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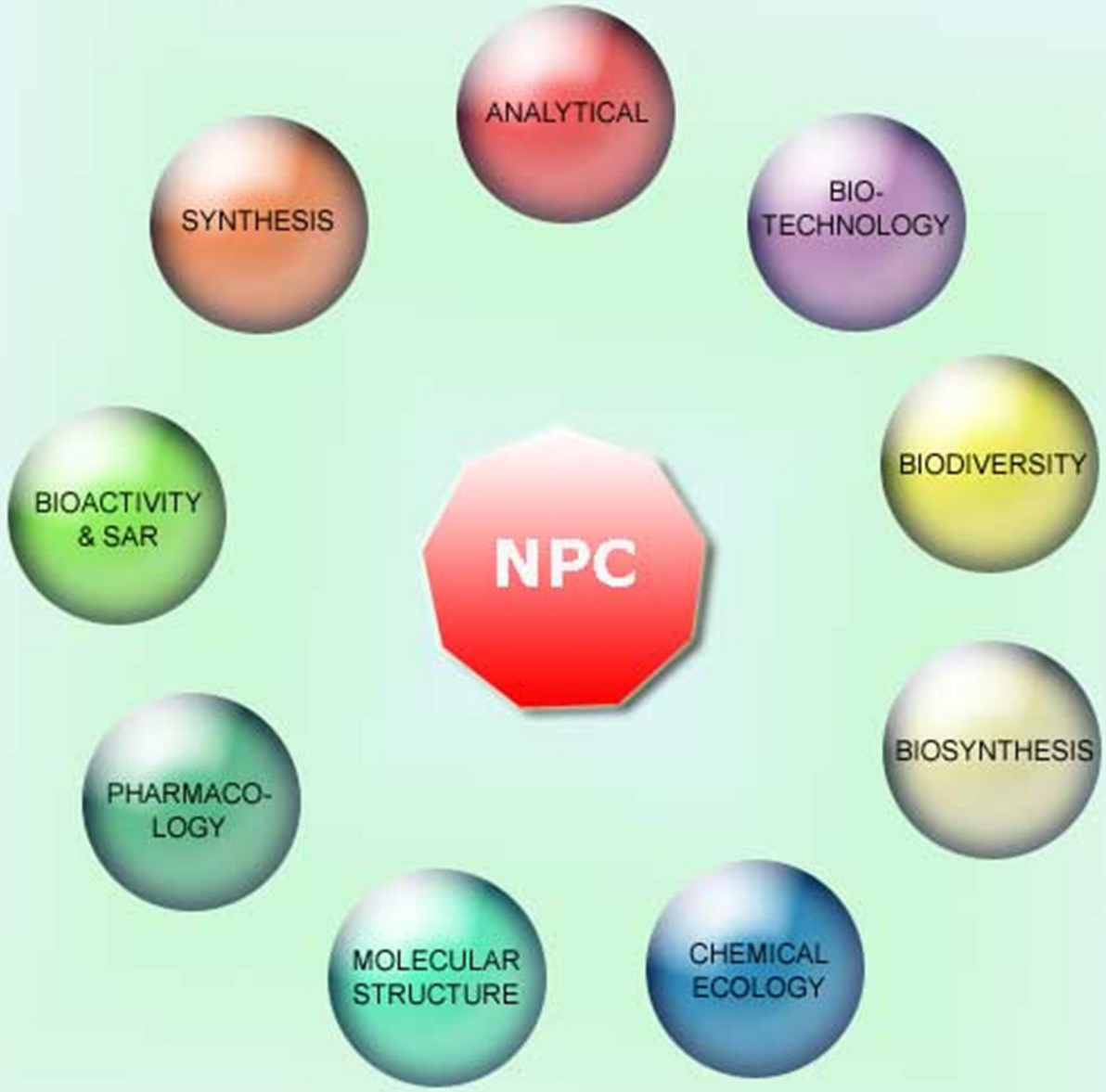

