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Research Article

Preclinical Effects of Ethiopian Medicinal Plants against *Neisseria gonorrheae* Clinical Isolates and Standard Strain

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ABSTRACT

Drug-resistant gonorrhea and the absence of a gonococcal immunization have prompted a renewed emphasis on medicinal plants as the main source of innovative treatments. In Ethiopia, gonorrhea has been treated using medicinal plants such as *Phytolacca dodecandra*, *Ranunculus multifidus*, *Clematis longicauda*, and *Impatiens ethiopica*. However, these plants' ability to combat gonococci hasn't been assessed. The purpose of this investigation was to confirm the *in-vitro* antigonococcal properties and examine the phytochemical constituents of these plants. A lab-based investigation was conducted. The dry, coarsely crushed plant samples were extracted by maceration with 80% methanol. Standard procedures were used to perform phytochemical screening of the crude extracts. The anti-gonococcal activity test was performed employing the agar well-diffusion method on gonococcal agar at extract concentrations of 200, 100, and 50 mg/mL. The standard treatment used was ceftriaxone 0.05 mg/disc. Using the broth dilution method, the minimum inhibitory and minimum bactericidal concentrations of the crude extract were ascertained. The plants contain secondary metabolites like terpenoids, alkaloids, tannins, saponins, flavonoids, and steroids. *P. dodecandra* roots showed promising anti-gonococcal activity against *Neisseria gonorrheae* clinical isolate-03 at a dose of 200 mg/mL, with the largest growth zone of inhibition (21.67 mm). The maximum concentration of all the plant extracts demonstrated remarkable growth inhibition in comparison to the placebo control. The methanolic extracts from the experimental plants have an MIC range of 6.125 to 100 mg/mL. The results showed that the MBC ranged from 25 to 200 mg/mL. The investigated herbs exhibited anti-gonococcal effects. Thus, thorough animal and *in-vitro* experiments are required to ascertain the phytotherapeutic benefits of these plants in treating gonorrhea.

Introduction

Neisseria gonorrheae (gonococcus), a gram-negative diplococcus, is the sole human pathogen that causes gonorrhea, a sexually transmitted infection (STI).[1,2] Around the world, gonorrhea poses a serious hazard to public health. This bacterial STI is the second most common in the world, after *Chlamydia trachomatis*. In 2016, the World Health Organization (WHO) reported that the number of new *N. gonorrheae* infections worldwide increased to 87 million from 78 million in 2012.[2] According to reports, direct contact between the germs and mucosal membranes in the oropharynx, anal canal, urogenital tract, and eyes occurs during coitus. For females, this frequently leads to cervicitis and, for males, urethritis. Following medical therapy, the majority of males with symptomatic gonococcal urethritis are no longer considered contagious.[3] While dysuria and purulent urethral discharge can occur in both males and females with gonorrhea, the majority of cases, such as pharyngeal or rectal gonorrhea, are asymptomatic.[2] About 50 to 80% of infected females and 1 to 40% of infected males experience asymptomatic infections, which

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can have negative effects if undetected or mistreated.[1] Since *N. gonorrheae* is not known to produce any strong exotoxins, the negative effects are primarily caused by damage due to innate immune response activation at the colonization sites. Untreated infections of the female ascending genital tract can lead to many complications, such as ectopic pregnancy, pelvic inflammatory disease, and infertility. Neonatal blindness may arise from mothers transferring these microorganisms to their unborn children. Disseminated gonococcal infection (known to cause infectious arthritis) and endocarditis can result from untreated *N. gonorrheae*. In rare instances, *it* may enter the bloodstream and spread, resulting in infections of the skin, tendons, or joints, as well as endocarditis or meningitis. Gonococcal infections that affect the eyes (ophthalmia neonatorum) or, in rare cases, cause extensive sickness can be passed on to babies born to infected women during childbirth. Furthermore, gonococcal infection has been demonstrated to enhance HIV acquisition and transmission.[3]

In both wealthy and poor nations, gonorrhea causes serious health problems that result in high annual costs for diagnostics and treatment (Rx). Antimicrobial resistance (AMR) poses an immediate threat to public health. Fighting AMR has become a major global concern due to *N. gonorrhoeae*'s development or acquisition of resistance to the last-line antibiotics used in empirical therapy. Because AMR infections are on the rise and there are currently no effective vaccines, the WHO recognized *N. gonorrheae* as a high-priority illness in 2017. To fight this infection, more research and development of novel antibiotics is required, in addition to the creation of a vaccine. The Centers for Disease Control and Prevention (CDC) updated the United States list of urgent concerns in 2019 to include gonococcus.[3, 4]

Rapidly emerging strains of *N. gonorrhoeae* have become resistant to all recommended Rxs, including extended-spectrum cephalosporins, penicillin, tetracyclines, fluoroquinolones, and macrolides. Despite an increase in reports of azithromycin resistance and decreased ceftriaxone susceptibility worldwide, injectable ceftriaxone combined with oral azithromycin is currently the recommended therapy in several nations.[4] Currently, there is no gonococcal vaccine available on the market. There are no novel antibiotics in late development, although more combinations of presently available antibiotics are being investigated.[1] It's critical to modify the antibiotic medications currently available, discover innovative substitutes, and create vaccines to combat the high prevalence and increasing resistance of *N. gonorrheae*.[2] Improved global action and research efforts to retain gonorrhea as a readily treatable infection are essential.[5] In this regard, exploring claimed medicinal plants (valuable resources) is crucial in novel drug discovery.

Medicinal plants (MPs) are the source of a wide variety of phytocompounds with biological uses. Science has identified over 12,000 active chemicals, and 20 to 25% of drugs used in contemporary medicine are chemically extracted from plants.[6] Like other underdeveloped nations, Ethiopia has long utilized MPs as Rxs, and they continue to be the primary therapeutic option in traditional medicine. Many traditional Ethiopian MPs are employed to treat infectious diseases, particularly STIs, such as syphilis and gonorrhea. These diseases are significant among the various pathologies caused by infectious agents.[7,8]

The effectiveness of various MPs recommended by traditional healers in the nation as anti-gonorrhea agents remains unproven. The legitimacy of these plants is disputed, and their use would continue to be confined to small areas without scientific proof of their efficacy and understanding of the ingredients responsible for any potential biological effects. Given this, it is intriguing and crucial to determine whether their customary uses are based solely on tradition or are supported by pharmacological efficacy.[9, 10]

