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The efficacy of *Artemisia annua* crude extracts on *Anopheles dirus* in the laboratory and whole plants on other mosquitoes in the field

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Evaluation of the efficacy of an aromatic plant *Artemisia annua* against *Anopheles dirus* mosquito (a major vector of forest malaria in Myanmar) was conducted in the laboratory and in the field situation. Repellent effect on adult mosquito was observed at the concentration as low as 0.5% *Artemisia* extract solution. At the concentration of 6.0 %, the repellency was markedly increased and the protection provided was found to be 94.74 % during an exposure period of six hours ($r = 0.95$, $df = 6$, $p = 0.001$). The larvicidal effect on 3rd and 4th instar larvae of *An. dirus* showed that the LC_{50} and LC_{90} were 0.04 % and 0.14 % respectively based on the dose-effect probit analysis ($\chi^2 = 3.05$, $df = 3$, $p<0.05$). The residual effect persisted for at least 6 days at 0.25% and 0.50% concentrations. Regarding the ovicidal effect, a series of concentrations starting from 0.025% were tested and at 0.40% concentration, the relative reduction in egg hatching rate was 93.85% compared to that of control ($r = 0.92$, $df = 4$, $p = 0.026$). However, protection from mosquito bites was not observed when *Artemisia annua* plants were placed around baits under field situation ($\chi^2 = 1.69$, $df = 1$, $p>0.05$). The prospects for the use of indigenous plants and its extracts for personal protective measures in prevention and control of vector borne diseases are also discussed.

INTRODUCTION

Anopheles dirus is one of the major vectors of malaria in mainland Southeast Asia, including Myanmar, Cambodia, Bangladesh and Thailand [1]. Despite the importance of this species as a vector of malaria, there is no published record of its response to repellent, larvicidal and ovicidal actions of *Artemisia annua* plant and plant extracts. The use of repellents and other personal protective measures to minimize contact with malaria vectors in Southeast Asia is especially important for two reasons. Firstly, the region is the center for the occurrence of *Plasmodium falciparum* drug resistance causing ineffective chemotherapy in many areas [2]. Secondly, *Anopheles dirus* is largely exophilic and therefore unaffected by interior spraying of houses with residual insecticides [3]. Synthetic insecticides that have been developed in agriculture, forestry and public health against plant pests and vectors of human diseases, do not fulfill the requirements for integrated pest management [4]. For these reasons as well as the increasing problems

of insecticide resistance, interest in insecticidal properties of plants has grown rapidly during recent years.

During the last decade, extracts and compounds of pyrethroid have attracted the special interest of entomologists and phytochemists resulting in the wide use of bed nets treated with pyrethrum insecticide as personal protective measures in malaria control. *Artemisia annua* plantations have been encouraged due to the parasiticidal effect of its derivatives such as artesunate and artemether. In parallel to this, it seems appropriate and worthwhile to conduct efficacy studies of *Artemisia annua* and its extract on mosquitoes for personal protection and other properties.

Thus, the general objective is to explore the repellent effect and other insecticidal properties of indigenous plant *Artemisia annua* against malaria vector mosquitoes with special reference to the primary malaria vector *An . dirus* for the development of future alternative vector control measures. The specific objectives are: (i) to determine the repellent action of *Artemisia annua* plant extract on *Anopheles dirus* adult mosquitoes under laboratory conditions; (ii) to assess the larvicidal and ovicidal effects of *Artemisia annua* extract on *An. dirus* under laboratory condition and (iii) to investigate the repellent action of *Artemisia annua* on *Anopheles* mosquitoes under field condition.

MATERIALS AND METHODS

Study area

The laboratory studies were carried out in the Medical Entomology Research Division, Department of Medical Research (DMR) (Lower Myanmar) and the field study was undertaken at Do-gwin plantation site, Myaingyi village, Pyin-Oo-Lwin Township, Mandalay Division.

