

Anti-Proliferative and Anti-Plasmodia Activity of Quinolactacin A2, Citrinadin A and Butrecitrinadin co-isolated from a Ghanaian Mangrove Endophytic Fungus *Cladosporium oxysporum* strain BRS2A-AR2F

Keywords: Mitochondria; Adhesive cells; Suspension cells; Apoptosis; Buruli ulcer

Abstract

The compounds quinolactacin A2 (2) and citrinadin A (13) which are originally considered to be the compounds of fungi genus *Penicillium*, were co-isolated for the first time from the new fungal strain *Cladosporium oxysporum* strain BRS2A-AR2F (Genebank number KX257257) which is an endophyte of the Ghanaian mangrove plant *Rhizophora racemosa*. A derivative of citrinadin A, which was named butrecitrinadin (12) was also isolated from fermentation cultures of this new fungal strain. The structures of quinolactacin A2 (2), citrinadin A (13) and butrecitrinadin (12) were determined using a combination of mass spectrometry with 1D and 2D NMR data analysis. The anti-proliferative activities of the three compounds 2, 12 and 13 were tested using human cancer cell lines HL-60, Jurkat cells, LNCap, PC3, HepG2, and MCF7. The compounds were found to show some level of anti-proliferative activity against some of the cell lines compared to curcumin as a positive control. The three compounds however, showed no activity against Jurkat and PC-3 cells. Compounds 2, 12 and 13 were also tested against the chloroquine sensitive *Plasmodium falciparum* 3D7 strain for anti-plasmodial activity. Only 2 showed anti-plasmodial activity within the concentration range tested with an EC₅₀ value of 24.80 μM compared to artesunate 0.074. Since this represents the first study of the anti-plasmodial activity of quinolactacin A2 (2), the apoptotic activity of 2 on 3D7 plasmodia strain was evaluated by studying the effect of the compound on the mitochondrion membrane potential of the parasite. The results indicated the dissipation of the mitochondrial membrane potential (ΔΨm) and abolition of probe accumulation in a concentration dependent manner. Hence 2 may provide a drug development scaffold for apoptotic death in the stages of *Plasmodium falciparum* development. The three compounds 2, 12 and 13 were also tested for anti-buruli ulcer activity on *Mycobacterium ulcerans* MN209 characterized isolates but, were found to be inactive.

Introduction

The quinolactacins are novel pyrrolo [3,4-b] quinolone-type compounds isolated from fungi and are mostly methylated at the nitrogen of the quinolone moiety [1,2]. Amongst the quinolactacin compounds isolated to date, it is only quinolactacide (11) that appears to be missing the N-methyl substituent on the quinolone moiety [3]. Also, only quinocitrinine A (9) and B (10) bear additional substituents in the form of a hydroxyl group on the quinolone which is

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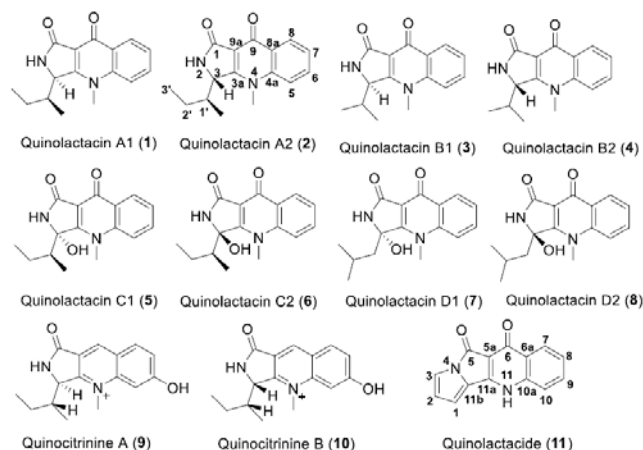
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otherwise free of further substitution [4]. The γ-lactam ring however, normally bears substituents at the C-3 position. These substituents are 2-butyl in the case of quinolactacin A1/A2 (1/2) [5] and quinocitrinine A/B (9/10) [4] but, in the case of quinolactacins C1/C2 (5/6) [6] there is a hydroxyl group at the same position in addition

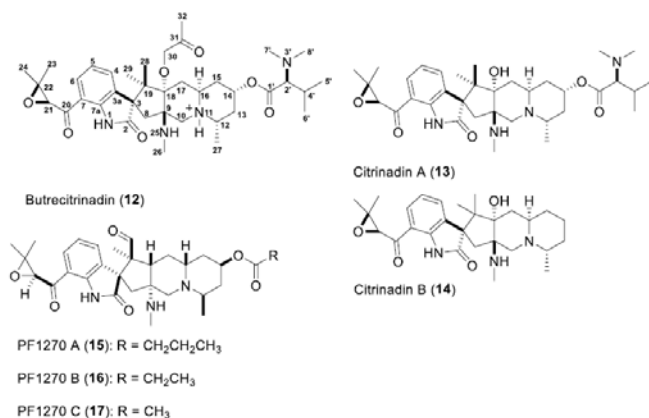


to the 2-butyl substituent. Quinolactacins D1/D2 (7/8) [6] have an isobutyl substituent at C-3 of the lactam ring while quinolactacins B1/B2 (3/4) [6] have an isopropyl at same position of the γ-lactam ring. In the structure of quinolactacide (11) [3], the γ-lactam ring is fully substituted to give a tetracyclic quinolone backbone which is numbered differently.

For compounds that contain a quinolone moiety, which can be

found in the structures of many very important and potent antibiotics currently in the clinic [7-11], no significant antimicrobial data has been reported for the 11 compounds presently characterized in this group. Biological activity studies have focused mainly on cytotoxicity, acetyl cholinesterase and tumour necrosis factor inhibition and insecticidal activity [1-6]. So far, the primary source organisms for the quinolactacins has been *Penicillium* sp. and *Penicillium citrinum* [1-6].

The citrinadins are a small group of pentacyclic alkaloids that have been found to be associated with fungi [12]. All the citrinadins possess a spirooxindole which is fused to a tricyclic system containing a quinolizidine moiety [12]. Furthermore, the substituent on the spirooxindole portion for these naturally occurring citrinadins, which is an α,β -epoxy carbonyl unit is the same [13]. The only structural difference between these compounds lies in the kind of



substituents on the quinolizidine containing tricyclic ring system. In citrinadin A (13), there is an N,N-dimethyl valine residue at C-14 [12] which is absent in the structure of citrinadin B (14) [13]. For the citrinadin-type compounds PF1270 A (15), B (16) and C (17), there exist butyrate, propionate and acetate substituents respectively at the C-14 position [14].

Generally, owing to their inherent three-dimensional structure and novelty, spiro scaffolds have gained increased attention in drug discovery. In particular; chiral spirooxindoles represent a class of lead compounds for a variety of biologically diverse diseases. These include inflammation, cancer and parasitic diseases [15-17]. Both citrinadin A (13) and citrinadin B (14) have also demonstrated cytotoxicity against murine leukemia L1210 cells with IC₅₀ values of 6.2 and 10.0 $\mu\text{g/ml}$ respectively [13]. Furthermore, citrinadin A (13) has demonstrated activity against human epidermoid carcinoma KB cells with IC₅₀ value of 10.0 $\mu\text{g/ml}$ [13]. PF1270 A (15), B (16) and C (17) have also shown high affinity for both rat and human histamine H₃ receptor ligands by functioning as potent agonists with EC₅₀ values of 0.12, 0.15 and 0.20 μM respectively [14]. As a result of the fact that, H₃ receptor ligands play a role in the release of neurotransmitters such as serotonin, noradrenalin and dopamine, it is envisaged that, they may hold the potential for the treatment of diabetes, obesity and central nervous system disorders [14].

