical tests on dried leaf powder of C. pulcherrima showed that it contained carbohydrates, ?-amino acids, phenolic compounds, tannins, saponins, steroids, alkaloids, glycosides and reducing sugar. Those of E. coronaria also had similar compounds except saponins (Table 1). All mice tested with both plant extracts were still alive and active at Day 14 without any toxic signs. Similarly in skin irritation tests there were no signs of irritation, erythema,escharand oedema formations in all tested guinea pigs at 72 hr.

9 b) Field trial results

Test larvicides of ethyl acetate extract of C. pulcherrima and E. coronaria were introduced into larvainfested minor water containers in surveyed houses separately andall larvae in treated containers were found to be dead at 24 hr. Total number of mosquito species caught during two days was 154 [Ae. aegypti (89.6%), Ae. albopictus (8.4%), Culex quinquefasciatus (1.6 %) and Anopheles vagus (0.7 %)]. Mosquito landing/biting rates were much lower in repellent treated skin than control and it was statistically significant (p ? 0.05).

Percentage protection was 98.3% by C. pulcherrima repellent and 97.8% by E. coronaria repellent during 90 min (Table 5). Different from control: * marginally significant (p =0.05), **significant (p $<$ 0.05) c) Indepth interviews Ten local residents (two ten-household leaders, three housewives, two dependents, one businessman, one labourer and one basic health staff midwife) wereindepth interviewed at ward religious centre.

One of two ten-household leaders stated his opinion like: 'DHF is caused by mosquito bite. This year about 10 -15 children in our ward were affected by DHF. We need more drugs to prevent mosquito bite. The currently

tested larvicides and repellents are known to be effective in mosquito control. We want to use them.' (60 year old male ten-household leader) One of three housewives expressed as follow:

'DHF is a mosquito-borne disease. We use mosquito coils and bednets to avoid mosquito bite. When we know the good effect of currently tested larvicides and repellents, we want to use them at our homes.'(35 year old housewife).

IV.

10 Discussion

The present pilot study is the first and foremost study of its kind ever in Myanmar. C pulcherrima Linn. and E coronaria (Jacq) Stapf. -were searched locally, collected and investigated for their larvicidal and repellent activities under laboratory and field conditions followed by evaluation of public acceptance on the use of these botanical control tools in a selected community.

C. pulcherrima (Family Fabaceae) known as Seinbangale is cultivated as ornamental trees in and around human dwellings and has several medicinal properties, for instance, anti-inflammatory. It has more than fifty chemical compounds like ?-pinene and ?terpinene. In the present study nine secondary metabolites were detected as a qualitative determination in its leaves including saponins which are anti-feedant and toxic to cold blooded organisms and insects 9 .

properties such as antioxidant and anti-infection in animal model 10 . In the present study its leaves also contained same secondary metabolites as in C. pulcherrima except saponin as a qualitative determination. These metabolites have larvicidal properties damaging the tissues of mid gut and cuticle of mosquito larvae. Plantphenolics, terpenoids, alkaloids and saponins are larvicidal against Aedes mosquitoes and also have pesticidal actions.They also has repellent action against mosquito by acting locally or at distance from the human body by molecules that alter the functioning of mosquito's sensory motor systems and block its sense of smell from the host or have neurotoxic effects 11 .

In larvicidal bioassays LC 50 of C. pucherrima extract (3.21 mg/l) against Aedes larvae was lower than that of E. coronaria (4.46 mg/l). It may be due to presence of saponins in the former. When compared to other studies, LC 50 values (mg/l) were 97.53 for ethyl acetate extract E. coronaria and 144.67 for ethyl acetate extract C. pucherrimia 12 . Therefore LC 50 values of two test extracts of the present study were lower than those repellents. In repellent bioassays, ED 50 of C. pucherrima extract (0.02 mg/cm 2) against female Aedes adults was higher thanthat ofE. coronaria(0.01 mg/cm 2). Therefore the latter is more effective than the former. Regarding percentage protection, C. pucherrima repellent and E. coronaria had 88.4% at 1.6 mg/cm 2 and 82.1% at 0.4 mg/cm 2 respectively at 90 min post application. In this case the latter is also more effective than the former in terms of the dose at 90 min. When compared to 25% DEET (N,N-diethyl-3-methylbenzamide)its complete protection time at 0.83 mg/cm 2 (25mg/30cm 2) was for 6.25 hr 13 .In animal acute toxicity tests and skin irritation tests due to the lack of toxic symptoms till 14 day observations and no skin adverse effects till post application 72 hr the test plant extracts are considered safe for human use.

