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Original Article

Anti-adhesion potential of non-polar compounds and extracts from *Ficus natalensis*

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ABSTRACT

Four triterpenoids, ergosta-4,6,8(14),22-tetraene-3-one **1**, stigma-4-ene-3-one **2**, 3β-hydroxy-21β-*H*-hop-22(29)-ene **3**, sitosterol and a quinone, tectoquinone **4**, were isolated from the leaf, stem bark and fruit extracts of *Ficus natalensis* subsp. *natalensis*, Moraceae, a medicinal fig found in Africa. The pure compounds **1**–**4** and crude extracts were tested for their antibacterial activity against five Gram-negative and seven Gram-positive strains and for their potential anti-biofilm activity. Antimicrobial susceptibility was observed with all pure compounds tested at 250 μg against the majority of Gram-negative and Gram-positive strains. The dichloromethane-soluble fruit extract was active against sensitive and resistant *Staphylococcus aureus* strains, *Enterococcus faecalis* and *Staphylococcus xylosus*. Compounds **2**, **3** and **4** demonstrated broad-spectrum antibiotic effects against eight of the twelve bacterial strains tested. In the anti-biofilm assay, exposure to ethyl acetate, methanol and aqueous methanol leaf, stem bark and fruit extracts decreased adhesion with a biofilm reduction of ≥100% for all three tested organisms: *Escherichia coli*, *Pseudomonas aeruginosa* and *S. aureus*. The methanol leaf extract demonstrated the most potent anti-adhesion potential against *E. coli* (218% biofilm reduction). The greatest ability to decrease adhesion was observed with compounds **2**, **3** and **5** against *P. aeruginosa* at the lowest concentration tested (100 μg ml^{-1}).

Introduction

Adhesion of microorganisms to diverse surfaces and to other microbes is essential for biofilm development and establishment of infections. When bacteria colonise and adhere to surfaces, they are associated with severe, recurrent infections and become 10–1000 times more resistant to antimicrobial agents (Borges et al., 2015). Pharmaceuticals or pharmaceutical extracts capable of preventing bacterial adhesion to surfaces are therefore desirable antibacterial agents. One source of these are medicinal plants used in traditional medicine, since many plant-based remedies are known to have therapeutic antimicrobial activity against clinical pathogens and an increasing number have even been identified as having anti-adhesion properties (Borges et al., 2016).

Ficus natalensis Hochst. subsp. *natalensis*, Moraceae, is a rock splitter tree which grows up to almost 20 m in height. It is geographically situated in South Africa, Zimbabwe, Zambia, Malawi, Mozambique and Kenya. The different parts of the tree have various applications varying from cultural, decorative, and commercial applications to folk medicine, is a major source of bark cloth and a potential natural dye (Burrows and Burrows, 2003). The root, bark, leaves and latex are used in several African countries to treat a variety of medical ailments including malaria, influenza, whooping cough, dysentery and guinea worm (Hutchings et al., 1996; Burrows and Burrows, 2003).

In previous studies on the plant, antibacterial activity has been reported for the methanol extract of the root (Rabe and van Staden, 1997). Apart from this, Olaokun et al. (2013) also reported the anti-diabetic potential of the acetone extract of the leaves.

Materials and methods

The fruits, leaves and stem bark from *Ficus natalensis* Hochst. subsp. *natalensis*, Moraceae, were collected in Durban, KwaZulu-Natal, South Africa in December 2012. The plant was identified at the Ward herbarium of the University of KwaZulu-Natal, where a voucher specimen was deposited under the collector number G.V. Awolola & H. Baijnath 3. The ground leaves, stem bark and fruits were extracted twice sequentially with organic solvents of increasing polarity, hexane (Hex), dichloromethane (DCM), ethyl acetate