The first plant examined in the current study is a Phytolaccaceae family member, Phytolacca dodecandra L'Herit. In Ethiopia, P. dodecandra is referred to as "endod" in Amharic, "haranje" in Sidama, "handoode" in Afaan Oromo, and "shibti" in Tigrigna.[11, 12] P. dodecandra has a variety of therapeutic uses in different countries. The plant extracts have also been traditionally utilized as a purgative, anthelmintic, laxative, emetic, diuretic, and anti-diarrheal agent. It is also employed to treat headaches, rheumatism, skin conditions, malaria, and rabies. Additionally, it is employed to manage tuberculosis, asthma, epilepsy, otitis media, uterine tumors, snakebite, and urinary illnesses.[11,13-17] The plant powders have been utilized in the manufacturing of laundry detergents and in the management of parasites like mice and houseflies as well as vectors like mosquito larvae.[18]

Traditional Ethiopian medicine makes extensive use of all components of *P. dodecandra*. Many human and animal diseases can be treated with berries, leaves, and roots of the plant. *P. dodecandra* is mostly used to treat gonorrhea, leeches, intestinal worms, anthrax, and rabies. It is also frequently used to treat abortion and skin irritations (ringworm).[12,19] Moreover, it is used to manage stomach pain, herpes virus infection, and liver disease.[13] This plant has been used in Ethiopia as a Rx for schistosomiasis.[18] *P. dodecandra* has several scientifically proven activities such as anti-rabies,[20,21] abortifacient,[22] analgesic, anti-inflammatory,[16] anti-bacterial,[23-25] anti-fungal/anti-mycelial,[11,24,26,7] anti-oxidant,[13,25] hepatoprotective,[13] molluscicidal,[28-33] anti-malarial,[34] spermicidal,[35] anthelminthic,[11] insecticidal,[36] pesticidal,[18] larvicidal,[11,14,37,38] and pupicidal effects.[14]

A phytochemical study of *P. dodecandra* leaf, fruit, and stem extracts indicated that they comprised alkaloids,

flavonoids, triterpenoids, saponins, steroids, total phenols, and tannins.[15, 24] Lemmatoxin and 3-*O*-(*O*-α-l-rhamnopyranosyl-[l,2]-*O*-[β-d-galactopyranosyl-(1,3)]-β-d-glucopyranosyl) oleanolic acid saponins isolated from *P. dodecandra* could be responsible for the molluscicidal activity of the plant. Additionally, saponin compounds isolated from this plant include *O*-acetyl oleanolate (I), 3-*O*-(2′,4′-di-*O*-[β-d-glucopyranosyl]-β-d-glucopyranosyl) 2-β-hydroxyoleanolic acid, methyl tri-*O*-acetyl bayogenin, 3-(2,4-di-*O*-[β-d-glucopyranosyl]-β-d-glucopranosyl)-olean-l2-ene-28-oic acid, and 3-*O*-(3′-*O*-[β-d-galactopyranosyl]-β-d-glucopyranosyl) 2β-hydroxyoleanolic acid. Further, from the plant endod, genins (hederagenin, oleanolic acid, 2β-hydroxyoleanolic acid, bayogenin, dodecandral, and dodecandralol), olean-12-ene dicarboxylic acids (serjanic acid, phytolaccagenin, and phytolaccagenic acid), and glycosides (esculentoside L1 and esculentoside) are identified.[11] The primary constituents of the aromas include aldehydes and ketones, such as sulcatone, 2-nonanone, benzaldehyde, and phytone. In addition, the essential oils of *P. dodecandra* included significant amounts of phytone, phytol, and hexadecanoic acid.[19]

Ranunculus multifidus Forsk., is the second plant studied for its anti-gonococcal activity in the present investigation. *R. multifidus* is a widely distributed species of flowering plant within the Ranunculaceae family. It is well recognized in Ethiopia by local appellations such as "*Hogioo*" in Kaffa, "*Etse siol*" in Geez, "*Gundi*" in Amharic, "*Tuche* or *Aysmamata*" in Gamo, and "*Abba warqe*" in Afaan Oromo. "Etse siol" literally translates to "a plant of hell" in Geez, referring to the burning feeling that is experienced while applying it.[39,40]

Various ethnobotanical uses of *R. multifidus* have been reported in African countries. The species is used to treat amenorrhea, dysmenorrhea (as an antispasmodic), morning sickness, and wounds.[39] The plant is also used to treat genital warts, gonorrhea,[41] STIs, tuberculosis, genital sores, hemorrhoids, and infertility. During pregnancy, it is used to facilitate labor and cleanse the blood.[40] A tea made of fresh leaves is used to alleviate stomach pain and malaria.[40,41] Urinary tract infections (UTIs) are treated using root decoction.[39] Rubefacient and anti-rheumatic are two common uses for the plant.[42] *R. multifidus* is used in the traditional medicine system of Ethiopia to remove intestinal worms and to heal leishmaniasis. Many illnesses, including tonsillitis, cataracts, eye infections, asthma, amoebiasis, hemorrhoids, external tumors ("nekersa") or cancer, dysentery, skin conditions, and toothaches, can be treated using the plant's leaves. Fresh *R. multifidus* leaves are chopped and filtered, and the juice is given to cattle orally to cure trypanosomiasis and edema in the Gamo zone of south Ethiopia.[43]

Ethnopharmacological studies have reported that *R. multifidus* has various bioactivities, including antileishmanial, antischistosomal,[43] anti-inflammatory,[44, 45] and antimalarial effects.[40] These properties of the plant may be attributed to its chemical constituent, anemonin. Anemonin has demonstrated antibacterial, anti-inflammatory, wound-healing, and antioxidant activities in multiple studies.[43] Anemonin isolated from *R. multifidus* also showed anti-plasmodial activity.[40] The essential oil obtained from the aerial parts (possessing *p*-mentha-2, 8-dien-1-ol) showed efficacy against *Streptococcus pyogenes* and *Staphylococcus aureus.*[42] *R. multifidus* leaf extracted with both petroleum and chloroform showed efficaciousness against *S. aureus* and *E. coli*.[45] Aqueous and organic leaf extracts showed promising activity against *N. gonorrheae* with a 2 mg/mL minimum inhibitory concentration (MIC).[46] Zinc oxide nanoparticles biosynthesized by aqueous *R. multifidus* aerial part extracts demonstrated antioxidant and antibacterial activities.[47]

