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LARVICIDAL PROPERTIES OF SIMALIKALACTONE D FROM *QUASSIA AFRICANA* (SIMAROUBACEAE) BAILL AND BAILL, ON THE MALARIA VECTOR *ANOPHELES GAMBIAE*

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Abstract

Background: Botanical and microbial insecticides have been increasingly used for the control of mosquito given their efficacy and documented non-toxic effects on non-target organisms. The discovery of new insecticides is imperative because of the development of resistance by the mosquitoes to the readily available insecticides. The aim of this study was therefore to isolate and characterize compounds from a local medicinal plant, *Quassia africana* Baill and Baill (Simaroubaceae) that were toxic to *Anopheles gambiae*.
Material and methods:The methanol extracts of the leaves, stem and roots of *Quassia africana* were tested against fourth instar larvae of *An. gambiae*. The root extract was partitioned into hexane, chloroform and ethyl acetate and the resulting extracts screened for larvicidal properties. The extracts and the fraction with the highest bioactivity were subjected to repeated column chromatography and isolated compounds evaluated for potential toxicity to *An. gambiae* larvae. The structure of the active compound was elucidated using spectroscopic techniques.
The root extract showed the strongest activity profile (LC_{50} = 17.58 µg/mL). The chloroform soluble fraction obtained after partitioning the crude extract into solvents based on polarities was the most toxic. Further bio-activity-guided chromatographic separation of the chloroform fraction of the root extract led to the identification and isolation of a simalikalactone D as the larvicidal compound in *Q. africana* (LC_{50} = 1.25 µg/mL).
Results: Results suggest that *Q. africana* may serve as a source for vector control agent for malaria.
Conclusion: Simalikalactone D was identified as the larvicidal compound in *Q. africana* (LC_{50} = 1.25 µg/mL).

Key words: vector control, larvicidal activity, spectroscopy

Introduction

Malaria remains a serious and growing health problem in many developing countries, particularly in the Afro-tropical region (sub-Saharan Africa) (WHO, 2000). One of the programs designed to reduce the transmission of important vector-borne diseases, such as malaria, is the use of chemicals in reducing the risk of human-vector contact (Najera & Zaim, 2003). The number of chemicals for such uses is becoming increasingly limited due to various factors (e.g., bans, environmental concerns, potential adverse effects on human health, resistance, loss of licensure). These issues in conjunction with increasing resistance to anti-malarial drugs and insecticides, inadequate health care systems, population displacement and declining community acceptance have further reduced the effectiveness of the malaria control approache/s developed in the eradication era; prompting a need to identify and develop alternative vector control methods as well as the production of new chemicals (Zaim & Guillet, 2002).

Quassia africana Baill & Baill (Simaroubaceae) is a shrub that grows in swampy areas in tropical Africa from Nigeria to Angola. All parts of the plant especially the roots are used medicinally (Daziel, 1937). The root bark of the plant is claimed by many traditional healers of this region to have anthelminthic and analgesic (Ayafor *et al*., 1993), anti-malarial and anti-diarrhea (Otshudi *et al*., 2000) properties. It is also used as a condiment for the treatment of gastrointestinal disturbances, as a vermifuge, a remedy for bronchial pneumonia, for venereal diseases, for wound dressing, as a febrifuge and as an anti-inflammatory (Iwu, 1993).

Species of the Simaroubaceae family are known to contain bitter substances called quassinoids and these quassinoids display a wide range of biological activities *in vivo* and *in vitro* including anti-feedant and insecticidal (Guo *et al*., 2005). Quassin, which was one of the first quassinoids to be isolated and structurally elucidated (Guo *et al*., 2005), was inactive in most biological activities but active as an aphid anti-feedant (Polonsky *et al*., 1989).

Furthermore, our studies of simaroubaceae plants and the systemic search for natural products that are toxic to *Anopheles gambiae* (Ajaiyeoba *et al*., 2008; 2009), the malaria vector, we present in this paper an activity-led isolation of a larvicidal quassinoid.

