Biflavonoids from an Ethno-Medicinal Plant *Ochna holtzii* Gilg

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**Abstract**

*Ochna holtzii* Gilg is a medicinal plant used extensively at the Kenya Coast for the treatment of various ailments. From solvent extracts of the root and stem barks of *O. holtzii*, seven constituents were isolated by standard chromatographic techniques (CC, VLC, prep-TLC and Sephadex LH-20). Their structures were analyzed by MS, UV,IR,1D and 2D NMR spectroscopy. All were found to be biflavonoids, including three novel compounds: dehydrate of lophirone C, holzizol, and tri-O-methyl lophirone A. Crude methanol extracts of *O. holtzii* and the isolated biflavonoids were tested for antimicrobial activities against two Gram-positive bacteria, *Staphylococcus aureus* and *Bacillus subtilis*, two Gram-negative bacteria, *Salmonella typhi* and *Pseudomonas aeruginosa*, and the diploid fungus *Candida albicans*. The extracts and the isolated constituents showed varying levels of activities against the microbes. Afzelone D, lophirone A and the novel tri-O-methyl lophirone A showed strong activities against *P. aeruginosa* and *S. aureus*, with the latter being more susceptible. Calodenin B and the novel dehydrolophirone C also showed strong activities against these bacteria, but were more active against *P. aeruginosa*. However, none of these matched those of the antibiotic Chloramphenicol or the antifungal Fluconazole. This represents the first study on phytochemical and antimicrobial profiles of *O. holtzii*.

**Keywords:** Ochnaholtzii Gil; Ochnaceae; Biflavonoids; Dehydrolophirone C; Holzizol, Afzelone D O-methyl ether

**Introduction**

*Ochna holtzii* Gilg (Ochnaceae) is one of eight plant species of the genus *Ochna* that are found in Kenya [1]. Plants in this genus are known to be rich in biflavonoids, isoflavonoids and anthranoids [2-4]. Flavonoids exhibit a wide range of biological activities, and have been of particular interest as potential anticancer [5], anti-HIV-1 [6,7] and antibacterial agents and analgesics, as well as insecticides and insect antifeedants [8]. Moreover, flavonoids are effective antioxidants and antidotes for snake bites [9], and are considered to provide protection from cardiovascular diseases [10], certain forms of cancer and age related degeneration of cell components [11]. *Ochnaholtzii* is a plant found mainly at the coastal region of Kenya. The local communities use various parts of the plant (stem and root bark) to treat different health problems, including high fever, headaches and coughs, and to soothe persistent backaches, especially in old age [12]. It is also used extensively with other plant extracts in treating microbial infections [1]. The bio-activities of this plant and associated phytochemicals have not been systematically investigated. In the present study, we undertook chromatographic separation of solvent extracts of the stem and root bark, isolated and structurally characterized some of the constituents and compared their antimicrobial and antifungal activities with those of crude extracts. Herein we report our findings.

**Discussion**

Compound 1 was obtained as yellow crystals from the EtOAc stem bark extract of *O. holtzii*. The UV spectrum exhibited absorption peaks at \(\lambda_{max} \) 201, 223 and 274 nm, characteristic of compounds with highly conjugated system [13]. EI-MS (70eV) gave m/z at 526 [M+H]+. The 13C NMR spectrum had 24 distinct signals for 30 carbon atoms, 2 of which were olefinic with \(J_{\text{HS}}\) 156.2 and 114.5 at positions \(\alpha 2\) and \(\beta 2\), respectively, in compound 1. The \(1^H\) NMR spectrum displayed 15 signals of which thirteen (13) appeared in the aromatic region and 2 were olefinic with \(J_{\text{CH}} = 15.4\) Hz characteristic of a trans-configuration. The \(1^3C\) NMR spectrum had 24 distinct signals for 30 carbon atoms, 2 of which were carbolnyl carbon atoms and over 12 associated with several aromatic rings. Chemical shifts assignments were made from the analysis of \(1^H\)-\(1^H\) COSY, HMBC and HQMRC spectra.

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The 1H NMR spectrum of DEPT) and 2D NMR (COSY, HSQC, HMBC and NOESY) spectra identical to those of calodenin B [3].

Compound 6 was obtained from the root bark that gave m/z 539 [M+H]+ corresponding to C18H16O8 with EIMS (70eV), MS fragmentation pattern, 1D NMR (1H, 13C and DEPT) and 2D NMR (COSY, HSQC, HMBC and NOESY) spectra identical to those of afzelone D [16].

