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Skin and Oviposition Deterrent, Ovicidal and Deleterious Delayed Mortality Effects of α-amyrin Acetate Against the Malarial Vector *Anopheles stephensi* (Diptera: Culicidae)

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ABSTRACT

Selective discriminating behaviour of the ovipositing female for an appropriate oviposition habitat selection and the substances involved in oviposition site choice by vector mosquitoes have recently become a focal point of interest in the concept of integrated vector control management. In the current study, we isolated and identified α-amyrin acetate from *Catharanthus roseus* Linn (Apocynaceae) and assessed the skin repellency, oviposition deterrency, ovicidal, gravid mortality and deleterious delayed mortality against the malarial vector *Anopheles stephensi* Liston (Diptera:Culicidae). Water treated with the α-amyrin acetate had a high deterrent activity in ovipositing females: oviposition activity index values for the test species were -0.22, -0.38, -0.42 and -0.52 for α-amyrin acetate at concentrations of 0.007, 0.015, 0.025 and 0.050 p.p.m., respectively. High degrees of mortality were observed with various concentrations of α-amyrin acetate: 1.12 (control) to 7.20 for gravid females, and 0.62 (control) to 9.05 for oviposited females. The highest mortality in both gravid and oviposited females was observed soon after they came in contact with oviposition medium treated with the α-amyrin acetate, and this was found to be significant at doses higher than 0.015 p.p.m., suggesting possible contact toxicity of the α-amyrin acetate. The α-amyrin acetate had the most effective skin repellency with ED_{50} and ED_{90} values of 0.6659 and 1.7720μg/cm^2 with biting protection time of 3.5 h. The α-amyrin acetate caused moderate ovicidal activity against various age groups of *A. stephensi* but it inflicted delayed effects such as high larval, pupal and adult mortality. The age of the eggs and the duration of the α-amyrin acetate treatment influenced the ovicidal activity observed. It is clear that α-amyrin acetate of *C. roseus* can affect the oviposition cycle of *A. stephensi* Liston, thereby suppressing the vector population and adversely influencing transmission of the disease pathogen.

Key words: *Anopheles stephensi*, *Catharanthus roseus*, oviposition deterrent, delayed mortality, gravid mortality, oviposition activity index.

INTRODUCTION

The distribution of larvae depends mainly on the selection performed by the female mosquito for an appropriate oviposition site called oviposition habitat selection an important phenomenon in mosquitoes, and the substances involved in oviposition site choice by vector mosquitoes have recently become a focal point of interest in the concept of integrated vector control management (Graham-Bryce, 1987). Substances

originating from the egg (Bruno et al*.,* 1979), larva (Kalpage *et a*l*.,* 1973) and pupa (Andreadis, 1977), as well as from other sources (Rockett, 1987), have produced highly selective oviposition attractancy for vector mosquitoes. Oviposition behavior is a plastic trait and can be affected by the availability of cues (Betley and Day, 1989; Isoe *et al.,* 1996; Yu *et al.,* 2004). The ability of the parent to distinguish among oviposition sites is important for many insects because habitat quality is often the major determinant of larval survival (Thompson, 1988). Many synthetic insecticides and naturally occurring chemical cues have been shown to influence mosquito oviposition (Miller *et al.,* 1992; Olagbemiro *et al.,* 1999; Geetha *et al.,* 2003). A few insecticides in common use have also exhibited high deterrent activity, causing negative ovipositional response (Moore, 1977). Botanical phytochemicals with mosquitocidal potential are now recognized as potent alternative insecticides to replace synthetic insecticides in mosquito control programs due to their excellent larvicidal, pupicidal and adulticidal properties.

