

Inhibitory Effects of Extracts and Isolates from *Kigelia africana* Fruits against Pathogenic Bacteria and Yeasts

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Abstract—*Kigelia africana* (Lam.) Benth. (Bignoniaceae) is a reputed traditional remedy for various human ailments such as skin diseases, microbial infections, melanoma, stomach troubles, metabolic disorders, malaria and general pains. In spite of the fruit being widely used for purposes related to its antibacterial and antifungal properties, the chemical constituents associated with the activity have not been fully identified. To elucidate the active principles, we evaluated the antimicrobial activity of fruit extracts and purified fractions against *Staphylococcus aureus*, *Enterococcus faecalis*, *Moraxella catarrhalis*, *Escherichia coli*, *Candida albicans* and *Candida tropicalis*. Shade-dried fruits were powdered and extracted with hydroalcoholic (1:1) mixture by soaking at room temperature for 72 h. The crude extract was further fractionated by column chromatography, with successive elution using hexane, dichloromethane, ethyl acetate, acetone and methanol. The dichloromethane and ethyl acetate fractions were combined and subjected to column chromatography to furnish a wax and oil from the eluates of 20% and 40% ethyl acetate in hexane, respectively. The GC-MS and GC×GC-MS results revealed that linoleic acid, linolenic acid, palmitic acid, arachidic acid and stearic acid were the major constituents in both oil and wax. The crude hydroalcoholic extract exhibited the strongest activity with MICs of 0.125-0.5 mg/mL, followed by the ethyl acetate (MICs = 0.125-1.0 mg/mL), dichloromethane (MICs = 0.250-2.0 mg/mL), hexane (MICs = 0.25-2.0 mg/mL), acetone (MICs = 0.5-2.0 mg/mL) and methanol (MICs = 1.0-2.0 mg/mL), whereas the wax (MICs = 2.0-4.0 mg/mL) and oil (MICs = 4.0-8.0 mg/mL) showed poor activity. The study concludes that synergistic interactions of chemical constituents could be responsible for the antimicrobial activity of *K. africana* fruits, which needs a more holistic approach to understand the mechanism of its antimicrobial activity.

Keywords—*Kigelia Africana*, traditional medicine, antimicrobial activity, *Candida albicans*, palmitic acid, synergistic interaction.

I. INTRODUCTION

KIGELIA africana (Lam.) Benth. (syn. *Kigelia pinnata* DC.), commonly known as the sausage tree, belongs to the family Bignoniaceae and is native to Africa. The plant is cultivated in various other tropical countries, and is used as an ornamental tree in Australia, USA and many parts of Southeast Asia. It is a tree growing up to 20 m tall with grey bark which is smooth at first but peeling on older trees. The wood is pale brown or yellowish, undifferentiated and not

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prone to cracking. The fruits are huge and cylindrical in shape up to 0.6 m in length and 4 kg in weight [1], [2]. The fruit of *K. africana* is a reputed traditional remedy for skin diseases, microbial infections, hepatic diseases boils, psoriasis, eczema, leprosy, syphilis, cancer, ulcers, dysentery, post-partum haemorrhage, malaria, diabetes, pneumonia, constipation and general pains in various African and Asian countries [3], [4]. Dried powdered fruit is also used to increase the flow of milk in lactating women [5]. Baked fruits are used to ferment beer and boiled ones yield a red dye [6]. The plant showed various biological activities like anti-implantation, molluscicidal, antimicrobial, cytotoxic, antibacterial and antifungal activities [7].

K. africana plant has many medicinal properties due to the presence of numerous secondary metabolites. Previous phytochemical examinations have resulted in the isolation of iridoids [8], [9] and naphthaquinoids [10], [11] as major secondary metabolites. In addition, flavonoids [12], coumarins [13], lignans [14] and volatile constituents [15] have also been isolated from various parts of the plant.

