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Antimicrobial compounds from the Kenyan *Ganoderma adspersum* (Schulz.) Donk species

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ABSTRACT

The emergence of antibiotic resistant pathogens has continuously increased, leading to a growing worldwide health threat due to infectious diseases. And therefore in our search for antibacterial and antifungal compounds from the polypore *Ganoderma adspersum*, the dried, ground fruiting bodies of *G. adspersum* were extracted with methanol and solvent removed in a rotary evaporator. The extract was suspended in distilled water, then partitioned using ethyl acetate solvent to obtain an ethyl acetate extract. The extract was fractionated and purified using column chromatographic method and further purification on sephadex LH20. The chemical structures were determined on the basis of NMR spectroscopic data from ^{1}H and ^{13}C NMR, HSQC, HMBC, ^{1}H-^{1}H COSY, and NOESY experiments. Antimicrobial activity against clinically important bacterial and fungal strains was assessed and zones of inhibition were recorded. Compound (**1**), ergosta-7,22-dien-3-one weakly inhibited the growth of Gram positive bacteria *Streptococcus pneumonia* and a fungus *Cryptococcus neoformans*. Compounds ergosta-7,22-dien-3-ol (**2**) and ergosta-5,7,22-trien-3-ol (**3**) also inhibited gram positive *Streptococcus pyogenes* bacteria.

Keywords: Polypores, steroid compounds, antimicrobial activity.

INTRODUCTION

The genus *Ganoderma* is a group of wood degrading polypores with hard fruiting bodies and an abundant producer of novel compounds. A number of *Ganoderma* species have been used in Asia for many centuries for their medicinal properties and were cultivated at an industrial scale (Wasser and Weis, 1999; Lai et al., 2004). Thus, some of the *Ganoderma* products have previously attained a broad economic value (Lai et al., 2004). Most of the metabolites known from *Ganoderma* have been isolated from specimens assigned to "*Ganoderma lucidum*" (Paterson and Russell, 2006). The secondary metabolism of other *Ganoderma* species has been studied less extensively, and for some species such as *Ganoderma adspersum*, scanty data seem to be available (Tel-Çayan et al., 2015). The aim of this study was to search for antimicrobial compounds from *Ganoderma adspersum*. The wood-decaying polypore (bracket fungi) occurs in a very wide range of tree species including deciduous trees

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and conifers throughout the world. The species has more frequently been detected in trees growing near human habitations, gardens, parks, and planted sites (De Simone and Annesi, 2012; Tel-Çayan et al., 2015). *Ganoderma adspersum* is a pathogen of roots and butts of living trees causing white rot, and can continue to grow saprophytically on non-living tissue such as stumps of felled trees (De Simone and Annesi, 2012). The polypore is a saprotroph, it excretes enzymes into their environment to degrade their substrate, before using it as a source of nutrients for their growth and development. Natural products have played a pivotal role in antibiotic drug discovery with most antibacterial drugs being derived from a natural product or natural product lead. But, the rapid onset of resistance to most antibacterial drugs diminishes their effectiveness considerably and necessitates a constant supply of new antibiotics for effective treatment of infections (Butler and Buss, 2006). Majority of, if not all, higher basidiomycetes contain different types of biologically active high-molecular weight and low-molecular weight compounds (triterpenes, lectins, steroids, phenols, polyphenols, lactones, statins, alkaloids, and antibiotics) in fruit bodies, cultured mycelia, and cultured broth (Wasser, 2010; Chang and Wasser, 2012; De Silva et al., 2013; Lindequist, 2013). In our search for antimicrobial agents from this polypore mushroom, the ethyl acetate extract from fruiting bodies of *G. adspersum* were fractionated and the possible antimicrobial activities of isolated compounds determined.

MATERIALS AND METHODS

Fungal material

The fruiting bodies of *Ganoderma adspersum* (Schulz.) Donk were collected in the month of July 2013 from Kabarnet forest, Baringo County in Kenya. The identification of the polypore mushroom was done through the examination of morphological features and further molecular identification by Dr. Leung Siu Han from Mushroom Initiative, Hong Kong. The voucher specimen number JO 13066 of *Ganoderma adspersum* species was kept as herbarium in Integrated Biotechnology Research Laboratory at Egerton University.