So far, all the known naturally occurring citrinadin compounds

have been isolated from *Penicillium citrinum* and *Penicillium waksmanii* [12-14].

Herein we report for the first time, the co-isolation of quinolactacin A2, citrinadin A and butrecitrinadin from a Ghanaian mangrove plant-derived endophytic fungus *Cladosporium oxysporum* strain BRS2A-AR2F (KX257257). To the best of our knowledge, this represents the first report of citrinadin compounds from a genus other than *Penicillium* and butrecitrinadin is a new compound. Using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, the antiproliferative activity of the three compounds were tested against six human cell lines namely; Jurkat (Human T-lymphoblastic leukemia cells-suspension cells), HepG2 (Human hepatocellular carcinoma-adhesive cells), HL-60 (Human promyelocytic leukemia cells-suspension cells), LNCap (Human prostate cancer-adhesive cells), MCF-7 (Human breast cancer-adhesive cells) and PC-3 (Human prostate cancer-adhesive cells). We also evaluated the anti-plasmodia activity of the three compounds on 3D7 plasmodia strain using the SYBR Green I assay and went further to evaluate the plasmodia apoptotic activity of quinolactacin A2. The compounds however, did not show any anti-buruli ulcer activity when tested against *Mycobacterium ulcerans* MN209.

Materials and Methods

Mangrove plant sample collection

The Western Region of Ghana is noted for its bio-diversely rich indigenous mangrove plants. Out of the six indigenous plants that are characteristic of the mangroves found in Ghana, three of them are particularly common in the Western Region and these are *Conocarpus erectus*, *Laguncularia racemosa* and *Rhizophora racemosa* (coordinates: 4°49'43.73'N and 1°54'50.84'W). Collection of mangrove plants was done along the banks of the River Butre (Figure S1) with five sampling sites chosen at 100 m apart from the shore to where the river meets the sea. The three main plant samples collected were *Conocarpus erectus*, *Laguncularia racemosa* and *Rhizophora racemosa* but, different plant parts were sampled at different sections

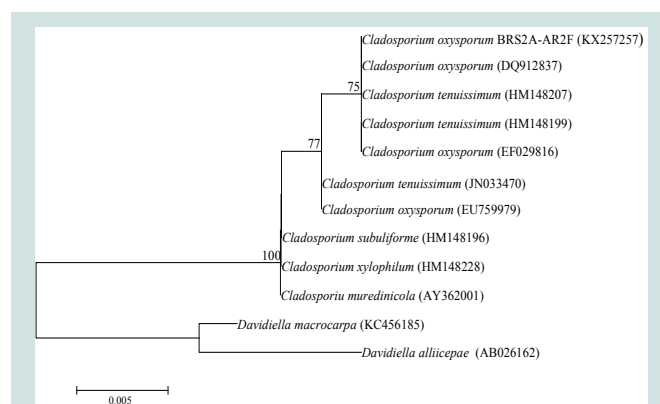
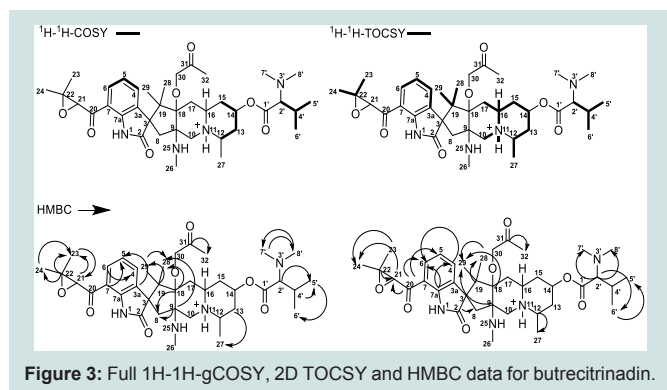
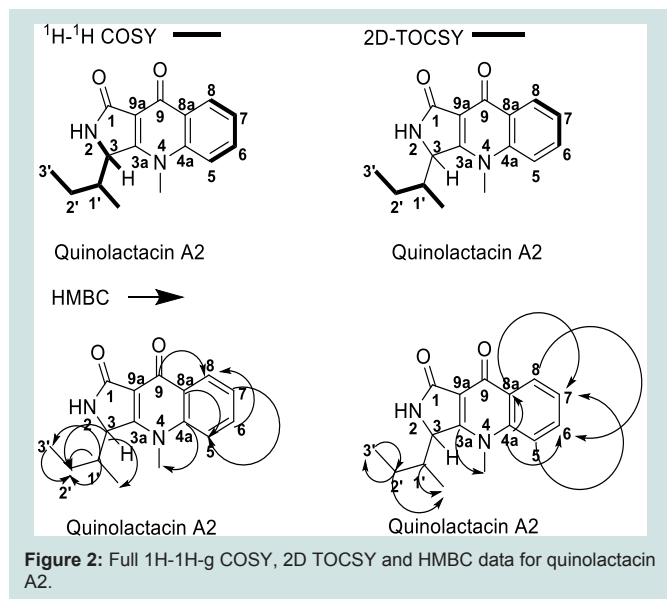


Figure 1: Neighbour-joining phylogenetic tree based on ITS1-5.8S-ITS2 gene sequences showing the relationship between strain BRS2A-AR2F and closely related species. Numbers at nodes indicate levels of bootstrap support (percentages of 1000 resamplings); only values above 50% are shown. GenBank accession numbers are given in parentheses. Bar, 0.005 substitutions per site.



of the river. The plant parts sampled were leaves, buds, submerged roots, aerial roots, aerial stems, fruit shoots and flowers. The exact positions of plants from which different parts were sampled were stored in a GPS and the data uploaded into Google Earth satellite database. The samples were videoed, photographed, bagged, labelled and stored at 4 °C in an ice-chest and transported to the Department of Chemistry, University of Ghana, Legon. Identification of the plants was done at the Department and specimens of all plant parts collected were dried in newspapers for onward submission to the herbarium.

Preparation of culture media

Malt extract solid media: Approximately 15 g of malt extract and 15 g of bacteriological agar were weighed into a 1 L autoclave bottle containing 900 ml of water and stirred to mix with a magnetic stirrer. The mixture was autoclaved after which it was cooled to about 55 °C and then poured into petri dishes (90 mm) to fill about 2/3 of the volume of each Petri dish under sterile conditions (25 plates for each 900 ml preparation). The plates were left half-open under a clean bench until almost all the water had evaporated from the plates. Dry plates were parafilm and kept in a fridge at 4 °C until needed.

Malt extract liquid media: Approximately 15 g of malt extract

was weighed into a 1 L autoclave bottle containing 900 ml of water and stirred to mix with a magnetic stirrer. The mixture was autoclaved and allowed to cool and then stored in a fridge at 4 °C until needed.

Treatment of *Rhizophora racemosa* and culture of BRS2A-AR2F

In the laboratory, pieces of the aerial roots of *Rhizophora racemosa* was surface sterilized under sterile conditions by first rinsing them with sterile artificial sea water (SASW) and then immersing them in 70% ethanol for 1 minute. The plant parts were then cut transversely at all sides into a smaller piece with a pair of flame sterilized scissors in a bio-safety cabinet. The pieces were again sterilized under sterile conditions by first rinsing them with SASW and then immersing them in 70% ethanol for 1 minute. The pieces were again cut at all sides into much smaller pieces with a flame sterilized scalpel. These pieces were again sterilized by rinsing with SASW, then immersing in 2% sodium hypochlorite for 1 minute and then rinsed again with SASW 3 times. The treated aerial roots were afterwards placed on malt extract agar plates (parent or master plates) and the plates were labelled, parafilm and incubated at 28 °C for three weeks with daily observations to detect the appearance of new fungal colonies.

