

PREVALENCE, ANTIBIOTIC RESISTANCE TRENDS, VIRULENCE AND
EFFECT OF SOME MEDICINAL PLANTS ON *STAPHYLOCOCCUS* FROM
SCHOOL CHILDREN IN THE MARIENTAL DISTRICT, NAMIBIA

A DISSERTATION SUBMITTED IN FULFILMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY (MICROBIOLOGY)

OF

THE UNIVERSITY OF NAMIBIA

BY

Sunette Walter

201023792

April 2018

MAIN SUPERVISOR: Dr. Ronnie Böck (Biological Sciences Department, University
of Namibia)

CO-SUPERVISORS: Dr. Mervyn Beukes (Biochemistry Department, University of
Pretoria)

Prof. Davis Mumbengegwi (Multidisciplinary Research
Centre, University of Namibia)

Abstract

The main aim of this study was to investigate potentially pathogenic community-associated staphylococci in school children. Objectives were: To determine the prevalence of nasal *Staphylococcus aureus* and coagulase-negative staphylococci (CoNS) in school children from the Mariental District; to characterize the bacteria in terms of their antibiograms and drug resistance patterns; to screen bacterial isolates for their ability to produce enterotoxins and produce biofilms as potential virulence factors; and to assess the antimicrobial and anti-biofilm activity of crude methanolic extracts of *Aptosimum albomarginatum* (Marloth and Engl.) roots, *Albizia anthelmintica* (A. Rich Brongn.) twigs and *Dicoma schinzii* (O. Hoffm.) against staphylococci (including multi-drug resistant strains) isolated from the learners. To our knowledge, this is the first study undertaken to report on the prevalence, antibiotic resistance trends and virulence characteristics of potentially pathogenic staphylococci among school children in the Mariental District.

This was a cross-sectional study involving five schools. With informed consent from parents/guardians, nasal specimens (swabs) were obtained from 272 randomly selected learners aged 6-14 years. Specimens from swabs were enriched for *Staphylococcus* in brain heart infusion broth prior to isolation on *Staphylococcus* medium no. 110 and tryptone soy agar. Pure cultures were obtained from mixed cultures, Gram-stained and biochemically tested for identification of *S. aureus* and CoNS. Methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant coagulase-negative staphylococci (MRCoNS) were identified by their resistance towards cefoxitin using Kirby-Bauer disk diffusion assay.

By disk diffusion assay, 352 *S. aureus* and 81 coagulase-negative staphylococcal isolates underwent susceptibility testing against the antibiotics ampicillin, cefoxitin, ciprofloxacin, erythromycin, gentamicin, rifampicin and tetracycline. The American Type Culture Collection reference strains *S. aureus* ATCC 25923 and *S. aureus* ATCC 33591 were used for quality control. Isolates were classified as multi-drug resistant when they displayed resistance towards three or more classes of antibiotics.

Twenty-two multi-drug resistant MRSA nasal isolates and the multi-drug resistant MRSA reference strain *S. aureus* ATCC 33591 were screened for production of enterotoxins A-D, using a SET-RPLA toxin detection kit. The microtiter plate assay

was employed to determine biofilm production in 10 nasal *S. aureus* isolates (including one MRSA isolate), as well as *S. aureus* ATCC 25923 and *S. aureus* ATCC 33591. Crude methanolic plant extracts were prepared by maceration, filtration, rotary evaporation and freeze-drying. Qualitative chemical assays and thin layer chromatography (TLC) were used to screen for flavonoids, saponins and anthraquinones in the plant material. To test for antimicrobial activity of the crude methanolic extracts, disk diffusion assays were used. The antibiotics gentamicin and chloramphenicol were used as positive controls, and discs flooded with dimethyl sulfoxide (DMSO) as negative control. A microtiter plate assay determined if extracts could inhibit and/or eradicate *S. aureus* and MRSA biofilms.

Four hundred and thirty-three isolates from 272 swabs were morphologically and biochemically identified as *Staphylococcus* bacteria. Of these isolates, 352 (81.3%) were *S. aureus*, while 81 (18.7%) were CoNS. Furthermore, 51/433 (11.8%) isolates were MRSA and 7/433 (1.6%) MRCoNS. The overall prevalence of *S. aureus* in the study population of 272 learners was 80.5%, and that of CoNS was 25.0%. Methicillin-resistant *S. aureus* was isolated from 48/272 (17.6%) learners and MRCoNS in only 7/272 (2.6%) learners. From the study participants who reported getting nosebleeds, 75.4% were colonized with *S. aureus*, whereas 81.4% who were exposed to cigarette smoke from a household member carried this bacterium.

Out of 433 staphylococcal isolates, 96.0% *S. aureus* and 66.7% CoNS were resistant to ampicillin, with this resistance being significantly higher in *S. aureus* ($P < 0.0001$). Ciprofloxacin and gentamicin were most effective against *S. aureus* isolates, with 99.7% and 93.2% of isolates that were susceptible to these drugs, respectively. Ciprofloxacin was also the most effective drug against isolates of CoNS, with 100.0% susceptibility. Of 352 *S. aureus* isolates, 51 (14.5%) were ceftiofur/methicillin-resistant. Only seven (8.6%) of 81 CoNS isolates were ceftiofur/methicillin-resistant. In total 31 antibiotic resistance patterns were observed, 27 for *S. aureus* and 14 for CoNS. Overall, the three most frequently observed patterns were AP, AP-E, and AP-T. Of all isolates, 12.5% were multi-drug resistant. These include 50 isolates of *S. aureus* and four CoNS. From the 51 MRSA isolates, 43.1% were multi-drug resistant. One of these MRSA isolates showed resistance towards 6/7 antibiotics tested with only ciprofloxacin that was effective against it. Methicillin-resistant CoNS was not multi-drug resistant, with the most common resistance pattern being AP-RP-FOX.

Twenty-three multi-drug resistant MRSA isolates were screened for enterotoxins A-D. Of these, seven were enterotoxigenic. Enterotoxin A was the most prevalent, produced by five isolates. Enterotoxin B was found in one isolate, while enterotoxin C was produced by two isolates. One isolate tested positive for both enterotoxins B and C. Enterotoxin D was not detected in the isolates screened. All 12 *S. aureus* isolates, including two MRSA strains, which were evaluated for biofilm formation were strong biofilm formers in microtiter plates. *Aptosimum albomarginatum* root extract was a moderately active antimicrobial agent against 7/12 *S. aureus* isolates, including two MRSA isolates. Moderate antimicrobial activity was also observed with this extract in 9/54 multi-drug resistant isolates, of which two were MRSA with the same antibiotic resistance pattern of AP-T-RP-FOX. These two isolates may therefore be the same *S. aureus* strain. The root extract was the best biofilm inhibition agent, with highly active inhibition (86.0%) observed in *S. aureus* ATCC 33591 (MRSA), and moderate activity in four other isolates. This extract eradicated the biofilm of *S. aureus* isolate S110 S73 Pure by 40.0% (moderate activity). Flavonoids and saponins/triterpenes may contribute to the root's antimicrobial and anti-biofilm properties. Root extract from *D. schinzii* displayed moderate antimicrobial activity against 8/12 *S. aureus* isolates, while the plant's leaf extract was moderately active against 2/12 *S. aureus* isolates. The leaf extract also moderately inhibited biofilms in three isolates. Flavonoids, coumarins, saponins or triterpenes in the leaves may contribute to its antimicrobial and anti-biofilm properties.

In conclusion, the high prevalence of *S. aureus* and presence of MRSA (including multi-drug resistant bacteria) among study participants calls for improvement in current hygiene practices at schools in the Mariental District to prevent staphylococcal disease. Nosebleeds and exposure to cigarette smoke were identified as possible risk factors for colonization with *S. aureus* in the children. Overall, multi-drug resistance in our study was relatively low. However, almost half of the MRSA isolates were multi-drug resistant, which is of concern. Results from toxin screening indicated that multi-drug resistant MRSA may produce enterotoxins, whereas *S. aureus* and MRSA can produce strong biofilms. Self-infection by these bacteria poses various health risks for the children. Nevertheless, learners should be encouraged to frequently wash their hands to prevent spread of antibiotic-resistant bacteria within the community. Ciprofloxacin and gentamicin may effectively be used to treat staphylococcal infections in this study population.

Antimicrobial activity was observed with *A. albomarginatum* roots and *D. schinzii* roots and leaves, indicating potential use against staphylococcal infections. Noteworthy is this activity of *A. albomarginatum* root extract against multi-drug resistant strains, including MRSA. The root extract was the best anti-biofilm agent against *S. aureus*. It was highly active in inhibiting biofilm formation in one MRSA isolate, and moderately active in inhibiting formation in four isolates. The extract moderately eradicated the biofilm in one isolate. This activity may to some extent be attributed to the presence of the secondary metabolites flavonoids, saponins or triterpenes in the roots. The *D. schinzii* leaf extract moderately inhibited biofilms in three isolates. Flavonoids, coumarins, saponins or triterpenes may play a role its anti-biofilm activity. The present work thus supports the traditional medicinal use of *A. albomarginatum* roots and *D. schinzii* roots and leaves as natural antimicrobial agents, and *A. albomarginatum* roots and *D. schinzii* leaves as anti-biofilm agents, in infections involving the bacteria under study.

Keywords: Prevalence, antibiotic resistance, virulence, *Staphylococcus*, school children, *Aptosimum albomarginatum*, *Dicoma schinzii*, Mariental

List of Publications and Conference/Symposium Proceedings

The following papers resulted from this research:

Walter, S., Beukes, M., Mumbengegwi, D. and Böck, R. (2017) Medicinal value of *Aptosimum albomarginatum* (Marloth and Engl.), *Albizia anthelmintica* (A. Rich Brongn.) and *Dicoma schinzii* (O. Hoffm.) to a small community living at Gochas, southern Namibia. *Journal of Medicinal Plants Research* [online]. **11**(46), pp. 742-748. Available from DOI: 10.5897/JMPR2017.6527 (See Appendix D).

Walter, S., Beukes, M., Mumbengegwi, D. and Böck, R. (2017) Antibiograms and resistance patterns of nasal *Staphylococcus* from Namibian school children. *In preparation*.

Walter, S., Beukes, M., Mumbengegwi, D. and Böck, R. (2017) Antimicrobial and anti-biofilm properties of *Aptosimum albomarginatum* (Marloth and Engl.) and *Dicoma schinzii* (O. Hoffm.) crude methanolic extracts against *S. aureus* and MRSA. *In preparation*.

The following oral/poster presentations resulted from this research:

Oral presentation: Prevalence, antibiotic resistance trends and virulence of *Staphylococcus* among school children in the Mariental District, Namibia (SABINA Annual Meeting, 27-29 October 2014, Dar es Salaam, Tanzania).

Oral presentation: Antimicrobial behaviour of three Namibian ethnomedicinal plants against *Staphylococcus aureus* ATCC 25923 (SABINA Annual Meeting, 27-29 May 2015, Durban, South Africa).

Oral presentation: Prevalence of *Staphylococcus* among school children in the Mariental District, Namibia and effects of traditional medicinal plant extracts on the bacteria (Green Medicine Symposium in collaboration with the University of the Western Cape, 30 July 2015, Windhoek, Namibia).

Poster presentation: Antimicrobial properties of a medicinal plant, *Aptosimum albomarginatum* (!Guxa), against staphylococcal infections (SABINA International Natural Products Conference, 28 August-1 September 2016, Kruger National Park, South Africa).

Table of Contents

Title Page	i
Abstract	ii
List of Publications and Conference/Symposium Proceedings	vi
Table of Contents	viii
List of Tables	xv
List of Figures	xvii
List of Abbreviations and/or Acronyms	xix
Acknowledgements	xxi
Dedication	xxii
Declarations	xxiii
Chapter 1	
Introduction	1
1.1 General introduction	1
1.2 Orientation of the study	2
1.3 Statement of the problem	3
1.4 Hypothesis of the study	4
1.5 Research aim and objectives of the study	4
1.6 Significance of the study	5
1.7 Limitations of the study	6
1.8 Statistical analysis of data	6
1.9 Funding	6
1.10 Ethical statement	7

1.11 Duration	7
References	8
 Chapter 2	
Literature study	12
2.1 An overview of the <i>Staphylococcus</i> genus	12
2.1.1 Antimicrobial resistance in <i>Staphylococcus</i>	13
2.2 The prevalence of <i>Staphylococcus</i> in Namibia	14
2.3 Prevalence of <i>Staphylococcus</i> in school-aged children in other countries ...	15
2.4 Risk factors for nasal carriage of <i>Staphylococcus</i> in children	18
2.5 Antibiotic resistant staphylococci in Namibia	20
2.6 Staphylococcal virulence factors	22
2.6.1 Exotoxins	22
2.6.1.1 Superantigens: Staphylococcal enterotoxins A, B, C and D	22
2.6.2 Enzymes: Coagulase and catalase	24
2.6.3 Biofilm formation	24
2.7 Antibacterial and anti-biofilm activity of traditional medicinal plants against <i>Staphylococcus</i>	25
2.7.1 Phytochemical compounds that may be involved in the activity of medicinal plants against staphylococci: Flavonoids, saponins and anthraquinones	26
References	29

Chapter 3

Nasal colonization of <i>Staphylococcus</i> among apparently healthy school children in the Mariental community, southern Namibia.....	41
Abstract.....	41
3.1 Introduction.....	42
3.2 Materials and methods.....	43
3.2.1 Study area, population and sample collection.....	43
3.2.2 Isolation and identification of bacteria.....	44
3.2.3 Statistical analysis.....	45
3.3 Results and discussion.....	46
3.3.1 Study population and sample collection.....	46
3.3.2 Prevalence of <i>S. aureus</i>, MRSA, CoNS and MRCoNS.....	48
3.4 Conclusion.....	53
References.....	53

Chapter 4

Antibiograms and resistance patterns of nasal staphylococcal isolates from Namibian school children.....	59
Abstract.....	59
4.1 Introduction.....	60
4.2 Materials and methods.....	61

4.2.1 Bacterial cultures used.....	61
4.2.2 Antibiotic susceptibility testing.....	62
4.2.3 Statistical analysis.....	64
4.3 Results and discussion.....	64
4.3.1 Antibiotic susceptibility/resistance of isolates.....	64
4.3.2 Resistance patterns and multi-drug resistance.....	69
4.4 Conclusion.....	72
References.....	73
 Chapter 5	
Enterotoxin production and biofilm formation as virulence factors of <i>S. aureus</i> and multi-drug resistant MRSA from nasal specimens of Namibian school children.....	78
Abstract.....	78
5.1 Introduction.....	79
5.2 Materials and methods.....	81
5.2.1 Bacterial cultures used.....	81
5.2.2 Staphylococcal enterotoxins A-D.....	81
5.2.3 Biofilm formation.....	83
5.3 Results and discussion.....	84
5.3.1 Production of enterotoxins A-D.....	84

5.3.2 Biofilm formation.....	86
5.4 Conclusion.....	88
References.....	88
 Chapter 6	
 Antimicrobial activity of <i>Aptosimum albomarginatum</i> (Marloth and Engl.) and <i>Dicoma schinzii</i> (O. Hoffm.) crude methanolic extracts against <i>S. aureus</i> and MRSA.....	
	94
Abstract.....	94
6.1 Introduction.....	95
6.2 Materials and methods.....	98
6.2.1 Selection of traditional medicinal plants and collection of plant material.....	98
6.2.2 Plant extracts used.....	99
6.2.3 Preparation of crude extracts.....	99
6.2.4 Phytochemical screening for flavonoids, saponins and anthraquinones.....	100
6.2.5 Antimicrobial activity.....	101
6.2.5.1 Microorganisms used.....	101
6.2.5.2 Disk diffusion (inhibition) assays.....	101
6.3 Results and discussion.....	102
6.3.1 Yields of extracts.....	102
6.3.2 Phytochemical screening.....	102

6.3.3 Antimicrobial activity	103
6.3.3.1 <i>Aptosimum albomarginatum</i> root extract	103
6.3.3.2 <i>Albizia anthelmintica</i> twig extract	106
6.3.3.3 <i>Dicoma schinzii</i> root extract	106
6.3.3.4 <i>Dicoma schinzii</i> leaf extract	108
6.4 Conclusion	109
References	109
 Chapter 7	
Anti-biofilm properties of <i>Aptosimum albomarginatum</i> (Marloth and Engl.) and <i>Dicoma schinzii</i> (O. Hoffm.) crude methanolic extracts against <i>S. aureus</i> and MRSA	114
Abstract	114
7.1 Introduction	115
7.2 Materials and methods	116
7.2.1 Bacteria and extracts used	116
7.2.2 Microtiter plate assay for biofilm inhibition	117
7.2.3 Microtiter plate assay for biofilm eradication	119
7.2.4 Thin layer chromatography (TLC) for screening of phytochemical compounds	120
7.3 Results and discussion	122

7.3.1 Biofilm inhibition	122
7.3.2 Biofilm eradication	124
7.3.3 Detection of flavonoids and saponins by TLC	124
7.4 Conclusion	126
References	127
 Chapter 8	
 Conclusions and prospects	131
 Appendices	133

List of Tables

Table 3.1 School children screened for nasal carriage of staphylococci and possible risk factors for colonization.....	47
Table 3.2 Nasal carriage of <i>S. aureus</i> , MRSA, CoNS and MRCoNS among learners aged 6-14 years attending five schools in the Mariental area.....	49
Table 4.1 Antibiotics used in this study and interpretation of inhibition zones of test cultures. Adapted from CLSI (2013) and EUCAST (2017).....	63
Table 4.2 Antibigram for <i>S. aureus</i> isolates (n = 352).....	65
Table 4.3 Antibigram for CoNS isolates (n = 81).....	66
Table 4.4 Resistance patterns for <i>S. aureus</i> and CoNS, including methicillin-resistant isolates, against seven antibiotics.....	71
Table 5.1 Enterotoxin production in multi-drug resistant MRSA from nasal specimens of school children in Mariental, southern Namibia.....	85
Table 5.2 Significant differences in biofilm formation between isolates according to single-factor ANOVA and the Student t-Test.....	88
Table 6.1 Antimicrobial activity of <i>A. albomarginatum</i> root extract against 12 <i>S. aureus</i> isolates, according to mean inhibition zones and standard deviations of triplicate assays.....	105
Table 6.2 Antimicrobial activity of <i>D. schinzii</i> root extract against 12 <i>S. aureus</i> isolates, according to mean inhibition zones and standard deviations of triplicate assays.....	107
Table 6.3 Moderate and weak antimicrobial of <i>D. schinzii</i> leaf extract against <i>S.</i>	

<i>aureus</i> , according to mean inhibition zones and standard deviations of triplicate assays.....	109
Table 7.1 Mobile phases (solvents), controls and spraying reagents used for TLC, adapted from Wagner and Bladt (1996).....	122
Table 7.2 Screening for flavonoid compounds in plant extracts based on R_f values and colour changes on chromatograms before and after spraying with 1.0% Antimony (III) chloride reagent.....	125
Table 7.3 Screening for saponin compounds in plant extracts based on R_f values and colour changes on chromatograms before and after spraying with Vanillin- Sulphuric acid reagent.....	126

List of Figures

- Figure 3.1** Duplicate Kirby-Bauer disk diffusion assay on Mueller-Hinton agar showing reduced inhibition zones of multi-drug resistant MRSA (Resistance pattern AP-T-RP-FOX) from a 9-year-old girl recruited from school D.....52
- Figure 5.1** 24-hour biofilm formation in microtiter plates according to mean absorbance values at 595nm over eight assays. Formation was classified as strong/high with mean OD values > 0.240 (Christensen *et al.*, 1985). Error bars indicate standard deviations.....87
- Figure 6.1** *A. albomarginatum* (A) (Image credit: southafricanplants.net), *A. anthelmintica* (B), and *D. schinzii* (C) growing in the veld at Gochas (Image credit: Sunette Walter).....98
- Figure 6.2** Duplicate inhibition assay with *A. albomarginatum* root extract displaying moderate antimicrobial activity at 60mg/ml (MIC 5mg/ml), against the multi-drug resistant *S. aureus* with the resistance pattern AP-RP-E. This isolate colonized the nose of a 7-year-old girl.....104
- Figure 6.3** Moderate antimicrobial activity of *D. schinzii* root extract at 60mg/ml and 30mg/ml concentrations, with inhibition zones of 13.1 ± 1.0 mm and 11.67 ± 0.58 mm, respectively.....108
- Figure 7.1** Schematic representation of the plate layout..... 119
- Figure 7.2** Percentage biofilm inhibition in microtiter plates by treatment with *A. albomarginatum* crude methanolic root extract. Classification of activity

according to Manner *et al.* (2013, p. 19438): $\geq 85.0\%$ inhibition - highly
active; $\geq 40.0\%$ inhibition - moderately active; $< 40.0\%$ inhibition -
inactive.....123

List of Abbreviations and/or Acronyms

AKBA	Acetyl-II-keto- β -boswellic acid
ANOVA	Analysis of variance
AOM	Acute otitis media
AP	Ampicillin
CA-MRSA	Community-associated methicillin-resistant <i>Staphylococcus aureus</i>
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CoNS	Coagulase-negative staphylococci
CSF	Cerebrospinal fluid
DMSO	Dimethyl-sulfoxide
E	Erythromycin
ETA	Enterotoxin A
ETB	Enterotoxin B
ETC	Enterotoxin C
ETD	Enterotoxin D
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FOX	Cefoxitin
GM	Gentamicin
HCl	Hydrochloric acid
IHO	Industrial hog operation
MDRSA	Multi-drug resistant <i>Staphylococcus aureus</i>

MICs	Minimum inhibitory concentrations
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MRCoNS	Methicillin-resistant coagulase-negative staphylococci
MSSA	Methicillin-susceptible <i>Staphylococcus aureus</i>
NaOH	Sodium hydroxide
NBRI	National Botanical Research Institute
NIP	Namibia Institute of Pathology
ODs	Optical densities
PVL	Panton-Valentine leukocidin
R _f	Retention factor
RP	Rifampicin
SCC _{mec}	Staphylococcal chromosome <i>mec</i>
<i>spa</i>	Surface protein A
STs	Sequence types
Sub-MICs	Sub-minimum inhibitory concentrations
T	Tetracycline
TLC	Thin layer chromatography

Acknowledgements

I would like to express my sincere gratitude towards the following people for their contribution throughout this study:

- My main supervisor, Dr. Ronnie Böck, and co-supervisors, Dr. Mervyn Beukes and Prof. Davis Mumbengegwi for their mentorship, help, support and guidance.
- SABINA (Southern African Biochemistry and Informatics for Natural Products) in collaboration with Carnegie-SIG/RISE (Carnegie-Science Initiative Group/Regional Initiative in Science and Education) for funding of this research.
- Dr. Martha Kandawa-Schulz, for her support and management of funds.
- The personnel at the Ministry of Education, Windhoek and Mariental for their time and assistance.
- The principals, teachers, parents and learners for their cooperation and willingness to participate in this study.
- Mr. Sakkie Coetzee and Ms. Anna Frederick, for sharing their knowledge regarding the use of traditional medicinal plants.
- Ms. Medried Muyongo and Ms. Celine Mukakalisa, for ordering of lab consumables.
- My co-workers at the Biomedical Research Laboratory for advice and help with some experiments.
- Everyone in the Biological Sciences Department and Multidisciplinary Research Centre (MRC) who lend a helping hand from time to time.
- The Chemistry and Biochemistry Department for allowing me to use some of their equipment.
- Everyone at the Biochemistry Department, University of Pretoria for your input during my exchange visit.
- My examiners, for their willingness to evaluate my manuscript and their valuable feedback.
- Friends, family and acquaintances who supported me and believed in me.
- God, for daily blessings and strength to keep going.

Dedication

This study is dedicated to my loving parents, Fritz and Alta Walter.

**“Science is magic that works.” – Kurt Vonnegut
Jr., Cat’s Cradle**

Declarations

I, Sunette Walter, hereby declare that this study is my own work and is a true reflection of my research, and that this work, or any part thereof has not been submitted for a degree at any other institution.

No part of this dissertation may be reproduced, stored in any retrieval system, or transmitted in any form, or by means (e.g. electronic, mechanical, photocopying, recording or otherwise) without the prior permission of the author, or the University of Namibia in that behalf.

I, Sunette Walter, grant the University of Namibia the right to reproduce this dissertation in whole or in part, in any manner or format, which the University of Namibia may deem fit.

Name of Student

Signature

Date

Chapter 1

Introduction

1.1 General introduction

Staphylococcus are Gram-positive cocci ranging from 0.5 to 1.5µm in diameter (Prescott *et al.*, 2002, p. 919), and can occur as single cocci, pairs, tetrads, short chains or irregular clusters that resemble bunches of grapes (Lowy, 2010, p. 386; Brooks *et al.*, 2013, p. 199). These bacteria are catalase-positive, non-motile, aerobic, facultatively anaerobic and non-spore forming (Lowy, 2010, p. 386). Some strains have polysaccharide capsules that contribute to their pathogenicity (Brooks *et al.*, 2013, p. 200).

The *Staphylococcus* genus belongs to the family *Micrococcaceae*, order *Eubacteriales* (Dorland's illustrated medical dictionary, 2003, p. 1754). It contains at least 40 species. *Staphylococcus aureus*, *Staphylococcal epidermidis*, *Staphylococcus lugdinensis* and *Staphylococcus saprophyticus* are the most frequently found ones that are of clinical significance (Brooks *et al.*, 2013, p. 199). More than 30 staphylococcal species are pathogens. Among the staphylococcal species, *S. aureus* is by far the most pathogenic and virulent for humans (Lowy, 2010, p. 386), while *S. epidermidis* is often responsible for biofilm infections (Brooks *et al.*, 2013, p. 205).

Though known as commensal bacteria in most people (Brooks *et al.*, 2013, p. 202 and p. 204), *Staphylococcus* have been known to be opportunistic as well (Dorland's illustrated medical dictionary, 2003, p. 1754). Infections can occur when colonizing strains enter normal sterile sites in the body through damaged skin or mucosal surfaces (Forbes *et al.*, 2007, p. 254). Staphylococci from an asymptomatic carrier or diseased person can be transmitted by the hands, released from the respiratory tract, or transported in or on living and non-living objects (Prescott *et al.*, 2002, p. 919). Of

concern is the spread of antibiotic resistant bacteria within hospitals and communities. According to Byarugaba (2010, p. 17), antibiotic resistance within communities are increasing due to widespread suboptimal use of antibiotics in outpatient settings and use of antibiotics in farming activities. Cleanliness, hygiene and aseptic management of wounds are effective ways to control staphylococcal disease (Prescott *et al.*, 2002, p. 923). Handwashing in particular is very important in disease control (Brooks *et al.*, 2013, p. 205).

1.2 Orientation of the study

The human nasal cavity contains mostly *S. aureus* and *S. epidermidis* as normal microflora (Forbes *et al.* 2007, p. 255) but is also a reservoir for transmission of other *Staphylococcus* species and predisposes one to potential self-infection (Pynnonen *et al.*, 2011, p. 2). Results obtained by Arali *et al.* (2016, p. 625) showed that healthy school children under 16 years are potential carriers of *S. aureus*, especially methicillin-resistant *S. aureus* (MRSA) and multi-drug resistant strains. According to Dukic *et al.* (2013, p. 1) MRSA emerged in the 1960s when it was not really considered a public health threat. This changed in the 1990s when community-associated MRSA (CA-MRSA) strains started circulating. Nowadays, CA-MRSA infections are common, causing serious and sometimes deadly infections in otherwise healthy individuals. According to Alzoubi *et al.* (2014, p. 114), children are asymptomatic reservoirs for CA-MRSA which enables these bacteria to rapidly spread within communities.

Bacterial infections are normally treated with antibiotics, which is often necessary. People who are frequently exposed to antibiotics are however at risk of becoming colonized with resistant strains. With drug-resistance being a never-ending problem,

one should look into alternative treatment options, such as use of natural products, for example plant-derived products with antimicrobial and/or anti-biofilm activity. Such natural products may in some instances also be used as adjuvants together with antibiotics. The present study portrays the prevalence of *Staphylococcus* species among school children in and around the town of Mariental, southern Namibia. It also investigates antibiotic resistance trends and some virulence factors associated with these isolated bacteria. Lastly, three traditional medicinal plants currently being used in Namibia to treat infections, including staphylococcal infections, are investigated.

1.3 Statement of the problem

The bacterium *S. aureus* causes illness and death worldwide, including sub-Saharan Africa. Nasal carriage of *S. aureus* is a major risk factor for becoming infected with this bacterium (Reid *et al.*, 2017, p. 795). Although national data and studies on staphylococcal infections in Namibia are very limited, there are indications of the presence of potentially pathogenic staphylococci and MRSA in Namibia (Lauschke and Frey, 1994; Mengistu *et al.*, 2013; PathCare Namibia, 2015). Globally, there is an increase in *Staphylococcus*-related health problems and resistance of these bacteria to currently used antibiotics (Okwu *et al.*, 2012; Wang *et al.*, 2012; Dinić *et al.*, 2013; Perveen *et al.*, 2013).

A study by Arali *et al.* (2016, p. 625) showed that healthy school children younger than 16 years can be colonized by *S. aureus*, especially MRSA and multi-drug resistant strains. According to Alzoubi *et al.* (2014, p. 114) children are asymptomatic reservoirs for CA-MRSA and this enables these bacteria to rapidly spread within communities. There is currently a lack of published evidence-based research and

statistics about the prevalence, antibiotic resistance profiles and virulence of disease-causing staphylococci (including MRSA) in Namibian school children.

Namibian people are using plants as natural medicine in the traditional setting to treat various illnesses, including those caused by staphylococci. To rule out the possibility of placebo effects, these plants should be tested in the laboratory to validate their medicinal value. This research therefore also aimed to investigate the effectiveness of three such plants currently being used for medicinal purposes.

1.4 Hypothesis of the study

The hypothesis for the present research is that there will be a high prevalence of *Staphylococcus* nasal colonization in school children in the Mariental District, with some of these bacteria being resistant against currently used antibiotics. Unlike in other countries with high prevalence of MRSA, the MRSA rate in this study is expected to be low, since the latest available PathCare data (PathCare Namibia, 2015) (Appendix C) indicates only 8.0% MRSA for 2015. It is hypothesized that traditional medicinal plants that are used in Namibia can be effective against staphylococcal-related infections.

1.5 Research aim and objectives of the study

The main aim this study was to investigate potentially pathogenic community-associated staphylococci in school children. Objectives were:

- To determine the prevalence of nasal *S. aureus* and CoNS in school children from the Mariental District.

- To characterize the bacteria in terms of their antibiograms and drug resistance patterns.
- To screen bacterial isolates for their ability to produce enterotoxins and form biofilms as potential virulence factors.
- To assess the antimicrobial and anti-biofilm properties of crude methanolic extracts from *Aptosimum albomarginatum* (Marloth and Eng.) roots, *Albizia anthelmintica* (A. Rich Brongn.) twigs and *Dicoma schinzii* (O. Hoffm.) against staphylococci (including multi-drug resistant strains) isolated from the learners.

1.6 Significance of the study

To our knowledge, this is the first study undertaken to report on the prevalence, antibiotic resistance trends and virulence characteristics of potentially pathogenic staphylococci among school children in the Mariental District. Information obtained from this research can be useful in assisting school health programmes. Also, the study will generate new knowledge and add to existing knowledge on traditional use of plants to treat staphylococcal-related diseases. It will give scientific evidence on the usefulness of these plants against bacterial infections. Van Wyk (2015, p. 10) explains the purpose and worth of ethnobotanical studies. Two key points in his article pertain to the present study. Firstly, since indigenous knowledge are passed on by word of mouth, ethnobotanical studies can ensure that this knowledge is kept for future generations in the form of publications. Secondly, ethnobotanical research can help to identify commercial plants which can potentially be a source of income for people if the development process is successful.

1.7 Limitations of the study

Only nasal samples were taken (staphylococci can colonize other anatomical sites as well). Since this was a cross-sectional study, children were swabbed only once, and it was not possible to determine the carrier status of each child, in other words if they are persistently or intermittently (transiently) colonized by *Staphylococcus*. Only crude plant extracts (not specific phytochemical compounds) were tested against bacteria.

1.8 Statistical analysis of data

A chi-square test for comparison of proportions with MedCalc statistical software (MedCalc statistical software version 16.4.3 {MedCalc software bvba, Ostend, Belgium; <https://www.medcalc.org>; 2016}) was used to compare percentage differences of *S. aureus*, MRSA, CoNS and MRCoNS between age groups and gender. This test was also used to compare percentage differences in antibiotic resistance between *S. aureus* and CoNS. Single-factor ANOVA and the Student t-Test (two-sample assuming equal variances) Microsoft Excel was used to determine significant differences in biofilm formation between isolates. Statistical significant differences was indicated by a P-value of ≤ 0.05 and F-value $> F$ critical value.

1.9 Funding

This research was funded by SABINA (Southern African Biochemistry and Informatics for Natural Products) in collaboration with Carnegie-SIG/RISE (Carnegie-Science Initiative Group/Regional Initiative in Science and Education). Funding was awarded annually (2014 - 2016) on the basis of satisfactory progress reports.

1.10. Ethical statement

Permission to conduct this study and ethical clearance was obtained from the Ministry of Education, school principals and parents (Appendices A1-A3). Learners were randomly selected from class lists. Parents or guardians of these learners were asked to complete consent forms, but were under no obligation to do so. No one was forced to take part in this study and participation was completely voluntary; the children were asked if they want to donate nasal samples. Participants did not receive any financial compensation. Names of learners and schools were not mentioned within reported results. Specific “codes” were assigned to them. Personal information was kept confidential. Colonization with *Staphylococcus* does not imply disease, therefore children that tested positive for *Staphylococcus* were not isolated (they kept attending school) because it is not necessary to keep them away from other children. Instead an effort was made to educate all teachers, learners and parents in general on preventative measures (for example encouraging good hygiene practices such as regular hand washing) by giving them information leaflets (Appendix B). Parents were informed verbally in English or a local language (local staff) about the project and were allowed to ask any questions they had. All questions were answered by the Principal Investigator. It remains a sensitive and private issue and care was taken not to upset learners or parents.

Plants were collected with a permit from the Ministry of Environment and Tourism and collection was done in a sustainable manner.

1.11 Duration

This study was conducted and completed on full-time basis within four years.

References

- Alzoubi, H.M., Agel, A.A., Al-Sarayreh, S.A. and Al-Zayadneh, E. (2014) Methicillin-resistant *Staphylococcus aureus* nasal carriage among primary school-aged children from Jordan: Prevalence, antibiotic resistance and molecular characteristics. *Journal of Egyptian Public Health Association* [online]. **9**(3), pp. 114-118. Available from DOI: 10.1097/01.EPX.0000454671.83406.e8 [Accessed 4 June 2016].
- Arali, S.M., Kulkarni, V., Manjula, N.G., Gaddad, S.M., Jayaraj, Y.M. and Shivannavar, C.T. (2016) Prevalence and antibiotic susceptibility profile of MRSA isolated from the anterior nares of school going children in Gulbarga. *International Journal of Medical Science and Public Health* [online]. **5**(4), pp. 620-626. Available from DOI: 10.5455/jmsph.2016.0805201592 [Accessed 7 December 2016].
- Brooks, G.F., Carroll, K.C., Butel, J.S., Morse, S.A. and Mietzner, T.A. (2013) *Jawetz, Melnick & Adelberg's Medical Microbiology*. 26th ed. s.l.: McGraw-Hill.
- Byarugaba, D.K. (2010) Mechanisms of antimicrobial resistance. In: Sosa, A., Byarugaba, D.K., Amábile-Cuevas, C.F., Hsueh, P., Kariuki, S. and Okeke, I.N. (eds.) *Antimicrobial resistance in developing countries*. New York: Springer, pp. 15-26.
- Dinić, M., Vuković, S., Kocić, B., Stanković-Đorđević, D. and Bogdanović, M. (2013) Nasal carriage of *Staphylococcus aureus* in healthy adults and in school children. *Acta Facultatis Medicae Naissensis* [online]. **30**(1), pp. 31-36. Available from DOI: 10.2478/v10283-012-0033-3 [Accessed 29 July 2015].
- Dorland's illustrated medical dictionary* (2003) 30th ed. Philadelphia: Saunders.

- Dukic, V.M., Lauderdale, D.S., Wilder, J., Daum, R.S. and David, M.Z. (2013) Epidemics of community-associated methicillin-resistant *Staphylococcus aureus* in the United States: A meta-analysis. *PLOS ONE* [online]. **8**(1). Available from DOI: 10.1371/journal.pone.0052722 [Accessed 29 July 2015].
- Forbes, B.A., Sahm, D.F. and Weissfeld, A.S. (2007) *Bailey and Scott's Diagnostic Microbiology*. 12th ed. St. Louis: Mosby.
- Lauschke, F.H.M. and Frey, C.T. (1994) Hematogenous osteomyelitis in infants and children in the northwestern region of Namibia: Management and two-year results. *Journal of Bone and Joint Surgery* [online]. **76**(4), pp. 502-510. Available from:
<http://journals.lww.com/jbjsjournal/pages/articleviewer.aspx?year=1994&issue=04000&article=00004&type=abstract> [Accessed 23 March 2017].
- Lowy, F.D. (2010) Staphylococcal infections. In: Kasper, D. and Fauci, A. (eds.) *Harrison's infectious diseases*. s.l.: McGraw-Hill, pp. 386-399.
- MedCalc (2016) *MedCalc statistical software version 16.4.3*. Available from:
<https://www.medcalc.org>.
- Mengistu, A., Gaeseb, J., Uaaka, G., Ndjavera, C., Kambyambya, K., Indongo, L., Kalemeera, F., Ntege, C., Mibirizi, D., Joshi, M.P. and Sagwa, E. (2013) Antimicrobial sensitivity patterns of cerebrospinal fluid (CSF) isolates in Namibia: Implications for empirical antibiotic treatment of meningitis. *Journal of Pharmaceutical Policy and Practice* [online]. **6**(4). Available from DOI: 10.1186/2052-3211-6-4 [Accessed 29 July 2015].
- Okwu, M., Bangbala, S. and Aborisade, W. (2012) Prevalence of nasal carriage of community-associated methicillin-resistant *Staphylococcus aureus* (CA-

MRSA) among healthy primary school children in Okada, Nigeria. *Journal of Natural Sciences Research* [online]. 2(4), pp. 61-65. Available from: <https://www.scribd.com/document/99264977/Prevalence-of-Nasal-Carriage-of-Community-Associated-Methicillin> [Accessed 29 July 2015].

PathCare Namibia (2015) Antimicrobial susceptibility data. PathCare [online]. Available from: http://196.25.36.50/webfiles/files/ANTIMICROBIAL_SUSCEPTIBILITY_DATA_Staphylococcus_aureus.pdf [Accessed 23 November 2015].

