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ANTIBACTERIAL AND ANTI-BIOFILM ACTIVITY OF FLAVONOIDS AND TRITERPENES ISOLATED FROM THE EXTRACTS OF *FICUS SANSIBARICA* WARB. SUBSP. *SANSIBARICA* (MORACEAE) EXTRACTS

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Abstract

Background: *Ficus* species are used in African traditional medicine in the treatment of a wide variety of ailments and diseases such as convulsive disorder, wound healing, gonorrhea, tuberculosis, diabetes, diarrhoeal infections, dysentery, malaria and HIV. The aim of this study was to isolate the phytochemical constituents in the plant and test them for their antibacterial activity.
Materials and methods: The fruits, leaves and stem bark were extracted with organic solvents and the compounds in the extracts separated and purified by column chromatography before being identified by NMR spectroscopy and by comparison of the NMR data against values reported in the literature. The antibacterial activity of the pure compounds and extracts were tested using the disk diffusion method.
Results: Three triterpenes and three flavonoids: lupeol acetate (**1**); cycloart-23-ene-3,25-diol (**2**); β-sitosterol (**3**); 5,7,4'-trihydroxyflavan-3-ol (**4**); epicatechin (**5**); and isovitexin (**6**) were isolated in this study. Antimicrobial activity was observed at 8 mg mL^{-1} for *Staphylococcus aureus* ATCC 29213 with four of the six isolated compounds, with no activity being observed at 1 – 4 mg mL^{-1} against *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218 and *S. aureus* ATCC 43300. Epicatechin (**5**) was found to decrease adhesion of *E. coli* ATCC 25922 and *S. aureus* ATCC 29213. Decreased adhesion of *S. aureus* ATCC 29213 was also observed with 5,7,4'-trihydroxyflavan-3-ol (**4**) and isovitexin (**6**).
Conclusions: The results of this study provide baseline information on *F. sansibarica*'s potential validity in the treatment of infections associated with Gram-positive microorganisms.

Key words: Moraceae, *Ficus sansibarica*, antimicrobial, anti-adhesion properties, flavonoids, triterpenoids.

Introduction

Primary healthcare in Africa still remain inaccessible to the vast majority of its populace due to inadequate transport systems to and from hospitals and clinics; the cost of drugs and the high rates charged for in-hospital treatment. In most cases, ~80% of the world's population, especially those living in the rural areas of African countries, rely on traditional medicine for the healing and treatment of various ailments from coughs and colds to HIV, malaria and tuberculosis (WHO, 1996; Street and Prinsloo, 2013). Research into pharmacologically active African medicinal plants is being carried out with the aim of providing scientific evidence for the use of the plants in traditional medicine, commercialization and pharmaceutical application of the active components. This has primarily centered on antimicrobial activity but is now being targeted towards microbial virulence mechanisms such as biofilm formation and/or quorum sensing. Microbial biofilm formation of microorganisms is associated with persistent tissue and foreign body infections which are resistant to treatment with antimicrobial agents. Up to 80% of human bacterial infections are biofilm-associated; with infections most frequently being caused by *Staphylococcus aureus*, *S. epidermidis*, *Pseudomonas aeruginosa*, and *Escherichia coli* (Römling and Balsalobre, 2012).

In tropical and subtropical regions of the world, the genus *Ficus* (Moraceae), of the Mulberry family constitutes one of the largest genera of angiosperms with over 900 species consisting of trees, shrubs and epiphytes (Lansky and Paavilainen, 2011). Among these, about 110 *Ficus* species are indigenous to Africa (Berg and Wiebes, 1992; Singh *et al*., 2011). Many *Ficus* species have been used in African traditional medicine either in the treatment or management of a variety of ailments and diseases such as convulsive disorder, wound healing, gonorrhea, tuberculosis, diabetes, diarrheal infections, dysentery, malaria and HIV (Wakeel *et al*., 2004; Yadav *et al*., 2006; Parekh and Chanda, 2007; Annan and Houghton, 2008; Abdulla *et al*., 2010: Lamorde *et al*., 2010; Nadembega *et al*., 2011). *Ficus* species have also been used in Indian ayurvedic and traditional medicine (Joseph and Justin Raj, 2010). Extracts of *Ficus* species have also been documented to have antioxidant, anti-diabetes, antibacterial, antifungal, antiviral, anti-protozoal, anticancer, cytotoxic, anti-ulcer, anti-inflammatory, anti-hyperglycemic, anti-diarrhoea, hepato-protective, muco-protective and gastro-protective activity (Kuete *et al*., 2009). Phytochemical studies on species from the genus *Ficus* have shown these plants to be rich sources of flavonoids, lignans, terpenoids, alkaloids, coumarins steroids, and ceramides (Ragasa *et al*., 1999; Kuete *et al*., 2008; Ramadan *et al*., 2009; Ueda *et al*, 2009; Chen *et al*., 2010; Kiem *et al*., 2011; Naressi *et al*., 2012). However, although there are many phytochemical and pharmacological reports on species within the genus *Ficus*, there are many species that have not been studied and whose ethno-botanical relevance is yet to be investigated. *Ficus sansibarica* Warb. subsp. *sansibarica* is a large tree with a spreading crown which is up to 20 × 30 m and is commonly known as the Zanzibar fig, Angola fig or knobbly fig. It is usually without aerial roots and contains milky latex. The knobby fig is distributed in South Africa (Mpumalanga and Northern Province), Swaziland, Mozambique, Zimbabwe, Zambia, Malawi, Tanzania and Kenya. It has also been visually recorded in Zululand (Burrows and Burrows, 2003). In Botswana, both adults and children eat the fresh ripe fruits (Daniel and Topo, 2012). The ripe fruits are also consumed by birds, fruit bats, baboons, monkeys and other animals as well as insectivorous birds which feed upon the wasps that pollinate the figs. The leaves are browsed by nyala, kudu, elephant and giraffe (Venter and Venter, 1996; Boon, 2010). The only biological study reported so far was the analysis of the fresh ripe fruits for its nutrients, total phenols and antioxidant activity (Daniel and Topo, 2012). This prompted us to investigate the phytochemical composition of *Ficus sansibarica* which to the best of our knowledge has not been studied previously, and to test the isolated compounds for antimicrobial and anti-biofilm activity with the aim of providing a scientific rationale for the use of the plant in the treatment of some bacterial infections.

