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Effect of Annona formulations on non-target invertebrates and on physicochemical water parameters at semi-field condition

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ABSTRACT
Plant extracts from the genus *Annona* exhibit insecticidal properties and thus, may offer an alternative to synthetic insecticides. In the present study larvicidal efficacy of formulations of *Annona squamosa* and *A. montana* were evaluated against Anopheline and Culicine larvae in the laboratory and semi-field conditions. In the laboratory there was no significant difference ($t=0.5313$, $p>0.05$) in the effectiveness of the *A. squamosa* formulation at 50 µg/ml and above between *An. gambiae* and *Culex quinquefasciatus* by day 4. Significant difference ($t= 2.649$, $p<0.05$) was observed for *A. montana* formulation at higher concentration than 50 µg/ml since *An. gambiae* was more susceptible than *Cx. quinquefasciatus.* In semi-field condition the efficacy of the two formulations against Anopheline was not quite significant ($t= 2.504$, $p>0.05$). The activity of *A. montana* leaf powder against Culicines was significantly lower compared to *A. squamosa* ($t=3.827$, $P<0.05$). The variation in water parameters had no significant effect on the efficacy of the formulations. The formulations did not affect the survival of the 9 invertebrate groups tested during the period of study except for the Order Ephemeroptera (Mayflies) whose mortalities was more than 85%. The formulations have proven to offer an alternative to synthetic insecticides.

Keywords: *Annona squamosa, Annona montana*, larvicidal formulations, environmental parameters, non-target invertebrates.

1. Introduction
Diseases spread by mosquito are the main health problems around the world; malaria being the most killer disease [1-2]. Others include filariasis, dengue, yellow fever and encephalitis. Vector control is one of the methods employed in the control of the diseases. However the synthetic chemicals used are toxic to non-target fauna and also harmful to human health [3-5]. Increase of vector resistance pose more challenge to conventional chemical insecticides [6-7]. Chemicals derived from plant are eco-friendly, biodegradable and some can offer third generation insecticides that are more specific to vectors and therefore could be integrated with other vector control initiatives for effective management protocols causing less harm to the environment and non- target species [8]. Worldwide there are over two thousand species of plants known to have insecticidal activity [9].
In Tanzania, a number of plant species have demonstrated major activity to insect vectors in the laboratory and in full field evaluations [10-12]. Recent investigations of leaf extracts and some formulations from family Annonaceae particularly *Annona squamosa* L., reveal that they perform excellently in laboratory setting against mosquito vectors [13-14]. Given the fact that most people in malaria endemic areas have poor socio-economic status which denied them access to protective tools against mosquito vectors such as use of insecticide treated bed nets (ITNs), Insecticide Residual Sprays (IRS) and application of topical repellents [15-16]. Simple and cheaply available tools have to be designed so as to save lives of people in this group. Therefore it was the aim of the present study to evaluate formulations of *Annona* species in the semi-field environment, to determine the effect of variation of water parameters to the formulation and to assess the impact of the formulations on selected non-target invertebrates.

2. Material & Methods
2.1 Plant materials
The leaves of *Annona montana* (voucher specimen number ASM-T-VI) were collected from mpunguso Village in Tukuyu District while those of *A. squamosa* (ASS-T-II) were collected from

Yombo village in Bagamoyo District in January, 2012. The plant species were identified by Mr. Frank Mbago, a Botanist from the Department of Botany, University of Dar es Salaam. The voucher specimens are kept in the Herbarium of Institute of Traditional Medicine (ITM), Muhimbili University of Health and Allied Sciences (MUHAS), Tanzania.

2.2 Preparation of formulation of leaf powders

Fine clay soil, starch and rice husks were used in preparation of formulations at 10, 30 and 70 percent. The shade dried plant materials and the rice husks were ground by motor and pestle into fine particles and then sieved by laboratory test sieve with mesh size of 2.00 mm. Ethanol and DMSO were used as moulding solvents for both formulations. In the present communication, we describe the efficacy of 70% Annona leaf powders in clay soil being moulded using ethanol as the best combination that was lighter than the rest as it could float over longer time but also spread homogeneously to cover more surface area of water bodies.

2.3 Mosquitoes

Eggs of *An. gambiae s.s.* (Kisumu strain) were obtained from NIMR-Ubwari field station in Muheza, Tanga, then reared in the insectary at MUHAS until the appropriate larval instar stage was reached to conduct the experiments. Larvae of *C. quinquefasciatus* was obtained from the MUHAS insectary which originated from wild in Dar es Salaam but having been reared since 2007. Anopheline and culicine larvae for experiments in semi-field environment were collected from the wild habitats from four villages of Magila, Madaba, Jembe and Genge in Muheza disrict-Tanga region, Tanzania (Table 1).

