

ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES



**Ethanollic extracts of *Warburgia ugandensis* against some
test microorganisms.**

By
Solomon Mekonnen

April, 2010

ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES
SCIENCE FACULTY
Department of Biology

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**A Thesis Submitted to the School of Graduate, Addis Ababa University
in Partial Fulfillment of the Requirement for the Degree of Masters of
Science in Biology**

Under the supervision of
Fassil Assefa (Ph.D.)
Sisay Feleke (Ph.D.)

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SCHOOL OF GRADUATE STUDIES**

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*A Thesis Presented to the School of Graduate Studies of the Addis Ababa University in
Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology
(Botanical Sciences Stream)*

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Table of Contents

Page

Acknowledgement.....	i
List of Figures	ii
List of Tables.....	iii
Abbreviations	iv
Abstract	v
1. INTRODUCTION.....	1
2. LITRATURE REVIEW	4
2.1. The Genus <i>Warburgia</i>	4
2.2. Antimicrobial test organisms	8
2.2.1. Bacterial test organisms	8
2.2.1.1. <i>Bacillus subtilis</i>	8
2.2.1.2. <i>Escherichia coli</i>	10
2.2.1.3. <i>Pseudomonas aeruginosa</i>	13
2.2.1.4. <i>Shigella boydii</i>	15
2.2.1.5. <i>Staphylococcus aureus</i>	17
2.2.1.6. <i>Streptococcus pneumoniae</i>	18
2.2.2. Fungal test organism.....	20
2.2.3. <i>Candida albicans</i>	20
3. OBJECTIVES	22
3.1. General Objective	22
3.2. Specific Objectives	22
4. MATERIAL AND METHODS	23
4.1. Plant Material.....	23
4.1.1. Collection And Identification.....	23
4.1.2. Extraction Preparation.....	23
4.2. Test Organisms	23
4.2.1. Bacterial Strains	23
4.2.2. Fungal Strains	24
4.2.3. Standard Antibiotics	24

4.3. Antibacterial And Antifungal Test	24
4.3.1. Disk Diffusion	25
4.3.2. Agar well Diffusion	26
4.3.3. Minimum Inhibitory Concentration.....	26
4.4. Data Analysis	28
5. RESULT AND DISCUSSION.....	29
5.1.1. Disk Diffusion	29
5.1.2. Agar Well Diffusion	35
5.1.3. Minimum Inhibitory Concentration.....	41
6. CONCLUSION AND RECOMMENDATION	45
7. REFERENCES.....	47
8. Annex	54

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List of Figures

Page

Figure 1. <i>Bacillus subtilis</i> with leave extraction	54
Figure 2. <i>Bacillus subtilis</i> with Heartwood extract.....	54
Figure 3. <i>E. coli</i> with Leaf extract	54
Figure 4. <i>E. coli</i> with Heartwood extract.....	54
Figure 5. <i>Pseudomonas aeruginosa</i> with Leaf extract.....	54
Figure 6. <i>Pseudomonas aeruginosa</i> with Heartwood extract	54
Figure 7. <i>Shigella boydii</i> with Leaf extract.....	55
Figure 8. <i>Shigella boydii</i> with Heartwood extract	55
Figure 9. <i>Staphylococcus aureus</i> with Leaf extract	55
Figure 10. <i>Staphylococcus aureus</i> with Heartwood extract.....	55
Figure 11. <i>Streptococcus pneumoniae</i> with Leaf extract.....	55
Figure 12. <i>Streptococcus pneumoniae</i> with Heartwood extract	55
Figure 13. <i>Candida albicans</i> with Leaf extract.....	56
Figure 14. <i>Candida albicans</i> with Heartwood extract	56
Figure 15. Broth dilution in a concentration gradient.....	56
Figure 16. Plating the broth dilution in their respective concentration gradient	57
Figure 17. <i>Warburgia ugandensis</i>	57
Figure 18. <i>Warburgia ugandensis</i> leaves and fruits	58
Figure 19. <i>Warburgia ugandensis</i> in ice box.....	58
Figure 20. Pouring liquid nitrogen in the ice box	58
Figure 21. Büchi vacuum rotary evaporator	59

List of tables

Page

Table 1. The effect of the leaf and heartwood extracts of <i>W. ugandensis</i> on test microorganisms using disk diffusion method (inhibition zone in mm).....	29
Table 2 Comparison of <i>Warburgia ugandensis</i> with <i>Mimusops elengi</i> and <i>Ricinus communis</i> extracts in Disk diffusion against some bacterial isolates.....	31
Table 3. Antifungal activity of <i>Cassia tora</i> and <i>Warburgia ugandensis</i> extracts on <i>Candidia albicans</i> determined by diameter of inhibition zones using disk diffusion method.....	33
Table 4. The relative efficacy of the reference drug on the test organisms compared to the different extracts (Disk diffusion) at 25mg/ml.....	34
Table 5. Antimicrobial activities of the leaf and heartwood extracts of <i>W. ugandensis</i> at different concentration against test organisms using Agar well diffusion (inhibition zone in mm).	36
Table 6. Antibacterial activities of <i>Betula utilis</i> and <i>Warburgia ugandensis</i> extracts on human pathogenic bacteria.	36
Table 7. Antibacterial activities of <i>Croton zambesicus</i> , <i>Urena lobata</i> and <i>Warburgia ugandensis</i> extracts on human pathogenic bacteria.	37
Table 8. The relative efficacy of the reference drug on the test organisms compared to the different extracts (Agar well diffusion).....	39
Table 9. Minimum inhibitory concentrations (MIC) of the leaf and heartwood extracts of <i>W. ugandensis</i> against test organisms on Nutrient broth plating after 24hrs incubation.	41
Table 10. Comparison of <i>W. ugandensis</i> with different plants in the Minimum inhibitory concentrations (MIC) for the leaf and heartwood extracts of against some test organisms.	43
Table 11. Comparison of Minimum Inhibitory Concentration (MIC) for <i>Warburgia ugandensis</i> with six traditional medicinal plants against <i>Candida albicans</i> from different parts of the plants	44

Abbreviations

ATTC – American Type Culture Collection

WAFC – World Agro Forestry Center

EHNRI – Ethiopian Health and Nutrition Research Institute

MIC – Minimum Inhibitory Concentration

Abstract

Warburgia ugandensis Sprague (Family: Canellaceae) is a tree that is highly utilized in traditional medicine in tropical and warm subtropical countries of Africa. Crude ethanol extracts of leaves and heartwood of *W. ugandensis* were tested for in-vitro antimicrobial activity against six bacterial and a fungi test organisms using the disk diffusion method, agar well diffusion and broth dilution for minimum inhibitory concentration. *E. coli* was found to be the most susceptible bacterial isolate in the Agar well diffusion and Broth dilution. Meanwhile *Shigella boydii* was the most resistant bacterial isolate showing MIC value of 10mg/ml for the leaf extract. *Bacillus subtilis* was the second resistant bacterial isolate giving 0.0mm and 11.0mm inhibition diameter at disk diffusion assay and agar well diffusion, respectively. *Streptococcus pneumoniae* gave 2.5mg/ml and 5mg/ml MIC value for heartwood and leaf extracts, respectively. The same Minimum Inhibitory Concentration (MIC) value was recorded for *B. subtilis*. *Pseudomonas aeruginosa* showed the highest inhibition diameter (16.7mm) in the agar well diffusion assay for the heartwood extract. *Staphylococcus aureus* and *Pseudomonas aeruginosa* showed the same MIC value of 2.5mg/ml in leaf and heartwood extract. *Candida albicans* showed the highest inhibition diameter of 10.0mm and 30.7mm in disk diffusion and agar well diffusion, respectively at the highest test concentration (25mg/ml). In the broth dilution, a MIC value of 1mg/ml concentration of the leaf and heartwood extracts of *W. ugandensis* inhibited the growth of *Candida albicans*. It can be concluded that *W. ugandensis* has the potential as an antimicrobial agent in the future especially against *C. albicans* and *E. coli*.

Key words: antimicrobial activity, *Warburgia ugandensis*, medicinal plants, leaf and heartwood extracts

1. INTRODUCTION

There is increasing interest in natural products as a source of raw material for use in the pharmaceutical, functional foods and inputs for industries in product development and commercialization of products derived from plants (Rhone, 2004).

Essential drugs from medicinal plants such as morphine, digoxin, aspirin, emetine, and ephedrine were introduced into modern therapeutics several centuries ago (Angeh, 2006).

For the last two decades a series of stilbenes and dihydrostilbenes (the combretastastins) with potent cytotoxic activity, and acidic triterpenoids and their glycosides with molluscicidal and antimicrobial activity, has been isolated from different species of *Combretum* (Rogers, 1989).

Similarly, antineoplastic agents include taxol and several derivatives of camptothecin have been isolated from *Taxus brevifolia* and *Camptoteca acuminata* (Pettit and Shigh, 1987; Angeh, 2006). Apart from their antimicrobial and antineoplastic activities, plants possess antioxidant compounds responsible for control of heart diseases, stroke, arteriosclerosis and cancer (Borchardt *et al.*, 2008; Mothana *et al.*, 2008).

Literally, thousands of plant products with inhibiting effect on microorganisms have shown *in-vitro* activity, and many of them have been used for centuries by various cultures in the treatment of different diseases. However, these plants are used at a very high concentration, with serious side-effects on the patient. This requires the evaluation of the concentration against the standard antibiotics that have been already on the market (Angeh, 2006).

Thus, higher plants remain important and reliable sources of potentially useful chemical compounds for direct use as drugs and to synthesize prototypes for synthetic analogues in terms of drug efficacy (Fernsworth, 1984; Iwu *et al.*, 1999).

Generally, medicinal plants are important sources of traditional medicine for millions of people and additional inputs to modern medicine in terms of exploring and producing new drugs to meet the need for the overgrowing population of the planet (Abad *et al.*, 2007). Although, herbal medicine represents one of the most important fields of traditional medicine all over the world, the search for the active ingredients for the synthesis of new drugs has not been extremely undertaken (Kumaraswamy *et al.*, 2008a). In addition to the increasing demand for new drugs, overuse and misuse of antibiotics have contributed to the development and spread of microorganisms that are resistant to treatment.

Tropical forests and many other tropical ecosystems are rich sources of diversity of plant species which produces different and diverse chemical compounds as a means of defense mechanism against herbivores and pathogens in the ecosystem (Fyhrquist, 2007).

Ethiopia is a tropical country that endowed with rich plant biodiversity. There are many plant species found in Ethiopia for medicinal purposes, which is often quoted as one of the six countries of the world where about 60% of the plants are said to be indigenous with a healing potential (Bannerman *et al.*, 1983; Mirgessa, 1996). On record there are 600 species of medicinal plants constituting a little over 10% of Ethiopia's vascular flora (Girma, 1998; Desalegn and Binggeli, 2002).

