Published in final edited form as:

Acta Trop. 2019 February 01; 190: 304–311. doi:10.1016/j.actatropica.2018.12.009.

Growth-disrupting *Murraya koenigii* leaf extracts on *Anopheles gambiae* larvae and identification of associated candidate bioactive constituents

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Abstract

Plant-based constituents have been proposed as eco-friendly alternatives to synthetic insecticides for control of mosquito vectors of malaria. In this study, we first screened the effects of methanolic leaf extracts of curry tree (*Murraya koenigii*) growing in tropical (Mombasa, Malindi) and semi-arid (Kibwezi, and Makindu) ecological zones of Kenya on third instar *An. gambiae* s.s. larvae. Extracts of the plant from the semi-arid region, and particularly from Kibwezi, led to high mortality of the larvae. Bioassay-guided fractionation of the methanolic extract of the leaves of the plants from Kibwezi was then undertaken and the most active fraction (20 fold more potent than the crude extract) was then analyzed by Liquid chromatography quadrupole time of flight coupled

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Author's contributions

C.M.M., A.H., F.M.K. and P.O.M. conceived and designed experiments, F.M.K. and W.L. contributed experiment reagents, materials and analysis tools, C.M.M. performed the experiments, C.M.M. analyzed the data, C.M.M., A.H. and P.O.M. developed the manuscript, C.M.M., A.H., F.M.K., M.K.R., W.L., C.M. and P.O.M. reviewed the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

with mass spectrometry (LC-QtoF-MS) and a number of constituents were identified, including a major alkaloid constituent, Neplanocin A (**5**). Exposure of the third instar larvae to a sub-lethal dose (4.43 ppm) of this fraction over 7-day periods induced gross morphogenetic abnormalities in the larvae, with reduced locomotion, and delayed pupation. Moreover, the few adults that emerged from some pupae failed to fly from the water surface, unlike in the untreated control group. These results demonstrate subtle growth-disrupting effects of the phytochemical blend from *M. koenigii* leaves on aquatic stages *An. gambiae* mosquito. The study lays down some useful groundwork for the downstream development of phytochemical blends that can be evaluated for integration into eco-friendly control of *An. gambiae* vector population targeting the often overlooked but important immature stages of the malaria vector.

Keywords

Anopheles gambiae; *Murraya koenigii*; Larval growth disruption; Mosquito control

1 Introduction

Anopheles gambiae mosquito is a major vector of malaria and is responsible for about 216 million infections and about 446,000 deaths in humans, mainly in sub-Saharan Africa (WHO, 2017) despite significant progress in the fight against the disease (Slutsker and Kachur, 2013). Effective management of the vector by current mosquitocides has been challenging due to widespread resistance to current insecticides, especially pyrethroids, in the vector across Sub-Saharan Africa (Gnankiné et al., 2013; Namountougou et al., 2013; Yunta et al., 2016). The resistance to pyrethroids presents a real and immediate challenge to efficacy of otherwise successful insecticide treated nets (ITN) based malaria control intervention against adult vector (Hightower et al., 2010). Resistance development has also hampered larvae control, a viable alternative control of the vector populations (Bisset et al., 2014). Recent phytochemical research has begun to reveal a variety of blend effects in the bioactivities of plant natural products. Two principal blend effects have been demonstrated: (1) enhanced biological activity resulting from synergistic or other additive effects of moderately active or individually inactive compounds to give mixtures that are more active than a linear summation of individual activities (Bekele and Hassanali, 2001), and (2) mitigating effects of structurally related or unrelated compounds against rapid resistance development that characterizes most single-component bioactive compounds (Abu Hasan et al., 2017; Casida, 2009). Most plants produce a variety of secondary metabolites, which may or may not be structurally related, with multiplicity of defense and non-defense functions against different pathogens and herbivores. This phytochemical and functional diversity has arisen from sustained selective forces in response to succession of attacks by pathogens and herbivores and other selective pressures over evolutionary time. The diversity is additionally influenced by ecological factors that include precipitation, temperature and soil factors, with the climatic factors having a major influence (Liu et al., 2015). Moreover, phytochemical blends rarely demonstrate acute toxicity to vertebrates and are eco-friendly. They also have subtle longer-term deleterious effects on plant pests and disease vectors. Extracts from Meliaceae plants, for example, have a wide range of activities against *An. gambiae* that include anti-feedant, anti-oviposition (Su and Mulla, 1998), repellent and growth-regulating