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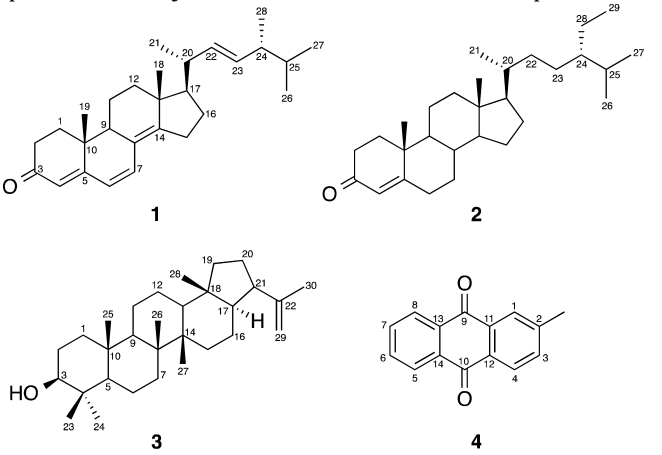
(EtOAc) and methanol (MeOH). Details of the extraction are given in the supplementary data.

The crude extracts were subjected to vacuum column chromatography (CC) on silica gel using a Hex-DCM-EtOAc-MeOH gradient elution, to afford five major fractions (A–E) by TLC. Compound **3** (250.6 mg), identified as 3β-hydroxy-21β-*H*-hop-22(29)-ene crystallised out of fraction A of the DCM and Hex crude extract of the leaves. Fraction B of the same crude extracts contained **1** ergosta-4,6,8(14),22-tetraene-3-one (140.5 mg). Sitosterol (80.6 mg) was isolated from fraction B of the EtOAc fraction of the leaves. Compound **4**, tectoquinone (70.8 mg) was obtained by purification of fraction B of the Hex and DCM crude extracts of the stem bark. Stigma-4-ene-3-one (**2**) (70.4 mg) was obtained from fraction C of the same extracts. All compounds were isolated and purified by silica gel CC using various ratios of Hex:EtOAc and identified by their ^{1}H and ^{13}C NMR data and verified by data in the literature, **1** (Fangkrathok et al., 2013), **2** (Liu et al., 2014), **3** (Sousa et al., 2012), sitosterol (Rasoanaivo et al., 2014) and **4** (Cheng et al., 2008).

Antibacterial susceptibility testing (AST) was carried out in duplicate on 12 bacterial indicator strains (given in the supplementary material) using the disc diffusion method according to standard procedures (CLSI, 2007). Bacterial adhesion on three bacterial strains: *Escherichia coli* ATCC 35218, *Staphylococcus aureus* ATCC 43300 and *Pseudomonas aeruginosa* ATCC 27853 based on the results from the AST according to the crystal violet microtitre plate assay were carried out in triplicate (Basson et al., 2008). Details of the assays are given in the supplementary data.

Results and discussion

The fractionation and purification of the leaf, stem bark and fruit extracts led to the isolation and identification of five non-polar compounds. To the best of our knowledge, this is the first report of secondary metabolites from *F. natalensis* subsp. *natalensis*.



The crude extracts of the different organs of *F. natalensis* were tested along with compounds **1**–**4** against the bacterial strains to determine whether they had any antibacterial activity to substantiate the reported use of the plant as an antibacterial agent (Rabe and van Staden, 1997). There has been extensive antibacterial studies on the ubiquitous sitosterol and therefore its antibacterial properties was not tested again.

The DCM extract of the fruit was the most active of all extracts demonstrating activity against several of the Gram-positive strains. The antibacterial activity of all extracts are reported in the supplementary material. Rabe and van Staden (1997) have previously observed MIC of 4 mg ml^{-1} for *S. aureus* and *Staphylococcus epidermidis* and 8 mg ml^{-1} for *Bacillus subtilis*, using methanolic root extracts of *F. natalensis*.