Study design

One arm of this study consisted of experimental laboratory studies namely: (i) repellency (ii) larvicidal (including persistency test) and (iii) ovicidal tests on *An. dirus*. This is the major component of the present study. Another arm of this study involved cross-sectional field comparative study comparing the total number of mosquitoes caught on cattle-baits with or without *A. annua* plants regardless of whether *An. dirus* are present or not.

Sample size

This study was not based on human population but on mosquitoes and therefore minimum sample size determination was not applicable for this situation. Nevertheless, enough mosquitoes had been utilized for each experiment.

1. Laboratory studies

The required plants were obtained from the Agricultural Department in Yangon. The *An. dirus* colonies reared in the insectary were utilized. *Artemisia annua* crude extract was obtained with the assistance provided by the Pharmacology Research Division (DMR-LM). A 10 percent emulsified concentration (EC_{10}) stock solution of *Artimisia annua (*Methanol extract) was prepared by dissolving it in acetone. It was tested in the laboratory for repellent, larvicidal and ovicidal properties using fifteen concentrations ranging from 0.01 percent to 6.00 percent. Detailed testing was according to the W.H.O larval susceptibility standard method[5], Frances [6], Abdulcada and Sasamananabu [7] and Swaroop [8].

1. 1 Repellent testing

Tests were carried out on two human volunteers based on a method described by Schreck [9]. Laboratory reared 6 to 7-day-old nulliparous female *An. dirus* mosquitoes were used. For each test, 50 mosquitoes were placed overnight in a screen wire cage

measuring 30cm x 30cm x 30cm and 10% sugar solution was used for feeding. Mosquitoes were starved for 12 hours before testing. Three replicates were carried out.

Tests were conducted by exposing untreated and repellent- treated human forearms to the mosquitoes according to the method of Frances [6]. Two millilitres of the solution was applied because this volume evenly covered the surface area. The treated arm was then exposed in the test cage for 15 minutes and subsequently exposed at 30-minute intervals. A number of bites for untreated and treated arms in each trial were recorded and percent protections were calculated using standard formula [10] and [11].

1.2 Larvicidal action testing

Larvicidal effect of *Artemisia annua* plant crude extract on *Anopheles dirus* was conducted in the laboratory at 27 ± 1°C, 75-80% Relative Humidity (RH). A series of five *Artemisia annua* plant crude extract concentrations were used. In each replicate ten late-third and fourth instar larvae were exposed to 30 ml of each concentrations for 24 hours and for control purpose, the same number of larvae was exposed using 100 ml polythene beakers. A total of 250 larvae was utilised.

Five replicates were carried out and mortalities were recorded and checked daily. The lethal concentrations of LC_{50} and LC_{90} values were determined using dose-effect probit analysis.

1.3 Ovicidal action testing

Beakers (100 ml) containing a series of *Artemisia annua* plant crude extract concentrations (30 ml each) were used. One hundred eggs were exposed to each concentration for 48 hours to 72 hours and for control purpose, the same number of eggs were exposed using distilled water. After 48 hours and 72 hours, the number of hatched eggs were counted and recorded. Six replicates were carried out using a total of 3,600 eggs.

2. Field studies

The plants were collected from Pyin-Oo-Lwin Botanical and Agricultural Garden and the field study site was conducted in Pyin-Oo-Lwin Township where *Artemisia annua* plants were grown by Agricultural Department. Mosquitoes caught were identified according to Rattanarithikul [12].

There were two catching stations. In one catching station, there were two big mosquito-net traps. One net had ten *Artemisia annua* plants around the inner periphery of the net and a cattle-bait was tied at the centre. Another net had no *Artemisia annua* plants but only cattle- bait. Mosquitoes that came into the net were caught hourly from dusk to dawn. To exclude bias in site, the positions of the plants were reversed in the next catching station. Cattle-baits were used because of the pronounced attraction to mosquitoes in comparison to human baits. The total number of mosquitoes caught for 8-collection nights was recorded separately according to the respective catching station.

Data analysis

Data entry and processing were made using data base software and data analysis was carried out using Epi-info version 6 software. Probit analysis (dose effect analysis) in determining effect of insecticide concentration on mortality was drawn manually and calculated according to Swaroop [8].