effects on the larval stages (Ndung'u et al., 2004; Okumu et al., 2007). Other plant extracts larvicidal to *An. gambiae* mosquito include those from *Cryptomeria japonica* (Mdoe et al., 2014) and *Piper nigrum* (Samuel et al., 2016). Extracts from *Vitex trifolia* have been shown to disrupt *An. gambiae* larval growth (Nyamoita et al., 2013) and those from *Turraea abyssinica* are both larvicidal and adulticidal to *An. gambiae* (Owino et al., 2014).

Mosquitocidal effects of relatively high doses of curry tree plant (*M. koenigii*) extracts have been demonstrated against larvae of *Anopheles stephensi* (Arivoli, 2015; Srivastava et al., 2009), *Aedes aegypti* (Kovendan et al., 2012a; Patil et al., 2010) and *Culex quinquefasciatus* (Tennyson et al., 2012). However, none of these studies assessed the potential effects of longer-term exposure of larval stages to sub-lethal doses of the plant extracts and characterized the chemical basis of their activities to the mosquito. While curry tree is a native to Asian countries (Gahlawat et al., 2014), it was established in different locations in Kenya about 200–300 years ago by immigrants from India (Jedwab et al., 2017).

This present study was initiated to (1) compare the larvicidal potential of methanolic extract of *M. koenigii* growing in a few different ecological areas on *An. gambiae* larvae, (2) study the time-course effects of a sub-lethal dose of the most active fraction of the extract on the larvae, and (3) identify potential bioactive components of the extract. We hypothesized that chemotypic differences in the plant growing in different ecological areas could have variable effects on *An. gambiae* larvae (Mayeku et al., 2014).

2 Materials and methods

2.1 Field collections of curry tree (*M. koenigii*) leaf samples

Murraya koenigii leave samples were selected on the basis of ethnobotanical information, such as their uses in traditional medicine and protection against biting insects. The leaves were collected from Malindi (35 °E, 1 °N), Mombasa (36 °E, 1 °S), Makindu (36 °E, 0°) and Kibwezi (35 °E, 1 °N) regions in Kenya (Fig. 1). The Malindi and Mombasa regions are located in the coastal lowland semi-humid ecological regions of Kenya with an elevation of 100–200 m above sea level (asl), with an average annual precipitation of 1000–1500 mm and mean temperatures of 25–27 °C. Makindu and Kibwezi on the other hand are located in semi-arid inland regions of Kenya at an elevation at 900–1000 m asl with 600–650 mm annual precipitation and 21–22 °C annual mean temperatures. The soil types across all the sites are similar and mainly consist of sandy clay loam (MoALF, 2016). The leaves were collected after the flowering period in February, placed in separate, well-labeled gunny bag and transported to the drying site within three days. Taxonomic classification of leaf samples were done using morphological keys of Parmar and M.K.K. (2008) and voucher specimens were subsequently deposited at the National Museums of Kenya.

2.2 Preparation of crude leaf extracts from curry tree (*M. koenigii*)

The leaf samples collected separately from the three sites at each location (at Malindi, Mombasa, Makindu and Kibwezi) were separately dried under shade for 7–14 days at ambient environment temperature (27–37 °C), and were subsequently powdered using commercial electrical stainless steel blender (Retsch Muhle, Haan, Germany). Each powder

sample was exposed to absolute methanol (Sigma Aldrich, St. Lois, USA) at 45–50 °C for eight hrs in a Soxhlet apparatus (Verran et al., 2014). The extracts were then filtered using a Whatman No.1 filter paper (Whatman Inc., Haverhill, USA), for 30 min with a vacuum pump and then concentrated on a rotary evaporator (Laborota 4000 efficient, Heidolph, Germany) at 40–50 °C as described by (Kovendan et al., 2012b). The residue obtained were then stored at 4 °C and later solubilized in absolute ethanol until required for experimental work.

