UNIVERSITY OF KWAZULU-NATAL

PHYTOCHEMICAL ANALYSES AND BIOLOGICAL ACTIVITIES OF FOUR SOUTH AFRICAN FICUS SPECIES (MORACEAE)

2015

GBONJUBOLA VICTORIA AWOLOLA
PHYTOCHEMICAL ANALYSES AND BIOLOGICAL ACTIVITIES OF FOUR SOUTH AFRICAN FICUS SPECIES (MORACEAE)

GBONJUBOLA VICTORIA AWOLOLA

2015

A thesis submitted to the School of Chemistry, College of Agriculture, Engineering and Science, University of KwaZulu-Natal, for the degree of Doctor of Philosophy.

This Thesis has been prepared according to Format 4 as outlined in the guidelines from the College of Agriculture, Engineering and Science which states:

This is a thesis in which chapters are written as a set of discrete research papers, with an overall introduction and final discussion, where one (or all) of the chapters have already been published. Typically these chapters will have been published in internationally recognized, peer-reviewed journals.

As the candidate’s supervisor, I have approved this thesis for submission.

Supervisor: Prof. Neil A. Koorbanally

Date: 8th December 2015
ABSTRACT

Four *Ficus* species used in African traditional medicine and indigenous to KwaZulu-Natal, *sansibarica, bizanae, natalensis* and *glumosa* were used in this study, where a phytochemical investigation and biological evaluation of the extracts and compounds were carried out. A range of compounds belonging to the triterpenoid, flavonoid, coumarin, polyphenol, megastigmane, cinnamic acid and anthraquinone classes of compounds were isolated.

Six compounds; three triterpenoids and three flavonoids; lupeol acetate (P1-1), cycloart-23-ene-3,25-diol (P1-2), sitosterol (P1-3), 5,7,4’-trihydroxyflavan-3-ol (P1-4), epicatechin (P1-5) and isovitexin (P1-6) were isolated from *F. sansibarica*. This is the first phytochemical investigation of *F. sansibarica*. Cycloart-23-ene-3,25-diol (P1-2), a cycloartane type triterpenoid, is reported herein for the first time in the genus *Ficus*. The *trans* configuration of the double bond at $\Delta^{23}$ on the side chain of the compound was clearly indicated by coupling constants close to 16 Hz. To the best of our knowledge only the *cis* isomer has been reported previously. The compounds exhibited moderate dose-dependent and selective antibacterial activity against both Gram-negative and Gram-positive bacteria. Exposure of the flavonoids; 5,7,4’-trihydroxyflavan-3-ol (P1-4), epicatechin (P1-5) and isovitexin (P1-6) at varying concentrations, decreased adhesion indicating good anti-biofilm activity against the bacteria tested. No previous studies have examined the effect of these compounds on microbial adhesion.

A total of fourteen compounds were isolated from *F. bizanae*, which included two coumarins, 7-methoxycoumarin (P2-1) and psoralen (P2-2), one anthraquinone, tectoquinone (P2-3), one flavonoid glycoside, quercetin-3-O-β-D-glucopyranoside (P2-4), two megastigmane derivatives, vomifoliol (blumenol A) (P2-5) and dehydrovomifoliol (P2-6), a cinnamic acid
derivative, dihydroferulic acid (P2-7), two polyphenolic compounds, 4-methoxy catechol (P2-8) and protocatechuic acid (P2-9) and five triterpenoids, taraxerol (P2-10), 3β-acetoxy-9(11)-fernene (P2-11), lupeol acetate (P1-1), β-amyrin acetate (P2-12) and sitosterol (P1-3).

This is the first phytochemical and pharmacological report on F. bizanae. A strain- and extract-specific potentiative synergistic anti-bacterial effect of F. bizanae extracts in combination with the antibiotics, azithromycin, gentamicin, sulphamethoxazole, nalidixic acid and cephalosporin was observed with all tested bacteria.

A comprehensive phytochemical analysis of F. natalensis yielded four triterpenoids, ergosta-4,6,8(14),22-tetraene-3-one (P3-1), stigma-4-ene-3-one (P3-2), a hopane triterpenoid, 3β-hydroxy-21β- H-hop-22(29)-ene (P3-3), sitosterol (P1-3) and an anthraquinone, tectoquinone (P2-3). This is the first report of triterpenes and anthraquinones in F. natalensis. Essential oil were previously reported in the plant. A broad spectrum antimicrobial effect was demonstrated by P3-2, P3-3 and P2-3 against the majority of Gram-negative and Gram-positive strains while only the dichloromethane fruit extract was active against selected strains. In the anti-biofilm assay, the methanol leaf extract demonstrated the most potent anti-adhesion potential whereas compounds P3-2, P3-3 and P2-3 showed the greatest ability to decrease adhesion at the lowest concentration tested against selected bacterial strains.

A phytochemical investigation of F. glumosa resulted in the isolation of 13 compounds, three polyphenolic compounds, p-hydroxybenzoic acid (P4-1), 3,4-dihydroxybenzoic acid (protocatechuic acid) (P2-9) and vanillic acid (P4-2), two coumarins, peucedanol (P4-3) and (-)-marmesin (P4-4) together with three flavonoid glycosides, quercetin-7-O-glucopyranoside (P4-5), quercetin-3-O-β-D-glucopyranoside (P2-4) and quercetin-3-O-β-galactopyranoside (P4-6). The five triterpenoids isolated were simiarenol (P4-7), 3β-
hydroxy-21β-H-hop-22(29)-ene (P3-3), α-amyrin acetate (P4-8), stigma-4-ene-3-one (P3-2) and sitosterol (P1-3). With the exception of (P4-2), all the compounds were isolated from *F. glumosa* for this first time. The methanol soluble leaf extract of the plant exhibited the best gastroprotective activity when compared with standard drugs. This is the first report of the anti-ulcerogenic activity of *F. glumosa*.

The bioactivity of the isolated compounds and extracts from the Ficus species used in this study support the use of the four *Ficus* species in African traditional medicine. This work suggests that *F. bizanae* could be considered a potential source of resistance-modifying agents (RMA), which if used together with current antimicrobial agents, can improve their efficacy. This work also indicates that the extracts of *Ficus glumosa* could be potential anti-ulcer agents.
List of isolated compounds

Numbers below may not be consecutive since compounds were isolated in more than one plant. The numbers below will allow one to match a number to one of the structure in the summary of compounds.

Compounds are numbered consecutively in each of the chapters.

**Chapter 2 Compounds from *Ficus sansibarica***

- **P1-1** lupeol acetate
- **P1-2** cycloart-23-ene-3,25-diol
- **P1-3** sitosterol
- **P1-4** 5,7,4’-trihydroxyflavan-3-ol
- **P1-5** epicatechin
- **P1-6** isovitexin

**Chapter 3 Compounds from *Ficus bizanae***

- **P1-1** lupeol acetate
- **P1-3** sitosterol
- **P2-1** 7-methoxy coumarin
- **P2-2** psoralen
- **P2-3** tectoquinone
- **P2-4** quercetin-3-O-β-D-glucopyranoside
- **P2-5** vomifoliol (blumenol A)
- **P2-6** (+)-dehydrovomifoliol
- **P2-7** dihydroferulic acid
- **P2-8** 4-methoxy catechol
- **P2-9** protocatechuic acid
- **P2-10** taraxerol
- **P2-11** 3β-acetoxy-9(11)-fernene
- **P2-12** β-amyrin acetate
Chapter 4 Compounds from *Ficus natalensis*

P1-3 sitosterol
P2-3 tectoquinone
P3-1 ergosta-4,6,8(14),22-tetraene-3-one
P3-2 stigma-4-ene-3-one
P3-3 3β-hydroxy-21β-H-hop-22(29)-ene

Chapter 5 Compounds from *Ficus glumosa*

P1-3 sitosterol
P2-4 quercetin-3-O-β-glucopyranoside
P2-9 protocatechuic acid (3, 4-dihydroxy benzoic acid)
P3-2 stigma-4-ene-3-one
P3-3 3β-hydroxy-21β-H-hop-22(29)-ene
P4-1 p-hydroxyl benzoic acid
P4-2 vanillic acid
P4-3 peucedanol
P4-4 (-) – marmesin
P4-5 quercetin-7-O-glucopyranoside
P4-6 quercetin-3-O-β-galactopyranoside
P4-7 simiareno
P4-8 α-amyrin acetate
Summary of isolated compounds

Structures in subsequent chapters are not repeated

Chapter 2

P1-1

P1-2

P1-3

P1-4: R = H

P1-5: R = OH

P1-6
Chapter 3

P1-1 lupeol acetate and P1-3 sitosterol were also isolated in this chapter.
Chapter 4

P1-3 sitosterol and P2-3 tectoquinone were also isolated in this chapter.
Chapter 5

**P4-1**: $R_1 = H$, $R_2 = OH$

**P4-2**: $R_1 = OCH_3$, $R_2 = OH$

**P4-3**

**P4-4**

**P4-5**: $R_1$ = glucopyranoside, $R_2 = H$

**P4-6**: $R_1 = H$, $R_2$ = galactopyranoside

**P4-7**

**P4-8**

**P1-3** sitosterol, **P2-4** quercetin-3-O-$\beta$-glucopyranoside, **P2-9** protocatechuic acid, **P3-2** stigma-4-ene-3-one and **P3-3** 3$\beta$-hydroxy-21$\beta$-H-hop-22(29)-ene were also isolated in this chapter.
LIST OF ABBREVIATIONS

$^1$H NMR  proton nuclear magnetic resonance spectroscopy
$^{13}$C NMR  C-13 nuclear magnetic resonance spectroscopy
COSY  correlated spectroscopy
DEPT  distortionless enhancement by polarization transfer
HMBC  heteronuclear multiple bond coherence
HSQC  heteronuclear single quantum coherence
GCMS  mass spectrometry
NOESY  nuclear overhauser effect spectroscopy
CDCl$_3$  deuterated chloroform
CD$_3$OD  deuterated methanol
DMSO-d6  deuterated dimethyl sulfoxide
UV  ultraviolet
IR  infrared
LCMS  liquid chromatography- mass spectrometry
EtOAc  ethyl acetate
Me  methyl
MeOH  methanol
br  broad resonance
tlc  thin layer chromatography
c  concentration
cc  column chromatography
p.o  per oral dose
SEM  standard error of the mean
ANOVA  analysis of variance
OECD  Organization of Economic Co-operation and Development
DECLARATION 1: PLAGIARISM

I, Gbonjubola Victoria Awolola, declare that:

1. The research reported in this thesis is my original research, except where otherwise indicated.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other persons’ data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted then;
   (a) Their words have been re-written but the general information attributed to them have been referenced.
   (b) Where their exact words have been used, then their writing has been placed in italics and inside quotation marks, and referenced.
5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

Signed: ..............................................
DECLARATION 2 - PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, in press and published and give details of the contributions of each author to the experimental work and writing of each publication)

Chapter 2


Contributions: All experimental work was carried out by me. The antimicrobial analysis was carried out under the supervision of Dr Hafizah Chenia. I wrote the manuscript. Based on each co-authors field of expertise, they verified the interpretation of my data, checked the scientific contents and edited the manuscript as a whole.

Chapter 3

3. Awolola, G. V., Chenia, H. Y., Bajnath, H. and Koorbanally, N. A. Identification of secondary metabolites and resistance modifying activity of *Ficus bizanae* leaf, stem bark and fruit extracts, manuscript is ready and intended to be submitted to *Acta Poloniae Pharmaceutica*.

Contributions: I carried out all the experimental work. Dr Hafizah Chenia supervised the resistant modifying aspect of the work. I wrote the manuscript. All other authors were supervisors and editors.

Chapter 4

5. Awolola, G. V., Chenia, H. Y., Baijnath, H. and Koorbanally, N. A. *Ficus natalensis*: Anti-adhesion potential of non-polar compounds and extracts, manuscript is ready and intended to be submitted to *Natural Product Research*.

Contributions: All experimental work was carried by me. Antimicrobial and anti-adhesion analysis was done under the supervision of Dr Chenia Hafizah. I compiled the data and wrote the manuscript. Each co-author contributed to the manuscript based on their field of expertise and edited the manuscript.

Chapter 5

6. Awolola, G. V., Sofidiya, M. O., Baijnath, H., Noreen, S. and Koorbanally, N. A. The phytochemistry and gastroprotective activities of the leaves of *Ficus glumosa*, manuscript is ready and intended to be submitted to the *Journal of Ethnopharmacology*.

Contributions: My role included the preparation of extracts, isolation, and characterisation of all phytoconstituents and writing of the manuscript. The anti-ulcer analysis and data interpretation was carried out by Dr. Margaret O. Sofidiya and Miss Noreen Seun. All other authors carried out supervision and editorial contributions.

Signed: …………………………………………………
DEDICATION

This thesis is dedicated to the ALMIGHTY GOD and my husband Oluwafemi Paul Awolola together with my sons, Oluwanifemi and Ifeoluwa Awolola.
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Chapter 1. Introduction

1.1 Introduction to the genus Ficus

The genus Ficus is one of the well-known members of the Angiosperms, belonging to the Moraceae family (also known as Mulberry family), comprising over 750 species which includes woody trees, shrubs, vines, epiphytes, and hemi-epiphytes. They are collectively identified as fig trees or figs distributed in tropical and subtropical regions worldwide. Australasia has approximately 500 diverse species, while Madagascar and Africa have approximately 110 species (Lansky and Paavilainen, 2011; Burrows and Burrows, 2003). The different species can be categorised as rock-splitters, free-standing, terrestrial or stranglers (Burrows and Burrows, 2003). Ficus species have the distinctive characteristics of having exceptional inflorescence and a unique pollination syndrome with the wasps’ family, Agaonidae (Ronsted et al., 2008; Abraham, 1989).

Ficus leaves are always simple, alternating with visible stipules that envelope the leaf bud, though they sometimes have irregular margins and are lobed and incised. They possess male and female flowers in the receptacles from which the fruits grow up to 1.5 cm in diameter, with or without a stalk on the axils of the leaves, stem or branches. The fruit (figs) grow as heavy fleshy spheres in pairs or dense clusters and are mostly edible (Palgrave, 1983). Generally, the bark, leaves, branches and unripe fruits of the different species of Ficus produce a rubber-like, non-toxic latex within their vasculatures (Lansky and Paavilainen, 2011; Burrows and Burrows, 2003).
1.2 Ethnobotanical relevance of *Ficus* species

The fruits, twigs, leaves, latex, stem, root and bark of *Ficus* species have been traditionally used as medicine for the treatment and control of many diseases and ailments (Lansky and Paavilainen, 2011; Burrows and Burrows, 2003). They are used to treat bacterial, fungal, parasitic and gastrointestinal infections, venereal diseases, gynaecological diseases, diarrhoea, respiratory disorders, liver problems, malaria, epilepsy, uterine disorders and hypertension and are used as anti-poison, and for the control of childhood convulsions (Table 1-1 on page 30). Some species are used against snake bites as antivenom and others as insecticides (Bhalerao and Sharma, 2014; Igoli et al., 2005). In general, the difference in ethnomedicinal use of the species vary geographically and with different local populations. They are mostly prescribed by traditional healers with no scientific knowledge (Nadembega et al., 2011; Bafor et al., 2010; Burrows and Burrows, 2003).

The plant part preparation depends on the application. For instance, stem bark and leaf decoctions of *Ficus carica*, *Ficus exasperata*, *Ficus ovata* and *Ficus polita* are mostly used for the treatment of numerous ailments and in some cases, the decoction is prepared in milk or alcohol (Lansky and Paavilainen, 2011). There have also been reports of the plant parts being used in combination (synergistically) with other plants for treatment (Lansky and Paavilainen, 2011). Table 1-1 (page 30) depicts the ethnomedicinal uses and biological activities of some common species within the genus.

There have been diverse reports on the biological activity of different species of *Ficus*, ascribing the activities to the variety of phytoconstituents contained in the plant. Antibacterial, synergistic antibacterial, antimycobacterial, antifungal and antioxidant activities of a number of species have been reported (Joshi et al., 2014; Madikizela et al., 2012; 2014; Dangarembizi et al., 2013; Chauke et al., 2012; Mbakwem-Aniebo et al., 2012; Ramde-
Some species of *Ficus* have also been reported to have uter tonic activity, which supports their ethnomedicinal use of easing childbirth (Bafor et al., 2009; 2010). Others have been shown to increase sperm production (Bafor et al., 2013; Igbokwe et al., 2009). Several *Ficus* species have been used in the management of pain, fever, inflammation, ulcers, sores and wound healing (Sabiu et al., 2015; Bhalerao et al., 2015; Bhalerao and Sharma 2014; Dangarembizi et al., 2013; Mawa et al., 2013; Devi et al., 2012; Gregory et al., 2009; Lansky et al., 2008; Rao et al., 2002).

Antiparasitic, antiplasmodial and anthelmintic activities of extracts and isolated phytoconstituents of *Ficus sycomorus, Ficus sur, Ficus thonningii, Ficus religiosa, F. carica* and *F. polita* have been reported in some studies (Dangarembizi et al., 2013; Mawa et al., 2013; Muregi et al., 2003; 2007; Sanon et al., 2003; Gbeassor et al., 1990). Cytotoxic, anticancer and antidiabetic activity of *Ficus* species and some related symptoms were demonstrated by *F. carica, F. sycomorus, F. exasperata, Ficus arnottiana, Ficus palmata, F. lutea, F. racemosa, Ficus craterostoma, F. glumosa* and *Ficus benghalensis* (Joshi et al., 2014; Chaturvedi and Murthy 2013; Kazeem et al., 2013; Mawa et al., 2013; Olaokun et al., 2013; 2014; Tamokou et al., 2013; Nana et al., 2012; Ahmed and Urooj, 2010; Lansky et al., 2008; Mazumder et al., 2008).

The use of *Ficus* species for the treatment of coughs, hypertension and the control of body water and lipids have been validated by the antitussive, antiasthmatic, expectorant, cardioprotective, anti-diuretic and hypolipidaemic activities of some species of *Ficus*
(Bhalerao and Sharma 2014; Dangarembizi et al., 2013; Liu et al., 2009; Rao et al., 2003; Ratnasooriya et al., 2003). The hepatoprotective and nephroprotective ability (protection of liver and kidney damage) of *Ficus ingens*, *F. exasperata*, *F. palmata* and *Ficus racemosa* has been documented in the literature (Enogieru et al., 2015; Joshi et al., 2014; Donia et al., 2013; Mandal et al., 1999). The anticonvulsant potential observed by the extracts of *F. sycomorus*, *Ficus sur* and *F. religiosa* has validated the plants' use in the management of epilepsy (Ishola et al., 2013; Patil et al., 2011; Sandabe et al., 2003).

Other activities such as proteolytic, antispasmodic, anticoagulant, antiplatelet, anti-amnesia, nematicidal, insecticidal, genotoxic, anti-mutagenic, chemomodulatory and immunomodulatory effects have also been reported for other *Ficus* species (Table 1-1 on page 30). Studies on crude plant extracts has mostly been used for the validation of the efficacy of the various biological activities, however there has been some cases where phytoconstituents such as flavonoids and their glycosides, triterpenoids, coumarins, phenolic acids, and ceramides and their derivatives have been isolated and found to be active in assays related to the plant's use (Table 1-1 on page 30).

1.3 A phytochemical review of 23 South African *Ficus* species with antibacterial or anti-ulcer activity

Plants from the genus *Ficus* are rich sources of diverse classes of compounds such as polyphenolic compounds (flavonoids, coumarins, phenolic acids, phenylpropanoids and anthocyanins), triterpenes, steroids, alkaloids, megastigmanes, lignanoids, ceramides, tannins and in some cases stilbenes and volatile compounds.

This mini review covers 23 South African *Ficus* plants, selected since they have been reported to have antibacterial or anti-ulcer activities. Triterpenoids and polyphenolic
compounds such as flavonoids, flavonoid glycosides, coumarins and phenolic acids were the major classes of isolated phytoconstituents. Triterpenoids were found in eighteen of the 23 plants reviewed, while polyphenolic compounds were found in fourteen. The names of the species under review are contained in Table 1-1 (on page 30). Table 1-2 (on page 35) contains the names and molecular formulae of all the compounds isolated in these 23 plants.

**Terpenoids and sterols**

Lupeol (1), lupeol acetate (2), lupenone (3), lupeol hexanoate (4) and betulinic acid (5) with different substituents at positions 3 and 28 were found in *F. benghalensis, F. carica, Ficus cordata, F. glumosa, F. microcarpa, F. nervosa, F. ovata, F. polita, F. racemosa, F. religiosa* and *F. thonningii*. 3β-Hydroxy-20-oxo-29(20→19) abeolupane (6), with an added methyl group at C-19 compared to compounds 1-5 and 29,30-dinor-3β-acetoxy-18,19-dioxo-18,19-secolupane (7), with an open cyclopentane ring were also isolated from *F. microcarpa*.

![Chemical structures of terpenoids and sterols](image)

<table>
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<th>Name</th>
<th>R₁</th>
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</tr>
<tr>
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<td>CH₃</td>
</tr>
<tr>
<td>3</td>
<td>lupenone</td>
<td>O</td>
<td>CH₃</td>
</tr>
<tr>
<td>4</td>
<td>lupeol hexanoate</td>
<td>OCO(CH₂)₄-CH₃</td>
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</tr>
<tr>
<td>5</td>
<td>betulinic acid</td>
<td>OH</td>
<td>COOH</td>
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</table>
β-amyrin (8), its acetate derivate, β-amyrin acetate (9) and oleanolic acid (10) of the oleanane triterpenoids with a double bond at Δ^{12}, and differing in their substituents at positions 3 and 28 were contained in nine of the nineteen Ficus species, F. benghalensis, F. carica, F. cordata, F. glumosa, F. lutea, F. microcarpa, F. nervosa, F. ovata and F. religiosa. A dihydroxy ester, methyl maslinate (11) with an extra hydroxy group at C-2 and an ester moiety at C-28, an acetylated triterpene, 3β-acetoxy-12,19-dioxo-13(18)-oleanene (12) with a double bond at the 13(18) position and 3β-acetoxy-11α-hydroxy-11(12→13)abeooleanan-12-al (13) with an unusual cyclopentane ring C in the basic oleanane skeleton were found in F. carica and F. microcarpa.

The ursane triterpenoids 14-19 with the same oleanane skeleton but with methyl groups on ring E on adjacent carbons (C-19 and C-20) and different substituents at C-3 and C-17 were found in five of the Ficus species reviewed (Jain et al., 2013; Ragasa et al., 2013; Singh et al., 2011; Chiang et al., 2005). Ficus nervosa and F. religiosa contains α-amyrin (14) with an alpha hydroxy group and methyl group attached at C-3 and C-17 respectively. α-Amyrin acetate (15) is contained in F. racemosa and F. sur (Singh et al., 2011; Feleke and Brehane 2005.), while α-amyrin octacosanoate (16) containing a long chain fatty acid at C-3 was also found in F. racemosa (Jain et al., 2013). The three carboxylic acids, ursolic acid (17), its
acetylated derivative, acetylursolic acid (18) and ursonic acid (19) with a keto group at C-3, were found in *Ficus microcarpa* and *F. polita* (Kuete et al., 2011; Chiang et al., 2005).

![Chemical structures](image)

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<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>18</td>
<td>acetylursolic acid</td>
<td>OAc</td>
<td>COOH</td>
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</table>

Taraxarane triterpenoids with a double bond at 20(30) as in taraxasterol (20) and 20(30)-taraxastene-3\(\beta\),21\(\alpha\)-diol (21) were isolated from *F. benghalensis* and *F. microcarpa* (Patil and Patil 2010; Kuo and Chiang 1999). 20-Taraxasten-3\(\beta\)-ol (22), its oxidised form, 20-taraxastene-3\(\beta\),22\(\beta\)-diol (23), and the three C-22 ketones, 22-oxo-20-taraxasten-3\(\beta\)-ol (24), 3,22-dioxo-20-taraxastene (25) and 3\(\beta\)-acetoxy-20-taraxasten-22-one (26), all with a double bond at \(\Delta^{20}\) were found in *F. microcarpa* (Kuo and Chiang 1999).
*F. microcarpa* also contained 3β-acetoxy-19(29)-taraxasten-20α-ol (27) with a double bond at position 19(29) and three related epoxides, 20α,21α-epoxytaraxastan-3β-ol (28), ptiloepoxide (29), and 3β-acetoxy-21α,22α-epoxytaraxastan-20α-ol (30) (Chiang et al., 2005; Kuo and Chiang 1999).