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Anti-Vancomycin-resistant *Enterococcus faecium* and *E. faecalis* Activities of (-)-Gossypol and Derivatives from *Thespesia garckeana*

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The root extract of *Thespesia garckeana* yielded three known oxidatively coupled sesquiterpenoids, namely (-)-gossypol (**1**) and two of its derivatives (-)-6-methoxygossypol (**2**) and (+)-6,6′-dimethoxygossypol (**3**), and the stem bark afforded (*E*)-docosyl-3-(3,4-dihydroxyphenyl) acrylate (**4**), stigmasterol (**5**) and betulinic acid (**6**). The structures of the isolated compounds were determined on the basis of full spectral data (1D and 2D NMR and HRMS) and comparison with literature values. Compound **1** showed potent antibacterial activity against vancomycin-resistant *Enterococcus faecium* (VRE) with IC_{50}/MIC/MBC values of 1.71/4.82/19.31 μM, respectively, whereas the reference standard vancomycin was found to be inactive. The mono- and di-methoxylated derivatives of this compound, (-)-6-methoxygossypol (**2**) and (+)-6,6′-dimethoxygossypol (**3**), were less active with respective IC_{50}/MIC/MBC values of 2.73/4.70/9.40 μM and 6.14/18.32/18.32 μM against this microbe. Compound **2** was more potent than **1** against the low level VRE strain with IC_{50}/MIC/MBC values of 4.34/9.40/9.40 μM (*vs* 5.23/19.31/19.31μM for **1**). This compound also showed interesting activities against *Candida glabrata* with an IC_{50} value of 2.97 μM, but was less active against methicillin-resistant *S. aureus* (MRSA) exhibiting an IC_{50} value of 17.33 μM. Compound **1** demonstrated modest activity against the other microbes tested including *C. glabrata*, *S. aureus* and MRSA with IC_{50} values of 0.73, 9.15 and 8.99 μM, respectively.

Keywords: *Thespesia garckeana*, (-)-Gossypol, (-)-6-Methoxygossypol, (+)-6,6′-Tetramethoxygossypol, Antimicrobial, Vancomycin-resistant *Enterococcus faecium*, *Enterococcus faecalis*.

The use of antibiotics inevitably leads to the emergence and rapid spread of multi-drug resistance in microbes. These strains that are resistant to almost all current antibiotic agents of choice compromise the treatment of bacterial, viral, fungal and parasitic infections posing potential threats to human health [1-3]. This phenomenon is exemplified by vancomycin-resistant *Enterococcus* (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) strains which have developed resistance to most first-line antibiotics and currently there are very few antibiotics that can be used to combat infections caused by these microbes [2, 3].

Bacteria of the genus *Enterococcus* are considered part of the normal flora of the gastrointestinal and genitourinary tracts of humans, and have emerged as one of the most important nosocomial pathogens, causing 12% of all hospital acquired infections [4]. *E. faecalis* and *E. faecium* are responsible for the majority of human enterococcal infections [3, 5]. Colonization and infection by vancomycin-resistant *Enterococci* are associated with multidrug resistance and up to 75% mortality rate of those infected [6]. *Staphylococcus aureus* is another persistent bacterium that colonizes the nasal mucosa of approximately 30% of individuals. It is associated with a wide range of infections including skin and soft tissue infections, systemic or fatal infections, endocarditis, pneumonia and toxin-associated diseases [7, 8]. MRSA is also a major cause of nosocomial infections and thus is alternatively called healthcare-associated MRSA (HA-MRSA). In addition to this strain, an emerging class of MRSA in the community, known as community-acquired MRSA (CA-MRSA), is also becoming a major concern worldwide [9, 10]. This calls for concerted efforts by researchers in the field of drug discovery to search continuously for newer and more effective anti-microbial agents that can combat the resistance menace. These two categories of multi-drug resistant microbes were tested for their viability against three compounds from *Thespesia garckeana* that were isolated in sufficient yields.