Materials and Methods

Collection of plant materials

The fruits, leaves and stem bark of *Ficus sansibarica* were collected in 2011, in KwaZulu-Natal, South Africa. The plant was identified and a voucher specimen (G. V Awolola & H. Baijnath 1), was deposited in the Ward herbarium at the University of KwaZulu-Natal.

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General experimental procedure

The ^{1}H, ^{13}C and 2D NMR spectroscopy were recorded at room temperature either using deuterated chloroform ($CDCl_3$), dimethyl sulfoxide (DMSO), or methanol (MeOH), on a Bruker Avance^{111} 400 and 600 MHz spectrometer with tetramethylsilane (TMS) as internal standard. All GC-MS analyses of samples were recorded on an Agilent GC-MSD apparatus equipped with DB-5SIL MS (30 m × 0.25 mm i.d., 0.25 µm film thickness), fused-silica capillary column. The carrier gas was Helium (2ml min^{-1}). The MS was operated in the EI mode at 70 eV. The LC-MS sample analyses were recorded on an Agilent LC-MSD apparatus equipped with a UV detector using a mobile phase of 95% acetonitrile, 10% water, and both containing 1.1% formic acid at a flow rate of 1ml min^{-1}. Infrared (IR), spectral data was obtained using a Perkin Elmer Spectrum 100 Fourier transform infrared spectroscopy (FT-IR), spectrometer with universal attenuated total reflection (ATR) sampling accessory. All UV spectra were obtained using a Varian Cary UV-VIS spectrophotometer. Optical rotations were measured at room temperature using a 10 cm flow tube in a PerkinElmer^{TM}, Model 341 Polarimeter. Melting points were obtained using an Ernst Leitz Wetziar micro-hot stage melting point apparatus. For column chromatography, Merck silica gel 60 (0.040-0.063 mm), was used while Merck 20 cm × 20 cm silica gel 60 F_{254} aluminum sheets were used for TLC. The TLC plates were analyzed under UV (254 and 366 nm) before being visualized by spraying the plate with a 10% sulphuric acid in MeOH solution followed by heating.

Extraction and Isolation

Freshly crushed *F. sansibarica* fruits (516 g) were subjected to cold extraction using MeOH, while air-dried and ground leaves (520 g), and stem bark (1.5 kg), were subjected to exhaustive extraction using solvents of increasing polarity: hexane, dichloromethane (DCM), ethyl acetate (EtOAc), and MeOH by maceration with continuous shaking on an orbital shaker for 48 hrs, each time at room temperature. The different solvent extracts were concentrated using a rotary evaporator and the obtained crude extracts in each case were stored for further analysis. Using suitably sized columns the crude extracts were subjected to column chromatography for fractionation and the fractions collected were monitored using TLC. Similar fractions were combined and concentrated using a rotary evaporator.

The MeOH extract from the fruits was partitioned with EtOAc and the EtOAc portion concentrated yielding 1 g of extract. This was separated on a 1.5 cm diameter column over silica gel (0.040-0.063 mm), using a gradient elution of n-hexane:EtOAc (with 10% increments of EtOAc), and finally with 100% MeOH, collecting 20 mL at each stage. The fractions were combined on the basis of TLC to afford six main fractions (I-VI). Fraction IV was purified using CC over Sephadex LH-20 (MeOH), to afford isovitexin **6** (25 mg), a dark yellow solid.

The crude hexane extract of the leaves (10.3 g) was separated using CC in a similar manner with a hexane:EtOAc step gradient (with 10% increments every 100 mL). Compound **2**, cycloart-23-ene-3,25-diol (13 mg) was eluted with hexane:EtOAc (9:1) and evaporated to a white powder. Lupeol acetate **1** (995 mg), was eluted with hexane:EtOAc (8:2), and re-crystallized in MeOH to give a white powder. The hexane extract (10.4 g), from the stem bark was separated in the same manner and yielded sitosterol **3** (305 mg), with hexane:EtOAc (8:2). The EtOAc extract (20.3 g), from the stem bark afforded two flavonoids using both a hexane:EtOAc step gradient and a DCM:MeOH step gradient, collecting 100 mL at each stage as stated above. With hexane:EtOAc (8:2), a brownish solid compound, 5,7,4'-trihydroxyflavan-3-ol **4** (85 mg) was obtained. Epicatechin **5** (124 mg) was eluted with 10% MeOH in DCM. The structures of **1-6** are given in Figure 1.

