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The antimicrobial investigation of 40 Southern African medicinal plants against some selected gut bacterial pathogens

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2019

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The antimicrobial investigation of neglected pathogens of the gut against Southern African medicinal plants used for stomach ailments



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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine

May 2019

DECLARATION

I, Hlambani Shirinda, declare that this dissertation is my own work. It is being submitted in fulfilment for the degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. This work has not been submitted before for any other degree or examination at this or any other University.

H. Shirinda Date

ACKNOWLEDGEMENTS

- Firstly, I would like to thank the Almighty God, for without his wisdom I wouldn't have made it.
- I would like to thank my supervisor Professor Sandy van Vuuren: My Masters wouldn't have been this amazing without your great supervision skills and encouragement, thanks for believing and looking at me as a potential candidate. I benefited from your advice, particularly so when exploring new ideas. Your positive attitude and confidence in my project inspired me and gave me strength. I consider myself lucky for having such an amazing supervisor like you.
- To my co–supervisor Professor Geoffrey Candy for all the effort, input and partial financial support. No words can explain how grateful I am for your contribution towards this study.
- To our laboratory manager and technician Mrs Phumzile Maorane; for making sure that our working environment (laboratories) was always clean and work friendly. You are an easy person to work with. You provided a most efficient and friendly environment. I found it very easy to work with you.
- My sincere gratitude to Mr Andrew Hankey the specialist horticulturalist for plant identification and authentication.
- The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed, and the conclusions arrived at are those of the author and are not necessarily to be attributed to the NRF.
- To Postgraduate Merit ward (PMA) office for bursary provision.
- To the Faculty Research Committee (FRC) for financial assistance.
- A very big thank you to my fellow students and colleagues Miss Khadijah Kharsany and Miss Evah Mahlatse Lediga for support throughout this project.
- A special thanks to my family for emotional support, prayers, and financial support.

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ABSTRACT

Intra–abdominal infections (IAI) are the infections of the abdomen and the major cause of morbidity and mortality especially in developing countries. In these countries especially in rural areas, most people rely on traditional medicine for their primary health care due to factors such as culture, availability and affordability. The popular use of Southern African medicinal plants has already been reviewed, however, the scientific evidence against neglected pathogens of the gut including *Helicobacter pylori*, *Fusobacterium varium* as well as *Bacteroides*, and *Clostridium* species has not been adequately studied. The aim of this study was thus to evaluate the antimicrobial activity of extracts from selected medicinal plants traditionally used to treat stomach ailments, against neglected gut pathogens responsible for IAI, to investigate activity against biofilms (using *Clostridium perfringens* as a model) and to determine toxicity of antimicrobially active plant extracts.

To test the antimicrobial effects of plant extracts against neglected gut pathogens, a total of 102 aqueous and organic extracts were prepared from 40 different plant species. These plant samples were screened for antimicrobial efficacy against anaerobes (*Bacteroides fragilis* (ATCC 25285), *Bacteroides ovatus* (ATCC 8483) *Bacteroides thetaiotaomicron* (ATCC 29741), *Bactericides vulgatus* (29327), *C. perfringens* (ATCC 13124), *C. difficile* (ATCC 43593), *Fusobacterium nucleatum* (ATCC 25586) *F. varium* (ATCC 27725), and microaerophiles (*H. pylori* clinical and *H. pylori* (B8) (reference strain) of the gut using the minimum inhibitory concentration (MIC) assay. The results from MIC assay revealed that the organic extracts were more active when compared to the aqueous extracts. The organic extract of *Lippia javanica* leaf showed the best antimicrobial activity overall with an MIC value of 0.5 µg/ml against *C. perfringens*, this value was comparable to that of positive control ciprofloxacin (0.2 µg/ml). Aqueous extracts of *Gunnera perpensa* leaf and rhizome were the most active with MIC values of 130 µg/ml against the *Clostridium* species. Leaf extracts from seven out of ten plants showed better activity when compared to the other plant parts.

Plant extracts that displayed noteworthy antimicrobial activity against *C. perfringens* were further evaluated for their antibiofilm activity using the crystal violet (CV) staining assay.

Approximately 90% of the extracts were active on one or more stages of biofilm development. Plant extracts disrupted biofilm development stage (24 h) where 57% of the extracts reduced the development of biofilms by at least 50%. The organic extract of *Salvia africana–caerulea* displayed the best antibiofilm activity overall, where it prevented the cell attachment (4 h) with inhibition percentage of 83%.

The toxicity profiles of plants that displayed noteworthy antimicrobial activity were further evaluated using the brine shrimp lethality (BSLA) assay to confirm some degree of safety. The BSLA results revealed that 82% of the tested plant extracts were non–toxic. Only 18% of the organic extracts exhibited toxic effects to the brine shrimp with >50% mortality. All the aqueous extracts tested possessed no toxic effects.

The results obtained from this study favours the traditional use of some of the tested plants for the treatment of IAI. This study demonstrates that studies should not only focus on common pathogens, but also on neglected pathogens which may yield excellent results not previously reported. The study contributes to the knowledge of antimicrobial properties of plants commonly found in Southern Africa.

DEDICATION

I dedicate this dissertation to my family

Particularly, my late father Mkhatshane Daniel Shirinda;

my mother Tintswalo Dorris Chauke;

my siblings, Ndongwe, Tivani, Dennis, Tshamiseka, Yingisa, Basisa, Somani, Shongani, and Xoliswa

I wouldn't have made it without your prayers, support, encouragement,

and unconditional love.

LIST OF ABBREVIATIONS

Aquo	Aqueous
ATCC	American type culture collection
BHI	Brain heart infusion
CFU	Colony forming units
CLSI	Clinical and laboratory standard institute
CV	Crystal violet
°C	Degrees Celsius
DMSO	Dimethyl sulfoxide
g	Grams
GAC	Gastric adenocarcinoma
GI	Gastrointestinal
h	Hours
IAI	Intra–abdominal infections
INT	*p*–Iodonitrotetrazolium violet
LC_{50}	Concentration of test substance causing 50% brine–shrimp death
MALT	Mucosa–associated lymphoid tissue lymphomas
mg	Microgram
µg/ml	Micrograms per millilitre
µl	Microliters
MIC	Minimum inhibitory concentration
ml	Millilitres
NHLS	National Health Laboratory Service
Org	Organic
%	Percent
PCR	Polymerase chain reaction
PPI	Proton pump inhibitor
RHIN	Random Harvest Indigenous Nursery
spp.	Species

TSA	Tryptone Soya agar
TSB	Tryptone Soya broth
WHO	World Health Organisation
WSBG	Walter Sisulu Botanical Gardens

CHAPTER 1

General introduction

1.1. Intestinal microbiota as a normal factor of life

The human gastrointestinal tract comprises of a complex ecosystem containing approximately 1000 different microbial species, among which anaerobes outnumber the aerobic bacteria by a factor 1000:1 (Simon and Gorbach, 1984; Borriello, 1986; Quigley, 2013; Sobhani *et al*., 2013). The stomach of a new born is firstly inhabited by maternal and environmental bacteria during birth and continues to be colonized through feeding and is affected by factors such as gestational age, mode of delivery, diet, level of sanitation, and exposure to antibiotics (Qin *et al*., 2010; Fouhy *et al*., 2012). The intestinal microbiota of a new born is colonised by bacteria such as Proteobacteria (*Helicobacter*, *Vibrio*, *Yersinia*, *Salmonella*, *Escherichia* species) and Actinobacteria. As the child develops, the above–mentioned phyla are later joined by Firmicutes (*Bacilli* and *Clostridium* species) and *Bacteroides* (*Bacteroides fragilis*, *Bacteroides ovatus*, *Bacteroides vulgatus*, *Bacteroides thetaiotaomicron*) species, which characterizes the adult microbiota (Wolf *et al*., 2004; Eckburg *et al*., 2005; Martinko and Madigan, 2005; Qin *et al*., 2010).

1.2. Pathogenesis

Although microbiota normally prevent infection by interfering with pathogens, they may cause infections when the mucosal barrier is injured. When this happens, bacteria enters the sterile peritoneal cavity and the surrounding organs through the intestinal wall (Gorbach, 1996). This leads to poor blood circulation, reduced oxygen supply, and dead tissues. Consequently, it promotes abscess formation and the growth of pathogenic anaerobic bacteria. This may lead to intra–abdominal infections (IAI) (Gorbach, 1996).

1.3. Intra–abdominal infections

Intra–abdominal infections are the diseases of the stomach and they are regarded as the common causes of morbidity and mortality worldwide (Marshall, 2004). Intra–abdominal infections include peritonitis, intra–abdominal abscesses, appendicitis, colorectal cancer, ulcerative colitis, food poisoning, chronic atrophic gastritis, peptic ulceration and stomach cancer (Ohkusa *et al*., 2003; Farnbacher *et al*., 2010; Castellarin *et al*., 2012; Bosques–Padilla *et al*., 2018). Sartelli *et al*. (2017) reported IAI as the most common gastrointestinal emergency and as major contributors to non–trauma related deaths in the medical emergency departments of hospitals worldwide.

1.4. Organisms responsible for intra–abdominal infections.

The most commonly isolated aerobic bacteria in the IAI are *E. coli*, *E. faecalis* and the Streptococci (Mazuski and Solomkin, 2009; Nagy, 2010). The anaerobe mostly isolated from IAIs is *B. fragilis*, and other members of *Bacteroides* genus including *B. thetaiotaomicron*, *B. distasonis*, *B. vulgatus*, *B. ovatus*, and *B. uniformis*. Other anaerobic pathogens that play a role in IAI include, *Clostridium*, *Eubacteria*, *Fusobacterium*, *Peptococcus*, and *Peptostreptococcus* (Mazuski and Solomkin, 2009, Nagy, 2010). *H. pylori* is a microaerophile and is one of the common gut pathogens that receives more attention due to its relevance and severity in causing gut infections and gastric cancer (Zaidi *et al*., 2009; Farnbacher *et al*., 2010; Bosques–Padilla *et al*., 2018). Most IAI are polymicrobial, however, clinical laboratories barely identified the micro–organisms implicated, specifically the anaerobic bacteria (Mazuski and Solomkin, 2009). This may be due to their fastidious nature. Table 1.1 details several neglected gut pathogens of importance and the infections they cause.

Table 1.1: Neglected pathogens associated with gut infections.

Anaerobic bacteria	Associated gut infection	References
Clostridium difficile	Pseudomembranous colitis (PMC) *Clostridium difficile*–associated diarrhoea	Thomas *et al*., 2003
Clostridium perfringens	Food poisoning, antibiotic–associated diarrhoea	Sparks *et al*., 2001 Varga *et al*., 2008
Bacteroides group: *B. fragilis* *B. ovatus* *B. thetaiotaomicron* *B. vulgatus*	Intra–abdominal infections	Ho *et al*., 2017
Fusobacterium nucleatum	Colorectal cancer	Castellarin *et al*., 2012
Fusobacterium varium	Ulcerative colitis	Ohkusa *et al*., 2003
H. pylori	Chronic atrophic gastritis, peptic ulceration, intestinal metaplasia, dysplasia, gastric adenocarcinoma (GAC), mucosa-associated lymphoid tissue lymphomas (MALT)	Farnbacher *et al*., 2010 Bosques-Padilla *et al*., 2018

1.5.1. *Clostridium* species as etiological agents for IAI

1.5.1.1. *C. difficile* infections

C. difficile is a Gram–positive anaerobe which was first isolated in 1935 (Theriot *et al*., 2014). *C. difficile* can live harmlessly in the colon, however, when an individual takes an antibiotic, the *C. difficile* can overgrow (Nelson *et al*., 2017). *C. difficile* infection may be caused by the intake of broad–spectrum antibiotics such as clindamycin, penicillin and cephalosporins (Kelly *et al*., 1994). The broad–spectrum antibiotics succeed by tempering with the structure and normal function of the gastrointestinal tract (Theriot *et al*., 2014). Once the antibiotics temper with gut microbiota, it results in the over growth of *C. difficile* (Theriot *et al*., 2014). As the

pathogenic strain of *C. difficile* is established in the colon, it produces toxins (TcdA and TcdB) which result in epithelial damage (Kelly *et al*., 1994; Dupuy *et al*., 1998). This leads to infections including pseudomembranous colitis (PMC) and *C. difficile*–associated diarrhoea (Thomas *et al*., 2003). *C. difficile*–infections are termed hospital acquired infections because they are mostly isolated in hospital equipment including the cutlery, cleaning equipment, wash rooms, and furniture, especially in wards where patients with *C. difficile* infection have recently been treated (Kelly *et al*., 1994). Patients who are admitted in the hospital are primary targets of *C. difficile* due environmental contamination with *C. difficile* spores is common (Fekety *et al*., 1981; McFarland, *et al*., 1989; Johnson *et al*., 1990).

1.5.1.2. *C. perfringens* infections.

C. perfringens is a Gram–positive opportunistic bacterium that cause diseases in both humans as well as animals (Markey *et al*., 2013; Charlebois *et al*., 2014). *C. perfringens* is widely distributed pathogenically in nature. It is fast growing and produces a varied diversity of toxins (Lindström *et al*., 2011). In humans, *C. perfringens* is responsible for gut infections such as Clostridial necrotizing enteritis, food poisoning, antibiotic–associated diarrhoea, sporadic diarrhoea, and sudden infant death syndrome (Knox and MacDonald, 1943; Brett *et al*., 1992; Smedley *et al*., 2004; Myers *et al*., 2006, Banaszkiewicz *et al*., 2013; Varga *et al*., 2008; Markey *et al*., 2013). Figure 1.1 is *C. perfringens* colonies on Tryptone Soya agar supplemented with sheep blood.

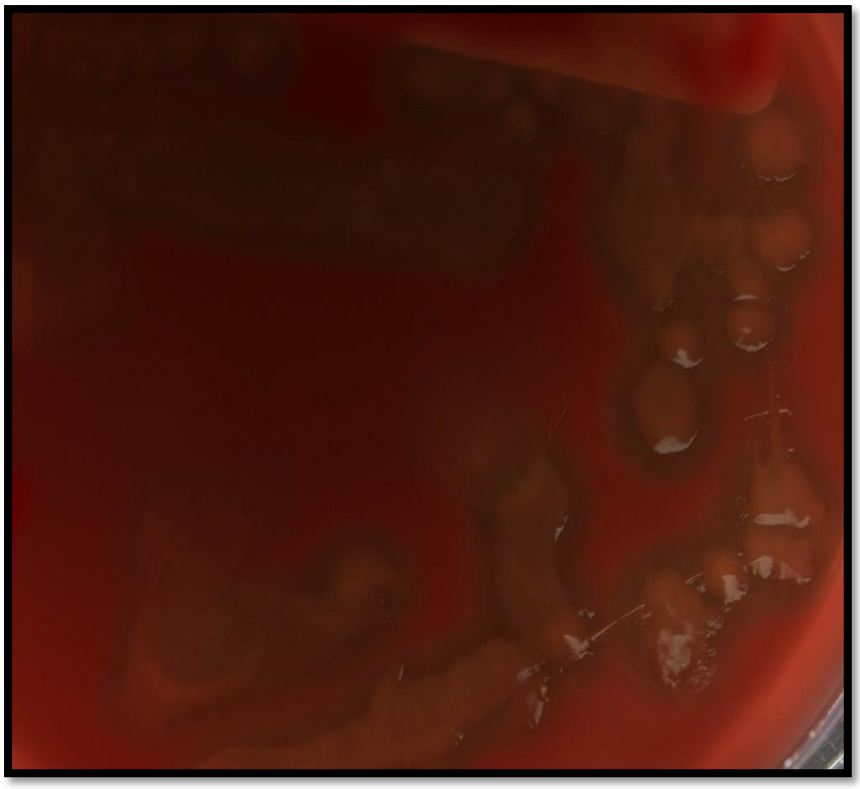


Figure 1.1: *C. perfringens* colonies on blood agar incubated for 48 h at 37 °C under anaerobic conditions.

1.5.2. *Bacteroides* species as aetiological agents for IAI

Bacteroides species are Gram–negative opportunistic bacteria, that form part of normal microbiota and often associated with polymicrobial infections such as intra–abdominal, pelvic, genital, complicated skin and soft tissue, and bloodstream infections (Wexler, 2007; Mazuski and Solomkin, 2009; Ho *et al*., 2017; Schwensen *et al*., 2017). In clinical specimens, the most frequently recovered and virulent species is *B. fragilis* followed by *B. thetaiotaomicron* (Wexler, 2007). Figure 1.2 is *B. thetaiotaomicron* colonies on Brucella blood agar supplemented with hemin and vitamin K.



Figure 1.2: *B. thetaiotaomicron* colonies on blood agar plate incubated for 48 h at 37 °C under anaerobic conditions.

1.5.3. *Fusobacterium* species as causative agents of IAI.

F. nucleatum is an invasive, adherent, pro–inflammatory, Gram–negative obligate anaerobe and is associated with oral diseases such as periodontitis and gingivitis, as well as colorectal cancer and appendicitis (Shang and Liu, 2018). Colorectal cancer is one of the most prevalent cancer and the most frequent cause of cancer–related mortality globally and is associated with raised mortality in areas with poor medical conditions (Center *et al*., 2009; Siegel *et al*., 2017; Shang and Liu, 2018). *F. varium* is associated with ulcerative colitis (Ohkusa *et al*., 2003). Figure 1.3 is *F. nucleatum* colonies on Tryptone Soya agar supplemented with sheep blood.

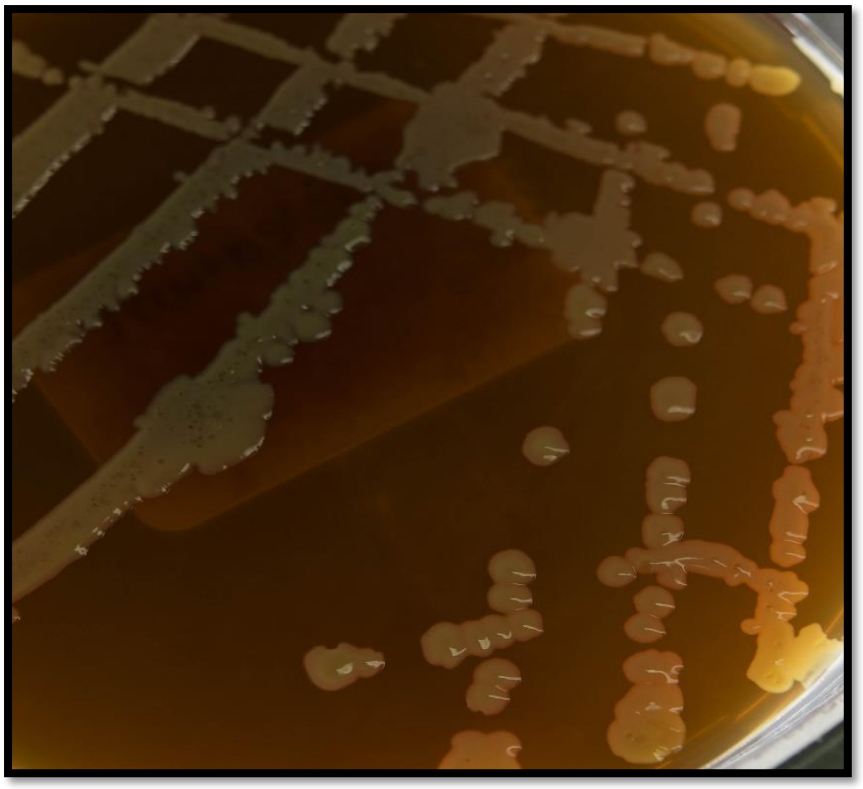


Figure 1.3: *F. nucleatum* colonies on blood agar plate incubated for 48 h at 37 °C under anaerobic conditions

1.5.4. *H. pylori* as an etiologic agent for IAI.

is a micro–aerophilic bacteria that lives in the human gastric mucosa, and was first discovered by Warren and Marshall in 1983 (Ohsaki *et al*., 1999; Abadi and Yamaoka, 2018). infects more than 50% of the world's population, however, only a small percentage of patients develop severe disorders (Abadi and Yamaoka, 2018). People that are most likely to be infected are people from developing countries (Zaidi *et al*., 2009). passes from one patient to the other through contaminated food and water, and through mouth–to–mouth contact (Espinoza *et al*., 2017). is associated with chronic active gastritis and severe gastric diseases, such as chronic atrophic gastritis, peptic ulcers and stomach cancer (Farnbacher *et al*., 2010; Bosques–Padilla *et al*., 2018). Risk factors associated with infection include type of diet, genetic susceptibility, and consumption of alcohol and tobacco (Bosques–Padilla *et al*., 2018). This is the only bacteria that can tolerate the acidic environment of the human stomach due to its genetical and phenotypical characteristics (Abadi and Yamaoka, 2018). Eradication of *H. pylori* is directly proportional to ulcer healing, prevention of peptic ulcer recurrence and may also reduce the

occurrence of gastric cancer in high–risk populations (Zaidi *et al*., 2009). Figure 1.4 is a colony on Columbia blood agar.



