Pharmacogn. Commn. 2019; 9(3): 91-95
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcogcommn.org

Original Article

Oleanolic Acid and other Compounds Isolated from *Cordia africana* Lam which Inhibit Vancomycin Resistant Enterococcus

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ABSTRACT

Introduction: Treatment of microbial infections has become complicated due to increased resistance of microbes to the current drugs. The current study investigates crude extracts and seven compounds from root and stem bark of *Cordia africana* Lam. for antimicrobial and cytotoxic activity. **Methods:** Extraction was done using 50% methanol in dichloromethane, followed by chromatographic separation of compounds, whose structures were established by interpretation of spectroscopic data. The *in vitro* susceptibility of selected microbes to the crude extracts and pure compounds was determined. Cytotoxicity of **1, 6** and **7** was determined against the drug sensitive, CCRF-CEM and resistant CEM/ADR-5000 cells, with doxorubicin used as the standard. **Results:** The root bark extract of *C. africana* yielded six known compounds: oleanolic acid **(1)**, 3-β-lup-20(29)-en-3-ol **(2)** stigmast-5,22-dien-3β-ol **(3)**, 2-(2*Z*) -(3-hydroxy-3,7-dimethylocta-2,6-dienyl)-1,4-benzenediol **(4)**, 4-hydroxy-3-methoxy- benzaldehyde **(5)** and 7-hydroxy-4′-methoxyisoflavone **(6)**. The stem bark extract resulted to **1** and **2** alongside, ubiquinone-8 **(7)** and 1-octacosanol **(8)**. Compound **1** showed moderate activity against *Enterococcus faecium* (IC_{50} of 14.44 µg/mL), with vancomycin being inactive. Compounds **1, 6** and **7** showed cell viability >50% against CEM/ADR5000 and CCRF-CEM cells at 10 µM and therefore were considered inactive. Surprisingly, **1** was relatively more active compared to the standard, with cell viability of 57.93% against CEM/ADR5000, versus 78.97% for doxorubicin. **Conclusion:** To the best of our knowledge, this is the first report of the eight compounds from *C. africana*. The cytotoxicity of **1, 6** and **7** are reported here for the first time. Traditional use of the plant extract in management of various infections may be attributed to presence of 1, which displayed moderate antimicrobial activity.

Key words: *Cordia africana*, Ubiquinone-8, Oleanolic acid acid, 7-hydroxy-4′-methoxyisoflavone, VRE.

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DOI: 10.5530/pc.2019.3.19

INTRODUCTION

Vancomycin resistant Enterococci (VRE) infections are caused by *E. feacalis* and *E. feacium*, which account for 80-90% of clinical cases.[1] VRE infections mostly occur in intra-abdominal sites, in the urinary tract, bloodstream and at surgery sites, thus increasingly affect immune-compromised patients and those with prolonged hospitalization.[2,3] Treatment of these infections involve combinations of antibiotics, but this is challenged by the fact that these microbes have developed resistance to all currently available antibiotics, including vancomycin which for thirty years had been used without notable resistance.[4,5]

Cordia africana Lam syn. *C. abyssinica* R.Br. (Boraginaceae) belongs to genus *Cordia*, which has about 250 plant species. The genus is widely used to treat many diseases, including microbial infections.[6] Different classes of compounds including hydroquinones, terpenoid quinones, phenolic compounds, terpenoids, flavonoids, lignans, porphyrins, phenyl propanoids and saponins have been reported from the genus *Cordia*.[6,7] Some of these compounds and crude extracts have exhibited potential anticancer, antioxidant, antimicrobial and anti-inflammatory activities.[7]

Commonly known as Sudan teak or East African *Cordia*, the evergreen small to medium sized tree that grows to a height of 4-15 m is found in tropical and sub-tropical climate areas including Central and South America, India, Asia and Africa.[8] Despite the ethno-medical use of *C. africana* to treat coughs, asthma, tuberculosis, wounds, toothache, tonsillitis and eye infections,[8,9] little phytochemical investigations have been performed to establish active principles. Previous screening was performed on the methanol, ethyl acetate and chloroform extracts of Sudan species against *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Aspergillus niger* and *Candida albicans*. The stem-bark extracts inhibited growth of most of the tested organisms, with zones of inhibition ranging from 12 to 22 mm.[10] Further attempts to authenticate *C. africana* medicinal use led to chromatographic separation and purification, of the root bark and stem bark extracts, yielding eight pure compounds. Antimicrobial potential and cytotoxicity of the isolated compounds have also been reported in the current study.