Previously, fatty acids exhibiting antibacterial effects were reported from the fruits [16]. Moreover, the methanolic extract from the bark showed significant activity against *Salmonella typhi* and *Proteus vulgaris* but poorly active against *Escherichia coli*, *Enterobacter aerogens*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus cereus* [17]. Some researchers demonstrated its antimicrobial activity as mild [18]. Even though, many reports presenting the antimicrobial activity of crude extracts from the fruits of *K. africana*, still the responsible constituents are unknown. Hence, we studied hydroalcoholic crude extract, various column fractions as well as the oil and wax obtained from the fruits of *K. africana* for their antimicrobial activities against various pathogenic bacteria and yeasts to investigate the responsible antimicrobial principles.

II. MATERIAL AND METHODS

A. General

Column chromatography (CC) was performed using silica 60 (Macherey-Nagel, GmbH & Co. KG, Germany). Thin layer chromatography (TLC) was done with aluminium plates precoated with silica gel 60 (Macherey-Nagel, GmbH & Co. KG, Germany). The spots on the TLC plate were visualised with I₂ vapours, spray of 7% H₂SO₄ in MeOH followed by heating and UV lamp (254/360 nm, CAMAG, Switzerland). Melting points were measured with the help of a Stuart digital melting point apparatus, Model SMP10. GCMS analysis was

carried out using Agilent 6890N GC system coupled to a 5973 MS (Agilent Technologies, USA) whereas GC×GCMS was performed with Pegasus 4D GCGC/TOF-MS (LECO Corporation, USA). UPLC-MS analysis was done with Waters Acquity Ultra Performance Liquid Chromatographic system with PDA detector (Waters, Milford, MA, USA). Mid-infrared analysis was carried out with Alpha-P Bruker spectrophotometer (Bruker OPTIK GmbH, Germany).

B. Extraction

Fresh fruits of *K. africana* were collected from Cape Town, South Africa during July, 2013. Shade-dried fruits (1 kg) were powdered and extracted with water-ethanol (1:1) mixture by soaking at room temperature for 72 h. The vacuum dried crude extract (143 g) was further fractionated by column chromatography, with successive elution using hexane, dichloromethane, ethyl acetate, acetone and methanol. The fractions thus obtained were dried under reduced pressure using a rota-evaporator to yield 7 g hexane, 19 g dichloromethane, 17 g ethyl acetate, 32 g acetone and 46 g methanol extracts.

C. Ultra-High Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS) Analysis

Each extract (1 mg) dissolved in 1 mL methanol (HPLC grade) was filtered using micro-filter syringe before injecting to UPLC-MS. The UPLC analyses were performed on a Waters Acquity Ultra Performance Liquid Chromatographic system with PDA detector (Waters, Milford, MA, USA). UPLC separation was achieved on an Acquity UPLC BEH C18 column (150 mm × 2.1 mm, i.d., 1.7 μm particle size, Waters) maintained at 40 °C. The mobile phase consisted of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B) at a flow rate of 0.5 ml/min; a gradient elution was as follows: 75% A: 25% B to 40% A: 60% B in 5 min, to 15% A: 85% B in 6 min, to 5% A: 95% B in 1 min and back to initial ratio in 0.5 min. The total running time was 15 min. The injection volume was 1.0 μl (full-loop injection). Data were collected by chromatographic software Masslynx 4.1. The UPLC system was interfaced with a Xevo G2QTOF mass spectrometer (Waters, USA). The same column, elution gradient and flow rate were used during the UPLC-MS analysis. Mass spectrometry was operating in a positive ion electrospray mode. N₂ was used as the desolvation gas. The desolvation temperature was set to 350 °C at a flow rate of 800 L/h and the source temperature was 100°C. The capillary and cone voltages were set to 3500 and 30 V, respectively. Data were collected between 50 and 1000 m/z.