Varying antibacterial activity was demonstrated by the isolated compounds, which ranged from resistant to susceptible against both Gram-negative and Gram-positive organisms (Table 1). The antibacterial activity was dose-dependent, being more active at 250 μg than at 100 μg. Compounds **1**–**4** showed good activity against β-lactam resistant *E. coli* ATCC 35218 and the extended spectrum β-lactamase producing *Klebsiella pneumoniae* ATCC 700603 but was only moderately active against *P. aeruginosa*. Amongst the Gram-positive bacteria, the best activity was seen by the methicillin-resistant *S. aureus* (MRSA) strain ATCC 43300 by all four compounds following 250 μg exposure (Table 1). *B. subtilis* and *Enterococcus faecalis* strains demonstrated varying levels of susceptibility to all four compounds. All compounds, **1**–**4**, showed broad-spectrum antibiotic effects with as much as 10 of the 12 indicator bacterial strains being susceptible to them. Tectoquinone **4** showed high zones of inhibition of 25 mm and 23 mm against *Staphylococcus sciuri* and *K. pneumoniae*, respectively and stigma-4-ene-3-one **2** at 250 μg demonstrated a high zone of inhibition against *Staphylococcus xylosus* of 23 mm.

Biofilms in body tissues can be formed by bacteria such as *Acinetobacter baumannii*, *E. coli*, *P. aeruginosa*, and *S. aureus* (Borges et al., 2016). Decreased adhesion was observed for all EtOAc, MeOH and aq/MeOH leaf, stem bark and fruit extracts against three resistant bacterial strains, *E. coli* ATCC 35218, *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 43300 (see supplementary data).

The MeOH leaf extract displayed the greatest anti-adhesion potential against *E. coli*, while majority of the extracts, with the exception of the DCM and Hex extract (leaves and fruit) showed reduced biofilm formation by *P. aeruginosa*. For *S. aureus*, the aq/MeOH leaf, EtOAc stem bark and fruit and MeOH stem bark extracts reduced biofilm formation. All the polar extracts decreased adhesion suggesting interference by the extracts on the ability of these microorganisms to adhere to polystyrene surfaces. There are varying reports on anti-adhesion effects of polar extracts against different bacterial strains in the literature, which corroborate our findings. The anti-adhesion effects of polar extracts have been reported for *E. coli* and *Bacillus cereus* (Bräunlich et al., 2013), oral bacteria *Streptococcus mutans* and *Streptococcus sobrinus* (Rahim and Khan, 2006), both *S. aureus* and MRSA (Chusri et al., 2012) and *P. aeruginosa* (Sarkar et al., 2014).

The activity of the isolated compounds with regard to bacterial adhesion was variable. All four compounds increased adhesion of *E. coli* ATCC 35218 at all concentrations (Table 2). Stigmast-4-en-one (**2**) decreased adhesion of *P. aeruginosa* ATCC 27853 at all concentrations, with 100 μg ml^{-1} being the most effective. Ergost-4,6,8(14),22-tetraen-3-one (**1**) increased adhesion at 100 μg ml^{-1}, but decreased adhesion at inhibitory and supra-inhibitory concentrations. For **3** and **4**, decreased adhesion was only observed at sub-inhibitory (100 μg ml^{-1}) concentrations, while increased adhesion was observed at inhibitory and supra-inhibitory concentrations. Increased adhesion of *S. aureus* ATCC 43300 was observed with all four compounds at all concentrations tested (Table 2).

The variable activity observed with the isolated compounds was in accordance with the effects of oleanolic acid and β-amyrin acetate from *Vernonia auriculifera* against biofilm formation by seven different bacterial strains (Kiplimo et al., 2011). Strain-specific effects have been observed with the triterpenes ursolic and betulinic acids, from the liverwort *Lepidozia chordulifera* which inhibited biofilm formation and elastolytic activity of *P. aeruginosa* ATCC 27853, but increased adhesion of *S. aureus* ATCC 6538 (Gilabert et al., 2015). The increased adhesion being observed with some of the isolated compounds may be due to promotion of microbial adhesion by providing a suitable conditioning film for enhancement of cell attachment (Selim et al., 2014).

The crude extracts did not demonstrate a significant antibacterial effect. It is, however, quite possible that the compounds isolated

Table 1
Antibacterial activity of isolated compounds (zones of inhibition in mm) from *Ficus natalensis* subsp. *natalensis*.