Taraxar-14-ene (31) and taraxeryl acetate (32) with a double bond at Δⁱ⁴ and the 11,12-epoxy taraxarenes, 3β-acetoxy-11α,12α-epoxy-14-taraxerene (33) and 3β-acetoxy-11α,12α-epoxy-16-oxo-14-taraxerene (34) were found in *F. microcarpa* (Chiang et al., 2005). In addition, a germanicol acetate (35) with a double bond at Δ¹⁸ was reported in *F. palmata* (Alqasoumi et al., 2014a).
Bauerenol (36), a friedoursane triterpenoid was isolated from *F. carica* and contained a double bond at the $\Delta^7$ position (Mawa et al., 2013). Four friedelane triterpenoids (37-40) were also isolated within the twenty-three species reviewed. Friedelin (37) and 3-friedelanol (38) were found in *F. benghalensis*, 3,7-dioxofriedelane (39) with keto groups at C-3 and C-7 was isolated from *F. exasperata* and the carboxylic acid, 3-oxofriedelan-28-oic acid (40) was reported in *F. microcarpa*.
The 9,10-cyclopropyl triterpenoids, 27-nor-3β-hydroxy-25-oxocycloartane (41), (23E)-27-nor-3β-hydroxycycloart-23-en-25-one (42) and (22E)-25,26,27-trinor-3β-hydroxycycloart-22-en-24-al (43) were isolated from *F. microcarpa* (Chiang et al., 2001). Cycloeucalenol (44), a common cycloartane with a 9,19 cyclopropyl group together with the 13,27-cyclopropyl cycloursanes, 3β-acetoxy-15α-hydroxy-13,27-cyclours-11-ene (45) and 3β-acetoxy-12α-formyloxy-13,27-cycloursan-11α-ol (46) were found in *F. nervosa*.

The tetracyclic triterpenes, 3β-acetoxy-25-hydroxylanosta-8,23-diene (47) and 3β-acetoxy-25-methoxylanosta-8,23-diene (48) were isolated from *F. microcarpa*. The lanosterol compounds, lanosta-7,24-dien-3-one (49), lanosta-8,24-dien-3-one (50), lanost-20-en-3β-acetate (51), 24,25-dihydroparkeol acetate (52) and gluanol acetate (53) were isolated from *F. glumosa* and *F. racemosa*. The euphol-3-O-cinnamate (54) was found in *F. polita*. 
Three ubiquitous steroids, stigmasterol (55), sitosterol (56) and sitosterol-3-O-β-D-glucopyranoside (57) were found in eleven of the twenty-three *Ficus* species reviewed. Daucosterol (58), sitosterol-3-O-(6'-O-heptadecanoyl)-β-D-glucopyranosyl (59), sitosterol-3-O-(6'-octadecanoyl)-β-D-glucopyranoside (60), 6β-hydroxystigmast-4-en-3-one (61), 6β-hydroxystigmasta-4,22-dien-3-one (62) were found in *F. microcarpa* and campesterol (63), 28-isofucosterol (64), ergosterol peroxide (65) and dongnoside E (66) were isolated in *F. religiosa* and *F. glumosa*. 
Flavonoids

Flavonoids were isolated from fourteen *Ficus* species, forming the second largest class of compounds isolated in the review (Table 1-2). Eleven flavonoids (67-77) of the flavonol skeleton with different substitution patterns on the A, B and/or C ring of the nucleus with hydroxy and/or sugar moieties have been isolated from several of the *Ficus* species.
Patuletin-3′-O-methyl-3-O-rutinoside (75) with methoxy substituents was isolated from *F. ingens* only (Donia et al., 2013) and an ester of isoquercetin, isoquercitrin-6′-O-4-hydroxybenzoate (77) from *F. exasperata* (Taiwo and Igbenehu 2014).

![Image](67-77)

<table>
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<tr>
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<td>H</td>
<td>rhamnosyl</td>
<td>H</td>
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<tr>
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<td>OH</td>
<td>H</td>
<td>hydroxybenzoate</td>
<td>H</td>
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</table>

The flavones 78-84, all with hydroxy groups at C-5 and C-7 and with either hydroxy or glucose moieties at the other positions on the aromatic rings were isolated in *F. exasperata*, *F. glumosa*, *F. microcarpa*, *F. nervosa* and *Ficus sarmentosa* (Table 1-2). The glucose moieties are substituted at positions 6, 8 or 4′. Compound 85 contains a prenyl substituent at C-6 while 86 has a linear pyran group attached to ring A of the flavone.
Dihydroflavanols were also present in the species reviewed. Naringenin (87), eriodictyol (88) and homoeriodictyol (89) were found in *F. nervosa* and *F. sarmentosa*, while dihydroquercetin (90) was isolated from *F. thonningii* along with aromadendrin (91), a 3,4',5,7-tetrahydroxyflavanol. The prenylated 6-prenylpinocembrin (92) was obtained from *F. glumosa*, while shuterin (93), (2R,3R)-3,5,4'-trihydroxy-7-methoxy-6-(3,3-dimethylallyl)-dihydroflavonol (94) and conrauflavonol (95) with a linear pyran ring was isolated from *F. thonningii*. 
Three common catechins, catechin itself (96), epiafzelechin (97) and epicatechin (98) were isolated from *F. cordata* and *F. microcarpa* (Table 1-2).

Five species of *Ficus*, *F. glumosa*, *F. microcarpa*, *F. nervosa*, *F. ovata* and *F. thonningii* contained isoflavonoids (Table 1-2). Compounds 99-104 were simple hydroxy or methoxy isoflavonoids.
The isoflavonoids, 5,7,2′,4′-tetrahydroxy-3′-(3-hydroxy-3-methylbutyl)-isoflavone (thonningiisoflavone) (105), luteone (106) and wighteone (107) contained prenyl or oxidised prenyl groups at C-3′ or C-6 while parvisoflavone B (β-isoluteone) (108), alpinumisoflavone (109), 4′-O-methylalpinumisoflavone (110) and hydroxyalpinumisoflavone (111) contained a pyran ring. Ficusisoflavone (112) have dihydropyran rings, while compounds 113 and 114 have dihydrofuran rings. The pyran rings are arranged in a linear fashion for compounds 111 and 112.
Coumarins

Simple coumarins, coumarin itself (115), umbelliferone (7-hydroxycoumarin) (116), and scopoletin (117) were isolated in *F. carica*, *F. nervosa* and *F. sarmentosa* (Mawa et al., 2013; Wang et al., 2011; Chen et al., 2010). The linear pyranocoumarins, 118-120 were isolated in *F. nervosa* (Chen et al., 2010) and the furano coumarins (121-127) were isolated in a number of *Ficus* species (Alqasoumi et al., 2014a, 2014b; Dongfack et al., 2012; Nana et al., 2012; Liu et al., 2011; Singh et al., 2011; Patil and Patil 2010; Deraniyagala et al., 1998). The benzopyranone, 7-hydroxy-2,5-dimethylchromen-4-one (128) and the benzofuran, trans-psoralenoloside (129) were isolated from *F. ingens* and *F. palmata* respectively (Alqasoumi et al., 2014a; Donia et al., 2013).
Anthocyanins

Six anthocyanins were found in three of the *Ficus* species reviewed. Three of these were the polyhydroxylated anthocyanidins, leucodelphinidin (130), and its 3-β-D-glycosides, 131-132, which were isolated from *F. benghalensis*. The cyanidin-3-rhamnoglucoside (133) was reported in *F. carica* and the anthocyanidin dimers, procyanidin B1 (134) and procyanidin B3 (135) were found in *F. microcarpa*. 
Phenolic compounds

The *Ficus* species reviewed also contained several phenolic acids. Protocatechuic acid (136), gallic acid (137), vanillic acid (138) and 2,4,5-trihydroxybenzoic acid (139) were found in *F. glumosa*, *F. microcarpa*, *F. ovata*, *F. sycomorus* and *F. palmata*. The cinnamic acid derivative, ferulic acid (140) was isolated in *F. carica* and *F. microcarpa* along with three caffeoyl acid derivatives, 3-*O*-caffeoylquinic acid (141), methyl chlorogenate (142) and 5-*O* -caffeoylquinic acid (143). The phenylpropanoid, syringin (144) was also found in *F.*
microcarpa and an isochroman-1-one ester glycoside, bergenin (145) was isolated from F. racemosa.

![Chemical structures](image)

Three phenylpropanoids, (7S,8R)-syringoylglycerol (146) and its glucoside derivatives, syringoylglycerol-7- O-β-D-glucopyranoside (147) and 2’,7-epoxysyringoylglycerol-8- O-β-D-glucopyranoside (ficuscarpanoside B) (148) were isolated from F. microcarpa, together with an aromatic glycoside, icariside D2 (149). A macrolide, lasiodiplodin (150) together with the
benzaldehydes, 4-hydroxybenzaldehyde (151) and vanillin (152) were found in *F. nervosa* and a prenylated stilbene, chiricanine A (153) and a glucosidic stilbene, *(E)-3,5,4'-trihydroxystilbene-3,5-O-β-D-diglucopyranoside* (154) were isolated from *F. glumosa* and *F. polita* respectively (Nana et al., 2012; Kuete et al., 2011).

Five lignans, ficusal (155), polystachyol (156), lyoniresinol-2a-O-β-D-xylopyranoside (157) ficusesquilignan A (158) and ficusesquilignan B (159) were found in *F. glumosa* and *F. microcarpa* ([Table 1-2](#)).
Miscellaneous compounds

Apart from the classes of compounds mentioned above, six ceramides, ficusamide (160), glumoamide (161), glumoside (162), benjaminamide (163), lutaoside (164) and politamide (165) were found in *F. exasperata*, *F. glumosa*, *Ficus lutea* and *F. polita*. The two anthraquinones, chrysophanol (166) and aloe emodin-**O**-glucoside (167) were isolated from *F. ingens*. Pheophorbide-**a** (168), pheophorbide-**b** (169) and pyropheophorbide-**a** (170) were the three pheophytins reported in *F. exasperata*. 
Five megastigmanes, 4,5-dihydroblumenol (171), (3S,5R,6R,7E,9S)-megastigman-7-ene-3,5,6,9-tetraol (172), bridelionoside B (173), dihydroalangionoside A (174), ficumegasoside (175), two apo carotenoids, ficusone (176) and (7E,9Z)-dihydrophaseic acid-3-O-β-D-
glucopyranoside (177), three γ-lactone derivatives, ficuspirolide (178), ficusolide (179) and ficusolide diacetate (180), three vitamin E like α-tocopheroids (181-183), an ether, 2,2′-dihydroxyl ether (184) and an acid, ficuscarpanic acid (185) were all isolated from *F. microcarpa.*
Some long chain fatty acids or fatty acid esters (186-188), alkanes (189-190), ketones and aldehydes (191-194), alcohols (195-198), esters (199-201) together with monoterpenes (202-204), sesquiterpenes (205-209) and some other miscellaneous compounds (210-214) were isolated in *F. carica*, *F. exasperata*, *F. ovata* and *F. religiosa* (Table 1-2).
(189) nonane
(190) n-hexacosane, n= (CH₂)₂₂
(191) 3-pentanone
(192) 6,10,14-trimethyl-2-pentadecanone
(193) 2-methylbutanal
(194) (E)-2-hexenal
(195) 3-methylbutanol
(196) (Z)-3-hexenol
(197) (E)-phytol
(198) n-octacosanol
(199) methyl butanoate
(200) (Z)-3-hexenyl acetate
(201) 3-O-glycerolacetate
(202) limonene
(203) (E)-ocimene
(204) (1R,4R,5R)-5-isopropyl-2-methylcyclohex-2-ene-1,4-diol*
* indicates relative stereochemistry
(205) β-bourbonene
(206) β-caryophyllene
(207) caryophyllene oxide
(208) β-elemene
(209) α-cubenene
1.4 A brief introduction to the plants used in this study

The phytochemical investigation of four *Ficus* species, *sansibarica, bizanae, natalensis* and *glumosa* was carried out in this study and the isolated compounds tested for their bioactivity, focusing on their antibacterial activity. These plants were chosen since all were found in KwaZulu-Natal in South Africa and were easily accessible. Although *Ficus glumosa* and *Ficus natalensis* had been studied phytochemically previously, these studies did not seem comprehensive and hence these plants were reinvestigated.

1.5 Methodology used in this study

The fresh leaves, stem bark and fruits of *Ficus sansibarica, Ficus bizanae, F. natalensis* and *F. glumosa* were collected from the province of KwaZulu-Natal in South Africa, identified, and voucher specimens deposited in the herbarium. The different parts of the plants were air dried, ground and extracted sequentially with organic solvents of increasing polarity (hexane < dichloromethane < ethyl acetate < methanol). The crude extracts were fractionated and purified using chromatographic techniques with the aim of isolating pure phytoconstituents. The isolated pure phytoconstituents were subjected to different spectroscopic techniques, mainly nuclear magnetic resonance spectroscopy (1D and 2D NMR), mass spectrometry,
infrared (IR) and ultra violet (UV) spectroscopy to identify the structures of the isolated phytoconstituents. Once identified, the phytoconstituents were verified by comparison with NMR and mass spectral data in the literature.

Appropriate bioassays were carried out on both the crude and isolated phytoconstituents in an attempt to support the indigenous medicinal uses of the plants. In particular, antibacterial studies were carried out on the isolates as well as anti-ulcerogenic studies. The cost of healthcare in Africa is in many cases unaffordable and inaccessible, especially to the indigenous population living in the rural areas. Plants with antibacterial constituents and extracts which offer gastroprotective effect can be used as substitutes for expensive antibiotics and anti-ulcer drugs which in many cases are inaccessible to the rural population.

This thesis reports the phytochemical investigation of four South African species, *F. sansibarica*, *F. bizane*, *F. natalensis* and *F. glumosa* and the antibacterial and anti-ulcerogenic activity of the crude extracts and some isolates from the plants.

1.6 Hypothesis used in this study

Many species of the genus *Ficus* have long been used in African indigenous medicine for the treatment and management of many disease conditions. It is therefore hypothesised that these plants must therefore contain active extracts or phytoconstituents responsible for the biological activity which are either acting alone or in synergy.
1.7 Objectives

The research objectives were:

1. To extract and isolate the phytoconstituents from extracts of the various morphological parts of *F. sansibarica*, *F. bizanae*, *F. natalensis* and *F. glumosa*.

2. To identify and characterise the isolated phytoconstituents using 1D and 2D NMR, IR, UV and MS spectroscopic techniques.

3. To identify appropriate bioassays based on the isolated phytoconstituents present in the plants and to test the extracts and phytoconstituents for their biological activity in these assays and validate their ethnomedicinal uses.
### Table 1-1 Species of some African Ficus used in ethnomedicine and their biological activities

