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Knockdown effect of crude ethanol extracts of *Phytolacca dodecandra* on *Anopheles gambiae* adults

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Abstract

Introduction: This study reports on knockdown effect of ethanol extracts of leaf and mature green fruits of Endod (*Phytolacca dodecandra*) against laboratory and wild laboratory reared *Anopheles gambiae* mosquitoes.

Materials and Methods: Three modeled surfaces of the size 26 x 26 cm^2 cut from plywood were used. The first was smeared with a mixture of mud and cow dung, the second was smeared with cement and the third left as it was (plain or unsmeared). The surfaces were sprayed with an approximate of 33 mls of different concentrations (80, 40, 20, 10, 5, 2.5mls) of ethanol extracts of mature green fruits and leaves of Endod, leaves of Neem and deltamethrin. Rain water alone was used as control. Three to five day old unfed female *An. gambiae* were exposed for five minutes to the treated surfaces, withdrawn, placed in paper cups and observed for knockdown and recovery.

Results: The highest number of knockdown for the test extracts was observed for Endod leaf extracts on cement and plain modeled surfaces (100%) against wild lab reared mosquitoes and mature green fruits on modeled plain surfaces (71%) for against lab reared mosquitoes. Extracts of Neem knocked down the highest number of wild lab reared mosquitoes (90%) on mud smeared surfaces. Deltamethrin knocked down all while rain water knocked down none of exposed mosquitoes.

Conclusion: Ethanol extracts of Endod knocked down *An. gambiae* mosquitoes rapid enough showing great potential as a future malaria vector control tool. Further purification and tests are however recommended.

Keywords *Anopheles gambiae*; *Phytolacca dodecandra*; Neem; Ethanol

Background

Malaria is a number one killer disease and available synthetic products that are the current tool of choice against the main vector *Anopheles gambiae* show reduced sensitivity (Enayati and Hemingway, 2010; Tiwari et al., 2010). This has made it necessary to seek alternate insect pest control tools such as botanicals (Montasser et al., 2011).

Botanicals are believed to be target specific, eco-friendly, biodegradability, cost-effectiveness and their complex chemistries limit development of pest resistance (Miresmailli et al., 2006). Botanicals of pyrethrum, rotenone, neem, and essential oils were the first against insect pests (Isman, 2006). Since then several other extracts from various botanical sources have demonstrated effectiveness against mosquitoes (Govindarajan, 2010; Govindarajan and Karuppannan, 2011). Examples of such botanicals have included ethanol extracts of citrus oil against *Aedes albopictus* (Faisal et al., 2010), crude hexane, ethyl acetate, benzene, chloroform, and methanol extracts of leaf of *Eclipta alba* and *An. paniculata* against *Cx. Quinquefasciatus* and *Ae. aegypti* (Govindarajan and Sivakumar, 2012) and *Delonix elata* against *Aedes aegypti* (Mohan and Marimuthu, 2014).

Phytolacca dodecandra which is from the family Pytolaccaceae (Walter, 1909) is native to sub-Saharan Africa and Madagascar (Schemelzer and Gurib-Fakim, 2008). It is useful in the treatment of various human as well as animal ailments (Nalule et al., 2011) and as soap, shampoo and poison to stun fish (Esser et al., 2003). It is commonly known as Endod, Gopo Berry,

or African soapberry in Ethiopia (Esser et al., 2003). Extracts of Endod have also been used against *Bulinus abyssinicus* snails (Erko et al., 2002; Abebe et al., 2005) and *An. gambiae* larvae (Were, 2008). However, the extracts have not been tested against *An. gambiae* adults. This study reports for the first time knockdown effects of crude ethanol extracts of Endod on laboratory reared and wild laboratory reared female *An. gambiae* adults on modeled wall surfaces in the laboratory.

1 Result

This experiment was done for a period of twelve days and a total of 22,400 laboratory and 2,520 wild laboratory reared *An. gambiae* mosquitoes used. Only 2-5 day old female unfed mosquitoes were exposed. For the lab reared mosquitoes, the following knockdown percentage was observed within the first ten minutes. Ethanol extracts of mature green fruits of Endod knocked down 71% on modeled plain walls and 63% on modeled cement and mud smeared walls respectively (Table 1). Endod leaf extracts knocked down 66% on modeled cement walls, 56% on modeled mud smeared walls and 57% on modeled plain surfaces (Table 2). Neem leaf extracts knocked down 85% on modeled cemented walls, 83% on modeled mud smeared walls and 79% on modeled plain walls (Table 3).