Catharanthus roseus Linn, commonly known as periwinkle, is a perennial, evergreen plant that occurs naturally in most warm regions of the world, including India. The plant has been historically used as a folk remedy, as well as an insecticide (Singh *et al.,* 2003). The leaves of *C. roseus* contain α-amyrin acetate and oleanolic acid, which have insect growth regulator (IGR) properties against the malarial vector *Anopheles stephensi*, tobacco caterpillar *Spodoptera litura* F., green pod borer (*Helicoverpa armigera* Hub.), and various other agricultural pests (*Patel et al*., 1990; Singh *et al*., 2003; Govindarajan *et al*., 2008 and 2012). Ovicidal and ovipositional deterrent effects of phytochemicals have not been investigated to any great extent, and once mosquito eggs have hardened, they become rather impervious, although this is perhaps truer for anophelines than for culicines and aedines. Studies conducted on freshly laid eggs have been limited. Repellents are a practical and economical means for preventing the transmission of mosquito borne diseases to humans. By definition, repellents are substances that act locally or at a distance, deterring an arthropod from flying to, landing on or biting human or animal skin (or a surface in general) Choochote *et al*. 2007). Usually, insect repellents work by providing a vapour barrier deterring the arthropod from coming into contact with the surface (Brown and Hebert, 1997). Repellents, such as the “gold standard” *N, N*-diethyl-3-methylbenzamide (DEET), have shown significant repellency against mosquitoes and other biting arthropods (Yap 1986; Coleman *et al*., 1993; Walker *et al*., 1996). However, toxicity reactions for humans after applications of DEET can be severe (Zadikoff ,1979; Robbins and Cherniack, 1986; Edwards and Johnson, 1987; Qiu *et al*. 1998). To avoid these adverse effects, many laboratories have tried to replace DEET with repellents that are derived from plant extracts. Various plant extracts, such as neem (from *Azadirachta indica* A. Juss), basil oil (*Ocimum basilicum* L., *O. basilicum*L. fa. *citrtum* Bach, *O.* gratissimum L., *O. americanum* L., *O. tenuiflorum* L.), citronella grass (*Cymbopogon nardus* Rendle), galingale (*Alpinia galanga* L.), clove (*Syzygiumaromaticum* L.), and thyme (*Thymus vulgaris* L.), have been recorded as mosquito repellents (Sukumar *et al*. 1991; Sharma *et al*. 1993; Chokechaijaroenporn *et al*. 1994; Suwonkerd and Tantrarongroj, 1994; Boonyabancha *et al*. 1997; Barnard, 1999). These natural repellents have demonstrated good efficacy against some mosquito species but

some were evaluated only by olfactometry or by using laboratory conditions. However, the evaluation of repellency should preferably be carried out using human subjects because laboratory animals may inadequately simulate the condition of human skin to which repellents will be eventually applied (WHO, 1996). Therefore, the objective of the present study was to assess the ovipositional and skin deterrency, mortality of gravid and oviposited females, ovicidal efficiency, and deleterious delayed mortality effects of α-amyrin acetate from *C. roseus* against *Anopheles stephensi* Liston, which is a vector of malaria in India, Iraq, Iran and some Asian countries, and for which control has not yet been established (Gad, 1967).

MATERIALS AND METHODS

Mosquito culture

Anopheles stephensi Liston were maintained in the laboratory from lines obtained from the National Institute of Communicable Diseases field station, Cunnoor, India. The colonies of mosquitoes were maintained at conditions of 27 ±2°C and 80% ± 5 relative humidity under a 14 h light : 10 h dark (LD 14:10) cycle. The larvae were reared in enamel trays and were fed finely ground dog biscuits and yeast at a 60:40 ratio. Water in rearing containers was refreshed every 2 days. Pupae were transferred from the trays to a cup filled with dechlorinated tap water and placed in screened cages where adults emerged. The adult mosquitoes were maintained in a net cage (90 ×90×90 cm) and were continuously provided with 10% sucrose solution in a jar with a cotton wick. For continuous culture, a selected number of mosquitoes were allowed to feed on chicken blood and every third day thereafter, moist filter paper was kept in a beaker in the cages for mosquitoes to lay their eggs on. Eggs laid on the filter paper were immersed in larval basins containing water for the maintenance of the colony.

Extraction, isolation and characterization

The leaves of *C. roseus* were collected locally from the foothills of the Western Ghats area adjacent to Bharathiar University, Coimbatore, Tamil Nadu, India. The leaves were washed with double distilled water and were shade dried at room temperature. The dried parts were chopped into small pieces of approximately 1 cm size by a falcon stem cutter (Biocraft Scientific India, Uttar Pradesh, India) and powdered with the help of an electric blender. The dried powder was subjected to acetone in a Soxhlet apparatus (Borasil, Mumbai, India) for 72 h (Saxena *et al.,* 1994). The solvent was then filtered and evaporated under reduced pressure in a rotary vacuum evaporator to afford crude extract (41.28gm), as greenish orange mass. The compound α-amyrin acetate was isolated from the acetone extract by column chromatography (Backett and Stenlake, 1986) on silica gel of 60-120 mesh size. The column was first eluted with n-hexane: ethyl acetate (1:5) and then polarity of the solvent system was gradually increased and 50 ml were collected in each fraction. The fractions 3 to 6 showed almost identical spots on TLC. These fractions were combined and subjected to preparative Thin Layer Chromatography (Egon and Stahi, 1969) using solvent system chloroform: methanol (7:1) to afford the compound α-amyrin acetate

as yellowish syrup. The compound α-amyrin acetate was characterized on the basis of its ^{1}H-NMR, ^{1}H-^{1}HCOSY 90^{0} spectrum, ^{13}C complete decoupling, ^{13}C DEPT 135^{0} spectra and long range correlation spectra.*(*Chenniappan *et al.,* 2011)

The volume (20 mL) of 1% stock solution was obtained by weighing 200 mg of the material and adding 20 mL ethanol to it and was kept in a screw-cap vial with aluminum foil over its mouth. The stock solution was then serially diluted ten-fold in ethanol (2 mL solution to 18 mL solvent) and test concentrations were obtained by adding 0.1-1.0 mL of the appropriate dilution to 100 mL distilled water (WHO, 2005).

