PharmacologyOnLine
April 30, 2012
Special ISSUE • 2012 • vol.1 • 75 - 83

In vitro anti-Neisseria gonorrhoeae activity of *Albizia gummifera* and *Croton macrostachyus*

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Abstract

Neisseria gonorrhoeae is a sexually transmitted bacterial pathogen for which there is an increasing antimicrobial resistance concern. Frequency of isolation of multi drug resistant strains is increasing rendering a potential 'Super bug' status for *N. gonorrhoeae.* Agar dilution method was used to evaluate *in vitro* anti *N. gonorrhoeae* activity of crude and solvent fractions of *Albizia gummifera* and *Croton macrostachyus* plants widely used in the Ethiopian folk medicine for the treatment of gonorrhea or syphilis. 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. Sequential solvent-solvent extraction method was then used to partition crude extracts of each plants into chloroform, n-butanol and aqueous fractions and their anti *N. gonorrhoeae* activity was determined. Chloroform and n-butanol fractions were identified to be 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 fraction of *A. gummifera* exhibited activity at MIC values of 500-1000 µg/ml. However, aqueous fraction of *C. macrostachyus* had no growth inhibition effect when tested at 125-1000 µg/ml gradient of concentrations. Chemical screening indicated the presence of secondary metabolites mainly saponins (in *A. gummifera*), alkaloids, phenols, sterols/terpens and glycosides in both plants. This implies presence of bio active compounds and the need for further activity-guided fractionation and purification of the most active fraction(s) to localize and identify active constituents from the complex matrix by excluding ubiquitous and non active components. Our results also substantiate the ethno-botanical use of these medicinal plants for the treatment of gonococcal infections.

Keywords/ Phrases: Antimicrobial resistance, Medicinal plants, Natural products, Neisseria gonorrhoeae

Introduction

Neisseria gonorrhoeae the etiologic agent of gonorrhea is an exclusive human pathogen with no other ecological niche [1]. Spectrum of infection ranges from local infection to complications such as pelvic inflammatory disease (PID) and related sequale (infertility and ectopic pregnancy), disseminated gonococcal infection (DGI) and ophtalmia neonatorum. Infections with *N. gonorrhoeae* also enhance the risk of sexual transmission and acquisition of human immunodeficiency virus (HIV) by three to five folds through increasing the prevalence of HIV shedding and viral load in genital secretions and appear to be fueling the HIV pandemic [1,2].

Infection due to *N. gonorrhoeae* is wide spread in many regions of the world however, it disproportionately affect people living in sub Saharan Africa, south and south east Asia and south and central America with overall lower socioeconomic status and limited or no access to health care facilities [2, 3].The frequency of acquiring gonorrhea and other co infecting sexually transmitted pathogens is very high with an increasing antimicrobial resistance concern [1-4]. Gonococcal strains may be resistant to the common drugs like Penicillins, Tetracyclines, Spectinomycin, and recently to the Fluoroquinolones and to the macrolide Azithromycin rendering a potential 'Super bug' Status for *N. gonorrhoeae*. At present, the third generations Cephalosporin are the only classes of antimicrobial agents to which gonococci have not developed confirmed resistance but the cost of these agents limits their use in many developing countries. According to recent studies; few clinical isolates were found to exhibit decreased susceptibility even to the oral agent Cefixime [5-7].

The increasing incidence of antibiotic resistant strains has triggered urgent need of developing an effective vaccine; unfortunately the search for an effective gonnococcal vaccine has not yet bore fruit due to in part to the high frequency of variation of gonococcal surface components investigated as a possible vaccine candidate [8]. In the context of resource poor countries, the prohibitively expensive cost or non availability of efficacious antibiotics and the continuing evolution of single and multiple antibiotic resistant *N. gonorrhoeae* strains call for the development of safe, affordable and pharmacologically active new agents with anti *N. gonorrhoeae* activity. Screening of medicinal plants which are potential source of a variety of secondary metabolites with diverse chemical structure and novel mechanism of action constitute an obvious choice for such study. Pharmacological and phytochemical studies done on traditionally used medicinal plants 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. At present, scientists of the world from divergent fields are investigating plants anew with an eye to their antimicrobial usefulness. A sense of urgency accompanies the search of novel anti-infective compounds from natural products as the clinical importance of drug resistant pathogens increases and the pace of plant species extinction continues at a faster rate [9, 10].