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<th>Plant Part</th>
<th>Ethnomedicinal Uses</th>
<th>Biological activity</th>
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<td>1.</td>
<td><em>F. arnottiana</em> Miq.</td>
<td>leaves, bark</td>
<td>demulcent (relief of burning sensations), inflammation, wounds and skin diseases, leprosy scabies, astringent (shrinkage of body tissues) aphrodisiac (sexual stimulantion), fertility.</td>
<td>anti-inflammatory, analgesic, hypoglycaemic, antioxidant, ulcer preventive</td>
<td>Amol and Rajsekhar 2011a, 2011b, Gregory et al., 2009; Mazumder et al., 2008; Singh 2006</td>
</tr>
<tr>
<td>2.</td>
<td><em>F. benghalensis</em> Linn</td>
<td>seed, fruit, latex, leaves, bark</td>
<td>diabetes, inflammation, ulcers (gastric problems), healing of wounds and sores, ringworm, leprosy, scabies, blood clots, diarrheoa, dysentery, gonorrhea, haemorrhoids (piles), leucorrhea (viginal complaints) aphrodisiac (sexual stimulantion), astringent (shrinkage of body tissues), antiseptic, toothache, obstinate hiccup</td>
<td>antidiabetic, anti-inflammatory, antitumor, antibacterial, anthelmintic (parasitic infections), anti diarrhoeal, antioxidant, antitherogenic (arteries protection), ameliorative effect (bearable), anti-stress</td>
<td>Bhalerao et al., 2015; Chaturvedi and Murthy 2013; Patil and Patil 2010; Nair and Chanda, 2007; Burrows and Burrows, 2003</td>
</tr>
<tr>
<td>3.</td>
<td><em>F. bizanae</em> Hutch. &amp; Burtt-Davy.</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>4.</td>
<td><em>F. carica</em> Linn</td>
<td>fruits, leaves, latex, root</td>
<td>tumours, anti-inflammatory, gastrointestinal, respiratory and cardiovascular disorders, diuretic, expectorant (mucus drainage from lungs), mild laxative</td>
<td>anticancer, hepatoprotective, hypoglycamic, anti tuberculosis, irritant potential (inflammations), antibacterial, anthelmintic (parasitic), antipyretic (fever), antifugal, anti-mutagenic (prevention of genes damage), oxidative stress, nematicidal, antispasmodic (suppresses muscle spasms) and antiplatelet (arterial circulation)</td>
<td>Mawa et al., 2013; Liu et al., 2011; Young-Soo and Cha, 2010; Gilania et al., 2008; Duke et al., 2002; Penelope, 1997</td>
</tr>
<tr>
<td>No</td>
<td>Plant species</td>
<td>Plant Part</td>
<td>Ethnomedicinal Uses</td>
<td>Biological activity</td>
<td>Reference(s)</td>
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<tr>
<td>5</td>
<td><em>F. cordata</em> Thunb.</td>
<td>Stem bark</td>
<td>tuberculosis, diarrhoea, filariasis (parasitic infection)</td>
<td>antimicrobial, hepatoprotective effect and antioxidant</td>
<td>Donfack et al., 2011; Kuete et al., 2008; Khare, 2007</td>
</tr>
<tr>
<td>6</td>
<td><em>F. craterostoma</em> Mildbr &amp; Burret</td>
<td>leaves</td>
<td>stomach pains</td>
<td>antibacterial, anti-inflammatory, antidiabetic and antioxidant</td>
<td>Olaokun et al., 2013; Madikizela et al., 2012; Bhat and Jacobs 1995</td>
</tr>
<tr>
<td>7</td>
<td><em>F. exasperata</em> Vahl.</td>
<td>leaves, bark, root, seed</td>
<td>hypertension, respiratory tract infections, ulcers, intestinal pains, stomach and chest pains, wounds, bleeding, gonorrhoea, uterine disorders, anti-abortifacient, rheumatism, arthritis, epilepsy, itching, colic, insecticide</td>
<td>antidiabetic (mellitus), hypoglycaemic and hypotensive, hepatoprotective, antiulcer, antioxidant, antimicrobial, antifungal, anticonvulsant, uterotonic and uterine stimulatory effect (fertility), oxytocin inhibiting effect (sexual reproduction), pesticidal</td>
<td>Enogieru et al., 2015; Sabiu et al., 2015; Taiwo and Igbenehu 2014; Bafor et al., 2013, 2011, 2010, 2009; Kazeem et al., 2013; Musa and Opadiran 2013; Lawal et al., 2012; Mbakwen-Aniebo et al., 2012; Adewole et al., 2011; Woode et al., 2011; Igoli et al., 2005; Mashana et al., 2000; Akah et al., 1998; Adjanahoun et al., 1991</td>
</tr>
<tr>
<td>8</td>
<td><em>F. glumosa</em> (Miq.) Delile</td>
<td>aerial parts</td>
<td>diabetes mellitus, hypertension, oedema, treating sores, upset stomach, menstrual pains, skin diseases, rheumatism, conjunctivitis, headache, as a mouthwash for toothache.</td>
<td>anticancer, antidiabetic, hypoglycaemic, anti-atherosclerotic (prevention of artery wall thickness), diuretic (control of body water), anti-diarrhoeal, hypolipidaemic (lipid-lowering), antioxidant, anti-diarrhoea</td>
<td>Niachapda et al., 2015; Onoja et al., 2014; Ntchapda et al., 2014, Olaokun et al., 2013, 2014; Nana et al., 2012; Madubunyi et al., 2012; Tanko et al., 2012; Burkhill, 1985</td>
</tr>
<tr>
<td>9</td>
<td><em>F. ingens</em> (Miq.) Miq.</td>
<td>aerial parts</td>
<td>anaemia, diuretic, diarrhoea, piles, laxative</td>
<td>antipROTOzoal, antinociceptive (inhibits pain sensation), anti-inflammatory, hepatoprotective effect, antibacterial, radical scavenging activity</td>
<td>Donia et al., 2013; Al-Musayeib et al., 2012; Chauke et al., 2012; Aliyu et al., 2008; Ibrahim et al., 2006; Burrows and Burrows, 2003; Watt and Breyer-Brandwijk, 1962</td>
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<tr>
<td>No</td>
<td>Plant species</td>
<td>Plant Part</td>
<td>Ethnomedicinal Uses</td>
<td>Biological activity</td>
<td>Reference (s)</td>
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<tr>
<td>10</td>
<td><em>F. lutea</em> Vahl</td>
<td>leaves, bark, fruits, root, wood</td>
<td>sores, boils, wound healing, rables and madness in livestock</td>
<td>antidiabetic and hypoglycaemic activity, antimicrobial, antioxidant</td>
<td>Olaokun et al., 2014; 2013; Poumale et al., 2011; Marwah et al., 2007; Chifundera, 1998</td>
</tr>
<tr>
<td>11</td>
<td><em>F. microcarpa</em> L.</td>
<td>leaves, aerial root and bark</td>
<td>fever, pain relief, reduce perspiration</td>
<td>cytotoxic, analgesic, anti-inflammatory, antioxidant, antibacterial, hyaluronidase inhibitor effect, permeability of fluids, antitussive (relieves cough), expectorant effect, anti-asthmatic</td>
<td>Shripad et al., 2012; Kiem et al., 2011; Ao et al., 2010, 2008; Liu et al., 2009; Chiang et al., 2005; Bich et al., 2004</td>
</tr>
<tr>
<td>12</td>
<td><em>F. natalensis</em> Hochst.</td>
<td>malaria, influenza, ulcers, whooping cough, dysentery, treating wounds, warts and septic ears (skin conditions), irregular and painful menstruations, to ease childbirth, induces lactation, arthritis, eye cataract, headache, as an antivenom</td>
<td>antidiabetic, antibacterial</td>
<td></td>
<td>Olaokun et al., 2013; Burrows and Burrows, 2003; Kamatenesi-Mugisha et al., 2007; Rabe and Staden, 1997; Hutchings et al., 1996</td>
</tr>
<tr>
<td>13</td>
<td><em>F. nervosa</em> Heyne ex Roth</td>
<td>bark, root</td>
<td>diabetes, ulcers, rheumatism</td>
<td>antimycobacterial, anti-ulcer, hepatoprotective effect</td>
<td>Devi et al., 2012; Shireesha et al., 2012; Chen et al., 2010; Kokate et al., 1991</td>
</tr>
<tr>
<td>14</td>
<td><em>F. ovata</em> Vahl.</td>
<td>stem bark, leaves, latex</td>
<td>Infectious and gastrointestinal diseases, ring worm, anti-poison</td>
<td>antimicrobial</td>
<td>Kuete et al., 2009; Burrows and Burrows 2003; Katende et al., 1995</td>
</tr>
<tr>
<td>No</td>
<td>Plant species</td>
<td>Plant Part</td>
<td>Ethnomedicinal Uses</td>
<td>Biological activity</td>
<td>Reference(s)</td>
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<tr>
<td>15.</td>
<td><em>F. palmata</em> Forsk.</td>
<td>all parts</td>
<td>anti-diabetic, anti-tumour, lung and bladder diseases, lipid lowering effect, ulcers, gastrointestinal, constipation, antifungal activities, warts</td>
<td>anti-diabetic, antiplasmodial, anti-ulcer, anti-coagulant, antioxidant, anti-calcinogenic, hepatoprotective (prevention of liver damage), nephroprotective (kidney protection), genoprotective, antilipoperoxidative (functional stability), antimicrobial</td>
<td>Alqasoumi et al., 2014a; Joshi et al., 2014; Iqbal et al., 2014; Singh et al., 2014; Al-Musayeib et al., 2012; Parmar and Kaushal, 1982</td>
</tr>
<tr>
<td>16.</td>
<td><em>F. polita</em> Vahl</td>
<td>fruit, leaves, bark, roots</td>
<td>malaria, microbial infections, abdominal pain, dyspepsia (indigestion)</td>
<td>antiviral (HIV-1, HIV-2), antimalarial, anti-inflammatory, antimicrobial</td>
<td>Kuete et al., 2011; Kubmarawa et al., 2007; Ayisi and Nyadedzor, 2003; Recio et al., 1995; Gbeassor et al., 1990</td>
</tr>
<tr>
<td>17.</td>
<td><em>F. racemosa</em> Linn</td>
<td>all parts</td>
<td>fever, ulcers, dysentery, asthma, urinary diseases, gleet (gonorrhoea infections), piles, leucorrhea (vaginal disorders), menorrhagia (heavy mensturation), skin diseases, antiseptic, vermicidal</td>
<td>cytotoxic effect (brine shrimp), radical scavenging activity, antioxidant, wound-healing, anti-inflammatory, antiulcer, antiinflammatory (relieves cough), anti-pyretic (fever), hypoglycaemic, hypolipidaemic, hepatoprotective, anti-diabetic, chemomodulatory effect (renal anticarcinogenesis) antidiarrhoeal, larvicidal, antihelmintic and antifilarial (parasitic infection), antifungal, antibacterial, analgesic</td>
<td>Rao et al., 2015, 2003, 2002; Mehta et al., 2012; Jain et al., 2013; Hamid et al., 2011; Shikharthi and Mittal, 2011; Ahmed and Urooj, 2010; Abdul-Rahuman et al., 2008; Khan and Sultana, 2005; Ratnasooriya et al., 2003; Mandal et al., 1999; Deraniyagala et al., 1998</td>
</tr>
<tr>
<td>18.</td>
<td><em>F. religiosa</em> Linn.</td>
<td>all parts</td>
<td>antiviral, antiprotozoal, inflammation, ulcers, fever, asthma, epilepsy, nervous disorders, unconsciousness, drug addiction, sexual disorders, skin diseases, antivenom</td>
<td>antiviral, anticancer, hypoglycemic, hypolipidaemic, antidiabetic, acetylcholinesterase inhibitor, antidiarrhoeal, anti-convulsant, wound-healing, proteolytic (protein breakdown), nootropic effect (mental function), immunomodulatory, parasympathetic</td>
<td>Gregory et al., 2013; Patil et al., 2011; Singh et al., 2011; Chandrasekar et al., 2010</td>
</tr>
<tr>
<td>No</td>
<td>Plant species</td>
<td>Plant Part</td>
<td>Ethnomedicinal Uses</td>
<td>Biological activity</td>
<td>Reference(s)</td>
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<tr>
<td>20.</td>
<td><em>F. sarmentosa</em> var. <em>henryi</em></td>
<td>Leaves, stem</td>
<td>Acesodyne (pains), detumescence (subsiding tension)</td>
<td>antifungal, insecticidal</td>
<td>Wang et al., 2011; 2010; Lu, 2002</td>
</tr>
<tr>
<td>21.</td>
<td><em>F. sur</em> Forssk</td>
<td>Fruit, leaves, stem bark, root bark, twigs</td>
<td><em>Note</em>: tuberculosis (pulmonary), influenza (flu), antifungal, diarrhoea, wound healing, skin diseases, gonorrhoea, sterility (men), infertility (women), leprosy, epilepsy, memory loss, circumcision, rickets (bones, emetic (vomiting))</td>
<td>antibacterial, antimalarial, antituberculous, anti-inflammatory, anti-cholinesterase, mutagenic and genotoxic, sedative effect, anticonvulsant; antioxidant</td>
<td>Madikizela et al., 2014; Ishola et al., 2013; Ramde-Tiendrebeogo et al., 2012; Nadembega et al., 2011; Muregi et al., 2007, 2003; Eldeen et al., 2005; Igoli et al., 2005; Watt and Breyer-Brandwijk, 1962; Hutchings et al, 1996;</td>
</tr>
<tr>
<td>22.</td>
<td><em>F. sycamorus</em> L</td>
<td>stem bark, fruits, leaves, latex</td>
<td>malaria, fever, tuberculosis, liver problems, diarrhoea, uterine disorders, to treat coughs, chest pains, skin disease, boils, scabies, ringworm, epilepsy, toothache, fatigue, hiccups</td>
<td>antiplasmodial, antifungal; antioxidative, sedative and anticonvulsant effects, sperm producing effect</td>
<td>Nadembega et al., 2011; El-Sayed et al., 2010; Igboke et al., 2009; Hassan et al., 2007; Njoroge and Bussmann, 2007; Burrows and Burrows 2003; Sanon et al., 2003</td>
</tr>
<tr>
<td>23.</td>
<td><em>F. thonningii</em> Blume</td>
<td>all parts</td>
<td>malaria, pneumonia and gingivitis (inflammation), urinary tract infections, liver disorders, cough (tuberculosis) and chest pains, emphysema, antiabortion, sore throat, bronchitis, jaundice, skin diseases, athlete’s foot, hip disorders</td>
<td>cytotoxicity, reno- and cardioprotective effects, antiprotocoal, anti-inflammatory, hypoglycaemic, antidiarrheal, toxicity (short-term), antifungal, analgesic, antihelminthic, antimicrobial and antioxidant</td>
<td>Fongang et al., 2015; Dangarembizvi et al., 2013; Lansky and Paavilainen 2011; Nadembega et al., 2011; Aniagu et al., 2008; Musabayane et al., 2007; Njoroge and Kibunga, 2007; Burrows and Burrows 2003.</td>
</tr>
<tr>
<td>No.</td>
<td>Name of compound</td>
<td>Molecular formular</td>
<td>Plant species isolated from</td>
<td>References</td>
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</tr>
<tr>
<td>1.</td>
<td>Lupeol</td>
<td>C₃₀H₄₉O</td>
<td><em>F. benghalensis</em>, <em>F. cordata</em>, <em>F. glumosa</em>, <em>F. nervosa</em>, <em>F. polita</em>, <em>F. religiosa</em></td>
<td>Bhalerao et al., 2015; Ragasa et al., 2013; Nana et al., 2012; Singh et al., 2011; Donfack et al., 2011; Kamga et al., 2010</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Lupeol acetate</td>
<td>C₃₀H₅₁O₂</td>
<td><em>F. carica</em>, <em>F. racemosa</em>, <em>F. thonningii</em></td>
<td>Fongang et al., 2015; Mawa et al., 2013; Jain et al., 2013;</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Lupenone</td>
<td>C₃₀H₴₉O</td>
<td><em>F. nervosa</em>, <em>F. religiosa</em></td>
<td>Ragasa et al., 2013; Singh et al., 2011.</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Lupeol hexanoate</td>
<td>C₃₀H₵₁O₂</td>
<td><em>F. thonningii</em></td>
<td>Fongang et al., 2015</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Betulinic acid</td>
<td>C₃₀H₴₉O₃</td>
<td><em>F. benghalensis</em>, <em>F. lutea</em>, <em>F. microcarpa</em>, <em>F. ovata</em>, <em>F. polita</em></td>
<td>Bhalerao et al., 2015; Poumale et al., 2011; Kamga et al., 2010; Kuete et al., 2009; Chiang et al., 2005</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>3β-Hydroxy-20-oxo-29(20→19)abeolupane</td>
<td>C₃₀H₄₉O₂</td>
<td><em>F. microcarpa</em></td>
<td>Chiang and Kuo, 2002</td>
<td></td>
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<tr>
<td>7.</td>
<td>29,30-Dinor-3β-acetoxy-18,19-dioxo-18,19-secolupane</td>
<td>C₃₀H₴₹O₄</td>
<td><em>F. microcarpa</em></td>
<td>Chiang and Kuo, 2002</td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>β-Amyrin</td>
<td>C₃₀H₴₉O</td>
<td><em>F. benghalensis</em>, <em>F. cordata</em>, <em>F. glumosa</em>, <em>F. lutea</em>, <em>F. nervosa</em>, <em>F. religiosa</em></td>
<td>Bhalerao et al., 2015; Ragasa et al., 2013; Nana et al., 2012; Poumale et al., 2011; Singh et al., 2011; Kuete et al., 2009; Chiang et al., 2005</td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td>β-Amyrin acetate</td>
<td>C₃₀H₴₹O₂</td>
<td><em>F. cordata</em>, <em>F. lutea</em>, <em>F. racemosa</em>, <em>F. sur</em>, <em>F. thonningii</em></td>
<td>Fongang et al., 2015; Jain et al., 2013; Donfack et al., 2011; Poumale et al., 2011; Feleke and Breheane, 2005</td>
<td></td>
</tr>
<tr>
<td>10.</td>
<td>Oleanolic acid</td>
<td>C₃₀H₴₉O₃</td>
<td><em>F. carica</em>, <em>F. microcarpa</em>, <em>F. nervosa</em>, <em>F. ovata</em></td>
<td>Mawa et al., 2013; Chen et al., 2010; Kuete et al., 2009; Chiang et al., 2005</td>
<td></td>
</tr>
<tr>
<td>11.</td>
<td>Methyl maslinate</td>
<td>C₃₀H₴₉O₄</td>
<td><em>F. carica</em></td>
<td>Mawa et al., 2013</td>
<td></td>
</tr>
<tr>
<td>12.</td>
<td>3β-Acetoxy-12,19-dioxo-13(18)-oleanene</td>
<td>C₃₀H₴₉O₄</td>
<td><em>F. microcarpa</em></td>
<td>Chiang et al., 2005</td>
<td></td>
</tr>
<tr>
<td>13.</td>
<td>3β-Acetoxy-11α-hydroxy-11(12→13)abeooleanan-12-al</td>
<td>C₃₀H₴₉O₄</td>
<td><em>F. microcarpa</em></td>
<td>Chiang and Kuo, 2002</td>
<td></td>
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<tr>
<td>14.</td>
<td>α-Amyrin</td>
<td>C₃₀H₴₉O</td>
<td><em>F. nervosa</em>, <em>F. religiosa</em></td>
<td>Singh et al., 2011; Chen et al., 2010</td>
<td></td>
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<tr>
<td>15.</td>
<td>α-Amyrin acetate</td>
<td>C₃₀H₴₹O₂</td>
<td><em>F. racemosa</em>, <em>F. sur</em></td>
<td>Jain et al., 2013; Feleke and Breheane 2005</td>
<td></td>
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<tr>
<td>16.</td>
<td>α-Amyrin octacosanoate</td>
<td>C₃₀H₴₉O₂</td>
<td><em>F. racemosa</em></td>
<td>Jain et al., 2013</td>
<td></td>
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<tr>
<td>17.</td>
<td>Ursolic acid</td>
<td>C₃₀H₴₉O₂</td>
<td><em>F. microcarpa</em></td>
<td>Kuete et al., 2011</td>
<td></td>
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<tr>
<td></td>
<td>Chemical Name</td>
<td>Molecular Formula</td>
<td>Source</td>
<td></td>
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<tr>
<td>18</td>
<td>Acetyltursolic acid</td>
<td>C₁₀H₁₆O₅</td>
<td><em>F. polita</em></td>
<td>Chiang et al., 2005</td>
<td></td>
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<tr>
<td>19</td>
<td>Uronic acid</td>
<td>C₁₀H₁₆O₃</td>
<td><em>F. microcarpa</em></td>
<td>Chiang et al., 2005</td>
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<tr>
<td>20</td>
<td>Taraxasterol</td>
<td>C₃₀H₄₆O</td>
<td><em>F. benghalensis</em></td>
<td>Patil and Patil, 2010</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>20(30)-Taraxastene-3β,21α-diol</td>
<td>C₃₀H₄₆O₂</td>
<td><em>F. microcarpa</em></td>
<td>Kuo and Chiang, 1999</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>20-Taraxasten-3β-ol</td>
<td>C₃₀H₄₆O</td>
<td><em>F. microcarpa</em></td>
<td>Kuo and Chiang, 1999</td>
<td></td>
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<tr>
<td>23</td>
<td>20-Taraxastene-3β,22β-diol</td>
<td>C₃₀H₄₆O₂</td>
<td><em>F. microcarpa</em></td>
<td>Kuo and Chiang, 1999</td>
<td></td>
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<tr>
<td>24</td>
<td>22-Oxo-20-taraxasten-3β-ol</td>
<td>C₃₀H₄₆O₂</td>
<td><em>F. microcarpa</em></td>
<td>Kuo and Chiang, 1999</td>
<td></td>
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<tr>
<td>25</td>
<td>3,22-Dioxo-20-taraxastene</td>
<td>C₃₀H₄₆O₂</td>
<td><em>F. microcarpa</em></td>
<td>Chiang et al., 2005</td>
<td></td>
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<tr>
<td>26</td>
<td>3β-Acetoxy-20-taraxasten-22-one</td>
<td>C₂₃H₃₆O₃</td>
<td><em>F. microcarpa</em></td>
<td>Kuo and Chiang, 1999</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>3β-Acetoxy-19(29)-taraxasten-20α-ol</td>
<td>C₃₀H₄₆O₂</td>
<td><em>F. microcarpa</em></td>
<td>Chiang et al., 2005</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>20α,21α-Epoxytaraxasten-3β-ol</td>
<td>C₃₀H₄₆O₂</td>
<td><em>F. microcarpa</em></td>
<td>Kuo and Chiang, 1999</td>
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**FLAVONOIDS**

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<td>139. 2,4,5-Trihydroxy benzoic acid</td>
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<td>140. Ferulic acid</td>
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<td>141. 3-O-Caffeoylquinic acid</td>
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<td>142. Methyl chlorogenate</td>
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<td>152. Vanillin</td>
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<td>161. Glumoamide</td>
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<td>Caryophyllene</td>
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plants used in Kisii, Kenya against malaria and their chloroquine potentiation effects. J. Ethnopharmacol. 84: 235-239.


Chapter 2. 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

A phytochemical investigation of *Ficus sansibarica* Warb. subsp. *sansibarica* (Moraceae) resulted in the isolation of three triterpenes and three flavonoids: lupeol acetate (1); cycloart-23-ene-3,25-diol (2); β-sitosterol (3); 5,7,4′-tri hydroxyflavan-3-ol (4); epicatechin (5); and isovitexin (6). The compounds were identified by NMR spectroscopy and by comparison of the NMR data against values reported in the literature. This is the first phytochemical investigation of *F. sansibarica*. The antimicrobial activity of the crude extracts and isolated compounds were weak in comparison to ampicillin and tetracycline. 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′-tri hydroxyflavan-3-ol (4) and isovitexin (6). 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.
2.1. Introduction

Primary healthcare in Africa is still inaccessible to the vast majority of its population due to inadequate transport systems to and from hospitals and clinics, the cost of drugs and the high rates charged for in-hospital treatment. An estimated 80% of the world’s population, 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). In most rural areas, traditional medicine is the only form of healthcare available. Research into the 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 wide variety of ailments and diseases or symptoms of these such as convulsive disorder, wound healing, gonorrhea, tuberculosis, diabetes, diarrhoeal infections, dysentery, malaria and HIV (Wakeel et al., 2004; Yadav et al., 2006; Parekh and Chanda, 2007; Annan and Houghton,
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, antiprotozoal, anticancer, cytotoxic, anti-ulcer, anti-inflammatory, anti-hyperglycemic, anti-diarrhoea, hepatoprotective, 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 ethnobotanical 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 knobbly 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. Insectivorous birds which feed upon the wasps that pollinate the figs also eat the fruits. 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.

2.2. 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.

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$^{11}$ 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 (2 mL 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 1 ml 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 Wetzlar 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$_{254}$ aluminium sheets were used for TLC. The TLC plates were analysed 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 h 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 recrystallized 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).
Figure 2-1 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).

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 2-1.

**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 deionized 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\(^{-1}\)). Plates were then incubated for 24 h 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) $\geq$ 15 mm, Intermediate (I) = 11 – 14 mm, and Resistant (R) $\leq$ 10 mm (Okoth et al., 2013). Criteria for assigning susceptibility or resistance to AMP10 was as follows: (S) $\geq$ 17 mm, (I) = 14 – 16 mm, (R) $\leq$ 13 mm, while those for TE30 were: (S) $\geq$ 19 mm, (I) 15 – 18 mm, (R) $\leq$ 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 microtitre 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 microtitre plate assays and volumes were standardised 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 microtitre 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. Microtitre plates were washed three times with sterile deionised 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-solubilisation 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\textsubscript{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 \textit{t}-tests (SigmaStat V3.5, Systat Software, Inc).

2.3. Results and discussion

The phytochemical investigation of the leaves, stem bark and fruits of \textit{F. sansibarica} yielded six compounds: three triterpenoids and three flavonoids. The non-polar hexane extract of both the leaves (1 and 2) and the 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 \textsuperscript{1}H and \textsuperscript{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 (Khan et al., 2006; Abdel-Monem et al., 2008; Escobedo-Martínez et al., 2012), 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 5,7,4′-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 C₃₀H₅₀O₂ 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 multiplets at δH 5.60. In our spectra, we were able to clearly define the *trans* nature of the Δ²³ 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 2-1.

The ¹H-NMR spectrum of compound 2 displayed two pairs of doublets with resonances at δH 0.55 (1H, d, J = 3.4 Hz, H-19a) and at 0.30 (1H, d, J = 3.4 Hz, H-19b), characteristic of cycloartane-type triterpenes. In addition, there were the presence of seven methyl groups at δ 0.82 (s, CH₃-29), 0.89 (d, J = 6.5 Hz, CH₃-21), 0.90 (s, CH₃-28), 0.99 (s, CH₃-30, CH₃-18), 1.36 (s, CH₃-26/27), the typical H-3 resonance for triterpenoids at δ 3.26, a doublet of doublet of doublets (ddd) at δH 5.71 (1H, J = 15.7, 8.5, 6.1 Hz, H-23), a doublet at δH 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 ¹³C NMR spectrum was able to be 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 $\delta_C$ 82.3 and an oxygenated
methine resonance at $\delta_C$ 78.8, indicating two hydroxy groups in the molecule.

The splitting pattern of the two olefinic protons, which appeared as a ddd at $\delta_H$ 5.71 ($J = 15.7, 8.5, 6.1$ Hz, H-23) and doublet at $\delta_H$ 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 $\Delta^{23}$ 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 $\delta_H$ 2.26 and
1.81, both multiplets was also present. The corresponding carbon resonances of C-23 and C-24
were identified at $\delta_C$ 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 $\delta_H$ 1.36 and in turn
the C-25 oxygenated singlet resonance at $\delta_C$ 82.30 through a HMBC correlation between $\delta_H$
1.36 and $\delta_C$ 82.30. The H-23 proton resonance also showed a HMBC correlation to C-25. The
doublet of 3H-21 at $\delta_H$ 0.89 showed HMBC correlations to C-17, C-22 and C-20 at $\delta_C$ 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).
**Table 2-1** \(^1\)H and \(^{13}\)C NMR data of cycloart-23-ene-3,25-diol (2) (600 MHz, CDCl\(_3\))

<table>
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<th>Pos.</th>
<th>(^1)H(^a)</th>
<th>(^1)H(^bc)</th>
<th>(^1)H (2)*</th>
<th>(^{13})C(^a)</th>
<th>(^{13})C(^b)</th>
<th>(^{13})C (2)*</th>
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<td>31.96</td>
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\(a\) Escobedo-Martinez et al. (2012); \(b\) Gherraf et al. (2010), \(\Delta^{23}\ cis\) isomer; \(c\) It is highly likely that the \(^1\)H NMR spectrum in Gherraf et al. (2010) is offset by approximately \(\delta 0.33\); coupling constants are given in parenthesis; \(^*\) \(^1\)H and \(^{13}\)C NMR data of this work for compound 2.

**Antimicrobial susceptibility testing**

The isolated compounds and extracts were subjected to antimicrobial screening and the results are presented in Table 2-2. 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 2-2*). 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 2-2*). However, the flavonoids, 5, 7, 4',-trihydroxyflavan-3-ol 4 and isovitexin 6 were not active against the bacteria being tested (*Table 2-2*). 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 (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'-trihydroxy-stilbene-3,5-O-β-D-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-25-diol (2) in this study.

**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 pathogenicity 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\(^{\prime}\)-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 (Figure 2-2) at all concentrations tested (2 – 15 mg/ml).
### Table 2-2 Antimicrobial susceptibility profile of *Staphylococcus aureus* ATCC 29213 following exposure to six compounds isolated from *Ficus sansibarica* and two crude methanol extracts

<table>
<thead>
<tr>
<th>Compound/Extract</th>
<th>Concentration mg mL&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>Zone diameter (mm)</th>
<th>Zone diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>40 µL&lt;sup&gt;*&lt;/sup&gt;</td>
<td>80 µL&lt;sup&gt;#&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lupeol acetate (1)</td>
<td>8</td>
<td>0</td>
<td>13.5 (I)</td>
</tr>
<tr>
<td>Cycloart-23-ene-3,25-diol (2)</td>
<td>8</td>
<td>0</td>
<td>11.5 (I)</td>
</tr>
<tr>
<td>Sitosterol (3)</td>
<td>8</td>
<td>0</td>
<td>13 (I)</td>
</tr>
<tr>
<td>5,7,4′-trihydroxyflavan-3-ol (4)</td>
<td>8</td>
<td>0</td>
<td>0 (R)</td>
</tr>
<tr>
<td>Epicatechin (5)</td>
<td>8</td>
<td>0</td>
<td>13.5 (I)</td>
</tr>
<tr>
<td>Isovitexin (6)</td>
<td>1.6</td>
<td>0</td>
<td>0 (R)</td>
</tr>
<tr>
<td>MeOH leaf extract</td>
<td>8</td>
<td>0</td>
<td>0 (R)</td>
</tr>
<tr>
<td>MeOH stem bark extract</td>
<td>8</td>
<td>0</td>
<td>9 (R)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>10</td>
<td>25 (S)</td>
<td>25 (S)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>30</td>
<td>28 (S)</td>
<td>28 (S)</td>
</tr>
<tr>
<td>Chloroform</td>
<td>0</td>
<td>0</td>
<td>0 (R)</td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td>0</td>
<td>0 (R)</td>
</tr>
</tbody>
</table>

<sup>*</sup> Tested using disk-diffusion assay;  
<sup>#</sup> Tested using agar-well diffusion assay;  
S = susceptible (≥ 15mm), I = intermediate susceptibility (11-14mm), and R = resistant (≤ 10mm) (CLSI, 2007).

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<sup>-1</sup>), with 5 mg mL<sup>-1</sup> being the most effective (Figure 2-3) 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 (Figure 2-3). 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).

5,7,4′,3′-Trihydroxyflavan-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 (Figure 2-4 and Figure 2-5). 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 (Figure 2-4). 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.

