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Larvicidal activities of the stem bark extract and rotenoids of *Millettia usaramensis* subspecies *usaramensis* on *Aedes aegypti* L. (Diptera: Culicidae)

Carren M. Bosire [a], Tsegaye Deyou [b], Jacques M. Kabaru [a], Dennis M. Kimata [a], Abiy Yenesew [b,*]

[a] School of Biological Sciences, University of Nairobi, P.O. Box 30197-00100, Nairobi, Kenya
[b] Department of Chemistry, University of Nairobi, P.O. Box 30197-00100, Nairobi, Kenya

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ABSTRACT

The dichloromethane/methanol (1:1) extract of the stem bark of *Millettia usaramensis* subspecies *usaramensis* was tested for its larvicidal activity against the 4th instar *Aedes aegypti* larvae and demonstrated activity with LC_{50} value of 50.8 ± 0.06 μg/mL at 48 h. Compounds isolated from the extract were also tested for their larvicidal activities, and the rotenoid usararotenoid-A (LC_{50} 4.3 ± 0.8 μg/mL at 48 h) was identified as the most active principle. This compound appears to be the first rotenoid having a *trans*-B/C ring junction and methylenedioxy group at C-2/C-3 with high larvicidal activity. Related rotenoids with the same configuration at the B/C-ring junction did not show significant activity at 100 μg/mL.

Introduction

Some of the many insects that proliferate in tropical environments due to conduciveness of its weather conditions transmit diseases and affect the health of both man and livestock. Mosquitoes are associated with several public health problems. This includes malaria, yellow fever, filariasis, dengue fever and Japanese encephalitis, which cause millions of deaths every year (Vatandoost and Vaziri, 2001). *Aedes aegypti* (Linnaeus 1762) (Diptera: Culicidae) is a vector for an arbovirus responsible for yellow fever and dengue fever. The latter disease sometimes leads to a complex and life-threatening stage called dengue haemorrhagic fever and dengue shock syndrome which is fatal if not treated. It has unusual manifestations such as central nervous system involvement (Hendarto and Hadinegoro, 1992; Pancharoen et al., 2002). About two-fifths of the world's population are at risk of catching dengue (Kautner et al., 1997; Rigau, 1998).

A. aegypti originated from Africa but now is found in the tropics worldwide (Womack, 1993; Mousson, 2005). It prefers breeding in areas of stagnant water such as flower vases, uncovered barrels, buckets, and discarded tyres, as well as wet shower floors and toilet tanks in houses. Treating these breeding areas with larvicidal agents remains an attractive strategy for the control of mosquitoes.

The use of conventional chemical pesticides such as organochlorides and organophosphates has resulted in the development of resistance (Rawlins and Ragoonansingh, 1990; Severini et al., 1993; Wirth and Georghiou, 1999; Macoris et al., 2007), undesirable effects on non-target organisms and fostered environmental and human health concerns (Forget, 1989). Therefore, the development of plant-derived products that do not produce adverse effects on the non-target organisms and are easily biodegradable remains one of the top objectives of scientists in search of alternative vector control agents (Redwane et al., 2002; Ribeiro et al., 2009; Kannathasan et al., 2011; Sagnou et al., 2012).

Insecticidal plants comprise of an array of secondary metabolites that act in concert on both behavioural and physiological processes, and hence the chances of pests to develop resistance to such insecticides are less probable (Saxena, 1987). Furthermore, botanical insecticides are less likely to bio-accumulate, as they are biodegradable. Amongst the botanical insecticides, rotenone and other rotenoids are found in some taxa of the family Leguminosae (Fabaceae), including the genus *Millettia* Wight et Arn. (Ollis et al., 1967; Dagne et al., 1991). In our earlier work we have reported the larvicidal activities of the crude extract and the rotenoids from the seeds of *Millettia dura* (Yenesew et al., 2003a). The rotenoids of *Millettia dura* having a *cis*-B/C ring junction satisfy the structural requirement for insecticidal activity (Fukami and Nakajima, 1971). The presence of unique 12a-hydroxyrotenoids with a *trans*-B/C ring junction (Yenesew et al., 1998; Yenesew et al., 2003b) from the stem bark of *Millettia usaramensis*

* Corresponding author. Tel.: +254 733 832576; fax: +254 20 4446138.
E-mail address: ayenesew@uonbi.ac.ke (A. Yenesew).

