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 ¹H and ¹³C NMR, HSQC, HMBC, ¹H-¹H 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.

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**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.
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 CDCl3 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 Fr1, Fr2 and Fr3. Pooled fraction Fr1 was purified further on silica gel column chromatography (eluted with dichloromethane/ethyl acetate 90:10 to yield compound 1 (12 mg). Fractions Fr2 and Fr3 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 Fr3 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 °C 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≤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 1H-NMR spectrum (Figure 2) of 1 displayed key olefin resonance signals at δH 5.18-5.20 Hz and six characteristic ergostane-type steroidal methyl signals at δH 0.93 (d, J=6.8 Hz, 3H-28), 1.03 (d, J=6.2 Hz, 3H-21), 1.02(s 3H-19), 0.93 (d, J=6.4 Hz, 3H-26), 0.83 (d, J=6.4 Hz, 3H-27) and δH 0.58 (s, 3H-18). The 13C NMR spectrum of the compound displayed 28 carbons, including carboxyl carbon signal at δc 212.2 (C-3), and four olefin carbon signals at δc 139.9 (C-8), 117.2 (C-7), 132.8 (C-22) and 135.5 (C-23). The 1H and 13C NMR (Table 1) DEPT-135, and HSQC data for 1 supported the presence of one keto (C-3), two sp3 and one sp3 quaternary carbon, ten methine groups, eight methylene groups and six methyl groups. The keto group at δc 212.0 C-3 was further confirmed by key HMBC correlations from proton signals at δH 2.42 (H-2), δH 2.23 (H-1), δH 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 13C NMR (Table 1) spectra showed four typical olefinic carbons at δc 117.5. 132.1, 135.8 and 139.8 and a methine oxygenated carbon at δc 71.2. A 13C and DEPT spectra showed 28 carbon resonances, including six methyl groups, eight methylene groups, eleven methine groups (three sp3 methines, and one oxygenated methine) and three quaternary carbons. Typical signals of the side chain as observed in the 1H NMR (Figure 3) were of proton olefins δH 5.18 dd J=7.3, 15 Hz (H-22), δH 5.19 dd J= 7.4, 15.1 (H-23) and four doublet methyls δH 1.02 J=6.7 (H-21), δH 0.83 J=6.8, δH 0.83 J=6.7 and δH 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 ($\delta_{\text{H}}$, 0.94, 0.63) and four secondary methyl groups at $\delta_{\text{H}}$ 0.83, 0.84, 0.91 and 1.03. The other key proton resonances are vinyl protons at $\delta_{\text{H}}$ 5.57, 5.39 and 5.19-5.21. A broad deshielded proton signal at $\delta_{\text{H}}$ 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 $\delta_{\text{C}}$ 70.7 (C-3) and six olefin carbon signals $\delta_{\text{C}}$ 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 ($\delta_{\text{H}}$3.64) showed HMBC spectrum correlation with the fully substituted C-5 carbon resonance ($\delta_{\text{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.](image1)

![Figure 2: Proton NMR spectra of compounds 1 in CDCl$_3$.](image2)
Figure 3: Proton NMR spectra of compounds 2 in CDCl₃.

Figure 4: Proton NMR spectra of compounds 3 in CDCl₃.
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$_2$Cl$_2$, δ 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±0.58a    | 6.0±0.00c               | 9.7±0.33b               | 9.0±0.58b                 |
| S. pneumonia        | 30.0±0.58a    | 7.2±0.67b               | 6.0±0.00c               | 6.0±0.00c                 |
| K. pneumonia        | 32.7±1.45a    | 6.0±0.00b               | 6.0±0.00b               | 6.0±0.00b                 |
| E. faecalis         | 24.0±0.58a    | 6.0±0.00c               | 6.0±0.00c               | 6.0±0.00c                 |
| S. aureus           | 19.0±0.58a    | 6.0±0.00c               | 6.0±0.00c               | 6.0±0.00c                 |
| C. enterocolitica   | 22.0±1.15a    | 6.0±0.00b               | 6.0±0.00b               | 6.0±0.00b                 |
| Shigella            | 24.7±0.88a    | 6.0±0.00b               | 6.0±0.00b               | 6.0±0.00b                 |
| E. coli             | 22.3±0.88a    | 6.0±0.00b               | 6.0±0.00b               | 6.0±0.00b                 |
| C. albicans         | 27.7±0.33a    | 6.0±0.00b               | 6.0±0.00b               | 6.0±0.00b                 |
| C. neoformans       | 24.0±1.00a    | 8.0±0.58b               | 6.0±0.00c               | 6.0±0.00c                 |
| S. typhi            | 25.0±1.15a    | 6.0±0.00b               | 6.0±0.00b               | 6.0±0.00b                 |

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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