ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

IN VITRO EVALUATION OF ANTI MICROBIAL ACTIVITIES OF *ALBIZIA GUMMIFERA* AND *CROTON MACROSTACHYUS* AGAINST CLINICAL ISOLATES OF *NEISSERIA GONORRHOEAE*

By

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LIST OF ABBREVIATIONS

ATCC	American type culture collection
AIDS	Acquired immunodeficiency syndrome
CD4	Cluster designation 4.
CDC	Center for disease control and prevention
CSW	Commercial sex workers
DGI	Disseminated gonococcal infection
DNA	Deoxyribo nucleic acid
EHNRI	Ethiopian health and nutrition research institute
GC	Gonococcus
HAM	Homosexually active men
HIV	Human immunodeficiency virus
LOS	Lipooligosaccharide
MIC	Minimum inhibitory concentration
MTM	Modified Thayer Martin medium
NHS	Normal human serum
Opa	Opacity protein
OMP	Outer membrane protein
PID	Pelvic inflammatory disease
Rmp	Reduction modifiable protein
STD	Sexually transmitted disease
TNF	Tumor necrosis factor
WHO	World health organization

ABSTRACT

A total of 250 urogenital specimens were collected from male and female adult patients who were clinically symptomatic for uncomplicated gonococcal infection from eight health centers in Addis Ababa over a period of six months (May 2005-October 2005). Among the 250 urogenital specimens collected from both sexes, the etiologic agents were isolated from 19 patients, indicating an 8% prevalence of *N. gonorrhoeae* infection. The agar dilution method was used to evaluate *in vitro* anti bacterial effects of crude and solvent fractions of two traditionally claimed medicinal plants namely, *Croton macrostachyus* and *Albizia gummifera* against clinical isolates and reference strain of *N. gonorrhoeae* (ATCC 49226). Crude hydro-alcoholic (20-80%) extracts of both plants were effective against the test organisms and their minimum inhibitory concentrations (MICs) were between 250-500 µg/ml. Solvent-solvent partition method was then used to divide the crude extracts of these plants into three fractions and determined their anti *N. gonorrhoeae* activity. Chloroform and n-butanol fractions were identified to be the more active ones in *C. macrostachyus* with MIC values between 125-250 µg/ml. The most active fraction in *A. gummifera* was identified to be the n- butanol fraction, which also had MIC values between 125-250 µg/ml. Aqueous fractions of this plant exhibited activity at MIC values of 500-1000 µg/ml. However, aqueous fraction of *C. macrostachyus* had no growth inhibition effect. MIC values for Penicillin and Spectinomycin were 128 and 256 µg/ml respectively. With this aspect, these results indicate the presence of chemical compounds in *A. gummifera* and *C. macrostachyus* with anti *N. gonorrhoeae* activity comparable to Penicillin or Spectinomycin and the need for further investigation. The results also substantiate the ethno-botanical use of these medicinal plants for the treatment of gonococcal infections.

Key words/ Phrases: Antibacterial activity**,** *Albizia gummifera***,** *Croton macrostachyus***,** *Neisseria gonorrhoeae,* Minimum inhibitory concentrations

1. GENERAL INTRODUCTION

Infectious diseases are global health problems. Worldwide, they account for nearly one-third of the total 50 million annual death estimates. In developing countries, which include about 75% of the world population, infectious diseases account for nearly 40% of deaths. In the category of developed countries, these diseases represent only about 4% of the mortality rates (Brock, 1997; WHO, 1999). The existence of such a sharp contrast in the degree of importance of infectious diseases as a cause of death in developing versus developed nations is attributed to the general lower levels of public health protection, lack of economic resources, a lower over all standard of living and lack of awareness and education in developing regions of the world (Brock, 1997; WHO, 1999).

The current Acquired Immunodeficiency Syndrome (AIDS) pandemic, which has apparently spread worldwide in 20 years or less, is an example of the devastating consequences of infectious diseases in a global theater. Despite the existing differences in the degree of importance of infectious diseases in developed versus developing countries, clearly these diseases will remain an important public health problems throughout the world. Hence, eradication or even effective control of infectious diseases requires scientific, economic, political and educational solutions and ultimately global cooperation (Brock, 1997; WHO, 1999).

Of the common infectious diseases, Sexually Transmitted Diseases (STDs) are among the most important public health problems worldwide with major social, medical and economic consequences. STDs include the four classical venereal diseases (syphilis, gonorrhoea, lymphogranuloma venerum (LGV) and chanchroid) and about 20 other diseases often referred to as "second generation" Sexually Transmitted Infections (STIs), caused by bacterial, viral, parasitic and fungal agents (Workneh Feleke and Kloos, 1993).

On average, an estimated 685,000 people are infected every day with an STD and every year there are about 250 million new cases throughout the world (Khanna *et al.*, 1992). In developing

countries in particular, STDs are one of the most common causes of illness and have been ranked among the top ten diseases for which adults seek health care services (Dyck *et al*., 1999).

Ethiopia is also a country where STDs are highly prevalent. According to the 1993 Ministry of Health report, STDs rank 8th in the list of 12 most frequently reported diseases from hospitals and health centers. Like the situation in other African countries, an estimated 80-95% of all cases of STDs are transmitted by a risk defining behavior in Ethiopia. Particularly commercial sex workers (CSWs) play a significant role as a reservoir and high frequently transmitters of STD in the country (Workneh Feleke and Kloos, 1993). In addition to logistic and resource problems, widespread and persisting prostitution, the attitude of many people towards casual and unsafe sexual relation with CSW or other people, rapid socio-cultural changes and political upheaval are recognized to be major obstacles to effective control of STDs in Ethiopia (Workneh Feleke and Kloos, 1993).

Failure to diagnose and treat STDs at an early stage results in serious complications and sequelae including infertility, neonatal and infant infection, ectopic pregnancy, anogenital cancer and death (Gore Felton *et al*., 2003). STDs also facilitate transmission of Human Immunodeficiency Virus (HIV) by increasing infectiousness or susceptibility. In HIV positive individuals, STDs can pose serious health risks because STDs can be exacerbated by a compromised immune system. Conversely, the immune suppression from HIV infection increases the likelihood for the development of opportunistic infections including the acquisition and persistence of STDs. Such bi-directional and mutually enhancing interaction between HIV and other STDs often referred to as epidemiological synergy may account for alterations in the transmission and progression of these infections (Gore Felton *et al*., 2003).

On top of that, the global effort of STD control is hampered by the following reasons. Nearly, one third of all cases of STD involve sexually active individuals who are more likely to have multiple sex partners. Many STDs are also initially asymptomatic, or the symptom they develop may be confused with the symptom of other disease not of a sexually transmitted nature. The

social stigma and discrimination attached to many STDs also prevent individuals from seeking prompt medical care. Antimicrobial resistance in many of the curable STD pathogens is also emerging with further complicating the disease picture (Workneh Feleke and Kloos, 1993; Okeke, 2003).

Neisseria gonorrhoeae, the causative agent of gonorrhoea is a notable STD bacterial pathogen for which there are increasing antimicrobial resistance concerns. Antimicrobial resistance in the gonococcus has been the most significant challenge in controlling gonorrhoea (WHO, 1990). Many of its strains are resistant to the common drugs like Penicillins, Tetracyclines, Spectinomycin, and recently to the Fluoroquinolones (Ciprofloxacin and Ofloxacin) and to the macrolide Azithromycin. These days, the broad spectrum third generation Cephalosporins (Ceftriaxone, Cefixime etc.) are the only classes of antimicrobial agents to which gonococci have not developed confirmed resistance but the cost of these agents limit their use in many developing countries (WHO, 1990; Ison *et al*., 1998; CDC, 2002).

In the context of countries like Ethiopia, the prohibitively expensive cost of efficacious antibiotics and the emergence of single and multiple antibiotic resistant *N. gonorrhoeae* strains call for the search of alternative agents with possible antibacterial effects from natural products. One possible means to manage this problem is the search of pharmacologically active agents by screening traditionally claimed medicinal plants for their possible antibacterial effects.

The major pathogenesis arising from bacterial infection for which vast array of Ethiopian traditional medicinal plants are used include STDs, mainly gonorrhoea and syphilis (Dawit *et al*., 2003). However, efficacy of many of the medicinal plants referred by traditional healers of the country as an effective anti *N. gonorrhoeae* agent is not well documented. In the absence of scientific evidence of efficacy and knowledge of the constituents responsible for the possible biological effects, the validity of these plants as antibacterial agent is questionable and their use would remain locally restricted (Aberra *et al*., 2005).

Plants that have been used as remedies over hundred years in the traditional system of medicine in Ethiopia, constitute an obvious choice for the study. In view of this, it is both necessary and interesting to investigate whether their traditional uses are supported by actual pharmacological effects or merely based on folklore. Previous studies done on crude extracts of some indigenous plants of Ethiopia have shown antibacterial activity coherent with the use of the plants in folk medicine (Aberra *et al.,* 2005)

More recently, crude extracts of 67 traditionally used medicinal plants of Ethiopia were screened for their anti microbial property, where 44 plant species (66%) exhibited activity against standard bacterial strains of *Staphylococcus aurous* (ATCC 27853), *Streptococcus pneumonia* (ATCC 49619), *Eschericia coli* (ATCC 25922) and *Neisseria gonorhoeae* (ATCC 49226). In addition, these plant extracts were also found to be active against standard fungal strains of *Aspergillus flavas* (ATCC 48746) and *Aspergillus niger* (ATCC 10535)(Aberra *et al.,* 2005).

In the above mentioned preliminary antimicrobial activity screening of some Ethiopian medicinal plants, *Albizia gummifera* and *Croton macrostachyus* had exhibited an interesting profile of activity against reference strain of *N. gonorrhoeae* (ATCC 49226) when tested at crude extract level (Aberra *et al*., 2005). However, no work has been done to determine activity of the crude and solvent fractions of these plants against clinical isolates of *N. gonorrhoeae*. This study was therefore; initiated to evaluate the *in vitro* antibacterial activity of crude and solvent fractions of these two traditionally used medicinal plants against clinical isolates of *N. gonorrhoeae.*

2. LITERATURE REVIEW

2.1 Gonococcal disease, historical perspective

The first usage of the term gonorrhoea, by Galen in the second century implied the phrase "a flow of seed" combining the two Greek words gonos (seed) and rhoia (a flow) together. For centuries thereafter, gonorrhoea and syphilis were confused resulting from the fact that mixed infection of the two diseases were often present together in an infected individual (Abrham *et al*., 1986;Todar, 2004).

Parcelsus (1530) thought that gonorrhoea was an early symptom of syphilis. The confusion was further heightened by the classic blunder of English physician John Hunter, in 1767. Hunter intentionally inoculated himself with pus from a patient with symptom of gonorrhoea and ended up by giving himself syphilis. Several years later, the causative agent of gonorrhoea, *Neisseria gonorrhoeae* was first described by A. Neisser in 1879 using specimens taken from the pustular exudates of a case of gonorrhoea. The organism was grown in pure culture in 1885 and its etiological relationship to human disease was later established using human volunteers in order to fulfill the experimental requirements of Koch's postulates (Abrham *et al*., 1986; Todar, 2004).

Gonorrhoea (*chebt* in Amharic) and syphilis (*Kitign*) enjoy better recognition by Ethiopian population and the public awareness to these diseases appears to be fairly high compared to other STDs. However, in some rural highland areas of Ethiopia, gonorrhoea is traditionally considered to be a natural non-preventable disease expected to affect almost every adolescent male as a mark of maturity (Workneh Feleke and Kloos, 1993).

2.2 Epidemiology of gonorrhoea

Gonorrhoea is one of the most wide spread sexually transmitted disease (STD) caused by the bacterium *Neisseria gonorrhoeae* for which humans are the only natural hosts. It is also one of the most frequently reported bacterial STD in adults distributed worldwide probably followed by *Chlamydia trachomatis* (Gerbase *et al.,* 1998). In adults, it is almost invariably transmitted by sexual intercourse. On rare occasions, it can also spread through non-sexual contacts. For instance, infected women may pass the pathogen to their newborn infants during delivery causing an eye infection. Young girls can also contract the disease either from sexual abuse or intimate contact with recently contaminated objects such us dump towels (Lin *et al*., 1998).

Both organism dependent and organism independent factors affect the dynamics of transmission of gonorrhoea. Organism dependent factors like infectivity and virulence determine its intrinsic ability to colonize persist on and infect mucosal surfaces. Organism independent factors are host related factors and depend on complex events described as " risk behaviors in risk space". The recognition of organism independent factors on transmission dynamics also led to the concept of "core groups" of patients who are high frequency transmitters of gonorrhoea (Gerbase *et al*., 1998).

Gonorrhoea is more common in large metropolitan areas, inner city areas, in populations with over all lower socio-economic status, among homosexually active men (HAM), among long distance truck drivers, travelers and clients of commercial sex workers (CSWs). The practice of having unsafe and casual sex with multiple sex partners is considered the main risk factor for acquisition of gonorrhoea. Several studies also indicated that individuals of 15 to 34 years of age to be the most vulnerable groups for many of STDs including gonorrhoea (Workneh Feleke and Kloos, 1993; Aberra Geyid, 2002).

Recent global estimate by WHO suggests that there are about 62 million new cases of gonorrhoea worldwide each year. Of the estimated 62 million new cases, the highest rate of

gonorrhoea (in male and female) occurred in South and South East Asia (2.4%), Sub Saharan Africa (4.8%) and South and Central America (1.7%). Disease rates in developed countries were approximately one-tenth of those of the developing nations, *i.e.* Western Europe (0.27%), North America (0.5%) and Australia (0.5%) (Gerbase *et al*., 1998).

In the industrialized countries, the decline in gonorrhoea was less marked until the advent of AIDS. The publicity surrounding sexual transmission of HIV and the behavioral changes, which accompanied this, increased awareness and led to substantial decrease in the rates of gonorrhoea in Western Europe, Australia and the United states in 1980s and early 1990s (Weller *et al*., 1984; Carne *et al*., 1987). In developing countries, the publicity given to HIV has had less of an effect than in more developed countries. However, control programs in Thailand, which successfully targeted HIV transmission rates also led to decline in gonorrhoea and other STDs. From 1987 to 1993, the number of notified cases of gonorrhoea declined by more than 80%. Steady decline in STD cases was also observed in Costa Rica, Chile and Zimbabwe, but other countries have reported increasing number of cases since 1990 (Gerbase *et al*., 1998).

The prevalence of curable STDs in the general population of Ethiopia is unknown, owing to the lack of laboratory facilities, under reporting, self-treatments, and use of traditional healers or non-use of any health care options for such stigmatized health problems. However, gonococcal and chlamydial infections are assumed prevalent in both sexes of most age groups as more cases of these diseases than any other treatable STD have been reported to the Ministry of Health since the early 1970s (Workneh Feleke and Kloos, 1993).