Isolation of pure endophytic fungi

Observation of the parent or master plates obtained for the aerial roots of *Rhizophora racemosa* starting from day one (1) to day twenty-one (21) saw the appearance on the parent plates of several different colonies of marine endophytic fungi which were subsequently picked one at a time, re-cultured on fresh malt extract plates and kept at an incubation temperature of 28 °C. All the colonies initially sub-cultured from the parent or master plates were subsequently sub-cultured until very pure strains were obtained for each species, one of which was the endophytic fungi BRS2A-AR2F (Figure S2).

Taxonomic identification of strain BRS2A-AR2F (Genbank Number KX257257)

Genomic DNA of strain BRS2A-AR2F was extracted using ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. The ITS1-5.8S rDNA-ITS2 region of the rDNA was amplified using the forward (ITS1: 5'-TCC GTA GGT GAA CCT GCG G-3') and reverse (ITS4: 5'-TCC TCC GCT TAT TGA TAT GC-3') universal primers [18] with GoTaq Hot Start master mix (Promega Corp., Madison, WI, USA). Amplification was performed on a T100 Thermal Cycler (Bio-Rad, USA) as follows: 1 cycle of 2 minute at 95 °C (primary denaturation), 30 cycles of 1 minute at 95 °C (denaturation), 1 minute at 55 °C (annealing), 1 minute at 72 °C (extension) and finally 1 cycle of 5 minute at 72 °C. PCR-amplified products were sequenced using a DNA sequencer ABI PRISM 3730 XL automatic sequencer (Applied Biosystems, Foster City, CA, USA). The generated sequence of strain BRS2A-AR2F (507 bp) was compared with other fungal DNA sequences from NCBI's Gene Bank sequence database using a BLAST (Basic Local Alignment Search Tool) (<http://blast.ncbi.nlm.nih.gov/>) search. Multiple alignments with sequences from closely related species were performed by using the program CLUSTALW in the MEGA6 software package [19].

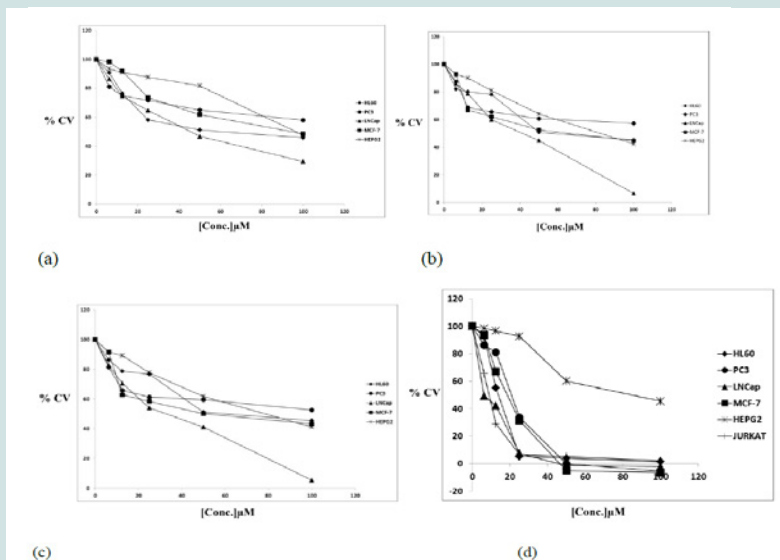


Figure 4: Anti-proliferative activities of compounds: a) quinolactacin A2, b) citrinadin A c) butrecitrinadin and d) curcumin on human cancer cell lines. The MTT Assay was used and the results are expressed as a mean of duplicate experiments done in triplicates

Phylogenetic tree was constructed with neighbour joining (NJ) [20]. Evolutionary distances were calculated using model of Jukes and Cantor (1969) [21]. Topologies of the resultant trees were evaluated by bootstrap analysis based on 1000 re-samplings [22].

Small scale culture of *Cladosporium oxysporum* strain BRS2A-AR2F

The malt extract liquid broth prepared earlier was thawed in a water bath and 100 ml was transferred into an already autoclaved 250 ml Erlenmeyer flasks with non-absorbent cotton wool stuck in the mouth under sterile conditions. A single colony from a *Cladosporium oxysporum* strain BRS2A-AR2F plate was picked and inoculated into the broth under sterile conditions. The inoculated flask was labelled and incubated in an incubator at 28 °C for two weeks. After two weeks, the culture broth was filtered by suction filtration to separate fungal mycelia from the broth itself. The broth was extracted once with ethyl acetate and the mycelia

repeatedly and alternatively extracted with methanol and dichloromethane. The ethyl acetate, methanol and dichloromethane extracts were dried under vacuum and subsequently combined to give a total crude extract (TCE).

Chemical profiling of *Cladosporium oxysporum* strain BRS2A-AR2F

About 1 mg of the TCE obtained for the small scale culture in malt extract liquid media of *Cladosporium oxysporum* strain BRS2A-AR2F was subjected to high resolution mass spectrometric analysis using a Thermo Instruments MS system (LTQ XL/LTQ Orbitrap Discovery) coupled to a Thermo Instruments HPLC system (Accela PDA detector, Accela PDA autosampler, and Accela pump). The following conditions were used: capillary voltage 45 V, capillary temperature 320 °C, auxiliary gas flow rate 10-20 arbitrary units, sheath gas flow rate 40-50 arbitrary units, spray voltage 4.5 kV, mass range 100-2000 amu (maximum resolution 30,000). HPLC separations were carried out using a Phenomenex reversed-phase (C18, 250 6 10 mm, L x i.d.) column connected to an Agilent 1200 series binary pump and monitored using an Agilent photodiode array detector. Detection was carried out at 227 nm. This data provided the photodiode array profile, mass ion counts, specific UV and fragmentation pattern of all the metabolites present in the crude fermentation extracts of strain BRS2A-AR2F (Figures S3 and S4). Input of this information into the MarinLit, AntiMarin and Antibase databases provided detailed information on the compounds contained in the extract.

Large scale culture of *Cladosporium oxysporum* strain BRS2A-AR2F

After high resolution electrospray ionization-HPLC with diode array coupled mass spectrometry analysis, it was detected that marine-derived fungus, *Cladosporium oxysporum* strain BRS2A-AR2F produced interesting compounds. Therefore large scale fermentation in about 1 L broth was carried out to isolate these compounds. This was done by inoculating two already autoclaved 1 L

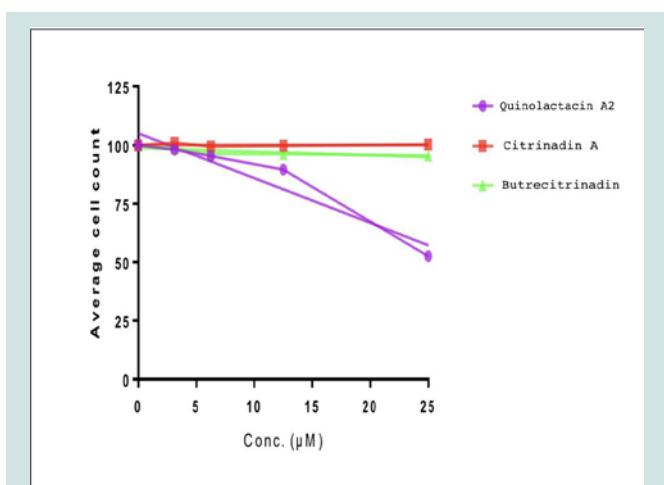


Figure 5: Anti-plasmodial activity of quinolactacin A2, citrinadin A and butrecitrinadin on 3D7 plasmodial strain. SYBR Green I Assay was used and the results are expressed as a mean of duplicate experiments.