2.4. 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 5,7,4′-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.
Figure 2-2 Anti-adhesion activity of 1 – 15 mg mL\(^{-1}\) of lupeol acetate (1) on biofilm formation by *E. coli* and *S. aureus* strains:

![Chart showing anti-adhesion activity of lupeol acetate](chart.png)

![Figure 2-2](chart.png)

**Figure 2-2** Anti-adhesion activity of 1 – 15 mg mL\(^{-1}\) of lupeol acetate (1) on biofilm formation by *E. coli* and *S. aureus* strains:

Figure 2-3 Anti-adhesion activity of 1 – 7.5 mg mL\(^{-1}\) of epicatechin (5) on biofilm formation by *E. coli* and *S. aureus* strains:

![Chart showing anti-adhesion activity of epicatechin](chart.png)

![Figure 2-3](chart.png)
Figure 2-4  Anti-adhesion activity of 1 – 2.5 mg mL\(^{-1}\) of 5,7,4’-trihydroxyflavan-3-ol (4) on biofilm formation by \textit{E. coli} and \textit{S. aureus} strains

Figure 2-5  Anti-adhesion activity of 0.2 – 0.5 mg mL\(^{-1}\) of isovitexin (6) on biofilm formation by \textit{E. coli} and \textit{S. aureus} strains
Acknowledgements

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2.5. References


Chapter 3. Identification of secondary metabolites and resistance modifying activity of *Ficus bizanae* leaf, stem bark and fruit extracts

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Abstract

A phytochemical investigation of the fruit, leaves and stem bark extracts of *Ficus bizanae* resulted in the isolation of fourteen compounds, 7-methoxy coumarin (1), psoralen (2), tectoquinone (3), quercetin-3-O-β-D-glucopyranoside (4), vomifoliol (blumenol A) (5), (+)-dehydrovomifoliol (6), dihydroferulic acid (7), 4-methoxy catechol (8), protecatehuiic acid (9), taraxerol (10), 3β-acetoxy-9(11)-fernene (11), lupanyl acetate (12), β-amyrin acetate (13) and sitosterol (14). The resistance-modifying effect of the hexane, dichloromethane, ethyl acetate and methanol extracts of the leaves, stem bark and fruit against ceftriaxone, cefotaxime, nalidixic acid, sulphamethoxazole, gentamicin and azithromycin was assessed against six Gram-positive and Gram-negative strains using a modified disc diffusion method. Most extract combinations demonstrated either potentiation, additive or indifferent interaction effects, in a strain- and extract-specific manner, against the tested bacteria with some antagonistic effects. Potentiation of azithromycin activity was observed for all Gram-positive and Gram-negative strains with varying extract combinations. Potentiation of gentamicin activity was observed with all extract combinations for both *Pseudomonas aeruginosa* strains tested, and this was not observed with any of the other antimicrobial agents tested. Potentiation of sulphamethoxazole and nalidixic acid activity was effective against staphylococci, while potentiation of cephalosporin activity was observed for majority of strains tested. Based on preliminary data, *F. bizanae* extracts could serve as resistance modifying adjuvants to improve efficacy of conventionally used antimicrobial agents.

**Keywords:** *Ficus bizanae*, dihydroferulic acid, resistance-modifying activity.
3.1 Introduction

South Africa has a wealth of flora, thus a wide variety of plants are available to be used for medicinal purposes (Lall and Kishore, 2014). Although many of the plants have been investigated for their medicinal uses and phytochemistry, there are still many others that are yet to be investigated. Furthermore, the phytochemical investigation of these plants can provide a rationale for their use in ethnomedicine.

*Ficus bizanae* Hutch. & Burtt-Davy, a tree of up to 15-18 m in height belongs to the Moraceae (mulberry) family and is one of the plants endemic to South Africa (IUCN 2014). It is commonly known as Pondo Fig or Pondoland Fig. In South Africa, the Afrikaners know it as ‘Pondovy’ while the Xhosa call it ‘umthombe’. It is distributed in the Butterwort district of Eastern Cape, and in the Ngoye Forest in KwaZulu-Natal, South Africa. Thus far, there is no record of either ethnobotanical or economic uses for this species. *Ficus bizanae* is documented to be morphologically similar to *Ficus polita* (Burrows and Burrows, 2003). In West Africa, the fruits are consumed by the indigenous people and bark cloth can be made from its fig (Irvine, 1961).

Various classes of compounds including steroids, triterpenes, coumarins, flavonoids, alkaloids, phenolic acids, megastigmanes, pheophytins and pheophorbides have been isolated from diverse *Ficus* species (Singh et al., 2011; Mawa et al., 2013; Salem et al., 2013; Barolo et al., 2014; Bunawan et al., 2014). Furthermore, extracts of many *Ficus* species have a variety of bioactivities including antibacterial, anti-diarrheal, antioxidant, antimalarial, anticoagulant, hepato-protective, nephro-protective, anti-inflammatory, anti-hyperglycemic, anti-gastric ulcer, anti-viral and anti-HIV (Kengap et al., 2011; Singh et al., 2011; Mawa et al., 2013; Barolo et al., 2014; Bunawan et al., 2014 ).
The search for new antimicrobial agents has increased recently due to the increase in multi-drug resistance demonstrated by bacterial pathogens globally. Plants produce a variety of structurally diverse bioactive compounds to protect themselves against a range of microorganisms including plant pathogens and environmental organisms (phytoalexins). Plants carry out combinatorial chemistry by using the variety of precursors it has at its disposal, creating a multitude of organic compounds (Ncube et al., 2012). Plant-derived compounds, while abundant and diverse, demonstrate limited antimicrobial activity compared to antibiotics of bacterial or fungal origin. Additionally, these compounds are more effective against Gram-positive rather than Gram-negative bacteria. Since plant-derived antimicrobials are less potent, plants adopt a different paradigm, i.e., synergy to combat microbial infections. Synergism is a phenomenon in which two different compounds are combined to enhance their individual activity, while if the combination results in a reduced effect; it is called antagonism (Chung et al., 2011).

While several bioactive compounds have significant antimicrobial activity, others are synergistic enhancers of antimicrobial agents, despite not having any antimicrobial properties themselves (Abreu et al., 2012). Many plant extracts or compounds with no antibacterial activity of their own can enhance the activity of antimicrobial agents to which bacteria are developing resistance, thus re-sensitising them. In medicinal chemistry, the “herbal shotgun” or “synergistic multi-target effects” strategy involves the use of plant extracts and antimicrobial compounds in a multi-targeted approach.

Synergistic interactions between drugs, plants and/or natural extracts can enhance their efficacy and bioactivity against a target, when their effectiveness as single agents is reduced/limited. Additionally, synergy reduces toxicity, lowers the dose regimen, decreases adverse side-effects, and combats antimicrobial resistance (Cottarel and Wierzbowski, 2007). Potentiation of antimicrobial activity can occur through several mechanisms, i.e., synergistic activity between
two compounds (antibiotic + antibiotic; antibiotic + non-antibiotic) through serial or orthogonal inhibition of vital physiological pathways; inhibition of resistance enzymes that degrade or covalently modify an antimicrobial agent to a non-active form; compounds that block antimicrobial efflux or enhance uptake into the cell; and/or dispersal of a biofilm to planktonically growing cells, resulting in increased susceptibility to an antimicrobial agent (Kalan and Wright, 2011).

Positive interactions that intensify the potency of a bioactive product are called potentiation, while additive and synergistic effects are subsets of potentiation when it is experimentally characterized and quantified. Negative interactions occur when certain components of the extract inhibit full biological activity of pharmacologically-active compounds by reducing their stability or bioavailability or by enhancing their metabolism (Lila and Raskin, 2005). Synergy indicates that the compounds in the extract act via different mechanisms and/or targets (Lila and Raskin, 2005). Potentiation may also be initiated by combined bioactivity interactions resulting in safety, stability, improved solubility, absorption rate, enhanced active constituent bioavailability or resistance-modifying effects i.e. mechanisms of bacterial resistance being either modified or inhibited (Abreu et al., 2012; 2014).

There are many examples of synergistic effects between plant extracts and/or their phyto-constituents and antimicrobial agents documented in the literature (Hemaiswarya et al., 2008; Biavatti, 2009; Abreu et al., 2012). Combinations of mono or multi-extracts not only affect a single target but several targets simultaneously in an agonistic-synergistic way (Hemaiswarya et al., 2008; Wagner and Ulrich-Merzenich, 2009). Thus combinational therapy (synergism) of plant extracts or its phyto-constituents with antimicrobial agents is being investigated for the development of new resistance-modifying agents (RMA), which can improve the efficacy of currently used antimicrobial agents (Biavatti, 2009; Abreu et al., 2012).
We herein report the isolation, identification and synergistic potential of secondary metabolites isolated from *F. bizanae* leaf, stem bark and fruit extracts to enhance the activity of six commonly used antimicrobial agents against Gram-positive and Gram-negative bacteria.

### 3.2 Results and discussion

The phytochemical investigation of various parts of *F. bizanae* led to the isolation of compounds 1-14 (Figure 3-1) which were characterised by 1D (\(^1\)H and \(^{13}\)C) and 2D NMR, IR and mass spectrometry and confirmed by comparison with data in the literature as the coumarins, 7-methoxy coumarin (1) (Mejia-Barajas et al., 2012) and psoralen (2) (Alqasoumi et al., 2014), an anthraquinone, tectoquinone (3) (Cheng et al., 2008; Endale et al., 2013), a flavonoid glycoside (quercetin-3-O-\(\beta\)-D-glucopyranoside 4) (Liu et al., 2008), two megastigmane derivatives, vomifoliol (5) (Afifi et al., 2014) and dehydrovomifoliol (6) (Serra et al., 2007), a reduced cinnamic acid derivative, dihydroferulic acid (7) (Cho et al., 2013), the polyphenols, 4-methoxycatechol (8) (Saito and Kawabata, 2005; Bernet and Seifert, 2006) and protocatechuic acid (9) (Ramadan et al., 2009), the sterol derivatives, taraxerol (10) (Viswanadh et al., 2006), 3\(\beta\)-acetoxy-9(11)-fernene (11) (Kathirgamanarth et al., 2006), lupanyl acetate (12) (Mahato and Kundu, 1994; Jamal et al., 2008), \(\beta\)-amyrin acetate (13) (Mahato and Kundu, 1994; Fingolo et al., 2013) and sitosterol (14) (Patra et al., 2010). Compounds 4, 10-11, 14 were isolated from the leaf, 1-3, 5-8 and 12 from the stem bark and 9 and 13 from the fruit. The spectroscopic data for the isolated compounds is contained in the supplementary material.

To the best of our knowledge, this is the first report of compounds 3, 6-8 and 11 in the genus *Ficus*. Tectoquinone (3) was reported in *Cryptomeria japonica* (Cupressaceae) (Cheng et al., 2008), dihydroferulic acid (7) from *Suaeda japonica* (Chenopodiaceae) and *Bulbophyllum vaginatum* (Orchidaceae) (Leong et al., 1999; Cho et al., 2013) and 3\(\beta\)-acetoxy-9(11)-fernene
(11) from *Picris hieracioides* (Compositae) (Shiojima et al., 1989), *Leprotria atrotomentosa* (Stereocaulaceae) lichen powder (Kathirgamanathar et al., 2006) and *Polypodium subpetiolatum* (Polypodiaceae) (Anderson et al., 1979). DellaGreca et al. (2004) and Leu et al. (2012) reported the isolation of dehydrovomifoliol (6) from *Chenopodium album* (Chenopodiaceae) and *Vigna vexillata* (Fabaceae), respectively, while 4-methoxycatechol (8) was reported in *Wedelia trilobata* (Asteraceae) (Qiang et al., 2011). The other compounds have been isolated previously in the genus *Ficus* (Table 3-1).

**Table 3-1** Occurrence of compounds isolated from *F. bizanae* within the genus *Ficus*

<table>
<thead>
<tr>
<th>Name</th>
<th><em>Ficus</em> species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-methoxy coumarin (1)</td>
<td><em>Ficus ramentacea</em></td>
<td>Yarosh and Nikonov (1973)</td>
</tr>
<tr>
<td></td>
<td><em>Ficus radicans</em></td>
<td>Naressi et al. (2012)</td>
</tr>
<tr>
<td>Psoralen (2)</td>
<td><em>Ficus beecheyana</em></td>
<td>Lee et al. (2004),</td>
</tr>
<tr>
<td></td>
<td><em>Ficus formosana</em></td>
<td>Huang et al (2013), Marrelli et al.</td>
</tr>
<tr>
<td></td>
<td><em>Ficus carica</em></td>
<td>al. (2014), Alqasoumi et al.</td>
</tr>
<tr>
<td></td>
<td><em>Ficus palmata</em></td>
<td>(2014)</td>
</tr>
<tr>
<td>Quercetin-3-O-β-D-glucopyranoside (4)</td>
<td><em>Ficus auriculata</em></td>
<td>El-Fishawy et al. (2011)</td>
</tr>
<tr>
<td>Vomifoliol (5)</td>
<td><em>Ficus platypoda</em></td>
<td>Afifi et al. (2014)</td>
</tr>
<tr>
<td>Protocatechuic acid (9)</td>
<td><em>Ficus ovata</em></td>
<td>Kuete et al. (2009)</td>
</tr>
<tr>
<td></td>
<td><em>Ficus pandurata</em></td>
<td>Ramadan et al. (2009)</td>
</tr>
<tr>
<td></td>
<td><em>Ficus mucuso</em></td>
<td>Bankeu et al. (2010)</td>
</tr>
<tr>
<td>Taraxerol (10)</td>
<td><em>Ficus thunbergii</em></td>
<td>Kitajima et al. (1994)</td>
</tr>
<tr>
<td></td>
<td><em>Ficus foveolata</em></td>
<td>Somwong et al. (2013)</td>
</tr>
<tr>
<td></td>
<td><em>Ficus tsiangii</em></td>
<td>Wang et al. (2014)</td>
</tr>
<tr>
<td>Lupanyl acetate (12)</td>
<td><em>Ficus cordata</em></td>
<td>Pounale et al. (2008)</td>
</tr>
<tr>
<td></td>
<td><em>Ficus mucuso</em></td>
<td>Bankeu et al. (2010)</td>
</tr>
<tr>
<td></td>
<td><em>Ficus bengalensis</em></td>
<td>Riaz et al. (2012)</td>
</tr>
<tr>
<td>β-amyrin acetate (13)</td>
<td><em>Ficus sur</em></td>
<td>Feleke and Brehane (2005)</td>
</tr>
<tr>
<td></td>
<td><em>Ficus conraui</em></td>
<td>Kengap et al. (2011)</td>
</tr>
<tr>
<td>Sitosterol (14)</td>
<td><em>Ficus mucuso</em></td>
<td>Bankeu et al. (2010)</td>
</tr>
<tr>
<td></td>
<td><em>Ficus radicans</em></td>
<td>Naressi et al. (2012)</td>
</tr>
<tr>
<td></td>
<td><em>Ficus nervosa</em></td>
<td>Ragasa et al. (2013)</td>
</tr>
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<td></td>
<td><em>Ficus sansibarica</em></td>
<td>Awolola et al. (2014)</td>
</tr>
</tbody>
</table>
Figure 3-1  Structures of compounds (1-14) isolated from *Ficus bizanae*
Since the compounds isolated in this work were all known compounds, where the antimicrobial activity has been extensively studied, only the antimicrobial susceptibility testing of the plant extracts of *F. bizanae* were assessed. All 10 crude plant extracts (4 mg mL\(^{-1}\)) were ineffective against both *P. aeruginosa* strains (Table 3-2). With the exception of the HLX extract, the β-lactamase-producing *Escherichia coli* ATCC 35218 was resistant to all extracts tested. *E. coli* ATCC 25922 was similar, with resistance to all extracts except HSX (Table 3-2). The leaf extracts were not effective against both methicillin-sensitive *Staphylococcus aureus* ATCC 29213 (MSSA) and methicillin-resistant *S. aureus* ATCC 43300 (MRSA). However, both *S. aureus* strains demonstrated inhibition zone diameters ranging from 11-15 mm with stem bark and fruit extracts (Table 3-2), suggesting intermediate susceptibility. The HFX extract was the most potent of the crude extracts, demonstrating activity against both *S. aureus* and *E. coli* strains, even though the concentration tested was 1.84 mg mL\(^{-1}\), which was ~5.4 × less concentrated than other tested extracts. The low antimicrobial activity of *F. bizanae* extracts is not surprising since other studies have reported antimicrobial inhibition zones with 2-250 mg mL\(^{-1}\) of extracts from diverse *Ficus* species including *F. carica* (Olufemi and Olusegan, 2013), *F. exasperata* (Takon et al., 2013) and *F. pseudopalma* (De Las Llagas et al., 2014). Since the crude extracts are a mixture of active and non-active compounds, inhibition is observed at much higher concentrations compared to conventional antimicrobial agents (Silva et al., 2013).

The 14 isolated compounds were not tested for their antibacterial activity alone in this work. A literature survey indicated that 10 of the 14 compounds isolated were reported to have antibacterial activity on its own (Table 3-3).
Table 3-2 Zones of inhibition (mm) of crude extracts of *Ficus bizanae* against six bacterial strains

<table>
<thead>
<tr>
<th>Test Strains</th>
<th>HLX*</th>
<th>DLX*</th>
<th>ELX*</th>
<th>MLX*</th>
<th>HSX*</th>
<th>DSX*</th>
<th>ESX*</th>
<th>MSX*</th>
<th>HFX §</th>
<th>EFX §</th>
</tr>
</thead>
<tbody>
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<td><em>S. aureus</em> ATCC 29213</td>
<td>0(R)</td>
<td>0(R)</td>
<td>10(R)</td>
<td>10(R)</td>
<td>11(I)</td>
<td>11(I)</td>
<td>12(I)</td>
<td>12(I)</td>
<td>15(I)</td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em> ATCC 43300</td>
<td>0(R)</td>
<td>8(R)</td>
<td>10(R)</td>
<td>8(R)</td>
<td>8(R)</td>
<td>11(I)</td>
<td>13(I)</td>
<td>12(I)</td>
<td>11(I)</td>
<td>13(I)</td>
</tr>
<tr>
<td><em>E. coli</em> ATCC 25922</td>
<td>8(R)</td>
<td>0(R)</td>
<td>8(R)</td>
<td>8(R)</td>
<td>14(I)</td>
<td>8(R)</td>
<td>0(R)</td>
<td>0(R)</td>
<td>10(R)</td>
<td>8(R)</td>
</tr>
<tr>
<td><em>E. coli</em> ATCC 35218</td>
<td>11(I)</td>
<td>10(R)</td>
<td>0(R)</td>
<td>0(R)</td>
<td>10(R)</td>
<td>0(R)</td>
<td>0(R)</td>
<td>0(R)</td>
<td>0(R)</td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em> ATCC 27853</td>
<td>0(R)</td>
<td>0(R)</td>
<td>0(R)</td>
<td>0(R)</td>
<td>0(R)</td>
<td>0(R)</td>
<td>0(R)</td>
<td>0(R)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em> ATCC 35032</td>
<td>0(R)</td>
<td>0(R)</td>
<td>0(R)</td>
<td>0(R)</td>
<td>0(R)</td>
<td>0(R)</td>
<td>0(R)</td>
<td>0(R)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*HLX, DLX, ELX, MLX = hexane, dichloromethane, ethyl acetate and methanol leaf extracts, respectively.*

*HSX, DSX, ESX, MSX = hexane, dichloromethane, ethyl acetate and methanol stem bark extracts, respectively.*

*HFX, EFX = hexane and ethyl acetate fractions of the methanol extract of the fruit, respectively.*

Concentration of extracts = 4 mg mL\(^{-1}\); HFX concentration = 1.84 mg mL\(^{-1}\).

S = susceptible \((≥ 15 \text{ mm})\), I = intermediate susceptibility \((11 – 14 \text{ mm})\), and R = resistant \((≤ 10 \text{ mm})\).

For the two coumarins, 7-methoxy coumarin (1) and psoralen (2), MIC values of 9.76 µg mL\(^{-1}\) and 156.25 µg mL\(^{-1}\) were obtained against *Bacillus subtilis* and *Bacillus megaterium*, respectively (Kuete et al, 2007). Quercetin glycoside (4) inhibited the growth of different *Staphylococcus* strains with MICs ranging from 1.2-2.5 mg mL\(^{-1}\) (Abedini et al., 2013). Pretto et al. (2004) demonstrated MICs of 100-1000 µg mL\(^{-1}\) for the phenolic protocatechuic acid (9) against both Gram-negative and Gram-positive bacteria. The triterpenes also demonstrated good activity, with MICs of 0.12-1 mg mL\(^{-1}\) for lupanyl acetate (12) and β-amyrin acetate (13) against nine bacterial strains (Kiplimo et al., 2011). This study was also aimed at exploring extracts that could potentiate the antibacterial activity of commonly used antimicrobial agents, which could easily be practiced as a home remedy to supplement the effect of these drugs. The coumarins, flavonoids, triterpenes and phenolic acid compounds isolated from this plant have previously demonstrated varying antimicrobial potency (Cowan, 1999).
Table 3-3 Literature survey of antibacterial activity attributed to 10 of the 14 isolated compounds as reported in the literature.

<table>
<thead>
<tr>
<th>Name</th>
<th>Bacterial species active against</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-methoxy coumarin (1)</td>
<td><em>B. megaterium</em>, <em>B. subtilis</em>, <em>Escherichia coli</em>, <em>P. aeruginosa</em>, <em>S. aureus</em>, <em>Kuete</em> et al. (2007)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Souza</em> et al. (2005)</td>
<td></td>
</tr>
<tr>
<td>Psoralen (2)</td>
<td><em>B. cereus</em>, <em>B. megaterium</em>, <em>B. subtilis</em>, <em>Citrobacter freundii</em>, <em>Enterococcus cloacae</em>, <em>Shigella flexneri</em>, <em>Streptococcus faecalis</em></td>
<td><em>Kuete</em> et al. (2007)</td>
</tr>
<tr>
<td>Tectoquinone (3)</td>
<td><em>E. coli</em>, <em>P. aeruginosa</em>, <em>S. aureus</em>, <em>S. epidermidis</em></td>
<td><em>Verdan</em> et al. (2010)</td>
</tr>
<tr>
<td>Quercetin-3-O-β-D-glucopyranoside (4)</td>
<td><em>Enterococcus faecalis</em>, <em>P. aeruginosa</em>, <em>S. epidermidis</em>, <em>S. warneri</em>, <em>Stenotrophomonas maltophilia</em></td>
<td><em>Abedini</em> et al. (2013)</td>
</tr>
<tr>
<td>Dihydroferulic acid (7)</td>
<td><em>B. subtilis</em>, <em>S. aureus</em></td>
<td><em>Chou</em> et al. (2008)</td>
</tr>
<tr>
<td>Protocatechuic acid (9)</td>
<td><em>B. cereus</em>, <em>E. cloacae</em>, <em>E. coli</em>, <em>P. aeruginosa</em>, <em>Proteus mirabilis</em>, <em>Salmonella typhimurium</em>, <em>S. aureus</em>, <em>S. saprophyticus</em>, <em>Strep. agalactiae</em>, <em>Strep. faecalis</em></td>
<td><em>Kuete</em> et al. (2009)</td>
</tr>
<tr>
<td>Taraxerol (10)</td>
<td><em>E. faecalis</em>, <em>E. coli P. aeruginosa</em>, <em>S. aureus</em></td>
<td><em>Mokoka</em> et al. (2013)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Amir</em> et al. (2013)</td>
</tr>
<tr>
<td>Lupanyl acetate (12)</td>
<td><em>B. subtilis</em>, <em>E. faecium</em>, <em>E. coli</em>, <em>Klebsiella pneumoniae</em>, <em>P. aeruginosa</em>, <em>S. aureus</em>, <em>S. epidermidis</em>, <em>S. saprophyticus</em>, <em>Stenotrophomonas maltophilia</em></td>
<td><em>Kiplimo</em> et al. (2011)</td>
</tr>
<tr>
<td>β-amyrin acetate (13)</td>
<td><em>B. subtilis</em>, <em>E. faecium</em>, <em>E. coli</em>, <em>K. pneumoniae</em>, <em>P. aeruginosa</em>, <em>S. aureus</em>, <em>S. epidermidis</em>, <em>S. maltophilia</em></td>
<td><em>Kiplimo</em> et al. (2011)</td>
</tr>
<tr>
<td>β-sitosterol (14)</td>
<td><em>Bacillus subtilis</em>, <em>Corynebacterium diphtheriae</em>, <em>S. typhii</em>, <em>Shigella dysenteriae</em>, <em>S. aureus</em>, <em>Vibrio cholerae</em></td>
<td><em>Kiprono</em> et al. (2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Awolola</em> et al. (2014)</td>
</tr>
</tbody>
</table>

Potentiation, additive or negative interactions were identified according to criteria described by Abreu et al. (2014) using a modified disk diffusion method (DDM). The DDM method was selected as Abreu et al. (2014) obtained good agreement between the checkerboard, Etest, and DDM assays (77.5%), as well as 86.3% agreement between checkerboard and DDM, and 82.5% between Etest and DDM. In addition to DDM having a good correlation with the other methods, it is a low-cost, easy, and rapid way of determining synergism between an extract and an antimicrobial agent (Abreu et al., 2014). The criteria for potentiation, additive and negative
was calculated as follows: Potentiation \((IZD_{a+p} - IZD_a) \geq 6\ \text{mm}\), additive \(4 \leq (IZD_{a+p} - IZD_a) < 6\ \text{mm}\), negative \((IZD_a - IZD_{a+p}) \geq 6\ \text{mm}\), indifferent interactions are considered between the limits imposed for additive and negative interactions, where \(a =\) antibiotic and \(p =\) phytochemical or extract (Abreu et al., 2014).