Recent studies also showed the prevalence of gonorrhoea to be high where up to 10% of pregnant women attending antenatal clinics in Addis Ababa were found asymptomatically infected, indicating the relative prevalence of gonorrhoea in the community (Elizabeth *et al*., 1995; Urassa *et al*., 1997). More recently, 250 positive cases of gonorrhoea were reported from a total of 350 STD patients examined at three study sites, Awassa, Nazareth and the EHNRI reception in Addis Ababa (Aberra *et al*., 2005).

The current recommended antimicrobial agents for the treatment of uncomplicated gonococcal infection are third generation Cephalosporins (Ceftriaxone, Cefixime etc.) and the Fluoroquinolones (Ciprofloxacin, Ofloxacin etc.). Since significant proportions of patients with gonorrhoea are also infected with *C. trachomatis,* Doxycycline or Erythromycin has been added to treat these concomitant infections. Condoms are effective in preventing gonorrhoea (CDC, 1993). The development of an effective vaccine has long been a strategy for the control of gonorrhoea. However, this long search for an effective gonococcal vaccine has not yet born fruit. A number of different components of the gonococcal surface structures have been investigated as possible vaccine candidates. The antigenic heterogeneity and apparent lack of strong immunogenicity pose difficulties for the development of an effective vaccine. Efforts are now continuing to produce an effective vaccine supported by the biology of the organism (Blake and Wetzler, 1995; Hedges *et al*., 1999).

2.3 General features of the gonococcus

N. gonorrhoeae is an exclusively human pathogen having no other ecological niche. It is an aerobe or facultative anaerobe Gram-negative diplococcus organism, 0.6 to 1.0 mm in diameter usually seen in pairs with adjacent flattened sides (Kihlstrom and Danielsson, 1994). It has a very short life cycle of 20 to 30 minute and is an intracellular organism that invades epithelial cells. In clinical specimens, the organism is frequently found intracellularly in polymorphonuclear leukocytes (neutrophils) of the gonorrhoea pustular exudates (Knapp, 1988).

The gonococcus is relatively fragile organism which survive poorly outside the human body being susceptible to temperature changes, drying, exposure to UV light and other environmental conditions (Catlin, 1973). It is closely related to and probably derived from *N. mengitidis* but has become highly adapted to survival in the genital tract (Sarafian and Knapp, 1989). It is a very successful pathogen in that it can evade host defenses persist without severely damaging the host and get transmitted to and infect other hosts there by maintaining it self (Knapp, 1988).

Strains of *N. gonorrhoeae* are variable in their cultural requirement when grown *in vitro*. Since gonococci are inhibited by normal microbial flora, antibiotics should be incorporated in to culture media. So highly selective and enriched media containing a combination of Vancomycin, Colistin, Trymethoprim, Nysatin, haemoglobin, yeast extract, sulfur in the form of cystine, and other media supplements like Isovitalex are advocated for successful isolation of the organism from clinical specimens. Cultures grow best at 35 to 37 ^{0}C in moist atmosphere ($\simeq$70% humidity) and elevated levels of $C0_2$ (3 to5%)(Knapp, 1988; Dyck *et al*., 1999).

2.4 Classification and antigenic types

The genus *Neisseria* consists of two important human pathogens *N. gonorrhoeae* and *N. mengitidis*, the later being a significant cause of acute bacterial meningitis (Knapp, 1988). *N. gonorrhoeae* has been typed employing different systems of classification. Typing of isolates on the basis of their growth requirement (auxotyping) for three growth factors, Arginine, Hypoxanthine and Uracil identified thirteen major types of
N. gonorrhoeae. Strains that require AHU for their growth (AHU^-) were found to be associated with bacteremic infections (DGI) (Aberra Geyid, 2002; Todar, 2004). Typing of *N. gonorrhoeae* isolates by antigenic differences in the porin protein using a panel of monoclonal antibodies specific for epitopes on protein I also identified nine types of *N. gonorrhoeae* strains. Typing of isolates using pilus protein also gave four major types as α, β, γ, and δ strains (Aberra Geyid, 2002). In general, a combined auxotype-serovar classification provides greater resolution for identifying strains among gonococcal isolates and useful in epidemiological investigations. More recently, restriction fragment length polymorphism in genes encoding ribosomal, rNA (ribotyping) and the separation of large DNA fragments by pulsed field gel electrophoresis have been used to type isolates (Knapp, 1988; Todar, 2004).

2.5 Determinants of virulence and pathogenesis

Studies of the gonococcus cell structure have helped to explain aspects of host- parasite interaction. Like the other pyogenic cocci, *N. gonorrhoeae* has a wide range of virulence determinants. The complex surface associated antigenic compositions of *N. gonorrhoeae* that are implicated in virulence and pathogenesis are grouped in to three major classes namely, the pilus protein antigens, the polysaccharide component of the cell wall and outer membrane proteins (Kihlstrom and Danielsson, 1994; Aberra Geyid, 2002).

2.5.1 Pili (Fimbriae)

Adherence to host tissue or mucosal surface can be a formidable event to microorganisms, due to a variety of physical and chemical barriers elaborated by the host system. These barriers are except for minor differences, essentially the same in all individuals. The similarity of barriers in the host led to the co-evolution of microorganisms that utilize mechanisms based on similar theme. Successful attachment (adherence) then requires the participation of a receptor on the host and a molecule on the surface of the microbe called adhesin. In chemical terms, host receptors are invariably carbohydrates, while adhesins are usually proteins (Boyd, 1995).

Pili are prominent adhesins that constitute one of the earliest and the most crucial events in the life cycle of many bacterial pathogens and function as a temporary ligand that mediate association of microbial surface with host target cells (Williams *et al*., 2001;Todar, 2004). Pili of *N. gonorrhoeae* are phase variable surface structures that mediate adherence to host target cells. Nearly all freshly isolated gonococci organisms have pili, fimbrial appendage that extends from the bacterial surface.

Each pilus is composed of thousands of major pillin protein subunits, *eg.* pilE proteins of 20 KDa, pilus associated protein pilC and possibly other components. *N. gonorrhoeae* has only one

copy of the complete pilin gene, PilE, which is a major sub unit protein expressed as a precursor (Propilin) with a seven amino acid leader sequence (Rytkonen *et al*., 2001). PilC is a 110 KDa pilus associated protein that is involved in biogenesis and adherence functions of pilus. PilC is found at the tip of the pilus fiber as well as being surface exposed on the bacterial membrane. Most strains carry two homologous but not identical pilC genes (Salyers and Whitt, 1994).

Piliated and non-piliated gonococcal clones may secrete a soluble smaller pilin (S-pilin). Change in the pilin sequence produces a spectrum of S-pilin production and pilus expression level that may influence epithelial cell surface adherence (Rytkonen *et al.,* 2001). S-pilin secretion has been proposed to allow the release of toxic pilin monomers that can not be efficiently assembled in to pili and have a role in pathogenesis. *In situ* studies of binding to formalin fixed tissue sections demonstrated that binding of S-pilin to human tissue but not to tissue from mouse or rat organs, showing the presence of specific human receptor binding domain in the S-pilin polypeptiable (Rytkonen *et al*., 2001).

The gonococcal pilus specifically interacts with a protein domain of CD46 also called membrane cofactor protein (MCP), a cell surface glycoprotein involved in complement regulation. This glycoprotein is found virtually in all human cell types except erythrocytes (Rytkonen *et al.,* 2001).

Gonococcal pili undergo both phase and antigenic variations and variant expression of pilin on the gonoccocal surface is reflected on colony morphology. *N. gonorrhoeae* uses a variety of mechanisms to undergo phase and antigenic variation of surface antigens. By employing pili alone, it can undergo an antigenic repertoire that may be as large as one million different antigenic variants. Thus, this process presumably accounts for the evasion of host response as well as the chronicity of gonorrhoea (Swanson, 1990; Adams and Seifert, 2000).

2.5.2 Outer membrane proteins

Like all other Gram-negative bacteria, the gonococcus species posses a cell envelope composed of three distinct layers, an inner cytoplasmic membrane, a middle peptidoglycan cell wall and an outer membrane. The outer membrane contains lipooligosaccharide (LOS), phospholipids and a variety of proteins. Among the different classes of proteins expressed on the outer membrane, Protein I, II and Protein III are the most important ones (Kihlstrom and Danielsson, 1994; Aberra Geyid, 2002).

2.5.2.1 Protein II

After adherence is made, some microorganisms do not remain on the epithelial surface instead penetrate to sub-epithelial layers where host defense mechanism awaits for them. Such ability to penetrate below the epithelium is referred to as invasiveness. Most invasive factors are enzymes, but not the only ones in the pathogenesis of infectious agents (Boyd, 1995; William *et al.,* 2001). Following initial attachment, the gonococcus becomes more tightly bound to the epithelial cell surface. This second stage of tight binding and phagocytic uptake is mediated in part by a family of bacterial outer membrane proteins called protein II. It is also called Opa (Opacity) protein, because colonies of bacteria expressing P. II on their surface have a more opaque surface on agar medium than those not expressing this protein. This easily visible phenotype has been very helpful in isolating mutants that have oberrant Opa expression (Gzassme *et al*., 1996; Aberra Geyid, 2002).

Opa is a necessary protein that mediates tight binding and invasion in *N. gonorrhoeae*, and the loss of P. II (Opa) by the gonococcus is associated with the loss of ability to adhere to and invade epithelial cells (Gzassme *et al*., 1996; Aberra Geyid, 2002). P. II also mediate binding of *N.*

gonorrhoeae cells to each other to form micro colonies, P. II from one bacterium binds to the exposed sugar of lipooligosaccharide (LOS) on another bacterium. Such micro colony formation that is functionally analogous to a biofilm also increases the adherence of the bacterium with each other and with different eukaryotic cells including phagocytes (Salyers and Whitt, 1994).

The loss of P.II from the strains cell makes the organisms less skim and less sticky and forms transparent colony on agar medium (Aberra Geyid, 2002). Several eukaryotic receptors for Opa have been identified. CD66, an immunoglobulin super-family cell adhesion molecule belonging to the carcinoembryonic antigen family, is an eukaryotic receptor for the majority of Opa proteins. Certain gonococcal Opa proteins also interact with cell surface associated heparin sulfate proteoglycan receptor (Rytkonen, *et al.,* 2001).
Each Opa protein is produced from its own *Opa* gene. Most strains of *N. gonorrhoeae* have multiple copies of the P. II genes as many as 12 in a single strain of gonococcus. Opa proteins have been characterized by their molecular mass and grouped in to seven OpaA, OpaB, OpaC, OpaD, OpaF, OpaH, and OpaI (Chen *et al.,* 1995). Presumably, homologous recombination events can occur between different copies of the P. II gene to produce antigenic variants. The most observed changes in P. II antigens however, appears to result from phase variation of the different Opa genes (Gzassme *et al*., 1996).

2.5.2.2 Protein I

Virtually all Gram-negative bacteria have porin protein (P.I). Many have multiple porins that fulfill different functions and are expressed under specific conditions. Mostly porin serves prime function of providing channels that allow low molecular weight nutrients to diffuse through outer membrane of bacteria (Salyers and Whitt, 1994). Porin (P.I) is the most abundant and principal protein in the outer membrane of the gonococcus having an estimated molecular weight of 32-39 KDa (Aberra Geyid, 2002).

The production of porin protein is stable genetically and each strain produces the same type of protein and P. I remain the only gonococcal porin described to date. When P. I is extracted from

the outer membrane, protein III (Rmp) co extracts unless special precautions are used. Radioimmuno precipitation using either anti P.I or anti P. III monoclonal antibodies (MAb) precipitates both proteins, implying a strong association between them. There is also evidence for the association of P.I with lipooligosaccharide (LOS) suggesting that P.I occurs in numerous patches in the outer membrane in association with P.III and LOS (Elkins and Sparling, 1990; Salyers and Whitt, 1994).

Based on analysis of proteolytic cleavage products, it is recognized that there are two major forms (alleles) of P.I, which exhibit different orientations in the membrane and possess both common and unique peptides. With the application of monoclonal antibody (MAb) technology, it became clear that there are two stable serotypes of P.I designated as P.IA and P.IB, each of which can be divided in to serovars (Elkins and Sparling, 1990; Hobbs *et al.,* 1999). All gonococci strains produce P.I of either P.IA or P.IB type, no strain produces both serotypes and no hybrid (P.IA – PIB) porins exist in natural isolates. This serotyping scheme along with auxotyping has been a useful epidemiological tool (Elkins and Sparling, 1990).

Strains with the P.IA variants are usually associated with systemic gonorrhoea, where as the P.IB variant is almost associated with infection that remains localized. The reason for this association between P.IA variants and invasiveness of the disease is unknown (Elkins and Sparling, 1990). The role of P.I in the pathogenesis of gonorrhoea has been suggested by studies of several groups. In addition to being an anion selective porin forming protein, it also appears to have another function that may contribute to the ability of some gonococci to survive inside phagocytes (Bjerkenes, 1995). When purified form or in intact bacteria P.I is added exogenously to cultured epithelial cells (artificial lipid bilayers) it inserts into the membrane and collapse the membrane potential probably by making pores. The relative ability to insert in to model membrane is correlated with ability of pathogenic isolates to cause invasive disease (Elkins and Sparling, 1990).

A role of P.I in resistance to complement dependent killing by normal human serum has also been suggested. Resistance to normal human serum is a stable *in vitro* property of nearly all

gonococci that cause disseminated infection and is strongly associated with expression of P.IA in clinical isolates (Elkins and Sparling, 1990; Aberra Geyid, 2002). Early genetic transformation studies showed very close linkage between serum resistance and a gene for P.I. Unlike the case of pili and P.II, there is only one gene for P.I and P.I does not have system for high frequency variation. Nevertheless, variation in the outer membrane porin protein (P.I) affects permeability and penetration of antibiotics in the organisms as well as evasion of host defenses (Elkins and Sparling, 1990; Hobbs *et al*., 1999).

2.5.2.3 Protein III (Rmp)

Protein III (Rmp) is a reduction modifiable, surface exposed, highly immunogenic and conserved protein found in all gonococci isolates. It has a molecular weight of 30-31 KDa and it is found in complex with porin (P.I) protein and LOS. It is structurally related and shares partial homology with ompA protein found in *E. coli* that has a predicted protein size of 24 KDa (Elkins and Sparling, 1990; Todar, 2004). Antibodies to Rmp (P.III) induced either by a neisserial infection or by colonization with *E. coli*, block bactericidal antibodies directed against Por (P.I) and LOS, with this respect, anti Rmp antibodies may increase susceptibility to infection by *N. gonorrhoeae*. Hence, the role of P. III in pathogenesis relates largely to its ability to elicit blocking antibodies that reduce the serum bactericidal activity against *N. gonorrhoeae* (Todar, 2004).