In field trials larvicidal efficacy of both test larvicides are satisfactory as the result of 100% mortality at 24 hour of Aedes larvae in the treated minor water containers. Similarly the larvicide can be used to treat the ant-traps as well as the miscellaneous containers like unused tires in the areas where solid waste disposal is not easily available. Like wise both test repellents were also found to be effective with percentage protection of approximately 98% against Aedes mosquitoes in 90 min. In the study by M Govindarajan et al 6 C. pulcherrima (dose, 5mg/cm 2) and E. coronaria (dose, 5mg/cm 2) gave 100% protection at 90 min and at 120 min respectively under the laboratory conditions. If higher percentage protection is desired, the treated dose should be double or treble. Botanical repellents are better than mosquito coils because these coils can cause indoor air pollution and subsequent development of respiratory tract disorders especially in children and sensitive individuals due to their ingredients of synthetic chemicals and coconut husk or saw dust.Regarding public acceptance, almost all householders representing the study area were found to be interested in and accepted and demanded these new control tools.

In conclusion, the present study highlighted that new larvicides and repellents were found to be very promising to be safely and effectively used to control Aedes mosquitoes -vector of deadly DHF.

11 V. Acknowledgements

We acknowledge Dr Kyaw Zin Thant, Director General of DMR for his kind providing laboratory and insectary facilities and Prof May Than Su, Head of Department of Botany, University of Magway for her authentication of test plants. We are also thankful to all volunteers who enthusiastically took part in repellent tests under laboratory and field conditions. Finally we are indebted to local and health authorities and all householders of Ward Aungmingala, Magawy for their active participation in field trials. The present study was conducted with financial aids of WHO/TDR. [1]

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1

Test for	Extract	Test reagent			Observation
Carbohydrates	H 2 O	10%	?-napthol	+	Pink ring
		concentrated H 2 SO 4			
?-Amino acids	H 2 O	Ninhydrin reagent			Red
Phenolic compounds	H 2 O	Ferric chloride solution			Deep brown
Flavonoids	Methanol	HCl/Mg			No colour
Tannins	H 2 O	Ferric chloride solution			Blue black
Saponins	H 2 O	Distilled H 2 O			Frothing
Steroids	Petroleum ether	Acetic concentrated H 2 SO 4 anhydride		+	Deep green
Alkaloids	10% acetic acid and EtOH	(i) Mayer's reagent			White precipitate
		(ii) Dragendroffs reagent			Orange precipitate
Glycosides	H 2 O	10% Lead acetate			White precipitate
Reducing sugar	Diluted H 2 SO 4 + 5N NaOH	Benedict's solution			Brick red precipitate
Cyanogenic glycoside	H 2 O	H 2 SO 4 + Sodium picrate solution			No colour

*[Note: * + = present, -= absent Table(2) shows larvicidal activity of crude ethyl acetate extracts of C. pulcherrima and E. coronariaa gainst Ae. aegypti under laboratory conditions. Test mosquitoes were not in heterogeneity in the former (p = 0.577) and in heterogeneity in the latter (p = 0.009).]*

Figure 1: Table 1 :

2

Concentration (mg/l)	C. pulcherrima Mean mortality ± SD (%)	LC 50 and LC 90 (95% FCI) (mg/l)*	E.coronaria Mean mortality ± SD (%)	LC 50 and LC 90 (95% FCI) (mg/l)**
1.563	12.7 ± 5.9	3.21 (2.95-3.48)	6.0 ± 3.3	4.46 (3.16-6.05)
3.125	50.0 ±10.4	7.2 (6.42-8.29)	34.7 ± 7.9	12.71 (8.81-25.03)
6.25	82.7 ±7.4		74.0 ± 20.2	
12.5	99.3 ± 1.6		88.7 ± 10.3	
25.0	100.0 ± 0.0		96.0 ± 3.8	
Control	1.3 ± 2.1		1.3 ± 2.1	

* p = 0.577, ** p = 0.009

Figure 2: Table 2 :

3

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Test repellent	ED 50 (95% FCI)*	ED 90 (95% FCI)*
C. pulcherrima	0.02 (0.01 - 0.03)	0.48 (0.28 - 1.35)
E. coronaria	0.01 0.005 - 0.02)	0.12 (0.08 - 0.16)

mg extract / cm 2 skin
Table (4) expresses percentage protection of crude ethyl acetate extracts of both test repellents against Ae. aegypti under laboratory conditions.

[Note: K]

Figure 3: Table 3 :

4

Test repellent	Concentration (mg/cm 2)	% protection (mean ± SD) Time post application of repellent (min)					
		0	30	60	90	120	150
C. pulcherrima	0.8	78.1± 13.3	67.8± 20.3	54.7± 21.0	43.7 ± 26.0	35.2 ± 24.3	32.2± 17.7
	1.6	94.4± 6.9	91.3± 9.7	88.7± 14.8	88.4± 13.3	84.6± 18.9	84.3± 21.4
E. coronaria	0.2	88.9± 4.8	63.1± 13.8	58.4± 17.0	51.4± 25.3	50.1± 26.9	50.0± 24.5
	0.4	93.3± 3.3	87.7± 4.9	83.4± 8.8	82.1± 6.4	82.1± 9.6	76.7± 10.8

Figure 4: Table 4 :

5

field trials

Figure 5: Table 5 :

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