The antimicrobial susceptibility of the tester strains to the selected six antimicrobial agents is shown in Table 3-4. The data for the combined synergistic effect of the ten \(F.\ bizanae\) extracts and antimicrobial agents (ceftriaxone (CRO) 30 \(\mu g\ \text{mL}^{-1}\); cefotaxime (CTX) 30 \(\mu g\ \text{mL}^{-1}\); nalidixic acid (NA): 30 \(\mu g\ \text{mL}^{-1}\); sulphamethoxazole (SXT): 25 \(\mu g\ \text{mL}^{-1}\); gentamicin (CN): 10 \(\mu g\ \text{mL}^{-1}\); azithromycin (AZM): 15 \(\mu g\ \text{mL}^{-1}\)) is shown in Table 3-4.

Most of the \(F.\ bizanae\) extracts demonstrated synergistic and/or antagonistic interactions with the antimicrobial agents in a strain- and extract-specific manner (Table 3-4). Extracts on their own had limited activity (Table 3-2) and tested strains were variable in their susceptibility to the six antimicrobial agents (Table 3-4), since susceptible and resistant strains were compared.

\textit{Staphylococcus}: Potentiation of CRO, AZM and SXT activity was observed with the fruit extract HFX for methicillin-susceptible \(S.\ aureus\) ATCC 29213 (MSSA), while HFX had an additive effect with CTX and NA (Table 3-4). No potentiation of CN and CTX activity was observed with any of the extracts (Table 3-5). Potentiation of NA and SXT activity was observed with three (HLX, MLX and HSX) and six (HLX, DLX, MLX, HSX, DSX and HFX) extracts, respectively (Table 3-5). Negative interactions were observed primarily for CN when targeted with 70% of extracts and NA with 50% of extracts (Table 3-5).
Table 3-4 Antimicrobial susceptibility of tester strains to antimicrobial agents and difference in inhibition zone diameter (IZD) of antimicrobial agents (AA) alone and antimicrobial agents combined with F. bizzanae extracts.

<table>
<thead>
<tr>
<th>Test Strains</th>
<th>Effect of the extract on the antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. aureus</strong></td>
<td></td>
</tr>
<tr>
<td>ATCC 29213</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>AA alone</td>
</tr>
<tr>
<td>S. aureus</td>
<td>ATCC 43300</td>
</tr>
<tr>
<td>NA30</td>
<td>24(8)</td>
</tr>
<tr>
<td>CN10</td>
<td>26(8)</td>
</tr>
<tr>
<td>SXT2.5</td>
<td>12(8)</td>
</tr>
<tr>
<td>NA30</td>
<td>26(8)</td>
</tr>
<tr>
<td>CN10</td>
<td>23(8)</td>
</tr>
<tr>
<td>S. aureus</td>
<td>ATCC 43300</td>
</tr>
<tr>
<td>NA30</td>
<td>14(8)</td>
</tr>
<tr>
<td>SXT2.5</td>
<td>10(8)</td>
</tr>
<tr>
<td>E. coli</td>
<td>ATCC 25922</td>
</tr>
<tr>
<td>NA30</td>
<td>15(8)</td>
</tr>
<tr>
<td>SXT2.5</td>
<td>11(8)</td>
</tr>
<tr>
<td>E. coli</td>
<td>ATCC 35218</td>
</tr>
<tr>
<td>NA30</td>
<td>17(8)</td>
</tr>
<tr>
<td>SXT2.5</td>
<td>9(8)</td>
</tr>
<tr>
<td>P. aeruginos</td>
<td>a ATCC 27853</td>
</tr>
<tr>
<td>NA30</td>
<td>19(8)</td>
</tr>
<tr>
<td>SXT2.5</td>
<td>12(8)</td>
</tr>
<tr>
<td>P. aeruginos</td>
<td>a ATCC 35032</td>
</tr>
<tr>
<td>NA30</td>
<td>18(8)</td>
</tr>
<tr>
<td>SXT2.5</td>
<td>10(8)</td>
</tr>
</tbody>
</table>

AA: Ceftriaxone (CRO30); cefotaxime (CTX30); nalidixic acid (NA30) - 30 μg/ml; sulphamethoxazole (SXT2.5) - 25 μg/ml; gentamicin (CN10) - 10 μg/ml; azithromycin (AZM15): 15 μg/ml.

R = resistant (≥ 10); Int – intermediate susceptibility (11-14); S = susceptible (≥ 15) (CLSI, 2007).

HLX, DLX, ELX, MLX: hexane, dichloromethane, ethyl acetate, methanol leaf extracts, respectively; HSX, DSX, ESX, MSX: hexane, dichloromethane, ethyl acetate, methanol stem bark extracts, respectively; HFX, EFX: hexane and ethyl acetate fruit extracts, respectively. Concentration of extracts = 4 mg mL⁻¹; HFX concentration = 1.84 mg mL⁻¹.

P = Potentiation (IZDₚ – IZDₚ) ≥ 6 mm, A = additive (blue) 4 ≤ (IZDₚ – IZDₚ) < 6 mm, N = negative (yellow) (IZDₚ – IZDₚ) ≥ 6 mm, I = indifferent (green) indifferent interactions are considered between the limits imposed for additive and negative interactions, a = antibiotic; p = phytochemical or extract (Abreu et al., 2014).
Potentiation of all antimicrobial agents was observed with as low as one (NA) or as high as eight (CTX) of the extracts when the methicillin-resistant *S. aureus* ATCC 43300 (MRSA) was targeted (Table 3-4). Potentiation of CTX activity was observed with 80% of extracts (HLX, DLX, ELX, MLX, HSX, DSX, HFX, and EFX). This suggests that *Ficus* extracts have potential as adjuvants for cephalosporin-based treatment of MRSA infections. Negative interactions were observed for CRO, NA and CN with 20%, 80% and 60% of extracts (Table 3-5).

*Escherichia:* Potentiation of CTX, NA and AZM activity was observed with all 10 extracts when susceptible *E. coli* ATCC 25922 was assessed (Table 3-4). Potentiation of CRO, SXT and CN was also observed with 80%, 30% and 70% of extracts, respectively (Table 3-5). No negative interactions were noted.

CRO activity potentiation was observed with all 10 extracts against the β-lactam-resistant *E. coli* ATCC 35218 (Table 3-4). CTX potentiation was observed with the fruit extracts HFX and EFX and additive interactions were observed with 40% of extracts (Table 3-5). These potentiation/additive interactions with the cell-wall inhibitor cephalosporins suggest that the *F. bizanae* fruit extracts might be suitable to counter β-lactamase-producing Enterobacteriaceae. No potentiation of NA, SXT, and CN was observed and negative interactions were observed with DLX and DSX extracts.
Table 3-5 Summary of interactions obtained when six conventional antimicrobial agents were combined with 10 crude *F. bizanae* leaf, stem bark and fruit extracts

<table>
<thead>
<tr>
<th>Strain</th>
<th>Potentiation</th>
<th>Additive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em> ATCC 29213</td>
<td>CRO with HFX</td>
<td>CRO with HFX</td>
<td>CTX with HFX</td>
</tr>
<tr>
<td></td>
<td>NA with HLX, MLX, HSX</td>
<td>CTX with ELX</td>
<td>NA with DLX, ELX, ESX, MSX, EFX</td>
</tr>
<tr>
<td></td>
<td>SXT with HLX, DLX, MLX, HSX, DSX, HFX</td>
<td>NA with HFX</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>CN with HLX, DLX, ELX, ESX, MSX, HFX, EFX</td>
</tr>
<tr>
<td></td>
<td>AZM with HFX</td>
<td>-</td>
<td>ELX, MLX, ESX, MSX</td>
</tr>
<tr>
<td><em>S. aureus</em> ATCC 43300</td>
<td>CRO with HSX, DSX, HFX, EFX</td>
<td>-</td>
<td>CRO with ELX, MLX</td>
</tr>
<tr>
<td></td>
<td>CTX with HLX, DLX, ELX, MLX, HSX, DSX, HFX, EFX</td>
<td>CTX with ESX, MSX</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NA with ELX</td>
<td>-</td>
<td>NA with HLX, DLX, MLX, DSX, ESX, MSX, HFX, EFX</td>
</tr>
<tr>
<td></td>
<td>SXT with HLX, DLX, MLX, DSX, HFX, EFX</td>
<td>-</td>
<td>CN with HLX, ELX, DSX, ESX, MSX, EFX</td>
</tr>
<tr>
<td></td>
<td>CN with DLX, MLX, HSX</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AZM with DLX, MLX, HSX, DSX, HFX</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> ATCC 25922</td>
<td>CRO with HLX, DLX, ELX, MLX, ESX, MSX, HFX, EFX</td>
<td>CRO with HSX</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CTX with all 10 crude extracts</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NA with all 10 crude extracts</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SXT with HLX, ELX, MLX</td>
<td>SXT with MSX</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CN with HLX, ELX, MLX, HSX, ESX, MSX, EFX</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AZM with all 10 crude extracts</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> ATCC 35218</td>
<td>CRO with all 10 crude extracts</td>
<td>CRO with ELX, MLX, ESX, MSX</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CTX with HFX, EFX</td>
<td>CTX with ELX, MLX, ESX, MSX</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>NA with DSX</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>SXT with DLX, DSX</td>
</tr>
<tr>
<td></td>
<td>AZM with MLX, ESX, EFX</td>
<td>AZM with MSX</td>
<td>CN with DLX, DSX, HFX</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> ATCC 27853</td>
<td>CRO with all 10 crude extracts</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CTX with HLX</td>
<td>CTX HSX, MSX, HFX, EFX</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NA with EFX</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CN with all 10 crude extracts</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AZM with HFX</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em> ATCC 35032</td>
<td>CRO with DLX, ELX, MLX, ESX</td>
<td>-</td>
<td>CRO with DLX, ELX, MLX, ESX</td>
</tr>
<tr>
<td></td>
<td>CN with all 10 crude extracts</td>
<td>-</td>
<td>CTX with ELX, MLX, ESX</td>
</tr>
<tr>
<td></td>
<td>AZM with HLX, MLX, HSX, DSX, EFX</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

*HLX, DLX, ELX, MLX; hexane, dichloromethane, ethyl acetate, and methanol leaf extracts, respectively; HSX, DSX, ESX, MSX: hexane, dichloromethane, ethyl acetate, and methanol stem bark extracts, respectively; and HFX, EFX: hexane and ethyl acetate fruit extracts, respectively.*

*Ceftriaxone (CRO30); cefotaxime (CTX30); nalidixic acid (NA30) - 30 µg/ml; sulphamethoxazole (SXT2.5) - 25 µg/ml; gentamicin (CN10) - 10 µg/ml; azithromycin (AZM15): 15 µg/ml.*
*Pseudomonas*: Potentiation of CRO and CN activity was observed with all 10 extracts for the non-mucoid *P. aeruginosa* ATCC 27853 (Table 3-4). CTX, NA and AZM activity was potentiated with 1 extract each (Table 3-5). No potentiation of SXT activity was observed. No negative interactions were observed. No potentiation of CRO, CTX, NA and SXT activity was observed for *P. aeruginosa* ATCC 35032. However, CN activity was potentiated with all 10 extracts (Table 3-4), while potentiation of AZM was observed for 50% of extracts (HLX, MLX, HSX, DSX and ESX).

Ncube et al. (2012) observed varying efficacies when they targeted diverse plant extract-antimicrobial agent combinations, with most extract combinations demonstrating either a synergistic, additive or indifferent interaction effect against the test bacteria with only a few exhibiting antagonistic effects. Since plant extracts contain multiple diverse chemical compounds, their combinational interaction effects are expected to be equally diverse. The observed variations in the activity of the extract-antimicrobial agent combinations against susceptible and resistant strains might be indicative of ultrastructural changes, in addition to the diverse structures and mechanisms of action of the active substance(s) in the crude extracts (Darwish and Aburjai, 2010).

Overall, CRO and CTX (cephalosporins: cell-wall inhibitors) potentiation was observed for both Gram-positive and Gram-negative bacteria (susceptible and resistant strains) tested with the exception of *P. aeruginosa* ATCC 35032. The *F. bizanae* extracts possibly allowed better penetration of the cephalosporin through the outer membrane of Gram-negative *E. coli* and *P. aeruginosa* ATCC 27853 to the cell wall, which is the target site for the cephalosporins (Darwish and Aburjai, 2010). In the resistant *E. coli* and *P. aeruginosa*, the extracts could also be acting against β-lactamase enzymes and/or efflux pumps. Potentiation
of NA (DNA gyrase inhibitor) activity with all 10 extracts was only effective for *E. coli* ATCC 25922. SXT (folic acid inhibitor) activity was potentiated primarily for the Gram-positive staphylococci tested and no potentiation was observed for resistant Gram-negative strains. It was interesting to note that CN (protein synthesis inhibitor) activity was potentiated with all 10 *F. bizanae* extracts for both *P. aeruginosa* strains.

Potentiation of AZM (protein synthesis inhibitor) was observed with varying extracts for all Gram-positive and Gram-negative strains examined. The potentiation of gentamicin and azithromycin activity suggests the presence of efflux-inhibitory plant-derived compounds or RMA. Aiyegoro et al. (2009) suggested that the methanolic leaf extract of *H. pedunculatum* could contain potential broad-spectrum, efflux pump inhibitors, since the synergistic effect was observed against both Gram-positive and Gram-negative organisms. This could hold for the *F. bizanae* extracts since potentiation of aminoglycoside, macrolide, cephalosporin and quinolone activity was observed for Gram-negative and Gram-positive susceptible and/or resistant strains.

Jeong et al. (2009) observed the synergistic effects of the *F. carica* leaf MeOH extract against oral bacteria in combination with ampicillin or gentamicin (4-8-fold reduction of MIC). Sharma et al. (2014) demonstrated the synergistic effect of the methanolic extract of *F. carica* against *E. coli* and *S. aureus* when combined with gentamicin and tetracycline, which they attributed to the presence of coumarins and triterpenoids. Documentation of the pharmacological activities of the phyto-constituents from the aqueous extract of ripe dried fruit of *F. carica* fig includes classes of compounds such as alkaloids, flavonoids, saponins, and terpenes, with the furanocoumarins like psoralen and bergapten, flavonoids like rutin,
quercetin, and luteolin, phenolic acids like ferulic acid, and also phytosterols like taraxasterol being reported by Lee and Cha (2010).

The MeOH extract in combination with oxacillin or ampicillin, when targeted against MRSA induced a $\geq$4-8-fold reduction in MIC, suggesting a synergistic effect (Lee and Cha, 2010). When Odunbaku et al. (2008), examined the synergistic interactions of the ethanol leaf extract of *F. exasperata* on *E. coli* and *S. albus*, they observed that the highest inhibitory activity was obtained by combining the crude plant extract with protein synthesis inhibitors (gentamicin, tetracycline, chloramphenicol and erythromycin), followed by the folic acid inhibitor combination, and then with cell wall inhibitors. These studies thus support the potentiation activity observed with *F. bizanae* extracts in combination with protein synthesis inhibitors (azithromycin and gentamicin), cell wall inhibitors (ceftriaxone and cefotaxime), DNA gyrase inhibitors (nalidixic acid), with the folic acid inhibitor (sulphamethoxazole) combination being the least effective. The coumarins, triterpenoids, flavonoids and polyphenolic compounds isolated from crude extracts have been documented previously to possess antimicrobial activity against different bacterial strains (Sato et al., 2004; Cushnie and Lamb, 2005; Nitiema et al., 2012; Mawa et al., 2013). 7-methoxy coumarin (1), quercetin-3-O-β-D-glucopyranoside (4), protocatechuic acid (9), lupanyl acetate (12) and β-amyrin acetate (13) isolated from this plant are likely to play a role in the synergistic effect experienced with these antimicrobial agents.

The findings mentioned above suggest the possible scientific rationale for *F. bizanae* to be used in ethnomedicine and the efficacy of the combination therapy of the extracts with antimicrobial agents as a source of alternative treatment of infections caused by Gram-negative microbes. Combination antimicrobial therapy might be the solution in targeting the
emergence of resistant bacterial strains, by reducing therapeutic dosages and increasing clinical efficacy, thus providing a broader spectrum of antimicrobial activity compared to monotherapy. The implications of this are that antibacterial drugs could be used in conjunction with some \textit{F. bizanae} extracts as a natural remedy, resulting in increased efficacy and probable reduction of side-effects associated with antimicrobial agents.

3.3 Experimental

General Experimental Procedures

Optical rotations were carried out on a Model 341 Polarimeter at room temperature. Melting points were determined on an Ernst Leitz Wetziar micro-hot stage melting point apparatus and are uncorrected. A Varian Cary UV-VIS Shimadzu spectrophotometer was used to obtain UV spectra and infrared spectral data was obtained on a Perkin Elmer Spectrum 100 (FT-IR) spectrometer. Mass spectra were obtained on a Shimadzu gas chromatograph mass spectrometer. NMR spectra were acquired using tetramethylsilane (TMS) as an internal standard on Bruker Avance III 400 and 600 MHz spectrometers. Silica gel 60 (Merck, 70-230 mesh) and Sephadex LH-20 (Pharmacia) were used as the stationery phase for column chromatography (CC). TLC analysis was carried out on Merck aluminium backed sheets precoated with silica gel 60 F$_{254}$ and visualised using 10% sulphuric acid in MeOH or under UV (254 and 366 nm).

Plant material

Fresh leaf, stem bark and fruit of \textit{Ficus bizanae} Hutch. & Burtt-Davy, were collected from Durban, KwaZulu-Natal, South Africa. A voucher specimen (G. V. Awolola & H. Baijnath 2) was deposited in the School of Life Sciences Ward herbarium of the University of KwaZulu-Natal.
**Extraction**

Powdered dried leaves (1.1 kg) and stem bark (2.5 kg) of *F. bizanae* were subjected to cold extraction using different organic solvents of increasing polarity: hexane (hex), dichloromethane (DCM), ethyl acetate (EtOAc), and methanol (MeOH) sequentially on an orbital shaker for 48 hours each. The extracts were evaporated using a rotary evaporator and yielded 26.86 g hexane, 18.21 g DCM, 10.59 g EtOAc and 45.56 g MeOH extract for the leaves and 9.26 g hexane, 20.43 g DCM, 6.39 g EtOAc and 45.39 g MeOH for the stem bark. The MeOH extracts of both the leaves and stem bark were dissolved in methanol and water (1:1) and partitioned with DCM and ethyl acetate to yield 4.59 g methanol derived DCM extract of the leaves, 3.28 g methanol derived DCM extract of the stem bark, 5.05 g methanol derived EtOAc extract of the leaves and 5.00 g methanol derived EtOAc extract of the stem bark.

The dried ground fruits (570 g) were macerated in methanol for 48 hours with continuous shaking on an orbital shaker at room temperature. The MeOH extract (85.45 g) was redissolved in methanol and water (1:1) and then partitioned with DCM and ethyl acetate to yield 3.23 g DCM and 8.24 g EtOAc extract.

**Isolation and Purification of compounds 4, 10, 11 and 14 from the leaves**

The hexane and DCM crude extracts of the leaves were combined as a result of similar TLC profiles. A 30.0 g sample of this combined extract was subjected to CC on a 5 cm column and eluted with a stepwise gradient of hexane and DCM (10%, 20%, 30%, 50%, 70%, 90% and 100% DCM in hexane) and then 20%, 50% and 100% EtOAc in DCM, collecting fractions of 100 mL for each step resulting in a total of 280 fractions. This was further combined into 6 fractions (A: fr. 71-78 (150 mg); B: fr. 79-83 (850 mg); C: fr. 84-110 (920mg); D: fr. 118-127 (1.00g); E: fr.128-141 (1.20g) and F: fr. 153-167 (2.5 g)).
Taraxerol (10) (40.1 mg) crystallized out of solution of fraction A as a white powder and was recrystallized in methanol. 3β-Acetoxy-9(11)-fernene (11) (10.7 mg) was purified with hex: EtOAc (19:1) from fraction B by CC (1 cm column) where 11 eluted in fractions 18-26. Fraction F was separated with hex: EtOAc (8:2) on a 2 cm column where sitosterol 14 (40.5 mg), was obtained as a white powder from fractions 7-17.

The methanol derived EtOAc fraction (5.05 g) partitioned from the MeOH extract of the leaves was fractionated on a 3 cm column with a hexane, EtOAc and MeOH gradient elution, increasing the polarity by 10% each time gradually to 100% MeOH. Fractions of 100 mL each were collected and similar fractions combined (based on TLC profiles) to afford four major fractions (A: 42-78; B: 79-117; C: 118-153 and D: 154-187). Quercetin-3- O-β-D-glucopyranoside 4 (90.8 mg) was obtained by purification on a 2 cm column using Sephadex LH-20 as the stationary phase and MeOH as the eluent and finally purified on a 1.5 cm column using DCM: MeOH (9:1) as the eluent to yield 4 in fractions 82-91.

**Isolation and Purification of compounds 1, 2, 3, 5, 6, 7, 8 and 12 from the stem bark**

The hexane and DCM crude extracts of the stem bark showed similar TLC profiles and were therefore combined. A 25.0 g sample of this combined extract was fractionated by CC with a gradient elution of hex: DCM and then DCM:EtOAc increasing the polarity by 10% and collecting 10×100 ml fractions for each step to afford six major fractions (A: 1-19; B: 20-23; C: 24-42; D: 43-52; E: 64-73; F: 74-89) containing similar compounds as indicated by TLC.

Fraction B (2.00g) was separated by CC on a 1.5 cm column using Hex:DCM (1:9) and collecting 20 mL fractions, which yielded 7-methoxy coumarin 1 (480.6 mg), a whitish powder in fractions 18-23. Fraction C was separated similarly but using Hex: EtOAc (9:1),
which yielded tectoquinone 3 (15.1 mg) as an orange powder in fraction 15-18. On increasing the polarity of the solvent to Hex:EtOAc (8:2), lupanyl acetate 12 (95.8 mg) was eluted in fractions 24-35 and was recrystallized from MeOH to obtain a white powder.

The EtOAc extract (6.00 g) of the stem bark was fractionated by CC on a 3cm column using hexane, EtOAc and finally MeOH as the eluent, increasing the polarity in a stepwise gradient by increments of 10% after each 1L of solvent and collecting 100 mL fractions. The fractions were combined according to TLC analysis as A: 16-36, B: 37-47, C: 48-60, and D: 61-79. Fraction B (950 mg) was subject to repeated CC with Sephadex LH-20 eluted with MeOH, and yielded dihydroferulic acid (7) (140.3 mg) as needle like crystals in fractions 55-85.