Perveen, I., Majid, A., Knawal, S., Naz, I., Sehar, S., Ahmed, S. and Raza, M.A. (2013) Prevalence and antimicrobial susceptibility pattern of methicillin-resistant *Staphylococcus aureus* and coagulase-negative staphylococci in Rawalpindi, Pakistan. *British Journal of Medicine & Medical Research* [online]. 3(1), pp. 198-209. Available from: <http://imsear.li.mahidol.ac.th/bitstream/123456789/162809/1/bjmmr2013v3n1p198.pdf> [Accessed 29 July 2015].

Prescott, L.M., Harley, J.P. and Klein, D.A. (2002) *Microbiology*. 5th ed. New York: McGraw-Hill.

Pynnonen, M., Stephenson, R.E., Schwartz, K., Hernandez, M. and Boles, B.R. (2011) Hemoglobin promotes *Staphylococcus aureus* nasal colonization. *PLOS Pathogens* [online]. 7(7). Available from DOI: 10.1371/journal.ppat.1002104 [Accessed 23 March 2017].

Reid, M.J.A., Fischer, R.S.B., Mannathoko, N., Muthoga, C., McHugh, E., Essigmann, H., Brown, E.L. and Steenhoff, A.P. (2017) Prevalence of *Staphylococcus aureus* nasal carriage in human immunodeficiency virus-infected and uninfected children in Botswana: Prevalence and risk factors. *The American Journal of*

Tropical Medicine and Hygiene [online]. **96**(4), pp. 795-801. Available from DOI: <https://doi.org/10.4269/ajtmh.16-0650> [Accessed 14 July 2017].

Van Wyk, B-E. (2015) Die kulturele en praktiese waarde van inheemse kennis oor plantgebruike in die 21ste eeu. *Suid-Afrikaanse Tydskrif vir Natuurwetenskap en Tegnologie* [online]. **34**(1). Available from DOI: 10.4102/satnt.v34i1.1349 [Accessed 19 January 2017].

Wang, L., Liu, Y., Yang, Y., Huang, G., Wang, C., Deng, L., Zheng, Y., Fu, Z., Li, C., Shang, Y., Zhao, C., Sun, M., Li, X., Yu, S., Yao, K. and Shen, X. (2012) Multidrug-resistant clones of community-associated methicillin-resistant *Staphylococcus aureus* isolated from Chinese children and the resistance genes to clindamycin and mupirocin. *Journal of Medical Microbiology* [online]. **61**, pp. 1240-1247. Available from DOI: 10.1099/jmm.0.042663-0 [Accessed 29 July 2015].

Chapter 2

Literature review

2.1 An overview of the *Staphylococcus* genus

The *Staphylococcus* genus belongs to the family *Micrococcaceae*, order *Eubacteriales* (Dorland's illustrated medical dictionary, 2003, p. 1754). These bacteria are spherical in shape, 0.5 to 1.5µm in diameter (Prescott *et al.*, 2002, p. 919), and can occur as single cocci, pairs, tetrads, short chains or irregular clusters that resemble bunches of grapes (Lowy, 2010, p. 386; Brooks *et al.*, 2013, p. 199). Staphylococci grow well on many types of agar, with optimal growth occurring at 37°C. Colonies on solid media produce pigments that vary from white to deep yellow, and are round, smooth, raised and glistening (Brooks *et al.*, 2013, p. 199). Furthermore, they are catalase-positive (as opposed to streptococci), non-motile, aerobic, facultatively anaerobic and do not form spores (Lowy, 2010, p. 386). They are relatively resistant to drying, heat and up to 9.0% sodium chloride (Brooks *et al.*, 2013, p. 199). Staphylococci are potentially pathogenic, causing harmful changes in organ tissue and serious opportunistic infections (Dorland's illustrated medical dictionary, p. 1754). Some strains have polysaccharide capsules that aid with their pathogenicity (Brooks *et al.*, 2013, p. 200). *Staphylococcus* is widespread in the environment. It is found in the air, dust, sewage, water, milk and food, on food equipment, environmental surfaces, humans and animals (Food and Drug Administration, 2012). Staphylococci are able to exist as commensals of the skin, nose and throat of most people, but can however become pathogenic, causing infections when the opportunity arises (Brooks *et al.*, 2013, p. 204). As explained by Lowy (2010, p. 388) infections can occur when colonizing strains enter normally sterile sites in the body through damaged skin or mucosal surfaces. The human nasal cavity contains mostly *Staphylococcus aureus* and *Staphylococcus*

epidermidis as normal microflora (Forbes *et al.*, 2007, p. 255) but is also a reservoir for transmission of other *Staphylococcus* species and predisposes one to potential self-infection (Pynnonen *et al.*, 2011, p. 2). Of all species, *S. aureus* is considered the most virulent (Lowy, 2010, p. 386), while *S. epidermidis* is often responsible for biofilm infections (Brooks *et al.*, 2013, p. 205).

Of concern is the spread of antibiotic resistant bacteria within hospitals and communities. According to Byarugaba (2010, p. 17), antibiotic resistance within communities are increasing due to widespread suboptimal use of antibiotics in outpatient settings and use of antibiotics in farming activities. Findings by Arali *et al.* (2016, p. 625) showed that healthy school children under 16 years are potential carriers of *S. aureus*, especially methicillin-resistant *S. aureus* (MRSA) and multi-drug resistant strains. According to Alzoubi *et al.* (2014, p. 114) children are asymptomatic reservoirs for community-associated MRSA (CA-MRSA) which enables these bacteria to rapidly spread within communities.

Staphylococci from an asymptomatic carrier or diseased person can be released from the respiratory tract, transmitted by the hands, or transported in or on living and non-living objects. Cleanliness, hygiene and aseptic management of wounds are effective ways to control staphylococcal disease (Prescott *et al.*, 2002, p. 919 and p. 923). Handwashing in particular is very important in disease control (Brooks *et al.*, 2013, p. 205).

2.1.1 Antimicrobial resistance in *Staphylococcus*

According to Cowan *et al.* (2012, p. 346) bacteria can become resistant to an antibiotic mainly as a result of one of the following: (1) Mutations in chromosomal genes. (2) Acquisition of drug resistant genes from other species. (3) Becoming dormant when

exposed to an antibiotic, slowing or stopping their metabolism to not be harmed by the drug.

Staphylococcal susceptibility to antibiotics can vary, with resistance caused by several mechanisms. Resistance towards aminoglycosides, macrolides and tetracyclines, for example, is plasmid-mediated and common in staphylococci. Resistance to penicillins, such as ampicillin and penicillin G, is because of the enzymatic action of β -lactamase in the bacteria and under plasmid control (Brooks *et al.*, 2013, p. 200), whereas resistance to methicillin is due to the presence of *mecA* or *mecC* genes (Kerschner *et al.*, 2015).

The mechanism for resistance to penicillins and some cephalosporins is drug inactivation: The bacteria produce β -lactamases and these enzymes hydrolyze the β -lactam ring of the antibiotic, making the drug inactive (Cowan *et al.*, 2012, p. 346). Methicillin resistance on the other hand, is encoded and regulated by a sequence of genes located in a region of the chromosome known as the staphylococcal cassette chromosome *mec* (*SCCmec*). The *mecA* gene on this locus encodes a penicillin-binding protein (PBP2a) that has a low affinity for β -lactam antibiotics, causing the resistance (Brooks *et al.*, 2013, p. 200).

2.2 The prevalence of *Staphylococcus* in Namibia

Studies on the prevalence of staphylococci and MRSA in Namibia are scarce. Lauschke and Frey (1994, p. 505) detected penicillin-resistant *S. aureus* in children with osteomyelitis in northwestern Namibia. A more recent study by Mengistu *et al.* (2013) also revealed the presence of *Staphylococcus* in Namibia. In a cross-sectional descriptive study, these investigators analysed data from the Namibia Institute of Pathology (NIP) MEDITECH database 2009-2012. They found that

Staphylococcus, including those species that cause meningitis, was amongst the most common isolates from clinical samples of cerebrospinal fluid (CSF) from 33 state hospitals throughout Namibia, comprising 7.2% of all organisms isolated. Species from the *Staphylococcus* genus that were detected in CSF include *S. aureus*, *S. epidermidis*, *S. capitis*, *S. haemolyticus*, *S. hominis*, *S. saprophyticus* and *S. simulans*. Other cocci found in the fluid specimens were from the genera *Micrococcus*, *Neisseria* (Gram-negative cocci), *Enterococcus* and *Streptococcus*. Similar to the study by Lauschke and Frey (1994) 25 staphylococcal isolates (73.5%) were resistant to penicillin, while one isolate (2.9%) was intermediately resistant to this antibiotic. Three of the CSF staphylococcal isolates (33.3%) were resistant to oxacillin. Although not mentioned as such by these authors, the three resistant isolates could possibly be MRSA, since oxacillin resistance can be an indication of methicillin resistance (Sina *et al.*, 2013, p. 2; Reta *et al.*, 2015, p. 2). Methicillin-resistant strains may be multi-drug resistant, which is of great concern. Antimicrobial susceptibility data (PathCare 2015) indicated a MRSA prevalence of 8.0% for 2015. The PathCare data is provided in Appendix C and discussed further in Section 2.5.

2.3 Prevalence of *Staphylococcus* in school-aged children in other countries

In Egypt, Alzoubi *et al.* (2014) collected 210 nasal swabs from children aged 6-11 years. They detected 7.1% CA-MRSA.

In a cross-sectional study by Erami *et al.* (2014) 350 healthy children in Kashan, Iran were screened for nasal colonization with *S. aureus*. The overall prevalence for *S. aureus* was 26.3%, with a much higher prevalence in boys (67.4%) than girls (32.6%). The mean age of children that tested positive was 6.65 ± 4.46 years. Of 92 positive

cultures, 33 (35.9%) were MRSA. Of the MRSA isolates, 57.6% were multi-drug resistant.

Tangchaisuriya *et al.* (2014) isolated 95/217 (44.0%) CoNS and 78/217 (36.0%) *S. aureus* from the noses of healthy Thai primary school learners aged 3-12 years. Seventy-three learners (34.0%) were colonized with MSSA, while only five (2.0%) had MRSA in their noses.

In Songpan County, southwest China, Deng *et al.* (2014) detected *S. aureus* in only 16 of 673 nasal samples (2.4%) from Tibetan school children aged 7-18 years. Most isolates came from the age group 7-13 years and prevalence decreased with increasing age. Although more boys (nine) than girls (seven) carried *S. aureus*, this difference in carriage rate was not significant ($P = 0.889$). No MRSA could be found.

In India, Bharathi *et al.* (2014) determined the nasal carriage rate of *S. aureus*, MSSA and CA-MRSA among 230 school-going children aged 5-10 years. *Staphylococcus aureus* was present in 51 children (22.2%), MSSA in 34 (14.8%) and MRSA in 17 (7.4%). Colonization with staphylococci was less in girls (19.1%) than boys (28.2%), while colonization with MRSA was almost the same (7.2% and 7.7%). This MRSA prevalence is similar to the prevalence (7.1% CA-MRSA) found in Egypt (Alzoubi *et al.*, 2014).

Esposito *et al.* (2015) took nasal swabs from healthy children and teenagers aged 6-17 years attending school in Milan, Italy. They screened 497 subjects and isolated 128 (25.7%) *S. aureus*. Only three (2.3%) of the isolates were MRSA and came from the age group 15-17 years.

In a cross-sectional study conducted in India by Jeyakumari *et al.* (2015) participants were healthy school children aged 3-8 years. Nasal carriage of *S. aureus* was 26.0% while that of MRSA was 2.6%. *Staphylococcus aureus* carriage here is 3.8% higher

and MRSA carriage 4.8% lower compared to the Indian study by Bharathi *et al.* (2014). Furthermore, MRSA prevalence is similar to that of Tangchaisuriya *et al.* (2014) (2.0% in Thai children aged 3-12 years), and Esposito *et al.* (2015) (2.3% in Italian children aged 6-17 years).

Another cross-sectional study by Nikfar *et al.* (2015) determined the frequency of MRSA carriage in healthy children in Ahvaz city in Iran. These researchers took nasal swabs from 864 children under the age of 14 years attending school and daycare centers. Methicillin-resistant *S. aureus* was identified by 1µg oxacillin disk method and detection of the *mecA* gene by PCR. The prevalence of CA-MRSA in this study was only 0.8% and seemed to be significantly higher in females (P = 0.005). The MRSA rate in this study (0.8%) is much lower than the rate reported by Erami *et al.* (2014), who reported a MRSA prevalence of 35.9% for children in Kashan, Iran.

A community based cross-sectional study involved children from nine primary schools in Bahir Dar Town, Ethiopia. In this study, Reta *et al.* (2015) recruited 300 participants aged 6-12 years. Frequency of *S. aureus* isolation from nasal specimens was 123/300 (41.0%), while 17/123 isolates (13.8%) were resistant to cefoxitin (MRSA). Similar to the findings by Bharathi *et al.* (2014) and Erami *et al.* (2014) *S. aureus* carriage was higher in males. From a total of 17 (13.8%) MRSA isolates, 12 isolates came from males and five from females.

Arali *et al.* (2016) determined the prevalence of *S. aureus* in children aged 5-15 years, attending school in Gulbarga, Karnataka, India. Out of 131 nasal swabs, 77.9% harboured *S. aureus*. Of 102 *S. aureus* isolates, 4 (3.9%) were MRSA. Females were colonized more frequently (82.3%) than males (73.9%). This contradicts the Indian study by Bharathi *et al.* (2014) who found significantly more staphylococci in males (28.2%) than females (19.1%).

In Zakho city, Iraq, Assafi *et al.* (2017) conducted a community-based cross sectional study to assess the occurrence and antibiotic susceptibility pattern of nasal *S. aureus* and MRSA in 300 primary school children, aged 8-12 years. In this study, *S. aureus* prevalence was relatively low at 30.0%, while MRSA comprised 4.0%, which is also low. *Staphylococcus aureus* colonization was significantly higher in females (37.7%) than males (25.3%). Methicillin-resistant *S. aureus* colonization was also slightly higher in females (4.4%) than males (3.8%).

Gong *et al.* (2017) isolated nasal *S. aureus* from 314 healthy Tibetan school children in the age group 6-11 years. *Staphylococcus aureus* was present in 16 (5.1%) children, and higher in boys, but not significantly higher than in girls ($P = 0.7060$). Methicillin-resistant *S. aureus* was found in 3/314 (0.96%) participants. The *S. aureus* prevalence was 2.7% higher than that previously reported in Tibetan school children (Deng *et al.*, 2014). Similar to Deng *et al.*, this prevalence was not significantly different between genders. Methicillin-resistant *S. aureus* was detected, while Deng *et al.* did not find any.

2.4 Risk factors for nasal carriage of *Staphylococcus* in children

Nasal carriage of *S. aureus* is a major risk factor for becoming infected with this bacterium (Reid *et al.*, 2017, p. 795). The presence of the blood component haemoglobin in nasal secretions is a host factor that can modulate *S. aureus* gene expression and increase nasal colonization with this bacterium (Pynnonen *et al.*, 2011, p. 1). In the present study, parents of study participants had to complete a questionnaire giving an indication of whether their child was suffering from frequent nosebleeds. According to Pynnonen *et al.* (2011, p. 2), persons who experience frequent nosebleeds are prone to *S. aureus* colonization. Parents also had to state whether they were

smokers. Children who are exposed to second-hand cigarette smoke may suffer from smoke-enhanced bacterial infections. In an American study, Kulkarni *et al.* (2012, p. 3805 and p. 3807) showed that cigarette smoke exposure caused increased biofilm growth and enhanced fibronectin binding in pathogenic *S. aureus*.

A survey by Erami *et al.* (2014, p. 3-4) revealed the following risk factors for nasal colonization with *S. aureus*: Recent antibiotic usage (for nasal colonization with *S. aureus*, MRSA and multi-drug resistant *S. aureus*, abbreviated MDRSA). Antibiotic usage during the last three months and being of the male gender (for nasal colonization with MRSA). Antibiotic usage in the last three months, family size of more than four members and parents that smoke (for nasal colonization with MDRSA).

In addition to the above (Soltani *et al.*, 2014, p. 3) mention the following risk factors for nasal colonization with MRSA: Hospitalization in the past three months. Being less than four years old. Sleeping with parents. Shetty *et al.* (2014, p. 13) mentioned two statistically significant risk factors for nasal colonization with *S. aureus*: Children younger than six years. Joint families (six or more family members). Alzoubi *et al.* (2014, p. 116) identified allergic rhinitis as a significant risk factor for MRSA nasal colonization in children.

Reta *et al.* (2015, p. 3) identified the following epidemiological risk factors associated with MRSA nasal carriage among school children: Recurrent acute otitis media (AOM) – three ear infections in six months or more than three infections in 12 months. Taking antibiotics repeatedly for these recurrent infections may lead to development of resistant bacteria. Also, frequently touching the ear and nose without washing hands can allow for MRSA from the ear to enter the nose and the nose can become colonized with these bacteria. It is then possible for MRSA to spread further into the body.

Taking an antibiotic in the previous 12 months. A child in the household taking an antibiotic in the previous 12 months.

In recent years, a new risk factor for staphylococcal nasal colonization have emerged. Hatcher *et al.* (2017) were first to investigate parental or caregiver occupation in industrial hog operations as a risk factor of child *S. aureus* nasal carriage and infection in the United States. These researchers found *S. aureus* nasal carriage prevalence to be higher in North Carolina children living with industrial hog operation (IHO) workers compared to a community referent group. In IHO children, 49.0% carried *S. aureus*, compared to 31.0% in the referent group. Furthermore, 14.0% MRSA and 23.0% multi-drug resistant *S. aureus* was isolated from IHO children, compared to only 6.0% and 8.0% in community referent children. They ascribed these findings mainly due to children coming into contact with bacteria living on personal protective equipment taken home by workers. It is possible for some of the participants in the present study to be exposed to such bacteria-harboring protective equipment, since there is a pig farm (Mariental Piggery) located south of Mariental.

2.5 Antibiotic resistant staphylococci in Namibia

As stated in Section 2.2, Lauschke and Frey (1994, p. 505) isolated penicillin-resistant *S. aureus* from children with the bone disease osteomyelitis in northwestern Namibia and Mengistu *et al.* (2013, p. 6) analysed antibiotic susceptibility data of 36 staphylococci isolated from CSF and found that 73.5% of these bacteria were resistant to penicillin. Resistance towards other antibiotics (Mengistu *et al.*, 2013) were as follows: amikacin (0%), vancomycin (0%), fusidic acid (2.9%), ciprofloxacin (19.0%), cephalothin (25.0%), clindamycin (22.2%), tetracycline (29.6%), erythromycin (32.3%), oxacillin (33.3%), cloxacillin (34.5%), cefuroxime (40.0%) and gentamicin

(52.9%). Although not indicated as such by these investigators, the oxacillin-resistant isolates could be suspected to be MRSA.

Antimicrobial susceptibility data (PathCare Namibia, 2015) for *S. aureus* for the time period 2001-2015 (Appendix C) was retrieved from the PathCare Namibia website (pathcarenamibia.com). There was however no indication of whether this data was community-associated, hospital-associated, and the age-groups involved. Similar to observations by Lauschke and Frey (1994, p. 505) and Mengistu *et al.* (2013, p. 6), the presence of penicillin-resistant *S. aureus* is seen in the PathCare data across the period 2001-2015 (a mean resistance of 92.0%). The most likely explanation for this observation is that these bacteria produce the enzyme penicillinase/ β -lactamase, which destroys the action of penicillin, making them resistant to this drug (Akindele *et al.* 2010, p. 232; Dinić *et al.*, 2013, p. 34).

Staphylococcus aureus was in general highly susceptible to the following antibiotics tested by PathCare (PathCare Namibia, 2015): Cloxacillin, cotrimoxazole, clindamycin, erythromycin, fusidic acid, rifampicin, tetracycline, vancomycin, teicoplanin, gentamicin, linezolid, ofloxacin, ciprofloxacin and moxifloxacin. Noteworthy is the very high susceptibility towards vancomycin (99.9% susceptible). Vancomycin is often used for treatment of staphylococcal (including MRSA) infections. The PathCare data furthermore showed that the average prevalence of MRSA for 2001-2005 was 5.2%. For the time period 2010-2015 the average MRSA prevalence was 9.1%. For 2015 alone the MRSA prevalence was 8.0%. It was evident that there was an increase of 3.9% in MRSA from 2001-2005 to 2010-2015.

Mengistu *et al.* (2013, p. 6) did not mention methicillin-resistant strains in their analysis of NIP susceptibility data for 2009-2012. From the susceptibility/resistance percentages supplied by them, one can see that 3/9 (33.3%) of staphylococci were

resistant to the antibiotic oxacillin. This could possibly also indicate methicillin-resistance (Reta *et al.*, 2015, p. 3).

2.6 Staphylococcal virulence factors

More than 30 staphylococcal species are pathogenic (Lowy, 2010, p. 199 and p. 388). *Staphylococcus aureus* is considered the most virulent species. Many factors contribute to its ability to be infectious and cause disease, including the production of toxins and enzymes, as well as biofilm formation (Forbes *et al.*, 2007, pp. 254-255). *Staphylococcus epidermidis* is often implicated in biofilm infections (Brooks *et al.*, 2013, p. 205). Virulence factors that were investigated in the present study are discussed hereafter.

2.6.1 Exotoxins

Exotoxins are proteins that are strongly specific for a target cell. Their effects can be very powerful and may cause death. They generally affect cells by causing damage to the cell membrane and initiating lysis (bursting) or by disrupting functions inside the cells (Cowan, 2012, p. 374).

2.6.1.1 Superantigens: Staphylococcal enterotoxins A, B, C and D

When *S. aureus* strains become stressed by the host, for example when they must grow in the presence of antimicrobial therapy, they specialize in producing virulence factors, including cytolysins and superantigens, with CA-MRSA that may produce more superantigens than their community-associated methicillin-sensitive *S. aureus* (CA-MSSA) counterparts (Schlievert *et al.*, 2010, pp. 10-11).

There are several – A-E, G-J, K-R and U, V – enterotoxins that are superantigens. Around 50.0% of *S. aureus* strains can produce one or more of these toxins (Brooks *et al.*, 2013, p. 202), and those strains with a combination of different enterotoxin genes

can increase the occurrence and severity of *S. aureus* infections (Dağı *et al.*, 2015, p. 174). Enterotoxins are heat stable and resistant to the working of gut enzymes. These toxins are important in food poisoning and are produced by *S. aureus* growing in carbohydrate and protein foods. Ingestion of as little as 25µg of enterotoxin B (ETB) causes vomiting and a runny stomach (Brooks *et al.*, 2013, p. 202). Enterotoxins are also associated with other infections. In an African study, Sina *et al.* (2013, p. 2 and p. 4) collected 136 *S. aureus* isolates from five different types of infection (furuncles, pyomyositis, abscesses, Buruli ulcers and osteomyelitis) from hospital admissions and out-patients in Benin. Among the enterotoxins, ETB was found most frequently. Enterotoxins A and B were associated with pyomyositis, furuncles, osteomyelitis and Buruli ulcers, whereas enterotoxins A, B and C were associated with abscesses. Wang *et al.* (2016, p. 6) detected the genes encoding enterotoxins A-D from CA-MRSA isolated from paediatric patients in China with respiratory and bloodstream infections. Just like in the study by Sina *et al.* (2013), toxin B was the most prevalent enterotoxin in their study.

Hassanien and Abdel-Aziz (2017, p. 110) screened 23 *S. aureus* isolates from nasal swabs of food handlers in Egypt for enterotoxins, and amplified *sea*, *seb*, and *sec* genes. In a Japanese study, Uemura *et al.* (2004, p. 22) found enterotoxins in 21/50 nasal *S. aureus* isolates from healthy volunteers. Out of the 21 isolates, 7 had ETA, 11 had ETB, 8 had ETC and 3 had ETD. In Ireland, Collery *et al.* (2008, p. 350) used 48 *S. aureus* isolates obtained from the noses of healthy students to screen for enterotoxins, and detected genes encoding ETA, ETB and ETC. In China, Chen *et al.* (2017, p. 5) tested nasal *S. aureus* from a healthy population for enterotoxin genes. Their results showed that 56.5% of all isolates carried both the *sea* and *seb* genes, 26.1% carried only *eta*, and 13.0% carried only *seb*.

2.6.2 Enzymes: Coagulase and catalase

Staphylococcus aureus can be highly resistant towards both normal immune responses and antimicrobials. Its resistance is partly due to the production of coagulase (Leboffe and Pierce, 2011, p. 65). Coagulase can be defined as a bacterial enzyme that reacts with a cofactor present in blood plasma to catalyse the formation of fibrin from fibrinogen (Dorland's illustrated medical dictionary, 2003, p. 380). This enzyme works together with normal plasma components to form protective fibrin barriers around individual bacterial cells or groups of cells, which shields them from phagocytosis and other types of ambush by the immune system (Leboffe and Pierce, 2011, p. 65). *Staphylococcus aureus* is coagulase-positive whereas other staphylococcal species (with a few exceptions in animal strains) are coagulase-negative (Lowy, 2010, p. 386).

2.6.3 Biofilm formation

Some strains of staphylococci are able to form biofilms. Hutcherson *et al.* (2015, p. 1) define biofilms as dense, surface-attached communities of bacteria or fungi encased within a microbial-derived matrix that helps with colonization and survival. According to Mack *et al.* (2013, p. 25) and Nazzari *et al.* (2014), this formation helps the bacterium to withstand the host's natural immune defence mechanisms and to resist antibiotic treatment. In other words, as stated by Speziale and Geoghegan (2015, p. 1), this is a survival strategy adapted by these bacteria. The nasopharynx, heart valves, lungs and oral cavity are all sites for biofilm growth involving staphylococci and streptococci. Such growth is also problematic during infection of implanted medical devices (Speziale and Geoghegan, 2015, p. 1).

Biofilm-producers are expected to be more resistant to antibiotics than non-producers (Cabrera-Contreras *et al.*, 2013, p. 3). Similar to Cabrera-Contreras *et al.* (2013),

Samant Sharvari and Pai Chitra (2012, p. 731) saw that biofilm producers displayed a higher degree of multi-drug resistance than non-producers. This finding between the two types of bacteria was statistically significant ($P = 0.04$). According to Samant Sharvari and Pai Chitra (2012, p. 732), detection of biofilm-related infection by staphylococci can help to alter antibiotic therapy and prevent infection.

In a school-based study in Thailand, Tangchaisuryia *et al.* (2014) screened 78 *S. aureus* isolates that were obtained from the noses of healthy primary school children for biofilm formation. Of the 78 isolates, 28 (36.0%) were classified as low grade biofilm positive in microtiter plates with optical density (OD) values of ≤ 0.1 at 570nm. In other words, these bacteria were weak biofilm producers. Forty-six (59.0%) of isolates that were screened could be classified as highly biofilm positive (strong biofilm producers) with OD values of ≥ 1.0 at 570nm.

2.7 Antibacterial and anti-biofilm activity of traditional medicinal plants against *Staphylococcus*

Most cultures across the globe have developed knowledge of local plants, enabling them to use these plants for medicinal purposes (Silvério and Lopes, 2012, pp. 110-11). African Traditional Medicine, which probably dates back to the origins of humankind, represents the most diverse of all medicinal systems, but is also the least systemized and most poorly documented of these systems. Traditional or folk medicine may be just as effective as conventional drugs, but its effectiveness is often questioned by scientists (van Wyk and Wink, 2015, p. 8 and p. 16). In some regions people are poor and do not always have access to modern medicine and antibiotic-based therapies. Certain plants utilized in some regions are unknown to western medicine and thus studied in the field of ethnopharmacology to investigate their

antimicrobial properties. The concentration of pharmacologically active compounds depends on the season that plants were harvested, how mature they were and the conditions in which growth took place. Due to lack of regulation, the same plant product that was bought at different times can possess different biological activities (Silvério and Lopes, 2012, pp. 110-11).

2.7.1 Phytochemical compounds that may be involved in the activity of medicinal plants against staphylococci: Flavonoids, saponins and anthraquinones

Kamonwannasit *et al.* (2013) examined the antibacterial activity of *Aquilaria crassna* leaf extract (which contains flavonoids and saponins) against *S. epidermidis*. Their disk diffusion results showed inhibition zones of 12.0 ± 1.0 mm with 4mg of aqueous leaf extract, 15.0 ± 0.4 mm with 4mg/ml extract and 18.0 ± 1.0 mm with 6mg/ml extract. The leaves were thus moderately to strongly active against the bacterium. At 6mg/ml the leaf extract was almost as effective as vancomycin (inhibition zone of 21.0 ± 1.0 mm). They also observed that the extract could inhibit biofilm formation in *S. epidermidis* and explained that destruction of the bacterial cell wall by the plant extract prevents bacteria from growing and creating primary biofilm structures. Their proposed mechanism of action were thus disruption of the bacterial cell wall.

In a large study by Manner *et al.* (2013) 500 commercially bought flavonoids were screened for anti-biofilm activity against the clinical strains *S. aureus* ATCC 25923 and the Newman strain. After initial screening, 443 flavonoids were classified as inactive (caused less than 40.0% biofilm inhibition), 47 as moderately active (caused more than 40.0% inhibition) and 10 as highly active (caused more than 85.0% inhibition). The 10 compounds that were highly active in inhibiting and eradicating biofilms consisted of one isoflavone (company ID ST014848), three flavonones (ST024709, ST056204, ST098360), three flavans (ST075672, ST081006, ST093738),

and three chalcones (ST092293, ST095411, ST095417). The two most potent flavonoids against staphylococcal biofilms were two synthetic flavans (ST075672 and ST081006).

Awolola *et al.* (2014) investigated the phytochemical composition of *Ficus sansibarica* and tested the isolated compounds (including flavonoids) for antimicrobial and anti-biofilm activity with the purpose to give a scientific rationale for using the plant to treat some bacterial infections. Three flavonoids, namely 5, 7, 4'-trihydroxyflavan-3-ol, epicatechin, and isovitexin were tested against MSSA ATCC 29213 and MRSA ATCC 43300. None of the flavonoids displayed antimicrobial activity against MRSA. Epicatechin was moderately active against MSSA when 80µl of the compound was used at a concentration of 8mg/ml in agar well diffusion (a zone of inhibition of 13.5mm was formed). The other two flavonoids were inactive. Results for anti-biofilm activity showed that epicatechin decreased the adhesion of MSSA in microtiter plates at all concentrations tested (1.0-7.5mg/ml), with 5.0mg/ml being most effective. This indicated that epicatechin is able to interfere with adhesion of bacteria to polystyrene surfaces. 5, 7, 4'-trihydroxyflavan-3-ol (1.0-2.5mg/ml) and isovitexin (0.2-0.5mg/ml) also decreased adhesion of MSSA. A concentration of 2.5mg/ml of 5, 7, 4'-trihydroxyflavan-3-ol almost completely inhibited adhesion of *S. aureus* ATCC 29213. The three compounds did not have anti-biofilm activity against MRSA. In fact, adhesion of MRSA was increased at all concentrations tested. The study showed potential validity of using *Ficus sansibarica* to treat staphylococcal biofilm-associated infections, despite its limited antimicrobial properties.

Carranza *et al.* (2015) observed promising anti-staphylococcal potential of methanolic extracts from two native Californian medicinal plants, namely *Rhamnus californica* and *Umbellularia californica*. Strong antimicrobial activity against MRSA ATCC

BAA-1683 was seen with 10mg/ml *R. californica* leaf extract, and moderate activity against *S. aureus* ATCC 25923. Bark extracts were strongly active against *S. aureus* ATCC 25923 at concentrations of 10 and 25mg/ml, and weakly active against MRSA at a concentration of 10mg/ml. Chemical profiling revealed the presence of flavonoids and saponins in all extracts tested. These may be involved in the extracts' anti-staphylococcal activity.

Monte *et al.* (2014) combined three antibiotics (tetracycline, erythromycin and ciprofloxacin) with saponin. Tetracycline-saponin combinations were antagonistic against *S. aureus* CECT 976, and synergistic against *S. aureus* XU 212 (MRSA). Erythromycin-saponin was additive against *S. aureus* CECT 976, and synergistic against *S. aureus* RN 4220. Ciprofloxacin-saponin was antagonistic against *S. aureus* CECT 976, and synergistic against *S. aureus* SA 1199 B.

Duraipandiyan *et al.* (2016) isolated the anthraquinone compound 6,6¹ – bis (1,5,7 – trihydroxy – 3 – hydroxymethylanthraquinone from *Streptomyces* spp. isolate ERI – 26. This compound had good antimicrobial activity against *S. aureus* ATCC 25923 and *S. epidermidis* MTCC 3615, with minimum inhibitory concentrations (MICs) of 62.5µg/ml and 15.62µg/ml, respectively.

In another study by Lee *et al.* (2016) eleven anthraquinone-related compounds were investigated for anti-biofilm activity against *Staphylococcus* species. These compounds were alizarin, anthraflavic acid, anthraquinone, (+) – catechin, 1,8 – dihydroxyanthraquinone, emodin, 1 – hydroxyanthra – 9,10 – quinone, hydroquinone, purpurin, pyrocatechol, and quinalizarin. Their results showed that alizarin, emodin, purpurin, and quinalizarin at 10µg/ml markedly inhibited *S. aureus* MSSA 6538 biofilm formation by $\geq 70.0\%$, compared to untreated controls. The other seven compounds did not have a significant effect. Addition of alizarin at concentrations of

0, 1, 2, 5, 10, 50, and 100µg/ml at the start of bacterial culture dose-dependently inhibited biofilm formation in three *S. aureus* strains (MSSA 6538, MSSA 25923, and MRSA MW 2) as well as a *S. epidermidis* strain (ATCC 14990). Alizarin (10µg/ml) decreased biofilm formation in all three *S. aureus* strains by $\geq 90.0\%$. For *S. epidermidis*, 50µg/ml was needed to inhibit biofilm formation by $\geq 70.0\%$. The investigators saw that alizarin at concentrations of up to 20µg/ml did not delay growth of *S. aureus* planktonic cells – at 200µg/ml it slightly inhibited growth. This indicates that the reduced biofilm formation by alizarin was because of its anti-biofilm activity and not its antimicrobial activity. Lastly, results of their molecular work indicated that alizarin significantly repressed the expression of biofilm-related genes, including the surface protein A (*spa*) gene.

References

- Akindele, A.A., Adewuyi, L.K., Adefioye, O.A., Adedokun, S.A. and Olaolu, A.O. (2010) Antibiogram and beta-lactamase production of *Staphylococcus aureus* isolates from different human clinical specimens in a tertiary health institution in Ile-ife, Nigeria. *American-Eurasian Journal of Scientific Research* [online]. **5**(4), pp. 230-233. Available from: [http://www.idosi.org/aejsr/5\(4\)10/2.pdf](http://www.idosi.org/aejsr/5(4)10/2.pdf) [Accessed 29 July 2015].
- Alzoubi, H.M., Agel, A.A., Al-Sarayreh, S.A. and Al-Zayadneh, E. (2014) Methicillin-resistant *Staphylococcus aureus* nasal carriage among primary school-aged children from Jordan: Prevalence, antibiotic resistance and molecular characteristics. *Journal of Egyptian Public Health Association* [online]. **9**(3), pp. 114-118. Available from DOI: 10.1097/01.EPX.0000454671.83406.e8 [Accessed 4 June 2016].

- Arali, S.M., Kulkarni, V., Manjula, N.G., Gaddad, S.M., Jayaraj, Y.M. and Shivannavar, C.T. (2016) Prevalence and antibiotic susceptibility profile of MRSA isolated from the anterior nares of school going children in Gulbarga. *International Journal of Medical Science and Public Health* [online]. **5**(4), pp. 620-626. Available from DOI: 10.5455/jmsph.2016.0805201592 [Accessed 7 December 2016].
- Assafi, M.S., Polse, R.F., Hussein, N.R., Haji, A.H. and Issa, A.R. (2017) The prevalence of *S. aureus* nasal colonization and its antibiotic sensitivity pattern amongst primary school pupils. *Science Journal of University of Zakho* [online]. **5**(1), pp. 7-10. Available from DOI: <https://doi.org/10.25271/2017.5.1.291> [Accessed 14 July 2017].
- Awolola, G.V., Koorbanally, N.A., Chenia, H., Shode, F.O. and Baijnath, H. (2014) Antibacterial and anti-biofilm activity of flavonoids and triterpenes isolated from the extracts of *Ficus sansibarica* warb. subsp. *sansibarica* (Moraceae) extracts. *African Journal of Traditional, Complementary and Alternative Medicines* [online]. **11**(3), pp. 124-131. Available from DOI: <http://dx.doi.org/10.4314/ajtcam.v11i3.19> [Accessed 19 January 2017].
- Bharathi, M., Lakshmi, N., Kalyani, C.S. and Padmaja, I.J. (2014) Nasal carriage of multidrug-resistant MSSA and MRSA in children of municipality schools [Letter to the editor]. *Indian Journal of Medical Microbiology* [online]. **32**(2). Available from: <http://www.ijmm.org/text.asp?2014/32/2/200/129840> [Accessed 29 July 2015].
- Brooks, G.F., Carroll, K.C., Butel, J.S., Morse, S.A. and Mietzner, T.A. (2013) *Jawetz, Melnick & Adelberg's Medical Microbiology*. 26th ed. s.l.: McGraw-Hill.

- Byarugaba, D.K. (2010) Mechanisms of antimicrobial resistance. In: Sosa, A., Byarugaba, D.K., Amábile-Cuevas, C.F., Hsueh, P., Kariuki, S. and Okeke, I.N. (eds.) *Antimicrobial resistance in developing countries*. New York: Springer, pp. 15-26.
- Cabrera-Contreras, R., Morelos-Ramírez, R., Galicia-Camacho, A.N. and Meléndez-Herrada, E. (2013) Antibiotic resistance and biofilm production in *Staphylococcus epidermidis* strains, isolated from a tertiary care hospital in Mexico City. *ISRN Microbiology* [online]. Available from DOI: <http://dx.doi.org/10.1155/2013/918921> [Accessed 29 July 2015].
- Carranza, M.G., Sevigny, M.B., Banerjee, D. and Fox-Cubley, L. (2015) Antibacterial activity of native California medicinal plant extracts isolated from *Rhamnus californica* and *Umbellularia californica*. *Annals of Clinical Microbiology and Antimicrobials* [online]. **14**(29). Available from DOI: 10.1186/s12941-015-0086-0 [Accessed 19 January 2017].
- Chen, B.J., Xie, X.Y., Ni, L.J., Dai, X.L., Lu, Y., Wu, X.Q., Li, H.Y., Yao, Y.D. and Huang, S.Y. (2017) Factors associated with *Staphylococcus aureus* nasal carriage and molecular characterization among the general population at a medical college campus in Guangzhou, south China. *Annals of Clinical Microbiology and Antimicrobials* [online]. **16**. Available from DOI: 10.1186/s12941-017-0206-0 [Accessed 22 September 2017].
- Collery, M.M., Smyth, D.S., Twohig, J.M., Shore, A.C., Coleman, D.C. and Smyth, C.J. (2008) Molecular typing of nasal carriage isolates of *Staphylococcus aureus* from an Irish university student population based on toxin gene PCR, *agr* locus types and multiple locus, variable number tandem repeat analysis.