Clematis longicauda Steud. ex A. Rich. (Ranunculaceae) is the third experimental plant investigated for activity against gonorrhea in this study. It is locally known as "*fitti*"/"*hida adi*" in Afaan Oromo and "*zina-charo*"/"*wusho-charo*" in Sheko. Traditionally, the plant is utilized to treat rheumatism, fever, blackleg, and leeches.[48, 49] It is an indigenous species in Ethiopia that has long been used to heal skin and ear ailments. People treat eczema and otorrhea with *C. longicauda* leaves.[48] The plant leaf is also used for managing skin itching, toothaches, and wounds.[50] In the Andracha District, Sheka Zone, Southwest Ethiopia, local healers use the plant's roots to treat gonorrhea. In terms of pharmacological effects, *C. longicauda* displayed wound healing,[51] antifungal, and antibacterial.[48] The genus Clematis has a variety of chemical components, including alkaloids, flavonoids, steroids, tannins, triterpene saponins, coumarins, phenolic glycosides, macrocyclic compounds, anemonins, lignans, fixed oils, and volatile oils.[48, 56]

Impatiens ethiopica Grey-Wilson is the fourth plant we studied for its effectiveness against *N. gonorrheae*. This plant, which belongs to the Balsaminaceae family, is native to Ethiopia and South Sudan.[53] In Ethiopia, *I. ethiopica* is called "insosla" in Amharic. Roots of the plant are traditionally used orally to suppress cough.[54] The plant root is also used to manage wounds.[55] According to tribal healers in Sheka Zone's Andracha District, the locals use insosla roots to cure gonorrhea. To the best of our knowledge, there have been no prior pharmacological investigations conducted on *I. ethiopica* due to its indigenous nature.

Realizing the trends in AMR, a phytochemical screening accompanied by *in-vitro* antibacterial testing of commonly used MPs against *N. gonorrheae* is crucial to providing opportunities for treating gonorrhea. Therefore, taking the traditional claims and reports of the earlier studies,

this work was aimed at determining the *in-vitro* anti-gonococcal activities and phytochemical constituents of *P. dodecandra* roots, *R. multifidus* leaves, *C. longicauda* roots, and *I. tinctoria* roots collected from the Sheka zone, southwest Ethiopia.

Materials And Methods

Chemicals, Materials and Reagents

Distilled water (Ethiopian Pharmaceutical Manufacturing), absolute methanol (Carlo Erba, France), and normal isotonic saline (Aculife Health Care, India) were the chemicals and solvents employed in this research. The experiment was carried out using an orbital shaker, Whatman filter paper No. 1 (Maidstone, UK), a water bath and dry oven (Okhla industrial area, India), antibiotic discs (Ceftriaxone (BioLab.) 100 µg/mL, colistin 10 µg (BD BBSensi-Disc)), plates, Thayer Martin Medium (MTM) (OXOID, UK), fastidious broth (Hardy Diagnostics, USA), oxidase (tetramethyl-para-phenylenediamine dihydrochloride) test reagent (BBL/Difco Diagnostic, USA), Gonococcal agar (BBL/Difco Diagnostic), and superoxol (30%H_2O_2). Every drug, chemical, and reagent used in this investigation was of analytical quality.

Collection and Authentication of the Experimental Plants

The Sheka Zone, located 562 kilometers southwest of Addis Ababa (the capital city of Ethiopia), is where the plant components were collected. The leaves were protected from outside influences by a plastic covering during transit. A taxonomist employed by the Addis Ababa University, College of Natural and Computational Sciences, National Herbarium, verified the harvested plant materials as *Phytolacca dodecandra* L. ' Herit (TW 001/2022), *Ranunculus multifidus* Forsk. (TW 002/2022), *Clematis longicauda* Steud. ex A. Rich. (TW 003/2022), and *Impatiens ethiopica* Grey-Wilson (TW 004/2022). For later use, the voucher specimens were documented.

Preparation of the Plant Material

Following their collection and identification, the fresh plant parts were examined for extraneous materials, cleaned with tap water to eliminate any contaminants, allowed to dry at room temperature in the shade, and then pulverized with a manual crusher, mortar, and pestle to produce coarse-sized particles. The resulting coarse powder was packed in a plastic bag to prevent the mixing of any impure materials before the experiment.

Extraction of the Study Plants

One of the most important factors in successfully extracting bioactive compounds from plant samples is the type of solvent employed in the extraction procedure. The majority of the time, traditional healers use water as the extracting solvent; however, in the maceration method of plant extraction, most plant compounds are better extracted using a mixture of water and alcohol. [56] After the dried materials were weighed, 600 g of *P. dodecandra* roots, 600 g of *R. multifidus* leaves, 361 g of *C. longicauda*, and 380 g of *I. ethiopica* were soaked in 1200, 1200, 722, and 760 mL of methanol (80%), respectively, for three days. An orbital shaker was used to periodically shake macerated samples at 120 rpm. Subsequently, gauze and Whatman filter paper No. 1 (Maidstone, UK) were used to filter the macerated plant material in turn. The residues were extracted again using the same solvent in order to increase the yield. The filtrates were collected into a single container, and a rotary evaporator (R 200, Switzerland) operating at 45 rpm and below 40°C was used to remove the methanol. Following that, a lyophilizer was used to further dry the concentrated material.[57] In the end, the dehydrated 80% methanolic extracts were stored at -20°C in a deep freezer until the experiment was conducted (Fig. 1).

Phytochemical Screening

The presence of alkaloids, terpenoids, saponins, tannins, phenols, flavonoids, anthraquinones, cardiac glycosides, and coumarins was determined by qualitative phytosubstances analysis of crude extracts using standard techniques.[58-61]

Test Bacteria

Four *N. gonorrheae* were isolated out of 38 clinical samples collected from patients suspected of gonorrhea, who were presenting with definitive urethral discharge as well as urethritis and cervicitis syndromes at Mizan-Tepi University Teaching Hospital. Endocervical swabs or urethral secretions were collected using sterile cotton