Table 1 Mean KDT on laboratory cultured *An. gambiae* mosquitoes from exposure to ethanol extracts of mature green fruits of Endod

KDT	N	Treatments applied on the modeled wall surface				
		Cement	Mud smeared	Plain	Deltamethrin	Control
10	40	67.29±5.49^{c}	62.71±5.03^{c}	71.15±4.61^{b}	100.00±0.00^{a}	0.00±0.00^{d}
20	40	9.69±2.05^{a}	13.65±2.45^{ab}	11.15±2.06^{a}	0.00±0.00^{b}	0.00±0.00^{b}
30	40	4.79±1.08^{a}	9.17±1.91^{a}	7.19±1.41^{a}	0.00±0.00^{b}	0.00±0.00^{b}
40	40	3.23±1.15^{a}	2.08±0.58^{a}	1.25±0.43^{b}	0.00±0.00^{c}	0.00±0.00^{c}
50	40	0.31±0.3^{ab}	0.10±0.10^{b}	0.21±0.14^{b}	0.00±0.00^{b}	0.00±0.00^{b}
60	40	14.69±3.22^{b}	12.29±3.61^{b}	9.06±3.25^{c}	0.00±0.00^{d}	0.00±0.00^{a}

Note: 1. KDT means knockdown time in minutes; 2. Mean percent knockdown time with standard error of mean followed by superscripts of different letters differ significantly at $p < 0.05$

Table 2 Mean KDT on laboratory cultured *An. gambiae* mosquitoes from exposure to ethanol extracts of leaves of Endod

KDT	N	Treatments applied on the modeled wall surface				
		Cement	Mud smeared	Plain	Deltamethrin	Control
10	40	66.46±4.68^{c}	56.25±4.49^{c}	56.88±5.00^{c}	100.00±0.00^{a}	0.00±0.00^{d}
20	40	15.00±3.06^{a}	17.40±2.78^{a}	13.13±2.37^{a}	0.00±0.00^{b}	0.00±0.00^{b}
30	40	5.10±1.32^{a}	4.90±1.09^{b}	7.40±1.71^{a}	0.00±0.00^{b}	0.00±0.00^{b}
40	40	0.94±0.47^{b}	3.02±0.60^{a}	2.29±0.67^{a}	0.00±0.00^{c}	0.00±0.00^{c}
50	40	0.83±0.47^{a}	1.46±0.45^{a}	1.46±0.56^{a}	0.00±0.00^{b}	0.00±0.00^{b}
60	40	11.67±3.60^{b}	16.98±3.88^{b}	18.85±4.35^{b}	0.00±0.00^{d}	0.00±0.00^{a}

Note: 1. KDT means knockdown time in minutes; 2. Mean percent knockdown time with standard error of mean followed by superscripts of different letters differ significantly at $p < 0.05$

Table 3 Mean KDT on laboratory cultured *An. gambiae* mosquitoes from exposure to ethanol extracts of Neem leaves

KDT	N	Treatments applied on the modeled wall surface				
		Cement	Mud smeared	Plain	Deltamethrin	Control
10	40	84.90±2.25^{b}	82.50±2.91^{b}	78.85±3.13^{b}	100.00±0.00^{a}	0.00±0.00^{d}
20	40	10.42±1.67^{a}	10.21±1.61^{b}	13.44±1.79^{a}	0.00±0.00^{b}	0.00±0.00^{b}
30	40	3.96±1.25^{a}	6.04±1.80^{ab}	6.67±1.86^{a}	0.00±0.00^{b}	0.00±0.00^{b}
40	40	0.42±0.33^{b}	0.52±0.37^{b}	0.10±0.10^{c}	0.00±0.00^{c}	0.00±0.00^{c}
50	40	0.00±0.00^{b}	0.00±0.00^{b}	0.21±0.21^{b}	0.00±0.00^{b}	0.00±0.00^{b}
60	40	0.31±0.31^{c}	0.73±0.73^{c}	0.73±0.44^{d}	0.00±0.00^{d}	0.00±0.00^{a}

Note: 1. KDT means knockdown time in minutes; 2. Mean percent knockdown time with standard error of mean followed by superscripts of different letters differ significantly at $p < 0.05$

For the wild laboratory cultured mosquitoes, ethanol extracts of mature green fruits of Endod knocked down 41% on modeled cement and plain walls and 38% on modeled mud smeared walls (Table 4). Endod leaf extracts knocked down all exposed mosquitoes on modeled cement and plain wall surfaces and 94% on modeled mud smeared walls (Table 5). Neem leaf extracts knocked down 89% on modeled cement wall surfaces, 90% on modeled mud smeared walls and 79% on modeled plain wall (Table 6). Deltamethrin and rain water (control) knocked down 100% and 0% mosquitoes respectively. Knockdowns were reported as significant at $p < 0.05$.