The methanol derived EtOAc fraction (5.00 g) partitioned from the MeOH extract of the stem bark was separated as above with hexane, EtOAc and then MeOH after which seven major fractions were obtained after fractionation (A: 8-10; B: 11-15; C: 16-18; D: 19-27; E: 36-50; F: 51-54; G: 57-80). Fraction B (40.6 mg) was purified by CC on a 1 cm column and eluted with DCM: hex (8:2) to yield psoralen 2 (20.4 mg) in fractions 11-15. Fraction F (280.2 mg) was purified using CC on a 2 cm column and eluted with hex: EtOAc (1:1) where (+)-dehydrovomifoliol 6 (10.9 mg) was obtained in fractions 4-11 and purified further on Sephadex LH-20 with MeOH as the mobile phase to yield 6 as colourless needles in fractions 27-36. Fraction G (1.12 g) was separated with EtOAc: Hex (8:2) using a 2.5 cm column where vomifoliol (blumenol A) 5 (10.3 mg) was eluted in fractions 15-22 and further purified using sephadex LH-20 (eluted with MeOH), to obtain colourless crystals in fractions 41-44. 4-Methoxy catechol 8 (2.1 mg) was obtained from fractions 3-5 after further purification by Sephadex LH-20 (eluted with MeOH) of fractions 25-32 of Fraction G.
Isolation and Purification of compounds 9 and 13 from the fruit

A 2.13 g sample of the methanol derived Hex fraction partitioned from the MeOH extract of the fruit was fractionated using CC using Hex:DCM and then DCM:EtOAc in a stepwise gradient elution by 10% increments after each 1L of solvent. A 2 cm column was used for the separation and 50 mL fractions were collected. The fractions were combined as follows, A: 13-17; B: 18-20; C: 23-77 and D: 78-120. β-Amyrin acetate 13 (20.0 mg), a white powder, precipitated out of fraction B and was recrystallized with MeOH. The methanol derived EtOAc fraction (8.00 g) partitioned from the MeOH extract of the fruit was fractionated in a similar manner and resulted in fractions A: 17-19; B: 20-39; C: 55-61; D: 62-85. Fraction C (1.00 g) was purified using Sephadex LH-20 (eluted with MeOH), which yielded an amorphous white powder, protocatechuic acid 9 (80.2 mg) in fractions 29-34.

Antimicrobial susceptibility testing

Four Gram-negative strains: E. coli ATCC 25922 and ATCC 35218 and P. aeruginosa ATCC 27853 and ATCC 35032, and two Gram-positive strains: S. aureus ATCC 29213 and ATCC 43300, were selected for the antimicrobial testing. Four F. bizanae leaf extracts, hexane (HLX), dichloromethane (DLX), ethyl acetate (ELX), and methanol (MLX), four F. bizanae stem bark extracts, hexane (HSX), dichloromethane (DSX), ethyl acetate (ESX), and methanol (MSX), as well as two F. bizanae fruit extracts, hexane (HFX) and ethyl acetate (EFX), were assessed for their antimicrobial potential.

Inhibition zone diameters (IZD) of the crude leaf, stem bark and fruit extracts and antimicrobial agents individually were determined using the disc diffusion assay (CLSI, 2007). Extracts were solubilised in DCM or DMSO to a concentration of 100 mg mL\(^{-1}\). Sterile, 6 mm disks (MAST, UK), impregnated with 4 mg mL\(^{-1}\) of each respective extract,
were placed on Mueller-Hinton (MH) agar plates swabbed with the respective microbial strains. Discs impregnated with DCM or DMSO were used as negative controls. Plates were incubated at 37 °C for 24 h. After incubation, IZD (in mm) of extracts were measured and their means determined.

**Synergistic effect determination**

The resistance modifying effects of *F. bizanae* extracts with antimicrobial agents were assessed using the modified DDM described by Abreu et al. (2014), with some further modifications. Commercially available, 6 mm antimicrobial agent discs (Oxoid, UK) [ceftriaxone (CRO30; 30 µg mL\(^{-1}\)), cefotaxime (CTX30; 30 µg mL\(^{-1}\)), nalidixic acid (NA30; 30 µg mL\(^{-1}\)), sulphamethoxazole (SXT25; 25 µg mL\(^{-1}\)), gentamicin (CN10; 10 µg mL\(^{-1}\)) and azithromycin (AZM15; 15 µg mL\(^{-1}\))] were impregnated with 40 µL (4 mg mL\(^{-1}\)) of respective crude extracts and placed on MH agar plates seeded with the respective bacteria. Discs of the six antimicrobial agents without extracts were used as positive controls, while discs impregnated with DCM and DMSO were used as negative controls. Following incubation at 37 °C for 24 h, IZD (in mm) were recorded and interpreted according to criteria (Table 3-6) described by Abreu et al. (2014). There are four possible effects when a combination of antibacterial products is used (Abreu et al., 2012): potentiation (when a combination of the antibacterial agents exceeds the sum of the effects of the individual products); additive (when a combination of antibacterial agents is equal to that of the sum of the effects of the individual products); negative (when the combination of antibacterial products promotes a reduced effect compared to the effect of the most efficient individual product), and indifference (when the combination of antibacterial products promotes equal effects to those of the most active product).
Table 3-6 Classification of interactions obtained when plant extracts were combined with antimicrobial agents (Abreu et al., 2014)

<table>
<thead>
<tr>
<th>Interaction</th>
<th>Disc diffusion method</th>
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<tr>
<td>Potentiation</td>
<td>$(IZD_{a+p} - IZD_{a}) \geq 6$ mm</td>
</tr>
<tr>
<td>Additive</td>
<td>$4 \leq (IZD_{a+p} - IZD_{a}) &lt; 6$ mm</td>
</tr>
<tr>
<td>Negative</td>
<td>$(IZD_{a} - IZD_{a+p}) \geq 6$ mm</td>
</tr>
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*a* = antibiotics, *p* = plant extract/phytochemical. Indifferent interactions are considered between the limits proposed for additive and negative interactions.

### 3.4 Conclusions

Fourteen compounds were isolated from the various parts of *F. bizanae*. Weak to moderate antimicrobial activity was observed against the test strains with crude extracts alone. Potentiation was observed when antimicrobial agents were combined with *F. bizanae* extracts in an extract- and strain-specific manner. While extracts potentiated activity of sulphamethoxazole (SXT) against staphylococci, potentiation of ceftriaxone (CRO) was effective against both *E. coli* strains and (gentamicin) CN activity was potentiated against *P. aeruginosa* strains. The results suggest the potential application of *F. bizanae* in ethnomedicine as resistance-modifying agents for the treatment of bacterial infections. Further studies are required to determine the mechanisms of action of this combinational therapy, as well as the exact dosages regimen that would be effective for each antimicrobial agent-extract combination.

### Acknowledgements

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3.5 References


Chapter 4. *Ficus natalensis*: Anti-adhesion potential of non-polar compounds and extracts

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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 4 and a quinone, tectoquinone 5, have been isolated from the leaf, stem bark and fruit extracts of *Ficus natalensis*, a medicinal fig found typically in South Africa. The pure compounds 1-3 and 5 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. For compounds 2, 3 and 5, broad spectrum antibiotic effects were shown with as much as ten of the twelve bacterial strains. Compound 3, 3α-hydroxy-21α-H-hop-22(29)-ene was active against all Gram-positive test organisms. The crude extracts showed no appreciable antimicrobial activity and only the DCM fruit extract was active against sensitive and resistant *Staphylococcus aureus* strains, *Enterococcus faecium* and *Staphylococcus xylosus*. In the anti-biofilm assay, exposure to EtOAc, MeOH and aqueous MeOH 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 MeOH leaf extract demonstrated the most potent anti-adhesion potential against *E. coli* (218.11% biofilm reduction). The greatest ability to decrease adhesion was observed with stigma-4-ene-3-one 2, 3α-hydroxy-21α-H-hop-22(29)-ene 3 and tectoquinone 5 against *P. aeruginosa* at the lowest concentration tested (100 µg mL⁻¹).

**Key words:** *Ficus natalensis*, fig, bacterial adhesion, biofilm reduction
4.1 Introduction

Bacterial adhesion to tissues is one of the initial stages in bacterial infections (Ofek et al., 2003). When these bacteria colonise and adhere to surfaces, they become more resistant to antibiotics. Further to this, colonisation and adherence make it easier for these microbes to gain access to nutrients needed for their survival (Ofek and Doyle, 1994). As such, pharmaceuticals or pharmaceutical extracts capable of preventing the adherence of bacteria to surfaces are desirable antibacterial agents. One source of these are medicinal plants used in traditional medicine, since many plant based remedies are known to have antibacterial properties (Saranraj and Sivasakthi, 2014; Hemalatha et al., 2013; Cowan, 1999; Rabe and van Staden, 1997) and some have even been identified as having anti-adhesion properties (Bräunlich et al., 2013; Chusri et al., 2012; Palombo, 2011).

*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, the Democratic Republic of Congo, Senegal, Kenya, Angola and Sudan and is well known as the Natal-fig or bark-cloth fig, and also known as natalvy, natou, umthombe, umdende and uluzi in South Africa (Burrows and Burrows, 2003).

The different parts of the tree have various applications varying from cultural, decorative, and commercial applications to folk medicine (Wanyama et al., 2011; Burrows and Burrows, 2003). The bark is a major source of bark cloth, as the name implies and a potential source of natural dye (Rwawiire et al., 2013; Wanyama et al., 2011; Burrows and Burrows, 2003). The root, bark, latex and leaves are used in several African countries such as Senegal, Uganda, Tanzania, Sierra Leone and South Africa to treat a variety of ailments such as lumbago, arthritis, headaches, toothaches, cataracts, for irregular and painful menstruations, to induce
labour, to ease the pain during childbirth, for retained placenta, and as a galactagogue, as an antivenom, to treat malaria, influenza, whooping cough, dysentery, and guinea worm, for ulcers, wounds, warts and septic ears and for colic and hiccups (Kamatenesi-Mugisha et al., 2007; Burrows and Burrows, 2003; Hutchings et al., 1996).

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. In a previous investigation on essential oils from *F. natalensis*, *(E)-phytol and 6,10,14-trimethyl-2-pentadecanone were found in the leaves (Sonibare et al., 2009). In our on-going phytochemical and antibacterial research on species belonging to the genus *Ficus* used in African traditional medicine, we have carried out a phytochemical investigation of the leaves, stem bark and fruit of *F. natalensis* and tested both the extracts and pure isolates for their antibacterial and anti-adhesion activity against several Gram-positive and Gram-negative bacteria.

4.2 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 (Figure 4-1): Ergosta-4,6,8(14),22-tetraen-3-one 1 (Fangkrathok et al., 2013), stigma-4-en-3-one 2 (Liu et al., 2014), 3α-hydroxy-21α-H-hop-22(29)-ene 3 (Sousa et al., 2012), sitosterol 4 (Rasoanaivo et al., 2014) and tectoquinone 5 (Cheng et al., 2008) using ¹H and ¹³C and 2D (COSY, NOESY, HMBC and HSQC) NMR spectroscopy and confirmed by comparison with values from the literature.
The $^1$H and $^{13}$C NMR spectrum of compound 3 for example revealed the presence of an oxygenated methine proton H-3 at $\delta_H$ 3.18 (dd, $J = 7.6, 3.2$ Hz), two protons at C-29 (m, $\delta_H$ 4.67-4.64), an isopropenyl methyl group at $\delta_H$ 1.64 (3H, s) and six tertiary methyls between $\delta_H$ 0.67-0.96 with corresponding methyl carbon resonances at $\delta_C$ 27.5 (C-23), 215.3 (C-24), 15.9 (C-25), 16.7 (C-26), 16.6 (C-27) and 15.1 (C-28). HMBC correlations between the methyl group proton resonances of H-23 and H-24 allowed these methyl resonances to be assigned two bonds away from C-3. These assignments compare well with that reported in literature (Kiem et al., 2004, Sousa et al., 2012). The coupling constant for H-3 double doublet ($J = 7.64$ Hz and 3.16 Hz) at C-3 for H-3 indicated that the stereochemistry of the hydroxy group at position 3 must be in the $\beta$ position. Based on the NMR data and comparison with literature (Kiem et al., 2004, Sousa et al., 2012), compound 3 was confirmed as $3\beta$-hydroxy-$21\alpha$-H-hop-22(29)-ene 3.

To the best of our knowledge, this is the first report of secondary metabolites from *F. natalensis*. 
Antibacterial activity

The crude hexane, dichloromethane, ethyl acetate and methanol extracts of the leaves, stem bark and fruits of *F. natalensis* were tested along with compounds 1-3 and 5 against both Gram-negative and Gram-positive bacterial strains to determine whether or not the crude extracts and the pure compounds have any antibacterial activity to substantiate the reported use of the plant as an antibacterial agent (Rabe and van Staden, 1997). Sitosterol 4 has previously been tested for its antibacterial properties, and was tested in our laboratory.
previously and was not tested again in these assays (Kiprono et al., 2000; Awolola et al., 2014).

In general, the leaf, stem bark and fruit extracts were relatively ineffective against all Gram-negative test organisms at 2 and 4 mg mL\(^{-1}\) (Table 4-1). Several of the extracts had strain-specific activity at an increased dosage of 4.0 mg; *S. sciuri* was susceptible to the aq/MeOH extract of the leaves, *Enterococcus faecalis* (ATCC 29212) was susceptible to both the EtOAc and MeOH extracts of the stem bark and hexane and DCM extracts of the fruit. Both *S. aureus* strains and *S. xylosus* were susceptible to the DCM extract of the fruit. This extract also showed intermediate activity against *S. sciuri*. The DCM extract of the fruit was thus the most active of all the extracts being active against several of the Gram-positive strains. This is an important point to note as incorporating these fruits into the diet could help fight against these pathogens.

The selective activity toward Gram-positive bacteria and poor activity against Gram-negative bacteria was consistent with a study done by Suffredini et al. (2006) on 1220 plant extracts from the Amazon and Atlantic rain forests. In their study, only selected extracts showed activity against Gram-positive strains while none of the extracts showed any significant activity against the Gram-negative bacteria. The poor activity against Gram-negative bacteria is not surprising since they are very resistant to antimicrobial compounds as a result of the outer membrane which prevents penetration of antimicrobial compounds (Nikaido, 2001). Most phytochemicals are effective against Gram-positive bacteria, however, small molecular weight phytochemicals with high activity against Gram-negative species are limited (Lewis, 2013). Rabe and van Staden (1997) have previously observed MICs of 4 mg mL\(^{-1}\) for *S. aureus* and *S. epidermidis* and 8 mg mL\(^{-1}\) for *B. subtilis*, using methanolic root extracts of *F. natalensis*. This is in keeping with the inhibition zone results obtained using crude extracts in this study. Varying antimicrobial activity has been reported for other *Ficus* species extracts,
with differences related to the *Ficus* species tested, part of the plant sampled, the extraction solvent and antimicrobial testing assay (Salem et al., 2013). Other *Ficus* species showing antibacterial activity were *F. exasperata* and *F. racemosa*; the *F. exasperata* methanol extract of the root showed antibacterial activity against *S. aureus* and *P. aeruginosa* and antifungal activity against *Candida glabrata* (Adebayo et al., 2009; Lawal et al., 2012), and the *F. racemosa* methanolic fruit extract showed a broad spectrum of activity against *S. aureus, B. subtilis, P. aeruginosa* and *Klebsiella* species (Hossain et al., 2014).

Varying antibacterial activity was demonstrated by the compounds tested which ranged from resistant to susceptible against both the Gram-negative and Gram-positive organisms (Table 4-1). The antibacterial activity was dose-dependent, being more active at 250 µg than at 100 µg. Both sensitive and resistant Gram-negative bacteria demonstrated varying levels of susceptibility to compounds 1-3 and 5 at 250 µg (Table 4-1). Compounds 1-3 and 5 showed good activity against β-lactam resistant *E. coli* ATCC 25922 and the extended spectrum β-lactamase producing *K. pneumoniae* ATCC 700603 (being susceptible to all of 1-3 and 5) but was only moderately active against *P. aeruginosa* (both strains being susceptible to only 1 and showing strain specific susceptibility to 2, 3 and 5). For the Gram-positive bacteria, the best activity was seen against all four staphylococci tested, particularly the MRSA strain (ATCC 43300) by all four compounds following 250 µg exposure (Table 4-1). *Bacillus subtilis* and *E. faecalis* strains demonstrated varying levels of susceptibility to all four compounds. All the tested compounds, 1-3 and 5, showed broad spectrum antibiotic effects where as much as ten of the twelve bacterial strains were susceptible to them. Tectoquinone 5 showed high zones of inhibition of 25 mm and 23 mm against *S. sciuri* and *K. pneumoniae* respectively. Stigma-4-ene-3-one 2 at 250 µg also demonstrated a high zone of inhibition.
against *S. xylosus* of 23 mm. The worst activity was shown against the Gram-negative *P. aeruginosa* and Gram-positive *E. faecalis* with the lowest recorded zones of inhibition.

Weak to strong activity has been previously reported in the literature for the isolated compounds. Ergosta-4,6,8(14),22-tetraen-3-one 1, previously isolated from a *Colletotrichum* culture has inhibited the growth of *S. aureus*, *P. aeruginosa* and *B. subtilis* at MICs between 25-75 µg mL\(^{-1}\) (Lu et al., 2000). Stigmast-4-en-3-one 2 isolated from *Pycnarrhena manillensis* showed comparable activity to chloramphenicol against *P. aeruginosa*, with antibacterial activity being demonstrated against *E. coli* and *S. aureus* (Ragasa et al., 2009). No antibacterial activity for 3β-hydroxy-21β-H-hop-22(29)-ene 3 could be found in the literature, however a similar hopane derivative, 22,29-epoxy-30-norhopane-13-ol, isolated from *Adiantum lunulatum* showed moderate activity against Gram-negative *Salmonella typhi* (Reddy et al., 2001). This is indicative that Gram-negative bacteria must be susceptible to this type of skeleton, which is supported by our study since the Gram-negative *E. coli*, *P. aeruginosa* and *K. pneumonia* were also susceptible to 3. Similarly, antibacterial activity for tectoquinone (2-methylantracene-9,10-dione) 5 could not be found in the literature, but 1,3,8-trihydroxy-6-methylantracene-9,10-dione, a similar anthraquinone isolated from *Cassia alata* was active against *S. aureus* and methicillin-resistant *S. aureus* (MRSA) with MIC values ranging from 4-16 µg mL\(^{-1}\) (Promgool et al., 2014).
Table 4-1 Antibacterial activity of isolated compounds and extracts (zones of inhibition in mm) from Ficus natalensis

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<th>Compounds&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>Ampicillin (AMP10)</td>
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<tr>
<td>Tetracycline (TE20)</td>
<td>28&lt;sup&gt;c&lt;/sup&gt;</td>
<td>23&lt;sup&gt;c&lt;/sup&gt;</td>
<td>15&lt;sup&gt;c&lt;/sup&gt;</td>
<td>nt&lt;sup&gt;c&lt;/sup&gt;</td>
<td>nt&lt;sup&gt;c&lt;/sup&gt;</td>
<td>28&lt;sup&gt;c&lt;/sup&gt;</td>
<td>36&lt;sup&gt;c&lt;/sup&gt;</td>
<td>25&lt;sup&gt;c&lt;/sup&gt;</td>
<td>26&lt;sup&gt;c&lt;/sup&gt;</td>
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</table>

<sup>a</sup>Crude extracts – 100 mg mL<sup>-1</sup> – 20 µl = 2 mg; 40 µl = 4 mg.

<sup>b</sup>Purified compounds 1 - 4: ergost-4, 6, 8(14), 22-tetra-3-one 1, stigmast-4-en-3-one 2, 3α-hydroxy-21α-H hop-22(29)-ene 3 and tectoquinone 4) – 10 mg mL<sup>-1</sup> stock – 10 µl = 100 µg; 25 µl = 250 µg.

<sup>c</sup>nt = not tested; <sup>R</sup>R = resistant (<10 mm); I = intermediate susceptibility (11-14 mm); S = susceptibility (≥ 15 mm); Negative control not used. AMP10 and TE20 refer to 10 and 20 µg discs.
Anti-adhesion activity

Biofilms in body tissues can be formed by bacteria such as *E. coli*, *P. aeruginosa*, *Haemophilus influenzae* and *S. aureus* (D’Abrosca et al., 2013). Due to this growing research area and in our continuing efforts to identify antimicrobial agents that can prevent or treat infections caused by biofilm-forming bacteria, the anti-adhesion potential of crude extracts (10 mg mL\(^{-1}\)) and isolated compounds (0.1, 0.25 and 0.5 mg mL\(^{-1}\)) from *F. natalensis* were evaluated against three bacterial strains, *E. coli* ATCC 35218, *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 43300.

Percentage biofilm reduction was calculated as follows:

\[
\text{Percentage reduction} = \left[ \frac{(C-B)-(T-B)}{C-B} \right] \times 100,
\]

where B denotes the average absorbance per well for blank wells (no biofilm, no treatment), C denotes the average absorbance per well for control wells (biofilm, no treatment), and T denotes the average absorbance per well for treated wells (biofilm and treatment).

A negative value therefore indicates that the extract or pure compound increases biofilm formation and a positive value indicates reduction of biofilm formation compared to the control.

Increased adhesion \((p < 0.05)\) was observed with the Hex and DCM crude leaf and fruit extracts (Figure 4-2). Decreased adhesion was observed for all EtOAc, MeOH and aq/MeOH leaf, stem bark and fruit extracts (Figure 4-2; Table 4-2).
The MeOH leaf extract displayed the greatest anti-adhesion potential against *E. coli* (Table 4-2), while most of the extracts except the DCM and hexane extract of the 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 (Table 4-2). All the polar extracts (EtOAc, MeOH, aq/MeOH leaf, stem bark and fruit extracts) decreased adhesion while the non-polar (Hex and DCM leaf and fruit extracts) increased adhesion of the three bacterial strains. The decreased adhesion of *E. coli*, *P. aeruginosa* and *S. aureus* of the polar crude extracts suggests interference of the extracts on the ability of these microorganisms to adhere to polystyrene surfaces. The opposite effect was observed for the non-polar extracts, which had the ability to promote biofilm formation. Unfortunately, no compounds were isolated from these polar extracts to find a link between the polar extracts and their anti-biofilm activity. However, Samoilova et al. (2014) observed
that extracts from medicinal plants *Arctostaphylos uvaursi, Vaccinium vitis-idaea, Tilia cordata, Betula pendula* and *Zea mays* stimulated *E. coli* biofilm formation up to three-fold. According to Samoilova et al., (2014), bacteria treated with polyphenol-rich extracts reduced more biofilm than with those treated with antibiotics alone. The differential adhesion activity by polar and non-polar crude extracts suggests perhaps differential selection of phytochemicals with varying biofilm reduction efficacy. There are varying reports on anti-adhesion effects of polar extracts against different bacterial strains in the literature, which corroborate our findings. Anti-adhesion inhibitory effects of polar extracts on *E. coli* and *Bacillus cereus* (Bräunlich et al., 2013; Tao et al., 2011), the oral bacteria, *Streptococcus mutans* and *Streptococcus sobrinus* (Song et al., 2006; Rahim and Khan, 2006), both *S. aureus* and methicillin-resistant *S. aureus* (MRSA) (Chusri et al., 2012; Hobby et al., 2012), *P. aeruginosa* (Sarkar et al., 2014) and *Proteus mirabilis* (Taqi, 2013) were reported in the literature.

**Table 4-2** Percentage biofilm reduction following exposure to 2 mg mL\(^{-1}\) crude leaf, stem bark and fruit extracts of *F. natalensis*

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Percent biofilm reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>E. coli</em> ATCC 35218</td>
</tr>
<tr>
<td>Dichloromethane (leaf)</td>
<td>-1511.09</td>
</tr>
<tr>
<td>Ethyl acetate (leaf)</td>
<td>212.57</td>
</tr>
<tr>
<td>Hexane (leaf)</td>
<td>-1139.56</td>
</tr>
<tr>
<td>Methanol (leaf)</td>
<td>218.11</td>
</tr>
<tr>
<td>Aqueous methanol (leaf)</td>
<td>159.43</td>
</tr>
<tr>
<td>Ethyl acetate (stem bark)</td>
<td>200.86</td>
</tr>
<tr>
<td>Methanol (stem bark)</td>
<td>210.41</td>
</tr>
<tr>
<td>Dichloromethane (fruit)</td>
<td>-1293.44</td>
</tr>
<tr>
<td>Ethyl acetate (fruit)</td>
<td>200.25</td>
</tr>
<tr>
<td>Hexane (fruit)</td>
<td>-1479.67</td>
</tr>
<tr>
<td>Methanol (fruit)</td>
<td>199.63</td>
</tr>
</tbody>
</table>
All four compounds increased adhesion of *E. coli* ATCC 35218 (*p* < 0.001) at sub-inhibitory (100 µg mL\(^{-1}\), *p* = 0.047), inhibitory (250 µg mL\(^{-1}\), *p* < 0.001) and supra-inhibitory (500 µg mL\(^{-1}\)) concentrations (Figure 4-3). The results with *P. aeruginosa* were variable. Stigmast-4-en-one (2) decreased adhesion of *P. aeruginosa* ATCC 27853 at all three concentrations, with 100 µg mL\(^{-1}\) being the most effective (Figure 4-4).