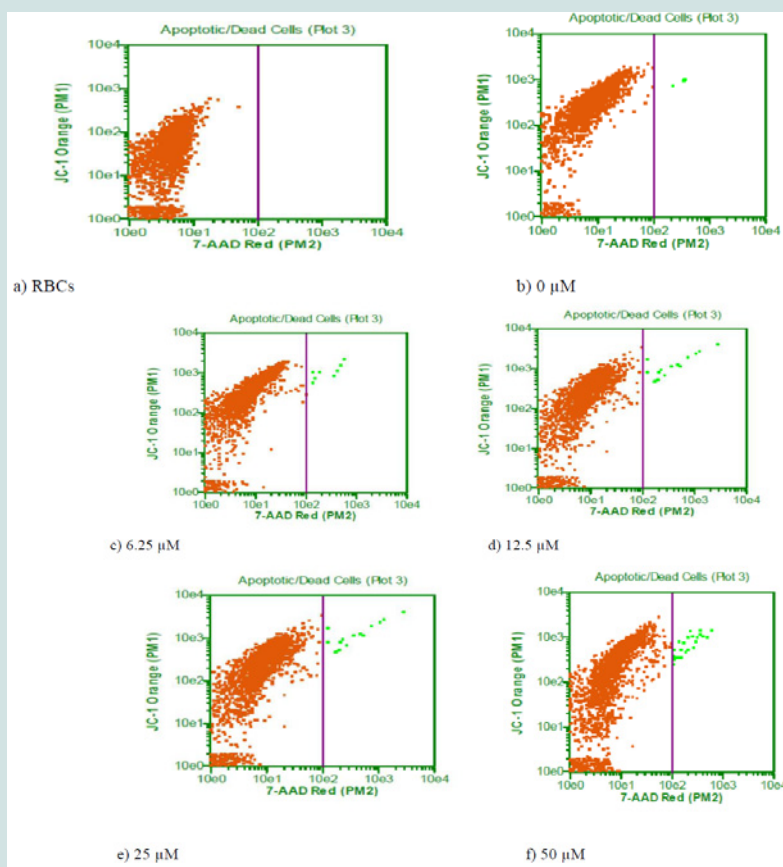


Figure 6: Effect of quinolactacin A2 on the loss of mitochondrion membrane potential. *Plasmodium falciparum* 3D7 strain were treated with different concentrations of quinolactacin A2: a) RBCs only b) 0 μM c) 6.25 μM d) 12.5 μM e) 25 μM f) 50 μM for 24 hours and analysed by flow cytometry after staining with JC-1.

Erlenmeyer flasks, sealed with non-adsorbent cotton wool, containing 500 ml of malt extract liquid broth with strain BRS2A-AR2F under sterile conditions. These flasks were labelled and incubated at 28 °C for a period of 2 months. At the end of the incubation period, the cultures filtered under suction and the mycelia separated from the broth. The broths were extracted once with ethylacetate and the mycelia combined and repeatedly and alternatively extracted with methanol and dichloromethane. The extracts obtained after drying under vacuum from the ethylacetate, methanol and dichloromethane fractions were combined to give a TCE (1.300 g).

Solvent partitioning by a modification of Kupchan's method

Following solvent extraction of the liquid broth and mycelia of BRS2A-AR2F was solvent partitioning. The procedures adopted were a modification of Kupchan's method (1973) [23] employing a combination of solvent systems with polarities ranging from the relatively non-polar hexane to the extremely polar water solvent. First, the TCE was suspended in water and extracted with an equal volume of dichloromethane three times. The result was two fractions, an aqueous fraction and a dichloromethane fraction. The aqueous fraction was extracted with an equal volume of *sec*-butanol once. Two separate fractions were obtained, the aqueous fraction from this end was discarded and the *sec*-butanol layer was rotary evaporated to dryness and labelled BRS2A-AR2F-WB (390.040 mg). The

dichloromethane fraction (908.075 mg) obtained initially from the first partitioning process was rotary evaporated to dryness under reduced pressure. This fraction was then suspended in 90% methanol:water and extracted with an equal volume of hexane (1:1) three times. The resulting hexane layer was rotary evaporated to dryness and labelled BRS2A-AR2F-FH (248.780 mg). The 90% methanol:water layer was then phase adjusted to 50% methanol:water and extracted with an equal volume of dichloromethane (1:1) three times. Both resultant fractions were rotary evaporated to dryness under reduced pressure and labelled BRS2A-AR2F-FD dichloromethane fraction (396.955 mg) and BRS2A-AR2F-FM 50% methanol:water fraction (256.488 mg) accordingly. Fractions that separated out in the solvent partitioning process possessed defined polarities.

Purification of BRS2A-AR2-FM and isolation of compounds

High resolution electrospray ionization-HPLC with diode array coupled mass spectrometry analysis of the Kupchan solvent partitioning extracts showed that, most of the compounds of interest were in BRS2A-AR2F-FM. Hence, this fraction was subjected to a size exclusion column chromatography using Sephadex LH-20 as stationary phase and methanol as mobile phase. Five Sephadex LH-20 fractions were collected and these fractions were concentrated under vacuum, transferred into vials and labelled BRS2A-AR2F-FM-SF1-5. Analysis of the Sephadex extracts showed that, SF2, SF3,

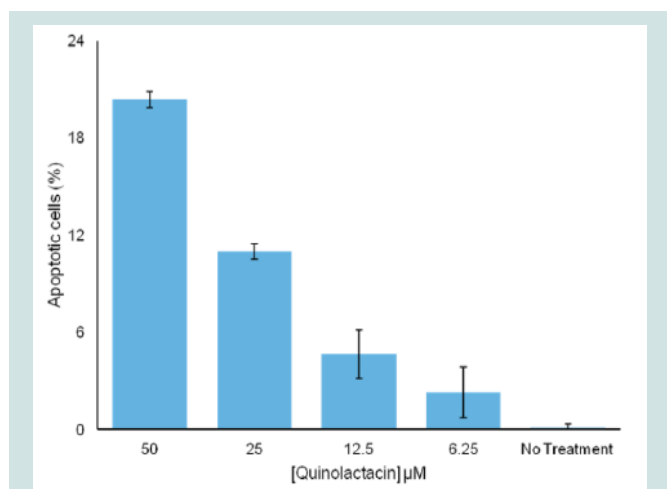


Figure 7: Percent apoptotic effect of quinolactacin A2 on the plasmodial strain after staining with JC-1. The results are expressed as a mean of triplicate readings.

SF4 and SF5 fractions all contained interesting compounds but the quinolactacins and citrinadins were found to be concentrated in the SF2 fraction (58.00 mg). Fraction BRS2A-AR2-FM-SF2 was subjected to alternating HPLC separation and purification using a Phenomenex Luna C18 column (C18 250 × 10 mm, L × i.d.) and column chromatography by gravity using silica as stationary phase and hexane-ethyl acetate mixtures as eluents. Gradients of H₂O:CH₃CN (1:1 in 30 minutes and hold for 20 minutes) were used as eluents for HPLC with column flow rates set at 1.5 mL/min to afford 3.900 mg of quinolactacin A2, 1.205 mg of butrecitrinadin and 3.030 mg of citrinadin A from 58.00 mg of sample BRS2A-AR2-FM-SF2.