Compound 7 was obtained as yellow crystals from the EtOAc root bark extract. It gave m/z at 534 [M+H]+ corresponding to C19H17O8, which was confirmed by NMR data, including 1D NMR (1H, 13C and DEPT) and 2D NMR (COSY, HSQC, HMBC and NOESY) spectra. The 1H NMR spectrum of 7 displayed 19 signals. Two singlets at δ 3.76 and 3.66, integrated for three and six methoxyl protons, respectively. The six protons singlet indicated that the two methoxyl groups are symmetrical positioned. The other 17 signals were very similar with respect to shifts and splitting pattern with those of lophirone A [15] and afzelone D [16] reported earlier. The existence of three methoxyl groups implied that compound 7 was tri-methoxy derivative of lophirone A, that is, lophirone A trimethyl ether (Figure 1 and Table 1).

### Biological activity

The root methanolic crude extract exhibited higher antimicrobial properties against S. aureus, P. aeruginosa and B. subtilis compared to those of the stem bark extract (Table 2). The isolated compounds showed varying levels of activities against the two gram positive and two gram negative bacteria, as well as against the diploid fungus. Lophirone A, afzelone D and lophirone Atrimethyl ether also showed strong activities against these bacteria. Likewise, calodenin B and dehydrolophirone C showed also strong activities against the bacteria as well as against C. albicans. Further studies on related compounds that may be isolated from other Ochna and related plant species are expected to shed some light on the structural requirements for antimicrobial activities of this group of natural products. In addition, it would be interesting to extend the study to other bioactivities of these bioflavonoids. For example, calodenin B was previously found to exhibit cytotoxicity against breast cancer cells [17], and Lophirone A was reported to inhibit Epstein-Barr virus [18]. This biflavonoid has also been reported to exhibit potent cytotoxicity activity against

| Position | Compound 1 | Compound 3 | Compound 7 |
|----------|------------|------------|------------|
| δC       | δH (ppm), m*, (J [Hz]) | δC | δH (ppm), m*, (J [Hz]) |
| B–1      | 114.7 s | 114.7 s | 117.0 s |
| B–2      | 167.6 s | 167.7 s | 159.2 s |
| B–3      | 103.8 d | 6.20 (1H, d, J=2.2 Hz) | 103.9 d | 6.27 (1H, d, J=2.2 Hz) | 103.3 d | 6.69 (1H, d, J=2.2 Hz) |
| B–4      | 166.7 s | 166.7 s | 165.8 s |
| B–5      | 109.3 d | 6.34 (1H, dd, J=9.0, 2.2 Hz) | 108.8 d | 6.42 (1H, dd, J=2.2, 9.1 Hz) | 116.1 d | 6.83 (1H, dd, J=2, 8.9) |
| B–6      | 133.7 d | 7.91 (1H, d, J=9.0 Hz) | 133.2 d | 8.10 (1H, d, J=9.1 Hz) | 128.2 d | 7.85 (1H, d, J=8.9 Hz) |
| C–1      | 193.3 s | 192.8 s | 177.1 s |
| α–1      | 121.4 d | 7.70 (1H, d, J=15.4 Hz) | 119.3 d | 7.72 (1H, d, J=15.4 Hz) | 122.4 s |
| β–1      | 145.2 d | 7.83 (1H, d, J=15.4 Hz) | 144.8 d | 7.85 (1H, d, J=15.4 Hz) | 157.4 d | 8.22 (1H, s) |
| A–1      | 130.8 s | 129.3 s | 135.7 s |
| A–2      | 122.1 d | 7.71 (1H, d, J=2.0 Hz) | 126.9 s | 7.16 (2H, d, J=8.4 Hz) | 129.8 d |
| A–3      | 132.5 s | 132.1 s | 116.1 d | 6.56 (2H, d, J=8.4 Hz) |
| A–4      | 158.2 s | 158.9 s | 157.4 s |
| A–5      | 112.8 d | 7.58 (1H, d, J=8.8 Hz) | 111.0 d | 6.95 (1H, d, J=8.8 Hz) | 116.1 d | 6.56 (2H, d, J=8.4 Hz) |
| A–6      | 126.8 d | 7.74 (1H, d, J=8, 2.0 Hz) | 132.1 d | 7.50 (1H, d, J=8.8 Hz) | 128.9 d | 7.16 (2H, d, J=8.4 Hz) |
| C–2      | 196.1 s | 196.5 s | 204.8 s |
| α–2      | 114.5 s | 113.3 s | 44.6 d | 6.02 (1H, d, J=12.2 Hz) |
| β–2      | 156.2 s | 153.3 s | 54.3 d | 4.70 (1H, d, J=12.2 Hz) |
| A–1      | 121.4 s | 135.8 s |
| A–2, 6   | 130.6 d | 7.48 (2H, d, J=8.4 Hz) | 130.5 d | 7.16 (2H, d, J=8.4 Hz) |
| A–3, 5   | 116.7 d | 6.71 (2H, d, J=8.4 Hz) | 114.6 d | 6.56 (2H, d, J=8.4 Hz) |
| A–4      | 160.8 s | 164.5 s |
| B–1      | 114.6 s | 115.3 s | 115.0 s |
| B–2      | 167.5 s | 166.1 s | 166.8 s |
| B–3      | 103.8 d | 6.27 (1H, d, J=2.2 Hz) | 103.3 d | 6.38 (1H, d, J=2.2 Hz) | 101.7 d | 6.28 (1H, d, J=2.3 Hz) |
| B–4      | 167.3 s | 166.1 s | 168.0 s |
| B–5      | 109.5 d | 6.05 (1H, dd, J=8.8, 2.2 Hz) | 109.0 d | 6.49 (1H, dd, J=2.2, 8.0 Hz) | 108.5 d | 6.34 (1H, dd, J=2.3, 9.0) |
| B–6      | 136.7 d | 7.23 (1H, d, J=8.8 Hz) | 136.5 d | 7.97 (1H, d, J=8.0 Hz) | 134.4 d | 8.14 (1H, d, J=9.0 Hz) |
| A–4 - OCH3 | 49.5 q | 3.76 (3H, s) |
| A–4 - OCH3 | 56.1 q | 3.76 (3H, s) |
| B–4 - OCH3 | 55.5 q | 3.66 (3H, s) |