General experimental procedures

Column chromatography was performed on silica gel (Kieselgel 60, 70-230 mesh, Merck) and Sephadex LH-20 (GE Healthcare, Uppsala, Sweden). Thin-layer chromatography (TLC) was performed on Silica gel 60 F254 plates and TLC spots were detected under UV-254-nm light and also were visualized by spraying with anisaldehyde reagent and heating. NMR analysis was performed on a Bruker 500 MHz NMR spectrophotometer and spectra were recorded in $CDCl_3$ at the University of Surrey, United Kingdom. Structures of compounds were elucidated and were confirmed by comparison of their NMR data against literature values. All the chemicals were obtained from Sigma Chemical Co. (Sigma–Aldrich GmbH, Sternheim, Germany).

Extraction and isolation

The dried material (823 gm) of *Ganoderma adspersum* was extracted four times with 3 L methanol, (4 ×3 L) at room temperature to give a deep brown syrup after concentration in vacuum. The crude extract was then suspended in water and subjected to liquid/liquid partition using ethyl acetate to obtain 5.0 gm ethyl acetate extract. The ethyl acetate extract was fractionated on silica gel column chromatography using gradient of hexane and dichloromethane increasing polarity (100:0 to 0:100) to afford sub fractions. Similar sub-fractions were combined on basis similar spot patterns on thin-layer chromatographic plates to give fractions Fr_1, Fr_2 and Fr_3. Pooled fraction Fr_1 was purified further on silica gel column chromatography (eluted with dichloromethane/ethyl acetate 90:10 to yield compound **1** (12 mg). Fractions Fr_2 and Fr_3 were also repeatedly purified on a small silica gel column chromatography (eluted with dichloromethane/hexane 80:20 and 70:30 respectively to afford compound **2** (6.4 mg). The fraction Fr_3 was further purified on Sephadex LH20 to yield compound **3** (8 mg).

Antimicrobial activity

Antimicrobial activity assay was carried out at the Kenya Medical Research Institute (KEMRI, Nairobi). The pure compounds were evaluated for their antimicrobial activity by using agar diffusion method against clinically important strains that included gram negative strains; *Salmonella typhi*, *Shigella*, *Escherichia coli*, *Citobacter enterocolitica* and *Klebsiella pneumonia*, gram positive bacteria; *Streptococcus pyogenes*, *Streptococcus pneumonia, Staphylococcus aureus* and *Entero feacalis*. The antifungal test organisms were *Candida albicans* and *Cryptococcus neoformans*. The zone of inhibition values were evaluated in accordance to (CLSI, 2012). Circular paper discs of equal size were impregnated with compounds broth (50 mg/ml) and air dried. The compounds treated discs were placed on plates where the organisms were cultured according to (CLSI, 2012) with the aid of sterile forceps and a wire loop (Hudzicki, 2009). Cover slip was placed on the petri dishes then incubated at 37 ºC for 24 hours. Absence of bacteria growth around the impregnated disc indicated antimicrobial activity of the test compounds. The level of inhibition was measured as the distance between the bacterial growth and the disc expressed in millimeters. Phosphate buffered saline and ciprofloxacin were used as the negative and positive control respectively.

To guarantee the reproducibility of the results, individual experiments were performed in triplicates and mean values expressed as mean ± standard error of mean. Zones of inhibition by the antimicrobial agents were measured, recorded and tabulated in Microsoft excel® spreadsheet. The data was exported to Minitab statistical software v18.0 upon which descriptive statistics were derived and expressed as mean ± standard error of mean (SEM). Significant difference between the means of different treatment groups was determined by One-way ANOVA. Tukey's post hoc test was then carried out for pairwise comparison of means. The values of $p \leq 0.05$ were considered.