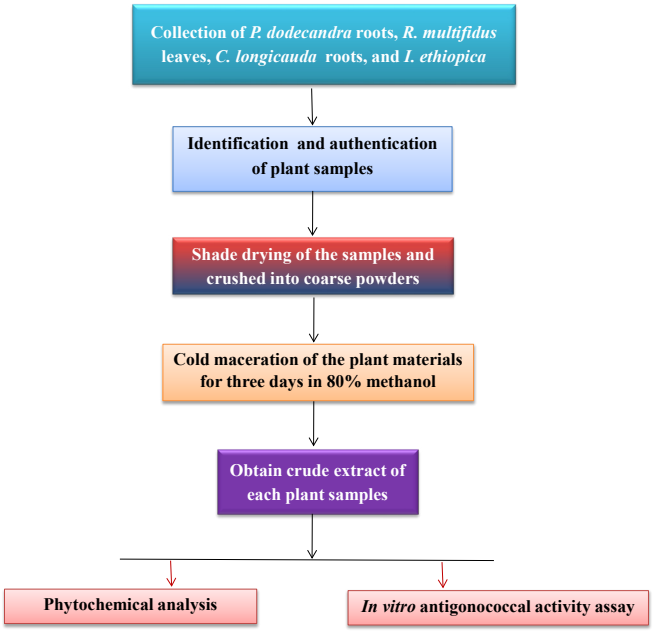

Fig. 1: Flowchart showing the process of getting the plant extracts

swabs. The collected specimens were immediately immersed in an Amies transport medium in sterile test tubes. The samples were taken to the microbiology laboratory at Mizan Campus to identify and culture the bacteria. The samples were inoculated in modified Thayer Martin Medium (MTM) (OXOID, UK) and incubated for 24 to 48 hours at 36°C in an atmosphere with 5% CO_2 enrichment. *N. gonorrheae* was first isolated on selective media, and its colony morphology—pinkish-brown, translucent, smooth, and with defined margins—led to a tentative diagnosis. The presence of characteristic pairs of gram-negative diplococci upon gram stain, a positive superoxide (30% H_2O_2) or catalase test, a positive oxidase test, and a positive colistin resistance test served as additional confirmations (Fig. 2). To evaluate antigonococcal effects and ensure the quality of each laboratory activity, the Ethiopian Public Health Institute (EPHI) reference strain of *N. gonorrheae* from the American Type Culture Collection (ATCC 49226) was also employed.[62]

Inoculum Preparation

Following the instructions provided by the Clinical and Laboratory Standards Institute (CLSI, 2018)[63], a bacterial suspension of clinical isolates and a standard strain was prepared. To make the 0.5 McFarland standards, also known as the turbidity standards, 0.5 mL of 1.175% w/v barium chloride and 99.5 mL of 1% v/v sulfuric acid were combined. It is expected that the turbidity standard has

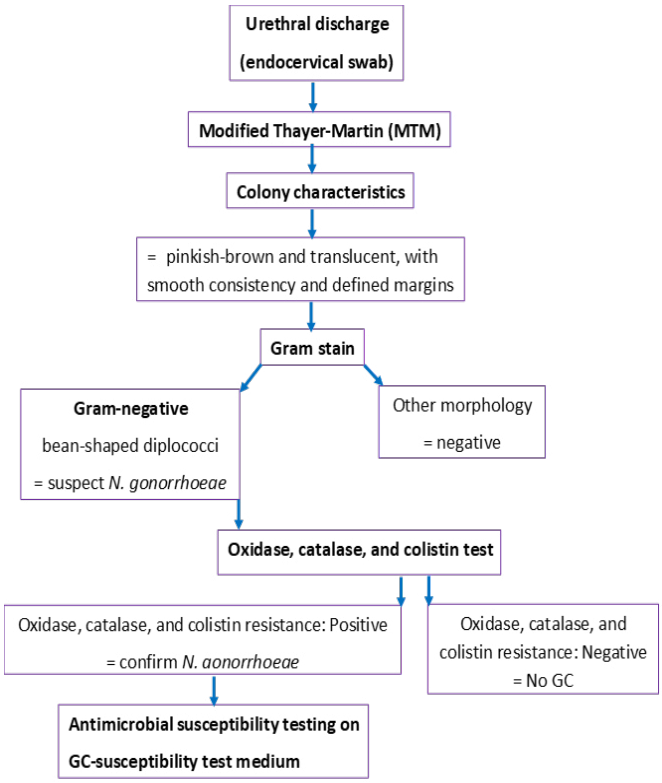

Fig. 2: Schematic diagram showing a summary of gonococcal culture and antimicrobial susceptibility testing method (GC: gonococcal agar)

a bacterial concentration of $1x10^8$ CFU/mL. Fresh agar plates of the bacterial culture were used to collect well-isolated pure colonies (3–5) of the isolated bacterium and the reference strain. The colonies were transferred in an aseptic manner into pre-labeled test tubes having 5 mL of normal saline using a sterile wire loop. By visual inspection against a white paper background and contrasting black lines in the presence of sufficient light, the turbidity of the bacterial suspension in each tube and that of the 0.5 McFarland standards were adjusted.[62,63]

Antibacterial Activity Assay

Agar well diffusion method

The assay was carried out using guidelines provided by the Clinical and Laboratory Standards Institute (CLSI, 2018). The suspension was streaked on the sterile gonococcal agar supplemented with 1% (v/v) IsoVitaleX agar by rotating at 45^0 in three different directions. The thickness of the gonococcal medium was first ensured by checking the depth to be 4 mm in a 150 mm plate. A 6 mm diameter sterilized borer was used to create five equally spaced wells on each plate, and the wells were numbered to indicate the concentration of extracts added. To produce test solutions with final stock concentrations of 200, 100, and 50 mg/mL, respectively, plant extracts weighing 0.2, 0.1, and 0.05 g were dissolved in distilled water. About 100 µL of the extracts at 200, 100, and 50 mg/mL concentrations were added to the respective wells (treatment groups). Distilled water was utilized as the negative control, and an antibiotic disc containing 100 µg/mL of ceftriaxone (BioLab) was employed as the positive control. The plates were placed in a gas jar with 5% CO_2 and incubated for 20 hours at 36°C. Millimeters (mm) were used to measure and describe the resulting zones of inhibition.[63,64]

Broth Dilution Method

Through the two-fold serial dilution approach, the MIC values were determined using the broth dilution method. The fastidious broth was first made according to the manufacturer's instructions. The crude extract was prepared at 800 mg/mL and kept in a sterile beaker. Then, 10 sterile test tubes were arranged in a row on the rack for each organism. One ml of the broth was filled into all the test tubes using a micropipette. Then, 1-mL of 800 mg/mL extract was added to the first test tube using a micropipette and serially diluted up to test tube number 8. Over 2 mL of diluted extract from test tube number 8 was discarded. The tip of the pipette was changed for subsequent test tubes. The 0.5 McFarland standardized bacterial inoculum was diluted to 1:150 by using normal saline (0.9%) to obtain 5×10^5 CFU/mL within 15 minutes after standardization. Next, 1-mL of the inoculum was introduced into every test tube, with the exception of test tube number 10, which was utilized to verify the broth's

sterility. Test tube number 9 served as the positive control. The final bacterial density became 1×10^6 CFU/mL, whereas the final concentration of the extract became 200, 100, 50, 25, 12.5, 6.25, 3.125, and 1.5625 mg/mL from test tubes 1 to 8, respectively. The test tubes were then loosely sealed and left to incubate for 20 hours at 36°C with 5% CO_2. The minimum concentration of the crude extract that totally stopped the organism's development in the tube (a clear broth or a broth with no discernible turbidity) as seen by an unaided eye was the MIC endpoint. For every group, three duplicates of each experiment were run. The MIC of the test plant material was determined using the producible values.