Table 4-3. 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 (250 and 500 mg mL\(^{-1}\) respectively) (Figure 4-4). For 3α-hydroxy-21α-H-hop-22(29)-ene (3) and tectoquinone (5), decreased adhesion was only observed at sub-inhibitory (100 µg mL\(^{-1}\)) concentrations, while increased adhesion was observed at inhibitory and supra-inhibitory concentrations (250 and 500 µg mL\(^{-1}\)) (Table 4-3). Increased adhesion of *S. aureus* ATCC 43300 was observed with all four compounds at all concentrations tested (Figure 4-5; Table 4-3).
Figure 4-3 Effect of 100 -500 µg mL\(^{-1}\) of ergost-4, 6, 8(14), 22-tetraen-3-one (1), stigmast-4-en-3-one (2), 3β-hydroxy-21β-H-hop-22(29)-ene (3) and tectoquinone (5) isolated from \textit{F. natalensis} on biofilm formation of \textit{E. coli} ATCC 35218, using microtitre plate assays. Data represents the mean standard deviation of three replicates on three separate occasions.

Table 4-3 Percentage biofilm reduction following exposure to 100-500 µg mL\(^{-1}\) of four compounds isolated from \textit{F. natalensis}.

<table>
<thead>
<tr>
<th>Compound</th>
<th>\textit{E. coli} ATCC 35218</th>
<th>\textit{P. aeruginosa} ATCC 35218</th>
<th>\textit{S. aureus} ATCC 43300</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100 250 500</td>
<td>100 250 500</td>
<td>100 250 500</td>
</tr>
<tr>
<td>Ergost-4,6,8(14), 22-tetraen-3-one</td>
<td>-315.62 -1456.10 -1176.99</td>
<td>-64.04 28.16 18.05</td>
<td>-445.34 -338.40 -437.78</td>
</tr>
<tr>
<td>Stigmast-4-en-3-one</td>
<td>-783.27 -1168.21 -1151.57</td>
<td>59.65 8.59 19.45</td>
<td>-1.81 -330.97 -381.66</td>
</tr>
<tr>
<td>Tectoquinone</td>
<td>-593.81 -1220.89 -1189.00</td>
<td>54.69 -6.49 -48.49</td>
<td>-51.30 -395.19 -416.02</td>
</tr>
</tbody>
</table>
**P. aeruginosa**

![Graph showing effect of compounds on biofilm formation](image)

**Figure 4-4** Effect of 100 -500 µg mL$^{-1}$ of ergost-4, 6, 8(14), 22-tetraen-3-one (1), stigmast-4-en-3-one (2), 3α-hydroxy-21α-H-hop-22(29)-ene (3) and tectoquinone (5) isolated from *F. natalensis* on biofilm formation of *P. aeruginosa* ATCC 27853, using microtitre plate assays. Data represents the mean standard deviation of three replicates on three separate occasions.
Variable concentration dependent activity observed with the isolated compounds were in accordance with the findings of Kiplomo et al., (2011), where they reported variations in the adhesion activities at MIC, sub-MIC (0.5xMIC) and supra-MIC (2xMIC) concentrations with some triterpenic class of compounds isolated from *Vernonia auriculifera* against seven different bacterial strains. A low inhibitory concentration (IC$_{50}$) 16 μg mL$^{-1}$ (range of 8 × MIC) has also been documented in literature for a pentacyclic triterpenoid, acetyl-11-keto-β-boswellic acid derived from *Boswellia serrata* against two oral pathogens, *S. mutans* ATCC 25175 and *Actinomyces viscosus* ATCC 15987 (Raja et al., 2011). 3β,6β,16β-
trihydroxylup-20(29)-ene from the plant *Combretum leprosum* Mart has been reported to inhibit the biomass of *S. mutans* and *S. mitis* by 97% and 90% respectively at a concentration as low as 7.8 µg mL\(^{-1}\) (Evaristo et al., 2014), which further supported our findings. Nevertheless, increased adhesion being observed at higher concentrations with some of the isolated compounds may probably be as a result of promotion of microbial adhesion caused by compounds that provides suitable conditioning film for enhancement of cell attachment (Selim et al., 2014).

4.3 Experimental

General Experimental Procedures

Silica gel 60 (Merck, 70-230 mesh and 200-300 mesh) was used for vacuum column chromatography (VCC) and column chromatography (CC) respectively. Preparative TLC was carried out using glass plates 10 × 20 cm, kieselgel 60 F\(_{254}\) (Merck). Aluminium sheets (20 × 20 cm) pre-coated with silica gel 60 F\(_{254}\) (Merck) were used for TLC analysis and visualised with 10% sulphuric acid in MeOH or under UV light at 254 and 366 nm. Melting points were determined on an Ernst Leitz Wetzlar micro-hot stage melting point apparatus and are uncorrected. IR spectra were obtained on a Perkin Elmer Universal ATR spectrometer. Optical rotation was carried out on a Perkin Elmer™, Model 341 Polarimeter at 20 °C with a 10 cm flow tube. UV spectra were obtained on a Shimadzu Varian Cary UV-VIS spectrophotometer. \(^1\)H, \(^{13}\)C and 2D NMR spectra were acquired on either Bruker Avance\(^{III}\) 400 or 600 MHz spectrometers at frequencies of 400.22 MHz for \(^1\)H and 100.63 MHz for \(^{13}\)C and referenced to TMS. GC-MS analysis was carried out on a Shimadzu GC-MSD apparatus using a fused-silica capillary column DB-5SIL MS (30 m × 0.25 mm i.d., 0.25 µm film thickness) with Helium as a carrier gas and operated in the EI mode at 70 eV.
Plant material
The fruits, leaves and stem bark 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 reference number G. V Awolola & H. Baijnath 3.

Extraction and purification
Ground fruits (970 g), ground leaves (1.1 kg) and ground stem bark (1.5 kg) were extracted twice by maceration, sequentially with organic solvents of increasing polarity, Hexane (Hex), dichloromethane (DCM), ethyl acetate (EtOAc) and methanol (MeOH). Yields of 20.75 g (Hex), 9.33 g (DCM), 4.05 g (EtOAc) and 9.97 g (MeOH) of the fruits was obtained after evaporation of the organic solvents. For the leaves, 15.05 g (Hex), 12.44 g (DCM), 7.06 g (EtOAc) and 14.41 g (MeOH) was obtained while for the stem bark, 8.58 g (Hex), 18.36 g (DCM), 10.41 g (EtOAc) and 11.49 g (MeOH) was obtained.

According to the TLC profile, similar components were observed for both the hexane and DCM crude extracts of the leaves. They were both combined and 24.09 g was subjected to vacuum column chromatography (VCC) on a silica gel column with a hex:DCM, DCM:EtOAc and EtOAc:MeOH step gradient, increasing the polarity each time in increments of 20% until 100% MeOH was reached. Fractions of 250 mL each were collected and 2L of solvent were used for each step in the gradient. A total of 122 fractions were eluted. The fractions were combined into five major fractions after analysis of each of the fractions by TLC (A: 28-35; B: 38-52; C: 53-69; D: 70-102 and E: 103-122). Compound 3, 3β-hydroxy-21β-H-hop-22(29)-ene (250.6 mg), was obtained from fraction A (1.56 g) after washing with hexane and recrystallized in MeOH. Fraction B (3.60 g) was further purified on a column (2 cm in diameter) by elution with hexane:EtOAc (9:1) to obtain 1 ergosta-
4,6,8(14),22-tetraene-3-one. Final purification of 1 was achieved by preparative TLC (hex: EtOAc; 9:1) to obtain 140.5 mg of 1.

The EtOAc fraction of the leaves (6.50 g) was separated in a similar manner as above, but collecting 150 mL fractions and using a volume of 1.2 L for each step in the gradient. Five major fractions were combined after TLC analysis (A: 21-23; B: 24-29; C: 30-48; D: 49-70 and E: 71-97). Compound 4, sitosterol (80.6 mg) was obtained from fraction B (210 mg), which was eluted with hex:EtOAc (8:2).

Similar to the leaves, the hexane and DCM crude extracts of the stem bark were combined based on TLC analysis. The combined extract (hex and DCM; 26.94 g) was separated similar to the leaves to yield six fractions (A: 5-16; B: 17-19; C: 24-26; D: 33-38; E: 50-54 and F: 66-75). Compound 5, tectoquinone (70.8 mg) was obtained by purification of fraction B (280.5 mg) on a silica column and eluted with hex:EtOAc (19:1). Fraction C (390.9 mg) was further purified with a hex:EtOAc (17:3) solvent system, which yielded stigma-4-ene-3-one (2) (70.4 mg).

Separation of the different extracts of the fruit was carried out as above, however no secondary metabolites were isolated from these extracts.

**Microbial strains**

A total of twelve species of microorganisms were used in this study: seven Gram-positive strains; *Staphylococcus aureus* ATCC 29213, *S. aureus* ATCC 43300, *Staphylococcus xylosus* ATCC 35033, *Staphylococcus sciuri* ATCC 29062, *Enterococcus faecalis* ATCC 51299, *Enterococcus faecium* ATCC 29212 and *Bacillus subtilis* ATCC 6633; and five
Gram-negative strains; *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *P. aeruginosa* ATCC 35032 and *Klebsiella pneumoniae* ATCC 700603 for antibacterial activity. Bacterial strains were all maintained on tryptic soy agar (TSA) at 4 °C. Prior to the antibacterial tests, the strains were sub-cultured on freshly prepared (TSA) plates at 37°C.

**Antibacterial susceptibility testing (AST)**

Using the disc diffusion method, the antibacterial susceptibility of compounds 1-3 and 5 and the crude extracts from *F. natalensis* was determined (CLSI, 2007). Sitosterol 4 was not subjected to the antibacterial tests as this compound has been studied extensively. Stock solutions of 10 mg mL\(^{-1}\) for compounds 1-3 and 5 and 100 mg mL\(^{-1}\) for crude extracts were prepared using appropriate solvents (DMSO or chloroform). Sterile, blank discs (6 mm diameter; MAST, UK), were impregnated with 100 µg or 250 µg of the isolated compounds and 2 mg or 4 mg of the crude extracts.

Mueller Hinton agar plates were swabbed with bacterial inocula (equivalent to a 0.5 McFarland standard), and impregnated discs were placed on the inoculated agar surface. For the negative control, chloroform and DMSO-impregnated discs were used while tetracycline (TE30) and ampicillin (AMP10) discs (Oxoid, UK) were used as standard antibacterial agent controls. Zones of inhibition, following incubation at 37 °C for 24 h, were measured in mm and averaged. All testing was done in duplicate.

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. Criteria for assigning susceptibility or resistance to TE30 were: (S) ≥
19 mm, (I) 15 – 18 mm, (R) ≤ 14 mm, while those for AMP10 was as follows: (S) ≥ 17 mm, (I) = 14 – 16 mm, (R) ≤ 13 mm (CLSI, 2007).

**Bacterial adhesion**

Based on the disc diffusion assay, three bacterial strains; *E. coli* ATCC 35218, *S. aureus* ATCC 43300 and *P. aeruginosa* ATCC 27853 were selected for the anti-adhesion study. The anti-adhesion effect of sub-inhibitory exposure (2 mg mL\(^{-1}\)) of *F. natalensis* crude extracts, as well as ergost-4,6,8(14),22-tetraen-3-one 1, stigma-4-ene-3-one 2, 3β-hydroxy-21β-H-hop-22(29)-ene 3 and tectoquinone 4 [100 µg mL\(^{-1}\) (sub-inhibitory), 250 µg mL\(^{-1}\) (inhibitory), 500 µg mL\(^{-1}\) (supra-inhibitory)] were determined using the crystal violet microtitre plate assay (Basson et al., 2008). Bacterial strains were cultured on TSA overnight, and re-suspended in sterile distilled water. Following centrifugation 12000 rpm for 2 min, 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 microtitre plates were filled with 90 µL of tryptic soy broth medium and 10 µL of the selected bacterial strains. Respective volumes of the isolated compounds (20, 50, 100 µL of a 1 mg mL\(^{-1}\) stock solution) and crude extracts (40 µL of a 10 mg mL\(^{-1}\) stock solution) were added to the TSB medium at the time of inoculation in microtitre plates and all volumes were standardized to 200 µL with sterile distilled water for the investigation of bacterial adhesion. TSB broth inoculated with the respective bacterial cultures, but without the tested compounds and extracts were used as positive controls, whilst wells containing uninoculated TSB broth only were used as a negative control to test for sterility and non-specific binding of media. Plates were incubated aerobically at 37 °C for 24 h with agitation on an Orbit P4 micro-titre plate shaker (Labnet).
The supernatant in each well, after incubation, was aspirated and the micro-titre plates washed three times with 250 µL of sterile distilled water to remove planktonic bacteria. The adherent bacteria were fixed with 200 µL of MeOH for 15 min. After the removal of methanol, the plates were left to air-dry. Subsequently, 150 µL of 2% Hucker’s crystal violet was added to the wells for 5 min. Thereafter, wells were rinsed gently and thoroughly with water for the removal of excess stain, air-dried and 150 µL of 33% (v/v) of glacial acetic acid was added for re-solubilization of dye bound to adherent cells (Basson et al., 2008). Using a Fluoroskan Ascent F1 spectrophotometer (Thermolabsystems), the optical density (OD) of the destained solution was measured at 600 nm. Assays were carried out in triplicate, performed twice and the data averaged. OD$_{600}$ values of treated cells were compared to untreated cells to investigate the increase or decrease in adhesion as a result of phytochemical and crude extract exposure as antimicrobial agents. Treated and untreated samples were compared statistically using Paired t-tests (SigmaStat V3.5, Systat Software, Inc).

A measure of efficacy called Percentage reduction was calculated from the blank, control, and treated absorbance values (Pitts et al., 2003):

$$ \text{Percentage reduction} = \left( \frac{C - B - (T - B)}{C - B} \right) \times 100, $$

where B denotes the average absorbance per well for blank wells (no biofilm, no treatment), C denotes the average absorbance per well for control wells (biofilm, no treatment), and T denotes the average absorbance per well for treated wells (biofilm and treatment).

### 4.4 Conclusions

Five non-polar compounds were isolated and identified from extracts of *F. natalensis*. Variable antibacterial activities were reported for both crude extracts and isolated compounds
with greater activity against Gram-positive bacteria, albeit at high concentration (4 mg mL\(^{-1}\)). Decreased adhesion with >100% biofilm reduction was demonstrated by all the crude polar extracts against all bacterial strains. The isolated compounds exhibited strain-specific anti-adhesion potential. This is the first report of the anti-adhesion potential of crude extracts and isolated compounds of *F. natalensis*. It is quite possible that the isolates from *F. 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 shown good antibacterial activity in the assays used in this work. The same isolates also showed anti-biofilm activity, which could also contribute to the antibacterial activity. Surprisingly though, the crude extracts did not show a significant antibacterial effect, however, probably if administered over a period of time, the concentrations of the isolates in the patient could increase resulting in the desired antibacterial effect shown by them in this study and may possess the potential to be developed into agents that can be used be prevent bacterial biofilm formation. Further research is required to identify specific compounds that will be able to inhibit biofilm formation by both Gram-negative and Gram-positive bacterial pathogens at sub-inhibitory concentrations that will prevent the evolution of antimicrobial resistance.

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4.5 References


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Chapter 5. The phytochemistry and gastroprotective activities of the leaves of *Ficus glumosa*

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Abstract

*Ficus glumosa* is reputed for the treatment and management of various health conditions, including diarrhoea, dysentery, oedema, headache, stomach ailments and ulcers. Three phenolic acids (1-3), two coumarins (4 and 5), three flavonoid glycosides (6, 7a, 7b) and five triterpenoids (8-12) were isolated from the aerial parts of *F. glumosa*. Gastroprotective activity of the methanol soluble extract of the leaves was apparent at 200 mg kg\(^{-1}\) with significant protection in ethanol, indomethacin and pylorus-induced experimental ulcer in rat models. The extract was shown to be non-toxic at 2000 mg kg\(^{-1}\). Quercetin-3-O-β-D-glucopyranoside (isoquercitrin) 7a, quercetin-3-O-β-D-galactopyranoside 7b and p-hydroxybenzoic acid 1 isolated from the leaf extract probably play a role in the observed gastroprotective activity. The findings in this study validate the use of *F. glumosa* in the treatment of ulcers in African ethnomedicine.

**Key words:** *Ficus glumosa*, toxicity, cytoprotection, ulcer models.
5.1 Introduction

The genus *Ficus* (Moraceae) comprises over 800 species of woody trees, shrubs and vines, with over 100 species found in Africa (Serrato et al., 2004). *F. glumosa* (Miq.) Delile is a medium sized tree which grows up to 5-10 m and has a smooth pale brown bark. The branches are thick and hairy while the leaves are thick with silky white oblong hairs. The fruit grow into hairy red figs when ripe and grow on the axils of the leaves either singly or in pairs (Burrows and Burrows, 2003). It is commonly known as “African rock fig” and is reputed for the treatment and management of various health conditions in African traditional medicine, including dysentery, oedema, hypertension, headache, stomach ailments, menstrual pains, skin diseases, rheumatism, diabetes mellitus and to treat female sterility (Burkill, 1985; Arbonnier, 2000; Orwa et al., 2009; Madubunyi et al., 2012). A mixture of the latex and bark are applied as mouthwash for toothache while the bark decoction is used for the treatment of sores and ulcers (Burkill, 1985). The fruits are eaten when ripe.

Pharmacologically, *F. glumosa* has been described for its antidiabetic, hypolipidaemic, hypoglycaemic and antioxidant properties (Madubunyi et al., 2012; Olaokun et al., 2013; 2014; Onoja et al., 2014). The methanol leaf extract of the plant had antidiarrheal activity (Tanko et al., 2012) and the aqueous leaf extract showed diuretic activity, supporting its traditional use in treating hypertension (Ntchapda et al., 2014) as well as hypolipidemic and anti-atherosclerotic properties (Ntchapda et al., 2015). Additionally, Nana et al. (2012) reported 23 phytochemical compounds from the stem bark including ceramides, flavonoids and their glycosides and sterols and their glycosides. One of the compounds isolated, dongnoside E showed good cytotoxic activity against the prostate cancer PC-3 cell line (Nana et al., 2012).
Several plant extracts, including those of *Ficus* have been shown to have gastroprotective effects (Sumbul et al., 2011; Srinivas et al., 2013; Alqasoumi et al., 2014; Ishikawa et al., 2014; Yesilada et al., 2014), which prompted us to investigate the aerial parts of *F. glumosa* for its gastroprotective potential. Thus far, despite various documented pharmacological studies on *F. glumosa*, there is no report on the gastroprotective activity and detailed phytochemical studies of the aerial parts of the plant. We herein report the phytochemical study of the stem bark, fruit and leaves and gastroprotective activity of *F. glumosa* leaves.

5.2 Results and discussion

The phytochemical investigation of the aerial parts of *F. glumosa* resulted in the isolation of 13 known compounds (Figure 5-1), which were identified by their $^1$H and $^{13}$C NMR spectra and by comparison of their physical and spectroscopic data with that reported in the literature. They were identified as $p$-hydroxybenzoic acid (1) (Refaat et al., 2015); 3,4-dihydroxy benzoic acid (2) (Zhu et al., 2010, Nana et al., 2012); vanillic acid (3) (Refaat et al., 2015); peucedanol (4) (Ikeshiro et al., 1994); (-) – marmesin (5) (Znati et al., 2014); quercetin-7-0-glucopyranoside (6) (quercimeritrin) (Shin et al., 2010); quercetin-3-O-β-glucopyranoside (7a) (Atay et al., 2015); quercetin-3-O-β-galactopyranoside (7b) (7a & 7b - were isolated as a mixture) (Atay et al., 2015); simiaremol (8) (Yoo et al., 2008); 3β-hydroxy-21β-H-hop-22(29)-ene (9) (Sousa et al., 2012); α-amyrin acetate (10) (Ali, 2013); stigma-4-ene-3-one (11) (Tanemossu et al., 2014) and sitosterol (12) (Rasoanaivo et al., 2014). Compounds 1, 7a & 7b, 9, 11 and 12 were isolated from the leaves while 2-5, 8 and 10 were isolated from the stem bark and 6 was found in the fruits. Eight of the isolated compounds have been previously reported in related *Ficus* species (Chang et al., 2005; Chen et al., 2010; Nana et al., 2012; Park et al., 2012; Tsai et al., 2014), including 3β-hydroxy-21β-H-hop-22(29)-ene (9), which was isolated from *Ficus natalensis* in this work (Chapter 4). This is
the first report of peucedanol (4), quercetin-7-O-glucopyranoside (6), quercetin-3-O-β-galactopyranoside (7b) and simiarenol (8) in the genus *Ficus*.

The structural elucidation was carried out using $^1$H, $^{13}$C, HMBC and NOESY data. For example, the $^1$H NMR spectrum of 4 indicated two characteristic coumarin doublets at $\delta_H$ 7.84 and $\delta_H$ 6.18 (H-3, $J = 9.2$ and H-4, $J = 9.4$). Two aromatic one-proton singlets indicating their *para* orientation to each other at $\delta_H$ 7.40 and $\delta_H$ 6.73 (H-5 and H-8) was evident. There were three other observable proton signals, two-proton multiplets at $\delta_H$ 2.53, $\delta_H$ 3.07 (H-1'a and H-1'b) attributable to a benzylic methylene and a one-proton double doublet at $\delta_H$ 3.62 (H-2'). Two three-proton singlets resonating at $\delta_H$ 1.29 and $\delta_H$ 1.28 (H-4' and H-5') were assigned to the gem-dimethyl groups at C-3'. The point of attachment of the isoprenyl unit was indicated by HMBC correlations between H-1'a and H-1'b to C-5 and C-7. The structure of Peucedanol (4) was confirmed by comparison with the data from literature and the configuration at C-2' was also determined as *R* as 4 had an optical rotation similar in sign and magnitude to an authentic *R* isomer of the compound (Ikeshiro et al., 1994).
There are many different experimental models used in evaluating the gastroprotective activity of a plant extract or compound, such as the ethanol and indomethacin induced ulcer models.
Gastric lesions induced by ethanol results in gastric mucosal injury which is as a result of aggressive and protective imbalance (Oliveira et al., 2011; Amandeep et al., 2012). The methanol soluble extracts of the aerial parts (leaves, stem bark and fruits) of *F. glumosa* was screened for their gastroprotective potential at the dose of 100 and 200 mg kg\(^{-1}\). The extracts showed varying degrees of ulcer inhibition compared to the control (Table 5-1). In particular, the gastroprotective activity of the leaf extract (FLM) was found to be very high. The leaf extract reduced ulcer lesions by 75 and 90%, respectively at a dose of 100 and 200 mg kg\(^{-1}\) significantly (P< 0.01). Misoprostol, the standard drug used in this experiment, showed protection of 85% at a dose of 0.1 mg kg\(^{-1}\) compared to the control. Since the methanol leaf extract showed a gastroprotective effect of 90% at 200 mg kg\(^{-1}\), it was thus selected for further studies in the indomethacin and pylorus ligation-induced ulcer models.

The long term administration of non-steroidal anti-inflammatory drugs (NSAID) is another important factor in the pathology of gastric ulcers. NSAID such as indomethacin caused therapeutic and toxic effects by inhibiting cyclooxygenase and decreasing the levels of prostaglandins in the gastric mucosa (Yoshikawa et al., 1993; Takeuchi et al., 1997). In the indomethacin induced ulcer assay, the extract showed significant and marked protection with 50, 61 and 100% protection at doses of 50, 100 and 200 mg kg\(^{-1}\) respectively (Table 5-2). The standard drug omeprazole, showed 96% protection at 100 mg kg\(^{-1}\). The results obtained suggest that the cytoprotective effect of *F. glumosa* may be mediated by prostaglandins.