2.3 Rearing and maintenance of *An. gambiae* s.s

Late third instar (L₃) larvae of *An. gambiae* s.s. (Koutsos et al., 2007) used in the experiment were obtained from mosquito colony maintained at the International Centre of Insect Physiology and Ecology (*icipe*), Nairobi, Kenya. The colony was established from *An. gambiae* s.s. mosquitoes originally collected from Mbita field station (00025'S, 34013'E), Homa Bay county in western Kenya in 2000. The mosquitoes were maintained in the standard operating procedure for rearing *Anopheles* mosquitoes where all life stages were reared at 28 ± 2 °C, 52-72% Relative Humidity, 12 L: 12 D photoperiod (Costa-da-Silva et al., 2013). From the day of emergence, adult mosquitoes were provided with a 10% sugar solution soaked in cotton wool and at three days post emergence, and female mosquitoes were subsequently allowed to feed on anaesthetized mice. Approval for feeding mosquitoes on mice was obtained from the Kenya National Ethical Review Board (protocol number KEMRI/RES/7/3/1); the protocol was reviewed and approved by the KEMRI Animal Care and Use Committee (ACUC). Fully engorged females were allowed to lay eggs on funnel-shaped filter paper placed over oviposition cups (4 cm diameter, 2 cm depth) inside the cages. Oviposited eggs were dispensed into hatching trays (39 cm long × 28 cm wide × 14 cm high) filled to a depth of 8 cm with dechlorinated water and larval densities maintained at approximately 100-150 larvae per tray. The larvae were fed on fish food Tetramin® three times daily (0.3 g Tetramin/100 larvae/ day).

2.4 Comparison of crude extracts effects on the mortality of *Anopheles gambiae* s.s. larvae

The most potent *M. koenigii* leaf extracts from all sampling sites was identified through larval (L₃) mortality bioassay. Briefly, five replicates were prepared from each of the three set of extracts. Each replicate was composed of 100 ppm of the crude extract in distilled water. In each assay, 1% of ethanol in aqueous medium was used as the control. Larvae (n = 20) were then placed in each replicate and control and maintained under the insectary conditions for 72 hs, when the mortality of the larvae in each replicate was recorded. Larval mortalities in each set of replicates were compared with the control by one-way analysis of variance (ANOVA). Mean larval mortalities of sets of extracts from different locations that were significantly different were identified using Tukey's HSD (Honestly Significant Difference) post-hoc analysis. The results revealed that the extracts from the location with the most larvicidal potential, which was then selected for dose response experiments to determine its median lethal concentration (LC₅₀). Briefly, different concentrations of the most potent extract (0, 25, 50, 100, 250 and 500 ppm) were prepared in distilled water with each of the three biological replicates. Larvae (n = 20) were similarly placed in each replicate and control, and maintained under the insectary and assessed for mortality 72 hs

post exposure (pe). Mortality data were corrected by Abbott's formula (Akçay, 2013) and then transformed to probits (Finney, 1971) for linear regression analysis. The LC_{50} values on the transformed data was determined using XLSTAT® 2017 (Addinsoft, Paris, France) and GraphPad Prism version 7.0 (GraphPad Software, San Diego, USA).

2.5 Bioassay guided fractionation of crude plant extracts on *An. gambiae* larvae

The most potent crude extract (site) was fractionated on a silica-packed column into its components based on their differences in polarity of the components as described by Jandera et al. (1985). Briefly, the extract was placed in the column packed with Silica (200 g, Kiesegel 60 M [0.004-0.063 mm mesh size], Macherey-Nagel GmbH & Co. KG, Germany) in a 50 × 410 mm column and pre-conditioned with analytical grade n-hexane (Sigma Aldrich, USA) for three hrs. Components of the extract were then fractionated by gradient mobile phase of n-hexane and ethyl acetate (Sigma Aldrich, USA) of increasing polarity (100:0, 80:20, 60:40, 50:50, 40:60, 20:80 and 0:100 ratios). Fractions with similar retention factor (Rf) values (matching polarities) identified by thin layer chromatography (Howard et al., 2009) were pooled into 17 major fractions. The fractions were rotor evaporated to remove the solvent and concentrate the blends of components (Kovendan et al., 2012b). The acute toxicity of each concentrate of the 17 fractions was then evaluated in five replicates (+ control) at 10 ppm for 24 hs. The most potent fraction was subsequently separately selected for dose response experiments to identify its median lethal concentration (LC_{50}), effect on regulation of larvae growth and chemical components.