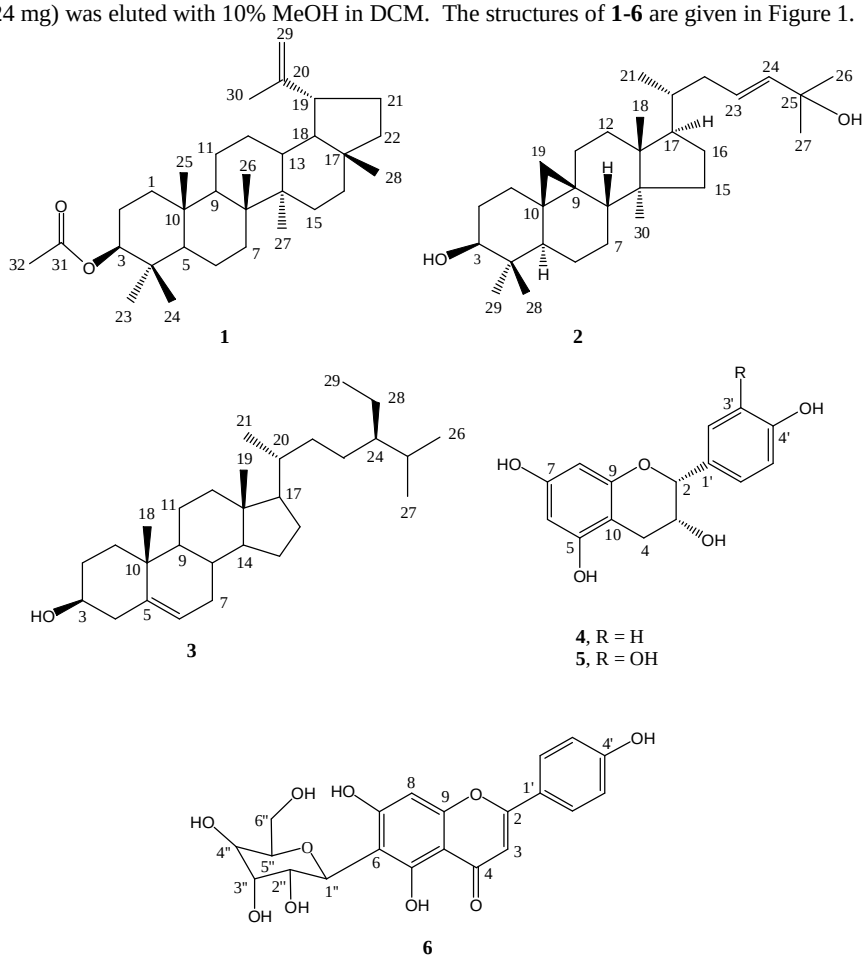

Figure 1: Chemical structures of compounds isolated from *Ficus sansibarica*. Lupeol acetate (**1**), cycloart-23-ene-3,25-diol (**2**), sitosterol (**3**), 5,7,4'-trihydroxyflavan-3-ol, R = H, (**4**), epicatechin, R = OH (**5**), isovitexin (**6**).

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Antimicrobial susceptibility testing

Antimicrobial susceptibility of the crude MeOH leaf and stem bark extracts and compounds **1-6** were determined using the disk-diffusion method. Crude extracts and isolated compounds were dissolved in chloroform or DMSO to a final concentration of 100 mg mL^{-1}. Blank discs (6 mm; MAST, UK), were impregnated with 10 µL (1 mg), 20 µL (2 mg) and 40 µL (4 mg) and allowed to dry.

Bacterial isolates (Gram-negative: *Escherichia coli* ATCC 25922 and *E. coli* ATCC 35218; Gram-positive: *Staphylococcus aureus* ATCC 29213 and *S. aureus* ATCC 43300), were grown overnight on tryptic soy agar (TSA) plates. Cell suspensions were prepared with sterile de-ionized water and the turbidity adjusted equivalent to that of a 0.5 McFarland standard. These were used to inoculate Mueller-Hinton (MH) agar plates by streaking swabs over the entire agar surface followed by the application of the respective phytochemical extract discs (CLSI, 2007). Plates were then incubated for 24 h at 37 °C. Testing was done in duplicate with tetracycline (TE30; 30 µg mL^{-1}), and ampicillin (AMP10; 10 µg mL^{-1}) discs being used as standard antimicrobial agent controls, in addition to DMSO and chloroform.