Pyloric ligation is a method used to induce ulcers in experimental rat models. It does this by the accumulation of gastric juice and by interfering with gastric blood circulation (Shay et al., 1945). This test allows measurement of parameters such as gastric secretion volume, pH and total acidity ([H\(^+\)]) in the gastric juice (Miranda, et al., 2015). The ulcer index and percent protection against ulcers in the pylorus ligated-induced ulcer model are shown in Table 5-3. The extract significantly reduced ulcer indices compared to the tween 20 treated pylorus-
ligated control group. The percentages of ulcer protection at doses of 50, 100 and 200 mg kg\(^{-1}\) were 56, 60 and 62\%, respectively. The gastric volume and gastric acidity were significantly \((P<0.05, P<0.01)\) reduced at the dose of 100 and 200 mg kg\(^{-1}\), while the gastric pH was increased at all the three doses, compared to the control. The standard drug, cimetidine produced a significant decrease in ulcer index, gastric volume and total acidity with significant increase in the pH compared to the control. These findings suggest that the gastroprotective effect of the extract could involve anti-secretory action.

The phytochemical profile of the leaves of \textit{F. glumosa} yielded five compounds including two flavonoids (quercetin-3-\(O-\beta\)-D-glucopyranoside (isoquercitrin) 7\(a\) and quercetin-3-\(O-\beta\)-D-galactopyranoside (hyperoside) 7\(b\) and a phenolic derivative of benzoic acid, \(p\)-hydroxybenzoic acid 1. These compounds have been reported to have gastroprotective and antioxidant activities. Rodrigues et al. (2012) reported isoquercitrin 7\(a\) as one of the components in the anti-ulcerogenic active fraction of \textit{Byrsonima sericea} leaves, while Yesilada et al. (2014) identified this compound as the active anti-ulcerogenic compound in \textit{Sambucus ebulus}. Quercetin-3-\(O-\beta\)-D-galactopyranoside has also been reported as one of the components of \textit{Byrsonima crassa}, a medicinal plant used in Brazilian folk medicine for the treatment of gastric ulcers (Sannomiya et al., 2005).

Moreover, the flavonoid glycosides and \(p\)-hydroxybenzoic acid 1 have been reported to have antioxidant activity (Zocoler et al., 2009; Zhang et al., 2014; Cong et al., 2015). Antioxidant activity is one of the mechanisms suggested for anti-ulcerogenic effects (Kwiecien et al., 2002; Repetto and Llesuy, 2002). Other suggested mechanisms include stimulation of prostaglandin, thereby inhibiting leukotriene production, increasing the gastric hexosamine level and enhancing the strength of the gastric barrier either physically or by blocking the \(H^+\),
K⁺-ATPase pump (Okazaki et al., 2007). It could therefore be deduced that the presence of these phenolic compounds may contribute to the significant gastroprotective activity observed in the leaf extract of *F. glumosa*, which would validate the use of the plant in African traditional medicine.

**Table 5-1** Effect of the methanol extracts of *F. glumosa* in rats with ethanol-induced ulcers

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Mean ulcer score</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% Tween 20</td>
<td>10</td>
<td>5.10±0.51</td>
<td>-</td>
</tr>
<tr>
<td>Extract FLM</td>
<td>100</td>
<td>0.50±0.32**</td>
<td>75.49</td>
</tr>
<tr>
<td>FLM 200</td>
<td>0.30±0.20***</td>
<td>90.20</td>
<td></td>
</tr>
<tr>
<td>FLM 100</td>
<td>3.50±1.19</td>
<td>31.37</td>
<td></td>
</tr>
<tr>
<td>FSM 100</td>
<td>2.10±0.95*</td>
<td>42.35</td>
<td></td>
</tr>
<tr>
<td>FSM 200</td>
<td>2.60±0.97</td>
<td>36.27</td>
<td></td>
</tr>
<tr>
<td>FFM 100</td>
<td>3.50±1.19</td>
<td>31.37</td>
<td></td>
</tr>
<tr>
<td>FFM 200</td>
<td>2.60±0.97</td>
<td>36.27</td>
<td></td>
</tr>
<tr>
<td>Misoprostol</td>
<td>0.1</td>
<td>0.60±0.24**</td>
<td>85.29</td>
</tr>
</tbody>
</table>

Results are mean ± SEM, n=5. Statistical comparison was determined by one way ANOVA followed by Dunnett’s multiple comparison *post-hoc* tests. *P*<0.05, **P**<0.01, ***P**<0.001, statistically significant compared to the control (5% Tween 20).

FLM, FSM, FFM: *F. glumosa* leaves, stem bark and fruits crude methanolic extracts, respectively.
Table 5-2 Effect of the methanol extract of the leaves of F. glumosa in rats with indomethacin-induced ulcers

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Mean ulcer score</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% Tween 20</td>
<td>10</td>
<td>6.20±0.68</td>
<td>-</td>
</tr>
<tr>
<td>Extract</td>
<td>50</td>
<td>3.10±0.70**</td>
<td>50.00</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>2.40±0.60***</td>
<td>61.29</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>0.00±0.00***</td>
<td>100.00</td>
</tr>
<tr>
<td>Omeprazole</td>
<td>100</td>
<td>0.10±0.08***</td>
<td>96.24</td>
</tr>
</tbody>
</table>

Results are mean ± SEM, n=5. Statistical comparison was determined by one way ANOVA followed by Dunnett multiple comparison post-hoc tests. **P<0.01, ***P<0.001, statistically significant compared to the control (5% Tween 20).

Table 5-3 Effect of the methanol extract of the leaves of F. glumosa in pylorus ligated rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Ulcer index</th>
<th>% Protection</th>
<th>Gastric juice (mL)</th>
<th>Gastric pH</th>
<th>Gastric acidity (meq L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% Tween 20</td>
<td>10</td>
<td>3.00±0.76</td>
<td>-</td>
<td>0.82±0.50</td>
<td>3.88±0.06</td>
<td>9.80±0.05</td>
</tr>
<tr>
<td>Extract</td>
<td>50</td>
<td>1.30±0.18*</td>
<td>56.67</td>
<td>0.54±0.05</td>
<td>6.20±0.80**</td>
<td>7.80±0.11</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1.20±0.12*</td>
<td>60.00</td>
<td>0.35±0.04**</td>
<td>7.36±0.27***</td>
<td>6.20±0.08*</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>0.90±0.24**</td>
<td>62.50</td>
<td>0.40±0.08**</td>
<td>6.60±0.22**</td>
<td>5.00±0.09**</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>100</td>
<td>0.10±0.04***</td>
<td>95.83</td>
<td>0.22±0.13***</td>
<td>7.48±0.05***</td>
<td>4.00±0.02***</td>
</tr>
</tbody>
</table>

Results are mean ± SEM, n=5. Statistical comparison was determined by one way ANOVA followed by Dunnnett multiple comparison post-hoc tests. *P<0.05, **P<0.01, ***P<0.001, statistically significant compared to the control (5% Tween 20).

5.3 Experimental

General Experimental Procedures

Reagents and chemicals were purchased from Merck and Sigma-Aldrich, South Africa.

Organic solvents were redistilled and dried according to standard procedures prior to being
used. Optical rotation was recorded with a 10 cm flow tube using a Perkin Elmer™, Model 341 Polarimeter. Melting points were recorded on an Ernst Leitz Wetzlar micro-hot stage melting point apparatus and are uncorrected. IR spectra were determined using a Perkin Elmer Spectrum 100 Fourier-Transform Infra-Red (FT-IR) spectrometer with universal ATR sampling accessory. Ultraviolet absorption (UV) spectra were obtained on a Shimadzu Varian Cary UV/VIS Spectrophotometer in either methanol or chloroform. $^1$H and $^{13}$C NMR spectra were recorded in either CDCl$_3$ or CD$_3$OD on a Bruker Avance™ 400 or 600 MHz spectrophotometer at 400.22 MHz for $^1$H and 100.63 MHz for $^{13}$C at room temperature. Chemical shifts (δ) are reported in ppm and coupling constants (J) in Hz. The chemical shifts of the deuterated solvent were 7.24 and 77.0 for $^1$H and $^{13}$C NMR respectively referenced to the internal standard, tetramethylsilane (TMS). For GC-MS analyses, the samples were analysed on a Shimadzu GC-MSD apparatus equipped with a fused-silica capillary column DB-5SIL MS (30 m × 0.25 mm i.d., 0.25 µm film thickness) at a flow rate of 2 mL min$^{-1}$ using helium as a carrier gas. The MS was operated in the EI mode at 70 eV. The MALDI-TOF MS spectra were recorded on a Bruker Reflex III (Germany). Chromatography was carried out by vacuum column chromatography (VCC) and column chromatography (CC) using silica gel 60 (Merck, 70-230 mesh, 200-300 mesh and Sephadex LH-20 (Pharmacia)). Analytical TLC was performed on pre-coated silica gel 60 F$_{254}$ aluminium sheets (Merck 1.05554) and visualised by spraying with 10% sulphuric acid in MeOH followed by heating or under UV light at 254 and 366 nm.

**Plant collection and extraction**

Fresh leaves, stem bark and fruit of *F. glumosa* (Miq.) Del. was collected at Durban, KwaZulu-Natal, South Africa during the month of February 2013. The plant was identified by Professor Himansu Baijnath, a botanist at the University of KwaZulu-Natal and deposited
at the University of KwaZulu-Natal, Ward herbarium (voucher no. G.V. Awolola & H. Baijnath 4). The plant parts were air dried (at room temperature) and pulverized in a mechanical grinder (Thomas Wiley laboratory mill).

The powdered leaves (1.1 kg), stem bark (3.2 kg) and fruit (1.1 kg) material of *F. glumosa* were sequentially extracted with organic solvents of increasing polarity; hexane (Hex), dichloromethane (DCM), ethyl acetate (EtOAc) and methanol (MeOH) on an orbital shaker for 48 hours. The extracts were combined, filtered and concentrated to afford 8.27 g (Hex), 8.16 g (DCM), 5.43 g (EtOAc) and 8.55 g (MeOH) crude extracts for the leaves, 35.26 g (Hex), 9.32 g (DCM), 8.72 g (EtOAc) and 13.14 g (MeOH) crude extracts for the stem bark and 29.17 g (Hex), 15.54 g (DCM), 10.55 g (EtOAc) and 29.81 g (MeOH) crude extracts for the fruits. The MeOH extracts of the leaves, stem bark and fruits were used for the gastroprotective assay while phytochemical analysis was conducted on all extracts.

**Isolation and purification of the leaf extracts**

A 7.12 g sample of the crude hexane extract was subjected to CC on 2.5 cm column and eluted with a stepwise gradient of hexane, dichloromethane, ethyl acetate and methanol, increasing the polarity by 20% for each step and collecting 100 mL fractions each. Based on their TLC profiles, the fractions were combined into four major fractions (A: 18-27; B: 28-31; C: 32-49 and D: 50-82). 3β-Hydroxy-21β-H-hop-22(29)-ene 9 (40.56 mg) crystallized out of fraction B as a white powder. The DCM and EtOAc crude extracts were combined (12.40 g) based on their TLC profiles and subjected to VCC as above. Similar fractions were pooled together to afford five major fractions (A: 11-26; B: 27-31; C: 32-36; D: 62-69 and E: 70-81). Purification of fraction B (220.67 mg) led to the isolation of stigma-4-ene-3-one 11 (30.12 mg) (purified with hex: EtOAc (17:3)) and sitosterol 12 (20.92 mg) (purified with hex: EtOAc (8:2)).
The methanol extract (7.52 g) was partitioned with EtOAc yielding 510.05 mg and was separated using Sephadex LH-20 and eluted with MeOH. Fractions of 10 mL each were collected from a 1 cm column. The fractions were combined into four major fractions as follows, A: 7-14; B: 15-21; C: 22-30 and D: 31-45. Purification of fraction B (80 mg) with Sephadex LH-20 and using MeOH as the eluent yielded $p$-hydroxybenzoic acid $\mathbf{1}$ (9.01 mg). Fraction C (100.56 mg) was separated on a 1 cm column and eluted with DCM: MeOH (9:1) to yield a mixture of $\mathbf{7a}$ and $\mathbf{7b}$, which was identified as quercetin-3-\(O-\beta\)-D-glucopyranoside $\mathbf{7a}$ and quercetin-3-\(O-\beta\)-D-galactopyranoside $\mathbf{7b}$ (40.52 mg of the mixture) in fractions 33-37.

**Isolation and purification of compounds from the stem bark**

The hexane and DCM extracts of the stem bark were combined due to similar TLC profiles. A 40.23 g sample of this combined extract was fractionated by VCC with a gradient elution using hexane, DCM, EtOAc and MeOH, increasing the polarity by 20% each time, collecting 8 x 250 ml fractions for each step. Fractionation of the crude extract afforded six major fractions (A: 1-6; B: 7-14; C: 15-21; D: 25-27; E: 28-45 and F: 46-63). $\alpha$-Amyrin acetate $\mathbf{10}$ (5.37 g), crystallised out of fraction A and was recrystallized in hexane. Simiarenol $\mathbf{8}$ (20.41 mg) precipitated out as a white powder from fraction B. Fraction D (1.14 g) was further purified on a 1.5 cm diameter column by CC using Hex: EtOAc (3:2), collecting 20 mL fractions. A light yellow powder was obtained from fractions 47-50 as (-)-marmesin $\mathbf{5}$ (72.20 mg).

The EtOAc fraction (7.50 g) of the stem bark was fractioned on a 3 cm diameter column and eluted with a step gradient of hex: EtOAc (increasing the polarity by 20% for each step) and finally with 100% MeOH; 10 x 100 mL fractions were collected at each stage. Six major
fractions were obtained based on similar TLC profiles (A: 1-13; B: 14-23; C: 24-27; D: 28-39; E: 40-56 and F: 57-72). Further purification of fraction B (460.48 mg) with Hex: EtOAc (1:1) yielded vanillic acid 3 (20.17 mg) as a brown powder. \( \text{p-Hydroxybenzoic acid } 1 \) (20.35 mg) was isolated from the same column with (Hex: EtOAc, 2:3) as a yellow powder. Fraction C (240.67 mg) yielded tiny brown crystals on elution with Hex: EtOAc (1:9) and was identified as 3,4-dihydroxybenzoic acid 2 (20.48 mg).

The MeOH extract was partitioned with EtOAc. A 3.10 g sample of the EtOAc portion was separated using a gradient elution of Hex: EtOAc and MeOH, increasing the polarity by 10% at each increment and collecting 10 \( \times \) 20 mL fractions at each stage. Four major fractions were obtained (A: 10-15; B: 16-30; C: 32-59 and D: 60-72). Fraction B (460.25 mg) was separated with Sephadex LH-20 and eluted with MeOH. This yielded more of 3,4-dihydroxybenzoic acid 2 (22.31 mg) and vanillic acid 3 (10.12 mg). Fraction C (760.12 mg) was purified on a 1 cm diameter column using Hex: EtOAc (1:1) and yielded a brown powder, identified as peucedanol 4 (30.12 mg).

**Isolation and purification of compounds from the fruits**

The methanol extract of the fruits was partitioned as above. A 870.42 mg sample of the EtOAc portion was separated using Sephadex LH-20 with MeOH as the mobile phase, collecting 10 mL fractions. Three major fractions were obtained (A: 15-21; B: 22-26 and C: 27-39). Fraction A (250.34 mg) was purified with sephadex LH-20 (eluted with MeOH) and yielded 3,4-dihydroxybenzoic acid 2 (90.78 mg). Fraction C (330.54 g) was also purified using Sephadex LH-20 (eluted with MeOH), and yielded quercetin-7-\( \text{O-glucopyranoside } 6 \) (10.19 mg).
Animals

Healthy Male Albino Wister rats weighing between 120-140 g were used for the study and maintained under standard laboratory conditions in temperature controlled rooms (24-28 °C), under a 12:12 h light–dark cycle, with access to water and food ad libitum until use. Animal care and handling procedures were in accordance with the International Association for the Study of Pain guidelines for the use of animals in pain research (Zimmermann, 1983). The research protocol was approved by the College of Medicine, Institutional Ethical Committee of the University of Lagos, Nigeria (CM/COM/08/VOL.XXVI).

Acute toxicity

An acute toxicity test was performed according to the Organization of Economic Co-operation and Development guidelines for chemical testing (OECD, 2001). A single oral dose (2000 mg kg\(^{-1}\)) of the methanol extract of *F. glumosa* (leaf, stem bark and fruit extracts) was separately administered to 6 male Swiss albino mice, which underwent an overnight fast, by gavage using a suitable canula. Animal behaviour was observed from 1 h after the first administration of a single extract and monitored daily for 14 days.

Gastroprotective activity in rats

Preliminary screening using an ethanol-induced gastric ulcer model

The gastroprotective potential of the methanol extracts of the leaves, stem bark and fruit of *F. glumosa* against superficial haemorrhagic mucosal lesions was investigated in the rats. Male rats weighing between 110 and 130 g were fasted for 24 h prior to the experiment. Forty rats were divided randomly into 8 groups of 5 rats each and pre-treated orally with the vehicle (5% Tween 20, 10 ml kg\(^{-1}\)), misoprostol (0.1 mg kg\(^{-1}\)) or extract (100-200 mg kg\(^{-1}\)). After 1 h of pre-treatment, all the rats were gavaged with 1 mL of absolute ethanol. The rats were
sacrificed 1 h later by cervical dislocation and their stomachs were immediately excised, cut along greater curvature, rinsed with normal saline and ulcer scored according to the method of Galati et al. (2001).

**Indomethacin-induced gastric ulcer model**

Rats fasted for 24 h were treated with vehicle (tween 20, 5%) or extract (50, 100 and 200 mg kg\(^{-1}\)). After 1 hour, indomethacin was administered orally to the rats at a dose of 80 mg kg\(^{-1}\) to all the groups (Nwafor et al., 2000). The animals were sacrificed after 6 h by cervical dislocation, their stomachs removed, washed gently with saline (0.9%) and ulcer index scored as referenced in the ethanol-induced model (Galati et al., 2001).

**Pylorus ligation-induced gastric ulcer model**

The evaluation of the gastric anti-secretory potential of the extracts was determined according to the method described previously by Asif et al. (2013) with slight modifications. The animals were divided into 5 groups, each consisting of 5 rats. All animals underwent 24 h of fasting. Group I served as the control (negative) and received 5% tween 20 only. Group II, III and IV received the extract at the doses of 50, 100 and 200 mg kg\(^{-1}\) respectively, while group V received cimetidine at 100 mg kg\(^{-1}\) (positive control). After 1 h of treatment, pylorus ligation was done without causing any damage to the blood supply of the stomach. Six hours after the ligation, the animals were sacrificed and the stomach removed. The gastric contents were collected into a centrifuge tube, centrifuged for 15 min at 2000 rpm and the supernatant volume measured. The pH value was obtained with a pH meter (Elico, Hyderabad, India) and total acidity by titration (phenolphthalein as indicator). The ulcers formed in the gastric mucosa were measured and scored as described earlier.
Statistical analysis

The results were expressed as the mean ± SEM (Standard error of the mean). Statistical analysis was performed with Graph Pad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA, USA) using one way ANOVA followed by Dunnett’s multiple comparism post-hoc test. The significance level was set at $P<0.05$.

5.4 Conclusions

Toxicity and mortality was not observed with the rats in the acute toxicity testing of the methanol extracts (2000 mg kg$^{-1}$) of the aerial parts of *F. glumosa*. A dose dependent gastroprotective effect was observed with the methanol soluble extract of the leaves, stem bark and fruits in the ethanol induced ulcer model. In the indomethacin induced ulcer model, the methanol leaf extract showed 100% gastroprotective activity at 200 mg kg$^{-1}$. The isolation of the mixture of isoquercitrin 7a and hyperoside 7b as well as $p$-hydroxybenzoic acid 1 in the methanol extract of the leaves of *F. glumosa* probably contributed to the gastroprotective effects as these compounds were previously reported as having gastroprotective activity. These findings substantiate the use of the plant in African traditional medicine.

ACKNOWLEDGMENTS

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5.5 References


extracts of ten African Ficus species (Moraceae) used traditionally to treat diabetes.


Chapter 6. Conclusion

This work describes the phytochemical investigation of four South African *Ficus* species, *sansibarica, bizanae, natalensis* and *glumosa* and the bioactivities of extracts and isolated compounds from these species. All species are used in African traditional medicine and indigenous to KwaZulu-Natal.

There have been no recorded traditional uses of *Ficus sansibarica*, nevertheless the fruits are eaten in some parts of Africa while birds which feed upon the wasps pollinate the figs. Due to the vast ethnomedicinal uses of many *Ficus* species we decided to carry out a phytochemical investigation of *F. sansibarica*. The phytochemical investigation of *F. sansibarica* yielded sterols and flavonoids, classes of compounds which have been known to have antibacterial, antioxidant, antifungal, antimalarial, anti-inflammatory, anti-leishmanial and anti-arthrital activity. Both the crude extracts and isolated compounds of *F. sansibarica* exhibited weak antimicrobial activity against Gram-positive bacterial strains. The flavonoids isolated from the plant demonstrated significant anti-biofilm activity suggesting the use of the plant to prevent bio-film formation. Our findings also indicate that *F. sansibarica* could be considered a natural source of antibiotics and traditional healers may want to incorporate this plant into their traditional medicinal practices.

Similar to *F. sansibarica*, *Ficus bizanae* also has no documented reports on its ethnomedicinal use. We also examined this plant phytochemically based on the traditional uses of other *Ficus* species. The phytochemical study of *F. bizanae* led to the isolation of coumarins, anthraquinones, flavonoid glycosides, megastigmane derivatives, cinnamic acid derivatives, polyphenols and triterpenoids. Antibacterial, antioxidant and anti-inflammatory activities were reported for the compounds isolated in *F. bizanae*. The crude extracts
exhibited weak to moderate antimicrobial activity against the test strains. However, the
extracts of *F. bizanae* were shown to have a synergistic and potentiative effect with currently
used antibiotics. Azithromycin, gentamicin, sulphamethoxazole, nalidixic acid and
cephalosporin gave the best potentiative effect with the extracts. Our studies indicate that
extracts of *F. bizanae* can be used to enhance the effect of currently used antibiotics. Further
studies are needed to identify the mechanism of action of this combinational therapy and
dosages needed to be effective.

Various parts of *Ficus natalensis* is used for ailments such headache, toothache, irregular and
painful menstruations, as antivenom, to treat malaria, influenza, whooping cough, dysentery,
for ulcers and wounds. A phytochemical study of the plant led to the isolation of
triterpenoids, sterols and an anthraquinone. Both the crude extracts and isolated compounds
have shown antibacterial activity against Gram-positive bacteria. The polar crude extracts of
the plant was shown to decrease antibacterial adhesion. Similarly, stigma-4-ene-3-one \( \text{P3-2} \),
\( 3\beta\)-hydroxy-21\( \beta\)-H-hop-22(29)-ene \( \text{P3-3} \) and tectoquinone \( \text{P3-5} \) showed anti-adhesion
potential to the bacteria tested against. It is quite possible that these isolates are responsible
for the antibacterial activity of the plant.

*Ficus glumosa* is used in African traditional medicine in the treatment and management of
various health conditions such as stomach ailments and ulcers, diarrhoea, dysentery, oedema,
hypertension, headache, menstrual pains and skin diseases. Phytochemical studies showed
the plant to contain polyphenolic compounds, coumarins, flavonoid glycosides, triterpenoids
and sterols. The various extracts of the plant parts of *F. glumosa* showed a dose dependent
gastroprotective activity with the methanol of the leaves being the most active from these
extracts. Our findings corroborate the use of *F. glumosa* in traditional medicine for the management of ulcers.

Secondary metabolites belonging to diverse classes of compounds have been isolated from the four South African *Ficus species* studied in this work. Most of these have previously shown pharmacological activity in many biological assays. This information will be very useful for the traditional healers who use these plants in African ethnomedicine in that our findings could provide a rationale for their use for certain medical conditions. In addition, these plants are an alternative, easily accessible, less expensive source to synthetic pharmaceuticals.

Furthermore, the plants studied in the work have shown that they could be used as antibacterial, anti-adhesive, resistant-modifying and gastroprotective agents. Future work is needed to identify the specific compounds from the active extracts that inhibit biofilm formation of both Gram-negative and Gram-positive bacteria. *F. bizanae* is a possible source for enhancing the activity of antibiotics used in the pharmaceutical industry. Further studies defining the possible mechanisms of action of this combinational therapy may thus be required.