Naturally occurring antibodies that kill serum sensitive gonococci and immune serum that kills serum resistant gonococci contain antibodies directed mostly against LOS, *i.e.,* non-sialylated LOS and P.I on the bacterial surface are known to be effective target for bactericidal antibodies. However, if antibodies produced against P. III (Rmp) react with their antigenic sites on the gonococcal surface, the effect is to block bactericidal antibodies against LOS and P.I to protect the bacterium from complement mediated lyses P.III (Rmp) blocking antibodies may therefore function either by sterically inhibiting the ability of complement fixing bactericidal antibodies to

bind to their target or by diverting the necessary localization of complement from bactericidal sites (Elkins and Sparling, 1990; Salyers and Whitt, 1994).

2.5.3 Lipooligosaccharide (LOS)

Neisserial lipopolysaccharide(LPS) is distinct from enteric LPS by its highly branched oligosaccharide structure and the absence of repeating O- antigen subunits. For these reasons, *Neisserial* LPS is referred to as lipooligosaccharide (LOS) (Minor *et al.*, 2000). LOS has a profound effect on the virulence and pathogenesis of *N. gonorrhoeae*. During growth neisserial LOS and peptidoglycan fragments are released by autolysis of cells. These soluble polysaccharides trigger an intense inflammatory response, subsequent activation of complement, attraction of neutrophils to the site and feeding of the bacteria by phagocytes (Minor *et al.*, 2000; Todar, 2004).

LOS also stimulates the production of tumor necrosis factor (TNF∝) that causes cell damage. The local production of TNF elicited by LOS is thought to be the main cause of damage to the fallopian tubes, as evidenced by fallopian tube organ culture. Many gonococci are able to survive inside the phagocytes, at least until the neutrophils themselves die and release the ingested bacteria (Minor *et al.*, 2000; Todar, 2002). LOS of the outer membrane is thought to be responsible for most of the symptoms of gonorrhoea, although the lyses of the phagocytes themselves contribute to the purulent discharge (Todar, 2004).

Apart from the induction of mucosal damage, LOS also brings about the release of enzymes such as proteases and phospholipases that may be important in pathogenesis (Lindhal *et al.,* 2000). Several studies indicated that strains of *N. gonorrhoeae* produce two distinct extracellular IgA protease, which cleave the heavy chain of the human immunoglobulin at different points with in the hinge region. Split products of IgA_1 have been found in the genital secretions of women with gonorrhoea, suggesting that the neisserial IgA1 protease is present and active during genital

infection. The gonococcal LOS, thus play an indirect role in mediating tissue damage (Todar, 2004).

It is also involved in resistance of *N. gonorrhoeae* to the bactericidal activity of normal human serum (NHS). Specific LOS epitopes are known to be associated with serum resistant phenotypes of *N. gonorrhoeae*. Serum resistance is an important trait for strains that cause systemic infections (Minor *et al*., 2000; Todar, 2004). Strains that cause uncomplicated genital infections are usually killed by normal human serum and are called serum-sensitive. Strains that cause systemic infections are not killed by most normal human serum and are called serum resistant (Lindhal *et al*., 2000).

Changes in the length of carbohydrate chains or other changes in carbohydrate portion of LPS can render the bacterium serum resistant (Minor *et al*., 2000). Pathogenic *Neisseria* spp. can also take an activated form of N-acetylneuramic acid (Sialic acid) from human blood and covalently bind it to galactose residues on their LOS, forming a microcapsule of sialylated LOS. This process converts serum sensitive organism to a serum resistant one. This is because sialic acid is a ubiquitous host molecule and this does not activate complement and because the membranes attack complex (MAC) does not form productively around the altered LOS (Minor *et al*., 2000).

There is also antigenic similarity between *Neisserial* LOS and antigens present on human erythrocytes. This similarity to “self” (molecular mimicry) may preclude an effective immune response to LOS antigens (Todar, 2004). *N. gonorrhoeae* undergoes antigenic variation by sialylation of its LOS. However, the main antigenic variability in LOS (change in oligosaccharide composition) operates using unknown mechanisms. In all cases, the variability in LOS enables it to evade host immune response and contributes to stable resistance in *N. gonorrhoeae* (Minor *et al.,* 2000).

2.5.4 Iron acquisition proteins

In humans and other vertebrates, iron after being assimilated from dietary components by the mucosal cells of the jejunum, passes in to the blood stream and is transported there being attached to the ubiquitous iron binding glycoprotein called transferrins (Ratledge and Dover, 2000). Basically, there are three major classes of transferrins(Tf) namely, serum transferrin (serotransferrin), lactoferrin (Lf) or lactotransferrin, which is found in many extra cellular fluids and ovotransferrin (conalbumin) which is found in the albumin of eggs (Gray Owen and Shryvers, 1996; Raulston, 1997).

Tf is never fully saturated with iron and this "spare" capacity is important to take up any surplus free iron that may arise in the blood or other body fluids. With this respect Pathogenic bacteria seem limited in their capacity to multiply *in vivo* because of the "iron withholding" defense mechanisms of the host. Therefore invading pathogens need to have mechanisms for the acquisition of iron from their host. This is because the acquisition of iron is the key step in the development of any pathogen in the host and possibly the major determinant as to whether a microorganism that finds itself within an animal is able to maintain itself there in. Without this ability, the pathogen face difficulty to grow and will effectively be eliminated by direct host defense mechanisms or will die of nutrient starvation (Gray Owen and Schryvers 1996; Raulston, 1997).

Two systems have been developed by pathogens to obtain iron. These involve, direct contact of the bacterium with the source of iron (usually Tf or individual iron proteins). Alternatively, bacteria may synthesize an ultra high affinity compound that physically captures the iron from the host protein by virtue of its superior binding strength. Such compounds are called siderophores and play vital role in microbial iron acquisition (Biswas and Spariling, 1995). Gonococci lack the siderophore system however, they respond to iron-depleted environment with

the production of multiple iron-regulated proteins, (FeRps), several of which are outer membrane proteins in origin (Lee and Bryan, 1989). Gonococci can grow in the presence of human Transferrin (Tf) or Lactoferrin (Lf) as a sole source of iron, and these proteins are probably their major source of iron during infection. This specificity is thought to be the main reason why gonococci organisms are exclusively human pathogens (Todar, 2004). That is on iron limitation, many of the pathogenic members of the *Neisseriaceae* produce two highly conserved Tf binding proteins that specifically bind the Tf of their particular host organism. Gonococcal mutants that are devoid of these proteins are avirulent in humans (Lee and Bryan, 1989; Ratledge and Dover, 2000).

Basically, the gonococcus is able to form two transferrin (TbpA and TbpB) and lactoferrin receptors (LbpA and LbpB) on its outer membrane, which are induced under iron limited conditions and are able to directly extract iron from transferrin and lactoferrin respectively. These proteins can also extract iron from heme and haemoglobin (by destruction of erythrocytes and hydrolysis of hemoglobin it self (Genco and Desai, 1996; Chen *et al.*, 1997). TbpA are outer membrane (OM) receptors that are thought to serve as channels through which ferric ion crosses the OM after its release from its bound Tf, and TbpB actually recognizes Tf and mediates iron uptake from it. This is because TbpB lacks transmembrane sequence and is thought to reside to whole outer membrane surface attached by an N- terminal lipid fragment where as the TbpA function to help internalization of the captured molecule (Lee and Bryan, 1989).

Iron deficiency is an undesirable state, and accordingly bacteria have developed a serieus of responses to adapt to this state. In *Neisseria* spp. besides the elaboration of iron binding proteins, they respond to iron-limited environment by expressing a number of other genes like the *fur* (Ferric uptake regulation) genes that are other wise silent during iron sufficient growth. Other proteins of outer membrane origin are also identified with different pathogenic effects and immune responses (Genco and Desai, 1996; Chen *et al*., 1997; Ratledge and Dover, 2000).

2.6 Clinical spectrum of gonococcal infection

Except vulvovaginits in prepubertal girls and conjunctivitis in newborn, gonococcal infections are usually spread by sexual contact (vaginal, oral or anal) even without ejaculation. The most common sites of infection are mucosal surfaces lined with epithelial cells (urethra, cervix, rectum, pharynx and conjunctiva) causing symptomatic or asymptomatic infections (Lin *et al*., 1998). The pathogenic mechanism involves the attachment of the bacterium to non-ciliated epithelial cells via pilli (fimbrieae) and Opa (P.II) protein, penetrate them and multiply on the basement membrane. The inflammatory response then triggers phagocytosis of the organism by PMNs resulting in the formation of a purulent discharge (Aberra Geyid, 2002).

It is difficult to diagnose gonorrhoea on clinical grounds alone. This is because, the signs and symptoms of gonorrhoea may be absent or overlap to those caused by other agents, most notably *Chlamydia trachomatis.* Consequently, laboratory procedures are needed for diagnosis, case finding and test of cure, although the current trend is empirical diagnosis (syndromic approach) of many of the curable STDs (Dyck *et al*., 1999; Hedges *et al*., 1999).

2.6.1 Genital infections in male and female

Uncomplicated gonorrhoea in adult male is an inflammatory and pyogenic infection of the mucus membrane of the anterior urethra. The inflammatory response triggers a discharge which is the most common symptom that may range from a scanty, clear or cloudy fluid to one that is copious and purulent exudates which is more obvious in male than female (Boyd, 1995; Sherrard). From the anterior urethra infection can progress to the posterior urethra with in 14 days showing more advanced symptoms like increasing dysuria, polyuria and occasionally headache and fever as well as pain and intense burning upon urination (Boyd, 1995; Sherrard and Barlow, 1996).

More than 90% of men with urethral gonorrhea will develop symptoms with in 5 days. Males with asymptomatic urethritis are important reservoirs for transmission and are at increased risk for developing serious complications (Barlow and Phillips, 1978; Boyd, 1995). If left untreated, various complications such as chronic infections of the prostate (prostatitis), seminal vesicles,

epididmyces and urethral strictures may occur. The organism can also extend to testicles (orchitis) resulting in male sterility (Boyd, 1995; Sherrard and Barlow, 1996).

Endocervical infection is the most common form of uncomplicated gonorrhea in female. Acute gonorrhea in female may also involve urethra, Skene's and Bartholin's glands (Barlow and Phillips, 1978). Symptoms of acute infection of the lower tract are seldom severe. The most prevalent symptoms of acute infection are abdominal or pelvic pain increasing vaginal discharge and dysuria (Radcliffe *et al*., 2004). About 50% of women with cervical infection are asymptomatic who are at higher risk of developing pelvic inflammatory disease (PID) and disseminated gonococcal infection (Boyd, 1995). Women who experience an episode of PID are at increased risk of having ectopic pregnancy and tubal infertility. After one episode of PID, a woman's risk of becoming sterile is 12% and reach 50% if she has had three episodes of PID. Such asymptomatic carriers represent a major obstacle in controlling the spread of gonorrhoea (Boyd, 1995).

Chronic infections may be indicated by tenderness of the abdomen, backaches, low-grade inflammation of urethra and genitourinary tract associated structures and profuse menstrual flow. Squamous epithelium that lines the adult vagina is not susceptible to infection. However, the prepubertal vaginal epithlum that has not been keratinized under the influence of estrogen is vulnerable to gonococcal infections. Hence, young girls who contract gonorrhoea either from sexual abuse or intimate contact with recently contaminated object may develop a severe infection called vulvovaginits (Boyd, 1995; Radcliffe *et al*., 2004).

2.6.2 Extra genital gonococcal infections

Local infections: apart from the traditional sites, local infections occur in various areas of the body. Rectal infection (proctitis) with *N. gonorrhoea* occurs in about one third of women with cervical infection. It most often results from autoinoculation of the perianum with cervical exudates and the spread of the microorganism to the rectal mucosa (Boyd, 1995). Rectal infections in homosexual men usually result from anal intercourse and are often symptomatic.

Ocular infection in newborn (Ophtalmia neonatarum) can develop as the child passes through the birth canal of an infected mother. Such infections can have serious consequences like corneal scaring and perforation leading to blindness. Other extra genital infection takes the form of pharyngitis (Boyd, 1995).

Disseminated gonococcal infection (DGI): on rare cases gonococci from the primary site of infection can spread via blood stream to cause most common form of disseminated gonoccoal infection (DGI) like anthritis-dermatitis syndrome and occasionally mengitidis, endocarditis and other conditions. Between 50 and 70% of patients with DGI have dermatitis and more 90% have arthropathy. The arthritis is usually felt in several joints with the knee, ankles and wrists being the most frequently involved parts. Eventually joint destruction occurs if the infection is left untreated (Knapp and Holmes, 1975; Boyd, 1995).

2.6.3 Association with HIV

The presence of genital ulcer disease including genital herpes, syphilis and chanchroid as well as non ulcerative STDs such as gonorrhea, chlamydia and trichomoniasis, enhance the sexual transmission and acquisition of HIV and appears to be fueling the HIV epidemic (Fonk *et al*., 2000; Gore Felton *et al*., 2003). Non-ulcerative STDs increase the risk of acquiring HIV by three to five folds through increasing the prevalence of HIV shedding and viral load in genital secretions (Fonk *et al*., 2000).

In HIV infected men gonococal infection increase shedding of viral load in semen by ten fold. Inflammatory STDs also attract $CD4^+$ lymphocytes to the inflammatory surface, which disrupts epithelial and mucosal barriers to infection, thereby increasing susceptibility to HIV infection (Gore Felton *et al*., 2003). Control of inflammatory STDs like gonorrhoea may therefore offer a means of reducing the spread of HIV/AIDS epidemic (Fonk *et al*., 2000; Gore Felton *et al*., 2003).

2.7 Antibiotic resistance in *N. gonorrhoeae*

The emergence of resistance to antibiotics has undermined the idealistic hope that bacterial infection would cease to be an important cause of morbidity and mortality all over the world (Okeke, 2003). Among the etiological agents of treatable STDs, *N. gonorrhoeae* and *Haemophilus ducreyi* stand out to be the first group because of the extent to which antibiotic resistance compromises the effectiveness of individual case management and disease control programs (WHO, 1990).

Resistance of *N. gonorrhoeae* to antibiotics has been recognized since 1940s. This can be a result of either mutation at different loci of *N. gonorrhoeae* genome or by the transfer of plasmid from resistant strains of *N. gonorrhoeae* to sensitive ones, which leads to an increase of the MIC of the antibiotics for the organism. Eventually, treatment failure can occur (Ivens *et al*., 2000). The gonococcal strains continue to develop resistance both to older, less expensive antimicrobials and to more recently introduced new generation of the agents and multiple drug resistance problem is becoming more common (WHO, 1990). In response to the increasing frequency of isolation of Penicillin, Tetracycline, Spectinomycin and Azithromycin resistant strains of *N. gonorrhoeae*, the use of broad spectrum third generation Cephalosporin antibiotics (Cefixime, Ceftriaxone etc.) or Fluoroquinolones (Ciprofloxacin, Ofloxacin etc.) were recommended for the primary treatment of uncomplicated gonorrhoea (Lai-King *et al*., 2002).