RESULTS

The dried fruiting bodies of *Ganoderma adspersum* yielded three known steroids. Their structures were identified on the basis spectral data from NMR spectroscopic experiments as, ergosta-7,22-dien-3-one (**1**), ergosta-7,22-dien-3-ol **(2),** ergosta-5,7,22-trien-3-ol (**3**) (Figure 1). Compound **1** was obtained as a white amorphous solid (12 mg). The ^{1}H-NMR spectrum (Figure 2) of **1** displayed key olefin resonance signals at δ_H 5.18-5.20 Hz and six characteristic ergostane-type steroidal methyl signals at δ_H 1.03 (d, *J* =6.2 Hz, 3H-21), 1.02(s 3H-19), 0.93 (d, *J* =6.8 Hz, 3H-28), 0.83 (d, *J*= 6.4 Hz, 3H-26), 0.83 (d, *J*= 6.4 Hz, 3H-27) and δH 0.58 (s, 3H-18). The ^{13}C NMR spectrum of the compound displayed 28 carbons, including carbonyl carbon signal at δc 212.2 (C-3), and four olefin carbon signals at δ 139.9 (C-8), 117.2 (C-7), 132.8 (C-22) and 135.5 (C-23). The ^{1}H and ^{13}C NMR (Table 1**)** DEPT-135, and HSQC data for **1** supported the presence of one keto (C-3), two sp^3 and one sp^2 quaternary carbon, ten methine groups, eight methylene groups and six methyl groups. The keto group at δ_C 212.0 C-3 was further confirmed by key HMBC correlations from proton signals at δ_H 2.42 (H-2), δ_H 2.23 (H-1), δ_H 2.12 (H-4). The structure of compound **1** was concluded as an ergosta-7,22-dien-3-one and it was also affirmed by comparing its spectral data with the literature (Protiva et al., 1980).

Compound (**2**) was obtained as a white amorphous powder (6.4 mg). Analysis of its ^{13}C NMR (Table 1) spectra showed four typical olefinic carbons at δ_c 117.5. 132.1, 135.8 and 139.8 and a methine oxygenated carbon at δ_c 71.2. A ^{13}C and DEPT spectra showed 28 carbon resonances, including six methyl groups, eight methylene groups, eleven methine groups (three sp^2 methines, and one oxygenated methine) and three quaternary carbons. Typical signals of the side chain as observed in the ^{1}H NMR (Figure 3) were of proton olefins δ_H 5.18 dd *J*=7.3, 15 Hz (H-22), δ_H 5.19 dd *J*= 7.4, 15.1 (H-23) and four doublet methyls δ_H 1.02 *J*=6.7 (H-21), δ_H 0.83 *J*=6.8, δ_H 0.83 *J*=6.7 and δ_H 0.91 *J*=6.7

(H-28) were observed. The full assignments of the ^{1}H and ^{13}C NMR signals were achieved by detailed interpretations of 2D NMR data including $^{1}H–^{1}H$ COSY, HSQC, and HMBC. The structure was concluded as ergosta-7,22-dien-ol and it was also affirmed by comparing its spectral data with the literature (Lee et al., 2006).

Compound **3** was obtained as a white solid (8 mg). The ^{1}H NMR spectrum (Figure 4) of the compound was indicative of two tertiary methyls (δ_H, 0.94, 0.63) and four secondary methyl groups at δ_H 0.83, 0.84, 0.91 and 1.03. The other key proton resonances are vinyl protons at δ_H 5.57, 5.39 and 5.19-5.21. A broad deshielded proton signal at δ_H 3.64 was also observed. The ^{13}C-NMR spectrum together with DEPT-135 revealed 28 carbon signals that included one oxygenated methine carbon at δc 70.7 (C-3) and six olefin carbon signals δ_C 116.6 C-7, 119.8 C-6, 132.2 C-23, 135.8 C-22, 140.3 C-8 and 141.8 C-5. The H-3 resonance (δ_H3.64) showed HMBC spectrum correlation with the fully substituted C-5 carbon resonance (δ_C 141.8). On further comparison with reported values (Kwon et al., 2002; Gao et al., 2007), the structure of the compound was determined to be ergosta-5,7,22-trien-3β-ol.

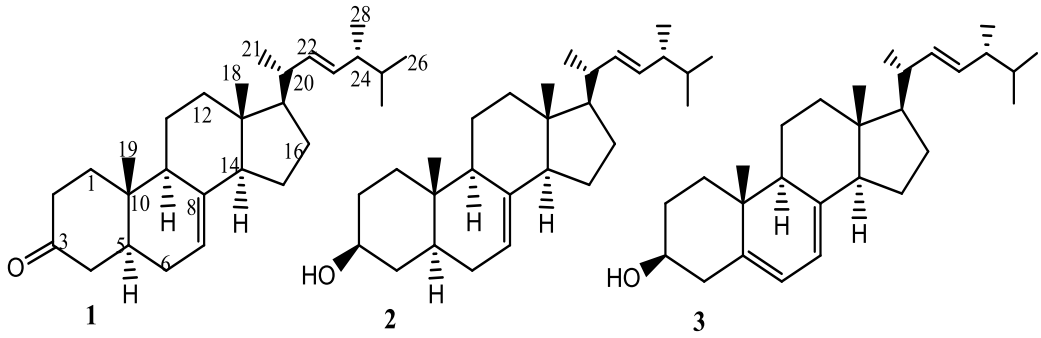

Figure 1: Structures of compounds 1-3.