Oviposition deterrence experiment

To study the ovipositional deterrence effect and the number of eggs deposited in the presence of α-amyrin acetate, a multiple concentration test was carried out. For this experiment, 50 males and 50 females were separated in the pupal stage (by size of the pupae) and were introduced into screen cages (23×23×23 cm) in a room at 27±2°C and with a photoperiod of LD 14:10. The pupae were allowed to emerge into adults in the test cages. Adults were provided continuously with 10% sucrose solution in a plastic cup with a cotton wick. They were blood fed on day five after emergence. In the multiple concentration test, four cups, each containing 100 ml distilled water with a 9 cm piece of white filter paper for oviposition as well as α-amyrin acetate at a concentration of 0.007, 0.015, 0.025 and 0.050 p.p.m., were placed in each cage. A fifth cup without α-amyrin acetate (i.e. distilled water only) served as a control. The treatments were replicated four times using four cages, and the positions of the cups were changed every time. The oviposition test was carried out in the cages on day four after blood feeding. The test was conducted in the late afternoon and the eggs were counted on the following morning.

Determination of oviposition activity index (OAI)

The results of the oviposition experiment were expressed as mean number of eggs, and oviposition activity index (OAI), which was calculated using the formula

$$OAI = \frac{NT - NS}{NT + NS}$$

Where NT is the total number of eggs in the test solution and NS is the total number of eggs in the control solution.

Index values lie within the range +1 to -1. Positive values indicate that more eggs were deposited in the test cups than in the control cups and that the test solutions were attractive. Conversely, negative values indicate that more eggs were deposited in the control cups than in the test cups and that the test solutions were a deterrent.

Repellent bioassay

Two different treatment methods (dose-response study and protection time determination) were used to determine the repellent activity of α-amyrin acetate against laboratory-reared *Anopheles stephensi* Liston after they were applied to human skin. In the dose-response test, the procedure for determining effective dosages of the plant

extract against hungry mosquitoes was a modification of the American Society for Testing and Materials Standard ED 951-83 (ASTM 1983). Tests were based on the variable dose-fixed time, "free choice method" described by Buescher *et a*l. (1982) and were similar to the method described by Coleman *et al*. (1993, 1994)., the timing of tests was between 20.00 h and 6.00 h. Evaluations were carried out in a 10x10x3 m room, at 25-30°C, and relative humidity of 60-80%. Five circles (29 mm in diameter) were outlined on the ventral surface of the volunteer's forearm using a plastic template and permanent marker. Applications of 25 ml of the diluent (control) and four serial dilutions of α-amyrin acetate in absolute ethanol were applied randomly on the marked areas. After air drying for 5 min, a plastic cage (4x5x18 cm), divided into 5 compartments, was secured over the area with rubber bands. Each compartment of the plastic cage, with a matching cutout on its floor, contained 10 blood-starved 5-7-d-old *Anopheles stephensi* females. The number of mosquitoes biting on each test site was recorded each minute for 5 min. Tests were carried out three times on each repellent-treated area and completed within 25 min of repellent application. The experiments were conducted four times on each subject of four human volunteers (2 ♀♀, 2 ♂♂).

Laboratory repellent tests were also conducted to determine the repellent protection time using the human bait technique of the WHO (1996) standard method, with some modifications. A plastic sleeve was wrapped around each forearm, with a hole cut in alignment with a 3x10 cm area on the inside part of the forearm, and attached with double-sided tape. Thus, only a restricted area of skin was exposed to the mosquitoes, with the hand being protected with a rubber glove. Approximately 0.1 ml of 25 g % α-amyrin acetate dissolved in absolute ethanol was spread as evenly as possible on the 30 cm2 test area of one forearm of each volunteer. The other forearm, acting as a control, was treated with absolute ethanol by the same procedure as that for the test repellent. After air drying for 1 min, the test arm was put into a 30x30x30 cm3 cage containing 200 unfed mosquitoes for 3 min. The mosquitoes that landed and attempted to probe and imbibe any blood were recorded. If no mosquito bites occurred in the initial 3 min, the arm was withdrawn from the cage and re-tested every 30 min. The period of repellent protection was then calculated as the time between α-amyrin acetate application and the time when at least two mosquitoes bit in the same 3-min exposure or the time when only one mosquito bit in one exposure period if another bit in the next exposure period (30 min later). When no confirming bites were observed in the period after the initial bite, the treated arm resumed the test until a confirming bite was recorded. During the experiment, successive introductions of the control arm were made in the same manner, prior to inserting the treated arm, in order to confirm the readiness of the mosquitoes to bite. The same test was repeated on each subject of 4 human volunteers (2 females, 2 males). The median complete-protection time was used as a standard measure of the α-amyrin acetate ct repellency against adult female *Anopheles stephensi* in the laboratory.