By removing 0.1 ml of the broth from each test tube containing extract concentrations higher than the MIC value, the Minimum Bactericidal Concentration (MBC), or the lowest extract concentration that totally eliminated the bacterial population, was determined. Accordingly, the broth (0.1 mL) was supposed to contain the reduced bacterial cells and none from each test tube was sub-cultured by the spread plate technique on the surface of modified Thayer Martin medium. The plates were incubated at 37ºC for 24 hours for bacterial growth observation. The MBC was defined as plates that did not exhibit colony growth or a 99.9% reduction in CFU on the solid agar medium following subculture. The reproducible values were used to determine the MBC of the test plant material, and the experiments were conducted in triplicate.

Quality Control

To improve the repeatability of the results, standard operating procedures were adhered to when screening phytochemicals, and all materials utilized in the experiment were of analytical grade. To ensure that the results could be reproducible, antibacterial activity tests were carried out three times. The lab technician, who was working under supervision, measured the diameter of the zone of inhibition.

Ethical Statement

The Mizan-Tepi University Research Directorate office provided ethical clearance by formal letter. The Mizan-Tepi University School of Pharmacy's Research and Ethics Committee thoroughly examined and approved the procedures, issuing permission number SOP3/10/14 in the process.

Statistical Analysis

SPSS version 22.0, a statistical tool for social research, was used to organize, edit, and analyze the data. The assay findings for agar well diffusion's antibacterial activity were shown as mean ± standard error of the mean (SEM). The diameters of the extracts' zones of inhibition against the clinical isolates and control strain were compared, and statistical significance was established using the one-way analysis of variance (ANOVA) Tukey post hoc test. At a 95% confidence interval, a *p-value* of less than 0.05 was deemed statistically significant.

Results

Extract yields

Here in Table 1, we show the calculated percentage yields.

Phytochemical Constituents

All of the tested metabolites were present, according to the preliminary phytochemistry study results on the methanolic (80%) extracts (Table 2). Flavonoids and coumarins were present in all study plants. Alkaloids and saponins were revealed in all experimental plants except *R. multifidus*. Tannins were not present only in *P. dodecandra*, while terpenoids were not present only in *C. longicauda*. Anthraquinones were detected only in *R. multifidus* and *C. longicauda* samples.

Antibacterial Activity Tests

The antibacterial activities of 80% methanolic extracts of *P. dodecandra (Fig. 3)*, *R. multifidus (Fig. 4)*, *C. longicauda (Fig. 5)*, and *I. ethiopica (Fig. 6)* against *N. gonorrheae* are evaluated. The vehicle employed to dissolve the extract, known as the negative control, was unable to exhibit any noticeable inhibition against either the standard strain or tested clinical isolates of *N. gonorrheae* across all groups. On the contrary, the standard drug produced much higher effects than all doses of all plant extracts in all test groups. The extracts demonstrated varying zones of inhibition at different concentrations in their anti-gonococcal efficacy against the test organisms, which included clinical isolates and control strains. The plant extracts showed dose-dependent antibacterial effects against *N. gonorrheae* at all dosages. The best antibacterial activity with a maximum inhibition zone was recorded at 200 mg/mL (21.67 mm) of *P. dodecandra* against *N. gonorrheae* clinical isolate-03 (NGCI-03), followed by 21.33 mm growth inhibition at the same dose of *P. dodecandra against the* standard strain (Fig. 3) and *C. longicauda* against clinical isolate-04 (NGCI-04) (Fig. 5).

At 200 mg/mL, the root extracts of *P. dodecandra* exhibited the maximum zone of inhibition (17 mm) in their antibacterial activity against NGCI-01 (Fig. 3). The

Table 1: Yields for hydroalcoholic *P. dodecandra roots, R. multifidus* leaves, *C. longicauda* roots, and *I. ethiopica* roots extracts

S. No.	*Extract type*	*Actual yield in grams*	*Percent yield (%)*
1	*P. dodecandra*	181.8	30.3
2	*R. multifidus*	159.1	26.52
3	*C. longicauda*	68.7	19.03
4	*I. ethiopica*	116.4	30.63

Table 2: Phytochemical constituents of 80% hydromethanolic extracts of *P. dodecandra* roots, *R. multifidus* leaves, *C. longicauda* roots, and *I. ethiopica* roots

S. No.	*Plant extract*	*Alkaloids*	*Anthraquinones*	*Flavonoids*	*Tannins*	*Steroids*	*Terpenoids*	*Saponins*	*Coumarins*
1	PD	+	-	+	-	-	+	+	+
2	RM	-	+	+	+	+	+	-	+
3	CL	+	+	+	+	-	-	+	+
4	IE	+	-	+	+	+	+	+	+

PD: P. dodecandra extract, RM: R. multifidus extract, CL: C. longicauda extract, IE: I. ethiopica extract, + = present, – = absent.