Following the preliminary disk-diffusion assays, the agar-well diffusion assay was also used for *S. aureus* ATCC 29213, in order to accommodate a greater concentration of the isolated compounds. Instead of using impregnated discs, agar wells with a diameter of ~6 mm were cut into MH agar plates swabbed with *S. aureus* ATCC 29213. Wells were filled with 80 µL of 100 mg mL^{-1} stock solutions of compounds **1**, **2**, **4** and **5** (8 mg mL^{-1}), and 80 µL of 20 mg mL^{-1} stock solution of compound **6** (1.6 mg mL^{-1}) (Table 2). Plates were then incubated for 24 hrs at 37 °C. Testing was done in duplicate and tetracycline (TE30) and ampicillin (AMP10), discs were used as standard antimicrobial agent controls, in addition to DMSO and chloroform as negative controls. Zone diameters were determined and averaged. The following zone diameter criteria were used to assign susceptibility or resistance to the phytochemicals tested: Susceptible (S) ≥ 15 mm, Intermediate (I) = 11 – 14 mm, and Resistant (R) ≤ 10 mm (Okoth *et al*., 2013). Criteria for assigning susceptibility or resistance to AMP10 was as follows: (S) ≥ 17 mm, (I) = 14 – 16 mm, (R) ≤ 13 mm, while those for TE30 were: (S) ≥ 19 mm, (I) 15 – 18 mm, (R) ≤ 14 mm (CLSI, 2007).

Biofilm microtitre plate assays

In order to ascertain the anti-biofilm potential of the isolated compounds, *E. coli* ATCC 29922, *E. coli* ATCC 35218, *S. aureus* ATCC 29213 and *S. aureus* ATCC 43300 were exposed to lupeol acetate **1** (100 mg mL^{-1} stock; 2, 5, 10 and 15 mg mL^{-1}); 5,7,4'-trihydroxyflavan-3-ol **4** (100 mg mL^{-1} stock; 1 and 2.5 mg mL^{-1}); epicatechin **5** (100 mg mL^{-1} stock; 1, 2.5, 5 and 7.5 mg mL^{-1}); and isovitexin **6** (20 mg mL^{-1} stock; 0.2 and 0.5 mg mL^{-1}) using microtitre plate assays (Basson et al., 2008). The purified cycloart-23-ene-3,25-diol **2** could not be tested due to unavailability of sufficient material. All isolates were cultured overnight in tryptic soy broth (TSB), and centrifuged for 2 min at 12000 rpm. Cell pellets were washed and re-suspended to a turbidity equivalent to a 0.5 McFarland standard. Each well of the sterile 96-well U-bottomed polystyrene micro-titre plates contained 90 µL of TSB medium and 10 µL of culture after standardization. To investigate the effect on adherence, respective volumes of extracts were added to the TSB medium at the time of inoculation in the micro-titre plate assays and volumes were standardized to 200 µL with sterile distilled water. Positive control wells contained TSB broth and respective cultures without the tested compounds whilst the negative control wells contained un-inoculated TSB broth only to test for sterility and non-specific binding of media. Plates were incubated aerobically for 24 h with agitation on an Orbit P4 micro-titre plate shaker (Labnet), at 37 °C to allow cell attachment and biofilm formation (Basson *et al*., 2008).

Following incubation, the crystal violet assay was performed. The contents of each well were aspirated. Micro-titre plates were washed three times with sterile de-ionized water to remove loosely attached cells. The remaining cells were fixed with 200 µl of MeOH for 15 min and air-dried. Following drying, the wells were stained with 150 µl of 2% Hucker's crystal violet for 5 min. Thereafter, wells were rinsed gently with water, air-dried and 150 µl of 33% (v/v) of glacial acetic acid was added for re-solubilization of dye bound to adherent cells. Plates were then read using the GloMax®-Multi+ Detection System (Promega) at an optical density of 600 nm. Tests were done in triplicate on two separate occasions and the results were averaged (Basson *et al*., 2008). $OD_{600\ nm}$ values of treated cells were compared to untreated cells to investigate the increase or decrease in adhesion as a result of phytochemical exposure. Treated and untreated samples were compared statistically using Paired *t*-tests (SigmaStat V3.5, Systat Software, Inc).

Results and Discussion

The phytochemical investigation of the leaves, stem bark and fruits of *F. sansibarica* yielded six compounds: three triterpenoids and three flavonoids. The non-polar hexane extract of both leaves (**1** and **2**) and stem bark (**3**) contained triterpenoids while the flavonoids were isolated from the more polar EtOAc extract of both the stem bark (**4** and **5**), and the fruits (**6**). The isolated compounds were identified based on their ^{1}H and ^{13}C NMR spectra and 2D NMR spectra and compared with literature values. Thus, they were identified as lupeol acetate **1** (Mahato and Kundu, 1994), cycloart-23-ene-3,25-diol **2** (Abdel-Monem et al., 2008; Escobedo-Martínez et al., 2012; Khan et al., 2006), sitosterol **3** (Chaturvedula and Prakash, 2012), 5,7,4'-trihydroxyflavan-3-ol **4** (Dafalla, 2005; Iida et al., 2007), epicatechin **5** (Hubert et al., 2011; Markham and Ternai, 1976) and isovitexin **6** (Lin et al., 2009).