Gravid mortality effect assay

To study the gravid mortality effects of the α-amyrin acetate on ovipositing and gravid females, the multiple concentration test (described above) was carried out.

The forced oviposition techniques were used to compare the mortality rates of the ovipositing and gravid females, and observation of mortality was made after 16-20 h.

Ovicidal effect assay

In our preliminary studies, we noted that the hatching rates of eggs laid over 24 h by gravid females in α-amyrin acetate was lower than those of eggs laid in water. On this basis, we initiated further studies to determine the non-larvicidal effects of α-amyrin acetate in a multiple concentration test of ovicidal activity. Freshly laid eggs were collected by providing ovitraps in mosquito cages from 10.00 to 16.00 hours for collecting 0-6 h old eggs, and 16.00 to 10.00 the next morning for collecting 0-18 h old eggs. Ovitraps were kept in the cages 2 days after the female mosquitoes were given a blood meal. The eggs were laid on filter paper lining provided in the ovitrap. After scoring, the filter papers containing the eggs were exposed to graded doses of α-amyrin acetate (0.007, 0.015, 0.025 and 0.050 p.p.m.) or control solution. A minimum of 100 eggs was used for each treatment, and the experiment was replicated four times. Eggs of the age group 0-6 h were exposed to the α-amyrin acetate for 18 h, while eggs of the age group 0-18 h were exposed for 6 and 18 h. After exposure, the eggs were sieved through muslin cloth, thoroughly rinsed with tap water, and left in enamel bowls filled with dechlorinated water for hatching. The hatching rate of eggs was assessed 3 days later. The percent egg mortality was calculated on the basis of non-hatchability of eggs with unopened opercula.

Delayed mortality assay

Delayed mortality and survival rates of larvae from 0-6 h and 0-18 h exposures were studied 3 days after hatching. The live larvae were transferred to individual enamel pans (50×25×7 cm) containing 2 l tap water and they were fed on yeast and finely ground dog biscuits in a ratio of 3:2. Larval mortality was assessed every 24 h until pupation. The number of pupae formed was scored and pupae were kept in small enamel bowls in empty mosquito cages (23×23×32 cm) for adult emergence. The number of adults that emerged was counted (those incompletely emerged were considered to be dead). From the data, the larval, pupal/adult, and cumulative mortality were calculated.

Statistical analysis

One-way analysis of variance (Anova) was used for the multiple concentration tests and for percent mortality to determine significant treatment differences ($P<0.05$). Before analysis, the percent data were transformed (Arcsine $\sqrt{p}$) to normalize variance and to make them homogenous (Sokal and Rohlf, 1981). The repellency effect was analyzed by means of probit analysis (SPSS Version 10.0) yielding $ED_{50,}$ ED_{90} and 95% confidence intervals. The median complete protection time was used as standard repellency measures of the plant samples against *Anopheles stephensi*.

RESULTS

In the oviposition deterrence assay, gravid *A. stephensi* preferred to lay eggs in the distilled water control cups than in the cups treated with α-amyrin acetate of *C. roseus*

(Table 1). There was also a marked difference in the number of eggs laid. The 0.050 p.p.m. treated cups received a mean number of 29.17 eggs per cup while the control cups received a mean number of 92.89 eggs per cup. A paired *t*-test confirmed that water treated with α-amyrin acetate of *C. roseus* received significantly fewer eggs (P<0.05) than the distilled water control (Table 1). The OAI value of α-amyrin acetate of *C. roseus* at 0.050 p.p.m. was -0.52. The OAI values revealed that the α-amyrin acetate of *C. roseus* has a deterrent effect: it caused a remarkable negative response resulting in oviposition of very few eggs. In the multiple concentration tests, oviposition cups of control and of 0.007, 0.015, 0.015 and 0.050 p.p.m. α-amyrin acetate of *C. roseus* received a mean number of 92.89, 59.28, 41.17, 37.28 and 29.17eggs, respectively (Table 1). As the concentration of α-amyrin acetate of *C. roseus* increased from 0.007 to 0.050 p.p.m., the number of eggs per cup decreased. The OAI values for 0.007, 0.015, 0.025 and 0.050 p.p.m. treatment were -0.22, -0.38, -0.42 and-0.52 respectively: the OAI values were inversely proportional to the concentrations of α-amyrin acetate of *C. roseus*. The mortality rates of gravid and oviposited females, respectively, were 1.12 and 0.60 for control; 4.10 and 2.10 for 0.007 p.p.m.; 7.05 and 6.01 for 0.015 p.p.m.; 7.05 and 6.01 for 0.025 p.p.m.; and 7.20 and 9.02 for 0.050 p.p.m. α-amyrin acetate of *C. roseus* treatment. The mortality rates of gravid females increased from 1.12 (control) to 7.20 at 0.050 p.p.m., which implies the possible contact toxicity of the α-amyrin acetate of *C. roseus* received through sensitive parts of the test species that help them in oviposition. The repellency of α-amyrin acetate against female adults of *An.stephensi* were shown in figure 1. α-amyrin provided biting protection with ED_{50} and ED_{90} values of 0.6659 and 1.7720 µg/cm2 respectively. The investigations on repellency of median complete protection time revealed that α-amyrin acetate were found to exhibit excellent repellent activity against *An.stephensi.* The median complete protection time of α-amyrin acetate against *An.stephensi* was 3.5 h.