MATERIALS AND METHODS

General Information

Thin layer chromatography (TLC) was performed on analytical TLC alumina plates pre-coated with silica gel 60 PF 254+366 (Merk, 0.25 mm thick) and spots visualized using UV lamp at wavelength of 254, 366 nm and exposure to iodine vapor. Column chromatography (CC) was run using silica gel 60 (Merk, 0.063-0.200 mm) and preparative thin layer chromatography on glass plates (0.5 mm thick): silica gel (Merk) 60 GF_{254} on 20 × 20 cm^2. Crystallization and Sephadex LH-20 on glass column were applied for purification of isolated compounds. ^1H and ^{13}C NMR (600, 500 MHz) were recorded on Brucker Avance DPX 300 spectrometer. Chemical shifts are given in δ values relative to internal standard TMS whereas coupling constant (*J*) is given in hertz (Hz). Multiplicity of the signals is defined using the following abbreviations: *s* (singlet), *d* (doublet), *t* (triplet), *dd* (double doublet), *q* (quartet). Melting points were determined on Stuart scientific apparatus fitted with a thermometer, with temperature range of 0-250^0 C. Electron impact (EI) mass spectrometry was done on MAT SSQ 7000 single quadrupole instrument at 70 eV.

Plant Material

The root bark and stem bark of *Cordia africana* Lam. were collected from Muthetheni, Machakos county, Kenya (approximately 40 km from

Nairobi city) and identified by a botanist Mr. Patrick Mutiso, from the School of Biological Studies (SBS), University of Nairobi, herbarium where a voucher specimen labelled, RWK 2017/01 is deposited.

Extraction and Isolation

The shade dried plant materials were separately ground into fine powder using RRH-1000A(K) multifunctional grinder and then exhaustively extracted by cold percolation in 1:1 methanol (MeOH)/dichloromethane (CH_2Cl_2) followed by concentration *in vacuo* to produce 80 g and 110 g root bark and stem bark extracts, respectively.

About 70 g of root-bark extract was loaded onto a column (70 g, SiO_2 gel) and elution was achieved with *n*-hexane (*n*-C_6H_{12}), CH_2Cl_2:*n*-C_6H_{12} and MeOH/CH_2Cl_2 solvent mixtures in increasing polarity. This yielded six (RWK 3A-3F) combined fractions, based on similarity on analytical TLC profiles.

Compound **2** (30.8 mg) was obtained as white needle-shaped crystals (R_f = 0.21, under 60% CH_2Cl_2/ *n*-C_6H_{12}, m.p. = 214-216°C, Lit. = 213.8-215.2[11]), from the fraction RWK 3B (1.0g) of the main column eluted with 80% CH_2Cl_2/*n*-C_6H_{12}, followed by crystallization in CH_2Cl_2/*n*-C_6H_{12}. Fraction RWK 3C (1.02 g) afforded **3** and **4** upon repeated CC using EtOAc/*n*-C_6H_{12} mixture in increasing polarities. Compound 3 (30 mg) was isolated as white needle shaped crystals, (R_f = 0.39 under 10% EtOAc/*n*-C_6H_{12}, m.p. = 172-174°C, Lit. = 174-176°C.[12] The sub-fraction obtained from RWK 3C was subjected to CC, followed by preparative TLC using 10% EtOAc/*n*-C_6H_{12} affording yellow solid of **4** (11.5 mg, R_f = 0.36, under 15% EtOAc/*n*-C_6H_{12}). CC of the combined fractions RWK 3D and 3E (2.93 g) using CH_2Cl_2/*n*-C_6H_{12} in increasing polarity gave yellow powder of 5 (8.6 mg, R_f = 0.34 under 80% CH_2Cl_2/ *n*-C_6H_{12}) and white powder (5 mg) of **1** (R_f = 0.31 under 1% MeOH/CH_2Cl_2, m.p. = > 250 °C, Lit. = 336°C).[13] Compound **6** (4.5 mg, R_f = 0.5 under 4% MeOH/CH_2Cl_2, m.p. = > 250 °C, Lit. = 255-256°C)[14] was obtained as yellow powder from fraction RWK 3F (0.9 g) through CC using MeOH/CH_2Cl_2 solvent mixture, followed by crystallization and separation by size exclusion using Sephadex-LH 20.