However, resistance to Fluoroquionolones particularly to Ciprofloxacin and Ofloxacin has now also been emerged and spread recently in areas with high burden of gonococcal disease combined with antibiotic over use or misuse (Knapp *et al*., 1997; Ivens *et al*., 2000; CDC, 2002). This days, the broad spectrum third-generation Cephalosporins (Ceftriaxone, Cefixime etc.) are the only classes of antimicrobial agents to which gonococci have not developed confirmed resistance, but the cost of these agents limit their use in many developing countries. According to reports of recent studies, few clinical isolates were found to exhibit decreased susceptibility even to the oral agent Cefixime (WHO, 1990; Ison *et al*., 1998; CDC, 2002).

Several studies conducted in Ethiopia have shown emergence and spread of antibiotic resistance properties in many bacterial pathogens, which increasingly hamper effective treatment of infectious diseases like gonorrhoea (Dodge and Wallace, 1975; Ashenafi Belihu and Lindtijorn, 1999; Aberra Geyid, 2002; Zeleke Wolde Tenssay, 2002). The first case of penicillin resistant *N. gonorrhoeae* was reported from Ethiopia in 1969. There after numerous investigators reported the occurrence of penicillinase producing *N. gonorrhoeae* (PPNG) and betalactamase producing strains, due to indiscriminate use of Penicillin Tetracycline and other antibiotics for numerous health problems (Dodge and Wallace, 1975; Eyasu Habtegabrel *et al*., 1983,1987; Workneh Feleke and Kloos, 1993).

The increasing antimicrobial resistance in *N. gonorrhoeae* has ultimately affected the control of gonorrhoea. This call for alternative approaches like the development of new anti-infective agents that could replace those antibiotics that have been losing their effect against *N. gonorrhoeae*. In addition to appropriate use of efficacious antibiotics, the search of new pharmacologically active agents by screening of traditionally used medicinal plants for their possible antibacterial effects could be a possible alternative (Shu 1998; Guillemot *et al*., 2001).

2.8 Application of medicinal plants

The use of plants as remedies to treat various forms of an ailment has been a common practice all over the world. According to the WHO report, nearly 70-90% of the world population depends on herbal medicine, which caters for most of African population (Akerele, 1993; Nair and Nathan, 1998). Pharmacological and phytochemical studies done with medicinal plants used traditionally in other countries have led either to isolation of novel structures for the manufacture of new drugs or templates that served for the production of synthetically improved therapeutic agents (Aberra *et al*., 2005).

Out of 104 new drugs developed over 37 years, 60 originated from plants used in traditional medicine of China. Modern drugs discovered from natural products such as Quinine,

Vincristine, Digoxin, Digitoxin, Emetine and Artemisine to mention just a few, exemplifies the huge potential that still exists in plants for the production of many more novel pharmaceuticals (Plotkin, 1988). Pharmacological studies done with essential oils from 15 species of aromatic plants obtained in Brazil have shown activity coherent with the use of the medicinal plants in folk medicine. These studies have dealt with the effects of these oils on muscle contraction, with their anti inflammatory and anti bacterial activity (Holetz *et al*., 2002).

Laboratories of the world have also literally found thousands of phytochemicals, which have inhibitory effect on all type of microorganisms *in vitro*. However, their effectiveness in whole organisms, toxicity assays and their possible effects on the beneficial micro biota are not yet conducted for many of these compounds (Cowan, 1999). The prohibitively expensive cost of modern antibiotics to treat various infections and the clinical importance of drug resistant pathogens, particularly in third world countries makes the search of alternative anti-infective agents from natural products more urgent (Shu, 1998). People in developed countries are also shifting back to herbal medicine because of the serious side effects of the modern drugs (Nair and Nathan, 1998).
At present, scientists of the world from divergent fields are investigating plants anew with an eye to their antimicrobial usefulness. The search for dietary supplements derived from plants has also been accelerated in recent years. A sense of urgency accompanies the search of novel compounds as the pace of plant species extinction continues (Cowan, 1999).

Ethiopia is one of the six regions of the world where about 60% of plant with healing potential are said to be indigenous in origin (Kapa, 1996; Balcha Abera, 2003). The major pathogenesis arising from bacterial infection for which various preparations of traditional medicinal plants are employed in Ethiopia include STDs (mainly gonorrhoea and syphilis), tuberculosis, leprosy, cholera, tropical ulcer, anthrax etc. (Dawit *et al*., 2003).

Garlic (*Allium sativum*) (garlic juice), Endod (*Phytolaca dodecandra*) (root powder), Bisanna (*Croton macrostachyus*) (actively growing leaf powder, unripe fruits, bark, sap of the plant and

decoctions of the roots), Sessa (*Albizia gummifera*) (root/bark powder cooked with meat and soup), Gibira (*Lobelia rhyncopetalum*) (various forms of preparation of the plant) are some of the common medicinal plants referred by traditional healers of the country as an effective anti *N. gonorrhoeae* agent (Azene Bekele, *et al*., 1993; Dawit *et al*., 2003). However, their efficacy against the pathogen *N. gonorrhoeae* is not well documented. In the absence of scientific evidence of efficacy and knowledge of the constituents responsible for the possible biological effects, the validity of these plants as anti infective agents is questionable and their use would remain locally restricted (Aberra *et al*., 2005).

Previous studies conducted in Ethiopia have pinpointed the antibacterial activity of some indigenous plants used in traditional medicine (Mintesnot Ashebir and Mogessie Ashenafi 1999; Dawit *et al*., 2002a, 2002b; Hirut *et al.,* 2002). Recently various preparation of *Lobelia rhyncopetalum* was reported to possess anti *N. gonorrhoeae* effect. Extracts of this plant were reported to contain several alkaloids that were effective against *N. gonorrhoeae* at very low concentration. Root extracts of *Plumbago zeylanica* has also been reported to be effective against a range of pathogens including *N. gonorrhoeae.* Chloroform extracts of this plant showed significant activity against penicillin resistant and penicillin sensitive strains of *N. gonorrhoeae* (Hirut *et al*., 2002; Dawit *et al*., 2003).

More recently, Aberra *et al* (2005) have screened 67 traditionally used medicinal plants collected from several regions of Ethiopia in the wild for their anti bacterial and antifungal activity, where 44 plant species (66%) exhibited activity against one or more of the organisms tested. In this screening study, *Albizia gummifera* and *Croton macrostachyus* crude hydro alcoholic extracts showed an interesting profile of activity against reference strain of *N. gonorroeae* (ATCC 49226). However, no work has been done to determine activity of the crude and solvent fractions

of these plants against clinical isolates of *N. gonorrhoeae.* This study was therefore, initiated to evaluate *in vitro* antibacterial activities of crude and solvent fractions of these two medicinal plants against clinical isolates of *N. gonorrhoeae* and further justify whether their traditional uses are supported by actual pharmacological effects or merely based on folklore. A short description of the two medicinal plants is given below.

Croton macrostachyus Hocht. (Euphorbiacease):

C. macrostachyus is a deciduous shrub or tree widely spread on forest margins, along roadsides and secondary woodlands. It grows mostly on soils of volcanic origin and it is more common in west of Guinea, South Angola, Zambia, Malawi and Mozambique. In Ethiopia it occurs in Dry, Moist and Wet Weynadega and Dega as well as in upper altitudes of Dry Kolla agro climatic zones in Tigray, Gondar, Gojam, Wollo, Bale Shewa, Illubabor, Kefa, Sidamo and Hararge regions at 1,100-2,500m above sea level. Apart from its medicinal uses, the tree is also used as firewood, timber, forage (young leaves) and as mulch. Means of propagation include, sowing of the seeds or the use of seedlings (Gilbert, 1989; Azene Bekele *et al*., 1993).

Albizia gummifera (J.F. Gmel) (CA.sm (Fabaceae or Leguminosae):

It is a deciduous large or medium tree preferring forest margins and open forest throughout mountainous regions. It occurs mainly in Eastern Tropical Africa and also Cameroon, Nigeria, Madagascar, Congo and South Africa at an altitudinal range of 1000 to 2300 meters. In Ethiopia, this tree is distributed in upland reverine forest at 1700-2400m in Gojam, Wollega, Illubabor and Kefa regions. Means of propagation include, direct sowing of seeds or the pod of the tree. Other uses of the tree include for making beehives, boats, soil amendment (N_2-fixing), as mulch and shade tree, (Thulin, 1989; Azene Bekele *et al*., 1993).

3. OBJECTIVES OF THE STUDY

3.1 General objective

The aim of this study was therefore to evaluate *in vitro* anti-bacterial activity of crude and fractionated extracts of *Albizia gummifera* (seeds) and *Croton macrostachyus* (leaves) against clinical isolates of *Neisseria gonorrhoeae*.

3.2 Specific objectives

- To collect and characterize clinical isolates of *Neisseria gonorrhoeae* from STD Patients.
- To collect plant species of *Albizia gummifera* and *Croton macrostachyus* and prepare crude extracts and solvent fractions of these plants.
- To evaluate the efficacy of the crude and fractionated extracts of these medicinal plants against the clinical isolates and reference strains (ATCC 49226) of *N. gonorrhoeae.*
- To determine the minimum inhibitory concentrations (MICs) of the crude extracts and solvent fractions of these plants against the test organisms, and

compare their alternativeness for the use of common antibiotic drugs.

- To give scientific evidence of efficacy and select good candidate medicinal Plant(s) for further investigation through *in vivo* experiment models.

4. MATERIALS AND METHODS

4.1 Study site

The experiment was conducted at the Clinical Bacteriology Laboratory and Drug Research Department of the Ethiopian Health and Nutrition Research Institute (EHNRI) in collaboration with Addis Ababa University (AAU), Biology Department.

4. Specimen collection site

After a preliminary survey of the flow of STD patients in selected health institutions in Addis Ababa, a total of eight health centers were selected for sampling. Namely, Arada, Kazanches, Kotebe, Entoto, Addis Ketema & Lideta government health centers and one private clinic and the EHNRI reception were included.

4.3 Characteristics of the Study Population

Male and female adult STD patients (15-49 years of age) attending STD clinics and those who fulfill the criteria for clinical diagnosis of uncomplicated gonorrhoea (Defined by the presence of a mucopurulent endocervical or urethral discharge on physical examination, dysuria, polyuria, burning on micturation and sexual exposure to a person infected with *N. gonorrhoeae*) (CDC, 2004)) were enrolled for participation in the study. Suggestive

diagnosis made by the physician for the presence of gonococcal infection was based on routine clinical and syndromic criteria.

The examination of patients (who come to STD clinics to get treatment for the specified disease and with the required clinical criteria) at the out patient department (OPD) was done by attending physicians. Among all attending STD patients with the required clinical criteria for uncomplicated gonococcal infection, actual patient selection was based on the criteria that no antimicrobial drug was taken during the period of one or two weeks prior to visiting the STD clinics. A pre-tested questionnaire (Annex I) was used to collect relevant information at the time of physical examination regarding the patient's clinical status and history. Patients were also inquired whether they had used any traditional medicinal plants or visited traditional healers before coming to clinics.

4.4 Ethical considerations

Before conducting this study, ethical clearance was obtained from Addis Ababa University, Biology Department and the thesis proposal was reviewed and approved by Research and Ethical Clearance Committee (RECC) of EHNRI for its scientific and academic merits. Volunteer study subjects who fulfill the criteria for clinical diagnosis of uncomplicated gonorrhoea were informed about the study objectives and written consent was obtained from each patient prior to participation in the study (Annex II).

While taking urogenital specimens from volunteer STD patients, standard clinical procedures for STD diagnosis were followed in accordance with the ethical standards of the country (EHNRI). Assessment of the case, suggestion of gonococcal infection and sample taking was done by attending medical officers under proper settings using standard and safe equipment. Patients name and clinical history were kept confidential. Volunteer participants were provided with free laboratory confirmatory test results and antibiotics free of charge by the investigator.

4.5 Collection and transport of clinical specimens

The primary symptomatic or sexually exposed anatomic sites, urethra in men and the endocervix in women were routinely sampled and cultured for isolation of *N. gonorrhoeae*. Urogenital specimen was collected from a total of 250 male and female STD patients at different study sites in Addis Ababa over a period of six months (May 2005- October 2005). In women and heterosexual men, clinical specimens were collected following the procedure described by Dyck *et al* (1999).

Endocervical specimens were collected according to the following procedures. Sterile vaginal speculum was moistened with sterile warm water and inserted in to the vagina. The exocervix was then cleaned with a cotton ball moistened with a sterile physiological saline using forceps. A sterile Dacron swab was then passed 20-30 mm in to the endocervical canal and gently rotated against the endocervical wall for up to 5-10 seconds to allow absorption of exudates.

Urethral specimens were collected at least one hour after the patient has urinated. For patients with evident discharge, the material was collected directly on a swab. For patients without visible discharge at the time of examination, the area around the urethral opening was cleaned using a swab moistened with physiological saline. The urethra was then gently massaged from above downwards and a sample of discharge was collected using a sterile Dacron swab. Swab collected specimens were then immediately immersed in a sterile test tube containing Amies transport medium, labeled and transported from the STD clinics to the clinical bacteriology laboratory of EHNRI by the investigator for analysis.

4.6 Laboratory examination of urogenital specimens

4.6.1 Microscopy and bacteriological culture

Prior to inoculating each specimen directly on to a culture medium, a smear for microscopy was prepared by rolling the swab on to a clean glass slide and allowed to air-dry for some time. The smear was then fixed by passing the slide rapidly through a flame three times while keeping the slide upper most, to avoid overheating and cell damage. The fixed smear was then Gram stained and examined under a bright light microscope with a x100 objective. The observation of Gram-negative intracellular diplococci /extra cellular diplococci organism was taken as a presumptive identification of *N. gonorrhoeae* (Knapp, 1988; Dyck *et al*., 1999).

For isolation of *N. gonorrhoeae* the recommended highly selective and enriched media *i.e*. Modified Thayer Martin Medium (MTM) prepared by the use of GC agar base medium (Oxoid CM 367), 1% Isovitalex supplement (Oxoid SK 0090A), 1% (w/v) soluble bovine haemoglobin powder (Oxoid L53) and VCNT (Oxoid) selective inhibitors (code SR 091E) containing Vancomycin, Colistin methane sulphate, Nystatin and Trimethoprim was used (Knapp, 1988; Dyck *et al*., 1999; Todar, 2004).