Figure 1.4: *H. pylori* colonies on blood agar plate incubated for four days at 37 °C under microaerophilic conditions.

1.6. Available treatment options for IAI

The management of IAI involves controlling the source of the infection and antibiotic treatment which is directed to the aetiologic agent (Mazuski and Solomkin, 2009). The current eradication regimens for *C. difficile* infection include metronidazole and vancomycin (Shigefuku *et al*., 2017; Roshan *et al*., 2018). *Bacteroides species* infections are currently treated using imipenem and metronidazole (Ho *et al*., 2017). *H. pylori* infection is treated using proton pump inhibitors (PPIs) and two antibiotics (any two amongst clarithromycin, amoxicillin and metronidazole), and bismuth–containing quadruple therapy consists of a PPI, bismuth and two antibiotics, such as tetracycline, clarithromycin and metronidazole (Thung *et al*., 2016). However, the effectiveness of the currently used antibiotics is related to undesirable factors such as strength of antibiotics, unaffordability, side effects, duration, and the inability of the patient to tolerate the antibiotics, as well as bacterial resistance. Increased antibiotic

resistance is the main cause of treatment failure (Thung *et al*., 2016; Ho *et al*., 2017; Shigefuku *et al*., 2017).

1.7. Antimicrobial resistance among neglected gut pathogens

Gut anaerobes and microaerophiles are mostly resistant to a wide range of conventional antimicrobials. An example is *B. fragilis*, which has been associated with the high resistance to antimicrobials. The resistance of this micro–organism is reported to reside on resistance genes (cfiA gene). This gene complicates treatment of other infections when passed to other infectious bacteria in the large intestine (Shoemaker *et al*., 2001; Schwensen *et al*., 2017).

Another example is , whose current eradication regimens include the use of two–three antimicrobial agents, along with proton pump inhibitors or bismuth–based adjuvant therapies (Mosites *et al*., 2018). This pathogen has a moderately–high resistance to the currently used antibiotics amoxicillin and clarithromycin (Njume *et al*., 2011; Mosites *et al*., 2018). As a result of high antibiotics resistance arising from gut pathogens, there is an increased and urgent demand for alternative treatment especially from natural products including medicinal plants (Zaidi *et al*., 2009, Njume *et al*., 201; Shigefuku *et al*., 2017; Roshan *et al*., 2018).

1.8. Medicinal plants as alternative route for treatment of gastro–intestinal infections

1.8.1. Global perspective

Phytomedicine has proved to be a readily available treasure as an alternative treatment for different diseases, including gastrointestinal disorders. Medicinal plants are one lead source for drug discovery (Zaidi *et al*., 2009). Several antimicrobial studies have focused on evaluating the activity of traditional medicinal plants against neglected gut pathogens from various parts of the world, including Brazil, Cameroon, Czech Republic, Iran, Mexico, and Turkey with promising antimicrobial activities against the neglected gut pathogens (Sokmen *et al*., 1999; Neto *et al*., 2002; Kloucek *et al*., 2005; Kloucek *et al*., 2007; Ndip *et al*., 2007; Zaidi *et al*., 2009; da Silva *et al*., 2014; Chauhan et al., 2017; Dora *et al*., 2017). Some of the antimicrobial studies on medicinal plants used traditionally against neglected fastidious gut pathogens are included in Table 1.2.

1.8.2. Southern African medicinal plants and gastro–intestinal infections

In Southern Africa, traditional healers use different types of medicinal plants to treat stomach ailments and the popular use of the Southern African medicinal plants in infectious diseases has previously been reported in literature (Watt and Breyer–Brandwijk, 1962; Hutchings *et al*., 2006; van Wyk *et al*., 1997; McGaw *et al*., 2000; von Koenen, 2001; Njume *et al*., 2011). Several antimicrobial studies have focused on evaluating the antimicrobial efficacy of medicinal plants against commonly studied gut pathogens such as *Staphylococcus aureus, Shigella flexineri, Candida albicans, E. coli, E. faecalis* and (van Vuuren, 2008; van Vuuren and Holl, 2017). Recently, Saheed and Tom. (2017) investigated the antimicrobial activity of *Elephantorrhiza elephantina* against common gut pathogens responsible for diarrhoea.

However, the scientific evidence against pathogens such as *Clostridium* species, *Bacteroides* species, *Fusobacterium* species as well as *H. pylori* has not been adequately studied. A review from a period dating over almost twenty years demonstrated very few, if any antimicrobial studies of Southern African medicinal plants and the gut pathogens selected for this study (van Vuuren, 2008; van Vuuren and Holl, 2017). The main obstacle for recognizing the need for antimicrobial studies against this pathogens was associated with the inability to successfully culture this pathogens due to their fastidious nature. Hence they are considered neglected pathogens of the gut. Table 1.3 details some of the plants that are commonly used traditionally in Southern Africa for the treatment of stomach ailments, and the plants in bold type phase are the plants that were selected for this study. These plants were selected based on availability and accessibility.

Table 1.2: Antimicrobial studies of medicinal plants against fastidious pathogens of the gut

Plant species	Neglected pathogen	Outcome	References
Thirty–Five Turkish plants were assayed for their antibacterial activities against five pathogenic bacteria and a yeast.	*C. perfringens*	Organic extracts of *Hypericum capitatum, Hypericum scabrum* and *Perganum harmala*	Sokmen *et al*., 1999

Plant species	Neglected pathogen	Outcome	References
Nine Peruvian medicinal plants	*B. fragilis*	Inhibition diameter ranging between 0.5 – 0.7 cm	Neto *et al*., 2002
Nine Peruvian medicinal plants were tested against	*B. fragilis*	The organic extracts of *Equisetum giganteum*, *Brunfelsia grandiflora*, *Caesalpinia spinose*, *Phyllanthus amarus*, and *Terminalia catappa* displayed antimicrobial activity against *C. perfringens* with MIC values ranging between 4000 and 16000 μg/ml	Kloucek *et al*., 2005
Six medicinal plant extracts	*B. fragilis*	The tested organic plant extracts displayed good antimicrobial activities with MIC values ranging between 130– 4000 μg/ml against *B. fragilis*	Kloucek *et al*., 2007
Ten medicinal plants from Cameroon		The organic extracts of the tested plant extracts showed good antimicrobial activity with MIC ranging between from 32 – 1000 μg/ml, of which *Ageratum conyzoides, Scleria striatinux, Lycopodium cernua* showed the best activities	Ndip *et al*., 2007
Anti–bactericidal activities of medicinal plants against 50 medicinal plants against strains		More than 50% of the tested extracts inhibited the growth of eight at the concentration of 500 μg/ml. The 70% aqueous–ethanol extracts of *Curcuma amada, Mallotus phillipinesis*, *Myrisctica fragrans*, and *Psoralea corylifolia* demonstrated the best anti–	Zaidi *et al*., 2009

Plant species	Neglected pathogen	Outcome	References
		activity with Minimum bactericidal concentration values ranging from 15.6 – 62.5 μg/ml	
The antimicrobial activity of *Pinus elliottii* was tested against eleven anaerobic bacteria that cause endodontic infection	*B. fragilis* *B. thetaiotaomicron* *F. nucleatum*	Oleoresins extracted from *P. elliottii* was active against the tested pathogens with MIC values ranging from 0.4 to 50 μg/ml	Da Silva *et al.*, 2014
Nerium indicum	*C. perfringens*	Methanol, Chloroform, Hexane extracts of *N. indicum* were active against *C. perfringens* with inhibition diameters of 15, 16, and 14 mm respectively	Chauhan *et al.*, 2017
Mikania glomerata	*F. nucleatum*	The organic extracts of *M. glomerata* displayed good activities *F. nucleatum* with MIC values ranging between 50 and 200 μg/ml	Dora *et al.*, 2017

Table 1.3: Southern African medicinal plants used traditionally to treat stomach ailments

Botanical and family name	Family name	Common name	Plant part used	Traditional use	References
Acacia nilotica var. *kraussiana* (Benth.)	Fabaceae	Scented thorn	Root, bark.	Root for stomach ailments; bark is used for dysentery	Hutchings *et al*., 1996
Acokanthera oppositifolia (Lam.) Codd.	Apocynaceae	Bushman's poison	Leaf, root, and ripe fruit	Leaf decoction for stomach ache, diarrhoea, anthelmintic. Root or leaf for abdominal pain. Ripe fruit for gastritis	Watt and Breyer–Brandwijk, 1962; Hutchings *et al*.,1996; Bisi–Johnson *et al*., 2010; Wintola *et al*., 2017
Agapanthus campanulatus Leighton	Agapanthaceae	Bell agapanthus	Root	Stomach problems in children	Watt and Breyer–Brandwijk, 1962
Agave L.	Asparagaceae	Centaury plant	Leaf	Stomach and intestinal disorders	Hutchings *et al*., 1996
Agrimonia bracteata E. Mey. ex C. A. Mey.	Rosaceae	Agrimony	Leaf	Abdominal pain	Van Wyk *et al*., 2009
Albizia adianthifolia (Schum.) W.Wight	Fabaceae	Flat–crown	Root	Stomach ache	Hutchings *et al*., 1996

Botanical and family name	Family name	Common name	Plant part used	Traditional use	References
Albizia petersiana (Bolle) Oliv. subsp.	Leguminosae	Many–Stemmed False–Thorn	Root	Stomach ailments.	Hutchings *et al*., 1996
Alepidea amatymbica Eckl. & Zeyh	Apiaceae	Larger tinsel flower	Rhizomes and root	Abdominal cramps	Van Wyk *et al*., 2009
***Aloe arborescens* Mill.**	**Aloaceae**	**Krans aloe**	**Leaf**	**Stomach ache**	**Hutchings *et al*., 1996; McGaw *et al*., 2000 Wintola and Afolayan, 2010; Wintola *et al*., 2017**
***Aloe ferox* Mill.**	**Aloaceae**	**Bitter aloe**	**Leaf**	**Stomach ache**	**Bhat, 1985; van Wyk, 2008; Wintola and Afolayan, 2010; Wintola *et al*., 2017**
***Aloe marlothii* Berger**	**Aloaceae**	**Mountain aloe**	**Root, leaf**	**Round worms stomach ailments**	**Hutchings *et al*., 1996; McGaw *et al*., 2000**
***Aloe tenuior* Lam.**	**Aloaceae**	**Slender aloe**	**Leaf**	**Peptic ulcer**	**Wintola *et al*., 2017**

Botanical and family name	Family name	Common name	Plant part used	Traditional use	References
Anthemis cotula L.	Asteraceae	Stinking chamomile	Leaf	Stomach pains	Watt and Reyer–Brandwijk, 1962
Antidesma rufescens Tul.	Portulacaceae	Sand Rose	Fruit, leaf and branches	Abdominal pain	Von Koenen, 2001
***Antidesma venosum* E. Mey. ex Tul.**	**Euphorbiaceae**	**Tossel berry**	**Leaf**	**Abdominal cramps, dysentery**	**Hutchings *et al*., 1996; Fawole *et al*., 2009**
Antizoma angustifolia (Burch.) Miers ex Harv.	Menispermaceae	Narrow–leaved arctotis	Root	Stomach ailments, diarrhoea	Von Koenen, 2001
Argemone ochroleuca Sweet	Papaveraceae	Mexican poppy	Whole plant	Stomach pains, colic	Von Koenen, 2001
Artemisia afra Jacq. ex Willd.	Asteraceae	African wormwood	Leaf	Stomach pain	Van Wyk, 2008; Wintola and Afolayan, 2010
Asclepias fruticosa L.	Asclepiadaceae	Modimolo	Leaf	Gastro–intestinal problems, stomach pain and diarrhoea	Hutchings *et al*., 1996; Von Koenen, 2001
***Boophone disticha* Herb.**	**Amaryllidaceae**	**Bushman poison bulb**	**Bulb scales**	**Abdominal pain, Gastric ulcers**	**Verschaeve and van Staden, 2008; Van Wyk *et al*., 2009**

Botanical and family name	Family name	Common name	Plant part used	Traditional use	References
Bridelia cathartica **G. Bertol.**	**Euphorbiaceae**	**Blue sweet berry**	**Root**	**Stomach ache**	**Hutchings *et al*., 1996**
Bridelia micrantha **Baill.**	**Euphorbiaceae**	**Coastal golden leaf**	**Bark**	**Stomach ache**	**Hutchings *et al*., 1996; Lin *et al*., 2002 Maroyi, 2017**
Carissa edulis Vahl	Apocynaceae	Simple spined Num–num	Root	Stomach ulcers	Von Koenen, 2001
Catha edulis **(Vahl) Forssk. ex Endl.**	**Celastraceae**	**Bush man's tea**	**Leaf**	**Gastrointestinal tract problems, gastritis stomach ailments**	**Hutchings *et al*., 1996; Al–Habori, 2005**
Chenopodium ambrosioides **L.**	**Chenopodiaceae**	**Mexican tea**	**Whole plant**	**Infusions of the whole plant are used for intestinal ulcers.**	**Hutchings *et al*., 1996**
Citrullus lanatus (Thunb.) Matsum. & Nakai	Cucurbitaceae	Wild watermelon	Fruit	Abdominal pain	Von Koenen, 2001
Clematis brachiate	Ranunculaceae	Traveller's joy	Leaf and stem	Abdominal disorders	Hutchings *et al*., 1996

Botanical and family name	Family name	Common name	Plant part used	Traditional use	References
Ker Gawl.					
Cucumis hirsutus Sond	Cucurbitaceae	Hairy Wild Cucumber	Leaf and root	Abdominal pain and diarrhoea	Hutchings *et al*., 1996
Cussonia paniculata Eckl. & Zeyh	Araliaceae	Mountain cabbage	Bark	Stomach ulcers	Hutchings *et al*., 1996
Cyperus textiles Thunb.	Cyperaceae	Umbrella sedge	Root	Stomach pains and cramps	Hutchings *et al*., 1996
***Dichrostachys cinereal* (L.) Wight & Arn.**	**Fabaceae**	**Sickle bush**	**Root**	**Abdominal pain**	**Hutchings *et al*., 1996**
Diospyros lycioides Desf.	Ebenaceae	Bluebush	Bark and root	Decoctions are taken for bloody faeces and dysentery	Hutchings *et al*., 1996
Dissotis princeps (Kunth) Triana	Melastomataceae	Royal dissotis	Leaf	Dysentery, diarrhoea	Hutchings *et al*., 1996
***Dodonaea viscosa* Jacq.**	**Sapindaceae**	**Sand olive**	**Leaf and tips of the twigs**	**Stomach trouble**	**Van Wyk, 2008; Van Wyk *et al*., 2009**

Botanical and family name	Family name	Common name	Plant part used	Traditional use	References
Dombeya rotundifolia Planch.	Sterculiaceae	Wild plum	Bark, root, stems, Leaf	Leaf for internal ulcers,bark for ulcerative colitis, intestinal ulceration; root for abdominal pain.	Watt and Breyer–Brandwijk, 1962; Von Koenen, 2001; Verschaeve and van Staden, 2008
Drimiopsis maculate Lindl. & Paxton	Hyacinthaceae	Little white soldiers	Bulbs	Stomach ailments	Hutchings *et al*., 1996 Du Toit *et al*., 2007
Ekebergia capensis Sparrm.	Meliaceae	Cape ash	Root, leaf	Dysentery, acute gastritis	Verschaeve and Staden, 2008 Van Wyk *et al*., 2009
Elephantorrhiza elephantina (Burch.) Skeels.	Fabaceae	Elephant's root	Root	Diarrhoea, dysentery, stomach disorders, peptic ulcers	Van Wyk *et al*., 2009; Bisi–Johnson *et al*., 2010
Elytropappus rhinocerotis (L.f.) Less.	Asteraceae	Renosterbos	Young tips of the branches	Stomach cancer, indigestion, ulcers	Van Wyk *et al*., 2009
Eucomis autumnalis (Mill.) Chitt.	Hyacinthaceae	Pineapple lily	Bulb	Abdominal problems. stomach ache	Van Wyk, 2008; Van Wyk *et al*., 2009;

Botanical and family name	Family name	Common name	Plant part used	Traditional use	References
					Bisi–Johnson *et al*., 2010
***Gunnera perpensa* L.**	**Gunneraceae**	**River pumpkin**	**Rhizomes**	**Root are used for stomach ailments; unspecified plant part is for stomach bleeding**	**Van Wyk, 2008; Van Wyk *et al*., 2009**
Haworthia limifolia Marloth	Asphodelaceae	Horse teeth	Leaf	Stomach trouble	Hutchings *et al*., 1996
***Heteromorpha arborescens* Cham. & Schltdl**	**Apiaceae**	**Parsley tree**	**Root**	**Abdominal pain Dysentery**	**Verschaeve and Van Staden, 2008; van Wyk *et al*., 2009**
***Ipomoea purpurea* (L.) Roth.**	**Onvolvulaceae**	**Morning glory**	**Stems**	**Stomach disorders**	**Hutchings *et al*., 1996**
***Kigelia Africana* (Lam.) Benth.**	**Bignoniaceae**	**Sausage tree**	**Leaf, fruit, stem, bark**	**Fruit for ulcers, fruit and ground bark for stomach ailments**	**Hutchings, 1996; Verschaeve and Van Staden, 2008; van Wyk *et al*., 2009**

Botanical and family name	Family name	Common name	Plant part used	Traditional use	References
Lepidium africanum (Burm. f.) DC.	Brassicaceae	African pepperwort	Seed	Stomach ulcers	Von Koenen, 2001
Lippia javanica **Spreng.**	**Oxalidaceae**	**Fever tea**	**Leaf**	**Diarrhoea, stomach disorders**	**Hutchings *et al*.,1996; van Wyk, 2008; van Wyk *et al*., 2009**
Mentha longifolia **Huds.**	**Verbenaceae**	**Mint**	**Leaf, twigs**	**Stomach ache**	**Hutchings *et al*., 1996; Wintola *et al*., 2017**
Osmitopsis asteriscoides **Cass.**	**Lamiaceae**	**Mountain daisy**	**Leaf**	**Colic**	**Van Wyk *et al*., 2009**
Osyris lanceolata Hochst. & Steud.	Santalaceae	Rock tannin–bush	Root	Stomach ulcers	Von Koenen, 2001
Oxalis corniculate **L.**	**Asteraceae**	**Creeping wood**	**Leaf**	**Peptic ulcers, stomach ache**	**Wintola *et al*., 2017**
Peltophorum africanum **Sond.**	**Leguminosae**	**African blackwood**	**Root, leaf, bark**	**Diarrhoea, dysentery, and abdominal pain**	**Watt and Breyer–Brandwijk, 1962; Bizimenyera *et al*., 2006**