The LC_{50} was determined as above where five concentrations of the most potent fraction (0, 1, 2, 4, 6 and 10 ppm) were prepared in distilled water in three replicates. Larvae (n = 20) were exposed to the treatments for 72 hs whereby their rates of mortality were observed in 24 h intervals pe, corrected by Abbott's formula (Abbott, 1925), transformed to probits (Finney, 1971) for linear regression analysis and their LC_{50} values determined using XLSTAT® 2017 (Addinsoft, Paris, France) and GraphPad Prism version 7.0. (GraphPad Software, San Diego, USA). Time-course effects of LC_{50} of the most potent fraction on three replicates (n = 20 each) of the larvae was then observed at 24 hs intervals for physiological changes in the larvae, pupation and adult emergence until the last larvae died or pupated and the adult(s) subsequently eclosed. The larvae were provided with 10 mg/l of Tetramin® fish food at two-day interval until the end of the observation period. Images of resulting morphological defects on mosquito developmental stages were recorded with a dissecting light microscope (Leica Corporation, Switzerland) at ×20 magnification. Harley mean index was used for estimating growth and survival rates of the treated larvae (Harley, 1967). The index was calculated as follows: Harley mean index = (Percentage of individuals pupating + Percentage of individuals reaching adulthood)/Median day of pupation). The experiment was replicated five times.

2.6 LC-QtoF-MS Chemical analysis of bioactive fraction from *Murraya koenigii*

The chemical components of the most potent fraction were identified using Liquid chromatography quadruple time of flight coupled to mass spectrometer (LC-QtoF-MS) as described by Cheseto et al. (2017). Briefly, 1.5 ml of the most potent fraction was dissolved in 1ml of 0.01% formic acid/methanol (95:5, v/v) LC-MS grade, vortexed for 30 s, and

centrifuged at 14 000 rpm for 5 min, after which 0.2 µL of the supernatant was analyzed by LC-QtoF-MS. Chromatographic separation of the components was achieved on a Waters Acquity UPLC (ultra-performance liquid chromatography) I-class system (Waters Corporation, Milford, MA, USA). The UPLC was fitted to a 250 mm × 4.6 mm i.d, 5 µm particle size ACE C-18 column; (Advance Chromatography Technologies, Aberdeen, Scotland) with the heater turned off and an auto-sampler thermostat at 5 °C. The mobile phases used were deionized water with 0.1% of formic acid (A) and methanol with 0.1% of formic acid (B). A programmed gradient was used as follows: begin with 5% B, change from 5% to 100% B in 18 min and constant 100% B for 2 min more. A post-run of 5 min was programmed to equilibrate the column between analyses. The UPLC system was interfaced with electrospray ionization (ESI) to a Synapt G2-Si QtoF-MS (Waters) operated in full scan MSE in positive mode. Data were acquired in resolution mode over the m/z range 100–700 with a scan time of 1 s using a capillary voltage of 0.5 kV, sampling cone voltage of 40 V, source temperature 100 °C and desolvation temperature of 350 °C. The nitrogen desolvation flow rate was 500 L/h. For the high-energy scan function, a collision energy ramp of 25–45 eV was applied in the T-wave collision cell using ultra-high purity argon (99.999%) as the collision gas. A continuous lock spray reference compound (leucine enkephalin; $[M + H]^+ = 556.2766$) was sampled at 10 s intervals for centroid data mass correction. The mass spectrometer was calibrated across the 50–1200 Da mass range using a 0.5 mM sodium formate solution prepared in 90:10 2-propanol/water (v/v). MassLynx version 4.1 SCN 712 (Waters Corporation, Maple Street, MA) was used for data acquisition and processing. The elemental composition was generated for every analyte. Potential assignments were calculated using mono-isotopic masses with a tolerance of 10 ppm deviation and both odd- and even-electron states possible. The number and types of expected atoms was set as follows: carbons 50; hydrogens 100; oxygens 50; nitrogens 10; chlorines 10; and sulfur 10. The empirical formulae, published literature and online database (ChemCalc, PubChem and Chempider) were used to propose the structures (Kim et al., 2016; Patiny and Borel, 2013; Pence and Williams, 2010).