1D and 2D NMR analysis of quinolactacin A2, citrinadin A and butrecitrinadin

NMR data were acquired on a Varian VNMR 600 MHz and Bruker 500 MHz spectrometers with either CDCl₃ or CD₃OD as solvents. Data acquired include ¹H, ¹³C, DEPT-135°, ¹H-¹H-COSY, HSQC, HMBC and gHSQC-TOCSY.

Chemicals and reagents

RPMI-1640, Dulbecco Modified Eagle's culture Media (DMEM), Foetal Bovine Serum (FBS), Trypsin, Gentamycin, Penicillin-Streptomycin-L-Glutamine (PSG), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Curcumin, Artesunate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye, Dimethyl sulphoxide (DMSO), Sodium citrate, Adenine, Sodium bicarbonate (NaHCO₃), AlbuMax II, Sodium chloride (NaCl), Potassium chloride (KCl), Sodium Phosphate Dibasic (Na₂HPO₄), Sodium Phosphate Monobasic (KH₂PO₄), Sodium bicarbonate (NaHCO₃), Sodium hydroxide (NaOH), Hydrochloric acid (HCl), 5,5',6,6'-tetrachloro-1, 1',3,3'-tetramethyl-benzimidazolylcarbocyanine iodide and 7-Aminoactinomycin D, Phosphate-buffered saline (PBS) were purchased from Sigma-Aldrich, USA. Malt extract, and other chemicals and reagents used were of analytical grade or of the highest purity commercially available.

Cell lines tested in the cytotoxicity assay

The under listed human cancerous cell lines used in the cytotoxicity assays conducted during the course of this project were kind gifts from Dr. Takuhiro Uto of Nagasaki International University, Japan.

Jurkat cells (Suspension cells)	Human T-lymphoblastic leukemia cells
HepG2 (Adhesive cells)	Human hepatocellular carcinoma
HL-60 (Suspension cells)	Human promyelocytic leukemia cells
LNCap cells)	Human prostate cancer (Adhesive cells)
MCF-7	Human breast cancer (Adhesive cells)

Preparation of compounds for bioactivity testing

Stock solutions of the compounds were prepared at a concentration of 10 mM. This was achieved by drying the compounds with nitrogen gas, weighing to ascertain the mass of the compound and dissolving in appropriate amount of DMSO in order to attain desired concentration. The solutions were vortexed and filter sterilized into vials through 0.45 μm millipore filters under sterile conditions and stored at -20 °C until use.

Cytotoxicity studies

Cell culture and cell treatments: Jurkat, HL-60, LNCap and PC-3 cells were cultured in RPMI-1640 medium, while the MCF-7 and HepG2 cells were maintained in DMEM. All cell cultures were supplemented with 10% FBS, 1% Penicillin-Streptomycin-L-Glutamine and incubated at 37 °C in 5% CO₂ under humidified atmospheric conditions. All the compounds in addition to curcumin (used as positive control) were dissolved in DMSO and stored at -20 °C until used. The DMSO concentration in the test wells did not exceed 0.1% (v/v), and the control cells were treated with the same amount of DMSO.

Cell viability assay

The growth inhibition of the cell lines due to the test compounds or standard was examined using the tetrazolium-based colorimetric assay (MTT Assay) to determine the viability of the cells at any particular time.

Principle: The assay was based on the capacity of the cellular mitochondrial reductase enzyme in living cells to reduce the yellow water-soluble substrate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into purple formazan crystals which was soluble in acidified isopropanol. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells. The colour development from yellow to purple was monitored at 570 nm using a spectrophotometer.

Procedure: The procedures described by Ayisi et al. were followed with modifications [24]. Dilution of the 10 mM stock solution of

each compound was made in 1% DMSO to obtain five different concentrations ranging from 0 to 100 μ M. Cultured suspension cells (HL-60 and Jurkat cells) were transferred into centrifuge tubes. The mono-layer adhesive cells (LNCap, PC3, HepG2, and MCF7) in the culture flasks were washed with PBS, detached with trypsin solution and transferred into centrifuge tubes. Tubes were centrifuged at 1000 rpm for 5 minutes and the pellets were re-suspended in growth media. Cells were counted using a haemocytometer (MARIENFELD, Germany) and a cell suspension of 1×10^5 cells/ml was prepared by diluting with growth media. 100 μ L (1×10^5 cells/mL) of cell suspensions were seeded into 96-well plates and incubated overnight before treatment. 10 μ L of the compounds at different concentrations were each added to the wells with the cell suspensions and incubated under the condition stated above for 72 hours. Curcumin was used as a positive control in all assays and a colour control plate was also setup for each compound. 20 μ L of 2.5 mg/mL MTT solution was added to the wells and incubated further for 4 hours in the CO₂ incubator. 150 μ L of acidified isopropanol containing Triton-X was then added to stop the reaction. The reaction plates were incubated in the dark at room temperature overnight and absorbance read at 570 nm using a micro-plate spectrophotometer (Tecan Infinite M200 Pro plate reader, Austria).

Percent Cell viability was determined as:

$$\% \text{ Cell viability} = \frac{[A_{\alpha} - A_{\beta}]}{[A_{\gamma} - A_{\beta}]} \times 100$$

A _{α} = Mean absorbance of treated cells

A _{β} = Mean absorbance of blank

A _{γ} = Mean absorbance of untreated cell^S

The average percentage cell viability determined at each concentration was plotted as a dose response curve using Graph Pad Prism Version 5.02. The inhibition concentration at fifty percent (IC₅₀) values, that is, concentration of compounds or standard drug inducing 50% inhibition of cells was determined from the dose response curve by nonlinear regression analysis.

Anti-malaria activity study

Blood collection and erythrocytes preparation: Erythrocytes were obtained from blood of consented volunteers (Blood group O⁺). Venous blood was drawn and collected into containers with citrate phosphate dextrose (CPD) solution and kept at 4 °C overnight. This was centrifuged at 2,000 rpm for 10 minutes to separate the serum and buffy coat. Packed erythrocytes were washed three times with parasite washing medium (RPMI 1640, buffered with, 50 μ g/mL gentamicin and 2 mM L-glutamine). Each washing step involved addition of wash medium, pipetting up and down thrice, centrifuging at 2,000 rpm for 10 minutes and then discarding suspended medium. After washing, medium was added to the packed erythrocytes and stored at 4 °C until use. Washed red blood cells were stored and used for up to 2 weeks maximum after which new blood had to be collected.

Giemsa-stained thin blood smear and parasitaemia determination

A drop of infected erythrocyte cultured medium was placed on a microscope slide and spread with the aid of another slide. Slides were air dried, dipped into methanol for some seconds to fix and air dry

again. A 10% Giemsa stain was added to cover completely the surface of the fixed slides for at least 10 minutes and then gently rinsed off with running water. Stained slides were air dried and then viewed under a light microscope (OLYMPUS CK30) (with immersion oil at 100x objective) to determine parasitaemia. Percent parasitaemia was determined by counting the number of infected cells in a total of 500 erythrocytes in the Giemsa-stained thin blood smear. Parasitaemia was expressed as a percentage of the number of infected erythrocytes to the total number of erythrocytes counted.