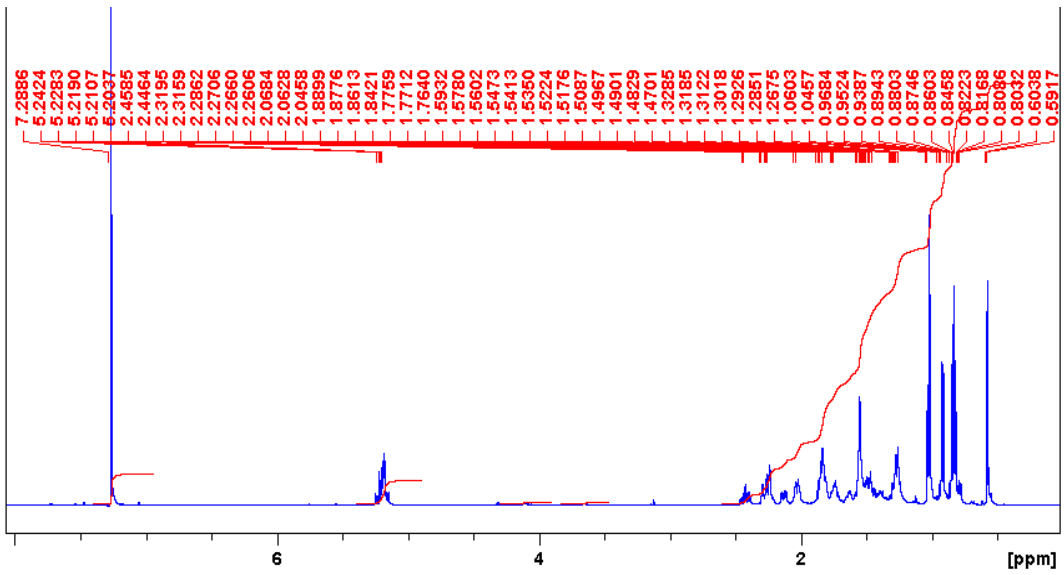

Figure 2: Proton NMR spectra of compounds **1** in $CDCl_3$.