D. Isolation

UPLC-MS analysis indicated that the compositions of the dichloromethane and ethyl acetate fractions were similar. These fractions were combined and subjected to column chromatography. The extract (25 g) obtained from these fractions was first pre-adsorbed with silica (1:1, w/w) and then applied to the top of the column packed with silica. The elution was first started with hexane followed by increasing polarity of ethyl acetate. Eluates were collected at 25 mL and

monitored on TLC plates with different combinations of hexane and dichloromethane as mobile phase. Fractions of similar TLC profiles were combined and concentrated at room temperature. The column fractions from 40 to 47 obtained from 20% ethyl acetate in hexane were combined to yield a semi-solid transparent wax, which was collected and washed with ethanol. Similarly, fractions from 65 to 85 obtained from 40% ethyl acetate in hexane were also combined and concentrated at room temperature to furnish oil. The oil was also washed with ethanol.

E. Mid-Infrared (MIR) Spectrometry Analysis

The attenuated total reflectance diamond crystal surface of an Alpha-P Bruker spectrophotometer was covered with 1 mg each of oil and wax. The MIR spectra in absorbance mode with the wavelength range of 550–4000 cm⁻¹ were recorded using an OPUS[®] software.

F. Esterification of Fatty Acids

The methyl derivatives of oil and wax constituents were prepared by following the method described by Badoni and coworkers [19]. Briefly, the sample (50 mg in each case) was added to 0.5 mL of KOH-CH₃OH solution (0.5 mol/L) and heated at 60°C for 30 min. Then it was added to 1.0 mL of BF₃-CH₃OH solution and again heated for 30 min at 60°C. To it petroleum ether and saturated NaCl (1 mL each) were added. Finally, this mixture was centrifuged for 10 min at 3000 rpm and the supernatant fluid was subjected to GC-MS and GC×GC-TOFMS analyses.

G. GC-MS Analysis (One Dimensional)

Esterified oil and wax were analysed by an Agilent 6890N GC system coupled directly to a 5973 MS. A volume of 1 μL was injected using a split ratio (200:1) with an autosampler at 24.79 psi and an inlet temperature of 250°C. A HP-5MS 5% phenyl methyl siloxane column (30 m×250 μm i.d.×0.75 μm film thickness) was used. The oven temperature was 100°C for first 10 min, rising to 220°C at a rate of 4°C/min and held for 10 min and then rising to 240°C at a rate of 1°C/min. He was used as a carrier gas at a constant flow of 1.2 mL/min. Spectra were obtained on electron impact at 70 eV, scanning from 35 to 550 m/z. The percentage composition of the individual components was obtained from the electronic integration measurements using flame ionization detection (FID, 250°C). Compounds identification was made by comparing mass spectra with NIST[®], Mass Finder[®] and Flavour[®] libraries.

H. GC×GC-MS Analysis (Two Dimensional)

The GC×GC-TOFMS system comprised an Agilent 7890 A (Agilent Technologies, USA) gas chromatograph, a Gerstel autosampler and a Pegasus 4D TOFMS (LECO, USA) equipped with a liquid nitrogen quadrupole cold jet modulator and a secondary oven. Stabilwax[®] polyethylene glycol (30 m × 0.25 mm i.d. × 0.25 μm film thickness (Restek, USA) and Rxi[®]-5Sil MS; 0.79 m × 0.25 mm i.d. × 0.25 μm film thickness (Restek USA) were used as first and second column, respectively. The two columns were connected by a universal press-tight connector, and were installed in the main GC oven.

Helium (high purity) was used as carrier gas at a constant flow rate of 1.5 ml/min and the split injector was set to 1:200. The main oven temperature was set at 60°C for 2 min and then ramped to 120°C at 5°C/min and hold for 2 min thereafter ramped to 260°C at 20°C, with a final isothermal period of 2 min at 260°C. The secondary oven installed inside the main GC oven was programmed with a +20°C offset above the primary oven. The best modulation period was 2 sec and the hot pulse duration was set at 0.6 s with the peak width of 12. The mass spectrometer was operated on acquisition rate of 100 spectra/sec. A solvent acquisition delay of 120 sec was set to protect the MS analyzer from excessive solvent exposure. The ion source temperature and the transfer line to the TOF MS were set to 200°C and 280°C, respectively. The detector voltage 1650 V and the ionization electron energy 70 eV were used. The mass spectra were acquired in the range 30–450 m/z and 1 µl (Insert concentration used here) of each sample was injected using the Gerstel multipurpose sampler.