RESULTS

Repellent action

Artemisia annua extract in the concentrations of 0.5%, 1.0%, 2.0%, 3.0%, 4.0%, 5.0% and 6.0% provided 15.78%, 36.36%, 56.52%, 75.0%, 80.0%, 86.6% and 94.74% protection respectively during the exposure period of six hours for the principal malaria vector *An. dirus*. There was a strong

correlation between *Artemisia annua* methanolic extract concentration and percentage protection from *An. dirus* bites (r = 0.95, df = 6, p = 0.001) (Fig 1).

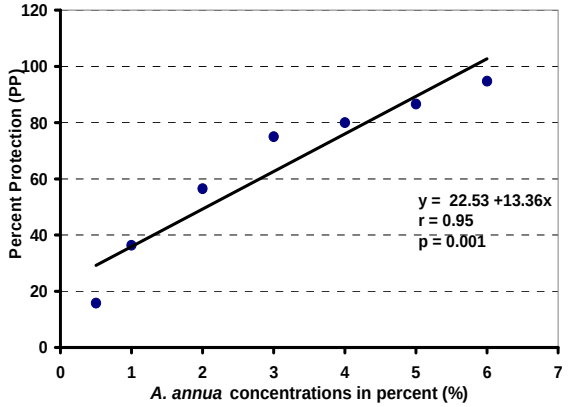

Fig. 1. Regression analysis of *Artemisia annua* concentrations against percent protection (PP) from *An. dirus* adult mosquito bites

Table 1. Larvicidal effect of *Artemisia annua* extract on the immature stages of *An. dirus* mosquitoes

A. annua concentration (%)	Dead/ Tested	Observed mortality (%)	Expected mortality (%)	(O-E)	Contribution to χ^2
0.01 %	4/50	8%	4.75	3.25	0.023
0.025 %	12/50	24%	28	4	0.007
0.05 %	27/50	54%	58	4	0.006
0.10%	45/50	91%	86	5	0.021
0.25%	50/50	100%	99.1	0.64	0.004
				Total	0.061

Larvicidal action

The summary of dose-effect mortality and testing the goodness of fit for A*rtemisia annua* methanolic extract concentrations against *An. dirus* larvae are shown in Table1& Fig, 2. The LC_{50} value and the LC_{90} value were found to be 0.04% and 0.14% concentrations respectively based on the dose-effect probit analysis ($\chi^2 = 3.05$, df = 3, $p<0.05$). There was a strong correlation between *Artemisia annua* methanolic extract concentrations and *An. dirus* larval mortality. The persistent effect of *Artemisia annua* extract concentrations on the immature 3rd and 4th stage of *An. dirus* larvae is shown in Table 2. The effects of *Artemisia annua* extract 0.25 percent and above persisted for 6 days.

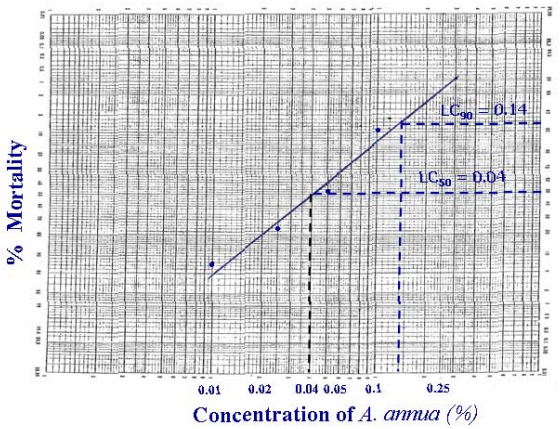

Fig . 2. Testing the goodness of fit for dose - effect analysis of *A. annua* concen ration and *An. dirus* mortality

Ovicidal action

Artimisia annua extract in the concentrations of 0.025%, 0.05%, 0.10%, 0.20% and 0.40% reduced *An. dirus* egg hatching by 10.07%, 37.59%, 50.12%, 76.16% and 93.85% respectively. There was a strong correlation between *Artemisia annua* methanolic extract concentrations and percent reductions in eggs hatching (r= 0.92, df = 4, p = 0.026) (Fig. 3).