Epicatechin **5** has been isolated from several *Ficus* species like *F. microcarpa* and *F. spragueana* (Kiem *et al*., 2011; Ragab *et al*., 2013). In contrast, isovitexin **6** was only reportedly isolated from *F. microcarpa* (Kiem et al., 2011), while 574',-trihydroxyflavan-3-ol **4** was isolated from *F. capensis* and *F. spragueana* (Owolabi et al., 2009; Ragab et al., 2013). Lupeol acetate **1** and sitosterol **3** are ubiquitous to plants. To the best of our knowledge, this is the first report of cycloart-23-ene-3,25-diol **2**, a cycloartane type triterpenoid with molecular formula $C_{30}H_{50}O_2$ in the genus *Ficus*. Furthermore, a comparison of the NMR data obtained for **2** with that published in the literature (Abdel-Monem et al., 2008; Escobedo-Martinez et al., 2012, Khan et al., 2006) matched the NMR data of the cycloartane backbone only and there were discrepancies in the NMR data of the side chain, which we have unambiguously assigned from HMBC data. The *cis* isomer of **2** is also reported (Gherraf et al., 2010), and is also included here for comparison. We speculate that the reports of **2** in Abdel-Monem et al. (2008), Escobedo-Martinez et al. (2012), and Khan et al. (2006), are also that of the *cis* isomer as the H-23 and H-24 resonances were always reported as overlapping multi-plets at d_H 5.60. In our spectra, we were able to clearly define the *trans* nature of the δ^{23} double bond by clear coupling constants. A full NMR structural elucidation is given below and a comparison with that in the literature given in Table 1.

The ^{1}H-NMR spectrum of compound **2** displayed two pairs of doublets with resonances at δ_H 0.55 (1H, d, J = 3.4 Hz, H-19_a) and at 0.30 (1H, d, J = 3.4 Hz, H-19_b), characteristic of cycloartane-type triterpenes. In addition, there were the presence of seven methyl groups at δ 0.82 (s, CH_3-29), 0.89 (d, J = 6.5 Hz, CH_3-21), 0.90 (s, CH_3-28), 0.99 (s, CH_3-30, CH_3-18), 1.36 (s, CH_3-26/27), the typical H-3 resonance for triterpenoids at δ 3.26, a doublet of doublet of doublets (ddd) at δ_H 5.71 (1H, J = 15.7, 8.5, 6.1 Hz, H-23), a doublet at δ_H 5.53 (1H, J = 15.7 Hz, H-24) and one proton of the H-22 methylene group at δ 2.26 which could clearly be distinguished. The ^{13}C NMR spectrum was able to be

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resolved into 7 methyl, 10 methylene, 7 methine and 6 singlet carbon resonances, including two olefinic methine resonances for the double bond in the side chain, an oxygenated singlet resonance at δ 82.3 and an oxygenated methine resonance at δ 78.8, indicating two hydroxy groups in the molecule.

Table 1: ^{1}H and ^{13}C NMR data of cycloart-23-ene-3,25-diol **2** (600 MHz, $CDCl_3$)

Pos.	$^{1}H^{a}$	$^{1}H^{b,c}$	^{1}H (**2**)	$^{13}C^{a}$	$^{13}C^{b}$	^{13}C (**2**)
1	1.56 (m), 1.24 (m)	1.30 (m), 0.90 (m)	1.58 (m), 1.26 (m)	31.98	31.93	31.96
2	1.76 (m), 1.56 (m)	1.40 (m), 1.23 (m)	1.78 (m), 1.58 (m)	30.40	30.33	30.38
3	3.24 (m)	3.10 (m)	3.26 (m)	78.86	78.80	78.84
4	-	-	-	40.50	40.45	40.48
5	1.30 (m)	0.91 (m)	1.30 (m)	47.11	47.10	47.09
6	1.59 (m), 0.79 (m)	1.26 (m), 0.45 (dd, 12.5, 8)	1.60 (m), 0.80 (m)	21.12	21.08	21.09
7	1.33 (m), 1.08 (m)	1.65 (m), 1.40 (m)	1.35 (m), 1.10 (m)	26.02	28.04	25.99
8	1.51 (m)	1.16 (dd, 5, 13)	1.51 (m)	47.98	48.00	47.94
9	-	-	-	20.02	20.00	19.98
10	-	-	-	26.11	25.96	26.10
11	1.99 (m), 1.20 (m)	0.77 (m, 2H)	2.00 (m), 1.15 (m)	26.46	26.07	26.45
12	1.61 (m, 2H)	0.93 (m, 2H)	1.63 (m, 2H)	32.80	35.55	32.80
13	-	-	-	45.32	45.29	45.33
14	-	-	-	48.83	48.80	48.83
15	1.30 (m, 2H)	1.27 (m, 2H)	1.30 (m, 2H)	35.59	32.76	35.56
16	1.91 (m), 1.30 (m)	1.60 (m, 2H)	1.90 (m), 1.30 (m)	28.09	26.42	28.08
17	1.57 (m)	1.24 (m)	1.58 (m)	52.02	52.00	52.07
18	0.97 (s, 3H)	0.62 (s, 3H)	0.99 (s, 3H)	18.10	18.04	18.07
19	0.57 (d, 4.2), 0.34 (d, 4.2)	0.22 (d, 4.5), 0.01 (d, 4.5)	0.57 (d, 3.9), 0.35 (d, 3.9)	29.90	30.00	29.86
20	1.46 (m)	1.10 (m)	1.50 (m)	36.40	36.66	36.29
21	0.86 (d, 6.5, 3H)	0.53 (d, 3.5, 3H)	0.89 (d, 6.5, 3H)	18.30	18.25	18.22
22	2.18 (m), 1.74 (m)	1.88 (m)	2.26 (m), 1.81 (m)	39.05	39.00	39.36
23	5.59 (m)	5.26 (d, 8.0)	5.71 (ddd, 15.7, 8.5, 6.06)	125.63	139.31	130.74
24	5.60 (m)	5.26 (d, 8.0)	5.53 (d, 15.7)	139.36	125.57	134.45
25	-	-	-	70.77	70.75	82.30
26	1.31 (s, 3H)	0.98 (s, 3H)	1.36 (s, 3H)	30.00	29.66	24.40
27	1.32 (s, 3H)	0.98 (s, 3H)	1.36 (s, 3H)	29.90	29.83	24.34
28	0.89 (s, 3H)	0.56 (s, 3H)	0.89 (s, 3H)	19.31	19.26	19.30
29	0.81 (s, 3H)	0.42 (s, 3H)	0.82 (s, 3H)	14.02	13.9	13.99
30	0.97 (s, 3H)	0.62 (s, 3H)	0.98 (s, 3H)	25.45	25.41	25.43