Materials and methods

General

One-dimensional (1D) NMR (^{1}H NMR, ^{13}C NMR, DEPT) and 2D NMR spectra were recorded in CD_3OD on a Bruker AM-300 MHz spectrometer. Chemical shifts were measured in ppm (δ) and coupling constants (*J*) are given in Hz. Mass spectra (Electron Impact), (EIMS) was recorded on Varian MAT 312 double Focusing spectrometer or on a Finnigan MAT 311 with MASS PEC data system. Peak matching and field adsorptions (FD) experiments were performed on Finnigan MAT 312X mass spectrometer. Fast atom bombardment mass spectra (FAB MS) were recorded on Jeol HX 600 mass spectrometer. Exact molecular formulae were determined by high resolution mass spectrometry (HRMS). Glycerol was used as reference compound for FAB (+ve). TLC on silica gel 60 F_{254}. Spot detection: UV light at 254 nm and spraying with ferric sulphate..

Plant material

Q. africana roots, stem and leaves were collected from Kribi, Cameroon and authenticated by Mr. Mezili at the National Herbarium of

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Cameroon where voucher specimens were deposited. The plant was identified under HNC 65074.

Extraction and isolation

The dried ground leaves (439 g), stem bark (640 g) and root bark of *Q. africana* (950 g) were extracted by maceration in redistilled methanol for 72 hrs at room temperature. The solvent was evaporated to dryness using a rotary evaporator at 40 ºC to afford 75, 56 and 31.5 g of root, stem and leaves extracts respectively. The root extract, which was the most toxic to the mosquitoes, was further fractionated into hexane (9 g), $CHCl_3$ (12 g), EtOAc (6 g) and CH_3OH (48 g).

The $CHCl_3$ soluble fraction of *Q. africana* (12 g) was chromatographed on silica gel G (60-40 mm) using column chromatographic technique and eluted with Hex:EtOAc and then EtOAc:MeOH mixtures of increasing polarity. Fractions (89) of 100 mL each were collected. After monitoring with TLC (silica gel, Hex/EtOAc, 3:5), fractions 55-68 that eluted with 20% MeOH in EtOAc, were pooled together.

Compound **1** (200 mg) R_f 0.5 (Silica gel, Hex:EtOAc:CH_3OH 3:7:1), was identified as the larvicidal compound. It crystallized from ethylacetate to give off a white powder that was visible as a dark blue spot when sprayed with ferric sulphate reagent. Melting point was 225-230°C. NMR spectra (Bruker AM-300 MHz spectrometer) were identical to those reported previously (Tresca *et al*., 1971; Moher *et al*., 1992; Apers *et al*., 2002).

Preparation of stock solutions for larval toxicity assay

Stock solutions of hexane, chloroform and ethyl acetate soluble extracts were prepared by dissolving 50 mg of each extract in 1 mL of ethanol. Other tests solutions of 25 and 12.5 mg/mL were prepared by diluting the stock solution in appropriate volumes of ethanol. Stock solution of the fraction obtained by column chromatography was prepared at 15 mg of each fraction in one mL of ethanol. Tests solutions of between 0.3 and 15 mg/mL were prepared by diluting the stock solution in appropriate volumes of ethanol. Stock solution of the compound was prepared at 2.5 mg in 1 mL of ethanol. Tests solutions of 0.31, 0.625 and 1.25 mg/mL were prepared by diluting the stock solution in appropriate volumes of ethanol.

Collection of mosquito larvae

Larvae were collected from mosquito breeding sites in Oyo state of Nigeria. Collected larvae were washed in clean water and reared in several plastic bowls in the insectaries of the Zoology Department, University of Ibadan. Identification of mosquito larvae was done at the Centre de Researche Entomologique de Cotonou (CREC), Cotonou, Benin Republic by Mr. Djouaka Rousseau. Larvae were fed with dog biscuit until they were mature enough to be used for experiments.

Larval toxicity assay

Sterile disposable cups were used for the study. Twenty, fourth instar, larvae were released into each cup containing 99 mL of clean well water and 1 mL of the extract fraction or compound to be screened at different concentrations. All experiments were done in duplicates. After 24 hrs contact, the number of dead larvae in each cup was counted. The larvae were considered dead if unable to reach the water surface and if there was no movement when pricked with a needle. Control experiments without the extract, with 1% ethanol and with DEET were run in parallel.

Statistical analyses

The lethal concentration was determined using the non-linear regression analysis, GraphPad Prism, and corrections for mortality when necessary were done by using Abbot's (1925) formula.