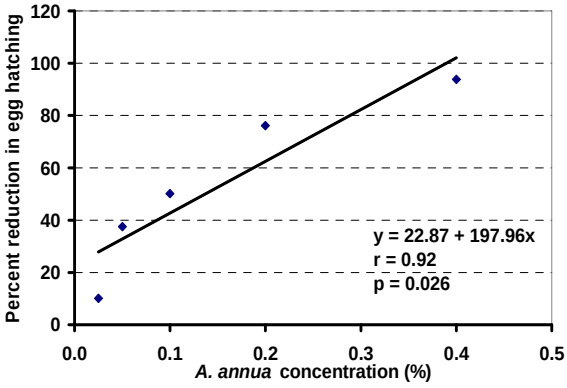

Fig. 3. Regression analysis of *Artemisia annua* concentration(%) against *An. dirus* percent reduction in eggs hatching

Table 2. The effective persistancy of *A. annua* extract EC_{10} application on immature stages of *An. dirus* mosquitoes

A. annua concentr-ation (%)	Effect of *A. annua* methanolic extract EC_{10} on *An. dirus* immature stages (% mortality in days)												Remarks
	Day (1)		Day (2)		Day (4)		Day (6)		Day (8)		Day (10)		
	T *	C **	T	C	T	C	T	C	T	C	T	C	
0.05	63	0.0	40	0.0	23.3	0.0	13.3	0.0	6.6	0.0	3.3	0.0	*A. annua* methanolic extract 0.25% persists up to 6 days. Percent mortality was still 50% on Day 6.
0.075	76.6	0.0	66.6	0.0	46.6	0.0	26.6	0.0	13.3	0.0	6.6	0.0	
0.10	93	0.0	70	0.0	56.6	0.0	30	0.0	16.6	0.0	10	0.0	
0.25	100	0.0	93.3	0.0	70	0.0	50	0.0	26.6	0.0	13.3	0.0	
0.50	100	0.0	96.6	0.0	80	0.0	73.3	0.0	46.6	0.0	23.3	0.0	

* T = Test ** C = Control

Field studies

Species and number of adults biting females mosquitoes collected from cattle baits were during field study were found in catching station (1). A total of 1011 mosquitoes were collected. Out of these 501 mosquitoes were caught where the plants were present along with cattle baits and 510 mosquitoes were caught where the plants were absent. Similarly, in catching station 2 out of 2290 mosquitoes collected, 1191 mosquitoes were caught where *Artemisia annua* plants were present and 1099 mosquitoes were caught where the plants were absent.

This field study revealed that the primary vector *An. minimus* and other secondary vectors *An. annularis* and *An. culicifacies* were present in the study area. There were no significant differences in collected mosquitoes species between catching stations and presence or absence of *A. annua* plants ($\chi^2 = 1.69$, df = 1, $p>0.05$).

DISCUSSION

The primary objective of the present study is to gain more knowledge on mosquito control with insecticides extracted from plant ingredients which are locally available. A notable species was found to be *Artemisia annua* plant [13]. *Artemisia annua* species (composite *Anthemideae*) are a group of aromatic plants with worldwide distribution.

Essential oils like Lemon grass, Thyme, Citronella etc. have mosquito repellent action. The fractionation of these essential oils has been carried out and the repellent effect of these fractions has been studied. Often, many unrelated plants have similar repellent constituents. Eucalyptus, alpine and camphor are the most commonly used natural oils as mosquito repellents before the artificial compounds are not available [14]. The problem with the natural oils is that they provide repellent action only for a short duration often not exceeding a few hours.

Moreover, a number of synthetic repellents have been developed. Some promising ones are DEET (N, N-diethyl-meta-toluamide) and DMP (dimethyl phthalate). These are most commonly used throughout the world to protect human against biting insects. The discovery of DEET in 1954 has ushered a new era in repelling mosquitoes.