Several studies have been undertaken in Ethiopia regarding medicinal plants that have been screened for their antimicrobial activities (Abera *et al.*, 2004). Bruck *et al.* (2004) reported that *Inula confertiflora*, *Clematis simensis*, *Zehneria scabra* and *Pycnostachys abyssinica* gave an antimicrobial activity for against common bacterial and fungal pathogens.

A study conducted by Mesfin *et al.* (2006) showing that *Jasminum abyssinicum*, *Solanecio gigas* and *Lagenaria siceraria* were active against some bacterial and fungal pathogens. Additionally, four Ethiopian medicinal plants, *Cleodendrum myrcoides*, *Ficus plamata*, *Grewia ferruginea* and *Perploca linearifolia* were active against some selected bacterial and fungal strains (Asmamaw *et al.*, 2007). Likewise, Tesfaye *et al.* (2006) reported about some medicinal plants of Ethiopia for the purpose of human and livestock disorder remedy.

Warburgia ugandensis is one the medicinal plants that is traditionally used as herbal medicine in some parts of Ethiopia (Friis, 1995). It has been used by the traditional medicinal practitioners to treat malaria, tuberculosis, bronchitis, pneumonia, hepatitis, tapeworm, gonorrhoea, and asthma in Dollo Menna, Bale region.

Although, there are few studies made on the leaf and bark of this species in some African countries like Ngure *et al.* (2009) on *in-vitro* antileishmanial activity, Olila *et al.* (2001) on antibacterial and antifungal activity; Abraham *et al.* (2005) on antimycobacterial activity. There was little information about the antimicrobial property of the heartwood of *W. ugandensis*. The present work was aimed at evaluating the antimicrobial properties of the heartwood extracts in reference to leaf extracts of *W. ugandensis* against some common disease causing microorganisms.

2. LITRATURE REVIEW

2.1. The Genus *Warburgia*

The genus *Warburgia* is one of the flowering plants classified in the family *Canellaceae*. The family in general contains sixteen species in six genera. The genus is named after Dr. Otto Warburg (1859-1938), born in Hamburg, lecturer in botany at the University of Berlin and author of numerous botanical papers (WAFC, 2002).

Most of the species are highly aromatic evergreen plants; with leaves having pellucid dots mostly trees and rarely shrubs, which produce essential oils (Friis, 1995). The flowers are bisexual, and bracts are overlapping on each other. Petals have the same numbers as the sepals or more. Stamens are found above the ovary. They have fruits with a berry type, with many seeds. The protective outer layer of the seeds (testa) is hard and shiny (Friis, 1995; WAFC, 2002).

In this genus, there are five species; *Warburgia breyeri*, Pott, *Warburgia elongata*, Verdc., *Warburgia salutaris*, (Bertol.f.) Chiov., *Warburgia stuhlmannii*, Engl., *Warburgia ugandensis*, Sprague. These trees have leaves which are alternate and leathery. Their flowers have ovaries which are elongated, and ovules are found in the top. The perinth have five stigmas. Fruits are fleshy with waxy covering (Friis, 1995; WAFC, 2002). The genus is native to Africa. In the East Africa tropical flora, the upland taxon referred to as *Warburgia ugandensis* Sprague and the coastal taxon referred to as *Warburgia salutaris* (Bertol. F.) Chiov. *W. ugandensis* occurs in lowland rainforest, upland dry evergreen forest and its relicts in secondary bush land and grassland; also on termitaria in swamp forest. It is native to Democratic Republic of Congo, Ethiopia, Kenya, Malawi, South Africa, Swaziland, Tanzania, and Uganda (Friis, 1995; WAFC, 2002).

Warburgia ugandensis Sprague. is a spreading evergreen tree 4.5-30 m tall with a diameter up to 70 cm. Its bark is scaly, has pale green, brown or slashes pink color. The leaves have dotted glands. Its flowers are solitary and bisexual; its kidney shaped bracts only covers the young buds. The petals are dotted with glands. The tree has a berry fruit, which at first is green and later turns purplish with a leathery glandular skin. The seeds are yellow-brown (Friis, 1995; WAFC, 2002).

This plant is a species of evergreen tree native to Africa. The wood is resistant to insect attack and very strong. The flavor is fiercely hot and finely different to chilies (Ngigi and Ndalut, 2005).

In Ethiopia, this species is limited to transitional montane forest, adjacent woodland often on termite mounds, with an altitude between 1400-1600m.a.s.l. It was only known from a small area at Dollo Menna, Bale region (Friis, 1995). This plant has been valued as useful timber tree elsewhere in Africa, especially in Ethiopia the wood has been studied by Wood Utilization and Research Center, Addis Ababa (Friis, 1995).

Traditionally, it is used to treat cough and rabies in Ethiopia (Aberra *et al.*, 2004). Its fruits are edible, in which all parts have a hot peppery taste. The leaves and seeds are sometimes used to add flavor. Its leaves; pods and seeds can be used as fodder to livestock. As a fuel source, the wood has high oil content and burns well with an incense-like smell. As a timber: the heartwood is yellow or greenish and becomes brown on exposure. It releases a very fragrant smell when freshly cut, the scent somewhat resembling that of sandalwood. It is a good timber for building and furniture, but not resistant to termite. The resin is used locally as glue to fix tool handles (WAFC, 2002).

The heartwood contains sesquiterpenoids such as bemadienolide, cinnamide, drimenol, muzigadial, polygodial, warburganal, warburgiadione, warburgin, ugandensidial and ugandensolide (WAFC, 2002); where some of these compounds are present in the stem bark (Abraham *et al.*, 2005). These compounds exhibit anti-feedant activity against armyworm (*Spodoptera littoralis* and *S. exopta*), widely occurring in African crop pests. The anti-feedant activities of warburganal and muzigadial are comparable (Oilia *et al.*, 2001). These two compounds belong to the strongest group of anti-feedants against African armyworm found so far (Ngigi and Ndalut, 2005).

In addition, they exhibit very potent antifungal, anti-yeast and plant-growth regulating activity (Oilia *et al.*, 2001; WAFC, 2002). Its dried bark is commonly chewed and the juice swallowed as a remedy for stomach-ache, constipation, toothache, cough, fever, muscle pains, weak joints and general body pains (WAFC, 2002). The inner bark is reddish, bitter and peppery and has a variety of applications (Ngigi and Ndalut, 2005). It provides treatment for the common cold and is used to clear sinuses. It can be chewed, or inhaled the smoke from the burning bark as a remedy for chest complaints. The bark, roots or leaves can be boiled in water and the decoction drunk is used to treat malaria and the decoction baths can cure several diseases (WAFC, 2002).

Oilia *et al.* (2001) has evaluated the extracts from the stem bark of *Warburgia ugandensis*. Leaf part of this plant was found to possess polyphenoles, unsaturated sterol/triterpens and tannins. Abraham *et al.* (2005) reported the antimycobacterial activity against *Mycobacterium aurum*, *M. fortuitum*, *M. phlei* and *M. smegmatis* from the stem bark of *W. ugandensis*. Extracts from stem bark of *Warburgia ugandensis* were tested antileishmanial activity *in vitro* at different concentrations against

Leishmania major and *Leishmania donovani* promastigotes and amastigotes (Ngure *et al.*, 2009).

Generally, different provenances of *W. ugandensis* are rich in a wide variety of secondary metabolite, such as tannins, terpenoids alkaloids and flavonoids, which have been found *in vitro* to have antimicrobial properties. Thousands of secondary plant products have been identified and it is estimated that thousands of these compounds still exist, which have been elaborated within living systems, they are often perceived as showing more “drug-likeness and biological friendliness than totally synthetic molecules” making them good candidates for further drug development (Kumaraswamy *et al.*, 2008b).

2.2. Antimicrobial test organisms

2.2.1. Bacterial test organisms

2.2.1.1. *Bacillus subtilis*

The genus *Bacillus* contains aerobic, gram positive rods catalase-positive and motile bacteria producing thermo-resistant endospores. They are active and versatile producers of enzymes and can utilize a wide variety of substances. In nature, they are widely distributed in soil, dust, water and in decaying vegetation.

An endospore is a dense survival unit that develops in a vegetative cell in response to nutrient deprivation. The extreme resistance to heat, drying, radiation, and chemicals accounts for the survival, longevity, and ecological niche of spore formers, and it is also relevant to their pathogenicity (Talaro and Talaro, 2002).

Bacillus subtilis, known as the hay bacillus or grass bacillus, is commonly found in soil. *Bacillus subtilis* is associated mainly with meat or vegetables in pastry, cooked meat or poultry products, and occasionally with bakery items such as bread or crumpets, sandwiches, and ethnic meat or seafood dishes. *Bacillus subtilis* is one of the species that is incriminated with the cause of mild disease and food poison in human kind. It forms spores during unfavorable growth conditions. These spores are heat-resistant and can survive cooking. If the food is cooled slowly or kept warm before serving they will germinate. The bacteria will then multiply rapidly at such temperatures and produce a toxin in the food. This toxin is very stable and will not be destroyed by subsequent re-heating, and thereby causes *Bacillus subtilis* food poison (intoxication) (ECDC, 2003). *Bacillus subtilis* causes food contamination, which nausea, diarrhea and vomiting could be resulted after ingestion within 10 mins - 4 hours (ECDC, 2003).

Food intoxication results from the ingestion of exotoxin secreted by bacterial cells growing in food. The absorbed toxin disrupts a particular target such as the intestine (if an enterotoxin) or the nervous system (if a neurotoxin). The symptoms of intoxication vary from bouts of vomiting and diarrhea to severely disrupted muscle function (botulism) (Talaro and Talaro, 2002).

Although, many species of bacteria are opportunists and are capable of causing disease, especially if present in large numbers, *B. subtilis* and *B. cereus* are singled out to cause invasive infections (lung and blood infection) in people with weakened immunity (Loutit and Reiman, 2001; Talaro and Talaro, 2002).

2.2.1.2. *Escherichia coli*

Escherichia coli is the best-known coliform, largely because of its use as a subject for laboratory studies. It is called the colon bacillus and sometimes regarded as the predominant species in the intestine of humans. Its prevalence in clinical specimens and infections is due to its being the most common aerobic and non-fastidious bacterium in the gut (Talero and Talero, 2002).

Because of its prominence as a normal intestinal bacterium in most humans, *E. coli* is currently one of the indicator bacteria to monitor fecal contamination in water, food, and dairy products. According to this rationale, if *E. coli* is present in a water sample, fecal pathogens such as *Salmonella*, viruses, or even pathogenic protozoa may also be present. Coliforms such as *E. coli* are used because they are present in larger numbers, can survive in the environment, and are easier and faster to detect than true pathogens. If a certain number of coliforms are detected in a sample, the water is judged unsafe to drink (Geissler *et al.*, 2000).