3 Results

3.1 Larvicidal effects of crude extracts from Kibwezi, Makindu, Malindi and Mombasa

Mean percentage mortalities of *An. gambiae* L₃ larvae exposed to 100 ppm crude extracts of *M. koenigii* leaves sampled from the four sites are summarized in Table 1.

Over 72 h pe (post-exposure), the two extracts from the semi-arid zone (Kibwezi and Makindu) showed higher larvicidal effects compared to those from the coastal area (Malindi and Mombasa), with that from Kibwezi being the most potent ($F_{(3, 16)} = 27.40$, $p < .0001$). Bioassay-guided fractionation of this extract (into 17 fractions) identified the most potent fraction with LC₅₀ of 2.43 ppm 72 hs pe (Table 2). Longer term exposure of larvae to 4.43 ppm of this fraction induced several morphogenetic effects, such as: 1) darkening of larval midgut, hindgut (Fig. 2B) and gastric caeca (Fig. 2C); 2) arrest of pupation of the larvae giving larval-pupal intermediates (Fig. 2C); 3) distended adult formation from underdeveloped pupae (Fig. 2B); and 4) partially emerged adults with deformed tarsi (Fig. 2B).

The exposure also delayed pupation five folds longer and the few adults that emerged showed grossly impaired flight ability compared to the non-exposed controls. Median durations of pupation for the exposed and control larvae were 10 and 2 days, respectively. Overall, less than 2% adults emerged from the exposed larvae compared to the control where most (> 91%) emerged (Table 3).

3.2 Identification of chemical constituents of the most larvicidal fraction of Kibwezi extract

LC-QTOF-MS analysis of the fraction revealed nine prominent peaks (Fig. 3). The major peak (retention time 4.73 min, Fig. 3), gave a molecular ion peak $[M + H]^+$ at m/z 264.1021, with molecular formula of $C_{11}H_{13}N_5O_3$ (**5**) (Table 4). ChemCalc, Chemspider and PubChem (Kim et al., 2016; Patiny and Borel, 2013; Pence and Williams, 2010) online databases were used to reveal the identity of the peaks. The most prominent peak showed fragmentation pattern associated with the alkaloid neplanocin A. Similar comparative search identified the other constituents as 3-(1-naphthyl)-L-alanine (**1**), lumiflavine (**3**), terezine C (**7**) and agelaspongine (**8**), murrayazolinol (**9**) as additional constituents (Table 4, Fig. 3). Neplanocin A was previously functionally associated with insecticidal and growth regulation in juvenile stages of mosquito (Sharma et al., 2015).

4 Discussion

In this study, we explored larvicidal potential of methanolic extracts of *M. koenigii* growing in different ecological areas on *An. gambiae* larvae, as well as time-course effects of a sub-lethal dose of the most active fraction of the extract on the larvae and respective emergent pupae and adults, and potential bioactive components of the extract. We found significant and consistently higher larvicidal potential in the extracts from semi-arid than from those from the coastal zones, suggesting a significant influence of the ecology on *M. koenigii* toxicity to *An. gambiae* larvae. The differential potency of the extracts within the semi-arid zones points to micro-geographic ecological influences on the constituent profiles and toxicity of the extract. These findings suggest that a further analysis of *M. koenigii* from more diverse agro-ecological zones of eastern Africa may provide more comprehensive insights on ecological factors associated on *M. koenigii* ecotypes with more potent phytochemical profiles on mosquito larvae.

Longer-term exposure of larvae to sub-lethal doses of the most potent fraction of the Kibwezi extract showed subtle time-course growthdisrupting effects with several morphogenetic consequences to the larvae, and negative effects on pupation and emergence of adults. Interestingly, this fraction was about 1.5 and 2 folds more larvicidal than those reported from green tea (*Camellia sinensis*) leaves (Muema et al., 2016) and Sickie Senna (*Cassia tora*) seeds (Mbatchou et al., 2017). The morphogenetic larvae-pupae developmental effects and decreased survival may be due to dysregulation of juvenile hormone in the mosquito by the *M. koenigii* phytochemicals, as evidenced by documented impact of phytochemicals in *An. gambiae* (Lee et al., 2015; Nyamoita et al., 2013) and other insects (Tusun et al., 2017), and/or midgut damage as observed in *Culex quinquefasciatus* larvae exposed to Garlic Vine extracts (Granados-Echegoyen et al., 2014; Procópio et al., 2015).

