CHAPTER FOUR

Antimicrobial activities of the plant extracts against potential diarrhoeal pathogens

4.0. Introduction

Infectious disease defined as an illness caused by a specific pathogen or its toxins that result from transmission of the causative agent or its virulence effectors from an infected person, animal or reservoir to a vulnerable host. The susceptibility of host to infectious pathogens, disease development, progression and severity depends on the age, gender, genetic, immune and nutritional status. Infectious diseases represent a leading cause of morbidity and mortality worldwide despite the advancement in orthodox medicine accounting for more than 26% of all death with developing countries carrying the major burden (Becker *et al*., 2006). Infections are also considered to be a major contributing factor associated with reduced performance in food animals during growth. Particularly, persistent infections account for slow growth, suboptimal feeding efficiency and economic loss in the livestock industry (Borghetti *et al*., 2009). The infective pathogens include bacteria, fungi, viruses, protozoa and parasites which manifest their virulence through different mechanisms (see section 2.2 for detailed discussion).

The discovery of antibiotics in 1928 and subsequent development in 1940 as medical treatment provides effective and efficient therapeutic agents for controlling almost all infectious diseases including many feared and contagious infections. Antibiotics are effective in curing many infectious diseases, but they also enhance selection of resistant microbes as some pathogens rapidly became resistant to many of the originally susceptible drugs (Barbour *et al*, 2004).

At present, the pharmaceutical drugs available to control antibiotic-resistant bacteria are becoming limited. The indiscriminate use and abuse of antibiotics has led to the development of antimicrobial resistance strains and toxicity of some drugs to human and animals (Barton, 2000; Parekh and Chanda, 2007). As a result of these problems, European Union (EU) with EU-directive 1831/2003 imposed ban on the use of antibiotics as growth factor in animal production with effect from 2006 to avoid cross resistance problem with human pathogens and chemical residues in foods (Makkar *et al*, 2007).

Drug resistance of human and animal pathogenic microbes and parasites has created a serious problem worldwide as previously treatable ailments such as diarrhoea (including dysentery and cholera), and tuberculosis are now more difficult and expensive to treat. The mechanisms of microbial resistance to antibiotic include (Dwyer *et al*., 2009):

- Genetic alterations which involved the physical exchange of genetic material with another organism (via plasmid conjugation, phage-based transduction, or horizontal transformation), the activation of latent mobile genetic elements (transposons or cryptic genes), and the mutagenesis of its own DNA.

- Chromosomal mutagenesis arises directly from interaction between the chromosome and the antibacterial agent or antibiotic-induced oxidative stress, or indirectly from the bacterium's error prone DNA polymerases during the repair of a broad spectrum of DNA lesions.

The situations have complicated by the treatment of infectious diseases in immunocompromised patients. These negative health trends necessitate for a new prevention and treatment of infectious diseases including diarrhoea.

Medicinal plants have also featured as therapeutic agents used by the world population for basic health care needs and to combat many kinds of infectious diseases worldwide (Voravuthikunchai and Limsuwan, 2006). Medicinal plants have curative properties due to the presence of complex mixture of phytochemicals acting individually or synergistically to exert the associated therapeutic effects. Some of the plant compounds may be novel bioactive substances that can be effective as therapeutic agents for treating ailments such as infectious diarrhoea. These phytochemicals exhibit their antidiarrhoeal effects through various mechanisms such as antimicrobial (Lutherodt *et al*, 1999), increasing colonic water and electrolytic re-absorption, inhibition of intestinal motility (Oben *et al*, 2006) and anti secretory effects (Rao *et al*, 1997). There is considerable research in the screening of natural products from extracts of edible and medicinal plants for the development of alternative drugs to prevent and curtail the emergence of drug-resistance pathogens or other forms of ailments.

4.1. Qualitative antimicrobial (Bioautography) assay

This refers to the direct bioactivity test on developed TLC plates as a means of localizing the biological activity such as microbial growth inhibition, enzymatic inhibition or antioxidative properties of extracts to the particular active compound(s). This helps in focusing attention on the relevant components of an extract (Saxena *et al*., 1995). Fractionation of medicinal plant extracts in combination with bioautography provides an efficient and relative cheap method for bioactivity-guided isolation of target compound(s) (Hostettman *et al*., 1997). Practical application of bioautography in activity guided isolation includes enzyme inhibition assay such as the Ellman method for cholinesterase inhibitors (Ellman *et al*., 1961). In this method, the developed TLC plate is sprayed with a substrate, enzyme and indicator to determine the inhibition by colour variation (white zone against yellow background) (Rhee *et al*., 2001). In the antimicrobial bioassay, two bioautography methods are available. Firstly, the agar diffusion method involves pouring a layer of inoculated agar solution of the microbes on the developed TLC plate and allowed to set, and the bioactive zone(s) are transferred to the agar gel by diffusion where they can inhibit the growth of the microorganism (Fig 4.1). Secondly, the direct method involves spraying of microorganism broth inocula onto the TLC plate (Homans and Fuchs, 1970) and incubating in humid conditions

to facilitate the growth of the organism.

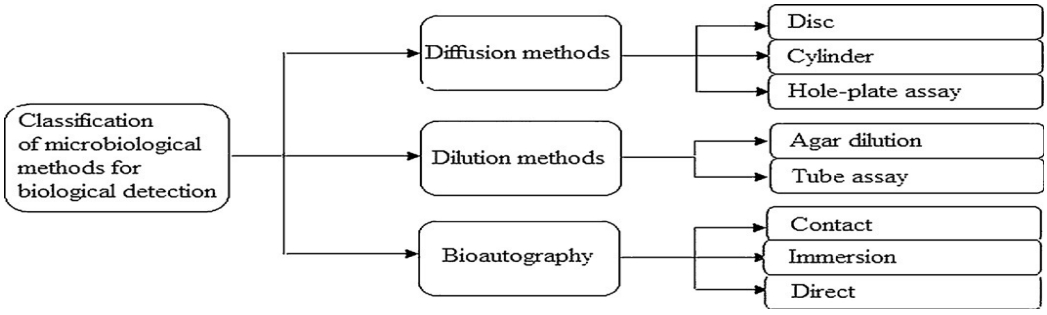

Fig 4.1. The classification of microbiological methods for biological detection (Adopted from Choma and Grzelak, 2011)

Microbial growth inhibitions are recognized based on the ability of the living microorganism to transform tetrazolium salts to a coloured formazan product. White spots against an intense purple coloured background indicate the compound(s) that kill the tested microorganism (Hostettman and Martson, 2002).

4.2. Quantitative antimicrobial activity (Minimum inhibitory concentration (MIC)) assay

Among the quantitative antimicrobial methods used in evaluating plant extract activity, agar diffusion assay (Greenwood, 1989) and two-fold serial micro-dilution assay (Eloff, 1998) are the most common in phytomedicine research. Sensitivity of the two protocols and their mechanisms varied widely. The mechanism of agar diffusion is the movement of bioactive compounds through the solid agar medium to kill or inhibit the growth of organism it may come in contact with. However, agar diffusion assays may sometimes lead to a false negative result, due to influence of the agar type, salt concentration, incubation temperature molecular size of the antimicrobial components (Greenwood, 1989), and limited diffusion of bioactive component in agar medium. The two-fold serial micro-dilution assay depend on direct contact between the test sample and organism is adjudged to be 30 times more sensitive than the other methods used to screen plant extracts for antimicrobial activity (Eloff, 1998). Although, the effective solubility and miscibility of the bioactive component in the test medium such as the non-polar compounds like terpenes, alkaloid and highly methoxylated phenolics is a limiting factor.