Similar procedures were performed on the stem bark extract (100 g) to yield **1** (60 mg) and **2** (56 mg) initially isolated from root bark extract. In addition, compound **7** (25 mg, R_f = 0.28 under 50% CH_2Cl_2/*n*-C_6H_{12}) was obtained as orange resin from a fraction of the main column eluted with 35% CH_2Cl_2/*n*-C_6H_{12}. Further chromatographic separation with CH_2Cl_2 *n*-C_6H_{12} mixtures of increasing polarity yielded **8** (30 mg, R_f= 0.28 under 60% CH_2Cl_2/*n*-C_6H_{12}, m.p. = 78-80°C). Structures of the pure compounds were elucidated by use of HRMS, 1D and 2D ^{1}H-NMR, ^{13}C-NMR spectral data and with close comparison with published data for related compounds.

Antimicrobial Activities

A slightly modified serial dilution technique,[15] was applied to determine *in vitro* susceptibility of the crude extracts and pure compounds against the microorganisms. The test organisms which included fungi *Candida albicans* ATCC 90028, *Aspergillus fumigatus* ATCC 204305, *Cryptococcus neoformans* ATCC 90113 bacteria, *Klebsella pneumonia* ATCC 2146, methicillin resistant *Staphylococcus aureus* MRSA 33591, *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853 and vancomycin resistant *Enterococcus* (VRE) *Enterococcus faecium* ATCC 700221 were obtained from American Type Culture Collection. The standard drug amphotericin B (92% pure) from MP Biomedical (CA, USA) was used as positive control in antifungal tests. Vancomycin (90% pure) from Sigma-Aldrich (MO, USA), served as positive control in the antibacterial assays. Crude extracts were tested against all strains at 200 µg/mL in duplicate to determine % inhibition. Pure compounds were dissolved to make a stock solution of 2 mg/mL. They were tested at concentrations of 20, 4 and 0.8 µg/mL and IC_{50}'s determined. The IC_{50} values were calculated using XLFit fit curve fitting software.

Cytotoxicity Assay

The resazurin assay[16] was applied to determine *in vitro* cell viability of the pure compounds against doxorubicin sensitive CCRF-CEM and resistant CEM/ADR-5000 lymphoblasts obtained from Germany collection for microorganisms and cell culture. The standard anticancer drug doxorubicin (98% pure) from Sigma-Aldrich, Schnelldorf, Germany) was used as positive control. Pure compounds were dissolved to make a test concentration of 10 µM. Samples that showed cell viability below 50 % had their IC_{50} values obtained from dose response curves.

RESULTS AND DISCUSSION

Chromatographic separation of the root bark extract of *C. africana* yielded three triterpenoids; oleanolic acid acid **(1)**,[13] 3-β-lup-20(29)-en-3-ol **(2)**[11] and stigmast-5,22-dien-3β-ol **(3)**[12] a hydroquinone derivative, 2-(2*Z*) -(3-hydroxy-3,7-dimethylocta-2,6-dienyl)-1,4-benzenediol **(4)**,[17] benzaldehyde derivative, 4-hydroxy-3-methoxy-benzaldehyde **(5)**[18] and isoflavone, 7-hydroxy-4′-methoxyisoflavone **(6)**[14] (Figure 1). Similarly, the stem bark extract led to re-isolation of **1** and **2** together with terpenoid benzoquinone ubiquinone-8 **(7)**[19] and an alcohol, 1-octacosanol **(8)**.[20] The structures of these compounds were elucidated based on interpretation of ^{1s}H, ^{13}C-NMR, IR and mass spectrometry data, as well as comparison with published data.