Results

Crude extracts of *Q. africana* root, stembark and leaves and *Q. amara* leaves were evaluated for their larvicidal activity. Results showed that all crude extracts tested were toxic to the mosquito larvae but the larvae were more susceptible to *Q. africana* root extract, which had an LC_{50} value of 17.58 µg/mL. The crude methanol extracts of all plant parts were further partitioned into hexane, chloroform and ethlyacetate and the resulting extracts subjected to larval toxicity assay. The chloroform extract of the root bark of *Q. africana* displayed the highest toxicity to *Anopheles* larvae (Figure 1).

Table I: LC_{50} values of the fractions of chloroform extract of roots of *Q. africana* obtained from the column.

Fractions	LC_{50} (µg/mL)
B	22.66
C	18.00
D	4.11
F	14.37
H	38.6
L	43.10
M	37.5

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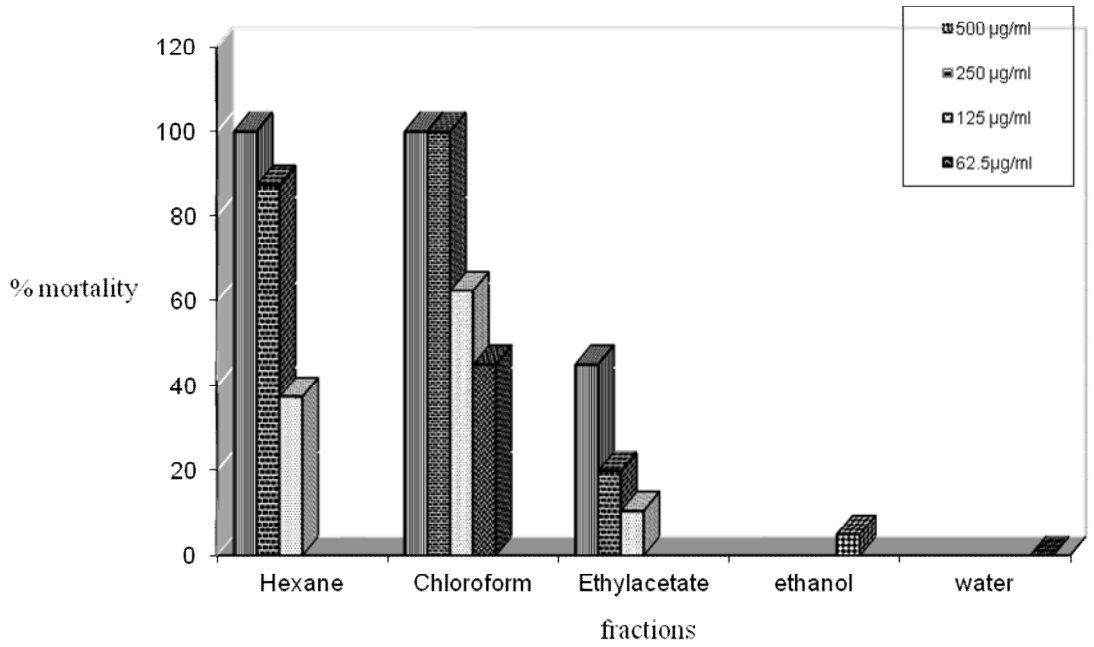

Fig 1: Larvicidal activity of fractions obtained from the root bark of Q. africana at 24h of exposure

Subjecting the chloroform extract to repeated column chromatographic technique and eluting with Hex:EtOAc and then EtOAc:MeOH mixtures of increasing polarity yielded 89 fractions. Fractions D which was a combination of fractions 55-68 was the most active fraction with an LC_{50} of 4.11 µg/mL (Table 1), and contained compound **1** which crystallized in ethylacetate to give off a white powde

Fractions A,E,G,I,J and K were not sufficient for larvicidal studies. Compound **1,** when evaluated for its toxicity to *An. gambiae* larvae displayed high toxicity with an LC_{50} of 1.25 µg/mL. 200 mg of compound **1**, R_f = 0.5 (Silica gel G, Hex:EtOAc:CH3OH 3:7:1), precipitated from ethylacetate to give off a white powder that was visible as a dark blue spot when sprayed with ferric sulphate reagent. Melting point was 225-230°C. By comparing NMR signals of proton and especially of the ^{13}C NMR spectrum of compound **1** with literature (Apers *et al*., 2002), the structure of this compound was elucidated as Simalikalactone D (**1**).

