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Original Research Article

Action of Ethanolic Extract of *Holerrhena pubescens* on Bacterial Strains

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Abstract: The need to screen efficient, safe, affordable, and readily available antimicrobial agents from various medicinal plants, for their potential antimicrobial activity has become critical in light of the rapid development of microbial resistance against chemotherapeutic drugs (mainly antibiotics). Despite notable advances in the biological and phytochemical evaluation in recent years, comprehensive reviews of *Holarrhena pubescens* are limited. The main objective of this study is to evaluate the antimicrobial activities of this plant extract. One significant use of plant extracts can be in treating bacterial infections and develop potential antibacterial agents against multi drug resistant (MDR) and susceptible bacteria. If antimicrobial activities are present, these extracts will provide useful information for discovering new compounds with better activity and more effectiveness against resistant and susceptible bacteria responsible for infections than currently available. The ethanolic extract of leaves of *Holarrhena pubescens* was prepared and tested against multidrug resistant (MDR) and American type culture collection strains of bacteria. We have obtained two types of results in this experiment. The extract showed antimicrobial properties against *Staphylococcus aureus* (ATCC 23235), *Escherichia coli* (MDR) and *Acinetobacter* (MDR), while, it showed no antimicrobial activities along with some growth promoting role against *Escherichia coli* (ATCC 25922), and *Klebsialla pneumoniae* (MDR), which may be due to chemicals other than secondary metabolites.

Keywords: *Holarrhena pubescens*, phytochemical, chemotherapeutic, antibacterial, multidrug resistance (MDR), American type culture collection (ATCC) bacterial strains.

INTRODUCTION

Holarrhena pubescens belongs to the Apocynaceae family. It is a native plant which mainly grows in the southern and central Africa, Indian subcontinent and the Indochina region. The plant is widely employed in Ayurveda and other traditional medical systems. It is widely known for its anti-diabetic action which makes it economically significant. Additionally, despite many reports of the plant's traditional applications by African and Asian people, neither in vitro nor in vivo research has been done to support its use in treating any specific diseases (Zahara *et al*., 2020).

H. pubescens leaves are not thought to have any therapeutic benefit in Ayurveda. Unani medicine uses leaves as an aphrodisiac, tonic, astringent, and galactagogue to treat conditions like chronic bronchitis, urine discharges, wounds, and ulcers as well as to relax muscles and control menstruation (Dubey *et al*., 2012).

Alkaloids such holamine, kurchamine, holaphyllidine, holaromine, mitiphylline, and holadysenterine as well as non-alkaloidal ingredients like kurchinin, kurchinicin, and holarrheno are some recently described substances that have been reported from *Holarrhena pubescens* (Siddiqui *et al*., 2014). This plant's seeds have been proven to have an excellent aqueous extract for treating diabetes. Although no scientific study has been published in the literature, the bark powder of this plant is frequently used as an efficient medication in numerous formulations against diabetes in various districts of Nepal (Bhusal *et al*., 2014).

The emergence of antibiotic resistance in bacterial strains poses a significant and urgent

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problem.The rapid occurrence of genetic changes can lead to the loss of effectiveness in standard antibiotics within a mere five-year timeframe (Chandra *et al*., 2017). The World Health Organization (WHO) reports that traditional medicines continue to be utilized by over 80% of the global population for treating various ailments. Given the escalating resistance of multidrug-resistant microorganism strains to antibiotics and other medications, there is an urgent need for alternative approaches (Dan *et al*., 2018)

In developing nations, surgical site infections are the most typical hospital acquired illnesses. Other wound infections include infections of the soft tissues, diabetic foot ulcers, pressure ulcers, bite wounds, and infections of the diabetic foot (Bhalchandra *et al*., 2018). The most frequent pathogenic bacteria recovered from wounds aregram-negative bacilli like *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Proteus* speciesand gram-positive cocci like *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Streptococcus spp*. (Pallavali *et al*., 2017).

The majority of Gram-positive bacteria have peptidoglycan, which has a strong structural foundation, as the main component of their cell walls. The transit of tiny chemicals like antibiotics does appear to be somewhat resistant in this form, but (Nikolaidis *et al*., 2014). Gram-negative bacteria, including Escherichia coli, have an outer membrane that serves as a strong barrier in contrast (Atef *et al*., 2019). It is essential to learn more about the antibacterial properties of plant crude extracts against both spoilage and pathogenic microbes.