Botanical and family name	Family name	Common name	Plant part used	Traditional use	References
***Polygala fruticose* P.J. Bergius**	**Polygalaceae**	**Petite butterfly**	**Whole plant**	**Intestinal sores**	**Van Wyk *et al*., 2009**
Protea repens L.	Proteaceae	Sugarbushes	Nectar or fruit	Bleeding stomach ulcers	Van Wyk *et al*., 2009
***Rapanea melanophloeos* Mez**	**Myrsinaceae**	**Cape beech**	**Bark**	**Stomach ache**	**Hutchings *et al*., 1996**
***Rauvolfia caffra* Sond.**	**Apocynaceae**	**Kinaboom**	**Bark**	**Bark for abdominal pain**	**Hutchings *et al*., 1996**
Ricinus communis L.	Euphorbiaceae	Castor oil plant	Root	Ulcers, diarrhoea, stomach ache,	Hutchings *et al*., 1996
Salvadora persica L	Salvadoraceae	Mustard bush	Root, bark.	Bark is used for gastritis and worms; root tea is used for gastro intestinal complaints and blood–stained diarrhoea.	Von Koenen, 2001
Salvia Africana L. caerulea	**Lamiaceae**	**Purple sage**	**Young stems and leaf**	**Stomach pain**	**Van Wyk *et al*., 2009**

Botanical and family name	Family name	Common name	Plant part used	Traditional use	References
Scadoxus puniceus **(L.) Friis & Nordal**	**Amaryllidaceae**	**Paintbrush lily**	**Bulbs and root**	**Abdominal pain stomach ailments, diarrhoea, and nausea.**	**Van Wyk *et al*., 2009**
Solanum incanum **Ruiz & Pav.**	**Solanaceae**	**Bitter apple**	**Unspecified plant part**	**Abdominal pains**	**Watt and Breyer–Brandwijk, 1962, Von Koenen, 2001**
Spirostachys Africana **Sond.**	**Euphorbiaceae**	**Jumping–bean tree**	**Root and bark**	**Stomach ulcers, acute gastritis, stomach pain, dysentery, diarrhoea**	**Hutchings *et al*., 1996, Mathabe *et al*., 2008**
Syzygium cordatum **Hochst**	**Myrtaceae**	**Water berry**	**Bark**	**Stomach ache, diarrhoea**	**Watt and Breyer–Brandwijk, 1962 Hutchings *et al*., 1996; Van Wyk *et al*., 2009**
Tarchonanthus camphoratus **L.**	**Asteraceae**	**Camphor bush**	**Leaf and twigs**	**Abdominal pains**	**Hutchings *et al*., 1996; Van Wyk *et al*., 2009**

Botanical and family name	Family name	Common name	Plant part used	Traditional use	References
***Tetradenia riparia* (Hochst.) Codd**	**Lamiaceae**	**Ginger bush**	**Leaf**	**Stomach ache, diarrhoea.**	**Watt and Breyer–Brandwijk, 1962; Hutchings *et al*., 1996; Van Wyk *et al*., 2009**
Vernonia natalensis Sch. Bip. exWalp.	Asteraceae	Silver Vernonia	Leaf and stems	stomach cramps, nervous spasms of the stomach	Hutchings *et al*., 1996; Van Wyk *et al*., 2009
Wahlenbergia undulata (L.f.) A.DC.	Campanulaceae	African bluebell	Whole plant	Intestinal ulceration	Hutchings *et al*., 1996
Warburgia salutaris (Berto.f.) Chiov.	Canellacea	Fever tree	Bark or root bark	Gastric ulcers	Hutchings *et al*.,1996
Watsonia tabularis Bak	Iridaceae	Table Mountain watsonia	Corms	Diarrhoea	Hutchings *et al*., 1996
***Zanthoxylum capense* (Thunb.) Harv**	**Rutaceae**	**Small knob wood**	**Leaf**	**Gastric and intestinal disorders**	**Hutchings *et al*., 1996**

"a" Bold type phase denote plant that were used in this study.

1.9. Biofilms as a physical barrier for penetration of antimicrobials.

Biofilms are defined as organized bacterial communities surrounded in an extracellular matrix attached to living or non–living surfaces (Macia *et al*., 2014). Biofilm formation usually occurs as a survival mechanism for micro–organisms on several different surfaces, such as natural aquatic and soil environments, living tissues, medical devices or industrial or potable water piping systems (Flemming and Wingender, 2001; Donlan, 2002). Biofilm formation happens in four stages. In the first stage, micro–organisms initiate adhesion to a solid surface using flagella and pili for Gram–negative bacteria, while the Gram–positive bacteria use external proteins for this purpose. This stage can either be reversible or irreversible (O'Toole *et al*., 2000; Cucarella *et al*., 2001; Bjarnsholt *et al*., 2013; Pantaléon *et al*., 2014). The second stage is maturation and growth of the biofilm. Here, the biofilm proliferates and produces an extracellular matrix (O'Toole *et al*., 2000; Cucarella *et al*., 2001; Pantaléon *et al*., 2014; Bjarnsholt *et al*., 2013). The extracellular matrix is a complex chemical and physical crosslinked network of proteins and glycosaminoglycans (Hubbell, 2014). In stage three, the biofilm develops fully, and in stage four, sessile bacteria are released from mature biofilms to colonize new surfaces and start the cycle again (Joo *et al*., 2012).

The ability of bacteria to aggregate and form biofilms makes it difficult to treat bacterial infections since biofilms enhance the bacteria's ability to resist the host's immune system response, and thus contribute to antibiotic resistance (Chen *et al*., 2013). Biofilms possess complex structures which reduce the ability of conventional antibiotics to attack infected cells (Chen *et al*., 2013; Agarwal and Gayathri, 2017). Biofilms present therapeutic barriers to many antibiotics, as they can tolerate higher concentrations of antibiotics that are enough to eliminate free floating cells (Macia *et al*., 2014). Many antimicrobial studies have focused on the effects of plant extracts against planktonic bacteria, although many bacterial pathogens including fastidious pathogens of the gut occur as biofilms. Although antibiofilm activities of medicinal plants against other micro–organisms were investigated, no previous study was sourced for the antibiofilm activity of medicinal plants against *C. perfringens*.

1.10. Toxicity effects of medicinal plants

Plants commonly used in traditional medicine are often assumed to be harmless. However, scientific research has shown that many of them can be lethal, mutagenic and carcinogenic (Fennell *et al*., 2004; Steenkamp *et al*., 2009; Verschaeve *et al*., 2014; Akhalwaya *et al*., 2018). Compounds that are found in plants interact with each other or with cells, and that can cause toxicity, which may lead to acute poisoning. Toxicity arising from interaction of compounds that are found in medicinal plants have been known to result in numerous deaths in South Africa (Malangu and Ogunbanjo, 2009; Otang *et al*., 2013). It has been emphasised by van Vuuren and Hall (2017) that phytotherapy research should not only focus on the antimicrobial studies, but also on the toxicity of medicinal plants to test for some degree of safety.

11. Aims and objectives

This study aimed to investigate the antimicrobial efficacy of indigenous Southern African plants used for the treatment of stomach ache against anaerobic bacteria responsible for gastro–intestinal infections. This was be achieved by addressing the following objectives:

Objective 1: To determine the antimicrobial activity of selected plants to treat stomach ailments using the broth dilution minimum inhibitory concentration (MIC) assay.

Objective 2: To determine the antibiofilm activities of selection of medicinal plants against the most active pathogen (*C. perfringens*)

Objective 3: To determine the toxicity profiles of the most antimicrobially active plant species using the Brine shrimp lethality (BSLA) assay

CHAPTER 2

Antimicrobial activity of plants used for stomach ailments

2.1. Introduction

The minimum inhibitory concentration assay is the preferred method for antimicrobial screening (van Vuuren, 2008; van Vuuren and Holl, 2017). The MIC is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a micro–organism (Andrews, 2001). The most recent review by van Vuuren and Holl (2017) revealed that activity will only be considered noteworthy for plant extracts if an MIC of ≤ 160 µg/ml is obtained. In the current study antimicrobial activity was considered noteworthy for plant extracts when MIC values were ≤ 160 µg/ml, moderate values considered for activities >160 µg/ml – ≤ 1000 µg/ml; weak activity classified as MIC values of >1000 – < 8000 µg/ml; and poor activity shown with MIC values > 8000 µg/ml (Pauw and Eloff, 2014; Freires *et al*., 2015; van Vuuren and Holl, 2017).

2.2. Materials and methods

2.2.1. Plant selection

An ethnobotanical literature review was conducted to identify the Southern African medicinal plants used traditionally to treat stomach ailments (Table 1.3, Chapter 1). Medicinal plant–based books such as: The Medicinal and Poisonous Plants of Southern and Eastern Africa (Watt and Breyer–Brandewijk, 1962), The Medicinal Plants of South Africa (van Wyk *et al*., 2009); Zulu Medicinal Plants (Hutchings *et al*., 1996); and Poisonous Medicinal Plants (von Koenen, 2001) were used. Scientific articles from Databases including ScienceDirect, Scopus, Google

Scholar and PubMed were also used. Search keywords included “stomach ailments”, “gastro–intestinal ailments”, “medicinal plants”, and “South Africa”.

2.2.2. Plant material collection

The selected plants which were available were collected from the Walter Sisulu Botanical Gardens (WSBG) where the chief horticulturist, Mr. Andrew Hankey, granted permission and assisted in plant identification. All “transfer of materials for research purposes” documents were signed accordingly. Medicinal plants materials that were not available at WSBG were purchased from Random Harvest Indigenous Nursery (RHIN). Following collection, voucher specimens were prepared for each species as detailed in Table 2.1 and were housed in the Department of Pharmacy and Pharmacology, University of the Witwatersrand.

2.2.3. Preparation of plant samples

The collected plant samples were left to dry at room temperature. Drying period varied based on the plant part being used, leaves took three to fourteen days, whereas the bulbs, roots, and stems took three to six weeks. Dried plant materials were separated and then crushed to powder using the high-speed Fritsch Pulverisette grinder (Labotec) or using a pounder (purchased at Faraday supermarkets) for harder stems and barks.

2.2.4. Preparation of organic extracts

In order to prepare organic extracts, plant powder was transferred to a previously weighed conical flask. Dichloromethane: methanol (1:1) that was twice the quantity of plant material was added and placed at the platform shaker incubator (Labcon) at 25 ºC for 24 h. Thereafter, the solvent was filtered and left in a fume hood to evaporate. The samples were re–extracted with a fresh solvent for another 24 h. Depending on the plant being used, the filtered solvent was left to evaporate for one to three days. Once the solvent had evaporated, the extract was transferred into suitable amber bottles for storage at ambient temperature.

2.2.5. Preparation of aqueous extracts

Aqueous extracts were prepared by immersing plant powder material in distilled water. This was followed by incubation in platform shaker incubator, overnight at 30 ºC. Thereafter, the liquid extracts were strained and stored at –80 ºC for 24 h before lyophilization. Aqueous extracts were lyophilized using a freeze dryer (Virtis) for 3–7 days. Before use, the aqueous extracts were placed under UV light overnight to eliminate possible microbial contaminants. All plant samples were stored in appropriate containers at room temperature.

2.2.6. Antimicrobial screening

2.2.6.1. Plant sample preparation

Samples were prepared by weighing out the crude extracts and calculating the volume of solvent to be added to create a sample concentration of 32 mg/ml. Acetone was used as the solvent of choice for organic samples as it has minimal antimicrobial effects. Sterile water was used to solubilize aqueous extracts.

2.2.6.2. Test micro–organisms

Test pathogens were selected according to their ability to cause stomach ailments. The micro–organisms that were mostly used are the American Type Culture Collection (ATCC) and were purchased from Davies Diagnostics. Eight members of the Gram–negative anaerobic bacilli *B. fragilis* (ATCC 23745), *B. ovatus* (ATCC 8483), *B. thetaiotaomicron* (ATCC 29741), *B. vulgatus* (ATCC 8482), *F. nucleatum* (ATCC 25586), *F. varium* (ATCC 27725), *H. pylori* (B8) reference strain, clinical strain; and two members of Gram–positive species *C. difficile* (ATCC 43593) and *C. perfringens* (ATCC 13124) were selected. Two non–fastidious pathogens *E. coli* (ATCC 8739) and *E. faecalis* (ATCC 29212) were included as a comparator of activity. These micro–organisms were cultured in the respective media and incubation conditions as per Clinical laboratory standards institute (CLSI, 2012) guidelines with slight modifications (see Table 1.3).

For , the clinical strain was obtained from Chris Hani Baragwanath Academic Hospital. Methodology as used by Tanih *et al*. (2010) was used to isolate the strains from patients. This

was achieved by obtaining two antral and corpus biopsy using, these specimens were then placed in sterile bijou bottles containing a mixture of cysteine (200 mg/ml) and glycerol (20%) in brain–heart infusion (BHI) broth and transported in ice to the laboratory within 2 h of collection. *H. pylori* isolates were then confirmed by polymerase chain reaction (PCR) using glmM as the target gene; colony morphology, characteristic spiral morphology on Gram staining; positive catalase, urease and oxidase tests. Confirmed isolates were suspended in 20% glycerol and stored at – 80ºC in a freezer for future use. A reference strain (B8) was also tested. This strain was obtained from the Ludwig Maximilian University of Munich (Germany) Medical microbiology lab, through the University of the Witwatersrand Surgery department. The two strains (clinical and reference strains) were then cultured using the standard operating procedure (SOP) as detailed in Appendix D. Two ethics waivers for the use of these micro–organisms were applied for from the University of the Witwatersrand Human Research Ethics Committee (Reference No. W–CBP–180509–01 for anaerobes and aerobic bacteria; and M170582 for strains, see Appendix C).

2.2.6.3. Evaluation of antimicrobial activity

Antimicrobial susceptibility was evaluated using the minimum inhibitory concentration (MIC) assay with specific modifications to facilitate fastidious growth of pathogens (Eloff, 1998; CLSI, 2012). The plant extracts were prepared as described in Section 2.1 and made up to a concentration of 32 mg/ml in acetone. Using aseptic techniques, 100 µl of selected broth depending on the micro–organism being tested (Table 2.1) were introduced to all the wells of the 96 well micro–titre plates. Thereafter 100 µl of respective samples to be tested were placed into the top row of the micro–titre plate.

Controls (positive, negative and culture) were included in all assays. The role of the negative control was to ensure that the solvent (acetone) exerted no or minimal antimicrobial effect. The positive controls at starting concentration 0.01 mg/ml were used to validate the microbial susceptibility, where ciprofloxacin was used for *E. coli*, *E. faecalis, C. perfringens* and *Fusobacterium* species; an equal ratio mix of clarithromycin and amoxicillin was used for species; imipenem for *Bacteroides* species; and metronidazole for *C. difficile*. Ciprofloxacin was used because is a broad–spectrum antibiotic. Metronidazole, imipenem, and clarithromycin and amoxicillin were chosen based on their relevance.

A culture control was added to ensure the broths ability to support microbial growth. Serial dilutions were then performed, and the plant extracts were diluted to concentrations of 16, 8, 4, 2, 1, 0.5, 0.25, 0.13, and 0.06 mg/ml. A 100 µl of a standardized culture suspension (1 x 10^8 CFU/ml) prepared as a 0.5 McFarland's standard was added to all the wells of the micro–titre plate. This resulted in two–fold dilution descending each row. Assays were undertaken at least in duplicate to ensure accuracy. The micro–titre plates were incubated at optimal conditions without an adhesive seal film to allow the exposure of the cultures to required atmospheric conditions (Table 2.1).

Table 2.1: Growing conditions for cultures

Pathogen name	Agar	Broth	Incubation conditions
***Bacteroides* species:** *B. fragilis* (ATCC 23745) *B. ovatus* (ATCC 8483) *B. thetaiotaomicron* (ATCC 29741) *B. vulgatus* (ATCC 8482)	Tryptone Soya agar (TSA) (Oxoid) with 5% defibrinated sheep blood (NHLS)	Muller–Hinton broth with 5% yeast (Oxoid), and *Haemophilus* supplement (Oxoid)	37 ºC for 24–48 h using anaerobic gas packs (Oxoid)
***Clostridium* species:** *C. difficile* (ATCC 43593) *C. perfringens* *(*ATCC 13124)	TSA with 5% defibrinated sheep blood	Thioglycolate broth (Oxoid)	37 °C for 24 h using anaerobic gas packs
E. coli *(ATCC 8739)* *E. faecalis* *(ATCC 29212)*	Tryptone Soya agar (TSA)	Tryptone Soya broth (Oxoid)	37 °C for 24 h using anaerobic gas packs
***Fusobacterium* species:** *F. nucleatum* (ATCC 25586) *F. varium* (ATCC 27725)	Todd' Hewitt broth (Oxoid) 5% defibrinated sheep blood	Muller–Hinton broth (Oxoid) supplemented with 5% yeast (Oxoid) and *Haemophilus* supplement	35–37 °C for 48–96 h using anaerobic gas packs
***H. pylori* strains:** (B8) (reference strain) (clinical strain)	Columbia agar base (Oxoid) supplemented with: 7% fetal bovine serum /sheep blood (Davies diagnostics),	Brain Heart Infusion broth (BHI) (Oxoid) supplemented with: 7% fetal bovine serum (Davies diagnostics), 10 ml Vitox,	37 °C, 4–9 days using Pack Microaero (Camphylo) generating kit (Oxoid)

Pathogen name	Agar	Broth	Incubation conditions
	10 ml Vitox (Oxoid), 2 ml *Helicobacter pylori* selective supplement (Dent) (Oxoid)	2 ml *H. pylori* supplement (Oxoid)	

2.3. Results and discussion

2.3.1. Extraction yield

After the extracts were prepared from sterile water and dichloromethane: methanol, the percentage yield was calculated for each plant sample. Sterile water extracted a greater amount of extracts for 33 (63.5%) of the 51 plant samples tested whereas dichloromethane: methanol extracted the greater amount of material for 19 (36.5%) of the 52 plant samples. There was no clear connection noted amongst the plant part tested and the amount of extracts obtained. The percentage yield ranged from 1.9 –38.8%. The highest percentage yield for the organic extracts was that of *A. tenuior* leaf (38.8%), and the lowest was *K. africana* fruit (1.9%). The highest percentage yield for the aqueous extracts was observed for the leaf of *A. arbroscens* (32.3%) and the lowest percentage was obtained by the leaf of *H. arborescens* (2.4%)**.** The calculated percentage yields of all plant extracts are displayed in Table 2.2.