Table 1. Oviposition deterrent and gravid mortality effects of α-amyrin acetate from *Catharanthus roseus* L. against the malarial vector *Anopheles stephensi* Liston in the multiple concentration experiment.

Dose (ppm)	Mean no of eggs (±SE)	OAI	GF	OF
Control	92.89±3.0	NA	1.12*	0.62
0.007	59.28±4.8	-0.22	4.10	2.10
0.015	41.17±5.1	-0.38	7.05	6.01
0.025	37.28±5.0	-0.42	7.20	9.02
0.050	29.17±7.0	-0.52	0.41	9.05

*Not significant ($P \geq 0.05$). OAI- oviposition activity index; GF- gravid females; OF- oviposited females; NA- not calculated.

To study the ovicidal activity of the α-amyrin acetate, the hatching rate of eggs of the age group 0-6 h exposed for 18 h and 0-18 h eggs exposed for 6 h and 18 h in various concentrations of α-amyrin acetate was assessed every 24 h until pupation. The hatching rates of eggs exposed to various concentrations of α-amyrin acetate were significantly lower than those of control eggs (Table 2). At 24 h after transfer,

64.23% of the eggs laid in the control group had hatched, and by 48 and 72 h after transfer, 96.21% of the control group eggs had hatched. Treatment of eggs of 0-6 h was more effective in inducing higher rates of mortality than for eggs of 0-18 h exposed for 6 h and 18 h. The hatching rates for 0-6 h laid eggs (18 h exposure) at 24, 48 and 72 h, respectively, were 20.04, 33.37 and 64.08% for 0.007 p.p.m. α-amyrin acetate treatment; 19.74, 32.64 and 62.64% for 0.015 p.p.m.; 16.65, 27.75 and 53.28% for 0.025 p.p.m.; and 13.8, 22.87 and 43.92% for 0.050 p.p.m. The hatching rates for 0-18 h (6 h exposed) at 0.007 p.p.m. treatment was 22.27% at 24 h, increasing to 71.28% at 72 h. The same trends in time were shown for other concentrations of α-amyrin acetate: the hatching rates increased from 24 to 72 h from 21.82 to 69.84% at 0.015 p.p.m.; 20.09 to 64.08% at 0.025 p.p.m. and 19.21 to 61.2% at 0.050 p.p.m. The hatching rates of eggs of 0-18 h (18 h exposed) at 24, 48 and 72 h, respectively, were 21.90, 36.37 and 69.84% for 0.007 p.p.m. α-amyrin acetate treatment. For 0.025, 0.025 and 0.050 p.p.m. at 72 h, the hatching rates were the 69.12, 59.76 and 56.88%, respectively.

Table 2. Egg, larval and pupal/adult mortality of *Anopheles stephensi* Liston when eggs of either 0-6 or 0-18h were treated with α-amyrin acetate from *Catharanthus roseus* L. for 6h and 18h.