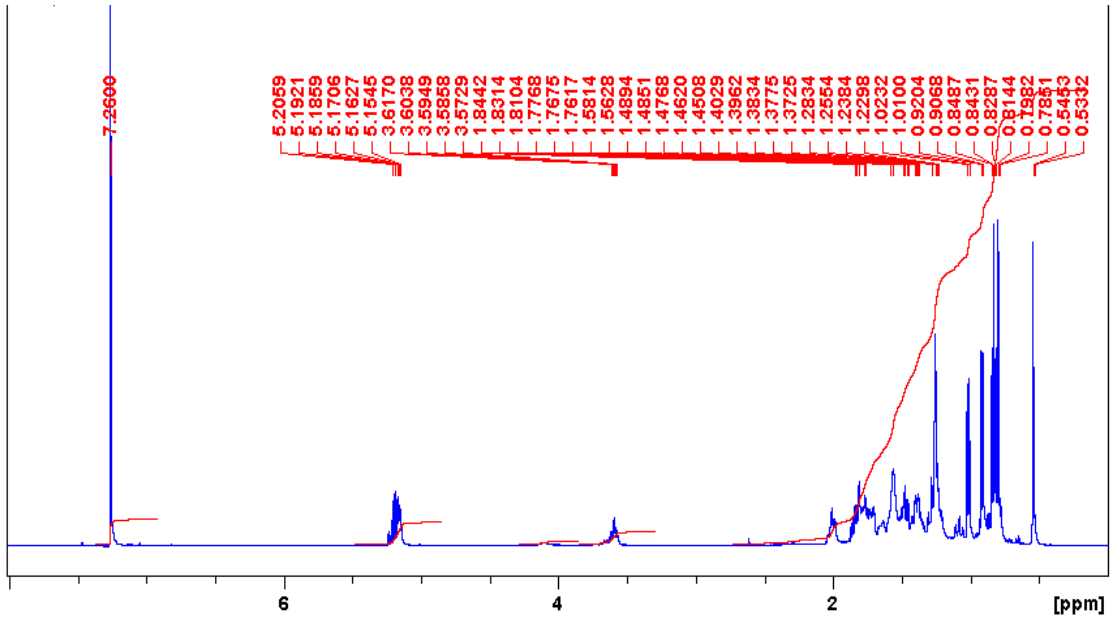

Figure 3: Proton NMR spectra of compounds **2** in $CDCl_3$.

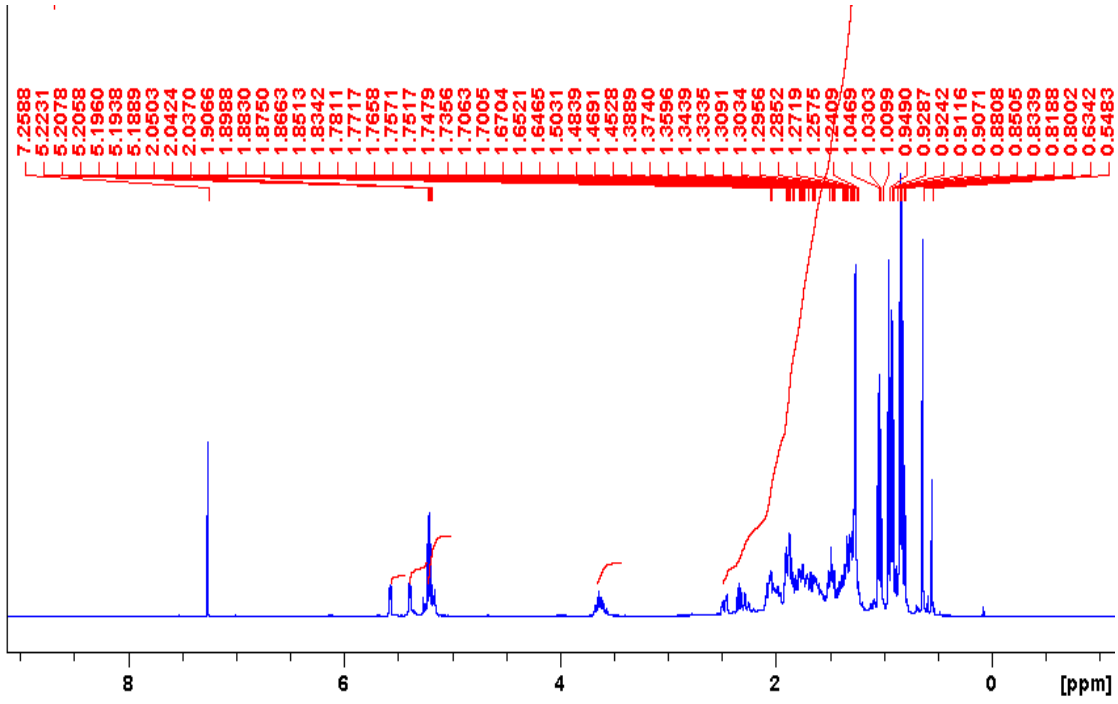

Figure 4: Proton NMR spectra of compounds **3** in $CDCl_3$.

Table 1: ^{13}C NMR chemical shifts of the steroids from *Ganoderma adspersum.*

C	1	2	3	C	1	2	3
1	39.0	37.6	38.6	**15**	23.1	23.1	23.0
2	38.4	28.3	32.2	**16**	28.3	31.7	29.9
3	212.2	71.3	70.7	**17**	56.2	56.2	56.0
4	44.5	38.2	41.0	**18**	12.4	12.3	12.3
5	43.5	40.5	141.8	**19**	12.7	13.2	16.3
6	30.3	29.9	119.8	**20**	40.6	40.7	40.6
7	117.0	117.2	116.6	**21**	21.3	21.3	21.3
8	139.7	139.7	140.3	**22**	135.8	135.9	135.8
9	49.2	49.7	46.6	**23**	132.3	132.3	132.2
10	34.5	34.4	37.2	**24**	43.0	43.0	43.1
11	21.9	21.8	21.3	**25**	33.3	33.3	33.4
12	39.6	39.7	39.3	**26**	20.2	20.2	20.1
13	43.5	43.5	43.1	**27**	19.9	19.9	19.9
14	55.2	55.3	54.8	**28**	17.7	17.7	17.8