I. Data Processing and Analysis

Two dimensional GC data were processed automatically using LECO ChromaTOF® software version 4.50. Automatic peak finding with mass deconvolution were used to create a raw peak table, based on minimum signal to noise ratio (S/N) cut off > 100 based on “unique mass” the most specific mass usually extracted for analyte after deconvolution of the mass spectra signal. Identification of peaks was based on NIST Mass Spectral Library (NIST 11), and Adams library. Library similarity factors were reported on a scale ≥ 800 (high match is associated with better match) for both forward and reverse search. Relative abundance (% area) calculations of were based on the ratio between the peak area of each compound.

J. Antimicrobial Susceptibility Assay

The antimicrobial efficacy of various fractions and isolates (oil and wax) of *K. africana* was determined by the broth microdilution method as approved by the guidelines of Clinical and Laboratory Standards Institute [20]. Briefly, all the fractions and isolates were added to the microtitre plates and serially diluted. Cultures of two Gram-positive bacteria (*Staphylococcus aureus* ATCC126000 and *Enterococcus faecalis* ATCC29212), two Gram-negative bacteria (*Moraxella catarrhalis* ATCC23246 and *Escherichia coli* ATCC8739) and two yeasts (*Candida albicans* ATCC10231 and *Candida tropicalis* ATCC201380) previously sub-cultured on Tryptone Soya agar (TSA) plates were added and the plates were incubated at optimal temperatures for each pathogen. Positive controls (Sigma Fluke), ciprofloxacin (0.01 mg/mL) for bacteria and amphotericin B (0.1 mg/mL) for yeasts and a negative vehicle control (acetone/water solution 32 mg/mL) were included in all experiments. Media and culture controls were included to check the sterility and viability, respectively. All assays were repeated (n=3) to ensure reproducibility.

III. RESULTS AND DISCUSSION

In order to identify the antimicrobial principles of *K. africana*, the crude extract and various other column fractions

were screened for their activities against two Gram-positive bacteria (*S. aureus* and *E. faecalis*), two Gram-negative bacteria (*M. catarrhalis* and *Escherichia coli*) and two yeasts (*C. albicans* and *C. tropicalis*). The preliminary results showed that crude hydro-alcoholic extract exhibited the strongest activity which justified its traditional use in microbial infections. Dichloromethane and ethyl acetate column fractions showed strong activities as compared to other fractions. The UPLC-MS analysis showed that the chemical profiles of both of these fractions were same. Hence, these fractions were combined and considered for further isolation work. The column chromatographic isolation of these fractions furnished an oil (light yellow liquid; boiling point 185°C; hexane and petroleum ether soluble; lighter than water) and a wax (transparent semi-solid; melting point 224–226°C (uncorr.); partially soluble in acetone).

MIR spectroscopy of these isolates suggested the presence of fatty acids in both wax and oil. The MIR spectrum of oil (Fig. 1) displayed the strong bands at 2923 and 2885 cm⁻¹ (C-H stretching of methylene groups), 1738 cm⁻¹ (carbonyl groups), 1472 (C-H bending) and 1185 cm⁻¹ (C-O stretching), characteristic for fatty acids [21]. The MIR spectrum of wax was found somewhat similar to that of oil except the hydroxyl region which was more intense and broaden in the case of wax (Fig. 2).