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subspecies *usaramensis* Taub (Gillet et al., 1971) has also been reported. The larvicidal activities of the extract and rotenoids obtained from the stem bark of this plant against 4th instar larvae of *Aedes aegypti* are presented here.

Materials and methods

General

Column chromatography was performed on silica gel (70–230 mesh). Analytical TLC was performed on Merck pre-coated silica gel 60F_{254} plates. ^{1}H NMR (200 MHz) and ^{13}C NMR (50 MHz) were recorded on a Varian 200 MHz spectrometer using the residual solvent peak as a reference.

Plant material

The stem bark of *Millettia usaramensis* subspecies *usaramensis* Taub. was collected in February 2008 from Diani along the Kenyan coast, geographical coordinates 4° 19′ 20″ S, 39° 34′ 30″ E. The plant was identified by Mr Simon Mathenge of the Herbarium, School of Biological Sciences of the University of Nairobi, where a voucher specimen (AYT-038-2008) was deposited. The sample was dried under shade and ground to fine powder using a mill.

Extraction of plant material

The stem bark of *M. usaramensis* subspecies *usaramensis* (0.6 kg) was extracted with dichloromethane/methanol (1:1) by cold percolation (3 × 24 h). The extract was filtered and concentrated in a rotary evaporator at 35 °C to afford crude extract (38 g).

Isolation of compounds

The constituents of the stem bark of *M. usaramensis* subspecies usaramensis including rotenoids (12a-epimillettosin, usararotenoid-A, 12-dihydrousararotenoid-A and usararotenoid-B), chalconoids (α,4,2′-trihydroxy-4′-*O*-geranyldihydrochalcone, 4′-*O*-geranylisoliquirtigenin and isoliquirtigenin) and isoflavones (jamaicin, norisojamaicin, barbigerone and maximaisoflavone G) were isolated and identified as described in Yenesew et al. (1998, 2003b). Deguelin was isolated from the seeds of *Millettia dura* according to (Yenesew et al., 2003a).

Preparation of derivatives

To a solution of 12-dihydrousararotenoid-A (10 mg, in 50 mL of acetone) four drops of conc. HCl were added and the solution was kept overnight. Purification of the product on PTLC (eluent: dichloromethane/*n*-hexane; 4:1) yielded 6 mg of colourless solid of 12a-deoxyusararotenoid-A. ^{1}H NMR ($CDCl_3$, 200 MHz): δ 6.69 (1H, s, H-1), 6.44 (1H, s, H-4), 4.18 (1H, dd, J = 3.6, 12.0 Hz, H-6α), 4.65 (1H, dd, J = 3.0, 12.0 Hz, H-6β), 4.98 (1H, t, J = 3.8 Hz, H-6a), 6.58 (1H, d, J = 8.4 Hz, H-10), 7.60 (1H, d, J = 8.4 Hz, H-11), 3.86 (1H, d, J = 3.8 Hz, H-12a), 5.83 (1H, d, J = 1.0 Hz, 2-OCH_2O-3), 5.87 (1H, d, J = 1.0 Hz, 2-OCH_2O-3), 6.01 (1H, d, J = 1.2 Hz, 8-OCH_2O-9), 6.08 (1H, d, J = 1.2 Hz, 8-OCH_2O-9). ^{13}C NMR ($CDCl_3$, 50 MHz): δ 107.0 (C-1), 144.8 (C-2), 148.2 (C-3), 99.2 (C-4), 148.8 (C-4a), 66.3 (C-6), 72.8 (C-6a), 142.5 (C-7a), 134.6 (C-8), 154.9 (C-9), 104.0 (C-10), 123.7 (C-11), 115.7 (C-11a), 188.9 (C-12), 45.6 (C-12a), 105.4 (C-12b), 101.4 (2-OCH_2O-3), 103.0 (8-OCH_2O-9).

A solution of 12-dihydrousararotenoid-A (15 mg) was refluxed in methanol containing 10 drops of concentrated HCl over water bath for 1 h. The product was purified by column chromatography (silica gel, using dichloromethane/hexane as eluent) to give colourless solid of 6a,12a-dehydrousararotenoid-A (9 mg). ^{1}H NMR ($CDCl_3$, 200 MHz): δ 8.27 (1H, s, H-1), 6.57 (1H, s, H-4), 7.09 (1H, d, J = 8.4 Hz, H-10), 7.80 (1H, d, J = 8.4 Hz, H-11), 5.08 (2H, s, H-6), 6.01 (2H, s, 2-OCH_2O-3), 6.32 (2Hs, 8-OCH_2O-9).