Table 2.2: Southern African medicinal plants selected for this study and the percentage yields

Plant name	Plant part	Voucher no	% yield	
			Aquo	Org
A. oppositifolia	Leaf	[b]HS245	9.5	19.5
	Root	[b]HS245	25.9	7.7
A. arborescens	Leaf	[a]HS214	32.3	6.3
A. ferox	Leaf	[a]HS215	10.5	3.3
A. marlothii	Leaf	[a]HS216	12.6	7.7
A. tenuior	Leaf	[a]HS217	19.6	38.8
A. venosum	Leaf	[a]HS218	7.5	8.3
A. afra	Leaf	[a]HS219	12.0	16.1

Plant name	Plant part	Voucher no	% yield	
			Aquo	Org
B. disticha	Bulb	[b]HS244	15.0	11.6
B. cathartica	Leaf	[a]SVV2013.1	14.4	7.6
B. micrantha	Leaf	[a]HS220	14.8	5.8
	Stem	[a]HS220	15.1	2.6
C. edulis	Leaf	[a] HS221	10.2	11.7
C. brachiate	Leaf + Stems	[a]HS243	13.8	11.8
D. cinereal	Bulb	[a]HS223	6.5	14.1
D. viscosa	Leaf	[a]HS222	16.8	9.6
D. rotundifolia	Leaf	[a]HS224	8.3	7.0
	Stem	[a]HS224	6.8	4.9
D. maculate	Bulb	[a]HS225	13.4	4.9
E. capensis	Leaf	[a]HS226	4.6	12.7
E. elephantina	Root + Rhizome	[a]UM172	15.9	10.8
E. automnalis	Leaf	[a]HS229	32.4	13.2
G. perpensa	Leaf	[a]UM168	26.9	11.8
	Rhizome	[a]UM176	27.5	14.1
H. arborescens	Leaf	[a]HS246	2.4	11.3
I. purpurea	Stem	[a]HS230	3.1	3.3
K. africana	Fruit	[a]HS231	7.4	1.9
	Leaf	[a]HS231	5.1	5.0
	Stem	[a]HS231	4.8	2.0
L. javanica	Leaf	[a]HS232	9.9	9.0
	Twigs	[a]HS232	15.2	3.3
M. longifolia	Leaf	[a]UM148	14.3	15.3
O. asteriscoides	Leaf	[a]HS234	14.3	9.3
O. corniculate	Leaf	[a]HS232	16.2	11.3
P. africanum	Leaf	[a]HS235	15.5	7.8
P. fruticose	Leaf	[a]SVV2013.2	12.4	22.4
R. melanophloeos	Leaf	[a]HS236	7.3	6.5

Plant name	Plant part	Voucher no	% yield	
			Aquo	Org
	Stem	[a]HS236	10..3	4.9
R. caffra	Leaf	[a]UM137	14.5	6.6
S. africana–caerulea	Leaf + twigs	SWC AV 875	17.4	12.4
S. puniceus	Root + rhizome	[a]UM143	9.8	4.3
S. incanum	Leaf	[a]UM158	25.8	9.7
S. africana	Leaf	[a]HS247	28.6	11.1
	Stem	[a]HS247	5.8	4.6
S. cordatum	Leaf	[a]HS237	10.0	8.9
T. camphoratus	Leaf	[a]SVV1100	10.2	10.8
T. riparia	Leaf	[a]HS238	10.4	13.4
W. salutaris	Leaf	[a]HS239	10.6	10.0
	Stem		3.2	4.0
Z. capense	Leaf	[a]HS240	8.9	8.0

[a]Walter Sisulu Botanical Gardens. [b]Random Harvest Indigenous Nursery.

2.3.2. Antimicrobial activity of organic plant extracts

A total of 52 organic extracts were prepared from 41 different plant species and screened against 12 pathogens. The results of the antimicrobial assay for the organic extracts expressed as MIC values are represented in Table 2.3. As in many other plant–based antimicrobial studies, organic extracts proved to have higher antimicrobial activity compared with the aqueous extracts (Fawole *et al*., 2009; Bussmann *et al*., 2010; van Vuuren *et al*., 2010; Mabona *et al*., 2013; van Vuuren *et al*., 2015; Akhalwaya *et al*., 2018). Values in bold denote noteworthy activity; values in italics denote moderate activity, >8000: MIC falls above the concentration of sample that was tested, denoting very weak activity.

Table 2.3: MIC (µg/ml) results for organic plant extracts

Plant extract	**Mean MIC value (µg/ml)**												
	Plant part used	**Gram–positive**		**Gram–negative**								**Commonly screened pathogens**	
		Clostridium Species		***B. fragilis* Group**				***Fusobacterium* species**		***Helicobacter Pylori***			
		C. d	**C. p**	**B. f**	**B. o**	**B. v**	**B.t**	**F. n**	**F. v**	**H. p c**	**H. p r**	**E. c**	**E. f**
A. oppositifolia	Leaf	>8000	**130**	2000	>8000	8000	2000	*1000*	4000	4000	*1000*	2000	4000
	Root	2000	*250*	*500*	>8000	>8000	2000	*1000*	4000	8000	2000	4000	4000
A. arborescens	Leaf	>8000	**30**	4000	4000	>8000	2000	2000	6000	>8000	**130**	4000	4000
A. ferox	Leaf	*750*	**130**	4000	2000	2000	2000	*1000*	2000	4000	*1000*	4000	2000
A. marlothii	Leaf	>8000	**130**	2000	>8000	4000	2000	4000	>8000	>8000	8000	4000	4000
A. tenuior	Leaf	2000	**2**	*380*	8000	4000	*1000*	2000	6000	2000	*500*	4000	2000
A. venosum	Leaf	3000	**60**	*1000*	4000	4000	2000	4000	6000	*500*	*250*	4000	2000
A. afra	Leaf	>8000	**8**	8000	2000	2000	*1000*	*500*	4000	*1000*	2000	2000	2000
B. disticha	Bulb	*1000*	*250*	*1000*	>8000	>8000	2000	4000	4000	>8000	>8000	4000	2000
B. cathartica	Leaf	2000	**130**	2000	8000	2000	2000	*1000*	2000	4000	>8000	*1000*	2000
B. micrantha	Leaf	>8000	*500*	*1000*	8000	8000	2000	2000	2000	*500*	*250*	2000	2000
	Stem	*750*	**130**	1500	*1000*	*1000*	*1000*	2000	4000	8000	8000	*1000*	2000
C. edulis	Leaf	>8000	*1000*	*380*	*1000*	4000	>8000	4000	2000	4000	2000	2000	*1000*

Plant extract	Plant part used	Mean MIC value (µg/ml)											
		Gram–positive		Gram–negative								Commonly screened pathogens	
		Clostridium Species		*B. fragilis* Group				*Fusobacterium* species		*Helicobacter Pylori*			
		C. d	**C. p**	**B. f**	**B. o**	**B. v**	**B.t**	**F. n**	**F. v**	**H. p c**	**H. p r**	**E. c**	**E. f**
C. brachiate	Stems	>8000	2000	2000	>8000	>8000	4000	4000	*1000*	*1000*	*1000*	4000	2000
D. cinereal	Bulb	>8000	2000	>8000	2000	4000	2000	2000	2000	*1000*	*500*	1000	2000
D. viscosa	Leaf	>8000	*250*	2000	>8000	8000	*1000*	*500*	>8000	*1000*	*500*	>8000	>8000
D. rotundifolia	Leaf	2000	*500*	>8000	2000	2000	4000	2000	2000	*500*	*1000*	4000	2000
	Stem	2000	2000	>8000	4000	4000	4000	2000	3000	>8000	>8000	4000	2000
D. maculate	Bulb	*750*	*250*	3000	2000	2000	*500*	*500*	1500	>8000	2000	2000	2000
E. capensis	Leaf	2000	2000	4000	>8000	4000	2000	2000	2000	8000	2000	2000	2000
E. elephantina	Roots	*500*	*500*	*1000*	*500*	1500	*1000*	*1000*	2000	>8000	8000	*500*	*500*
E. automnalis	Leaf	2000	>8000	4000	>8000	8000	2000	2000	4000	>8000	2000	4000	2000
G. perpensa	Leaf	**130**	**2**	*750*	*250*	*750*	*1000*	*500*	*250*	8000	*1000*	2000	*1000*
	Rhizomes	**130**	**60**	*1000*	*500*	*1000*	*1000*	*500*	2250	>8000	4000	2000	4000
H. arborescens	Leaf	*1000*	4000	8000	>8000	>8000	>8000	2000	3000	2000	2000	4000	2000
I. purpurea	Leaf	2000	2000	8000	>8000	>8000	4000	4000	2000	4000	2000	4000	4000
K. africana	Leaf	2000	*1000*	4000	8000	8000	*1000*	*1000*	2000	2000	*500*	*1000*	2000
	Fruit	3000	*1000*	4000	>8000	2000	*1000*	*1000*	2000	>8000	>8000	*1000*	2000

Plant extract	Plant part used	Mean MIC value (µg/ml)											
		Gram–positive		Gram–negative								Commonly screened pathogens	
		Clostridium Species		*B. fragilis* Group				*Fusobacterium* species		*Helicobacter Pylori*			
		C. d	**C. p**	**B. f**	**B. o**	**B. v**	**B.t**	**F. n**	**F. v**	**H. p c**	**H. p r**	**E. c**	**E. f**
	Stem	*1000*	*500*	4000	8000	4000	*1000*	*1000*	4000	>8000	8000	4000	4000
L. javanica	Leaf	*1000*	**0.5**	20	4000	20	*250*	*190*	*250*	2000	2000	4000	2000
	Twigs	2000	**2**	2000	4000	2000	*500*	*500*	*310*	>8000	4000	2000	*500*
M. longifolia	Leaf	**60**	*250*	2000	2000	2000	*1000*	*500*	*750*	*1000*	3000	2000	2000
O. asteriscoides	Leaf	*500*	*250*	2000	8000	2000	*500*	*500*	*750*	4000	2000	*1000*	*1000*
O. corniculate	Leaf	2000	2000	>8000	8000	>8000	2000	4000	2000	2000	*1000*	2000	2000
P. africanum	Leaf	1500	*1000*	4000	4000	6000	2000	2000	4000	2000	2000	4000	2000
	Stems	*1000*	*1000*	2000	1000	4000	2000	2000	4000	4000	4000	*1000*	2000
P. fruticose	Leaf	*250*	**20**	*500*	*250*	*1000*	**130**	**130**	*250*	*500*	*1000*	*500*	*500*
R. melanophloeos	Leaf	4000	*1000*	4000	>8000	8000	*1000*	2000	*500*	*500*	2000	2000	4000
	Stem	4000	*500*	4000	4000	6000	2000	4000	2000	4000	4000	2000	2000
R. caffra	Leaf	8000	2000	2000	>8000	8000	*1000*	*1000*	*1000*	*250*	*380*	*1000*	*1000*
S. africana–caerulea	Leaf	**130**	**30**	*380*	*250*	*380*	*1000*	*250*	1300	*500*	*750*	*500*	*500*
S. puniceus	Rhizomes	2000	2000	*1000*	2000	*1000*	**130**	*250*	2000	>8000	4000	2000	2000
S. incanum	Leaf	2000	**130**	8000	>8000	8000	*1000*	*1000*	3000	*1000*	*1000*	2000	2000

Plant extract	Plant part used	Mean MIC value (µg/ml)											
		Gram–positive		Gram–negative								Commonly screened pathogens	
		Clostridium Species		*B. fragilis* Group				*Fusobacterium* species		*Helicobacter Pylori*			
		C. d	**C. p**	**B. f**	**B. o**	**B. v**	**B.t**	**F. n**	**F. v**	**H. p c**	**H. p r**	**E. c**	**E. f**
	Leaf	**130**	**130**	*1000*	*1000*	2000	*1000*	*1000*	2000	*500*	2000	2000	4000
S. africana	Stems	*1000*	*500*	2000	2000	4000	*500*	*1000*	*250*	8000	4000	2000	4000
S. cordatum	Leaf	**130**	**130**	*1000*	2000	2000	2000	2000	*500*	*500*	1500	2000	2000
T. camphoratus	Leaf	*1000*	*500*	1500	2000	3000	*500*	*1000*	*250*	2000	2000	2000	2000
T. riparia	Leaf	**130**	**130**	*380*	2000	*250*	*250*	*250*	1300	2000	2000	*500*	*500*
	Leaf	1500	2000	3000	2000	4000	*500*	*1000*	1500	*1000*	*1000*	2000	2000
W. salutaris	Stem	2000	*1000*	2000	2000	4000	*500*	*1000*	*1000*	8000	4000	2000	2000
Z. capense	Leaf	>8000	4000	>8000	>8000	8000	8000	>8000	>8000	*500*	2000	4000	4000
Positive control		**0.156[a]**	**0.156[b]**	**0.04[c]**	**0.08[c]**	**0.08[c]**	**0.08[c]**	**0.08[b]**	**0.08[b]**	**0.08[d]**	**0.04[d]**	**0.04[b]**	**0.63[b]**
Negative control		>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000
Culture control		>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000

C. d: *C. difficile* (ATCC 43593); **C. p**: *C. perfringens* (ATCC 13124); **B. f**: *B. fragilis* (ATCC 23745); **B. o**: *B. ovatus* (ATCC 8483); **B. t**: *B. thetaiotaomicron* (ATCC 29741); **B. v**: *B. vulgatus* (ATCC 8482); **F. o**: *F. varium* (ATCC 27725); **F. n**: *F. nucleatum* (ATCC 25586); **H. p c**: (548) clinical strain; **H. p r**: (B8) reference strain; **E. c**: *E. coli*; **E. f**: *E. faecalis*; [a] metronidazole; [b] ciprofloxacin; [c] imipenem; [d] amoxicillin; clarithromycin. Values in bold = noteworthy activity; values in italics = moderate activity.

2.3.2.1. Antimicrobial activity of organic extracts against Gram–positive bacteria

This category included two *Clostridium* species namely *C. perfringens* and *C. difficile*. The Gram–positive bacteria were more vulnerable towards the extracts when compared to the Gram–negative bacteria. *C. perfringens* was the most susceptible. Approximately 10% of the extracts displayed noteworthy activities against *C. difficile*, whereas 39% of the extracts displayed moderate activities. Approximately 39% of the extracts displayed noteworthy activities against *C. perfringens* and another 39% displayed moderate activities *C. perfringens*. Figure 2.2 summarizes the activity or organic extracts against the Gram–positive bacteria.

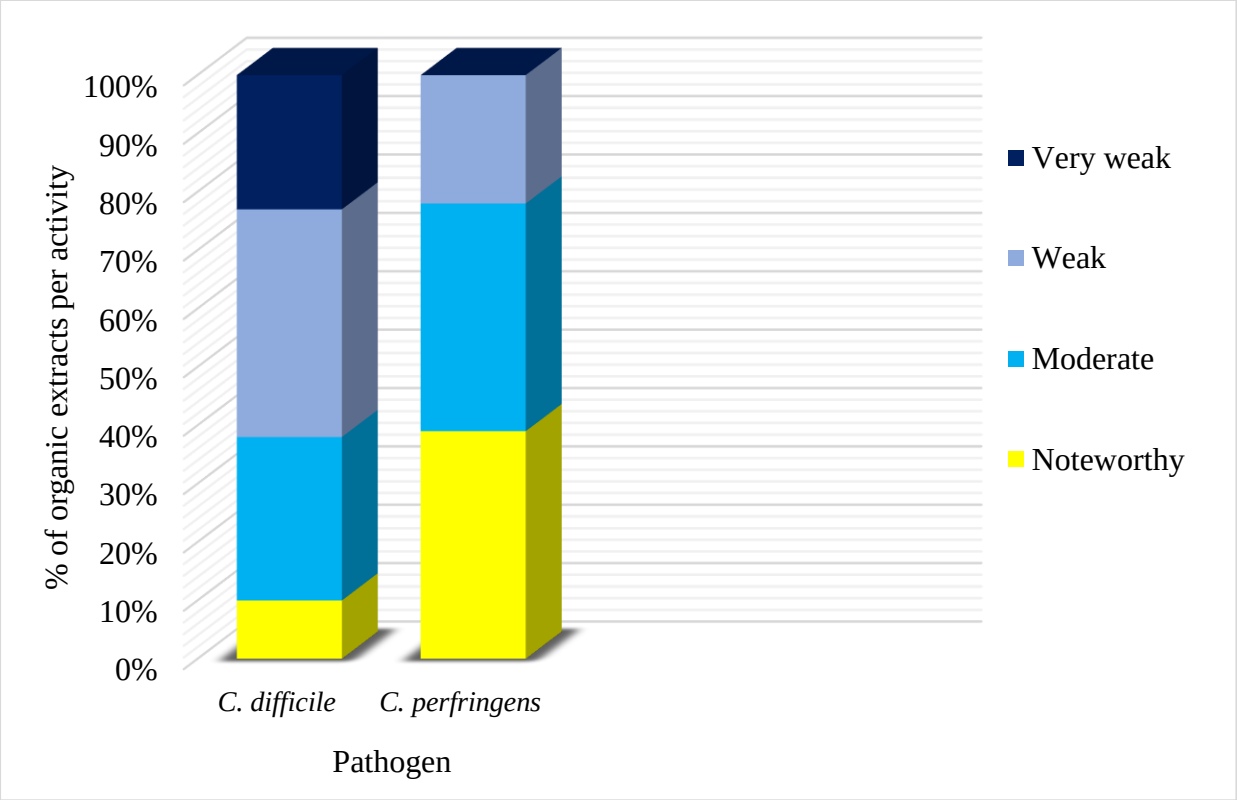


Figure 2.1: The antimicrobial activities of organic extracts against Gram–positive bacteria.

The organic extracts of *L. javanica* leaf showed the best antimicrobial activity with an MIC value of 0.5 µg/ml against *C. perfringens*. This value was comparable to the control antibiotics ciprofloxacin (MIC = 0.2 µg/ml). The organic extracts of *L. javanica* small twigs displayed noteworthy activity against *C. perfringens* with an MIC value of 2 µg/ml. The traditional use of *L. javanica* corroborates with its antimicrobial activity against *Clostridium* species as the leaf infusion is traditionally used for diarrhoea, which is one of the symptoms of food poisoning or pseudomembranous colitis (Hutchings *et al*., 1996; van Wyk, 2008; van Wyk *et al*., 2009;

Maroyi, 2017). Even though *L. javanica* displayed the best antimicrobial activity, to the best of our acquaintance no report on this plant against any of the neglected micro–organisms have been previously documented. Other studies have instead focused on the antimicrobial activity of this plant against common pathogens (e.g. *S. aureus*) of the gut (Samie *et al*., 2009; Shikanga *et al*., 2010). A study by Shikanga *et al*. (2010) demonstrated that the organic extracts showed noteworthy activity with a mean MIC value of 130 µg/ml against *S. aureus*.

The organic extracts of *G. perpensa* (leaf and rhizome) (2 and 130 µg/ml), as well as the leaf extracts of *S. africana–caerulea* (130 and 30 µg/ml), *S. africana* (130 µg/ml), *S. cordatum* (130 µg/ml) and *T. riparia* (130 µg/ml) displayed noteworthy activities against both *Clostridium* species.

Traditionally, unspecified parts of *G. perpensa* are used for the treatment of stomach bleeding and the roots are used for other stomach ailments (van Wyk, 2008; van Wyk *et al*., 2009; McGaw *et al*., 2010). To date, no previous studies have been reported on the antimicrobial effectiveness of this plant on neglected pathogens. Nevertheless, findings from the current study were comparable to those reported in literature, where Madikizela *et al* .(2017) reported good activity for the organic extracts of *G. perpensa* (leaf) against the gut pathogens *Campylobacter jejuni*, *E. coli*, *S. aureus*, and *Shigella flexineri*, with MIC values ranging between 0.39 mg/ml – 0.78 mg/ml. Mabona *et al*. (2013) highlighted good antimicrobial effects of the organic extract of *G. perpensa* leaf against fastidious skin pathogens.

Traditionally the twigs and leaf infusions of *S. africana–caerulea* are mixed with Epsom salts (magnesium sulphate) and lemon to treat stomach illness such as colic, diarrhoea, indigestion, stomach pain, and abdominal trouble (Kamatou *et al*., 2008; van Wyk *et al*., 2009). To the best of our knowledge, no antimicrobial study was found with regards to *S. africana–caerulea* and the neglected gut pathogens selected for this study. Several other studies, however, have reported on the antimicrobial findings of *S. africana–caerulea* other gut micro–organisms. An example of this is a study conducted by Nielsen *et al*. (2012), where the organic extracts of one of the subspecies of *Salvia africana* (*S. africana–lautea*) displayed good inhibitory activity against *S. aureus* and *E. coli* with a mean MIC value of 39.12 µg/ml.

S. africana is commonly known as the "jumping–bean tree" and it is traditionally used for the treatment of stomach ulcers, stomach pain, dysentery, acute gastritis and diarrhoea (Hutchings

et al., 1996, Mathabe *et al*., 2008). The antimicrobial effects of *S. africana* on other pathogens has also been reported (Amoo *et al*., 2013) where the leaf and twigs showed good activity against *S. aureus* with MIC value of 0.78 mg/ml.