The swabs containing the specimen were rolled approximately one quarter of the surface of the MTM plate. Using a sterile bacteriological loop, the inoculum was streaked over the remaining part of the medium to ensure growth of isolated pure colonies. The plates were immediately incubated at 35-37 ^{o}C in moist ($\simeq$70% humidity supplied by placing moist cotton at the bottom of the jar) and CO_2 enriched atmosphere (containing 3-5% CO_2, supplied by using the low tech. candle extinction jar).

The presence of growth was then examined after 18, 24, 48 or 72 hours of incubation. The observation of pinkish brown colonies having a diameter of 0.5-1mm varying from transparent to opaque and convex to flat with defined margins were presumptively identified as *N. gonorrhoeae* colonies (Knapp, 1988; Dyck *et al*., 1999; CDC, 2004).

4.6. 2 Presumptive and confirmatory identification of isolated colonies

Typical colonies with gonococcal like appearances on chocolate agar (Oxoid CN 0481) plate were further examined by Gram-staining and tested for oxidase, catalase and superoxol reactions before carrying out definitive identification tests.

Gram stain of culture colonies: from 24 hour old culture, a single suspect colony was taken and emulsified in a drop of saline on a glass slide, which was then air-dried, heat fixed and stained.

*Oxidase test***:** oxidase identification sticks (Oxoid BR0064A) were used to perform the test. Oxidase sticks along with their containers were removed from refrigerator and allowed to stand for five minutes at room temperature. A well separated representative colony from an overnight chocolate agar plate was touched by rotating the oxidase stick tips impregnated with a solution of N, N-, dimethyl-p- phenylenediamine oxalate, ascorbic acid and α-naphtol. A positive reaction was recorded when the impregnated end of the stick develop a blue-purple color with in 30 seconds (Knapp, 1988; CDC, 2004).

Catalase test: slide method was used to perform this test, where few colonies to be tested were picked up from the chocolate agar plate with a loop and emulsified directly in a drop of 3% hydrogen peroxide at the center of a clean glass slide. The observation of an immediate reaction in forming gas bubbles was taken as positive result (Knapp, 1988; CDC, 2004).

Superoxol test: few colonies to be tested were picked up from the chocolate agar plate with sterile write loop and emulsified directly in a drop of 30% w/v hydrogen peroxide placed at the center of a clean glass slide. Immediate and abundant production of bubbles or effervescence within two seconds was defined as a positive superoxol test result (WHO, 1998; CDC, 2004). The observation of Gram-negative diplococci organism on microscopic examination from genital specimens having positive result for catalase, superoxol and oxidase tests was taken as a presumptive identification of *N. gonorrhoeae* (Knapp, 1988; Dyck *et al*., 1999; CDC, 2004).

The presumptively identified *N. gonorrhoeae* organisms were confirmed by performing carbohydrate utilization tests using API-NH identification kit. Subculture was prepared by transferring typical colonies from primary isolation plates on to non-selective chocolate agar

(Oxoid CN 0481) and incubated for 18-24 hours. The subculture plates were then carefully examined to ascertain that the cultures are pure before carrying out the confirmatory testing.

Few well-separated pure colonies were picked up using a swab and inserted in to 2ml of NaCl 0.85% solution. The resulting bacterial suspension was adjusted to turbidity equivalent to McFarland standard #4($8x10^8$). The API-NH strips containing dehydrated substrate were then inoculated by distributing the prepared bacterial suspension. The strips were then covered and incubated for two hours at 35-37 ^{o}C under aerobic condition. After a two-hour incubation period, isolates producing acid only from glucose but not from lactose, maltose and sucrose and having a positive relation for ProA (Proline arylamidase) were definitely confirmed to be *N. gonorrhoeae* isolates.

The confirmed isolates were designated as UD1 - UD17 and VD1- VD2 to denote their source, number as well as to differentiate response of each isolate to the plant extracts and standard antibiotics (where UD, through UD17 refers 17 isolates obtained from urethral discharge and VD1 and VD2 refers two isolates obtained from cervical discharge). The isolates were then stocked for subsequent use by taking a loop full of pure gonococcal isolates from an overnight culture of chocolate agar and placing them in small tubes containing Trypticase Soya Yeast (TSY) + 20% glycerol stocking solution and kept frozen at **-**80°c until required (Dyck *et al*., 1999). Reference strains of *N. gonorrhoeae* ATCC 49226 obtained from the EHNRI were also maintained frozen at -80°c in tubes of TSY + 20% glycerol stocking solution. From the frozen stock, the organisms were sub cultured on chocolate agar for different assays.

4.6.3 Antimicrobial susceptibility testing

In order to have basic susceptibility information, clinical isolates of *N. gonorrhoeae* were screened for antimicrobial suceptibility pattern using the agar disc diffusion method. The

following standard antibiotic discs, Erythromycin, Ciprofloxacin, Spectinomycin, Kanamycin, Cotrimoxazole, Ceftriaxone, Pencillin and Tetracycline were tested against the test organisms following the method defined by the NCCLS (2002) performance standards and with the NCCLS quality control strain *N. gonorrhoeae* ATCC 49226 as a reference

A 60 ml of standard sensitivity testing medium for *N. gonorrhoeae* (*i.e.* GC agar base medium with 1% Isovatalex supplement) was poured in to 150 mm diameter plate to a uniform depth of 3 to 4 mm and allowed to solidify. Bacterial Suspension was prepared by taking one or more gonococcal colonies from an overnight culture of chocolate agar (Oxoid CN 0481) and inserting in a tube with 2ml of Muller Hinton broth. Turbidity of the suspension was then adjusted to turbidity equivalent to McFarland standard # 0.5(10^8 cells/ml). A sterile swab was then dipped in to the suspension, rotated several times and then pressed firmly on the inside of the tube above the fluid level to remove excess fluid. The swab was then streaked (within 15-20 minutes of bacterial suspension preparation) across the surface of the standard test medium evenly by rotating the plate each time to ensure homogeneous distribution of the inoculum on to the surface of the agar plate. The antibiotic discs were then dispensed and pressed to stick on the standard sensitivity-testing medium. After 3 to 5 minutes of application, the plates were incubated in an inverted position in humid atmosphere containing 3-5% CO_2, at 35-37 $^{o}{}_{C}$ for 20 to 24 hours. Result for ATCC 49226 was read first and checked for agreement whether it is in the control range. Inhibition zones diameters for clinical isolates were then measured using a sliding caliper, recorded and compared to interpretive standards for disc diffusion inhibition zone for *N. gonorrhoeae*.

4.7 Plant materials

4.7.1 Collection and identification

Seeds of *Albizia gummifera* and leaves of *Croton macrostachyus* used in this study were collected in August 2005 from around Bedelle and Bale approximately 540 and 500km away from Addis Ababa respectively, having an altitudinal range of 900-3900m above sea level.

These plant species were selected on the basis of the available ethnomedical information as well as a preliminary screening study result on the effects of crude hydro-alcoholic (20-80%) extracts of these plants against reference strains of *N. gonorrhoeae* ATCC 49226 (Aberra *et al* 2005). The plants were authenticated as *A. gummifera* (Voucher No. AG-2110) and *C. macrostachyus* (Voucher No. CM-1194) by a taxonomist in the Department of Drug research, EHNRI, Addis Ababa.

During a preliminary investigation of antibacterial activity testing, the type of secondary metabolites in these plant extracts were also identified with possible biological effect which include saponins (in *A. gummifera* only), alkaloids, polyphenols, unsaturated sterols/triterpens and glycosides/carbohydrates in both *C. macrostachyus* and *A. gummifera* (Aberra *et al*., 2005).

4.7.2 Crude extract preparation and solvent – solvent partitioning

The test plants were subjected to crude hydro alcoholic (20- 80 %)extraction and solvent – solvent partitioning process (Fig.1). Seeds of *A. gummifera* and leaves of *C. macrostachyus* were air-dried at room temperature and ground to powder. About 300g powdered seeds of *A. gummifera* and 350g leave powder of *C. macrostachyus* were then submitted to maceration/percolation process with 80% methanol for 48 hours at room temperature being protected from sunlight. The hydro alcoholic extract obtained was filtered using What man No. 1. filter paper and then concentrated under reduced pressure in rotary evaporator to give 48 and 34g gummy residue total extracts of each plant species respectively. The crude extracts were then kept in a tightly stoppered bottle in a refrigerator (2-8°C) until tested against the organisms and subjected to solvent-solvent partitioning process (Holetz *et al*., 2002; Aberra *et al*., 2005).

Part of 80% methanol extract was suspended in about 200ml-distilled water. The water suspension was then shaken with trichloromethane ($CHCl_3$) and allowed to be partitioned in to chloroform-aqueous layer. The chloroform fraction was combined evaporated and labeled as fraction one. The remaining aqueous fraction was further shaken with n-butanol and allowed to be portioned in to n-butanol aqueous layer. The n-butanol layer was then combined, concentrated and evaporated to dryness (on water bath at 40°c) to give n-butanol fraction, which was labeled as fraction two (Fig. 1).

The aqueous residue that was left following the two solvents partitioning was filtered (Whatman No.1 filter paper) and lyophilized by a freeze dryer system to give a dried amorphous solid which was labeled as fraction three (Fig.1). The solvent fractions were then kept in a tightly closed bottle until tested for antibacterial effects and MIC determination (Asfaw Debella, 2002; Silva *et al*., 2002, Shokeen *et al*., 2005). Part of 80% methanol extract of *C. macrostachyus* was also suspended in 100ml of distilled water and acidified using 2% HCl (pH 3-4) and then basified using 10% ammonia (pH 7-8). The suspension was then shaken with chloroform to give basic aqueous and chloroform fraction that contained alkaloid component of *C. macrostachyus* (Asfaw Debella, 2002).

Based on the polarity of solvents, chloroform fraction contains intermediate polar compounds such as flavonoids, phenols, diterpens etc. Aqueous fraction contains very polar compounds (polar saponins, phenolic glycosides and terpens). The n-butanol fraction contains polar compounds including terpens, saponins, phenols etc. (Asfaw Debella, 2002). Of these, saponins (in *A. gummifera* only), alkaloids, phenols, terpens and glycosides were the semi purified test extracts in both *A. gummifera* and *C. macrostachyus.* The following crude extracts and solvent fractions shown in Table 1 were used for antibacterial activity test and MIC determination against the test organisms.

Table 1. Designation of crude plant extracts and solvent fractions tested.

Agc	*Albizia gummifera* crude extract
Ag-1	*A. gummifera* aqueous fraction
Ag-2	*A. gummifera* butanol fraction
Ag-chloroform*	*A. gummifera* chloroform fraction.
Cmc	*Croton macrostachyus* crude extract
Cm-1	*C macrostachyus* chloroform fraction
Cm-2	*C. macrostachyus* butanol fraction
Cm-3	*C. macrostachyus* basified chloroform fraction
Cm-aqueous*	*C. macrostachyus* basified and non-basified aqueous fractions.

*Fractions with no growth inhibition effect.

4.8 Antibacterial activity test and MIC determination

Antibacterial activity and MIC tests were conducted using the agar dilution method which is the reference (gold standard) method widely used to determine gonococcal MICs (Andrews, 2001). Antibacterial effects of the plant extracts on the standard organism and clinical isolates were determined in comparison with standard antibiotics using the general procedure outlined by Rios *et al* (1988).

4.8.1 Preparation of anti microbial Solutions and agar dilution plates

Stock solutions of crude and fractionated plant extracts were prepared by dissolving 100 mg of each plant sample in 5 ml final volume of appropriate solvents. These stock solutions contained antimicrobial product at a concentration of 2mg/ml. Crude 80% methanol extract of *A. gummifera* and *C. macrostachyus* were dissolved using 9% ethanol (1 ml) + sterile distilled water (4ml) for both samples. Chloroform and butanol fractions of *A. gummifera* were solublized using 9% ethanol (4ml) + 2% Tween 20 (1 ml) and 9% ethanol (1ml) + sterile distilled water (4ml) respectively. Likewise chloroform and butanol fractions of *C. macrostachys* were solublized using 9% ethanol (1ml) + 2% Tween 20 (4ml) and 9% ethanol (2ml)+ 20% Tween 20 (3ml) respectively. Aqueous extracts of both samples were solublized using sterile distilled water alone.

In addition, 128mg of the following standard antibiotic powders (Sigma), Spectinomycin (Lot No. 40464), Gentamycin sulphate (Lot No 9706201), Ciprofloxacin hydrochloride (Lot No. 09867), Tetracycline hydrochloride (Lot No. 3383), and crystalline sodium Penicillin G (Lot No. 809169) used as a positive control were solublized using sterile distilled water (5-10 ml). They were then diluted further with sterile distilled water to an exact final volume of 25 ml. These stock solutions contained antimicrobial product at a concentration of 5.12mg/ml. From the primary stock solutions, a serious of two fold stock solutions were prepared for each antibiotic containing antimicrobial concentration ten times higher than the final concentration to be obtained in the agar dilution plates (Dyck *et al*., 1999;Andrews, 2001).

Standard susceptibility testing medium to determine gonococcal MICs, *i.e*. GC-agar base medium (Oxoid) enriched with 1% Isovitalex (Oxoid) was used. The dehydrated GC-agar base medium and distilled water were dispensed into a container after being adjusted to an appropriate volume for the number of dilution plates to be prepared for each antimicrobial concentration to be tested. It was then autoclaved (121°C, 15lb) in a tightly closed container and allowed to cool down to a temperature of 50-55°C in a water bath before adding and mixing the sterile supplement (1% Isovitalex (Oxoid) supplement) (Dyck *et al*., 1999; Andrews, 2001).

A 2 ml of each antimicrobial solution was then incorporated in to 18 ml of enriched GC agar base medium using a scheme in which one part of the antimicrobial solution was added to nine parts of agar *i.e*. in each sterile petridishes appropriate concentration of plant extracts and standard drugs were dispensed and labeled prior to adding the molten enriched agar. In each plate, the following gradients of concentrations of plant extracts were included. 1000, 500, 250 and 125 μg/ml. Tetracycline HCl, Penicillin G, Spectinomycin and Gentamycin were added to the enriched GC agar giving a final drug concentration of 256, 128, 64, 32, 16, 8, 4, 2, 1, and 0.5 μg/ml. Ciprofloxacin was tested in concentrations of 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.062 and 0.031 μg/ml (Dyck *et al*., 1999; Andrews, 2001).

4.8.2 Inoculation of plates, incubation and MIC determination

Direct colony suspension method recommended for fastidious microorganisms was used for the preparation of inoculum (Andrews, 2001) where a small quantity of inoculum from an overnight subculture on chocolate agar was taken and suspended in Muller Hinton Broth (MHB). A vortex mixer homogenized the suspension and its turbidity was then adjusted to that of McFarland standard #0.5 to give a concentration of approximately 10^8 colony-forming units (CFU) per ml.