Mosquito cultures and larval rearing conditions

The larvae of *Aedes aegypti* (L) were obtained from the School of Biological Sciences, University of Nairobi. They were maintained at 25 ± 2 °C, 70–80% relative humidity (RH) and 12:12 light and dark photo period cycle. The larvae were fed on ground dog biscuit and yeast powder in the ratio of 3:1. The adults were fed on 10% sucrose solution and allowed to take blood meals from the blood vessels of the ears of immobilized rabbits.

Larvicidal assays

The larvicidal bioassays followed the guidelines for laboratory and field-testing of mosquito larvicides (WHO, 2005) with slight modifications. Initially, twenty 4th instar mosquito larvae were exposed to 5–1000 μg/mL of test solutions of *M. usaramensis* subspecies usaramensis crude stem bark extract and the control. After determining the mortality of larvae in this wide range of concentrations, a narrower range of 8 concentrations, yielding between 10% and 95% mortality in 24 h or 48 h was used to determine LC_{50} values. Batches of twenty 4th instar larvae were transferred by means of a dropper to glass jars each containing 100 mL of tap water. The appropriate volume of stock solution, where each of the crude extract and pure compound was dissolved in DMSO, was added to 100 mL water in the glass jars to obtain 0, 5, 10, 25, 50, 100, 200, 400, and 800 μg/mL (for crude extract), and 0, 6.25, 12.5, 25, 50, and 100 μg/mL (for pure compounds) dose levels. Six replicates were set up for each crude extract concentration (triplicates for pure compounds) and an equal number of controls were set up simultaneously with tap water, to which 1 mL DMSO was added. Each test was run three times on different days. Same quantity (20 mg) of larval food was added to each glass jar. The photoperiod was 12 h light followed by 12 h dark (12 L:12D). Larval mortality was recorded at 24 h and 48 h after exposure.

Statistical analysis

The average larval mortality data were subjected to probit analysis for calculating lethal concentrations (LC_{50} and LC_{90}) and ANOVA at 95% confidence limits using SPSS 16.0 software. Results with $p < 0.05$ were considered to be statistically significant. When the mortality in control was between 5 and 20%, Abbot's formula was used to correct the mortality to remove error due to other factors other than the toxicity of the test samples extracts (Abbot, 1925).

Corrected %mortality = [(T − C) / (100 − C)] × 100 where, T = % mortality in test concentration.

C = % mortality in control.

The values were expressed as mean ± standard deviation of replicates. Log dosage-probit mortality was plotted with Minitab® statistical software.

Results and discussion

The dichloromethane/methanol (1:1) extract of the stem bark of *Millettia usaramensis* subspecies *usaramensis* was tested for lethality against the 4th instar larvae of *Aedes aegypti*. This crude extract showed marginal activity with LC_{50} value of 167.0 μg/mL at 24 h, which improved at 48 h to LC_{50} value of 50.8 ± 0.06 μg/mL. The constituents of the stem bark (Yenesew et al., 1998, 2003b), including rotenoids (12a-epimillettosin, usararotenoid-A, 12-dihydrousararotenoid-A and usararotenoid-B, Fig. 1), chalconoids (α,4,2′-trihydroxy-4′-*O*-geranyldihydrochalcone, 4′-*O*-geranylisoliquirtigenin and isoliquirtigenin) and isoflavones (jamaicin, norisojamaicin, barbigerone and maximaisoflavone G)