The antimicrobial activity of *S. cordatum* corroborates with the traditional use as the bark is crumpled and transferred to water and boiled, then the mixture is taken orally three times a day, until diarrhoea is treated (van Wyk *et al*., 2009; de Wet *et al*., 2010). Mathabe *et al*. (2006) reported *S. cordatum* to be effective against a wide variation of diarrhoeal pathogens, including *S. aureus, E. coli*; *S. typhi*, *Vibrio cholerae* as well as *Shigella* species with MIC values ranging between 0.16 – 0.31 mg/ ml.

In previous studies, *T. riparia* showed good antimicrobial activity against common pathogens of the gut (Amoo *et al*., 2013; Akhalwaya *et al*., 2018). *Tetradenia riparia* is a multiple branched shrub or small tree plant of which the leaf infusions are traditionally used for stomach ache and diarrhoea (van Wyk *et al*., 2009). No study was found on the antimicrobial activity of *T. riparia* and *Clostridium* species. In a previous study by Amoo *et al*. (2013), *T. riparia* was found to be active against *S. aureus* with an MIC value of 0.78 mg/ml. Good antimicrobial activity of *T. riparia* was also noted against oral pathogens (Akhalwaya *et al*., 2018).

Other extracts that displayed noteworthy activity against *C. perfringens* include *A. oppositifolia* (MIC 130 µg/ml), *A. arborescens* (MIC 30 µg/ml), *A. marlothii* (MIC 130 µg/ml), *A. tenuior* (MIC 2 µg/ml), *A. venosum* (MIC 60 µg/ml), *A. afra* (MIC 8 µg/ml), *B. micrantha* (MIC 130 µg/ml), *P. fruticosa* (MIC 20 µg/ml), *S. incanum* (MIC 130 µg/ml), *S. cordatum* (MIC 130 µg/ml). The noteworthy antimicrobial activity, obtained from the organic extracts from *L. javanica, G. perpensa, and A. tenuior* (Table 2.3), may imply that dichloromethane: methanol could be a better solvent for the extraction of anti–*C. perfringens* compounds from these plants.

2.3.2.2. Antimicrobial activity of organic extracts against Gram–negative bacteria.

This category included the eight bacteria which were further divided into two classes namely Gram–negative anaerobes (*B. fragilis, B. ovatus, B. thetaiotaomicron, B. vulgatus, F. nucleatum* and *F. varium*) and Gram–negative microaerophiles (reference and the clinical strain).

2.3.2.2.1. Gram–negative anaerobes.

Three extracts displayed pathogen specific noteworthy activity against this category. A total of 37 of the organic extracts displayed moderate activities against one or more Gram–negative anaerobes. The organic extracts of *L. javanica* (leaf) exhibited the best antimicrobial activity in this category, where it was active against *B. fragilis and B. vulgatus*, with MIC values of 20 µg/ml. Other plant extracts that were active in this category include *P. fruticosa*, which was active against *B. thetaiotaomicron* and *F. nucleatum* with MIC value of 130 µg/ml. and *S.* puniceus which was active against *B. thetaiotaomicron* with an MIC value of 130 µg/ml. *Polygala fruticosa* (roots) are used traditionally for the management of intestinal sores (van Wyk *et al*., 2009). In a previous study undertaken by van Vuuren and Naidoo (2010), activity of *P. fruticosa* against the sexually transmitted pathogen *Gardnerella vaginalis* was reported with a mean MIC value of 200 µg/ml. Figure 2.3 summarizes the activity or organic extracts against Gram–negative anaerobes.

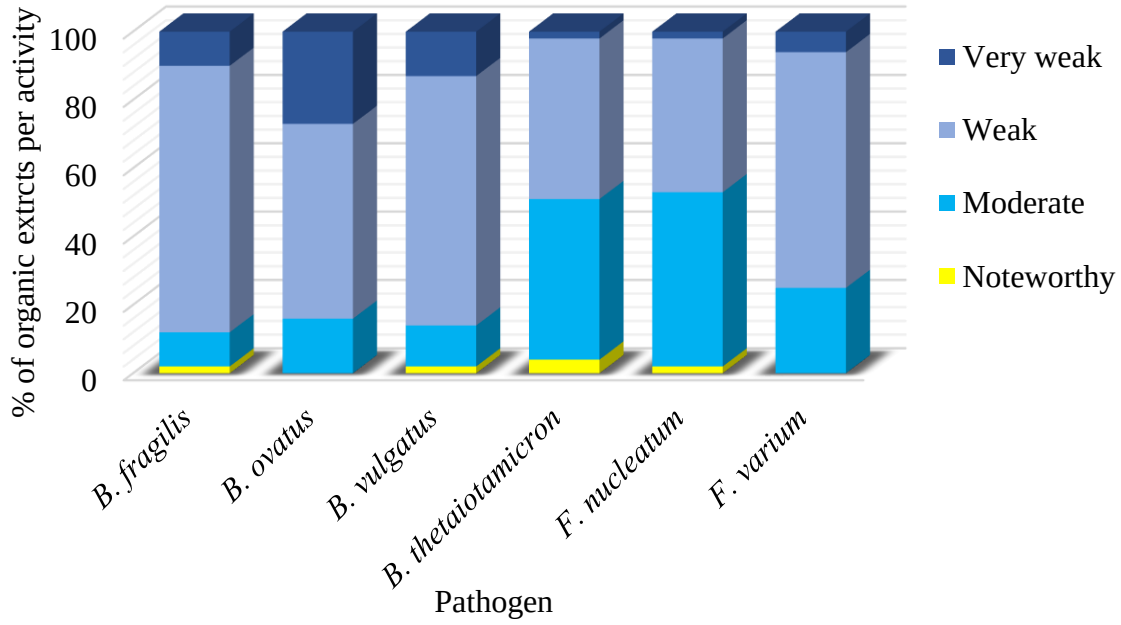


Figure 2.2: Antimicrobial activity if organic extracts against Gram–negative anaerobes

2.3.2.2.1. Gram–negative microaerophiles

Microaerophiles included the *Helicobacter* spp., which are a group of micro–organisms that require oxygen to survive, but need atmospheres containing lower amount of oxygen than are (Prescott *et al*., 1996; Hogg, 2005). With regards to susceptibility, this category was the least

susceptible and the reference strain was more susceptible to the medicinal plant extracts when compared to the clinical strain. The organic extracts of *A. arborescens* displayed the best antimicrobial activity with the MIC value of 130 µg/ml against the reference strain. *Aloe arborescens* is widely cultivated and used for its therapeutic and cosmetic purpose (Jia *et al*., 2008). Comparative studies regarding the anti–*Helicobacter* activities of *A. arborescens* was not found in the literature, however, it is not surprising that this species displayed good activities against *H. pylori*, because a decoction of the fresh leaves of *Aloe* species are traditionally used for management of related infections (Njume *et al*., 2011; Wintola *et al*., 2017). Figure 2.3 summarizes the antimicrobial activity if organic extracts against Gram–negative microaerophiles.

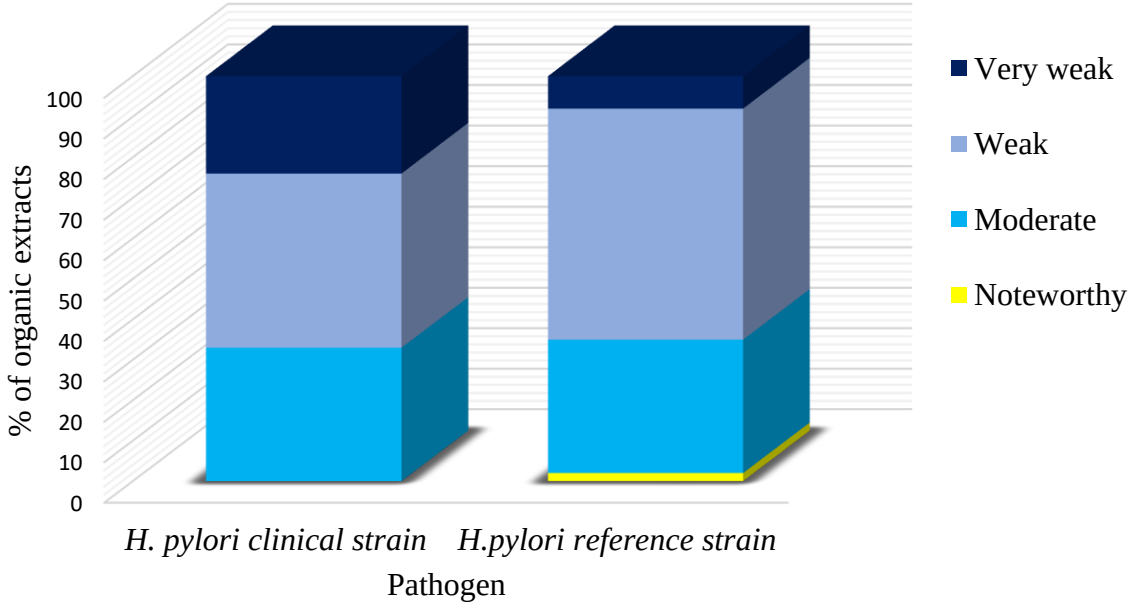


Figure 2.3: The antimicrobial activities of organic extracts against Gram–negative microaerophiles (*H. pylori*)

The difference in sensitivity between the Gram–positive and Gram–negative bacteria may be due to the morphological differences between these micro–organisms (Paiva *et al*., 2010). Gram–negative bacteria have an outer phospholipidic membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to lipophilic solutes, while porins constitute a selective barrier to the hydrophilic solutes (Paiva *et al*., 2010). The Gram–positive bacteria are more susceptible because they only consist of an outer peptidoglycan layer which is not an effective permeability barrier (Arias *et al*., 2004).

2.3.3. Overall antimicrobial effects of organic extracts on neglected gut pathogens.

About 41% of the tested extracts displayed pathogen specific noteworthy activities, whereas 59% displayed moderate to poor activities. Figure 2.4 represent the overall antimicrobial effects of organic extracts on neglected gut pathogens.

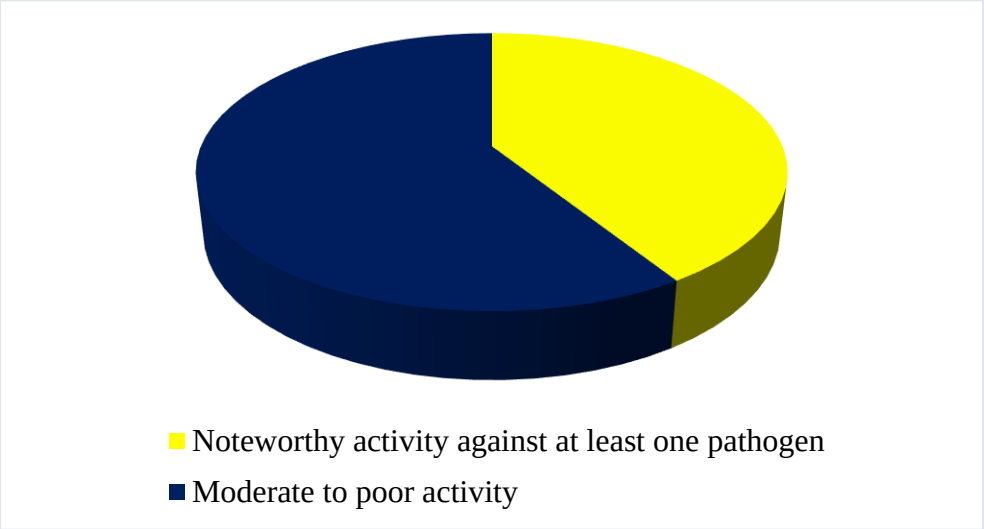


Figure 2.4: Overall antimicrobial effects of organic extracts on neglected gut pathogens

2.3.4. Comparative analysis of the antimicrobial efficacies of organic extracts of leaf vs other plant parts.

On occasions where leaf and other plant parts were tested, leaf extracts proved to have the best antimicrobial activities. *Lippia javanica* leaf proved to have the best antimicrobial activity with mean MIC value of 970 µg/ml, followed by *S. africana* leaf which had the mean MIC value of 1000 µg/ml. The mean MIC value of both leaf and rhizomes of *B. micrantha* displayed the

same mean MIC values (MIC 2700 µg/ml). The leaf extracts of *P. africanum* and *W. salutaris* displayed poor mean MIC results when compared to the other plant parts. The mean MIC values for various plant parts can be seen in Figure 2.1. Lewu *et al*. (2006) recommended that leaf must be used to substitute the traditionally used bark or root to promote sustainable harvesting. With exceptions of *P. africana* and *S. africana –caerulea* (other plants had better activity as compared to leaf material) as well as *B. micrantha* (both plants part displayed equal mean MIC value), leaf material displayed better activities. The current findings revealed that most leaf material had better activity as compared to other plant parts, it can be strongly recommended that traditional formulations which utilizes *A. oppositifolia* (root), *B. micrantha* (stem), *D. rotundifolia* (stem), *K. africana* (fruit and stem), *L. javanica* (twigs) *R. melanophloeos* (stem), *W. salutaris* (stem) be substituted with leaf material. Figure 2.5: is the average antimicrobial activity of leaf extracts vs other plant parts (leaf, roots, stem, twigs against neglected gut pathogens).

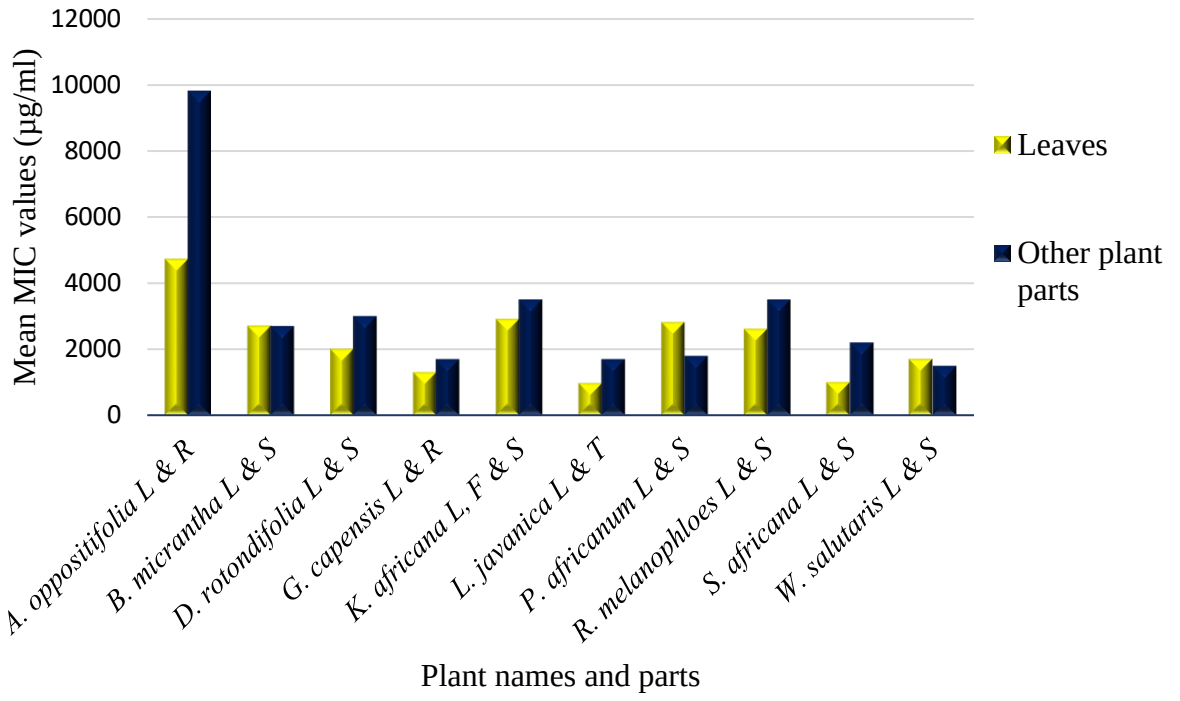


Figure 2.5: Average antimicrobial activity organic extracts of leaf and other plant parts i.e. roots, stem, twigs against neglected gut pathogens.

2.3.5. Aqueous extracts

A total of 52 aqueous extracts were screened against 12 pathogens studied. The results of the antimicrobial assay for the aqueous extracts expressed as MIC values are represented in Table 2.4. Values in bold denotes noteworthy activity, whereas values in italics denote moderate activity. Gram–positive bacteria were the most susceptible to aqueous extracts. Most of the extracts displayed weak–very poor activities. Only two aqueous extracts displayed noteworthy activities. This was seen in on *Clostridium* species. *C. perfringens* was the most vulnerable to the effects of organic extracts with 13 of the extracts displaying moderate activities. It was seconded by *C. difficile* in which 6 of the extracts displayed moderate activities. The aqueous extracts of *P. fruticosa* displayed moderate activities against three of the 10 pathogens studied: this included both the Gram–positive and Gram–negative pathogens.

The Gram–positive bacteria were the most susceptible to aqueous extracts. Most of the extracts displayed weak–very poor activities. Only two aqueous extracts displayed noteworthy activities. This was seen in on *Clostridium* species. *C. perfringens* was the most vulnerable to the effects of aqueous extracts with 13 of the extracts displaying moderate activities. It was seconded by *C. difficile* in which 6 of the extracts displayed moderate activities. The aqueous extracts of *P. fruticosa* displayed moderate activities against three of the 10 pathogens studied: this included both the Gram–positive and Gram–negative pathogens.

The aqueous extracts of *G. perpensa* rhizome displayed noteworthy activity against both *C. perfringens* and *C. difficile* with MIC values of 130 µg/ml. These were the only aqueous extracts that showed noteworthy activity against any of the pathogens studied. Buwa and van Staden (2006) reported the antimicrobial properties of *G. perpensa* root where the aqueous extracts of the plant were screened against various other bacterial pathogens. It was reported that the aqueous extracts of *G. perpensa* root demonstrated antimicrobial activity with a MIC value of 0.78 mg/ml. Mabona *et al*. (2013) examined the antimicrobial efficacy aqueous extracts of *G. perpensa* leaf and rhizomes against pathogens responsible for skin infections. They reported that the aqueous extract of *Gunnera perpensa* leaf exhibited antimicrobial activity against *S. aureus* with MIC values between 0.5 and 1.00 mg/ml (Mabona *et al*., 2013).