Journal of Medical Microbiology [online]. **57**, pp. 348-358. Available from DOI: 10.1099/jmm.0.47734-0 [Accessed 22 September 2017].

Cowan, M.K. (2012) *Microbiology: A systems approach*. 3rd ed. New York: McGraw-Hill.

Dagi, H.T., Fındık, D., Demirel, G. and Arslan, U. (2015). Detection of methicillin resistance and various virulence factors in *Staphylococcus aureus* strains isolated from nasal carriers. *Balkan Medical Journal* [online]. **32**, pp. 171-175. Available from DOI: 10.5152/balkanmedj.2015.150186 [Accessed 9 January 2017].

Deng, J.J., Xiao, G.G., Zhu, Y., Zhou, W. and Wan, C.M. (2014) *Staphylococcus aureus* nasal carriage and its antibiotic resistance profiles in Tibetan school children in southwest China. *Hong Kong Journal of Paediatrics (New Series)* [online]. **19**, pp. 75-78. Available from: <http://www.hkjpaed.org/details.asp?id=952&show=12> [Accessed 29 July 2015].

Dinić, M., Vuković, S., Kocić, B., Stanković-Đorđević, D. and Bogdanović, M. (2013) Nasal carriage of *Staphylococcus aureus* in healthy adults and in school children. *Acta Facultatis Medicae Naissensis* [online]. **30**(1), pp. 31-36. Available from DOI: 10.2478/v10283-012-0033-3 [Accessed 29 July 2015].

Dorland's illustrated medical dictionary (2003) 30th ed. Philadelphia: Saunders.

Duraipandiyani, V., Al-Dhabi, N.A. and Ignacimuthu, S. (2016) New antimicrobial anthraquinone 6,6¹ - bis (1,5,7 - trihydroxy - 3 - hydroxymethylantraquinone) isolated from *Streptomyces* sp. isolate ERI – 26. *Saudi Journal of Biological Sciences* [online]. **23**, pp. 731-735. Available from

DOI: <http://dx.doi.org/10.1016/j.sjbs.2016.02.008> [Accessed 2 February 2017].

Erami, M., Soltani, B., Ardakani, A.T., Moravveji, A., Rezaei, M.H., Soltani, S. and Moniri, R. (2014) Nasal carriage and resistance pattern of multidrug resistant *Staphylococcus aureus* among healthy children in Kashan, Iran. *Iranian Red Crescent Medical Journal* [online]. **16**(9). Available from DOI: 10.5812/ircmj.21346 [Accessed 16 December 2015].

Esposito, S., Terranova, L., Ruggiero, L., Ascolese, B., Montinaro, V., Rios, W.P., Galeone, C. and Principi, N. (2015) *Streptococcus pneumoniae* and *Staphylococcus aureus* carriage in healthy school children and adolescents. *Journal of Medical Microbiology* [online]. **64**, pp. 427-431. Available from DOI: 10.1099/jmm.0.000029 [Accessed 7 December 2016].

Food and Drug Administration (2012) *Bad bug book: Handbook of foodborne pathogenic microorganisms and natural toxins*. 2nd ed. Washington: Center for Food Safety and Applied Nutrition.

Forbes, B.A., Sahm, D.F. and Weissfeld, A.S. (2007) *Bailey and Scott's Diagnostic Microbiology*. 12th ed. St. Louis: Mosby.

Gong, Z., Shu, M., Xia, Q., Tan, S., Zhou, W., Zhu, Y. and Wan, C. (2017) *Staphylococcus aureus* nasal carriage and its antibiotic resistance profiles in children in high altitude areas of Southwestern China. *Archivos Argentinos de Pediatría* [online]. **115**(3), pp. 274-286. Available from DOI: <http://dx.doi.org/10.5546/aap.2017.eng.274> [Accessed 13 July 2017].

Hassanien, A.A. and Abdel-Aziz, N.M. (2017) *Staphylococcus aureus* and their enterotoxin gene in fast food and food handlers from different food premises

in Sohag City, Egypt. *International Journal of Agricultural Sciences and Veterinary Medicine* [online]. **5**(1). Available from: https://www.ijasvm.com/ijasvadmin/upload/IJASVM_58a403b503a72.pdf [Accessed 22 September 2017].

Hatcher, S.M., Rhodes, S.M., Stewart, J.R., Silbergeld, E., Pisanic, N., Larsen, J., Jiang, S., Krosche, A., Hall, D., Carroll, K.C. and Heaney, C.D. (2017) The prevalence of antibiotic-resistant *Staphylococcus aureus* nasal carriage among industrial hog operation workers, community residents, and children living in their households: North Carolina, USA. *Environmental Health Perspectives* [online]. **125**(4), pp. 560-569. Available from DOI: <http://dx.doi.org/10.1289/EHP35> [Accessed 14 July 2017].

Hutcherson, J.A., Scott, D.A. and Bagaitkar, J. (2015) Scratching the surface – tobacco-induced bacterial biofilms. *Tobacco Induced Diseases* [online]. **13**. Available from DOI: 10.1186/s12971-014-0026-3 [Accessed 1 June 2016].

Jeyakumari, D., Nagajothi, J., Menon, R. and Tiroumourougane, S.V. (2015) Prevalence of nasal carriage of *Staphylococcus aureus* and its antibiotic susceptibility pattern among primary school children in a rural area of Puducherry, India. *International Journal of Current Research* [online]. **7**(7), pp. 17930-17933. Available from: http://www.gmferd.com/journalcra.com/sites/default/files/9697_0.pdf [Accessed 7 December 2016].

Kamonwannasit, S., Nantapong, N., Kumkrai, P., Luecha, P., Kupittayanant, S. and Chudapongse, N. (2013) Antibacterial activity of *Aquilaria crassna* leaf extract against *Staphylococcus epidermidis* by disruption of cell wall. *Annals of*

Clinical Microbiology and Antimicrobials [online]. **12**(20). Available from DOI: 10.1186/1476-0711-12-20 [Accessed 19 January 2017].

Kerschner, H., Harrison, E.M., Hartl, R., Holmes, M.A. and Apfalter, P. (2015). First report of *mecC* MRSA in human samples from Austria: Molecular characteristics and clinical data. *Microbes and New Infections* [online]. **3**, pp. 4-9. Available from DOI: <http://dx.doi.org/10.1016/j.nmni.2014.11.001> [Accessed 29 February 2016].

Kulkarni, R., Antala, S., Wang, A., Amaral, F.E., Rampersaud, R., LaRussa, S.J., Planet, P.J. and Ratner, A.J. (2012). Cigarette smoke increases *Staphylococcus aureus* biofilm formation via oxidative stress. *Infection and Immunity* [online]. **80**(11), pp. 3804-3811. Available from DOI: 10.1128/IAI.00689-12 [Accessed 29 July 2017].

Lauschke, F.H.M. and Frey, C.T. (1994) Hematogenous osteomyelitis in infants and children in the northwestern region of Namibia: Management and two-year results. *Journal of Bone and Joint Surgery* [online]. **76**(4), pp. 502-510. Available from: <http://journals.lww.com/jbjsjournal/pages/articleviewer.aspx?year=1994&issue=04000&article=00004&type=abstract> [Accessed 23 March 2017].

Leboffe, M. and Pierce, B.E. (2011) *Photographic atlas of the Microbiology laboratory*. 4th ed. Englewood: Morton.

Lee, J-H., Kim, Y-G., Ryu, S.Y. and Lee, J. (2016) Calcium-chelating alizarin and other anthraquinones inhibit biofilm formation and the hemolytic activity of *Staphylococcus aureus*. *Scientific Reports* [online]. **6**. Available from DOI: 10.1038/srep19267 [Accessed 1 February 2017].

- Lowy, F.D. (2010) Staphylococcal infections. In: Kasper, D. and Fauci, A. (eds.) *Harrison's infectious diseases*. s.l.: McGraw-Hill, pp. 386-399.
- Mack, D., Davies, A.P., Harris, L.G., Jeeves, R., Pascoe, B., Knobloch, J.K.-M., Rohde, H. and Wilkinson, T.S. (2013) *Staphylococcus epidermidis* in biomaterial-associated infections. In: Moriarty, F., Zaat, S.A.J. and Busscher, H.J. (eds.) *Biomaterials associated infection: Immunological aspects and antimicrobial strategies*. New York: Springer.
- Manner, S., Skogman, M., Goeres, D., Vuorela, P. and Fallarero, A. (2013) Systemic exploration of natural and synthetic flavonoids for the inhibition of *Staphylococcus aureus* biofilms. *International Journal of Molecular Sciences* [online]. **14**, pp. 19434-19451. Available from DOI: 10.3390/ijms41019434 [Accessed 29 July 2015].
- Mengistu, A., Gaeseb, J., Uaaka, G., Ndjavera, C., Kambyambya, K., Indongo, L., Kalemeera, F., Ntege, C., Mibirizi, D., Joshi, M.P. and Sagwa, E. (2013) Antimicrobial sensitivity patterns of cerebrospinal fluid (CSF) isolates in Namibia: Implications for empirical antibiotic treatment of meningitis. *Journal of Pharmaceutical Policy and Practice* [online]. **6**(4). Available from DOI: 10.1186/2052-3211-6-4 [Accessed 29 July 2015].
- Monte, J., Abreu, A.C., Borges, A., Simões, L.L. and Simões, M. (2014) Antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* and their biofilms. *Pathogens* [online]. **3**, pp. 473-498. Available from DOI: 10.3390/pathogens3020473 [Accessed 29 July 2015].
- Nazzari, E., Torretta, S., Pignataro, L., Marchisio, P. and Esposito, S. (2014) Role of biofilm in children with recurrent upper respiratory tract infections. *European*

Journal of Clinical Microbiology and Infectious Diseases [online]. Available from DOI: 10.1007/s10096-014-2261-1 [Accessed 13 February 2016].

Nikfar, R., Shamsizadeh, A., Kajbaf, T.Z., Panah, K., Khaghani, S. and Moghddam, M. (2015) Frequency of methicillin-resistant *Staphylococcus aureus* in healthy children. *Iranian Journal of Microbiology* [online]. **7**(2), pp. 67-71. Available from: <http://www.researchgate.net/publication/281999247> [Accessed 16 December 2015].

PathCare Namibia. (2015) Antimicrobial susceptibility data. PathCare [online]. Available from: http://www.196.25.36.50/webfiles/files/ANTIMICROBIAL_SUSCEPTIBILITY_DATA_Staphylococcus_aureus.pdf [Accessed 23 November 2015].

Prescott, L.M., Harley, J.P. and Klein, D.A. (2002) *Microbiology*. 5th ed. New York: McGraw-Hill.

Pynnonen, M., Stephenson, R.E., Schwartz, K., Hernandez, M. and Boles, B.R. (2011) Hemoglobin promotes *Staphylococcus aureus* nasal colonization. *PLOS Pathogens* [online]. **7**(7). Available from DOI: 10.1371/journal.ppat.1002104 [Accessed 23 March 2017].

Reid, M.J.A., Fischer, R.S.B., Mannathoko, N., Muthoga, C., McHugh, E., Essigmann, H., Brown, E.L. and Steenhoff, A.P. (2017) Prevalence of *Staphylococcus aureus* nasal carriage in human immunodeficiency virus-infected and uninfected children in Botswana: Prevalence and risk factors. *The American Journal of Tropical Medicine and Hygiene* [online]. **96**(4), pp. 795-801. Available from DOI: <https://doi.org/10.4269/ajtmh.16-0650> [Accessed 14 July 2017].

Reta, A., Gedefaw, L., Sewunet, T. and Beyene, G. (2015) Nasal carriage, risk factors and antimicrobial susceptibility pattern of methicillin resistant *Staphylococcus*

aureus among school children in Ethiopia. *Medical Microbiology and Diagnosis* [online]. **4**(1). Available from DOI: 10.4172/21610703.1000177 [Accessed 15 September 2015].

Samant Sharvari, A. and Pai Chitra, G. (2012) Evaluation of different detection methods of biofilm formation in clinical isolates of staphylococci. *International Journal of Pharma and Bio Sciences* [online]. **3**(4), pp. 724-733. Available from:
https://www.researchgate.net/publication/279770601_Evaluation_of_different_detection_methods_of_biofilm_formation_in_clinical_isolates_of_Staphylococci [Accessed 29 July 2015].

Shetty, V., Trumbull, K., Hegde, A., Shenoy, V., Prabhu, R., Sumathi, K., Palavecino, E. and Shetty, A.K. (2014). Prevalence of community-acquired methicillin-resistant *Staphylococcus aureus* nasal colonization among children. *Journal of Clinical and Diagnostic Research* [online]. **8**(12). Available from DOI: 10.7860/JCDR/2014/9986.5276 [Accessed 24 November 2015].

Schlievert, P.M., Strandberg, K.L., Lin, Y-C, Peterson, M.L. and Leung, D.Y.M. (2010) Secreted virulence factor comparison between methicillin-resistant and methicillin-susceptible *Staphylococcus aureus*, and its relevance to atopic dermatitis. *Journal of Allergy and Clinical Immunology* [online]. **125**(1). Available from DOI: 10.1016/j.jaci.2009.10.039 [Accessed 25 July 2017].

Silvério, A. and Lopes, M. (2012) Antimicrobial activity of condiments. In: Bobbarala, V. (ed.) *Antimicrobial agents*. Croatia: Intech, pp. 109-128.

Sina, H., Ahoyo, T.A., Moussaoui, W., Keller, D., Bankolé, H.S., Barogui, Y., Stienstra, Y., Kotchoni, S.O., Prévost, G. and Baba-Moussa, L. (2013) Variability of antibiotic susceptibility and toxin production of *Staphylococcus*

- aureus* strains isolated from skin, soft tissue, and bone related infections. *BMC Microbiology* [online]. **13**. Available from DOI: 10.1186/1471-2180-13-188 [Accessed 14 January 2017].
- Soltani, B., Ardakani, A.T., Moravveji, A., Erami, M., Rezaei, M.H., Moniri, R. and Namazi, M. (2014) Risk factors for methicillin-resistant *Staphylococcus aureus* nasal colonization of healthy children. *Jundishapur Journal of Microbiology* [online]. **7(9)**. Available from DOI: 10.5812/jjm.20025 [Accessed 29 July 2015].
- Speziale, P. and Geoghegan, J.A. (2015) Biofilm formation by staphylococci and streptococci: Structural, functional, and regulatory aspects and implications for pathogenesis. *Frontiers in Cellular and Infection Microbiology* [online]. **5**. Available from DOI: 10.3389/fcimb.2015.00031 [Accessed 7 August 2015].
- Tangchaisuriya, U., Yotpanya, W., Kitti, T. and Sitthisak, S. (2014) Distribution among Thai children of methicillin-resistant *Staphylococcus aureus* lacking *cna*, *fnbA* and *icaAD*. *Southeast Asian Journal of Tropical Medicine and Public Health* [online]. **45(1)**. Available from: <http://www.mahidol.ac.th/seameo/2014-45-1-full/19-5911-15.pdf> [Accessed 17 August 2015].
- Uemura, E., Kakinohana, S., Higa, N., Toma, C. and Nakasone, N. (2004) Comparative characterization of *Staphylococcus aureus* from throats and noses of healthy volunteers. *Japanese Journal of Infectious Diseases* [online]. **57**, pp. 21-24. Available from: <http://www0.nih.go.jp/JJID/57/21.pdf> [Accessed 22 September 2017].
- Van Wyk, B.-E. and Wink, M. (2015) *Phytomedicines, herbal drugs & plant poisons*. Pretoria: Briza.

Wang, X., Li, X., Huang, W., Fu, Q. and Li, M. (2016) Molecular characteristic and virulence gene profiles of community-associated methicillin-resistant *Staphylococcus aureus* isolates from pediatric patients in Shanghai, China. *Frontiers in Microbiology* [online]. **7**. Available from DOI: 10.3389/fmicb.2016.01818 [Accessed 10 January 2017].

Chapter 3

Nasal colonization of *Staphylococcus* among apparently healthy school children in the Mariental community, southern Namibia.

Abstract

Nasal colonization with *Staphylococcus* is a major risk factor for becoming infected with this bacterium. Self-infection with colonized strains may lead to infections of the skin, ears and throat. To our knowledge, this is the first study to report on the prevalence of nasal staphylococci among apparently healthy pupils in and around the town of Mariental, Namibia. Such information may be useful in school health programmes.

This was a cross-sectional study in the Mariental District. With informed consent from parents/guardians, nasal specimens (swabs) were collected from randomly selected learners aged 6-14 years attending school in the area during the months of March, September and October 2016. Specimens were enriched for staphylococcal growth in brain heart infusion broth prior to isolation on *Staphylococcus* medium no. 110 and tryptone soy agar. Pure cultures were Gram-stained and biochemically tested for identification of *Staphylococcus aureus* and coagulase-negative staphylococci (CoNS). Methicillin-resistant isolates were identified by their resistance towards cefoxitin (30µg) using the Kirby-Bauer disk diffusion assay.

Four hundred and thirty-three isolates from 272 swabs were morphologically and biochemically identified as *Staphylococcus* species by Gram staining and a positive catalase test. Of these isolates, 352 (81.3%) were identified as *S. aureus* by a positive coagulase test, while 81 (18.7%) were coagulase-negative (CoNS). Methicillin-resistant *S. aureus* (MRSA) comprised 11.8% of all isolates and methicillin-resistant CoNS (MRCoNS) 1.6% as indicated by a cefoxitin inhibition

zone diameter < 22mm. The overall prevalence of *S. aureus* in the study population of 272 learners was 80.5%, and that of CoNS was 25.0%. Methicillin-resistant *S. aureus* was isolated from 48 (17.6%) learners and MRCoNS in only seven (2.6%). From the study participants who reported getting nosebleeds, 75.4% were colonized with *S. aureus*, whereas 81.4% who were exposed to cigarette smoke from a household member carried this bacterium.

In conclusion, the high prevalence of *S. aureus* and presence of MRSA (including multi-drug resistant bacteria) among apparently healthy pupils calls for improvement in current hygiene practices at schools in the Mariental District to prevent staphylococcal disease. Nosebleeds and exposure to cigarette smoke are possible risk factors for colonization with *S. aureus* in the children.

Keywords: Prevalence, *Staphylococcus*, MRSA, school children, Mariental, nosebleeds, cigarette smoke

3.1 Introduction

Staphylococcus can asymptotically colonize humans and most people have staphylococci as commensals of the skin and upper respiratory tract (Brooks *et al.*, 2013, p. 202 and p. 204). The human nasal cavity predominantly contains *Staphylococcus aureus* and *Staphylococcus epidermidis* as normal microflora (Forbes *et al.*, 2007, p. 255). These bacteria are however opportunistic pathogens, causing infections when conditions are favorable (Prescott *et al.*, p. 704). According to Lowy (2010, p. 388) infections occur when colonizing bacteria enter normally sterile body sites through damaged skin or mucosal surfaces. Skin infections (Jenney *et al.*, 2014, p. 7), ear infections (Elmanama *et al.*, 2014, p. 89) and tonsillitis (Zautner *et al.*, p. 4) with *S. aureus* as a causative agent are common among school-aged children.

Reid *et al.* (2017, p. 795) regard nasal carriage of *S. aureus* as a major risk factor for becoming infected with this bacterium. Healthy school children under 16 years are potential carriers of *S. aureus*, especially methicillin-resistant *S. aureus* (MRSA) and multi-drug resistant strains (Arali *et al.*, 2016, p. 625). Alzoubi *et al.* (2014, p. 114) describe children as asymptomatic reservoirs for community-associated MRSA (CA-MRSA) which enables these bacteria to rapidly spread within communities.

There is currently a lack of published evidence-based research and statistics on the prevalence of disease-causing staphylococci (including methicillin-resistant strains) in Namibian school children. Our study therefore aimed to determine the prevalence of nasal *S. aureus*, coagulase-negative staphylococci (CoNS), MRSA and methicillin-resistant CoNS (MRCoNS) among healthy-looking school children in the Mariental community, southern Namibia. To our knowledge, this is the first study undertaken to report on the prevalence of *Staphylococcus* among school children in the Mariental District. Information obtained from this research can be useful in assisting school health programmes.

3.2 Materials and methods

3.2.1. Study area, population and sample collection

The town of Mariental is located southeast of Windhoek on the B1 national highway. It is the capital of the Hardap Region, which has a population of approximately 80 000 people (Graig and Hoff, 2016, p. 14). The present study was a cross-sectional one in and around the town.

Approval to conduct this study was obtained from the Director, Hardap Regional Council Directorate of Education, Arts and Culture, Mariental, and the Permanent Secretary at the Windhoek Head Office, Ministry of Education, Arts and Culture

(Appendix A1). With written consent from their parents/guardians (Appendix A3), the population that was screened for nasal staphylococci consisted of randomly chosen learners attending five schools in the Mariental District. Four of these schools are public schools while one is private school. Assuming that half of the study participants would carry staphylococci in their noses, sample size was calculated using the formula for sample size calculation for cross-sectional studies by Charan and Biswas (2013):

$$\begin{aligned}\text{Sample size} &= Z_{1-\alpha/2}^2 p (1-p)/d^2 \\ &= 1.96^2 \times 0.5 (1-0.5)/0.05^2 \\ &= 384 \text{ learners}\end{aligned}$$

Four hundred and eleven parents/guardians were approached and asked to complete consent forms and questionnaires. They were also provided with leaflets explaining the importance of the study (Appendix B). Only 66.2% of them consented, making the final number of study participants 272 (8.8%) of the total population (3 086 learners) aged 6-14 years, according to 15th School Day Statistics 2017. Children were divided into two age-groups: 6-10 years and 11-14 years, and consisted of 126 boys and 146 girls. Since not all people wanted to participate, the sample size was less than expected.

Sample collection was done during March, September and October 2016. One nasal specimen was obtained from each child by gently rotating a sterile Amies transport medium swab (Labocare™, Johannesburg, South Africa) thoroughly around the perimeter of both nostrils (Harley and Prescott, 2002, p. 336). Specimens were kept frozen at -20°C until transporting them to the laboratory for processing.

3.2.2 Isolation and identification of bacteria

To enrich for staphylococcal growth, specimen-containing swabs were cut shorter with flamed scissors and placed into test tubes each containing 2ml sterile brain heart

infusion broth (Merck, Darmstadt, Germany). These were incubated at 37°C for 24-48 hours, until sufficient growth was observed. Enriched cultures were then inoculated onto selective medium *Staphylococcus* medium no. 110 (Oxoid, Basingstoke, England) and tryptone soy agar (Scharlau Microbiology, Spain) and incubated at 37°C for 24 hours. Pure cultures were obtained by streaking different colonies from mixed culture plates onto *Staphylococcus* medium no.110 and tryptone soy agar. These were incubated at 37°C for 24 hours. Pure culture plates were parafilm and refrigerated at 4°C for further assays.

Identification of *S. aureus* (coagulase-positive) and CoNS was done by standard microbiological procedures (Harley and Prescott, 2002), including observation of distinctive characteristics of isolates on agar plates, Gram-positive stains, and production of the enzymes catalase and coagulase. Detection of MRSA and MRCoNS was done by Kirby-Bauer disk diffusion assay (Harley and Prescott, 2002, p. 258) on Mueller-Hinton agar (Mast Diagnostics, Merseyside, UK) using 0.5 McFarland standards of test cultures and the antibiotic cefoxitin (30µg) (Mast Diagnostics, Merseyside, UK). According to EUCAST Clinical Breakpoint Tables v. 7.1 (2017, p. 22), a zone diameter breakpoint of < 22mm was taken as resistance towards cefoxitin/methicillin. Ten percent glycerol stocks of pure cultures were prepared for each isolate in 2-ml Eppendorf tubes (Eppendorf, Hamburg, Germany). The stocks were labeled, parafilm at the lids to prevent them from popping open in the fridge, and placed in labeled plastic zipper bags to be stored at -86°C for long-term preservation. All procedures were performed under sterile conditions.

3.2.3 Statistical analysis

A chi-square test for comparison of proportions with MedCalc statistical software software (MedCalc statistical software version 16.4.3 {MedCalc software bvba,

Ostend, Belgium; <https://www.medcalc.org>; 2016}) was used to compare percentage differences of *S. aureus*, MRSA, CoNS and MRCoNS between age groups and gender. Statistical significant differences was indicated by a P-value of ≤ 0.05 .

3.3 Results and discussion

3.3.1 Study population and sample collection

Two hundred and seventy-two nasal specimens were collected from children aged 6-14 years whose parents/guardians gave permission for their children to take part in the study. Schools A, B, C and D were visited in March 2016, while school E was visited in September and October 2016. Table 3.1 gives information on the study participants, and includes potential risk factors for being colonized with staphylococci, as obtained from questionnaires completed by parents/guardians (See Appendix A3). School A did not have learners from the age-group 6-10 years. There was no significant difference in the percentage of males and females swabbed ($P = 0.08$).

Table 3.1 School children screened for nasal carriage of staphylococci and possible risk factors for colonization.

School	Age group	Males (%)	Females (%)	Children (%) who get nosebleeds	Children (%) who are exposed to cigarette smoke in the household
A (Private)	6-10 years	-	-	-	-
	11-14 years	3 (60.0)	2 (40.0)	4 (80.0)	2 (40.0)
B (Public)	6-10 years	11 (37.9)	18 (62.1)	8 (27.6)	6 (20.7)
	11-14 years	12 (52.2)	11 (47.8)	4 (17.4)	12 (52.2)
C (Public)	6-10 years	12 (46.2)	14 (53.8)	11 (42.3)	6 (23.1)
	11-14 years	16 (51.6)	15 (48.4)	14 (45.2)	8 (25.8)
D (Public)	6-10 years	18 (43.9)	23 (56.1)	5 (12.2)	15 (66.3)
	11-14 years	9 (47.4)	10 (52.6)	2 (10.5)	9 (47.4)
E (Public)	6-10 years	13 (43.3)	17 (56.7)	4 (13.3)	11 (36.7)
	11-14 years	32 (47.1)	36 (52.9)	13 (19.1)	28 (41.2)
Total (%)		126/272 (46.3)	146/272 (53.7)	65/272 (23.9)	97/272 (35.7)

According to Pynnonen *et al.* (2011, pp. 1-2) the blood component haemoglobin in nasal secretions is a host factor that can modulate *S. aureus* gene expression, increasing nasal colonization with this bacterium. Children that experience frequent nosebleeds are thus prone to *S. aureus* colonization. In the present study, 23.9% of the study population experienced nosebleeds, as indicated in completed questionnaires. Furthermore, 49/65 (75.4%) study participants with nosebleeds were colonized by *S. aureus*, 6/65 (9.2%) with nosebleeds did not have *S. aureus*, but had CoNS, while 8/65 (12.3%) with nosebleeds had no staphylococci in their noses. Methicillin-resistant *S. aureus* was found in 11/65 (16.9%) of these children, while MRCoNS was isolated

from one (1.5%). From these findings, it seems that getting nosebleeds may play a role in colonization with *S. aureus* in Mariental children.

Children who are exposed to second-hand cigarette smoke may suffer from smoke-enhanced bacterial infections. In a study by Kulkarni *et al.* (2012, p. 3805 and p. 3807) cigarette smoke exposure led to increased biofilm growth and enhanced fibronectin binding in pathogenic *S. aureus*. In the present study, 35.7% of learners were exposed to cigarette smoke from family members. Also, 79/97 (81.4%) of study participants that were exposed to cigarette smoke in the household were colonized with *S. aureus*, 10/97 (10.3%) did not have *S. aureus* but had CoNS, 20/97 (20.6%) were colonized with both *S. aureus* and CoNS, whereas 7/97 (7.2%) did not have staphylococci. Methicillin-resistant *S. aureus* was detected in 17/97 (17.5%) of the children, and MRCoNS in only 2/97 (2.1%). These results suggest cigarette smoke exposure to be a risk factor for nasal colonization with *S. aureus* in study participants.

3.3.2 Prevalence of *S. aureus*, MRSA, CoNS and MRCoNS

Nasal swabs were collected from 272 learners, randomly selected from five schools in the Mariental District. As indicated in Table 3.2, 433 isolates from the 272 swabs were morphologically and biochemically identified as *Staphylococcus* species by Gram staining and a positive catalase test. Of these isolates, 352 (81.3%) were identified as *S. aureus* by a positive coagulase test, while 81 (18.7%) were coagulase-negative (CoNS). Fifty-one (11.8%) isolates were MRSA and seven (1.6%) MRCoNS as indicated by cefoxitin resistance, in other words an inhibition zone diameter < 22mm. The overall prevalence of *S. aureus* in the study population of 272 learners was 80.5%, and that of CoNS was 25.0%. Methicillin-resistant *S. aureus* was isolated from 48 (17.6%) learners (Also see Figure 3.1) and MRCoNS in only seven (2.6%).

Table 3.2 Nasal carriage of *S. aureus*, MRSA, CoNS and MRCoNS among learners aged 6-14 years attending five schools in the Mariental area.

School	Age group	<i>S. aureus</i>	MRSA	CoNS	MRCoNS
		Females (%); Males (%)	Females (%); Males (%)	Females (%); Males (%)	Females (%); Males (%)
A (Private)	11-14 years	2 (100.0); 3 (100.0)	2 (100.0); 2 (66.7)	0 (0); 1 (33.3)	0 (0); 0 (0)
B (Public)	6-10 years	16 (88.9); 11 (100.0)	4 (22.2); 2 (18.2)	4 (22.2); 1 (9.1)	0 (0); 0 (0)
	11-14 years	10 (90.9); 12 (100.0)	2 (18.2); 5 (41.7)	4 (36.4); 1 (8.3)	0 (0); 0 (0)
C (Public)	6-10 years	12 (85.7); 8 (66.7)	2 (14.3); 1 (8.3)	6 (42.9); 3 (25.0)	2 (14.3); 1 (8.3)
	11-14 years	12 (80.0); 11 (68.8)	3 (20.0); 2 (12.5)	6 (40.0); 5 (31.3)	0 (0); 0 (0)
D (Public)	6-10 years	16 (69.6); 11 (61.1)	6 (26.1); 3 (16.7)	5 (21.7); 3 (16.7)	1 (4.3); 0 (0)
	11-14 years	8 (80.0); 4 (44.4)	3 (30.0); 1 (11.1)	3 (30.0); 1 (11.1)	1 (10.0); 1 (11.1)
E (Public)	6-10 years	13 (76.5); 11 (84.6)	3 (17.6); 1 (7.7)	3 (17.6); 3 (23.1)	1 (5.9); 0 (0)
	11-14 years	29 (80.6); 30 (93.8)	1 (2.8); 5 (15.6)	10 (27.8); 9 (28.1)	0 (0); 0 (0)
Total (%) Significance level		118/146; 101/126 (80.8); (80.2) P = 0.90	26/146; 22/126 (17.8); (17.5) P = 0.95	41/146; 27/126 (28.1); (21.4) P = 0.20	5/146; 2/126 (3.4); (1.6) P = 0.35

This very high prevalence of *S. aureus* in our study (80.5%) is comparable to that of Arali *et al.* (2016, p. 621) who observed 77.9% *S. aureus* nasal carriage in Indian school children aged 5-15 years. These high percentages can be due to isolation techniques such as enrichment for staphylococcal growth. We enriched for such growth using brain heart infusion broth, whereas Arali *et al.* also enriched using nutrient broth. In another study, Reta *et al.* (2015, p. 2 and p. 5) used enrichment broth and isolated 41.0% *S. aureus* from nasal swabs. Without enrichment, lower numbers of *S. aureus* may be detected, as seen with studies by Tangchaisuriya *et al.* (2014, p.

152), Erami *et al.* (2014, p. 4), Deng *et al.* (2014, p. 76), Jeyakumari *et al.* (2015, p. 17932), Assafi *et al.* (2017, p. 8) and Gong *et al.* (2017, p. 275), who isolated 36.0%, 26.3%, 2.4%, 26.0%, 30.0% and 5.1% *S. aureus* from nasal specimens of school children, respectively. Our CoNS prevalence (25.0%) among children was very low, much lower than the 44.0% reported in Thailand (Tangchaisuriya *et al.*, p. 152) and 71.7% in Iran (Abadi *et al.*, 2014, p. 3).

The percentage of MRSA among school children in our study (17.6%) is very close to the 18.8% reported for Ethiopian primary school children (Kejela and Bacha, 2013). Compared to 12 other studies involving school children from eight countries (three from India, two from China, two from Iran and one each from Thailand, Italy, Iraq, Egypt and Ethiopia), our MRSA prevalence ranked second highest, above an Ethiopian study with a prevalence of 13.8% (Reta *et al.*, 2015, p. 4), and below an Iranian study with a reported MRSA prevalence of 35.9% (Erami *et al.*, 2014, p. 3). At 2.6%, MRCoNS carriage in our study was very low, well below the 16.7% isolated from Iranian learners aged 7-19 years (Abadi *et al.*, 2014, p. 5).

In the present study, gender did affect nasal colonization with *Staphylococcus*, since there was no significant difference ($P > 0.05$) between males and females with regards to the prevalence of *S. aureus*, MRSA, CoNS and MRCoNS. There was also no significant difference between age groups and colonization with *S. aureus*, CoNS and MRCoNS. Methicillin-resistant *S. aureus* colonization was however significantly higher ($P = 0.003$) in the age group 11-14 years than in the group 6-10 years.

Similar to our study, Deng *et al.* (2014, p. 76) and Gong *et al.* (2017, p. 275) also found no significant difference for *S. aureus* nasal colonization and gender. In contrast to our study, Erami *et al.* (2014, p. 3), Bharathi *et al.* (2014, p. 1), Jeyakumari *et al.* (2015,

p. 17931), Nikfar *et al.* (2015, p. 69) and Reta *et al.* (2015, p. 3) all reported *S. aureus* nasal carriage to be much higher in males than females. In two other studies, Arali *et al.* (2016, p. 622) and Assafi *et al.* (2017, p. 8) found *S. aureus* carriage to be higher in females than males. Also similar to our study, Alzoubi *et al.* (2014, p. 115) and Bharathi *et al.* (2014, p. 1) observed no significant difference in MRSA nasal colonization between males and females. Studies by Erami *et al.* (2014, p. 3) and Reta *et al.* (2015, p. 3) showed higher percentages of MRSA among nasal isolates from males, whereas Nikfar *et al.* (2015, p. 69) reported a higher incidence of MRSA isolates from females.

In agreement with the present study, studies by Erami *et al.* (2014, p. 4), Deng *et al.* (2014, p. 76) and Esposito *et al.* (2015, p. 64) indicated no significant difference in *S. aureus* isolates among age groups. In contrast, Reta *et al.* (2015, p. 3) and Arali *et al.* (2016, p. 621) reported *S. aureus* carriage that differed with age groups. Our MRSA isolates was significantly higher in the age-group 11-14 years, which is contradictory to the findings of Alzoubi *et al.* (2014, p. 115), Reta *et al.* (2015, p. 3) and Arali *et al.* (2016, p. 623) who observed no big difference in nasal MRSA isolates among age groups.

Bacteria are multi-drug resistant if they display resistance towards three or more classes of antibiotics. Multi-drug resistant MRSA (isolate M25 TSA A) is shown in Figure 3.1. This isolate came from a 9-year-old girl from school D who did not get nosebleeds but was exposed to cigarette smoke. Apart from being resistant to ceftazidime (FOX 30µg) (methicillin-resistant), the isolate was also resistant towards ampicillin (AP 25µg), tetracycline (T 30µg) and rifampicin (RP 5µg), with mean inhibition zones of 7±0mm, 8.5±1.41mm, 12±0mm, respectively. In Jordan, Egypt, Alzoubi *et al.* (2014, p. 116) also detected some multi-drug resistant MRSA isolates from nasal

specimens of primary school children. Out of 15 MRSA isolates, five (33.3%) were resistant to erythromycin, three (20.0%) were resistant to trimethoprim-sulfamethoxazole, two (13.3%) were resistant to tetracycline and all isolates displayed resistance towards ceftiofloxacin. In Kashan, Iran, Erami *et al.* (2014, p. 3) observed multi-drug resistance in 19/33 (57.6%) children with MRSA nasal carriage. Bharathi *et al.* (2014, p. 1) reported an alarming multi-drug resistance carriage rate in municipality school children from India, with MRSA that was resistant to gentamicin (41.2%), doxycycline (58.8%), co-trimoxazole (88.2%), rifampicin (64.7%) and ciprofloxacin (52.9%). These antibiotics are commonly prescribed for treatment of *S. aureus* and/or MRSA infections.

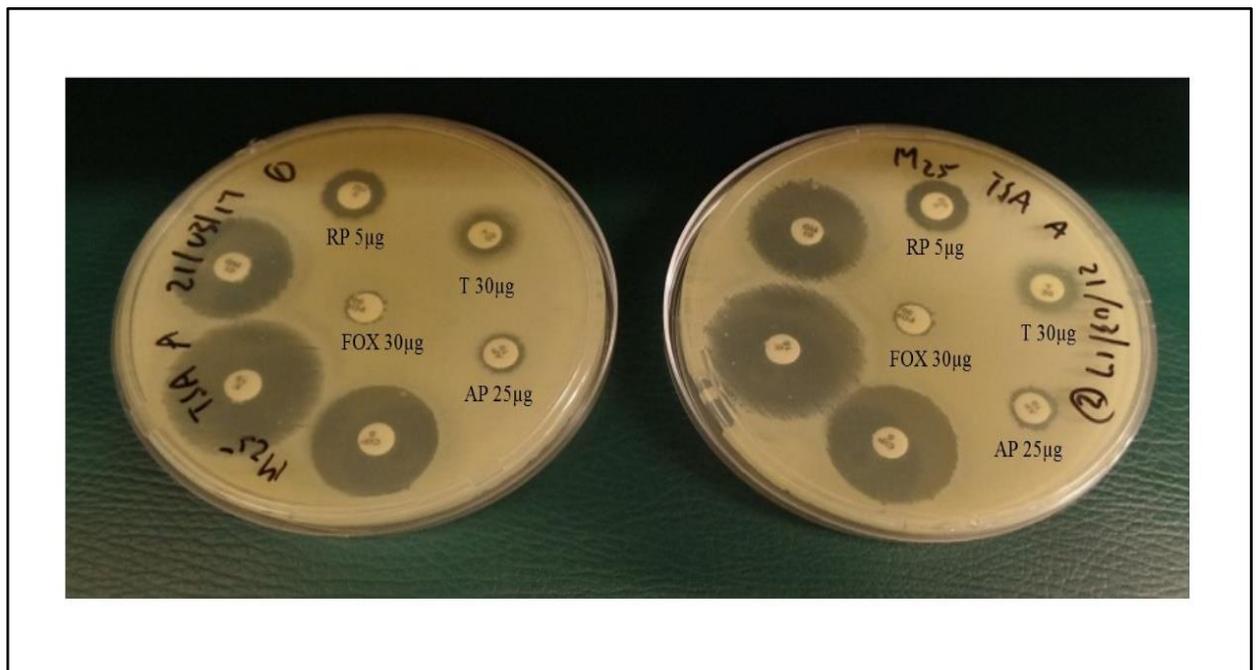


Figure 3.1 Duplicate Kirby-Bauer disk diffusion assay on Mueller-Hinton agar showing reduced inhibition zones of multi-drug resistant MRSA (Resistance pattern AP-T-RP-FOX) from a 9-year-old girl recruited from school D.