Concentration ppm	Eggs 0-6 h (Treated for 18h)				Eggs 0-18 h (Treated for 6h)				Eggs 0-18 h (Treated for 18h)			
	Egg mortality%	Larval mortality%	Pupal/adult mortality %	Cumulative mortality %	Egg mortality%	Larval mortality%	Pupal/adult mortality %	Cumulative mortality %	Egg mortality%	Larval mortality%	Pupal/adult mortality %	Cumulative mortality %
0.007	16.2±4.5^{d}	23.7±4.4^{d}	17.5±3.9^{d}	57.4±5.3^{d}	13.9±4.8^{d}	18.9±6.0^{d}	13.5±6.0^{d}	46.3±5.3^{d}	15.3±4.0^{d}	20.0±5.9^{d}	15.3±5.0^{d}	50.6±4.5^{d}
0.015	18.5±5.3^{c}	36.0±5.4^{cd}	21.3±4.5^{c}	75.8±5.2^{c}	14.5±5.2^{bc}	25.3±5.2^{c}	27.1±6.9^{bc}	66.9±6.0^{c}	17.5±5.1^{c}	27.5±5.0^{c}	25.7±6.7^{cd}	70.7±5.2^{c}
0.025	38.5±6.0^{b}	31.3±4.0^{ab}	18.4±5.0^{bc}	88.2±6.7^{b}	22.0±6.5^{b}	35.6±6.5^{b}	24.9±6.3^{b}	82.5±9.3^{bc}	32.5±6.2^{bc}	43.9±3.9^{b}	18.6±7.9^{b}	85.0±7.2^{bc}
0.050	41.7±7.6^{a}	52.5±2.5^{a}	4.5±6.0^{b}	98.7±7.8^{ab}	33.7±7.2^{a}	43.2±4.3^{ab}	10.2±4.2^{a}	87.1±4.8^{a}	37.5±6.9^{a}	45.3±6.9^{a}	10.2±6.9^{a}	93.0±5.6^{c}
Control	15.5±3.2^{f}	0.7±6.3^{f}	2.4±3.8^{f}	18.6±5.6^{f}	14.0±2.7^{f}	1.3±2.3^{f}	4.3±2.8^{f}	19.6±4.1^{f}	12.0±2.5^{f}	8.1±1.2^{f}	7.6±1.0^{f}	27.7±3.8^{f}
LC_{50} Values (F.L)	0.0565 (0.0432-0.0937)				0.7509 (0.0573-0.1268)				0.0654 (0.0507-0.1058)			
Chi Sq.	6.846				0.405				1.417			
Reg. Coff.	Y=18.1050X=-1.02365				Y=16.5300X=-1.2412				Y=16.4280X=-1.0756			

Means ± Standard error (SE) followed by the same letters (a-f) within columns indicate no significant differences.

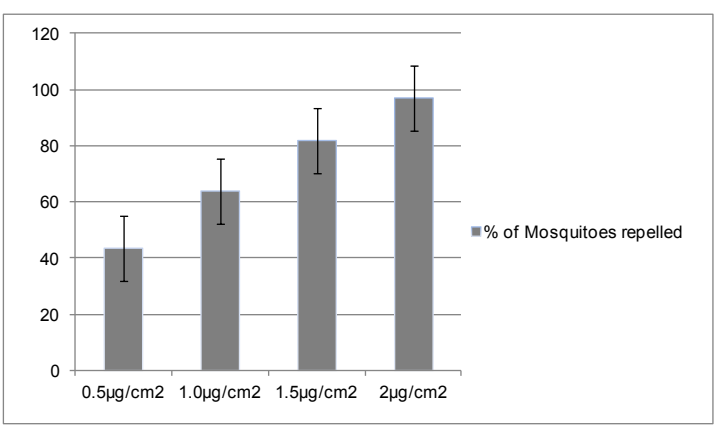

Fig. 1. Initial repellency of α-amyrin acetate against adult female of *Anopheles stephensi* Liston.

The survival rates of larvae were 61.82, 40.8, 27.2, 22.2, and 7.6% and of pupae/adults were 50.21, 23.3, 5.9, 3.8 and 3.1% for 0-6 h eggs exposed to 18 h control and 0.007, 0.015, 0.025 and 0.050 p.p.m. α-amyrin acetate, respectively. The highest cumulative mortality, 100%, was observed at 0.050 p.p.m. (Table 2). The survival rates of larvae from eggs that were exposed to α-amyrin acetate were significantly lower compared to the control group. The survival rates of larvae and pupae resulting from eggs collected at 0-18 h and exposed for 6 and 18 h were also studied: as the concentration of α-amyrin acetate increased, the survival rate decreased. The survival rates of pupae/adults were 62.55, 39.1, 17.9, 8.1 and 3.8% for 0-18 h eggs exposed for 6 h; and 62.01, 34.8, 16.0, 7.7 and 1.8% for 0-18 h eggs exposed for 18 h for control and 0.007, 0.015, 0.025 and 0.050 p.p.m α-amyrin acetate, respectively.

DISCUSSION

Changes in oviposition behavior, reduced egg hatchability, skin repellency, increased mortality of gravid and oviposited females, and decreased survival rates of individuals reduce the overall performance of the malarial vector *A. stephensi* Liston. The data from the present study support this hypothesis, and we conclude that α-amyrin acetate of *C. roseus* possesses various activities such as oviposition deterrent, ovicidal, gravid mortality and deleterious delayed mortality effects against the malarial vector *A. stephensi* Liston.