Although many of the strains are not infectious, some have developed greater virulence through plasmid transfer, and others are opportunists. *E. coli* is the predominant cause of both community and nosocomial urinary tract infection (UTI) (Bean *et al.*, 2008). *E. coli* often invade sites other than the intestine. For instance, it causes 50% to 80% of urinary tract infections (UTI) in healthy people. Urinary tract infections usually result when the urethra is invaded by its own endogenous bacterial colonists. The infection is more common in women because their relatively short urethras promote ascending infection to the bladder (cystitis) and occasionally the kidneys. Patients with bladder catheters are also at risk for *E. coli* urethritis (Talero and Talero, 2002).

Most clinical diseases of *E. coli* are transmitted exclusively among humans. Pathogenic strains of *E. coli* are frequent agents of infantile diarrhea, the greatest single cause of mortality among babies. In some areas of the world, about 15% to 25% of children 5 years or younger die of diarrhea as either the primary disease or complication of some other illness (Talero and Talero, 2002). The rate of infection is higher in crowded tropical regions where sanitary facilities are poor and water supplies are contaminated and adults carry pathogenic strains to which they have developed immunity. Other extraintestinal infections in which *E. coli* is involved are neonatal meningitis, pneumonia, septicemia, and wound infections. These often complicate surgery, endoscopy, tracheostomy, catheterization, renal dialysis, and immunosuppressant therapy (Talero and Talero, 2002). About 10% of patients develop hemolytic uremic syndrome (HUS), a severe hemolytic anemia that can cause kidney damage and failure. The highest risk is to young children and the elderly and to immunocompromised people. Antibiotic treatments do not seem to help, and there is really no prevention other than avoiding ingestion of the pathogen. *E. coli* is known to transfer R (resistance) plasmids to several other gut bacteria including *Klebsiella*, *Salmonella* and *Enterobacter*, as well as *Shigella* (Hogg, 2005).

The most serious *E. coli* infection comes from Enterotoxigenic *E. coli* that causes a severe diarrheal illness brought on by two exotoxins, termed heat-labile toxin (LT) and heat-stable toxin (ST), that stimulate heightened secretion and fluid loss. In this way it mimics the pathogenesis of cholera. This strain of bacteria also has fimbriae that provide adhesion to the small intestine. Enteroinvasive *E. coli* causes an inflammatory disease similar to *Shigella dysentery* that involves invasion and ulceration of the mucosa of the large intestine. Enteropathogenic

strains of *E. coli* are linked to a wasting form of infantile diarrhea whose pathogenesis is not well understood. (Talero and Talero, 2002). The newest strain to emerge, O157:H7 causes a hemorrhagic syndrome that can cause permanent damage to the kidney. But, unlike most new pathogens, it has no catchy nickname. It is known instead by its antigen profile—O (somatic) type 157 and H (flagellar) type 7. This bacterium is the agent of a spectrum of conditions, ranging from mild gastroenteritis with fever to bloody diarrhea. It has been variously associated with fast-food restaurants and supermarket hamburger (Mohsenzadeh, 2007), where studies showed that purchasing ground beef at a certain grocery and meatpacker, was the major risk factor for illness to a nationwide outbreak associated with *E. coli* O157:H7 illness that resulted in the second largest recall of beef in U.S. history at the time (Vogt and Dippold, 2005).

2.2.1.3. *Pseudomonas aeruginosa*

The genus *Pseudomonas* contains a very diverse group of bacteria that are gram negative, motile, rod shaped in morphology, able to grow and survive in almost any environment, soil, water and vegetation. This is due to their metabolic diversity of the genus in general, and the production of siderophore and fluorescein by some of the most dominant members of the group (Mayer *et al.*, 2002).

Pseudomonas aeruginosa is one of such members that are not only dominant in different habitats, but also associated with community-acquired and nosocomial infection in immuno-compromised patients and hospitals. As a result, the pathogen is considered as an opportunist bacterium (Delden and Iglewski, 1998).

P. aeruginosa is responsible for 16% of nosocomial pneumonia cases, 12% of hospital-acquired urinary tract infections, 8% of surgical wound infections, and 10% of bloodstream infections (Delden and Iglewski, 1998). Amongst the AIDS patients, *P. aeruginosa* bacteremia is associated with 50% of deaths (Delden and Iglewski, 1998).

Most *Pseudomonas* species are naturally resistant to penicillin and the majority of related beta-lactam antibiotics, but a number are sensitive to piperacillin, imipenem, tobramycin, or ciprofloxacin (Gailiené *et al.*, 2007).

One of the most striking characteristics of *P. aeruginosa* consists in its low antibiotic susceptibility. This low susceptibility is attributable to a concerted action of multidrug efflux pumps with chromosomally-encoded antibiotic resistance genes and the low permeability of the bacterial cellular envelopes. Besides intrinsic resistance, *P. aeruginosa* easily

develop acquired resistance either by mutation in chromosomally-encoded genes, or by the horizontal gene transfer of antibiotic resistance determinants (Srikumar *et al.*, 1998).

2.2.1.4. *Shigella boydii*

Shigella is a genus of gram negative, non-sporeforming rod shaped bacteria that causes human shigellosis in primates and humans. *Shigella boydii* is one of the most dominant member of the genus that causes dysentery that result in the destruction of the epithelial cells of the intestinal mucosa in the cecum and rectum. Some strains produce enterotoxin and Shiga toxin, are associated with causing hemolytic uremic syndrome (Braun, 2005).

Shigellosis is endemic throughout the world where it is held responsible for some 120 million cases of severe dysentery with the overwhelming majority of which occur in developing countries and involve children less than five years of age (WHO, 2009). In tropical developing countries, shigellosis is endemic and a major killer of children under 5 years of age. Shigellosis occurs following ingestion of a very small number (100–1000) of bacteria, thus permitting easy spread of the disease by person-to-person contact as well as by the drinking of contaminated water (Sasakawa, 2004; WHO, 2009).

The most common symptoms of shigellosis are fever, nausea, vomiting, stomach cramps, flatulence, and straining to have a bowel movement. The stool may contain blood, mucus, or pus (e.g. dysentery). Symptoms can take as long as a week to show up, but most often begin two to four days after ingestion. Symptoms usually last for several days, but can last for weeks (Popoff, 2005).

Shigella may remain alive in cultures for months or even years if moisture is retained. They may survive in tap water and sea water for two to five months. Their resistance to physical and chemical agents appears to be similar to other non-sporulating organism (Soltys, 1963).

Transferable antimicrobial resistance was described in *Shigella* and there has been a wide distribution of resistant strains and the development of resistance to an increasing number of antimicrobial agents (Sack *et al.*, 2001). Combined resistance to cotrimoxazole, chloramphenicol, ampicillin, and tetracycline has been widely described and resistance to nalidixic acid, the recent preferred treatment of choice, has become increasingly common. Resistance to nalidixic acid is associated with reduced sensitivity to ciprofloxacin and other fluoroquinolones, leaving few treatment options, particularly in low-income countries (Hogg, 2005; Shears, 2008).

2.2.1.5. *Staphylococcus aureus*

The genus *Staphylococcus* consists of spherical cells arranged in irregular grape-like clusters, gram-positive, nonmotile and nonsporing. It is frequently found in the nose and skin of the human population. Members of this genus are facultatively anaerobic (Precott, 2002). *Staphylococci* are quite resistant to desiccation and high-osmotic conditions. These properties facilitate their survival in the environment, growth in food, and communicability. Pathogenic staphylococci are usually opportunists and cause illness in compromised hosts.

Staphylococcus aureus is the most pathogenic species. It causes skin and tissue infections and can invade many other organs and some strains produce toxins. The major diseases are carbuncles, abscesses, osteomyelitis, endocarditis, toxic shock syndrome, bacterial pneumonia, common food poisoning, and other diseases (Yarwood, 2006). Food poisoning strains of *S. aureus* produce a heat-stable enterotoxin that has a direct effect upon the central nervous system (Celikel and Kavas, 2008; Heritage *et al.*, 1999). It is one of the four most common causes of nosocomial infection, often causing post surgical wound infection (Precott, 2002). *S. aureus* remains an important pathogen, particularly among people who are hospitalized. For example, in the United States, between 1999 and 2000, about one percent of hospital patients had or acquired *S. aureus* infection, and nearly half of these infections were caused by strains resistant to multiple antibiotics (Guilfoile, 2007).

In addition, some of these *Staphylococci* are resistant to penicillin (Precott, 2002). Several new antibiotics have recently become available for treating multiple antibiotic resistant *S. aureus*, but it may be only a matter of time before widespread resistance to these drugs develops as well (Guilfoile, 2007).

2.2.1.6. *Streptococcus pneumoniae*

Streptococcus is a genus of spherical gram-positive, non-motile, non-sporeforming, oxidase and catalase-negative bacteria belonging to the lactic acid bacteria group. Cellular division occurs along a single axis and they grow in chains or pairs. Most streptococci are facultative anaerobes, and some are obligate (strict) anaerobes (Patterson, 1996). They are also part of the normal commensal flora of the mouth, skin, intestine, and upper respiratory tract of humans.

Streptococcus is a diverse genus that contains non-pathogenic and pathogenic bacteria that infect a barrage of different animals, including humans, with diseases ranging from strep throat to necrotizing fasciitis. In addition to strep throat, certain *Streptococcus* species are responsible for many cases of meningitis, bacterial pneumonia, endocarditis, ear infection, erysipelas and necrotizing fasciitis (the 'flesh-eating' bacterial infections) (Hogg, 2005).

Streptococcus pneumoniae ("pneumococcus") is a bacterium commonly found in the back of the nose (nasopharynx) of healthy people. Most people have been carriers of *S. pneumoniae* at some point in their lives. Pneumococcal carriage is more common in young children, is usually transient and generally causes no illness. It develops into a serious infection when the host's defenses are depleted due to such factors as old age, illness (like AIDS), or malnutrition. *S. pneumoniae* is an exclusively human pathogen and is spread from person-to-person by respiratory droplets, meaning that transmission generally occurs during coughing or sneezing to others (Romney *et al.*, 2008).

Streptococcus pneumoniae was a leading cause of death in the United States and many other countries in the first half of the 20th century including many other parts of the world (Guilfoile, 2007). Despite the

introduction of antibiotics in the 1940s, and more recently, the development of polysaccharide and conjugate pneumococcal vaccines, *Streptococcus pneumoniae* remains a leading cause of morbidity and mortality worldwide. Most disease due to *S. pneumoniae* is sporadic, and outbreaks of invasive pneumococcal disease (IPD) are rare (Romney *et al.*, 2008).

2.2.2.Fungal test organism

2.2.2.1. *Candida albicans*

Candida is a genus of yeasts. While usually living as commensals, some *Candida* species have the potential to cause disease. Clinically, the most significant member of the genus is *Candida albicans*, which can cause oral and genital infections (called candidiasis moniliasis or thrush) in humans (Du and Calderone, 2009). *Candida albicans* is a common internal infection in human beings. *Candida* enters newborn infants during or shortly after birth. Usually, the growth of the yeast is kept in check by the infant's immune system and thus produces no overt symptoms. The fungus also can travel through the blood stream and affect the throat, lungs, intestines and heart valves. *C. albicans* becomes an infectious agent when there is some change in the body environment that allows it to grow out of control (Hay, 1991).