Standardized multi point inoculator (Steers inoculator) was used to inoculate MIC test plates where aliquots of each suspension (0.5 ml) was placed in the corresponding well of the inoculator that stamps approximately 1-2 µl of each suspension on the agar surface in circular areas with a diameter of 5-7 mm giving a final bacterial inoculum of 10^4 CFU per spot. Two plates with only enriched GC base medium but without the test compounds were prepared, where one plate was used to provide appropriate growth control, the other one was used to monitor antibiotic carryover effect .A third plate with out the test compounds but only with solvents used as diluents and enriched GC base medium was also used to monitor effects of solvents on growth of organisms.

Negative control plates were inoculated first, followed by the plates containing different antimicrobial products starting with the lowest concentration for each antimicrobial agent. Finally, the third negative control plates were inoculated to ensure that there has been no antimicrobial carry over or contamination during inoculation. Gonococcal reference strain ATCC 49226 was included for each antibiotic/plant extract tested.

After allowing the inoculated plates to dry for some time, the plates were then incubated in an inverted position in atmosphere containing 3-5% CO_2, with high humidity ($\simeq$70%) at a temperature of 35-37$^{o}_{C}$ for 18 to 24 hours (Dyck *et al*., 1999; Andrews, 2001). After an overnight incubation, the presence or absence of visible growth at each concentration of the plant extract and standard antibiotics was examined by direct visual comparison of the test cultures with the negative control plates having confluent bacterial growth.

MICs were defined as the lowest concentration of antimicrobial agent that inhibited visible growth of bacterial spots (Bosio *et al*., 2000) after an overnight incubation, disregarding a few single colonies or a fine barely visible haze (Andrews, 2001). MIC results of reference strain of *N. gonorrhoeae* (ATCC 49226) for the standard drugs were also checked.

4.9 Data analysis

All assays were repeated three times and the modal minimum inhibitory concentrations (MICs)(Bosio *et al*., 2000) were determined based on the triplicate tests done for each of the crude, fractionated plant extracts and standard drugs against clinical isolates and reference strains of *Neisseria gonorrhoeae* (ATCC 49226).

5. RESULTS

5.1 Results of clinical data and questionnaire survey

A total of 250 male and female adult patients who were clinically symptomatic for uncomplicated gonococcal infection were enrolled to participate in this study according to the written consent. Among the 250 urogenital specimens collected from both sexes, the etiologic agents were isolated from 19(8%) patients (Table 2).

The age-sex distribution and socioeconomic status of the totally examined 250 STD patients presenting with clinical symptoms of uncomplicated gonococcal infection are shown in Table 2 and 3 respectively. Questionnaire survey on the attitude of the study subjects towards the use of plants as remedies also showed that some of the patients about five (2%) had visited traditional healers prior to attending STD clinics (Data not shown).

Characterization of presumptively identified 19 clinical isolates, which were able to grow on the primary isolation medium (Modified Thayer Martin Medium) showed that all the isolates from genital sources to be Gram-negative diplococci, oxidase, catalase and superoxol positive. Confirmatory testing of these presumptively identified isolates for carbohydrate utilization pattern also showed that acid production from only glucose but not maltose, lactose or sucrose.

Antimicrobial drug sensitivity pattern of the 19 clinical isolates of *N. gonorrhoeae* against standard drugs by the agar disc diffusion method is indicated in Table 4, and the rates of the antibiogram and the multiple drug resistance pattern of these isolates are shown in Table 5.

5.2 Anti bacterial activity and MIC test results

Crude hydro-alcoholic (20-80%) extract of *Croton macrostachyus* (Cmc) completely inhibited growth of all the 19 clinical isolates and reference strain of *N. gonorrhoeae* (ATCC 49226) at a

sample concentration of 250 µg/ml. Whereas the crude hydro-alcoholic extract of *Albizia gummifera* (Agc) exhibited complete growth inhibition of both clinical isolates and reference strain of *N. gonorrhoeae* at a sample concentration of 500 µg/ml. (Table 6, Fig 2, 3).

Chloroform (Cm-1) and n-butanol fraction (Cm-2) of *C. macrostachyus* showed complete growth inhibition of the test organisms at the four concentration levels tested (1000, 500, 250 and 125 µg/ ml) MIC for both fractions was found to be 125 µg/ml for clinical and standard organisms (Table 6, Fig 2, 3). However, the aqueous fractions (Cm-aqueous) (basified and non basified) had no growth inhibition effect at the four concentration levels tested (Annex III, IV).

In n-butanol fraction of *A. gummifera* (Ag-2), complete growth inhibition was observed at MIC of 250 and 125 µg/ml for clinical isolates and standard organism of *N. gonorrhoeae*, respectively. The aqueous fraction (Ag-1) showed complete growth inhibition at a relatively higher concentration level of 1000 µg/ml for clinical isolates and 500 µg/ml for standard organisms (Table 6, Fig 2, 3). However, the chloroform fraction (Ag-chloroform) had no growth inhibition effect at the four concentration levels tested (Table 6, Annex III, IV).

The basified chloroform fraction of *C. macrostachyus* (Cm-3) completely inhibited growth of clinical isolates and standard organisms of *N. gonorrhoeae* at a sample concentration of 250 and 125 µg /ml respectively (Table 6, Fig. 2, 3). Growth inhibition even by the basified chloroform fraction was also observed in about 74% of the clinical isolates at a sample concentration of 125 µg/ml of *C. macrostachyus* (Annex III).

MIC values of the standard drugs used as positive controls ranged from 0.06 to 256 µg/ml against clinical isolates and standard organism of *N. gonorrhoeae* (Table 6, Fig. 2, 3). Detailed presentation of MIC results of the crude, solvent fractions of plant extracts and standard drugs against the tested organisms are shown in Annexes III-V. The comparative MIC values of plant extracts and standard drugs against clinical isolates and reference strain of *N. gonorrhoeae* is shown in Figures 2 and 3 respectively.

6. DISCUSSION

Analysis of the age-sex distribution of the STD patients indicated that out of the totally examined 250 patients, the majority (60%) of cases suspected for gonococcal infection were in the age group of 16-34 years (Table 2). The observed high STD cases in this age group in this study may reflect the sexually active nature and vulnerability of the individuals to many other STDs including HIV and can be considered as target groups for designing STD control strategies. Concordant findings indicating high STD cases in this age group was also reported by Aberra Geyid (2002) where out of the 350 patients examined, 210 were in the age group of 16-34 years. Fonc *et al* (2002) also reported that about 57% of STD clinic attenders in Kenya were in the above age group. In this study, more positive rate of the gonococcal isolates were observed in the above age group (12/19) compared to the isolates from higher aged group patients.

The age-sex distribution also indicated that significant proportions of genital specimens were collected from male patients and a male predominance of about 148 to 102 female cases were observed (Table 2). The male predominance with male to female ratio of 1:0.6 in this study might explain the greater frequency of asymptomatic carriers of STD cases in women (Kihlstrom

and Danielsson, 1994) such that fewer infected women could be identified. However, if contacts are traced carefully and women are screened thoroughly, the ratio of men to women may fall dramatically.

The results in this study also indicated that more positive cases of gonococcal infections were from male patients, 17/19 than female positive 2/19 patients (Table 2). Among the 250 urogenital specimens collected from both sexes, *N. gonorrhoeae* were identified from 19 patients. The observed low positive rate of gonococcal isolates in this study might indicate the presence of other co-infecting STIs, possibly *Chlamydia trachomatis* in the patients examined.

Analysis of socioeconomic status of the 250 patients presenting with clinical symptoms of uncomplicated *N. gonorrhoeae* infections (Table 3) showed that almost 66% of STD patients of both sexes were unmarried, 26% were civil servants, 32% were engaged in private business, 42% were unemployed and 26% were students. Similar results were reported in a questionnaire survey conducted at four health centers in Addis Ababa, where 67% of the STD patients of both sexes were unmarried, 21% of them were students and 24% civil servants (Workneh Feleke and Kloos, 1993). Questionnaire survey regarding patients attitude towards the use of herbal medicine for the treatment of gonococcal infection showed that significantly lower number of patients, about five (2%) had visited the near by traditional healers prior to attending the STD clinics. This finding may be due to the fact that most of the study subjects were urban dwellers having better access of visiting private clinics or government health centers.

Modified Thayer Martin Medium (MTM) successfully distinguished *N. gonorrhoeae* isolates using specimens taken from non-sterile sites (urethra in men and vagina in women). The observed morphological colony characteristics shown on culture media and results of oxidase, catalase, superoxol and carbohydrate utilization tests agreed with the characteristics described for the species *N. gonorrhoeae* (Knapp, 1988; CDC, 2004).

Drug sensitivity testing of the isolates performed by using the agar disc diffusion method showed that of all the antibiotics tested, three of them were resisted against the 19 clinical isolates tested

except for Erythromycin, Ciprofloxacin and Ceftriaxone which were 100% effective against the19 clinical isolates tested. These sensitivity test results agree with the results reported by Aberra Geyid (2002) where 90%, 70% and 93% of the tested isolates were resistant to Penicillin, Tetracycline and Cotrimoxazole respectively, although it is 100% resistance to all these three drugs in this study (Table 4, 5).

In this study, the 19 clinical isolates showed similar drug resistance pattern in multiples of only three drugs (Penicillin, Tetracycline and Cotrimoxazole). None of the resistant strains showed resistance to only one drug or even to only two combinations of drugs (Table 5). Among the 19 isolates tested, 8(42%) were intermediately susceptible to Kanamycin and the remaining 11 (58%) were sensitive for this drug. In addition, 6 (32%) of the isolates were sensitive for Spectionomycin and the remaining 13 (68%) of the isolates were intermediately susceptible to Spectinomycin (Table 4).

MIC determination using standard drug powders of Gentamycin, Penicillin, Tetracycline, Spectinomycin and Ciprofloxacin also showed that four of the tested drugs were resisted except for Ciprofloxacin that retained its activity at MIC of 0.06 μg/ml (Table 6, Annex V). Comparable results were reported from Ethiopia and some other African countries (Van Hall *et al*., 1991; West *et al*., 1995; Aberra Geyid, 2002).

Both clinical isolates and standard organism of *N. gonorrhoeae* were sensitive to extracts of *C. macrostacchyus* and *A. gummifera* at MIC range of 250 -500 μg/ml in our study. Preliminary screening study conducted by Aberra *et al* (2005) using crude extracts of *C. macrostachyus* and *A. gummifera* also showed antibacterial activity against *N. gonorrhoeae* (ATCC 49226) at MIC of 250 and 1000 μg/ml respectively. Chemical analysis done on these extracts also showed the presence of several Alkaloids, polyphenols, unsaturated sterols (triterpens), saponins (in *A. gummifera* only) and glycosides (carbohydrates) in both A. *gummifera* and *C. macrostachyus* (Aberra *et al*., 2005). It is possible that these compounds could be responsible for the antibacterial property reported in this study, possibly due to their synergistic effect on the test organisms (Cowan, 1999; Martini *et al*., 2004). In a similar study in West Africa crude ethanol-

water (80-20%) extract of *Terminalia macroptera* leaves and roots were found to be effective against clinical isolates and reference strain of *N. gonorrhoeae* with MIC range of 100-200 µg/ml (Silva *et al*., 2002).

In this study, the crude extracts and semi purified solvent fractions exhibited varied spectrum of anti-bacterial activity against the test organisms. Butanol and chloroform fractions of *C. macrostachyus* exhibited highest degree of antibacterial activity at MIC of 125 µg/ml on both clinical isolates and standard organisms of as compared to the basified chloroform fraction of the same plant which showed activity at MIC of 250 µg/ml on clinical isolates (Table 6, Fig. 2, 3) and the aqueous fractions (basified and non basified) which had no visible growth inhibition effect at all (Annex III, IV).

On the other hand, butanol fraction of *A. gummifera* showed highest degree of anti-bacterial activity at MIC of 250 and 125 µg/ml against clinical isolates and standard organisms respectively compared to the aqueous fraction that exhibited growth inhibition at MIC of 1000 and 500µg/ml on clinical and standard organisms respectively (Table 6, Fig. 2, 3). However, the chloroform fraction did not show any antibacterial activity in this study. Similar results were reported by Abayneh Unasho (2005) when the chloroform fraction is tested against clinical isolates and standard organisms of *S. pneumoniae* and *S. pyogenes*. Butanol fraction of this plant was reported to inhibit growth of clinical isolates and reference strain of *S. pyogens* and *S. pneumoniae* at MIC range of 500-1000 µg/ml (Abayneh Unasho, 2005).

The results in this study also indicated that solvent fractions (butanol and chloroform fractions of *C. macrostachyus* and butanol fraction of *A. gummifera*) were active at lower concentration level (125-250 µg/ml) than crude preparations of the same plant samples, which were effective at a relatively higher level of concentration (250-500 µg/ml) (Table 6, Fig. 2, 3). Anti-bacterial activity at lower concentration level in the semi-purified fractions in this result may indicate the partitioning and concentration of semi-purified bioactive compounds in the solvents (vehicles) used and the need for further partitioning and purification (Aberra *et al*., 2005; Shokeen, 2005).

In this study, the bacteriostatic activity of crude extract of *C. macrostachyus* against the test organisms was higher than *A. gummifera* crude extracts. In another study, similar findings were reported, where crude extracts of *C. macrostachyus* was found to active at MIC of 250 µg/ml than crude extract of *A. gummifera, which* was active at MIC of 1000 µg/ml (Aberra *et al.,* 2005). Marked difference was also observed in the activity of crude and fractionated extracts of both plants against the test organisms. The observed variation in the degree of anti-bacterial activity may be attributed to the qualitative or quantitative variation of secondary metabolites present in these medicinal plants (Santos *et al*., 2002; Martini *et al.*, 2004).

Aqueous fraction of *A. gummifera* was less effective than the butanol fraction of the same plant in our study. Compared to MIC of the crude extract (500 µg/ml), MIC of aqueous fraction *A. gummifera* was also higher which was 1000 µg/ml against clinical isolates of *N. gonorrhoeae* (Table 6, Fig. 2). In a similar study, aqueous fraction of *A. gummifera* did not show any anti bacterial activity even at a concentration as high as 2000 µg/ml against clinical isolates and reference strain of *S. pneumoniae* and *S. pyogenes* (Abayneh Unasho, 2005). In this study, aqueous fraction of *C.* m*acrostachyus* (basified and non basified) also did not show any anti bacterial activity (Table 6, Annex III, IV). Absence or reduced activity in aqueous phase may be explained by a study carried out by Rios *et al* (1988) where they observed high diffusion power and low anti bacterial activity in many of the exceptionally water soluble plant compounds. Recent studies have also reported similar findings (Cowan, 1999).