Compounds **6** and **7** are reported from the genus *Cordia* for the first time. ESI-mass spectrum of **6** showed a base peak at *m/z* 267 $[M-H]^+$. ^{1}H-NMR spectrum of **6** displayed six aromatic (δ_H 8.14, 8.04, 6.92, 6.84, 7.47×2, 6.98×2 ppm), one methoxy (δ_H 3.78 ppm) and one chelated hydroxyl proton (δ_H 10.8 ppm). One of the aromatic protons (δ_H 8.14 ppm), suggested its β-position attachment with respect to a carbonyl group (δ_c 178.1 ppm) as well as direct attachment to electron withdrawing oxygen atom at position C-1. This suggested an isoflavove skeleton and close study of COSY, HMQC and HMBC correlations, HRMS fragmentation pattern, together with comparison with published compounds led to identification of the compound as 7 -hydroxy-4′-methoxyisoflavone **(6)**, previously reported from the clover, *Trifolium repens*,[14] *Bolusanthus speciosus*[21] and *Eysenhardtia polystachya*.[22] Compound **7**, known to be

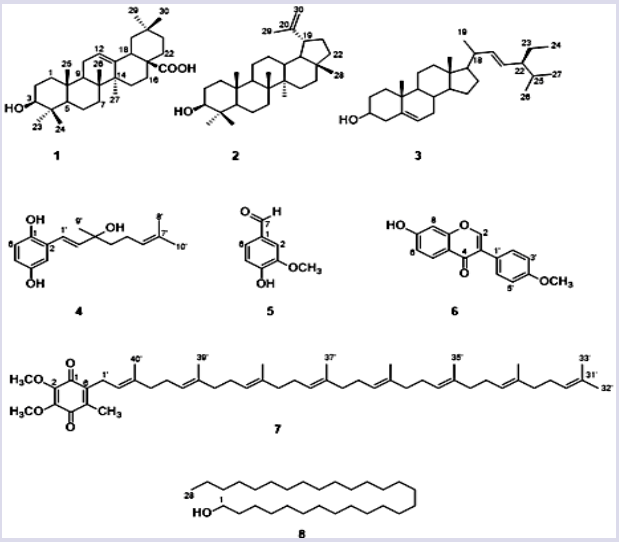

Figure 1: Structures of compounds isolated from the root bark (**1-6**) and stem bark (**7-8**) of *Cordia africana* Lam.

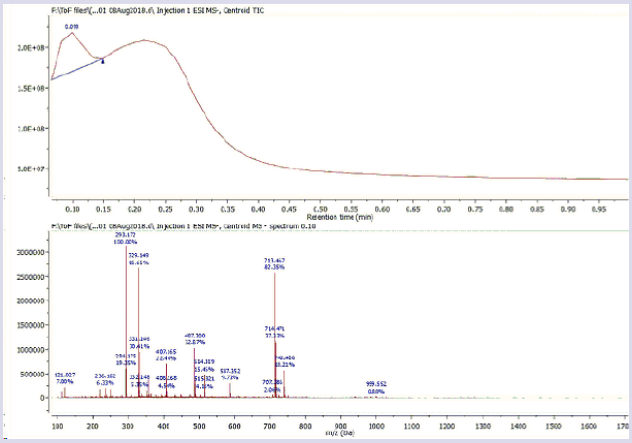

Figure 2: ESI-mass spectrum of compound **7**.

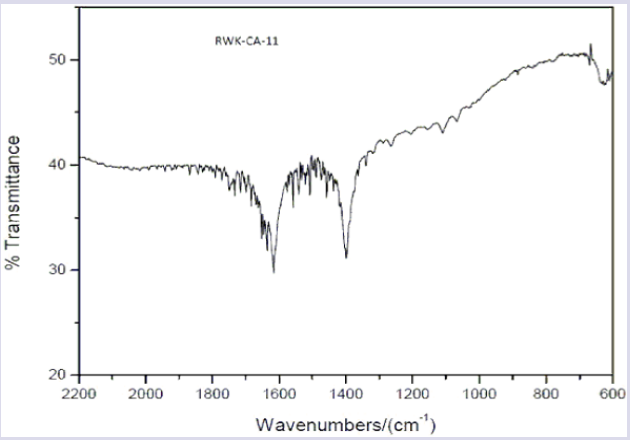

Figure 4: IR spectrum of compound **7**.

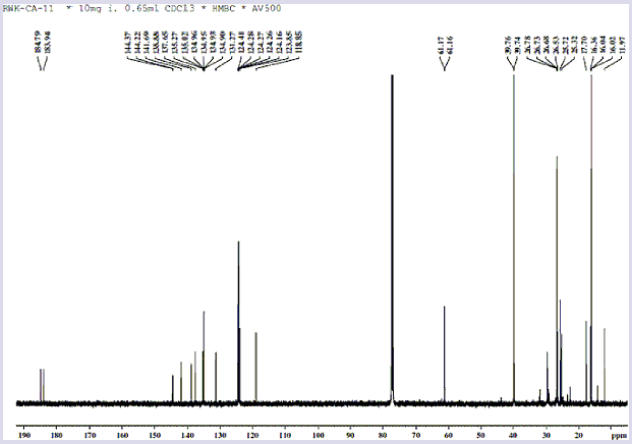

Figure 3: ^{13}C-NMR spectrum of compound **7**.

synthesized by *Escherichia coli*, was previously characterized from a predatory gram-negative bacterium, *Bdellovibrio bacteriovorus* 109J.[19] However, its occurrence in plants as well as detailed structural elucidation details are unknown and therefore the motivation to provide the spectroscopic data for this compound. The molecular formula of compound **7** was determined as $C_{49}H_{74}O_4$, from interpretation of ESI-mass spectrum (Figure 2) of the compound, which showed a key fragment at *m/z* 713.467 $[M+1]^+$ -CH_3), giving the compound a molecular mass of 727.467 amu. The molecular formula was supported by ^{13}C- NMR spectrum (Figure 3) which revealed a total of 49 signals, which were classified as, two carbonyls (δ_c 184.8, 184.0 ppm), two methoxy (61.2 × 2), ten methyl (12.0, 16.1×6, 16.4, 17.7 and 25.7 ppm), twelve quaternary (olefinic δ_c 131.3-144.4 ppm), eight olefinic methine (δ_c 118.8, 124.3×7 ppm) and fifteen methylene groups (δ_c 25.3, 26.8 × 7 and 39.8 × 7 ppm). IR spectrum (Figure 4) (Lit. unreported) displayed bands at 1398 (-CH_3, C-H bend), 1460 (-C-H bend) and 1620 (-C=C-C(O)-R stretch for ubiquinones[23] cm^{-1}. ^{13}C-NMR spectrum clearly revealed signals at δ_c 139.0, 141.9, 144.2, 144.4, 184.0, 184.0 ppm, which anticipated a tetra-substituted benzoquinone skeleton.[24] Study of long range correlations revealed two methoxy, methyl and isoprenyl substituents assigned to C-2, C-3, C-5 and C-6, respectively. This assignment matched that of a meroterpenoid benzoquinone, previously isolated from roots of *C. globosa*[24] but differed on substituents at C-5 and C-6 position. The presence of eight olefinic protons at δ_H 4.95-5.13 ppm, thirty methylene protons and nine methyl protons confirmed a C-40 isoprenoid group, which was attached at position C-6 of the benzoquinone. Close comparison of the observed values with literature spectral and physical data led to the identification of the compound as ubiquinone-8 (UQ-8), previously characterized from a predatory gram-negative bacterium, *Bdellovibrio bacteriovorus* 109J[19] through semi-prep HPLC analysis. This compound belongs to a group of compounds known as ubiquinones (UQs), identified by presence of benzoquinone ring attached to poly-isoprenoid side chain, with the length varying with source organism. The compounds UQ-8, with eight isoprene units is known to be synthesized in *Escherichia coli*, where it serves as a cellular radical scavenger as well as an electron carrier in respiratory chain.[25] It is reported that plant ubiquinones usually consist of nine to ten isoprene units, which is contrary to the present study which reports UQ-8 with eight units from the stem bark of *Cordia africana*. The genus *Cordia* has previously yielded reduced forms of benzoquinones as well as meroterpenoid benzoquinones. Alencar and coworkers (2005) reported a meroterpenoid benzoquinone from the roots of *C. globosa*.[24] The compound had a C-4 and C-5 methoxy substituted benzoquinone skeleton but the C-40 side chain observed in UQ-8 was missing, instead, it had two isoprene units, in form of fused rings. To the best of our knowledge this is the first report of **7** in the plant kingdom.

The crude extracts and all compounds except **5** were tested for antibacterial and antifungal activities against *C. albicans* ATCC 90028, *A. fumigatus* ATCC 204305, *C. neoformans* ATCC 90113, *K. pneumonia* ATCC 2146, methicillin resistant *Staphylococcus aureus* MRSA 33591, *E. coli* ATCC 35218, *P. aeruginosa* ATCC 27853 and vancomycin resistant *Enterococcus* (VRE) *E. faecium* ATCC 700221. The crude extracts were inactive towards the tested microorganisms as their IC_{50} values were >200 mg/mL which could be attributed to the inactivity of most constituent compounds. However, **1** showed moderate activity against VRE (*E. faecium* ATCC 700221) with IC_{50} value of 14.44 µg/mL (Table 1), versus vancomycin which was inactive at a concentration range of 4-100 µg/mL. The rest of the compounds did not show inhibition towards the growth of the microbes at concentration range of 0.8-20 µg/mL. Oleanolic acid (**1**), is found in a wide range of medicinal and edible plants.[26] Comprehensive studies have revealed its therapeutic potential as an antitumor,[27] anti-diabetic,[28] antihypertensive,[29] antioxidant,[30] anti-inflammatory,[31] anti-

Table 1: Antimicrobial activity of crude extracts and isolated compounds from *Cordia africana* Lam.

Samples	IC_{50} (µg/mL)							
	VRE*	*A. fumigatus*	*C. neoformans*	*C. albicans*	MRS**	*E. Coli*	*P. aeruginosa*	*K. Pneumoniae*
Root bark extract	-	-	-	-	-	-	-	-
Stem bark extract	-	-	-	-	-	-	-	-
1	14.43	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-
Vancomycin	-	-	-	-	1.43	72.80	-	-
Amphotericin B	-	0.72	0.29	0.12	-	-	-	-
Meropenem	-	-	-	-	-	-	85.90	-

- not active at concentration ranges of 0.8-20, 8-200 and 4-100 µg/mL for pure compounds, crude extracts and standard drugs, respectively.
*Vancomycin-resistant enterococci: *Enterococcus faecium*
**Methicillin-resistant *Staphylococcus aureus.*

leishmania[32] and antimicrobial[33] agent. Previous studies on antimicrobial activity of 1 towards *E. feacalis* ATCC 29212, methicillin resistant *S. aureus* ATCC 29213, *S. aureus* ATCC25293 and *P. aeruginosa* ATCC27853 reported MIC values of 8, 32, 64 and 256 µg/mL, respectively.[33] However, in the current study when the test concentration was lowered to 20 µg/mL, compound **1** was found selective towards VRE, showing activity towards only *E. feacalis* ATCC 700221 with an IC_{50} value of 14.44 µg/mL and inactive towards *S. aureus* and *P. aeruginosa*.