4.3. Selection of microorganisms used in the study

The selection of the microorganisms for antibacterial evaluation in this study was based on their known pathogenic effects in both human and animals with emphasis on diarrhoeal pathogens. Pathogenic *E. coli* has been implicated in diseases such as diarrhoea, hemorrhagic colitis, haemolytic uremic syndrome and thrombocytopenic purpura (Voravuthikunchai and Limsuwan, 2006). *Enterococcus faecalis* has been implicated in causing enteric infection with diarrhoeal effects (Butler, 2006). *Pseudomonas aeruginosa* strains cause diseases such as mastitis, abortions and upper respiratory complications (Masika and Afolayan, 2002). *Staphylococcus aureus* is one of the prominent microbes causing skin infection such as boils, abscesses, carbuncles and sepsis of wounds and it also produces toxins causing diarrhoea and vomitting (Maregesi *et al*,

2008). *Candida albicans* is a typical opportunistic pathogen causing diarrhoea (Gambhir *et al*, 2006), oral and vaginal candidiasis (Shai *et al*, 2008) especially in immunocompromise individuals due to unexpected opportunity by a failure of host defence. *Cryptococcus neoformans* has been implicated in causing life-threatening meningoencephalitis (Xue *et al*, 2007) and pneumonia in immunocompromise individuals (Hamza *et al*, 2006).

4.4. Material and Methods

4.4.1. Microorganism strains

Two standard strains of Gram-positive bacteria (*Staphylococcus aureus* ATCC 29213*, Pseudomonas aeruginosa* ATCC 25922) and two standard strains of Gram-negative bacteria (*Escherichia coli* ATCC 27853*, Enterococcus faecalis* ATCC 29212) were used for antibacterial assay. Three clinical pathogenic fungi namely yeasts (*Candida albicans, Cryptococcus neoformans*) and mould (*Aspergillus fumigatus*) (All fungal strains obtained from the Department of Veterinary Tropical Diseases, Faculty of Veterinary Sciences, University of Pretoria) were used.

4.4.2. Culturing of the Bacteria

The bacterial strains were maintained in Mueller Hinton agar (MHA) (Fluka, Spain) while the fungi were maintained in Sabouraud dextrose agar (Merck, Germany) at 4^{o}C under anaerobic conditions. All the organisms were subcultured every 2 weeks. Before testing, the bacterial inoculums were prepared and cultivated in Mueller Hinton broth for 12 h at incubation temperature of 37^{o}C. The fungi inoculums were prepared in Sabouraud dextrose broth (SDB,). The microbial cultures were serially diluted (10 fold increments) in sterile broth to obtain the cell suspension of 1.0 ×10^{5} CFU/ml.

4.4.3. Bioautography against some pathogenic microorganisms

Bioautography was undertaken to ascertain the number of active compound(s) present in crude extracts and fractions. TLC plates were developed as described in section 3.5.4 (Pp 43 - 44), and sprayed with overnight cultures of *E. coli, S. aureus, P. aeruginosa* or *E. faecalis* and incubated at 37^{o}C for 12-16 h prior to being spray with tetrazolium violet (INT). The inhibitory activity of any components was evident as clear white zones against the purple/red background.

4.4.4. Determination of Minimum Inhibitory Concentration (MIC) against the bacteria pathogens

The minimum inhibitory concentration (MIC) for the crude extract and fractions against bacteria were evaluated using the twofold serial dilution assay with tetrazolium violet added as growth indicator (Eloff, 1998). The extracts (100 µl) at an initial concentration of 1.0 × 10^{4} µg/ml was serially diluted with distilled water up to 50% in 96-well microtitre plate to prepare solution range between 5000 µg/ml first well and 40 µg/ml last well. The bacterial (100 µl) inoculants from 12 h broth cultures (section 4.4.2) diluted to 1:100 were added to each well to obtain final extract concentration range of 2500 µg/ml first well and 20 µg/ml last well. Gentamicin (25 µg/ml first well and 0.18 µg/ml last well) was used as positive control and the solvent used in dissolving the extract was used as

negative control. Final volume in each well was 200 µl. The plates were incubated for 24 h at 37°C and 100% relative humidity. The inhibition of the bacteria were visualised by adding 40 µl of aqueous p-iodonitrotetrazolium violet (INT) (Sigma) to each well (concentration 200 µg/ml). The plates were incubated for another 1 h and MIC was determined as the lowest concentrations of test sample before purple formazan colour were observed.

4.4.5. Determination of Minimum Inhibitory Concentration (MIC) against the fungal pathogens

Minimum inhibitory concentrations (MIC) against three pathogenic fungi were determined using twofold serial dilution assay as described above by Eloff, 1998 with the following modification of Masoko *et al*, 2005. The fungal inoculants (100 µl) were in fresh Sabouraud dextrose broth and positive controls was amphotericin B (50 µg/ml first well and 0.4 µg/ml last well) and negative controls was 70% acetone, final visualization of inhibitory activity was obtained after an incubation for 24 h at 37°C, and 100% relative humidity.

4.5. Results

4.5.1. Microbial bioautography

The TLC bioautography of the crude extracts and fractions of the 27 plant species tested against standard strain bacteria and clinical fungal isolates are presented in Fig 4.2-4.12.

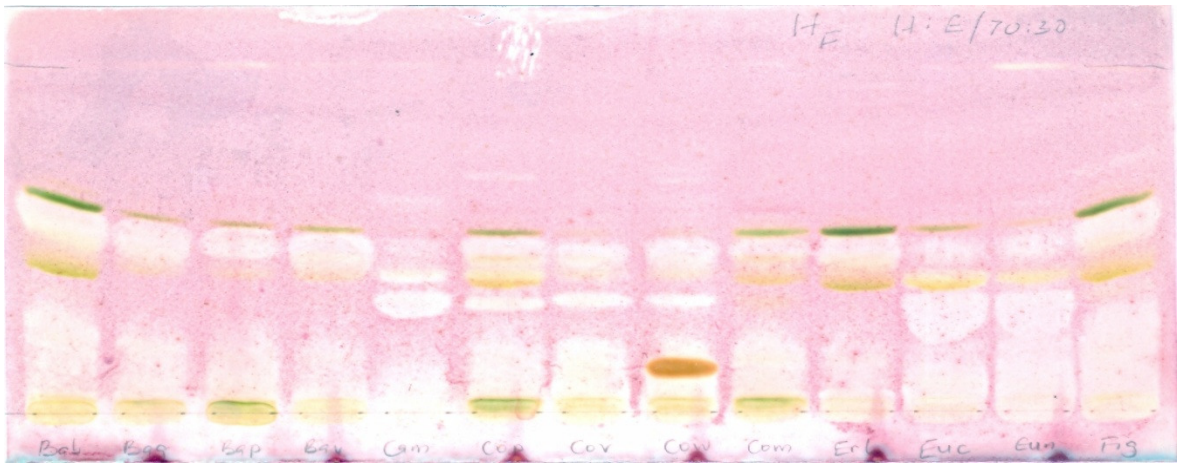

Fig. 4.2. Bioautography of hexane (upper) fraction of different plant species against *S. aureus* (Bab (*Bauhinia bowkeri*), Bag (*Bauhinia galpinii*), Bap (*Bauhinia petersiana*),Cam (*Carissa macrocarpa*), , Cop (*Combretum padoides*), Cov (*Combretum vendae*), Cow (*Combretum woodii*), Com (*Commiphora harveyi*), Euc (*Euclea crispa*), Eun (*Euclea natalensis*), Erl (*Erythrina latissima*), Fic (*Ficus craterestoma*), Fig (*Ficus glumosa*) developed with hexane: ethyl acetae: formic acid (70:30))

The antimicrobial activities of the extracts were concentrated on the non-polar-enriched hexane fraction while the polar enriched components no sign of microbial inhibition.