Table 1: 1H and 13C NMR data for compounds 1, 3 and 7.
melanoma (UACC62), renal (TK10) and breast (MCF10) cancer cell lines [19]. Recently, Ajiboye found that lophirone C has relatively high anticancer, antimutagenic, and antioxidant activities [20]. Thus, although our results with *O. holtzii* and its constituents provide some scientific rationale for the use of this ethno-medicinal plant by the communities of the coastal region of Kenya, it also suggests the need for extending the study to other *Ochna* species and other bioactivities.

### Experimental

#### Spectral measurements

$^1$H (1D, 2D COSY) and $^{13}$C spectra were recorded using Varian Gemini 400 MHz (NMR) instrument using CD$_3$OD as solvent. Peaks on $^1$H-NMR were recorded as singlet ($s$), doublet ($d$), doublet of doublet ($dd$), triplet ($t$), quartet ($q$) and (or) broad ($b$) using internal standard TMS as reference. The $^{13}$C-NMR multiplicity was determined by DEPT experiments. Chemical shifts were recorded in $\delta$ (ppm) and coupling constants, $J$, in Hertz (Hz). Standard sequences were used for COSY, HMQC, HMBC and NOESY experiments. IR: KBr pellets technique.

#### Chromatographic techniques

These include CC: silica gel 60 (0.063-0.200 mm, Merck); VLC and TLC: silica gel 60 F$_{254}$ (Merck) precoated aluminum plates; Sephadex LH-20 used as filter gel; and p-anisaldehyde spray with UV-Vis was used for visualization.

#### Disc diffusion assay

The crude extracts and the isolated compounds were tested for anti-microbial activity against *S. aureus*, *P. aeruginosa*, *B. subtilis*, *S. typhi* and *C. albicans* using the disc diffusion method.