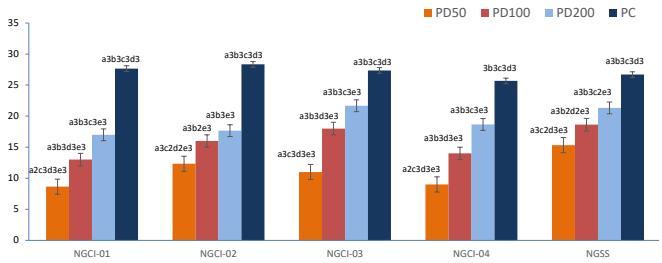

Fig. 3: *Growth inhibition zone (mm) by P. dodecandra root extract against clinical isolates and standard strain of N. gonorrhoeae. The values are expressed as mean ± SEM (n = 3). NTC: negative control; PD: P. dodecandra extract; PC: positive control, NAGA: no anti-gonococcal activity, NGCI: N. gonorrhea clinical isolate, NGSS: N. gonorrhea standard strain. [a] to NC; [b] to 50 mg/ml; [c] to 100 mg/ml; [d] to 200 mg/ml; [e] to PC; [1]p <0.05, [2]p <0.01, [3]p<0.001. Numbers 50, 100, and 200 next to the letters in the first column indicate the doses in mg/ml.*

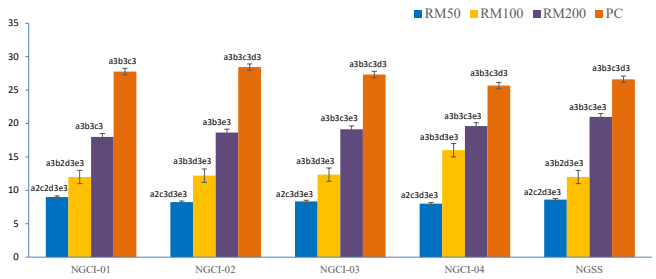

Fig. 4: Growth inhibition zone (mm) by *R. multifidus* leaf extract against clinical isolates and the standard strain of *N. gonorrhoeae*. The values are expressed as mean ± SEM (n = 3). NTC: negative control; RM: *R. multifidus* extract; PC: positive control; NAGA: no anti-gonococcal activity; NGCI: N. *gonorrhea* clinical isolate, NGSS: *N. gonorrhea* standard strain. [a] to NC; [b] to 50 mg/mL; [c] to 100 mg/mL; [d] to 200 mg/mL; [e] to PC; [1]p <0.05, [2]p <0.01, [3]p <0.001. Numbers 50, 100, and 200 next to the letters in the first column indicate the dose in mg/mL.

extract also showed relatively stronger antibacterial activity, exhibiting a higher zone of inhibition (21.67 mm) against NGCI-03 at a 200 mg/mL concentration, but was lower compared to the positive control (25.33 mm). The growth inhibition zone against NGCI-03 at a 100 mg/mL concentration was 18.00 mm. The antigonococcal activity of the crude extract against clinical isolate NGCI-04 is also promising (18.67 mm inhibition zone at the highest dose). All doses of *P. dodecandra* extract considerably ($p < 0.001$ at higher doses and $p < 0.01$ at the lowest dose) inhibited the growth of all clinical isolates in comparison to the placebo control. The entire dose of this extract also exhibited outstanding action ($p < 0.001$) against the control (standard) strain (NGSS) as compared to the placebo group.

The zone of inhibition exhibited by 200 mg/mL of *R. multifidus* leaf extract against clinical isolates NGCI-01, NGCI-02, NGCI-03, and NGCI-04 was found to be 18, 18.64, 19.13, and 19.62 mm, respectively (Fig. 4). The antigonococcal effect of this plant against the control (standard) strain was noted to be slightly higher (exhibiting a growth inhibition zone of 21 mm) at 200 mg/mL. All doses of *R. multifidus* extract appreciably ($p < 0.001$ at higher doses and $p < 0.01$ at the lowest dose) inhibited the growth of all clinical isolates and the standard strain as compared to the distilled water.

With a concentration of 100 mg/mL, the *C. longicauda* root extract displayed a smaller zone of inhibition against NGCI-01 (12 mm) and NGCI-02 (11.33 mm) than it did against NGCI-03 (18.33 mm) and NGCI-04 (18.67 mm) (Fig. 5). Unlike the effects seen with other extracts, the root extract of *C. longicauda* at 200 mg/mL showed a larger area of growth inhibition against the standard strain and most clinical isolates.

All doses of *C. longicauda* extract significantly ($p < 0.001$ at higher doses and $p < 0.01$ at the lowest dose) suppressed the development of all clinical isolates and the standard strain in comparison to the vehicle, similar to the results

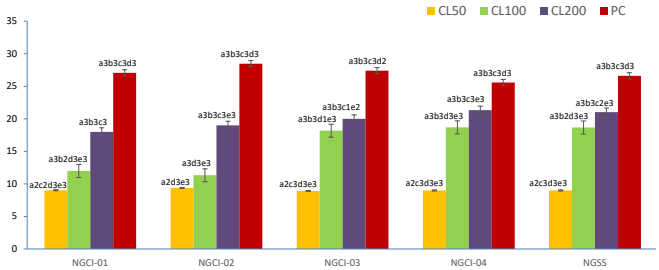

Figure 5: *Growth inhibition zone (mm) by C. longicauda root extract against clinical isolates and standard strain of N. gonorrhoeae. The values are expressed as mean ± SEM (n = 3). NTC: negative control; CL: C. longicauda extract; PC: positive control; NAGA: no anti-gonococcal activity, NGCI: N. gonorrhea clinical isolate, NGSS: N. gonorrhea standard strain. a to NC; b to 50 mg/ml; c to 100 mg/ml; d to 200 mg/ml; e to PC; 1p<0.05, 2p<0.01, 3p<0.001. Numbers 50, 100, and 200 next to the letters in the first column indicate the doses in mg/ml.*

shown in *R. multifidus*. It was demonstrated that the standard drug (positive control) had a zone of inhibition against NGCI-02 (28.48 mm), which was greater than the zones of inhibition the standard drug evoked against the standard strain and the remaining clinical isolates in the other test groups.

The zone of growth inhibition of the *I. ethiopica* root extract with 200 mg/mL was noted to be slightly lower against clinical isolates NGCI-01 and NGCI-02 (18 mm) as compared to NGCI-03 (19 mm), NGCI-04 (20 mm), and NGSS (21 mm). At 100 mg/mL, the zone of inhibition against NGCI-04 (18.69 mm) was greater than that against NGCI-01 (13 mm), NGCI-02 (12 mm), NGCI-03 (12 mm), and NGSS (13.67 mm) (Fig. 6). When compared to the effects of 100 mg/ml (18.67 mm) and 50 mg/mL (10 mm) concentrations, 200 mg/mL demonstrated the largest zone of growth inhibition (20 mm) against the isolate.

All doses of *I. ethiopica* extract drastically ($p < 0.001$ at higher doses and $p < 0.01$ at the lowest dose) inhibited the growth of NGCI-01, NGCI-02, and the standard strain in comparison to the placebo treatment. All concentrations of I. ethiopica extract also significantly increased the zone of inhibition against NGCI-03 and NGCI-04 ($p < 0.001$) when compared to the placebo substance. The growth inhibition zone for positive control against NGCI-04 (25.67 mm) was relatively lower than that noted against other isolates and control strains.

Fig. 6: Growth inhibition zone (mm) by *I. ethiopica* root extract against clinical isolates and standard strain of *N. gonorrhoeae*. The values are expressed as mean ± SEM (n = 3). NTC: negative control; IE: *I. ethiopica* root extract; PC: positive control, NAGA: no anti-gonococcal activity, NGCI: *N. gonorrhea* clinical isolate, NGSS: *N. gonorrhea* standard strain. a to NC; b to 50 mg/ml; c to 100 mg/mL; d to 200 mg/mL; e to PC; 1 p <0.05, 2 p <0.01, 3 p<0.001. Numbers 50, 100, and 200 next to the letters in the first column indicate the doses in mg/mL.

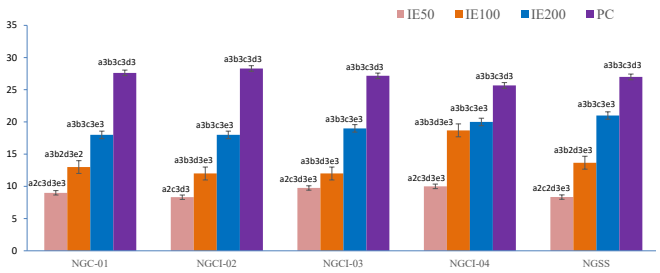

Figure 6: *Growth inhibition zone (mm) by I. ethiopica root extract against clinical isolates and standard strain of N. gonorrhoeae. The values are expressed as mean ± SEM (n = 3). NTC: negative control; IE: I. ethiopica root extract; PC: positive control, NAGA: no anti-gonococcal activity, NGCI: N. gonorrhea clinical isolate, NGSS: N. gonorrhea standard strain. a to NC; b to 50 mg/ml; c to 100 mg/ml; d to 200 mg/ml; e to PC; 1 p<0.05, 2 p<0.01, 3 p<0.001. Numbers 50, 100, and 200 next to the letters in the first column indicate the doses in mg/ml.*

Determination of MIC and MBC

P. dodecandra and *R. multifidus* extracts revealed a 50 mg/mL MIC and 200 mg/mL minimum bactericidal concentration (MBC) against NGCI-01. The two plants showed an MIC of less than 50 mg/mL and an MBC of less than 200 mg/mL (except for NGCI-04) against all the remaining isolates and the standard strain. The MIC and MBC of *C. longicauda* and *I. ethiopica* roots against the same clinical isolate NGCI-01 were found to be 100 mg/mL and greater than 200 mg/mL (no anti-gonococcal activity at 200 mg/mL extract concentration), respectively. All four plant extracts showed anti-gonococcal activities at lower concentrations against the standard strain (with 50 mg/mL MIC and 12.5 mg/mL MBC) than against the clinical isolates. The lowest and highest MICs of the four experimental plants, *P. dodecandra*, *R. multifidus*, *C. longicauda*, and *I.* ethiopica against *N. gonorrheae* were found to be 6.125 and 25, 12.5 and 50, 12.5 and 50, and 12.5 and 50, respectively. On the other hand, the lowest

Table 3: MIC and MBC of the crude extracts of *P. dodecandra* roots, *R. multifidus* leaves, *C. longicauda* roots, and *I. ethiopica* roots against clinical isolates and standard strain of *N. gonorrheae*

S. No.	*Study plants*	*Concentration (mg/mL)*	*N. gonorrheae*				
			NGCI-01	*NGCI-02*	*NGCI-03*	*NGCI-04*	*NGSS*
1	*P. dodecandra*	MIC	50	12.5	25	6.125	12.5
		MBC	200	50	100	25	50
2	*R. multifidus*	MIC	50	12.5	12.5	50	12.5
		MBC	200	50	50	200	50
3	*C. longicauda*	MIC	100	50	50	12.5	12.5
		MBC	–	200	200	50	50
4	*I. Ethiopica*	MIC	50	25	50	50	12.5
		MBC	200	100	200	200	50

MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration, NGCI: *N. gonorrheae* clinical isolate, NGSS: *N. gonorrheae* standard strain, –: MBC not determined at all until maximum concentration

and highest MBCs of the four experimental plants, *P. dodecandra*, *R. multifidus*, *C. longicauda*, and *I. ethiopica,* against *N. gonorrhoeae* were also found to be 25 and 200, 50 and 200, 50 and 200, and 50 and 200, respectively (Table 3).

DISCUSSION

The ongoing emergence of antibiotic resistance in pathogenic bacteria, especially *N. gonorrheae*, is a serious global health problem. Finding novel medications is largely dependent on screening plant materials and their separated constituents.[65] In this study, four plant extracts were found to suppress the specific bacteria responsible for STDs, suggesting that these plants may be used to make potent anti-gonococcal drugs.

Liquid mixtures—often alcohol and water—are used extensively in the extraction of plant constituents. Most of the polar and non-polar substances found in the plant sections can be extracted using a water and alcohol mixture.[56] Furthermore, compared to water extract, organic solvent extract has been shown to have promising antibacterial properties. Additionally, the majority of MPs' secondary metabolites include aromatic rings, making organic solvents the most effective for their extraction.[66, 67, 68] Therefore, the hydroalcoholic solvent was selected to act as a solubilizing agent in this experiment.

The presence of various secondary metabolites in the extracts (Table 2) of *P. dodecandra*, *R. multifidus*, *C. longicauda*, and *I. ethiopica* has been confirmed by qualitative phytoconstituent screening. It is commonly recognized that these metabolites, through various mechanisms, induce antibacterial actions in other plants. [69, 70] Plant secondary metabolites are usually resistant to illness. Therefore, the presence of flavonoids, alkaloids, terpenoids, steroids, and tannins in the extracts that act individually or synergistically may be attributed to the plants' activity in this study against clinical isolates and the standard strain of *N. gonorrheae* at all concentration levels examined.[71,72] Flavonoids isolated from *Piper lanceaefolium* crude extract exhibited activity against both susceptible and resistant *N. gonorrheae* strains.[73] The plant material used in the current experiment may contain anti-gonococcal metabolites besides those detected in this research.

Polyphenolic compounds, like flavonoids, exhibit various biological activities. The capacity of flavonoids to form complexes with bacteria's soluble extracellular proteins and cell walls may be the reason for reports of their antibacterial action. Tannins, a different class of polyphenolic chemicals with an astringent attribute, are thought to be crucial in the healing process of the inflamed, microbially related mouth and throat surface. They result in antimicrobial action by precipitating microbial protein.[48] The mechanism by which tannins exert their effects may be attributed to their ability to deactivate specific enzymes, disrupt transport proteins in the cell envelope, and inhibit microbial adhesion. The mode of action of saponins may be comparable to that of flavonoids.[74] Alkaloids also exhibit a propensity to hinder the development of gram-negative bacteria.[75]

When compared to the placebo control, the extracts of *P. dodecandra*, *R. multifidus*, *C. longicauda*, and *I. ethiopica* showed a significant growth inhibition against *N. gonorrheae*. In a prior study, extracts of various plants showed zones of growth inhibition against *N. gonorrheae* ranging from 8 to 22 mm,[73] which is consistent with the current findings. Despite the differences in the test bacteria, the zones of growth inhibition produced by the plant extracts in this study at all concentration levels are generally consistent with the zones of growth inhibition shown by methanol extracts of *Nigella sativa* seeds, *Echinops kebericho* roots,[76] *Commelina benghalensis*, *Cissus quadrangularis*, *Euphorbia prostrate*, and *E. heterophylla*.[74] Comparing extracts from the current study plants to the standard agent, the former produced reduced biological activity. The plant extracts' crude nature could be the cause of their reduced efficacy.[77]

Previous research that indicates the antibacterial effects of *P. dodecandra*, *R. multifidus*, and *C. longicauda* provides additional evidence of their current antigonococcal activity, even if the test organism used in the study was not *N. gonorrheae*.[23-25,42,45,48] This strengthens the present activity due to the similarity of their cell wall structure. The effects against gonorrhea exhibited by *P. dodecandra* might be contributed by the compounds previously isolated from this plant.[11, 19]

Several species of the genus *Ranunculus* have been shown to exhibit antibacterial activity against the gram-negative bacterium *E. coli*.[47,48] The current effects against gonorrhea by *R. multifidus* are also supported by the earlier investigation, which found that water and organic *R. multifidus* leaf extracts showed activity against *N. gonorrheae* standard strain with a 2 mg/mL MIC.[46] In this study, the MIC value for *R. multifidus* leaf extract is 12.5 mg/mL. The activity difference between the two studies might be ascribed to the differences in methods used, solvents employed for extraction, geographical location of the plant, collection season and time, etc. The antigonococcal activity of *R. multifidus* is mainly attributed to its bioactive compound anemonin, which displayed antibacterial efficacy against other bacteria. [40] Additionally, protoanemonin and anemonin isolated from *R. sceleratus* have shown antimicrobial activity.[78]

The present bioactivity of *C. longicauda* is asserted by the plant's previously confirmed activity against the gram-negative bacteria *P. aeruginosa*.[48,51] The antibacterial compound anemonin is also isolated from the genus Clematis.[52] In line with the past phytochemical screening results from leaf extracts,[48] antibacterial metabolites, saponins, tannins, and flavonoids were detected from

C. longicauda root extract in the present study (Table 2). The bioactivity and phytochemicals of *I. ethiopica* have never been the subject of an experimental study before, as far as the authors know. However, species of the same genus, *I. tinctoria,* have demonstrated antimicrobial effects against gram-negative bacteria in other studies.[79] In the current study, secondary metabolites having reported antibacterial efficacy like alkaloids, flavonoids, tannins, steroids, terpenoids, and saponins[69,70] are detected in hydroalcoholic *I. ethiopica* root extract (Table 2).

To determine how well the extracts worked to stop the tested bacteria from growing, an MIC assay was used. Plant extracts that are more active against a given organism typically have low MICs, whereas extracts that are less active have high MIC results.[74] According to this general claim, the extracts' MIC results in the current investigation matched their respective antigonococcal activities. This study's MIC scores were found to be lower than the corresponding MBC results, indicating that the plant extract was bactericidal at higher concentrations but bacteriostatic at lower ones.[80] Consistent with the findings in this study, extracts of *Blepharis subvolubilis*, *Jatropha zeyheri*, and *Peltophorum africanum* produced 12.5 mg/ml of MIC against *N. gonorrhoeae*. Additionally, extracts of *Abrus precatorius*, *Rhoicissus tridentata*, and *Senna petersiana* revealed activity against *N. gonorrheae* with a 6.3 mg/mL MIC value.[81]

Even though the tested bacteria are different, the MIC values of 50, 25, and 12.5 mg/mL observed in the current research are in agreement with the MIC values revealed by *Punica granatum* peels and *N. sativa* seeds.[76] Almost near the MIC values of 100, 50, 25, 12.5, and 6.125 mg/mL seen in the present study, MIC values of 97.5, 48.75, 24.38, 12.18, and 6.09 mg/mL by different plants against other bacteria were displayed in other studies.[74]

The present findings strongly suggest that the investigated plant extracts possess remarkable anti-gonococcal potential, positioning them as promising candidates in the search for novel therapeutics against antibiotic-resistant *N. gonorrhoeae*. The consistent growth inhibition observed across all tested concentrations, together with MIC values comparable to those reported for other bioactive medicinal plants, reinforces the robustness of the antibacterial activity. Moreover, the alignment of these results with previous studies on related plant species and compounds, such as anemonin and other polyphenolic metabolites, further substantiates the biological relevance of the findings. Although the crude extracts exhibited lower activity than standard drugs and relatively high MIC values, this is likely attributable to the lack of purification, suggesting that isolation of active principles could yield even more potent antibacterial agents. Collectively, these results highlight the substantial therapeutic promise of *the study plants* as rich sources of effective anti-gonococcal compounds, with significant potential for future drug development despite the current evidence being limited to *in-vitro* investigations. Therefore, more *in vivo* research on these therapeutic plants ought to be done in the future.

Conclusion And Recommendation

The methanolic (80%) extracts of *P. dodecandra* roots, *R. multifidus* leaves, *C. longicauda* roots, and *I. ethiopica* roots have *in-vitro* antigonococcal activities. Good anti-gonococcal activities were demonstrated by the extracts at the highest concentration. As a result, this research offers proof in favor of the plant's traditional use in treating gonorrhea. However, the number of clinical isolates is limited, and we have stressed the necessity of future studies involving a larger number of clinical isolates to strengthen the findings. To discover novel anti-gonococcal antibiotics, more *in-vitro* and *in vivo* research on the solvent fractions and isolated compounds, together with a thorough examination targeted at an explanation of the mechanism of action, should be conducted in the future.

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