Table 1: The larval sources and characteristics of their habitats

Larval source	Habitat type	Characteristics of the habitat
Magila road (**W1**) S 0510034 E 03846571	Burrow pit	Grass at edges, water clear, all stages of culicine and anopheline present, non-target invertebrates present
Madaba (**W2**) S 0510113 E038482	Neglected well	Water clear, all stages of culicine and anopheline present, non-target invertebrates present
Jembe (**W3**) S 051016.3 E0384701.6	Irrigation well	Water clear, Anopheline dominant, non-target invertebrates present
Genge (**W4**) S0510318 E03847241	Burrow pit	Covered by floating water plants, all stages of Culicine and Anopheline, non-target invertebrates present

2.4 Larvicidal evaluation of formulations in the laboratory

Formulations of *A. squamosa* and *A. montana* were evaluated using laboratory reared larvae of *An. gambiae s.s.* and *C. quinquefasciatus* at concentrations from 25, 50 and 100 µg/ml. The amount of each formulation to be added in water was estimated based on the yield of crude extract of the leaves from each plant species. For each species the amount of formulation to be added in 1000 ml of water to yield approximately required concentrations were worked out. Aluminum containers containing 1000 ml of distilled water and 50 third instar larvae ware used. Each test concentration was replicated and a control experiment (blank) was provided for each set of experiment. All experiments were carried out under controlled temperature (27±2 ^{0}C) and relative humidity of 75-85%. The mortality was determined after every 24 hours.

2.5 Sampling of Larvae and non-Target invertebrates

A survey to identify habitats for both species of mosquito was conducted in Muheza-Tanga. Among the habitats identified included abandoned burrow pits, slow moving streams, irrigation wells, abandoned fish ponds and drains. Several mosquito habitats were identified and chosen for collection of mosquito larvae depending on larvae density and dominant stage of desired Culicine, Anopheline and non-target invertebrates. Habitats where larvae can be seen without dipping or when every dip contained more than five mosquito larvae, were selected for sampling because these sites were defined as having a high mosquito density. The locations and characteristics of habitats from which larvae was collected are summarised in (Table 1). Larvae, water and small amount of soil were collected in buckets from the identified habitats and transferred to screen house. Also non-target invertebrates that co-habited with larvae from same source were collected. On collection of larvae and non-target invertebrates, temperature, pH, Total dissolved solute (TDS), and electrolytic conductivity (EC) were measured on-site in each habitat from which larvae were collected and in the artificial ponds in the screen house on the third day post treatment. GPS readings for each collection location were also recorded (Table 1).

2.6 Larvicidal evaluation of formulations in semi-field trials

In the screen house the samples were poured in plastic containers which were half buried. Each container contained 5000 ml of water from mosquito source habitat, 30 larvae and some representative invertebrates depending on the abundance of collected species. The containers were treated with the formulated botanical larvicides at concentration 100 µg/ml and for each formulation four replicates and controls (blank) were provided. The mortality of larvae and non-target invertebrates were scored after every 24 h post treatment until all larvae were dead or when no significant mortality was recorded. Temperature, pH, Total dissolved solutes (TDS) and electrolytic conductivity (EC) were recorded in each habitat from which larvae were collected. Mosquitoes and non-target invertebrates were re-collected from same source and re-introduced to the containers to determine residual activity of the larvicides

2.7 Data analysis

Percentage mortality was calculated using the Mulla's formula [17], which takes into account the natural factors of population change in an ecosystem. % Mortality = 100 – (C1/T1 xT2/C2) x100, where C1 and C2 represent average numbers of larvae in the control containers before and after treatment, respectively, while T1 and T2 represent larvae in treated containers before and after treatment, respectively. Percentage mortalities were statistically analyzed by one-way analysis of variance (ANOVA). Student t-tests with p<0.05 was considered statistically significant using Graph pad Instant™ software 1993. The cumulative percentage reduction of the abundance of non-target invertebrates was calculated using the same formulation. The bars in all the graphs are the standard errors obtained from four replicates.