It is known that outer membrane of Gram-negative bacteria present barrier to penetration of numerous antibiotic molecules. The periplasmic space also contains enzymes, which are capable of breaking down foreign molecules (Duffy and Power 2001), and appears to be less susceptible to plant extracts than Gram-positive bacteria. Under the conditions employed here, the crude extracts and semi-purified fractions exhibited an interesting profile of activity against the Gram-negative bacteria, N. *gonorrhoeae.* In another study, a lower MIC value of 250 µg/ml was reported by Holetz *et al* (2002) using crude extracts of *Piper regnellii* against the Gram-negative

organism *P. aerougonosa*. Silva *et al* (2002) also reported an MIC as low as 25 µg/ml using diethyl ether fraction of *Terminalia macroptera* leaf extract against *N. gonorrhoeae*.

Compared to MICs of Tetracycline, Gentamycin and Ciprofloxacin, the MICs of the semi-purified fractions were greater. However, the semi purified fractions (Ag-2, Cm-1, Cm-2 and Cm-3) exhibited comparable MIC values with Penicillin and Spectinomycin (Fig. 2). With this aspect, the results indicate the presence of chemical compounds in *A. gummifera* and *C. macrostachyus* with anti *N. gonorrhoeae* activity comparable to Penicillin or Spectinomycin.

In most plant extracts, the compounds responsible for biological activity are present with in a range of 1- 0.001%. This implies the need for further activity-guided fractionation and purification of the most active semi-purified fraction(s) to locate and identify marker compounds with MICs attainable *in vivo* (Berghe and Vlientinck, 1999). For instance, Martini *et al* (2004) isolated seven anti-bacterial flavonoids under bioassay-guided fractionation of chloroform fraction of *Combretum erythrophyllum* and reported a *good* anti-bacterial activity against *Vibrio cholerae*, *Enterococcus faecalis, Shigella sonei, Staphylococcus aureus* and *Micrococcus luteus* with MIC values in the range of 25-50 µg/ml.

Some components of extracts of *A. gummifera* and *C. macrostachyus* were detected by spraying thin layer chromatography (TLC) plates with spraying reagents. When *C. macrostachyus* extract applied TLC plates were sprayed with Dragondorff`s reagent, orange red spots were observed. This was taken as an indication of the presence of alkaloids in the extract. Similarly, spraying of *A. gummifera* extract applied TLC plates with vanillin sulfuric acid gave blue or blue black color, which was also taken as an indication of the presence of saponins in the extract.

7. CONCLUSIONS AND RECOMMENDATIONS

The major pathogenesis arising from bacterial infection for which vast array of Ethiopian traditional medicinal plants are used include STDs, mainly gonorrhoea and syphilis. Despite the existence of plethora of information regarding the prolonged and uneventful local use of

these plants, scientific evidences regarding their efficacy are less abundant. Large gaps also exist regarding the constituents of these traditionally claimed medicinal plants. In view of these, the medicinal flora of Ethiopia is an avenue yet to be explored and could be a potential source of chemical compounds with high therapeutic index that can be used at least at the primary health care level. Under the conditions employed in this study:

- Crude and semi purified solvent fractions of *A. gummifera* and *C. macrostachyus* have shown an interesting profile of antibacterial activity against clinical isolates and reference strain of *N. gonorrhoeae* (ATCC 49226). This finding may indicate the presence of bioactive compound(s) in *A. gummifera* and *C. macrostachyus* responsible for the observed activity.

- The semi-purified solvent fractions, in particular butanol fraction of *A. gummifera,* chloroform and butanol fractions of *C. macrostachyus* have shown an interesting anti *N. gonorrhoeae* activity with MIC values comparable to standard drugs of Penicillin and Spectinomycin. The potentially bio-active compounds present in these fractions could be identified if further bioassay guided fractionation and purification is undertaken

- Although the *in vitro* anti-bacterial activity of these plant extracts does not yet justify their use in the treatment of infections caused by *N. gonorrhoeae*, our results substantiate the ethno-botanical use of these medicinal plants for the treatment of gonococcal infection. In order to provide a clear rationale for the ethno medicinal use of *A. gummifera* and *C. macrostachyus, in vivo* data are highly valuable in determining the potential use fullness of these plants for the treatment of infections and mammalian toxicity effects.

Based on these findings, the following recommendations are given:

- Further research involving the search of new anti *N. gonorrhoeae* agents appears to be warranted in countries like Ethiopia where effective antibiotic therapy is unobtainable or prohibitively expensive, and much more number of isolates are recommended for the test of this nature.

- The standardization of the methods of plant extraction for an *in vitro* testing is highly recommended to make the search of anti microbial agents more systematic and interpretation of the results easier (Cowan, 1999).

- Clinical isolates of *N. gonorrhoeae* used in this study may contain strain of different susceptibility to standard drugs and active compounds present in the plant extracts. Thus, it is essential to confirm the effectiveness of these semi-purified fractions against known resistant strains of *N. gonorrhoeae*

- Further fractionation and purification of active fractions of both plants (particularly n-butanol and chloroform fractions of *C. macrostachyus*) using improved techniques is highly recommended.

- In addition, alternative mechanisms of infection prevention and treatment should be included in initial activity screenings of medicinal plants. Disruption of adhesion is one example of an anti-infection activity not commonly screened for currently (Cowan 1999).

- In terms of conservation, this study and similar earlier studies have shown that leaves of *C. macrostachyus* and seeds of *A. gummifera* have remarkable antibacterial uses; as they are renewable parts of the plant they can be used without any detrimental effect on the biodiversity of Ethiopian medicinal flora. However, traditionally various plant parts of *A. gummifera* and *C. macrostachyus* are used for the treatment of infectious diseases hence

carrying out comparative studies on the biological activity of different parts of these plants are highly recommended.

- Traditional medicinal plants, which have been used over centuries, constitute an obvious choice for any study involving the search of new anti-infective agents from natural products. Given effort and due attention, plants with high therapeutic value could be found and in the future, it may be possible to develop locally, pharmaceutical formulations for clinical trials.

8. REFERENCES

Abayneh Unasho (2005). Investigation of antimicrobial activities of *Albizia gummifera* and *Ferula communis* on *Streptococcus pneumoniae* and *Streptococcus pyogenes* causing upper respiratory tract infections in children. MSc thesis, Addis Ababa University, Ethiopia, pp. 37-79.

Aberra Geyid (2002). A study of surface proteins, other adhesions and iron acquiring mechanisms in the pathogenesis of *Neisseria gonorrhoeae* strains isolated from STD patients in Ethiopia. *Journal of Ethiopian Medical practice* **4**(1): 14-20.

Aberra Geyid, Dawit Abebe, Asfaw Debella, Zewdnesh Mekonnen, Frehiwot Aberra, Frehiwot Teka, Tesfaye Kebede, Kelbessa Urga, Kidist Yersaw, Tekle Biza, Bisrat H. Mariam and Mulugeta Guta (2005). Screening of some medicinal plants of Ethiopia for their anti microbial properties and chemical profiles. *Jorunal of Ethnopharmacology* **97**: 421-427.

Abraham, I., Braude, M.D., Davis, C. and Filuer, J. (1986). Gonococcal and Chlamydial genital infections. **In**: *Infectious Disease and Medical Microbiology*, pp.1030-1031, (Allen, J. and Cutchan, M.E., eds). WB. Sounders Press, USA.

Adams, B.H. and Seifert, H.S. (2000). Molecular models accounting for the gene conversion reactions mediating Gonococci pilin antigenic variation. *Molecular Microbiology* **37** (5): 1446-1158.

Akerele, O. (1993). Nature's medicinal bounties do not throw it away. *World Health Forum* **14**: 390-395.

Andrews, J.M. (2001). Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotherapy* **48**: 5-16.

Asfaw Debella (2002). *Manual for phytochemical screening of medicinal plants*. Ethiopian Health and Nutrition Research Institute, Addis Ababa, Ethiopia, pp. 1-83.

Ashenafi Belihu and Lindtjorn, B. (1999). Increasing incidence of resistance to antimicrobials in Sidamo. *Ethiop. Med. J.* **37**: 181-187.

Azene Bekele, Birnie, A. and Tengnas, B. (1993). *Useful trees and shrubs for Ethiopia*. Identification, propagation and management for agricultural and pastoral communities. RSCU, SIDA, pp.174-179.

Balcha Aberra (2003). Medicinal plants used in traditional medicine in Jimma zone, Oromia, South West Ethiopia. *Ethiop J. Health Sci*. **13** (2): 85-94.

Barlow, D. and Phillips, I. (1978). Gonorrhoea in women: diagnostic clinical and laboratory aspects. *Lancet* **I**: 761-764.

Berghe, V. and Vlientinck, A.J. (1991). Screening methods for antibacterial and antiviral agents from higher plants. **In**: *Methods in plant Biochemistry,* pp.47-69 (Dey, P.M. and Hatborne , J.B ., eds). Academic Press, London.

Biswas, G. and Sparling, PF. (1995). Characterization of LbpA the structural gene for a lactoferrin receptor in *Neisseria gonorrhoeae*. *Infection and Immunity* **63**: 2958-2857.

Bjerknes, R. (1995). Neisserial porins inhibit human neutrophil actin polymerization, degranulation opsonin receptor expression and phagocytosis but prime the neutrophils to increase their oxidative burst. *Infection and Immunity* **63**: 160-167.

Blake, M.S and Wetzler, L.M. (1995). Vaccines for gonorrhoea: where are we on the curve? *Trends in Microbiology* **3**(12): 469-474.

Bosio, K., Avanzini, C. Davolio, A., Ozino, O. and Savoia, D. (2000). *In vitro* activity of propolis against *Streptococcus pyogenes*. *Letters in Applied Microbiology* **310**(2): 174-177.

Boyd, R.F. (1995). *Basic Medical Microbiology*. Lippincott Williams and Wilkins, New York, pp. 264-271.

Brock, T.D. (1997). Sexually transmitted disease **In:** *Biology of Microorganisms*, pp.940-947, (Madigan, M.T., Martinko, J.M. and Parker, J., eds). Prentice Hall, USA.

Carne, C.A., Weller, I.V., Kohl, P.K. and Whittington, W. (1987). Prevalence of antibodies to human immunodeficiency virus, gonorrhoea rates and changed sexual behavior in homosexual men in London. *Lancet* **1**: 656-688.

Catlin, B.W. (1973). Nutritional profiles of *Neisseria gonorrhoeae, Neisseria menengitidis* and *Neisseria lactamica* in chemically defined media and the use of growth requirement for goncoccal typing. *J. Infect Dis* **128**: 178-194.

CDC: Center for Disease Control and Prevention (1993). Sexually transmitted diseases treatment guidelines. *MMWR* **42** (14): 5-8.

CDC: Center for Disease Control and Prevention (2002). Increase in Fluoroquinolone resistant *N. gonorrhoeae*, Hawai and California, *MMWR* **51** (46): 1041-1044.

CDC: Center for Disease Control and Prevention (2004). Identification of *N. gonorrhoeae*, National guideline, Division of AIDS, STD and TB laboratory research, CDC, pp. 1-9.

Chen, C., Elkins, C. and Sparling, P.F. (1997). Phase variation of haemoglobin utilization in *Neisseria gonorrhoeae*. *Infection and Immunity* **66**: 987-998.

Chen, T., Swanson, J. and Wilson, J. (1995). Heparin protects Opa+ *Neisseria gonorrhoeae* from the bactericidal action of Normal Human Serum. *Infection and Immunity* **63**: 1790-1795.

Cowan M.M. (1999). Plant Products as Anti Microbial Agents. *Clinical Microbiology Reviews* **12** (4): 564-582.

Dawit Abebe, Asfaw Debella and Kelbessa Urga (2003). *Medicinal plants and other useful plants of Ethiopi*a. (Illustrated Check list),Camerapix Publishers International, Singapore, pp. 8-38.

Dawit Dikasso, Hirut Lemma, Kelbessa Urga, Asfaw Debella, Getachew Addis, Ashenafi Tadele and Kidist Yirsaw (2002a). Investigation on the antibacterial properties of garlic *(Allium sativum*) on Pneumonia causing bacteria. *Ethiopian Medical Journal* **40**(3): 241-249.

Dawit Dikasso, Hirut Lemma, Kelbessa Urga, Chernet Ambaye, Ashenafi Ayale and Kidist Yirsaw (2002b). Investigation of the antibacterial effects of Papaya (*Carcica papaya*) seeds on three Pneumonia causing bacteria. *Ethiopian Journal of Health Science* **12** (1): 47-55.

Dodge, RW. and Wallace, C.K. (1975). *Neisseria gonorrhoeae* in Ethiopia, antibiotic sensitivity pattern. *Eth. Med. J.* **13** (1): 19-22.

Duffy, C.F. and Power, R.F. (2001). Antioxidant and antimicrobial properties of some Chinese plant extracts. *Int. J. of Antimicrob Agents* **17**: 527-529.

Dyck Van, E., Meheus, A.Z. and Piot, P. (1999). *Laboratory diagnosis of sexually transmitted diseases*, WHO, Geneva, pp. 1-21.

Elizabeth, D.M., Tibaus, G., Pelzer, A., Leterbirhan Mehari., Peutherer, J., Young, H., Yasmin Jamil., Darougar, S., Lind, I., Reimann, K., Piot, P., Roggen, E. and Perine,P.L. (1995). Prevalence and significance of sexually transmitted disease among Ethiopian women attending antenatal clinics in Addis Ababa. *Ethiop. J. Health Dev*. **9** (1): 31-40.

Elkins, C. and Sparling, P.F (1990). Outer Membrane Proteins of *Neisseria gonorrhoeae*. **In:** *Microbial Determinant of Virulence and Host Responses,* pp. 207-218, (Ayoub, E.M., Cassell., G.H., Brache, W.C. and Henry, T.J., eds). American Society for Microbiology, USA.

Eyasu Habtegabriel, Aberra Geyid and Dibaba Serdo (1983). Betalactamase producing *N. gonorrhoeae* in Addis Ababa. *Ethiop.Med. J.* **21**: 198-199.

Eyasu Habtegabriel, Aberra Geyid, Dibaba Serdo , Biddle, J. and Perine , P.L (1987). Single dose treatment of uncomplicated acute gonococcal urethritis in Ethiopian men, comparison of Rosoxacin, Spectinomycin, Penicillin and Ampicillin. *Sex.Tra.Dis.* **14**(3): 153-155.

Fonk, K., Kaul, K., Kimani J., Keli, F., Mac Donald, K.S., Ronald, A.R., Plummer, F.A., Kirui, P., Bwayo, J.J., Ngugi, E.N., Moses, S. and Temmerman, M. (2000). A randomized, placebo-controlled trial of monthly Azithromycin prophylaxis to prevent sexually transmitted infections and HIV-I in Kenya sex workers: Study design and baseline findings. *International Journal of STD and AIDS* **11**: 804-811.