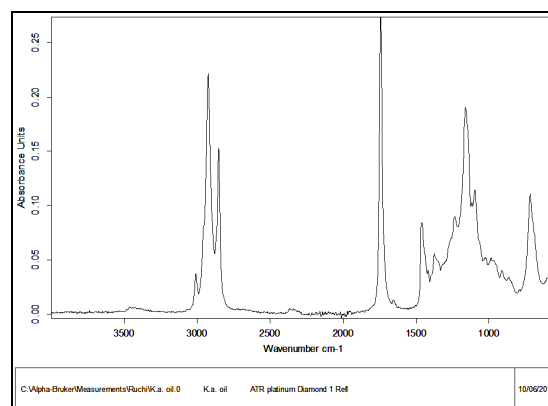


Fig. 1 MIR spectrum of oil

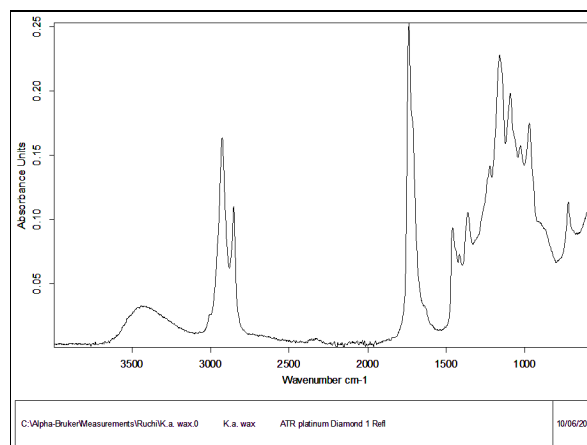


Fig. 2 MIR spectrum of wax

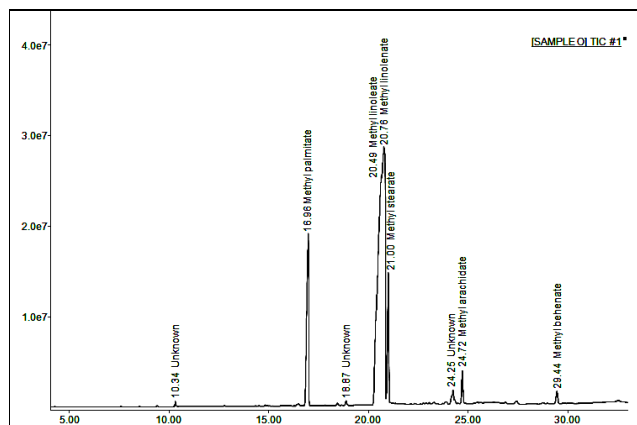


Fig. 3 Gas chromatogram of esterified fatty acids of oil

Results from the GC-MS and GC×GCMS analyses (Table I) revealed that linoleic acid, linolenic acid, palmitic acid, arachidic acid, stearic acid, elaidic acid and oleic acid were the major constituents in both oil and wax. However, the concentration of linolenic acid was found to be highest in both cases, followed by linoleic acid and palmitic acid in the case of oil whereas stearic acid and palmitic acid in the case of wax. The concentrations of linolenic acid and linoleic acid were found to be 17.90% and 14.09%, respectively, whereas

in the case of wax, their concentration was measured to be 21.68% and 8.56%, respectively. On the basis of GC-MS, only 6 major components (Fig. 3 and 4) were identified in both oil and wax whereas 2 dimensional GC×GC-MS was found to be most advanced and identified around 50 constituents, among them the major constituents are graphically shown in Fig. 5.