Compound	Zones of inhibition (mm)									
	Gram-negative bacteria									
	E. coli ATCC 25922		*E. coli* ATCC 35218		*P. aeruginosa* ATCC 27853		*P. aeruginosa* ATCC 35032		*K. pneumoniae* ATCC 700603	
	100 μg	250 μg	100 μg	250 μg	100 μg	250 μg	100 μg	250 μg	100 μg	250 μg
1	10^R	20^S	9^R	15^S	7^R	15^S	9^R	15^S	9^R	19^S
2	10^R	18^S	9^R	16^S	0^R	15^S	10^R	14^I	9^R	17^S
3	0^R	16^S	0^R	15^S	0^R	14^I	0^R	18^S	7^R	16^S
5	9^R	18^S	10^R	20^S	8^R	13^I	10^R	15^S	0^R	23^S
AMP10	22^S		0^R		0^R		0^R		0^R	
TE20	28^S		23^S		15^S		14^R		12^R	

Compound	Zones of inhibition (mm)													
	Gram-positive bacteria													
	B. subtilis ATCC 6633		*E. faecalis* ATCC 29212		*E. faecalis* ATCC 51299		*S. aureus* ATCC 29213		*S. aureus* ATCC 43300		*S. sciuri* ATCC 29062		*S. xylosus* ATCC 35033	
	100 μg	250 μg	100 μg	250 μg	100 μg	250 μg	100 μg	250 μg	100 μg	250 μg	100 μg	250 μg	100 μg	250 μg
1	0^R	20^S	0^R	13^I	0^R	13^I	9^R	16^S	9^R	15^S	9^R	15^S	11^I	16^S
2	15^S	18^S	0^R	14^I	0^R	16^S	10^R	18^S	8^R	22^S	10^R	19^S	0^R	23^S
3	0^R	0^R	0^R	17^S	0^R	16^S	7^R	20^S	7^R	19^S	0^R	19^S	0^R	17^S
5	0^R	20^S	0^R	16^S	0^R	11^I	9^R	17^S	0^R	19^S	9^R	25^S	16^S	18^S
AMP10	40^S		nt		25^S		25^S		20^S		34^S		0^R	
TE20	36^S		nt		0^R		28^S		36^S		25^S		26^S	

R, resistant; S, susceptible; I, intermediate; nt, not tested; AMP10, ampicillin (10 μg); TE20, tetracycline (20 μg).

Table 2
Percentage biofilm reduction following exposure to 100–500 μg ml^{-1} of four compounds isolated from *F. natalensis* subsp. *Natalensis*.

Compound	Percent biofilm reduction (%)[a]								
	E. coli ATCC 35218			*P. aeruginosa* ATCC 27853			*S. aureus* ATCC 43300		
	100	250 μg ml^{-1}	500	100	250 μg ml^{-1}	500	100	250 μg ml^{-1}	500
1	−315.62	−1456.10	−1176.99	−64.04	28.16	18.05	−445.34	−338.40	−437.78
2	−783.27	−1168.21	−1151.57	59.65	8.59	19.45	−1.81	−330.97	−381.66
3	−149.72	−1346.58	−1071.16	39.07	−26.45	−38.54	−54.09	−302.98	−474.39
5	−593.81	−1220.89	−1189.00	54.69	−6.49	−48.49	−51.30	−395.19	−416.02

[a] Biofilm reduction calculated according to Pitts et al. (2003). Negative values are indicative of an increase in attachment/biofilm formation.

from *F. natalensis* subsp. *natalensis* in this work could be responsible for the antibacterial activity experienced by those who use the plant for medicinal purposes since most of them have demonstrated promising antibacterial activity. Decreased adhesion with >100% biofilm reduction was demonstrated by all the crude polar extracts against resistant bacterial strains. The isolated compounds exhibited strain-specific anti-adhesion potential, with biofilm reduction against *P. aeruginosa*, but not *E. coli* or *S. aureus*.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Authors' contributions

GVA and HB collected the plant materials. All experimental work was carried by GVA under the supervision of NK. Antimicrobial and anti-adhesion analysis was done under the supervision of HC. GVA and NK wrote the manuscript with contributions from HC and HB in their fields of expertise. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2017.07.004.

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