Table 2.3: MIC (µg/ml) results for aqueous plant extracts

Plant extract	Plant part used	Mean MIC value (µg/ml)											
		Gram positive		Gram negative								Commonly screened pathogens	
		Clostridium Species		*B. fragilis* Group				*Fusobacterium* species		*Helicobacter pylori*			
		C. d	C. p	B. f	B. o	B. v	B.t	F. n	F. v	H. p c	H. p r	E. c	E. f
A. oppositifolia	Leaf	>8000	>8000	8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000
	Root	*500*	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	2000	2000
A. arborescens	Leaf	>8000	>8000	8000	>8000	8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000
A. ferox	Leaf	*1000*	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	2000
A. marlothii	Leaf	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000
A. tenuior	Leaf	2000	>8000	>8000	>8000	8000	>8000	>8000	>8000	>8000	>8000	2000	4000
A. venosum	Leaf	8000	>8000	8000	4000	8000	>8000	>8000	8000	4000	4000	4000	4000
A. afra	Leaf	>8000	>8000	8000	>8000	3000	>8000	>8000	>8000	>8000	>8000	4000	>8000
B. disticha	Bulb	1500	*1000*	>8000	2000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000
B. cathartica	Leaf	8000	*1000*	>8000	4000	4000	>8000	>8000	>8000	>8000	>8000	4000	>8000
B. micrantha	Leaf	3000	*250*	4000	2000	4000	>8000	>8000	>8000	>8000	>8000	4000	2000
	Stem	>8000	*190*	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000
C. edulis	Leaf	>8000	4000	>8000	>8000	8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000

Plant extract	Plant part used	Mean MIC value (µg/ml)											
		Gram positive		Gram negative								Commonly screened pathogens	
		Clostridium Species		*B. fragilis* Group				*Fusobacterium* species		*Helicobacter pylori*			
		C. d	**C. p**	**B. f**	**B. o**	**B. v**	**B.t**	**F. n**	**F. v**	**H. p c**	**H. p r**	**E. c**	**E. f**
C. ambrosioides	Leaf	3000	8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000
C. brachiata	Stems	2000	>8000	8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	2000
D. cinerea	Bulb	4000	*250*	8000	>8000	>8000	>8000	>8000	4000	8000	8000	4000	4000
D. viscosa	Leaf	>8000	4000	>8000	>8000	>8000	8000	>8000	>8000	8000	>8000	>8000	>8000
D. rotundifolia	Leaf	8000	*500*	>8000	>8000	4000	>8000	>8000	8000	>8000	>8000	2000	2000
	Stem	8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	*500*	*500*
D. maculata	Bulb	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	8000	>8000	>8000
E. capensis	Leaf	8000	*1000*	>8000	>8000	8000	>8000	>8000	>8000	>8000	8000	>8000	>8000
E. elephantina	Roots	*1000*	*500*	>8000	>8000	3000	>8000	4000	8000	8000	4000	>8000	>8000
E. automnalis	Leaf	8000	4000	>8000	>8000	3000	>8000	8000	>8000	>8000	>8000	>8000	>8000
G. perpensa	Leaf	**130**	>8000	>8000	*1000*	>8000	4000	4000	8000	2000	>8000	1000	1000
	Rhizomes	**130**	**130**	>8000	4000	8000	*1000*	>8000	>8000	8000	>8000	1000	1000
H. arborescens	Leaf	4000	8000	>8000	>8000	8000	8000	>8000	>8000	>8000	8000	>8000	>8000
I. purpurea	Leaf	>8000	8000	>8000	*500*	>8000	8000	>8000	>8000	>8000	>8000	>8000	>8000
K. africana	Leaf	3000	8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000

Plant extract	Plant part used	Mean MIC value (µg/ml)											
		Gram positive		Gram negative								Commonly screened pathogens	
		Clostridium Species		*B. fragilis* Group				*Fusobacterium* species		*Helicobacter pylori*			
		C. d	**C. p**	**B. f**	**B. o**	**B. v**	**B.t**	**F. n**	**F. v**	**H. p c**	**H. p r**	**E. c**	**E. f**
	Fruit	>8000	8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	8000	>8000	>8000
	Stem	*1000*	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000
L. javanica	Leaf	>8000	*250*	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	4000
	Twigs	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000
M. longifolia	Leaf	3000	8000	>8000	>8000	>8000	>8000	>8000	>8000	2000	4000	>8000	>8000
O. asteriscoides	Leaf	*1000*	4000	8000	>8000	>8000	8000	>8000	>8000	4000	4000	>8000	4000
O. corniculata	Leaf	*750*	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	4000	>8000	>8000
P. africanum	Leaf	>8000	2000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	2000	2000	2000
	Stems	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	8000	>8000	>8000
P. fruticosa	Leaf	>8000	*500*	8000	>8000	>8000	2000	*1000*	*1000*	4000	>8000	>8000	>8000
R. melanophloeos	Leaf	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	4000	4000
	Stem	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	8000	2000	4000
R. caffra	Leaf	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	4000	>8000	>8000
S. africana caerulea	Leaf	>8000	*1000*	>8000	2000	>8000	>8000	>8000	>8000	2000	8000	*1000*	*500*

Plant extract	Plant part used	Mean MIC value (µg/ml)											
		Gram positive		Gram negative								Commonly screened pathogens	
		Clostridium Species		*B. fragilis* Group				*Fusobacterium* species		*Helicobacter pylori*			
		C. d	C. p	B. f	B. o	B. v	B.t	F. n	F. v	H. p c	H. p r	E. c	E. f
S. puniceus	Rhizomes	>8000	>8000	>8000	8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000
S. incanum	Leaf	4000	4000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000
S. africana	Leaf	>8000	*1000*	>8000	>8000	2000	>8000	>8000	>8000	4000	8000	>8000	2000
	Stems	8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000
S. cordatum	Leaf	4000	*1000*	*500*	*1000*	8000	>8000	>8000	8000	2000	>8000	2000	*1000*
T. camphoratus	Leaf	>8000	8000	>8000	>8000	8000	>8000	>8000	>8000	4000	>8000	8000	4000
T. riparia	Leaf	>8000	2000	>8000	>8000	2000	>8000	>8000	>8000	2000	>8000	>8000	4000
W. salutaris	Leaf	>8000	>8000	>8000	>8000	3000	>8000	>8000	>8000	>8000	*1000*	>8000	>8000
W. salutaris	Stem	>8000	8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	*1000*	2000	2000
Z. capense	Leaf	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000
Positive control		**0.16[d]**	**0.16[c]**	**0.04[e]**	**0.08[e]**	**0.08[e]**	**0.08[e]**	**0.08[c]**	**0.08[c]**	**0.08[a]**	**0.04[a]**	**0.04**	**0.63**
Negative control		>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000
Culture control		>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000

C. d: *C. difficile* (ATCC 43593); C. p: *C. perfringens* (ATCC 13124); B. f: *B. fragilis* (ATCC 23745); B. o: *B. ovatus* (ATCC 8483); B. t: *B. thetaiotaomicron* (ATCC 29741); B. v: *B. vulgatus* (ATCC 8482); F. o: *F. varium* (ATCC 27725); F. n: *F. nucleatum* (ATCC 25586); H. p c: (548) clinical strain; H. p r: (B8) reference strain; E. c: *E. coli*; E. f: *E. faecalis*; [a] Metronidazole; [b] Ciprofloxacin; [c] Imipenem; [d] Amoxicillin: clarithromycin.

2.3.6. Overview of antimicrobial activity of aqueous extracts.

Most of the aqueous extracts displayed weak to very poor activities. Only 4% displayed pathogen specific noteworthy activities. Figure 2.6: Representation of overall antimicrobial effects of aqueous extracts on neglected gut pathogens.

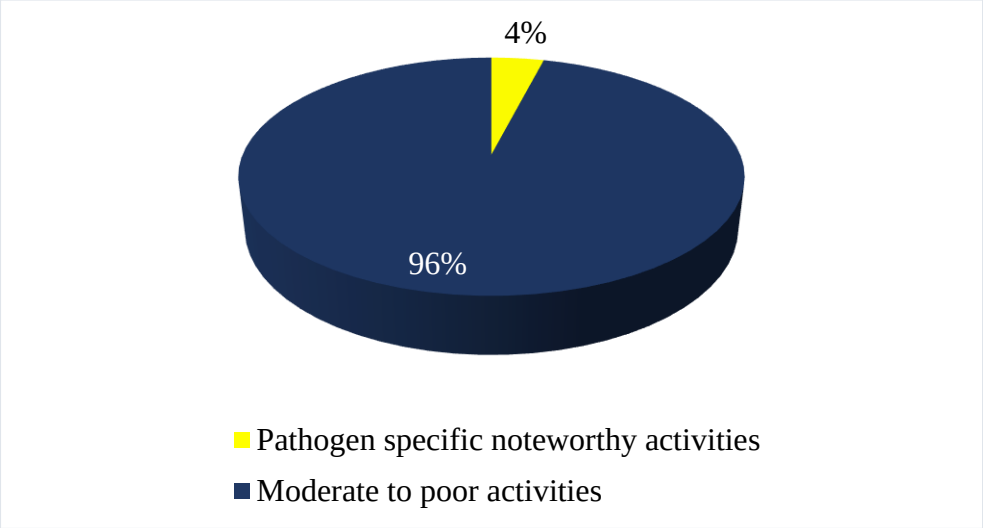


Figure 2.6: Overall antimicrobial effects of aqueous extracts on neglected gut pathogens

2.4. Antimicrobial activity of organic extracts against common pathogens.

Of all the 102 plant extracts tested, none of the plant extracts displayed noteworthy activity against the common pathogens. Which simply means that, some plants that have poor antimicrobial activity against common pathogens can yield good activity against fastidious pathogens.

2.5. Chapter Summary

- The organic extracts of *L. javanica* and *G. perpensa* demonstrated the most significant MIC activity of 0.5 µg/ml and 2 µg/ml against *C. perfringens*.
- *Gunnera perpensa* leaf and rhizomes displayed the best activity in the aqueous extract's category with MIC value of 130 µg/ml against *C. difficile* and *C. perfringens*.
- No activity was noted against neglected pathogens.
- Most extracts were active against *Clostridium* species.

CHAPTER 3

Clostridium antibiofilm of plants used for stomach ailments

3.1. Introduction

It was recently discovered in 2008, that *C. perfringens* has the ability to form biofilms (Varga *et al*., 2008). Type IV pilus together with the CcpA protein are the two factors responsible for maximal biofilm formation in *C. perfringens* (Varga *et al.*, 2008). Biofilms formed by *C. perfringens* are found problematic as they can tolerate the stress arising from antibiotics, disinfectants, as well as physical and environmental stresses (Varga *et al*., 2008; Charlebois et al., 2017). *C. perfringens*–biofilm increases the survival of bacterial cells by protecting them from atmospheric oxygen and from increased concentrations of antibiotics and anticoccidial agents (Charlebois *et al*. (2014). Although antibiofilm activities of medicinal plants against other micro–organisms were investigated, no previous study was found in the antibiofilm activity of medicinal plants against *C. perfringens*. In this study, anti–*C. perfringens*–biofilm effectiveness of selected medicinal plants were analysed.

3.2. Methods

3.2.1. Plant extract preparation

Plant extracts that exhibited noteworthy (≤160 µg/ml) MIC values against *C. perfringens* (Chapter 2, Table 2.3) were selected for biofilm studies. Samples were immersed in sterile water, 2 mg/ml was used as a stork concentration.

3.2.2. Antibiofilm analysis

The effects of plant extracts on preformed biofilm were tested using the method described by Sandasi *et al*. (2011), where testing the prevention of new biofilms and the dispersion of preformed biofilms were described. The method of testing was performed as follows; a few colonies of *C. perfringens* were transferred to a test tube containing Thioglycolate broth and incubated overnight according to optimal incubation conditions (Chapter 2). After 24 h, 100 µl of the overnight culture was transferred to wells of a 96–well micro–titer plates. Aabsorbance readings using a microplate reader (Universal microplate reader ELX 800) were undertaken to confirm biofilm formation. Acceptable absorbance is approximated at 0.02 absorbance units, as this absorbance indicates that the culture contains approximately 0.5 McFarland's standard as used for the MIC assay (Chapter 2, Table 2). If absorbance of the liquid cultures was found to be more than this, cultures were diluted in sterile broth until the desired absorbance values were reached. Once the desired absorbance was reached, the culture could be used as the stock culture in the assay.

Biofilms were formed in 96 well micro–titer plates by adding 100 µl of stock culture into the wells and incubated for 4 h, 24 h, 48 h, and 72 h at 37 ºC. A blank column containing sterile Thioglycolate broth, a negative control column containing sterile broth and culture, and a positive control column containing the ciprofloxacin (0.0025 mg/ml) were also included. After incubation, 100 µl of each plant extract was transferred to a final concentration of 1 mg/ml in the wells. Plates were incubated overnight at 37 ºC, after which the crystal violet (CV) assay was performed and the biofilm biomass was determined. The percentage inhibition was calculated using Equation 3.1 (Sandasi *et al*., 2011):

Equation 3.1: The percentage inhibition of Southern medicinal plants against *C. perfringens*

$$\% \text{ inhibition} = \frac{\text{Optical density (OD) Culture control} - \text{OD Experimental} \times 100}{\text{OD Culture control}}$$

3.2.3. Crystal violet staining assay

The crystal violet (CV) assay was done to evaluate the ability of the extracts to prevent and eradicate biofilms. This was done by washing the incubated plates with sterile water and oven–drying them at 60 ºC for 45 min. Once they have dried, all the wells were tainted with 200 µl of 1% crystal violet, they were then left at room temperature for 15 min, to allow for proper absorption of the stain. This was followed by washing the plates with sterile water three times to remove the unabsorbed stain. After that, an amount of 125 µl ethanol was added to remove the stain from the wells. A volume of 100 µl of the destaining solution was transferred to a new micro–titer plate and the absorbance was determined at 590 nm using a micro–plate reader (Universal micro – plate reader ELX 800). The mean absorbance of the extracts was determined prior to calculating the percentage inhibition. All tests were repeated four times for reproducibility.

3.3. Results and discussion

The antibiofilm activity of 21 medicinal plant extracts (19 organic and two aqueous extracts) that displayed antimicrobial activity (MIC ≤160 µg/ml) against *C. perfringens* (Chapter 2, Table 2.3.) were individually screened at 1 mg/ml. Results are categorized into four phases corresponding to biofilm developmental stages. Results for antibiofilm activities are categorized into four phases corresponding to biofilm developmental stages. Firstly, the initial attachment of biofilms is represented at 4 h; biofilm formation at 24 h; and development of a mature biofilm at 48 h and 72 h. The results are presented in Table 3.1 and are interpreted either as weak antibiofilm activity (0–49%) or good antibiofilm activity (50–100%) (Sandasi *et al*., 2010). Negative percentage inhibition results denote enhancement, rather than inhibition of biofilms. Values in bold typeface denote good antibiofilm activity.

At initial cell attachment stage (4 h), 19% of the extracts had antibiofilm inhibitory activities with at least 50% reduction in cell attachment. Approximately 57% of the extracts displayed good antibiofilm development (24 h) with percentage > 50% – many of the extracts had better activity than ciprofloxacin, whereas 38% of extracts displayed good antibiofilm activity stopping the development of mature biofilm (48 h and 72 h). Except for the organic extracts of *A. tenuior*, *B. cathartica*, and *B. micrantha* all the other extracts displayed good antibiofilm activities on at least one stage of biofilm development.

Table 3: The antibiofilm activity of plant extracts against *C. perfringens*

Plant extracts	% Inhibition				
	Plant part used	**4 h biofilm**	**24 h biofilm**	**48 h biofilm**	**72 h biofilm**
Organic extracts					
A. oppositifolia	Leaf	30.4	**64.8**	**52.1**	**59.6**
A. arborescens	Leaf	43.9	**62.9**	46.5	10.1
A. ferox	Leaf	31.4	**65.8**	45.8	**61.4**
A. marlothii	Leaf	43.4	39.8	**57.1**	**61.6**
A. tenuior	Leaf	38.9	40.4	14.0	07.9
A. venosum	Leaf	17.6	**59.9**	12.2	40.2
A. afra	Leaf	37.5	**53.7**	23.5	**75.1**
B. cathartica	Leaf	–95.1	–33.0	–31.3	21.7
B. micrantha	Stem	18.2	46,1	08,7	45.3
G. perpensa	Leaf	**57.7**	**77.6**	**50.1**	23.8
	Rhizomes	39.1	**78.8**	**60.9**	**55.8**
L. javanica	Leaf	45.4	**77.2**	34.4	42.7
	Small twigs	**59.5**	**57.5**	**52.6**	39.2
P. fruticosa	Leaf	42.4	41,3	**50.4**	38.9
S. africana	Leaf	**82.8**	49.1	37,1	16.2
S. incanum	Leaf	34.9	–5.5	**55.3**	15.6
S. africana	Leaf	30.8	**71.6**	16.8	**51.5**
S. cordatum	Leaf	24.2	4.0	32.6	**62.1**
T. riparia	Leaf	**51.9**	**73.2**	**77.9**	13.3
Aqueous extracts					
G. perpensa	Leaf	32.4	5.1	42.5	26.7
	Rhizomes	–35.9	–36.9	42.5	**73.8**

Ciprofloxacin	**70.4**	**58.7**	**68.5**	**68.3**

[a] Values in bold are the plants that displayed good antibiofilm activity per time interval

The organic extracts of *A. oppositifolia* (leaf), *G. perpensa* (leaf), *L. javanica* (twigs) and *T. riparia* (leaf) displayed good antibiofilm activities at least three biofilm developmental stages. Although there was no previous study on the antibiofilm activities *of A. oppositifolia,* in the current study *A. oppositifolia* displayed good antibiofilm at 24 ,48 and 72 h preventing biofilm formation and formation of mature biofilms. *A. oppositifolia* displayed poor activity at times 0 and 4 h. It can thus be concluded from this results that *A. oppositifolia* worked better on older biofilm. At 24 h the activity of *A. oppositifolia* was greater than that of ciprofloxacin (64.8% vs 58.7%).

The organic extracts of *G. perpensa* (leaf) was active at 4, 24 and 48 h, preventing the attachment, formation and development of mature biofilm. Whereas the organic extracts of *G. perpensa rhizomes* was active at 24, 48 and 72 h. *G. perpensa* extracts were mostly active against older biofilms.

L. javanica twigs was active at times 4, 24 and 48 h with similar inhibition percentages. This suggests that *L. javanica* is not incubation period dependent and can work at any stage of biofilm development. Concerning best activity in the MIC assay (Chapter 2, Table 2.2), it is very interesting to note that it did not only work best on planktonic cells of *C. perfringens* only but also worked best on the *C. perfringens*–biofilms as it was one of the extracts that displayed good antibiofilm activities at least three biofilms developmental stages. This findings support the finding by Andriani *et al*. (2017), in this study they found out that the same plant extracts that had good antibacterial activity also had good antibiofilm activity.

The organic extracts of *S. africana–caerulea* stood out with the highest antimicrobial activities at 4 h but had poor activities at 24, 48 and 72 h. At 4 h, *S. africana–caerulea* reduced cell attachment with better reduction percentage when compared to that of ciprofloxacin (82.79% vs 70.35%). The activities of *S. africana–caerulea* decreased with an increase in incubation period, thus it can be concluded that *S. africana–caerulea* worked better on new biofilms.

The organic extracts of *T. riparia* (leaf) demonstrated notable antibiofilm activity, at times 4, 24, and 48 h preventing cell attachment, stopping development of biofilm, and development of mature biofilm. At 72 h *T. riparia* displayed poor antibiofilm activities, meaning that it works better on premature biofilms than on matured biofilms.

There is little knowledge on the antibiofilm activities of the plant extracts tested in this study. This is the first study to report on the antibiofilm activity of plant extracts on *C. perfringens*–biofilms. Globally, most plant–based studies have focused on the antibiofilm activities of medicinal plants against biofilm formers such as *E. coli*, *S. aureus* and *Pseudomonas aeruginosa* (Quave *et al*., 2008; Varposhti *et al*., 2013; Costa *et al*., 2015; Bazargani and Rohloff, 2016). For southern African plant species, studies undertaken on antibiofilm activities have been neglected. Only a few relevant studies have been investigated (van Vuuren and Holl, 2017). Most of these have focused on the antibiofilm activities of Southern African medicinal plants against gut pathogens such as *Listeria monogenes*, *P. aeruginosa*, and *C. albicans* (Leonard *et al*., 2010; Sandasi *et al*., 2010; Sandasi *et al*., 2011; Nyila *et al*., 2012). Some antibiofilm activities of Southern African medicinal plants have been investigated against the oral pathogen *Streptococcus mutans* (Naidoo *et al*., 2012; Akhalwaya *et al*., 2018).

An example of a more recent antibiofilm studies is a study by Akhalwaya *et al*. (2018), who carried out a study to assess the activities of several Southern Africa medicinal plants including *S. africana* leaf, that are used traditionally to treat oral infections against *S. mutans*–biofilms. Where they yielded good activity with reduction percentage ranging from 50.21% – 97.57% for dichloromethane: methanol extracts including that of *S. africana* leaf.