No publications or data on the prevalence of nasal staphylococci among Namibian school children were found for comparison of our results. Given the high incidence of *S. aureus* (the most virulent of staphylococcal species depending on the strain) and presence of antibiotic resistant bacteria among school children in the Mariental community, it is recommended that school health programmes improve current hygiene practices in these schools. Learners must be educated on the importance of frequent handwashing to prevent staphylococcal disease such as skin, ear and throat infections and must have access to water and soap.

3.4 Conclusion

The high prevalence of *S. aureus* and presence of MRSA (including multi-drug resistant bacteria) among apparently healthy pupils aged 6-14 years attending five schools in the Mariental District calls for improvement in current hygiene practices in these schools to prevent staphylococcal disease. Nosebleeds and exposure to cigarette smoke are possible risk factors for colonization with *S. aureus* in the children.

References

- Abadi, M.I.M., Moniri, R., Khorshidi, A., Piroozmand, A., Mousavi, S.G.A., Dastehgoli, K. and Ghazikalayeh, H.M. (2015) Molecular characteristics of nasal carriage methicillin-resistant coagulase negative staphylococci in school students. *Jundishapur Journal of Microbiology* [online]. **8**(6). Available from DOI: 10.5812/jjm.18591v2 [Accessed 11 September 2017].
- Alzoubi, H.M., Agel, A.A., Al-Sarayreh, S.A. and Al-Zayadneh, E. (2014) Methicillin-resistant *Staphylococcus aureus* nasal carriage among primary school-aged children from Jordan: Prevalence, antibiotic resistance and molecular

characteristics. *Journal of Egyptian Public Health Association* [online]. **9**(3), pp. 114-118. Available from DOI: 10.1097/01.EPX.0000454671.83406.e8 [Accessed 4 June 2016].

Arali, S.M., Kulkarni, V., Manjula, N.G., Gaddad, S.M., Jayaraj, Y.M. and Shivannavar, C.T. (2016) Prevalence and antibiotic susceptibility profile of MRSA isolated from the anterior nares of school going children in Gulbarga. *International Journal of Medical Science and Public Health* [online]. **5**(4), pp. 620-626. Available from DOI: 10.5455/jmsph.2016.0805201592 [Accessed 7 December 2016].

Assafi, M.S., Polse, R.F., Hussein, N.R., Haji, A.H. and Issa, A.R. (2017) The prevalence of *S. aureus* nasal colonization and its antibiotic sensitivity pattern amongst primary school pupils. *Science Journal of University of Zakho* [online]. **5**(1), pp. 7-10. Available from DOI: <https://doi.org/10.25271/2017.5.1.291> [Accessed 14 July 2017].

Bharathi, M., Lakshmi, N., Kalyani, C.S. and Padmaja, I.J. (2014) Nasal carriage of multidrug-resistant MSSA and MRSA in children of municipality schools [Letter to the editor]. *Indian Journal of Medical Microbiology* [online]. **32**(2). Available from: <http://www.ijmm.org/text.asp?2014/32/2/200/129840> [Accessed 29 July 2015].

Brooks, G.F., Carroll, K.C., Butel, J.S., Morse, S.A. and Mietzner, T.A. (2013) *Jawetz, Melnick & Adelberg's Medical Microbiology*. 26th ed. S.l.: McGraw-Hill.

Charan, J. and Biswas, T. (2013) How to calculate sample size for different study designs in medical research? *Indian Journal of Psychological Medicine*

[online]. **35**(2), pp. 121-126. Available from DOI: 10.4103/0253-7176.116232
[Accessed 22 March 2015].

Deng, J.J., Xiao, G.G., Zhu, Y., Zhou, W. and Wan, C.M. (2014) *Staphylococcus aureus* nasal carriage and its antibiotic resistance profiles in Tibetan school children in southwest China. *Hong Kong Journal of Paediatrics (New Series)* [online]. **19**, pp. 75-78. Available from: <http://www.hkjpgaed.org/details.asp?id=952&show=12> [Accessed 29 July 2015].

Elmanama, A.A., Tayyem, N.E.A. and Allah, S.A.N. (2014). The bacterial etiology of otitis media and their antibiogram among children in Gaza Strip, Palestine. *Egyptian Journal of Ear, Nose, Throat and Allied Sciences* [online]. **15**, pp. 87-91. Available from DOI: <http://dx.doi.org/10.1016/j.ejenta.2014.03.002> [Accessed 7 November 2017].

Erami, M., Soltani, B., Ardakani, A.T., Moravveji, A., Rezaei, M.H., Soltani, S. and Moniri, R. (2014) Nasal carriage and resistance pattern of multidrug resistant *Staphylococcus aureus* among healthy children in Kashan, Iran. *Iranian Red Crescent Medical Journal* [online]. **16**(9). Available from DOI: 10.5812/ircmj.21346 [Accessed 16 December 2015].

Esposito, S., Terranova, L., Ruggiero, L., Ascolese, B., Montinaro, V., Rios, W.P., Galeone, C. and Principi, N. (2015) *Streptococcus pneumoniae* and *Staphylococcus aureus* carriage in healthy school children and adolescents. *Journal of Medical Microbiology* [online]. **64**, pp. 427-431. Available from DOI: 10.1099/jmm.0.000029 [Accessed 7 December 2016].

- European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2017) *Breakpoint tables for interpretation of MICs and zone diameters version 7.1*. Available from: http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_7.1_Breakpoint_Tables.pdf [Accessed 9 September 2017].
- Forbes, B.A., Sahm, D.F. and Weissfeld, A.S. (2007) *Bailey and Scott's Diagnostic Microbiology*. 12th ed. St. Louis: Mosby.
- Gong, Z., Shu, M., Xia, Q., Tan, S., Zhou, W., Zhu, Y. and Wan, C. (2017) *Staphylococcus aureus* nasal carriage and its antibiotic resistance profiles in children in high altitude areas of Southwestern China. *Archivos Argentinos de Pedriatría* [online]. **115**(3), pp. 274-286. Available from DOI: <http://dx.doi.org/10.5546/aap.2017.eng.274> [Accessed 13 July 2017].
- Graig, A. and Hoff, G. (eds.) (2016) *Namibia town of the year 2016*. Brochure. Windhoek: Newsprint Namibia.
- Harley, J.P. and Prescott, L.M. (2002) *Laboratory exercises in Microbiology*. 5th ed. New York: McGraw-Hill.
- Jenney, A., Holt, D., Ritika, R., Southwell, P., Pravin, S., Buadromo, E., Carapetis, J., Tong, S. and Steer, A. (2014) The clinical and molecular epidemiology of *Staphylococcus aureus* infections in Fiji. *BMC Infectious Diseases* [online]. **14** (160). Available from DOI: 10.1186/1471-2334-14-160 [Accessed 7 November 2017].
- Jeyakumari, D., Nagajothi, J., Menon, R. and Tiroumourougane, S.V. (2015) Prevalence of nasal carriage of *Staphylococcus aureus* and its antibiotic susceptibility pattern among primary school children in a rural area of

Puducherry, India. *International Journal of Current Research* [online]. **7**(7), pp. 17930-17933. Available from:

http://www.gmferd.com/journalcra.com/sites/default/files/9697_0.pdf

[Accessed 7 December 2016].

Kejela, T. and Bacha, K. (2013) Prevalence and antibiotic susceptibility pattern of methicillin-resistant *Staphylococcus aureus* (MRSA) among primary school children and prisoners in Jimma Town, southwest Ethiopia. *Annals of Clinical Microbiology and Antimicrobials* [online]. **12**. Available from DOI: 10.1186/1476-0711-12-11 [Accessed 29 July 2015].

Kulkarni, R., Antala, S., Wang, A., Amaral, F.E., Rampersaud, R., LaRussa, S.J., Planet, P.J. and Ratner, A.J. (2012) Cigarette smoke increases *Staphylococcus aureus* biofilm formation via oxidative stress. *Infection and Immunity* [online]. **80**(11), pp. 3804-3811. Available from DOI: 10.1128/IAI.00689-12 [Accessed 29 July 2017].

Lowy, F.D. (2010) Staphylococcal infections. In: Kasper, D. and Fauci, A. (eds.) *Harrison's infectious diseases*. S.l.: McGraw-Hill, pp. 386-399.

MedCalc (2016) *MedCalc statistical software version 16.4.3*. Available from: <https://www.medcalc.org>.

Nikfar, R., Shamsizadeh, A., Kajbaf, T.Z., Panah, K., Khaghani, S. and Moghddam, M. (2015) Frequency of methicillin-resistant *Staphylococcus aureus* in healthy children. *Iranian Journal of Microbiology* [online]. **7**(2), pp. 67-71. Available from: <http://www.researchgate.net/publication/281999247> [Accessed 16 December 2015].

- Prescott, L.M., Harley, J.P. and Klein, D.A. (2002) *Microbiology*. 5th ed. New York: McGraw-Hill.
- Pynnonen, M., Stephenson, R.E., Schwartz, K., Hernandez, M. and Boles, B.R. (2011) Hemoglobin promotes *Staphylococcus aureus* nasal colonization. *PLOS Pathogens* [online]. **7**(7). Available from DOI: 10.1371/journal.ppat.1002104 [Accessed 23 March 2017].
- Reta, A., Gedefaw, L., Sewunet, T. and Beyene, G. (2015) Nasal carriage, risk factors and antimicrobial susceptibility pattern of methicillin resistant *Staphylococcus aureus* among school children in Ethiopia. *Medical Microbiology and Diagnosis* [online]. **4**(1). Available from DOI: 10.4172/21610703.1000177 [Accessed 15 September 2015].
- Tangchaisuriya, U., Yotpanya, W., Kitti, T. and Sitthisak, S. (2014) Distribution among Thai children of methicillin-resistant *Staphylococcus aureus* lacking *cna*, *fnbA* and *icaAD*. *Southeast Asian Journal of Tropical Medicine and Public Health* [online]. **45**(1). Available from: <http://www.mahidol.ac.th/seameo/2014-45-1-full/19-5911-15.pdf> [Accessed 17 August 2015].
- Zautner, A.E., Krause, M., Stropahl, G., Holtfreter, S., Frickmann, H., Maletzki, C., Kreikemeyer, B., Pau, H.W. and Podbielski, A. (2010) Intracellular persisting *Staphylococcus aureus* is the major pathogen in recurrent tonsillitis. *PLoS ONE* [online]. **5**(3). Available from DOI: 10.1371/journal.pone.0009452 [Accessed 7 November 2017].

Chapter 4

Antibiograms and resistance patterns of nasal staphylococcal isolates from Namibian school children.

Abstract

Healthy school children under 16 years are potential carriers of methicillin-resistant *Staphylococcus aureus* (MRSA) and multi-drug resistant strains. Nasal colonization with these resistant strains puts children at risk of developing difficult-to-treat staphylococcal infections. Antibiotic resistance data is limited in Namibia and few reports on staphylococcal drug resistance exist. Our study thus aimed to provide antibiograms and resistance patterns for 433 nasal *S. aureus* and coagulase-negative staphylococci (CoNS) from school children in the Mariental District. To our knowledge, this is the first report on resistance trends of nasal staphylococci from Namibian school children.

By Kirby-Bauer disk diffusion assay, 352 *S. aureus* and 81 coagulase-negative staphylococcal isolates underwent susceptibility testing against the antibiotics ampicillin, cefoxitin, ciprofloxacin, erythromycin, gentamicin, rifampicin and tetracycline. The reference strains *S. aureus* ATCC 25923 (susceptible strain) and *S. aureus* ATCC 33591 (multi-drug resistant MRSA strain) were used for quality control. Antibiograms and resistance patterns were obtained and isolates were classified as multi-drug resistant when they displayed resistance towards three or more classes of antibiotics. Cefoxitin resistance - an inhibition zone of < 22mm - indicated MRSA/methicillin-resistant CoNS (MRCoNS).

Out of 433 staphylococcal isolates, 96.0% *S. aureus* and 66.7% CoNS were resistant to ampicillin. Ampicillin resistance was significantly higher in *S. aureus* than in CoNS ($P < 0.0001$). Ciprofloxacin and gentamicin were most effective against *S. aureus*,

with 99.7% and 93.2% of isolates that were susceptible to these drugs. Ciprofloxacin was also the most effective drug against CoNS, with 100.0% susceptibility. Cefoxitin/methicillin resistance was seen in 14.5% *S. aureus* isolates and 8.6% of CoNS. In total 31 antibiotic resistance patterns were observed. The three most frequently found patterns were AP, AP-E, and AP-T. Out of all isolates, 12.5% (50 *S. aureus* and four CoNS) were multi-drug resistant. From the 51 MRSA isolates, 43.1% were multi-drug resistant. One of these MRSA isolates showed resistance towards 6/7 antibiotics tested with only ciprofloxacin that was effective against it. Methicillin-resistant CoNS were not multi-drug resistant, with the most common resistance pattern being AP-RP-FOX.

In conclusion, multi-drug resistance in our study was relatively low. However, almost half of the MRSA isolates were multi-drug resistant, which is of concern. Nevertheless, learners should be educated on the importance of handwashing and judicious use of antibiotics to prevent spread of antibiotic-resistant bacteria within the community. Ciprofloxacin and gentamicin may effectively be used to treat staphylococcal infections in this study population.

Keywords: *Staphylococcus*, MRSA, MRCoNS, school children, multi-drug resistance, antibiograms, resistance patterns, Mariental

4.1 Introduction

Healthy school children under 16 years are potential carriers of *Staphylococcus aureus*, especially methicillin-resistant *S. aureus* (MRSA) and multi-drug resistant strains (Arali *et al.*, 2016, p. 625). Antibiotic resistance within communities is increasing due to widespread suboptimal use of antibiotics in outpatient settings and use of antibiotics in farming activities (Byarugaba, 2010, p. 17). According to Alzoubi *et al.* (2014, p.

114) children are asymptomatic reservoirs for community-associated MRSA (CA-MRSA) which enables these bacteria to rapidly spread within communities.

Being colonized with *Staphylococcus* puts one at risk of developing an infection (Reid *et al.*, 2017, p. 795). Most staphylococcal infections can easily be cleared with appropriate antibiotics, but bacteria that develop resistance towards certain antibiotics makes treatment options limited, especially if antibiograms for reference purposes are unavailable. Methicillin-resistant *S. aureus* is resistant to beta-lactam antibiotics, while some strains are multi-drug resistant and may produce disease-causing toxins. Drug resistant strains are often responsible for chronic, persistent and recurrent infections. This is problematic for healthcare practitioners.

Antibiotic resistance data is limited in Namibia and few reports on staphylococcal drug resistance exist. Our study aimed to make a contribution towards closing this gap in information by providing antibiograms and resistance patterns for 433 *S. aureus* and coagulase-negative staphylococci (CoNS) coming from nasal swabs of children attending schools in the Mariental District. To our knowledge, this is the first report on resistance trends of nasal staphylococcal isolates from Namibian school children.

4.2 Materials and methods

4.2.1 Bacterial cultures used

Two purchased reference strains, *S. aureus* ATCC 25923 (an antibiotic susceptible strain) and *S. aureus* ATCC 33591 (a multi-drug resistant MRSA strain) (Microbiologics[®], St. Cloud, US), as well as 433 staphylococcal nasal isolates from 272 Mariental school children were used in antibiotic assays. Of these isolates, 352 were *S. aureus* and 81 were CoNS.

4.2.2 Antibiotic susceptibility testing

Kirby-Bauer disk diffusion assay was employed to determine antibiograms for isolates (Harley and Prescott, 2002; BSAC, 2013; CLSI, 2013; EUCAST, 2017). Table 4.1 shows the antibiotics that were used and interpretation of inhibition zones. *Staphylococcus aureus* ATCC 25923, *S. aureus* ATCC 33591 (MRSA), 352 nasal *S. aureus* isolates and 81 nasal CoNS were tested to determine their antibiograms and resistance patterns. Resistance towards cefoxitin (30µg), in other words an inhibition zone diameter < 22mm, indicated MRSA or methicillin-resistant CoNS (MRCoNS), while resistance towards three or more different classes of antibiotics was an indication of multi-drug resistance in bacteria.

By direct colony suspension, three to five well-isolated colonies from overnight tryptone soy agar (Scharlau Microbiology, Spain) plate cultures were inoculated into 10ml sterile phosphate buffered saline pH 6.8-7.4 (Skylabs, Johannesburg, SA) and adjusted to 0.5 McFarland standard (1.5×10^8 CFU/ml, absorbance reading 0.08-0.13 at 625nm). Adjusted cultures were then swabbed onto Mueller-Hinton (Mast Diagnostics, Merseyside, UK) agar and left to dry for 5-10 minutes at room temperature before dispensing the antibiotic disks (Mast Diagnostics, Merseyside, UK) onto the plates. The “15-15-15 minute rule” (EUCAST 2017) was applied here. This rule states that the inoculum should be used within 15 minutes of preparation and always within 60 minutes. Also, disks should be applied within 15 minutes of inoculating the plates, and incubation should be within 15 minutes after application of disks. Plates were incubated at 35°C for 18-20 hours and diameters were measured using a ruler. Isolates were classified as susceptible, resistant or intermediate towards each antibiotic, according to the diameter (in millimetres) of their zones of inhibition. The reference strains *S. aureus* ATCC 25923 (susceptible) and *S. aureus* ATCC 33591

(multi-drug resistant MRSA) served as quality control strains. Clinical and Laboratory Standards Institute (CLSI) guidelines (2013) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoint tables version 7.1 (2017) were used to interpret results.

Table 4.1 Antibiotics used in this study and interpretation of inhibition zones of test cultures. Adapted from CLSI (2013) and EUCAST (2017).

Chemical class	Antibiotic	Disk symbol	Disk content	Resistant	Intermediate	Susceptible
Aminoglycosides	Gentamicin	GM	10µg	<18mm <i>S.aureus</i> <22mm CoNS	-	≥18mm <i>S.aureus</i> ≥22mm CoNS
β-lactams	Ampicillin	AP	25µg	<18mm	-	≥18mm
Cephalosporins (also a β-lactam)	Cefoxitin	FOX	30µg	<22mm	-	≥22mm
Fluoroquinolones	Ciprofloxacin	CIP	5µg	<20mm	-	≥20mm
Macrolides	Erythromycin	E	15µg	<18mm	18-20mm	≥21mm
Tetracyclines	Tetracycline	T	30µg	<19mm	19-21mm	≥22mm
Other	Rifampicin	RP	5µg	<23mm	23-25mm	≥26mm

4.2.3 Statistical analysis

A chi-square test for comparison of proportions with MedCalc statistical software (MedCalc statistical software version 16.4.3 {MedCalc software bvba, Ostend, Belgium; <https://www.medcalc.org>; 2016}) was used to compare percentage differences in antibiotic resistance between *S. aureus* and CoNS. Statistical significant differences was indicated by a P-value of ≤ 0.05 .

4.3 Results and discussion

4.3.1 Antibiotic susceptibility/resistance of isolates

Altogether 433 staphylococcal isolates, as well as two reference strains, underwent antibiotic susceptibility testing against seven antibiotics (Table 4.1). Excluding the reference strains, 352 of these were *S. aureus* and 81 were CoNS. Only 4.8% of isolates were susceptible to all antibiotics tested. As expected, *S. aureus* ATCC 25923 was susceptible, while *S. aureus* ATCC 33591 (MRSA) was resistant to ceftazidime and also multi-drug resistant.

Table 4.2 gives the antibiogram for *S. aureus*. Most isolates (96.0%) were resistant to ampicillin, rendering this antibiotic mostly ineffective against *S. aureus*. For CoNS, 66.7% of isolates were resistant to the antibiotic (Table 4.3). Resistance towards ampicillin was significantly higher in *S. aureus* than in CoNS ($P < 0.0001$). Namibia standard treatment guidelines (Ministry of Health and Social Services, 2011) recommends the use of ampicillin to treat septicaemia in newborns, and in combination with gentamicin to treat hospital-acquired pneumonia and bacterial meningitis (in babies < 3 months). Other researchers also detected high ampicillin resistance in *S. aureus*. In northeastern Brazil, for example, de Carvalho *et al.* (2017, p. 465) observed 80.0% ampicillin resistance in *S. aureus* from nasal secretions of children attending

public daycare. Although this drug was still effective against 33.3% of coagulase-negative isolates in the current study, it should not be the drug of choice for treating infections caused by *S. aureus* and CoNS, unless used in combination with other drugs. High resistance towards ampicillin can be expected since many staphylococci produce the enzyme penicillinase (a β -lactamase) which destroys the action of penicillins (Forbes *et al.*, 2007, p. 181; Akindele *et al.* 2010, p. 232; Dinić *et al.*, 2013, p. 34). According to Forbes *et al.* (2007, p. 181) approximately 90.0% or more of clinical staphylococcal isolates are resistant to penicillin because of β -lactamase production.

Table 4.2 Antibiogram for *S. aureus* isolates (n = 352).

Antibiotic	Susceptible (%)	Intermediate (%)	Resistant (%)
Ampicillin 10 μ g	14 (4.0)	-	338 (96.0)
Cefoxitin 30 μ g	301 (85.5)	-	51 (14.5)
Ciprofloxacin 5 μ g	351 (99.7)	-	1 (0.3)
Erythromycin 15 μ g	238 (67.6)	31 (8.8)	83 (23.6)
Gentamicin 10 μ g	328 (93.2)	-	24 (6.8)
Rifampicin 5 μ g	248 (70.4)	40 (11.4)	64 (18.2)
Tetracycline 30 μ g	232 (66.0)	60 (17.0)	60 (17.0)

Table 4.3 Antibiogram for CoNS isolates (n = 81).

Antibiotic	Susceptible (%)	Intermediate (%)	Resistant (%)
Ampicillin 10µg	27 (33.3)	-	54 (66.7)
Cefoxitin 30µg	74 (91.4)	-	7 (8.6)
Ciprofloxacin 5µg	81 (100.0)	-	0 (0)
Erythromycin 15µg	65 (80.2)	8 (9.9)	8 (9.9)
Gentamicin 10µg	72 (88.9)	-	9 (11.1)
Rifampicin 5µg	55 (67.9)	12 (14.8)	14 (17.3)
Tetracycline 30µg	60 (74.1)	14 (17.3)	7 (8.6)

As indicated in Tables 4.2 and 4.3, a total of 83 (23.6%) *S. aureus* isolates and nine (11.1%) CoNS were resistant to erythromycin. These resistance rates are not that high and erythromycin is expected to be effective against staphylococci in most instances. Compared to our study, Namibia Institute of Pathology (NIP) data showed higher resistance (32.3%) to this drug in *Staphylococcus* isolated from cerebrospinal fluid (CSF) for the period 2009-2012 (Mengistu *et al.*, 2013, p. 6). Also using NIP data, Iileka *et al.* (2016, p. 119) reported a lower percentage resistance (10.2%) than ours for *S. aureus* isolates obtained from various clinical samples over the time period 2012-2014. According to PathCare Namibia (2015) 11.0% *S. aureus* bacteria were resistant to erythromycin from 2014 to 2015, which is very close to the 10.2% indicated by Iileka *et al.* (2016). In a Brazilian study, de Carvalho *et al.* (2017, p. 465) observed a higher percentage (32.8%) erythromycin resistance for *S. aureus* compared to our study. Their percentage resistance is however almost the same as the 32.3% from Mengistu *et al.* (2013). In Namibia, erythromycin may be used to manage conditions

such as furuncles (boils) in the external ear canal; bacterial skin infections like acne, abscesses, cellulitis and erysipelas; eyelid infections such as preseptal cellulitis (inflamed eyelid); and in combination with ceftriaxone in neonatal conjunctivitis. It may also be used in some instances as an alternative when the patient is allergic to penicillin (Ministry of Health and Social Services, 2011). Children from the present study could have therefore acquired erythromycin-resistant staphylococcal strains after receiving treatment for some of the above-mentioned conditions.

In the current work, only 17.0% *S. aureus* and 8.6% CoNS were resistant to tetracycline, indicating its effectiveness against *Staphylococcus*. According to NIP data for 2009-2012, 29.6% of staphylococci from CSF displayed resistance towards tetracycline (Mengistu *et al.*, 2013, p. 6). In agreement with our results, Iileka *et al.* (2016, p. 119) reported 17.4% tetracycline resistance in clinical *S. aureus* strains across the period 2012-2014. The resistance for *S. aureus* in our study towards this drug is much higher than the 4.3% found in Brazil (de Carvalho *et al.*, 2017, p. 465). Tetracycline may be prescribed to treat eyelid infections such as recurrent chalazia (Ministry of Health and Social Services, 2011), and resistant strains may then colonize children receiving such treatment.

Rifampicin resistance was relatively low at 18.2% and 17.3% for our *S. aureus* and CoNS isolates, respectively, which is somewhat higher than the 7.0% indicated in PathCare data for 2014-2015 (PathCare Namibia, 2015). These findings justify its use in Namibia against some staphylococcal infections. In other countries, however, resistance may be higher. In India for example, Bharathi *et al.* (2014, p. 1) performed susceptibility testing against nasal MRSA bacteria from school children, and observed rifampicin resistance of 64.7%. According to them, rifampicin are among the antibiotics commonly used to treat MRSA infections in India. Brooks *et al.* (2013, p.

205) say that staphylococci can quickly develop resistance to rifampicin. Namibia standard treatment guidelines (Ministry of Health and Social Services, 2011) recommend the use of rifampicin in tuberculosis (TB) treatment (although TB does not involve staphylococci). The children in our study population could however have been exposed to rifampicin-resistant staphylococci in some way since the drug is used in Namibia.

We found that ciprofloxacin and gentamicin were most effective against *S. aureus*, with 99.7% and 93.2% of isolates that were susceptible to these drugs, respectively. Ciprofloxacin was also the most effective drug against CoNS, with 100.0% susceptibility. In line with our results, de Carvalho *et al.* (2017, p. 465) observed 92.9% *S. aureus* susceptibility towards ciprofloxacin in Brazil. Using NIP data, Mengistu *et al.* (2013, p. 6) indicated 19.0% ciprofloxacin resistance and 52.9% gentamicin resistance for *Staphylococcus* from CSF samples, whereas Iileka *et al.* (2016, p. 119) indicated a low 4.4% ciprofloxacin resistance for *S. aureus* clinical isolates. Namibian PathCare data for 2011-2012 showed *S. aureus* to be 96.0% susceptible to ciprofloxacin, while data for 2014-2015 indicated 95.0% susceptibility to gentamicin (Appendix C). According to Ministry of Health and Social Services (2011) ciprofloxacin may be used along with clindamycin to treat osteomyelitis, whereas gentamicin together with ampicillin may be used to manage hospital-acquired pneumonia and bacterial meningitis in infants <3 months. Gentamicin is also recommended for septicaemia in newborns. Based on our results, ciprofloxacin and gentamicin would be the drugs of choice to treat certain staphylococcal infections in our study population. Taking into consideration the 52.9% resistance towards gentamicin of CSF *Staphylococcus* that may cause meningitis in Namibia (Iileka *et al.*,

2016, p. 6), care should be taken by physicians when prescribing gentamicin for meningitis.

In this study, ceftazidime was used to detect methicillin-resistant bacteria. These bacteria are also resistant to all β -lactam antibiotics. A total of 51/352 (14.5%) *S. aureus* isolates were MRSA, and 7/81 (8.6%) of CoNS were MRCoNS. Our MRSA prevalence is close to the 13.8% ceftazidime-resistant *S. aureus* reported by Reta *et al.* (2015, p. 3) in a community based cross-sectional study that involved children aged 6-12 years from nine primary schools in Bahir Dar Town, Ethiopia. It is also in line with Namibia Institute of Pathology data that showed 13.6% MRSA (2010-2014) and 13.5% MRSA (2012-2014) obtained from various clinical specimens (Festus *et al.*, 2016, p. 1 and p 3; Iileka *et al.*, 2016, p 119). Our data and that of NIP however showed a higher MRSA prevalence than PathCare data which indicated only 8.0% MRSA for 2014-2015 (PathCare Namibia, 2015). Based on these relatively low percentages, MRSA does not seem to be a major problem in Namibia yet. In general, there are not many studies available on methicillin resistance in CoNS. According to Jayadev-Menon *et al.* (2015) MRSA and MRCoNS are found together in the human nose and have similar antibiotic resistance genes that can be transferred between the bacteria. In the present study, 10 resistance patterns were shared between *S. aureus* and CoNS isolates (see Table 4.4). Of these patterns, only one (AP-RP-FOX) was shared between MRSA and MRCoNS, with 12 *S. aureus* isolates and seven CoNS isolates that displayed this resistance pattern. Resistance patterns observed in our study are discussed in the next section.

4.3.2 Resistance patterns and multi-drug resistance

Altogether 31 antibiotic resistance patterns were observed (Table 4.4). Isolates with similar resistance patterns can be the same strain, unless they acquired these resistance

genes from other strains, therefore sharing the same resistance pattern. For *S. aureus*, 27 different resistance patterns were obtained. For CoNS, there were 14 different patterns. Ten patterns (AP, RP, AP-E, AP-GM, AP-RP, AP-T, AP-GM-RP, AP-RP-FOX, AP-T-E and AP-T-RP-E) were shared by *S. aureus* and CoNS. AP-RP-FOX was the only pattern shared between MRSA and MRCoNS. The three most frequently encountered patterns were AP, AP-E, and AP-T, with 194, 52 and 23 isolates having these patterns, respectively.

As indicated by their respective antibiograms in Tables 4.2 and 4.3, the majority of *S. aureus* isolates (96.0%) and more than half of CoNS isolates (66.7%) were resistant to ampicillin. A total of 23.6% of *S. aureus* isolates and 9.9% CoNS showed resistance against erythromycin, while 17.0% *S. aureus* and 8.6% CoNS were resistant against tetracycline. Resistance towards ampicillin is very common in staphylococci and can be ascribed to the action of beta-lactamase and is under plasmid control (Brooks *et al.*, 2013, p. 200). Plasmids containing resistance genes can be transferred from one bacterium to another. *Staphylococcus aureus* strains in our study were less resistant to tetracycline than the 29.6% *S. aureus* from CSF tested by NIP (Mengistu *et al.*, 2013, p. 6). They were equally resistant compared to other clinical NIP strains with 17.4% tetracycline resistance (Iileka *et al.*, 2016, p. 119). Our *S. aureus* was also less resistant to erythromycin than the CSF isolates which were 32.3% resistant, and more resistant than the 10.2% *S. aureus* from NIP. According to PathCare data for 2014-2015 (PathCare Namibia, 2015) found in Appendix C, the *S. aureus* isolates in our study were twice as resistant to erythromycin and tetracycline. The overall relatively low resistance against erythromycin and tetracycline in our study participants is an indication that they are currently not being over-exposed to these antibiotics.

Table 4.4 Resistance patterns for *S. aureus* and CoNS, including methicillin-resistant isolates, against seven antibiotics.

Number of isolates with this pattern	Number of <i>S. aureus</i> isolates with this pattern	Number of CoNS isolates with this pattern	Antibiotic resistance pattern
194	162	32	AP
2	0	2	E
2	0	2	GM
4	3	1	RP
2	0	2	T
52	50	2	AP-E
19	14	5	AP-GM
11	8	3	AP-RP
23	22	1	AP-T
8	8	0	AP-FOX
1	1	0	GM-E
2	2	0	T-RP
1	1	0	RP-E
1	1	0	AP-CIP-FOX
5	5	0	AP-E-FOX
3	3	0	AP-GM-E
5	4	1	AP-GM-RP
1	1	0	AP-GM-T
4	4	0	AP-RP-E
19	12	7	AP-RP-FOX
9	8	1	AP-T-E
7	7	0	AP-T-RP
3	3	0	AP-T-FOX
1	0	1	GM-T-E
1	1	0	AP-GM-RP-FOX
5	5	0	AP-RP-E-FOX
1	1	0	AP-T-E-FOX
2	1	1	AP-T-RP-E
11	11	0	AP-T-RP-FOX
3	3	0	AP-T-RP-E-FOX
1	1	0	AP-GM-T-RP-E-FOX

Key: Multi-drug resistant 

Multi-drug resistant MRSA 

Fifty-four out of 433 isolates (12.5%) were resistant against three or more classes of antibiotics and classified as multi-drug resistant. Of these, 50 isolates were *S. aureus* and four were CoNS. The most common multi-drug resistance pattern for methicillin-

susceptible isolates was AP-T-E, displayed by eight *S. aureus* isolates, and one coagulase-negative isolate. Of the 51 MRSA isolates, 22 (43.1%) were multi-drug resistant, with AP-T-RP-FOX as the most encountered resistance pattern among them. This is of concern, but is 14.5% less than the 57.6% multi-drug resistant MRSA isolated from Iranian children (Erami *et al.*, 2014, p. 1), and 20.5% less than the 63.6% from Brazilian children (de Carvalho *et al.*, 2017, p. 465). One of these multi-drug resistant MRSA isolates in our study showed resistance towards 6/7 antibiotics tested with only ciprofloxacin that was effective against it. Methicillin-resistant CoNS did not show multi-drug resistance, with the most common resistance pattern being AP-RP-FOX. In a French study, Lebeaux *et al.* (2012, p. 319) observed up to 40.0% rifampicin resistance in community-associated MRCoNS. Overall, our findings support the statement by Arali *et al.* (2016, p. 625) that healthy school children under the age of 16 years are potential carriers of MRSA and multi-drug resistant strains.

4.4 Conclusion

Our study showed the presence of multi-drug resistant MRSA and non-multi-drug resistant MRCoNS among healthy school children. Overall, multi-drug resistance was relatively low. However, almost half of the MRSA isolates were multi-drug resistant, which is of concern. Nevertheless, learners should be encouraged to frequently wash their hands to prevent spread of antibiotic-resistant bacteria within the community. They should also be educated on the judicious use of antibiotics, such as completion of antibiotic courses. Ciprofloxacin and gentamicin may effectively be used to treat staphylococcal infections in this study population.

References

- Akindede, A.A., Adewuyi, L.K., Adefioye, O.A., Adedokun, S.A. and Olaolu, A.O. (2010) Antibiogram and beta-lactamase production of *Staphylococcus aureus* isolates from different human clinical specimens in a tertiary health institution in Ile-ife, Nigeria. *American-Eurasian Journal of Scientific Research* [online]. **5**(4), pp. 230-233. Available from: [http://www.idosi.org/aejsr/5\(4\)10/2.pdf](http://www.idosi.org/aejsr/5(4)10/2.pdf) [Accessed 29 July 2015].
- Alzoubi, H.M., Agel, A.A., Al-Sarayreh, S.A. and Al-Zayadneh, E. (2014) Methicillin-resistant *Staphylococcus aureus* nasal carriage among primary school-aged children from Jordan: Prevalence, antibiotic resistance and molecular characteristics. *Journal of Egyptian Public Health Association* [online]. **9**(3), pp. 114-118. Available from DOI: 10.1097/01.EPX.0000454671.83406.e8 [Accessed 4 June 2016].
- Arali, S.M., Kulkarni, V., Manjula, N.G., Gaddad, S.M., Jayaraj, Y.M. and Shivannavar, C.T. (2016) Prevalence and antibiotic susceptibility profile of MRSA isolated from the anterior nares of school going children in Gulbarga. *International Journal of Medical Science and Public Health* [online]. **5**(4), pp. 620-626. Available from DOI: 10.5455/jmsph.2016.0805201592 [Accessed 7 December 2016].
- Bharathi, M., Lakshmi, N., Kalyani, C.S. and Padmaja, I.J. (2014) Nasal carriage of multidrug-resistant MSSA and MRSA in children of municipality schools [Letter to the editor]. *Indian Journal of Medical Microbiology* [online]. **32**(2). Available from: <http://www.ijmm.org/text.asp?2014/32/2/200/129840> [Accessed 29 July 2015].

- British Society for Antimicrobial Chemotherapy (BSAC). (2013) *BSAC methods for antimicrobial susceptibility testing version 6.0*.
- Brooks, G.F., Carroll, K.C., Butel, J.S., Morse, S.A. and Mietzner, T.A. (2013) *Jawetz, Melnick & Adelberg's Medical Microbiology*. 26th ed. s.l.: McGraw-Hill.
- Byarugaba, D.K. (2010) Mechanisms of antimicrobial resistance. In: Sosa, A., Byarugaba, D.K., Amábile-Cuevas, C.F., Hsueh, P., Kariuki, S. and Okeke, I.N. (eds.) *Antimicrobial resistance in developing countries*. New York: Springer, pp. 15-26.
- Clinical and Laboratory Standards Institute (CLSI). (2013) *Performance standards for antimicrobial susceptibility testing: Twenty-third informational supplement*. CLSI document M100-S23. Wayne: CLSI.
- de Carvalho, S.P., de Almeida, J.B., Andrade, Y.M.F.S., da Silva, L.S.C., de Oliveira, M.V., Timenetsky, J. and Marques, L.M. (2017) Community-acquired methicillin-resistant *Staphylococcus aureus* carrying SCCmec type IV and V isolated from healthy children attending public daycare in northeastern Brazil. *The Brazilian Journal of Infectious Diseases* [online]. 21(4), pp. 464-467. Available from DOI: <http://dx.doi.org/10.1016/j.bjid.2017.04.001> [Accessed 22 September 2017].
- Dinić, M., Vuković, S., Kocić, B., Stanković-Đorđević, D. and Bogdanović, M. (2013) Nasal carriage of *Staphylococcus aureus* in healthy adults and in school children. *Acta Facultatis Medicae Naissensis* [online]. 30(1), pp. 31-36. Available from DOI: 10.2478/v10283-012-0033-3 [Accessed 29 July 2015].
- Erami, M., Soltani, B., Ardakani, A.T., Moravveji, A., Rezaei, M.H., Soltani, S. and Moniri, R. (2014) Nasal carriage and resistance pattern of multidrug resistant

Staphylococcus aureus among healthy children in Kashan, Iran. *Iranian Red Crescent Medical Journal* [online]. **16**(9). Available from DOI: 10.5812/ircmj.21346 [Accessed 16 December 2015].

European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2017) *Breakpoint tables for interpretation of MICs and zone diameters version 7.1*. Available from: http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_7.1_Breakpoint_Tables.pdf [Accessed 9 September 2017].

Festus, T., Mukesil, M. and Moyo, S.R. (2016). The distribution of methicillin resistant *Staphylococcus aureus* isolated at the Namibia Institute of Pathology in Windhoek, Namibia. *Indian Journal of Medical Research and Pharmaceutical Sciences* [online]. **3**(7). Available from DOI: 10.5281/zenodo.58108 [Accessed 3 August 2017].

Forbes, B.A., Sahm, D.F. and Weissfeld, A.S. (2007) *Bailey and Scott's Diagnostic Microbiology*. 12th ed. St. Louis: Mosby.

Harley, J.P. and Prescott, L.M. (2002) *Laboratory exercises in Microbiology*. 5th ed. New York: McGraw-Hill.

Iileka, A.E.K., Mukesi, M., Engelbrecht, F. and Moyo, S.R. (2016). Antimicrobial susceptibility patterns of *Staphylococcus aureus* strains isolated at the Namibia Institute of Pathology 2012 to 2014. *Open Journal of Medical Microbiology* [online]. **6**, pp. 116-124. Available from DOI: <http://dx.doi.org/10.4236/ojmm.2016.63016> [Accessed 3 August 2017].

Jayadev-Menon, P., Humphreys, H. and Fitzgerald-Hughes, D. (2015) Could coagulase negative staphylococci be an evolutionary source of resistance genes

for *Staphylococcus aureus*? *BMC Proceedings* [online]. **9**(Suppl 1). Available from: <http://www.biomedcentral.com/1753-6561/9/S1/A43> [Accessed 17 September 2017].

Lebeaux, D., Barbier, F., Angebault, C., Benmahdi, L., Ruppé, E., Felix, B., Gaillard, K., Djossou, F., Epelboin, L., Dupont, C., Renard, M., Peroz, G., Vandenesch, F., Wolff, M., Andremont, A. and Ruimy, R. (2012) Evolution of nasal carriage of methicillin-resistant coagulase-negative staphylococci in a remote population. *Antimicrobial Agents and Chemotherapy* [online]. **56**(12), pp. 315-323. Available from DOI: 10.1128/AAC.00547-11 [Accessed 11 September 2017].

MedCalc (2016) *MedCalc statistical software version 16.4.3*. Available from: <https://www.medcalc.org>.

Mengistu, A., Gaeseb, J., Uaaka, G., Ndjavera, C., Kambyambya, K., Indongo, L., Kalemeera, F., Ntege, C., Mbirizi, D., Joshi, M.P. and Sagwa, E. (2013) Antimicrobial sensitivity patterns of cerebrospinal fluid (CSF) isolates in Namibia: Implications for empirical antibiotic treatment of meningitis. *Journal of Pharmaceutical Policy and Practice* [online]. **6**(4). Available from DOI: 10.1186/2052-3211-6-4 [Accessed 29 July 2015].

Ministry of Health and Social Services (2011) *Namibia standard treatment guidelines*. Windhoek: Ministry of Health and Social Services.

PathCare Namibia (2015) Antimicrobial susceptibility data. PathCare [online]. Available from: http://196.25.36.50/webfiles/files/ANTIMICROBIAL_SUSCEPTIBILITY_D ATA_Staphylococcus_aureus.pdf [Accessed 23 November 2015].

- Reid, M.J.A., Fischer, R.S.B., Mannathoko, N., Muthoga, C., McHugh, E., Essigmann, H., Brown, E.L. and Steenhoff, A.P. (2017) Prevalence of *Staphylococcus aureus* nasal carriage in human immunodeficiency virus-infected and uninfected children in Botswana: Prevalence and risk factors. *The American Journal of Tropical Medicine and Hygiene* [online]. **96**(4), pp. 795-801. Available from DOI: <https://doi.org/10.4269/ajtmh.16-0650> [Accessed 14 July 2017].
- Reta, A., Gedefaw, L., Sewunet, T. and Beyene, G. (2015) Nasal carriage, risk factors and antimicrobial susceptibility pattern of methicillin resistant *Staphylococcus aureus* among school children in Ethiopia. *Medical Microbiology and Diagnosis* [online]. **4**(1). Available from DOI: 10.4172/21610703.1000177 [Accessed 15 September 2015].

Chapter 5

Enterotoxin production and biofilm formation as virulence factors of *S. aureus* and multi-drug resistant MRSA from nasal specimens of Namibian school children.

Abstract

Colonization with *Staphylococcus aureus* does not necessarily imply disease, however, this bacterium has the potential to cause infections in healthy persons when the opportunity arises. It is capable of producing virulence factors that aid in its pathogenicity and virulence. Enterotoxin and biofilm studies involving strains from school children are limited and the current work thus aimed to screen *S. aureus* and multi-drug resistant MRSA isolates from nasal specimens of apparently healthy school children for these two virulence factors.

Twenty-two multi-drug resistant MRSA nasal isolates and the multi-drug resistant MRSA reference strain *S. aureus* ATCC 33591 were screened for production of enterotoxins A-D. This was done in V-shaped 96-well microtiter plates by reversed passive latex agglutination, using a SET-RPLA toxin detection kit. The microtiter plate assay was employed to determine biofilm production in 10 nasal *S. aureus* isolates (including one MRSA isolate), and *S. aureus* ATCC 25923 and *S. aureus* ATCC 33591.

Out of the 23 multi-drug resistant MRSA isolates that underwent toxin screening, seven (30.4%) were enterotoxigenic. Enterotoxin A was the most prevalent, produced by five isolates. Enterotoxin B was found in one isolate, while enterotoxin C was produced by two isolates. One isolate tested positive for both enterotoxins B and C. Enterotoxin D was not detected. All 12 isolates, including two MRSA strains, that

were tested for biofilm formation were strong biofilm formers in microtiter plates, as indicated by absorbance values larger than 2.40 at 595nm.

The findings of this study indicated that multi-drug resistant MRSA nasal isolates from healthy children can produce enterotoxins. Strong biofilm production was observed in *S. aureus* and MRSA strains. Biofilm-related infections may be difficult to treat due to resistance to the human immune response and antibiotics. Self-infection by these potentially virulent bacteria poses various health risks for the children in our study population.

Keywords: *S. aureus*, multi-drug resistant MRSA, virulence factors, enterotoxins, biofilms, school children, Mariental

5.1 Introduction

Staphylococcus is found in the noses, throats and on the hair and skin of 50.0% or more healthy persons (Food and Drug Administration, 2012) and colonization with this bacterium does not necessarily imply disease. According to Prescott *et al.* (p. 704 and 919) staphylococci are however opportunistic with the potential to cause disease when taken from their natural environment (such as the respiratory tract) and introduced into foreign locations (bloodstream and tissues) or compromised hosts. These hosts are less resistant to infections due to factors such as diabetes, cancer, malnutrition, other infectious disease, trauma from surgery or injury, altered normal microbiota from prolonged antibiotic treatment, and immunosuppression by a variety of factors (for example, drugs, the HIV virus, hormones and genetic deficiencies).

More than 30 staphylococcal species are pathogens. *Staphylococcus aureus* is the most virulent species (Lowy, 2010, p. 386) and a versatile pathogen with the ability to cause a wide range of human illnesses aided by a combination of toxin-mediated virulence,

invasiveness and antibiotic resistance. Staphylococcal infections can range from minor skin infections such as pimples to much more serious conditions such as sepsis, depending on its virulence factors (Ortega *et al.*, 2010, p. 2119).

When *S. aureus* becomes stressed by its host, for instance when growing in the presence of antibiotics, it specializes in producing virulence factors, including superantigens. Community-associated methicillin-resistant *S. aureus* (CA-MRSA) may produce more superantigens than their methicillin-sensitive counterparts (Schlievert *et al.*, 2010, pp. 10-11). Several enterotoxins are superantigens and around 50.0% of *S. aureus* strains can produce one or more of these toxins (A-E, G-J, K-R and U, V) that are heat stable and resistant to the working of gut enzymes (Brooks *et al.*, 2013, p. 202) such as trypsin and pepsin (Food and Drug Administration, 2012). Staphylococcal enterotoxins are normally associated with food poisoning but can also play a role in other infections (Ortega *et al.*, 2010, p. 2119).

Biofilm production is an important virulence factor in some staphylococcal strains. Hutcherson *et al.* (2015, p. 1) define biofilms as dense, surface-attached communities of bacteria or fungi encased within a microbial-derived matrix that helps with colonization and survival. Biofilm-associated infections lead to chronic disease with resistance towards host immune responses (Archer, *et al.*, 2011, p. 453 and p. 456) and antibiotics (Cabrera-Contreras *et al.*, 2013, p. 3). Staphylococcal biofilms can form in the nasopharynx, heart valves, lungs, oral cavity and implanted medical devices (Speziale and Geoghegan, 2015, p. 1). The ability of *S. aureus* to form biofilms in its host results in chronic, recurrent disease that is difficult to treat. Biofilm-associated infections that involve *S. aureus* include osteomyelitis, indwelling medical device infection, periodontitis and peri-implantitis, chronic wound infection, chronic rhinosinusitis, endocarditis, ocular infection and polymicrobial biofilm infections

(Archer *et al.*, 2011, pp. 449-456). Biofilm-related skin infections (Jenney *et al.*, 2014, p. 7), ear infections (Elmanama *et al.*, 2014, p. 89) and tonsillitis (Zautner *et al.*, p. 4) with *S. aureus* as a causative agent are common among school-aged children. According to Samant Sharvari and Pai Chitra (2012, p. 732), detection of biofilm-related infection by staphylococci can help to alter antibiotic therapy and prevent infection.

Enterotoxin and biofilm studies involving school children are limited and the present work thus aimed to screen *S. aureus* and multi-drug resistant MRSA isolates from nasal specimens of apparently healthy school children in Namibia for these two virulence factors.

5.2 Materials and methods

5.2.1 Bacterial cultures used

Twenty-two multi-drug resistant MRSA isolates originating from nasal specimens of school children, and the multi-drug resistant MRSA reference strain *S. aureus* ATCC 33591 (Microbiologics[®], St. Cloud, USA) were screened for production of enterotoxins A-D. Ten *S. aureus* isolates (including one MRSA isolate) from the nasal specimens, as well as *S. aureus* ATCC 25923 and *S. aureus* ATCC 33591 (Microbiologics[®], St. Cloud, USA) were tested for biofilm production.

5.2.2 Staphylococcal enterotoxins A-D

Twenty-three multi-drug resistant MRSA isolates were tested for production of one or more of the enterotoxins A-D. This was done in V-shaped 96-well microtiter plates (Thermo Fisher Scientific, Newport, UK) by reversed passive latex agglutination with a SET-RPLA toxin detection kit (Oxoid, Basingstoke, England) as per manufacturer's instructions.

A control plate was prepared as per instructions to provide references for positive tests. Each reconstituted control caused agglutination with its respective sensitized latex. Samples were prepared as follows: Each isolate was inoculated into 2-ml Eppendorf centrifuge tubes (Eppendorf, Germany) containing 1.5ml sterile tryptone soy broth (Mast Group, Merseyside, UK) and incubated at 37°C for 18-24 hours. After incubation, broth cultures were refrigerated to 4°C and centrifuged at 900g for 20 minutes. The filtrate was retained for assay of toxin content.

The plate was arranged so that each row consisted of eight wells. Each sample needed the use of five such rows. Using a pipette, 25µl of diluent was dispensed in each well of the five rows. Thereafter, 25µl of test sample (filtrate) was added to the first well of each of the five rows. Starting at the first well of each row, 25µl was picked up and double dilutions were made along each of the five rows, stopping at the seventh well (negative control well) that contained diluent only. To each well in the first row, 25µl of latex sensitized with anti-enterotoxin A was added. To each well of the second row, 25µl of latex sensitized with anti-enterotoxin B was added. This was repeated for the next two rows using latex sensitized with anti-enterotoxin C and D. To each well of the fifth row, 25µl of latex control was added. The contents of each well were mixed by gentle agitation by hand. Plates were covered with lids to avoid evaporation. After 20-24 hours incubation at room temperature, each plate was examined for agglutination against a black background. If staphylococcal enterotoxins A, B, C or D were present, agglutination occurred, resulting in the formation of a lattice structure. Upon settling, this formed a diffuse layer on the base of the well. If staphylococcal enterotoxins were absent or at a concentration below the assay detection level, a lattice structure could not be formed and a tight button was observed at the bottom of each well.

5.2.3 Biofilm formation

The ability of 12 *S. aureus* isolates to form biofilms over 24 hours and the extent of this formation (no, weak, moderate and strong biofilm production) was determined by means of the microtiter plate assay, using the methods and classification of Christensen *et al.* (1985, p. 998), Merritt *et al.* (2011), and Monte *et al.* (2014). The assay was repeated eight times.

Isolates were inoculated into 50-ml centrifuge tubes (Greiner Bio-One, Kremsmünster, Austria) containing 5ml brain heart infusion broth (Merck, Darmstadt, Germany) and grown to stationary phase at 37°C for 24 hours. Stationary-phase cultures were diluted 1:100 (a 0.5 McFarland standard) and 100µl of diluted culture was added to each of six wells of a sterile flat-bottomed 96-well microtiter plate (Thermo Fisher Scientific, Newport, UK). Eight wells each contained 100µl sterile brain heart infusion broth only as control. The plates were parafilm-ed at the lids to prevent them from drying out and incubated at 37°C for 24 hours.

After incubation, planktonic cells were removed by placing the microtiter plate upside down on towel paper and allowing for the paper to soak up any liquid. To remove remaining planktonic cells, each well was washed three times by pipetting 400µl distilled water into it and inverting the plate onto towel paper. The biofilms in the wells were fixed by oven-drying the microtiter plates for 45 minutes at 60°C.

Wells were stained with 125µl of 0.1% crystal violet, incubated for 15 minutes at room temperature and the crystal violet discarded. Excess stain was removed by washing (pipetting) three times with 400µl distilled water. Plates were air-dried for a few hours or overnight. Wells were de-stained with 200µl of 33.0% glacial acetic acid (Merck, Darmstadt, Germany) for 10-15 minutes. The contents of each well was briefly mixed

by pipetting, and 125µl was transferred to corresponding wells of a new clean microtiter plate. The optical densities (ODs) of stained biofilms were determined with a SpectraMax M2 Multi-mode Microplate Reader (Molecular Devices, China) at 595nm. Readings from broth control wells were averaged and subtracted from the test readings. Test readings were averaged and standard deviations calculated.

Results for biofilm formation were interpreted using the classification by Christensen *et al.* (1985). According to this classification, mean OD values of ≤ 0.120 indicated non-adherence to the plastic wells and non/weak biofilm formation. Values of 0.121 – 0.240 indicated moderate adherence and moderate biofilm formation. Optical density readings of > 0.240 meant that there was strong bacterial attachment to the wells and strong/high biofilm formation. Single-factor ANOVA and the Student t-Test (two-sample assuming equal variances) in Microsoft Excel was used to determine significant differences in biofilm formation between isolates, as indicated by a P-value of ≤ 0.05 and F-value $> F$ critical value.

5.3 Results and discussion

5.3.1 Production of enterotoxins A-D

Enterotoxin assays were performed in V-shaped 96-well microtiter plates by reversed passive latex agglutination with a SET-RPLA toxin detection kit from Oxoid, Basingstoke, England.

Out of the 23 multi-drug resistant isolates screened in our study, seven (30.4%) were enterotoxigenic as indicated by agglutination reactions which caused the formation of lattice structures in plate wells. Enterotoxin A was the most prevalent toxin, found in five of the isolates (Table 5.1). Only one isolate produced enterotoxin B, whereas two

isolates produced enterotoxin C. One isolate was able to produce both toxins B and C. Enterotoxin D was not detected.

Table 5.1 Enterotoxin production in multi-drug resistant MRSA from nasal specimens of school children in Mariental, southern Namibia.

Isolate name	Source	Enterotoxin(s)
<i>S. aureus</i> ATCC 33591	American type culture collection reference strain. Clinical isolate.	A
<i>S. aureus</i> M37 S110 A	9-year-old male	A
<i>S. aureus</i> M39 S110 A	9-year-old female	A
<i>S. aureus</i> M42 S110/TSA A	14-year-old male	A
<i>S. aureus</i> S1 S110 Pure	7-year-old female	B and C
<i>S. aureus</i> S29 S110 Pure	13-year-old male	C
<i>S. aureus</i> DJ25 S110 A	10-year-old female	A

From literature, it is evident that it is not uncommon for nasal staphylococci from healthy persons to harbor enterotoxins. In Egypt, Hassanien and Abdel-Aziz (2017) detected the genes for enterotoxins A, B and C in nasal *S. aureus* from food handlers. Similar to our results, one of their isolates carried both enterotoxins B and C. According to Brooks *et al.* (2013, p. 202) ingestion of as little as 25µg of enterotoxin B causes vomiting and a runny stomach. In Ireland, Collery *et al.* (2008) amplified *sea*, *seb* and *sec* genes in nasal *S. aureus* from healthy students. In accordance with our results and that of Collery *et al.* (2008), one of their isolates carried enterotoxins B and C simultaneously. According to Dağı *et al.* (2015, p. 174) *S. aureus* strains with a combination of different enterotoxin genes can increase the incidence and severity

of *S. aureus* infections. These researchers performed toxin assays on 104 *S. aureus* isolates from nasal swabs of healthy university students and found that 95.2% of isolates tested positive for at least one enterotoxin gene. In Japan, Uemura *et al.* (2004) also used the SET-RPLA kit to screen for enterotoxins A-D in *S. aureus* isolated from the noses of healthy volunteers. They detected all four toxins. The absence of enterotoxin D in our study is consistent with another African study conducted in Benin, where Sina *et al.* (2013, p. 2) also did not find this toxin in *S. aureus*. Chen *et al.* (2017) found that out of all nasal *S. aureus* from healthy persons, 56.5% carried both *sea* and *seb* genes, 26.1% carried only *sea*, while 13.0% carried only the gene encoding enterotoxin B, in China. De Carvalho *et al.* (2017, p. 465) amplified the *seb* gene in two MRSA isolates, and the *sec* gene in two methicillin-susceptible *S. aureus* (MSSA) strains. These enterotoxigenic isolates were recovered from nasal secretions of Brazilian daycare-attending children in the age-group 1-6 years.

5.3.2 Biofilm formation

The microtiter plate assay was used to determine if isolates could form biofilms in plastic wells. Biofilm formation in 12 *S. aureus* isolates over 24 hours is graphed in Figure 5.1. All isolates were strong producers (optical density at 595nm > 2.40), with *S. aureus* S110 S73 Pure being the strongest one. The isolate colonized the nose of an 11-year-old male. There was a statistical difference in biofilm formation between isolates ($P = 0.02$; $F = 2.18$; $F_{crit} = 1.90$) as presented in Table 5.2. When involved in infections, these biofilm-producing bacteria may resist the immune response and antibiotic treatment (Nazzari *et al.*, 2014).

Strong biofilm production in *S. aureus* have also been detected by other researchers. In Brazil, de Carvalho *et al.* (2017, pp. 465-466) observed biofilm formation in all of their nasal *S. aureus* isolates, with 64.2% of the bacteria that were able to form strong

biofilms in microtiter plates. In a study undertaken in Thailand, Tangchaisuryia *et al.* (2014) screened 78 nasal *S. aureus* isolates from healthy primary school children for biofilm formation and found that 46 (59.0%) of these isolates were strong biofilm formers.

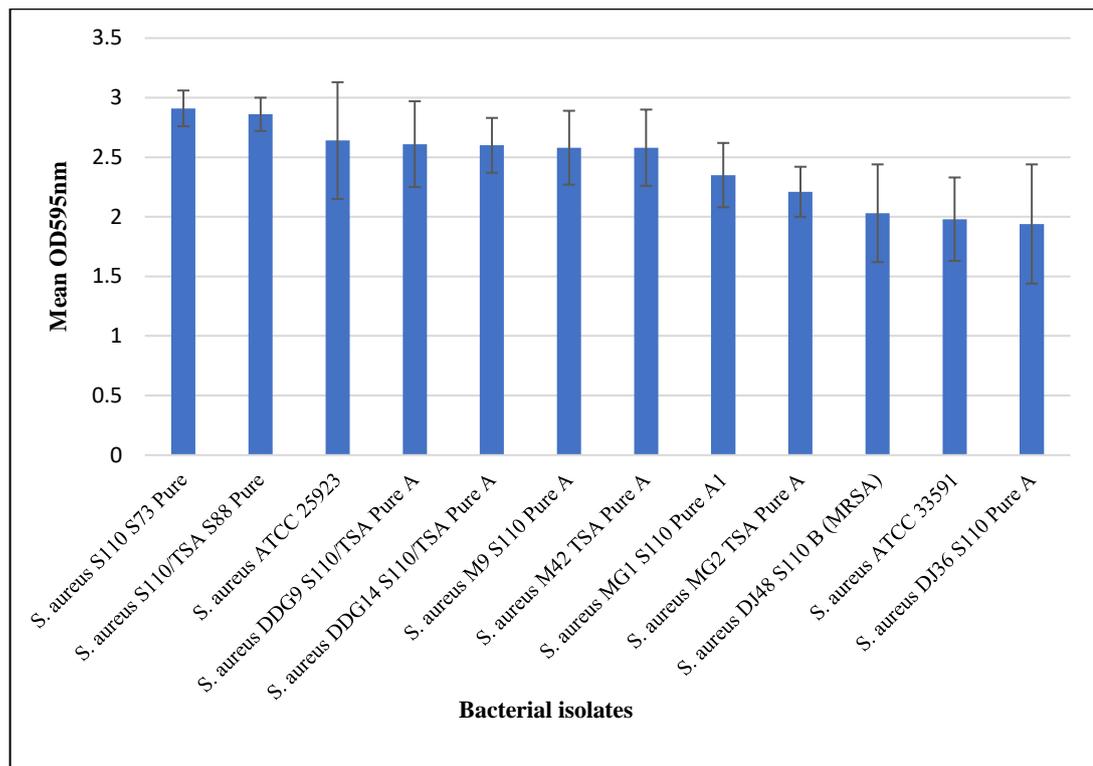


Figure 5.1 24-hour biofilm formation in microtiter plates according to mean absorbance values at 595nm over eight assays. Formation was classified as strong/high with mean OD values > 0.240 (Christensen *et al.*, 1985). Error bars indicate standard deviations.

Table 5.2 Significant differences in biofilm formation between isolates according to single-factor ANOVA and the Student t-Test.

Isolate 1	Isolate 2	P-value \leq 0.05
<i>S. aureus</i> S110/TSA S88 Pure	<i>S. aureus</i> DJ48 S110 B (MRSA)	0.02
	<i>S. aureus</i> ATCC 33591	0.02
	<i>S. aureus</i> DJ36 S110 Pure	0.02
<i>S. aureus</i> ATCC 25923	<i>S. aureus</i> DJ48 S110 B (MRSA)	0.04
	<i>S. aureus</i> ATCC 33591	0.03
	<i>S. aureus</i> DJ36 S110 Pure	0.04
<i>S. aureus</i> DDG9 S110/TSA Pure A	<i>S. aureus</i> ATCC 33591	0.05
<i>S. aureus</i> DDG14 S110/TSA Pure A	<i>S. aureus</i> DJ48 S110 B (MRSA)	0.05
	<i>S. aureus</i> ATCC 33591	0.04
	<i>S. aureus</i> DJ36 S110 Pure	0.05
<i>S. aureus</i> M42 TSA Pure	<i>S. aureus</i> DJ48 S110 B (MRSA)	0.03
	<i>S. aureus</i> ATCC 33591	0.02
	<i>S. aureus</i> DJ36 S110 Pure	0.03

5.4 Conclusion

The findings of this study indicated that multi-drug resistant MRSA nasal isolates from healthy children can produce enterotoxins. Strong biofilm production was observed in *S. aureus* and MRSA strains. Biofilm-related infections may be difficult to treat due to resistance to the human immune response and antibiotics. Self-infection by these potentially virulent bacteria poses various health risks for the children in our study population.

References

Archer, N.K., Mazaitis, M.J., Costerton, W., Leid, J.G., Powers, M.E. and Shirtliff, M.E. (2011). *Staphylococcus aureus* biofilms: Properties, regulation and roles

- in human disease. *Virulence* [online]. **2**(5), pp. 445-459. Available from DOI: <http://dx.doi.org/10.4161/viru.2.5.17724> [Accessed 24 September 2017].
- Brooks, G.F., Carroll, K.C., Butel, J.S., Morse, S.A. and Mietzner, T.A. (2013) *Jawetz, Melnick & Adelberg's Medical Microbiology*. 26th ed. S.l.: McGraw-Hill.
- Cabrera-Contreras, R., Morelos-Ramírez, R., Galicia-Camacho, A.N. and Meléndez-Herrada, E. (2013) Antibiotic resistance and biofilm production in *Staphylococcus epidermidis* strains, isolated from a tertiary care hospital in Mexico City. *ISRN Microbiology* [online]. Available from DOI: <http://dx.doi.org/10.1155/2013/918921> [Accessed 29 July 2015].
- Chen, B.J., Xie, X.Y., Ni, L.J., Dai, X.L., Lu, Y., Wu, X.Q., Li, H.Y., Yao, Y.D. and Huang, S.Y. (2017) Factors associated with *Staphylococcus aureus* nasal carriage and molecular characterization among the general population at a *Microbiology and Antimicrobials* [online]. **16**. Available from DOI: 10.1186/s12941-017-0206-0 [Accessed 22 September 2017].
- Christensen, G.D., Simpson, W.A., Younger, J.J., Baddour, L.M., Barrett, F.F., Melton, D.M. and Beachey, E.H. (1985) Adherence of coagulase-negative staphylococci to plastic tissue culture plates: A quantitative model for the adherence of staphylococci to medical devices. *Journal of Clinical Microbiology* [online]. **22**(6), pp. 996-1006. Available from: <http://jcm.asm.org/content/22/6/996.long> [Accessed 29 July 2015].
- Collery, M.M., Smyth, D.S., Twohig, J.M., Shore, A.C., Coleman, D.C. and Smyth, C.J. (2008) Molecular typing of nasal carriage isolates of *Staphylococcus aureus* from an Irish university student population based on toxin gene PCR, *agr* locus types and multiple locus, variable number tandem repeat analysis.

Journal of Medical Microbiology [online]. **57**, pp. 348-358. Available from DOI: 10.1099/jmm.0.47734-0 [Accessed 22 September 2017].

Dagi, H.T., Fındık, D., Demirel, G. and Arslan, U. (2015) Detection of methicillin resistance and various virulence factors in *Staphylococcus aureus* strains isolated from nasal carriers. *Balkan Medical Journal* [online]. **32**, pp. 171-175. Available from DOI: 10.5152/balkanmedj.2015.150186 [Accessed 9 January 2017].

de Carvalho, S.P., de Almeida, J.B., Andrade, Y.M.F.S., da Silva, L.S.C., de Oliveira, M.V., Timenetsky, J. and Marques, L.M. (2017) Community-acquired methicillin-resistant *Staphylococcus aureus* carrying SCCmec type IV and V isolated from healthy children attending public daycare in northeastern Brazil. *The Brazilian Journal of Infectious Diseases* [online]. 21(4), pp. 464-467. Available from DOI: <http://dx.doi.org/10.1016/j.bjid.2017.04.001> [Accessed 22 September 2017].

Elmanama, A.A., Tayyem, N.E.A. and Allah, S.A.N. (2014) The bacterial etiology of otitis media and their antibiogram among children in Gaza Strip, Palestine. *Egyptian Journal of Ear, Nose, Throat and Allied Sciences* [online]. **15**, pp. 87-91. Available from DOI: <http://dx.doi.org/10.1016/j.ejenta.2014.03.002> [Accessed 7 November 2017].

Food and Drug Administration (2012) *Bad bug book: Handbook of foodborne pathogenic microorganisms and natural toxins*. 2nd ed. Washington: Center for Food Safety and Applied Nutrition.

Hassanien, A.A. and Abdel-Aziz, N.M. (2017) *Staphylococcus aureus* and their enterotoxin gene in fast food and food handlers from different food premises

in Sohag City, Egypt. *International Journal of Agricultural Sciences and Veterinary Medicine* [online]. **5**(1). Available from: https://www.ijasvm.com/ijasvadmin/upload/IJASVM_58a403b503a72.pdf [Accessed 22 September 2017].

Hutcherson, J.A., Scott, D.A. and Bagaitkar, J. (2015) Scratching the surface – tobacco-induced bacterial biofilms. *Tobacco Induced Diseases* [online]. **13**. Available from DOI: 10.1186/s12971-014-0026-3 [Accessed 1 June 2016].

Jenney, A., Holt, D., Ritika, R., Southwell, P., Pravin, S., Buadromo, E., Carapetis, J., Tong, S. and Steer, A. (2014) The clinical and molecular epidemiology of *Staphylococcus aureus* infections in Fiji. *BMC Infectious Diseases* [online]. **14** (160). Available from DOI: 10.1186/1471-2334-14-160 [Accessed 7 November 2017].

Lowy, F.D. (2010) Staphylococcal infections. In: Kasper, D. and Fauci, A. (eds.) *Harrison's infectious diseases*. S.l.: McGraw-Hill, pp. 386-399.

Merritt, J.H., Kadouri, D.E. and O'Toole, G.A. (2011) Growing and analyzing static biofilms. *Current Protocols in Microbiology* [online]. Available from DOI: 10.1002/9780471729259.mc01b01s00 [Accessed 6 August 2015].

Monte, J., Abreu, A.C., Borges, A., Simões, L.L. and Simões, M. (2014) Antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* and their biofilms. *Pathogens* [online]. **3**, pp. 473-498. Available from DOI: 10.3390/pathogens3020473 [Accessed 29 July 2015].

Nazzari, E., Torretta, S., Pignataro, L., Marchisio, P. and Esposito, S. (2014) Role of biofilm in children with recurrent upper respiratory tract infections. *European*

Journal of Clinical Microbiology and Infectious Diseases [online]. Available from DOI: 10.1007/s10096-014-2261-1 [Accessed 13 February 2016].

Ortega, E., Abriouel, H., Lucas, R. and Gavez, A. (2010) Multiple roles of *Staphylococcus aureus* enterotoxins: Pathogenicity, superantigenic activity, and correlation to antibiotic resistance. *Toxins* [online]. **2**, 2117-2131. Available from DOI: 10.3390/toxins2082117 [Accessed 22 February 2018].

Samant Sharvari, A. and Pai Chitra, G. (2012) Evaluation of different detection methods of biofilm formation in clinical isolates of staphylococci. *International Journal of Pharma and Bio Sciences* [online]. **3**(4), pp. 724-733. Available from: https://www.researchgate.net/publication/279770601_Evaluation_of_different_detection_methods_of_biofilm_formation_in_clinical_isolates_of_Staphylococci [Accessed 29 July 2015].

Schlievert, P.M., Strandberg, K.L., Lin, Y-C, Peterson, M.L. and Leung, D.Y.M. (2010) Secreted virulence factor comparison between methicillin-resistant and methicillin-susceptible *Staphylococcus aureus*, and its relevance to atopic dermatitis. *Journal of Allergy and Clinical Immunology* [online]. **125**(1). Available from DOI: 10.1016/j.jaci.2009.10.039 [Accessed 25 July 2017].

Sina, H., Ahoyo, T.A., Moussaoui, W., Keller, D., Bankolé, H.S., Barogui, Y., Stienstra, Y., Kotchoni, S.O., Prévost, G. and Baba-Moussa, L. (2013) Variability of antibiotic susceptibility and toxin production of *Staphylococcus aureus* strains isolated from skin, soft tissue and bone related infections. *BMC Microbiology* [online]. **13**. Available from DOI: 10.1186/1471-2180-13-188 [Accessed 14 January 2017].

- Speziale, P. and Geoghegan, J.A. (2015) Biofilm formation by staphylococci and streptococci: Structural, functional, and regulatory aspects and implications for pathogenesis. *Frontiers in Cellular and Infection Microbiology* [online]. **5**. Available from DOI: 10.3389/fcimb.2015.00031 [Accessed 7 August 2015].
- Tangchaisuriya, U., Yotpanya, W., Kitti, T. and Sitthisak, S. (2014) Distribution among Thai children of methicillin-resistant *Staphylococcus aureus* lacking *cna*, *fnbA* and *icaAD*. *Southeast Asian Journal of Tropical Medicine and Public Health* [online]. **45**(1). Available from: <http://www.mahidol.ac.th/seameo/2014-45-1-full/19-5911-15.pdf> [Accessed 17 August 2015].
- Uemura, E., Kakinohana, S., Higa, N., Toma, C. and Nakasone, N. (2004) Comparative characterization of *Staphylococcus aureus* from throats and noses of healthy volunteers. *Japanese Journal of Infectious Diseases* [online]. **57**, pp. 21-24. Available from: <http://www0.nih.go.jp/JJID/57/21.pdf> [Accessed 22 September 2017].
- Wang, X., Li, X., Huang, W., Fu, Q. and Li, M. (2016) Molecular characteristic and virulence gene profiles of community-associated methicillin-resistant *Staphylococcus aureus* isolates from pediatric patients in Shanghai, China. *Frontiers in Microbiology* [online]. **7**. Available from DOI: 10.3389/fmicb.2016.01818 [Accessed 10 January 2017].
- Zautner, A.E., Krause, M., Stropahl, G., Holtfreter, S., Frickmann, H., Maletzki, C., Kreikemeyer, B., Pau, H.W. and Podbielski, A. (2010) Intracellular persisting *Staphylococcus aureus* is the major pathogen in recurrent tonsillitis. *PLoS ONE* [online]. **5**(3). Available from DOI: 10.1371/journal.pone.0009452 [Accessed 7 November 2017].

Chapter 6

Antimicrobial activity of *Aptosimum albomarginatum* (Marloth and Engl.) and *Dicoma schinzii* (O. Hoffm.) crude methanolic extracts against *S. aureus* and MRSA.

Abstract

Phytomedicine may be just as effective as conventional drugs, but its effectiveness is often questioned by scientists. This study therefore aimed to test crude methanolic extracts from three traditional medicinal plants currently used in Namibia for antimicrobial activity against *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA), including multi-drug resistant strains.

Aptosimum albomarginatum (Marloth and Engl.) roots, *Albizia anthelmintica* (A. Rich Brongn.) twigs and *Dicoma schinzii* (O. Hoffm.) roots and leaves were used to prepare crude methanolic extracts through the process of maceration, filtration, rotary evaporation and freeze-drying. Qualitative chemical assays were used to detect flavonoids, saponins and anthraquinones in the plant material. To test for antimicrobial activity, *S. aureus* reference strains and staphylococcal nasal isolates from school children were used in disk diffusion assays with crude methanolic plant extracts.

With observed inhibition zone diameters in the range of 11-14mm, *A. albomarginatum* root extract was moderately active against 7/12 *S. aureus* isolates, including two MRSA isolates. Moderate antimicrobial activity was also observed with this extract in 9/54 multi-drug resistant isolates, of which two were MRSA with the same antibiotic resistance pattern. Flavonoids and saponins may contribute to the root's activity. *Dicoma schinzii* root extract had moderate activity against 8/12 *S. aureus* isolates, while the plant's leaf extract was moderately active against 2/12 *S. aureus* isolates. This activity may partly be ascribed to saponins in the leaves.

In conclusion, *A. albomarginatum* roots and *D. schinzii* roots and leaves displayed anti-staphylococcal activity, indicating potential use against staphylococcal infections. Noteworthy is the activity of *A. albomarginatum* root extract against multi-drug resistant strains, including MRSA. The present work thus supports the traditional medicinal uses of *A. albomarginatum* roots and *D. schinzii* roots and leaves as natural antimicrobial agents in infections involving the bacteria under study.

Keywords: *Aptosimum albomarginatum*, *Dicoma schinzii*, crude methanolic extracts, antimicrobial activity, flavonoids, saponins, *S. aureus*

6.1 Introduction

Globally many people still rely on traditional medicine to remain healthy and treat a variety of ailments. African Traditional Medicine represents the most diverse of all medicinal systems. Traditional or folk medicine may be just as effective as conventional drugs, but its effectiveness is often questioned by scientists (van Wyk and Wink, 2015, p. 8 and p. 16).

Aptosimum albomarginatum (Marloth and Engl.) shown in Figure 6.1 (A) is commonly known as “!Guxa” by the Nama tribe in Namibia. The roots are pulverized, boiled as a tea and drunk to purify the blood and cleanse the uterus. Some believe that it can cure women who experience difficulty in conceiving. It also helps to relieve the symptoms of colds (S. Coetzee, personal communication, February, 2015; A. Frederick, personal communication, February, 2015). *Staphylococcus* may be associated with infection of the uterus, for example in the medical condition known as endometritis (inflammation of the endometrium). One form of this condition is known as bacteriotoxic endometritis, where it is caused by the toxins of bacteria rather than the presence of the pathogens themselves (Dorland’s illustrated medical dictionary,

2003, p. 614). Colds are due to viruses, not bacteria. However, when one sneezes a lot as a result of cold symptoms, lots of bacteria (including staphylococci in the nose) can quickly be spread to a person's surroundings and other people. In a study to assess the effects of sneezing on airborne spread of *S. aureus* and other bacteria, Bischoff *et al.* (2006, p. 1119) concluded that nasal *S. aureus* carriers can disperse a great number of these bacteria into the air by sneezing.

Albizia anthelmintica (A. Rich Brongn.) in Figure 6.1 (B) has many common names in different languages, including Kersieblombloom, Worm-cure albizia (Orwa *et al.*, 2009, p. 1; Hoffmann, 2014), Aruboom, Oumahout, Wurmindenbaum, Kirschblütenbaum and Omuama. According to local people at Gochas, the outer part of the twigs is scraped off and the inner part is used as a chewing stick or toothbrush to clean the teeth and tongue (S. Coetzee, personal communication, February, 2015; A. Frederick, personal communication, February, 2015). Staphylococci may be associated with mouth infections, where they can be part of biofilm communities as dental plaque.

The bark, wood or root is boiled and milk is added to treat an upset stomach or intestinal worms. Tea made from the roots and bark is drunk to treat malaria. The Samburu pastoralists in Kenya treat gonorrhoea by boiling the roots, bark and leaves, mixing it with sheep fat and giving it as an enema. Otherwise the boiled bark and roots are consumed with milk (Sullivan, 1998, p. 46; du Pisani, 1983; Fratkin, 1996, p. 75). The boiled bark, wood and roots can also be used to de-worm livestock (Fratkin, 1996, p. 81). The stem bark is widely used as a purgative (Orwa *et al.*, 2009, p. 3).

Dicoma schinzii (O. Hoffm.) in Figure 6.1 (C) is also known as “Gu-!aru” in the Nama language (S. Coetzee, personal communication, February, 2015) or the “Kalahari fever bush” (Dugmore and van Wyk, 2008). The roots and leaves are pulverized, boiled as

tea and drunk or used to steam yourself in the treatment of measles, chickenpox, the flu, colds and a blocked nose (Coetzee, 2015). Unspecified parts are used to treat febrile convulsions in babies in the Kalahari, hence the name “Kalahari fever bush” (van Wyk and Gericke, 2000; Dugmore and van Wyk, 2008). Measles, chickenpox, the flu and colds are caused by viruses, but staphylococci can be involved in congested nose or sinus infections.

There is an interesting folk tale (“Dicoma’s shadow”) behind the plant’s traditional use in the Kalahari to treat febrile convulsions in babies. Van Wyk (2015) explains the story in short. It is said that if the shadow of the black shouldered kite (*Elanus caeruleus*) falls on a baby, the child will get sick, and this illness will be recognized by the spastic movements of the baby’s arms, similar to the movements made by the bird’s feathers when it’s hanging over its prey. It is furthermore said that if the condition is not treated, the infant can develop feathers on its arms. An extract of the plant can be given both topically and internally, which will counteract the symptoms and cure the child. In the traditional African context, the symbol of the bird represents fever, since birds have a higher natural body temperature (40°C) compared to that of humans (37°C). “The condition of the bird” refers to fever. Referral to feathers on the arms is actually “the gooseflesh of fever” – one of the symptoms of febrile convulsions in infants.

The present work aimed to test *A. albomarginatum* roots, *A. anthelmintica* twigs and *D. schinzii* roots and leaves for antimicrobial activity against *Staphylococcus aureus*, including methicillin-resistant *S. aureus* (MRSA) and multi-drug resistant strains.

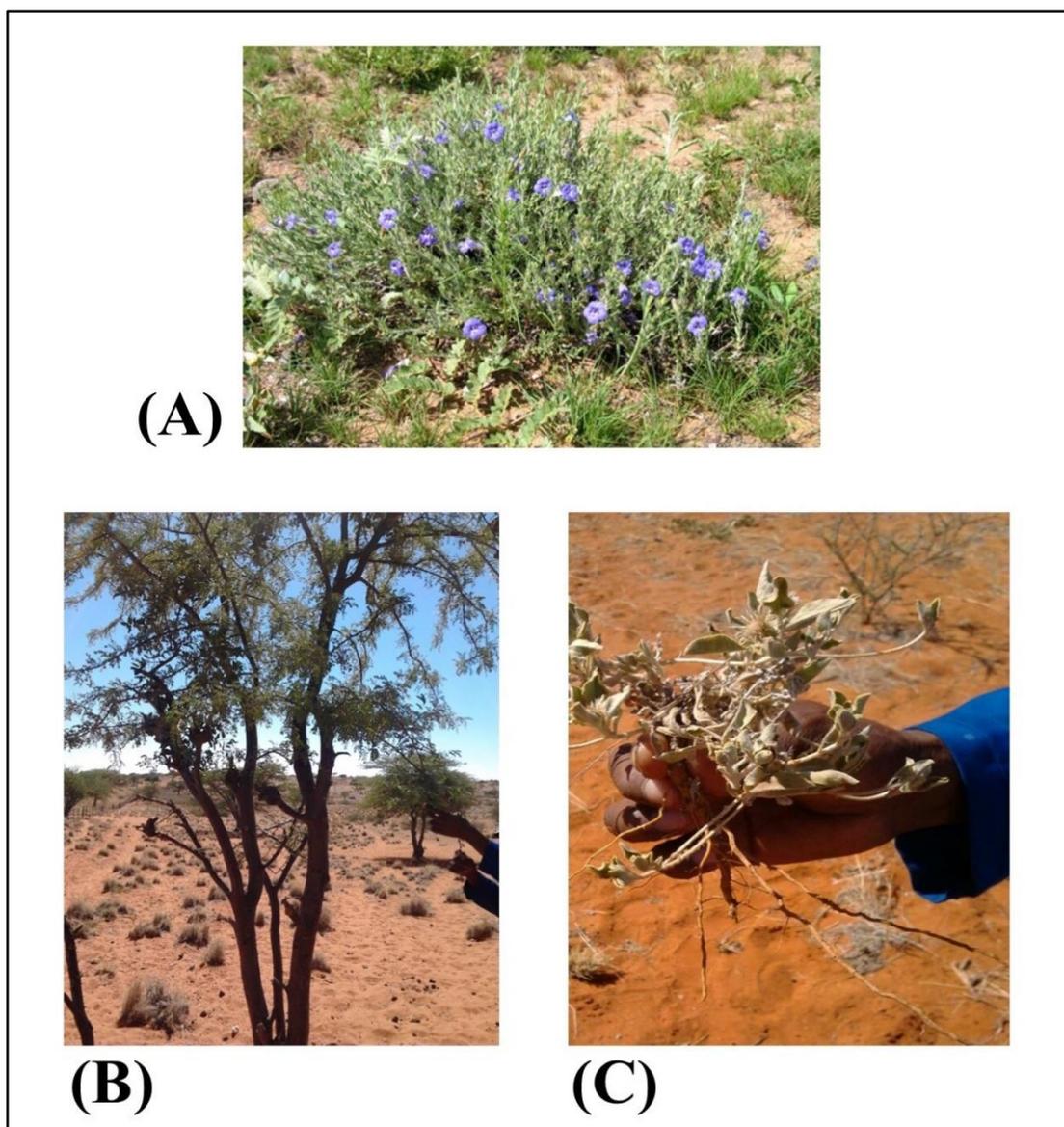


Figure 6.1 *A. albomarginatum* (A) (Image credit: southafricanplants.net),
A. anthelmintica (B), and *D. schinzii* (C) growing in the veld at Gochas
(Image credit: Sunette Walter).

6.2 Materials and methods

6.2.1 Selection of traditional medicinal plants and collection of plant material

Plant material was collected from the veld at Gochas (Altitude: 1139m; GPS coordinates: 24°47'S, 18°49'E), located in the Karas Region, southern Namibia in February 2015. Plants were selected based on indigenous knowledge of local people

about their medicinal value in the traditional setting. Voucher specimens were prepared under the voucher numbers CID 58, CID 59 and CID 60 and submitted to the herbarium at the National Botanical Research Institute (NBRI) in Windhoek for scientific identification of the plants (Appendix D). The selected plants as identified by the NBRI were the shrub *Aptosimum albomarginatum* (Marloth and Engl.), the tree *Albizia anthelmintica* (A. Rich Brongn.), and the shrub *Dicoma schinzii* (O. Hoffm.).

6.2.2 Plant extracts used

Crude methanolic extracts were prepared at the Biomedical Research Laboratory, Biological Sciences Department at the University of Namibia. Plant parts used were *A. albomarginatum* roots, *A. anthelmintica* twigs and *D. schinzii* roots and leaves.

6.2.3 Preparation of crude extracts

To prepare crude methanolic extracts for bioassays, the maceration methods of Njateng *et al.* (2013) were followed, with some modifications. Ten grams of plant material from the different plant parts was macerated in 100ml methanol (Skylabs, Johannesburg, SA). Flasks containing the extracts were parafilm, placed in a cupboard and left to stand for three days with occasional swirling. After three days, the extracts were gravitationally filtered through Whatman 110mm filter papers. The extracts were rotary evaporated in round bottom flasks at reduced pressure (91 mbar) and temperature (45°C) to evaporate the methanol, and to dry and concentrate them. To avoid thermal decomposition of compounds in the plant material, the temperature set for the rotary evaporator (Heidolph, Schwabach, Germany) did not exceed 45°C. The flasks were labeled, sealed with parafilm and frozen at -86°C for a few hours. Thereafter, the frozen extracts were connected to an Alpha 1-2 LD Plus freeze-dryer (Christ®, Osterode, Germany) for two to four days to further dry and concentrate them. Dried extracts were scraped off with a spatula, weighed and stored in labeled 50-ml

centrifuge tubes (Greiner Bio-One, Kremsmünster, Austria) at -86°C for further use. Yields of extracts were calculated using the formula used by Osungunna and Adedeji (2011, p. 215): Percentage yield = Quantity of dried extract (g) / Quantity of powdered sample (g) x 100. Fresh crude extracts were prepared four times as needed over the course of the study.

6.2.4 Phytochemical screening for flavonoids, saponins and anthraquinones

Antimicrobial activity of plant extracts may be attributed to the presence of secondary metabolites such as flavonoids, saponins and anthraquinones. These compounds were screened for based on the methods for qualitative chemical assays described by Farnsworth (1966) and Nyambe (2014, p. 39 - 40).

Flavonoids: 0.5g of powdered plant material was extracted with 15ml water and methanol mixture in the ratio 2:1. The mixture was left to stand for 30 minutes after which it was filtered using Whatman 110mm filter paper. Thereafter some magnesium turnings were added to the filtrate and concentrated hydrochloric acid (HCl) (SMM Instruments, Midrand, SA) was added dropwise. A yellow colour development indicated the presence of flavonoids.

Saponins: 0.5g powdered plant material was mixed with 15ml distilled water. The mixture was then heated in a water bath (Techne, Lasec, SA) at 100°C for 30 minutes, and the filtrate was left to cool down to room temperature. It was then vigorously shaken in a test tube for 10 seconds and observed for formation of froth. Froth measured 2cm or higher that persisted for 10 minutes or more confirmed the presence of saponins.

Anthraquinones: 0.5g plant material was extracted with 10ml ether-chloroform (Merck, Modderfontein, SA) in the ratio 1:1 for 15 minutes. The mixture was filtered and 1ml of the filtrate was treated with 1ml of 10.0% (w/v) sodium hydroxide (NaOH)

(Saarchem/Merck, Wadeville, SA) solution. Development of a red colour indicated the presence of anthraquinones.

6.2.5 Antimicrobial activity

6.2.5.1 Microorganisms used

Ten *S. aureus* isolates (including one MRSA isolate) originating from nasal specimens of school children in the Mariental District, Namibia, as well as purchased *S. aureus* ATCC 25923 and *S. aureus* ATCC 33591 (MRSA) (Microbiologics[®], St. Cloud, USA) were used in disk diffusion assays with crude methanolic plant extracts mentioned in Section 6.2.2. The *A. albomarginatum* root extract was also tested against 54 multi-drug resistant staphylococcal isolates from the children.

6.2.5.2 Disk diffusion (inhibition) assays

Disk diffusion assays based on the Kirby-Bauer technique (Harley and Prescott, 2002, p. 258) were performed to determine the antimicrobial effects of the different plant extracts on staphylococci. Extract concentrations of 60mg/ml, 30mg/ml, 10mg/ml, 5mg/ml, 0.5mg/ml, and 0.1mg/ml were prepared in 2-ml Eppendorf tubes (Eppendorf, Germany) each containing 1ml undiluted Dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany) and vortexed well to dissolve. A tube with only DMSO was kept to use as control.

The assay was done in triplicate for each isolate. Whatman filter paper disks (110mm) were used to punch out smaller 6mm disks. The small disks were autoclaved in a screwed cap test tube before use. Bacterial-saline suspensions adjusted to 0.5 McFarland standard were swabbed onto Mueller-Hinton agar (Mast Diagnostics, Merseyside, UK) and the plates were left to dry for 5 minutes before applying the filter paper disks with a flamed tweezer onto the agar. Extracts (10µl) were pipetted onto the disks. Gentamicin or chloramphenicol antibiotic disks (Mast Diagnostics, Merseyside, UK) were applied as positive controls and disks containing DMSO as

negative controls. Plates were incubated at 37°C for 18-20 hours and inhibition zones measured with a ruler to the nearest millimeter. The classification by Nematollahi *et al.* (2011, p. 1733) was used to interpret results: ≤ 7 mm inhibition (negative), 8-10mm (weak activity), 11-14mm (moderate activity), 15-24mm (strong activity), and ≥ 25 mm (very strong activity). Results were compared with the antibiotic susceptibility profiles of isolates to see if the extracts have potential to be used as antimicrobial agents. Minimum inhibitory concentrations (MICs) were taken as the lowest concentration of extract that were able to inhibit bacterial growth on each plate.

6.3 Results and discussion

6.3.1 Yields of extracts

Yields of extracts were calculated using the formula used by Osungunna and Adedeji (2011, p. 215): Percentage yield = Quantity of dried extract (g) / Quantity of powdered sample (g) x 100. Fresh crude extracts were prepared four times as needed for the duration of the study. The highest average yield (12.5%) was obtained with *A. albomarginatum* roots, followed by *D. schinzii* leaves (7.3%), *D. schinzii* roots (6.1%), and *A. anthelmintica* twigs (3.5%). Similar studies on these plants could not be found for comparison of yields obtained.

6.3.2 Phytochemical screening

Antimicrobial activity of plant extracts may be ascribed to secondary metabolites such as flavonoids, saponins and anthraquinones. These compounds were screened for based on the methods for qualitative chemical assays described by Farnsworth (1966) and Nyambe (2014, p. 39 - 40). Flavonoids were detected only in *A. albomarginatum* roots, as indicated by a yellow pigmentation. In another study, Bama *et al.* (2012, p. 559) observed flavonoids as an intense yellow colour from the methanolic extract of

Tridax procumbens leaves. Saponins were found in *A. albomarginatum* roots, *A. anthelmintica* twigs, and *D. schinzii* leaves, as indicated by formation of persistent foam. Hassan *et al.* (2015, p. 22) observed an abundance of saponins as froth in the methanolic leaf extract of *Senna siamea lam.* Anthraquinones were not detected in any of the plant material.

6.3.3 Antimicrobial activity

6.3.3.1 *Aptosimum albomarginatum* root extract

Disk diffusion assays were carried out to assess antimicrobial activity. Such activity for six concentrations of *A. albomarginatum* root extract against 12 *S. aureus* isolates is shown in Table 6.1. Based on the highest (60 mg/ml) concentration, the extract was moderately active against 7/12 isolates, including two MRSA isolates. The largest inhibition zone ($13.67 \pm 0.58\text{mm}$) was observed against *S. aureus* DJ36 S110 A. This isolate was obtained from the nose of a 9-year-old male attending school in the town of Mariental. Based on inhibition zone size, the root extract was not more effective than the antibiotic gentamicin (GM) 10 μg .

The extract was also tested against 54 multi-drug resistant isolates from the Mariental school children. At 60mg/ml, moderate antimicrobial activity was observed with nine multi-drug resistant *S. aureus* isolates as shown in Figure 6.2. Noteworthy is that two of these nine isolates were multi-drug resistant MRSA from children attending different schools, and the two isolates shared the same resistance pattern of AP-T-RP-FOX. The isolates may therefore be the same *S. aureus* strain. Three of the nine isolates from children from the same school had the resistance pattern AP-GM-RP; two isolates coming from learners of two different schools both had the pattern AP-RP-E. As seen in other studies (Kamonwannasit *et al.*, 2013, p. 4; Carranza *et al.*,

2015, p. 3) flavonoids and saponins may play a role in the root extract's anti-staphylococcal activity.

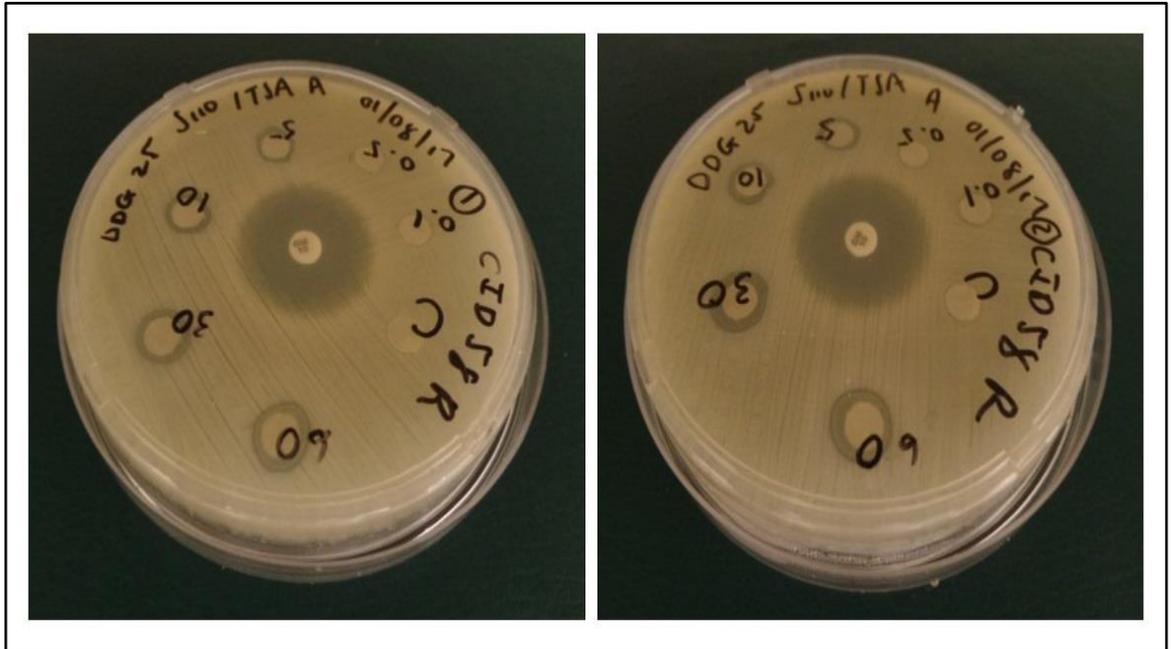


Figure 6.2 Duplicate inhibition assay with *A. albomarginatum* root extract displaying moderate antimicrobial activity at 60mg/ml (MIC 5mg/ml), against the multi-drug resistant *S. aureus* with the resistance pattern AP-RP-E. This isolate colonized the nose of a 7-year-old girl.

Table 6.1 Antimicrobial activity of *A. albomarginatum* root extract against 12 *S. aureus* isolates, according to mean inhibition zones and standard deviations of triplicate assays.

Isolate (Most susceptible to least susceptible)	60mg/ml Inhibition zone (IZ) in mm plus standard deviation (SD)	30mg/ml IZ in mm plus SD	10mg/ml IZ in mm plus SD	5mg/ml IZ in mm plus SD	0.5mg/ml IZ in mm plus SD	0.1mg/ml IZ in mm plus SD	GM 10µg antibiotic control IZ in mm plus SD	Classification of extract activity, based on the 60mg/ml concentration
DJ36 S110 Pure A	13.67 ±0.58	12.67 ±1.15	11.0 ±1.0	10.33 ±0.58 (MIC)	No activity	No activity	26.67 ±1.15	Moderate activity
<i>S. aureus</i> ATCC 33591 (MRSA)	13.48 ±0.64	12.9 ±0.53	12.0 ±0.47	9.39 ±0.29	9.17 ±0.19	8.75 ±0.68 (MIC)	29.0 ± 0	Moderate activity
<i>S. aureus</i> ATCC 25923	12.95 ±1.01	12.24 ±0.5	11.71 ±0.72	10.71 ±0.79	8.61 ±0.46	8.33 ±0.58 (MIC)	25.67 ± 0.58	Moderate activity
DDG9 S110/TSA Pure A	12.61 ±0.45	12.24 ±0.47	11.28 ±0.29	10.06 ±0.36	8.11 ±0.26 (MIC)	No activity	13.0 ± 0	Moderate activity
DDG14 S110/TSA Pure A	12.33 ±0.58	11.91 ±0.55	11.1 ±0.33	10.38 ±0.41 (MIC)	No activity	No activity	26.0 ± 0	Moderate activity
M9 S110 Pure A	11.67 ±0.61	11.0 ±0.56	10.29 ±0.33	9.86 ±0.56	8.47 ±0.23 (MIC)	No activity	24.0 ± 0	Moderate activity
DJ48 S110 B (MRSA)	11.67 ±1.15	10.0 ±0	10.0 ±0	9.33 ±0.58 (MIC)	No activity	No activity	19.0 ± 1.0	Moderate activity
MG1 S110 Pure A1	10.67 ±0.58	10.0 ±0	10.0 ±0	9.67 ±0.58 (MIC)	No activity	No activity	25.0 ± 0	Weak activity
S88 S110/TSA Pure	10.33 ±0.58	10.0 ±1.0	9.67 ±0.58	9.0 ±0	7.67 ±0.58	7.0 ±0 (MIC)	20.67 ± 1.53	Weak activity
M42 TSA Pure A	10.0 ±0.56	9.33 ±0.47	9.29 ±0.64	8.95 ±0.39	8.57 ±0.41	7.5 ±0 (MIC)	22.67 ± 1.15	Weak activity
S73 S110 Pure	9.67 ±0.58	8.67 ±1.15	8.33 ±1.53	8.33 ±0.58	7.33 ±0.58	7.33 ±0.58 (MIC)	17.67 ± 0.58	Weak activity
MG2 TSA Pure A	8.67 ±1.15	8.33 ±0.58	8.33 ±0.58 (MIC)	No activity	No activity	No activity	24.33 ± 0.58	Weak activity

6.3.3.2 *Albizia anthelmintica* twig extract

Although the twigs from the *A. anthelmintica* tree are used as chewing sticks for oral hygiene as well as other ailments (A. Frederick, personal communication, February, 2015), methanolic twig extract in the present work did not have antimicrobial activity against *S. aureus*.

6.3.3.3 *Dicoma schinzii* root extract

The antimicrobial activity for six concentrations of *D. schinzii* root extract against the 12 *S. aureus* isolates is shown in Table 6.2. Based on the highest (60 mg/ml) concentration, this extract was moderately active against 8/12 isolates. Such activity is depicted in Figure 6.3. The extract displayed weak activity against the two MRSA isolates. The largest inhibition zone ($14.0 \pm 0\text{mm}$) was observed against *S. aureus* M9 S110 Pure A, isolated from the nose of a 7-year-old female. The root extract was not more effective than the antibiotics chloramphenicol (C) 30 μg or gentamicin (GM) 10 μg .

Table 6.2 Antimicrobial activity of *D. schinzii* root extract against 12 *S. aureus*

isolates, according to mean inhibition zones and standard deviations of triplicate assays.

Isolate (Most susceptible to least susceptible)	60mg/ml Inhibition zone (IZ) in mm plus standard deviation (SD)	30mg/ml IZ in mm plus SD	10mg/ml IZ in mm plus SD	5mg/ml IZ in mm plus SD	0.5mg/ml IZ in mm plus SD	0.1mg/ml IZ in mm plus SD	C 30µg or GM 10µg* antibiotic control IZ in mm plus SD	Classification of extract activity, based on the 60mg/ml concentration
M9 S110 Pure A	14.0 ±0	11.0 ±1.0	8.67 ±0.58	7.33 ±0.58	7.0 ±0	7.0 ±0 (MIC)	25.33 ± 0.58	Moderate activity
S88 S110/TSA Pure	13.67 ±1.15	10.33 ±0.58	8.0 ±1.73	8.0 ±1.73	7.0 ±0	7.0 ±0 (MIC)	21.0* ±0	Moderate activity
S73 S110 Pure	13.1 ±1.0	11.67 ±0.58	10.0 ±0	9.5 ±0.71	7.5 ±0.71	7.5 ±0.71 (MIC)	16.67* ±0.58	Moderate activity
<i>S. aureus</i> ATCC 25923	12.0 ±0	10.67 ±0.58	9.33 ±0.58	8.33 ±0.58	8.0 ±0	8.0 ±0 (MIC)	23.67 ±0.58	Moderate activity
MG2 TSA Pure A	12.0 ±0	10.0 ±0	7.67 ±0.58	7.0 ±0	7.0 ±0	7.0 ±0 (MIC)	24.0 ±1.0	Moderate activity
MG1 S110 Pure A1	11.67 ±0.58	10.0 ±0	9.33 ±0.58	7.33 ±0.58	7.0 ±0	7.0 ±0 (MIC)	21.0 ±0	Moderate activity
DDG14 S110/TSA Pure A	11.67 ±0.58	10.0 ±0	9.0 ±0	8.67 ±0.58	7.67 ±0.58	7.67 ±0.58 (MIC)	20.33 ±0.58	Moderate activity
DDG9 S110/TSA Pure A	11.67 ±0.58	10.0 ±0	9.0 ±0	8.67 ±0.58	7.67 ±0.58	7.67 ±0.58 (MIC)	26.67 ±0.58	Moderate activity
DJ36 S110 Pure A	10.67 ±1.53	9.0 ±1.0	7.67 ±0.58 (MIC)	No activity	No activity	No activity	24.33 ±0.58	Weak activity
<i>S. aureus</i> ATCC 33591 (MRSA)	10.0 ±1.0	9.0 ±1.0	8.0 ±1.0	7.33 ±0.58 (MIC)	No activity	No activity	10.0 ±0.58	Weak activity
DJ48 S110 B (MRSA)	9.67 ±0.58	8.67 ±0.58 (MIC)	No activity	No activity	No activity	No activity	27.67 ±0.58	Weak activity
M42 TSA Pure A	9.67 ±0.58	8.33 ±0.58 (MIC)	No activity	No activity	No activity	No activity	24.67 ±0.58	Weak activity



Figure 6.3 Moderate antimicrobial activity of *D. schinzii* root extract at 60mg/ml and 30mg/ml concentrations, with inhibition zones of $13.1 \pm 1.0\text{mm}$ and $11.67 \pm 0.58\text{mm}$, respectively.

6.3.3.4 *Dicoma schinzii* leaf extract

Indicated in Table 6.3, *D. schinzii* leaf extract was moderately active against only 2/12 *S. aureus* isolates. It showed very weak activity against multi-drug resistant MRSA. Its activity may partly be due to the presence of saponins in the leaves. The extract was less active than the antibiotic control chloramphenicol (C) 30 μg .

Table 6.3 Moderate and weak antimicrobial of *D. schinzii* leaf extract against *S. aureus*, according to mean inhibition zones and standard deviations of triplicate assays.

Isolate (Most susceptible to least susceptible)	60mg/ml Inhibition zone (IZ) in mm plus standard deviation (SD)	30mg/ml IZ in mm plus SD	10mg/ml IZ in mm plus SD	5mg/ml IZ in mm plus SD	0.5mg/ml IZ in mm plus SD	0.1mg/ml IZ in mm plus SD	C 30µg antibiotic control IZ in mm plus SD	Classification of extract activity, based on the 60mg/ml concentration
DDG14 S110/TSA Pure A	12.33 ±0.58	11.67 ±0.58	9.33 ±0.58	9.33 ±0.58	8.33 ±0.58	8.0 ±1.0 (MIC)	21.33 ±1.15	Moderate activity
MG1 S110 Pure A1	12.33 ±0.58	11.0 ±0	8.67 ±0.58	8.0 ±0	7.0 ±0	7.0 ±0 (MIC)	22.0 ±1.0	Moderate activity
<i>S. aureus</i> ATCC 33591 (MRSA)	8.67 ±0.58 (MIC)	No activity	No activity	No activity	No activity	No activity	10.33 ±1.53	Weak activity

6.4 Conclusion

Aptosimum albomarginatum roots and *D. schinzii* roots and leaves displayed anti-staphylococcal activity, indicating potential use against staphylococcal infections. Noteworthy is the activity of *A. albomarginatum* root extract against multi-drug resistant strains, including MRSA. The present work thus supports the traditional medicinal uses of *A. albomarginatum* roots and *D. schinzii* roots and leaves as natural antimicrobial agents in infections involving the bacteria under study.

References

Bama, S.S., Kingsley, J.S., Sankaranarayanan, S. and Bama, P. (2012) Antibacterial activity of different phytochemical extracts from the leaves of *T. procumbens* Linn.: Identification and mode of action of the terpenoid compound as

antibacterial. *International Journal of Pharmacy and Pharmaceutical Sciences*. **4** (Suppl 1), pp. 557-564.

Bischoff, W.E., Wallis, M.L., Tucker, B.K., Reboussin, B.A., Pfaller, M.A., Hayden, F.G. and Sherertz, R.J. (2006) “Gesundheit!” Sneezing, common colds, allergies, and *Staphylococcus aureus* dispersion. *Journal of Infectious Diseases* [online]. **194**(8), pp. 1119-1126. Available from DOI: <https://doi.org/10.1086/507908> [Accessed 7 September 2017].

Carranza, M.G., Sevigny, M.B., Banerjee, D. and Fox-Cubley, L. (2015) Antibacterial activity of native California medicinal plant extracts isolated from *Rhamnus californica* and *Umbellularia californica*. *Annals of Clinical Microbiology and Antimicrobials* [online]. **14**(29). Available from DOI: 10.1186/s12941-015-0086-0 [Accessed 19 January 2017].

Coetzee, S. (2015) Personal communication, Gochas, Namibia.

Dorland's illustrated medical dictionary (2003) 30th ed. Philadelphia: Saunders.

Dugmore, H. and van Wyk, B-E. (2008) *Muthi and myths from the African bush*. Pretoria: Briza.

Du Pisani, E. (1983) Past and present plant utilization in Namaland and the lower Kuiseb River valley, South West Africa (Namibia): A preliminary report. *Khoisis*. **4**.

Farnsworth, N.R. (1966) Biological and phytochemical screening of plants. *Journal of Pharmaceutical Science* [online]. **55**(3), pp. 225-276. Available from DOI: 10.1002/jps.2600550302 [Accessed 2 August 2015].

Fratkin, E. (1996) Traditional medicine and concepts of healing among Samburu pastoralists of Kenya. *Journal of Ethnobiology* [online]. **16**(1), pp. 63-97.

Available from: <https://ethnobiology.org/sites/default/files/pdfs/JoE/16-1/Fratkin1996.pdf> [Accessed 18 January 2017].

Frederick, A. (2015) Personal communication, Gochas, Namibia.

Harley, J.P. and Prescott, L.M. (2002) *Laboratory exercises in Microbiology*. 5th ed. New York: McGraw-Hill.

Hassan, I.A., Nasiru, I.A., Malut, A.M., Abdulkadir, I.S. and Ali, A.S. (2015) Phytochemical studies and thin layer chromatography of leaves and flower extracts of *Senna siamea lam* for possible biomedical applications. *Journal of Pharmacognosy and Phytotherapy* [online]. **7**(3), pp. 18-26. Available from DOI: 10.5897/JPP2014.0337 [Accessed 21 November 2017].

Hoffmann, L. (2014) Meet the trees of Namibia: The worm-cure albizia (*Albizia anthelmintica*). *The Namibian* [online]. Available from: <http://www.namibian.com.na/> [Accessed 18 January 2017].

Kamonwannasit, S., Nantapong, N., Kumkrai, P., Luecha, P., Kupittayanant, S. and Chudapongse, N. (2013) Antibacterial activity of *Aquilaria crassna* leaf extract against *Staphylococcus epidermidis* by disruption of cell wall. *Annals of Clinical Microbiology and Antimicrobials* [online]. **12**(20). Available from DOI: 10.1186/1476-0711-12-20 [Accessed 19 January 2017].

Nematollahi, A., Aminimoghadamfarouj, N. and Wiart, C. (2011) Anti-bacterial, antioxidant activity and phytochemical study of *Diospyros wallichii* – an interesting Malaysia's endemic species of Ebenaceae. *International Journal of PharmTech Research* [online]. **3**(3), pp. 1732-1736. Available from: https://www.researchgate.net/publication/265014833_Antibacterial_antioxida

[nt activity and phytochemical study of diospyros wallichii an interesting malaysia%27s endemic species of ebenaceae](#) [Accessed 29 July 2015].

Njateng, G.S.S., Gatsing, D., Mouokeu, R.S., Lunga, P.K. and Kuate, J.-R. (2013) In vitro and in vivo antidermatophytic activity of the dichloromethane-methanol (1:1 v/v) extract from the stem bark of *Polyscias fulva* Hiern (Araliaceae). *BMC Complementary and Alternative Medicine* [online]. **13**(95), pp. 1-10. Available from DOI: <https://doi.org/10.1186/1472-6882-13-95> [Accessed 20 February 2015].

Nyambe, M.M. (2014) *Phytochemical and antibacterial analysis of indigenous chewing sticks, Diospyros lyciodes and Euclea divinorum*. Master's thesis. University of Namibia, Windhoek. Available from: <http://repository.unam.edu.na/handle/11070/783> [Accessed 2 August 2015].

Orwa, C., Mutua, A., Kindt, R., Jamnadass, R. and Anthony, S. (2009) Agroforestry database: A tree reference and selection guide version 4.0 [online]. Available from: <http://www.worldagroforestry.org/sites/treedbs/treedatabases.asp> [Accessed 18 January 2017].

Osungunna, M.O. and Adedeji, K.A. (2011) Phytochemical and antimicrobial screening of methanol extract of *Heliotropium indicum* leaf. *Journal of Microbiology and Antimicrobials*. **3**(8), pp. 213-216.

Sullivan, S. (1998) *People, plants and practice in drylands: Socio-political and ecological dimensions of resource-use by Damara farmers in north-west Namibia*. Unpublished doctoral thesis. University College, London. Available from: <http://discovery.ucl.ac.uk/id/eprint/1317514> [Accessed 18 January 2017].

- Van Wyk, B-E. (2015) Die kulturele en praktiese waarde van inheemse kennis oor plantgebruike in die 21ste eeu. *Suid-Afrikaanse Tydskrif vir Natuurwetenskap en Tegnologie* [online]. **34**(1). Available from DOI: 10.4102/satnt.v34i1.1349 [Accessed 19 January 2017].
- Van Wyk, B.-E. and Gericke, N. (2000) *People's plants*. Pretoria: Briza.
- Van Wyk, B.-E. and Wink, M. (2015) *Phytomedicines, herbal drugs & plant poisons*. Pretoria: Briza.

Chapter 7

Anti-biofilm properties of *Aptosimum albomarginatum* (Marloth and Engl.) and *Dicoma schinzii* (O. Hoffm.) crude methanolic extracts against *S. aureus* and MRSA.

Abstract

Biofilm bacteria can withstand their host's natural immune defence mechanisms and resist antibiotic treatment, often leading to chronic infections. This is a significant health problem and the search for novel sources of antimicrobial agents is a global challenge. Plant extracts are being screened by scientists with the goal to discover new compounds effective for treatment of bacterial infections. The present work aimed to assess the biofilm inhibition and eradication properties of crude methanolic extracts from three traditional medicinal plants against *S. aureus* and MRSA. Extracts were also screened for secondary metabolites that can play a role in anti-biofilm activity.

The microtiter plate assay with crystal violet stain was used to determine if crude methanolic extracts from *Aptosimum albomarginatum* (Marloth and Eng.), *Albizia anthelmintica* (A. Rich Brongn.) and *Dicoma schinzii* (O. Hoffm.) could inhibit and/or eradicate *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) biofilms. The extent of biofilm growth and inhibition/eradication was quantified by measuring the absorbance of bacterial cells at 595nm. Phytochemical compounds that may play a role in the extracts' anti-biofilm activity were screened for by thin layer chromatography (TLC).

Aptosimum albomarginatum root extract was the best biofilm inhibition agent, with highly active inhibition (86.0%) observed in *S. aureus* ATCC 33591 (MRSA), and moderate activity in four other isolates. This extract eradicated the biofilm of *S. aureus* isolate S110 S73 Pure by 40.0% (moderate activity). Flavonoids and

saponins/triterpenes in the roots may play a role the extract's activity. *Dicoma schinzii* leaf extract moderately inhibited biofilms in three isolates. Flavonoids, coumarins, saponins or triterpenes in the leaves may contribute to its anti-biofilm properties.

In conclusion, *A. albomarginatum* root extract was the best anti-biofilm agent against the tested *S. aureus* isolates. It was highly active in inhibiting biofilm formation in one MRSA isolate, and moderately active in inhibiting formation in four isolates. The extract moderately eradicated the biofilm in one isolate. This activity may to some extent be attributed to the presence of the secondary metabolites flavonoids, saponins or triterpenes in the roots. *Dicoma schinzii* leaf extract moderately inhibited biofilms in three isolates. Flavonoids, coumarins, saponins or triterpenes may play a role its anti-biofilm activity. The current study supports the traditional medicinal uses of *A. albomarginatum* roots and *D. schinzii* leaves as anti-biofilm agents in infections involving the bacteria under study.

Keywords: *Aptosimum albomarginatum*, *Dicoma schinzii*, crude methanolic extracts, phytochemical compounds, biofilms, inhibition, eradication, *S. aureus*, MRSA

7.1 Introduction

Some strains of staphylococci can form biofilms. Hutcherson *et al.* (2015, p. 1) define biofilms as dense, surface-attached communities of bacteria or fungi encased within a microbial-derived matrix that helps with colonization and survival. According to Mack *et al.* (2013, p. 25) and Nazzari *et al.* (2014), this formation helps the bacterium to withstand the host's natural immune defense mechanisms and to resist antibiotic treatment. According to Speziale and Geoghegan (2015, p. 1) the nasopharynx, heart valves, lungs and oral cavity are all sites for biofilm growth involving staphylococci

and streptococci. Such growth is also problematic during infection of implanted medical devices.

Cowan (2012, p. 187 and p. 342) explains that biofilm bacteria behave differently than their free-floating counterparts and are associated with chronic infections. They are often resistant to the same antimicrobials that work against them when they are free-floating. When attached to surfaces their gene expression is altered, leading to different antibiotic susceptibility profiles. According to Stefanović *et al.* (2012, p. 2) bacterial resistance to antibiotics is a significant health problem. Solving this problem and the search for novel sources of antimicrobial agents is a global challenge and the aim of many researchers. One approach is to test biologically active compounds from plants. Scientists have been screening plant extracts with the goal to discover new compounds effective for treatment of bacterial infections. Plant extracts display direct antibacterial activity exhibiting effects on bacterial growth and metabolism. They also display indirect activity as antibiotic resistance modifying compounds which, in combination with antibiotics, enhance their effectiveness.

The present work aimed to assess the biofilm inhibition and eradication properties of crude methanolic extracts from three traditional medicinal plants against *S. aureus* and MRSA. Extracts were also screened for secondary metabolites that can play a role in anti-biofilm activity.

7.2 Materials and methods

7.2.1 Bacteria and extracts used

Ten biofilm-producing *S. aureus* isolates (including one MRSA isolate) originating from nasal specimens of school children in the Mariental District, Namibia, as well as purchased *S. aureus* ATCC 25923 and *S. aureus* ATCC 33591 (MRSA) strains

(Microbiologics[®], St. Cloud, USA) were used in disk diffusion assays with crude methanolic plant extracts biofilm assays with plant extracts.

Crude methanolic extracts were prepared from *A. albomarginatum* roots, *A. anthelmintica* twigs and *D. schinzii* roots and leaves.

7.2.2 Microtiter plate assay for biofilm inhibition

For biofilm assays, the methods of Christensen *et al.* (1985), Merrit *et al.* (2011), and Monte *et al.* (2014) were used, with some modifications. Cultures were inoculated into 50-ml centrifuge tubes (Greiner Bio-One, Kremsmünster, Austria) containing 5ml brain heart infusion broth (Merck, Darmstadt, Germany) and grown to stationary phase at 37°C for 24 hours. The stationary phase cultures were then diluted 1:100 (a 0.5 McFarland standard) and 100µl of diluted culture was added to each of six wells in a sterile flat-bottomed 96-well microtiter plate (Thermo Fisher Scientific, Newport, UK). Figure 7.1 shows the plate layout. Three of these six wells were each inoculated with 10µl of previously determined sub-minimum inhibitory concentrations (sub-MICs) of plant extract. Eight wells each contained 100µl sterile brain heart infusion broth only as control.

After incubation, planktonic cells were removed by placing the microtiter plate upside down on towel paper and allowing for the paper to soak up any liquid. To remove remaining planktonic cells, each well was washed three times by pipetting 400µl sterile distilled water into it and inverting the plates onto towel paper. The biofilms in the wells were fixed by oven-drying the microtiter plates for 45 minutes at 60°C.

Wells were stained with 125µl of 0.1% crystal violet, incubated for 15 minutes at room temperature and the crystal violet discarded. Excess stain was removed by washing (pipetting) three times with 400µl sterile distilled water. Plates were air-dried for a few hours. Wells were de-stained with 200µl of 33.0% glacial acetic acid (Merck,

Darmstadt, Germany) for 10-15 minutes. The contents of each well was briefly mixed by pipetting, and 125µl was transferred to corresponding wells of a new clean microtiter plate. The optical densities (ODs) of stained biofilms were determined with a SpectraMax M2 Multi-mode Microplate Reader (Molecular Devices, China) at 595nm. Readings from the sterile brain heart infusion broth control wells were averaged and subtracted from the test readings. Test readings were averaged and standard deviations calculated. Results for biofilm formation/inhibition were interpreted using the classification by Christensen *et al.* (1985, p. 998).

The equation $I\% = [1 - (A_{595} \text{ of test} / A_{595} \text{ of non-treated control}) \times 100]$ was used to calculate percentage inhibition (Kawsud *et al.* 2014, p. 1497). According to Manner *et al.* (2013, p. 19438) selection criteria (activity-based) for antimicrobials are as follows: Highly active - $\geq 85.0\%$ inhibition; moderately active - $\geq 40.0\%$ inhibition; inactive - $< 40.0\%$ inhibition.

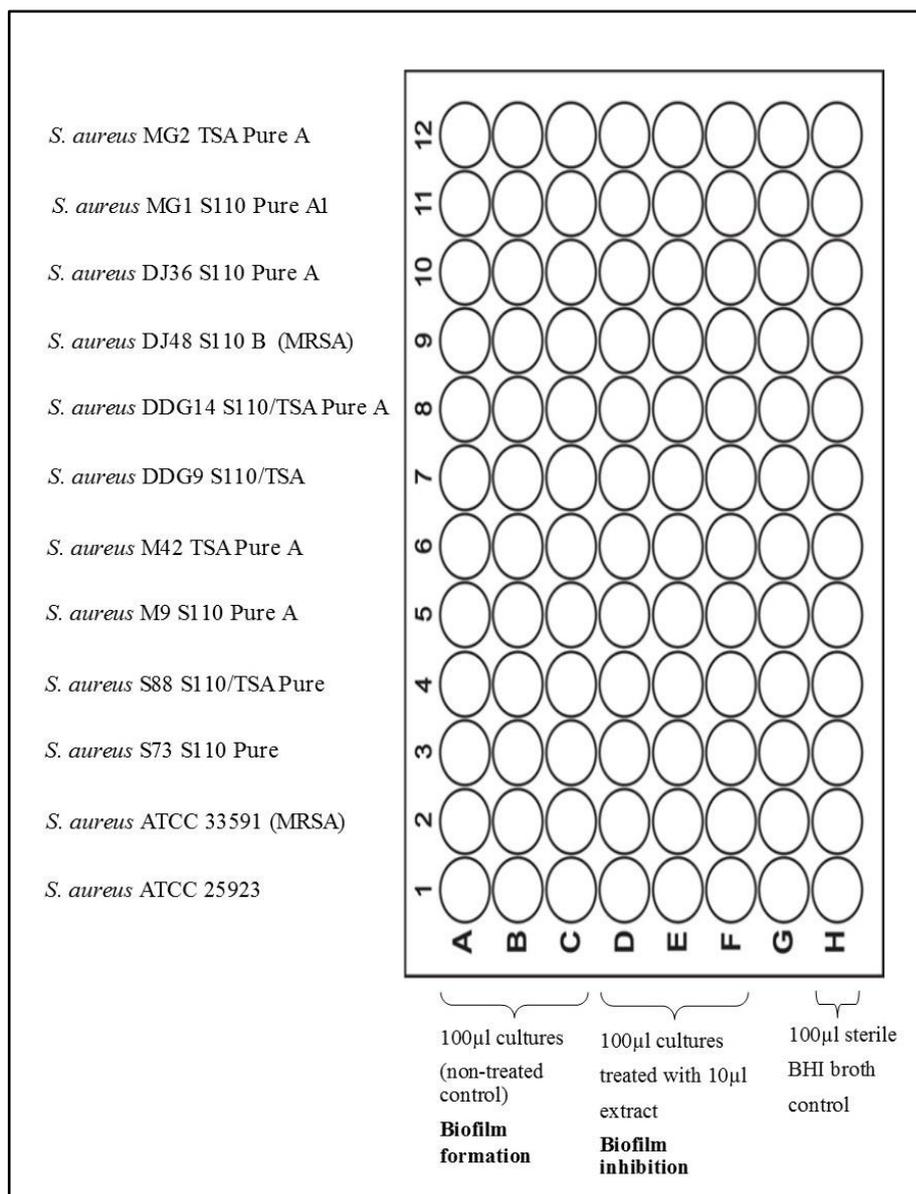


Figure 7.1 Schematic representation of the plate layout.

7.2.3 Microtiter plate assay for biofilm eradication

Stationary-phase cultures were diluted 1:100 and 100µl of diluted culture was added to each of six wells of a sterile flat-bottomed 96-well microtiter plate. Eight wells each contained 100µl sterile brain heart infusion broth only as control. The plates were parafilmmed at the lids to prevent them from drying out and incubated at 37°C for 24 hours.

After incubation, planktonic cells were removed by decanting onto towel paper. To remove remaining planktonic cells, each well was washed three times by pipetting 400µl sterile distilled water into it and inverting the plate onto towel paper. Three wells with the grown biofilms were inoculated with 10µl of extract (just below MIC) and 190µl sterile distilled water. The remaining wells were filled with 200µl sterile distilled water and plates were incubated at room temperature for another 24 hours. After the second incubation and removal of liquid in the wells, the same steps for fixing, staining and de-staining were followed as for the inhibition assay. The equation $E\% = [1 - (A_{595} \text{ of test} / A_{595} \text{ of non-treated control}) \times 100]$ (Kawsud *et al.*, 2014) was used to calculate percentage eradication. The classification of activity for inhibition by Manner *et al.* (2013) was also used to interpret eradication results.

7.2.4 Thin layer chromatography (TLC) for screening of phytochemical compounds

Six 2-ml Eppendorf tubes (Eppendorf, Germany) were used, four for the plant extracts and two for positive controls, and 500µl methanol was pipetted into each tube. Then 2-3mg extract and 2mg of the phytochemical standards (positive controls) were added to the respective tubes.

Two Erlenmeyer flasks were labelled for flavonoids and saponins. Mobile phases (solvents) were prepared as 100ml volumes, according to the ratios given in Table 7.1. Spraying reagents for confirmation of compounds belonging to the two phytochemical classes were also prepared (Table 7.1).

A TLC gel 60 F₂₅₄ aluminium sheet 20 x 20cm (TLC plate) (Merck, Darmstadt, Germany) was cut in half. Pencil origins were drawn 2cm from the bottom of the plate. Using a capillary tube, the control was spotted 1cm from the left side and right side of

the plate on the origin line, three times with drying in between. The four plant extracts were also spotted three times, evenly spaced on the origin.

Two clean TLC glass tanks were labelled and the different solvents were poured into each. The plates were carefully inserted into the tanks and the tanks were covered with lids. The plates were run until the solvent was approximately 1cm from the top of the plate. Pencil markings were made quickly where the solvent stopped while the plates were still in the tanks, otherwise the plates dry too fast when taken out and the end-point cannot be measured accurately. The plates were removed from the tanks and straight pencil lines were drawn where the solvent stopped.

The plates were viewed at visible and under UV light at 366nm (blue light, long wavelength). A pencil was used to trace around the most prominent spots/bands and observed colours were recorded. The chromatograms were placed on paper towels in a fume hood and sprayed with the appropriate spraying reagents. The plates were dried in the fume hood, and viewed again under UV light. Any colour changes and new spots/bands were circled and recorded. As described by Maobe *et al.* (2012, p. 33) distances travelled by the solvents and the spots/bands were measured with a ruler and used to calculate retention factor (R_f) values: $R_f = \text{distance travelled by compound} / \text{distance travelled by solvent}$.

Table 7.1 Mobile phases (solvents), controls and spraying reagents used for TLC, adapted from Wagner and Bladt (1996).

Phytochemical class and standards (controls) used	Mobile phase	Spraying reagent and expected result
Flavonoids Control: Quercetin dihydrate (97%)	Ethyl acetate : Formic acid : Acetic acid : Water 100 : 11 : 11 : 27	1.0% Antimony (III) chloride in chloroform. Dark yellow, orange, green, or blue fluorescent spots at 366nm (intensified by spraying). Also detectable in visible light. Quercetin is orange-yellow or yellow-green. Flavonoid extracts often contain coumarins, which form blue, light blue or green fluorescent zones.
Saponins Control: Saponin	Butanol : Ethyl acetate : Acetic acid : Water 10.8 : 3.6 : 0.2 : 2.7	Vanillin-Sulphuric acid reagent; sprayed plates heated at 110°C for 5-10 minutes. Blue, blue-violet, red, or yellow-brown zones in visible light. With some exceptions, saponins are not detectable under UV light and need spraying reagents.

7.3 Results and discussion

7.3.1 Biofilm inhibition

Overall, *A. albomarginatum* root extract was the best biofilm inhibition agent, with highly active inhibition (86.0%) observed in *S. aureus* ATCC 33591 (MRSA), and moderate activity in four other isolates (Figure 7.2). Flavonoids and saponins/triterpenes were detected in the plant's roots (Tables 7.2 and 7.3). These secondary metabolites may play a role in its activity. Manner *et al.* (2013) observed

10 commercially bought flavonoids to be highly active, causing more than 85.0% biofilm inhibition and eradication against the clinical strains *S. aureus* ATCC 25923 and the Newman strain. In another study, Kamonwannasit *et al.* (2013) found that *Aquilaria crassna* leaf extract, containing flavonoids and saponins, could inhibit biofilm formation in *Staphylococcus epidermidis*. They explained that destruction of the bacterial cell wall by the plant extract prevents bacteria from growing and creating primary biofilm structures. Results from a study by Raja *et al.* (2011) indicated that acetyl-II-keto- β -boswellic acid (AKBA), a pentacyclic triterpene, significantly inhibited biofilm formation and eradicated preformed *S. aureus* and *S. epidermidis* biofilms.

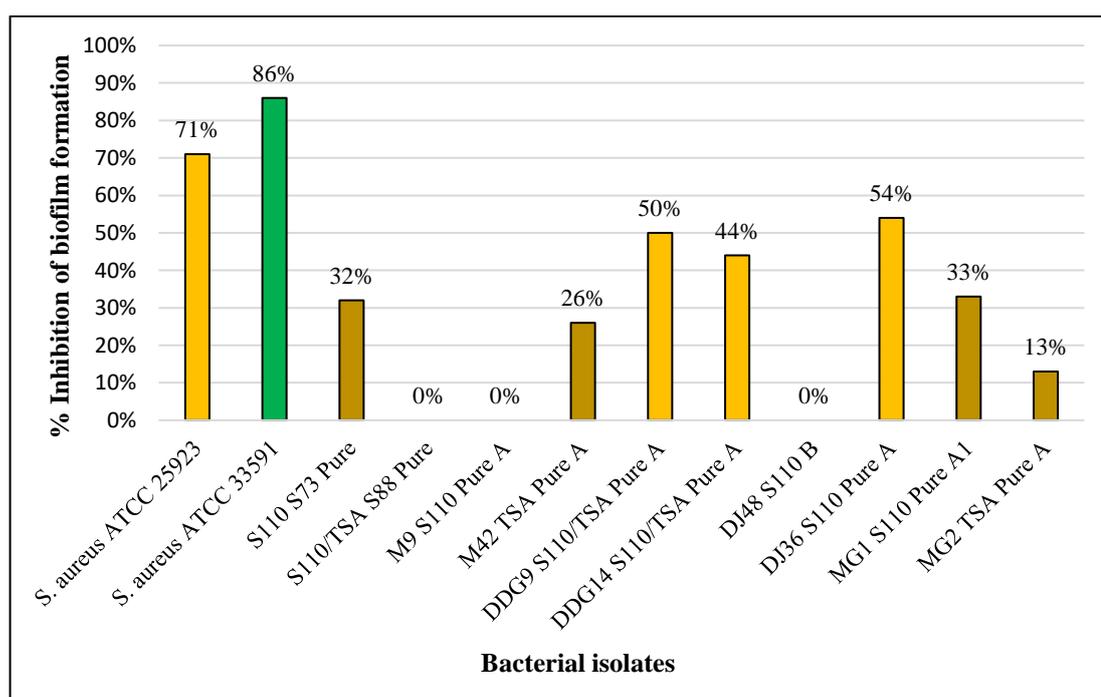


Figure 7.2 Percentage biofilm inhibition in microtiter plates by treatment with *A. albomarginatum* crude methanolic root extract. Classification of activity according to Manner *et al.* (2013, p. 19438): $\geq 85.0\%$ inhibition - highly active; $\geq 40.0\%$ inhibition - moderately active; $< 40.0\%$ inhibition - inactive.

Dicoma schinzii leaf extract moderately inhibited biofilm formation in 3/12 isolates. Flavonoids, coumarins, saponins or triterpenes in the leaves (Tables 7.2 and 7.3) may aid in the extract's activity. Bahar *et al.* (2017) saw that the aqueous extract from *Quercus brontii* subsp. *persica* fruits containing coumarins, caused up to 61.0% biofilm inhibition in *S. aureus*. *Albizia anthelmintica* twigs and *D. schinzii* roots were classified inactive against all 12 isolates.

7.3.2 Biofilm eradication

Biofilm eradication was observed only with *A. albomarginatum* root extract in 1/12 isolates. It broke down the biofilm of *S. aureus* isolate S110 S73 Pure by 40.0% (moderate activity). As said previously, this activity may partly be attributed to the presence of flavonoids, saponins or triterpenes in the plant's roots.

7.3.3 Detection of flavonoids and saponins by TLC

Phytochemical profiles for the four crude methanolic plant extracts are given in Tables 7.2 and 7.3. *Aptosimum albomarginatum* root extract was the most effective anti-biofilm agent in this study. For this extract, one flavonoid spot with a R_f value 0.92 was detected. Close to our results, Hussain *et al.* (2011, p. 13310) observed a blue flavonoid spot with a R_f value of 0.94 at UV 365nm for methanolic extract of *Figonia critica*. Three saponin/triterpene spots with R_f values of 0.15, 0.49 and 0.94 were also seen in the current study. After derivatization with Vanillin-Sulphuric acid reagent and heating at 105°C, Priya *et al.* (2014, p. 71) obtained a purple saponin spot with R_f 0.94 from chloroform extract of *Milagathi chooranam*.

For *D. schinzii* leaf extract, which inhibited biofilms in three *S. aureus* isolates, there were three flavonoid spots with R_f values of 0.43, 0.69 and 0.96. One spot with R_f value 0.59 can either be a flavonoid or coumarin compound. For saponin screening,

four spots with R_f values 0.22, 0.50, 0.71 and 0.96 were observed. Two of these spots were identified as saponins, one as a saponin/triterpene, and one as a flavonoid, coumarin or triterpene. Olivier (2012) detected steroids, terpenoids, bitter principles, saponins and flavonoids in *Dicoma* species (including *D. schinzii*), using qualitative colour tests and TLC.

Table 7.2 Screening for flavonoid compounds in plant extracts based on R_f values and colour changes on chromatograms before and after spraying with 1.0% Antimony (III) chloride reagent.

	Compound number and R_f in brackets	Colour before spraying	Colour after spraying	Identification
Extract 1: <i>A. albomarginatum</i> roots	1. (0.92)	Light fluorescent blue at UV 366nm	Yellow in visible light	Flavonoid
Extract 2: <i>A. anthelmintica</i> twigs	1. (0.20)	Light fluorescent blue at UV 366nm	Became colourless	Flavonoid or coumarin
	2. (0.33)	Light fluorescent blue at UV 366nm	Became colourless	Flavonoid or coumarin
	3. (0.97)	Light fluorescent blue and pink mixture at UV 366nm	Green in visible light	Flavonoid
Extract 3: <i>D. schinzii</i> roots	1. (0.08)	Light fluorescent blue at UV 366nm	Became colourless	Flavonoid or coumarin
	2. (0.28)	Light fluorescent blue at UV 366nm	Became colourless	Flavonoid or coumarin
	3. (0.57)	Light fluorescent blue at UV 366nm	Stayed the same	Flavonoid or coumarin
	4. (0.97)	Light fluorescent blue at UV 366nm	Yellow in visible light	Flavonoid
Extract 4: <i>D. schinzii</i> leaves	1. (0.43)	Light fluorescent blue at UV 366nm	Yellow in visible light	Flavonoid
	2. (0.59)	Light fluorescent blue at UV 366nm	Stayed the same	Flavonoid or coumarin
	3. (0.69)	Fluorescent yellow at UV 366nm and yellow in visible light	Stayed the same	Flavonoid
	4. (0.96)	Light fluorescent blue, pink and orange mixture at UV 366nm	Yellow in visible light	Flavonoid
Control/standard: Quercetin dihydrate (97%)	1. (0.97)	Fluorescent yellow-green at UV 366nm and yellow-green in visible light	Brighter yellow-green	Quercetin dihydrate (flavonol)

Table 7.3 Screening for saponin compounds in plant extracts based on R_f values and colour changes on chromatograms before and after spraying with Vanillin-Sulphuric acid reagent.

	Compound number and R_f in brackets	Colour before spraying	Colour after spraying	Identification
Extract 1: <i>A. albomarginatum</i> roots	1. (0.15)	Colourless	Purple in visible light	Triterpene or saponin
	2. (0.49)	Colourless	Purple-brown in visible light	Triterpene or saponin
	3. (0.94)	Light fluorescent blue at UV 366nm	Purple-brown in visible light	Triterpene or saponin
Extract 2: <i>A. anthelmintica</i> twigs	1. (0.25)	Fluorescent blue at UV 366nm	Stayed the same	Flavonoid, coumarin or triterpene
	2. (0.32)	Colourless	Became blue in visible light	Saponin
	3. (0.45)	Fluorescent blue at UV 366nm	Stayed the same	Flavonoid, coumarin or triterpene
	4. (0.96)	Fluorescent orange-pink mixture at UV 366nm	Green in visible light	Flavonoid
Extract 3: <i>D. schinzii</i> roots	1. (0.23)	Light fluorescent blue at UV 366nm	Stayed the same	Flavonoid, coumarin or triterpene
	2. (0.49)	Light fluorescent blue at UV 366nm	Stayed the same	Flavonoid, coumarin or triterpene
	3. (0.71)	Colourless	Purple-brown in visible light	Triterpene or saponin
	4. (0.96)	Light fluorescent blue at UV 366nm	Purple-brown in visible light	Triterpene or saponin
Extract 4: <i>D. schinzii</i> leaves	1. (0.22)	Light fluorescent blue at UV 366nm	Stayed the same	Flavonoid, coumarin or triterpene
	2. (0.50)	Yellow-brown in visible light	Stayed the same	Saponin
	3. (0.71)	Yellow-brown in visible light	Stayed the same	Saponin
	4. (0.96)	Fluorescent orange-pink mixture at UV 366nm	Green at UV 366nm and purple in visible light	Triterpene or saponin
Control/standard: Saponin	1. (0.1)	Colourless	Red in visible light	Saponin

7.4 Conclusion

Aptosimum albomarginatum root extract was the best anti-biofilm agent against *S. aureus*. It was highly active in inhibiting biofilm formation in one MRSA isolate, and moderately active in inhibiting formation in four isolates. The extract moderately

eradicated the biofilm in one isolate. This activity may to some extent be attributed to the presence of the secondary metabolites flavonoids, saponins or triterpenes in the roots. *Dicoma schinzii* leaf extract moderately inhibited biofilms in three isolates. Flavonoids, coumarins, saponins or triterpenes may play a role its anti-biofilm activity. The current study supports the traditional medicinal uses of *A. albomarginatum* roots and *D. schinzii* leaves as anti-biofilm agents in infections involving the bacteria under study.

References

- Bahar, Z., Ghotaslou, R. and Taheri, S. (2017) *In vitro* anti-biofilm activity of *Quercus brantii* subsp. *persica* on human pathogenic bacteria. *Research Journal of Pharmacognosy* [online]. **4**(1), pp. 67-73. Available from: http://www.rjpharmacognosy.ir/article_41185_7cbe2ab8dc2758b06446668197c5ed26.pdf [Accessed 4 September 2017].
- Christensen, G.D., Simpson, W.A., Younger, J.J., Baddour, L.M., Barrett, F.F., Melton, D.M. and Beachey, E.H. (1985) Adherence of coagulase-negative staphylococci to plastic tissue culture plates: A quantitative model for the adherence of staphylococci to medical devices. *Journal of Clinical Microbiology* [online]. **22**(6), pp. 996-1006. Available from: <http://jcm.asm.org/content/22/6/996.long> [Accessed 29 July 2015].
- Cowan, M.K. (2012) *Microbiology: A systems approach*. 3rd ed. New York: McGraw-Hill.

- Hussain, I., Ullah, R., Khan, J., Khan, N., Zahoor, M., Ullah, N., Khattak, M.R., Khan, F.A., Baseer, A. and Khurram, M. (2011) Analysis of chemical constituents of medicinal plants of selected districts of Pakhtoonkhwa, Pakistan. *African Journal of Biotechnology* [online]. **10**(61), pp. 13307-13312. Available from DOI: 10.5897/AJB10.2128 [Accessed 21 November 2017].
- Hutcherson, J.A., Scott, D.A. and Bagaitkar, J. (2015) Scratching the surface – tobacco-induced bacterial biofilms. *Tobacco Induced Diseases* [online]. **13**. Available from DOI: 10.1186/s12971-014-0026-3 [Accessed 1 June 2016].
- Kamonwannasit, S., Nantapong, N., Kumkrai, P., Luecha, P., Kupittayanant, S. and Chudapongse, N. (2013) Antibacterial activity of *Aquilaria crassna* leaf extract against *Staphylococcus epidermidis* by disruption of cell wall. *Annals of Clinical Microbiology and Antimicrobials* [online]. **12**(20). Available from DOI: 10.1186/1476-0711-12-20 [Accessed 19 January 2017].
- Kawsud, P., Puripattanavong, J. and Teanpaisan, R. (2014) Screening for anticandidal and antibiofilm activity of some herbs in Thailand. *Tropical Journal of Pharmaceutical Research* [online]. **13**(9), pp. 1495-1501. Available from DOI: <http://dx.doi.org/10.4314/tjpr.v13i9.16> [Accessed 3 August 2015].
- Mack, D., Davies, A.P., Harris, L.G., Jeeves, R., Pascoe, B., Knobloch, J.K.-M., Rohde, H. and Wilkinson, T.S. (2013) *Staphylococcus epidermidis* in biomaterial-associated infections. In: Moriarty, F., Zaat, S.A.J. and Busscher, H.J. (eds.) *Biomaterials associated infection: Immunological aspects and antimicrobial strategies*. New York: Springer.
- Manner, S., Skogman, M., Goeres, D., Vuorela, P. and Fallarero, A. (2013) Systemic exploration of natural and synthetic flavonoids for the inhibition of *Staphylococcus aureus* biofilms. *International Journal of Molecular Sciences*

[online]. **14**, pp. 19434-19451. Available from DOI: 10.3390/ijms41019434 [Accessed 29 July 2015].

Maobe, M.A.G., Gitu, L., Gatebe, E. and Rotich, H. (2012) Phytochemical analysis of phenol and flavonoid in eight selected medicinal herbs used for the treatment of diabetes, malaria and pneumonia in Kisii, Kenya. *Academic Journal of Cancer Research* [online]. **5**(2), pp. 31-39. Available from DOI: 10.5829/idosi.ajcr.2012.5.2.66210 [Accessed 24 November 2015].

Merritt, J.H., Kadouri, D.E. and O'Toole, G.A. (2011) Growing and analyzing static biofilms. *Current Protocols in Microbiology* [online]. Available from DOI: 10.1002/9780471729259.mc01b01s00 [Accessed 6 August 2015].

Monte, J., Abreu, A.C., Borges, A., Simões, L.L. and Simões, M. (2014) Antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* and their biofilms. *Pathogens* [online]. **3**, pp. 473-498. Available from DOI: 10.3390/pathogens3020473 [Accessed 29 July 2015].

Nazzari, E., Torretta, S., Pignataro, L., Marchisio, P. and Esposito, S. (2014) Role of biofilm in children with recurrent upper respiratory tract infections. *European Journal of Clinical Microbiology and Infectious Diseases* [online]. Available from DOI: 10.1007/s10096-014-2261-1 [Accessed 13 February 2016].

Olivier, D.K. (2012) *The ethnobotany and chemistry of South African traditional tonic plants*. PhD thesis. University of Johannesburg.

Priya, F., Shakila, R., Sathiyarajeswaran, P. and Pitchiahkumar, M. (2014) Standardization of Milagathi Choornam. *International Journal of Herbal Medicine*. **1**(6), pp. 69-74.

- Raja, A.F., Ali, F., Khan, I.A., Shawl, A.S., Arora, D.S., Shah, B.A. and Taneja, S.C. (2011) Antistaphylococcal and biofilm inhibitory activity of acetyl-11-keto- β -boswellic acid from *Boswellia serrata*. *BMC Microbiology* [online]. **11**(54). Available from DOI: 10.1186/1471-2180-11-54 [Accessed 4 October 2017].
- Speziale, P. and Geoghegan, J.A. (2015) Biofilm formation by staphylococci and streptococci: Structural, functional, and regulatory aspects and implications for pathogenesis. *Frontiers in Cellular and Infection Microbiology* [online]. **5**. Available from DOI: 10.3389/fcimb.2015.00031 [Accessed 7 August 2015].
- Stefanović, O., Radojević, I., Vasić, S. and Ćomić, L. (2012) Antibacterial activity of naturally occurring compounds from selected plants. In: Bobbarala, V. (ed.) *Antimicrobial agents*. Croatia: InTech, pp. 1-24.
- Wagner, H. and Bladt, S. (1996) *Plant drug analysis: A thin layer chromatography atlas*. 2nd ed. Germany: Springer.

Chapter 8

Conclusions and prospects

Staphylococcus aureus, methicillin-resistant *S. aureus* (MRSA) and multi-drug resistant staphylococci cause illness and death in both adults and children around the globe, including sub-Saharan Africa. Research on *Staphylococcus* is being done in other countries, but not in Namibia. The present study is a step in trying to close this gap in information by being the first to report on the prevalence, antibiotic resistance and virulence of *Staphylococcus* among Namibian school children. The findings support the hypothesis.

There was a high prevalence of *S. aureus* among children aged 6-14 years attending five schools in the Mariental District. The coagulase-negative staphylococci (CoNS) prevalence and that of methicillin-resistant bacteria was relatively low. A total of 31 antibiotic resistance patterns were observed, 27 for *S. aureus* and 14 for CoNS. The three most encountered patterns were AP, AP-E, and AP-T. Multi-drug resistance occurred in only 12.5% of all isolates. Out of 51 MRSA isolates, 43.1% was multi-drug resistant. Ciprofloxacin and gentamicin were the most effective drugs against staphylococcal isolates. Seven multi-drug resistant MRSA isolates were enterotoxigenic. Twelve representative *S. aureus* isolates were able to form strong biofilms on plastic surfaces of microtiter plates. Self-infection by these potentially virulent bacteria poses various health risks for the children. The present work supports the traditional medicinal uses of *A. albomarginatum* roots and *D. schinzii* roots and leaves as natural antimicrobial agents, as well as *A. albomarginatum* roots and *D. schinzii* leaves as anti-biofilm agents in infections involving the bacteria under study. *Aptosimum albomarginatum* is of particular interest since its crude methanolic root extract displayed antimicrobial activity against multi-drug resistant isolates and

MRSA. This extract also had anti-biofilm activity against MRSA, showing promise for future research on MRSA strains.

Prospective studies should screen staphylococcal isolates for additional virulence factors, such as the Panton-Valentine leukocidin (PVL) toxin. Multi-drug resistant MRSA isolates from nasal specimens must be identified according to their sequence types (STs), staphylococcal protein A (*spa* types), and staphylococcal chromosome *mec* (SCC*mec*) types. Instead of using only crude plant extracts, specific phytochemical compounds should be extracted and identified. Such compounds must be evaluated for their activity against multi-drug resistant *Staphylococcus* and the mechanism of action must be determined. Compounds should also be tested in combination with antibiotics to determine possible synergistic interactions. Finally, it can be concluded that the aim and various objectives were achieved as set out in Section 1.5.

Appendices

Appendix A1: Permission letters from the Ministry of education, Windhoek and Mariental.


REPUBLIC OF NAMIBIA

MINISTRY OF EDUCATION, ARTS AND CULTURE

Tel: +264 61-2933 278
Fax: +264 61-2933 922
Email: ayeshawentworth@yahoo.com

Private Bag 13186
Windhoek
Namibia

Ms S. Walters
Sunette.walter8@gmail.com

Dear Ms Walter

RE: PERMISSION TO COLLECT NASAL SAMPLES (SWABS) FROM PRIMARY SCHOOL CHILDREN IN THE HARDAP REGON, MARIENTAL DISTRICT

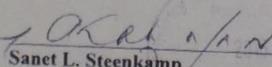
The Ministry of Education, Arts and Culture acknowledges receipt of the communication dated 11 February 2016 requesting permission to enter randomly selected schools in the Hardap Region, Mariental District. The Ministry supports research initiatives as a way of enhancing knowledge and directing evidence based interventions.

Kindly be advised the permission to conduct the study has been granted under the following conditions:

1. That consent be obtained from the Regional Directors of Education in the Hardap Region;
2. That the teaching and learning in schools should not be disrupted;
3. That consent is received from all school Principals of sampled schools;
4. That informed consent is received from all parents of learners participating in the study, and ensure that no learner without consent participate;
5. That the confidentiality of all learners be strictly adhered to;
6. That all children who do test positive for Staphylococcus will be assisted in the appropriate manner, that maintains confidentiality.

Should you require any further information, please contact Ms A. Wentworth on 061 2933278 or ayeshawentworth@yahoo.com.

Yours sincerely


Sanet L. Steenkamp
PERMANENT SECRETARY

24/2/16
Date

All official correspondences must be addressed to the Permanent Secretary

Appendix A2: Copy of consent form for school principals.

Prevalence, antibiotic resistance trends and virulence of *Staphylococcus* among school children in the Mariental District, Namibia

CONSENT FORM (PRINCIPALS)

1. WHAT THE PROJECT IS ABOUT

The project aims to isolate *Staphylococcus* bacteria (germs) from children's noses. In many other countries, researchers have found staphylococci in school children. So now we want to get an idea of how many children in the Mariental District carry *Staphylococcus* in their noses. If these bacteria are found in a child's nose it does not mean that the child is sick and he/she must keep attending school. *Staphylococcus* can be part of the normal nasal bacteria in the child and not cause any harm. It is only when it enters a wound on the skin that it can possibly cause an infection. Parents and children are therefore encouraged to wash their hands regularly and cover wounds to avoid spreading of the bacteria.

2. WHAT WILL HAPPEN TO MY LEARNERS IF I AGREE TO PARTICIPATE IN THE STUDY?

Sampling will be done in March and June 2016. On the day of the school visit, a nasal sample (swab) will be collected from the front part of the learner's nose using a sterile (clean) cotton swab. This will not hurt at all – it is like when you clean your child's nose with a cotton bud. Sampling will only take up 5 minutes of the child's time and can be done during break-time so that there won't be any disruption of classes.

3. ARE THERE SERIOUS RISKS?

No, taking the swab is safe and will not hurt the child at all.

4. ETHICAL STATEMENT

Permission to conduct this study and ethical clearance will be obtained from the Ministry of Education, school principals and parents. Learners will be randomly selected from class lists. Parents or guardians of these learners will be asked to complete consent forms, but are under no obligation to do so. Participation in this study is completely voluntary; the children will be asked if they want to donate nasal samples. Participants will not receive any financial compensation. Names of learners and schools will not be mentioned within reported results. Specific "codes" will be assigned to them. Personal information will be kept confidential. Children that test positive for *Staphylococcus* will not be isolated because it is not necessary (they will keep attending school). Instead an effort will be made to educate all teachers, learners and parents in general on preventative measures (for example encouraging good hygiene practices such as regular hand washing) by giving them information leaflets. All questions will be answered by the main researcher. In cases where a particular virulent or multi-drug resistant strain is isolated (which is unlikely among healthy children), the parents will be informed and advised to take the child to a doctor or clinic. It remains a sensitive and private issue and care will be taken not to upset learners or parents.

6. I HAVE READ THROUGH THE GIVEN INFORMATION AND AGREE TO PARTICIPATE IN THE STUDY IF THE FOLLOWING CONDITIONS ARE MET:

- I understand the study and what will be expected of me and my learners.
- I understand my learners' safety if agreed to take part in the study.
- I agree that nasal samples can be collected from my learners for the study.

If you still have questions about anything, feel free to contact the Principal Investigator:

Miss Sunette Walter, University of Namibia Main Campus Windhoek, Faculty of Science, Department of Biological Sciences, Biomedical Research Lab W059, Cell nr.: 0814151015, e-mail: sunette.walter8@gmail.com

Signature of School Principal Date.....

Signature of Witness 1 Date.....

Signature of Witness 2 Date.....

Signature of Principal Investigator Date.....

THANK YOU FOR YOUR TIME AND HELP

Appendix A3: Copy of consent form and questionnaire for parents/guardians.

Prevalence, antibiotic resistance trends and virulence of *Staphylococcus* among school children in the Mariental District, Namibia

CONSENT FORM

1. WHAT THE PROJECT IS ABOUT

The project aims to isolate *Staphylococcus* bacteria (germs) from children's noses. In many other countries, researchers have found staphylococci in school children. So now we want to get an idea of how many children in the Mariental District carry *Staphylococcus* in their noses. If these bacteria are found in your child's nose it does not mean that your child is sick and he/she must keep attending school. *Staphylococcus* can be part of the normal nasal bacteria in the child and not cause any harm. It is only when it enters a wound on the skin that it can possibly cause an infection. Parents and children are therefore encouraged to wash their hands regularly and cover wounds to avoid spreading of the bacteria.

2. WHAT WILL HAPPEN TO MY CHILD IF I AGREE TO PARTICIPATE IN THE STUDY?

On the day of the school visit, a nasal sample (swab) will be collected from the front part of your child's nose using a sterile (clean) cotton swab. This will not hurt your child at all – it is like when you clean your child's nose with a cotton bud. Sampling will only take up 5 minutes of your child's time.

3. DO I HAVE THE RIGHT TO SAY NO IN PARTICIPATING?

Yes, your child's participation is voluntary and you are allowed to withdraw your child from the study at any time, but then the researcher must be informed.

4. ARE THERE SERIOUS RISKS?

No, taking the swab is safe and will not hurt your child at all.

5. WILL MY INFORMATION BE KEPT CONFIDENTIAL?

Yes, personal information will be kept confidential. Only four researchers will have access to it and when the results of the study are published, it will be presented in group form and your child's name will not be mentioned. Your information will be destroyed after the study is complete.

6. ARE THERE COSTS OR PAYMENTS INVOLVED?

No, you will not be charged or get paid to give nasal samples.

7. I HAVE READ THROUGH THE GIVEN INFORMATION AND AGREE TO PARTICIPATE IN THE STUDY IF THE FOLLOWING CONDITIONS ARE MET:

- I understand the study and what will be expected of me and my child.
- I understand my child's safety if agreed to take part in the study.
- I know that I can withdraw my child from the study when I feel like it without having to explain myself.
- I agree that nasal samples can be collected from my child for the study.

If you still have questions about anything, feel free to contact the Principal Investigator:

Miss Sunette Walter, University of Namibia Main Campus, Faculty of Science, Department of Biological Sciences, Windhoek, Cell nr.: 0814151015, e-mail: sunette.walter8@gmail.com

Signature of parent/guardian..... Date.....

Signature of witness 1..... Date.....

Signature of witness 2..... Date.....

Signature of Principal Investigator..... Date.....

QUESTIONNAIRE

Please answer the following questions:

1. What is your child's name and surname?
 2. What age is your child?
 3. Is your child staying in a hostel?
 4. Does your child get regular nosebleeds?
 5. Are you a smoker, or does anyone in your household smoke cigarettes?
-

THANK YOU FOR YOUR TIME AND HELP

Appendix B: Information leaflets for parents/guardians and teachers.

Thank you for your time and helping us by allowing your child/learner to take part in this study!

This study is supported and funded by UNAM, SABINA and Carnegie SIG/RISE.




For any questions, please contact :
 The Principal Investigator, Miss Sunette Walter
 The University of Namibia
 Department of Biological Sciences/Faculty of Science, Main Campus, Windhoek
 Cell: 0814151015
 E-mail: sunette.walter8@gmail.com



Department of Biological Sciences

.....

Information leaflet for parents and schools on the study: Prevalence, antibiotic resistance trends and virulence of *Staphylococcus* in school children in the Mariental District, Namibia.

.....

This leaflet will help you understand what the study is about, and what is expected from you. Please take your time to read through it and if there are any more questions, feel free to contact the Principal Investigator (Contact details on the back).

This leaflet was prepared by Sunette Walter.

About the study

- *Staphylococcus* is a bacterium (germ) that can live in the nose, on the skin or other parts of the body in some people and animals.
- In this project we will learn more about the different types of staphylococci that may be found in the noses of healthy school children in the Mariental District.
- This information can be useful in assisting school health programmes.

What is expected from you

On the first day of the school visit:
 Homeroom teachers for Grades 1-7 will be asked to help us randomly select possible participants for the study from the class list of names.
 The selected learners will then be given letters (this information leaflet and a consent form) to take home to the parents.
 The Principal Investigator will answer all questions you may have.
 If you agree that your child can participate in the study, you will have

to sign the consent form.
 Please give the signed form to your child to return to the homeroom teacher the following day.
 The Principal Investigator will collect the forms from the teachers.