In vitro cultivation of malaria parasites

Erythrocytic stages of the malaria parasite (*Plasmodium falciparum*-chloroquine sensitive strain 3D7) were cultured in 25 ml flasks using the method of Trager and Jensen (1976) with modifications [25]. Erythrocytes were maintained at 2% haematocrit (v/v) cell suspension in complete malaria parasite medium (RPMI 1640, buffered with 25 mM HEPES, supplemented with 7.5% NaHCO₃, 25 μ g/ml gentamycin, 5% heat-inactivated human O⁺ serum (from consented subject) and 5 mg/ml AlbuMax II) and incubated at 37 °C under gas condition of 2% O₂, 5% CO₂ and 93% N₂. Parasite growth and development were monitored with Giemsa stained thin blood smear. Parasite culture was purified by using 5% sorbitol to obtain matured erythrocyte parasitic stages (late trophozoites and schizonts) from uninfected cells. The matured erythrocyte parasitic stages (purity > 90%) obtained were used to screen the compounds for anti-malaria activity.

Screening for anti-malaria activity using the SYBR Green I assay

Quinolactacin A2, citrinadin A and butrecitrinadin were screened for anti-malaria activity by using the SYBR Green I fluorescence assay as described by Smilkstein et al. with some modifications [26]. Serial dilution of the standard (artesunate) which served as an experimental control and stock solution of compounds to yield final concentrations ranging from 100 ng/ml to 400 ng/ml and 3.13 μ g/ml to 25 μ g/ml respectively were prepared. The matured erythrocyte parasitic stages were treated with compounds and washed in 96 well plates (Nunc) and incubated with complete malaria parasite medium for 24 hrs. Slides were then prepared and percent parasitaemia was determined by counting the number of infected cells in a total of 500 erythrocytes in the Giemsa-stained thin blood smear. Briefly, an aliquot of 5 μ L per each concentration of the standard drug and compounds was dispensed into test wells. 95 μ L of complete malaria parasite medium with washed erythrocytes at 2% haematocrit and the purified matured erythrocyte parasitic stages (1% parasitaemia) were added, and incubated at 37 °C under gaseous conditions as stated above and untreated erythrocytes were used as control. Wells containing erythrocytes at 2% haematocrit, infected erythrocytes at 2% haematocrit and complete parasite medium alone served as negative controls, positive controls and blank controls respectively. Furthermore, wells containing infected parasites and 0.1% DMSO served as reference controls. Final volume per well was 100 μ L. Plates were then incubated for 24 hrs as described above for the cultivation of malaria parasites. 100 μ L aliquot of 2.5x buffered SYBR Green I (0.25 μ L of SYBR Green I/mL of phosphate buffer saline) was added to each well after the incubation period and incubated in the dark for 30 minutes at 37 °C. Fluorescence was detected by Guava EasyCyte

HT FACS machine (Millipore, USA).

Principle: The contrast between host erythrocytes which lack DNA and RNA and the malaria parasites which have DNA and RNA form the basis of the SYBR Green experiment. The plasmodium parasites can easily be stained with the SYBR Green dye and quantified by fluorescence spectroscopy.

Apoptosis study

Mitochondrial potential: Determination of apoptosis-inducing capabilities of compounds as indication of high anti-malaria activities was done by the use of the Guava MitoPotential[®] kit (Guava Technologies, USA) as described by the manufacturer's instructions. A cationic dye, 5,5',6,6'-tetrachloro-1,1',3,3'-tetrathyl benzimidazolyl carbocyanine iodide (JC-1), was used to evaluate mitochondrial membrane potential changes and 7-Aminoactinomycin D (7-AAD), a cell-impermeant DNA intercalator, was also used to monitor cell membrane permeability changes. Synchronized culture parasites and preparation of 96-well micro-titer plates were taken through the same procedure described above for the screening of the compounds. 2x staining solution (4 µL of 50x staining solution/100 µL of complete parasite medium) of the Guava MitoPotential[®] kit was then added to each well in a ratio of 1:1 (v/v) and incubated for 30 minutes at 37 °C in the dark. Stained cells were analyzed on a Guava EasyCyte HT system.

Anti-buruli ulcer activity assay

Resazurin Microtiter Assays (REMA)

Preparation of inoculum: The Resazurin based assay as described

by Yemoa et al. was followed [27]. The assay used in detecting growth inhibition of microorganisms including mycobacteria was used to access the ability of the compounds to exhibit inhibition against *Mycobacterium ulcerans*. The inoculum was prepared by making a direct broth suspension of a full loop of isolated colonies selected from a 6 weeks Middlebrook 7H9 solid medium. Fresh *Mycobacterium ulcerans* MN209 characterized isolate culture was adjusted to achieve a turbidity of 1.0 McFarland turbidity standard which is equivalent to 3 x 10⁸ colony forming units (CFU)/ml. The adjusted inoculum suspension was further diluted 1:1000 in 7H9 broth up to approximately 1 x 10⁵ CFU/ml.

Inoculation: Within 15 minutes after the inoculum has been standardized as described above, 100 µL of the adjusted inoculum was added to each tube containing 4 µL of the compounds in the dilution series with a positive control well containing only broth and the plates were incubated.

Procedure: After 15 days of incubation, resazurin was added and a colour change observed after 48 hours of incubation.

Results

Discussion

According to BLAST analysis of the ITS region, the strain BRS2A-AR2F displays the 100% nucleotide similarity with *Cladosporium oxysporum* and *Cladosporium tenuissimum* and ITS data is not sufficient to discriminate for the strain species (Figure 1). *Cladosporium tenuissimum* conidiophores are up to 310(-460) µm long and are often subnodulose or nodulose with a head-

Table 1: ¹H and ¹³C NMR data of butrecitrinadin in CDCl₃.

ATOM	δ ¹³ C (ppm)	¹³ C mult	δ ¹ H (ppm)	Mult (Hz)	¹ H- ¹ H COSY	HMBC	2D-TOCSY
1-NH			9.65	s, br.			
2	185.3	C				H8	
3	64.4	C					
3a	134.6	C				H5	
4	133.2	CH	7.65	d, 7.2	H5	H6	H6
5	122.6	CH	7.2	ov.	H6, H4		
6	127.5	CH	7.76	d, 7.2	H5	H4	H4
7	117.6	C				H5	
7a	142.7	C				H6, H4	
8	41.7	CH ₂	2.21, 2.13	ov. ov.			
9	63.2	C				H8', H29, H28	
10	50.8	CH ₂	3.75, 3.26	ov. d, 11.6	H10, H10'		11-NH
11-NH			11.23	s, br.			H16, H10, H10', H15, H15', H17, H17'
12	56.5	CH	3.75	ov.	H13, H15	H27	H14, H15, H13
13	33.0	CH ₂	1.94, 1.91	m, m	H14, H12	H27	H14, H16, H12, H27, H17
14	65.7	CH	5.28	m	H13, H15		H16, H12, H27, H17, H15, H13
15	34.3	CH ₂	1.94, 1.91	m, m	H14, H12		H14, H16, H12, H27, H17, 11-NH
16	47.5	CH	4.02	ov.			11-NH, H14, H17, H15, H13

17	31.5	CH2	1.71, 1.67	m, m			H14, H16, H15, H13, 11-NH
18	82.3	C				H8, H29, H28	
19	51.2	C				H29, H28	
20	194.8	C				H6, H5, H21	
21	64.1	CH	4.06	s		H23, H24	
22	61.6	C				H21, H23, H24	
23	24.3	CH3	1.61	s		H24, H21	H24
24	18.5	CH3	1.25	s		H23	H23
25-NH							
26	30.0	CH3	2.45	s			
27	15.1	CH3	1.55	m			H14, H15', H13'
28	22.1	CH3	1.04	s		H29	H29
29	28.1	CH3	1.39	s		H28	H28
30	70.6	CH2	3.65	s		H10	
31	206.2	C				H32	
32	32.4	CH3	2.16				
1'	166.8	C					
2'	74.4	CH	2.75	d, 10.8	H4'	H7', H8', H5', H6'	H6', H5', H4'
3'-N							
4'	27.5	CH	2.04	ov.	H2', H5', H6'	H5'	H2', H6', H5'
5'	19.2	CH3	1	d, 6.4	H4'	H6'	H2', H4', H6'
6'	20.0	CH3	0.9	d, 6.8	H4'	H5'	H2', H4', H5'
7'	41.4	CH3	2.33	s		H8'	
8'	41.4	CH3	2.33	s		H7'	

like swollen apex and sometimes a few additional nodes on a lower level but, most conidiophores are neither geniculate nor nodulose. *Cladosporium oxysporum* conidiophores are up to 720 μm or longer and are always nodulose to nodose, with conidiogenous loci restricted to swellings on synthetic nutrient-poor agar (SNA). Loci on *C. tenuissimum* are often situated on swellings but, are not restricted to them. In intercalary conidiogenous cells, loci often sit at about the same level on the stalk, but are not connected with swellings as in *C. oxysporum*. They are on potato-dextrose agar (PDA) and oatmeal agar (OA) conidiophores that are darker and often with swellings [28]. Microscopic observations of isolate were made from colonies cultivated on SNA, PDA and OA for 7 days at 25 °C using the method of Bensch et al. [28]. Morphological features were consistent with the fact that strain BRS2A-AR2F belong to *Cladosporium oxysporum*. The sequence was deposited in GenBank nucleotide sequence database under KX257257.

Quinolactacin A2 was isolated as a very light yellow substance which on drying under nitrogen gas turned into very white and flaky bits that easily became airborne. Apparently, the light yellow portion was only remnants of impurities from the crude extracts. The compound was soluble both in dichloromethane and methanol. The UV profile was very interesting with wavelength absorption maxima at λ_{max} 221, 248, 255, 315 and 328 nm in 0.1% formic acid methanol solution (Figure S4). The mass spectrometry data of quinolactacin A2 was also very interesting and actually played a part to conceal

the identity of this compound until late in the structure elucidation process. Two peaks 293.1262 and 563.2631 were very consistent in the mass spectrometry data of the crude extracts as well as pure fractions containing quinolactacin A2. Initially, during the dereplication stage, these masses were entered into the databases but they returned with no hits and rightfully so. It turned out after the analysis of 1D and 2D NMR data that, 293.1262 was $[\text{M} + \text{Na}]^+$ and 563.2631 was $[2\text{M} + \text{Na}]^+$ (Figure S3). The molecular weight of the isolated quinolactacin was therefore 270.1362 representing a molecular formula of $\text{C}_{16}\text{H}_{18}\text{N}_2\text{O}_2$ ($\Delta = 0.1$ ppm) and nine (9) degrees of unsaturation.

The ^1H , ^{13}C and multiplicity edited gHSQC spectra, suggested the presence of 6 quaternary carbons, 6 methine, 1 methylene and 3 methyl carbons. The δ_{H} 7.50 (1H, dd, $J = 8.8, 4.4$ Hz, H-5), 7.68 (1H, ddd, $J = 8.0, 7.2, 4.0$ Hz, H-6), 7.40 (1H, ddd, $J = 8.0, 7.2, 6.8$ Hz, H-7) and 8.39 (1H, ddd, $J = 8.8, 6.4, 4.8$ Hz, H-8) were direct indication of a spin system belonging to a di-substituted aromatic ring. Analysis of the gCOSY correlations H-6/H-5, H-6/H-7, H-8/H-7, H-7/H-8, H-7/H-6 and H-5/H-6 fully confirmed the di-substituted benzene aromatic portion of the quinolone moiety. The gHMBC correlations C-5/H-7, C-6/H-8, C-7/H-5, C-8/H-6 and 2D-TOCSY correlations H-6 to H-8 and H-8 to H-6 was consistent with this first substructure. Furthermore, the gHMBC correlations C-8a/H-5, H-7, C-4a/H-6, H-8 and C-9/H-8 confirmed C-8a and C-4a showing at δ_{C} 128.2 and 141.3 as the two quaternary carbons in the quinolone benzene aromatic ring. C-9 at δ_{C} 173.5 was therefore the ketone functionality

Table 2: Cell growth-inhibitory potencies of pure compounds expressed as IC₅₀ values. Data presented are mean of two experiments done in triplicates. ND: Not Determined IC₅₀ (μM) = Concentration that inhibits cell growth by 50%.

Cell Line	Origin	Compound IC ₅₀ (μM)			
		Quinolactacin A2	Citrinadin A	Butrecitrinadin	Curcumin
HEPG2	Human Hepatocellular Carcinoma	96.54	82.51	78.57	61.38
HL-60	Human Promyelocytic Leukemia	54.47	57.23	60.31	13.78
Jurkat	Human T-Lymphoblastic Leukemia	ND	ND	ND	8.91
MCF-7	Human Breast Cancer	94.49	66.07	51.32	20.68
LNCap	Human Prostate Cancer	45.71	41.42	32.94	6.15
PC-3	Human Prostate Cancer	>100	>100	>100	20.72

present in the quinolone skeleton. The two quaternary olefinic carbons that are α,β to C-9 could easily be assigned as δ_C 110.9 and 164.1 corresponding to carbons C-9a and C-3a respectively with the gHMBC correlation C-3a to 4-CH₃ confirming their placements. The δ_H 2.12 (1H, ov., H-1'), 0.53 (3H, d, J = 6.8 Hz, 1'-CH₃), 1.63 (1H, m, H-2'), 1.49 (1H, m, H-2''), 1.05 (3H, t, J = 7.2 Hz, H-3'), 4.76 (1H, s, H-3) and 5.98 (1H, d, J = 45.2, 2-NH) along with gCOSY correlations H-1'/2-NH, H-3, 1'-CH₃, H-2'/H-3, H-3/H-2', H-2'', 2-NH/H-1' and 1'-CH₃/H-1' confirmed the attachment of a 2-butyl substituent at position C-3 of the γ-lactam ring. 2D-TOCSY correlations provided confirmation for all the spin systems present in the compound (Figure 2). All the remaining 1D and 2D NMR data are summarised in Table S1 and raw data are as shown in Figures S5-S10.

Butrecitrinadin was isolated as a very deep yellow crystalline substance that was soluble in both dichloromethane and methanol. This compound showed prominent wavelength absorption maxima at λ_{max} 221, 250, 270 and 332 nm in a 0.1% formic acid methanol solution (Figure S4). The HRESIMS of this compound gave m/z = 681.4223 for [M + H]⁺ corresponding to a molecular formula of C₃₈H₅₇N₄O₇ (Δ = 0.2 ppm) with thirteen (13) degrees of unsaturation. Butrecitrinadin shows the same fragmentation pattern as citrinadin with a difference of m/z = 57.0335 corresponding to the formula C₃H₅O⁺ which is a substituent on the C-18 hydroxyl group of the quinolizidine containing tricyclic ring system (Figures S11 and S12).

The δ_C chemical shifts of butrecitrinadin were extracted from the gHSQC, HMBC and DEPT-135° data. Analysis of the ¹H, ¹³C and multiplicity edited gHSQC spectra, suggested the presence of 12 quaternary carbons, 9 methine, 7 methylene and 10 methyl carbons. The huge molecular weight of 681.4223 with about 31% of the carbon atoms being quaternary alongside the observation of only few correlations in the gCOSY spectrum was direct indication of the presence of several very short spin systems which is typical of the butrecitrinadin backbone. The only substructures obtained by analysis of gCOSY data are as shown in Figure 3. The aromatic protons at δ_H 7.65 (1H, d, J = 7.2, H-4), 7.20 (1H, ov., H-5) and 7.76 (1H, d, J = 7.2, H-6) were direct indication of a tri-substituted

benzene ring and gCOSY correlations H-5/H-6, H-4 confirmed this spin system. Furthermore, gCOSY correlations from H-14/H-13, H-13', H-15, H-15' and H-12/H-13, H-13', H-15, H-15' established the only spin system present in the quinolizidine containing tricyclic ring system (Figure 3). The δ_H 2.04 (1H, ov., H-4'), 2.75 (1H, d, J = 10.8, H-2'), 1.00 (3H, d, J = 6.4, H-5') and 0.90 (3H, d, J = 6.8, H-6') and gCOSY correlations H-4'/H-2', H-5' and H-6' established the spin system present in the N,N-dimethyl valine residue at C-14. Diastereotopic protons were observed at positions C-10, C-8, C-13, C-15 and C-17. Unlike the gCOSY data, the gHMBC was very detailed and provided a lot of correlations to complete a large part of the structure. The aromatic benzene ring portion of the spirooxindole was completed with the aid of gHMBC correlations from C-6/H-4, C-7/H-5, C-3a/H-5, C-7a/H-6, C-7a/H-4 and C-4/H-6. The δ_C 185.3 (C-2) and 64.4 (C-3) along with the gHMBC correlation C-2/H-8 provided concrete evidence of the existence of an indolinone ring. The remaining part of the cyclopenta [b] quinolizidine moiety was also extended with the aid of crucial HMBC correlations C-18/H-29, C-19/H-28, C-9/H-29, C-9/H-28, C-28/H-29, C-18/H-28, C-9/H-8 and C-18/H-8 (Figure 3). gHMBC correlations C-24/H-23, C-23/H-24, C-22/H-23, C-22/H-24, C-21/H-23 and C-23/H-21 provided proof of the presence of the epoxy propane ring and its substituents while HMBC correlations C-2'/H-7', C-2'/H-8', C-2'/H-6', C-2'/H-5', C-5'/H-6', C-7'/H-8', C-8'/H-7' and C-4'/H-5' made it obvious that, the N,N-dimethylvaline residue was present in this structure. In order to confirm the structural assignments made by gCOSY and gHMBC data, a detailed interpretation of the 2D-TOCSY of butrecitrinadin was made. Here further confirmation of all the spin systems made it obvious that, the structure in question was butrecitrinadin. A detailed illustration of the data obtained from the 2D-TOCSY is shown in Figure 3. All 1D and 2D NMR data are summarized in Table 1 and raw data can be found in Figures S13-S17.

The anti-proliferative activities of the three compounds quinolactacin A2, citrinadin A and butrecitrinadin were tested on human cancer cell lines and the results are as shown in Figure 4. The compounds showed some level of anti-proliferative activity against some of the cell lines compared to curcumin as a positive control. The cell growth-inhibitory potencies of compounds, expressed as IC₅₀ values, are shown in Table 2. Quinolactacin A2, citrinadin A and butrecitrinadin, showed no activity against Jurkat and PC-3 cells but, the three compounds showed moderate anti-proliferative activity against LNCap and HL-60 cells. However, the activity of the three compounds towards HEPG2 and MCF-7 was good when compared to curcumin, a well-known potent inhibitor of cancer cell lines.

Table 3: The half maximal effective concentration of quinolactacin A2, citrinadin A and butrecitrinadin for 3D7 plasmodial strain.

Strain	Compounds EC ₅₀ (μM)			
	Quinolactacin A2	Citrinadin A	Butrecitrinadin	Artesunate
3D7	24.80	>25	>25	0.074

Table 4: Minimum inhibition concentration of quinolactacin A2, citrinadin A and butrecitrinadin on *Mycobacterium ulcerans* MN209.

Strain	MIC (μM)			
	Quinolactacin A2	Citrinadin A	Butrecitrinadin	Rifampicin
Mu MN209	>10	>10	>10	<1 μM

Quinolactacin A2, citrinadin A and butrecitrinadin were also tested against the chloroquine sensitive *Plasmodium falciparum* 3D7 strain for anti-plasmodial activity as shown in Figure 5. Only quinolactacin A2 showed anti-plasmodial activity within the concentration range tested. Table 3 shows the half maximal effective concentration (EC_{50}) of quinolactacin A2, citrinadin A and butrecitrinadin for 3D7 plasmodial strain. Quinolactacin A2 gave an EC_{50} of 24.80 μ M, while citrinadin A and butrecitrinadin gave values higher than 25.00 μ M. This represents the first study of the anti-plasmodial activity of quinolactacins in general and the results proved very encouraging.

The apoptotic activity of quinolactacin A2 on 3D7 plasmodia strain was evaluated by studying the effect of the compound on the mitochondrion membrane potential of the parasite. The effect of the compound on the loss of mitochondrion membrane potential of this strain after treatment with 0 μ M, 6.25 μ M, 12.5 μ M, 25.0 μ M and 50.0 μ M concentrations of quinolactacin A2 for 24 hours were analysed by flow cytometry after staining with JC-1. Figure 6 shows the percentage of apoptotic cells for the parasite after treatment with the compound and a histogram displacement is plotted in Figure 7 which indicates dissipation of the mitochondrial membrane potential ($\Delta\Psi_m$) and abolition of probe accumulation in a concentration dependent manner. Mitochondria play an essential role in apoptosis induction by a variety of death stimuli and therefore, the ability of quinolactacin A2 to compromise mitochondrial changes will lead to the loss of the $\Delta\Psi_m$ which will result in cytochrome C release from the mitochondria to the cytosol. Literature provides evidence that one of the mechanisms of malaria drug resistance is mediated through a decrease in apoptosis susceptibility and hence quinolactacin A2 may provide a scaffold to apoptotic death in the stages of *Plasmodium falciparum* development.

The compounds quinolactacin A2, citrinadin A and butrecitrinadin were tested for Anti-buruli ulcer activity on *Mycobacterium ulcerans* MN209 characterised isolates and the minimum inhibitory concentrations (MICs) are shown in Table 4. All the compounds showed no interesting MICs within the concentration range tested.

Conclusion

The co-isolation of 2, 12 and 13, from the same species suggests that the biosynthetic gene clusters for making quinolactacins and citrinadins which have been independently isolated and characterized from different species of *Penicillium* can be found in the same genome. While the three compounds show no activity against Jurkat and PC-3 cells, each of them possess different but moderate levels of anti-proliferative activities against HL-60, LNCap, HepG2, and MCF7 when tested in comparison to curcumin. Also, while the citrinadins, 12 and 13 possess no activity against the chloroquine sensitive *Plasmodium falciparum* 3D7 strain, 2 was found to be active within the concentration range tested with an EC_{50} value of 24.80 μ M compared to artesunate 0.074. 2 cause dissipation of the mitochondrial membrane potential ($\Delta\Psi_m$) and abolition of probe accumulation in a concentration dependent manner. Hence 2 may provide a drug development scaffold for apoptotic death in the stages of *Plasmodium falciparum* development. 2, 12 and 13 are inactive towards the *Mycobacterium ulcerans* MN209.

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