The delayed pupation and impaired flight in emerging adults can potentially expose the larval mosquitoes to undue predation, which together with the minimal (< 2%) adult emergence would substantially reduce the population sizes of the mosquito in successive generations, and significant collapse in mosquito populations (Athrey et al., 2012; O'Loughlin et al., 2016). In addition, this phenomenon of undue predation may minimize the risk of fueling selection toward resistance by naturally reducing the number of survivors in field conditions (Kweka et al., 2011). Semi-field assays targeting diverse larval stages derived from different co-existing broods of mosquito larvae can shed some light on the levels of survival of mosquitoes. Moreover, any possibility of risk of resistance can be evaluated by sampling mosquitos from the site of application and monitoring incidences of any resistant strains (Swale et al., 2018).

LC–MS analysis of the potent fraction led to the identification of 3-(1-naphthyl)-L-alanine (**1**), lumiflavine (**3**), neplanocin A (**5**), tereazine C (**7**), agelaspongins (**8**), and murrayazolinol (**9**) as the major constituents. Compounds **1**, **3** and **5** have previously been linked to growth-disruptive effects in insects, such as darkening of gastric caeca at the anterior end of the midgut and reduced locomotion (Arrese and Soulages, 2010; Atella and Shahabuddin, 2002; Lorenz and Gáde, 2009), delayed pupation and incomplete adult development (Hauerland, 1996; Magee et al., 1994; Singh and Brown, 1957), and disruption of growth and high mortality in immature *An. gambiae* respectively (Lu et al., 2013; Sharma et al., 2015). The phenotype observed from our growth disrupting experiments (Fig. 2B, C) is consistent with chitin synthesis inhibition (Fontoura et al., 2012) similar to No-valuron, an insect growth regulator already in use against *Aedes* and *Anopheles* mosquito larvae (Arredondo-Jiménez and Valdez-Delgado, 2006; Su et al., 2003). The effects of inhibition of chitin synthesis could persist beyond juvenile stages of the mosquito (Swale et al., 2018) suggesting possible reduced survivability and longevity of adult mosquitoes, and thus greatly reducing their vectorial capacity. Subtractive assays with blends of sub-lethal doses of the major constituents can shed some light on the contribution of each compound on the growth disruption of the larval stages of mosquitoes and on the most effective blend for potential downstream applications.

5 Conclusion

In conclusion, this exploratory study shows the presence of a blend of secondary metabolites in the leaves of *M. koenigii* growing in a semiarid region of Kenya that has subtle growth-disrupting effects on *An. gambiae* larvae at a low sub-lethal dose. Some of these metabolites were previously shown to have negative morphogenetic effects on different insects. For effective down-stream exploitation of the plant in the control of malaria vectors, two sets of studies need to be undertaken: first, the role of the different constituents in the most active fraction needs to be elucidated in a series of subtractive assays and the active blend fully characterized; second, the effect of a broader profile of agroecologies in Eastern Africa on the levels of the active constituents in the plant needs to be comprehensively established to see if the active blend can be produced on a large scale.

Acknowledgements

We thank Mr. John Bwire, Mr. Levi Ombura, Mr. Jackson Muema and Mr. Xavier Cheseto (*icipe*) for the technical and operational support during the project lifespan and Mr. Richard Otieno (*icipe*) for assisting in maintenance of the mosquito colony. We also gratefully acknowledge logistical support for this research from Kenyatta University and *icipe*.

Funding

The work was supported by Grand Challenges Canada www.grandchallenges.ca/ (grant number: 02691). The views expressed herein do not necessarily reflect the official opinion of the donors.

Availability of data and material

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Fig. 1.
Map showing geographical sites of *M. koenigii* plant collection in Kenya.

Fig. 2.

A morphometric aspect of *Anopheles gambiae* specimen after exposure to the bioactive fraction of *M. koenigii* observed at $\times 20$ magnification. Panel A - control specimen of Larvae, pupa, and adult. Panel B and C - treated with the most larvicidal fraction (Kibwezi).

Fig. 3.
LC-QToF-MS analysis profile of bioactive fraction of *Murraya koenigii* leaf extract.
Neplanocin A (m/z 264.1021) was the most abundant compound (40.03%).

Table 1

Mortality (mean % $\pm$ SE) of L₃/L₄ instars of *Anopheles gambiae* over 72 h upon exposure to acute toxic levels of *M. koenigii* extract. Means with the same letters within a column are not significantly different at 5% level.

Time	Concentration 100ppm		
	24hrs	48hrs	72hrs
Kibwezi	18.3 $\pm$ 1.74 a	43.0 $\pm$ 2.75 a	80.3 $\pm$ 1.65 a
Makindu	26.7 $\pm$ 1.80 a	40.3 $\pm$ 1.90 a	55.7 $\pm$ 2.58 b
Malindi	15.7 $\pm$ 2.50 a	27.7 $\pm$ 3.90 ab	28.7 $\pm$ 3.43 c
Mombasa	13.0 $\pm$ 2.43 ab	18.3 $\pm$ 3.73 bc	26.7 $\pm$ 4.19 c
Control	0.0 $\pm$ 0.00 b	0.3 $\pm$ 0.00 c	1.3 $\pm$ 0.59 d

Table 2

Median toxicity (LC₅₀) responses of L₃ *An. gambiae* to crude and fractionated *M. koenigii* Kibwezi extract over 72 h. LC₅₀ were determined for each dose at their 95% confidence intervals. All χ^2 values presented are significantly different at 0.05 levels of p and represent goodness of fit of the regression lines in the probit analyses.

Extract type	Time	LC ₅₀ (ppm)	95% CI	Slope (± SE)	χ^2
Crude	24 hrs	159.80	96.90 - 263.67	1.84 ± 0.11	0.92
	48 hrs	76.40	49.60 - 116.84	2.29 ± 0.09	0.70
	72 hrs	66.56	46.96 - 94.34	3.14 ± 0.78	0.98
Fraction	24 hrs	4.43	3.30 - 6.00	3.35 ± 0.06	0.71
	48 hrs	3.62	2.68 - 4.87	3.31 ± 0.07	0.72
	72 hrs	2.43	1.86 - 3.17	4.37 ± 0.06	0.97

Table 3

Effects of *M. koenigii* fractionated bioactive leaf extracts at concentration of LC₅₀ on the growth and development of fourth instar of *An. gambiae* mosquito larvae. Means with the same letters within a column are not significantly different at 5% level.

	Inhibition of adult emergence (%)	Larval period (days)	Median day of pupation	Harley mean Index (Hi)
Bioactive fraction	98.70 ± 0.42 ^a	14.50 ± 0.71 ^a	10	0.71 ± 0.69 ^a
Control	8.81 ± 0.29 ^b	7.58 ± 0.87 ^b	2	61.41 ± 0.85 ^b

Table 4

Constituents of *M. koenigii* bioactive fraction. Data show retention time (Rt), peak area percentage, monoisotopic mass m/z , chemical formula, and putative compound identification from published literature and publicly accessible online databases.

No.	R _t (mins)	Peak Area %	m/z [M + H] ⁺	Chemical formula	Putative ID
1	0.73	0.87	216.1015	C ₁₃ H ₁₃ NO ₂	3-(1-Naphthyl)-L-alanine
2	2.56	15.26	197.1171	C ₅ H ₁₆ N ₄ O ₄	?
3	3.56	3.83	257.1048	C ₁₃ H ₁₂ N ₄ O ₂	Lumiflavine
4	4.00	4.83	181.1230	C ₅ H ₁₆ N ₄ O ₃	?
5	4.73	40.03	264.1021	C ₁₁ H ₁₃ N ₅ O ₃	Neplanocin A
6	5.35	13.92	277.2169	C ₁₄ H ₃₀ NO ₄	?
7	5.67	9.08	323.1522	C ₁₆ H ₂₂ N ₂ O ₅	Terezine C
8	5.90	4.81	248.1079	C ₁₁ H ₁₃ N ₅ O ₂	Agelaspongine
9	6.81	6.42	348.1968	C ₂₃ H ₂₅ NO ₂	Murrayazolinol