Table 4 Mean KDT on wild laboratory cultured *An. gambiae* mosquitoes from exposure to ethanol extracts of mature green fruits of Endod

KDT	N	Treatments applied on the modeled wall surface				
		Cement	Mud smeared	Plain	Deltamethrin	Control
10	40	41.11 ± 13.52^{b}	37.78 ± 10.60^{b}	41.11 ± 11.11^{b}	100.00 ± 0.00^{a}	0.00 ± 0.00^{c}
20	40	6.67 ± 3.33^{a}	6.67 ± 0.00^{a}	11.11 ± 4.44^{a}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
30	40	3.33 ± 1.92^{a}	1.11 ± 1.11^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
40	40	0.00 ± 0.00^{a}	1.11 ± 1.11^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
50	40	0.00 ± 0.00^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
60	40	48.89 ± 12.37^{b}	53.33 ± 10.72^{b}	47.78 ± 9.69^{b}	0.00 ± 0.00^{c}	0.00 ± 0.00^{a}

Note: 1. KDT means knockdown time in minutes; 2. Mean percent knockdown time with standard error of mean followed by superscripts of different letters differ significantly at $p < 0.05$

Table 5 Mean KDT on wild laboratory cultured *An. gambiae* mosquitoes from exposure to ethanol extracts of leaves of Endod

KDT	N	Treatments applied on the modeled wall surface				
		Cement	Mud smeared	Plain	Deltamethrin	Control
10	40	100.00 ± 0.00^{a}	94.44 ± 5.56^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	0.00 ± 0.00^{c}
20	40	0.00 ± 0.00^{b}	5.56 ± 5.56^{a}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
30	40	0.00 ± 0.00^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
40	40	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
50	40	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
60	40	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{a}

Note: 1. KDT means knockdown time in minutes; 2. Mean percent knockdown time with standard error of mean followed by superscripts of different letters differ significantly at $p < 0.05$

Table 6 Mean KDT on wild laboratory cultured *An. gambiae* mosquitoes from exposure to ethanol extracts of Neem leaves

KDT	N	Treatments applied on the modeled wall surface				
		Cement	Mud smeared	Plain	Deltamethrin	Control
10	40	88.89 ± 11.11^{a}	90.00 ± 8.39^{a}	78.89 ± 11.60^{a}	100.00 ± 0.00^{a}	0.00 ± 0.00^{c}
20	40	1.11 ± 1.11^{b}	2.22 ± 2.22^{a}	2.22 ± 1.11^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
30	40	0.00 ± 0.00^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
40	40	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.0 ± 00.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
50	40	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
60	40	10.00 ± 10.00^{c}	7.78 ± 6.19^{c}	18.89 ± 10.60^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{a}

Note: 1. KDT means knockdown time in minutes; 2. Mean percent knockdown time with standard error of mean followed by superscripts of different letters differ significantly at $p < 0.05$

2 Discussions

Extracts of many plants are now known to have effects on anopheles mosquitoes. The effects are either of broad spectrum toxicity, short and long term (Tripathi et al., 2004). None of these effects however, examines the ability of extracts of Endod on adult *Anopheles gambiae* upon direct contact. Our study explored this and has shown that *An. gambiae* is sensitive to extracts from different parts of Endod.

Female laboratory reared mosquitoes exposed to ethanol extracts from mature green fruits of Endod were knocked down more rapidly compared to those

exposed to extracts from leaves. The observed knockdown percent were less rapid compared to those observed for extracts of Neem leaves or deltamethrin. Knockdown of wild female *An. gambiae* mosquitoes from exposure to extracts of Endod leaves was total (100%) when exposure was done on cement and plain modeled wall surfaces. The rapidity with which the exposed adult female *An. gambiae* mosquitoes were knocked down were higher compared to other botanicals like essential oils of orange against *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* of that recorded percentages of 27.44%, 26.22% and 29.91% knockdown per min (Manimaran et al., 2012).

Wild mosquitoes were observed to be stunned more by the effects of phyotochemical from Endod leaves than mature green fruits with knockdown on exposure on cement and plain modeled wall surfaces occurring faster than modeled mud smeared. This observation was consistent to that of essential oils of *Casicum annuum* (Solanaceae) on *Anopheles gambiae* (Dadji et al., 2011). It was also observed that only knockdown at the 10th, twentieth and sixtieth minute after exposure differed at $p < 0.05$. The observation again was consistent with that made by Etang et al., (2011) who in addition also demonstrated differences in activities of the extracts spatially.

In all the cases plant metabolites are produced for use by the plants for a variety of function among which is protection from herbivores. The most vulnerable parts of the plant have the highest concentration to offer maximum protection by deterring consumption by herbivores. From the observation on knockdown, leaves of Endod had the highest concentration of phytochemicals and hence the appropriate part to source for extraction of phytochemicals.

Knockdown effect is a key factor in preventing mosquito bites as it is an indicator of toxicity for the product (Duvallet and De Gentille, 2012). Knockdown observed in this study is important in three ways. First it demonstrates that extracts of Endod parts are toxic to adult *An. gambiae* adults and therefore a biodegradable, environmentally friendly (Regnault-Roger et al., 2012) candidate for future malaria vector control. Second most residence in the rural Kenya live either in permanent (stone walled with plastered interior), semi permanent (mud walled with both interior and exterior cemented and plastered) or in wooden walled houses and this demonstrates that the extracts will be effective as these are the surfaces they will be used on in future. Thirdly, like all current insecticides approved for ITNs, our Endod extracts knocked down more that ¾ of the exposed mosquitoes within the first ten minutes of contact. This was rapid and show that extracts of Endod have great potential to reduce mosquito lifetime reproductive success.

In conclusion, crude ethanol extracts of Endod has the ability to knock down *An. gambiae* mosquitoes fast enough and this demonstrates the potential of Endod as an adulticide against malaria vector *An. gambiae*. However, more refining and further studies need be done before commercial exploitation is done.

3 Materials and methods

3.1 Study area

The experiments were conducted at the Entomology laboratory at Center for Global health Research/Kenya medical Research Institute (CGHR/KEMRI). The climatic conditions within the insectary and the laboratory were maintained at temperatures of 28 – 30 ℃ and relative humidity of 70−80% while photoperiod was of 12h light (06.30−18.30 hours) alternating with 12h darkness (18.30−06.30 hours).

3.2 Experimental design

A completely randomized informal ‘after-only with control’ experimental design (Kothari, 2004) was used to investigate the knockdown effect of crude ethanol extracts of Endod on the mature stages of *An. gambiae* mosquitoes. Crude ethanol extracts of Endod parts and leaves of Neem and deltamethrin were used as treatments against *An. gambiae* adults on modeled wall surfaces. Extracts of Neem leaves and preparations of deltamethrin were used as positive control while water alone was used as negative control. The treatments were taken as independent variables while the time and number of adult *An. gambiae* knocked down from exposures were taken as dependent variables.

3.3 *An. gambiae* mosquitoes for the experiment

Pink eyed *An. gambiae* mosquitoes maintained at the Entomology laboratories of CGHR/KEMRI were used in these experiments. The mosquitoes were reared following standard techniques (Das, 2007). Wild *An. gambiae* mosquito were sourced as larvae

from Ahero rice fields and transported to the laboratory at CGHR/KEMRI for further processing and rearing to adult stages.

3.4 Modeling experimental wall surfaces

Standard WHO procedures (WHO, 2006) with slight modification were used to model wall surfaces to represent common human residential wall surfaces to which endophagic mosquitoes rest. The surfaces were created from plywood measuring 26 cm × 26 cm and were either used as it were (plain) to depict wooden walls, smeared with cement to depict permanent or semi permanent cement plastered walls or smeared with a mixture of mud with cow dung to depict local homesteads of mud walled interiors (Figure 1).

Figure 1 Modeled wall residential surfaces

3.5 Endod plants

Leaves (shoot and midsection) and mature green fruits of Endod (Figure 2) obtained from high and lowlands were dried in a shade to a constant weight and ground to powder (mesh size, 200μ) before active chemical contents extracted using ethanol and the extract kept in airtight glass bottles under refrigeration (4^{O}C) to serve as stocks' quantity.

Figure 2 Male and female Endod plants showing leaves and flowers

3.5.1 Ethanol extract of Endod

Two hundred grams of each grounded botanical were soaked separately in 400ml of distilled water for one hour to dissolve the active components (Tilahun et al., 2003). The suspensions were filtered using Whitman's No. 1 filter paper and the filtrates freeze-dried using the Edwards Modulyo Freeze-drying machine to remove the water solvent. From the freeze-dried stock, 80mg were weighed and serial dilutions made to obtain different concentrations of 80, 40, 20, 10, 10, 5 and 2.5 mg/100mls of rain harvested water. Rain harvested water was chosen for two reasons. First, it makes the natural environment for aquatic stages of *An. gambiae* and second it is in a form that is available to most rural Kenyan.

3.6 Neem (*Azadirachta indica*)

Neem (*Azadirachta indica*) used in the experiments was sourced from Nyando in Kisumu County, Kenya. The leaves were harvested, dried in a shade to a constant weight and ground to powder (mesh size, 200μ) and the active chemical contents from the powder extracted using ethanol and distilled water. Ethanol extracts were obtained and diluted in a similar way as that of Endod.

3.7 Deltamethrin

A tablet of deltamethrin **(**KOTab 1-2-3®) of synthetic origin was obtained from a local retail chemist. Each tablet weighed 1.6g and contained 0.4g deltamethrin [pyrethroid (250g/kg)]. The tablets were crushed and 80mgs weighed and diluted in a similar way as that of Endod.

3.8 Impregnating the surfaces and bioassay

The modeled surfaces were sprayed with different concentration (80mg, 40mg, 20mg, 10mg, 5mg and 2.5mg in 100mls of rain harvested water) of mature green fruit and leaf extracts of Endod, Neem and deltamethrin and left to stand for an hour to dry in a shade before use. WHO exposure cones were placed on the surfaces and clipped (Figure 3) to create a confinement space for the mosquitoes to enable exposure. Ten 2-5 day old female unfed *An. gambiae* mosquitoes were aspirated from the mother colony, introduced into each cone and the inlet sealed.

To score on knockdown, a modified version of the median knockdown technique (Skovmand et al., 2008) was used and the WHO threshold of >95% knockdown

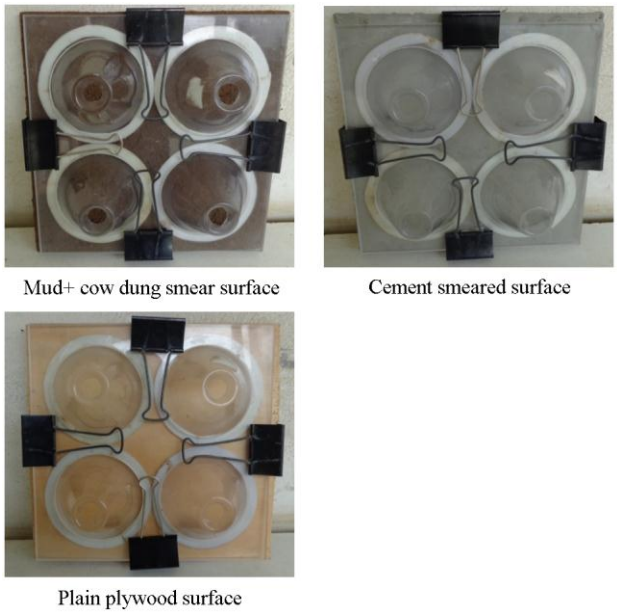

Figure 3 Modeled wall surfaces ready for exposure experiment

(WHO, 2006) assumed to assess the effectiveness of the extracts. Exposure was done for five minutes, exposed mosquitoes removed, put in a clean dry paper holding cups and observed for knockdown effect. Knockdown was defined as mosquito collapsed against the netting or fallen to the base of the cup and not moving. The exposed mosquitoes were observed for 1 hour and each time Knockdown occurred the mosquito was aspirated immediately out of the paper cup and into a second clean one to avoid a recount of the same mosquito (Skovmand et al., 2008). The knocked down mosquitoes were provided with 10% sugar solution and observed for recovery and mortality recorded after 24 hours.

3.9 Statistical Analysis

Data obtained from the bioassays was entered in excel spreadsheets and the relationship between the knockdown effect of the extracts with source (part of Endod plant used) and modeled wall surface used on mature stages of *An. gambiae* determined using descriptive statistics. One way analysis of variance (ANOVA) was used to determine the level of significance of the knockdown time and percentage. Statistical analysis was performed using SAS statistical package version 20.

Authors' contribution

YJO conceived the concept, conducted the experiments and wrote the manuscript. YJO and O-OJB designed the experiments and sourced for funds. O-OJB, VJM and AFK supervised and guided the experiments. O-OJB, VJM and AFK, read and corrected the manuscript. YJO and AR sourced for the wild mosquito larvae and cultured the mosquitoes.

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