3. Results

The percentage yield of crude extracts upon extraction of leaves of powders from *A. montana* was 16.1 g while that of *A. squamosa* was 21.5 g. The effective concentration was estimated based on extractable amount of crude extract from known amount of plant powders. Thus, exposure of known amount of 70% leaf powder formulations at 25, 50 and 100 µ/ml indicate increased larvae reduction with increase in number of days post treatment for both species of mosquito. However none of the formulations could attain 100% larvae mortality in 5 days period of exposure as beyond this period larvae started to pupate. (Figure 1 and Figure 2)

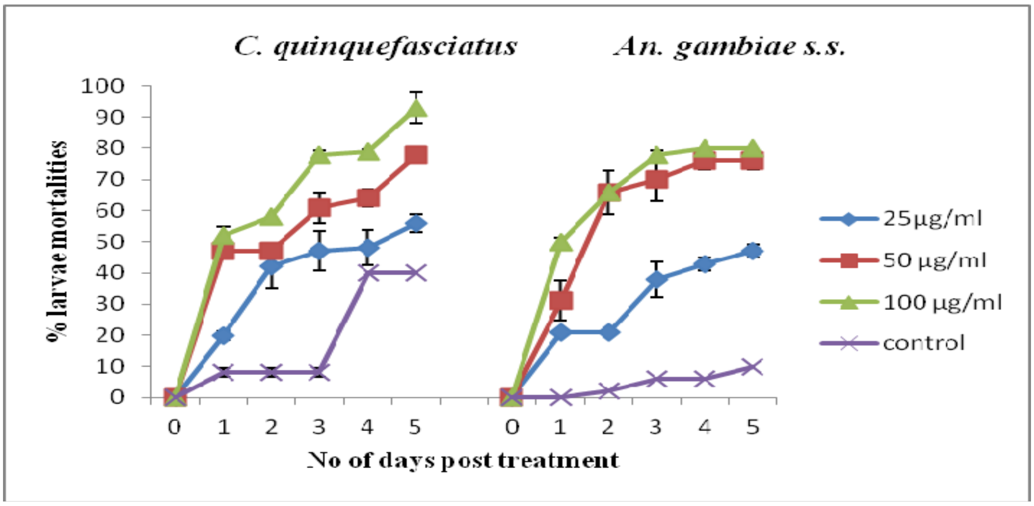

Fig 1: Efficacy of the formulations containing 70% *A. squamosa* leaf powder in clay against *C. quinquefasciatus* and *An. gambiae s. s.* larvae

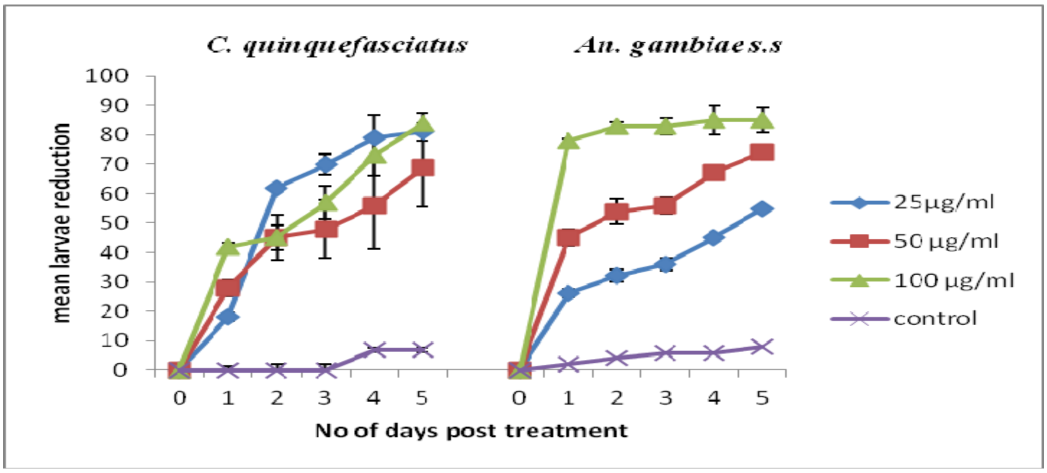

Fig 2: Efficacy of the formulations containing 70% *A. montana* leaf powder in clay against *C. quinquefasciatus* and *An. gambiae* s.s. larvae

An. gambiae s.s. were more susceptible to the two formulations of *A. squamosa* and *A. montana* than *Culex quinquefasciatus* (Figure 1 and figure 2). However, there was no significant difference (t=0.5313, p>0.05) in the effectiveness of the *A. squamosa* formulation at 50 mg/ml and above between *An. gambiae* and *Culex quinquefasciatus* by day 4 since both attained more than 70% reduction. On the other hand, significant difference (t=2.649, p<0.05) was observed for *A. montana* formulation at higher concentration than 50 µg/ml since *An. gambiae* seemed more susceptible than *Culex quinquefasciatus* (Figure 2).