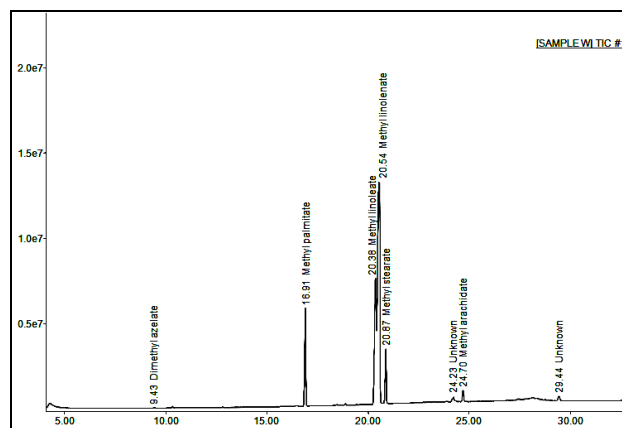


Fig. 4 Gas chromatogram of esterified fatty acids of wax

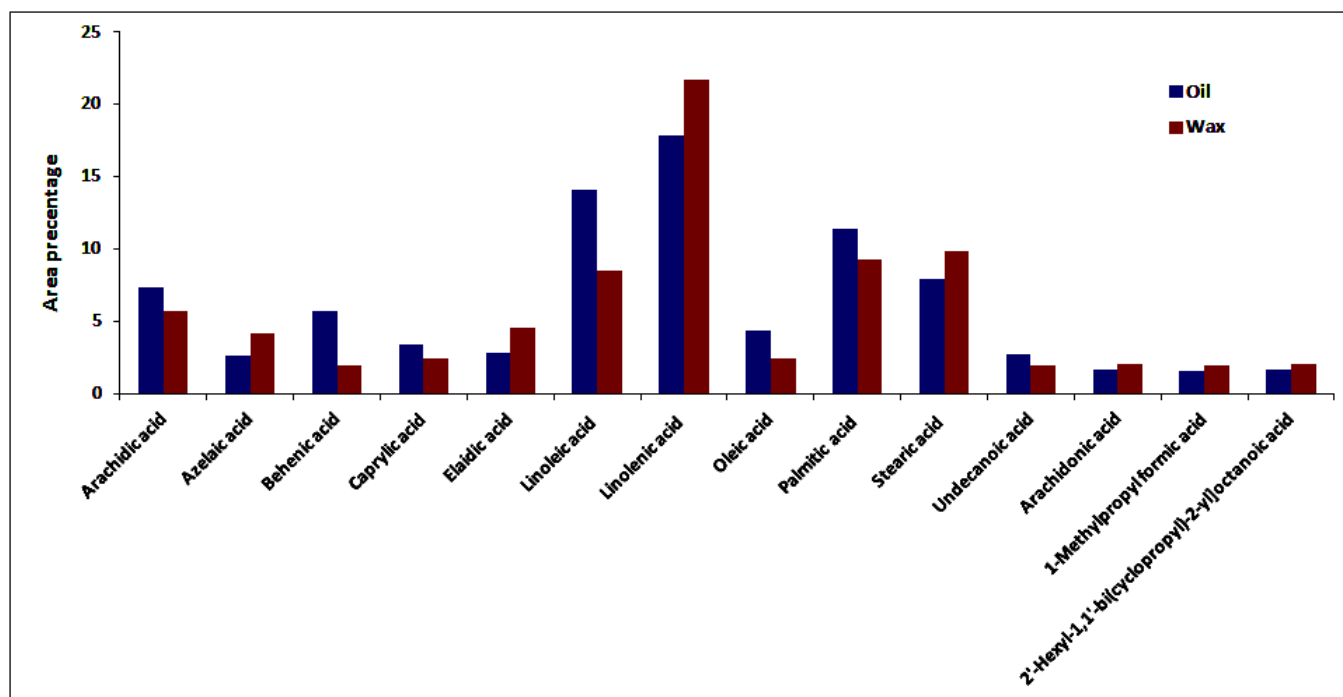


Fig. 5 Graphical presentation of fatty acids identified from the oil and wax

All the fractions and isolates (oil and wax) of *K. africana* produced antimicrobial effects against the tested Gram-positive, Gram-negative and yeast pathogens with MIC values ranging from 0.0625 to 8.0 mg/mL. The crude hydro-alcoholic extract exhibited the strongest activity with MICs of 0.125-0.5 mg/mL, followed by the ethyl acetate (MICs=0.125-1.0

mg/mL), dichloromethane (MICs=0.250-2.0 mg/mL), hexane (MICs=0.25-2.0 mg/mL), acetone (MICs=0.5-2.0 mg/mL) and methanol (MICs=1.0-2.0 mg/mL) column fractions. The wax (MICs=2.0-4.0 mg/mL) and oil (MICs=4.0-8.0 mg/mL) were less effective. MIC values of test samples and standards against pathogenic bacteria and yeasts are given in Table II.