The main alkaloid that has been shown to have antibacterial properties is conessine. It has not yet been established whether the antibacterial action is brought on by a single alkaloid or by a combination of alkaloids found in this plant. Although this method has been published throughout the previous four decades, bioassay-guided purification is still absent (Zahara *et al*., 2020).

MATERIALS AND METHODS

Collection and procurement of plant material:

At 22.6792° N and 86.5004° E, in the Basadera forest near Dharagiri Falls, Ghatshila, Jharkhand, the leaves of the shrub *Holarrhena pubescens* were collected on December 19, 2022.

Fig 1: Leaf of *H. pubescens*

Preparation of Mueller Hinton Broth

100 ml of MH Broth is prepared by dissolving 2.1 grams in 100 ml of distilled water in a sterile glass conical flask. It was boiled so that the medium dissolves completely and sterilized by autoclaving at 15 lbs pressure for 15 minutes at 121°C.

Preparation of Plant Extract:

The ethanolic extract of *Holerrhena pubescens* was prepared by dissolving 1 gram of dried leaves into 5 ml of ethanol. After this, the mixture was vortexed.

Fig 2: Ethanolic extract of *Holarrhena pubescens*

Microorganisms to be Tested:

The strains of *Staphylococcus aureus* (ATCC 23235), *Escherichia coli* (ATCC 25922), *Escherichia coli* (MDR, Table 1), *Klebsialla pneumoniae* (MDR, Table 2) and *Acinetobacter* (MDR, Table 3) are chosen for this experiment. It was collected from the Department of Microbiology, Peerless Hospitex Hospital & Research Centre Limited, Kolkata, West Bengal, India. The Table 1, 2 and 3 show their antibiotic sensitivity.

Table 1: Antibiotics sensitivity of tested *Escherichia coli* (MDR) in VITEK 2 automated system

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Amoxicillin/ clavulanic acid	>= 32	R	**Meropenem**	>=16	R
Piperacillin/ Tazobactam	>=128	R	**Amikacin**	>=64	R
Cefuroxime	>=64	R	**Gentamicin**	>=16	R
Cefuroxime Axetil	>=64	R	**Ciprofloxacin**	>=4	R
+Cefixime	>=64	R	**+Levofloxacin**	>=4	R
Ceftriaxone	>=64	R	**Tigecycline**	<=0.5	S
Cefoperazone/ Sulbactam	>=64	R	**Fosfomycin**	<=16	S
Cefepime	>=32	R	**Colistin**	<=0.5	I
+Doripenem	>=8	R	**+Polymyxin B**	<=0.5	I
Ertapenem	>=8	R	**Trimethoprim/ Sulfamethoxazole**	>=320	R
Imipenem	8	R			

R- Resistant; S- Sensitive; I- Intermediate

Table 2: Antibiotics sensitivity of tested *Klebsialla pneumoniae* (MDR) in VITEK 2 automated system

Antimicrobial	**MIC**	**Interpretation**	**Antimicrobial**	**MIC**	**Interpretation**
Amoxicillin/ clavulanic acid	>= 32	R	**Meropenem**	>=16	R
Piperacillin/ Tazobactam	>=128	R	**Amikacin**	>=64	R
Cefuroxime	>=64	R	**Gentamicin**	>=16	R
Cefuroxime Axetil	>=64	R	**Ciprofloxacin**	>=4	R
+Cefixime	>=64	R	**+Levofloxacin**	>=8	R
Ceftriaxone	>=64	R	**Tigecycline**	1	S
Cefoperazone/ Sulbactam	>=64	R	**Fosfomycin**	>=256	R
Cefepime	>=32	R	**Colistin**	<=0.5	I
+Doripenem	>=8	R	**+Polymyxin B**	<=0.5	I
Ertapenem	>=8	R	**Trimethoprim/ Sulfamethoxazole**	>=320	R
Imipenem	8	R			

R- Resistant; S- Sensitive; I- Intermediate

Table 3: Antibiotics sensitivity of tested *Acinetobacter* (MDR) in VITEK 2 automated system