Compounds **1, 6** and **7** were isolated in sufficient quantities and were also tested for their cytotoxicity against drug sensitive CCRF-CEM and resistant CEM/ADR 5000 human leukemic lymphoblast. These compounds exhibited cell viability > 50% at the tested concentration of 10 µM and therefore were considered inactive. Nevertheless, oleanolic acid **(1)** was relatively more active than doxorubicin against CEM/ADR 5000 as it displayed cell inhibition of 42.07% versus 21.03%, respectively.

CONCLUSION

The current study has revealed eight compounds **1-8** for the first time in this plant. Compound **7** is reported for the first time in plant kingdom, earlier known to be produced by gram negative bacteria only. Oleanolic acid **(1)** showed moderate activity (IC_{50} =14.44 µg/mL) towards VRE (*Enterococcus faecium* 700221), compared to the standard drug, vancomycin, which was inactive at the same concentration. Compounds **1, 6** and **7** were inactive (cell viability > 50% at 10 µM) towards CCRF-CEM and CEM/ADR 5000 human leukemic lymphoblast. The traditional use of the plant extracts in the management of various infections would be attributed to the presence of oleanolic acid which displayed moderate antimicrobial activity.

ACKNOWLEDGEMENT

The authors thank DAAD-NAPRECA for scholarship to Rahab Kamau, Dr. Melissa Jacob, National Center, for National Products Research, School of Pharmacy, University of Mississippi and Dr. Thomas A. Efferth, Department of Pharmaceutical Biology, Johannes Gutenberg University Mainz, Germany for Bioassays and International Science Programme, Uppsala University, Sweden for financial support through KEN-02 project. This work was partially supported by the USDA-ARS Specific Cooperative Agreement No. 58-6408-1-603.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

ABBREVIATIONS

ATCC: American Type Culture Collection; **CCRF-CEM:** Doxorubicin sensitive leukemia lymphoblasts; **CEM/ADR 5000:** Doxorubicin resistant leukemi lymphoblasts; **IC_{50}:** Concentration that causes 50% inhibition of growth; **NMR:** Nuclear Magnetic Resonance; **HRMS:** High Resolution Mass Spectrometry; **COSY:** Correlation spectroscopy; **HMQC:** Heteronuclear multiple quantum coherence; **HMBC:** Heteronuclear multiple bond correlation.

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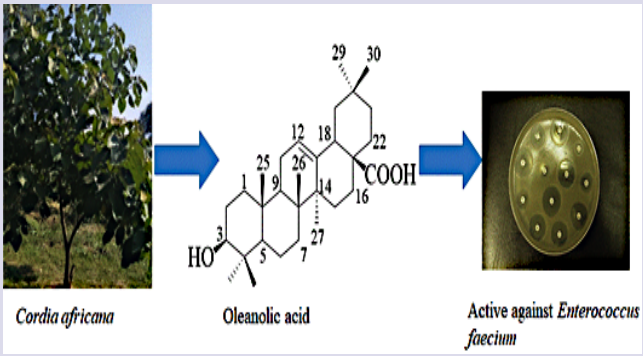
PICTORIAL ABSTRACT



SUMMARY

Eight compounds were isolated from *C. africana* for the first time. Oleanolic acid (1) isolated from both the stem-bark and root-bark showed moderate (IC_{50}=14.44 μg/ml) antibacterial activity against *Enterococcus faecium*, whereas standard drug vancomycin showed no inhibition. Additionally, compound 1 exhibited better activity against drug resistant CEM/ADR 5000 with a cell inhibition of 42.07 %, compared to 21.03 % by doxorubicin. This is the first report of compound 7 (ubiquinone-8) from plants, having been previously reported in gram-negative bacteria, only.

ABOUT AUTHORS

Rahab Kamau has an interest in researching Kenyan medicinal plants for bioactive phytochemicals. To test their efficacy, the characterized phytochemicals are tested for antibacterial, antifungal and anticancer activities.