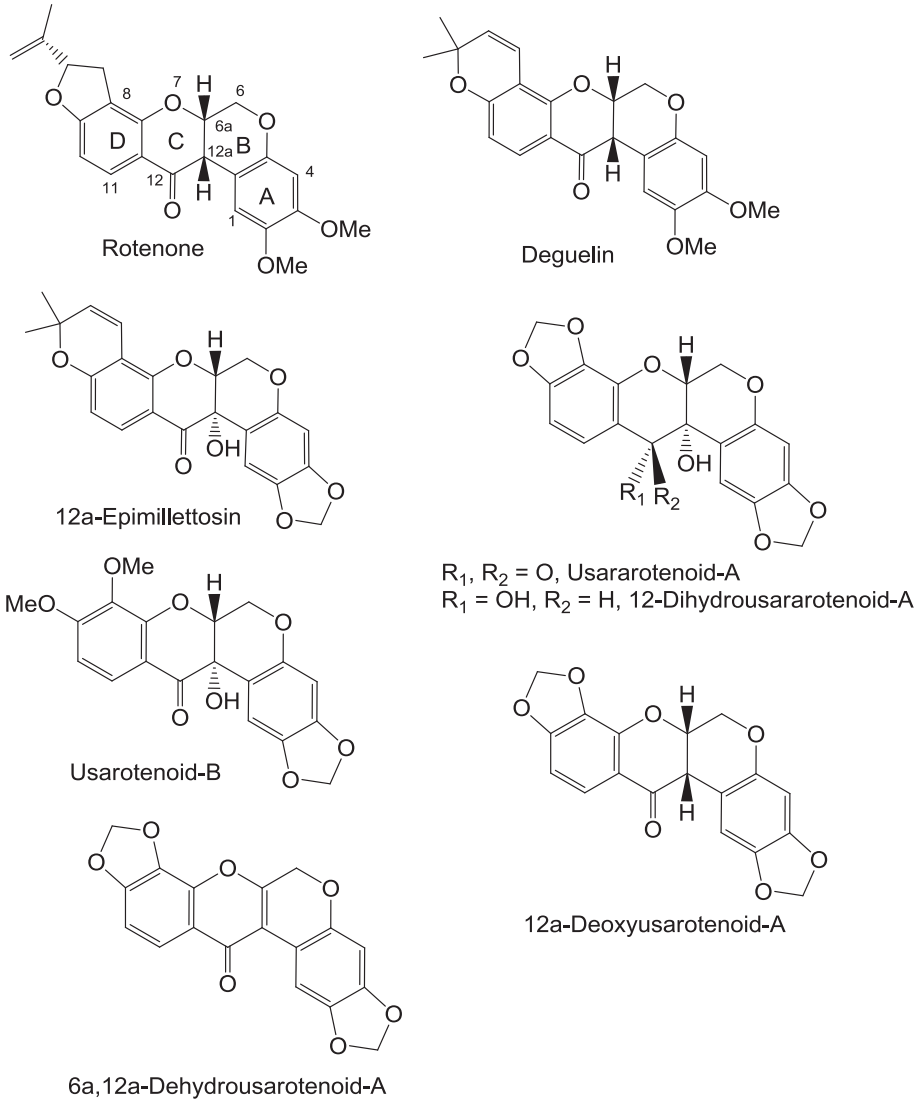

Fig. 1. Structures of rotenoids.

were also tested against the 4th instar larvae of *A. aegypti*. Amongst these, the rotenoid usararotenoid-A caused 100% mortality at 100 μg/mL at 48 h. The rest of the compounds did not show significant activities at this concentration at 48 h.

Usararotenoid-A along with 12a-epimillettosin (the major compound of the stem bark of this plant) and deguelin (insecticidal rotenoid with *cis*-B/C ring junction) was tested at different concentrations; the LC_{50} values with corresponding 95% confidence limits were calculated for each bioassay and are shown in Table 1. The slopes of the concentration–mortality curves of the results varied considerably between 24 h and 48 h post exposure (Fig. 2). The slopes were greater for the 48 hour than the 24 hour post exposure period indicating homogeneity of response to the tested larvicides.

It has been reported that the activity of rotenoids such as rotenone (Fig. 1), against insects is associated with the fused four-ring system (rings A, B, C and D) – a chromanochromanone known as 6a,12a-dihydrorotoxen-12(6H)-one – where the B/C ring junction is *cis*. Furthermore rotenoids with modified rings and a *trans*-B/C ring junction were shown to be less insecticidal (Fukami and Nakajima, 1971; Joseph and Casida, 1992). We have earlier demonstrated the importance of the methoxyl groups at C-2 and C-3 of ring A for larvicidal activity amongst rotenoids with *cis*-B/C ring junction (Yenesew et al., 2003a). In this study, it was of no surprise then that 12a-epimillettosin (and other related rotenoids of the stem bark), having *trans*-B/C ring junction and methylenedioxy at C-2/C-3, did not show significant larvicidal activity at 100 μg/mL. The major rotenoid of this plant, 12a-epimillettosin, was further tested at higher concentrations to determine the LC_{50} value and was found to be 2037 ± 8.3 μg/mL at 48 h, against the 4th instar larvae (Table 1), which is over 800 times less active than deguelin (LC_{50} 2.6 ± 0.9 μg/mL at 48 h), a rotenoid with known larvicidal activity (Yenesew et al., 2003a).

