IN VITRO ANTIMICROBIAL ACTIVITY OF *AVICENNIA MARINA* (MANGROVE)STEM BARK

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2020

DECLARATION

I declare that this dissertation is my original work and it has not been presented to any other university for a similar or any other degree award.

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DEDICATION

This dissertation is dedicated to my parents Mr. Stanley Njenga Muthiora and Mrs. Lorna Njeri Muthiora

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ABBREVIATIONS

AMR	Antimicrobial resistance
MRSA	Multidrug resistant *Staphylococcus aureus*
VRSA	Vancomycin resistant *Staphylococcus aureus*
ESBL	Extended spectrum beta lactamase
MDR	Multidrug resistant
XDR	Extreme drug resistant
STI	Sexually transmitted infection
ESC	Extended spectrum cephalosporins
USD	US dollar
MIC	Minimum inhibitory concentration
MBC	minimum bactericidal concentration
KWS	Kenya wildlife services
KFS	Kenya forestry services
Ha	Hectares
CFU	Colony forming units
CLSI	Clinical and lab standards institute
DMSO	Dimethylsulfoxide
CFR	Case fatality rate

ABSTRACT

Background: There has been a rising global antimicrobial resistance (AMR) pandemic in the recent past with far-reaching health and socio-economic implications.For example, there has been an emergence of Methicillin resistant *Staphylococcus aureus* (MRSA), Vancomycin resistant *Staphylococcus aureus*(VRSA) andextended spectrum beta lactamase-(ESBL) producing strains of *Klebsiella* spp., *E. coli,* and *Pseudomonas* spp. which have caused longer hospital stays and higher cost of care. Search for new approaches inthe treatment of infectious diseases is therefore imperative.

Africa has a wide unexploited diversity of plants with bioactive molecules that can be used as antibiotics. For instance, local information shows that mangrove trees, were decades ago being used in the treatment of various ailments along the Kenyan coast. In the recent past,mangrove species such as *Rhizohphora* sp, *Avicennia* sp, and *Sonneratia* sp havebeen shown to have anti-*Pseudomonas* spp. activities based on which more studies on its activities against other important microbial pathogens such as*Staph aureus, Vibrio cholerae, Salmonella*typhi, *E. coli* and *Candida albicans* need to be carried out.

Objective: To determine the*in vitro* activity of *Avicennia marina* extracts against *Staphylococcus aureus, Vibrio cholerae,Salmonella* typhi*, E. coli and Candida albicans*

Methodology: Barks of *Avicennia marina*were collected from Gazi bay in Kwale county. They were washed, air dried, milled and weighed. The material was then used to prepare extracts using different organic solvents(methane, ethyl acetate, dichloromethane, and chloroform) throughmaceration. The crude extractswere rotary evaporated, whereof the pure extractsweresubjected to*in vitro*antimicrobial susceptibility tests. The zones of inhibition were measured and the minimum inhibitory concentration and minimum bactericidal concentration for eachextract determined.

Results: The results from this study showed that the bark of *Avicennia marina* has significant antimicrobial and antifungal activity. The MIC and MBC values also revealed that at lower concentrations, the extracts exhibited bacteriostatic properties and at higher concentrations exhibited bactericidal properties.

CHAPTER 1: BACKGROUND AND LITERATURE REVIEW

1.0BACKGROUND

Infectious diseases present a major public health problem and remainthe leading cause of death globally, with a rising global antimicrobial resistant pandemic [1-3].Owing to this, antibiotics have a critical role in the reduction of the infectious disease burden [1]. There has however been a rising global antimicrobial resistance (AMR) pandemic in the recent past, with far-reaching health andsocio-economic implications [2]. New approaches, including novel drugs, to treat infectious diseases are therefore necessary [3].

Various plants have been used for centuries for medicinal purposes and are an indispensable and valuable source of natural products. They have great potential for sources of new drugs that are considered safer as they are environmentallyfriendly andare less toxic [4].Mangroves have been shown to contain bioactive compounds such as steroids, tannins, triterpenes, saponins, alkaloids and flavonoids with varying degree of antimicrobial activities [4-6]. However, their potential as therapeutic agents have not been fully assessed. A few residents from Gazi Bay and Msambweni in Kwale county were interviewed. They averred that when they were younger, they witnessed their parents and grandparents treating abdominal ailments using mangrove trees. According to the residents the community no longer used mangrove trees for medicinal purposes and the knowledge was not common among them.

The present study was designedto assessthe activity of crude extracts of*Avicennia marina*, a mangrove species found in the Kenya coast,against *Staphylococcus aureus, Salmonella* typhi, *E. coli, Vibrio cholerae* and *Candida albicans*.

1.1 PROBLEM STATEMENT

Antibiotics have an important role to play in the fight against infectious diseases [1]. AMR is however a global public health threat. Human infections with these AMR strainshave led to longer hospital stays, higher mortality rates and higher cost of care due to availability of very few alternative therapies. For example, there has been rising AMR by *Staph aureus, E. coli, Salmonella spp, Vibrio spp* as well as *C. albicans*which are of public health importance due to their propensity to cause severe, life threatening illness.

1.2LITERATURE REVIEW

It is estimated that over 80% of the global population relies on traditional medicine partly due to its accessibility and affordability as a source of treatment in primary health care in resource

poor settings [7,8].Natural products from terrestrial and marine organisms have for long been used in the treatment of human diseases. In recent years there has been renewed interest in the role of plants in drug development due to factors such as unmet therapeutic needs, diversity of structure and biological activity of natural compoundscoupled with new technologies in phytochemistry [7].

There is increasing convergence between traditional and modern medicine, and it has been suggested that the inclusion of traditional medicine in provision of essential health services will contribute towards Universal Health Care, as well as attainment of Sustainable Development Goal #3 geared towards ensuring healthy lives and promoting wellbeing for all at all ages [9,10]. There is therefore the need to explore the pharmaceutical potential of various plant species to contribute to the achievement of this goal.

Plants have been used for centuries for medicinal purposes and are a valuable source of natural products withhistorical evidence on the use of plants in the preparation of drugs dating back over 5000 years [4,8].

1.3MANGROVE SPECIES

Mangroves are evergreen, salt tolerant trees growing in sheltered tidal and intertidal waters between 30° N and 30°S of the equator. Extensions to these limits however occur in Japan (31°32'N), Bermuda (32°20'N), New Zealand (38°03'S), Australia (38°45'S) and South Africa (32°59'S)[11,12].Globally, there are approximately 60 -70 species of mangroves which are divided into 12 families [11]. These cover approximately 25% of Earth's coastline in about 112 countries and cover close to 181,000km² of the earth's surface [12,13].

Mangrove forests cover 61,271Ha of land in Kenya and account for approximately 3% of the country's natural forest cover and less than 1% of the national land area where they are protected bylaw and managed by the Kenya Forest Service (KFS) singly or in collaboration with Kenya Wildlife Service (KWS) where they occur in marine protected areas. These forests are found along the 536km coastline, extending from the Kenya-Tanzania border in the south to the Kenya-Somalia border in the north (latitudes 1°40´S and 4°25´S and longitudes 41°34´E and 39°17´É) [14]. This areaspans Kwale, Kilifi, Lamu, Tana River and Mombasa Counties. Within Kwale County, the forests are found in Vanga-Funzi, Gazi Bay and River Mwachema in Diani (Figure 1).

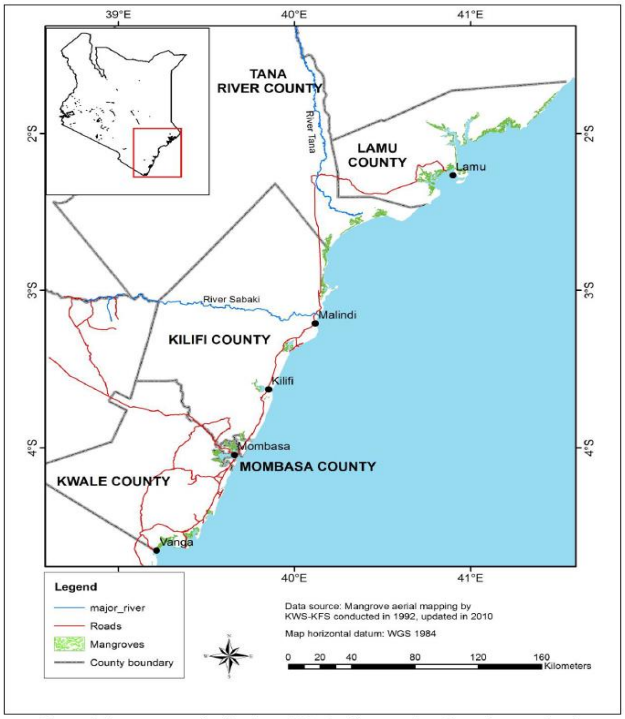

Figure 2.1: Mangrove distribution within the five counties along the coastal strip

Figure 1 Mangrove distribution within the 5 counties along the coastal strip (Mangrove and management, 2017)

Mangroves exist in a unique habitat and therefore need to develop different metabolic processes to survive thus leading to the synthesis of secondary metabolites includingtannins, saponins, flavonoids, alkaloids, steroids and triterpenes that may have pharmaceutical value [4,5,15]. It has been suggested that they have been used to treat ailments such as gastric distress, skin disease, elephantiasis, sores, leprosy and headache [16].

1.4LOCAL USE OF MANGROVE

Mangrove trees have a myriad of uses locallyalthough they are mainly used as fuel wood (Table 1)[17].The tree stems are also used for medical purposes. For example,*Rhizopora mucronata*is said to provide relief from constipation, fertility problems and menstrual disordersin women whereas*Xylocarpus granatum*is known to relieve muscle aches and skin disorders. These ailments are usually caused by different viral, fungal, and bacterial pathogens. The bark of *Rhizophora* spp. is used to make local dyes whereasthe smoke from logs of *Avicennia marina* is said to have insect repelling properties.

Table 1 Mangrove species found in Kenya and their uses (mangrove and mangrove management 2017)

SPECIES	LOCALNAME(SWAHILI/DIGO)	MAIN USE
Rhizophora mucronata	Mkoko	Poles, dye, firewood, fencing, charcoal
Bruguiera gymnorhiza	Muia	Poles, firewood, charcoal
Ceriops tagal	Mkandaa	Poles, firewood, charcoal
Sonneratia alba	Mlilana	Boat ribs, poles, firewood
Avicennia marina	Mchu	Firewood, poles
Lumnitzera racemose	Kikandaa	Fencing poles, firewood
Xylocarpus granatum	Mkomafi	Furniture, poles, firewood
Heritiera littoralis	Msikundazi	Timber, poles, boat masts
Xylocarpus moluccensis	Mkomafi dume	Fencing poles, firewood

1.5ANTIMICROBIAL ACTIVITY OF MANGROVES

Several studies have been conducted on effects of mangrove extracts on various bacterial, fungal and viral pathogens using differentextraction methods includingwater, methanol, ethanol, ethyl acetate, chloroform and petroleum ether [4,5,18,19].

Previous work [4]has showed that extracts of *Rhizophora mucronata* were active against hepatitis B whereas *Avicennia marina, Avicennia officialis, Bruguiera sexangular*and *Lumnitzera racemose* were activeagainst *Staphylococcus aureus* and *Proteus*spp.

Sahoo *et al*(2012) conducted a study on the leaf extracts of *Rhizophora mucronata,Avicennia marina*and *Exoecaria agallocha* and demonstrated no activity against *Salmonella typhi* and *Proteus vulgaris*. Theyhad varying degrees of activity against *Staphylococcus aureus, Streptococcus pneumoniae* and*Proteus mirabillis.*Lotilkar *et a*l ((2016)studied the activity of dried and powdered leaf extracts of *Acanthus illicifolius, Ceriops tagal* and *Sonneratia caseolaris* against *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae* and *Bacillus subtillis.*These mangrove species were active against these bacteria but*C. tagal*had no activity against *Klebsiella pneumoniae.*

Two studies on the activity of *Lumnitzera littorea* and *Avicennia marina*againsthuman pathogenic fungi C*andida albicans, Aspergillus* spp. and *Cryptococcus neoformans* showed

that mangrove have no activity against these three pathogens[11,19]. However, no information is available on the microbicidal activities of *A. marina* from Kenya.

1.6*AVICENNIA MARINA*

*Avicennia marina*is locally knownas "Mchu" among the Digo. It is classified as a true mangrove due to the formation of pure stands, presence of morphologically specialized adaptive forms and physiological mechanisms to control salt. Mature trees stand at a height ranging from 7 – 18 metres. The tree trunk is grey to greyish brown, with a diameter of 30 – 35cm and cracks in the bark [20]. The trees grow in 2 zones: seaward and land ward. Seaward trees grow up to 12-18m tall while landward groves grow up to 2-5m tall. The roots appear vertically on the surface of the soil and are light to dark brown in color. The leaves occur singly and are round with no anglesas shown in the Figure below.

Figure 2 Leaves of Avicennia marina. Source

(Photo taken by author at Gazi bay on 26/11/2019)

1.7*VIBRIO CHOLERAE*

Vibrio cholera is a Gramnegative, non-sporing bacillus inhabiting estuarine aquatic habitats. It is a flagellated, facultative anaerobe, curved rod, capable of respiratory and fermentative metabolism. Its growth is stimulated by 1% NaCl but is able to grow on nutrient agar without NaCl. This bacterium is oxidase positive and reduces nitrate to nitrite [21,22].

Vibrio cholerae has re-emerged as a major cause of disease, causing diarrhea that affects both rural and urban populations. Locally, between January 2019-April 2019 about 1,350 new cases were identified of which 72 were confirmed positive and 6 reported deaths (CFR 0.4%) [21,23]. Disease occurrence is usually due to ingestion of contaminated and undercooked sea and freshwater fish, contaminated water supplies and rarely exposure of wounds to contaminated water. Once ingested, enterotoxin is produced which disrupts ion transport within the intestinal epithelium leading to water and electrolyte loss through watery diarrhea. Other symptoms may include bloody diarrhea, necrotizing fasciitis and primary septicemia in immunocompromised patients [21,22].

The drugs of choice for treatment of the infection include tetracyclines, fluoroquinolones and azithromycin, which until recently remained active against the bacteria [24]. There have however been reports in the recent past of strains exhibiting reduced susceptibility to these agents leading to treatment failure [24,25]. Reports of multidrug resistant strains date back as far as the 1970's to tetracyclines, streptomycin and chloramphenicol, with increased resistance over the years [25,26]. Resistance is either acquired through gene mutations or through horizontal gene transfer through plasmids. The bacteria may then be able to transfer these genes of resistance to gut commensals and pathogens thus complicating treatment [27]. With resistance to all classes of drugs documented, and no new drugs under development, it is imperative that novel and alternative strategies are devised for disease management [28].

1.8`*STAPHYLOCOCCUS AUREUS*

Staph. aureus is a Gram positive non-motile and non-sporing coccus, appearing in grape-like clusters under a microscopy. Itrequires 30-37℃, pH 5-7.5 and up to 15% NaCl to grow. Biochemically it is coagulase and catalase positive[29,30].

Staph aureus is a common commensal of skin, oral cavity and the human gut, with an estimated asymptomatic carrier rate of 30-60%[31]. It is however themost common cause of gastroenteritis and food borne infection, with a CFR of 0.03% in adults and 4.4% in paediatrics and geriatrics. Symptoms of the infection include nausea, vomiting, diarrhea, and abdominal pain. It can also cause infections of the skin, bones, soft tissues, urinary tract, respiratory tract and disseminate blood infections[29,31,32].

Staph. aureus is a major pathogen associated with hospital and community acquired disease, Methicillin resistant Staph. aureus has long since been considered a nosocomial bacterium, but in the recent past has been documented in the community as an emerging public health

threat [33,34]. Drugs used in the treatment of staphylococcal infection include aminoglycosides, tetracyclines, macrolides, oxazolidinones, lincosamides and streptogramins. There has however been documentation of resistance to multiple classes of antimicrobial agents due to decades of recurrent exposure to the agents [35,36]. Antimicrobial resistance is therefore a major challenge to the treatment of both nosocomial and community acquired infections due to the limited treatment options available [33,34].

1.9*SALMONELLA* TYPHI

Salmonella spp.are flagellated, Gram-negative rods. They are facultative anaerobes capable of respiratory and fermentative metabolism. Biochemically they are indole positive. They reduce nitrates to nitrites and ferment carbohydrates to produce acid. Identification is by culture and biochemistry, as serological tests have low sensitivity and low specificity[37,38].

Salmonellosis is of public health importance with the most common manifestation being gastroenteritis,bacteremia,and enteric fever where the spectrum of clinical manifestations ranges from asymptomatic carriage to fatal disease[39,40].Enteric fever is caused by typhoidal species *Salmonella enterica* serovar typhi and *salmonella enterica* serovar paratyphi A, with an estimated 22million cases occurring each year resulting in approximately 200,000 deaths annually [39-41]. Non typhoidal salmonella (*Salmonella* typhimurium, *Salmonella* enteritidis, *Salmonella* Heidelberg and *salmonella* Newport)., has become a major cause of illness. The common causes of disease in Africa are *S.* typhimurium and *S.* enteritidis. Symptoms of illness include nausea, diarrhea, vomiting, and abdominal pain. Invasive non typhoidal salmonellosis may also occur in immunosuppressed individuals [38,39,42].

The first line drugs in the treatment of salmonellosis were chloramphenicol, ampicillin and cotrimoxazole, with chloramphenicol being the drug of choice. Due to the inappropriate use of antibiotics, there has been selection pressure within bacterial populations with the rise of resistant strains [43,44]. Second line drugs subsequently introduced included fluoroquinolones and azithromycin, to which resistance has also arisen, leading to the introduction of third generation cephalosporins for the treatment of multidrug resistant infections [43-45].

1.10*ESCHERICHIA COLI*

*E.coli*is a Gram-negative rod that ferment sorbitol and grow at a wide range of temperatures[46]. The bacterium is considered to be normal flora inhabiting the gastrointestinal tracts of humans, cattle and deer[47].

Several strains of the bacterium such enteroinvasive *E. coli*(EIEC), enterohaemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC) and enteroaggregative *E. coli* (EAgg.EC) exist which cause human disease. Subtypes are based on the O and H antigens, with E.coli O157:H7 being the most important cause of food borne epidemics[48]. This subtype is also the most studied due to reasons such as its widespread diffusion, tolerance of a diverse physical and chemical environments, the low infective dose required to cause illness as well as the severity of illness it causes [46].

Pathogenic strains of *E. coli* cause a wide spectrum of disease ranging from diarrheal disease to meningitis, wound infections, septicemia, and hemolytic uremic syndrome[47]. Infection with *E. coli* occurs through direct contact from shed bacteria on surfaces, direct person-person contact or through ingestion of contaminated food and water[49].

Antimicrobial studies have shown regional variation in susceptibility. Kibret and Aberashowed that strains of *E. coli* resistant to amoxycillin, erythromycin and tetracycline while susceptible to nitrofurantoin, norfloxacin, gentamycin and ciprofloxacin exist [50]. Vramic and Uzunovic demonstratedthe presence of *E. coli*strains resistant to ampicillin and trimethoprim-sulfamethoxazole, while susceptible to gentamicin, ciprofloxacin, nitrofurantoin, cephazolin and nalidixic acid[51].

1.11*CANDIDA ALBICANS*

Candida albicans is a non-fastidious yeast that is dimorphic in nature (exhibits both yeast-like and fungal forms). On gram stain it appears as large, budding, gram-positive cells with peudohyphae [52,53].

Candida albicans is a common commensal of oral and gastrointestinal flora, with asymptomatic colonization of the mouth, gastrointestinal tract, and reproductive tract of healthy individuals. The gastrointestinal tract acts as a reservoir for disease causing organisms and especially so for *Candida* sp which do not have a significant environmental reservoir[54-56]. *Candida* sp are opportunistic pathogens, causing mucocutaneous, vulvovaginal and hematogenous infections in persons with compromised immunity [57,58].s

Candida sp are an important pathogen due to their predilection to cause opportunistic infections especially in immune compromised populations. There are relatively few classes of antifungals therefore treatment options are often limited in diseases. This particular problem is compounded by the emergence of drug resistant strains which emerge as a result of frequent exposure to antifungals leading to selection pressure and tolerance[59].

Azoles and topical treatments are the first choice in oropharyngeal and cutaneous infections, while invasive disease, candidemia and hematogenous candidiasis are treated with amphotericin B, intravenous azoles and echinocandins[59].

As with most microbes, there has been documentation of drug resistant *Candida* sp which may arise as a consequence of long term antifungal use in recurrent candidiasis [58,60]. Due to the public health burden of disease caused by *Candida* sp and especially in immune compromised populations, it is imperative that new agents are developed to reduce this burden of disease.

1.12JUSTIFICATION

The continued misuse and overuse of antimicrobial agents has led to selection pressure among microbial populations, with emergence of antimicrobial resistant phenotypes[61]. The emergence of drug resistant microbes has thus become a serious global public health threat, with treatment failures reported in both nosocomial and community settings[34,43]. With the upward trend in antimicrobial resistance, and an ever-shrinking pool of antimicrobial classes to choose from, it is imperative that the development of new agents is forefront in the global health agenda.

Several studies have been done elsewhere on the antimicrobial and anticancer properties of mangrove species and specifically *Avicennia marina*[62-64], however there is very little information and very few studies done on the antimicrobial activity of any mangrove species in Kenya. *Avicennia marina* was chosen as the mangrove of choice as it is found on the landward side. Several microbial species were chosen on the basis of biological importance and potential to cause disease; *Staphylococcus aureus, Vibrio cholerae, E. coli, Salmonella* typhi and *Candida albicans*. These are species known to cause serious human infection and have in the recent past been reported to exhibit antimicrobial resistance to their treatment of choice. This resistance has necessitated the development of novel compounds for their treatment, hence the present study

The microorganisms studied in this work were chosen due to their great disease causing potential especially in the background of immune suppression. Chronic infection with *C. albicans* could lead to hematogenous candidiasis as well as predispose to other opportunistic infections. Failed treatment of *V. cholerae* could lead to death due to dehydration. It is therefore imperative to test the antimicrobial properties of *A. marina* which has been shown to be effective against a myriad of bacteria species.

The present study was designedto evaluatethe antimicrobial activity of the bark extracts of *A. marina*and to establish the MIC and MBC of the extracts.

1.13RESEARCH QUESTION

1. Does the species *Avicennia marina* possess biologically active substances with antimicrobial activity and microbial selectivity?

1.14OBJECTIVES

1.14.1GENERAL OBJECTIVE

To determinethe antimicrobial activity of *A. marina* against *C. albicans, E. coli, S.* typhi, *Staph. aureus* and*V. cholerae*

1.14.2SPECIFIC OBJECTIVES

1. To evaluatethe *in vitro*bactericidalactivity of different extracts of *A. marina* against *C. albicans, E. coli, S.* typhi, *Staph. aureus* and*V. cholerae*
2. To determine the minimum inhibitory concentrations of extracts of different extracts of *A.marina* against *C. albicans, E. coli, S.*typhi, *Staph. aureus* and*V. cholerae*
3. To determine the minimum bactericidal concentrations of extracts of different extracts of *A. marina* against*C. albicans, E. coli, Salmonella* typhi, *Staph. aureus* and*V. cholerae*

CHAPTER 2:METHODOLOGY

2.0 INTRODUCTION

The present study was an experimental study employing solvent extraction. Different solvents were used for the extraction of phytochemicals from *A. marina*. The bark of *A. marina*was harvested and subjected to pre-washing, drying, and milling to obtain a homogenous substance that was exposed to the different solvents through a process of maceration. The solvents used were methanol, ethyl acetate, water,dichloromethane, chloroform, and hexane. The plant extracts were then tested against *Staph.aureus,V.cholerae, E. coli, Salmonella* Typhi and*C.albicans*using agar well diffusion method for antimicrobial susceptibility testing and broth dilution for determination of the minimum inhibitory concentration and minimum bactericidal concentrations.

2.1PLANT COLLECTION AND IDENTIFICATION

Plant material (*A. marina*) was obtained from the Gazi bay mangrove ecosystem in Kwale county (Figure 3) following approvalfrom Kenya Forest Services (KFS)forthe same. Gazi is located at 4°25'S,39°30'E and approximately 50km from Mombasa. It experiences a diurnal tidal pattern with a bimodal rainfall pattern which peak in May and November. The forests cover approximately 6.61km^2 with all 8 species of mangrove present. Four species are however dominant: *Rhizophora mucronata, Avicennia marina, Ceriops tergal* and *bruguieria* spp.

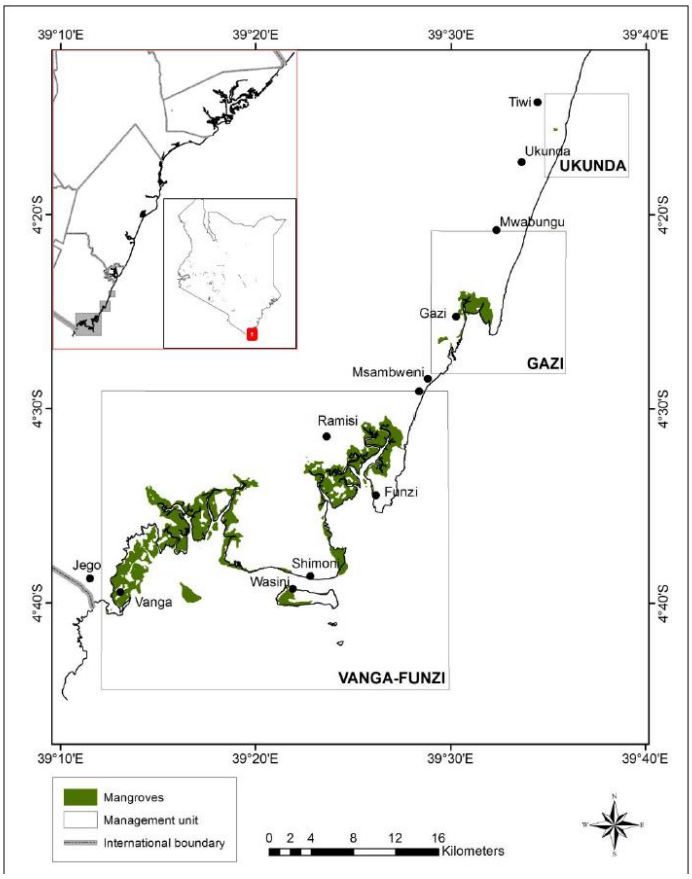

Figure 3 Distribution of mangrove forests in Kwale county (Image courtesy of Mangrove ecosystem management plan 2107-2027)

The barks were obtained from mature *A.marina* trees from the Gazi bay ecosystem in Kwale County of coastal Kenya.The pieces of bark werespread on a flat surface under a shade to dry overnight in preparation for transport and stored in clean containers and transported to the Department of Medical Microbiologylaboratory, University of Nairobi. The barkswerethen spread into a single layer on a bench top to air dry for five days under shade.

Mature leaves, fruits and bark material were also collected and prepared by mounting and pressing in between sheets of mounting paper until they were dried. These specimens werethen shipped to the Department of Botany herbarium, school of biological sciences, University of Nairobi for identification. A voucher specimen was also deposited at the herbarium for documentation (voucher number JNM 2019/02).

2.2EXTRACTION OF PLANT MATERIAL

2.2.1 SOLVENTS AND REAGENTS

The solvents used were methanol, ethyl acetate, chloroform, water, hexane, and dichloromethane(Finar Ltd, Ahmedabad, India). Dimethylsulphoxide (Finar Ltd, Ahmedabad, India) was used to prepare the suspensions of the crude extracts used for antimicrobial activity testing.

2.2.1.1 MATERIALS AND EQUIPMENT

Filtration of the crude extracts before rotary evaporation was done using Whatman filter paper no. 1 (Whatman international Ltd, Maidstone, England).

A Heidolph VV2000® rotary vacuum evaporator (Heidolph Electro GmbH & Co. K.G., Kelheim, Germany) connected to a diaphragm vacuum pump (KNF Neuberger GmbH, Freiburg, Germany) was used to dry the crude extracts to dryness.All glassware used was sterilized at 150℃ for 1hour using a Memmert universal oven (Memmert GmbH & Co, KG, Schwabach, Germany). The culture media was sterilized using a portable autoclave (Dixon's surgical instruments Ltd, Essex, England) at 121℃ for 15 minutes.

2.2.2PREPARATION OF CRUDE EXTRACTS IN ORGANIC SOLVENTS

The dried plant material was milled to fine powder to increase the surface area for efficient extraction[65]. For this purpose, cold maceration technique with continuous stirringwasused (figure 4)

Two hundred grams (200g) of the powdered crude extract was placed in a stoppered container with 1200ml of the different organic solvents, stirredand extracted for 48 hours with frequent agitation until the soluble matter had dissolved. The mixture was then strained and filtered through Whatman filter paper No.1, after which the filtrate was collected in a stoppered conical flask.

2.2.3 PREPARATION OF AQUEOUS EXTRACTS

Cold maceration was used to prepare the cold aqueous extract (figure 4). Two hundred grams of the plant material was mixed with 1200ml of distilled waterat room temperature. The mixture was extracted for 48 hours with continuous agitation using a magnetic stirrer. It was then strained and filtered through Whatman filter paper No.1 and the filtrate collected in a stoppered conical flask.

For hot decoction, 200 g of the plant material was mixed with 1200ml of distilled waterat room temperature and the mixture was allowed to come to a boil for 20 minutes. The mixture was allowed to cool then strained and filteredthrough Whatman filter paper No.1 and the filtrate collected in a stoppered conical flask.

2.2.4 CONCENTRATION OF THE EXTRACTS

This was done *in-vacuo*using a rotary evaporator which contains a vacuum chamber that produces a reducing pressure thatreduces the boiling point of the solvent, allowing it to condense in a separate flask, leaving behind the crude extract (figure 4).

The aqueous extracts were separately freeze dried to leave behind a dry residue which was stored in tightly sealed containers under refrigeration at 4℃until use.

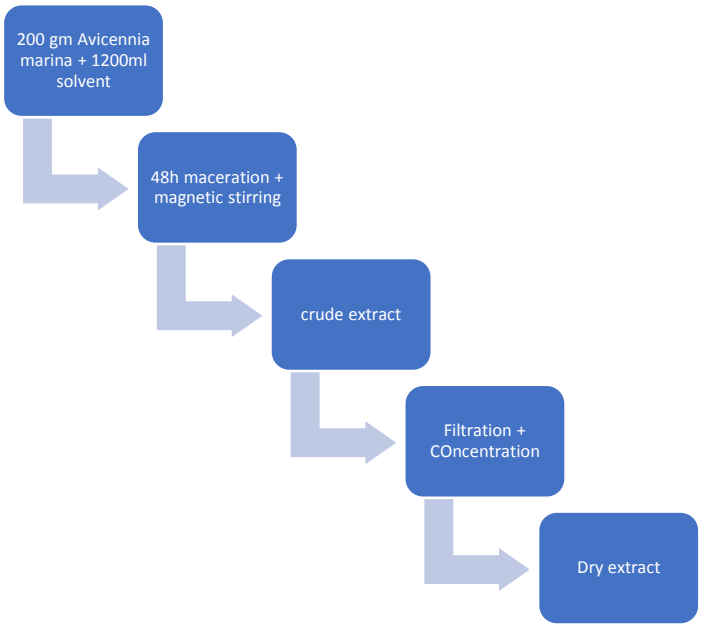

Figure 4 Flow chart of extraction process

2.2.5DETERMINATION OF PERCENTAGE YIELD

The percentage yield was calculated as:

$$\text{Percentage yield} = \frac{W2-W1}{W0} \times 100$$

Where $W_1$was the weight of the empty container; W_2 was the weight of the container plus the weight of the plant extract; $W_0$the weight of the milled extract (200g)

2.3ANTIMICROBIAL SUSCEPTIBILITY TESTING

The current study used the agar well diffusion method and macro dilution method to determine the antimicrobial activity of *Avicennia marina.*

2.3.1 TEST ORGANISMS

*Staphylococcus aureus, E. coli, Vibrio cholerae, Salmonella*Typhi and *Candida albicans* strains were obtained from stock culture at theDepartment of Medical Microbiology laboratory,University of Nairobi.

2.3.2ANTIMICROBIAL SUSCEPTIBILITY TESTS

Antimicrobial susceptibility testing was carried out as per CLSI guidelines and protocols (2012) using Kirby Bauer disc diffusion method.

Table 2: Standard antibiotics

	Antibiotic	**Zone of inhibition (mm)**
Vibrio spp	Ciprofloxacin	13.5mm
Staph. Aureus	Ciprofloxacin	13.5mm
C. albicans	Fluconazole	0mm
S. Typhi	Ciprofloxacin	25mm
E. coli	ciprofloxacin	13mm

The strain of *C. albicans* used was a strain obtained from a clinical specimen and was found to be resistant to Fluconazole antimicrobial disc.

2.3.3 AGAR WELL DIFFUSION

Antimicrobial susceptibility screening of the different extracts was carried out as per CLSI guidelines using agar well diffusion technique.

2.3.4PREPARATION OF PLANT EXTRACTS

The prepared plant extracts were dissolved in dimethylsulfoxide to yield a stock solution of 100mg/ml. Double serial dilutions of the stock solution were carried out to yield concentrations of 50mg/ml, 25mg/ml and 12.5mg/ml.Fortymicrolitres(40 µl)of each plant extract concentration was then inoculated into each agar well.

2.3.5 INOCULUM AND INOCULUM PROCEDURE

A 0.5 McFarland standard wasprepared with a 1.5×10^8CFU/ml inoculum concentration. Two to five singlecolonies from each microorganism culture agar plate were suspended in 2ml of Mueller Hinton broth and incubated as per their growth requirements until turbidity was visible. The turbidity was adjusted as per the McFarland standard.

A sterile swab was used to transfer the standard suspension onto already prepared culture plates. Nutrient agar plates were used for all the organisms. Asterile swab was used to streak the dried media and the plate rotated 60°to ensure even inoculation on the media.

2.3.6 PREPARATION OF AGAR WELLS

Sterile cork borers (6mm) were used to punch wells into the agar plates. Each plate comprised 4 wells equidistant to each other to avoid overlap of the zones of inhibition. Each of the fourwells wasinoculated with a different concentration of the plant extract. Another plate was designated for the controls. A standard antibiotic disc and DMSO were used as the positive control and negative control, respectively. Forty microlitres of the different concentrations of extract was inoculated into each well as well as 40µl of the DMSO into the negative control well.

The plates were left to dry, then incubated overnight at 37℃. After 24 hours the plates were examined for visible zones of inhibition and a caliper used to measure the diameter of the zones to the nearest millimeter.

All tests were conducted in triplicate for reliability.

2.4DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION AND MINIMUM BACTERICIDAL CONCENTRATION

2.4.1DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION

Minimum inhibitory concentration was defined as the highest dilution of plant extract inhibiting growth of the different microorganisms.

Plant extracts showing zones of inhibition on the agar well plates were subjected to further tests to determine their MIC values by microbroth dilution method.

2.4.1.1METHOD OF DILUTION

The different dilutions were obtained using double serial dilution of the stock plant extracts in sterile Mueller Hinton broth.

2.4.1.2 GROWTH TECHNIQUE

Four to six colonies of the microorganisms with identical morphological characteristics were identified. Two to three of these colonies were used to prepare the 0.5 MacFarland turbidity standard, using aseptic transfer method, into test tubes containing 5ml of fresh MuellerHinton broth and incubation until visibly turbid. Two to three of the colonies were used to perform grams staining to reveal the characteristics of the microorganisms as shown in Table 3.

Table 3 Table of gram stain characteristics of different microorganisms

Organism	Gram stain characteristics
Staph. Aureus	Gram positive cocci in clusters
Vibrio cholerae	Gram negative curved bacillus
E. coli	Gram negative bacilli
Salmonella Typhi	Gram negative bacilli
C. albicans	Gram positive budding yeast cells

2.4.1.3 DILUTION TECHNIQUE

Ten test tubes, eachcontaining 2 ml of Mueller Hinton broth,were allocated to each plant extract concentration, and arranged in a rack. A stock solution of 100mg/ml of plant extract was prepared in a separate tube. Double serial dilutions were conducted until a concentration of 12.5mg/ml was achieved. The first test tube in each set of 10 contained 2ml of the 12.5mg/ml of plant extract. Double serial dilutions were then carried out to yield the following concentrations: 6.25mg/ml, 3.125mg/ml, 1.562mg/ml, 0.781mg/ml, 0.39mg/ml, 0.195mg/ml and 0.098mg/ml. The final 2 tubes acted as positive and negative controls and contained 2ml Mueller Hinton broth, 40µl McFarland standard, 2ml Mueller Hinton agar and 40µl sterile water. The tubes were incubated for 18–24h at 37°C.

2.4.1.4MINIMUM INHIBITORY CONCENTRATION

After overnight incubation, the test tubes were assessed for bacterial growth visualized as turbidity. To confirmthat each test tube did or didnot have growth, a standard loop of suspension from each tube was subcultured on nutrient agar. After overnight incubation, the plates were examined for growth. The lowest concentration for the extracts showing no visible growth was regarded as the MIC.

2.4.2 MINIMUM BACTERICIDAL CONCENTRATION

Minimum bactericidal concentration was defined as the lowest concentration of extract that will kill the microorganism after overnight incubation.The tube within the series of 12 that immediately followed the tube with the MIC was considered as the MBC. After overnight incubation, the test tubes were assessed for bacterial growth as visualized by turbidity. To ensure that each test tube did/didnot have growth, a standard loop of suspension from each tube was subculture on appropriate agar.

CHAPTER 3:RESULTS AND DISCUSSION

3.0 INTRODUCTION

This chapter describes the results from the study-percentage yield of crude extract acquired, the antibiotic susceptibility pattern of the organisms, mean diameter of the zones of inhibition of the crude extracts against the different microorganisms, minimum inhibitory concentration and minimum bactericidal concentrations of the crude extracts.

3.1 PERCENTAGE YIELD

This study used cold maceration as the solvent extraction technique. This technique facilitated the extraction of the biologically active components required without altering their functional properties. Seven solvents were used for this technique- methanol, ethyl acetate, hot water, cold water, chloroform, hexane, and dichloromethane. Solvents used in extraction of bioactive compounds are usually chosen on the basis of the polarity of the compound of interest whereby solvents should be of similar polarity to the compounds to facilitate almost complete dissolution [66]. Table 4 below shows the polarity index of the different solvents.

Table 4: Polarity index of different solvents (Snyder)

Solvent	**Polarity index**
Water	9.0
Methanol	6.6
Ethyl acetate	4.4
Chloroform	4.1
Dichloromethane	3.7
Hexane	0.1

The polarity of the biologically active compounds found in Avicennia marina was unknown to us, therefore we chose the seven solvents, each with differing polarity, to increase the probability of getting good results. The percentage yields from the different solvents are shown in table 5 below.

Table 5: Percentage (%) yield of extract from each solvent

Solvent	W_1 (grams)	W_2(grams)	W_3 (grams)	% yield
Methanol	150	207	57	28.5
Ethyl acetate	150	160	10	5
Hot aqueous	150	158	8	8
Cold aqueous	150	157.8	7.8	7.8
Dichloromethane	50.34	52.64	2.3	1.15
Chloroform	46.43	47.03	0.6	0.3
Hexane	48.78	49.28	0.5	0.25

Where:

W^1 is the weight of the empty container

W^2 is the weight of the container plus the weight of the extract

W^3 is the weight of the extract ($W^2 – W^1$)

The percentage yield ranged from 28.5% to 0.25%. The highest yield, was obtained from methanol at 28.5%, followed by hot aqueous decoction at 8%, cold aqueous maceration at 7.8%, ethyl acetate at 5%, dichloromethane at 1.15%, chloroform at 0.3% and hexane at 0.25%.

The different solvents had differing yields, with the more polar solvents having higher yields. Methanol, hot aqueous decoction, cold water and ethyl acetate had the best yields at 28.5%, 8%, 7.8% and 5% respectively. Dichloromethane, chloroform, and hexane had lower yields at 1.15%, 0.3% and 0.25% respectively. The variation in yields from the different solvents could have been due to the varying chemical structures of the bioactive compounds, which would affect the solubility of the compounds in the different solvents [67]. The choice of solvent therefore affected what biologically active compound was extracted.

3.2 ANTIMICROBIAL SUSCEPTIBILITY PATTERN

The 5organisms were subjected to susceptibility testing using standard antibiotic discs. Ciprofloxacin 30µg/ml was used for *Staph.aureus, Vibrio cholerae, E.coli* and *Salmonella* typhi, while fluconazole 25µg/ml was used against *C.albicans*.

Table 6 below shows the average zones of inhibition for the various antibiotics against the microorganisms

Table 6: Antimicrobial susceptibility to Standard antibiotic discs

	Antibiotic	**Antibiotic concentration (µg)**	**Zone of inhibition (mm)**	**CLSI breakpoint**		
				Susceptible (Zone greater than)	**Intermediate**	**Resistant (zone smaller than)**
Vibrio spp	Ciprofloxacin	30	13.5mm	21mm	16-20mm	15mm
Staph. Aureus	Ciprofloxacin	30	13.5mm	14mm	-	13mm
C. albicans	Fluconazole	25	0mm	19mm	15-18mm	14mm
S. Typhi	Ciprofloxacin	30	25mm	20mm	17-19mm	16mm
E. coli	ciprofloxacin	30	13mm	20mm	17-19mm	16mm

Ciprofloxacin was tested against *Vibrio cholerae, Staphylococcus aureus, E. coli* and *Salmonella* typhi and was found to be active against all. Fluconazole was tested against a strain of *Candida albicans*obtained from a clinical specimen. The strain used was found to be resistant to fluconazole. All tests were conducted in triplicate

3.3 ANTIMICROBIAL ACTIVITY OF THE CRUDE EXTRACTS

3.3.1 METHANOL

The crude extracts of methanol at the various concentrations were used to carry out antimicrobial susceptibility testing against *Staph aureus, E. coli, Vibrio cholerae, Salmonella* typhi and *Candida albicans*. The results of the effect of the extract against the different organisms is shown in figure 5 below and appendix I.

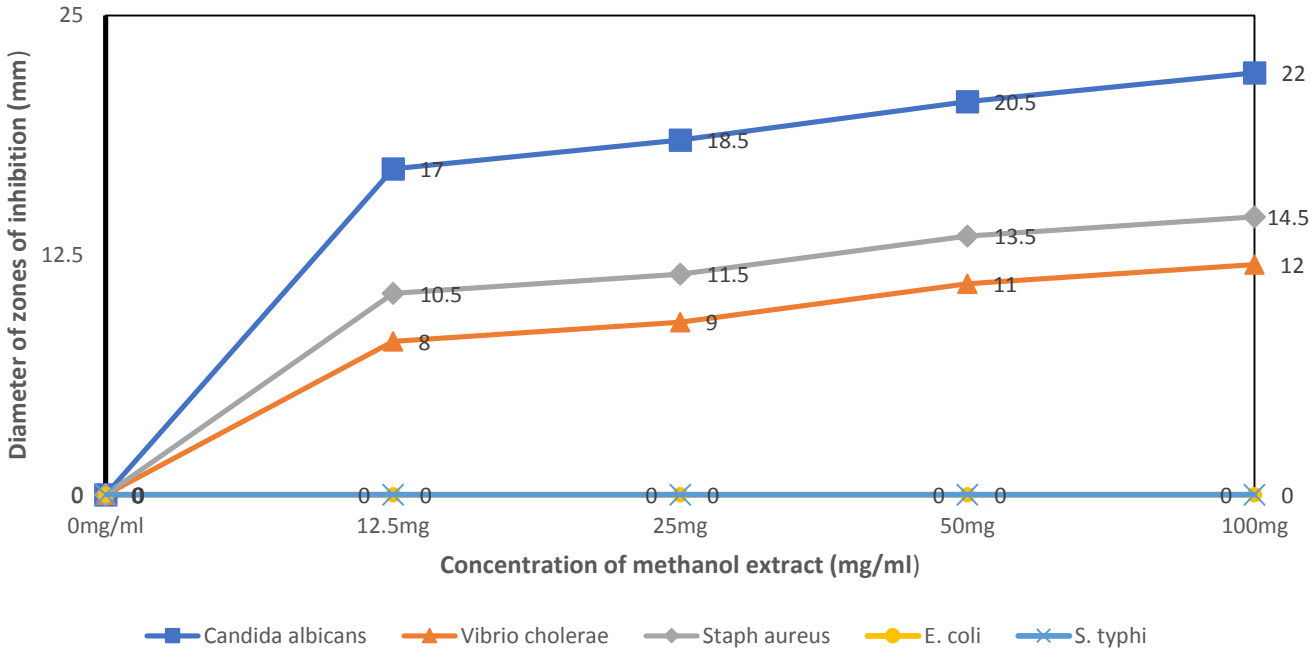

Figure 5: Diameter of zones of inhibition (mm) against concentration of methanol extract (mg/ml) for the different microorganisms

The extract showed varying results for each organism at each concentration. The extract showed the highest activity against Candida albicans at all concentrations, such that at for example 100mg/ml, the diameter of the zone of inhibition in mm was 22 as compared to 14.5, 12, 0 and 0 for *Staph aureus, Vibrio cholerae, Salmonella* typhi and *E. coli* respectively. This trend was replicated for all the different concentrations. The activity of the extract was noted to be concentration dependent, with the highest activity noted at the highest concentration of 100mg/ml. It was also observed that the methanol extract had no effect against *E. coli* or *Salmonella* typhi.

Polarity of a solvent influences the biologically active substances extracted. Methanol has a high polarity index of 6.6 and is used frequently as a solvent in extraction as it tends to extract several compounds. The high polarity in this case could have been a contributing factor to the success of extraction of active compounds from *A. marina*.

The results thus obtained were in line with results from Rao et al who showed that *A. marina* has antibacterial and antiviral activity.

3.3.2 ETHYL ACETATE

The crude extract of *Avicennia marina* using ethyl acetate as a solvent, at concentrations of 100mg/ml, 50mg/ml, 25mg/ml and 12.5mg/ml were used to carry out antimicrobial

susceptibility testing against *Staph aureus*, *Vibrio cholerae*, *E. coli*, *Salmonella* typhi and *Candida albicans*. The results are shown in figure 6 below and appendix II.

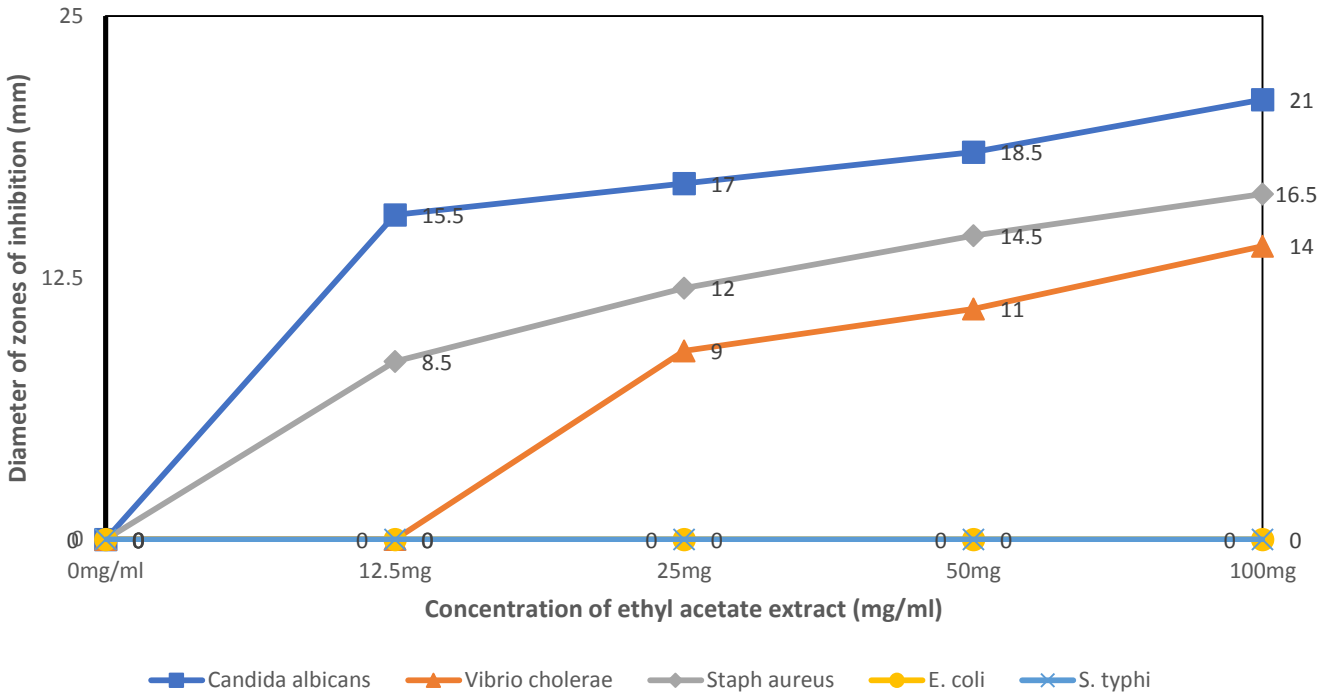

Figure 6: Diameter of zones of inhibition mm) against concentration of ethyl acetate extract (mg/ml) for the different microorganisms

The results varied for each organism at each given concentration. The extract showed the best activity against *Candida albicans* at all concentrations, compared to the other organisms. At 100mg/ml, the zones of inhibition for *Candida albicans* was an average of 21mm, while for *Staph aureus* it was 16.5mm, for *Vibrio cholerae* it was 14mm and 0 for both *E. coli* and *Salmonella* typhi. At 12.5mg, the average diameter of the zone of inhibition against *Candida albicans* was 15.5mm, for *Staph aureus* was 8mm and 0 for the rest of the organisms. The activity of the ethyl acetate extract was thus noted to be concentration dependent, having greater activity at higher concentrations.

The polarity index of ethyl acetate is 4.4, which made it a good solvent for extraction of compounds from *Avicennia marina*. The findings on the activity of ethyl acetate extract of *Avicennia marina* bark were congruent with findings from a study conducted on the same by Rao et al

3.3.3 AQUEOUS EXTRACTS

The crude aqueous extracts of *Avicennia marina* were used to carry out antimicrobial susceptibility testing against *Vibrio cholerae, Staph aureus, E. coli, Salmonella* typhi and *Candida albicans*. The figures 7 and 8, and appendix III show the results.

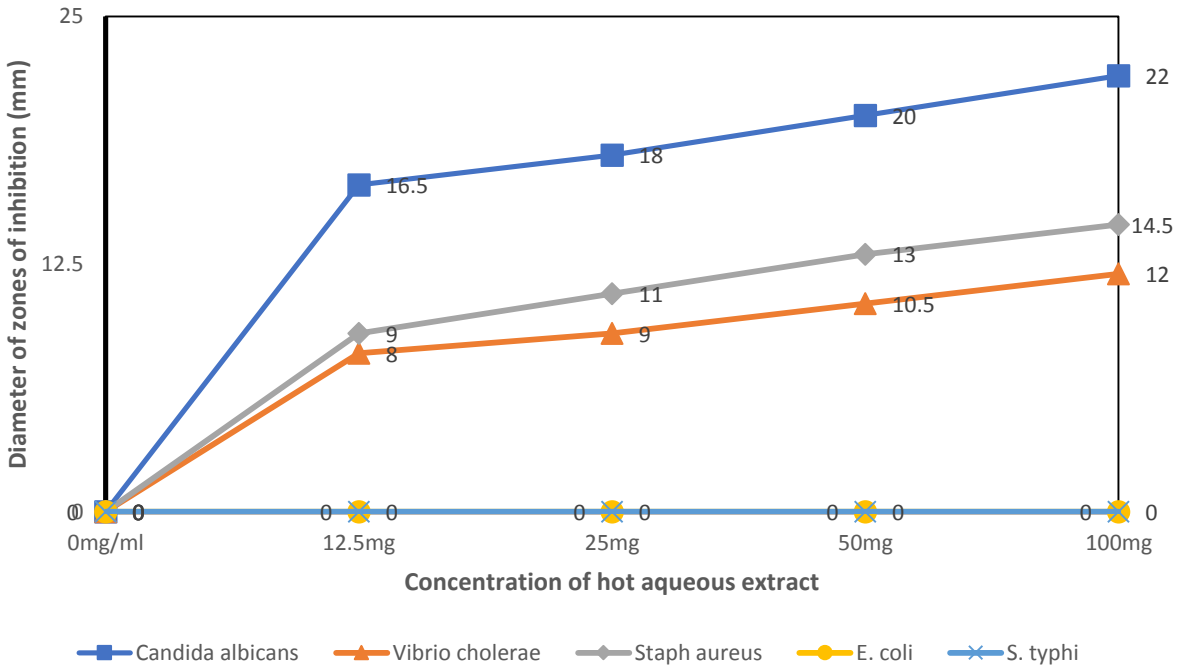

Figure 7: Diameter of zones of inhibition (mm) against concentration of hot aqueous extract (mg/ml) of the different microorganisms

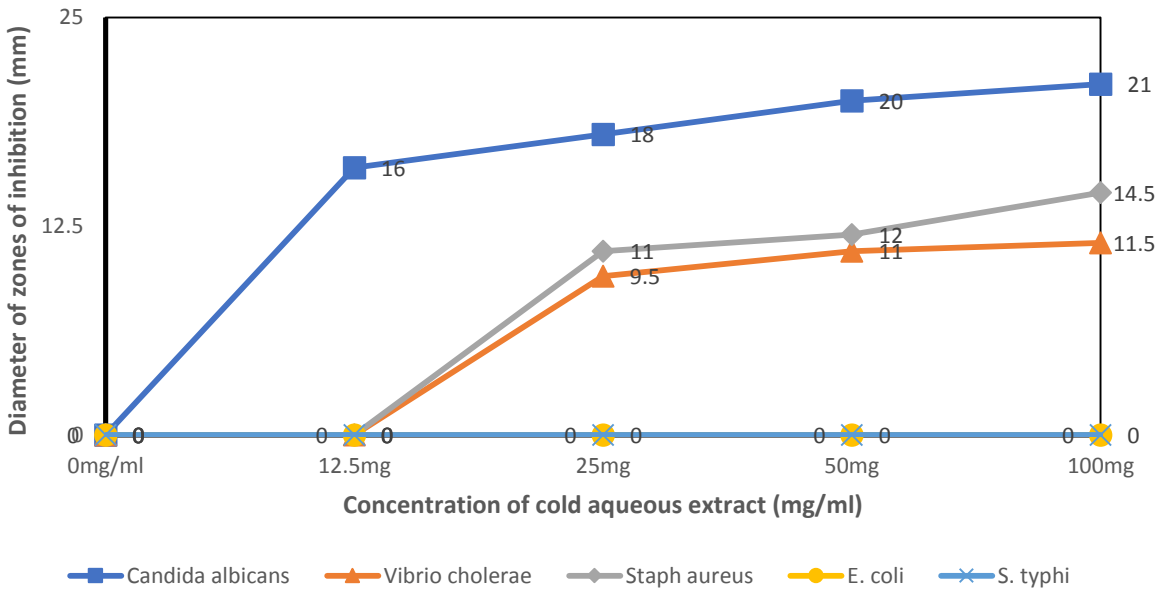

Figure 8: Diameter of zones of inhibition (mm) against concentration of cold aqueous extract (mg/ml) for the different microorganisms

For the hot aqueous decoction, the results varied for each organism at each concentration. The extract showed the best activity against *Candida albicans* at all concentrations. At 100mg/ml, the average diameter of the zones of inhibition against *Candida albicans* was 22mm, while for *Staph aureus* it was 14.5mm, *Vibrio cholera* was 12mm and 0 for *E. coli* and *Salmonella* typhi. The average diameter of the zones of inhibition at 12.5mg/ml for *Candida albicans* was 16.5mm, for *Staph aureus* 9mm, for *Vibrio cholerae* 8mm and 0 for *E. coli* and *Salmonella* typhi. The extract thus shows concentration dependent activity, similar to that seen with the methanol and ethyl acetate extracts.

Similarly, the results for the cold aqueous extract varied at each concentration, with the best activity noted against *Candida albicans*. At 100mg/ml the average diameter of the zones of inhibition were 21mm, 14.5mm and 11mm for *Candida albicans, Staph aureus* and *vibrio cholerae,* respectively. There was no activity noted at this concentration against *E. coli* and *Salmonella* typhi. The average diameters of the zones of inhibition at 12.5mg/ml were 16mm for *candida albicans* and 0 for all other microorganisms. Similar to the hot aqueous decoction, the cold aqueous extract showed concentration dependent activity.

Water has a polarity index of 9.0, making it a good solvent for extraction of polar compounds. The hot decoction was noted to have better activity against all the microbes compared to the cold aqueous extract.

Traditionally, the whole root and bark of *Avicennia marina* were prepared by boiling them and the decoction drank by patients exhibiting symptoms of stomach upset. The decoction was drank over a period of several days until symptoms alleviate. Results from the water extractions show antibacterial and antifungal activity against various bacteria and fungi, some of which are known to cause gastrointestinal illness, and thus validating its use in traditional medicine. Results from the water decoctions also illustrate that extracts of *Avicennia marina* are heat stable as both the hot and cold aqueous extracts exhibited antibacterial and antifungal activity.

3.3.5 DICHOLOROMETHANE, CHLOROFORM, HEXANE

The crude extracts of chloroform, dichloromethane and hexane were used to carry out antimicrobial susceptibility testing against *Vibrio cholerae, Staph aureus, E. coli, Salmonella* typhi and *Candida albicans*. The extracts were all inactive against all the microbes at all concentrations tested as illustrated in appendices IV-VI.

The polarity indexes for chloroform, dichloromethane and hexane are 4.4, 3.7 and 0.1, respectively. The polarity indices of these solvents may be low compared to what the polarity of the active compounds in *Avicennia marina* could be. This could be the reason for the low percentage yields from each of these solvents. This would have implications in further studies, as it would discourage further work using solvents of low polarity which have been shown to have low yields of inactive compounds.

3.4 MINIMUM INHIBITORY CONCENTRATION AND MINIMUM BACTERICIDAL CONCENTRATION

The minimum inhibitory concentration (MIC) was defined as the highest concentration of plant extract that inhibited growth of microorganism after overnight incubation, while the minimum bactericidal concentration was defined as the lowest concentration of subcultured MIC tube that showed no visible growth after overnight incubation. The results after overnight incubation are shown in the table below.

Table 7: Minimum inhibitory concentrations and minimum bactericidal concentrations

	MIC (mg/ml)		**MBC (mg/ml)**	
	Ethyl Acetate	**Methanol**	**Ethyl Acetate**	**Methanol**
Vibrio spp	6.25	6.25	3.125	3.125
Staph aureus	12.5	12.5	6.25	6.25
C. albicans	6.25	6.25	3.125	3.125

The range for MIC was between 12.5mg/ml and 6.25mg/ml, while the range for MBC was between 6.25mg/ml and 3.125mg/ml.

MIC and MBC were used to quantify the antibacterial and antifungal activity of *Avicennia marina*.The values differed for the different microorganisms-e.g. for methanol extract, the MBC for *Staph aureus* was 6.25mg/ml, for *Vibrio cholerae* was 3.125mg, while for *candida albicans* it was 3.125mg/ml. The MIC values for the same extract were 12.5mg/ml for *Staph aureus*, 6.25mg/ml for *Vibrio cholerae* and 6.25mg/ml for *Candida albicans*. This can be interpreted to mean that at low concentrations crude extracts have bacteriostatic and fungistatic properties, while at higher concentrations they have bactericidal and fungicidal properties. This would suggest that when used traditionally, due to the low concentrations consumed, the extracts are bacteriostatic.

CHAPTER 4: CONCLUSIONS AND RECOMMENDATIONS

4.0 CONCLUSIONS

The continued rise in antimicrobial resistance has necessitated increased research into novel compounds especially from plants. The WHO recommends that countries formulate standard protocols for validation of medicinal plant products, to facilitate their safe incorporation into mainstream medicine and healthcare [68].

There is limited data on the antimicrobial activity of the bark of *Avicennia marina*. Previous studies have used the leaves and roots, which have been found to be active. Studies have also shown the plant to contain anticancer, antiviral and antifungal properties [69].

This study used cold maceration as the solvent extraction technique. This technique facilitated the extraction of the biologically active components required without altering their functional properties. Seven solvents were used for this technique- methanol, ethyl acetate, hot water, cold water, chloroform, hexane and dichloromethane. Solvents used in extraction of bioactive compounds are usually chosen on the basis of the polarity of the compound of interest whereby solvents should be of similar polarity to the compounds to facilitate almost complete dissolution [66]. The polarity of the biologically active compounds found in Avicennia marina was unknown to us, therefore we chose the seven solvents, each with differing polarity, to increase the probability of getting good results.

The more polar solvents methanol, ethyl acetate and water showed both antibacterial and antifungal activity. The less polar solvents chloroform, dichloromethane and hexane exhibited neither activity. The choice of solvent therefore influenced the yield of bioactive compounds and on the activity against the bacteria and fungi.

The different solvents had differing yields, with the more polar solvents having higher yields. Methanol, hot aqueous decoction, cold water and ethyl acetate had the best yields at 28.5%, 8%, 7.8% and 5% respectively. Dichloromethane, chloroform, and hexane had lower yields at 1.15%, 0.3% and 0.25% respectively. The variation in yields from the different solvents could have been due to the varying chemical structures of the bioactive compounds, which would affect the solubility of the compounds in the different solvents [67]. The choice of solvent therefore affected what biologically active compound was extracted. Further phytochemical analyses would however be needed to identify the bioactive component from each solvent fraction.

The antibacterial and antifungal effects of bark extracts of *Avicennia marina* was also found to be concentration dependent i.e. the higher the concentration of extract used, the more the inhibitory activity exhibited.

The extracts of *Avicennia marina* also exhibited a degree of bio selectivity against the microorganisms, showing inhibitory action against *S. aureus, V. cholerae, N. gonorrhoea* and *C. albicans* but no such activity against *S. typhi* and *E. coli*.There are very few studies that look at the antimicrobial activity of *Avicennia marina* bark extracts against human pathogens. The results thus obtained were in line with what has previously been reported by V.U. Rao et al. that found bark extracts to have both antibacterial and antifungal activity [70].

The crude extracts of *Avicennia marina* were found to have bacteriostatic activity against *Staph aureus*, *Vibrio cholerae*and *Neisseria gonorrhoea* as well as fungistatic activity against *Candida albicans* at lower concentrations. At higher concentrations, the crude extracts were found to have bactericidal and fungistatic activity.

The crude extracts were also found to be inactive against *Salmonella* typhi and *E. coli*. This could possibly be due to presence of one or more mechanisms of resistance (drug efflux pump, reduced membrane permeability, mechanisms of drug inactivation or absence of drug target molecules). This resistance could also be due to the extract concentrations being too low to exhibit any antimicrobial activity against the two bacteria.

MIC and MBC were used to quantify the antibacterial and antifungal activity of *Avicennia marina*. The MIC values ranged from 12.5mg/ml to 6.25mg/ml, while those of MBC ranged from 6.25mg/ml to 3.125mg/ml. This can be interpreted to mean that at low concentrations crude extracts have bacteriostatic and fungistatic properties, while at higher concentrations they have bactericidal properties. This would suggest that when used traditionally, due to the low concentrations consumed, the extracts are bacteriostatic.

4.1 RECOMMENDATIONS

Following the results of this study, it is recommended that additional modes of extractions using additional solvents be carried out on the bark as well as other parts of the plant. The extracts should then be used to carry out antimicrobial susceptibility testing on different species of bacteria. This would help determine what part of the plant is most active against different bacteria.

It is also recommended that phytochemical studiesshould be conducted to determine the activecomponents of *Avicennia marina*. These compounds should then be used to carry out antimicrobial susceptibility testing to determine the biologically active components. Structural investigationwouldbe requiredto characterizethe bioactive compounds ineach fraction.

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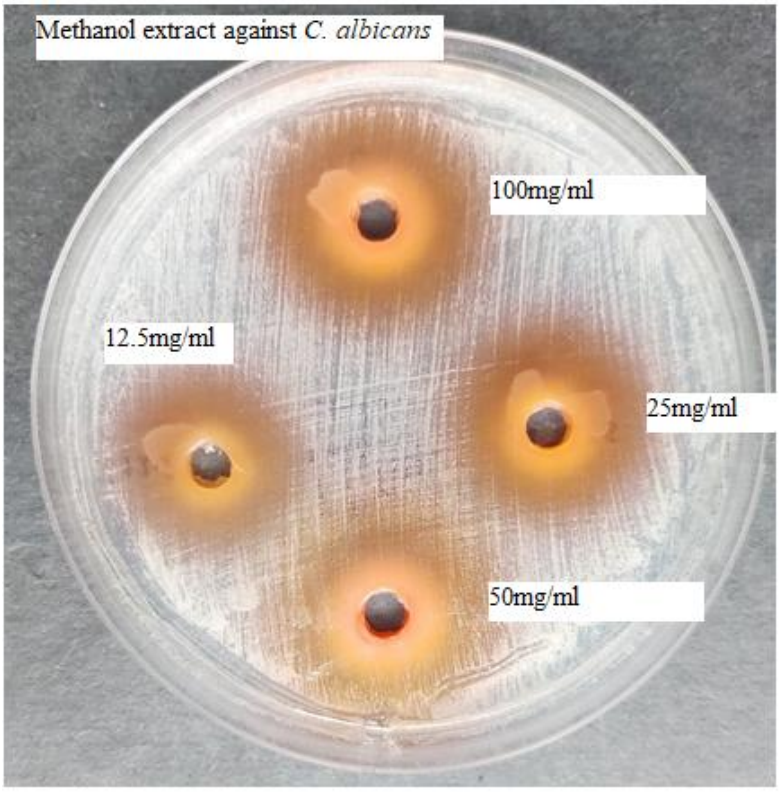
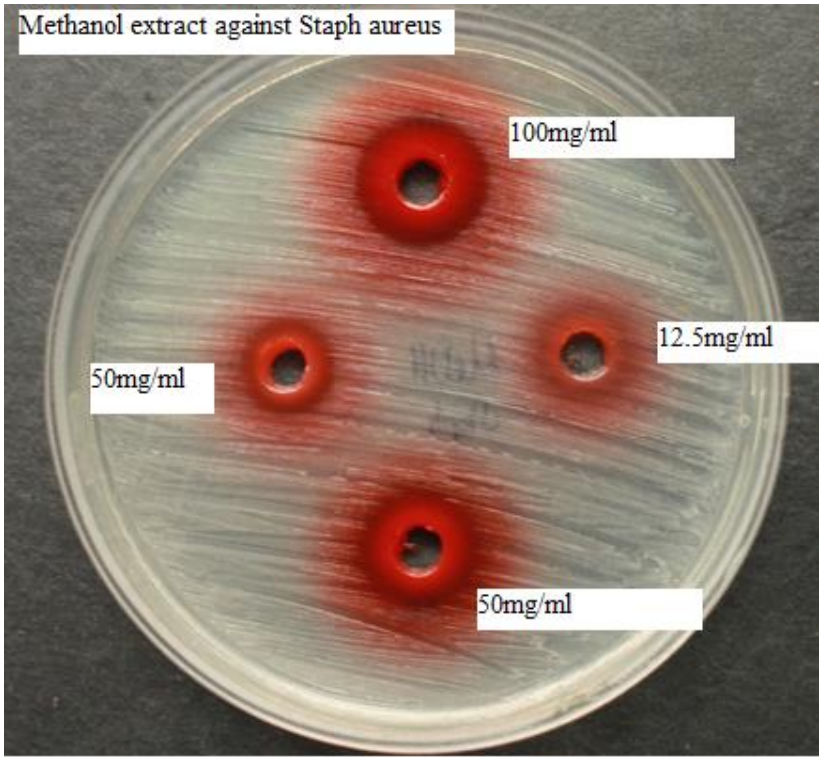
APPENDIX I: METHANOL EXTRACT PLATES

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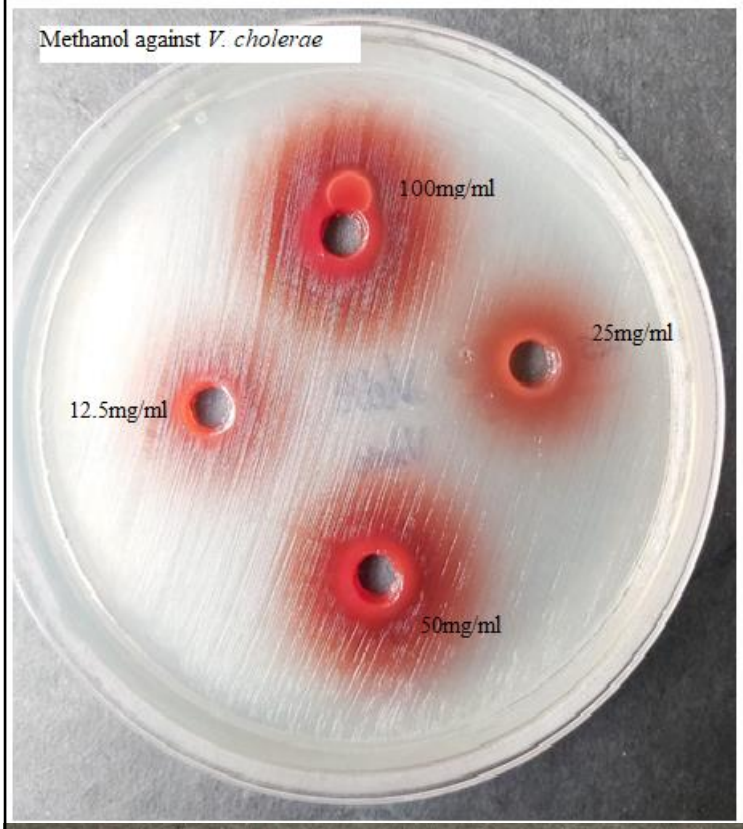
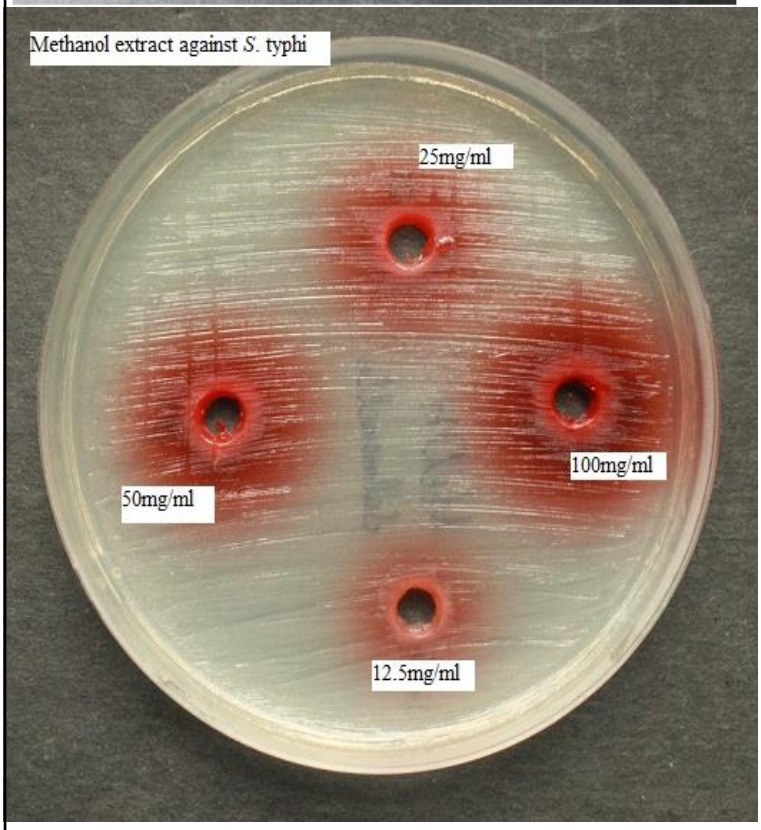
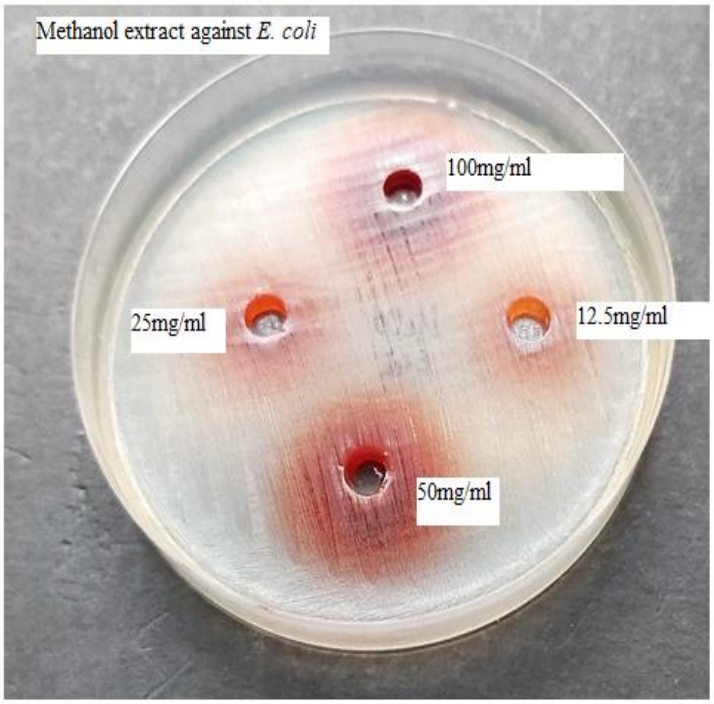
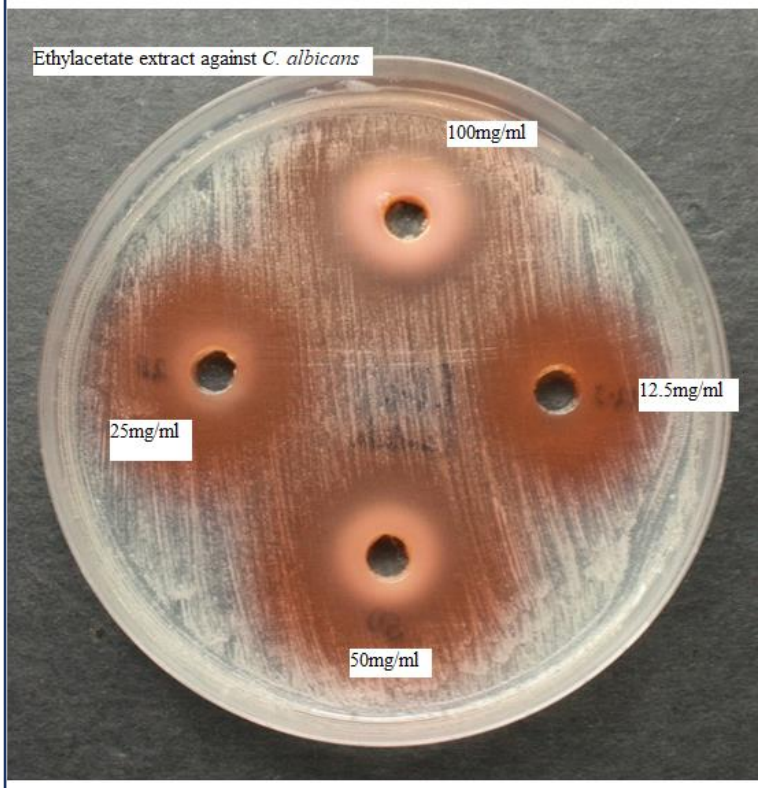
12.5mg/ml
50mg/ml
50mg/ml

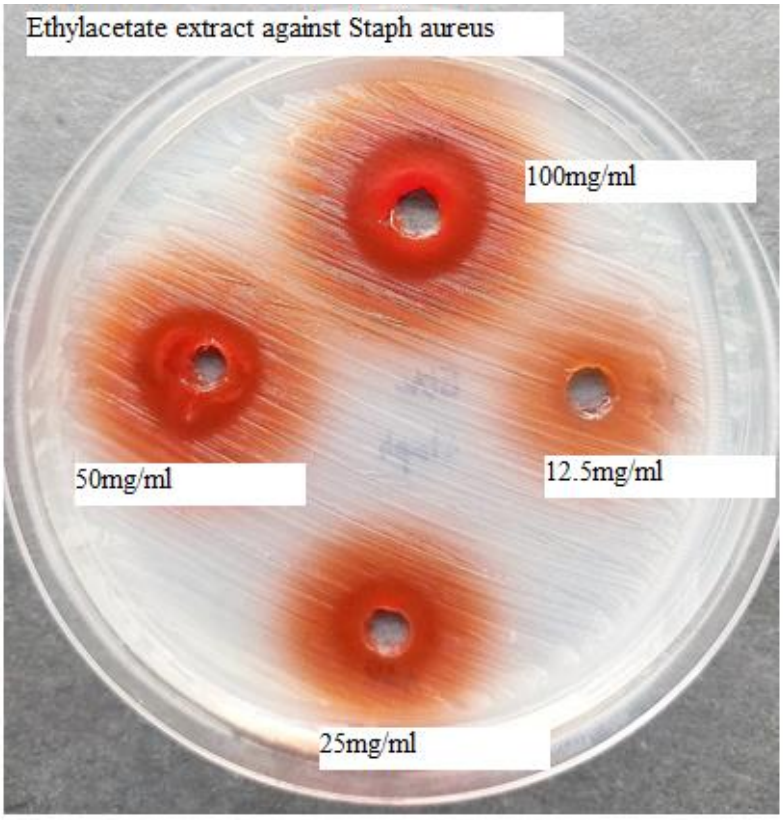

Methanol against *V. cholerae*
100mg/ml
25mg/ml
12.5mg/ml
50mg/ml

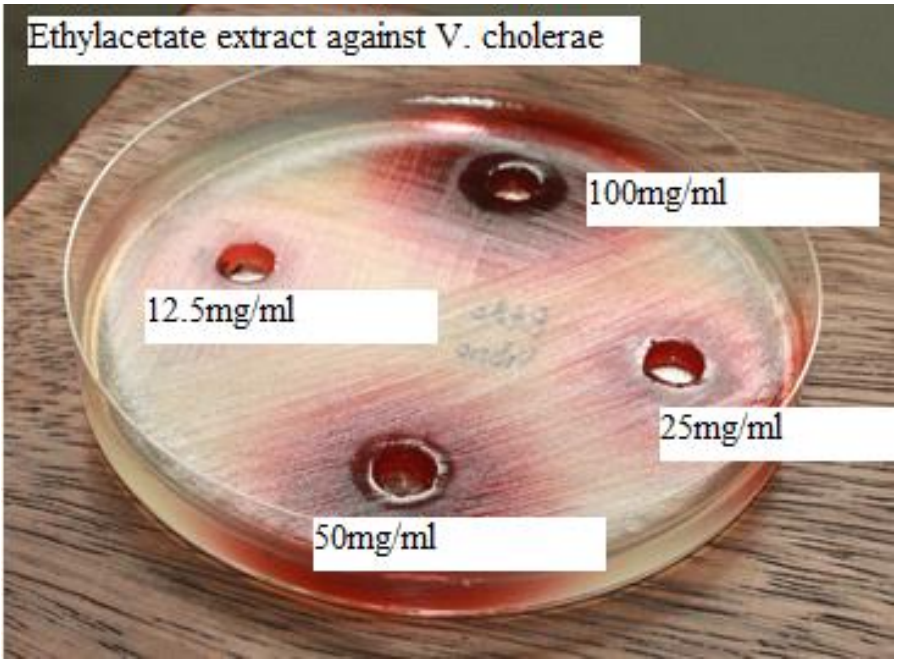

Methanol extract against *S.* typhi
25mg/ml
100mg/ml
50mg/ml
12.5mg/ml


Methanol extract against *E. coli*
100mg/ml
25mg/ml
12.5mg/ml
50mg/ml

APPENDIX II: ETHYLACETATE EXTRACT PLATES






Ethylacetate extract against V. cholerae
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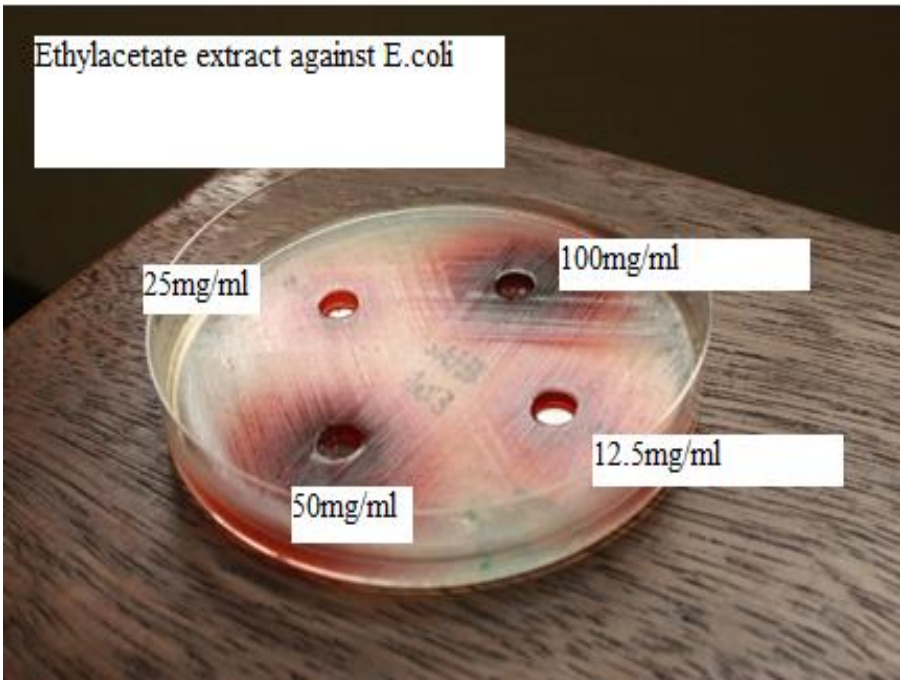
12.5mg/ml
25mg/ml
50mg/ml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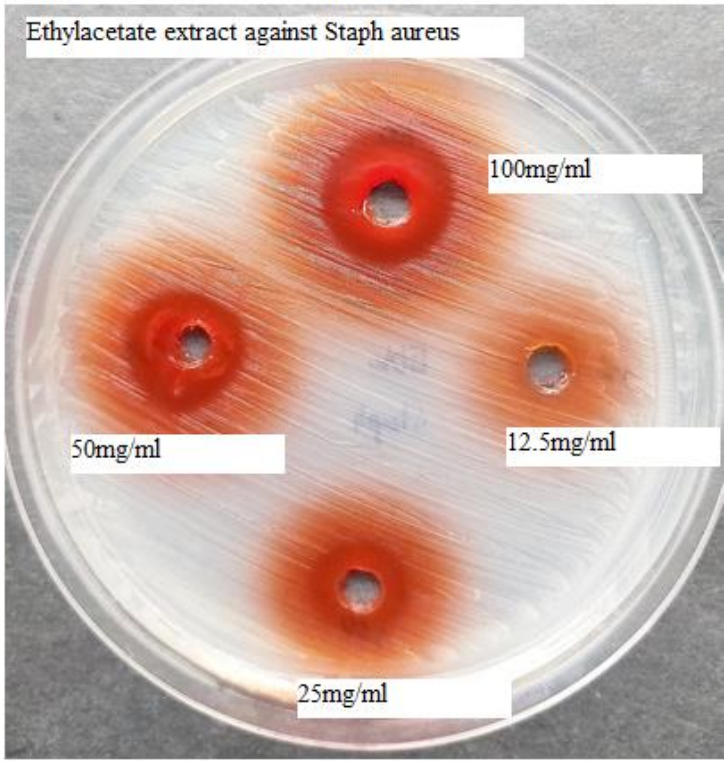
12.5mg/ml
50mg/ml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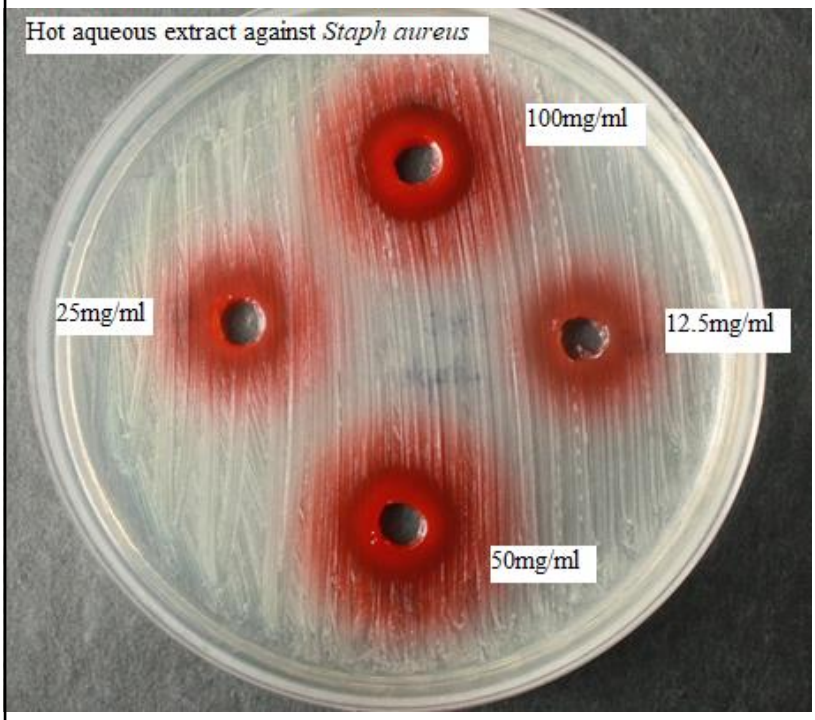
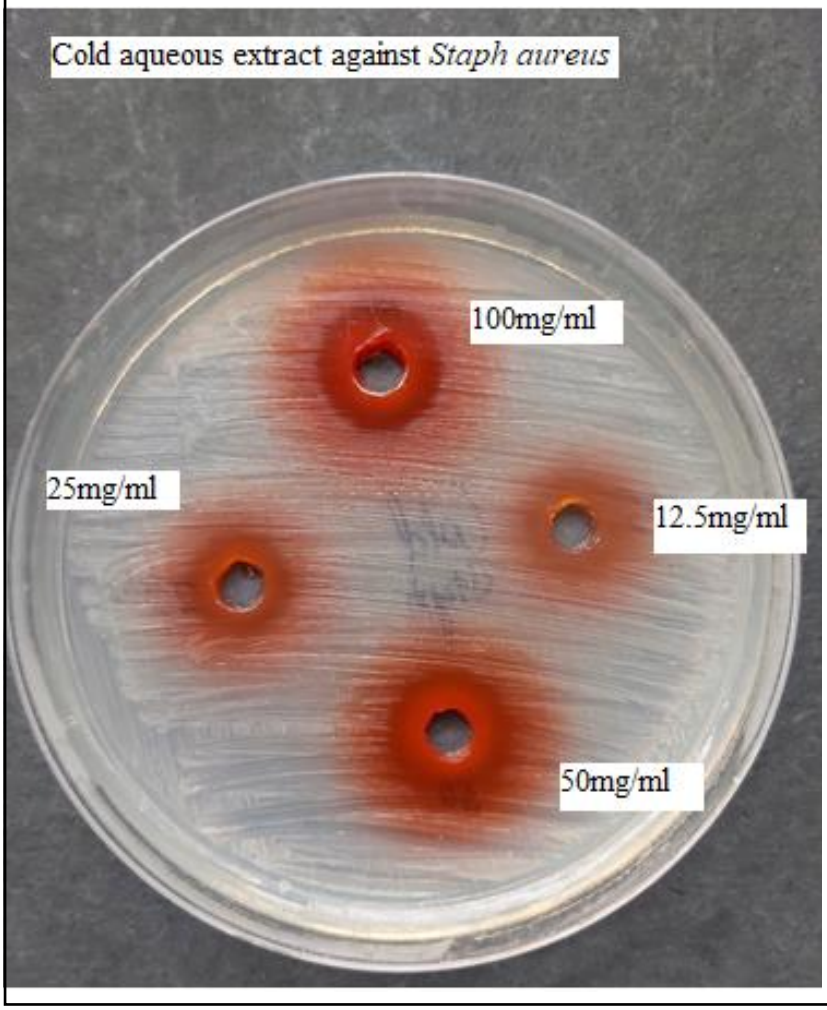
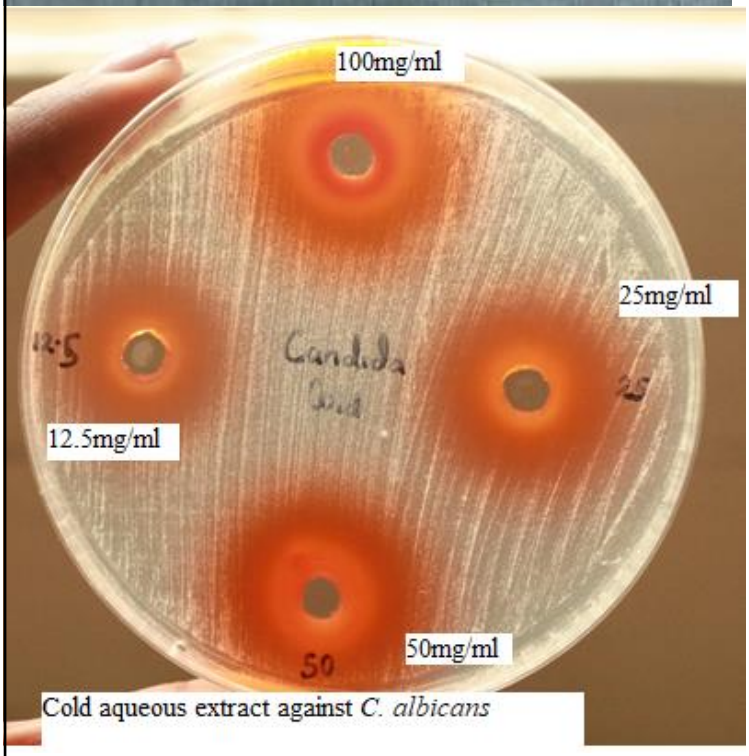
12.5mg/ml
25mg/ml

APPENDIX III: AQUEOUS EXTRACT PLATES

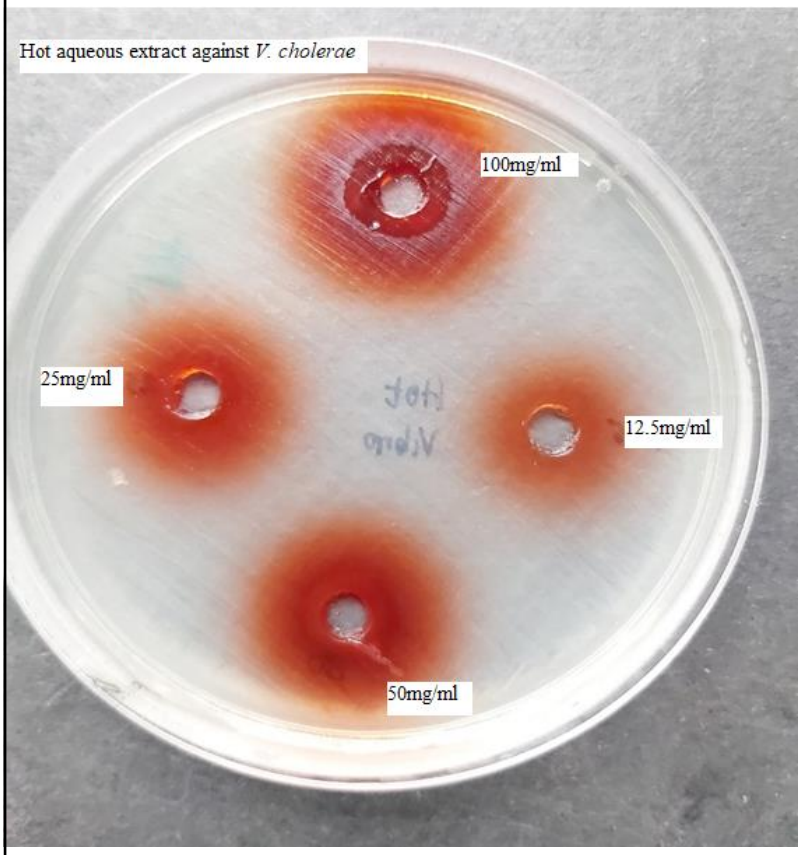


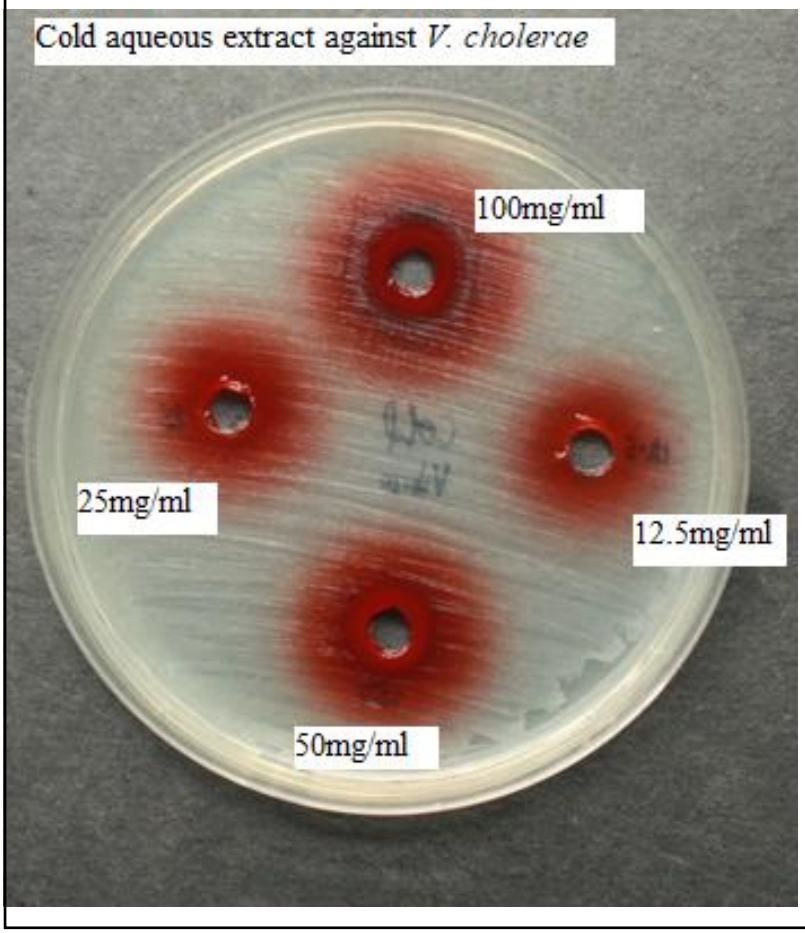


Hot aqueous extract against *C. albicans*



Cold aqueous extract against *C. albicans*


Hot aqueous extract against *V. cholerae*
100mg/ml
25mg/ml
12.5mg/ml
50mg/ml


Cold aqueous extract against *V. cholerae*
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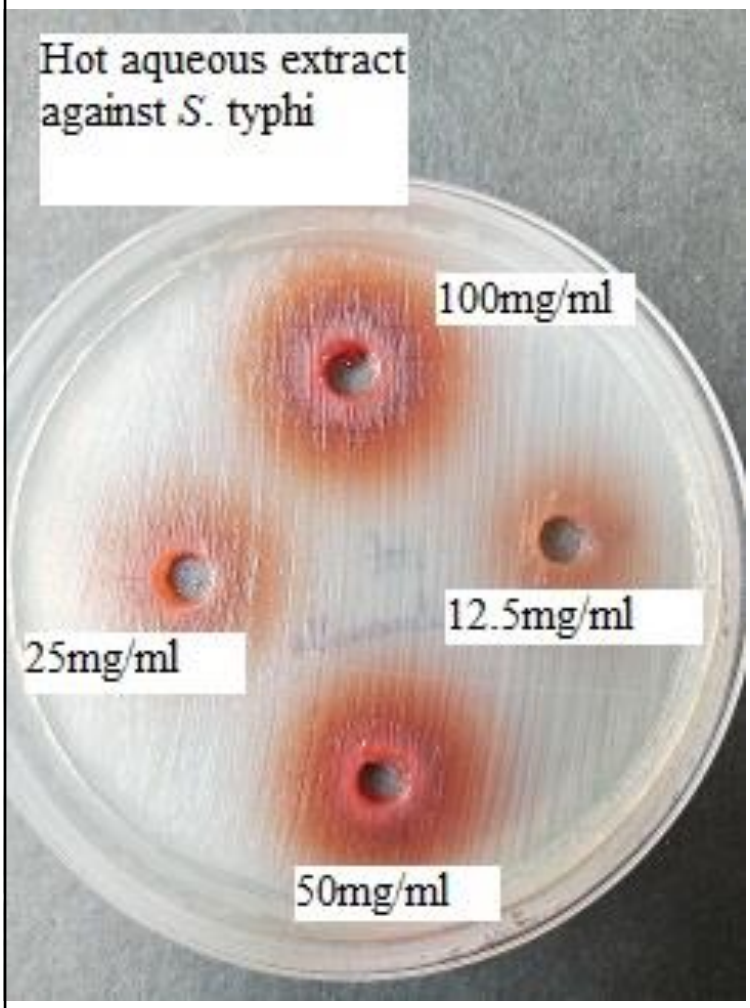
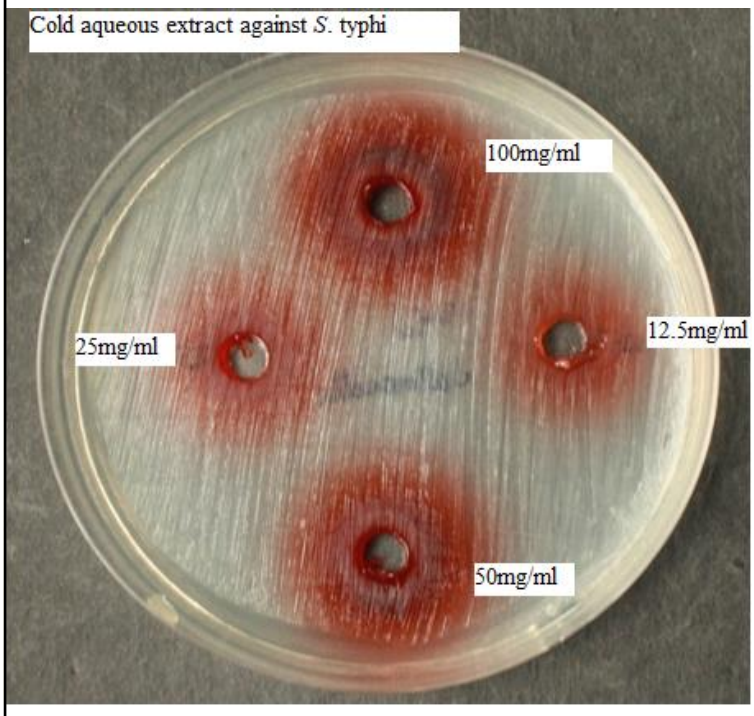
25mg/ml
12.5mg/ml
50mg/ml


Hot aqueous extract against S. typhi
100mg/ml
12.5mg/ml
25mg/ml
50mg/ml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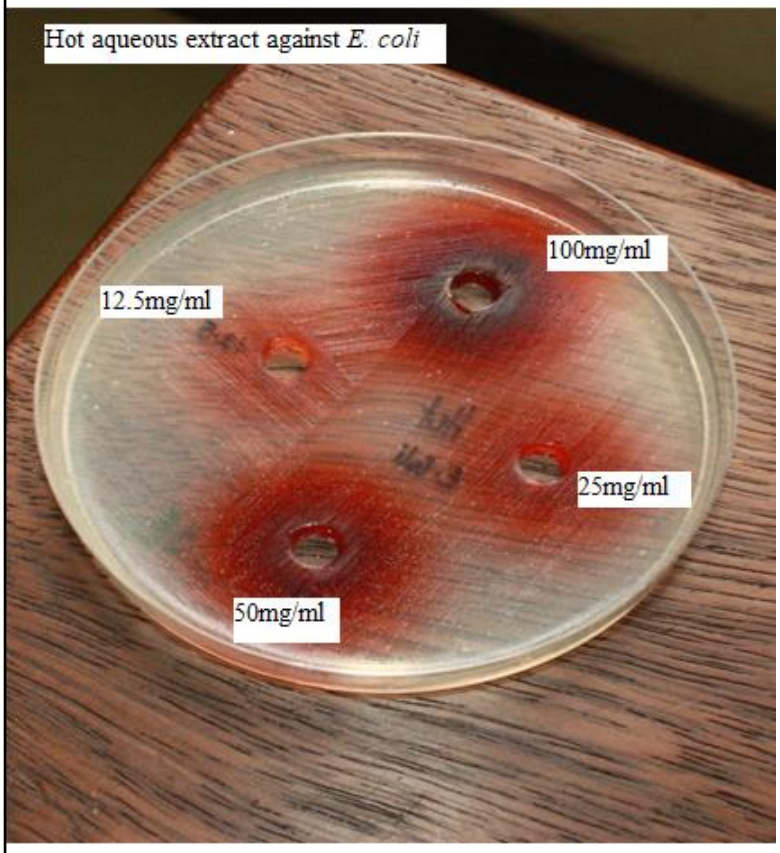
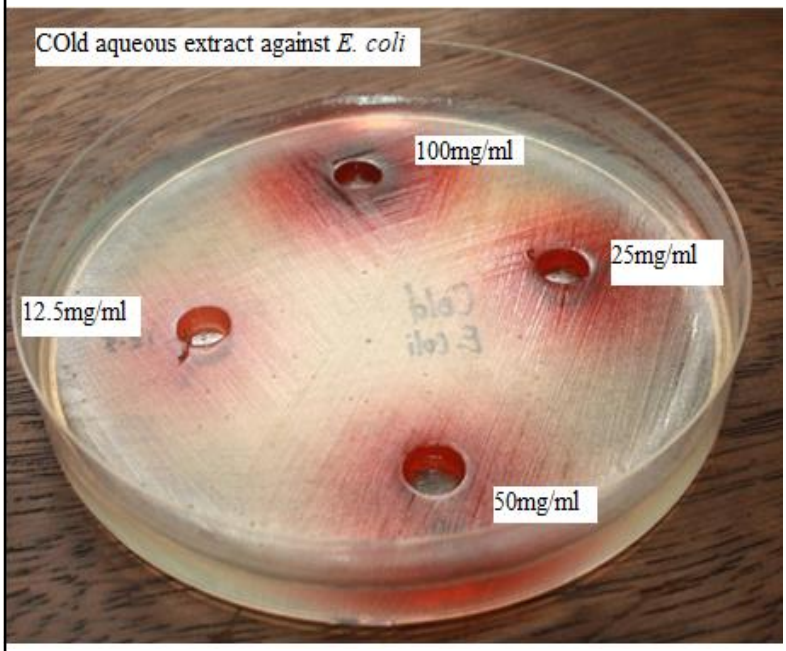
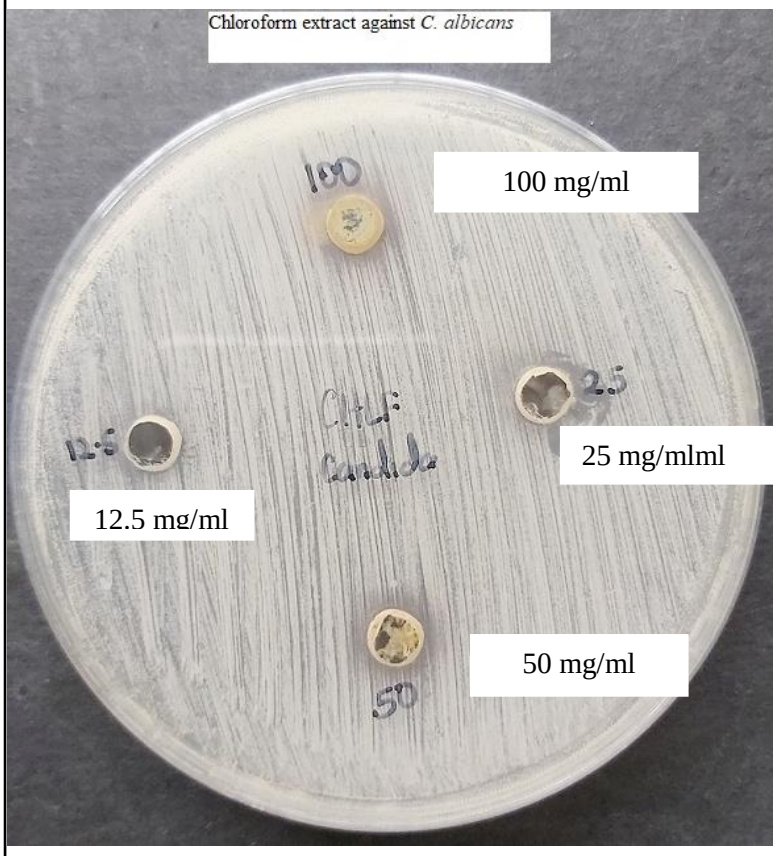
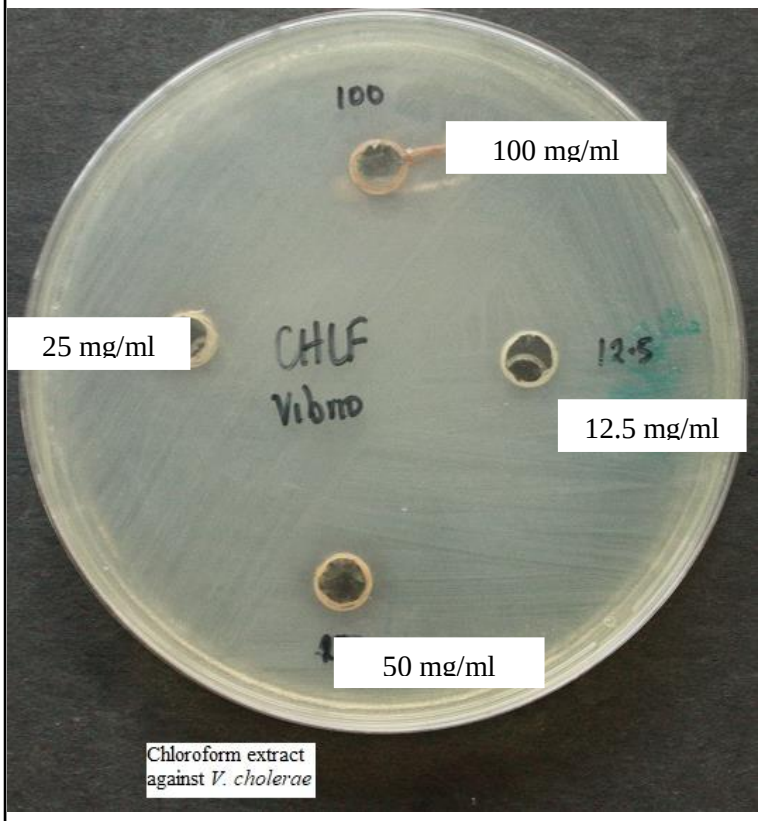
100mg/ml
12.5mg/ml
25mg/ml
50mg/ml

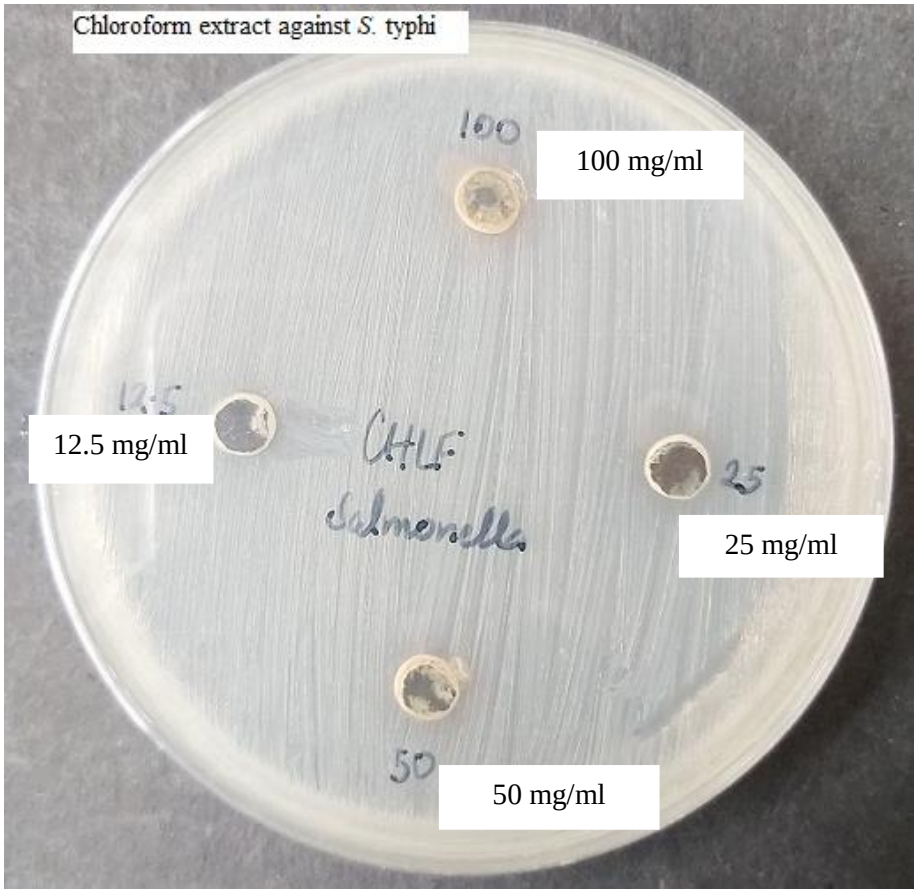

Hot aqueous extract against *E. coli*
100mg/ml
12.5mg/ml
25mg/ml
50mg/ml


COld aqueous extract against *E. coli*
100mg/ml
25mg/ml
12.5mg/ml
50mg/ml

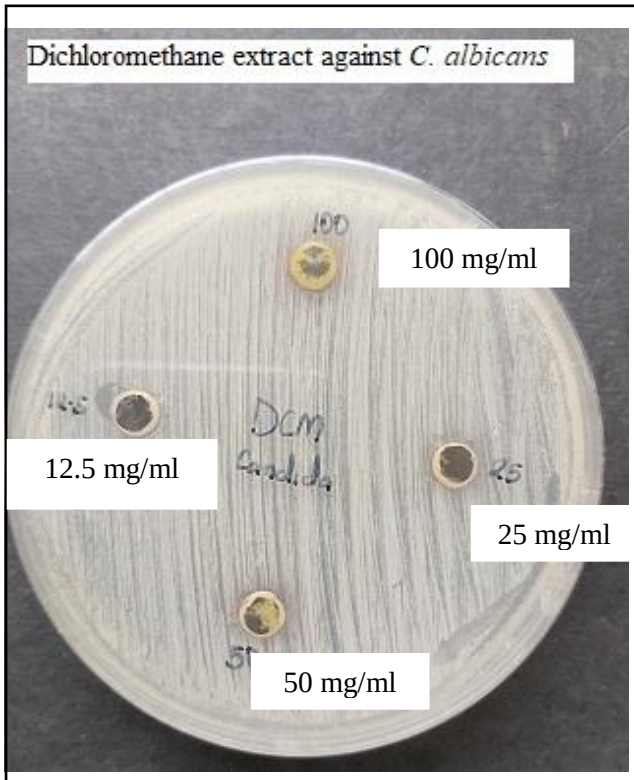
APPENDIX IV: CHLOROFORM EXTRACT PLATES

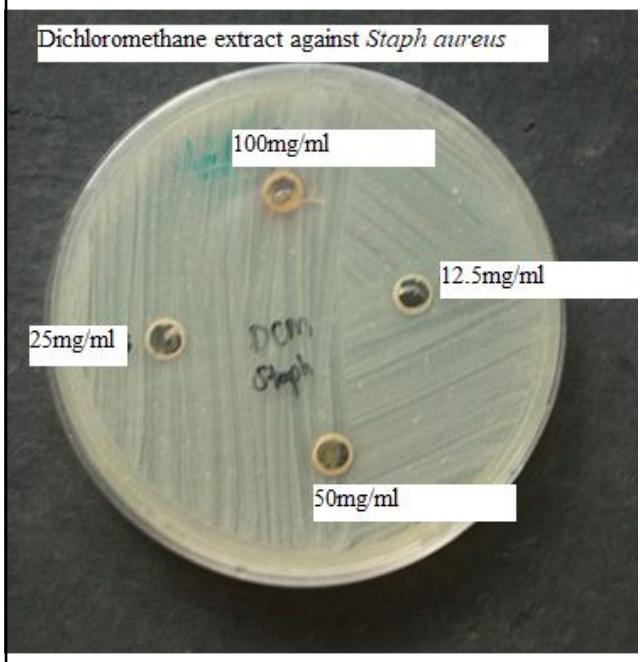


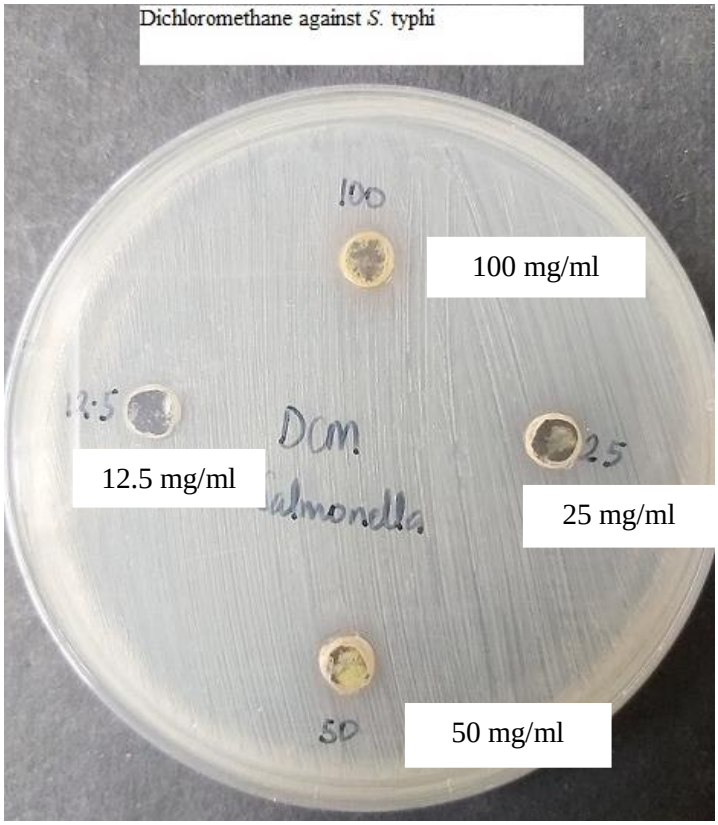


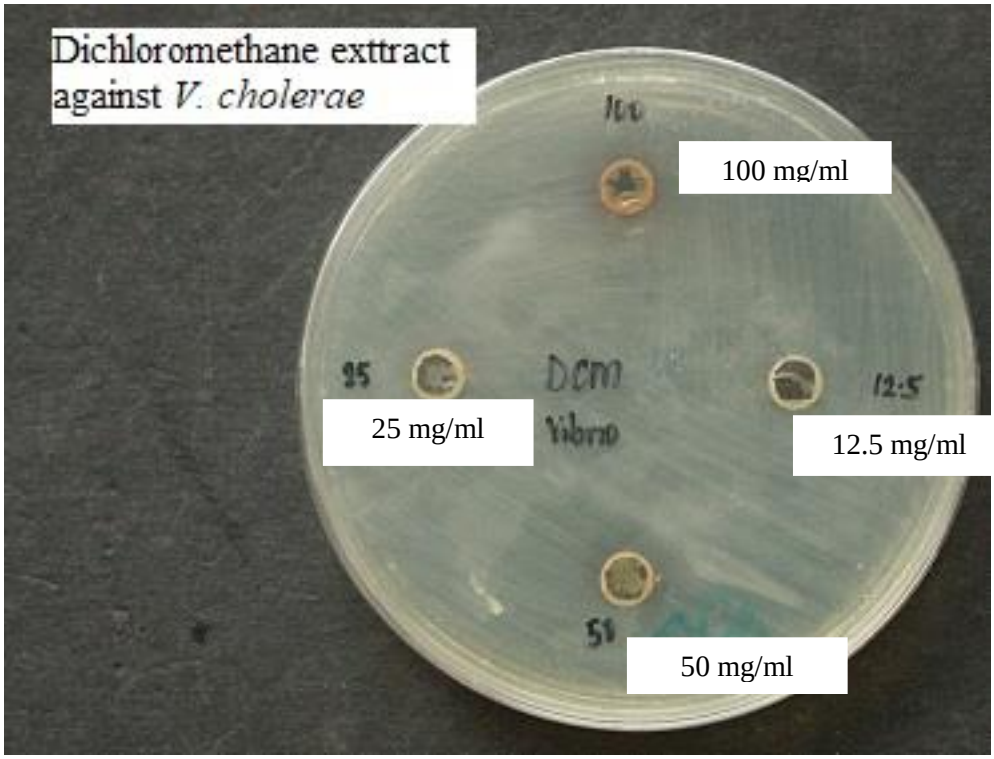

Chloroform extract against S. typhi
100
100 mg/ml
12.5
12.5 mg/ml
CHL:
Salmonella
25
25 mg/ml
50
50 mg/ml

APPENDIX V: DICHLOROMETHANE EXTRACT PLATES

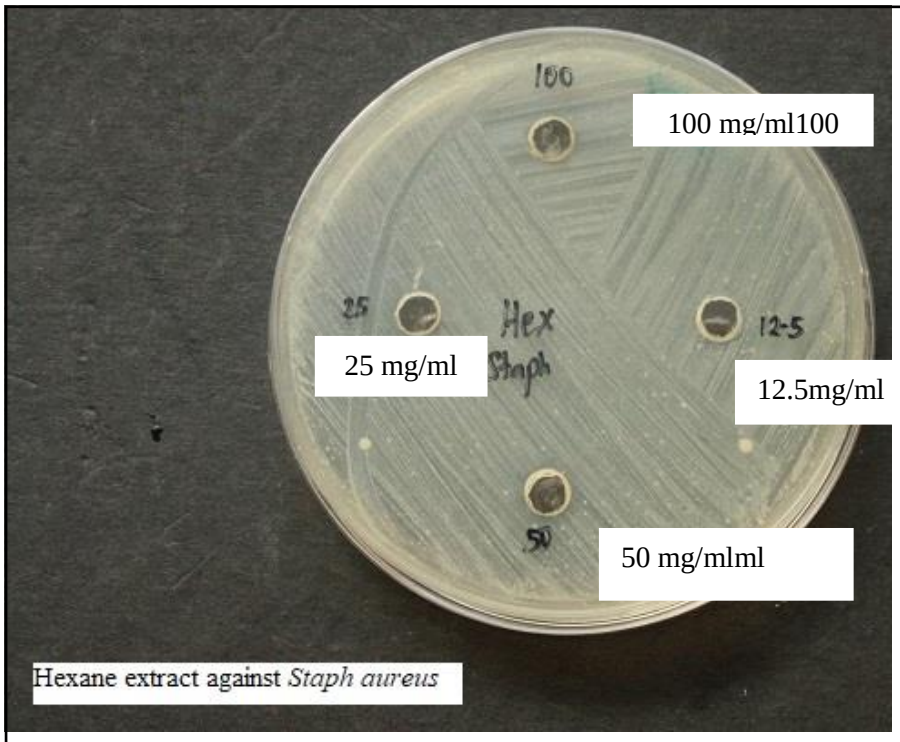


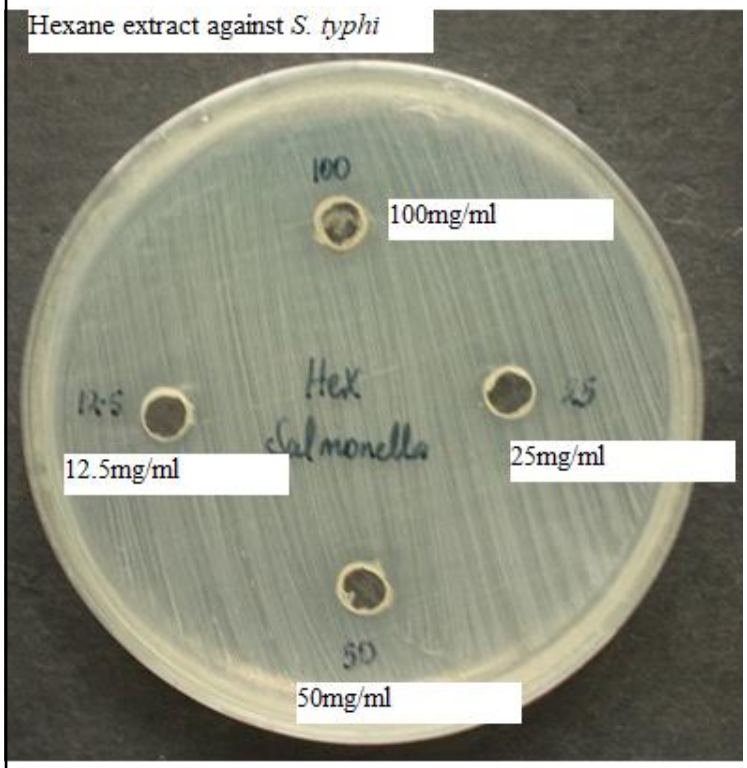


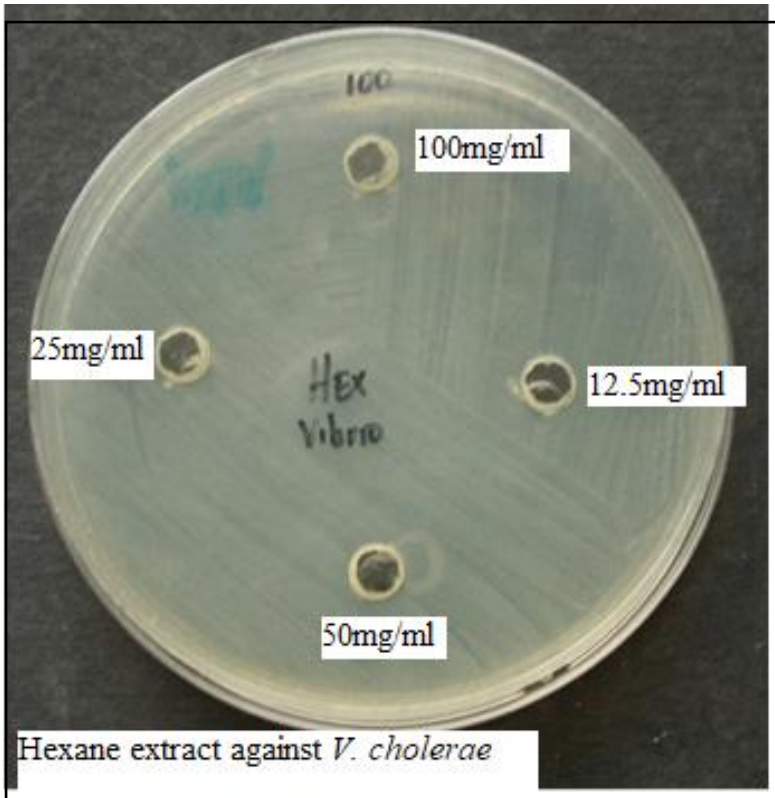

Dichloromethane against S. typhi
100
100 mg/ml
12.5
12.5 mg/ml
DCM
Salmonella
25
25 mg/ml
50
50 mg/ml


Dichloromethane exttract against V. cholerae
100 mg/ml
25
25 mg/ml
DCM
Vibrio
12.5
12.5 mg/ml
50
50 mg/ml

APPENDIX VI: HEXANE EXTRACT PLATES







Hexane extract against *V. cholerae*