In Ethiopia like other developing countries medicinal plants have been used as remedies for many years and still represent the main therapeutic tool in the traditional medicine. The major Pathogeneses arising from infectious agents for whom vast arrays of Ethiopian traditional medicinal plants are used includes sexually transmitted diseases (STDs), mainly gonorrhea and syphilis [10,11]. *Croton macrostachyus* (actively growing leaf powder, unripe fruits, bark, sap of the plant and decoctions of the roots), *Albizia gummifera* (root/bark powder cooked with meat and soup), *Allium sativum* (garlic juice), *Phytolaca dodecandra* (root powder), *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 gonorrhoeae agent [11].However, efficacy of many of the medicinal plants referred by traditional healers of the country, as an effective anti gonorrhoeae agent is not well documented. In the absence of scientific evidence of efficacy and knowledge of the consti-

tuents responsible for the possible biological effects, the validity of these plants is questionable and their use would remain locally restricted. In view of these, it is both necessary and interesting to investigate whether their traditional uses are supported by actual pharmacological effects or merely based on folklore [10-12].

Previous studies done on crude extracts of some indigenous plants of Ethiopia have shown anti microbial activity coherent with the use of the plants in folk medicine. More recently, crude extracts of 67 traditionally used medicinal plants of Ethiopia collected from different regions in the wild were screened for their anti microbial property, where 44 plant species (66%) exhibited activity against a range of clinically important bacterial and fungal strains. In this preliminary antimicrobial activity screening of some Ethiopian medicinal plants, *Albizia gummifera* J .F. Gmel,(*Fabaceae-Mimosoideae*) exhibited an interesting profile of activity against standard bacterial strains of *Neisseria gonorhoeae* (ATCC 49226) ,*Staphylococcus aurous* (ATCC 27853), *Streptococcus pyogenes* (ATCC 19615), *Streptococcus pneumonia* (ATCC 49619), *Eschericia coli* (ATCC 25922) and clinical isolates of *Bacillus cerus, Shigella dysentery*, *Shigella flexinery*, *Salmonella typhi* and *Salmonella typhimurium* when tested at crude extract level .*Croton macrostachyus* Hocht. (*Euphorbiaceae*) crude extract also demonstrated high activity against reference strain of *N. gonorrhoeae* (ATCC 49226) [10- 13]. However, no work has been done to determine activity of the crude and solvent fractions of these promising 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 promising traditionally used medicinal plants against wide range of clinical isolates of *N. gonorrhoeae.*

Material and Methods

Plant materials

Collection and taxonomic identification

C. macrostachyus leaves and *A. gummifera* seeds used in this study were collected from Bedelle and Bale regions approximately 540 and 500km away from Addis Ababa Ethiopia respectively, having an altitudinal range of 900-3900m above sea level. The plants were authenticated by a taxonomist in the Department of Drug research, Ethiopian Health and Nutrition Research Institute, Addis Ababa Ethiopia and Voucher specimens of *C. macrostachyus* (Voucher No. CM-1194) and *A. gummifera* (Voucher No. AG-2110) were deposited at the herbarium of the department for future reference.

Crude extracts preparation and sequential solvent – solvent partitioning

A. *gummifera* seeds and *C. macrostachyus* leaves 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 recovered hydro alcoholic extract of each plant was filtered (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 respectively.

Part (17g) of 80% methanol extract of the first plant was suspended in about 200ml-distilled water. The water suspension was then shaken with 50-80ml of trichloromethane ($CHCl_3$) each time until colorless and allowed to be partitioned in to chloroform-aqueous layer. The chloroform fraction was combined, filtered, evaporated (rotary evaporator) and labeled as fraction one. The remaining aqueous fraction was further shaken with n-butanol and allowed to be portioned into n-butanol-aqueous layer. The n-butanol layer was then combined, filtered, concentrated and evaporated to dryness (on water bath at 40°c) to give n-butanol fraction, which was labeled as fraction two. 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 (lyophilizer) to give a dried amorphous solid,

which was labeled as fraction three. The above procedure was repeated for the second plant to obtain the fractions. 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*.

The aqueous fractions were placed in a tightly closed bottle and kept in a desiccating environment. The remaining crude and solvent fractions were also kept in a tightly stopper bottle in a refrigerator (2-8C) until used for anti bacterial assay. 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. Of these, saponins (in *A. gummifera* only), alkaloids, phenols, terpens and glycosides were the semi purified test compounds in both *A. gummifera* and *C. macrostachyus.*

Test organisms

Organisms used for the antimicrobial activity tests were 19 clinical isolates of *N. gonorrhoeae* obtained from STD patients clinically symptomatic for uncomplicated gonorrhea. The standard *N. gonorrhoeae* organism used as a reference strain was obtained from American type culture collection of (ATCC 49226).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. The presumptively identified *N. gonorrhoeae* organisms were definitely confirmed by performing carbohydrate utilization tests using API-NH identification kit.

Antibacterial activity test

Antibacterial activity test and MIC determination were conducted using the agar dilution method which is the gold standard method used to determine gonococcal MICs [14]. An antibacterial effect of the plant extracts and fractions on the standard organism and clinical isolates was determined in comparison with standard antibiotics. Stock solutions of crude and fractionated plant extracts were prepared by dissolving 100 mg of plant extract in 5 ml final volume of appropriate solvent. In addition, 128mg of the following standard antibiotic powders Spectinomycin (Sigma) (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) and then diluted further with sterile distilled water to an exact final volume of 25 ml. From the primary stock solutions, a serious of two fold dilutions were prepared for each antibiotic containing antimicrobial concentration ten times higher than the final concentration to be obtained in the agar dilution plates [14, 15]

Standard susceptibility testing medium used 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)). 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 [14, 15]. In each plate, the following gradients of concentrations of plant extracts were included. 1000, 500, 250 and 125 ìg/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 ìg/ml. Ciprofloxacin was tested at concen-

trations of 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.062 and 0.031 ìg/ml.

Determination of minimum inhibitory concentrations

Direct colony suspension method recommended for fastidious microorganisms was used for the preparation of inoculum .In brief a small quantity of inoculum from an overnight subculture on chocolate agar was taken and suspended in Muller Hinton Broth (MHB). The suspension was homogenized with vortex mixer 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 [15,16]. Standardized multi point 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 ìl 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 solvent used as diluents and enriched GC base medium was also used to monitor effects of solvent on growth of organisms. Negative control plates were inoculated first, followed by the plates containing different antimicrobial products but starting with the lowest concentration for each antimicrobial agent. Finally, the third negative control plate was 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 (70%) at a temperature of 35-37C for 18 to 24 hours. 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 after an overnight incubation, disregarding a few single colonies or a fine barely visible haze .All assays were repeated three times and the modal MICs 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) [17, 18]

see Table 1.

see Table 2.

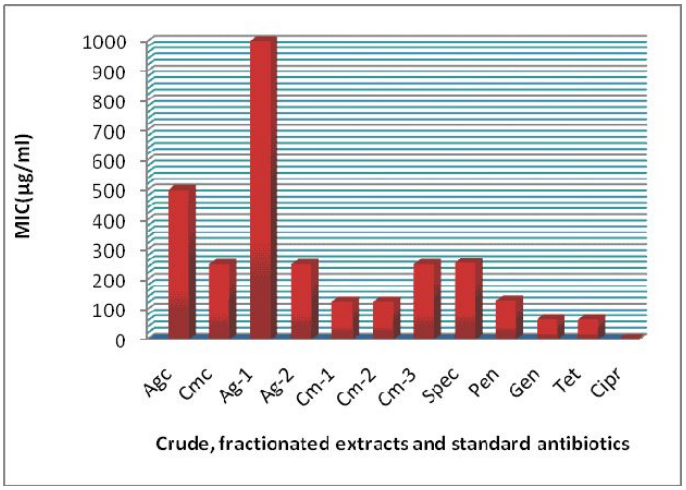

Fig. 1 MIC of crude extracts, solvent fractions and standard drugs against clinical isolates of *N. gonorrhea*

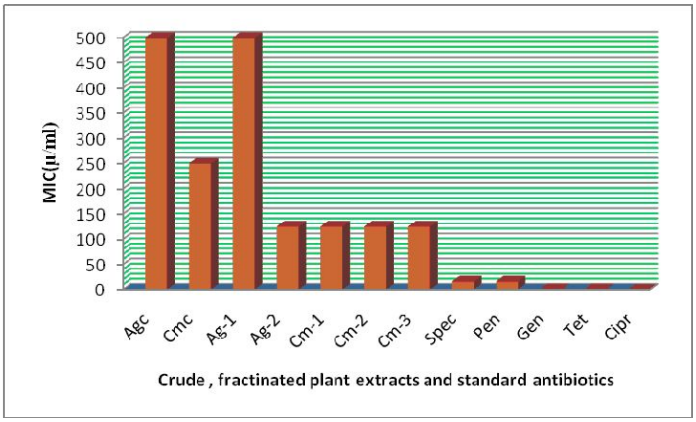

Figure 2. MIC of crude extracts, solvent fractions and standard drugs against reference strain of *N. gonorrhoeae*

Results and discussion

Crude hydro-alcoholic extract of *C. macrostachyus* (Cmc) completely inhibited growth of all the 19 clinical isolates and reference strain of *N. gonorrhoeae* at a sample concentration of 250 ìg/ml. Whereas the crude hydro-alcoholic extract of *A. gummifera* (Agc) exhibited complete growth inhibition at a sample concentration of 500 ìg/ml. (Table 1, Fig 1, 2).Comparable findings were reported by earlier studies[10,19] 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 ìg/ ml) MIC for both fractions was found to be 125 ìg/ml for clinical and standard organisms. However, the aqueous fractions (Cm-aqueous) (basified and none basified) had no growth inhibition effect. The basified chloroform fraction (Cm-3) completely inhibited growth of isolates and standard at a sample concentration of 250 and 125 ìg /ml respectively(Table 1, Fig 1, 2).Growth inhibition even by Cm-3 was also observed in about 74% of the clinical isolates at a sample concentration of125 ìg/ml (data not shown).

In n-butanol fraction of *A. gummifera* (Ag-2), complete growth inhibition was observed at MIC of 250 and 125 ìg/ml for clinical isolates and standard organism, respectively. In a similar study Ag-2 was reported to inhibit growth of clinical isolates and reference strain of *S. pyogens* and *S. pneumoniae* at MIC range of 500-1000 ìg/ml [19]. The aqueous fraction (Ag-1) showed complete growth inhibition at a relatively higher concentration level of 1000 ìg/ml for clinical isolates and 500 ìg/ml for standard organisms. However, the chloroform fraction (Ag-chloroform) had no growth inhibition effect at the four concentration levels tested.

The results in this study indicated that solvent fractions (Ag-2, Cm-1, Cm-2 and Cm-3) were active at lower concentration level (125-250 ìg/ml) than crude preparations of the same plant samples, which were effective at a relatively higher level of concentration (250-500 ìg/ml). This may indicate the partitioning and concentration of semi-purified bioactive compounds in the solvents used and the need for further partitioning and purification. In this study, the bacteriostatic activity of Cmc against the test organisms was higher than that of Agc. 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 [9, 20]. 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 and appears to be less susceptible to plant extracts than Gram-positive bacteria [21]. Under the conditions employed here, the crude extracts and semi-purified fractions exhibited an interesting profile of activity against *N. gonorrhoeae* .An MIC as low as 25 ìg/ml was also reported using diethyl ether fraction of *Terminalia macroptera* leaf extract against *N. gonorrhoeae* [22].

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 ìg/ml. Concordant findings were reported by other investigators from Ethiopia and some other African. 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 (Table, 2 Fig 1, 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 within 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* [21-24].

Conclusions and Recommendations

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* suggesting the presence of bioactive compound(s) in these plants. Bio-active compounds present in the plant extracts could be identified if further bioassay guided fractionation and purification is undertaken. Hence fractionation and purification of active fractions of both plants using improved techniques is highly recommended. 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 these plants, *in vivo* data are highly valuable in determining the potential use fullness of these plants for the treatment of infections and mammalian toxicity effects.

Further research with much more number of isolates involving known resistant strains of *N. gonorrhoeae* is recommended to confirm the effectiveness of these semi-purified fractions. In addition, alternative mechanisms of *N. gonorrhea* infection prevention and treatment should be included in activity screenings of such promising plants. Disruption of adhesion is one example of an anti-infection activity not commonly screened for currently. 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.

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Plant extract	Plant part	*N. gonorrhea*		MIC (µg/ml)	Class of compounds
		Isolates (N0.)	Standard(No.)		
Cmc	Leaf	19	1	250, 250	G, P, S, T
Cm-aqueous	,,	19	1	- -	
Cm aqueous (basified)	,,	19	1	- -	
Cm-1	,,	19	1	125, 125	
Cm-2	,,	19	1	125, 125	
Cm-3	,,	19	1	250, 125	
Agc	Seed	19	1	500, 500	A, G, P, S, T
Ag-chloroform	,,	19	1	- -	
Ag-1	,,	19	1	1000, 500	
Ag-2	,,	19	1	250, 125	

Table 1. In vitro anti N. gonorrhea activity of plant extracts

Key; A, G, P, S, T= Alkaloids, Glycosides, Phenols, Saponins, & Terpens, MIC, Minimum inhibitory concentration for clinical isolates standard organisms respectively,- no growth inhibition effect. Agc A. gummifera crude extract, Ag-1 A. gummifera aqueous fraction, Ag-2 A. gummifera butanol fraction, Ag-chloroform A. gummifera chloroform fraction, Cmc C. 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 & non-basified aqueous fractions.

Standard drugs	*N. gonorrhea* strains tested			
	Clinical Isolates		Standard organism (ATCC 49226)	
	No.	MIC (µg/ml)	No.	MIC (µg/ml)
Ciprofloxacin	19	0.06	1	0.06
Gentamycin	19	64	1	16
Penicillin	19	128	1	1
Tetracycline	19	64	1	1
Spectinomycin	19	256	1	16

Table 2. MIC test results of 19 isolates and reference strain of N. gonorrhea against standard drugs