### Table 2: Anti-microbial activities of methanolic extracts of root and stem barks of *O. holtzii* and isolated compounds.

| Isolate/extract            | *S. aureus* | *P. aeruginosa* | *B. subtilis* | *S. typhi* | *C. albicans* |
|----------------------------|-------------|-----------------|---------------|------------|--------------|
|                           | (Z-I) MIC   | (Z-I) MIC       | (Z-I) MIC     | (Z-I) MIC  | (Z-I) MIC    |
| Stem MeOH crude            | 20          | 500             | 19            | 1000       | 23           |
| Root MeOH crude            | 26          | 250             | 22            | 500        | 26           |
| Dehydrolophirone C (1)     | 21          | 250             | 24            | 500        | 23           |
| Holtzinol (3)              | 16          | 1000            | 14            | 1500       | 16           |
| Calodenin B                | 20          | 250             | 25            | 250        | 23           |
| Lophirone A                | 24          | 200             | 21            | 500        | 25           |
| Alzitone D                 | 23          | 200             | 20            | 500        | 25           |
| Tris-O-methyl/Lophirone A (7) | 23     | 200             | 20            | 500        | 24           |
| Chloramphenicol            | 32          | 36              | 31            | 59         | 32           |
| Fluconazole                | -           | -               | -             | -          | -            |

Z-I = Zone of inhibition in mm; MIC = Minimum Inhibitory Concentration in $\mu$g/ml

![Diagram of biflavonoids](image-url)

**Figure 1:** Biflavonoids isolated from the Ochna holtzii Gilg.
antimicrobial activities using agar diffusion technique against two Gram-positive, *Bacillus subtilis* and *Staphylococcus aureus*, two Gram-negative, *Salmonella typhi* (Type K [1]) and *Pseudomonas aeruginosa*, and a yeast *Candida albicans*. The plate diffusion method was used. Chloramphenicol was used as positive control. 14 g of nutrient agar was dissolved in 0.5 l of distilled water. Round filter paper MN 615 of 9 cm diameter was punched and the paper pieces sterilized. Nutrient agar (15 ml) was poured into Petri dishes in a laminar flow apparatus under sterile conditions. Then 0.1 ml of bacterial solution was added to it. Filter paper pieces containing 100 μg of the test extract were put on Petri dish and then finally incubated at 37°C for bacteria or 30°C for fungi. The results observed were recorded by measuring the diameter of the zone of inhibition from original 6 mm.

**Determination of MIC**

The MIC was determined using two-fold serial dilution method in a peptone water solution for bacterial and PDA broth for yeast and fungal of the active extracts. Each tube was then inoculated with 0.1 ml of standardized bacterial suspension (1×10⁸ CFU/ml) and fungal suspension (1×10⁸ spores/ml). The cultures were then incubated at 37°C for 24 hours for bacteria, 48 hours for yeast and at 30°C for 72 hours for fungi. The first tube showing no growth was the MIC.

**Plant materials**

The stem and root barks of *O. holtzii* were collected from Arabuko-Sokoke Forest, Malindi, Kilifi County of Coast province in Kenya in April 2010. The plant was authenticated by Mr. Lucas Karimi, Department of Pharmacy and Complementary Medicine for authenticating the plant. A voucher specimen (No. MM/002/10) was deposited at the herbarium of the Kenya National Museums, Nairobi.

**Method of extraction and isolation**

Air-dried stem and root barks of *O. holtzii* were separately ground to give fine powders (5.58 and 7.23 kg, respectively) and each was extracted separately using solvents of increasing polarity sequentially (starting with hexane, then DCM, followed by EtOAc and finally MeOH) for 48 hrs each with occasional swirling to ensure thorough extraction. The extracts were decanted and filtered through Whatman filter paper and the marcerate steeped in solvent again for 48 hrs. The extraction process was repeated 3 times when a clear extract was obtained. The filtrates were combined and concentrated using rotary evaporator under reduced pressure and a temperature of 45°C. A small portion of each crude extract was used for bioassays.

**Dehydrolophirone C (1)**

Yellow crystals, MP 198-200°C

IR (KBr): 2926 (br), 3356, 2962, 1620, 1512 cm⁻¹

UV λmax: 201, 223 and 274 nm

1H and 13C NMR: Table 1.

**Holtzinol (3)**

Yellow crystals, MP 178-180°C

UV λmax: 201, 224 and 274 nm

IR (KBr): 2926 (br), 3410, 2962, 1627, 1512 cm⁻¹

1H and 13C NMR: Table 1.

**Lophirone A trimethyl ether (7)**

Yellow crystals, MP 192-194°C.

UV λmax: 218, 282 nm

IR (KBr): 2926 (br), 3256, 2963, 1605, 1512 cm⁻¹

1H and 13C NMR: Table 1.

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