What will be done to your child

On the day of sampling at the school:

- Your child will be called away from his/her class for a few minutes.
- One nasal sample will be taken from his/her nose using a sterile (clean) cotton swab.
- This will not hurt at all (it's like cleaning the nose with a cotton bud).
- Sampling will not take up more than 5 minutes of your child's time and he/she will be given a lollipop to say thanks for the sample!
- Your child can return to his/her class immediately after sampling.
- The swab sample from your child will be taken to the University's laboratory in Windhoek to be analyzed.



What can be done to fight this germ?
 The very best way to fight *Staphylococcus* is by proper hand washing for 20 seconds with soap and warm water.
 We encourage you to teach your children/learners how important it is to wash their hands regularly.



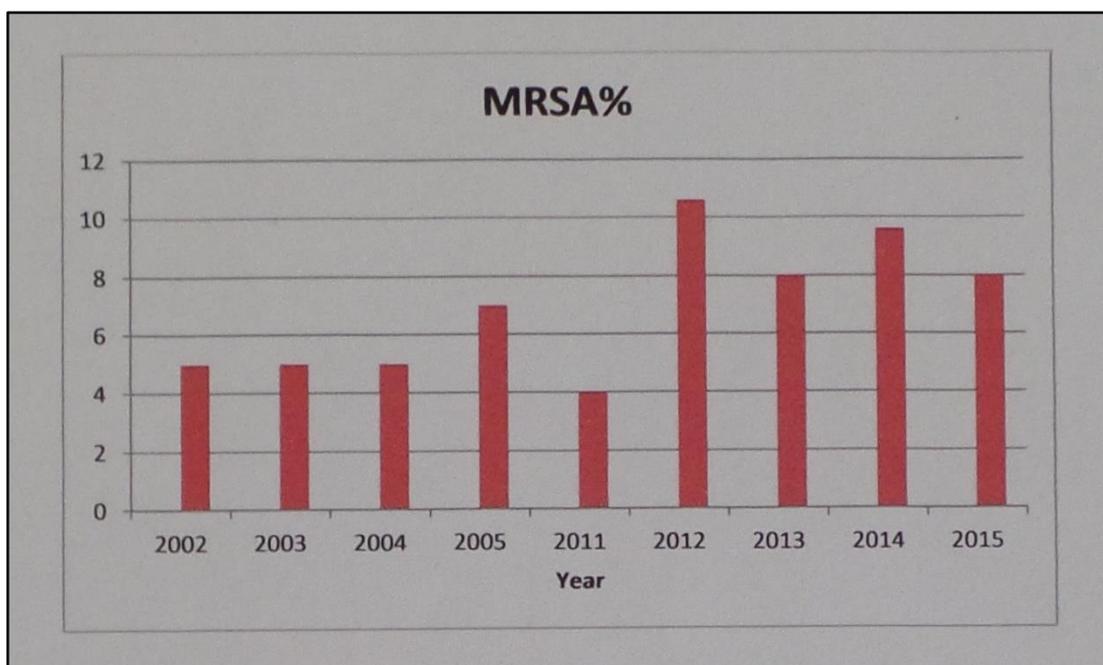
Appendix C: PathCare antibiotic susceptibility data for *S. aureus* 2001-2015

(PathCare Namibia, 2015).

ANTIMICROBIAL SUSCEPTIBILITY DATA (% Susceptible)

Staphylococcus aureus

Antibiotics	Year									
	2001	2002	2003	2004	2005	2010-2011	2011-2012	2012-2013	2014-2015	
Penicillin	12	7	9	10	9	8	7	5	5	
Cloxacillin	95	95	95	93	96	89.4	92	90.4	92	
Cotrimoxazole	92	93	92	90	63	85	81	72	64	
Clindamycin	68	96	96	98	99	87	91	90	91	
Macrolides										
Erythromycin	92	93	86	87	93	83	85	87	89	
Fucidic acid	95	98	97	95	98	93	96	98	97	
Rifampicin	-	-	-	-	-	95	95	94	93	
Tetracycline	86	87	89	88	91	87	87	87	91	
Glycopeptides										
Vancomycin	100	100	100	100	100	99	100	100	100	
Teicoplanin	-	-	-	-	-	99	99.9	100	100	
Aminoglycosides										
Gentamycin	-	-	-	-	-	92	90	91	95	
Linezolid										
Linezolid	-	-	-	-	-	100	99.8	100	100	
Quinolones										
Ofloxacin	99	98	97	94	97	94	-	-	-	
Ciprofloxacin	-	-	-	-	-	100 (19)	96	-	-	
Moxifloxacin	-	-	-	-	-	93	99	97	98	
Total organisms	315	428	404	495	520	420	962	814	893	
MRSA %	5	5	5	7	4	10.6	8	9.6	8	



Appendix D: Identification report from the National Herbarium, National Botanical Research Institute (NBRI).

Number	ID cat.	Identification
CID 60	1	<i>Dicoma schinzii</i> O.Hoffm.
CID 56 (99)	1	<i>Dicoma schinzii</i> O.Hoffm.
HS 0014	4	
CID 50	4	
CID 54	3	<i>Gnelum</i> sp.
CID 58	1	<i>Aptosimum albomarginatum</i> Marloth & Engl.
UNME 552	1	<i>Ziziphus mucronata</i> Willd.
UNME 551	1	<i>Sansevieria aethiopica</i> Thunb.
UNME 555	1	<i>Jatropha erythropoda</i> Pax & K.Hoffm.
UNME 560	1	<i>Aptosimum albomarginatum</i> Marloth & Engl.
UNME 566	1	<i>Sansevieria aethiopica</i> Thunb.
UNME 670	1	<i>Otoptera burchellii</i> DC.
UNME 582	1	<i>Melhania burchellii</i> DC.
UNME 581	1	<i>Harpagophytum procumbens</i> (Burch.) DC. ex Meisn.
UNME 580	3	<i>Harpagophytum</i> sp.
HS 007	1	<i>Aptosimum spinescens</i> (Thunb.) F.E.Weber
HS 002	1	<i>Fockea angustifolia</i> K.Schum.
UNME 656	1	<i>Antizoma angustifolia</i> (Burch.) Miers ex Harv.
UNME 571	1	<i>Antizoma angustifolia</i> (Burch.) Miers ex Harv.
UNME 554	1	<i>Antizoma angolensis</i> Exell & Mendonça
CID 55	1	<i>Cryptolepis decidua</i> (Planch. ex Hook.f. & Benth.) N.E.Br.
HS 005	4	
HS 003	1	<i>Indigofera auricoma</i> E.Mey.
UNME 553	1	<i>Acacia hebeclada</i> DC.
CID 53	1	<i>Pteronia acuminata</i> DC.
CID 56	1	<i>Nymanianthus capensis</i> (Thunb.) Lindb.
UNME 578	1	<i>Solanum lichtensteinii</i> Willd.
UNME 561	1	<i>Senna italica</i> Mill.
UNME 558	1	<i>Senna italica</i> Mill.
UNME 669	1	<i>Aptosimum albomarginatum</i> Marloth & Engl.
UNME 564	1	<i>Melhania virescens</i> (K.Schum.) K.Schum.
CID 59	1	<i>Albizia anthelmintica</i> (A. Rich.) Brongn.
UNME 57615	1	<i>Acacia mellifera</i> (Vahl) Benth.
UNME 5563	1	<i>Withania somnifera</i> (L.) Dunal
HS 006	1	<i>Nymanianthus capensis</i> (Thunb.) Lindb.

Identification categories: 1. Certain identification 2. Closest to 3. Certain to genus only 4. Unable to identify

Private Bag 13184, Windhoek Tel: +264 - 61 - 202 - 2021 Fax: +264 - 61 - 259 - 153 e-mail: essiek@nbri.org.na

Full Length Research Paper

Medicinal value of *Aptosimum albomarginatum* (Marloth and Engl.), *Albizia anthelmintica* (A. Rich Brongn.) and *Dicoma schinzii* (O. Hoffm.) to a small community living at Gochas, southern Namibia

Sunette Walter^{1*}, Mervyn Beukes², Davis Mumbengegwi³ and Ronnie Böck¹

¹Department of Biological Sciences, Faculty of Science, University of Namibia, Windhoek, Namibia.

²Department of Biochemistry, Faculty of Natural and Agricultural Sciences, University of Pretoria, Pretoria, South Africa.

³Multidisciplinary Research Centre, University of Namibia, Windhoek, Namibia.

Received 23 October, 2017; Accepted 17 November, 2017

Traditional medicine is widely used, but its effectiveness is often questioned. Biofilm-producing bacteria and fungi are important in difficult-to-treat persistent and recurrent infections. The present study investigated the anti-biofilm properties of crude methanolic extracts from three medicinal plants used in Namibia, namely *Aptosimum albomarginatum* (Marloth and Engl.), *Albizia anthelmintica* (A. Rich Brongn.) and *Dicoma schinzii* (O. Hoffm.). Biofilm formation, inhibition and eradication were determined using microtiter plate assay. Extracts were tested against *Escherichia coli* ATCC 700928, *Staphylococcus aureus* ATCC 12600, *S. aureus* U3300, *Bacillus subtilis* ATCC 13933, *Streptococcus mutans* ATCC 25175, *Streptococcus sanguinis* ATCC 10556, *Pseudomonas aeruginosa* and *Candida albicans*. All isolates were strong biofilm producers. *A. albomarginatum* root extract moderately inhibited biofilm formation in *S. mutans* ATCC 25175 (60.0%), *E. coli* ATCC 700928 (51.6%) and *P. aeruginosa* (49.1%). *A. anthelmintica* twigs caused 58.4% biofilm inhibition in *C. albicans* and eradicated *S. aureus* U3300 biofilm by 74.8%. *D. schinzii* leaf extract inhibited *P. aeruginosa* biofilm by 67.3%, and in addition broke down *S. mutans* ATCC 25175 biofilm by 44.2%. These results validate the usefulness of the three plants as traditional medicine in some instances.

Key words: *Aptosimum albomarginatum*, *Albizia anthelmintica*, *Dicoma schinzii*, traditional medicine, anti-biofilm activity, flavonoids, saponins.

INTRODUCTION

All cultures across the globe have developed knowledge of local plants, enabling them to use these plants for medicinal purposes (Silvério and Lopes, 2012). Globally,

many people still rely on such traditional medicine to remain healthy (van Wyk and Wink, 2015), because they often do not have access to modern medicine and

*Corresponding author. E-mail: sunette.walter8@gmail.com. Tel: +264) 814151015.

Author(s) agree that this article remain permanently open access under the terms of the [Creative Commons Attribution License 4.0 International License](https://creativecommons.org/licenses/by/4.0/)

antibiotic-based therapies (Silvério and Lopes, 2012).

African traditional medicine, which probably dates back to the origins of humankind, represents the most diverse of all medicinal systems, but is also the least systematized and most poorly documented of these systems. Traditional or folk medicine may be just as effective as conventional drugs, but its effectiveness is often questioned (van Wyk and Wink, 2015). Some strains of bacteria and fungi are able to form biofilms. Such biofilm producers are often responsible for difficult-to-treat persistent and recurrent infections. Hutcherson et al. (2015) define biofilms as dense, surface-attached communities of bacteria or fungi encased within a microbial-derived matrix that helps with colonization and survival. According to Mack et al. (2013) and Nazzari et al. (2014), this formation helps bacteria to withstand the host's natural immune defense mechanisms and to resist antibiotic treatment. In other words, as stated by Speziale and Geoghegan (2015), this is a survival strategy adapted by bacteria.

With drug-resistance being a never-ending problem, one should look into alternative treatment options, such as use of natural products, for example plant-derived products with antimicrobial and/or anti-biofilm activity. Such natural products may in some instances also be used as adjuvants together with antibiotics. Namibian people are using plants as natural medicine in the traditional setting to treat various illnesses, including biofilm-related infections. To rule out the possibility of placebo effects, such plants should be tested in the laboratory to validate their medicinal value.

The present study aimed to test crude methanolic extracts from three plants, namely, *Albizia albomarginatum* (Marloth and Engl.), *Albizia anthelmintica* (A. Rich Brongn.) and *Dicoma schinzii* (O. Hoffm.) for their anti-biofilm properties in seven bacterial strains and a fungus. Extracts were screened for phytochemicals that may contribute to these properties.

MATERIALS AND METHODS

Selection of traditional medicinal plants and collection of plant material

Plant material was collected from the veld at Gochas (Altitude: 1139m; GPS coordinates: 24°47'S, 18°49'E), located in the Karas Region, southern Namibia in the month of February 2015. Plants were selected based on indigenous knowledge of local people about their medicinal value in the traditional setting. Voucher specimens were prepared and submitted to the herbarium at the National Botanical Research Institute (NBRI) in Windhoek for scientific identification of the plants. The selected plants (Figure 1) as identified by the NBRI are the shrub *Aptosimum albomarginatum* (Marloth and Engl.), the tree *A. anthelmintica* (A. Rich Brongn.) and the shrub *D. schinzii* (O. Hoffm.).

Plant material/extracts used

Crude methanolic extracts were prepared at the Biomedical

Research Laboratory, Biological Sciences Department at the University of Namibia. Plant parts used were *A. albomarginatum* roots, *A. anthelmintica* twigs and *D. schinzii* roots and leaves.

Preparation of plant material

A. albomarginatum roots, *A. anthelmintica* twigs, and *D. schinzii* roots and leaves were prepared for extractions in the laboratory. Plant material was washed with tap water, placed on towel paper on the benches and left to air-dry at room temperature for two weeks. Thereafter, the material was cut and crushed to finer pieces and blended to powder form using a Philips Problend 5 household blender. Blended material was sieved, weighed, put into labeled 50 ml Falcon centrifuge tubes and stored in the freezer at -20°C.

Preparation of crude extracts

To prepare crude methanolic extracts, 10 g of plant material from the different plant parts was added to 100 ml methanol. Flasks containing the extracts were parafilm, placed in a cupboard and left to stand for three days (maceration) with occasional swirling. After three days, the extracts were gravitationally filtered through Whatman 110 mm filter papers. The extracts were rotary evaporated in round bottom flasks at reduced pressure (91mbar) and temperature (45°C) to evaporate the methanol, and to dry and concentrate them. To avoid thermal decomposition of compounds in the plant material, the temperature set for the rotary evaporator (Heidolph, Germany) did not exceed 45°C. The flasks were labeled, sealed with parafilm and kept at -86°C for a few hours. Thereafter, the frozen extracts were connected to a Christ Alpha 1-2 LD Plus freeze-dryer (Germany) for two to four days to further dry and concentrate them. Dried extracts were scraped off with a spatula, weighed and stored in labeled 50 ml centrifuge tubes, and kept at -86°C for further use. Yields of extracts were calculated using the formula used by Osungunna and Adedeji (2011):

Percentage yield = Quantity of dried extract (g) / Quantity of powdered sample (g) × 100.

Phytochemical screening for flavonoids, saponins and anthraquinones

Antimicrobial and anti-biofilm activity of plant extracts may be attributed to the presence of secondary metabolites such as flavonoids, saponins and anthraquinones. These compounds were screened for using the methods for qualitative chemical assays described by Farnsworth (1986), with minor modifications.

For screening of flavonoids, 0.5 g of powdered plant material was added to a conical flask and extracted with 15 ml water and methanol (Merck, Germany) mixture in the ratio 2:1. The mixture was left to stand at room temperature for 30 min after which it was filtered using Whatman 110 mm filter paper. Thereafter, some magnesium turnings were added to the filtrate, and concentrated hydrochloric acid (HCl) (Merck, Germany) was added dropwise. Appearance of a yellow color indicated the presence of flavonoids.

To screen for saponins, 0.5 g powdered plant material was mixed with 15 ml distilled water. The mixture was then heated in a water bath at 100°C for 30 min, and the filtrate was left to cool down to room temperature. It was then vigorously shaken in a test tube for 10 seconds and observed for formation of froth. Froth measured 2cm or higher that persisted for 10 minutes or more confirmed the presence of saponins.

To screen for anthraquinones, 0.5 g plant material was extracted with 10 ml ether-chloroform (Merck, Germany) in the ratio 1:1 for 15 min at room temperature. The mixture was filtered and 1 ml of the

Table 1. Screening for flavonoids, saponins and anthraquinones.

Variable	<i>A. albomarginatum</i> roots	<i>A. anthelmintica</i> twigs	<i>D. schinzii</i> roots	<i>D. schinzii</i> leaves
Flavonoids	+	-	-	-
Saponins	+++	++	-	+
Anthraquinones	-	-	-	-

Key: +++ High presence; ++ Moderate presence; + Low presence; - Absent.

filtrate was treated with 1 ml of 10% (w/v) sodium hydroxide (NaOH) (Sigma, USA) solution. Development of a red color indicated the presence of anthraquinones.

Biofilm assays

Microorganisms used

The following biofilm-producing strains were obtained from the University of Pretoria's Biochemistry Department:

Escherichia coli ATCC 700928, *Staphylococcus aureus* ATCC 12600, *S. aureus* U3300, *Bacillus subtilis* ATCC 13933, *Streptococcus mutans* ATCC 25175, *Streptococcus sanguinis* ATCC 10558, *Pseudomonas aeruginosa* and *Candida albicans*. Strains were inoculated into Falcon centrifuge tubes containing 5 ml brain heart infusion broth (Merck, Germany) and grown to stationary phase at 37°C for 24 h.

Biofilm formation and inhibition

The methods of Christensen et al. (1985), Merritt et al. (2011), and Monte et al. (2014) were used for biofilm assays, with some modifications. To obtain a 0.5 McFarland turbidity standard of 1.5×10^8 CFU/ml, stationary-phase cultures were diluted 1:100 and 100 µl of diluted culture was added to each of six 400 µl wells of a sterile flat-bottomed 96-well microtiter plate (Lasec, SA). Three of these six wells were each inoculated with 10 µl of crude methanolic extract dissolved in 100% Dimethyl sulfoxide (DMSO) just below minimal inhibitory concentration (MIC). MICs were determined by standard microbiological procedure, prior to this assay. Eight wells each contained 100 µl sterile brain heart infusion broth only as control. The plates were parafilmated at the lids to prevent them from drying out and incubated at 37°C for 24 h.

After incubation, planktonic cells were removed by placing the microtiter plate upside down on towel paper, and allowing for the paper to soak up any liquid. To remove remaining planktonic cells, each well was washed 3x by pipetting 400 µl triple distilled water into it, and inverting the plate onto towel paper. The biofilms in the wells were fixed by oven-drying the microtiter plates for 45 minutes at 60°C.

Wells were stained with 125 µl of 0.1% crystal violet, incubated for 15 min at room temperature and the crystal violet discarded. Excess stain was removed by washing (pipetting) 3x with 400 µl triple distilled water. Plates were air-dried for a few hours or overnight. Wells were de-stained with 200 µl of 33% glacial acetic acid (Merck, Germany) for 10 to 15 min. The contents of each well were briefly mixed by pipetting, and 125 µl was transferred to corresponding wells of a new clean microtiter plate. The optical densities (ODs) of stained biofilms were determined with a Multiskan Ascent plate reader (Thermo Labsystems, USA) at 595 nm. Readings from the broth control wells were averaged and subtracted from the test readings. Test readings were averaged and standard deviations calculated. Results for biofilm formation

were interpreted using the classification by Christensen et al. (1985).

The equation $1\% = (1 - (A_{595} \text{ of test} / A_{595} \text{ of non-treated control}) \times 100)$ was used to calculate percentage inhibition (Kawsud et al. 2014). According to Manner et al. (2013), selection criteria (activity-based) for antimicrobials are as follows:

Highly active: $\geq 85\%$ inhibition; moderately active: $\geq 40\%$ inhibition; inactive: $< 40\%$ inhibition.

Biofilm formation and eradication

Stationary-phase cultures were diluted 1:100, and 100 µl of diluted culture was added to each of six 400 µl wells of a sterile flat-bottomed 96-well microtiter plate (Lasec, SA). Eight wells each contained 100 µl sterile brain heart infusion broth only as control. The plates were parafilmated at the lids to prevent them from drying out, and incubated at 37°C for 24 h. After incubation, planktonic cells were removed by decanting onto towel paper. To remove the remaining planktonic cells, each well was washed 3x by pipetting 400 µl triple distilled water into it and inverting the plate onto towel paper. Three wells with the grown biofilms were inoculated with 10 µl of extract (just below MIC) and 190 µl sterile triple distilled water. The remaining wells were filled with 200 µl water and plates were incubated at room temperature for another 24 h. After the second incubation and removal of liquid in the wells, the same steps for fixing, staining and de-staining were followed according to inhibition assay. The equation $E\% = (1 - (A_{595} \text{ of test} / A_{595} \text{ of non-treated control}) \times 100)$ (Kawsud et al., 2014; Teanpaisan et al., 2014) was used to calculate percentage eradication. The classification of activity for inhibition by Manner et al. (2013) was also used to interpret eradication results.

RESULTS AND DISCUSSION

Average percentage yields for *A. albomarginatum* root extract, *A. anthelmintica* twig extract, *D. schinzii* root extract and *D. schinzii* leaf extract were 12.5, 3.5, 6.1 and 7.3%, respectively. The plant material (roots, leaves and twigs) were used for qualitative detection of flavonoids, saponins and anthraquinones. According to Kamonwannasit et al. (2013) and Lee et al. (2016), these secondary metabolites can aid in a medicinal plant's antimicrobial properties, including anti-biofilm activity. The presence or absence of the compounds is indicated in Table 1. Flavonoids were detected only in *A. albomarginatum* roots. Saponins were present in *A. albomarginatum* roots, *A. anthelmintica* twigs and *D. schinzii* leaves. Anthraquinones were not detected.

Biofilm formation over 24 h is shown in Figure 2. All isolates were classified as strong biofilm formers, with *B. subtilis* ATCC 13933 being the strongest one. Single-

factor analysis of variance (ANOVA) in Microsoft Excel revealed that there was a significant difference in biofilm formation between the eight strains ($P = 0.0002$; $F = 4.86$; $F_{crit} = 2.18$). A Tukey-Kramer Multiple Comparisons Procedure indicated that this difference was between *B. subtilis* ATCC 13933 and *S. sanguinis* ATCC 10556, *B. subtilis* ATCC 13933 and *E. coli* ATCC 700928, and *S. mutans* ATCC 25175 and *E. coli* ATCC 700928. The medicinal uses of the plants in this study, as well as anti-biofilm properties of their methanolic crude extract against seven bacterial strains and a fungus is discussed hereafter.

A. albomarginatum (Marloth and Engl.) as seen in Figure 1A is commonly known as "Guxa" in Namibia (Coetzee, personal communication, February, 2015; A. Frederick, personal communication, February, 2015), also spelled "Kuxa" (Sullivan, 1998) or "Khuxa" (Huggins et al., 2013). The roots are pulverized, boiled as a tea and drunk to purify the blood and cleanse the uterus. Some believe that it can cure women who experience difficulty in conceiving. It also helps to relieve the symptoms of colds (Coetzee, personal communication, February, 2015; A. Frederick, personal communication, February, 2015).

Bacteria such as staphylococci may be associated with infection of the uterus, for example in the condition known as endometritis (inflammation of the endometrium). One form of this condition is known as bacteriotoxic endometritis, where it is caused by the toxins of bacteria rather than the presence of the pathogens themselves (Allen, 2004). Phytochemical screening revealed the presence of saponins in the roots (Table 1), and according to Wink and van Wyk (2008) saponins have anti-inflammatory effects. It is thus possible that the plant may be effective to treat conditions of the uterus, and saponins can play a role in its effectiveness.

According to Sullivan (1998), du Pisani, (1983) and Steyn (1981), Nama people shred the plant's roots and use it as a spice or coffee substitute. A decoction from the crushed root is drunk by the Nama to treat chest complaints, stomach disorders and coughs. Huggins et al. (2013) say that tea made from the plant is used to treat headaches, to induce vomiting and as a general body cleanser. Flavonoids were detected in the roots (Table 1). According to Wink and van Wyk (2008), flavonoids act as antioxidants and free-radical scavengers. Flavonoids may therefore be partly responsible for the plant's cleansing properties.

At a sub-MIC concentration of 0.625mg/ml, *A. albomarginatum* root extract caused 60.0% inhibition of biofilm formation in *S. mutans* ATCC 25175, and at 1.25 mg/ml it inhibited *E. coli* ATCC 700928 and *P. aeruginosa* biofilms by 51.6 and 49.1%, respectively. This is moderate inhibition, according to Manner et al. (2013). With inhibition below 40.0%, it was inactive against the fungus *C. albicans*, as well as the bacteria *S. aureus* U3300, *S. aureus* ATCC 12600, *S. sanguinis* ATCC

10556, and *B. subtilis* ATCC 13933, and could not eradicate pre-formed biofilms.

From these inhibition results, it is evident that *A. albomarginatum* roots may be used as traditional medicine to treat biofilm-related infections involving *S. mutans* ATCC 25175, *E. coli* ATCC 700928, and *P. aeruginosa*. The roots are not expected to be effective against biofilm infections caused by *C. albicans*, *S. aureus* U3300, *S. aureus* ATCC 12600, *S. sanguinis* ATCC 10556, and *B. subtilis* ATCC 13933.

A. anthelmintica (A. Rich Brongn.) seen in Figure 1B have many common names including "kersieblombloom", "worm-cure albizia" (Orwa et al., 2009; Hoffmann, 2014), "aruboom" and "oumahout" (Hoffmann, 2014). In traditional practice, the outer parts of the twigs are scraped off and the inner part is used as a chewing stick or toothbrush to clean the teeth and tongue (Coetzee, personal communication, February, 2015; A. Frederick, personal communication, February, 2015). Both bacteria and fungi may be associated with mouth infections, where they can be part of biofilm communities as dental plaque.

The bark, wood or root from *A. anthelmintica* is boiled and milk is added to treat an upset stomach or intestinal worms. Tea made from the roots and bark is drunk to treat malaria. The Samburu pastoralists in Kenya treat gonorrhoea by boiling the roots, bark and leaves, mixing it with sheep fat and giving it as an enema. Otherwise the boiled bark and roots are consumed with milk (Sullivan, 1998; du Pisani, 1983; Fratkin, 1996). The stem bark is widely used as a purgative (Orwa et al., 2009). Consumption of this plant can also be hazardous. The seeds and bark contains alkaloids which have a toxic effect. Animal poisoning can occur in cattle and sheep; livestock can die from heart failure. Overdoses of the plant can also cause death in humans (Wink and van Wyk, 2008).

Twig extract (1.25 mg/ml) from *A. anthelmintica* showed inhibition of biofilm formation only in the fungus *C. albicans*, with moderate activity (58.4%). The twigs can thus be useful as a toothbrush to remove this fungus from the teeth and tongue. The extract eradicated biofilm also only in one strain – *S. aureus* U3300. The extract's activity may partly be due to saponins.

D. schinzii (O. Hoffm.) as seen in Figure 1C is also known as "Gu-laru" (Coetzee, personal communication, February, 2015) or the "Kalahari fever bush" (Dugmore and van Wyk, 2008). The roots and leaves are pulverized, boiled as tea and drunk or used to steam yourself in the treatment of measles, chickenpox, the flu, colds and a blocked nose (Coetzee, personal communication, February, 2015). Unspecified parts are used to treat febrile convulsions in babies in the Kalahari, hence the name "Kalahari fever bush" (Sobiecki, 2002; Dugmore and van Wyk, 2008).

Measles, chickenpox, flu and colds are caused by viruses, but bacteria can be involved in congested nose



Figure 1. *A. albomarginatum* (A), Image credit: southafricanplants.net; *A. anthelmintica* (B), and *D. schinzii* (C) growing in the veld at Gochas, Image credit: Sunette Walter

or sinus infections. There is an interesting folk tale behind the plant's traditional use in the Kalahari to treat febrile convulsions in babies. This tale known as "Dicoma's shadow" can be read in the book "Muthi and myths from the African bush" written by Dugmore and van Wyk (2008). van Wyk (2015) explains the story in short. It is said that if the shadow of the black shouldered kite (*Elanus caeruleus*) falls on a baby, the child will get sick, and this illness will be recognized by the spastic movements of the baby's arms, similar to the movements made by the bird's feathers when it is hanging over its

prey. It is furthermore said that if the condition is not treated, the infant can develop feathers on its arms. An extract of the plant can be given both topically and internally, which will counteract the symptoms and cure the child. In the traditional African context, the symbol of the bird represents fever, since birds have a higher natural body temperature (40°C) compared to that of humans (37°C). "The condition of the bird" refers to fever. Referral to feathers on the arms is actually "the gooseflesh of fever" – one of the symptoms of febrile convulsions in infants.

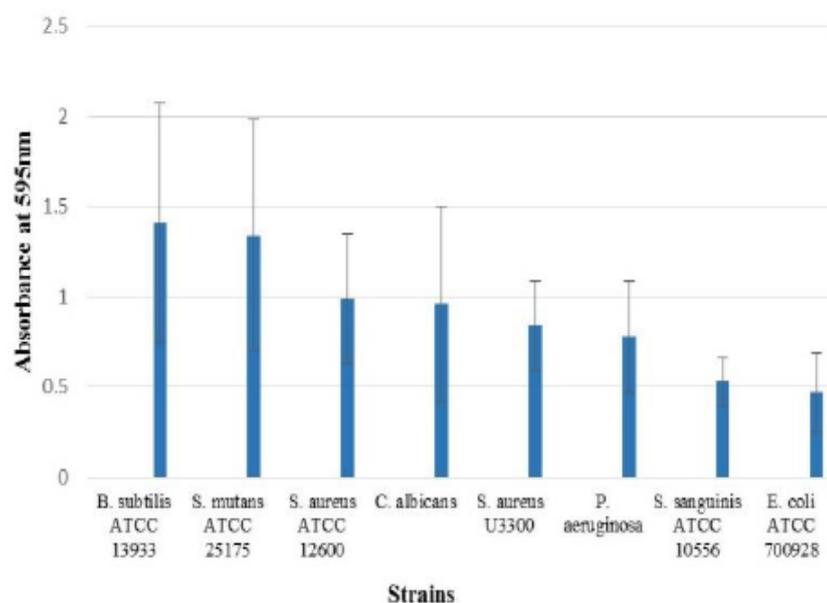


Figure 2. 24 h biofilm formation according to mean absorbance values at 595nm over eight microtiter plate assays. Formation was classified as strong/high with mean OD values > 0.240 (Christensen *et al.*, 1985). Error bars indicate standard deviations. There was a statistically significant difference in biofilm formation between *B. subtilis* ATCC 13933 and *S. sanguinis* ATCC 10556, *B. subtilis* ATCC 13933 and *E. coli* ATCC 700928, and *S. mutans* ATCC 25175 and *E. coli* ATCC 700928.

D. schinzii root extract was tested at a concentration of 1.25 mg/ml, and was unable to inhibit or eradicate biofilms. The plant's leaf extract (1.25mg/ml) however moderately inhibited biofilm formation in *P. aeruginosa* (67.3%) and is expected to help fight biofilm infections involving this bacterium. The extract eradicated the biofilm of *S. mutans* ATCC 25175 by 44.2%. Saponins in the leaves may be involved in biofilm eradication. *S. mutans* is normally not involved in the diseases mentioned by the locals at Gochas or in literature. The leaf extract may possibly be effective to treat other illnesses that are related to *S. mutans*, such as dental caries and endocarditis.

Conclusions

All strains in this study formed strong biofilms, with that of *B. subtilis* ATCC 13933 being the strongest. *A. albomarginatum* root extract moderately prevented biofilm formation in *S. mutans* ATCC 25175, *E. coli* ATCC 700928 and *P. aeruginosa*, and may therefore be effective as traditional medicine to treat biofilm infections involving these bacteria. *A. anthelmintica* twig extract inhibited *C. albicans* biofilm, and can thus be useful as a toothbrush or chewing stick to remove this fungus from the mouth. The twig extract may also be effective against

biofilm infections involving the strain *S. aureus* U3300, as it was able to remove some of the bacterium's pre-formed biofilm. *D. schinzii* leaf extract moderately inhibited *P. aeruginosa* biofilm and moderately eradicated *S. mutans* ATCC 25175 biofilm. The leaves from this plant may thus be used in the traditional setting to treat biofilm infections related to these two strains. Anti-biofilm properties of the extracts under study may partly be attributed to the presence of flavonoids and saponins. The present work supports the use of the three medicinal plants in some instances.

CONFLICTS OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors acknowledge and thank the University of Pretoria's Biochemistry Department, and the University of Namibia's Biomedical Research Laboratory, Biological Sciences Department where the work was carried out. We also acknowledge and thank the Southern African Biochemistry and Informatics for Natural Products (SABINA) and the Regional Initiative in Science and

Education (RISE) for financial support. Lastly, we thank the people at Gochas for sharing their knowledge on traditional medicinal plants with us. This work forms part of a Ph.D. study undertaken at the University of Namibia.

REFERENCES

- Allen T (2004). *Dorland's Illustrated Medical Dictionary*: WB Saunders, Philadelphia, 2003. ISBN 0-721-60146-4.
- Christensen GD, Simpson WA, Younger JJ, Baddour LM, Barrett FF, Melton DM, Beachey EH (1985). Adherence of coagulase-negative staphylococci to plastic tissue culture plates: A quantitative model for the adherence of staphylococci to medical devices. *J. Clin. Microbiol.* 22(6):996-1006.
- Coetzee S (2015). Personal communication, Gochas, Namibia.
- Du Pisani E (1983). Past and present plant utilization in Namaland and the lower Kuiseb River valley, South West Africa (Namibia): A preliminary report. *Khoisis* 4:1-19.
- Dugmore H, van Wyk BE (2008). *Muthi and myths from the African bush*. Pretoria: Briza Publications.
- Farnsworth NR (1986). Biological and phytochemical screening of plants. *J. Pharm. Sci.* 55(3):225-276.
- Fratkin E (1996). Traditional medicine and concepts of healing among Samburu pastoralists of Kenya. *J. Ethnobiol.* 16(1):63-97.
- Frederick A (2015). Personal communication, Gochas, Namibia.
- Hoffmann L (2014). Meet the trees of Namibia: The worm-cure albizia (*Albizia anthelmintica*). The Namibian. Available at: <http://www.namibian.com.na>
- Huggins G, Roberts C, Turpie J (2013). Consequences of scenarios on ecosystem services: Research project on environmental flow requirements of the Fish River and the Orange-Senqu river mouth (Technical report No. 26). Pretoria, South Africa. Available at: <http://www.the-eis.com/data/literature/OrangeSenqu%20Estuary%20Concept%20Pa-per-2.pdf>
- Hutcherson JA, Scott DA, Bagaikar J (2015). Scratching the surface – tobacco-induced bacterial biofilms. *Tob Induc. Dis.* 13(1):1.
- Kamonwannasit S, Nantapong N, Kumkrai P, Luecha P, Kupittayanant S, Chudapongse N (2013). Antibacterial activity of *Aquilaria crassna* leaf extract against *Staphylococcus epidermidis* by disruption of cell wall. *Ann. Clin. Microbiol. Antimicrob.* 12:20.
- Kawsud P, Puripattanaovong J, Teanpaisan R (2014). Screening for anticandidal and antibiofilm activity of some herbs in Thailand. *Trop. J. Pharm. Res.* 13(9):1495-1501.
- Lee JH, Kim YG, Ryu SY, Lee J (2016). Calcium-chelating alizarin and other anthraquinones inhibit biofilm formation and the hemolytic activity of *Staphylococcus aureus*. *Sci. Rep.* 6:19267.
- Mack D, Davies AP, Harris LG, Jeeves R, Pascoe B, Knobloch JK-M, Rohde H, Wilkinson TS (2013). *Staphylococcus epidermidis* in biomaterial-associated infections. In Moriarty F, Zaat SAJ, Busscher HJ (Eds.), *Biomaterials associated infection*. Springer New York, USA, pp. 25-56.
- Manner S, Skogman M, Goeres D, Vuorela P, Fallarero A (2013). Systemic exploration of natural and synthetic flavonoids for the inhibition of *Staphylococcus aureus* biofilms. *Int. J. Mol. Sci.* 14:19434-19451.
- Merritt JH, Kadouri DE, O'Toole GA (2011). Growing and Analyzing Static Biofilms. *Curr. Protocols Microbiol.* 22:B:1B.1:1B.1.1–1B.1.18.
- Monte J, Abreu AC, Borges A, Simões LL, Simões M (2014). Antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* and their biofilms. *Pathogens* 3:473-498.
- Nazzari E, Torretta S, Pignataro L, Marchisio P, Esposito S (2014). Role of biofilm in children with recurrent upper respiratory tract infections. *Eur. J. Clin. Microbiol. Infect. Dis.* 34(3):421-429.
- Orwa C, Jamnadass RH, Kindt R, Mutua A, Simons A (2009). *Agroforestry database: A tree reference and selection guide version 4.0*. Available at: <http://www.worldagroforestry.org/publication/agroforestry-database-tree-species-reference-and-selection-guide-version-40>
- Osungunna MO, Adediji KA (2011). Phytochemical and antimicrobial screening of methanol extract of *Heliotropium indicum* leaf. *J. Microbiol. Antimicrob.* 3(8):213-216.
- Silvério A, Lopes M (2012). Antimicrobial activity of condiments. In Bobbarala, V (Ed.), *Antimicrobial agents*. Available at: <https://cdn.intechopen.com/pdfs-wm/38847.pdf>
- Speziale P, Geoghegan JA (2015). Biofilm formation by staphylococci and streptococci: Structural, functional, and regulatory aspects and implications for pathogenesis. *Front. Cell. Infect. Microbiol.* 5:31.
- Steyn HP (1981). *Nharo plant utilization: An overview*. University of Stellenbosch.
- Sullivan S (1998). *People, plants and practice in drylands: Socio-political and ecological dimensions of resource-use by Damara farmers in north-west Namibia* (Unpublished doctoral thesis). University College, London. Available at: <http://discovery.ucl.ac.uk/1317514/>
- Teanpaisan R, Senapong S, Puripattanaovong J (2014). In vitro antimicrobial and antibiofilm activity of *Artocarpus lakoocha* (Moraceae) extract against some oral pathogens. *Trop. J. Pharm. Res.* 13(7):1149-1155.
- Van Wyk B, Wink M (2015). *Phytomedicines, herbal drugs and plant poisons*. Pretoria, South Africa:Briza.
- Van Wyk B-E (2015). Die kulturele en praktiese waarde van inheemse kennis oor plantgebruike in die 21ste eeu. *Suid-Afr. Tydskrif Natuurwetenskap Tegnol.* 34(1):1.
- Wink M, van Wyk B-E (2008). *Mind-altering plants of the world*. Pretoria, South Africa:Briza.