Thespesia garckeana F. Hoffm. (Malvaceae), also known by its synonym *Azanza garckeana* (F. Hoffm.) Exell & Hillc,, is an evergreen medium sized woodland tree of 3-10 m in height distributed in Eastern and Southern Africa and is endemic to South Africa, Kenya, Mozambique, Botswana, Zimbabwe, Malawi, Namibia, Tanzania, Zambia, and Sudan [11, 12]. In Kenya, it is found in coastal and semi-arid regions [13]. The most common genus in this family is *Gossypium*, used in the provision of fibers [14]. *Thespesia* is closely related to *Gossypium*, with which it shares the presence of gossypol glands. Plants from this genus are known to possess antibacterial, antiviral, antiallergic, anti-inflammatory, anticancer and immunostimulant properties, which could be attributed to the presence of polyphenols, including gossypol (**1**), in various plant organs [15,16]. Previous studies have shown that gossypol (**1**) is a promising male contraceptive [17, 18] and has also been reported to have potent activities against various types of cancer cell lines [18-20]. However, gossypol is toxic to non-ruminant animals and has limited the use of cottonseed meal as a dietary source of protein for mono-gastric animals [21]. Earlier interest in gossypol (**1**) was centered around its toxicity, but more interest has developed in its possible usefulness as a drug [22]. There is limited information on the anti-microbial activities of this compound and its mono- and dimethoxylated derivatives (**2**, **3**), especially against multi-drug resistant bacteria, and hence the motivation to carry out the current studies.

In a search for more effective antimicrobial principles from Kenyan ethnomedicinal flora, chromatographic separation of crude extracts of the roots and stem barks of *T. garckeana* obtained using 1:1 methanol (MeOH) in dichloromethane (CH_2Cl_2) yielded three oxidatively coupled sesquiterpenoids, namely (-)-gossypol (**1**) [23], (-)-6-methoxygossypol (**2**) [24, 25] and (+)-6,6′-dimethoxygossypol (**3**) [24, 25], a diterpenoid, (*E*)-docosyl-3-(3,4-dihydroxyphenyl)acrylate (**4**) [26], and two triterpenoids, stigmasterol (**5**) [27] and betulinic acid (**6**) [28]. The structures of these compounds were determined using 1D and 2D NMR spectroscopy and MS, and supported by literature values. (-)-Gossypol (**1**), (-)-6-methoxygossypol (**2**) and (+)-6,6′-dimethoxygossypol (**3**) were isolated in sufficient amounts to be investigated for *in vitro* antimicrobial potencies, especially against VRE and MRSA.

In vitro antibacterial and antifungal activities of compounds **1-3** were determined against the following microbes: *Candida glabrata,* two strains of *Staphylococcus* [*S. aureus*, methicillin resistant *S. aureus* (MRSA)] and three strains of *Enterococcus* [vancomycin-resistant *Enterococcus* (VRE), vancomycin-sensitive *Enterococcus* (VSE) and low level vancomycin-resistant *Enterococcus* (LLVRE)]. Compound **1** showed strong activity against vancomycin-resistant *Enterococcus faecium* (VRE) with IC_{50}/MIC (minimal inhibitory concentration)/MBC (minimal bactericidal concentration) values of 1.71/4.82/19.31 μM, respectively, whereas the reference standard, vancomycin, was inactive. The mono- and dimethoxylated derivatives of this compound, (-)-6-methoxygossypol (**2**) and (+)-6,6′-tetramethoxygossypol (**3**), were less active with IC_{50}/MIC/MBC values of 2.73/4.70/9.40 μM and 6.14/18.32/18.32 μM against this microbe. Compound **2** was more potent than **1** against low level VRE strain with IC_{50}/MIC/MBC values of 4.34/9.40/9.40 μM (*vs.* 5.23/19.31/19.31 μM for **1**). This compound also showed interesting activities against *C. glabrata* with an IC_{50} value of 2.97 μM, but was inactive against MRSA exhibiting an IC_{50} value of 17.33 μM. Compound **1** demonstrated modest activities against the other microbes tested including; *C. glabrata*, *S. aureus* and MRSA with IC_{50} values of 0.73, 9.15 and 8.99 μM, respectively.