[a] Escobedo-Martinez et al. (2012); [b] Gherraf et al. (2010), δ [23] *cis* isomer; [c] It is highly likely that the ^{1}H NMR spectrum in Gherraf et al. (2010) is offset by approximately δ 0.33; coupling constants are given in parenthesis.

The splitting pattern of the two olefinic protons, which appeared as a ddd at δ_H 5.71 (J = 15.7, 8.5, 6.1 Hz, H-23) and doublet at δ_H 5.53 (J = 15.7 Hz, H-24) arose as a result of H-23 coupling with H-24, and H-23 coupling with the two diastereotopic H-22 methylene protons. These coupling constants clearly indicated that the double bond at δ [23] was *trans* because of the large $J_{23,24}$ value of 15.7 Hz, typical of *trans* olefinic protons. Coupling in the COSY spectrum between H-23 and H-24 and between H-23 and the two H-22 proton resonances at δ 2.26 and 1.81, both multiplets was also present. The corresponding carbon resonances of C-23 and C-24 were identified at δ 130.74 and 134.45 respectively. The C-23 carbon resonance was used to identify the 3H-26/27 methyl resonances through a HMBC correlation with δ 1.36 and in turn the C-25 oxygenated singlet resonance at δ 82.30 through a HMBC correlation between δ 1.36 and δ 82.30. The H-23 proton resonance also showed a HMBC correlation to C-25. The doublet of 3H-21 at δ 0.89 showed HMBC correlations to C-17, C-22 and C-20 at δ 52.07, 39.36 and 36.29 respectively, completing the side chain assignment and linking it to the core skeleton.

The rest of the proton and carbon resonances were confirmed by COSY and HMBC correlations and matched well with that in Escobedo-Martinez et al. (2012).

Antimicrobial susceptibility testing

The isolated compounds and extracts were subjected to antimicrobial screening and the results are presented in Table 2. Initial disk-diffusion assays with 1 – 4 mg mL^{-1} of the six isolated compounds, as well as the crude methanolic leaf and stem-bark extracts proved ineffective against the four bacterial strains (Gram-negative: β-lactam susceptible *E. coli* ATCC 25922 and β-lactam resistant *E. coli* ATCC 35218; Gram-positive: methicillin-susceptible *S. aureus* ATCC 29213 and methicillin-resistant *S. aureus* ATCC 43300) tested, with no zones of inhibition being observed (Table 2). However, *S. aureus* ATCC 29213 demonstrated intermediate susceptibility to 8 mg mL^{-1} for four of the compounds: lupeol acetate **1,** cycloart-23-ene-3,25-diol **2**, sitosterol **3** and epicatechin **5**, using the agar-well diffusion assay (Table 2). However, the flavonoids, 5,7,4',-trihydroxyflavan-3-ol **4** and isovitexin **6** were not active against the bacteria being tested (Table 2). Most phytochemicals work best against Gram-positive bacteria and there are very limited small-molecule plant-derived antimicrobial compounds with high activity against Gram-negative species (Lewis, 2013).

The limited inhibition observed for the crude MeOH extracts is not surprising. Reports on the antimicrobial activity of extracts from other *Ficus* species have been variable, with differences related to the *Ficus* species tested, the part of the plant sampled, the extraction solvent and the method of antimicrobial testing (Salem et al., 2013). Lawal *et al.* (2012) observed *F. exasperata* root bark MeOH extract antimicrobial activity against three *S. aureus* strains at ≥ 12.5 mg mL^{-1}, while activity against *E. coli* strains required ≥ 25 mg mL^{-1}. They also noted the potency of the MeOH extract compared to the EtOAc extract. Murti and Kumar (2011) demonstrated that the *F. racemosa* ethanolic root extract

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(25, 50 and 75 mg mL^{-1}) showed maximum inhibition against *S. aureus* when compared with *F. benghalensis* ethanolic root extract. Crude extracts and compounds from *Ficus conraui* stem barks also demonstrated selective antimicrobial activity varying from weak to moderate (Kengap et al., 2011). In contrast, MICs of ≥ 625 µg mL^{-1} were obtained with *F. ovata* stem bark MeOH extracts from *S. aureus and E. coli.* (Kuete et al., 2009), while *F. polita* MeOH root extract and euphol-3-*O*-cinnamate and (*E*)-3,5,4'-trihydroxystilbene-3,5-*O*-β-δ-diglucopyranoside MICs ranged from 32 - ≥512 µg mL^{-1} against Gram-positive and Gram-negative bacteria (Kuete et al., 2011).

The antimicrobial potencies of the two classes (triterpenes and flavonoids), of compounds isolated have previously been established (Cowan, 1999). Akiyama et al. (2001), obtained epicatechin MICs of 8 mg mL^{-1} with 18 clinical *S. aureus* strains, since it causes limited damage to bacterial plasma membranes, while Mahmoud et al. (2013) observed no antimicrobial effect against methicillin-susceptible and –resistant *S. aureus* strains with 32 – 1024 µg mL^{-1} of epicatechin. The reported weak activity of some isolated cycloartane-type triterpenoids from the genus *Aphanamixis* against *S. aureus* (Wang et al., 2013) was consistent with the weak activity observed for cycloart-23-ene-3,25-diol **(2)** in this study.

Table 2: Antimicrobial susceptibility profile of *Staphylococcus aureus* ATCC 29213 following exposure to six compounds isolated from *Ficus sansibarica* and two crude methanol extracts

Compound/Extract	**Concentration mg mL^{-1}**	**Zone diameter (mm) 40 µL***	**Zone diameter (mm) 80 µL#**
Lupeol acetate (**1**)	8	0	13.5 (I)
Cycloart-23-ene-3,25-diol (**2**)	8	0	11.5 (I)
Sitosterol (**3**)	8	0	13 (I)
5,7,4'-trihydroxyflavan-3-ol (**4**)	8	0	0 (R)
Epicatechin (**5**)	8	0	13.5 (I)
Isovitexin (**6**)	1.6	0	0 (R)
MeOH leaf extract	8	0	0 (R)
MeOH stem bark extract	8	0	9 (R)
Ampicillin	10	25 (S)	25 (S)
Tetracycline	30	28 (S)	28 (S)
Chloroform		0	0 (R)
DMSO		0	0 (R)

* Tested using disk-diffusion assay;
# Tested using agar-well diffusion assay; and
S = susceptible, I = intermediate susceptibility, and R = resistant (CLSI, 2007).

Biofilm microtitre plate assays

In the face of increasing incidence of antimicrobial resistance and recalcitrance of bacteria to current antimicrobial therapy, drug discovery research is focusing on limiting the pathogenecity mechanisms demonstrated by bacteria. Given the focus on biofilms and their role in microbial pathogenicity, screening for the anti-biofilm activity of phytochemicals is imperative in identifying alternative therapeutic options. The anti-biofilm activity of lupeol acetate **1**; 5,7,4'-trihydroxyflavan-3-ol **4**; epicatechin **5** and isovitexin **6** against *E. coli* ATCC 29922, *E. coli* ATCC 35218, *S. aureus* ATCC 29213 and *S. aureus* ATCC 43300 was variable. Lupeol acetate (triterpene), exposures resulted in statistically significant increased adhesion for all strains (Fig. 2a) at all concentrations tested (2 – 15 mg/ml).

Epicatechin **5** exposures decreased adhesion of antimicrobial-susceptible *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 at all concentrations tested (1 – 7.5 mg mL^{-1}), with 5 mg mL^{-1} being the most effective (Fig. 2b) indicating that epicatechin has the ability to interfere with the adhesion ability of these microorganisms to polystyrene surfaces. El-Adawi (2012) obtained a 55-66% decrease in *Streptococcus mutans* biofilm formation upon exposure to 2-15% epicatechin. Previous reports have suggested that epicatechin was unable to inhibit biofilm formation as observed for *Eikenella corrodens*, a periodontopathogenic bacterium (Matsunaga et al., 2010) and for *P. aeruginosa* PAO1 biofilm formation after 18 h of growth (Vandeputte et al., 2010). Similarly, Nyila et al. (2012) observed that epicatechin from *Acacia karroo* did not reduce *Listeria monocytogenes* biofilms. The observed anti-biofilm activity of epicatechin was, however, strain-specific as adhesion of β-lactam resistant *E. coli* ATCC 35218 and methicillin-resistant *S. aureus* ATCC 43300 was increased at all concentrations tested (Fig. 2b). Biofilm formation of *P. aeruginosa* PAO1 was enhanced 3- to 7-fold under the action of epicatechin. Epicatechin caused an up to 5-fold enhancement of the *P. aeruginosa* PAO1 biofilm formation at sub-inhibitory concentrations up to 750 µg mL^{-1} (Plyuta et al., 2013).

574',-Trihydroxyfl3avan-3-ol **4** (1 – 2.5 mg mL^{-1}) and isovitexin **6** (0.2 – 0.5 mg mL^{-1}) exposures also decreased adhesion of methicillin-susceptible *S. aureus* ATCC 29213 (Figs. 2c - 2d). Exposure to 2.5 mg mL^{-1} of **4** almost completely inhibited adhesion of *S. aureus* ATCC 29213 and decreased adhesion of β-lactam susceptible *E. coli* ATCC 25922 (Fig. 2c). No previous studies have examined the effect of lupeol acetate, 5,7,4',-trihydroxyflavan-3-ol **4** and isovitexin **6** on microbial adhesion. The anti-adhesion activity of **4**, **5** and **6** was strain-specific, being most effective against *S. aureus* ATCC 29213, while lupeol acetate (**1**) increased adhesion of all *E. coli* and *S. aureus* strains tested.

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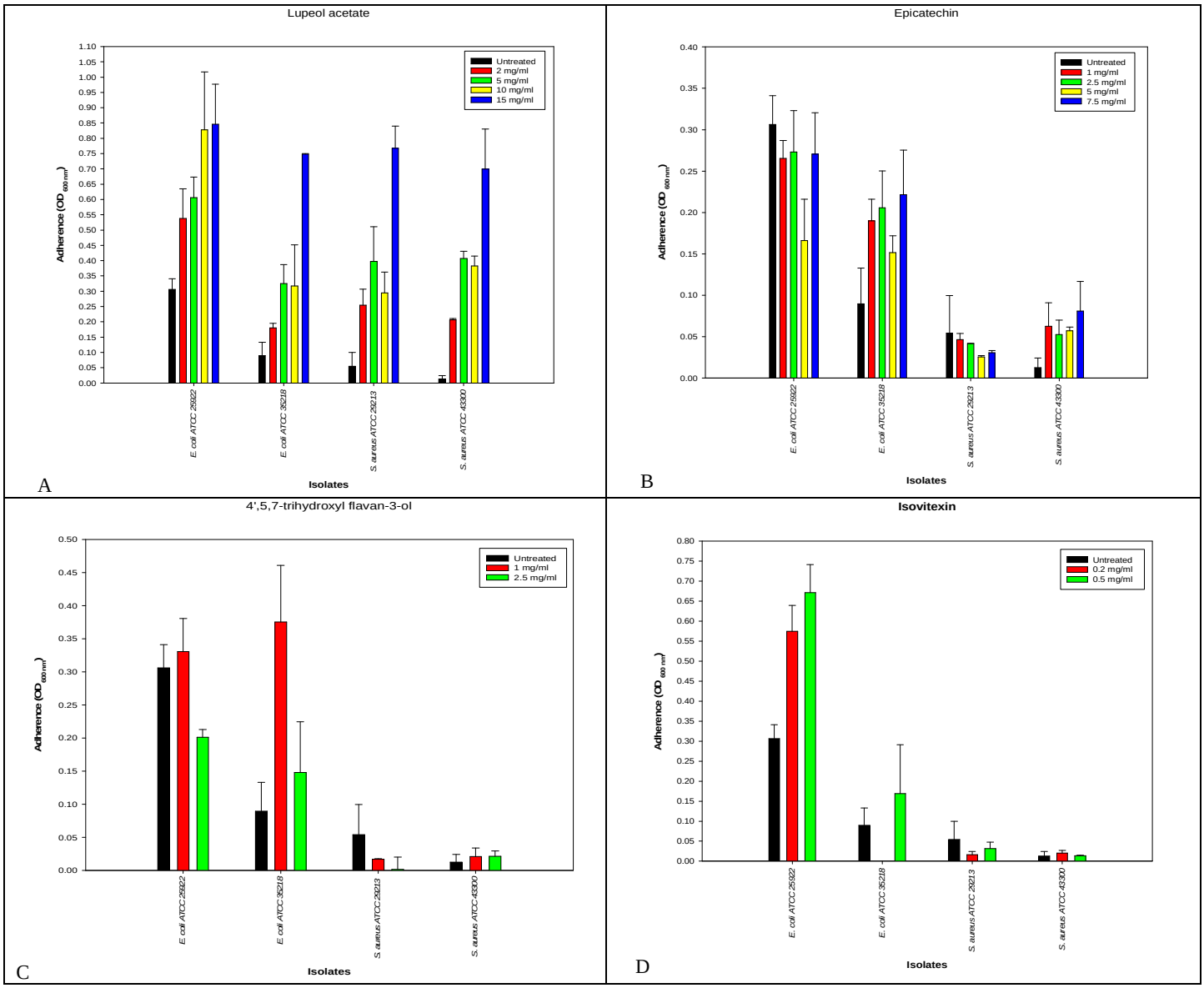

Figure 2: Anti-adhesion activity of 1 – 15 mg mL^{-1} of compounds **1** and **4-6** on biofilm formation by *E. coli* and *S. aureus* strains: (a) lupeol acetate (**1**); (b) epicatechin **(5)**; (c) 5,7,4'-trihydroxyflavan-3-ol **(4)**; (d) isovitexin (**6**).

Conclusion

The phytochemical investigation of *Ficus sansibarica* resulted in the isolation of three triterpenes and three flavonoids. The antimicrobial activity of the crude extracts and the isolated compounds were weak with a narrow-spectrum activity against Gram-positive bacteria. Most phytochemicals work best against Gram-positive bacteria and there are very limited small-molecule plant-derived antimicrobial compounds with high activity against Gram-negative species. The anti-biofilm activity demonstrated by the flavonoids 574',-trihydroxyflavan-3-ol, epicatechin and isovitexin (**4-6**), is most significant for *S. aureus* ATCC 29213. The results of this study provide baseline information on *F. sansibarica* potential validity in the treatment of Gram-positive biofilm-associated infections, despite its limited antimicrobial potential.

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