The current study showed that some plant extracts that showed good antimicrobial activities against *C. perfringens* on the MIC assay are capable of inhibiting *C. perfringens*–biofilms. Prevention of cell attachment proved to be more difficult to achieve than prevention of biofilm development and development of mature biofilm. It is very surprising that many extracts displayed better activity at biofilm development stage (24 h) than at cell attachment stages (4 h) as previous studies reported that inhibiting initial cell attachment is easier than inhibiting preformed biofilms (Sandasi *et al*., 2010).

3.4. Chapter summary

- Approximately 90% of the extracts were active on least at one – biofilm developmental stage.
- Plant extracts seemed to attack biofilms better at (24 h) where 57% of the extracts stopped the development of biofilms by at least 50%.
- *S. africana–caerulea* showed the best antibiofilm activity at cell attachment stage (4 h) where it exhibited the percentage inhibition of 82.79%.
- The organic extracts of *G. perpensa* leaf and rhizome displayed the best antibiofilm activities with percentage inhibitions of 77.6 and 78.8 respectively.
- Against 48 h biofilm, the organic extracts of *G. perpensa* leaf displayed the best antibiofilm activity inhibiting the mature biofilm by 60.9%.
- The organic extracts of *A. afra* displayed the best antibiofilm activity against 72 h biofilm inhibiting the mature biofilm by 75.1%
- Organic extracts of *A. oppositifolia* leaf, *G. perpensa* leaf, *L. javanica* twigs and *T. riparia* leaf displayed good antibiofilm activities at three different time frames.

CHAPTER 4

Toxicity analysis of plants used for stomach ailments

4.1. Introduction

The brine shrimp lethality assay (BSLA) is a simple method that is used primarily to test for toxicity of plant extracts and other natural products and it was first described by Michael et al. (1956). It was then successively employed by Meyer *et al*. (1982). The BSLA is considered a simple and cost effective method that requires a small amount of test material, and does not require special equipment (Sarah *et al*., 2017). Hence it was selected for the current study to screen for the toxic effects of plant extracts that demonstrated good antimicrobial activity (see Chapter 2).

4.2. Methods

4.2.1. Brine shrimp hatching.

In order to hatch brine shrimp larvae, artificial sea water was prepared by dissolving 16 g of Tropic Marine® salt in 500 ml of sterile water. Thereafter, 0.5 g of brine shrimp larvae (*Artemia franciscana*) (Ocean Nutrition) was added to the prepared sea water. Sea water was selected because it promotes the growth for brine shrimp larvae. A mixture containing the brine shrimp larvae and sea water was exposed to a constant light. Then larvae was aerated using a rotary pump (Kiho) to promote better hatch. The mixture was then left at room temperature (25 ºC) for one to two days

4.2.1.2. Preparation of plant samples

Toxicity for all extracts that displayed noteworthy antimicrobial activities (≤160 µg/ml) against any of the tested pathogens studied was investigated (Chapter 2; Table 2.3). Both the dichloromethane: methanol and aqueous plant extracts were prepared to a stock concentration of 2 mg/ml, then a starting concentration of 1 mg/ml was achieved after dilution. Organic extracts were dissolved in (2 % v/v) of Dimethyl sulfoxide (DMSO) and aqueous extracts in sterile water.

4.2.1.3. Brine shrimp lethality bioassay

Hatched shrimp were transferred into a shallow and four–sided container, then the lamp was placed next to the container facing the opening of the container. This allowed for maximum light exposure, which in turn allowed the shrimp to gather in one place for easy collection. An amount of 400 µl of sea water comprising of the brine shrimp (39–75) was transferred to each well of the 48 well micro–titre plate. The feasibility of brine shrimp was confirmed by observing the brine shrimp under a light microscope (Olympus) prior to adding samples. An amount 400 µl each organic and aqueous plant samples was added into the 48 well micro–titre plate. Each test was done in triplicate. This was followed by adding 32 mg/ml of sea water and 1.6 mg/ml potassium dichromate (Sigma) which were used as positive and negative controls respectively. All the shrimp that were found dead after 24 and 48 h incubation were counted under the light microscope. Figure 4.1 represents a view of the adult *Artemia franciscana* viewed under the light microscope.

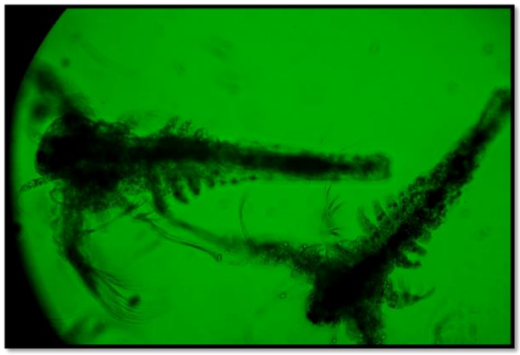


Figure 4.1: Adult *Artemia franciscana* when seen under the light microscope (Olympus) at 40x magnification.

After the 48 h count, 50 µl glacial acetic acid (lethal to brine shrimp) was introduced to each well so that the percentage mortality could be calculated. All plant samples that demonstrated mortality percentage of >50% were considered naturally toxic (Bussmann *et al*., 2011).

4.2.1.4. Generating LC_{50} values

All plant extracts that displayed toxic effects were further tested at six different concentrations (1, 500, 250, 125, 63 and 31 µg/ml) to generate LC_{50} values that were determined using IBM® SPSS statistics and probit analysis. The LC_{50} value is defined as the concentration of a test material possess a toxic effect on half 50% of the tested shrimp. A lower LC_{50} value indicates a higher toxic profile of a material. The LC_{50} values $\leq$ 249 µg/ml were considered highly toxic, 250–499 µg/ml (moderately toxic), 500–999 µg/ml (low toxic) and $\geq$ 1000 (non–toxic (Bussmann et al., 2011).

4.3. Results and discussion

The results of the BSLA for both the organic and aqueous extracts are shown in Table 4.1. At 24 h, none of the extracts displayed toxic effects. At 48 h, 82% of the tested extracts were non–toxic. None of the aqueous extracts displayed toxic effects. The lowest toxic effects were observed for the organic extracts of *B. cathartica, G. perpensa* leaf and aqueous extracts of *G. perpensa* leaf and rhizome, where the percentage mortalities of 0% were displayed at both 24 h and 48 h. Similar conclusions were reached in a study by McGaw *et al*. (2005). The authors found that the dichloromethane extracts of *G. perpensa* rhizome had no toxic effects on the brine shrimp where they exerted percentage mortality of 0 % at a concentration of 1000 µg/ml. Organic extracts of *A. oppositifolia, A. venosum, L. javanica,* and *T. riparia*, were toxic with percentage mortalities of 73.23%, 100%, 94.07%, and 59.65% respectively. Table 4.2 displayed the LC_{50} values displayed by extracts that were found to be toxic on the brine shrimp larvae.

Table 4.1: Brine shrimp lethality assay of organic and aqueous extracts

Plant species	Plant part used	Average (%) mortality	
		24 h	48 h
A. oppositifolia	Leaf	0.0	**73.2**
A. arborescens	Leaf	0.0	6.0
A. ferox	Leaf	0.0	1.4
A. marlothii	Leaf	0.0	0.0
A. tenuior	Leaf	0.0	0.0
A. venosum	Leaf	28.0	**100.0**
A. afra	Leaf	2.4	11.3
B. cathartica	Stem	0.0	0.00
G. perpensa	Leaf	0.0	0.00
	Rhizomes	2.1	5.2
L. javanica	Leaf	6.0	**94.7**
	Small twigs	0.0	3.4
M. longifolia	Leaf	0,0	10.6
P. fruticose	Leaf	5.2	6.6
S. africana–caerulea	Leaf	3.3	7.4
S. puniceus	Rhizomes	6.7	25.2
S. incanum	Leaf	2.8	11.2
S. africana	Leaf	3.6	16.9
S. cordatum	Leaf	1.6	2.4
T. riparia	Leaf	1.7	**59.7**
Aqueous extracts			
G. perpensa	Leaf	0.0	0.0
	Rhizomes	0.0	0.0
L. javanica	Leaf	2.0	41.0
Tropic marine water		0.0	0.0
Potassium dichromate		**100.0**	**100.0**

[a] Values marked in bold denote a % Mortality greater than 50% and are considered toxic.

Table 4.2: LC_{50} values of plants samples that displayed toxic effects.

Plant extract	LC_{50} (µg/ml)	
	24 h	at 48 h
A. oppositifolia Leaf	984	984
A. venosum Leaf	690	297
L. javanica Leaf	624	**88**
Tetradenia riparia Leaf	492	**77**

[a] Values marked in bold signify highly toxic medicinal plants ($LC_{50} \leq 249$ µg/ml)

A. oppositifolia displayed a LC_{50} value of 984 µg/ml. *A. venosum* was moderately toxic with an LC_{50} value of 297 µg/ml, whereas *L. javanica* and *T. riparia* were highly toxic with LC_{50} values of 88 µg/ml and 77 µg/ml respectively. The high percentage mortality exerted by *A. oppositifolia* leaf is supported by previous reports (van Wyk., 2002; Aremu *et al*., 2010). *A. oppositifolia* possesses no toxic effects on the brine shrimp after 24 h, however, it displayed greater toxic effect after 48 h. It is evident that *A. oppositifolia* (leaf material) is not toxic at lower concentrations. This is supported by the lower LC_{50} value. It is advisable that a dose lower than 984 µg/ml should be taken when *A. oppositifolia* is used for the treatment of stomach ailments. Investigation of toxicity effects of aqueous extracts of all the plants that showed toxic effects must be investigated, because some plants have only possess toxic effects when extracted using organic solvents.

Toxic effects were seen in the organic extracts of *A. venosum* leaf at 48 h. *A. venosum,* however, displayed moderate toxicity effects (297 µg/ml), when the LC_{50} was calculated. Similar conclusions were reached, when Steenkamp *et al*. (2009) investigated the toxic effects of *A. venosum* using *Vibrio fischeri* bioluminescence inhibition bioassay and *Poecilia reticulata* toxicity assay. *Antidesma venosum* inhibited the *P. reticulata* with a mortality percentage of 100%. Steenkamp *et al*. (2009), also noted the toxic effects of *A. venosum* to be dose dependent. Hence it can be advisable that lower doses (1000 µg/ml) be preferred when used in traditional medicine.

L. javanica is a volatile rich herbal tea which is used traditionally for colds, cough, fever or malaria, wounds, repelling mosquitos, diarrhoea, cholera, dysentery, chest pains, bronchitis, and asthma (Hutchings *et al*., 1996; van Wyk, 2008; van Wyk *et al*., 2009; Maroyi, 2017). In

the current study, *L. javanica* displayed minimal toxic effects on the brine shrimp at 24 h. However, at 48 h it showed a high toxic effect. Hübsch *et al*. (2014), reported similar results, in their study, the organic extracts of *L. javanica* exerted toxic effect against the brine shrimp larvae with LC_{50} value of 70.13 µg/ml. In another study, piperitenone isolated from *L. javanica* showed toxic effects (LC_{50} value of 265.6 µg/ml) against cultures of human (HCT–8) cells, which were derived from stomach cancer (Samie *et al*. (2009). In the previous studies, when the toxic effect of aqueous extracts of *L. javanica* were investigated, they showed non–toxic effects with mortality percentage less than 50% at both 24 and 48 h (Lediga, 2018). This validates the safety of this plant species when prepared traditionally using water. Hence aqueous extracts of *L. javanica* must be considered over organic extracts to monitor safety.

T. riparia is an aromatic medicinal plants that is used for the treatment of malaria, fever, coughs, diarrhoea, headaches and toothaches (Campbell *et al*., 1997; van Zyl *et al*., 2006). In this study, the BSLA revealed that the organic extracts of *T. riparia* leaf were toxic after 48 h. *T. riparia* was found to be highly toxic even when tested in lower doses effects when tested in lower concentrations. Lediga (2018) also reported that the organic extracts of *T. riparia* to be toxic at both 24 and 48 h. Further tests should be performed to evaluate the potential mammalian toxicity of *L. javanica* and *T. riparia* extracts, as McGaw *et al*. (2005) suggested that toxicity against brine shrimp larvae is a preliminary indication of the likely presence of toxic compounds in the extracts.

4.4. Chapter summary

- The BSLA results revealed that 82% of the tested plant samples were non–toxic.
- Lowest cytotoxic effects were observed for organic extracts of *A. marlothii, A. tenuior, B. cathartica, G. perpensa* leaf
- All aqueous extracts were non–toxic, *G. perpensa* leaf and Rhizome displaying as low as 0% percentage mortality.
- Only four organic extracts (*T. riparia, L. javanica, A. venosum, A. oppositifolia*) were toxic with percentage mortality greater >50%.
- *A. venosum* was the least toxic when tested in lower doses.
- *T. riparia* (77 µg/ml) and *L. javanica* (88 µg/ml) were highly toxic with concentrations lower than 250 µg/ml.

- It was noted that *L. javanica* which displayed the best antimicrobial activity, displayed high toxic profiles, however, the aqueous extracts which is used in traditional medicine had no toxic effects.

CHAPTER 5

Summary and conclusion

5.1.1. Summary

In Southern Africa, several studies have focused on evaluating the antimicrobial efficacy of medicinal plants against commonly studied gut pathogens. However, fastidious pathogens of the gut have been neglected. Recently, studies have investigated common pathogens such as *S. aureus, S. flexineri, E. coli*, *E. faecalis* and *C. albicans* even though most gut infections are caused by fastidious pathogens (van Vuuren and Holl, 2017). Hence, this study aimed to evaluate the antimicrobial activity of selected medicinal plants documented for stomach ailments against neglected gut pathogens responsible for IAI and to further investigate biofilm activity (using *C. perfringens* as a model) and to determine toxicity of extracts of these traditional remedies.

The organic and aqueous extracts of each medicinal plant were tested for their antimicrobial activity against neglected pathogens of *Bacteroides*, *Clostridium, Fusobacterium* species as well as . Organic extracts displayed higher antimicrobial activity when compared to the aqueous extracts. The organic extracts of *L. javanica* leaf showed the best antimicrobial activity with an MIC value of 0.5 µg/ml against *C. perfringens*. This value was comparable to the control antibiotic ciprofloxacin (MIC = 0.2 µg/ml). *G. perpensa* leaf and rhizomes (aqueous extract) displayed the best antimicrobial activity with a mean MIC value of 130 µg/ml against both Clostridial strains. In occasions where leaves were tested in comparison with other plant parts, the activity of leaf was superior in most cases (seven of the ten tested plants) thus justifying the practice of using leaf materials to prepare infusions for stomach ailments and promoting the sustainable harvesting. These results support the traditional knowledge, as traditional healers use aqueous extracts for the treatment of stomach ailments. *C. perfringens* which is well–known for causing necrotic enteritis, food poisoning, and antibiotic–associated diarrhea was the most susceptible to the activities of the tested plant extracts. All plants that

displayed noteworthy activity against fastidious gut pathogens were not effective against common pathogens.

The plant extracts that displayed noteworthy antimicrobial activity against *C. perfringens* were further evaluated for their antibiofilm activity against using the crystal violet (CV) staining assay. Many of the tested extracts showed good antibiofilm activity (19% of the extracts were active at 4 h interval, 57% were active at 24 h interval whereas 38% were active at 48 h and 72 h respectively. At 24 h interval, most of the extracts had better activity than ciprofloxacin. The organic extract of *S. Africana–caerulea* displayed the best antibiofilm activity overall, preventing biofilm attachment (4 h) by 82.8%. Biofilms results indicated that most of the tested plants were not only effective against planktonic form of *C. perfringens* but also against its biofilms.

The toxicity profiles of the plant species studied that showed noteworthy antimicrobial activity were evaluated to confirm some degree of safety using the BSLA. Most of the extracts tested were non–toxic. All the aqueous extracts tested possessed no toxic effects as their percentage mortalities were less than 50%. Lowest cytotoxic effects were observed for organic extracts of *A. marlothii, A. tenuior, B. cathartica, G. perpensa* leaf with mortality percentage as low as 0%. However, some of the organic extracts exhibited toxicity, namely *A. oppositifolia* (73.2% mortality, LC_{50} of 984 µg/ml), *A. venosum* (100% mortality, LC_{50} of 297 µg/ml), *L. javanica* (94.1% mortality, LC_{50} of 88 µg/ml) and *T. riparia* (59.7% mortality, LC_{50} of 77 µg/ml). The BSLA results revealed that most of the plant samples were not toxic in nature.

The low toxicity of some of the plant extracts and their good antimicrobial and antibiofilm activity against the neglected gut pathogens associated with IAI further validates and encourage their traditional use in the treatment of IAI. Table 5.1 display the overall summary of the study, these are the plants that were most significant, (They displayed good antimicrobial activity, good antibiofilm effects, with low toxicity).

Table 5.1: Overall summary of the study

Plant species	MIC ≤160 µg/ml	Antibiofilm > 50%	Toxicity Mortality < 50%
A. marlothii	130 µg/ml against	Active against mature biofilms	0.0

(L) (O)	*C. perfringens*		
G. perpensa (L) (O)	2 – 130 µg/ml against *Clostridium* species	Active against biofilm attachment, development and mature biofilm.	0.0
G. perpensa (R) (O)	60 and 130 µg/ml against *Clostridium* species	Active against biofilm development and mature biofilm	0.0
S. africana–caurulea (L and T) (O)	30 and 130 µg/ml against *Clostridium* species	Active against biofilm attachment	5.4

L= Leaf; R= Rhizomes; T=Twigs; O = Organic extracts

5.2. Limitations

Firstly, growing fastidious cultures was found to be challenging, as this was the first time some of them (*H. pylori*, *C. difficile*, *F. varium*, and all *Bacteroides* species) were studied at the University of Witwatersrand Pharmaceutical Microbiology division. I started working on *H. pylori* in March 2018, of which I had to attend a workshop on how it is grown then later had to design an SOP (See Appendix D). Another limitation was the investigation of antibiofilm effects of plant extracts on *H. pylori* due to longer incubation period. Anaerobes could not grow in CO2 incubator, and at that stage, CO2 seemed to be the only available option, the anaerobic gas packs and gas jars were later opted. Insolubility of organic extracts for biofilms and toxicity studies was also a problem, which was further solved by using a sonicator.

5.3. Future recommendations

The effects of the Southern African medicinal plants in combination shouldn't be overlooked, because some plants are traditionally used in combination for the treatment of stomach ache. An example of this is the use of *Acorus calamas in combination with Siphonochilus aethiopicus, Euclea divinorum* and *Croton megalocarpus, Lagenaria sphaerica* and *Bidens pilosa, Polygala leptophyla* and *Catophractes alexandri, Senna italica* and *Otopreta burchellii* (von Koenen, 2001). It would be interesting to determine how this combination inhibits the pathogens studied in this project. Antibiofilm activity of plant that displayed noteworthy activity against the planktonic forms of *Bacteroides*, *Fusobacterium* and *Helicobacter* species should be investigated because these pathogens are biofilm formers and biofilms weaken the effects of conventional antimicrobials hence increasing the level of resistance. Future studies

should consider toxicity studies using human cell lines human (HCT–8) cells derived from a human intestinal adenocarcinoma to verify the suitability of these extracts for this purpose. Antimicrobial investigation of the neglected gut pathogens against more Southern African medicinal plants needs to be explored, because in literature about 155 plants were documented to be used for stomach–ache but only 41 plants were investigated in this study. Researchers should not only focus on the commo n pathogens, but also test the effects of medicinal plants used on traditional medicine against fastidious pathogens as they might yield promising results.