From the above observations, (-)-gossypol (**1**), with free hydroxyl groups at positions C-6,6′,7,7′ was established to be the most potent, followed by its mono-methylated derivative (**2**) against the VRE and VSE strains of bacteria. Dimethylation of the hydroxyl groups substantially reduced antimicrobial activities against these two strains of bacteria, as observed in compound **3**. This trend was replicated against the two strains of *Staphylococcus* and *C. grabrata*, which were all insensitive to compound **3** at concentrations < 36.63 μM. However, mono-methoxylation substantially improved the antimicrobial selectivity of these compounds against *C. glabrata*, as exhibited by **2**, which showed the highest antifungal activity. The antibacterial activities tend to be attributed to the hydrophilicity of gossypol (**1**) and derivatives (**2**, **3**), while the antifungal activities require some degree of lipophilicity, as observed in **2**, which is in agreement with previous studies [29, 30].

This appears to be the first report of (-)-gossypol (**1**) from the genus *Thespesia*. However, (+)-gossypol had previously been isolated from *T. populnea*, which exhibited cytotoxic and elastase inhibitory activities [31-32]. The toxicity associated with natural gossypol (**1**) may be reduced through structural diversification to obtain analogs, some of which may exhibit improved antimicrobial activities. Since the mono-methylated derivative (**2**) of gossypol (**1**) showed strong activities against low level VRE and *C. glabrata*, further studies should be carried out to establish comprehensively their safety profiles towards antibiotic drug development.

Experimental

General experimental procedures: Melting points (uncorrected) were recorded using a Gallenkamp melting point apparatus with capillary tubes, and optical rotations were measured using an AUTOPOL polarimeter at ambient temperature. 1D and 2D NMR spectra were recorded in $CDCl_3$ on a 400 MHz Bruker AVANCE NMR instrument at room temperature. Chemical shifts, δ, were expressed in ppm and referenced against the solvent resonances at 7.26 and 77.23 ppm for ^1H and ^{13}C NMR, respectively. EIMS spectra were recorded at 70 eV on a SSQ 710 MAT mass spectrometer; HRMS were obtained by direct injection using a Bruker Bioapex-FTMS with electrospray ionization (ESI). Column chromatography was carried out using Merck silica gel 40 (70-230 mesh) and Fluka Sephadex LH-20 as stationary phases. Analytical TLC and Preparative TLC were conducted on Merck pre-coated 60 F_{254} and Merck 60 PF_{254}, respectively. Compounds were visualized by observing under UV light at 254 or 365 nm, followed by spraying with 1% vanillin-H_2SO_4 and also by placing the plates in iodine tanks to view the compounds that were not UV active.

Plant material: The roots and stem barks of *T. garckeana* were collected from Muthetheni, Machakos County in March, 2013. The plant materials were identified by Mr Patrick Mutiso from the University of Nairobi herbarium, School of Biological Sciences where a voucher specimen of the plant MVM 2013/UoN 02 is deposited.