TABLE I
MAJOR FATTY ACIDS IDENTIFIED FROM THE OIL AND WAX BY GC/MS AND GC×GC/MS

Name	Esterified product	Exact mass	Total area (%)	
			Oil	Wax
Arachidic acid	Methyl arachidate	326.3185	7.35	5.72
Arachidonic acid	Methyl <i>cis</i> -5,8,11,14-eicosatetraenoate	318.2559	1.7	2.1
Azelaic acid	Dimethyl azelate	216.2700	2.62	4.19
Behenic acid	Methyl behenate	354.6121	5.72	1.93
Caprylic acid	Methyl caprylate	158.1307	3.37	2.43
Elaidic acid	Methyl elaidate	296.4923	2.85	4.57
Linoleic acid	Methyl linoleate	294.2559	14.09	8.56
Linolenic acid	Methyl linolenate	292.2402	17.90	21.68
Oleic acid	Methyl oleate	296.4923	4.37	2.48
Palmitic acid	Methyl palmitate	270.2559	11.44	9.26
Stearic acid	Methyl stearate	298.2872	7.92	9.84
Undecanoic acid	Methyl undecanoate	200.1776	2.72	1.98
1-Methylpropyl formic acid	Formic acid 1-methylpropyl ester	102.0681	1.6	2.0
2'-Hexyl-1,1'-bi(cyclopropyl)-2-yl]octanoic acid	2'-Hexyl-[1,1'-bicyclopropyl]-2-octanoic acid, methyl ester	322.2872	1.7	2.1
Total			85.35%	78.84%

TABLE II
MINIMUM INHIBITORY CONCENTRATION (MIC) VALUES OF TEST SAMPLES AND STANDARDS AGAINST PATHOGENIC BACTERIA AND YEASTS

Test samples/ standards	Minimum inhibitory concentration (mg/mL)					
	<i>Staphylococcus aureus</i>	<i>Enterococcus faecalis</i>	<i>Moraxella catarrhalis</i>	<i>Escherichia coli</i>	<i>Candida albicans</i>	<i>Candida tropicalis</i>
Hydroalcoholic	0.250	0.500	0.500	0.500	0.125	0.500
Methanol	2.000	2.000	2.000	2.000	2.000	1.000
Acetone	1.000	1.000	1.000	2.000	1.000	0.500
Ethyl acetate	0.125	0.500	0.500	1.000	0.125	0.125
Dichloromethane	0.500	1.000	2.000	0.500	0.250	0.250
Hexane	2.000	2.000	2.000	2.000	2.000	0.250
Oil	4.000	4.000	4.000	8.000	8.000	4.000
Wax	4.000	4.000	2.000	2.000	2.000	4.000
Positive control*	0.0005	0.001	0.0002	0.0005	0.001	0.001

*Ciprofloxacin for bacteria and amphotericin B for the yeasts; acetone/water as negative control.

IV. CONCLUSION

The crude fruit extract of *K. africana* showed the strong antimicrobial activity, which justified its traditional use in microbial infections. The chemical constituents in the fruits of the plant, those are responsible for the antimicrobial activities, may have synergistic interactions, which are lost during compound isolation. A more holistic approach is needed to fully understand the antimicrobial activity of this important African remedy.

ACKNOWLEDGMENT

The work is financially supported by National Research Foundation and Tshwane University of Technology, South Africa.

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