Genco, C.A. and Desai, P.J. (1996). Iron acquisition in the pathogenic Neisseria. *Trends in Microbiology* **4**: 179-184.

Gerbase, A.C piot, P., Kohl, P.K. and Fox, K.K. (1998). Global prevalence and incidence estimate of selected curable STDs. *Sex Transm. Inf.* **74**: 12-16.

Gilbert, M.G. (1989). Euphorbiaceae. **In**: *Flora of Ethiopia and Eritrea* vol.2, pp. 326-327, (Edwards, S. Mesfin Tadesse and Hedberg, I.,eds). Upssala, Sweden.

Gore Felton, C., Vosvick, M., Bendel, T., Koopman, C., Das, B., Israelski, D., Herrera,M., Litzenberg, K. and Spiegel, D. (2003). Correlates of sexually transmitted disease infection among adults living with HIV. *International Journal of STD and AIDS* **14**: 539-546.

Gray Owen, S.O. and Schryvers, A.B. (1996). Bacterial transferrin and lactoferrin receptors. *Trends in Microbiology* **2**: 157-164.

Guillimot, D., Courvalin, P. and the French working party to promote research to control bacterial resistance (2001). Better Control of Antibiotic Resistance. *CID* **33**: 542-547.

Gzassme, H.V., Mirland, C.R. and Vanputteen, J.P.M. (1996). Gonococcal Opacity protein promotes bacterial entry associated rearrangement of the epithelial cell actin cytoskeleton. *Infection and Immunity* **63**: 1790-1795.

Hedges, S.R., Mayo, M.S., Mestecky, J., Hook, EW. and Russell, M.W. (1999). Limited local and systemic antibody response to *Neisseria gonorrhoeae* during uncomplicated genital infections. *Infection and Immunity* **67** (8): 3937-3946.

Hirut Lemma, Asfaw Debella, Getachew Addis, Olaf Kunert, Aberra Geyid, Ferehiwot Teka and Kidist Yersaw (2002). Antibacterial activity of *Plumbago zeylanica* L. roots on some Pneumonia causing pathogens. *SINET. Ethiop. J. Sci.* **25** (2): 285-294.

Hobbs, M.M., Alcorn, T.M., Davis, R.H., Fischer, W., Thomas, J.C., Marin, I, Ison, C., Sparling, P.F. and Cohen, M.S (1999). Molecular typing of *Neisseria gonorrhoeae* causing repeated infections: Evolution of porins during passage within a community. *The Journal of Infectious Disease* **179**: 371-381.

Holetz, F.B., Pessini, G.L., Sanches, N.R., Cortez, A.G., Nakamura,CV. and Filho, B.D .(2002). Screening some plants used in Brazilian folk medicine for the treatment of infectious diseases. *Mem Inst Oswaldo Cruz. Rio de Janeiro* **97** (7): 1027-1031.

Ison, C.A., Woodfod, P.J., Madders, H. and Claydon, E. (1998). Drift in susceptibility of *Neisseria gonorroheae* to Ciprofloxacin and emergence of therapeutic failure. *Antimicrobial Agents and Chemotherapy* **42**(11): 2919-2922.

Ivens, D., Martin, I. and Ison, C. (2002). *Neisseria gonorrhoeae* in London sexually transmitted infection clinic not fully sensitive to Quinolones are isolates imported and how effective is Ciprofloxacin as a first line therapy? *International Journal of STD and AIDS* **11**: 774-776.

Kapa, M. (1996). Utilization of plant medicine for the treatment of health problems. The case of Oromo, Illubabor zone Western Ethiopia. *Ethiop J. Health Devt.* **10**: 161-162.

Khanna, J. Vanlook, P.F.A, and Griffin, P.D. (1992). Reproductive Health: A study to a brighter future, Biennial report, 1990- 1991, WHO, Geneva, pp. 8-12.

Kihlstrom, E. and Danielsson, D. (1994). Advances in biology management and prevention of infections caused by *Chlamydia trachomatis* and *Nesseria gonorrhoeae*. *Curr. Opin. Infect. Dis***. 7**: 25-33.

Knapp J.S. and Holmes, K.K. (1975). Disseminated gonococcal infections caused by *Neisseria gonorrhoeae* with unique nutritional requirement. *J. Infect Dis.* **132**: 204-208.

Knapp, J.S. (1988). Historical perspective and identification of *Neisseria* and related species. *Clinical Microbiology Reviews* **1** (4): 415-431.

Knapp, J.S., Fox, K.K., Trees, D.L. and Whittington, W.L. (1997). Fluoroquinolone resistance in *Neisseria gonorrhoeae. Emerging Infectious Diseases* **3**(1): 465-77.

Lai- king, I., Martin, I., Liu, G. and Bryden, L. (2002). Mutation in 23S rRNA is associated with Macrolide resistance in *Neisseria gonorrhoeae*. *Antimicrobial Agents and Chemotherapy* **46** (9): 3020-3025.

Lee, B.C. and Bryan, L .E. (1989). Identification and comparative analysis of the lactoferrin and transferrin receptors among clinical isolates of Gonococci. *J. Med. Microbiol*. **28**: 199-2004.

Lin, J.H., Donegan, P., Heeren, T.C., Greenberg, M., Flaherty, E.C., Haivanis, R., Dean, D., Newhall, W.J., Knapp., J.S., Sarafian, S.K., Rice, R.J. Morse, S.A. and Rice, P.A. (1998).Transmission of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* among men with urethritis and their female sex partners. *Journal of Infectious Disease* **178:** 1707-1712.

Lindhal, G., Sjobring, U. and Johnsson, E. (2000). Human complement regulators a major target for pathogenic microorganisms. *Curr. Opin. Immunol.* **12**: 44-51.

Martini, N.D., Katerere, D.R.P. and Eloff, J.N. (2003). Biological activity of five antibacterial flavonoids from *Combretum erythrophyllum* (Combretaceae). *Journal of Ethnopharmacology* **93**: 207-212.

Mintesnot Ashebir and Mogessie Ashenafi (1999). Evaluation of the antibacterial activity of crude preparation of *Zingiber officinale* ("zinjibl"), *Echinop spp.* ("kebericho")

Cordianum sativum ("dimbilal") and *Cymbopogan citrantus* ("tej sar") on some food borne pathogens. *Ethiop. J. Hlth Sci*. **9**(1): 33-40.

Minor, SY., Banerjee, A. and Gotschlich, E.C. (2000). Effect of oligosaccharide phenotype of *Neisseria gonorrhoeae* strain MS-11 on invasion of chang conjunctival Hec-1-B endothelial and ME -180 cervical cells. *Infection and Immunity* **68**: 6526-6534.

Nair, M. and Nathan, G. (1998). Medicinal plants cure for the 21st century: Biodiversity, conservation and utilization of medicinal plants: Proceedings of the seminar UPM, Serdang, Malaysia, pp 162-170.

NCCLS: National Committee for Clinical Laboratory Standards (2002). International Standards of Antimicrobial Susceptibility NCCLS Approved Standards M100.59, NCCLS, Wayne, P.A.

Okeke, N.I. (2003). Antibiotic resistance in Africa discerning the enemy and plotting a defense. *MERA* **4**: 10-15.

Plotkin ,N.J. (1988). Conservation, ethno botany and the search for jungle medicine: pharmacology comes of age… again. *Pharmacotherapy* **8**: 257-262.

Radcliffe, K., Ghanmen, M. and Allan, P.(2004). The role of urethral samples in the diagnosis of gonorrhoea in women. *Int J. STD and AIDS* **15**: 45-47.

Ratledge, C. and Dover, L.G. (2000). Iron metabolism in pathogenic bacteria. *Annu. Rev. Microbiol.* **54**: 881-891.

Raulston, J.E. (1997). Response of *Chlamydia trachomatis* serovar E to iron restriction *in vitro* and evidence for iron regulated *Chlamydial* proteins. *Infection and Immunity* **65**: 4539-4547.

Rios, J.J., Recio, M.C. and Villar, A. (1988). Screening methods for natural products with antimicrobials activity: a review of the literature. *Journal of Ethno pharmacology* **23**: 127-149.

Rytkonen, A., Johansson, L.,Vendela, ASP., Albiger, B., and Johnson, A.B. (2001). Soluble pilin of *Neisseria gonorrhoeae* interacts with human target cells and tissues. *Infection and Immunity* **69** (10): 6419-6426.

Salyers, A.A. and Whitt, D.D. (1994). *Bacterial Pathogenesis: A molecular Approach*. American Society for Microbiology, New York, pp. 245-260.

Santos, F.A., Bastos, E.M.A., Uzeda,M., Carvallo, M.H.R., Farians,L.M., Moreira, E.S. and Braga, F. C. (2002). Antibacterial activity of Brazilian propolis and fractions against oral anaerobic bacteria. *Journal of Ethnopharmacology* **80**: 1-7.

Sarafian, S.K. and Knapp, J.S. (1989). Molecular epidemiology of gonorrhoea. *Clinical Microbiology Reviews* **2**(1): 49-55.

Sherrad, J. and Barlow, D. (1996). Gonorrhoea in men: Clinical and diagnostic aspects. *Genitourin Med* **72**: 22-26.

Shokeen, P., Ray, K., Bala, M. Tandon, VD. and Ambedkar, B.R. (2005). Preliminary studies on activity of *Ocimum sanctum*, *Drynaria quercifolia*, and *Anonna squamosa* against *Neisseria gonorrhoeae. Sex Transm Dis* **32** (2): 106-111.

Shu, Y.K. (1998). Recent Natural Products Based Drug Development: A pharmaceutical Industry Perspective. *J. Nat. Prod*. **61**: 1053-1071.

Silva, O., Ferreira, E., Pato, M.V., Canica, M. and Gomes E.T. (2002). *In vitro* anti *Neisseria gonorrhoeae* activity of *Terminalia macroptera* leaves. *FEMS Microbiology Letters* **217**: 271-274.

Swanson J. (1990). Pilus and outer membrane protein II variation in *N. gonorrhoeae* **In**: *Microbial Determinants of Virulence and Host Responses*, pp. 197-205, (Ayuob, E.M., Cassell, G.H., Branche, W.C. and Hennery, T.J., eds). American Society for Microbiology, USA.

Thulin, M. (1989). Fabaceae (Leguminosae). **In**: *Flora of Ethiopia and Eritrea* vol. 3, pp. 49-46, (Herdeng, I. and Edwards, S., eds). Upssala, Sweden.

Todar, K. (2004). Todar’s online Textbook of Bacteriology. The pathogenic Neisseriae. URL: www.kenethtodar.org/winconson.htm

Urassa, W., Lyamuya, E. and Mhalu, F. (1997). Recent trends on bacterial resistance to antibiotics. *East African Medical Journal* **74**(3): 129-133.

Van Hall, M.A., Petit, P.C., Van Hall, H.N., Mouton, R.P and Ndinya Acholo, J.O (1991). Prevalence of resistance of *N. gohorrhoeae* to Penicillin and three other antibiotics in rural areas in Kenya. *East Afr. Med. J.* **68**: 853-855.

Weller, I.V., Kohl, P.K. Piot, P. and Meyer, L. (1984). Gonorrhoea in homosexual men and media coverage of the acquired immunodeficiency syndrome in London 1982-3: *Br. Med J* **289**: 1041-1042.

West, B., Changalucha, J., Gross kurth, H., Mayaud, P., Gabone, R.M., Gina, K.A. and Mabey, D. (1995). Antimicrobial screening, auxotype and plasmid content of *Neisseria gonorrhoeae* in Northern Tanzania, emergence of high level plasmid Tetracycline resistance. *Genito urin med.* **71:** 9-12.

WHO: World Health Organization (1990). Global surveillance network for gonococcal antimicrobial susceptibility, WHO, Geneva, pp. 366-452.

WHO: World Health Organization (1998). Methods in the diagnosis of gonorrhoea, WHO, Regional office for South East Asia, pp.1-9.

WHO: World Health Organization (1999). World Health Organization reports on infectious disease. The leading infectious killers, millions of deaths world wide in all age groups. WHO, Geneva, pp. 1-31.

William, A., Petri J.R. and Barbara, J. (2001). Microbial adhesion. **In**: *Principles of Infectious Disease*, pp, 13-20, (Mendel, G.L., Douglas, RG. and Bennett, S.E., eds). Churchill Livingston, U.K

Workneh Feleke and Kloos, H. (1993). Sexually transmitted diseases**. In**: *The Ecology of Health and Disease in Ethiopia*, pp. 295-305, (Kloos, H. and Zein Ahmed Zein., eds). West View Press, USA.

Zeleke WoldeTensay (2002). Multiple anti microbial resistance in bacterial isolates from clinical and environmental sources of Jimma hospital, South West Ethiopia. *SINET: Ethiop. J. Sci*. **25** (2) 295-302.

9. ANNEXES

Annex I. Questionnaire

This questionnaire aims to assess clinical status and history of volunteer patients suspected for gonococcal infection.

Date ________

Card No.________

1. Patients Age ______years
2. Sex: Male, ☐ Female ☐
3. Address________________
4. Educational background
   - 4.1 Elementary ☐
   - 4.2 High school or above ☐
   - 4.3 Read & write ☐
   - 4.4 Illiterate ☐
5. Economical status
   - 5.1 Civil servants ☐
   - 5.2 private businesses ☐
   - 5.3 Unemployed ☐
6. Marital status
   - 6.1 Married ☐
   - 6.2 Un married ☐
   - 6.3 Other ☐
7. Presence of ☐
   - 7.1 Urethral/ vaginal discharge ☐
   - 7.2 Frequent urination ☐
   - 7.3 Difficulty /burning upon urination ☐
   - 7.4 Other ☐
8. Duration of infection
   - 8.1 Chronic ☐
   - 8.2 Acute ☐

9. Are antibiotics taken for the last two weeks?

9.1 No ☐ 9.2 yes ☐

If yes which antibiotics? ________________, _________________

10. Have you taken traditional medicinal plants to treat the infection?

10.1 No ☐ 10.2 yes ☐, If yes, type of medicinal plants and their mode of application

______________________ ________________ ___________________

Annex II. Study subjects consent form

Date___________________________

Health center___________________________

I _______________________________have fully understood the objective and scientific merits of the study and here by give my consent for STI examination and agree that Ato Mesfin Tefera can utilize the specimens (urethral/vaginal discharge) for the scientific investigation titled " *In vitro* evaluation of antibacterial activities of some medicinal plants against clinical isolates of *Neisseria gonorrhoeae*" which will be conducted at Addis Ababa university.

Participants' signature __________________

DECLARATION

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

Mesfin Tefera

Signature

Date:

This thesis has been submitted for examination with our approval as

Advisor: Aberra Geyid, Ph.D

Signature**:**

Date:

Advisor: Asfaw Debella, Ph.D

Signature

Date: