ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES
DEPARTMENT OF BIOLOGY
MASTER'S THESIS

INVESTIGATION OF THE ANTIMICROBIAL ACTIVITIES OF THREE MEDICINAL PLANTS ON THE GENUS *SHIGELLA* AND *SALMONELLA* CAUSING DIARRHOEA IN CHILDREN

BY

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A THESIS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES, ADDIS ABABA UNIVERSITY, IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF SCIENCE IN BIOLOGY

ADDIS ABABA

JANUARY, 2005

ACKNOWLEDGEMENTS

It's my pleasure to thank all of those who have participated in this work directly or indirectly. It would have been impossible to do this work without those individuals. First I would like to extend my deepest gratitude to my advisors, Dr. Abera Geyid, (former Director of Ethiopian Health and Nutrition Research Institute), Dr. Asfaw Debela (Ethiopian Health and Nutrition Institute, Drug Research Department) and Professor Selishi Lulseged from the Department of Pediatrics and Child Health from Addis Ababa University for their valuable and great guidance in doing this thesis work.

I would also like to thank all of the staff of the Drug Research Department at EHNRI, for their unreserved support in introducing me to the techniques of plant extraction, especially W/t Frehiwot Teka and W/t Asnakech Senbeta. My thanks also goes to the staff of Bacteriology, Research Team, without whom this work would have not come to an end. It's my pleasure to thank Dr. Eshitu Lemma, W/o Mekdes Gabyehu and Ato Endris Mohammed, Ato Kidanemariam Mamo, Ato Tesfaye Kebede, Ato Surafel Fentaw, Ato Nega Asamnew,Ato Redwane Muzaine, Ato Faris Kumsa, W/t Feven Girmachew W/o Shishg Chekol, W/o Meskerem Seifu, and Ato Tariku Yerfu from the Department of Bacteriology Research Team. I also would like to thank, Dr. Chris Watters from Middles Burry College for his contribution in sending relevant articles and Ato Mehirtu Tekeste, from Drug Quality and Control, Authority for giving us the relevant reference antibiotics.

Finally I sincerely acknowledge my families, who have supported me in all aspects. This work was financially supported in part by A.A.U., Department of Biology and E.H.N.R.I.. I would also like to thank the Department of Drug Research and Clinical Laboratory of Bacteriology, and in large the Department of infection and Diseases at E.H.N.R.I. for the technical and material support they provided me with, to do this research work.

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List of Abbreviations

ATCC--American Type Culture Collection

MIC--- Minimum Inhibitory Concentration

NCCLS-- National Committee for Clinical Laboratory Standards

EHNRI--Ethiopian Health and Nutrition Research Institute

WHO – World Health Organization

CDC, P--Centre for Disease Control and Prevention

AIDS –Acquired-Immuno Deficiency Syndrome

BSAC- British Society of Antimicrobial Chemotherapy

S----------Streptomycin

Su---------- Sulphonamide

T---------- Tetracycline

C---------- Chloramphinicol

MS---------- *Myrica salisfolia*

GL ---------- *Gardinea lutea*

OE---------- *Olea europea*

TP---------- Terpenoid

A---------- Alkaloid

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Abstract

Diarrhoea, particularly infectious diarrhoea in children less than 5 yrs of age is labeled as the second leading cause of mortality and morbidity throughout the world. This is especially true in developing countries like Ethiopia where there is poor sanitation and overcrowding. Among the leading causes of infectious diarrhoea, *Salmonella* and *Shigella* contributes a lot. Currently the chemotherapeutical treatment of salmonellosis and shigellosis is complicated as a result of drug resistance. Moreover, since the majority of the people who lives in these developing countries have no access for modern treatment, it has made them to look for other alternative therapies such as, the use of medicinal plants. Ethiopia is one of the well known countries of the world where medicinal plants are used widely. The major objective of this study is to evaluate the antibacterial activity of three medicinal plants (*Gardinea lutea, Olea europaea* subsp. *cuspidata*, *Myrica salisfolia)* against clinical isolates of *Shigella* and *Salmonella* and a control strain *E. coli* ATCC (25922).

The minimum inhibitory concentration (MIC) of the three medicinal plant extracts including their semi purified fractions, and modern antibiotics were determined, using the standard agar dilution method (NCCLS). Those fractionated extracts which have shown weak to high antimicrobial activity and the three antibiotics, (Chloramphinicol, Tetracycline and Norfloxacin) have been tested in three replicates.

From the three plants of both the crude and semi purified fractionate of *Olea europaea* subsp. *cuspidata* has shown weak activity against both *Shigella* and *Salmonella*. The MIC of *Olea europaea* subsp. *cuspidata* is $\geq$ 2000µg/ml for both clinical isolates. The other two plants

(*Gardinea lutea* and *Myrica salicfolia*) have shown relatively better MIC value, particularly against the clinical isolates of *Shigella* and *Salmonella* species. The range of MIC, where antishigella activity was recorded for both the crude and butanol fraction of *Gardinea lutea* was between 2000µg/ml - 250µg/ml and the range of MIC for both the crude and fractionated extracts *Myrica salisfolia* is greater than or equal 1000µg/ml for both clinical isolates of *Shigella* and *Salmonella*. As compared to the result of modern antibiotics, it can be suggested that, the plant extracts have shown weak activity with low MIC values. Among the antibiotics, tetracycline, has shown MIC value of ≥200 µg/ml, for both *Salmonella* and *Shigella*. While chloramphinicol has shown MIC value of <600µg/ml for *Salmonella* isolates and ≥150µg/ml for *Shigella* isolates. The least MIC value was obtained for norfloxacin with MIC value of ≥ 0.43µg/ml with 100% growth inhibition for *Shigella* and *Salmonella*. Further investigations (purifications) could enhance, especially for the antimicrobial activity of the semi purified butanol fractionates of *Gardinea lutea* and *Myrica salisfolia* which have shown relatively the best activity against the clinical isolates of *Salmonella* and *Shigella*.

Key words: Minimum Inhibitory Concentration (MIC), *Gardinea lutea*
Olea europaea subsp. *cuspidata* , *Myrica salisfolia*

1. General Introduction

Diarrhoea is defined as the passage of three or more loose or watery stools (i.e. stool, liquid enough to take the shape of the receiving container) within a period of 24 hour (WHO, 1996b)**.** Diarrhoea is known to be caused by several factors including the non infectious type of diarrhoea such as metabolic diseases, food allergy and other organic causes. On the contrary, infectious diarrhoea is a type of diarrhoea which is caused by an infectious agent (bacteria, fungus, parasites and viruses) due to the invasion and colonization of the host tissue. Infectious diarrhoea is characterized by an alteration in a normal bowel movement, an increase in the volume of water content, or frequency of stools, nausea, vomiting, cramps and/or abdominal discomfort (Sood and Pacheco*,* 2002).

An infectious diarrhoeal illness is the second leading cause mortality and morbidity throughout the world in children less than five years of age (Sood and Pacheco*,* 2002). Infectious diarrhoea poses a significant economic and societal burden throughout the world both in developing and developed countries such as the United States. This is especially true in developing countries where it's the leading cause of mortality and morbidity in children, where geographic, economic, political, and sociocultural factors interact to create devastating and continuing challenges to its prevention and control programm (Carl and William, 1989).

Annually, more than five million people, 80% of whom are less than one year of age, die from acute infectious diarrhoea (Guerrant, 1998). According to Mohammed (2001), diarrhoea is a major cause of mortality in 15 to 20% of the under 5-year-old children and it contributes

to approximately 5 to 10 million cases of mortality in Asia, Africa and Latin America countries. An estimated one billion episodes and 3.3 million deaths occur each year among children under five years of age. Generally, these children experience an average of 2.6 episodes of diarrhoea per child per year (Urio *et al.,* 2001).

Based on the report from health institution in Ethiopia (1989-91) diarrhoeal disease was one of the fifteen top causes of hospitalization and hospital visits in terms of morbidity and mortality. An investigation done by Ethio-Swedish Pediatric Clinic (1968-79), has indicated that diarrhoeal disease was second to respiratory disease in Ethiopia, and have accounted for 81.2% of patients visit to the clinic. In Ethiopia, various studies have invariably concluded diarrhoeal diseases to be major causes of infant and child mortality and morbidity. About 39,000,000 episodes of diarrhoea/year was estimated to occur in Ethiopia out of which 230,000 children below five years of age die (Mirgissa Kaba and Fekadu Ayele, 2000).

In recent years a number of bacteria have been added to the list of recognized etiologic agents causing acute diarrhoeal disease (Greenwood *et al.,* 1987). The etiological agents of infectious diarrhoea are known to be caused by a variety of enteric pathogens including bacterial, viral, and parasitic agents (WHO, 1992). Viral diarrhoea is acute, self-limiting illnesses and is the most common group. From viral caused diarrhoea rotaviruses, appears to be responsible for many of the serious sporadic diarrhoea in young children.

Among diarrhoea caused by a variety of parasites, *Entamoeba histolytica* and *Giardia lamblia* are the most common in developing countries (Black *et al.,* 1984) and currently HIV associated opportunistic parasites such as Cryptosporidia are coming in to the picture.

From the pathogens responsible for acute diarrhoea in developing countries, it is possible to observe that the contribution of Rotavirus is, 15-25 %, entrerotoxigenic *E. coli*, 10-20% *Shigella* species 5-15%, *Salmonella* species, 1-5% *C. jejuni*, 10-15% entropathogenic and *E.coli* accounting for 1-5 % (EHNRI, 2002). Among diarrhoea caused by bacteria in developing countries *E. coli* (EIEC) entroinvassive and enterohemorragic (EHEC), *Salmonella ,Shigella ,V. cholerea, Yersinia entrcolitica and Campylobacter* are the major bacterial pathogens most often responsible for causing pandemic and epidemic infectious diarrhoeal disease in developing countries (Black *et al.,* 1984). Shigellosis is said to be an endemic disease with special concern to developing countries like Ethiopia (Mekonnen Admassu and Abera Geyid, 1992). Ethiopia as a tropical and developing country is frequently subjected to shigellosis. According to Afeworki Gebreyohanes (1989) *S. dysentery* and *S. flexneri* are the most prevalent and accounts for about 80% of the outbreak of shigellosis. Shigellosis is primarily a pediatric disease, which accounts for 5.8% of the first year of life, 2% between the first and 15^{th} year and 15% in persons over 50 yrs of age (James, 1996). Shigellosis is the most common disease among children two to four years of age (Blake *et al.,* 1982*;* John *et al*., 1999). Transmission of the bacteria, that cause diarrhoea occurs through fecal-oral route as a result of direct person-to-person contact (such as hand-to-mouth contact) and exposure to contaminated food, water, and objects (Howard *et al*., 1996). Convalescent

carriers who pass infective stage of diarrhoeal agents after apparent recovery from the disease or asymptomatic or healthy carriers who don't show any sign of the disease serve as a constant source of diarrhoeal disease.

Among the different species of *Shigella, S. dysentrea* serotype I is known for its fatality, and life threatening disease that it causes. It is the most common cause of large-scaled, regional outbreaks of dysentery. *S. dysentrea* are the most important cause of acute bloody diarrhoea (dysentery). Shigellosis is characterized in patients with bloody stools, fever, abdominal cramps, and rectal pain. In almost half of the cases, however, *Shigella* causes acute non-bloody diarrhoea that cannot be distinguished clinically from diarrhoea caused by other enteric pathogens (WHO, 2001a).

Shigellae are generally resistant to most commonly used antibiotic and it is also reported to be a multiple drug resistant. Widespread outbreak of shigellosis due to multiple antibiotics resistant *Shigella* has been documented all over the globe. This pattern of resistance is believed to have originated from the normal flora of the patients intestine, such as *E.coli where* the resistant gene containing plasmid is transferred in the form of conjugation (Mitsuhashi, 1969).

The phenomena of R-factor mediated multiple drug resistance in *shigella* was first noted in Japan in 1950 (Tokumistu *et al.,* 1975). The reports of different studies conducted in Ethiopia also revealed high prevalence rate of antibiotics resistance of *S. flexenri* and *S. dysentrea* (1978-85) (Afeworki Gebreyohans, 1989).

Another type of bacterial caused diarrhoeal diseases is Salmonellosis which is caused by a group of bacteria called *Salmonella*. It is primarily transmitted through ingestion of contaminated food by infected faces from man or animal, through fecal oral route (James, 1996). Active cases of *Salmonella* in man are source of contamination and transmission to other human beings and to lower animals.

Strains of *Salmonella* species with resistance to antimicrobial drugs are now widespread in both developed and developing countries. In developed countries it is now increasingly accepted that for the most part such strains are zoontic in origin and acquire their resistance in the food-animal host before onward transmission to humans through the food chain. For *S. typhi*, multiple drug resistance is now the norm in strains originating in the Indian subcontinent and south–east Asia. In developed countries antimicrobial resistance is zoonotic and has been attributed to the injudicious use of antimicrobials in food-producing animals (John ,2002).

Hence, one way to control and lessen the problem drug resistance is through the use and development of traditional medicinal plants for their possible antibacterial effect. Ethiopia is one of the six countries of the world where medicinal plants with healing potential are said to be indigenous (Mintesnot Ashebir and Mogessie Ashenafi, 1999). Ethiopia where the majority of the population live in rural areas where there is limited access to modern medications and where the cost of drugs are unaffordable the use of self treatment with medicinal plants is the best solution.

2. Literature Review

2.1. Infectious Diarrhoea

Infectious diarrhoea is caused by infectious etiologies, often accompanied by symptoms of nausea, vomiting, or abdominal cramps. It is labeled into three major types, according to World Health Organization (1996 b). The first one is acute watery diarrhoea which is characterized by an increase in the frequency of defecation (three or more times per day or at least 200 g of stool per day) lasting for less than 14 days. The majority of the deaths in this group are due to dehydration. The second is persistent diarrhoea which persists for more than 14 days. It is almost invariably associated with severe malnutrition and affects children, who require intensive medical treatment. It usually arises as secondary follow up of acute diarrhoea caused by infections in the presence of complications such as malnutrition. The third type is, dysentery (mucoid diarrhoea with blood mixed through the stool) which accounts for relatively few cases but has a significant mortality. Up to 20-30% of the cases are associated with severe malnutrition affecting children.

Acute infectious diarrhoeal diseases can be further divided into two main clinical entities: inflammatory diarrhoea and, non inflammatory diarrhoea (Mitchael and Micheal, 1991). Patients with inflammatory diarrhoeal syndrome typically experience tenesmus, fever, abdominal pain, and frequent small-volume stools that may be bloody (dysentery) (Aranda and Gainella, 1999). The presence of these symptoms often helps to clinically differentiate inflammatory diarrhoea from non inflammatory diarrhoea. Fecal leukocytes are seen more commonly in inflammatory diarrhoeal syndromes.

The Food borne Diseases Active Surveillance Network (Food Net) of the Centers for Disease Control and Prevention (CDC) collects data on the incidence of diarrhoea attributable to nine enteropathogens in 13 % of the U.S. population (37.4 million people) living in nine states. Of these, the pathogens responsible for the most cases of diarrhoea in 2002 were *Salmonella* (16.1 cases per 100,000 population), *Campylobacter* (13.4 cases per 100,000 population), *Shigella* (10.3 cases per 100,000 population), *Escherichia coli* O157:H7 (1.7 cases per 100,000 population), and cryptosporidium (1.4 cases per 100,000 population); *Vibrio, Yersinia, Listeria*, and *Cyclospora* were reported in fewer than 1 person per 100,000. Other enteropathogens for which diagnostic testing are readily available includes *Clostridium difficile,* giardia, rotavirus, and *Entamoeba histolytica*. Additional agents of infectious diarrhoea for which clinical diagnostic testing is not routinely available include enterotoxigenic, enteropathogenic, enteroaggregative, and enteroinvasive strains of *E. coli,* toxin-producing *Clostridium perfringens, Staphylococcus aureus, Bacillus cereus,* and rotaviruses (Sood and Pacheco ,2002).

Infectious diarrhoea is one of the principal causes of morbidity and mortality among children in the developing world. An infectious diarrhoeal illness is the second leading cause of mortality and morbidity throughout the world in children less than five years of age (Sood and Pacheco, 2002). Infectious diarrhoea poses a significant economic and societal burden throughout the world both in developing and developed countries such as the United States. This is especially true in developing countries where it's the leading cause of mortality and morbidity in children, where geographic, economic, political and sociocultural factors

interact to create devastating and continuing challenges to its prevention and control program (Carl and William, 1989).

In the early 1980's diarrhoeal disorders were the biggest child killers, responsible for an estimated 4.6 million deaths worldwide every year. Despite widespread use of oral rehydration therapies (ORT) and an increased understanding of the pathogenesis of diarrhoea, 2.5 million children still die from these illnesses every year, almost all of them in developing countries.

2.2. The Genus *Shigella*

2.2.1. General Characteristics and Nomenclature

The genus *Shigella* is named after a Japanese bacteriologist K. Shiga, the first to discoverer the organism. It is found in the family *entrobacteriacea*, and the tribe E*scherichia.* According to the definition of Edward and Ewing (1972) *Shigellae* are Gram negative aerobic nonsporulating nonmotile and with few exceptions, nonproductive gas from fermentable substances. They do not utilize salicin, adonitol, or citrate or liquefy gelatin. Lactose is not fermented except *Shigella sonnei*. *Shigella* is rod shaped of 2.0-6.0 µm in length and 1.1.-1.5 µm in width with round ends.

Based on DNA–DNA hybridization studies the genus *E.coli* and *Shigella* are very closely related and could be combined into one genus. In contrast to the characters described for *E.coli, Shigella* are non motile, doesn't produce gas except *S. flexenri* serotype 6 from glucose and doesn't decarboxylate lysine / hydrolyse argininie, Sodium acetate is used only by certain serovars (Edward and Ewing, 1972).

Shigella survives for 5-46 days in dried linen and kept in dark and for 9-12 days at room temperature. Although *Shigella* perish in stool acidified by growth of coliforms other bacteria will remain alive for days if species are kept alkaline and preserved from drying (Rowe, 1990).

Shigella are facultative anaerobic and chemo-organotrophic having both respiratory and fermentative type of metabolism. They grow on ordinary media containing 1% peptone carbon and nitrogen source. According to the status of lipopolysaccharide of the outer membrane, growth on solid media is characterized by glistering smooth /dry, wrinkled and rough colonies. In pure culture they form circular glistering translucent /slightly opaque colonies on nutrient agar on Nutrient agar (Balows and Sussman, 2000).

They are classified into four species: *S. dysenteria*e (13 serotypes), *S. flexenri* (6 serotypes), *S. boydi*i (18 serotypes), and *S. sonne*i, (1 serotype) which contains only a single serotype. *Shigella* is grouped among enteric pathogens that can cause acute and bacillary dysentery in man and apes (WHO, 2001a). *Shigella* is highly adapted to man, with humans and primates being the only known natural hosts (Tropical Medicinal Centre, 2000). The proportion of each subgroup varies from country to country, though dysentreal outbreak in developing countries is usually caused by *S. dysenteriae* type I, a highly virulent pathogen. The hallmark of infection with *Shigella* is diarrhoea with blood, often termed “dysentery.”

The clinical syndrome of shigellosis is characterized by frequent, but small volume, loose stools, consisting largely of blood and mucus, fever, pain, and tenesmus, which are frequently present. Unlike secretory diarrhoea these syndromes are the result of invasion of the distal small bowel and/or colon by bacteria. Because dehydration is not as severe as in secretory diarrhoea, oral rehydration therapy does not significantly reduce the case-fatality rates for shigellosis (Bennish , 1991).

In shigellosis, bacterial invasion of colonic epithelial cells is one of the most significant events responsible for the clinical manifestation of the disease (Anthony, 1991). The invasive process encompasses complex features that include penetration into epithelial cells, intracellular multiplication, and spreading to adjacent cells and to the connective tissue of intestinal villi (Anthony, 1991). These events lead to a strong inflammatory reaction, which causes abscesses and ulcerations of the colon. Although severe, bacillary dysentrea is limited to the mucosal surface and does not spread significantly from the lamina propria to the sub mucosa (Butler *et al.*, 1986). The intensity of the inflammatory reaction may prevent systemic dissemination of the pathogen, thus accounting for the low frequency of bacteremia.

2.2.2. The Epidemiology of *Shigella*

According to Kotloff (1999), the average annual number of *Shigella* episodes throughout the world between 1966-97 was estimated to be 164.7 million, of which 163.2 million were in developing countries and 1.5 million in industrialized countries. The primary victims, being children under 5 years of age where by 69% of all episodes and 61% of all deaths were related to shigellosis.

In areas of the developing world, shigellosis is predominantly a pediatric disease, with the urban poor being the hardest hit (Cobra and Sack, 1996). In developed countries it occurs more commonly where there are overcrowding and poor sanitation (e.g. in institutionalized individuals, children in day-care centers, prisoners, military recruits and residents of Native American reservations).

In some regions, disease rates increase somewhat during the monsoon season, perhaps due to increased faecal contamination of drinking-water (Tropical Medicinal Centre, 2000). The four species of *Shigella* have different geographical distribution and the reasons for this are still unclear. *S. flexneri* is the most commonly isolated species in the developing world and the most frequent cause of morbidity and mortality. *S. dysenteriae* infections also occur in less developed countries, often in epidemics, with periodic pandemic outbreaks. In industrialized countries the predominant species is *S. sonnei*. In general, the illness caused by *S. sonnei* is less severe, but individual cases of infection with any of the *Shigella* species can be severe. Infection with *Shigella* protects against subsequent infection with the same serotype. However, because there are multiple serotypes, individuals may become infected several times. Worldwide, the incidence of shigellosis is highest among children 1 to 4 years old (WHO, 1996a). The predominant mode of transmission is via faecal-oral contamination, and the low infectious inoculum (bacterial load) (as few as 10 organisms) renders *Shigellae* highly contagious (Cobra *et al.,* 1996). The most important single reservoir of infection caused by *Shigella* is the human carrier, either convalescence or with an inapparent infection. The spread of the disease is due to the more or less direct transfer of the specific *Shigella* strain from infected intestinal discharges of the alimentary tract of another individual asymptomatic carriers with diarrhoea are primarily responsible for transmission (Carl and Wiliiams, 1989).

Polluted water and contaminated food can be the medium of transmission of shigellosis; however, the organism can generally survive poorly in the environment (John *et al.,* 1999). In certain settings where disposal of human faces is inadequate, houseflies can serve as a

mechanical vector for transmission (Levine *et al.,* 1991). In *S. dysentrea*, human to human contact is also associated with the epidemiology of the disease (WHO, 2001a). Excretion of *Shigella* in stool is highest during the acute phase of dysenteric illness. During this phase the environment is contaminated and the organism can survive for weeks in cool and humid conditions.

Epidemics of shigellosis are more common during the rainy season and shortly thereafter. During hot dry summers, epidemics are rare. In the rainy season in the tropics, people crowd together indoors and are more susceptible to chills. The rains inhibit people from defecating at a safe distance from their village and the damp soil allows the bacilli to flourish (Tropical Medicine Centre Resource, 2000). Under these conditions, shigellosis generally spreads rapidly. Excretion of *Shigella* in stool is highest during the acute phase of dysenteric illness. During this phase the environment would be contaminated and the organism can survive for weeks in cool and humid conditions.

In many tropical and subtropical areas devoid of modern sanitation, flies are effective in spreading infection as do contaminated fingers, utensils, water and food. The abundance of *Mollusca domestica sorbeus* that feeds in human faces is so much linked with poor sanitation and known to be the major vector of transmission. Dysentery in tropical parts of the world is most prevalent during peak fly breading season and bacteriologic studies of flies indicate their potential role in the disease transmission.

Recently carriers of shigellosis were found to be common in developed countries that have adopted an "alternative way of life style" and practiced orogenitial and oral anal sexual contact (Bovee *et al*., 2003). In this case *Shigella* has been most common in young people who have adopted this "alternative style of life". Many studies have been done regarding the prevalence of *S. sonnei* by orogenetial sex, but there is also the possibility of transmission of *other Shigella* species by orogential method of intercourse.

Regarding that a concentration of 10^6 -10^{10} organisms /gram of stool and a concentration of 10-10^2 organism /gram can cause up the disease, makes fecal contamination extremely dangerous especially for children. For example *S. dysentrea* serotype I showed that only as few as 10 organisms have produced the disease in human volunteers (Cobra and Sack, 1996).

Shigella flexneri and *S. sonnei* subgroups are the most common causes of bacillary dysentery in the United States, England, Europe, Egypt, and the Orient while *Shigella boydii* is found in India and Egypt. (Tropical Medicine Centre Resource, 2000) A microbiological survey in areas where diarrhoeal cases *S. flexeneri* 2a is usually the prevalent species and serotype in these areas. *S. boydii* is mainly prevalent in South Asia and Middle East. In Europe and North America, *S. sonnei* is by far predominant followed by *S. flexenri.*

2.2.3. Virulence factors and Pathogenesis of *Shigella*

Shigellosis is initiated by ingestion of the organism usually via fecal-oral contamination. An early symptom, of, diarrhoea (possibly elicited by enterotoxins and or cytotoxin), may occur

as the organisms pass through the small intestine (http//:www.edu/microbook/ch022.htm). The hallmarks of shigellosis are bacterial invasion of the colonic epithelium and inflammatory colitis. These are interdependent processes amplified by local release of cytokines and by the infiltration of inflammatory elements. Colitis in the recto-sigmoid mucosal, with concomitant malabsorption, results in the characteristic sign of bacillary dysentery, unformed stools tinged with blood and mucus.

Shigellosis can be characterized as an acute inflammatory bowel disease initiated by the uptake of only a few $\geq$ 10 cells depending on the immunity of the host. *Shigella* infection is initiated at the membranous (M) cells that are associated with macroscopic lymphoid follicles (Peyer's patches). During the early stages of infection, bacteria are transcytosed through the M cells into the subepithelial space. In the subepithelial space, the organisms are phagocytosed by resident macrophages. However, virulent *shigellae* are not killed and digested in the macrophage phagolysome. The bacteria lyse the phagosome and initiate apoptosis (programmed cell death). During this process, the infected macrophage releases the inflammatory cytokine IL-1, which elicits infiltration of PMN ingested by membranous (M) cells that are associated with lymphoid microfollicles in the colon. Eventually the infected macrophages undergo apoptosis (programmed cell death), and the bacteria are released onto the basolateral surface of adjacent colonic enterocytes. In addition, PMN transmigration through the epithelium disrupts tight junctions, allowing *Shigellae* to migrate into the subepithelial space. The bacteria infect enterocytes by induced endocytosis, and the endocytic vacuoles are subsequently degraded. (Thomas and Gerald, 2000).

The fundamental virulence characterstic of *Shigella* is its ability to invade epithelial cells and subsequently multiply therein (Plothin and Waldman, 1979). The multiplication of *Shigella* in intestinal mucosal ,stimulates infiltration of polymorph nuclear leucocytes into the areas of infected bowel , which result in ulcer through which red blood cells and plasma proteins leak into the bowel lumen (*http://gsbs.utmb.edu/microbook/ch022.htm*).

Several features of *Shigella* contribute to its invasiveness and Pathogencity. The bacteria is able to invade enterocytes in colonic and rectal epithelia and to lyse intracellular phagocytic vacuoles, thus escaping into the cytoplasm where they multiply and then invade adjacent cells. These virulence determinants are encoded by large extra-chromosomal elements (plasmids) that are functionally identical in all *Shigella* species.

Experiments with, an invasive isolate of *S. flexneri* serotype 5, have indicated that it's the virulence plasmid pWR100 which participates at every essential step of the invasive process in the HeLa cell model, including entry into cells, rapid intracellular multiplication, and early killing of host cells (Clerc *et al.,* 1987).

2.2.3.1. Role of the Virulence Plasmid

A noninvasive variant of *S. sonnei* isolates upon subculture led to the idea that plasmid-borne genes were responsible for the invasive phenotype (Anthony, 1991). A 180-kilobase (kb) plasmid present in invasive Form I strains was absent from noninvasive Form II strains (Kopecko *et al.,* 1980). This plasmid encodes the form somatic antigen and when

reintroduced into form II the bacterium restores both the invasive phenotype and the somatic specificity (Kopecko *et al.*, 1980).

The major role of the virulence plasmid is in controlling the invasion of eukaryotic cells is emphasized by the results of experiments in which the TnS-labeled virulence plasmid pWR110 of *S. flexneri* serotype 5 was mobilized into *E. coli* K-12. Transconjugants carrying pWR110 expressed high invasive potential for HeLa cells (Sansonetti *et al.,* 1986). Plasmid genes are also involved in early killing of host cells. Studies have shown that the intracellular fate of both an invasive strain and a noninvasive, plasmidless derivative of *S. flexneri,* plasmid pWR100 appeared to mediate killing of host cells (Anthony, 1991).

2.2.3.2. Role of Lipopolysaccharide in *Shigella* Virulence

The lipopolysaccharide (LPS) layer of Gram-negative bacteria is an important component of the cell surface and contributes to the virulence of many pathogens by providing resistance to certain host defenses such as serum killing and phagocytosis (Okamura *et al.,* 1983). The possession of a smooth LPS alone would not appear to be a sufficient for virulence. But also possible role of the chemical composition of the particular *Shigella* somatic antigen is implicated. Lipopolysaccharide also serves as bacterial endotoxin.

The pathogenesis of *Shigella* is also attributed to the possession of the potent endotoxin, which is associated with Lipopolysaccharide. *S. dysentery* in addition to this it contains theromolabile toxin-the *shiga* toxin, which is known to cause paralysis, diarrhoea and death, when injected to rabbits, mice, or guinea pigs and hemolytic uremic syndrome in human beings.

The enteric pathogen *S. dysenteriae* serotype I and Shiga toxin-producing *Escherichia coli* (STEC) cause bloody diarrhoeal diseases that may progress to life-threatening extraintestinal complications (Rama *et al.,*2003). Shiga toxin is composed of two subunits (subunit A and B). Subunit B(32-kd) possesses the main biological activities which is responsible for binding to cell-surface receptors. It has three main biological activities: enterotoxicity, neurotoxicity and cytotoxicity.

2.2.4. Drug Resistance in *Shigella*

The severity of symptoms and the length of time the stool contains *Shigella* can be reduced with antibiotics. Antibiotics are the mainstay of therapy of all cases of shigellosis. Yet, from the advent of shigellosis chemotherapy started in the 1950 with sulpha drugs being mainly used up to now the chemotherapeutical medication of shigellosis is a discouraging history due to the continual development of drug resistance. In the early 1940's *Shigella* acquired resistance to sulfa drugs, in 1950's to tetracycline, and chloramphinicol while in the 1970's to Ampicilin and in the 1990's to Trimethoprim and Sulfamethoxazole. In this era of the 21th century *Shigella* is becoming resistant to the latest antibiotics such as Nalidixic acid and other floroquinolones (Candian Communicable Disease Report ,CCDR, 1997).

For the first time in 1940 in Japan a multiple drug, resistant *S. dysentrea* has been investigated (Swapan et *al.,* 2003). By the end of 1967, in Japan about 80% of *Shigella* strains have developed resistance to two or more drugs. For the first time in 1968-1970 *S. dysentrea* type I showed multiple drug resistance in Central America to (TCSsU) (WHO, 2001a). *Shigellae* are generally resistant to most commonly used antibiotic and a number of strains have been reported to be a multiple drug resistant. Widespread outbreak of shigellosis due to multiple antibiotic resistant *Shigella* to Streptomycin, Ampicillin, tetracycline, chloramphinicol has been documented all over the world (Mache *et al.,* 2001).

Recent (1984 to 1992) trends in the antimicrobial resistance of *Shigella* isolates in Israel has increased from 59 to 92% to trimethoprim-sulfamethoxazole resistance to (TMP-SMX) and that to ampicillin increased from 13 to 86% (Ashkenazi, *et al.,* 1995). In Ethiopia, in 1988 , investigation have been carried for clinical isolates of *Shigella* and it was found that all or most of the strains were susceptible to Cephalothin, Gentamicin, kanamycin, Polymyxin and Trimethoprim-Sulphamethoxazole. Frequencies of susceptibility to Ampicillin, Carbenicillin and Chloramphenicol were, respectively, 79, 80 and 75% (Gedebou and Tassew, 1988).

2.3. The Genus *Salmonella*

2.3.1. General Characteristics and Nomenclature

The genus *Salmonella* are Gram-negative motile, rod-shaped bacteria found in the class Schizomycetes, order eubacteriales family Enterobacteriaceae and tribe Salmonellae (Robert *et al.,* 1957). They are discovered for the first time by Daniel E.Salmon in 1880 from porcine intestine. *Salmonellae* are common in the gastrointestinal tracts of mammals, reptiles, birds, and insect (http://www.emedicine.com/specialties.htm).

The genus *Salmonella* is composed of motile bacteria that conform to the definition of the family Entrobacteriacea and the tribe Salmonelleae. Urease is not produced, sodium malonate is not utilized, gelatin is not liquefied, and growth does not occur in medium containing potassium cyanide. Lysine, arginine, and ornithine are decarboxylated. Acid is produced in Jordan's tartarate medium. Dulictol is fermented and inositol is utilized by numerous strains. Sucrose, salicin, raffinose, and lactose are not fermented (Edward and Ewing, 1972).

The first member of this group to be studied was typhoid bacillus, originally observed in human tissue by Eberth in 1880 and cultured by Gaffky in 1884 (Burrows, 1959, sited in Heran *and* Virginia*,* 1999) Soon after Salmon and Smith reported an organism (*Bacillus choleraesuis*) isolated from diseased pigs in 1885. The name *Salmonella* is in memory of Salmon and his early work on these organisms.

The sereotypic identification and classification of *Salmonella* is based on the antigen present on cell surface (somatic antigen), which contain lipopolysaccharide, flagellar antigen. The flagella are composed of monomeric protein (flagellin) which consists of flagellar antigen.

The nomenclature for the genus *Salmonella* has evolved from the initial concept of one serotype-one species proposed by Kauffmann on the basis of the serologic identification of O (somatic) and H (flagellar) antigens. Each serotype was considered a separate species (for example, *S. paratyphi* A, *S. newport,* and *S. enteritidis*) this concept, if used today, would result in 2,463 species of *Salmonella* (Popoff *et al.,* 2000). It is generally accepted now that there is only a single species of *Salmonella* (*S. enterica*) rather than over 2,000 named serovars to *S. enetridis* species. According to the new nomenclature all *Salmonellae* that cause enteric fever in humans are grouped and named as *Salmonella enterica* and the previous species names are assigned as serotypes e.g., *Salmonella enterica* serotype Typhi, Paratyphi A, B, C, etc (Brenner *et al.*,2000). A proposal has been made to redefine the genus to consist of only one species. *S. enterica*. The one species concept would recognize the following subspecies based on DNA-DNA hybrid property.

S. enterica subsp. enterica
salamae
arizonae
diarizonae
houtenae
bongorii
indica

Biochemically, the genus *Salmonella* is characterized by the following characters. *Salmonella* are known for the production of hydrogen sulphide, although group A *Salmonella* is not able to produce it. Lactose is not fermented in all species, urease is negative, and motility is positive. The other unique biochemical characters that are exhibited by some species of this genus are:

- *S. gallinarum* and *S. Pullorum* cause "fowl typhoid" and "pullorum disease", respectively, in poultry. These serovars are unlike typical *salmonellae* in being non-motile.
- *S. typhi* causes "typhoid fever" in humans; this is the classic "enteric fever." Gas is not produced during glucose fermentation.
- *S. Paratyphi* A and *S. Paratyphi* B cause milder forms of enteric fever in humans. Most strains of *S. paratyphi* A do not produce hydrogen sulfide.

The common occurrences of *Salmonella* in a wide range of hosts and its pathogenesis activity in both human and other animal species have made *Salmonella* a typical zoonotic micro-organism. According to Sahilu Ayalew (1983) salmonellosis can be divided into three major groups depending on their host specificity and pathogenicity.

1. Pathogenic to man–producing typhoid and paratyphoid disease which include *Salmonella typhi* ,*Salmonella paratyphi* A,B,C and *S. sendas*. More than 90 % of culture comes from man, domestic animals also act as a reservoir.

2. *Salmonella* pathogenic for animal including *S. abortosquin* which causes abortion in horses, *S. abortsuis* cause abortion in sheep.

3. *Salmonella*, which are pathogenic for animals which include *S. chloresuisi* swine pathogen, *S. pullorn* and *S. gallinarum* pathogens of birds.

Salmonella species are not fastidious organisms requiring the supplement of nutrients. It can grow on common minimal nutrient media. As a human pathogen it requires optimum temperature of $\pm$ 37 ^{0} C with aerobic to facultative anaerobic conditions. *Salmonella* as a contaminant can be isolated from foods such as eggs and animal by products.

Salmonella serotypes are associated with three distinct human disease syndromes, bacteremia, typhoid fever, and enterocolitis. Among these bacteremia, a syndrome caused by the porcine-adapted *S. enterica* serotype Choleraesuis and the bovine-adapted *S. enterica* serotype dublin, is encountered least frequently in humans (Fang and Fierrer, 1991).

Under-reporting of cases and the presence of other diseases considered to be of high priority may have overshadowed the problem of salmonellosis in some developing countries including Ethiopia (Bayleyegn Molla *et al.,* 2003). The increased global population coupled with mass production of animal and human food and the rapid international trade in agriculture, aquaculture and food products has worsened the problem (Bayleyegn Molla *et al.,* 2003).

2.3.2. Epidemiology of *Salmonella*

For epidemiologic purposes *Salmonella* can be classified into three groups (WHO, 1988 sited in James, 1996):

1. Those that infect human only: These include *S. typhi, S. paratyphi* A, and *S. Paratyphi* C. This group includes the agents of typhoid and the paratyphoid fevers.

2. Those host adapted serovars (some of which are human pathogens and may be contracted from foods): these are *S. gallinarum* (poultry*), S. dublin* (cattle), *S. abortus-equi* (horses), *S. abortus-ovis* (sheep), and *S. choleraesuis* (swine).

3. Unadpeted serovars (no host reference). These are pathogenic for human and other animals, and they include most food borne serovars.

Both Non-typhoidal and Typhoid fever salmonellosis are a worldwide disease of humans. Contaminated food is the major mode of transmission for non-typhoidal *Salmonellae* because salmonellosis is a zoonotic and has an enormous animal reservoir. *Salmonella* that cause other enteric fevers spread mainly from person-to-person via the fecal-oral route (Ralph, 2000). The prominent epidemiologic Typhoid fever is a public health problem of which there are an estimated 33 million cases and a resulting 500 000 deaths each year worldwide (Levine, 1999). The disease is endemic in many developing countries of the Indian subcontinent, South and Central America, and Africa. Outbreaks of typhoid fever have also occurred in Eastern Europe.

The incidence of salmonellosis in the United States is greatest among children younger than 5 years (61.8 per 100,000 people), with a peak among those younger than 1 year. Infants and people older than 60 years are most susceptible and tend to have more severe infections (Zapor and Dolley, 2003).

In Ethiopia, from a total of 412 *Salmonella* isolates, 25 different serotypes were identified from slaughtered cattle (4.2%), camels (16.2%), slaughterhouse personnel (6.0%), minced beef (12.1%), chicken meat and giblets (23.6%). The predominant serovars were *S. braenderup*, *S. dublin* and *S. saintpaul* followed by *S. typhimurium* (including var. Copenhagen) and *S. anatum, Salmonella enteritidis* was detected from chicken, cattle and camel meat (Bayelegn Molla, 2003) .

2.3.3. Virulence Factors and Pathogenesis of *Salmonella*

A large inoculum is thought to be necessary to overcome stomach acidity and to compete with normal intestinal flora. After ingestion, *Salmonella* colonize the ileum and colon, invade the intestinal epithelium, and proliferate within the epithelium and lymphoid follicles. Invasion of the epithelium involves an initial binding to specific receptors on the epithelial cell surface (Ralph, 2000). After invading the epithelium, the organism multiply intracellular and then spread to mesenteric lymph nodes and throughout the body via the systemic circulation where they are taken up by the reticuloendothelial cells. *Salmonella* is able to survive and multiply within the mononuclear phagocytic cells of the lymphoid follicles, liver, and spleen (House *et al.,* 2001).

After invading the intestine, *Salmonellae* induce an acute inflammatory response, which can cause ulceration of the epithelial cells to synthesize and release various proinflammatory cytokines which evokes an acute inflammatory response (Ralph, 2000).

In addition the pathogenesis is also attributed to the possession of one or more enterotoxin-like substances which may stimulate intestinal secretion. However, the precise role of these toxins in the pathogenesis of *Salmonella* enterocolitis and diarrhoea has not been established (Chopra *et al.,* 1994).

2.3.3.1. Pathogencity Islands: These often contain multiple functionally related genes necessary for a specific virulence phenotype, suggesting that acquisition of a Pathognicity island during evolution (Groisman and Ochman, 1996). Pathognicity islands, encode specialized devices for the delivery of virulence proteins into host cells. *Salmonella* pathogenicity island-1 (SPI-1) encodes genes necessary for invasion of intestinal epithelial cells and induction of intestinal secretory and inflammatory responses (Watson and Paulin, 1995). In contrast, *Salmonella* pathogenicity island 2 (SPI-2) encodes genes essential for intracellular replication, and, in the mouse enteric fever model, is necessary for establishment of systemic infection beyond the intestinal epithelium (Cirillo *et al.,*1998) including SPI-1 and SPI-2 which, encode specialized devices for the delivery of virulence proteins into host cells, termed type III secretion systems (TTSSs). TTSSs are specialized virulence devices that have evolved to modify host-cell function through the direct translocation of bacterial virulence proteins into the host-cell cytoplasm (Hueck, 1998). These translocated bacterial proteins alter such basic host-cell functions such as signal transduction, cytoskeleton architecture, membrane trafficking, and cytokine gene expression. The delivery of bacterial

proteins into the host cell clearly represents a complex task, as translocated proteins must cross the bacterial inner and outer membrane as well as the host-cell plasma membrane.

Salmonella serotypes that can cause up salmonellosis in general, should be able to survive and must also be able to replicate within the host macrophage to establish systemic infection (Fields *et al.,* 1986). Among the virulence factors which is important for the survival of *Salmonella* that is also necessary for the establishment and to produce systemic infection in the macrophages is the TTSS (Uchiya *et al*., 1999). TTSS is located in *Salmonella* pathogenicity island II which activates within the phagosome and translocates bacterial effector proteins from the phagosome into the macrophage cytosol (Uchiya *et al*., 1999). Effector proteins are also other virulence factors involved in epithelial cell invasion and induction of enteritis (Behalu and Miller, 1998).

2.3.4. Drug Resistance in *Salmonella*

Sporadic cases of salmonellosis due to drug resistant *S. typhimurium* have been recorded worldwide to different antibiotics. Since 1968, plasmid-bearing, multidrug-resistant strains have emerged in various parts of the world (Threfall *et al.,* 1986).

Since 1990s up to now *Salmonella typhimurium* definitive phage type (DT) 104, is of particular importance displaying resistance to up to six commonly used antimicrobials (sylvia *et al*.,2004). *S.enteritidis* strains are also reported to be resistant to 12 different antimicrobials which have been obtained from different sources. The highest levels of

resistance were found for sulphonamides, 100% of poultry-related samples, 90.9% of isolates from broiler carcasses, 88.2% of human samples and 41.9% of food samples. High resistance was also found to nitrofurantoin (52.8%) (Sylvia *et al.,* 2004).

Since the introduction in 1948, Chloramphinicol has been the treatment of choice for typhoid fever. Although there were sporadic reports of resistance, the effectiveness of chloramphinicol remained satisfactory until 1989, when there was rapid emergence and spread of multi drug-resistant (MDR) *Salmonella enterica* serovar Typhi (resistant to ampicillin, Chloramphinicol, and trimethoprim-sulfamethoxazole) in several parts of India (Mandal and Mandal,2004). Alternative drugs for treatment are now required by the emergence of multidrug-resistant (MDR) *Salmonella enterica* serovar Typhi resistant to Ampicillin, Chloramphinicol, and Cotrimixazole (Talawadekar *et al.,*1989).

Among the, currently proven effective antibiotics for the treatment of typhoid fever caused by MDR strain, fluoroquinolones is the major and have become the drugs for the first line of treatment of typhoid fever. Recently there are also reports where by *Salmonella typhi and Salmonella paratyphi* A species are becoming increasingly resistant to fluoroquinolone from patients in the Indian subcontinent and South–East Asia. Quinolone and fluoroquinolone resistances in *Salmonella enterica* have mainly been due to single point mutations in *gyrA and o*ther mechanisms have also been proposed, e.g., mutations in the *parC* gene and decreased uptake of antimicrobial agents (Heddle *et al.,*2000).

2.4. Introduction to Medicinal Plants

Herbal medicine which is often referred to as herbalism or Botanical Medicine is the use of herbs for their therapeutic or medicinal value. A herb is a plant or plant part valued for its medicinal, aromatic or savory qualities (Ernest, 1999). Herbal plants produce and contain a variety of chemical substances that act upon the body. Herbal medicine is the oldest form of healthcare known system to mankind. The use of plants as medicines dates back before the written human history. Almost all countries in the world have an expertise concerned with the therapeutic properties of the local flora (Houghton, 1995).

Many drugs commonly used today are of herbal origin. About 25 percent of the prescriptions of drugs dispensed in the United States contain at least one active ingredient derived from plant material. Some are made from plant extracts; others are synthesized to mimic a natural plant compound. According to WHO (2001b) report 119 plant-derived pharmaceutical medicines, where by about 74% are used in modern medicine in many ways are correlated directly with their traditional uses as plant medicines by native cultures.

Plant products have played an important role in the discovery of new therapeutic agents since ancient times, e.g., quinine obtained from Cinchona has been successfully used to treat malaria (Sujata *et al.,*2004). In recent years pharmaceutical companies are spending a lot of time and money in developing natural products extracted from plants. Trials to produce more cost effective remedies that are affordable to the public are going on in different parts of the world.

2.4.1. Short Description of the Three Medicinal plants

2.4.1.1. *Myrica salisfolia,* Shinet (Amharic)

The genus Myrica is found in the family of Myretaceae. It contains more than 150 species, which are indigenous to tropical South America and the West Indies. The major species includes *saliscifoliauniflorus, salisfolia and sphaerocarpa.* It is also known as *Pedra* hume caá, in Brazi and Baybery in Europe (http://www.rain-tree.com/plantimages.htm).

Figure 1. *Myrica salisfolia*

Myrica salisfolia is a medium-sized shrub that grows in drier regions of the world. It has small, green leaves and large, orange-red flowers. *Myrica salisfolia* is a tall, branching, shrub or tree, attaining a height between 0.3 and 6 meters with a bark light grey to brown, and in some species often covered with lichen and moss (Sebsbe Demissew ,2003). Bayberry is dioecious, flowers appearing in springtime before the leaves have fully expanded. On the male plants, the flowers grow from the branches between the leaves in oblong, cylindrical

catkins. The female flowers are much smaller, with an ovate-shaped ovary and 2 filiform styles. In general *Myrica salisfolia* is characterized by a distinctively spicy aroma (http://www.rain-tree.com/plantimages.htm.). It prefers dry forests and sandy areas, and is often found in open fields reclaiming abandoned areas, and thus is considered by some to be an invasive weed. The major plant parts used for medicinal purpose are the Bark and leaves.

Historical Background: *Myrica salisfolia* has a long history of use in North America. Apart from its medicinal uses the mature *Myrica salisfolia* fruit was used as a source of wax, often for making candles, as its names 'Waxberry' or 'Candleberry'(http://www.rain-tree.com/plantimages.htm).

Myrica salisfolia contains Beta-amyrin, catechin, desman thin, gallic acid, ginkgoic acid, guaijaverin, mearnsitrin, myrciacitrin I-V, myrciaphenone A, myrciaphenone B, myricitrin, quercitrin(http://www.rain-tree.com/plantimages.htm).

USES :- The Indications and uses of Bayberry includes for the treatment of fever, respiratory, gastrointestinal tract, diarrhoea, dysentery, and other uses (http://www.rain-tree.com/plantimage.html). In northwest Amazon the Taiwanos tribe considers the leaves to be astringent and use it for persistent diarrhoea. The bark is employed as a remedy against gonorrhea and edema. Growth of *Pseudomonas aeroginosa*, *Salmonella typhi* and *Shigella boydii* has been inhibited by the alcoholic extract of the stem bark (Chhabara *et al*., 1990 sited in Dawit Abebe *et al.,* 2004).

2.4.1.2. *Gardinea lutea* Fres. (Gambilo Amharic)

Gardenia species belong to the Rubiaceae family commonly known as, the fourth largest family of flowering plants, with approximately 650 genera and 13,000 species (Robbrecht, 1988). The genus *Gardenia* contains more than 200 species native to tropical and subtropical Africa and Asia.

Description: It is a shrub or small tree up to 7m high, leaves are simple and fruits are with persistent calyx. *Gardenia* generally grows as a shrub or tree and has white and yellow tubular flowers, evergreen leaves and large, berry-like fruits often containing a sticky, orange pulp (Puff, 2003). Many species of the genus are used as an ornamental in tropical and sub-tropical countries and raw material in the perfume industry and others, like *G. latifolia* and *G. gummifera*, are used for manufacturing small handicraft products and light furniture (Ambasta, 1986).

It's also indicated that it's used for treating Syphilis; malaria. A pedunclum with bacterial has been isolated from *Gardinea* species. Although many *Gardenia* species are known for their medicinal properties and traditional applications (Ambasta, 1986). *G. jasminoides* is probably the most widely used. The fruit is used in Chinese medicine as a treatment for a wide range of diseases, including hepatitis, colds, fevers, insomnia, conjunctivitis, mouth ulcer, toothache, constipation, pulmonary and renal conditions (Ambasta,1986).

Figure 2. Gardinea lutea

2.4.1.3. *O. europaea* L. *subsp cuspidate* (Wall. ex. G.Don)

Olea europaea subsp. *cuspidate* is native to the Mediterranean region, widely cultivated throughout the world. *Olea* is a genus of about 20 tropical and subtropical species of the Mediterranean region, Africa, southern and eastern Asia, Malesia, eastern Australia, and New Caledonia (Wagner et al. 1999). The name is derived from *elaia*, the greek name for *Olea europaea*. It is distinguished by simple leaves with silver, green or brown scaly undersurface curved leaf tip fruit to 1 cm long, purple-black and succulent when ripe. It is an evergreen tree the leaves are opposite with simple entire (outside the flora area sometimes toothed) usually leathery inflorescent terminal or axillary, cymose, paniculate or dichasial(Dawit *et al.,* 2004). The flowers are usually bisexual petioles 0.5 – 1.5 cm long. Ellipsoid 5-7 mm long endocarp dry dark purple black when ripe.

Figure 3. *Olea europea* subsp. *cuspidata*

2.4.1.4. Objectives of the study

This study is initiated to investigate the antimicrobial potential effect of the above mentioned medicinal plants on diarrhogenic clinical strains of *Salmonella* and *Shigella.*

A. General objectives

The purpose of this study is to evaluate the in vitro antibacterial activity of the potential effect of the three medicinal plant (*Myrica salisfolia*, *Olea europaea* subsp. *cuspidata* and *Gardinea lutea*) extracts against the clinical isolates of *Shigella* and *Salmonella* and a standard organism *(E.coli* ATCC 25922).

B. Specific objectives

1) To clinically diagnose, collect and screen clinical isolates of *Salmonella* and *Shigella* from children with diarrhoea up to 14 yrs of age.

2) To point out the semi-purified plant fractionates which have the potential for anti *Salmonella* and/or anti *Shigella* activity, and use it as a basis for further investigations.

3) To compare the MIC of the semi-purified plant fractionates against the crude extract and the commonly used modern drugs on clinical isolates of *Shigella* and *Salmonella*.

3. Materials and Methods

3.1. Site of Sample Collection and Approach of Sampling

Collection of stool specimen have been done at the Departments of Pediatrics and Health Services at Black lion Specialized Referral Hospital (both at the Laboratory of pediatrics and Diarrhoeal Unit). It has been sufficient to get the target number and type of clinical isolates from this hospital, which is suitable as patients are referred to it which is received from different parts of the Capital city and even from different regions of the country.

The study subjects were both male and female patients in the range of age between 0-14 yrs, visiting the hospital because of having infectious diarrhoea. Background information regarding the health status and other related questions, which were useful for the study were asked and collected in a form of questionnaire either directly from the patients or their parents or guardians of the children.

3.2. Method of Specimen collection and Transportation

Fresh stool specimens were collected using cotton swabs from patients with diarrhoea clinically suspected of having shigellosis and salmonellosis. The swabs were then placed immediately into screw capped transport media (Cary Blair transport media) and then transported immediately to ENHRI Bacteriological Laboratory for further isolation and characterization of the causative agents. A total of 265 stool specimens have been collected for the purpose of isolation and identification.

3.3. Isolation and Characterization of Target Clinical Isolates of *Salmonella* and *Shigella*

Rectal (Fecal) swab specimens have been inoculated in two ways. The first was by inoculating Selenite-F broth, thereby enriching it, followed by incubation of the Selenite-F enrichment broth, for 16-24hrs after which, a loopful of bacteria have been streaked onto XLD (Oxoid) agar plates and incubated at 37 °C for 24h. The second one was by directly inoculating onto XLD (Oxoid) and DCA (Oxoid) agar plates. In all cases further purification, was made on the more selective media of Xylose lysine Deoxycholate (XLD) (Oxoid) for both clinical isolates while, only SS agar media was used for sub culturing *Salmonella.* Before the biochemical test, pure colonies have been sub cultured onto MaCkoncey. All Plates have been incubated at 37 °C for 24 hr aerobically. The plates were examined for the presence of *Shigella* or *Salmonella* colonies after 24 hours. After 18-24 hours of incubation *Shigella* appears as colorless to slightly pink colonies on MAC(MaCkoncey), SS (Salmonella-Shigella agar), and DCA (Desoxycolate Citrate Agar), while *Salmonella* appears as colorless colonies with black centre due to the production of hydrogen sulfide in SS , DCA and XLD, and in most of the cases with a unique type of odor. *S. sonnei* strains may form pink colonies because of the late lactose fermentation properties. Colonies which were positive were stocked at -70 ^{0}C for further future work using TSY mixed with glycerol.

3.4. Biochemical tests (Identifications)

To obtain the true picture of biochemical tests, pure colonies have been used as far as possible by sub culturing to maximize the process of identification. Carefully selected two to

three colonies were picked and inoculated into nutrient broth. A Loop full of inoculum had been taken from nutrient broth culture and used to inoculate the following biochemical test tubes.

- **Indole Test: -** The indole test is used to identify bacteria capable of producing indole using the enzyme tryptophanase. The enzyme tryptophanase can convert the amino acid, tryptophan, to indole, ammonia, and pyruvic acid. The by-product, indole, is the metabolite identified by this test. When Kovac's reagent, which contains hydrochloric acid and dimethylaminobenzaldehyde and amyl alcohol, a red layer will from when indole is present. No color in this layer is a negative result.

- **Kligler Iron slant agar**: - both the butt and the slant were streaked, to determine fermentation of glucose, lactose and to see the production of hydrogen sulfide. When incubating care had been taken not to tighten the screw cap, before placing the inoculated tubes in the incubator. *Shigella* and *salmonella species* characteristically produce an alkaline (red) slant and an acid (yellow) butt, little or no gas, and with no or with Hydrogen sulfide, respectively.

- **Urea slant agar: -** Only the slant was streaked to determine urea hydrolysis. Urea medium screens out urease-producing organisms (*e.g.*, *Klebsiella* and *Proteus*). Urea agar was inoculated heavily over the entire surface of the slant. Urease-positive cultures produced an alkaline reaction in the medium, evidenced by pinkish-red color formation. Urease-negative organisms did not change the color of the medium, which is a pale yellowish-pink. *Shigella* is always urease-negative.

- **Mannitol broth: -** Was inoculated to determine fermentation of mannitol. In case of *S.dysentery* most serotypes did not ferment it while the rest of *Shigella* fermented mannitol (Edward and Ewing, 1972).

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- **Motility test:** - To determine weather the organism is motile or not it was stabbed with a straight inoculating needle, making a single stab about 1–2 cm down into the medium. Motility was indicated by the presence of diffuse growth (appearing as clouding of the medium) away from the line of inoculation. All the biochemical test tubes were then incubated 24-48 at 35°–37°C.

- **Simmons citrate agar:** - only the slant was streaked to check utilization of citrate as a sole carbon source.
- **Lysine Desoxycholate:** – both the slant and the butt were inoculated, to check weather the organism was able to produce the enzyme lysine decarboxylase.

All biochemical test tubes had been incubated at 35-37 ^{0}C for 18-24 hrs. Results (interpretations of the biochemical changes or reactions were based on standard biochemical identification formats (charts) for *Salmonella* and *Shigella* species (Edward and Ewing, 1972).

3.5. Plant Materials and Test Micro-Organisms

The Plants used in this study were selected based on ethnomedicinal and preliminary study on other microorganisms, on the crude plant extract which was done in the Ethiopian Health and Nutrition Research Institute (Abera Geyid et al., 2002, In press).

The plants have been collected from their natural habitat, identified and authenticated by a taxonomist. A voucher specimen was deposited at the herbarium of EHNRI Drug Research department. The plants used in this study are: *Olea europaea* subsp cuspidata herbarium No, (OE-2042), *Myrica salisfolia*, herbarium No (MS-2039) and *Gardinea lutea* herbarium No (GL-2024) Clinical isolates of *Salmonella* and *Shigella* have been collected, from patients under 14 Yrs of old, suspected of having salmonellosis and shigellosis. A total of 14 clinical isolates of *Salmonella* and *Shigella* were used to determine the MIC value. For the purpose of quality control, a control strain of *E. coli* ATCC (25922) has been used from the laboratory of EHNRI to determine the minimum inhibitory concentration of the selected plant extracts and modern antibiotics.

3.6. Method of Plant Extraction and Screening for Antibacterial Activity

3.6.1. Preparation of Extracts (Crude and Semi-purified Fractions)

The bark of *Myrica saliscifolia* and *Gardinea lutea* while the leaves of *Olea europaea* subsp. *cuspidata* were shade-dried. These dried barks and leaves were coarsely powdered and subjected to maceration and percolation process with 80% methanol and water followed by filtration with Whatman paper No 1. After exhaustive extraction, the methanolic and aqueous

extracts were dried at low temperature (55–60 °C), using Rota evaporator and lyophilizer kept in desiccators and refrigerator.

The Crude extract was subjected to further fractionation using four solvent systems. Part of 80% methanol extract concentrate have been suspended in about 200 ml of water and taken by n-hexane (40-60 ^{0}C) by continuous vortexing for 30 minutes using separator funnel three times. The n-hexane fraction have been combined, evaporated and labeled as fraction **I**. The aqueous residue was taken and fractionated between water and n-butanol three times. The n-butanol layer have been combined and evaporated to dryness (fraction **II**) and the aqueous layer residue was lyophilized to dryness (fraction **III**)**.** The remaining aqueous residue was suspended in chloroform and evaporated to dryness (fraction **IV**)**.** While for extraction of alkaloids the remaining part of 80% methanol extract concentrate have been dissolved or suspended in about 100 ml, 2% citric acid (pH=3) and base freed with 10% NH_3 (pH=9) and extracted with dichloromethane exhaustively. The organic layer have been combined and evaporated to dryness to give fraction**.** The fraction **I** and **IV** (Lipohilic fractions) have been dissolved with a mixture of physiological buffer (pH =7.4) and or appropriate solvents. Fraction **II** and **III** (polar fractions) were dissolved with water or 2% tween-80 of water mixtures.

3.7. Standard (Reference) Antibiotics

The reference antibiotics which have been used in this study were obtained from Drug Quality and Control Authority located in Addis Ababa. The reference antibiotics (in the form of powder) were (positive control) selected based on the following criteria:

1. Antimicrobial agents which are active against Gram-negative bacteria.

2. Antimicrobials used because of having a broad spectrum property.

Standard (reference) Chloramphinicol, Tetracycline and Norfloxacin powders had been used in this study.

3.8. Method of Minimum Inhibitory Concentration Determination for Fractionated Extracts of Plant and Modern Antibiotics

3.8.1. Preparation of Agar Dilution plates

The MIC for the plant extracts as well as for the modern antibiotics was done using Agar dilution method. A series of concentrations ranging from 2000µg/ml to 250µg/ml were used for both the Semi-purified and crude extracts of the three medicinal plants. A 0.1g (100mg) of the tested semi-purified fractionates and crude extract was weighed in analytical balance. In a sterilized test tube, a 5ml of distilled water or 80% alcohol were used to solubilize the sample, depending on the type of the plant extract. The solution was thoroughly stirred for few minutes to get the stock solution (2000µg/ml). This was followed by a series of half dilutions using sterilized distilled water. Finally, the agar dilution plates were done on 90mm Petri dishes by adding 2ml of the bacterial and 18ml of the cool molten agar.

3.8.2. Preparation of Inocula

Pure colonies stored at -70 ^{0}C were thawed, and subcultured on XLD for 18-24hr. Four to five morphologically similar colonies were selected after 18-24hr incubation with a sterile loop and were transferred to TSY, and incubated at 35-37 ^{0}C, until the turbidity is equal to 0.5 McFarland standards. Before inoculation the suspension was diluted 1:10 by adding sterile distilled water. Finally, Inoculation with 1–2 ml spots containing approximately 10^4cfu of each organism was done using a multipoint replicator on the prepared plates. Based on the standard method of agar dilution it was done in 3 replicas. The agar dilution method followed that approved by the National Committee for Clinical Laboratory Standards (NCCLS, 2001). Mueller Hinton agar was used as a positive growth control. At the same time the solvent used also has been used as a negative control with the same amount and concentration added to dissolve the plant extract. Inoculated plates were incubated at 37 °C for 24 h. Minimum inhibitory concentrations (MIC's) were determined after 20hr. The MIC's were determined as the lowest concentration of plant extract inhibiting the visible growth of each organism on the agar plate. The presence of stunt growth was disregarded, according to Jennifer, (2001).

The same procedure was followed as that of the plant extracts for the determination of MIC for the modern antibiotics. Modern antibiotics were used as a reference to compare and contrast the result with the plant extract. Three standard reference antibiotics were used. These are Chloramphinicol, Tetracycline and Norfloxacin.

3.9. Data Analysis

The results were analyzed based on the three replicates done for each of the three modern antibiotics and each of the crude and semi-purified fractionates of the medicinal plants. Data analysis were made in terms of the percentage of the modal growth inhibition value obtained for each of the fourteen clinical isolates of *Salmonella,* and fourteen clinical isolates of *Shigella* in addition a control strain (E. coli ATCC 25922)was used by giving a code for each of the strains.

4. Results

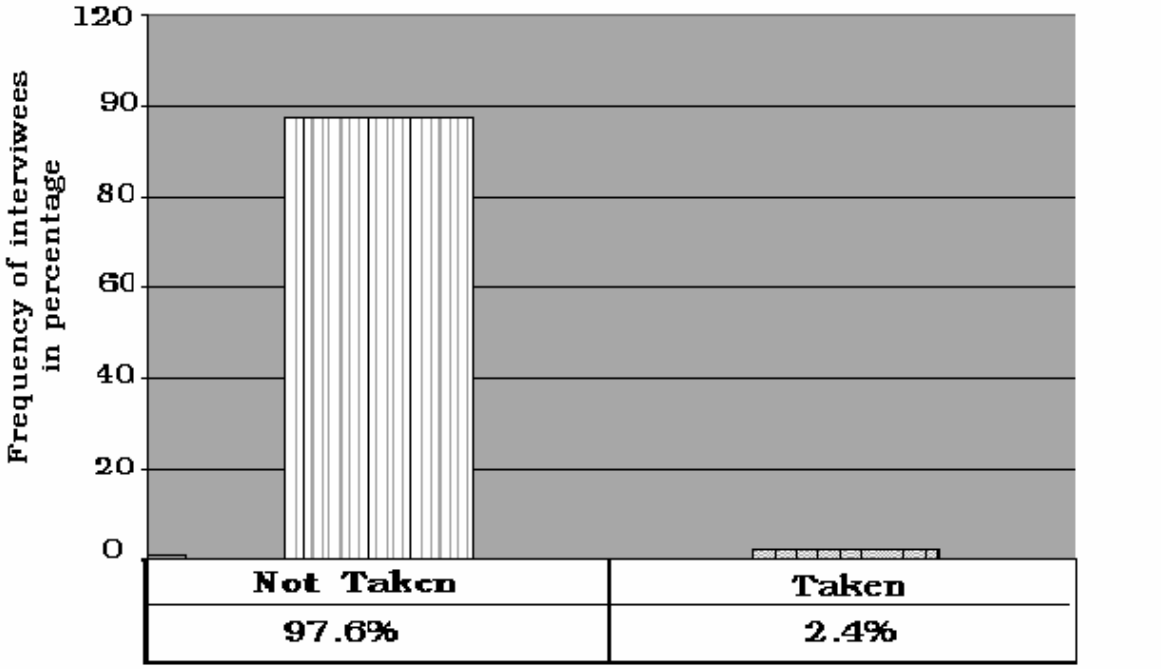
4.1. Total number of Target Clinical Isolates

A total of 265 samples have been collected of which a total of 45 positive clinical isolates of *Salmonella* and *Shigella*, were obtained. Among the total 265 samples 25(9.5%) species were *Salmonella*, while 20(7.5%) species were *Shigella* species.

4.2. History of interviewees (Patients) for the use of Medicinal Plants

Based on the reliable answers obtained from the interviewed patients or guardians using the pre-formatted questionnaires for which 250 interviewees responded, whether or not they had taken any other alternative treatment for their problem of diarrhoea, including herbal treatments. It was found that only 2% of the interviewees had used up medicinal plants (Figure 4). This includes the use of seeds of “Dibrk”, “tult”, boiled rice water, and the mixture of powdered coffee and honey together.

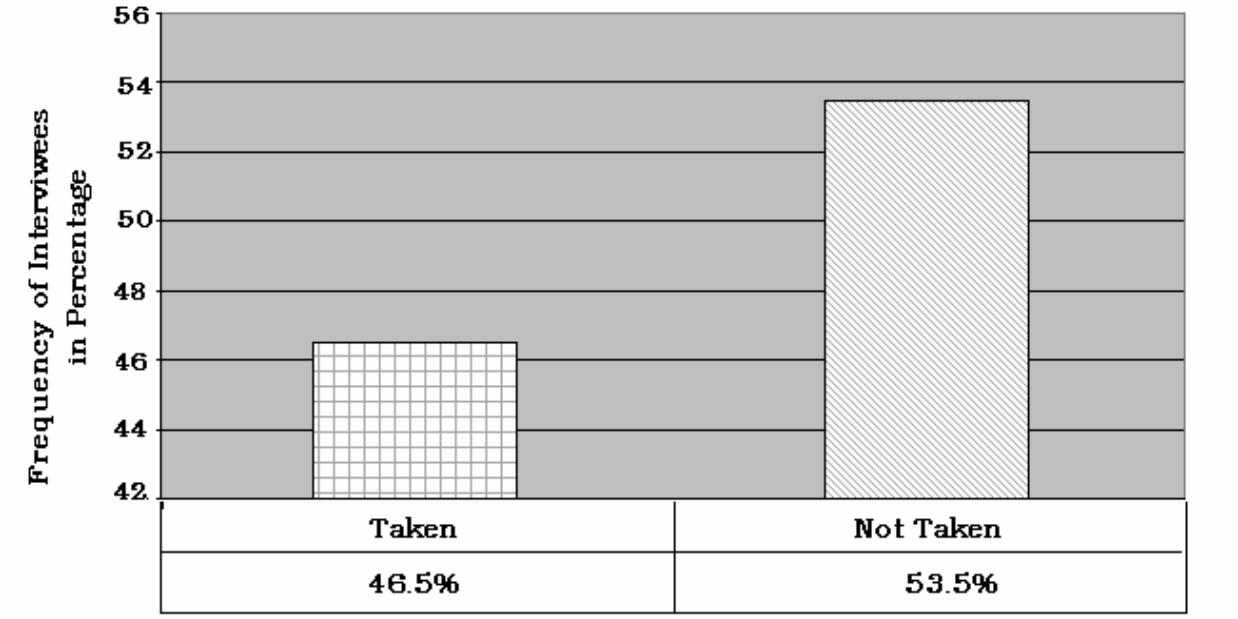
Figure 4. The use of medicinal plants by the interviwees for the case of diarrhoea



4.3. History of Interviewees (Patients) for The Use of Modern Antibiotics

In the case of modern antibiotics usage by the interviewees it was found out that out of a total of 237 patients 46%(110)had taken one or more antibiotics especially co-trimixazole in the form of syrup for the case of infants, as shown in figure 5. The rest 54% of the interviewed patients have reported that they had not taken any antibiotic at all before and while coming to the referral hospital.

Figure 5. The Use of one or more Antibiotics before and during Sample Collection



4.4. Age Vs Sex ratio of the Interviewees

A total of 265 infants and young children aged ≤ 14 years who were clinically symptomatic to infectious diarrhoea were screened for the study. Based on the results of age to sex relation of the study subjects where one can observe that out of the total interviewees (265 patients) 178(67.1%) were males and the rest 87(32.7%) were female with a male to female ratio of 2:1 on the average (Figure 6).

Figure 6 Total number of Male and Female Interviwees

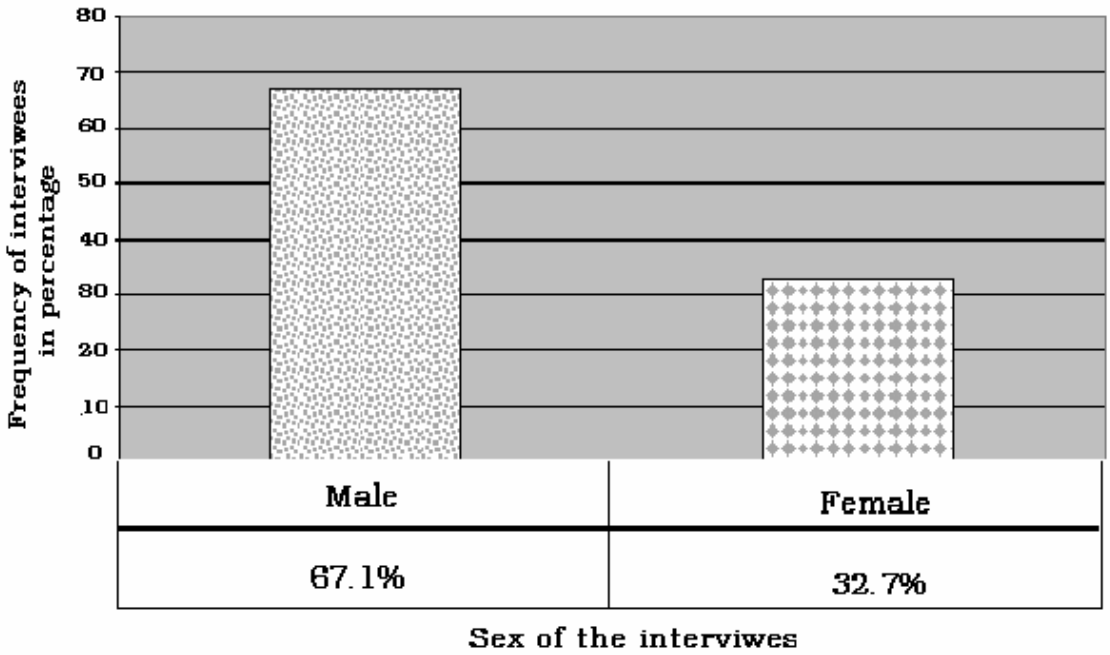
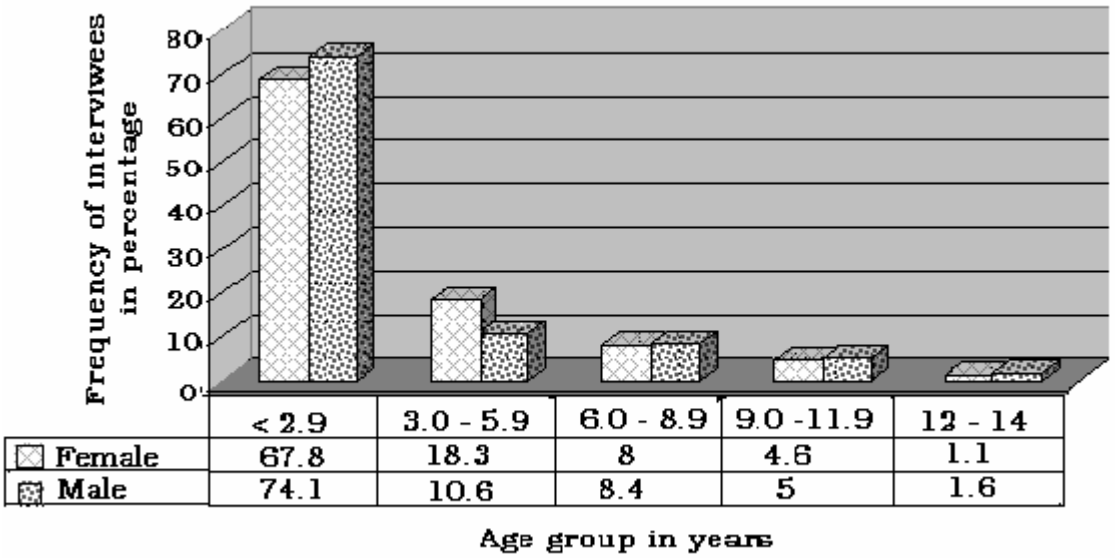


Figure 7,Age Vs sex of the Interviwees frequency distribution



	< 2.9	3.0 - 5.9	6.0 - 8.9	9.0 -11.9	12 - 14
Female	67.8	18.3	8	4.6	1.1
Male	74.1	10.6	8.4	5	1.6

Age groups and sex distribution of the patients (subjects) is shown in Figure 7. A detailed analysis of age to sex ratio has shown that the children with age ≤ 2.9 yrs in both sexes was with the highest frequency 67.8% and 74.1% with female and male, respectively. This was followed by interviewees in the age group of 3-5.9 yrs, in both sexes .

4.5. Minimum Inhibitory Concentration (MIC) of the Plant extracts

4.5.1. *Gardinea lutea*

The MIC value for the crude extracts and semi purified fractionates of *Gardinea lutea* has shown in Tables 1-3 and figure 8-10. From the figures and tables it can be observed that both the crude extracts and fractionates, especially (GL-TP-2) the butanol fraction of *Gardinea lutea,* has shown the highest antimicrobial activity for the clinical isolates of *Shigella* as compared to the other fractionates. It has MIC value as low as 250µg/ml for 21% of the clinical isolates of *Shigella*. This was followed by GL-TP-3 (water fraction) which has shown MIC value up to 500µg/ml for 21% clinical isolates of *Shigella.* In the case of *Salmonella,* only the butanol fraction of *Gardinea lutea* has shown strong activity (MIC values), whereby only 14% of the clinical isolates were inhibited at 2000µg/ml. No or less activity has been observed against the clinical isolates of *Salmonella* even at 2000µg/ml, (14%) for the water fraction of *Gardinea lutea.*

Table 1. MIC value for the crude extracts of *O. europaea* subsp. *cuspidate, M. salicsfolia and G. lutea* against the isolates of *Salmonella* and *Shigella*

	Percentage of Growth Inhibition			
Type of plant	Concentration in µg/ml	*Shigella*	*Salmonella*	*E.coli*(25922)
G.lutea (Crude extract)	2000	100NG	100NG	100NG
	1000	78NG	35NG	0NG
	500	42NG	0NG	0NG
	250	21NG	0NG	0NG
M.salicsfolia (Crude extract)	2000	100NG	71NG	100NG
	1000	42NG	35NG	0NG
	500	0NG	0NG	0NG
	250	0NG	0NG	0NG
O.europaea Ssp.cuspidata (Crude extract)	2000	50NG	42NG	0NG
	1000	0NG	0NG	0NG
	500	0NG	0NG	0NG
	250	0NG	0NG	0NG

Where NG = No growth

Table 2. MIC value for water fractionated extract of *Gardinea lutea*

Type of plant extract	Concentrationμg/ml	Percentage of Growth Inhibition		
		Salmonella	*Shigella*	*E.coli* (25922)
Water extract (GL-TP-3)	2000	14NG	71NG	0NG
	1000	0NG	42NG	0NG
	500	0NG	21NG	0NG
	250	0NG	0NG	0NG

Where NG = No Growth

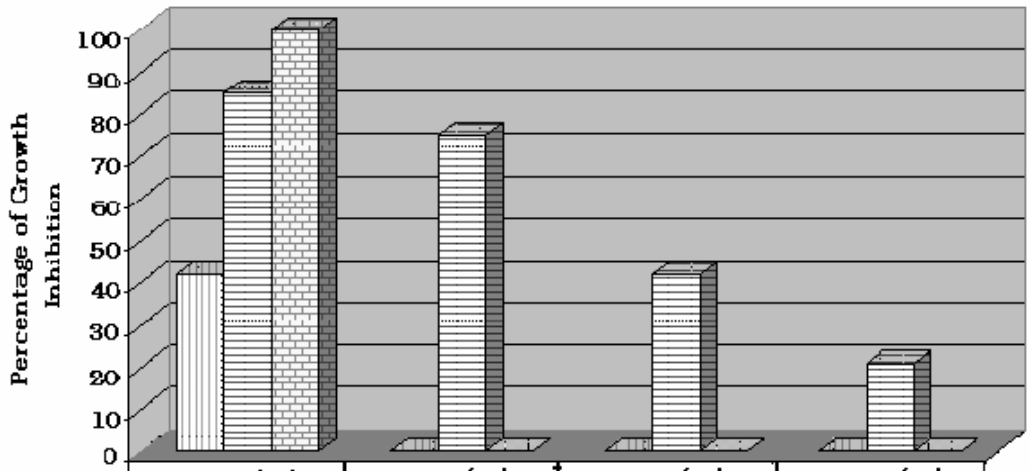
Table 3- MIC value for the Butanol fractionated extract of *G. lutea*

	Percentage of Growth Inhibition			
Type of extract	Concentration in μg/ml	*Salmonella*	*Shigella*	*E.coli25922*
G. lutea Butanol extract (GL-TP-2)	2000	42NG	85 NG	100 NG
	1000	0NG	75 NG	0NG
	500	0NG	42 NG	0NG
	250	0NG	21 NG	0NG

Where NG= No growth

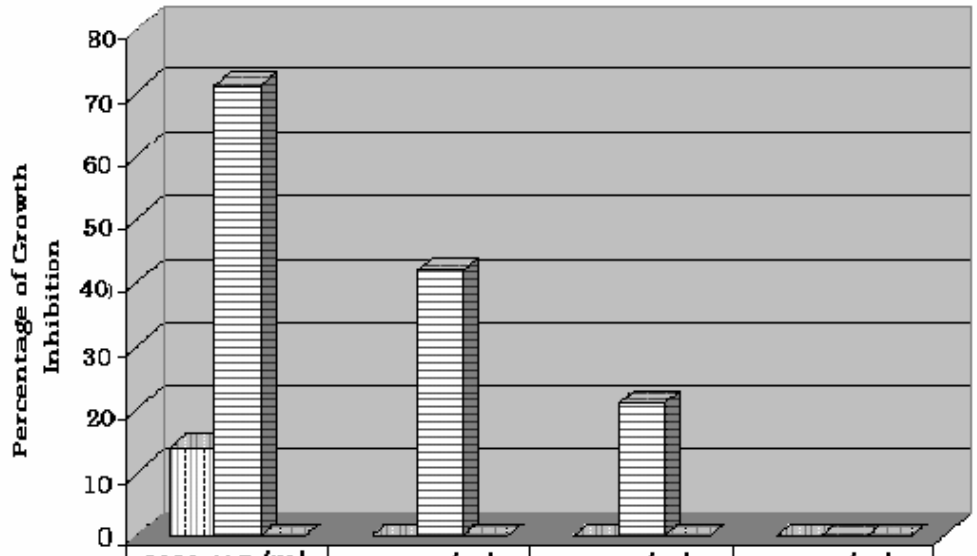
Note that zero NG (ZERO PERCENT No Growth = 100%G)

Figure 8.MIC value for G.lutea butanol fraction



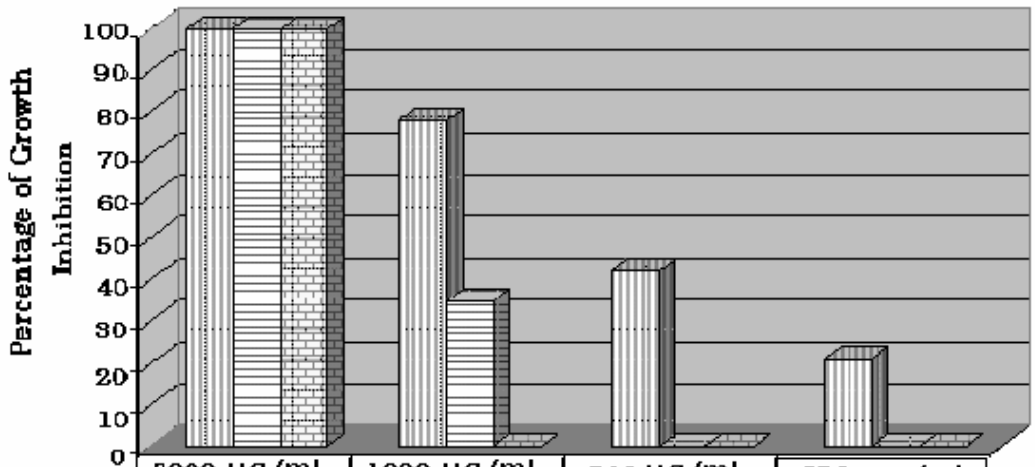
	2000 µg/ml	1000 µg/ml	500 µg/ml	250 µg/ml
Salmonella	42 %	0 %	0 %	0 %
Shigella	85 %	75 %	42 %	21 %
E.coli 25922	100 %	0 %	0 %	0 %

Figure 9. MIC value for G.lutea water fraction



	2000 µg/ml	1000 µg/ml	500 µg/ml	250 µg/ml
Salmonella	14 %	0 %	0 %	0 %
Shigella	71 %	42 %	21 %	0 %
E.coli 25922	0 %	0 %	0 %	0 %

Figure 10 . MIC value for the crude extract of G. lutea



	2000 µg/ml	1000 µg/ml	500 µg/ml	250 µg/ml
Salmonella	100 %	78 %	42 %	21%
Shigella	100 %	35%	0 %	0%
E.coli 25922	100 %	0 %	0 %	0%

4.5.2. *Myrica salisfolia*

Both the crude extract and semi purified fractionates of this plant has shown antimicrobial activity $\geq$ 1000µg/ml concentration. All of the crude and fractionated extracts haven't shown antimicrobial activity less than 500µg/ml for both *Salmonella* and *Shigella* clinical isolates. Among the semi purified fractionates of this plant the butanol fraction or (MS–TP-3) has shown relatively the best antisalmonella activity.

From this plant fractionates, the butanol fraction at 2000µg/ml has inhibited 71% of *Salmonella* and *Shigella* clinical isolates. At 1000µg/ml 57% of the clinical isolate of *Salmonella* and 35% of clinical isolates of *Shigella* species have been inhibited. The chloroform fraction has shown weak activity for both *Shigella* and *Salmonella* even at concentration of *2000* µg/ml. For concentrations less than 2000 µg/ml no activity have been observed (Tables 4,5 and Figure, 12-14*).* While no activity had been observed for water fractionate of this plant, hence no further replicas were done.

Table 4. MIC value for Chloroform fraction of *Myrica salisfolia*

	Percentage of Growth Inhibition			
Type of plant extract	Concentration µg/ml	*Salmonella*	*Shigella*	*E.coli (25922)*
Myrica salisfolia Chloroform fraction (Ms-Tp-2)	2000	14 NG	21 NG	0 NG
	1000	0 NG	0 NG	0 NG
	500	0 NG	0 NG	0 NG
	250	0 NG	0 NG	0 NG

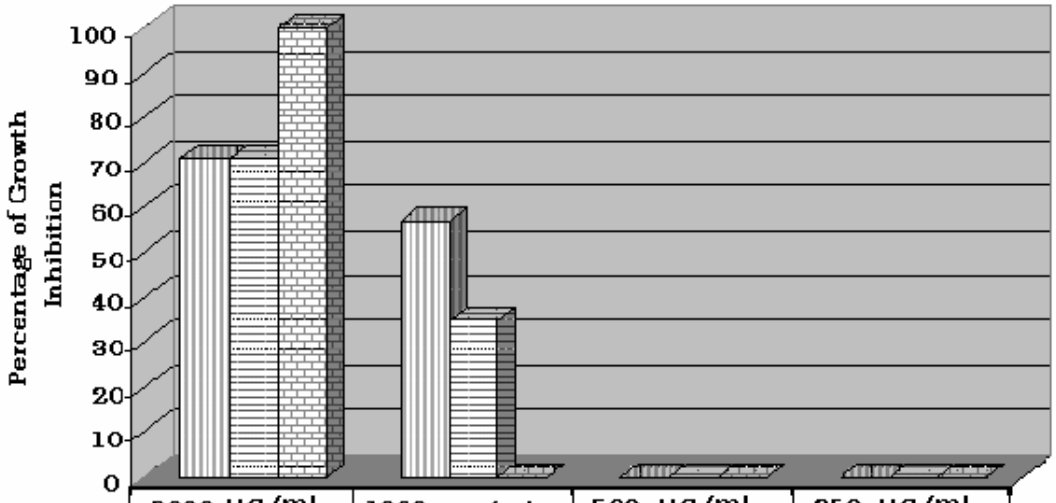
Where, NG = No Visible growth

Table 5. MIC value for the Butanol fraction of *M. salisfolia*

	Percentage of Growth Inhibition			
Type of plant extract	Concentration in µg/ml	*Salmonella*	*Shigella*	*E.coli (25922)*
Myrica salisfolia Butanol extract (Ms-Tp-3)	2000	71 NG	71 NG	100 NG
	1000	57 NG	35 NG	0 NG
	500	0 NG	0 NG	0 NG
	250	0 NG	0 NG	0 NG

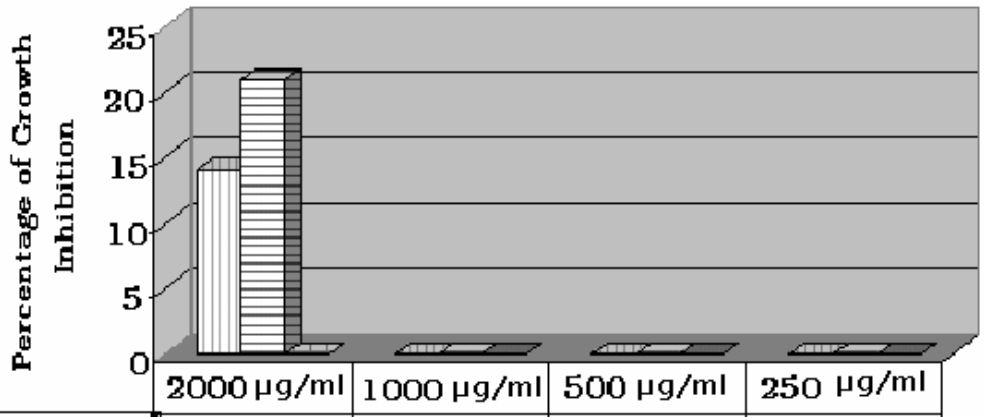
Where, NG = No growth

Figure 11 *MIC value for M. salisfolia butanol fraction*



	2000 µg/ml	1000 µg/ml	500 µg/ml	250 µg/ml
Salmonella	71%	57 %	0 %	0 %
Shigella	71%	35 %	0 %	0 %
E.coli 25922	100 %	0 %	0 %	0 %

Figure 12 *MIC value for M. salisfolia* **Chloroform** *fraction*



	2000 µg/ml	1000 µg/ml	500 µg/ml	250 µg/ml
Salmonella	14%	0 %	0 %	0 %
Shigella	21%	0 %	0 %	0 %
E.coli 25922	0 %	0 %	0 %	0 %

Concentrations in microgram/ml

Figure 13 MIC value for the crude extract of M. salisfolia

	2000µg/ml	1000µg/ml	500µg/ml	250µg/ml
Salmonella	100%	42 %	0 %	0%
Shigella	71 %	35 %	0 %	0%
E.coli 25922	100 %	0 %	0 %	0%

4.5.3. *Olea europaea* subsp *cuspidata*

The beginning of the *invitro* antimicrobial screening test was done using the crude extract of this plant which has shown a week activity, even at the higher concentration of 2000µg/ml, for both clinical isolates of *Salmonella* and *Shiglla.* For this plant the butanol and chloroform fractionates has shown relatively better MIC value for 35% of the clinical isolate of *Shigella* but for 0% of clinical isolates of *Salmonella at 2000*µg/ml. Second to the butanol fractionate, the chloroform fractionate which has shown growth inhibition for 21% of clinical isolates of *Salmonella* and 28% of *Shigella* .Table 6-8 and figure 11-14.

Table 6 .MIC value for the butanol extract of *O.europaea* subsp. *cuspidata*

	Concentration in µg/ml	Salmonella	Shigella	E.coli(25922)
OE-TP-2 Butanol fraction	2000	0 NG	35NG	0 NG
	1000	0 NG	0 NG	0 NG
	500	0 NG	0 NG	0 NG
	250	0 NG	0 NG	0 NG

Table 7.Modal value for water extract of *O. europaea* subsp. *cuspidate*

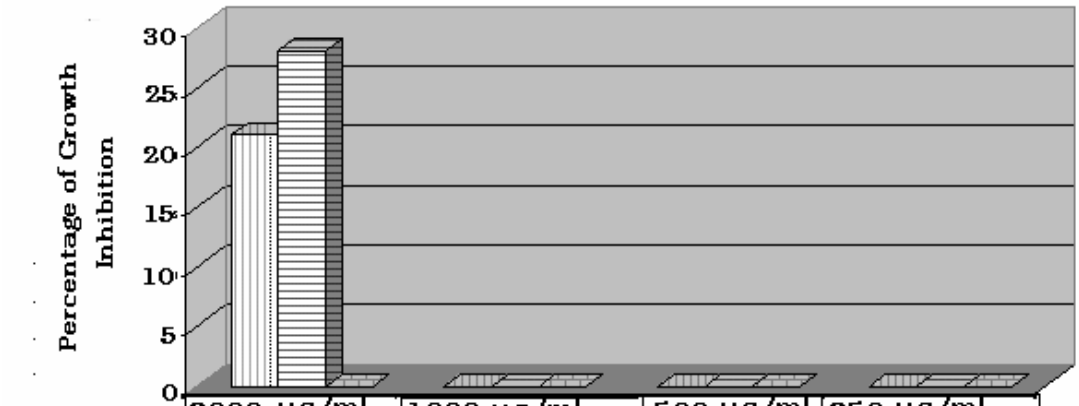
	Concentration µg/ml	Salmonella	Shigella	E.coli(25922)
OE-TP-3 Water fraction	2000	0NG	14NG	0 NG
	1000	0 NG	0 NG	0 NG
	500	0 NG	0 NG	0 NG
	250	0 NG	0 NG	0 NG

Table 8. MIC value for chloroform fraction of Olea europaea subsp. *cuspidata*

	Concentration µg/ml	Salmonella	Shigella	E.coli(25922)
OE-A-2 Water fraction	2000	21NG	28NG	0 NG
	1000	0 NG	0 NG	0 NG
	500	0 NG	0 NG	0 NG
	250	0 NG	0 NG	0 NG

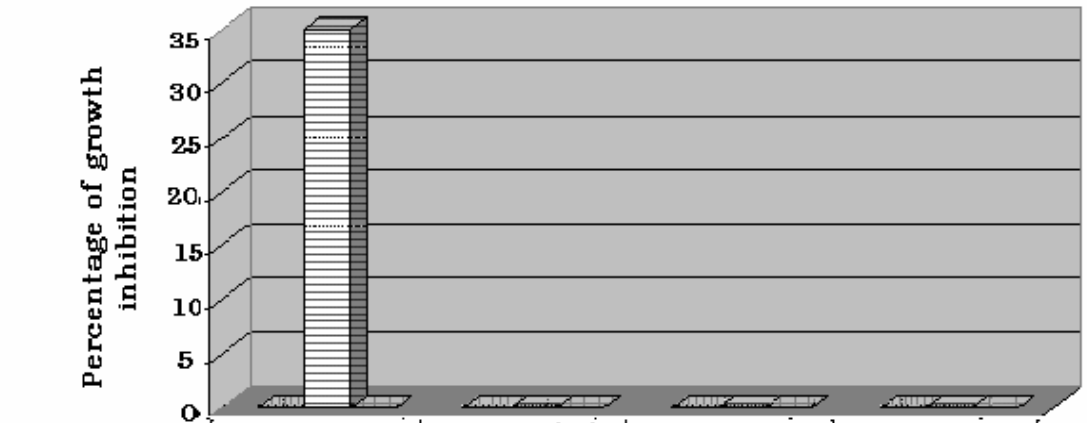
Where NG = No growth

Figure 14. MIC value for Chloroform fraction of O. europea subsp. africana



	2000 µg/ml	1000 µg/ml	500 µg/ml	250 µg/ml
Salmonella	21%	0%	0%	0%
Shigella	28%	0%	0%	0%
E.coli 25922	0%	0%	0%	0%

Figure 15. MIC value for water fraction of O. europea subsp. africana

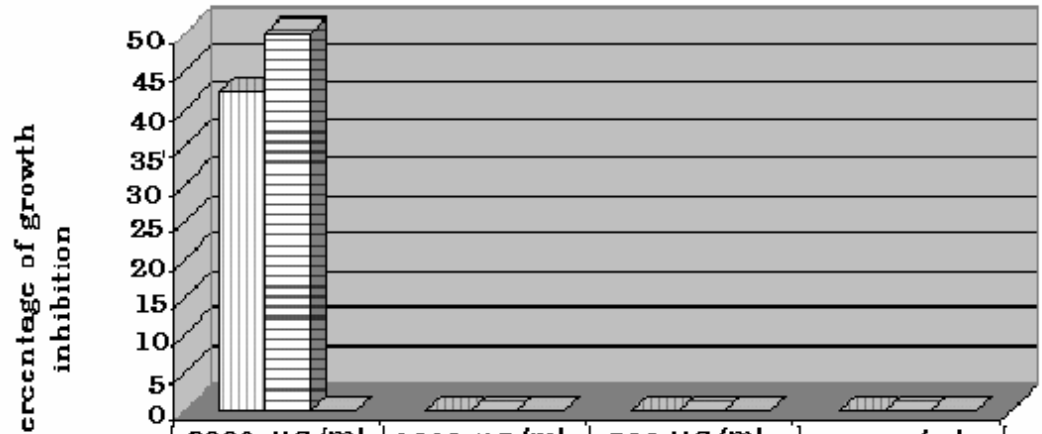


	2000 µg/ml	1000 µg/ml	500 µg/ml	250 µg/ml
Salmonella	0%	0%	0%	0%
Shigella	14%	0%	0%	0%
E.coli 25922	0%	0%	0%	0%

Figure 16. MIC value for Butanol fraction of O. europea subsp.africana

	2000 µg/ml	1000 µg/ml	500 µg/ml	250 µg/ml
Salmonella	0 %	0 %	0 %	0 %
Shigella	35 %	0 %	0 %	0 %
E.coli 25922	0 %	0 %	0 %	0 %

Figure 17. MIC value for the Crude Extract of O. europea subsp.africana



	2000 µg/ml	1000 µg/ml	500 µg/ml	250 µg/ml
Salmonella	42 %	0 %	0 %	0 %
Shigella	50 %	0 %	0 %	0 %
E.coli 25922	0 %	0 %	0 %	0 %

4.6. Minimum Inhibitory Concentration for the Modern Antibiotics

4.6.1. MIC for Chloramphinicol

The MIC value for Chloramphinicol against the clinical isolates of *Shigella* has been found to be less than or equal to 75µg/ml in terms of modal value where by only 14% growth is seen, while the rest 86% were inhibited at 75µg/ml (Table 9 and Graph 18). *The MIC* value, where 100% growth inhibition was seen was at 150µg/ml while at 1.17µg/ml 100% growth was observed. On the other hand for *Salmonella* at 300µg/ml concentration of this drug 78% growth was observed. It is at 4.7µg/ml concentration whereby 100% full growth was observed for the clinical isolates of *Salmonella*.

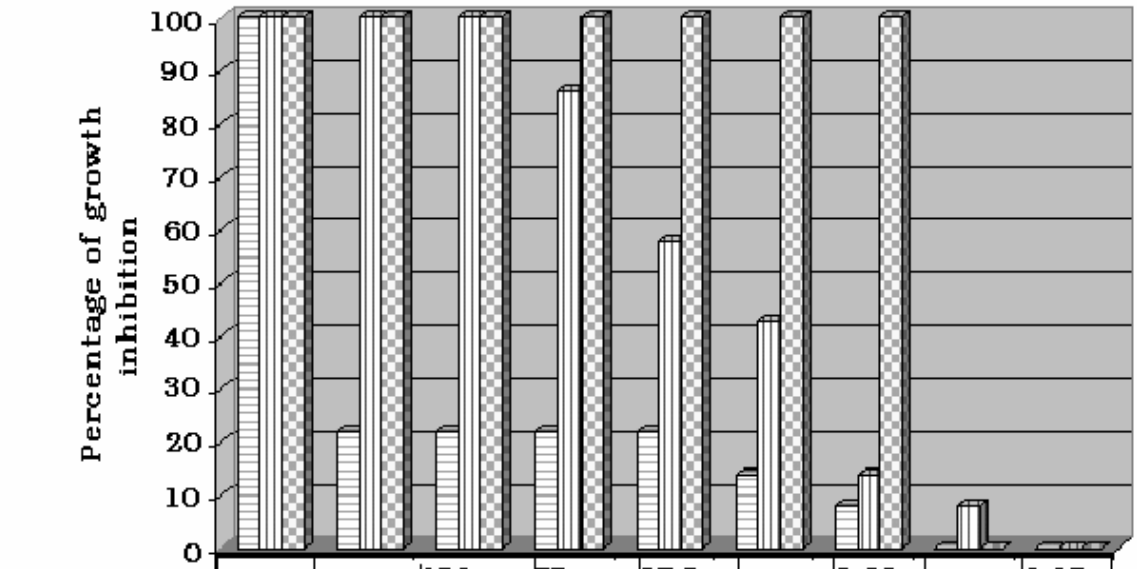
Table 9. MIC value for Chloramphinicol

Type of Antibiotics	Percentage of Growth Inhibition			
Chloramphinicol	Concentration in µg/ml	*Salmonella*	*Shigella*	*E.coli*(*25922*)
C0	600	100%NG	100%NG	100%NG
C1	300	22%NG	100%NG	100%NG
C2	150	22%NG	100%NG	100%NG
C3	75	22%NG	86%NG	100%NG
C4	37.5	22%NG	58%NG	100%NG
C5	18.75	14%NG	43%NG	100%NG
C6	9.375	8%NG	14%NG	100%NG
C7	4.6875	0%NG	8%NG	0%NG
C8	1.171875	0%NG	0%NG	0%NG

Where , NG = No growth

The MIC value of chloramphinicol for the control strain (*E.coli* ATCC 29522) in this work was ≤ 4.6875 µg/ml. Almost all of the clinical isolates have shown high value of MIC as compared to the control strain (*E. coli* ATCC 25922).

Figure 18. MIC value for Chloramphinicol



	600	300	150	75	37.5	18.8	9.38	4.69	1.17
Salmonella	100 %	22 %	22%	22 %	22 %	14 %	8 %	0 %	0 %
Shigella	100%	100 %	100 %	86 %	58 %	43%	14%	8 %	0%
E.coli 25922	100 %	100 %	100%	100%	100%	100%	100%	0 %	0%

4.6.2. Minimum Inhibitory Concentration for Tetracycline

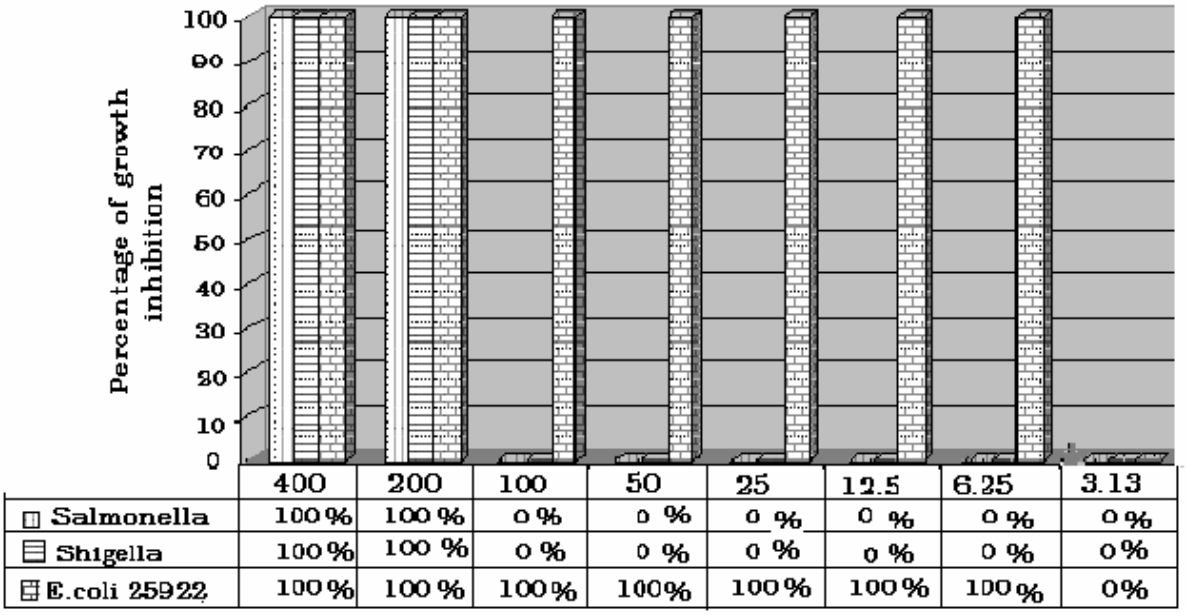
The MIC value for tetracycline has been found to be 200µg/ml for 100% of the clinical isolates of *Shigella* and *Salmonella*. The control strain (*E.coli* ATCC 25922) has been grown at concentration of 3.125µg/ml (Table 10, figure 19). As compared to the control strain (*E.coli* ATCC 25922), the clinical isolates of *Salmonella* and *Shigella* have shown resistance to tetracycline by more than 50 times than that of the control strain (*E.coli* ATCC 25922).

Table 10. MIC value for Tetracycline

Tetracycline	Concentration µg/ml	Percentage of Growth Inhibition		
		Salmonella	*Shigella*	*E.coli*(25922)
T0	400	100%NG	100%NG	100%NG
T1	200	100%NG	100%NG	100%NG
T2	100	0%NG	0%NG	100%NG
T3	50	0%NG	0%NG	100%NG
T4	25	0%NG	0%NG	100%NG
T5	12.5	0%NG	0%NG	100%NG
T6	6.25	0%NG	0%NG	100%NG
T7	3.125	0%NG	0%NG	0%NG

Where NG = No growth

Figure 19. MIC value for Tetracycline



4.6.3. Minimum Inhibitory Concentration for Norfloxacin

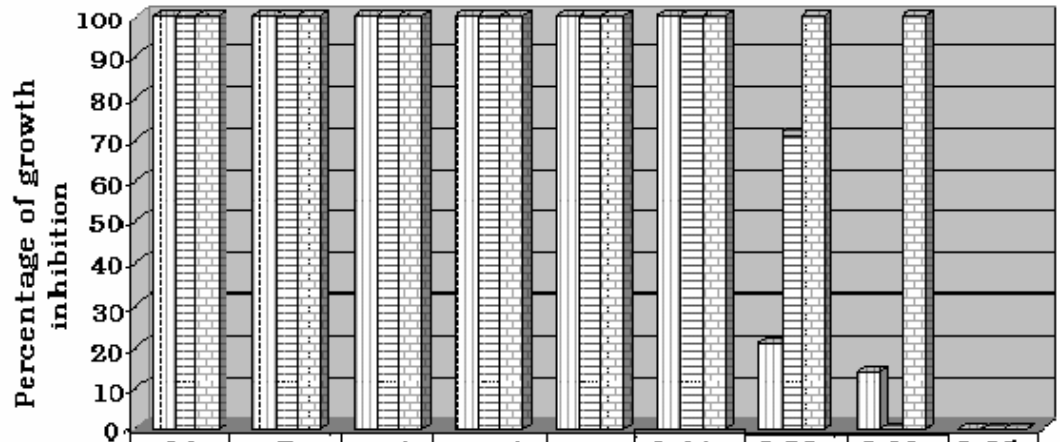
For norfloxacin a lower MIC value has been obtained for both the clinical isolates of *Salmonella* and *Shigella* as compared to Chloramphinicol and Tetracycline, as expected. At concentration of 0.21875µg/ml 79% and 29% growth have been observed for the clinical isolates of *Salmonella* and *Shigella*, respectively (Table 11, Figure 20). While for concentrations greater than 0.21875µg/ml 100% growth inhibition was observed for both of the clinical isolates of *Salmonella* and *Shigella.*

Table 11. MIC value results for Norfloxacin

Norfloxacin	Concentration (µg/ml)	Percentage of Growth Inhibition		
		Salmonella	*Shigella*	*E.coli*(25922)
N_0	14	100 NG	100 NG	100 NG
N_1	7	100 NG	100 NG	100 NG
N_2	3.5	100 NG	100 NG	100 NG
N_3	1.75	100 NG	100 NG	100 NG
N_4	0.875	100 NG	100 NG	100 NG
N_5	0.4375	100 NG	100 NG	100 NG
N_6	0.21875	21NG	71NG	100 NG
N_7	0.109375	14N G	0NG	100 NG
N_8	0.0546875	0NG	0NG	0NG

Where NG = No growth

Figure 20. MIC value for Norfloxacin



	14	7	3.5	1.75	0.88	0.44	0.22	0.11	0.05
Salmonella	100%	100 %	100%	100%	100%	100	21%	14%	0%
Shigella	100%	100%	100%	100%	100%	100	71 %	0 %	0%
E.coli 25922	100 %	100 %	100%	100 %	100	100%	100%	100%	0%

5. Discussion

5.1. Summary of some parameters from the interviewed Patients

Age VS Sex ratio: Most of the patients who visited during the five months of sample collection were found to be dominated by male from the total cases (265) interviewees, 67.1 % (178) were male while 32.7%(87) were females, showing male to female ratio of 2:1. From the total age groups, it was found that those children found in the age group ≤ 2.9 yrs were found to be the primary target of the case. In this age group male accounted for 74.1% and 67.8% were female case patients, respectively. As it is expected an inverse relationships of the frequency of case patients (interviewees) with that of the age groups have been found (i.e. as the age group increase a decreasing frequency of the cases has been obtained, for both sexes). The last age group with the least frequency was 12-14 yrs of age. In this study the mean age for male was 1.1yrs and for females it's 2.5yrs while for both sexes is 1.5 yrs.

Use of Antibiotics and Medicinal plants before and during sample collection: The result obtained from the interviewees has shown that more than 46% of the total (237) patients have used one or more antibiotics including gastrointestinal drugs. It was reported by Tenaw Andualem (2004) that from the most frequently requested category of drugs for self-medication by drug consumers from 780 individuals, in Addis Ababa 26.4% and 17.7% were antimicrobial drugs and gastrointestinal drugs respectively. Those patients, who have taken antibiotics, have complained that there was no improvement of their condition even after taking one or more of these antibiotics. This may be due to the development of drug resistance against these drugs they have used or due to the inappropriate chemotherapeutical treatment.

The other information which was important and used in this study was the use of medicinal plants by the patients. The result has shown that most of the patients have not used medicinal plants although they may know that it is important, this may be because in most of the cases people are used to go for herbalists for the cases like sexually transmitted disease (STD), hemorrhage and jaundice and few cases go to herbalists for the case of diarrhoea. The other reason can be due to the fear of adverse effect of the recurrently occurring, side effects of the prescribed medicine (in any form) by the herbalists. Out of the total number of patients included in this study only 2% had claimed that they had used medicinal plants. This figure may only represent urban people who have come to visit the hospital during the sample collection period and responded to the questionnaire correctly. It does not represent those who treated themselves with the medicinal plants or go to the herbalists directly.

5.2. Minimum Inhibitory Concentration for Gram Negative Bacteria (*Shigella* and *Salmonella)*

In general, and in most cases, Gram-negative bacteria are less sensitive to modern antibiotics as compared to Gram positive bacteria. This is also found to be true for most plant extracts and other toxic compounds. Different studies conducted using both Gram positive and Gram negative bacteria have revealed that Gram negative bacteria are less sensitive to most plant extracts as compared to Gram positive bacteria (Shelf, 1996).

Different factors account for Gram negative bacteria to be less sensitive to antimicrobial agents such as modern antibiotics and plant extracts. One of the major factors is the low permeability of the outer membrane which reduces the drug diffusion across the cell envelope

(Nikiaido , 1994). In some cases the permeability of the outer membrane can be further decreased by the loss of porins (Nikaido., 1994). However, once the drugs have entered the membrane cannot prevent the drugs from exerting their toxic action. The active efflux of drugs is another mechanism in Gram negative bacteria, which is essential to ensure significant levels of drug resistance (Figure 21). This is a mechanism which evades the toxic effects of antibiotics by the active extrusion of structurally unrelated drugs from the cell. Also, there are still unknown factors which help these bacteria to resist to different toxic and antimicrobial agents.

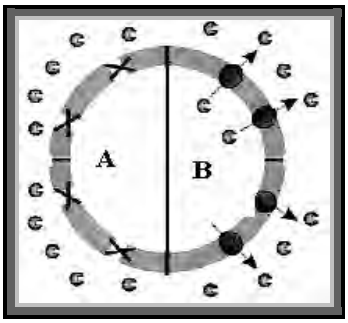


Figure 21. Resistance mechanisms in Gram negative bacteria
(A) Prevention of drug influx, (B) Active extrusion of drug from the cell.
Source: Monique et al., 2000.64(4). Molecular Biology Reviews

The result of this study has shown that, generally the plant extracts and modern antibiotics used in this study have high MIC values which may be due to the above explanations of resistance factors for Gram negative bacteria. It's more likely that the medicinal plants used in this study would show lower MIC value if they were tested using the Gram positive bacteria. Generally, the clinical isolates of *Salmonella* was found to be resistant for both the crude and fractionated extracts of the plants as well as to the modern antibiotics studied as compared to the clinical isolates of *Shigella.*

5.3. Minimum Inhibitory Concentration Comparison between the Fractionated Plant Extracts

The qualitative and quantitative antimicrobial screening of the crude and semi-purified fractionated extracts of the plant extracts was determined by agar dilution assay method. The three plant species tested have exhibited different antibacterial activities against the Gram negative pathogens (*Salmonella* and *Shigella* and the control strain *E.coli* ATCC 25229 species).

Generally, the crude extract have shown higher activity as compared to the semi-purified fractionates against both species of *Shigella* and *Salmonella,* which may be explained to be due to the synergetic effect of the total compounds found in the crude extract. The crude extracts of *G. lutea* and *M. salicfolia* had shown highest activities 100% growth inhibition at 2000µg/ml against *Shigella* and *Salmonella* species respectively as compared to their fractionated extracts. The least activity had been obtained from *O. europaea* subsp. *cuspidata* of both crude and semi purified fractionates. From the semi-purified fractionates the butanol fractionate of *Gardinea lutea* has shown its best activity against clinical isolates of *Shigella* and *Salmonella* where inhibition have been observed up to 250µg/ml for clinical isolates of *Shigella*. Similarly, the semi-purified butanol fractionate of *Myrica salisfolia* has shown highest activity for both *Salmonella* and *Shigella* at 2000µg/ml and 1000µg/ml while for concentration $\leq$ 500µg/ml no inhibition was observed for both clinical isolates of this plant.

From the whole fractionates it is mainly the butanol fractionates which has shown relatively the best antibacterial activity in all of the three medicinal plants as compared to the other fractionates (solvents). As to the water fractionate, the water fractionate of *Gardinea lutea* is the only fractionate which has shown higher antibacterial activity (Table 2 / Figure 9). Since the water fractionate of *Myrica saliscifolia* has shown no activity had been shown so no further replica was done. The same is true for the hexane fraction of the three plants.

There can be several factors which can affect or reduce the efficacy of the medicinal plants in their antimicrobial activity. This is beginning from the time of plant collection (it's recommended that the collection of plant parts in most cases, but not always should be done after the flowering stage of the plant) the state of plant processing and the state of storage of plant (Grrigs *et al*., 2001). The method of plant extraction is another factor which affects the antimicrobial activity of the medicinal plant.

Generally, the results reported from different studies are difficult to compare because of the use of different test methods, bacterial strains and sources of antimicrobial samples used. The overall antibacterial activity of the tested plants have however a comparable MIC values. By similar studies, done using three different Nigerian plants against the clinical isolates of *Shigella,* it was shown that the MIC values were in the ranges of 300-515.6µg/ml *S.dysentrea* , 309.4-543.8µg/ml for *S.sonnei* and 243.8-337.5µg /ml for *S.boydii* (Iwalokunl *et al.,* 2001). Where as in our study *Gardinea lutea* has a range of 2000µg/ml -250µg/ml against the tested clinical *Shigella* while the butanol fraction of *Gardinea lutea* has MIC $\geq$ 1000µg/ml for *Myrica salisfolia*.

It's was observed that the control strain *E. coli* ATCC (25922) has not been inhibited by the plant extracts while the clinical isolates were inhibited by the plant extract i.e. it has high MIC value as compared to the clinical isolates of *Shigella* and *Salmonella* for the plant extracts. In contrary the control strain of *E. coli* ATCC (25922) has the lowest MIC value for the three modern antibiotics (Tetracycline, Chloramphinicol and Norfloxacin) than all the tested clinical isolates. This result has shown that being a standard organism alone may not probably mean that the organism is less or more sensitive to plant extracts or other antimicrobial agents rather than modern antibiotics as compared to the clinical isolates.

5.4. Comparison between Minimum Inhibitory Concentration for the Modern Antibiotics

The MIC value of chloramphinicol for *Salmonella* have been reported to be greater than 256µg/ml in the work of Talwadkar *et al*., 1989, Hirose *et al*., 2001. Mandal *et al.,* (2004) has reported MIC value of 2000 µg/ml for *Salmonella typhi,* while the MIC for modern antibiotics value for *Salmonella* in this study for 22% of the clinical isolates was greater than 300 µg/ml. According to the standard interpretations of MIC, microorganisms with MIC value greater than 16 µg/ml for Chloramphinicol are considered to be resistant (Jenny, 2003). This implies that about 86% of the clinical isolates of *Salmonella* in our study were resistant to Chloramphinicol while 57% of the clinical isolates of *Shigella* were resistant to this drug (Table 9).

The clinical isolates of *Salmonella* in our study have shown that the MIC value for chloramphinicol is high. This can be due to the high intake of Chloramphinicol by most suspected cases of salmonellosis (for both typhoidal and non typhoidal cases) in developing countries like Ethiopia where, Chloramphinicol is used to be the first line of drug choice.

Tetracycline being an old antibiotic many microorganisms of both Gram positive and Gram negative bacteria have developed resistance against it. In this study it has been found that the MIC value for tetracycline, where 100% growth inhibition was seen for both *Salmonella* and *Shigella* was at 200µg/ml (Table 10). According to the standard MIC value of interpretation microorganisms with MIC value greater than 2µg/ml, for tetracycline are considered as being resistant (Jennifer, 2001). Hence 100% of both the clinical isolates *Salmonella* and *Shigella* are resistant, since their MIC value is 200µg/ml. No clinical isolate have been inhibited at concentration less than 200 µg/ml, except the control strain *E. coli* ATCC 25922*,* with MIC value of 3.125µg/ml.

Norfloxacin, in this study was mainly used to compare its activity with the old antibiotics as relatively it has better activity and there are few reports which exist for its development of resistance (Chloramphinicol and Tetracycline). The result obtained for norfloxacin was as expected that the range of concentration where growth inhibition was seen between 0.12µg/ml-0.05µg/ml. Norfloxacin is found in the family of the floroquinolone, which has currently better antimicrobial activities. Norfloxacin is analog of nalidixic acid with much greater intrinsic activity. The superiority of the activity of norfloxacin has been shown in both *Shigella* and *Salmonella* as compared to Chloramphinicol and Tetracycline, in this study.

According to Jenny (2003) coliforms with MIC < 4μg/ml are said to be sensitive, for Norfloxacin this implies that all (100%) of the clinical isolates of *Salmonella* and *Shigella* were sensitive to this antibiotic.

In this study, norfloxacin has shown the lowest MIC value as compared to all of the plant extracts and all of the other modern antibiotics. It had inhibited growth at concentration as low as 0.43μg/ml for clinical isolates of *Salmonella* and *Shigella*. There are no reports in our country Ethiopia, for the development of resistance against this drug by the clinical isolates of both *Shigella* and *Salmonella* up to now. As compared to the clinical isolates of *Shigella* the clinical isolates of *Salmonella* had been found to have relatively higher MIC value to Norfloxacin in this study (Table 11 and Graph 20).

It was found according to Jennifer (2001) that the MIC of norfloxacin for (*E. coli* ATCC 29522) is 4 μg/ml. This result is in consistent with our result obtained for the Control strain ATCC *E.coli* 25922 we had used for as quality control which also prove how much the work is standardized.

5.5. Comparison between Control Strain (*E. coli* ATCC 25922) and Clinical Isolates

The use of control strains of bacteria is important to monitor the accuracy, reproducibility and precision of antimicrobial susceptibility testing (Robert and Barnishan, 1979). The result of modern antibiotics has shown that the control strain that is used in this study (*E. coli* ATCC 25922) has closer MIC value (as it should be) compared to the standard MIC value set for

the three antibiotics (Tetracycline, Chloramphinicol and Norfloxacin) by the National Committee for Clinical Laboratory Standard, NCCLS (2001). As compared to the clinical isolates, the control strain (*E. coli* ATCC 25922) has shown lower MIC value for modern antibiotics. While, the control strain (*E. coli* ATCC 25922) was resistant (minimum MIC value) for the plant extracts as compared to the clinical isolates. This can be due to the genetical difference between the organisms and the control strain.

As compared to the clinical isolates, the control strain (*E. coli* ATCC 25922) has shown lower MIC value for modern antibiotics. While, the control strain (*E. coli* ATCC 25922) was resistant (minimum MIC value) for the plant extracts as compared to the clinical isolates. This can be due to the genetical difference between the organisms and the control strain.

MIC value for control strain (Jennifer, 2001):***Adapted from:Jennifer (2001) J.antim.Chem*.48 S1, 5-16**

Antibiotic	**Standard MIC *E. coli* ATCC 25922**	**MIC obtained in this study for *E. coli* ATCC 25922**
Chloramphinicol	4µg/ml	4.16µg/ml
Tetracycline	2µg/ml	3.125µg/ml
Norfloxacin	0.06µg/ml	0.05µg/ml

5.6. Comparison between Minimum Inhibitory Concentration value of Plant Extracts and Modern Antibiotics

It was found that, with the exception of Norfloxacin, the other antibiotics (tetracycline and chloramphinicol) relatively have a comparable MIC value for the clinical isolates, in general with that of the extract of *Gardinea lutea* and *Myrica salisfolia* which have relatively better MIC value for the clinical isolates of *Shigella* and *Salmonella,* respectively. At 2000 µg/ml the crude fractionates has 100% growth inhibition for both clinical isolates while butanol fraction of *Gardinea lutea* has inhibited 85% of the clinical isolates of *Shigella*. In other hand at 150µg/ml and 250 µg/ml 100% of the clinical isolates of *Shigella*, have been inhibited by Chloramphinicol and Tetracycline, respectively.

In the case of *Salmonella*, both the (butanol fraction) of *Myrica salisfolia* have shown relatively the best antimicrobial activity at MIC value of 2000µg/ml and 1000µg/ml whereby 71% and 51% of the clinical isolates of *Shigella* and *Salmonella* had been inhibited respectively. This MIC value of the plant extract as compared to 100µg/ml and 600µg/ml MIC values for tetracycline and chloramphinicol respectively, is said to be less active, but with further optimization and purification, the semi-purified fractionate of the method of extraction of the plant extracts more results can be achieved.

6. Conclusion and Recommendations

All of the plants have shown different activity towards the clinical isolates. The least antimicrobial activity had been observed by *O. europaea* subsp *cuspidata* of both the crude and semi purified fractionates. The highest activity has been observed from the crude and semi purified fractionates of *Gardinea lutea*. While *M. salisfolia* is in between of these two plants in terms of its activity.

Our study has shown that with further purifications of the semi purified fractionates particularly the butanol fraction of *Gardinea lutea* and *Myrica salisfolia* could have a better antimicrobial activity. Hence further chromatographic and bioautographic method of purification should be followed in order to identify to which active compound the antibacterial activity of this plant originates and to increase the efficiency of these semi-purified fractionates.

From the overall study one could observe that there can be several factors which can affect or reduce the efficacy of medicinal plants in their antimicrobial activities. Studies have to be conducted as to when and how to collect, store and extract the plant, as well as how to apply for the in vitro test against microorganisms. Some studies have shown that collecting some medicinal plants after their flowering period may be the best time to get their best effect as antimicrobial activities.

Based on the finding of this study the following major recommendations are made:

- ✪ Further work is needed to know and characterize the pure chemical compounds of the plant that are responsible for their antimicrobial properties. This includes such as bioautographic method and further pharmacological analysis for *Gardinea lutea* especially, on the butanol fraction against clinical isolates of *Shigella.*

- ✪ The butanol fraction of *G. lutea* and *M. salisfolia*, which are used for the treatment of diarrhoea in the native people, should be solublized by less polar solvents other than water to get their medicinal effect.

- ✪ The method or the protocol of MIC determination by agar dilution for the plant extract should be optimized so that better results could be obtained.

- ✪ This study has touched some investigations regarding diarrhoea in children less than 14 yrs, further investigation have to be carried on the why male children are more affected as compared to female children for the case of diarrhoea . Studies have also to be made on some of the medicinal plants used by the interviewees, for their antimicrobial activity.

- There can be cases where plants of the same species can have different medicinal property depending upon some intrinsic and extrinsic factors, therefore studies have to be conducted as to prove as to how much the diversity of the population of the plants can affect the medicinal property of plants.

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Annex One

A Clinical Survey for Diarrhoea

Underline Whenever There is a Need

Date----------.

Age ----------yrs.

Sex: M / F

1. **Address**

- Wereda: ----------
- Keble: ----------
- Region: ----------

2. Feeding pattern

- Breast feeding
- Formula
- Cow milk
- Additional food/ Semisolid foods

3. Appetite of the child

- More than 4 time a day.
- Four times a day
- Three times a day
- Two and less than two times a day

4. Onset of diarrhoea----------.

5. Frequency of deification in a day - ----------.

6. Antibiotics taken before (during) the last 5 days----------.

7. Types of diarrhoea.

- Watery+ blood
- Watery
- Mucoid
- Amoebic
- Other forms

8. Symptoms

- Vomiting----------
- Nausea ----------.
- Headache ----------.
- Fever----------.
- Abdominal pains ----------.
- Other symptoms ----------.

9. Height_____cm

10. Weight _____kg

11. Oral rehydration taken during last few days (ORS) ----------.

12. Onset of diarrhoea----------

. **13.** Frequency of deification in a day - ----------.

14. Information regarding parent----------

14.1. Educational Level Educated Not educated

If Educated Elementary High School College

14.2. Economic Status High Medium Low

15. Any herbal treatment/medicinal plants given to the child or the patient __