Oviposition deterrent effect

Our results show that the mean number eggs in control cups was greater than for each concentration of α-amyrin acetate. The OAI values also indicated that the gravid and oviposited females were repelled by α-amyrin acetate and the reduced oviposition was due to the greater mortality of adults, caused by α-amyrin acetate, before they oviposited. The total number of eggs laid by 50 gravid females in treatment cups was relatively lower than in the control cups. We noted that more adults of both gravid and oviposited females died on the surface of the α-amyrin acetate than in distilled water. The females that alighted on the treated water surfaces were unable to take off, and they eventually died. A few that managed to crawl or fly away died later. This confirmed that the α-amyrin acetate had contact toxicity for gravid and oviposited females. The present study results are favorably supported by the oviposition deterrent activity of acetone extract of *Solanum trilobatum* (Rajkumar *et al.,* 2005), ethyl acetate fraction of *Samadera indica* (Muthukrishnan *et al.,* 2001), ethyl acetate fraction of *Solanum suratense* (Muthukrishnan *et al.,* 2001), and alkaloids of *Annona squamosa* (Saxena *et al.,* 1993), which elicited 99, 45, 55 and 32% reduced egg laying, respectively, against *A. stephensi* Liston. The usefulness of α-amyrin acetate as a larvicide and insect growth regulator (Kuppusamy *et al.,* 2009) has been shown in mosquito control programs. Its role in reducing oviposition and causing adult mortality are additional advantages of α-amyrin acetate. The lower number of eggs deposited by gravid females in water treated with α-amyrin acetate produce deleterious delayed effects on growth and development of larvae are the additional advantages of using this plant derived compound in mosquito control programs.

Skin repellency effect

Personal protection measures, including repellents, are widely used to prevent the transmission of arthropod borne diseases by minimizing the contact between humans and vectors. In contrast to vaccines and chemoprophylaxis as means of personal protection, repellents are convenient, inexpensive and afford advantages in protection against a wide range of vectors (WHO, 1996). They are also the primary means of mosquito-borne diseases prevention available in areas where vector control is not practical (Gupta and Rutledge, 1994; Copeland *et al*. 1995; Govindarajan *et al.*, 2011). The repellent potential of plants to mosquitoes and other pest insects has been well known both prior to (Granett, 1940) and after (Thoresell *et al.*, 1998) the advent of synthetic chemicals. Various botanical substances *Cymbopogan spp*. (Rutledge *et al*., 1983; Ansari and Razdan, 1995). *Eucalyptus maculats citriodon* (Collins and Brady, 1993), *Azadirachta indica* (Sharma *et al*., 1993), *Pelargonium citrosum* (Matsudha *et al*., 1996), *Lantana camera* (Dua *et al*., 1996), and *Mentha piperita* (Ansari *et al*., 2000) have been reported as being repellent against adult mosquitoes.

Our results suggested that α-amyrin acetate possessed significant repellent activity against *An. stephensi* and exerted an effective biting protection time from 2.0 to 3.5h. The result is favorably supported by Bejawan pitasawat *et al*., (2003) demonstrated *Curcuma aromatica* extract exerted an effective biting protection time of 3.5h which is low compared to that reported for currently used synthetic compounds such as DEET, A13-37220, A13-35765 and CIC-4 (Schreck and McGovern, 1985; Coleman *et al*.,1993 and 1994). However, α-amyrin acetate exerted an effective repellent protection time and is considered satisfactory.

To our best knowledge, this is the first report of the potential of α-amyrin acetate as a repellent against *An. stephensi*. The results indicated that α-amyrin acetate had the most effective repellent activity (3.0 to 3.5h). Repellent properties of several essential oils appear to be associated with the presence of terpenoids, sesquiterpenes and lactones (Jaenson *et al*., 2006; Sukumar *et al*., 1991). The results obtained here in were similar to those reported by Twatsin *et al*., 2001, demonstrated under laboratory conditions that volatile oils derived from turmeric (*Curcuma longa*), Citronella grass (*Cymbopogon winterianus*), and hairy basil (*Ocimum americanum*), with the addition of 5' vanillin, were effective in repelling both diurnal and nocturnal mosquitoes for up to eight hours.

Repellent protection time in laboratory bioassays, however, can change depending on the biological characteristics of the mosquito test population. Differences in species and body size, sugar water availability, adult density in test cages, and mosquito age can affect test results (Gouck and Smith, 1962; Khan *et al*., 1975; Rutledge *et al*., 1983; Xue *et al*., 1995).No adverse effects on the skin or other parts of the body of the human volunteers were observed during the study period through the 2 mo after the application. No volunteers complained of the odor, stickiness or uncomfortable feeling of this extract.