Extraction and isolation of compounds: The roots were dried under shade, ground to powder and extracted by cold percolation with 1:1 MeOH in CH_2Cl_2. The extracts were concentrated under reduced pressure yielding 70 g of crude extract. A portion of the extract (65 g) was subjected to CC using a silica gel matrix and eluting with mixtures of *n*-hexane (*n*-C_6H_{12})/CH_2Cl_2/MeOH in order of increasing polarities. This yielded a total of 80 fractions of 200 mL each, which were subsequently combined depending on the similarities of their TLC profiles into 12 fractions. Crystallization of the fraction eluted with 100% CH_2Cl_2 from the main column resulted in the isolation of (-)-gossypol (**1**) [mp 177-182 °C (Lit. 183.7 °C); $[\alpha]_D^{25}$-346 (*c* 0.5, MeOH), Lit. -386 (*c* 0.5, $CHCl_3$)]. Purification of the fraction eluted with 1% MeOH/CH_2Cl_2 from the main column was achieved by further CC eluting initially with 5% ethyl acetate (EtOAc) in *n*-C_6H_{12} and subsequently with increasing amounts of EtOAc up to 40%. The fraction from the minor column eluted with 10% EtOAc in *n*-C_6H_{12} was recrystallized from 5% EtOAc in *n*-C_6H_{12} to yield (+)-6,6′-dimethoxygossypol (**3**) [mp 164-167 °C (Lit. 166.4 °C); $[\alpha]_D^{25}$+300 (*c* 0.6, MeOH), Lit. unreported]. The fraction that eluted with 20% EtOAc in *n*-C_6H_{12} was recrystallized from 10% EtOAc in *n*-C_6H_{12} resulting in yellow crystals of (-)-6-methoxygossypol (**2**) [mp 176-179 °C (Lit. 177.5 °C); $[\alpha]_D^{25}$-388 (*c* 0.5, MeOH), Lit. unreported]. Isolation of the constituents of the crude extract (75 g) of the stem barks was achieved by CC using silica gel as the stationary phase with gradient elution with increasing amounts of EtOAc in *n*-C_6H_{12} from 2.5 to 40%. The fraction of the major column eluted with 20% EtOAc in *n*-C_6H_{12} was recrystallized from 50% CH_2Cl_2/*n*-C_6H_{12} to yield a colorless solid of *E*-docosyl-3-(3,4-dihydroxyphenyl) acrylate (**4**), while that eluted with 15% EtOAc/*n*-C_6H_{12} was recrystallized from 50% CH_2Cl_2/*n*-C_6H_{12} to yield white needle-like crystals of stigmasterol (**5**) with the mother liquor recrystallized from the same solvent system giving betulinic acid (**6**). Compounds **1-6** were identified by comparing their NMR and ESIHRMS data (see *Supporting Information*) with those published in the literature.

Antimicrobial activities: The organisms were obtained from the American Type Culture Collection (Manassas, VA) and included the yeast *Candida glabrata* ATCC 90030 and the bacteria *Staphylococcus aureus* ATCC 29213, methicillin-resistant *S. aureus* ATCC 33591, *Enterococcus faecium* ATCC 700221, *E. faecalis* ATCC51299 and *E. faecalis* ATCC 29212. Drug controls/standards ciprofloxin, methicillin and vancomycin for bacteria and amphotericin B for fungi were included in each assay. The *in vitro* antimicrobial test was performed using a modified version of the CLSI methods as described [33], at the National Center for Natural Products Research, University of Mississippi. Briefly, pure compounds were dissolved in DMSO and serially diluted in 20% DMSO/saline to make 20, 10, 5, 2.5 up to 0.02 µg/mL and transferred, in duplicate, into 96-well flat bottom microplates. Microbial innocula were prepared in assay medium to afford target CFU/mL after addition to the samples. Growth, solvent and media controls were included in each test plate. Assay plates were read at 530 nm before and after incubation using the Biotek Power wave XS plate reader. Percent growth was plotted versus test concentration to afford the IC_{50} values or concentration that affords 50% growth relative to controls. The minimum bacterial or fungicidal concentrations (MBC/MFCs) were determined by removing 5 µL from each clear well, transferring to fresh media and incubating until growth is seen.

Supporting information: ^{1}H, ^{13}C NMR and ESIHRMS of **1**–**6**, and HMQC and HMBC spectra of **3**.

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Accounts/Reivews

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Contents

Original Paper

Continued inside backcover