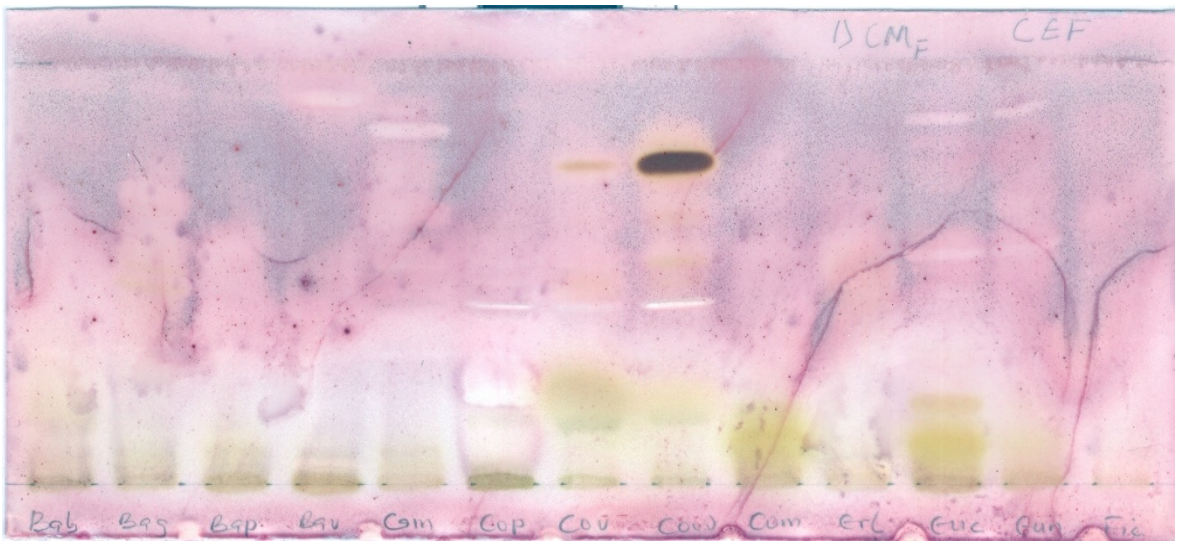

Fig. 4.3. Bioautography of dichloromethane fractions of different plant species against *S. aureus* (Bab (*Bauhinia bowkeri*), Bag (*Bauhinia galpinii*), Bap (*Bauhinia petersiana*),Bav (*Bauhinia variegata*) Cam (*Carissa macrocarpa*), , Cop (*Combretum padoides*), Cov (*Combretum vendae*), Cow (*Combretum woodii*), Com (*Commiphora harveyi*), Euc (*Euclea crispa*), Eun (*Euclea natalensis*), Erl (*Erythrina latissima*), Fic (*Ficus craterestoma*), Fig (*Ficus glumosa*) developed with chloroform: ethylacetate: formic acid (100:13:10)).

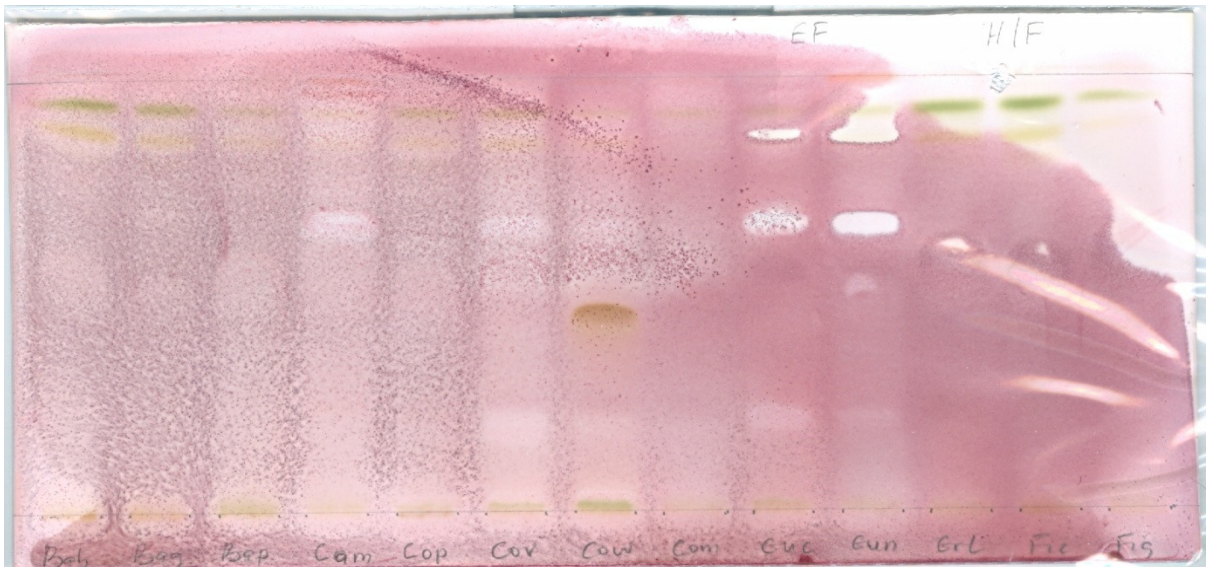

Fig. 4.4. Bioautography of hexane fractions of different plant species against *E. faecalis* (Bab (*Bauhinia bowkeri*), Bag (*Bauhinia galpinii*), Bap (*Bauhinia petersiana*),Cam (*Carissa macrocarpa*), Cop (*Combretum padoides*), Cov (*Combretum vendae*), Cow (*Combretum woodii*), Com (*Commiphora harveyi*), Euc (*Euclea crispa*), Eun (*Euclea natalensis*), Erl (*Erythrina latissima*), Fic (*Ficus craterestoma*), Fig (*Ficus glumosa*) developed with hexane: ethyl acetae: formic acid (70:30))

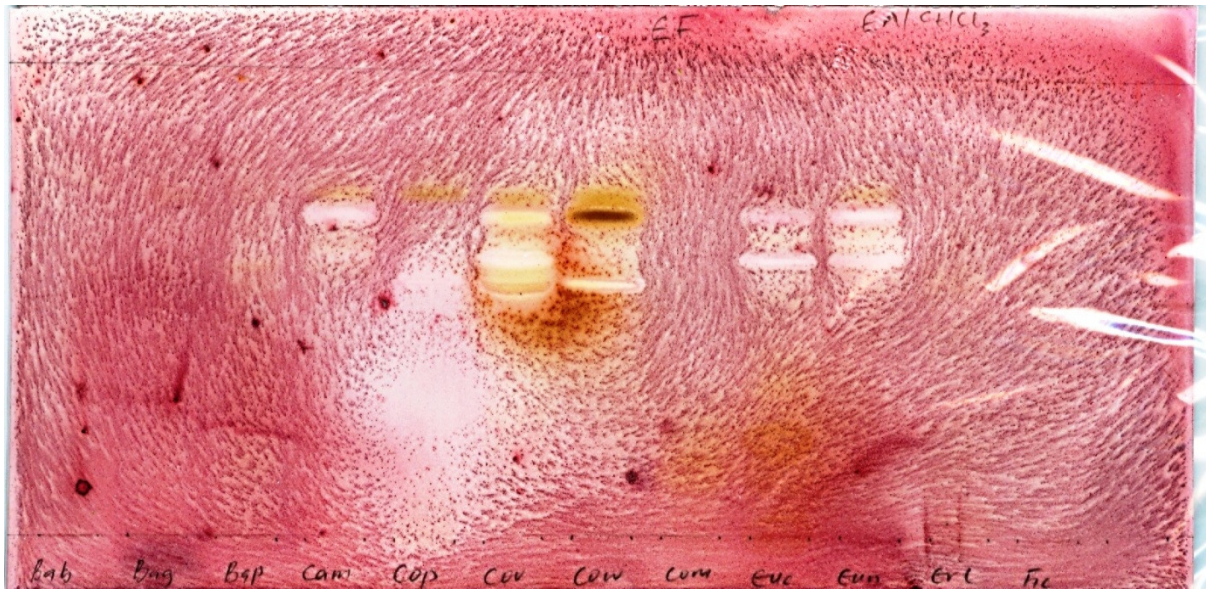

Fig. 4.5. Bioautography of dichloromethane fractions of different plant species against *E. coli* (Bab (*Bauhinia bowkeri*), Bag (*Bauhinia galpinii*), Bap (*Bauhinia petersiana*),Bav (*Bauhinia variegata*) Cam (*Carissa macrocarpa*), , Cop (*Combretum padoides*), Cov (*Combretum vendae*), Cow (*Combretum woodii*), Com (*Commiphora harveyi*), Euc (*Euclea crispa*), Eun (*Euclea natalensis*), Erl (*Erythrina latissima*), Fic (*Ficus craterestoma*) developed with chloroform: ethylacetate: formic acid (100:13:10)).

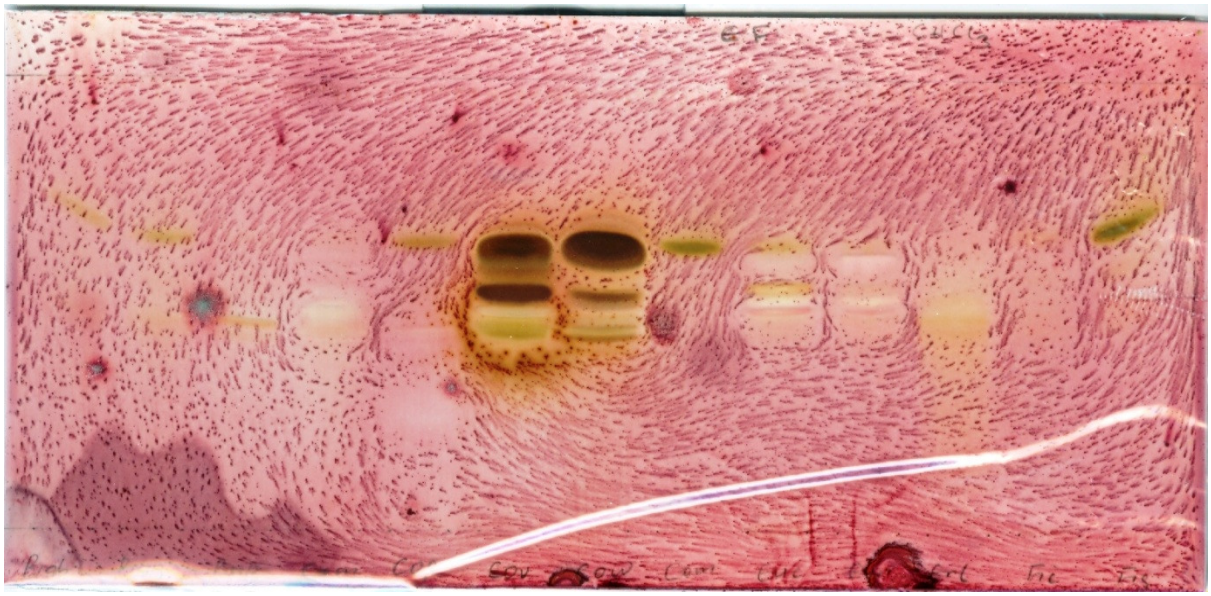

Fig. 4.6. Bioautography of dichloromethane fractions of different plant species against *E. faecalis* (Bab (*Bauhinia bowkeri*), Bag (*Bauhinia galpinii*), Bap (*Bauhinia petersiana*),Bav (*Bauhinia variegata*) Cam (*Carissa macrocarpa*), , Cop (*Combretum padoides*), Cov (*Combretum vendae*), Cow (*Combretum woodii*), Com (*Commiphora harveyi*), Euc (*Euclea crispa*), Eun (*Euclea natalensis*), Erl (*Erythrina latissima*), Fic (*Ficus craterestoma*), Fig (*Ficus glumosa*) developed with chloroform: ethylacetate: formic acid (100:13:10)).

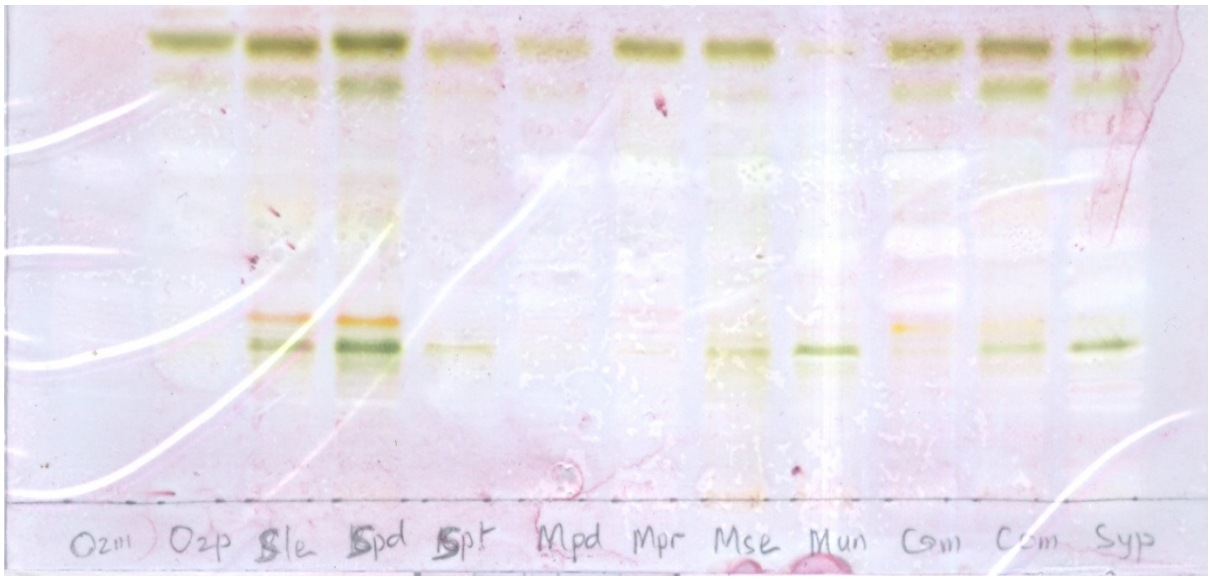

Fig. 4.7. Bioautography of hexane of different plant speicies against *C. neoformans* (Ozm (*Ozoroa mucronata*), Ozp (*Ozoroa paniculosa*), Sle (*Searsia leptodictya*), Spd (*Searsia pendulina*), Spt (*Searsia pentheri*), Mpd (*Maytenus peduncularis*), Mpr (*Maytenus procumbens*), Mse (*Maytenus senegalensis*), Mun (*Maytenus undata*), Cam (*Carissa macrocarpa*), Com (*Commiphora harveyi*), Syp (*Syzygium paniculatum*)).

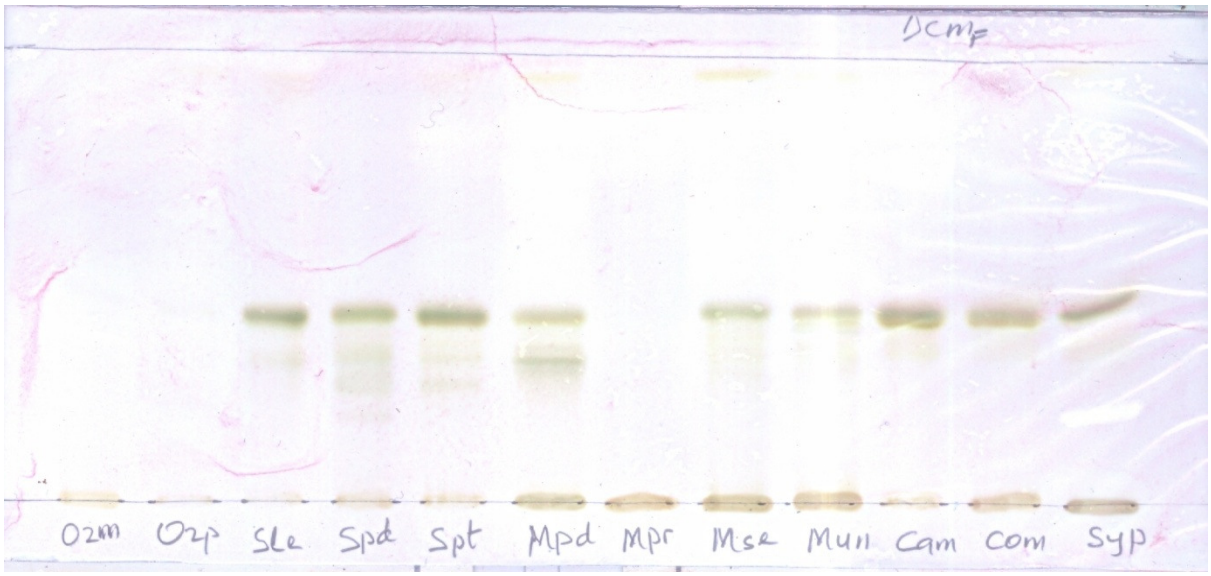

Fig. 4.8. Bioautography of dichloromethane fractions against *C. neoformans* (Ozm (*Ozoroa mucronata*), Ozp (*Ozoroa paniculosa*), Sle (*Searsia leptodictya*), Spd (*Searsia pendulina*), Spt (*Searsia pentheri*), Mpd (*Maytenus peduncularis*), Mpr (*Maytenus procumbens*), Mse (*Maytenus senegalensis*), Mun (*Maytenus undata*), Cam (*Carissa macrocarpa*), Com (*Commiphora harveyi*), Syp (*Syzygium paniculatum*))

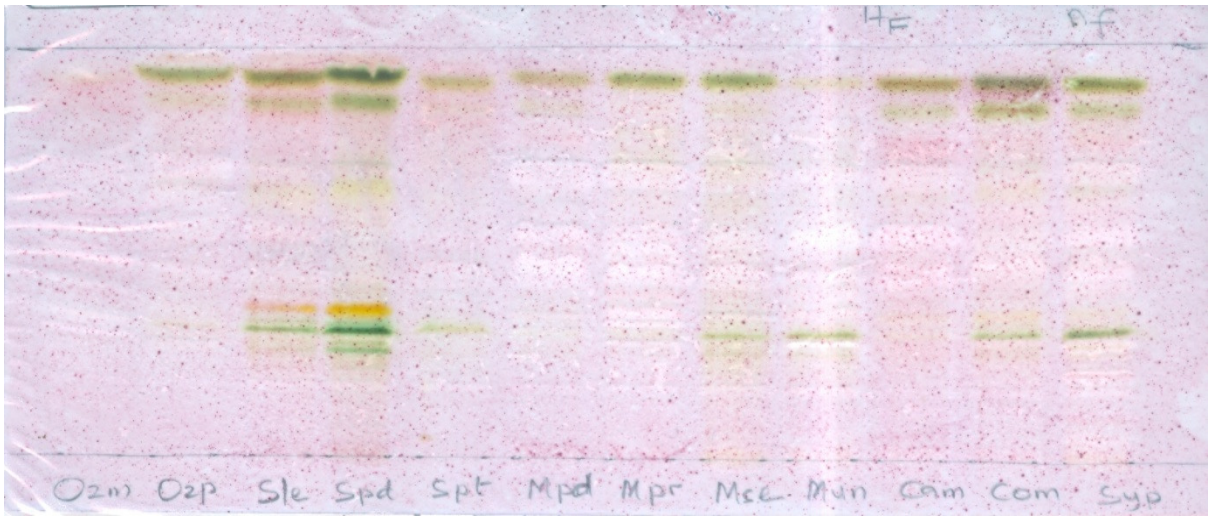

Fig. 4.9. Bioautography of hexane fractions against *A. fumigatus* (Ozm (*Ozoroa mucronata*), Ozp (*Ozoroa paniculosa*), Sle (*Searsia leptodictya*), Spd (*Searsia pendulina*), Spt (*Searsia pentheri*), Mpd (*Maytenus peduncularis*), Mpr (*Maytenus procumbens*), Mse (*Maytenus senegalensis*), Mun (*Maytenus undata*), Cam (*Carissa macrocarpa*), Com (*Commiphora harveyi*), Syp (*Syzygium paniculatum*))

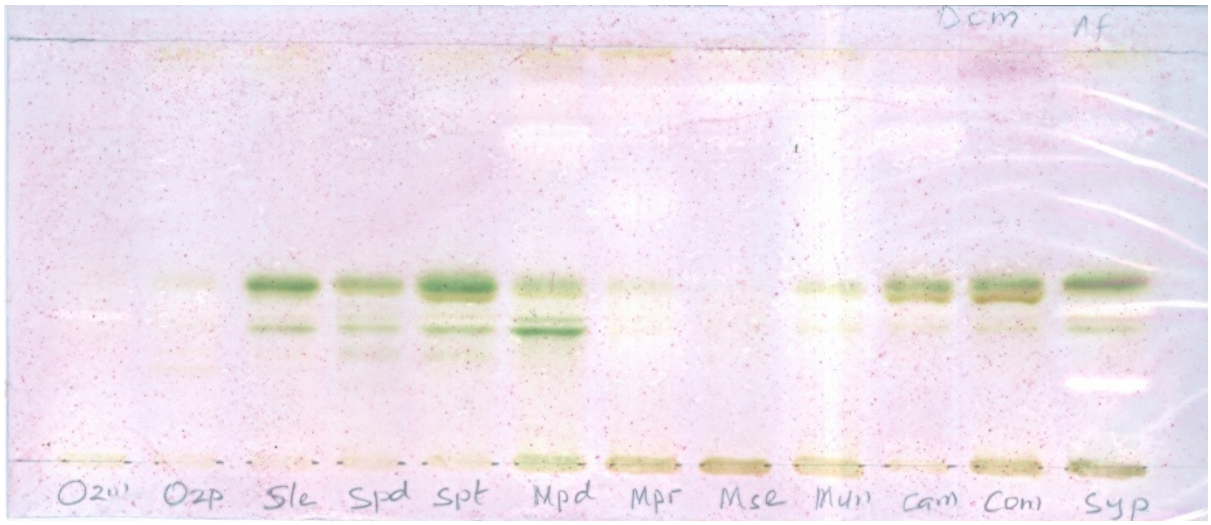

Fig. 4.10. Bioautography of dichloromethane fractions against *A. fumigatus* (Ozm (*Ozoroa mucronata*), Ozp (*Ozoroa paniculosa*), Sle (*Searsia leptodictya*), Spd (*Searsia pendulina*), Spt (*Searsia pentheri*), Mpd (*Maytenus peduncularis*), Mpr (*Maytenus procumbens*), Mse (*Maytenus senegalensis*), Mun (*Maytenus undata*), Cam (*Carissa macrocarpa*), Com (*Commiphora harveyi*), Syp (*Syzygium paniculatum*))

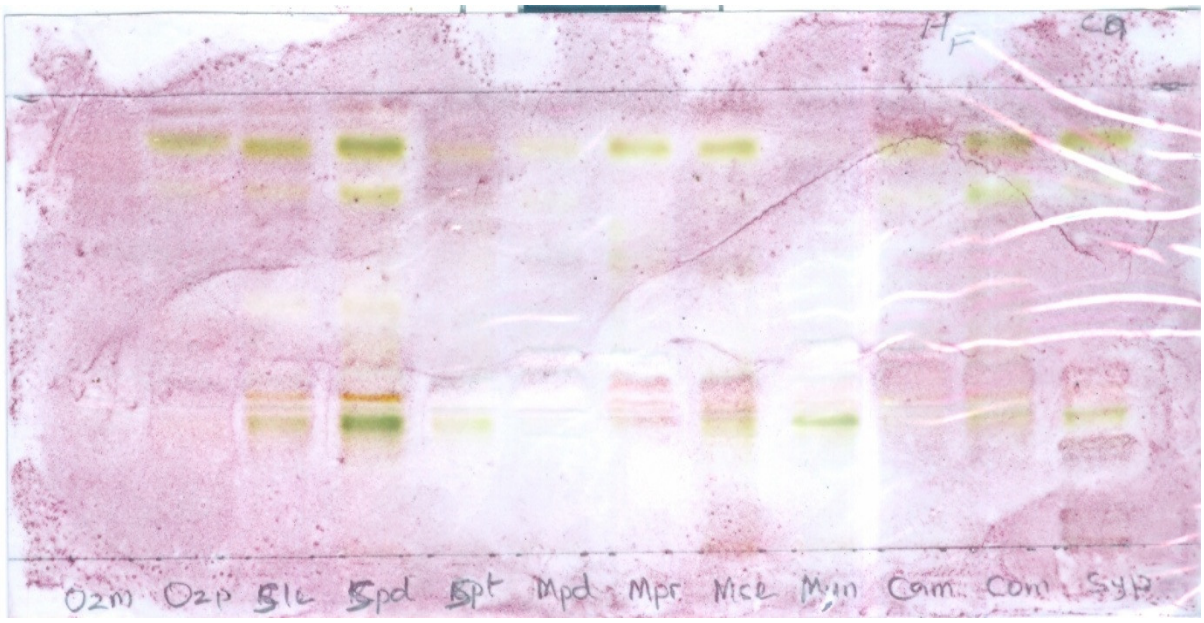

Fig. 4.11. Bioautography of hexane fractions against *C. albicans* (Ozm (*Ozoroa mucronata*), Ozp (*Ozoroa paniculosa*), Sle (*Searsia leptodictya*), Spd (*Searsia pendulina*), Spt (*Searsia pentheri*), Mpd (*Maytenus peduncularis*), Mpr (*Maytenus procumbens*), Mse (*Maytenus senegalensis*), Mun (*Maytenus undata*), Cam (*Carissa macrocarpa*), Com (*Commiphora harveyi*), Syp (*Syzygium paniculatum*)).

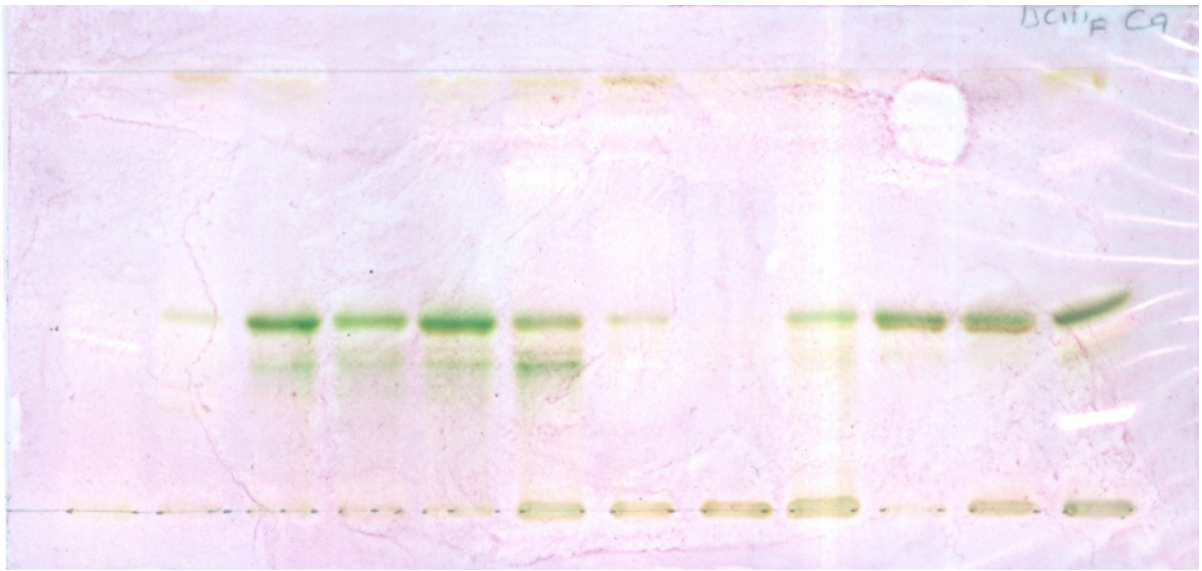

Fig. 4.12. Bioautography of dichloromethane fractions against *C. albicans* (Ozm (*Ozoroa mucronata*), Ozp (*Ozoroa paniculosa*), Sle (*Searsia leptodictya*), Spd (*Searsia pendulina*), Spt (*Searsia pentheri*), Mpd (*Maytenus peduncularis*), Mpr (*Maytenus procumbens*), Mse (*Maytenus senegalensis*), Mun (*Maytenus undata*), Cam (*Carissa macrocarpa*), Com (*Commiphora harveyi*), Syp (*Syzygium paniculatum*))

4.5.2. Minimum inhibitory concentration (MIC)

The antibacterial activities of the phenolic-enriched extracts, fractions and sub-fractions of different polarities of 27 plant species evaluated against 4 microorganisms (two gram positive and two gram negative bacteria) are presented in Table 4.1. The results are presented as minimum inhibitory concentrations (µg/ml) against tested bacteria and the fungi. The extracts and fractions of the plant species tested exhibited average to good degree of

inhibition against the growth of all the tested bacteria and fungi strains. The extracts and fractions exhibited high potency or growth inhibition at concentration between 19 and >2500 µg/ml for the various different organisms. In this investigation, hexane and dichloromethane fractions had significant and broad-spectrum antimicrobial activities against all tested microbial strains.

The most susceptible bacterium to the crude extract is *E. faecalis* with MIC ranged from 78 to 1250 µg/ml and the least susceptible bacterium to the crude phenolic-enriched extract is *E. coli* with MIC ranging from 312 to 2500 µg/ml. However, all the microorganisms tested are highly susceptible to hexane and dichloromethane fractions with MICs ranging from 19 to 1250 µg/ml. Some of the interesting results include the hexane and dichloromethane fractions of *C. padoides, C. vendae, C. woodii, B. galpinii, M. penducularis, M. procumbens, S. leptodictya* and *S. pendulina* with MICs of 19 - 39 µg/ml against *E. coli*. The hexane and dichloromethane fractions of *C. padoides, C. vendae, C. woodii, M. penducularis, M. procumbens, M. senegalensis, O. mucronata, O. paniculosa, S. leptodictya* and *S. pentheri* also exhibited good microbial growth inhibitory activity against *E. faecalis* with MICs between 19 - 78 µg/ml.

Growth inhibition activities against *S. aureus* of interest include hexane, dichloromethane, ethyl acetate, butanol fractions of *B. galpinii* and *C. vendae* (MIC 39 - 156 µg/ml); hexane and dichloromethane fractions of *C. padoides*, *O. mucronata* and *S. pentheri* (19 - 78 µg/ml), and hexane, dichloromethane, ethyl acetate fractions of *O. paniculosa, S. leptodictya* and *S. pendulina* (19 - 39 µg/ml).

Pseudomonas aeruginosa is also susceptible to growth inhibition by hydrogen and dichloromethane of *C. padoides, E. latissima, M. pendicularis, M. procumbens, and M. senegalensis* with MICs of 19 - 78 µg/ml; hydrogen, dichloromethane, ethyl acetate *M. undata, O. paniculosa, S. leptodictya, S. pendulina* and *S. pentheri* with MICs of 19 - 78 µg/ml. The reference antibiotic (gentamicin) exhibited good antibacterial activity against the four tested bacterial strains with MIC ranged between 0.18 and 1.56 µg/ml.

Table 4.1. Minimum inhibitory concentration (MIC) of the crude extracts and fraction against *E. coli* and *E. faecalis*

	E. coli						*E. faecalis*					
Plant spp	CRE	Hf	DCMf	ETOAc	Butanol	Water	CRE	Hf	DCMf	ETO Ac	Butanol	Water
Bab	625	39	156	312	156	>2500	78	312	312	39	78	312
Bag	312	39	156	312	156	312	156	156	39	156	78	312
Bap	312	78	156	312	625	312	78	156	312	78	39	312
Bav	625	78	156	156	156	312	312	312	625	39	39	312
Cam	312	156	156	156	312		312	312	312	78	156	
Cob	312	39	156	312	156	>2500	156	312	312	39	78	312
Cop	156	19	19	312	39	>2500	78	19	19	156	78	312
Cov	312	39	39	156	156	312	156	39	78	78	78	39
Cow	625	39	39	312	156	>2500	156	39	78	78	78	312
Cmh	625	312	39	78	312		156	156	156	78	156	
Erl	1250	156	19	156	156	1250	156	78	19	78	78	1250
Euc	312	625	78	78	156		156	312	156	156	78	
Eun	312	312	312	78	156		625	312	312	156	156	
Fic	2500	312	78	312	312		2500	78	312	312	312	
Fig	1250			1250	312		312			625	156	
Inc	1250				312		1250				312	
Mpd	312	39	39	78	625			39	39	156	1250	
Mpr	1250	39	39	156	1250		>2500	39	39	156	625	
Mse	2500	78	78	156	1250		2500	39	39	312	625	
Mun	1250	312	156	156	>2500		312	156	156	312	2500	
Ozm	1250	78	39	156	312		625	39	19	625	1250	
Ozp	1250	78	39	39	625		1250	39	19	156	78	
Sle	1250	39	39	78	2500		625	39	19	156	625	
Spd	1250	39	39	78	1250		625	156	19	312	625	
Spt	2500	39	39	156	2500		1250	39	19	156	1250	
Scb	625	312	156	39	156	1250	1250	78	312	39	78	2500
Syp	625	312	312	39	156	312	1250	312	156	39	156	1250

Table 4.1. Cont.....Minimum inhibitory concentration (MIC) of the crude extracts and fraction against *S. aureus* and *P. aeruginosa*

	S. aureus						*P. aeruginosa*					
Plant spp	CRE	Hf	DCMf	ETOAc	Butanol	Water	CRE	Hf	DCMf	ETOAc	Butanol	Water
Bab	625	78	312	625	78	312	312	78	312	156	312	312
Bag	625	39	78	39	78	312	312	312	78	312	156	312
Bap	312	156	312	78	78	78	78	312	625	625	312	625
Bav	625	78	1250	-	-	-	156	78	625	625	312	625
Cam	312	312	156	156	78		312	156	625	312	78	
Cob	625	78	312	156	78	312	312	78	312	625	156	312
Cop	156	19	39	156	156	312	156	78	39	312	19	312
Cov	156	39	78	78	39	39	156	156	156	156	39	312
Cow	312	39	78	156	156	312	625	312	156	312	312	312
Cmh	312	78	156	78	156	2500	1250	312	312	156	312	
Erl	1250	78	312	78	312		2500	78	78	156	78	1250
Euc	156	312	312	78	312		156	312	625	156	39	
Eun	312	312	625	312	312		625	625	625	312	78	
Fic	2500	156	312	1250	1250		2500	156	312	625	156	
Fig	625			312	625		1250			312	78	
Inc	625				156		625				312	
Mpd	>2500	78	39	312	78		312	19	19	156	312	
Mpr	1250	39	78	312	625		312	78	39	156	625	
Mse	625	78	39	156	625		625	39	39	156	1250	
Mun	625	156	156	156	312		312	78	78	78	2500	
Ozm	2500	19	19	625	1250		312	156	39	312	2500	
Ozp	156	19	19	78	156		156	39	39	78	156	
Sle	312	19	19	78	312		625	39	39	39	625	
Spd	312	78	78	39	625		625	78	78	19	2500	
Spt	312	19	19	156	312		156	19	19	78	2500	
Scb	156	1250	156	156	156	1250	156	312	156	156	156	1250
Syp	156	39	156	312	312	2500	156	156	156	78	312	1250

4.5.3. Minimum inhibitory concentration (MIC)

The phenolic-enriched crude extracts and fraction exhibited good to moderate growth inhibitory activities against the three fungal strains of different morphology with MICs ranging from 19 to 2500 µg/ml (Table 4.2). *Candida albicans* demonstrated resistance to all the crude extracts and fractions with the exception of dichloromethane and butanol fractions which had MICs of 19 - 78 µg/ml. In contrast, Cryptococcus neoformans was sensitive to majority of many crude extracts and fractions at the concentration ranging from 19 - 78 µg/ml. The fungi were susceptible to amphotericin B with the MIC ranges from 0.78 - 6.25 µg/ml.

Table 4.2. Minimum inhibitory concentration (MIC) of the crude extracts and fraction against *C. albicans*, *C. neoformans* and *A. fumigatus* Values below 100 µg/ml in different colour

	C. albicans						*C. neoformans*						*A. fumigatus*					
Plant spp	CRE	H	DCM	ETOAc	But	Water	CRE	H	DCM	ETOAc	But	Water	CRE	H	DCM	ETOAc	But	Water
Bab	625	625	78	156	39	39	156	312	78	78	39	39	2500	156	312	312	156	625
Bag	312	625	78	156	78	625	78	315	78	39	39	312	625	156	156	156	78	156
Bap	1250	156	78	156	156	625	78	625	39	39	156	78	625	312	78	78	156	156
Bav	312	-	-	156	78	625	78	-	-	78	78	78	625			312	156	156
Cam	625	156	625	312	19	156	78	78	156	19	19	1250	625	19	1250	625	312	156
Cob	625	625	312	156	156	1250	78	625	625	39	19	39	312	312	156	156	312	625
Cop	312	312	19	312	156	625	39	156	625	19	312	312	156	156	78	78	156	625
Cov	1250	625	312	78	312	312	78	19	156	78	156	312	156	156	156	156	156	625
Cow	1250	625	19	156	78	625	78	312	312	39	156	312	156	156	78	78	78	625
Cmh	625	156	39	156	39	625	156	39	19	39	19	1250	625	39	19	1250		312
Erl	1250	156	156			625	312	78	19			625	1250	156	156	312		625
Euc	625	156	156	156	78	156	78	156	78	39	39	156	312	625	156	625	156	156
Eun	625	156	78	78	78	625	78	78	39	156	78	625	312	625	625	312	78	312
Fic	2500	78	39	156	39	312	312	78	19	156	39	625	1250	312	156	156	312	156
Fig	625	78	39	78	39	312	78	78	19	78	39	1250	625	78	78	78	625	312
Inc	1250	78	19	156			78	78	39	39			1250	78	156			
Mpd	312	312	78	312	156	312	78	312	78	78	1250	2500	312	78	156	156	312	625
Mpr	1250	156	39		312	625	625	156	39		156	2500	1250	78	78		156	625
Mse	1250	78	39	156	156	625	156	156	39	78	78	1250	625	156	78	156	1250	312
Mun	1250	625	78	312	625	1250	156	1250	78	78	312		1250	625	625	156	625	625
Ozm	625	312	156	156	625	625	156	312	156	39	156	2500	625	39	156	39	156	1250
Ozp	312	156	78	156	312	625	78	312	39	39	156	1250	312	78	78	312	156	625
Sle	312	39	39		312	625	156	156	39		312	1250	625	78	39		156	312
Spd	625	39	78	156	625	625	312	156	39	39	156	625	625	78	39	156	78	312
Spt	2500	625	78	312	156	1250	312	1250	19	39	312	1250	2500	78	19	625	156	625
Scb	625	156	312			625	78	312	78			2500	312	78	19			625
Syp	312	156	156	625	312	625	156	78	39	78	39	1250	312	156	19	1250	2500	312

4.6. Discussion

4.6.1. Antimicrobial bioautography

The crude extracts and various fractions were screened qualitatively for growth inhibitory activity against 4 bacteria and 3 fungi representing different morphologies as yeasts and moulds. Many compounds present in the non-polar enriched hexane fractions inhibited the growth of the organisms tested with several zones of inhibitions.

However, the polar fractions exhibited poor individual inhibitory activities, and this may be due to the high solubility of polar compounds in water, like flavonoids resulting in washing and spread of the compounds on the TLC plate surface. Therefore, reducing the threshold inhibitory concentrations of the bioactive compounds on the spot of the chromatogram against the organisms tested. Other factors may include the disruption of synergistic effects of the individual compounds separated on TLC plates or the concentration of the bioactive components is not sufficient to inhibit microbial growth.

A number of methods have been developed for effective and quick screening of microbial growth inhibitory properties of compounds like the disc or agar diffusion assay adapted as agar-overlay methods (Rasoanaivo and Ratsimamanga-Urverg, 1993). However, the differential diffusion of the bioactive compounds from the TLC plate to the agar layer make the method unsuitable for certain class of compounds, especially the water-insoluble types like terpenoids and non-polar compounds (Eloff, 1998). The direct bioautography method allows the localization of a number of components with significant individual inhibitory activities against the tested organisms. The characteristic features of this method are its quickness, efficiency, simplicity, high sample throughput, small test sample size and no sophisticated equipment required. The method is adaptable and applicable to all extracts that can be separated on TLC, against any organism capable of growing directly on TLC plate surfaces.

4.6.2. Minimum inhibitory concentration (MIC)

In this investigation, in vitro antimicrobial efficacy of the crude 70% acetone leaf extracts and fractions derived from 27 plants (13 genera across 9 families) used in South African traditional medicine for treating diarrhoea and related ailments was quantitatively assessed on the basis of minimum inhibitory concentration (MIC). All the plants evaluated exhibited varying degree of inhibitory effect against the standard strain of human and animal pathogenic bacteria (Gram-positive as well as Gram-negative) and clinical isolate of pathogenic fungi. There have been no specific cut-off values as a reference or standard for categorizing antimicrobial activity of plant extracts and fractions. In this study, crude extracts and fractions with an MIC value less than 500 µg/ml were considered to have good activity and MIC value less than 100 µg/ml were considered to have significant antimicrobial activity of pharmacological interest according to the criterion by Rios and Recio (2005). A lower MIC values indicated high effectiveness of the compound as antimicrobial agent as little quantity which may be below toxicity level of the extracts can be applied without being harmful to the host.

Crude extracts of 4 out 27 had an MIC less than a 100 µg/ml (*Bauhinia bowkeri*, *Bauhinia galpinii*, *and Combretum padoides*) against *E. faecalis* and *Bauhinia petersiana* against *P. aeruginosa* (78 µg/ml). However, the antimicrobial activities were potentiated in the fractions with the non-polar fractions of hexane (MIC ranges from 19 to 312 µg/ml) and dichloromethane (MIC ranges from 19 to 625 µg/ml) enriched with terpenoids exhibiting more broad-based potency compared with the polar fractions of ethyl acetate (MIC ranges from 39 to 1250 µg/ml) and butanol (MIC ranges from 39 to <2500 µg/ml). The MIC value less than 100 µg/ml obtained for some fractions were significant although much higher than that of the control antibiotic (gentamicin with an MIC ranged from 0.18 to 1.56 µg/ml against bacteria and amphotericin B with an MIC ranged from 0.78 to 6.25 µg/ml against fungi).

The water fractions have relatively low antimicrobial activities (MIC ranges from 312 to <2500 µg/ml) except for the *C. vendae* with an MIC value 39 µg/ml against *E. faecalis* and *S. aureus*. In traditional medicine plant preparation, water is used as the major extractant. The poor antimicrobial activity of water fractions of most of the plants indicated that decoction or infusion may be less effective in infectious diseases. These observations are consistent with most of the findings in other studies.

From the phytochemical evaluation, the crude extracts contain high level of polyphenolic compounds. The activity of the extracts and polar fractions (ethyl acetate, butanol, and residual), though not exclusive to polyphenolic compounds only would be expected to correlate to the respective constituents, the structural configuration, functional groups and possible synergistic effects among the constituents. Members of this class of compounds are known to have either bacteristatic or bactericidal properties against most microorganisms depending on the structure and concentration used. The mechanism of their antimicrobial activity may be related to their fundamental properties of having the ability to form complex with protein and polysaccharides, thus the capacity to inactivate microbial adhesions, enzymes, and cell envelope transport protein. The presence and position of hydroxyl group in the phenolic structure determine and influence the antimicrobial activities of this class of compounds (Taguri *et al*, 2004). Phenolic compounds including tannins and flavonoids were found to have high antimicrobial activity (Majhenic *et al*, 2007; Vaquero *et al*, 2007). Some mechanisms of antimicrobial activity of phenolic compounds includes their ability to denature microbial proteins as surface-active agents (Sousa *et al*, 2006), ability to react with cellular membrane component which impairs both function and integrity of cells (Raccach, 1984), and the reducing property of phenolics can influence the redox potential (E_h) of microbial growth causing growth inhibition (Jay, 1996).

However, two methods are widely used in quantitative evaluation antimicrobial activities of plants extracts: Agar disc diffusion method (NCCLS, 2002) and serial dilution method (Eloff, 1998). Both methods depend on the effective solubility of the extracts in the test medium in order to obtain a maximum efficacy against the organisms. However, some phenolic compounds form complex with proteins and other macromolecules present in the test medium, therefore, get precipitated. While some extract components especially the non-polar are not readily soluble in test medium which is more than 90% water in most cases. These factors may at times cause

reduction in the effectiveness of the plant extracts to inhibit microbial growth. The antimicrobial profiles indicated that the extracts and fractions there from were active against Gram-positive and Gram negative bacteria, yeast and mould fungi. The susceptibility of both bacteria and fungi to the extracts may be indicative of the presence of broad-based bioactive compounds or general metabolic toxins.

Pathogenic enteric microorganisms present in contaminated food and water produces enterotoxins or irritants that cause intestinal disorder such as diarrhoea. *In vitro* antimicrobial assays against standard strains of the intestinal pathogens using the polyphenolic-rich crude extracts and fractions have demonstrated various degree of microbial growth inhibition. The plant extracts and fractions investigated have moderate to good activities against diarrhoeal standard strains such as *E. coli*, *S. aureus* and *C. albicans* and *P. aeruginosa*, thus validating their use in traditional medicine for treatment of diarrhoea symptoms. The mechanisms involved in diarrhoea symptoms are multifaceted and interwoven. It is, therefore, possible that extracts and fractions with moderate antimicrobial activities could still have good antidiarrhoeal effects by elaborating other biological activities such as antioxidant, anti-inflammatory, antisecretory, binding of toxins, and antimotility effects on the gastrointestinal tract.

4.7. Conclusion

In infectious diarrhoea many bacteria, protozoa, virus and parasites have been implicated as causative agents. These agents include *Vibrio cholera*, *Escherichia coli*, *Shigella dysenteriae*, *Bacillus cereus*, *Stapylococcus aureus*, *Clostridium difficile*, *Entamoeba histolytica, Salmonella typhi* and *Giardia lamblia*. Some viruses such as Rotavirus and adenovirus have also been implicated as causative agent of diarrheal diseases. The infectious mechanisms of the pathogenic strains of the enteric microbes include microbial adhesion and attachment, localized effacement of the epithelial mucosa lining, production and elaboration of secretory enterotoxins, production of cell-destroying cytotoxins, and direct epithelial cell invasion.

In this study, the emphases were on *E, coli*, *S. aureus*, *E. faecalis*, *C.albicans* as diarrhoeal pathogens. Seven virulence groups of diarrheagenic *E. coli,* namely enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAggEC), diarrhoea-associated haemolytic *E. coli* (DHEC) and cytolethal distending toxin (CDT)-producing *E. coli* have been classified (Clarke, 2001). On a global scale EPEC, EHEC, ETEC and EIEC are the most important diarrhoeal agents accounting for 4-8%, 0-1%, 12-20% and 0-2% respectively in terms of total episodes (Bhan, 2000). The virulence mechanisms of ETEC, EHEC, *S. aureus*, *E. faecalis* and some strains of *V. cholerae* include production of endotoxin, cytotoxins and reactive species. The use of antimicrobial therapy with microbicidal or microbistatic mechanisms may not be effective in the diarrhoea cases involving these organisms because the toxins if already present in contaminated food or water does not need the pathogens to exert activity. Therefore, non-antimicrobial therapy may be required in such cases but antitoxins which can antagonize

toxin and receptor interactions. More work is needed in evaluating the antitoxin and antiadhesion of medicinal plant extracts as other forms anti-infectious mechanisms.

Many of the plant extracts and fractions used have good activities especially the non-polar fractions of hexane and dichloromethane against the pathogens tested, and this may explain the traditional use of these medicinal plants.

Considering importance of oxidative burst such as ROS/RNS in the immune mechanisms and possible consequences of cellular damages, if the resultant oxidative stress is not resolved by the endogenous antioxidant system, the antioxidant potentials of the plants will be evaluated in the next chapter.