Solvent: CD_3Cl δ_c in ppm, 125 MHz

DISCUSSION

From this study the compound, ergosta-7,22-dien-3-one was found to weakly inhibit the growth of Gram positive bacteria *Streptococcus pneumonia* by 7.7±0**.**67 mm *and* a fungus *Cryptococcus neoformans* by 8.0±0.58 mm (Table 2). The compound had previously been found to exhibit antiviral activity against influenza A virus (Niedermeyer et al., 2005). Ergosta-7,22-dien-3β-ol, has been reported to have antiviral activity against influenza A virus and Herpes simplex virus type 1 (HSV)44 (Seo et al., 2009). In this study, it was found to inhibit the growth of gram positive *Streptococcus pyogenes* by 9.7±0.33. There was no inhibition against the test strains of gram negative bacteria and fungi. Compound ergosta-5,7,22-trien-3β-ol has also been reported previously to have several biological effects attributed to it including anti-inflammatory activity (Kobori et al., 2007), which could provide significant protection against the promotion of bladder tumour induced by many types of promoters in the environment and colon adenocarcinoma cell growth, as well as MCF-7 cell line proliferation *in vitro* and tumour growth in H-22 implanted mice in vivo (Yan et al., 2009). It is worth noting that all the compounds were not relatively potent inhibitors of Gram-negative bacteria, as seen in some of the literature (Quereshi et al., 2010). From the current study ergosta-5,7,22-trien-3β-ol was found to weakly inhibit only Gram positive *Streptococcus pyogenes* by 9.0±0.58 mm compared to positive control ciprofloxacin (31.0±0.58 mm).

Table 2: Mean±SEM (mm) diameter of inhibition zones in agar diffusion antimicrobial assay.

Test microorganisms	Ciprofloxacin	ergosta-7,22-dien-3-one	ergosta-7,22-dien-3β-ol	ergosta-5,7,22-trien-3β-ol
S. pyogenes	31.0±0.58[a]	6.0±0.00[c]	**9.7±0.33[b]**	**9.0±0.58[b]**
S. pneumonia	30.0±0.58[a]	**7.7±0.67[b]**	6.0±0.00[c]	6.0±0.00[c]
K. pneumonia	32.7±1.45[a]	6.0±0.00[b]	6.0±0.00[b]	6.0±0.00[b]
E. feacalis	24.0±0.58[a]	6.0±0.00[c]	6.0±0.00[c]	6.0±0.00[c]
S. aureus	19.0±0.58[a]	6.0±0.00[c]	6.0±0.00[c]	6.0±0.00[c]
C. enterocolitica	22.0±1.15[a]	6.0±0.00[b]	6.0±0.00[b]	6.0±0.00[b]
Shigella	24.7±0.88[a]	6.0±0.00[b]	6.0±0.00[b]	6.0±0.00[b]
E. coli	22.3±0.88[a]	6.0±0.00[b]	6.0±0.00[b]	6.0±0.00[b]
C. albicans	27.7±0.33[a]	6.0±0.00[b]	6.0±0.00[b]	6.0±0.00[b]
C. neoformans	24.0±1.00[a]	**8.0±0.58[b]**	6.0±0.00[c]	6.0±0.00[c]
S. typhi	25.0±1.15[a]	6.0±0.00[b]	6.0±0.00[b]	6.0±0.00[b]

[a,b] Means values followed by the same superscript row-wise are not significantly different at ($p<0.05$).

Conclusion

In conclusion, the compounds isolated from *Ganoderma adspersum* showed weak antimicrobial activity against Gram positive *Streptococcus pneumonia* and *Streptococcus pyogenes.* Equally, lower antimicrobial activity against a fungus *Cryptococcus neoformans* was observed. And finally no activity against Gram-negative bacteria and *Candida albicans* was observed.

COMPETING INTERESTS

No potential competing interest was reported by the authors.

AUTHORS' CONTRIBUTIONS

RKM was the principal investigator, MKL, AWN, JOO, PKC contributed fully to the work. All authors read and approved the manuscript.

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