The EI-MS and FAB-MS of compound **1** showed a molecular ion at m/z 478(1.5), 460(7.5), 358(38.1), 340(30.3), 301(34.0), 284(17), 273(15), 225(12.9), 165(33.6), 151(30.3), 135(26.6) 123(42.5), 85(55.6), 69(20.5), 57(100), calculated for C25H34O9.
IR Vmax cm-1 : 3458, 2971, 2369, 1722, 1437, 1377, 966, 944, 866, 803, 715, 695, 662, 605, 543, 401.

Detailed analysis of the 1D and 2D NMR spectra allowed complete ^{1}H- and ^{13}C- NMR assignments and the establishment of the structure of this compound (Fig. 1). These are represented in Table 2.

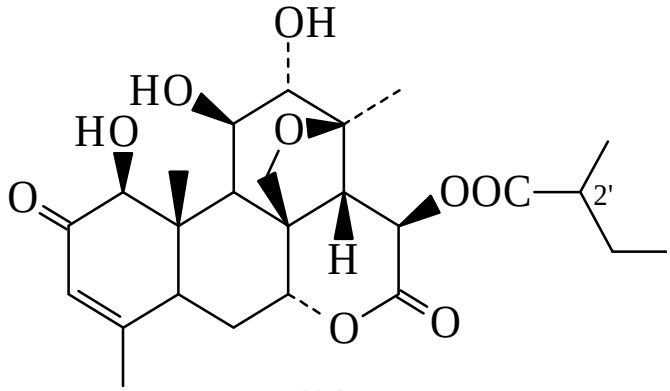

Figure 2: Simalikalactone D

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Table 2: ^{1}H- and ^{13}C-NMR assignments for simalikalactone D

Position	^{13}C (δ)	DEPT	^{1}H (δ, mult.)
1	83.23	CH	4.27, s
2	199.88	C	
3	125.28	CH	6.03, brd
4	165.36	C	
5	43.49	CH	2.98, d
6	29.12	CH_2	a: 2.29,d b: 1.92, m
7	85.24	CH	4.77, s
8	47.28	C	
9	44.76	CH	2.39, d
10	49.53	C	
11	75.79	CH	4.65, brd
12	80.23	CH	3.57, brs
13	81.93	C	
14	53.37	CH	2.53, d
15	69.33	CH	6.27, d
16	169.99	C	
17			
18	22.58	CH_2	1.96, brs
19	11.51	CH_3	1.18, s
29	23.73	CH_3	1.37, s
30	72.92	CH_3	a: 4.59, brd b: 3.53, brd
1´	176.75	C	
2´	42.39	CH	2.44
3´	27.92	CH_2	1.73, m
4´	11.92	CH_3	0.97, t
5´	16.92	CH_3	1.16, d

Discussion

Simalikalactone D had previously been characterized and described by several authors (Tresca *et al*., 1971; Moher *et al*., 1992; Apers *et al*., 2002). Anti-feedant properties of simalikalactone D have been investigated previously against larvae of stored grain pests. At concentration points between 10-500 ppm, simalikalactone D and other quassinoids including bruceantin, glaucarubinone and isobruceine A, displayed anti-feedant activity against the Mexican bean beetle 4th instar larvae and the Southern armyworm 5th instar larvae (Leskinen *et al*., 1984). Our present investigation showed that larvae of *An. gambaie* are susceptible to simalikalactone D. Triterpenoids are known to possess insect growth regulation activity against a variety of agricultural pests (Govindachari *et al*., 2001). Quassinoids, being modified triterpenoids can be expected to show similar activity; however, *Q. africana* had not so far been tested for activity against insects of medical importance. Fortunately, simalikalactone D, has been completely synthesized (Moher et al., 1992). This compound could provide a new lead to novel class of larvicidal compounds. Simalikalactone A, B, C and D and other quassinoids including quassin and quafrinoic acids have previously been isolated from *Q. africana* (Ajaiyeoba & Krebs, 2003). Simalikalactone D, was identified as the active anti-malarial compound, from *Q. amara* against FcB1 *Plasmodium falciparum* chloroquine resistant strain *in vitro* (Bertani *et al*., 2006).

Plant derived products have received increased attention from scientists and more than 200 plant species are already known to have insecticidal properties (Senthil Nathan *et al*., 2008). One advantage of targeting larvae is that they cannot escape from their breeding sites until the adult stage and unlike adult mosquitoes, cannot easily avoid control measures (Walker & Lynch, 2007). Simalikalactone D and other naturally occurring insecticides may play a more prominent role in mosquito control programs in the future.

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