Unexpectedly, usararotenoid-A, despite having a *trans*-B/C ring junction and methylenedioxy group at C-2/C-3 as 12a-epimillettosin, showed high larvicidal activity (LC_{50} 4.3 ± 0.8 μg/mL, at 48 h) against the 4th instar *A. aegypti* larvae which is comparable with that of deguelin (Table 1). On the other hand the structurally related compounds, 12-dihydrousararotenoid-A and usararotenoid-B (Fig. 1) were inactive even at 100 μg/mL showing the importance of the 12-keto group and the methylenedioxy group at C-8/C-9 for the activity of usararotenoid-A.

In order to explore further the structural requirement for larvicidal activity, 12a-deoxyusarotenoid-A and 6a,12a-dehydrousararotenoid-A (Fig. 1) were prepared and tested for larvicidal activity. Both derivatives were completely inactive at 100 μg/mL against the 4th instar *A. aegypti* larvae at 48 h. The fact that usararotenoid-A having a *trans*-B/C ring junction is active and the 12a-deoxyusararotenoid-A with *cis*-B/C ring

Table 1
Summary of Log probit analysis of the larvicidal activity of crude CH_2Cl_2/MeOH (1:1) stem bark extract and pure compounds from *M. usaramensis* subspecies *usaramensis* on the 4th instar *A. aegypti* larvae.

Sample	Exposure period (h)	Regression equations	R^2 (%)	LC_{50} (μg/mL)	95% CI (μg/mL)	
					LCL	UCL
Crude stem bark extract	24	Y = 2.41 + 1.17X	96.3	167.00[a]	142.82	196.28
	48	Y = 2.76 + 1.31X	91.2	50.82[a]	49.91	67.72
12a-Epimillettosin	24	Y = 2.83 + 0.571X	75.0	6350.31[b]	6348.76	6351.86
	48	Y = 2.97 + 0.612X	89.7	2037.00[b]	2035.68	2038.32
Usararotenoid-A	24	Y = 3.63 + 0.914X	80.9	31.77[b]	18.79	70.16
	48	Y = 3.48 + 2.42X	68.0	4.27[b]	0.799	8.05
Deguelin	24	Y = 2.91 + 2.75X	78.6	5.75[b]	2.15	9.21
	48	Y = 3.82 + 2.82X	89.0	2.63[b]	0.25	5.02

Y, probit; X, log conc; R, coefficient of regression equation; LC, lethal concentration; [a]represents mean of six replicates; [b]represents mean of triplicates; calculated log LC_{50} transformed to LC_{50}; CI, confidence interval; LCL, lower confidence limit; UCL, upper confidence limit.

junction is inactive is the exact opposite to what has been reported for rotenone and related rotenoids having methoxyl groups at C-1 and C-2 (Fukami and Nakajima, 1971; Joseph and Casida, 1992). In light of this finding, it is worth to investigate the insecticidal activity of usararotenoid-A against a variety of insect species. In relation to usararotenoid-A, the present finding supports the previous assertion that the structure–activity relationship amongst rotenoids and isoflavonoids is not entirely clear as structurally different isoflavonoids have shown some activity against insects (Sreelatha et al., 2010; Morel et al., 2013; Pluempanupat et al., 2013).

According to literature, the organophosphate synthetic insecticide temephos, has shown an LC_{50} value of 2.3 μg/mL in larvicidal activity assays performed against third-instar larvae of susceptible strains of *A. aegypti* (Macoris et al., 2007). In this study, usararotenoid-A (LC_{50} of 4.3 ± 0.8 μg/mL) showed high activity (in a 48 hour period), comparable to that of deguelin (LC_{50} 2.6 ± 0.9 μg/mL) and temephos. Therefore, usararotenoid-A may be considered as a promising natural mosquito larvicidal agent, in the era of resistance of *A. aegypti* populations to organophosphate and other synthetic organic pesticides, as well as the environmental safety and human health concerns associated to their use.