The risk of invasion increases with extreme youth, pregnancy, drug therapy, immunodeficiency, and trauma. Any situation that maintains the yeast in contact with moist skin provides an avenue for infection. Although candidiasis is usually endogenous and not contagious, it can be spread in nurseries or through surgery, childbirth, and sexual contact. *Candida albicans* and its close relatives account for nearly 80% of nosocomial fungal infections and 30% of deaths from nosocomial infections in general (Talaro and Talaro, 2002). A common cause of infection may be the use of antibiotics that destroy beneficial, as well as harmful, microorganisms in the body, permitting *Candida* to multiply in their place (Boekhout and Guého, 2002).

Candidiasis is often observed in immunocompromised individuals such as HIV-positive patients. Candidiasis also may occur in the blood and in the genital tract. Candidiasis is usually easily cured in people who are

not immunocompromised. When the body defenses are impaired due to malnutrition, after infections like measles, due to infection by human immunodeficiency virus (HIV) as found in AIDS, or during treatment with immunosuppressive drugs, the bowel may be colonized by organisms which do not normally cause disease. These include yeast the *Candida albicans* (Cutting, 1991).

3. OBJECTIVES

3.1. General objective

The aim of this study is to evaluate the efficacy of the leaf and heart wood ethanol crude extracts of *Warburgia ugandensis*, on some disease causing microorganism agents.

3.2. Specific objectives

- To test the antimicrobial effect of the different concentrations of the heartwood and leaf crude extracts of *Warburgia ugandensis* using disc diffusion and agar well diffusion methods
- To determine the Minimum Inhibitory Concentration (MIC) of the leaf and heartwood crude extract of *Warburgia ugandensis*

4. MATERIAL AND METHODS

4.1. Plant Material

4.1.1. Collection and Identification

The leaves of *Warburgia ugandensis* were collected from Bale region at 13 km. from Dolo Menna to Goba road, in Harena Forest, Southeastern Ethiopia in January 2009. The plant was identified by The National herbarium, Department of Biology of the Addis Ababa University. Voucher specimens (01) were recorded in the Herbarium.

4.1.2. Extract preparation

Sample materials (Leaves and heartwood) were kept under liquid nitrogen and transported to the laboratory. Then the specimens were immediately removed from storage and were processed. The leaves were crushed easily by hand and were soaked in ethanol (96%) overnight (24hours). The heartwood was chopped and dried. The dried specimen was milled by electric grinder into fine powder at 1200 rpm. The fine powdered heartwood was extracted with ethanol in a Soxhlet apparatus. The filtrates of the leaves and heartwood (ethanol and plant extract) were concentrated in Büchi rotary vacuum evaporator at 60°C.

The crude extracts from leaves and heartwood were prepared in four concentrations; 1mg/ml, 5mg/ml, 10mg/ml and 25mg/ml (w/v) for both extracts using ethanol as a solvent.

4.2. Test Organisms

4.2.1. Bacterial Strains

Bacterial test organisms; *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Shigella boydii* (Clinically isolated), *Staphylococcus aureus* (ATCC 25923), *Streptococcus*

pneumoniae (ATCC 49619) were obtained from Ethiopian Health and Nutrition Research Institute (EHNRI).

4.2.2.Fungal Strains

Candida albicans (Clinically isolated) was used as a fungal test organism in this study. It was brought from Ethiopian Health and Nutrition Research Institute.

4.2.3.Standard Antibiotics

Tetracycline and Ketoconazole were used as control for the bacterial and fungal test organisms, respectively (Olila *et al.*, 2001; Ahmad *et al.*, 2002).

4.3. Antibacterial and antifungal test

The antimicrobial tests of the crude extracts were undertaken in three replications, with incubation temperature of 37°C for 24 hrs and 24-72hrs for bacterial and fungal isolate, respectively.

Inocula Preparation

In order to determine the physically active state of the isolates, each isolate was grown in 100ml appropriate broth (nutrient broth for bacteria isolates and Sabouroud's broth for fungal isolate media) in 250ml Erlenmeyer flask on a rotary shaker (120 rpm) at 37°C. Samples were taken every 2 hrs for a total of 12 hrs and the optical density was used to determine the exponential phase using spectrophotometer at 660nm. The exponential phase of each isolate was made by making a growth curve of optical density against time (Lalitha, 2004).

After determining the active phase at which time it occurred, inoculum preparation was standardized by inoculating bacterial strains where inocula were then taken from the exponential phase and standardized

with 0.5 McFarland turbidity standard (prepared by adding a 0.5 ml aliquot of 0.048 mol/L BaCl₂ (1.175% w/v BaCl₂. 2H₂O) added to 99.5 ml of 0.18 mol/L H₂SO₄ (1% v/v)) (Lalitha, 2004).

For fungal strain, *C. albicans* was grown in Sabouroud's broth. After 24 hrs of incubation at 37°C, the isolate was then diluted 1:100 in normal saline, followed by a 1:100 dilution in Sabouroud's broth (Radetsky *et al.*, 1986). A volume of 0.1 ml of the final broth dilution contained approximately 10³ CFU of yeast, which was determined by plating onto Potato dextrose agar. For comparison, a McFarland 0.5 standard contains 10⁷ to 10⁸ cfu/ml. A 0.1ml portion of broth (10³ cfu) was used as the standard inoculum in agar well diffusion and broth dilution techniques (Radetsky *et al.*, 1986).

4.3.1.Disk diffusion

A preliminary screening for antibacterial activity was done by the disk diffusion method (Angeh, 2006). Disk diffusion susceptibility testing was performed using a filter paper discs (Whatman No. 1) with a diameter of 6 mm. The disks were impregnated with a 1mg/ml, 5mg/ml, 10mg/ml and 25mg/ml concentration of crude extracts. 0.025 mg/ml of Tetracycline was used as the standard drug for bacterial isolate and 25 mg/ml of Ketoconazole was used as the standard drug for fungal isolate. Cultures of the different test organism with 0.5 McFarland turbidity standard were placed by making a swab on the plates. The disks were placed in the center after drying for 5 min. The plates were left at room temperature for about 10 min to allow the extract or the compounds to diffuse from the disc into the medium, and were then incubated at 37°C for 24 hrs and 24-72hrs for bacterial and fungal isolates, respectively after which the inhibition zones were noted and their diameters were recorded as the zone of inhibition (Lalitha, 2004; Wanger, 2007).

4.3.2. Agar well diffusion

Bacterial strains were tested in Nutrient agar media by making a 6 mm well in the media using a sterile borer. Inoculum from exponential phase of each bacterial isolates was centrifuged using a vortex (Griffin & George Ltd.). The turbidity of the reconstituted organisms was adjusted to 0.5 McFarland standards. Both the standard and bacterial suspensions were agitated on a vortex mixer immediately prior to use. After inoculating the bacterial isolates, the plates were allowed to dry for 5 min after which the crude extracts and the controls were dispensed into each well. The plates were incubated at 37 °C for 24hrs. Inhibition zone sizes were measured in millimeters compared to standard Tetracycline (0.025 mg/ml) (Olila *et al.*, 2001).

For *Candida albicans*, the same method was used to make a well in Potato dextrose agar. Broth was expressed from the swabs by pressing and rotating the swabs firmly against the inside of the tube above the fluid level. The swab was then evenly streaked over the entire surface of the agar plate to obtain uniform inoculums. Each of the extract concentrations was applied into the well. All these activities were undertaken under aseptic condition. The plates were then incubated for 24-72 hrs at 37°C to determine the inhibition zone (Ito, 2004). Ketoconazole (25 mg/ml) was used as the standard drug.

4.3.3. Minimum inhibitory concentration

Broth dilution method

The Minimum inhibitory concentrations (MIC) was determined by broth dilution technique (Hogg, 2005; Boyanova *et al.*, 2005; Lalitha, 2004; Wanger, 2007), using nutrient broth for bacteria and Sabouroud's broth (Mycological peptone + Dextrose) media for the fungus. First, the leaf and heartwood extracts were prepared in different concentrations (0.025, 0.05, 0.1, 0.25, 0.5, 1.0, 1.75, 2.5, 5.0, 10.0 and 25mg/mL). A 1 ml of

each concentration from each extracts was dissolved in sterile capped test tubes containing 8.9mL of suitable microbial growth medium. Then, serial dilutions of the concentrations were inoculated with 0.1mL of standard size of bacterial and fungal inoculum from the exponential phase. Two test tubes containing broth without antimicrobial agent was added in each test. One of these tubes was inoculated with the test organism; the other was left uninoculated and served as a check for media sterility. The test tubes were incubated at 37°C for 24hrs for bacterial isolates and 24-72hrs incubation time at 37°C for fungi. Lowest concentration of compound that showed antimicrobial activity against test organisms was recorded as MIC value.

4.4. Data analysis

Data analysis were made by *Microsoft Excel* in terms of the mean of the growth inhibition value obtained from each of the six bacterial strains and one clinical isolate of fungal strains.

5. RESULT AND DISCUSSION

5.1.1. Disk Diffusion

The result of Table 1 has shown that the antimicrobial activity of the different concentrations of leaf and heartwood extracts of the plant against six test pathogenic bacterial and one fungal species.

As it has been indicated in Table 1 the growth inhibition that was recorded from all the test organisms at 25mg/ml concentration of the heartwood extract ranging from diameter of 2.0 – 10.0mm in inhibition zone. Similarly, the leaf extract showed at 25mg/ml concentration for all test organisms ranging diameter from 2.0 – 10.0mm inhibition zone except *B. subtilis* (0.0mm). *S. pneumoniae* was the only bacterial species that was inhibited by 10mg/ml concentration of the leaf extract. There was no bacterial species that was inhibited at concentrations less than 10mg/ml (except *S. pneumoniae*) and 25mg/ml of the leaf and heartwood extracts.

Table 1. The effect of the leaf and heartwood extracts of *W. ugandensis* on test microorganisms using disk diffusion method (inhibition zone in mm).

Test Organism	Leaf			Heartwood			Controls*
	5.0mg /ml	10mg /ml	25mg /ml	5.0mg /ml	10mg /ml	25mg /ml	
<i>Bacillus subtilis</i>	0.0	0.0	0.0	0.0	0.0	5.0	23.0
<i>E. coli</i>	0.0	0.0	4.0	0.0	0.0	6.1	22.0
<i>Pseudomonas aeruginosa</i>	0.0	0.0	2.8	0.0	0.0	4.1	21.0
<i>Shigella boydii</i>	0.0	0.0	2.3	0.0	0.0	2.0	25.0
<i>Staphylococcus aureus</i>	0.0	0.0	2.0	0.0	0.0	4.7	27.0
<i>Streptococcus pneumoniae</i>	0.0	2.0	6.2	0.0	0.0	3.7	20.0
<i>Candida albicans</i>	5.2	6.9	8.0	6.4	7.8	10.0	10.0

Standard drugs* - **Tetracycline** (0.025mg/ml) for Bacterial strains and **Ketoconazole** (25mg/ml) for *Candida albicans*

Different test bacteria species shown variations in their sensitivity/resistance to the leaf and heartwood extract (Table 1). Accordingly, the most sensitive test bacterium was *Streptococcus pneumoniae* that was inhibited at the lowest test concentration of 10mg/ml of the leaf extract with inhibition diameter of 2.0mm, and displayed the largest inhibition diameter of 6.2mm at a concentration of 25mg/ml (Table 1).

E. coli was found to be the second sensitive test bacterium with inhibition diameter of 4mm; where as, the next most resistant test bacterium to the leaf extract was *Staphylococcus aureus*, with inhibition diameter of 2.0mm at the highest test concentration of 25mg/ml (Table 1).

With regard to inter-bacterial differences in response to exposure to heartwood extract, *E. coli* was the most sensitive bacterium to the heartwood extract with the highest inhibition diameter of 6.1mm, followed by *B. subtilis* (5mm) and *S. aureus* (4.7mm). The relatively resistant test microorganisms were *S. boydii* followed by *S. pneumoniae* with small inhibition diameters of 2mm and 3.7mm, respectively (Table 1).

Mbwambo *et al.* (2009) also reported that *Escherichia coli*, and *Staphylococcus aureus* gave inhibition diameters of 15mm at a concentration of 5mg/ml of freeze-dried ethanolic leaf extracts of *W. ugandensis*. This shows that the extract of the same plant was 3-15 times more effective than the present study.

Although *B. subtilis* was the most resistant test bacterium that was not inhibited at 25mg/ml concentration of the leaf extract in this work. Ali *et*

al. (2008) reported that the leaf extracts from *Mimusops elengi* gave inhibition diameter of 16mm against *B. subtilis* at 30mg/ml concentration (Table 2).

Ali et al. (2008) reported that *S. boydii* and *S. aureus* gave 3 - 6 times higher inhibition zones from the test concentrations of 30mg/ml of the bark extracts of *Mimusops elengi* than *W. ugandensis* extracts on the same test microorganisms in the present study (Table 2).

All these differences may show that variations occur not only among different species of plants, but also among different varieties of the same species. The effectiveness may also be attributed to the difference in the inherent resistance of the test organisms in the different tests (Table 2).

Table 2 Comparison of *Warburgia ugandensis* with *Mimusops elengi* and *Ricinus communis* extracts in Disk diffusion against some bacterial isolates.

Species	Plant part	Concentration	Organism					Source
			<i>B. subtilis</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. boydii</i>	<i>S. aureus</i>	
<i>Mimusops elengi</i>	Bark	30mg/ml	0.0	–	–	9.0	11.0	Ali et al. 2008
	Leaf	30mg/ml	16.0	–	–	8.0	13.0	
<i>Warburgia ugandensis</i>	leaf	5mg/ml	13.0 (<i>B. cereus</i>)	15.0	–	–	15.0	Mbwambo et al. 2009
<i>Warburgia ugandensis</i>	Leaf	10mg/ml	0.0	0.0	0.0	0.0	0.0	Present study
		25mg/ml	0.0	4.0	2.8	2.3	2.0	
	Heartwood	10mg/ml	0.0	0.0	0.0	0.0	0.0	
		25mg/ml	5.0	6.1	4.1	2.0	4.7	

– not tested

Generally, the test organisms showed different pattern of resistance/sensitivity to the two extracts in that the heartwood extract was found to be three times more active on *E. coli* than *S. boydii*. The same pattern was also recorded with *S. aureus* and *S. pneumoniae* on the leaf extract. In most test organisms, heartwood extract at 25mg/ml concentration was relatively effective as compared to the leaf extract

based on the size of the inhibition diameter on the test organisms. Although Olila *et al.* (2001) reported that it was not possible to demonstrate the bactericidal effect of *W. ugandensis* bark extract in the paper disk assay at a concentration lower than 50mg/ml. The leaf and heartwood extracts showed inhibition at 25mg/ml concentrations in the present study.

The data also showed the inhibitory effect of the leaf and heartwood extracts was more pronounced on the test fungus, *C. albicans*, than the bacterial species. *C. albicans* was found to be sensitive to the tested concentrations of 5.0, 10.0 and 25.0mg/ml of leaf extracts with inhibitory diameters of 5.2, 6.9 and 8.0mm, respectively. Likewise, the heartwood extracts displayed slightly larger inhibitory diameters of 6.4, 7.8 and 10.0mm at concentrations of 5.0, 10.0 and 25mg/ml, respectively (Table 1).

Olila *et al.* (2001) reported that *W. ugandensis* stem bark water extracts showed antifungal activity against *Candida albicans* in disk diffusion assay at 100mg/ml, as opposed to the ethanolic extract of the same plant that did not show antifungal activity of the same test organisms. On contrary, the leaf and heartwood ethanolic extract of this plant in the present study showed the antifungal activity against *C. albicans* as low as 1mg/ml.

Mbwambo *et al.* (2009) reported that freeze-dried ethanolic leaf extract of *W. ugandensis* showed inhibition of *C. albicans* with a diameter of 15mm at a test concentration of 5mg/ml, indicating that the plant variety from Nigeria was more than 3 times effective than the Ethiopian variety. However, the inhibition diameter from *W. ugandensis* was better than the

leaf and heartwood extracts of *Cassia tora* that did not inhibit the fungus at the same concentration (25mg/ml) (Ahmad *et al.*, 2002) (Table 3).

Table 3. Antifungal activity of *Cassia tora* and *Warburgia ugandensis* extracts on *Candidia albicans* determined by diameter of inhibition zones using disk diffusion method (in mm).

Species	Plant part	Concentration	<i>Candida albicans</i>	Source
<i>Cassia tora</i>	Leaf	5mg/ml	0.0	Ahmad <i>et al.</i> 2002
		10mg/ml	0.0	
		25mg/ml	0.0	
	Seed	5mg/ml	0.0	
		10mg/ml	0.0	
		25mg/ml	8.8	
<i>Warburgia ugandensis</i>	Leaf	5mg/ml	15.0	Mbwambo <i>et al.</i> 2009
<i>Warburgia ugandensis</i>	Leaf	5mg/ml	5.2	Present study
		10mg/ml	6.9	
		25mg/ml	8.0	
	Heartwood	5mg/ml	6.4	
		10mg/ml	7.8	
		25mg/ml	10.0	

The inhibition diameters of the standard drugs (Tetracycline and Ketoconazole) showed variations amongst the test bacteria ranging from 20mm (*S. pneumoniae*) to 27mm (*S. aureus*). Comparing the efficacy of the reference drug to the leaf and heartwood extracts based on drug extract ratio, the drug was found to be more effective than the leaf extract ranging from 3.2 (*S. pneumoniae*), to 13.5 (*S. aureus*). Similarly, the drug was more potent than the heartwood extract to inhibit the test bacteria within the range of 3.6 (*E. coli*) and 12.5 (*S. boydii*). This means the extracts are promising on the test organisms found at the lower level such as *E. coli* and *S. pneumoniae* but not on *S. aureus* and *S. boydii*.

The reference drug (Ketoconazole) also showed the smallest inhibition diameter of 10mm which is similar to the inhibitory property of the heartwood extracts against *C. albicans*. Likewise, the drug to leaf and heartwood extract ratio on *C. albicans* were 1.3 and 1.0, respectively, indicating that the extracts were as effective as the test drug (Ketoconazole) (Table 4).

Ahmad *et al.* (2002) compared the effectiveness of the reference drug, Ketoconazole with ethanol extracts of *Cassia tora* seeds against *Candida albicans*, and showed that the drug did not inhibit the test fungus where as the seed extract from *Cassia tora* inhibited *C. albicans*, with inhibition diameters of 8.8 mm at 25 mg/ml (Table 3)

Table 4. The relative efficacy of the reference drug on the test organisms compared to the different extracts (Disk diffusion) at 25mg/ml.

Organism	Ratio (leaf)	Ratio (heartwood)
<i>Bacillus subtilis</i>	-	4.6
<i>E. coli</i>	5.5	3.6
<i>Pseudomonas aeruginosa</i>	7.5	5.1
<i>Shigella boydii</i>	10.9	12.5
<i>Staphylococcus aureus</i>	13.5	5.7
<i>Streptococcus pneumoniae</i>	3.2	5.4
<i>Candida albicans</i>	1.3	1.0

Standard drugs-**Tetracycline** (0.025mg/ml) for Bacterial strains and **Ketoconazole** (25mg/ml) for *Candida albicans*

It is interesting to note that the leaf and heartwood extracts of *W. ugandensis* were as effective as the standard drug (Ketoconazole) against *C. albicans*. This indicates that the crude extracts have the potential as source of drug in the future.

5.1.2. Agar Well Diffusion

The antimicrobial activity of the ethanol extracts of *W. ugandensis* was also tested using Agar well diffusion. Unlike that of the paper disc method, this method showed that the leaf and heartwood extracts induced inhibition on the different test organisms at concentrations as low as 1mg/ml with relatively larger diameters (Table 5). There was a steady increase in the degree of inhibition as the concentrations increased.

At the lowest test concentration of 1.0mg/ml, the leaf extracts inhibited the test bacteria species with inhibition diameters of 0.5mm-9.6mm, whereas the heartwood extracts reduced the growth of the test bacteria with inhibition diameters of 8.2mm-11.5mm without significant difference amongst them. Likewise, the leaf and heartwood extracts imposed reduced growth on the test organisms with inhibition diameters of 5.3mm-13.7, and 11.0mm-16.7mm, respectively at the highest test concentrations of 25mg/ml. This shows that the 25-fold increase in concentrations of the extracts only induced a maximum of two-fold increase in inhibition on the test bacteria.

The most sensitive bacterium to the lowest concentration (1mg/ml) of the leaf extract was *E coli* with inhibition diameter of 9.6mm, followed by *P. aeruginosa* with inhibition diameter of 8.2mm. On the contrary, *S. boydii* and *S. pneumoniae* were the most resistant to the leaf extract with inhibition diameters of 0.5mm and 4.6mm, respectively (Table 5). The same pattern of sensitivity/resistance to the test bacteria was displayed by the heartwood extract. In general, the leaf extract showed a variation in inhibition with significant difference amongst the test bacteria, whereas, the heartwood extract seem to be equally potent with larger diameters and little difference in inhibition diameters on the test organisms.

Table 5. Antimicrobial activities of the leaf and heartwood extracts of *W. ugandensis* at different concentration against test organisms using Agar well diffusion (inhibition zone in mm).

Organism	Leaf				Heartwood				Control*
	1.0mg/ml	5.0mg/ml	10mg/ml	25mg/ml	1.0mg/ml	5.0mg/ml	10mg/ml	25mg/ml	
<i>Bacillus subtilis</i>	7.0	7.8	8.5	9.0	8.7	9.3	9.9	11.0	36.5
<i>E. coli</i>	9.6	10.9	12.7	13.7	11.5	12.3	13.0	14.8	36.5
<i>Pseudomonas aeruginosa</i>	8.2	10.0	10.7	11.9	10.0	11.2	12.0	16.7	34.5
<i>Shigella boydii</i>	0.5	0.7	1.2	5.3	8.9	10.5	11.2	12.5	39.0
<i>Staphylococcus aureus</i>	7.3	8.2	8.8	10.2	8.2	11.9	12.8	14.3	40.5
<i>Streptococcus pneumoniae</i>	4.6	6.0	6.8	7.7	10.0	10.6	12.2	13.4	37.0
<i>Candida albicans</i>	19.4	23.3	25.4	28.0	21.0	25.3	28.8	30.7	15.3

Standard drugs* - **Tetracycline** (0.025mg/ml) for Bacterial strains and **Ketoconazole** (25mg/ml) for *Candida albicans*

Kumaraswamy *et al.* (2008a), also reported that *E. coli*, *P. aeruginosa*, *S. aureus*, and *S. boydii* displayed inhibition diameters of 15.3, 15.5 and 12.13mm, and 14.75mm at 100mg/ml to the bark extract of *Betula utilis*. This shows that heartwood extract of *W. ugandensis* was as equally effective as the bark extract of *B. utilis* at only a quarter of the test concentration of 25mg/ml (Table 6).

Table 6. Antibacterial activities of *Betula utilis* and *Warburgia ugandensis* extracts on human pathogenic bacteria.

Species	Plant part	Concentration	Organism				Source
			<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. boydii</i>	<i>S. aureus</i>	
<i>Betula utilis</i>	Bark	100mg/ml	15.13	15.5	14.75	12.13	Kumaraswamy <i>et al.</i> 2008a
<i>Warburgia ugandensis</i>	Leaf	10mg/ml	12.7	10.7	1.2	8.8	Present study
		25mg/ml	13.7	11.9	5.3	10.2	
	Heart wood	10mg/ml	13.0	12.0	11.2	12.8	
		25mg/ml	14.8	16.7	12.5	14.3	

From all the test organisms, the fungus *C. albicans* was the most susceptible to leaf and heartwood extracts. This is evidenced from the

inhibition diameters of 19.4mm and 21.0mm at the test concentrations of 1.0mg/ml leaf and heartwood extract, respectively (Table 5). There was also a steady increase in the inhibitory effect of leaf and heartwood extracts with inhibition diameters of 28mm and 30.7mm, respectively, with 25-fold increase in the test concentration.

Although, Olila *et al.* (2001) reported that *W. ugandensis* stem bark water extract showed antibacterial activity against *S. aureus* and *E. coli* in the agar well diffusion assay at 100mg/ml, the same activity was not detected from the ethanolic extract of the plant. Adeloye *et al.* (2007) also reported the antimicrobial activity of crude leaf extract of *Urena lobata* with inhibition diameters of 15, 27, 15 and 12mm against *B. subtilis*, *E. coli*, *P. aeruginosa* and *S. aureus* at a concentration of 20mg/ml, respectively. This was higher than the inhibition diameters displayed by the highest test concentrations of 25mg/ml in this work. However, this increase was only 1.3 - 2 times more than the inhibition diameters imposed by 10mg/ml of the leaf extract of *W. ugandensis* (Table 7).

Table 7. Antibacterial activities of *Croton zambesicus*, *Urena lobata* and *Warburgia ugandensis* extracts on pathogenic bacteria.

Species	Plant part	Concentration	Organism				Source
			<i>B. subtilis</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	
<i>Croton zambesicus</i>	Stem bark	300mg/ml	–	30.0	–	30.0	Reuben <i>et al.</i> 2008
		400mg/ml	–	30.0	–	28.3	
		500mg/ml	–	28.7	–	27.0	
<i>Urena lobata</i>	Leaf	20mg/ml	15.0	27.0	15.0	12.0	Adeloye <i>et al.</i> 2007
<i>Warburgia ugandensis</i>	Leaf	10mg/ml	8.5	12.7	10.7	8.8	Present study
		25mg/ml	9.0	13.7	11.9	10.2	
	Heart wood	10mg/ml	9.9	13.0	12.0	12.8	
		25mg/ml	11.0	14.8	16.7	14.3	

– not tested

Reuben *et al.* (2008) reported that stem bark extract of *Croton zambesicus* induced inhibition of 30mm on *E. coli* and *S. aureus* at a concentration of 300mg/ml. This generally indicates that *W. ugandensis* is more effective than the leaf and heartwood extracts of *Urena lobata*, *Betula utilis* and *Croton zambesicus* considering the effectiveness in terms of ratio of concentration to the inhibition diameters (Table 7).

In the leaf extract, *C. albicans* was 2 times more susceptible than the most sensitive bacterial species, *E. coli* (25mg/ml), and 5 times more susceptible than the most resistant bacterial species, *S. boydii* (25mg/ml). Likewise, the heartwood extract, *C. albicans* was almost 2 times more susceptible than the most sensitive test bacterium, *E. coli* (25mg/ml), and 3 times more susceptible than the most resistant test bacterium, *B. subtilis* (25mg/ml).

The inhibitory effects of the leaf and heartwood extracts of *W. ugandensis* were effective at a concentration of 1mg/ml (19.4-21mm) which was almost equivalent to the inhibition diameter of 24mm at 20mg/ml concentration of the leaf extract of *Urena lobata* (Adeloye *et al.*, 2007), and 25.3mm at concentrations of 300mg/ml of the stem bark extract of *Croton zambesicus* (Reuben *et al.*, 2008).

The efficiency of leaf and heartwood extract at the lower (1mg/ml) and higher concentration (25mg/ml) was also evaluated in relation to the reference drugs using agar diffusion method. In the case of the growth response of the bacterial species to the standard drug (Tetracycline), they showed sensitivity with inhibition diameters of 36.5 – 40.5mm compared to the inhibition diameters of the leaf and heartwood extracts of (0.5-16.7mm) (Table 5).

Accordingly, the reference drug to the leaf extract ratio of the test bacteria fell within 3.8-8.0 at a lower concentration of 1mg/ml, except *S boydii*, and 2.7-7.4 at the highest concentration of 25mg/ml for the test bacteria (Table 8).

Table 8. The relative efficacy of the reference drug on the test organisms compared to the different extracts (Agar well diffusion)

Organism	Drug-leaf ratio (1mg/ml)	Drug-leaf ratio (25mg/ml)	Drug- heartwood ratio (1mg/ml)	Drug- heartwood ratio (25mg/ml)
<i>Bacillus subtilis</i>	5.2	4.1	4.2	3.3
<i>E. coli</i>	3.8	2.7	3.2	2.5
<i>Pseudomonas aeruginosa</i>	4.2	2.9	3.5	2.1
<i>Shigella boydii</i>	78.0	7.4	4.4	3.1
<i>Staphylococcus aureus</i>	5.5	4.0	4.9	2.8
<i>Streptococcus pneumoniae</i>	8.0	4.8	3.7	2.8
<i>Candida albicans</i>	0.8	0.5	0.7	0.5

Standard drugs -**Tetracycline** (0.025mg/ml) for Bacterial strains and **Ketoconazole** (25mg/ml) for *Candida albicans*

Likewise, the heartwood extract showed the relative effectiveness of 3.2-4.9 and 2.1-3.1 at 1.0mg/ml and 25mg/ml test concentrations, respectively. Similarly, the relative efficacy of the reference drug (Ketoconazole) to the leaf extract was found to fall into 0.8 and 0.5 at the lowest (1.0mg/ml) and highest concentrations (25mg/ml); whereas the ratio of the reference drug to heartwood extracts was within 0.7 to 0.5 at the lowest and highest concentrations, respectively (Table 8).

It is very interesting to note that the test microorganisms showed variations in drug to the extracts ratio. The most sensitive test bacteria to the ethanolic extracts of *W. ugandensis* such as *E. coli* and *P. areuginosa* showed narrower drug to extract ratio than the relatively resistant bacteria, *S. boydii*. In the case of *C. albicans*, both extracts

showed a very narrow drug to extract ratio (<1) in the agar well diffusion method (Table 8).

The low extract to drug ratio displayed by test microorganisms is a reflection of either the efficacy of the extracts to inhibit the specific microorganisms or the resistance/sensitivity of the bacteria to the test drug (Mbwambo *et al.*, 2009).

The inhibitory effect of the extracts was detected at higher concentration in disk diffusion method; where as the same trait was recognized at a concentration as low as 1 mg/ml with agar well diffusion method. Although the inhibitory effect of the extracts increased as a function of concentration, the effectiveness was not as pronounced as the increase in concentration indicating that lower concentration performed well against the different test organisms. The performance of the test drugs on the test bacteria was also found to be 1.6 - 2 times greater in the agar well diffusion method than the disk diffusion method.

5.1.3. Minimum Inhibitory Concentration

The minimum inhibitory concentration assay was also employed to evaluate the effectiveness of the extracts to inhibit growth of the test microorganisms. All of them were subjected to concentrations of extracts ranging from 0.025mg/ml up to 25 mg/ml. The results are recorded on Table 9.

Table 9 Minimum inhibitory concentrations (MIC) of the leaf and heartwood extracts of *W. ugandensis* against pathogenic test microorganisms.

Bacteria	Part	1mg/ml	1.75mg/ml	2.5mg/ml	5.0mg/ml	10mg/ml
<i>Bacillus subtilis</i>	Leaf				†	
	Heartwood			†		
<i>E. coli</i>	Leaf		†			
	Heartwood		†			
<i>Pseudomonas aeruginosa</i>	Leaf			†		
	Heartwood			†		
<i>Shigella boydii</i>	Leaf					†
	Heartwood				†	
<i>Staphylococcus aureus</i>	Leaf			†		
	Heartwood			†		
<i>Streptococcus pneumoniae</i>	Leaf				†	
	Heartwood			†		
<i>Candida albicans</i>	Leaf	†				
	Heartwood	†				

† Absences of microbial growth

Within the bacterial isolate, *E. coli* was highly susceptible to the MIC of 1.75mg/ml of the leaf and heartwood extracts followed by *P. aeruginosa* and *S. aureus* which were inhibited to the concentration of 2.5mg/ml of both extracts. *E. coli*, *P. aeruginosa* and *S. aureus* were the bacterial species showing the same MIC result for both the leaf and heartwood extracts. Likewise, *B. subtilis* and *S. pneumoniae* were inhibited at 2.5mg/ml of the heartwood extracts. Similarly, *B. subtilis* and *S. pneumoniae* were inhibited at the MIC of 5.0mg/ml of the leaf extract;

whereas, the heartwood extract inhibited *S. boydii* at the same concentration. Generally, the result showed that the most resistant bacterial isolate was *S. boydii* followed by *B. subtilis* and *S. pneumoniae* (Table 9).

All taken together, the leaf extract required relatively higher MIC values to inhibit the test microorganisms than the heartwood extracts. The most sensitive bacterium, *E. coli* was found to be almost 6 times more susceptible to the leaf and heartwood extracts than the most resistant isolate, *S. boydii* (10.0mg/ml). The data also showed that, *S. boydii* was 4 times more resistant than *P. aeruginosa* and *S. aureus* to both extracts.

In Ethiopia, Aberra *et al.* (2005) reported that leaf extracts of *Warburgia ugandensis* inhibited *Bacillus cereus* and *S. aureus*, with MIC of 1mg/ml and 2mg/ml, respectively showing that the plant variety was more effective than the variety tested in this work. Aberra *et al.* (2005) also reported that leaf and bark extracts of *Syzygium guineense* and *Olea europea* inhibited *S. aureus* at MIC of 0.25 and 1mg/ml, respectively. However, the leaf extract of *Discopodium peninervum* and *Olea europea* showed MIC value of 2mg/ml against *S. aureus*, which was equivalent in effectiveness to the extracts of *W. ugandensis* in this work (Table 10).

Although the MIC result showed that *B. subtilis* was the second resistant bacterial isolate to have been inhibited at 5.0mg/ml concentrations of the heartwood extract (Table 9), Shahwa and Raza (2009) showed that the test organism can be inhibited at a concentration as low 0.6mg/ml of the *Mimosops eleongi* bark extract Reuben *et al.*, (2008) also reported that the stem bark extract of *Croton zambesicus* gave MIC values of 1.56, 6.25 and 1.56mg/ml on *E. coli*, *P. aeruginosa* and *S. aureus*, respectively. Comparing these results with the present study, extracts of *W.*

ugandensis were slightly lower in inhibiting the test microorganisms than the extracts of *C. zambesicus* (Table 10).

Table 10 Comparison of *W. ugandensis* with different plants in the Minimum inhibitory concentrations (MIC) for the leaf and heartwood extracts of against pathogenic microorganisms.

Species	Plant part	Organism					Source
		<i>B. subtilis</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>S. pneumoniae</i>	
<i>Mimusops elengi</i>	Bark	0.6 mg/ml	-	-	-	-	Shahwar and Raza 2009
<i>Croton zambesicus</i>	Stem bark	-	1.56 mg/ml	6.25mg/ml	1.56 mg/ml	-	Reuben <i>et al.</i> 2008
<i>Warburgia ugandensis</i>	Leaf	2.5 mg/ml	1mg/ml	2.5mg/ml	2.5 mg/ml	5mg/ml	Present study
	Heartwood	5.0 mg/ml	1mg/ml	2.5mg/ml	2.5 mg/ml	2.5mg/ml	
<i>Syzygium guineense</i>	Leaf	-	-	-	0.25 mg/ml	1 mg/ml	Aberra <i>et al.</i> 2005
	Stem bark	-	-	-	0.25 mg/ml	2 mg/ml	
<i>Discopodium peninervum</i>	Leaf	-	-	-	2mg/ml	2 mg/ml	
<i>Olea europea</i>	Leaf	-	-	-	2mg/ml	2 mg/ml	
	Stem bark	-	-	-	1mg/ml	1 mg/ml	
<i>Warburgia ugandensis</i>	Leaf	1 mg/ml (<i>B. cereus</i>)	-	-	2mg/ml	-	

- not tested

Ahmed *et al.*, (2007) also showed that the ethanolic extracts of *Sesame radiatum* and its combination with *Sesame indicum* leaves had strong inhibitory effects against *Streptococcus pneumoniae*, with a MIC of 76.2 µg/ml and 70.0µg/ml, respectively indicating that these plants had a highly significant bacterial activity as compared to leaf or heartwood extract of *W. ugandensis*.

The fungal isolate, *C. albicans*, showed the lowest MIC values (1mg/ml). Aberra *et al.* (2005) reported that *Ximenia americana*, *Syzygeum guineense*, *Euclea divinorum* and *Osyris quadripartite* leaf extracts inhibited the test fungus at MIC vales of 4mg/ml. On the contrary, leaf extracts of *Dovyalis abyssinica* were found to inhibit the test fungus at MIC of 1mg/ml. All taken together, the leaf extracts of *W. ugandensis* was better for its anti-candidal activity than the above plant extracts, except *Dovyalis abyssinica* (Table 11).

Table 11 Comparison of Minimum Inhibitory Concentration (MIC) for *Warburgia ugandensis* with six traditional medicinal plants against *Candida albicans* from different parts of the plants

Plant	Plant part	MIC	Source
<i>Warburgia ugandensis</i>	Leaf	1 mg/ml	Present study
	Heartwood	1 mg/ml	
<i>Dovyalis abyssinica</i>	Leaf	1 mg/ml	Aberra <i>et al.</i> , 2005
<i>Ximenia Americana</i>	Leaf	4 mg/ml	
<i>Syzygeum guineense</i>	Leaf	4 mg/ml	
<i>Euclea divinorum</i>	Leaf	4 mg/ml	
<i>Osyris quadripartita</i>	Leaf	4 mg/ml	

Given that many commercial antifungal drugs induce adverse drug reactions that include liver, kidney and gastrointestinal toxicities (Graybill, 1988 cited on Ahmad *et al.*, 2002), extracts of *W. ugandensis* may have the potential to be a candidate as an anti-candidal agent in the future provided that their active components can be further isolated and processed.

6. CONCLUSION AND RECOMMENDATION

- From the above results, it can be concluded that the traditional medicinal plant, *W. ugandensis* has a certain degree of efficacy against the test microorganisms.
- The different test bacteria showed some difference in the response to the different concentration and type of the extracts, there is a pattern of more effectiveness with the heartwood extracts than the leaf extracts.
- The pattern of inhibition, in general, also showed that *E. coli* and *P. aeruginosa* were sensitive, where as *S. boydii*, *S. pneumoniae* and *B. subtilis* were resistant to the extracts.
- The antimicrobial activity of the leaf and heartwood extracts was more revealed using agar well diffusion than the disk diffusion method. This was clearly indicated by the size of the inhibition diameter, and the concentrations at which the inhibition was detected.
- In this study, *Candida albicans* was the most sensitive test organism to the leaf and heartwood extracts. In addition, the leaf and heartwood extracts showed that the extract was 1.3 – 2 times more effective than the reference drug (Ketoconazole) using the agar well diffusion detecting method.
- The MIC value was recorded from the leaf and heartwood crude extract starting from 1mg/ml against *C. albicans* up to 10mg/ml against *S. boydii*.

Recommendations

- The different methods used in this test could not establish the same result. Moreover, some strains became more resistant in one

method and showed different response in the other. This necessitates for more detailed investigation using different solvent extracts to clearly establish the antimicrobial activities of the plant.

- The leaf and heartwood crude extracts of this plant may contain specific active components that may enhance effective antimicrobial activities. Thus, isolation of compounds using fractionation from these parts of the plant is important for future application.

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8. ANNEX

A. Agar well diffusion

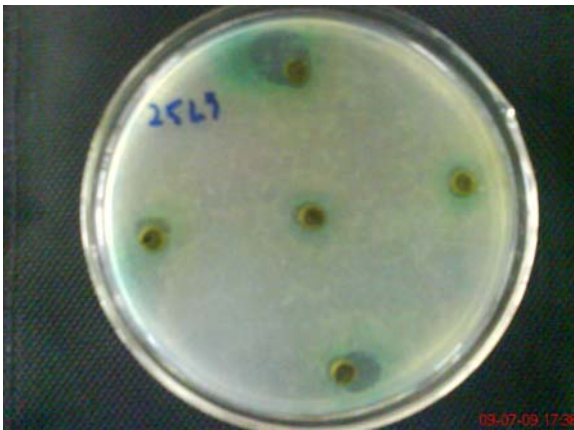


Fig. 1 *Bacillus subtilis* with leaf extraction

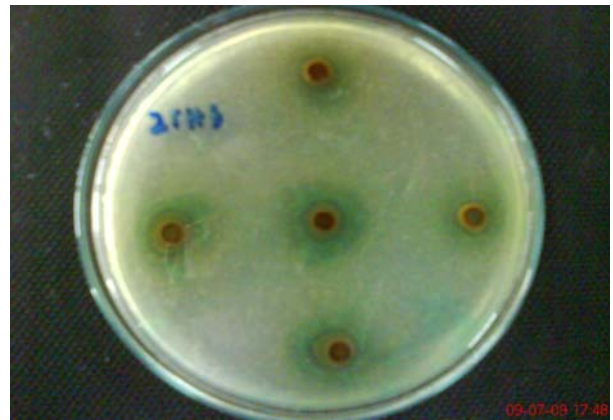


Fig. 2 *Bacillus subtilis* with heartwood extract

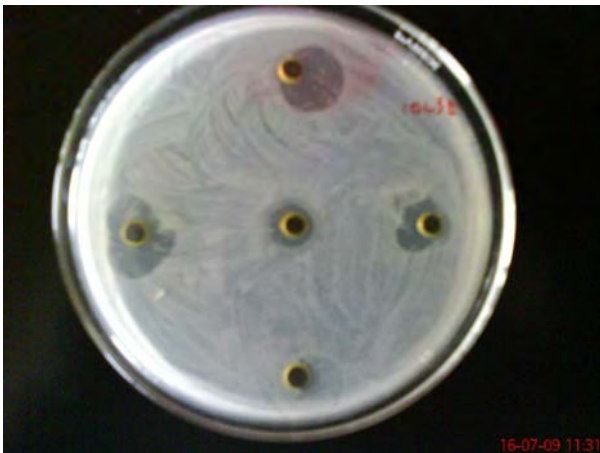


Fig. 3 *E. coli* with Leaf extract

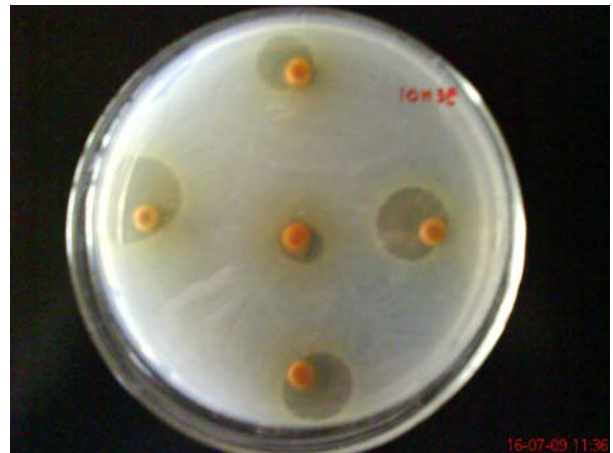


Fig. 4 *E. coli* with Heartwood extract

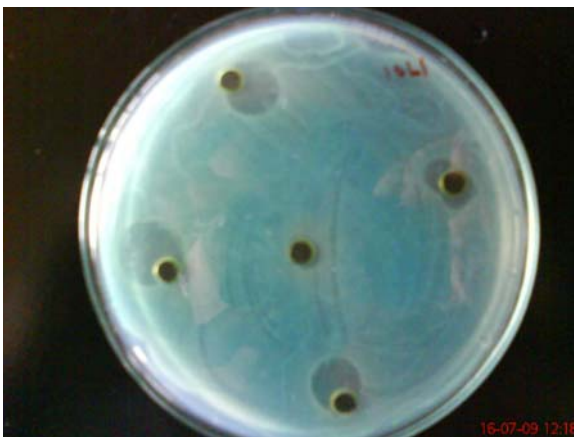


Fig. 5 *Pseudomonas aurogenosa* with leaf extract

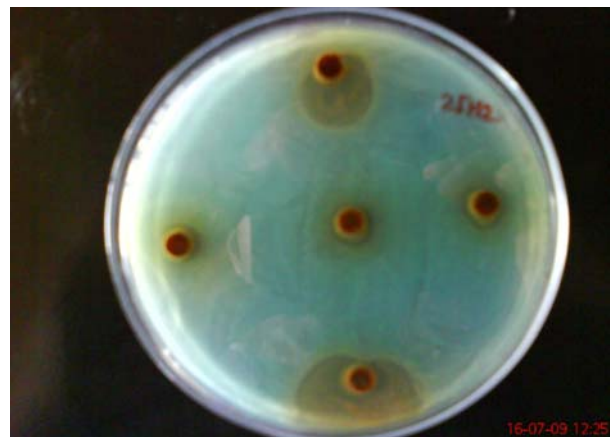


Fig. 6 *Pseudomonas aurogenosa* with heartwood extract

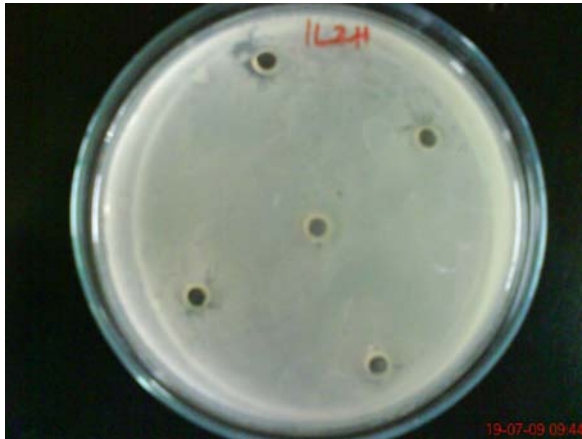


Fig. 7 *Shigella boydii* with leaf extract

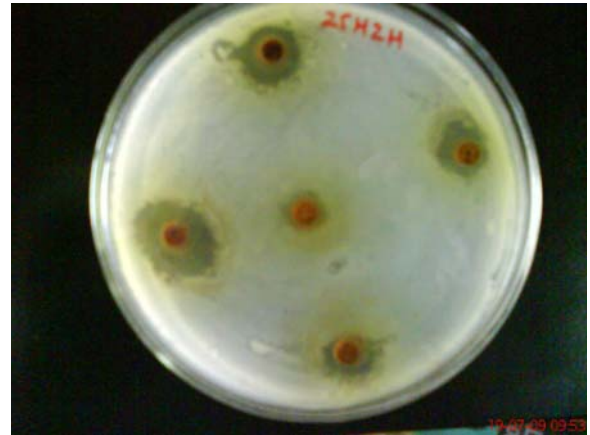


Fig. 8 *Shigella boydii* with heartwood extract

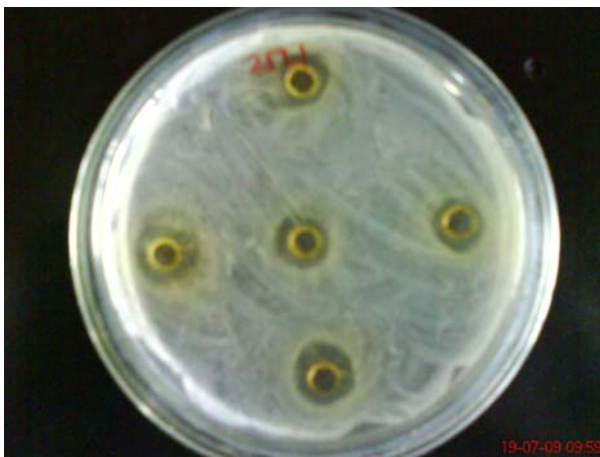


Fig. 9 *Staphylococcus aureus* with leaf extract

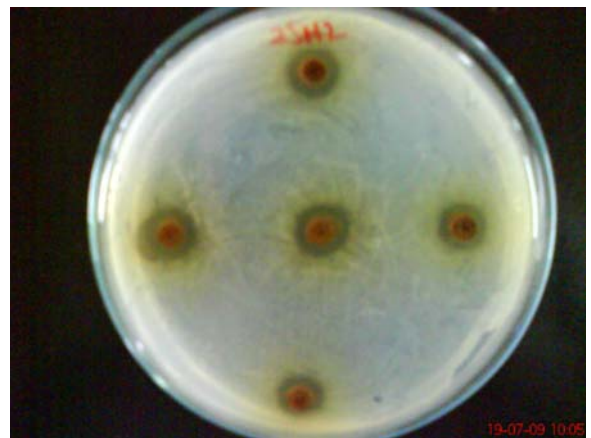


Fig. 10 *Staphylococcus aureus* with leaf extract

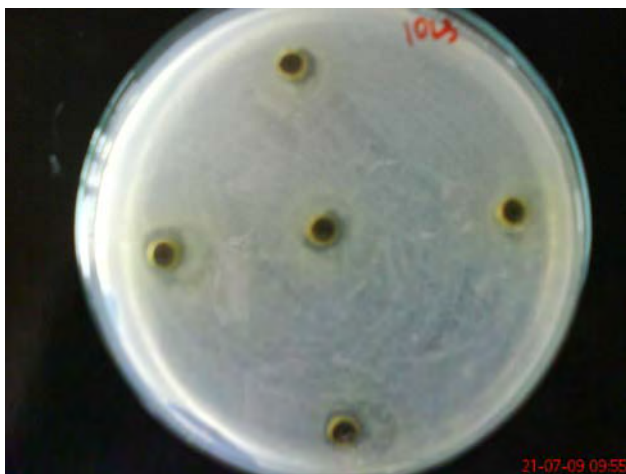


Fig. 11 *Streptococcus pneumoniae* with leaf extract

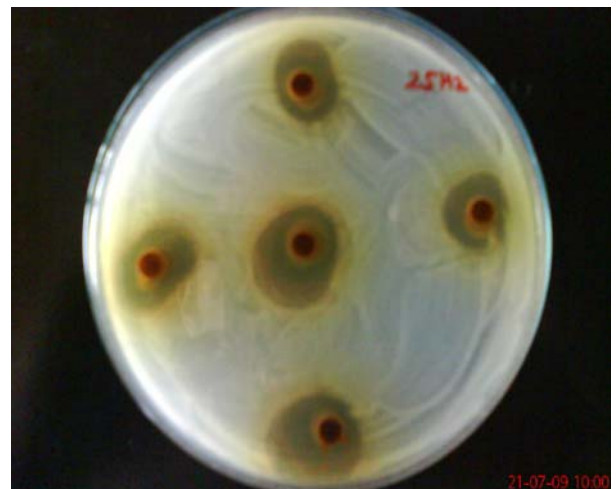


Fig. 12 *Streptococcus pneumoniae* with heartwood extract

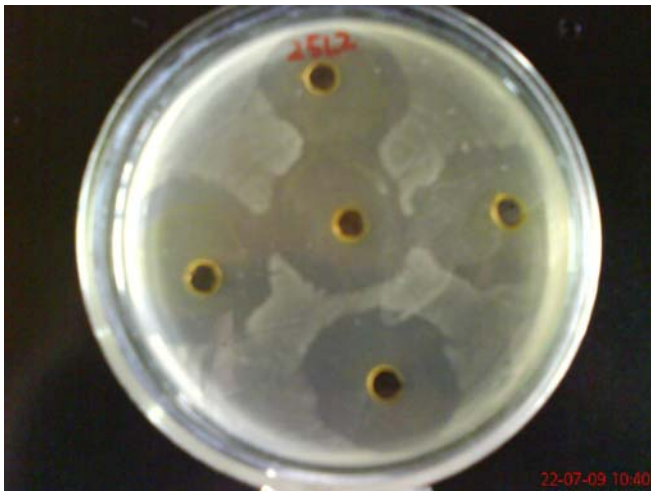


Fig.13 *Candida albicans* with lead extract

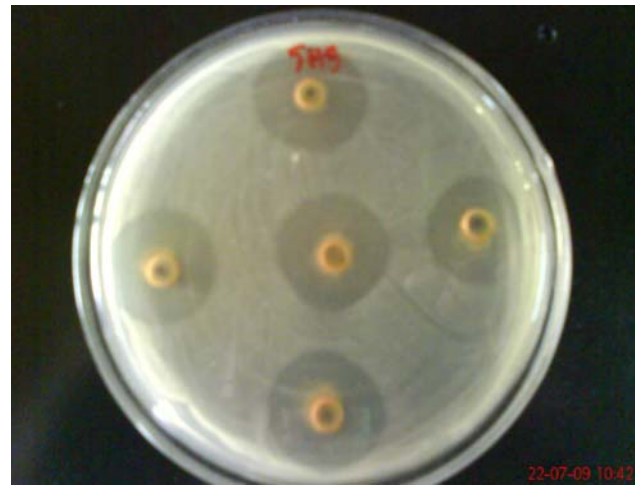


Fig. 14 *Candida albicans* with heartwood extract

B. Minimum Inhibitory Concentration (Broth dilution)



Fig. 15 Broth dilution in a concentration gradient



Fig. 16 Plating the broth dilution in their respective concentration gradient

C. Plant material collection and transportation



Fig 17 *Warburgia ugandensis*



Fig. 18 *Warburgia ugandensis* leaves and fruits



Fig. 19 *Warburgia ugandensis* in ice box



Fig. 20 Pouring liquid nitrogen in the ice box

C. Extraction process



Fig. 21 Büchi vacuum rotary evaporator

DECLARATION

I, the undersigned declare that this M.Sc. thesis is my original work and it has not been presented in any other institution/university for a similar degree or other purpose. I also declare that all the sources of materials used for this study have been fully acknowledged.

Solomon Mekonnen

Signature



Date: 02/06/10

This thesis has been submitted for examination with our approval as

Advisor: Fassil Assefa, Ph. D

Signature:



Date:

2/6/2010

Advisor: Sisay Feleke, Ph. D

Signature:



Date:

2/06/2010