These results gave an indication of effective dose to be used at semi-field studies. Evaluation of the *Annona* formulations at 100 µg/ml in the semifield conditions showed varied results against the two groups of mosquito larvae (Figure 3). Formulations containing 70% *Annona montana* leaf powder in clay soil showed high efficacy against Anopheline larvae reaching 100% larvae reduction after 3 days post treatment while 70% *Annona squamosa* in clay soil attained 89% reduction within same time of exposure. The difference in the efficacy of the two formulations against Anopheline was not quite significant (t= 2.504, P>0.05). However the activity of *A. montana* leaf powder against Culicine larvae was significantly lower compared to *A. squamosa* (t=3.827, P<0.05). The two formulations did not cause significant larvae reduction after re-introduction of the new batch of larvae.

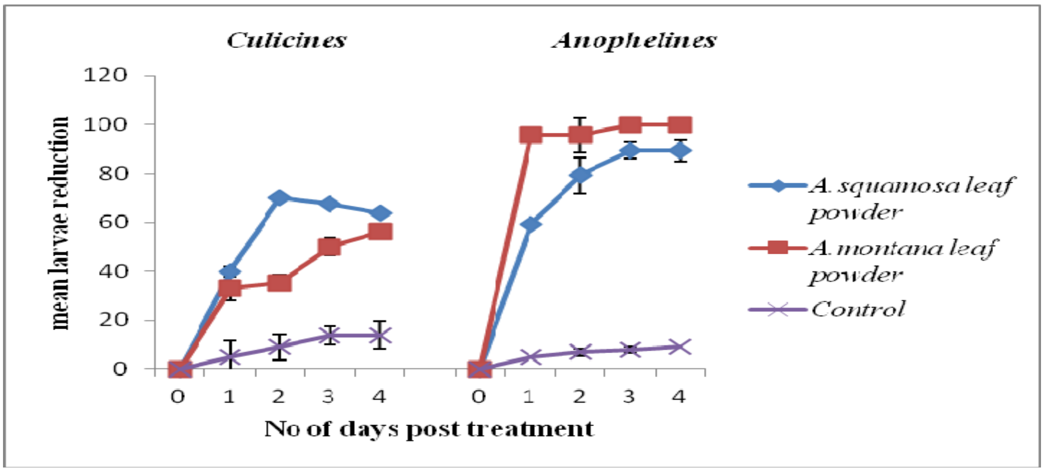

Fig 3: Efficacy of the formulations containing 70% *A. montana* leaf powder and 70% *A. squamosa* leaf powder in clay against Anopheline and Culicine at 100 µg/ml

Measurements of physicochemical parameters at water sources W_1-W_4 and in the screen house showed no much variations in temperature, total dissolved solutes (TDS) and Electrolytic conductivity (EC) during the whole period of study (Table 2). There was a slight increase in pH after treatment in anopheline habitats at semi-field condition (t=3.469, p<0.05). However, these parameters had low impact on the efficacy of the formulations since the laboratory and field results were comparable (t=0.5756, p>0.05) except for *A. montana* leaf powder against Culicine larvae, which was slightly lower at semifield as compared to laboratory results (t=3.697, p<0.05) (Table 2). All the formulations studied had no significant impact on most invertebrate groups studied except for the Order Ephemeroptera where mortalities were more than 85% (Table 3).

Table 2: Physicochemical parameters recorded at water sources and artificial ponds in the screen house 72 hrs of post exposure

	Mean value of physicochemical parameter in ponds treated with 70% *A. Montana* Leaf Powder						Mean value of physicochemical parameter in ponds treated with 70% *A. Squamosa* Leaf Powder				
	W1			W2			W3			W4	
	0 hrs	72 hrs		0 hrs	72 hrs		0 hrs	72 hrs		0 hrs	72 hrs
T ^{0}C	25.9	26±0.2		25.6	25.7±0		27.1	26.1±0		26.6	26.9±0.1
pH	7.37	7.9±0.1		7.79	8.1±0		8.02	8.3±0.1		8.01	8.25±0
TDS	362	386.5±2.1		703	728±19		531	557.5±2.1		434	478.7±4.5
EC	726	775±1.4		1416	1459.5±36.5		1074	1119.5±3.5		869	955.5±7.5