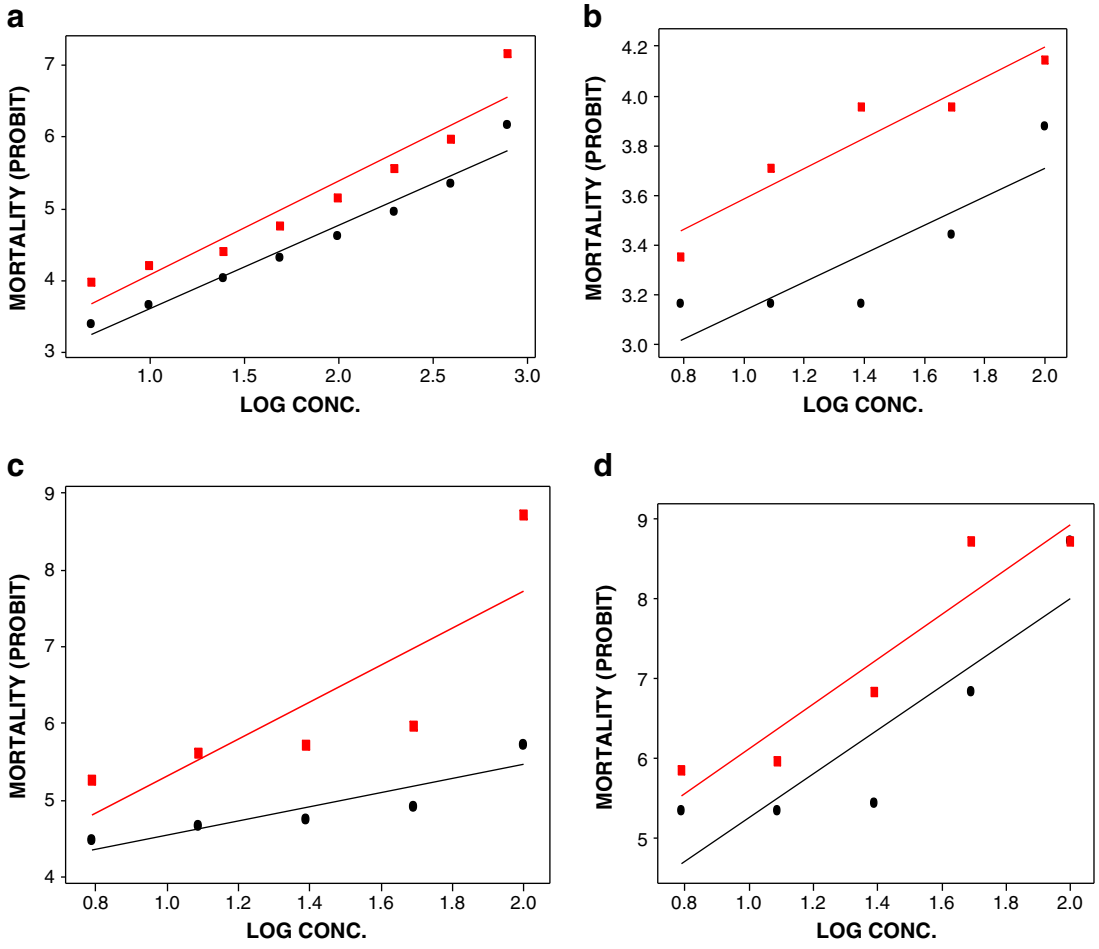

Fig. 2. Probit regression lines (LC-p lines) resulting from *A. aegypti* 4th instar larvae exposed to: *M. usaramensis* subspecies *usaramensis* crude stem bark extract (a), 12a-epimillettosin (b), usararotenoid-A (c), deguelin (d); ◆ 24 h and; ■ 48 h.

Conclusion

The crude extract of the stem bark of *Millettia usaramensis* is moderately larvicidal against the 4th instar larvae of *Aedes aegypti*, which could be attributed mainly to usararotenoid-A. This compound appears to be the first rotenoid with *trans*-B/C ring junction demonstrating remarkable insecticidal activity, and offers promise as a potential biocontrol agent against *A. aegypti.* However, further studies on the larvicidal mode of action, its effect on non-target organisms and environment, and formulations for improving the larvicidal potency and stability are needed for its practical use in mosquito control.

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