The present study indicated that methanolic extract of *A. annua* could be considered as a potential repellent. It possessed repellency property and at 6.0 percent concentration, 94.74% protection was achieved during 6 hours period. This period is long enough if the

repellent is applied during the peak biting period. However, its repellency could not be compared with chemicals such as DEET in both efficacy and residual duration since these chemical compounds provide better and longer protection against many biting insect (ED_{50} and ED_{95} level = 0.37-25.37 $\mu g/cm^2$, 3-8 hours) [15-17].

The previous report of the repellent tolerance of *Ae. aegypti* was higher than the present finding which showed that the repellent action concentration was much lower with *An. dirus* [18]. As mentioned earlier, *Artemisia annua* methanolic extract 6.0 percent provided the protection up to 6 hours. Thus, it was found that *Ae. aegypti* was less susceptible to *Artemisia annua* than *An. dirus* in the laboratory test.

The results of the larvicidal effect of *Artemisia annua* extract on *An.dirus* larvae showed that LC_{50} value was 0.040% and LC_{90} value was 0.14%. The LC values were within statistically insignificant range. The regression lines were linear and the range of concentration to get a kill of 50% to 90% mortality is nearly 14 times of the initial concentration. Using the statistical analysis adopted by Swaroop's chi square test, it was found that the test results were highly significant. The present study differed with the finding of Htay Aung [18] where *Artemisia annua* extract on *Ae. aegypti* larvae (LC_{90}) was found to be 0.75% compared to only 0.14% in our studies on *An. dirus.* This could be that *An. dirus* is more susceptible than *Ae. aegpti* under laboratory condition.

Studies on the persistence effect of *Artemisia annua* on *An. dirus* larvae showed *Artemisia annua* methanolic extract 0.25 percent and above persisted up to 6 days. These findings also differed from the finding of Htay Aung [18] who reported that persistence effect of *Artemisia annua* on *Ae. aegypti* larvae lasted for 30 days at 1 percent to 2 percent concentration. It must be noted that the concentration in our study was 40 times less. The lesser concentration is better in the sense that scarcity of the extract could be an operational problem.

Regarding ovicidal effect, observations at lower concentration showed that *Artemisia annua* 0.025 percent concentration provided 10.07 percent reduction in egg hatching, whereas in the higher concentration 0.40 percent concentration provided 93.85 percent reduction in egg hatching. The lack of data for comparison is due to the fact that this is the first time ovicidal effect has been studied.

Contrary to the findings under laboratory condition, our field results indicated that the repellent action of *Artemisia annua* plants on the field mosquito species was not promising. Although *An. dirus* were not caught, *An. minimus* (also primary vector of malaria) were caught. It showed that there were no significant differences in collected mosquito species between 2 catching stations with and without *A. annua* plants. These findings are similar to the finding of Htay Aung [18]. One possible explanation could be that "whole plants" are used in the field but the "pure extracts" are used in the laboratory. Further studies are needed to clarify these findings.

Employing *Artemisia* (EC_{10}) solution is safe to use as a larvicide, though with different characteristics and mode of action unlike other existing insecticides. *Artemisia* is not a conventional pesticides. It does not produce the non-discriminatory rapid and directly non-toxic effects that are associated with traditional medicine. The use of simple and cheap local indigenous *Artemisia* products such as crushed *Artemisia* leaves (plants), seems promising for treatment of temporary water pools in towns and villages in developing countries especially during the post-monsoon season for prevention of mosquitoes breeding.

The overall laboratory results indicated that *Artemisia* is an effective repellent against *An. dirus*. It can be useful and cost-effective in areas where insecticides can not be sprayed due to administrative and financial

reasons and also in areas where there is no effective method for protection against mosquito vectors. Night biting activity of *An. dirus* and other potential *anopheline* mosquitoes in Myanmar is during the 1st and 2nd quarters of the night. The application of *Artemisia* (6 percent) during this period is recommended as prophylaxis against bites of *An. dirus* mosquitoes. Thus, in the present context of malaria situation, application of *Artemisia annua*, an indigeneous plant, as mosquito repellent, larvicides or ovicides could be cost-effective for the communities.

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