Ovicidal effect

The ovicidal activity of the α-amyrin acetate, the hatching rate of eggs of the age group 0-6 h exposed for 18 h and 0-18 h eggs exposed for 6 h and 18 h in various

concentrations of α-amyrin acetate was assessed every 24 h until pupation. The treatment of eggs with various concentrations of α-amyrin acetate caused embryonic death, resulting in failure to hatch the eggs. Egg mortality in any of the treatments did not go beyond 50%, even with the highest concentration of 0.050 p.p.m. The treatment of eggs of 0-6 h was more effective in inducing higher rates of mortality than for eggs of 0-18 h treated for 6 h and 18 h. A shorter duration of treatment was decisively inferior to a longer exposure to the α-amyrin acetate at the egg stage. Exposure of freshly laid eggs was more effective than of older eggs. The age of the embryos and the exposure time played a crucial role (Miura *et al.,* 1976). The efficiency of the ovicide to act on the embryo inside the egg shell depends on its efficient penetration, which in turn is influenced by the exposure period (Grosscurt *et al.,* 1977). The total inhibition of egg eclosion when eggs were directly exposed to a high concentration of compound indicated more penetration of the chemical inside the egg shell, which affected embryogenesis (Broadbent *et al.,* 1984). Longer exposure periods also facilitated increased penetration of the compounds into the cells, thus increasing their effectiveness (Smith *et al.,* 1966). As the concentration of α-amyrin acetate increased, the hatching rates decreased: from 62% (control) to 13.8% at 24 h, 73 to 22.87% at 48 h, and 86 to 43.92% at 72 h for 0-6 h eggs exposed for 18 h; from 63.2% (control) to 19.21% at 24 h, 78 to 31.7% at 48 h, and 89 to 61.21% for 72 h for 0-18 h eggs exposed for 6 h; and from 64% (control) to 17.9% at 24 h, 79.6.1 to 29.62% at 48 h, and 90 to 56.88% at 72 h for 0-18 h eggs exposed for 18 h. There was an inverse relationship between concentration and the magnitude of the hatching rate. In general, the hatching rates increased with time from 24 to 72 h, but the hatching rate decreased with an increase in concentration. This ovicidal effect is comparable to alkaloids of *A. squamosa* (Saxena *et al*., 1993),ethyl acetate fractions (seeds) of *Calophyllum inophyllum* (Pushpalatha and Muthukrishnan 1999), petroleum ether fractions of *Rhinacanthus nasutus* (Muthukrishnan *et al.,* 2001) and ethyl acetate fractions of *S. suratense* (Muthukrishnan *et al.,* 2001), which caused 32, 54, 40 and 55% ovicidal activity against *A. stephensi* Liston.

Delayed mortality effect

In the delayed mortality assay, the results suggest that the α-amyrin acetate has deleterious delayed effects in causing high larval mortality and moderate pupal and adult mortality in the larvae hatched from the treated eggs. The larvae that hatched from the treated eggs showed much higher levels of mortality in all of the treatments. The treatments produced low to moderate levels of pupal mortality and adult mortality at the time of adult emergence. The exposure time and age of the eggs played a significant role in survival rates. Although ovicidal activity was only moderate, an important finding is that the larvae that hatched from the treated eggs immediately died. The pupal and adult mortality was significantly less than the larval and egg mortality. The hatching rates and survival rates of larvae had an inverse relationship with the concentration of α-amyrin acetate of *C. roseus*. Although the concentrations used in the present study were not higher than the normal application concentrations used in mosquito control programs, we still noted reductions in oviposition, number

of eggs, hatching rates and survivorship. This mechanism of action and observation is similar to the deleterious delayed effects of neem limonoids (Senthilnathan and Murugan, 2005) and acetone extracts of *Ipomoea carnea fistula* (Saxena *et al.,* 1985), which caused larval mortality, pupal mortality, pupal/adult intermediates and reduced emergence against *A. stephensi* Liston. The usefulness of phytochemicals as a larvicide in mosquito control programs has been known for a long time, however, reduction of egg number and reduced hatching and survival rates suggest additional advantages of phytochemicals such as α-amyrin acetate of *C. roseus* when used in mosquito control programs. These findings could open new areas of research into different mosquito species, other phytochemical extracts/compounds, and the mechanism of adult mortality and ovicidal activity, to elucidate the potential extra larvicidal effects of α-amyrin acetate of *C. roseus* in mosquito control programs. The present study revealed that α-amyrin acetate of *C. roseus* can be further developed as a potential eco-friendly phytopesticide and can be beneficial for vector control programs. Future work in this direction is necessary, because biopesticides from plant origin can be effective vector control tools. These new agents can preferentially be applied in integrated vector control strategies.

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