Values are mean ± SE from four ponds in the screen house; T =Temperature; TDS= Total Dissolved Solutes; EC=Electrolytic Conductivity; **W1-W4**=Water from source 1-4 as summarized in **Table 1.**

Table 3: Effect of *Annona* formulations on selected non-target invertebrates in 4 days of exposure at a concentration 100 µg/ml

	Water ponds treated with 70% An. montana formulation at 100 µg/ml		**Control**		**Water ponds treated with 70% An. squamosa formulation at 100 µg/m**		**Control**	
	Number exposed	**% mortality**	**Number exposed**	**% mortality**	**Number exposed**	**% mortality**	**Number exposed**	**% mortality**
1. Order Odonata Family: Zygoptera (Damsel flies)$^{\alpha}$	n=26	12.5	n=27	0	n=26	0	n=16	20
2. Order Ephemeroptera (May flies)$^{\alpha}$	n=45	88.5	n=49	20.40	n=32	100	n=21	36.36
3. Order Hemiptera- Family Corixidae (Water Boatmen)	n=30	40	n=29	6.89	n=18	0	n=15	33.3
4. Order Trichoptera (Caddis flies)$^{\alpha}$	n=17	0	n=12	0	n=4	0	n=5	0
5. Order Odonata Family:	n=20	10	n=28	0	n=7	0	n=5	0
6. Anisoptera (Dragon flies)$^{\alpha}$								
7. Order Coleoptera (Beetles)	n=16	33.3	n=16	0	n=3	0	n=3	0
8. Order Hemiptera- Family Notonectidae Back swimmers)	n=5	0	n=6	0		-	-	
9. Fresh-Water snails	--		-		n=3	0	n=4	0
10. Fresh-water crabs	--		-		n=4	0	n=3	0

n= number of individuals species exposed for testing; α= nymph

4. Discussion

In the present study, the formulations of clay soil with 70% leaf powders were recommended for further study due to their good performance in the preliminary laboratory evaluations which suggested an increased performance over extended observation period after application. Extended observations for these formulations was important in order to guide for a basis for residual activity studies and application intervals of the larvicide in the field setting. In addition, this gives a clue on the optimum dosage that will have maximum lethal impact on target species and minimum impact on non-target fauna at a given time of exposure.

The variations in the efficacies of the formulations exposed to wild Culicine mosquitoes could be attributed to the fact that larvae were collected from different sources; some were collected from very polluted habitats where larvae could have attained a certain level of resistance probably due to routine exposure to diverse toxic substances, and hence have a higher general tolerance. Another possibility is that the field strains mosquitoes were genetically more heterogeneous and so resistant to external selection pressures compared to laboratory reared mosquitoes. Previous studies reported resistance to insecticides in *Cx. quinquefasciatus* from Muheza District Tanzania [18-19], the same area where wild mosquitoes were collected for semi-field studies.

Apart from tolerable natural mortalities (Table 3), the survival of the 9 non-target invertebrate groups exposed in the treatments was not affected by the formulations during the period of study except for the Ephemeroptera (Mayflies) whose mortality was more than 85% (Table 3). Similar observations were reported by Kreutzweiser and co-workers where neem formulations caused significant mortality only on mayflies but not on other invertebrates tested [20-21]. Another similar study, reported higher susceptibility of mayfly nymphs to fenvalerate and permethrin than stoneflies, caddisflies, dipterans and snails [22]. This might be due to uniqueness of this group of insects as they are normally tiny and short-lived, therefore more susceptible to hazard environment [23].

Measurement of water parameters revealed that there was slight increase in pH of water in ponds in the screen house 72 h post treatment. However, all the measured parameters had no noticeable effect on the effectiveness of botanical formulations under study. The measurements of total dissolved solutes (TDS) and Electrolytic conductivity was high in ponds W2 and W3 since these water sources were natural wells, hence had direct contact with dissolved water soluble metals in the rocks compared to W1 and W4. Despite of differences in TDS and EC, no noticeable mortality effects were observed. In the literature there was no study of similar water parameters, and therefore conclusions is based on the present study.

5. Conclusion

The results from the present study indicates that formulations from *Annona* species have considerable efficacy and minimal effect on other non-target fauna. Therefore, they could be used as a more environmentally friendly alternative to synthetic larvicides. On the other hand the clay soil dust proved to be a good carrier of the larvicides due to the fact that, they were light and could float on the water surface. The incorporation of small of amount of clay soil dust in the leaf powder will render prolongation of the floating time as well as the release of the active compounds at the water surface where larvae of mosquitoes are usually found.

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