5.4. Conclusion

The results from the MIC assay favour the traditional use of several plant extracts in IAI. *Gunnera perpensa* organic extracts were the most significant of all the tested extracts, as they displayed the very good antimicrobial activity against *Clostridium* species (MIC value of 2 – 130 µg/ml). It also displayed good antibiofilm activity against new and older biofilms (Average inhibition = 52.3% for leaf extract and 58.7% for rhizome), with no cytotoxic effects (Mortality = 0%). A notable result was seen in *G. perpensa* (Leaf and rhizomes), extracts where noteworthy activity was observed against *Clostridium* species with MIC values of 130 µg/ml. In several occasions there was a connection between the antimicrobial activity and the traditional use. For example, *S. africana* is traditionally used for diarrhoea. In the current study the organic extract of the leaf displayed noteworthy activity against *C. difficile* and *C. perfringens*. Interesting to note, none of the plant extracts displayed noteworthy activity against the common pathogens. Biofilm results indicated that most of the plants that were active against *C. perfringens* were also effective against *C. perfringens* biofilms. Frequent intake of infusions made from selection of plants that had good antibiofilm activity may play a role in the prevention of biofilms development. The BSLA results revealed that most of the plant samples were naturally non–toxic. This study demonstrates that studies should not only focus on common pathogens, but also on neglected pathogens which may yield excellent results not previously reported. This study contributes to the knowledge of the antimicrobial properties of plants commonly found in Southern Africa. The low toxicity of some of the plant extracts and their good antimicrobial and anti–biofilm activity against the neglected gut pathogens associated with IAI validate their traditional use and safety in the treatment of IAI.

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APPENDIX A

Presentations

A1: **The 2018 Indigenous Plant Use Forum (IPUF) conference – Oudtshoorn, South Africa, 1–4 July 2018.**

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How do *H. pylori* and other neglected gut anaerobes respond to Southern African medicinal plants?

A number of studies have been undertaken on Southern African medicinal plant species and their antimicrobial efficacy against pathogens that cause stomach ailments. However, neglected anaerobes such as the *Helicobacter*, *Bacteroides* and *Clostridium* species have been neglected. The aim of this study was to investigate the antimicrobial efficacy of indigenous Southern African medicinal plants used for the treatment of stomach ailments against anaerobic bacteria responsible for intra–abdominal infections. A total of 112 aqueous and organic extracts were prepared from 43 different plants species. These plants samples were screened for antimicrobial efficacy against the following gut anaerobes; two strains, four *Bacteroides* species, two *Fusobacterium* species, and two *Clostridium* species using the minimum inhibitory concentration (MIC) assay. The toxicity profiles of the most active plant species were evaluated using the brine shrimp lethality assay (BSLA). The organic extracts of *Lippia javanica* (leaves and stems), *Artemisia afra* (leaves), *Aloe tenuior* (leaves), tenuior *Polygala fruticosa (*leaves), *Gunnera perpensa* (rhizomes and leaves) and *Aloe arborescens* (leaves) displayed the lowest MIC values ranging from 0.5 µg/ml to 0.06 mg/mL against *C. perfringens*. The leaves of *Salvia africana* and *Polygala fruticosa* demonstrated broad–spectrum activity. *Gunnera perpensa* (leaves) had noteworthy activity on almost all the groups of bacteria tested yet did not demonstrate noteworthy activity against common pathogens such as *E. coli* and *E. faecalis*. Most of plants tested in the BSLA were considered safe in the BSLA, however, the highest toxicity profiles were noted for the leaves of *Antidesma venosum* and the stems of *Warbugia salutaris* with 100% mortality. The study demonstrates that studies should not only focus on common pathogens, but also on neglected pathogens, which may yield excellent results not previously observed.

A2: School of Therapeutic Sciences Biennial Research Day – University of the Witwatersrand (Faculty of Health Sciences), Johannesburg, South Africa, 6 September 2018.



Efficacy of medicinal plants against *Helicobacter pylori* and gut anaerobes

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INTRODUCTION

Intra-abdominal infections (IAI) are one of the most common gastrointestinal emergencies, and have been reported as major contributors to non-trauma deaths in the medical emergency departments of hospitals worldwide[1]. These include peritonitis, intra-abdominal abscesses, diverticulitis, appendicitis, food poisoning, gastric ulcers and pelvic inflammatory disease[2,3,4]. The most commonly isolated micro-organisms in IAI include *Escherichia coli*, and *Fusobacterium*, *Bacteroides*, *Clostridium*, and *Helicobacter* species[5,6].

In South Africa, around 80% of the population relies mainly on traditional medicine for their primary health care needs. Numerous studies have been done in South Africa with regards to medicinal plants and stomach ailments[46] including diarrhoea, yet little attention has been given to gut anaerobes and the plant used to treat these conditions (Table 1).

Plant name	Plants part used	Traditional use
Chenopodium ambrosioides	Leaf	Intestinal ulcers
Dombeya rotundifolia	Leaf , Stems	ulcerative colitis
Elephantorrhiza elephantina	Roots	peptic ulcers
Gunnera perpensa	Leaf , Rhizome	Stomach bleeding
Oxalis cornalucata	Leaf	Peptic ulcers
Spirostachys africana	Leaf , Stems	stomach ulcers
Warburgia salutaris	Leaf , Stems	Gastric ulcers
Zanthoxylum capense	Leaf	Gastric and intestinal disorders

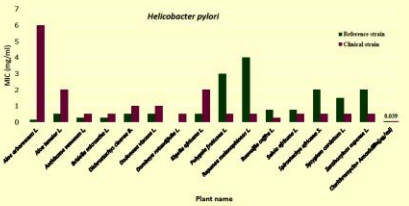
AIMS AND OBJECTIVES

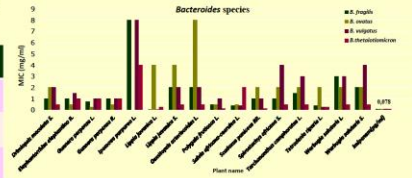
To investigate the antimicrobial activity of Southern African medicinal plants used in the treatment of intra-abdominal infections against *Helicobacter pylori* and gut anaerobes using the Minimum inhibitory concentration (MIC) assay.

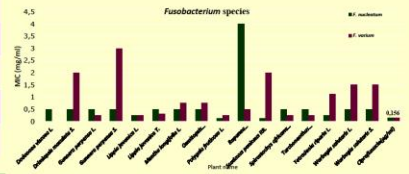
METHODOLOGY

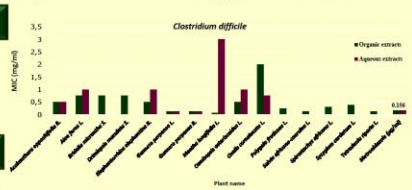
The micro-titre plate dilution assay[7] was used to determine the minimum inhibitory concentration (MICs) against *B. fragilis* (ATCC 23745), *B. ovatus* (ATCC 8483), *B. thetaiotaomicron* (ATCC 29741), *B. vulgatus* (ATCC 8482), *C. difficile* (ATCC 43593), *C. perfringens* (ATCC 13124), *F. nucleatum* (ATCC 25586) and *F. varium* (ATCC 27725), and *H. pylori*, where reference and clinical strains were used. *Enterococcus faecalis* (ATCC 29212) and *E. coli* (ATCC 8739) were included as a comparator of activity against common pathogens. A concentration of 0.01 mg/mL ciprofloxacin for *Fusobacterium* species and *C. perfringens*, 1:1 ratio of clarithromycin and amoxicillin for *H. pylori* species, imipenem for *Bacteroides* species, and metronidazole for *C. difficile* were used as positive controls to confirm the antimicrobial activity. Negative controls (sterile media and solvent) were included in all assays to monitor the antimicrobial susceptibility and sterility. All susceptibility assays were undertaken at least in duplicate.

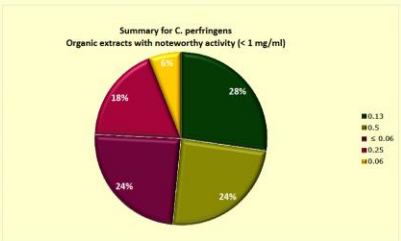
RESULTS











RESULTS OVERVIEW

A total of 42 plants were tested, but only the most active results are presented. Approximately 33% of the plants were active against *H. pylori*, 38% were active against *Bacteroides* and *Fusobacterium* species respectively, 73% were active against *Clostridium* species, of which 15 % aqueous extracts and 61 % organic extracts had noteworthy activity. The most active plant extract was *Lippia javanica* (organic extract) with a mean MIC value of 0.5 µg/ml against *C. perfringens*. *Salvia africana* displayed the broadest spectrum of activity where antimicrobial activities ranged from 0.50 - 0.03 mg/ml were found against nine of the ten pathogens studied.

Key results for *Clostridium perfringens*.



Lippia javanica MIC = 0.5 µg/ml



Gunnera perpensa MIC = 2 µg/ml

Ciprofloxacin MIC = 0.2 µg/ml



Artemisia afra MIC = 8 µg/ml



Aloe tenuior MIC = 2 µg/ml

CONCLUSION

It was shown that 83% of the medicinal plant extracts tested exhibited antimicrobial effects against one or more of the 10 different microbial strains. With the exception of *Chenopodium ambrosioides*, all the plants that were documented to be used specifically for ulcers (Table 1) displayed noteworthy activity against the causative microbes. This study provides some ethnopharmacological evidence that supports the traditional use of these plant species for infections associated with intra-abdominal infections.

ACKNOWLEDGEMENTS

- WITS Surgery department for providing us with *H. pylori* strains.
- National Research Foundation (NRF) for financial assistance
- Supervisors for running cost funding.
- WITS Postgraduate Merit Bursary (PMA).

REFERENCE LIST

1.) Sartelli et al., 2017. World. J. Emergency Surgery 2.) Intra-Abdominal Infection Market - Global Industry Analysis, Size, Share, Growth, Trends and Forecast, 2014 – 2020. Transparency Market Research. 3.) Gorbach, 1996. Med. Microbiology, 4th edition, Chapter 95. 4.) Njume et al., 2009. Afr. J. Pharmacol 5.) Eid et al., 2017. Front. Pharmacol. 6.) Van Vuuren and Holl, 2017. J. Ethnopharmacol. 7.) Eloff, 1998. J. Plants Med.

A3: Postgraduate Botany Symposium – University of Johannesburg (Department of Botany and Plant Biotechnology) Johannesburg, South Africa, 5 November 2018.

Antimicrobial activity of Southern African medicinal plants against neglected gut pathogens responsible for intra–abdominal infections.

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A number of studies have been undertaken on Southern African medicinal plant species and their antimicrobial efficacy against pathogens that cause stomach ailments. However, gut pathogens such as the *Helicobacter*, *Bacteroides* and *Clostridium* species have been neglected. This study aimed to investigate the antimicrobial efficacy of indigenous Southern African medicinal plants used for the treatment of stomach ailments against *H. pylori* and anaerobic bacteria responsible for intra–abdominal infections. A total of 104 aqueous and organic extracts were prepared from 41 different plant species. These plant samples were screened for antimicrobial efficacy against eight anaerobic (four *Bacteroides species*, two *Fusobacterium* species, two *Clostridium* species) and two microaerophilic (reference and clinical strains) gut pathogens using the micro–titre plate method. Common pathogens (*E. coli* and *E. faecalis*) were added as comparators of activity. Approximately 39% of organic extracts demonstrated noteworthy activity (MIC ≤160 µg/ml) against one or more neglected gut pathogens studied. The lowest minimum inhibitory concentration (MIC) value was displayed by the organic extract of *Lippia javanica* leaf with an MIC value of 0.5 µg/ml against *C. perfringens*. The leaves of *Aloe arborescens*, *Lippia javanica*, *Polygala fruticosa*, *Salvia Africana*– caerulea, *Tetradenia riparia* and leaves and rhizome of *Gunnera perpensa* displayed noteworthy activity against more than one neglected gut pathogens studied with MIC values ranging between 0.5µg/ml – 130 µg/ml. Aqueous extracts of *G. perpensa* leaf and rhizomes displayed noteworthy activity with an MIC value of 130 µg/ml. Interesting to note, none of the plants extracts displayed noteworthy activity against the common pathogens. The study demonstrates that studies should not only focus on common pathogens, but also on neglected pathogens which may yield excellent results not previously reported.

APPENDIX B

Publication

Antimicrobial activity of Southern African medicinal plants against *H. pylori* and other anaerobes of the gut.

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[a]*Department of Pharmacy and Pharmacology, University of the Witwatersrand, South Africa.*
[b]*Department of Surgery, University of the Witwatersrand, South Africa.*

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ABSTRACT

Intra–abdominal infections (IAI) are one of the most common of all gastrointestinal emergencies and have been reported as major contributors to non–trauma deaths in the medical emergency departments of hospitals worldwide. In the human gut, anaerobes outnumber the aerobic bacteria, and the most commonly isolated micro–organisms in IAI include *E. coli Peptostreptococcus micros* as well as the *Bacteroides* genus and *Clostridium* species. Several studies have been undertaken on Southern African medicinal plant species and their antimicrobial efficacy against pathogens that cause stomach ailments. However, pathogens such as *H. pylori*, *F. varium* as well as *Bacteroides*, and *Clostridium* species have been neglected. The aim of this study was thus to evaluate the antimicrobial activity of selected medicinal plants documented for stomach ailments against neglected gut pathogens responsible for IAI. A total of 102 aqueous and organic extracts were prepared from 40 different plant species. These plant samples were screened for antimicrobial efficacy against eight anaerobes of the gut namely: *B. fragilis* group [*B. fragilis* (ATCC 25285), *B. ovatus* (ATCC 8483), *B. vulgatus* (ATCC 29327), *B. thetaiotaomicron* (ATCC 29741)]; Two *Clostridium* species [*C. perfringens* (ATCC 13124), and *C. difficile* (ATCC 43593)]; two *Fusobacterium* species [*F. varium* (ATCC 27725), and *F. nucleatum* (ATCC 25586)] and two microaerophilic strains (clinical and reference strains) using the micro–dilution antimicrobial assay. Plant extracts that displayed noteworthy antimicrobial activity against *C. perfringens* were further evaluated for their anti–biofilm activity using the crystal violet (CV) staining assay. The cytotoxicity profiles of plants that displayed noteworthy antimicrobial activity were evaluated using the brine shrimp lethality assay (BSLA) to confirm some degree of safety. The organic extract of *Lippia javanica* leaf showed the best antimicrobial activity with a minimum inhibitory concentration (MIC) value of 0.5 µg/ml against *C. perfringens*. The organic extract of *Salvia africana–caerulea* displayed the best anti–biofilm activity overall, at cell attachment (4 h) biofilm developmental stage with inhibition percentages of 82%. The BSLA results revealed that most of the tested plant samples were non–cytotoxic in nature, and aqueous extracts proved to be safer.

APPENDIX C

Ethics waivers

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research & Post Graduate Affairs)

TO: Ms S Hlambani
School: Therapeutic Sciences
Department: Pharmacy and Pharmacology
Medical School
University
E-mail: Hlambani2@gmail.com

CC: Supervisor: Professor S van Vuuren <Sandy.vanVuuren@wits.ac.za>
and <HREC-Medical.ResearchOffice@wits.ac.za>

FROM: Iain Burns
Human Research Ethics Committee (Medical)
Tel: 011 717 1252

E-mail: Iain.Burns@wits.ac.za

DATE: 09/05/2018

REF: *R14/49*

PROTOCOL NO: *W-CBP-180509-01 (This is your ethics application study reference number. Please quote this reference number in all correspondence relating to this study)*

PROJECT TITLE: *The antimicrobial investigation of neglected pathogens of the gut against southern African medicinal plants used for stomach ailments*

Please find attached the Clearance Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to the Government funding of the University.

MSWorks2000\Iain0007\ClearScanWaiver.wps

R1449 Prof Geoffrey Candy

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M170582

NAME: (Principal Investigator)	Prof Geoffrey Candy
DEPARTMENT:	Surgery
PROJECT TITLE:	Effect of Helicobacter Pylori Eradication on Blood Pressure in Patients with Hypertension and without Type 2 Diabetes (NIDDM)
DATE CONSIDERED:	26/05/2017
DECISION:	Approved unconditionally
CONDITIONS:	Renewal for the period 26/05/2017 - 26/02/2022 Previously M120481
SUPERVISOR:	
APPROVED BY:	Prof P Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	02/06/2017

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 301, Third floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193. University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in May and will therefore be due in the month of May each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

APPENDIX D

SOP



Figure 1: on Columbia blood agar

Solid media preparation

Solid media: Columbia blood agar base (Oxoid)

Storage of solid media powder: Room temperature

Preparation: Weigh out and add 19.5 Columbia blood agar base powder to 500ml distilled and sterile water. Autoclave at 121°C for 15min. Allow cooling to 50°C before adding supplements.

Supplementation: 1 vial of *H. pylori* selective supplement (Dent) (Oxoid), Vitox (Oxoid), 7 % fetal bovine serum (Davies diagnostics) or Sheep blood (National Health Laboratory Science).

Preparation: Reconstitute *H. pylori* selective supplement by adding 2 ml of distilled and sterile water to supplement vial. Aseptically add 1 vial of Vitox Rehydration Fluid to 1 vial of Vitox. Melt the frozen fetal bovine serum (FBS) at room temperature and add it to media using sterile needle and filter.

Storage of supplements: Fridge (2–8 °C). FBS to be stored in –80 °C

Description of colonies: Tiny, round, transparent (colorless) colonies, that look like water drops.

Incubation period: 4 –9 days

Incubation temperature: 37 °C

Atmosphere: Microaerobic atmosphere using Pack Microaero (Camphylo) generating kit (Oxoid).

Working environment: Make use of the biohazard, and all the used consumables to be dumped into a biohazard bins including.

Storage: Freezing on anaerobic environment: –80°C, make use of dry ice when transporting the culture from the freezer to the biohazard and when using it to avoid in viability i.e. is too sensitive.

Liquid media Preparation

Liquid media: Brain Heart infusion (Oxoid)

Storage of liquid media powder: Room temperature

Preparation: Weigh out and add 37g of Brain Heart infusion broth powder to 1000ml sterile water. Autoclave at 121°C for 15min. Allow cooling to 55°C before adding supplement.

Supplementation: 1 vial of *H. pylori* selective supplement (Dent) (Oxoid), Vitox (Oxoid), 7 % fetal bovine serum (Davies diagnostics)

Supplement preparation: Reconstitute *H. pylori* selective supplement by adding 2ml of sterile water to supplement vial. Aseptically add 1 vial of Vitox Rehydration Fluid to 1 vial of Vitox. Melt the frozen fetal bovine serum at room temperature and add it to media using a needle and sterile filter.

APPENDIX E

Originality report

The antimicrobial investigation of neglected pathogens of the gut against Southern African medicinal plants used for stomach ailments.

ORIGINALITY REPORT

16%	6%	14%	3%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	S. Akhalwaya, S. van Vuuren, M. Patel. "An in vitro investigation of indigenous South African medicinal plants used to treat oral infections", Journal of Ethnopharmacology, 2018 Publication	3%
2	Unathi Mabona, Alvaro Viljoen, Emmanual Shikanga, Andrew Marston, Sandy Van Vuuren. "Antimicrobial activity of southern African medicinal plants with dermatological relevance: From an ethnopharmacological screening approach, to combination studies and the isolation of a bioactive compound", Journal of Ethnopharmacology, 2013 Publication	1%
3	M. Sandasi, C.M. Leonard, S.F. Van Vuuren, A.M. Viljoen. "Peppermint (Mentha piperita) inhibits microbial biofilms in vitro", South African Journal of Botany, 2011 Publication	1%
4	repository.up.ac.za Internet Source	1%
5	Mahumane, Gillian D., Sandy F. van Vuuren, Guy Kamatou, Maxleene Sandasi, and Alvaro M. Viljoen. "Chemical composition and antimicrobial activity of Eucalyptus radiata leaf essential oil, sampled over a year", Journal of Essential Oil Research, 2016. Publication	<1%
6	es.scribd.com Internet Source	<1%