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Piperacillin/ Tazobactam	>=128	R	**Gentamicin**	>=16	R
+Cefixime	>=64	R	**Ciprofloxacin**	>=4	R
Ceftriaxone	>=64	R	**+Levofloxacin**	>=4	R
Cefoperazone/ Sulbactam	>=64	R	**Tigecycline**	>=8	R
Cefepime	>=32	R			
+Doripenem	>=8	R	**Colistin**	<=0.5	I
Imipenem	>=16	R	**+Polymyxin B**	<=0.5	I
Meropenem	>=16	R	**Trimethoprim/ Sulfamethoxazole**	<=20	S
Amikacin	>=64	R			

Micro-dilution Test

We took a 96-well cell culture plate. 100 microliters of MHA broth are put in alternate rows from H to A. The vacant rows in between are filled with 100 microliters of alcohol. The alcohol filled wells are taken as control. On each alternate row filled with MH broth, 100 microliters of plant extract were added to the H column. No extract is added to the alcohol-filled wells. The plant extract and MH broth are properly mixed using the pipette, and then they are serially diluted throughout the row from H to A such that each well has 100 microliters of the combined product of MH broth and plant extract in it. Two rows each are assigned for each microorganism namely, *Staphylococcus aureus* (MDR), *Escherichia coli* (ATCC), *Escherichia coli* (MDR), *Klebsiella pneumoniae* (MDR), and *Acinetobacter* (MDR). The row containing the alcohol is the control and the row consisting of the plant extract is the experimental set up. A small amount of culture from the microbial culture plate was taken with an inoculation loop and then mixed with sterile saline water taken in an eppendorf and the microbial extract was prepared for each of the individual microorganisms we have already chosen. A specific microbe was allocated a set of test and control wells on the cell culture plate, and 10 microliters of its microbial extract were pipetted into those wells. After measuring the optical density (OD) at 0 hours, the plate is incubated at 37°C for 24 hours, and the optical density is then measured once again.Graphs are constructed using the measured optical density values to assess how each microorganism is affected by the ethanolic extract of Holarrena pubescens.

RESULTS

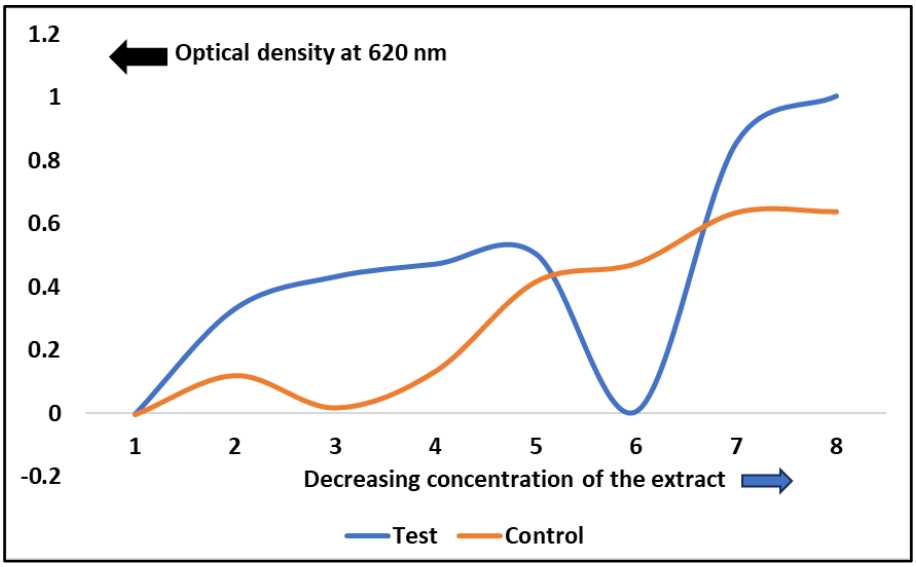

Fig 3: Effect of *Holarrhena pubescens* ethanolic extract on *Staphylococcus aureus* (ATCC 23235) showing MIC values of 3.125 mg/ml. Increased OD levels from 1-5 is due to high colour of the extract. Concentration of the extract 1: 100 mg/ml, 2: 50 mg/ml, 3: 25 mg/ml. 4: 12.5 mtg/ml. 5: 6.25 mg/ml, 6: 3.125 mg/ml, 7: 1.5625 mg/ml. 8: 0.78125 mg/ml

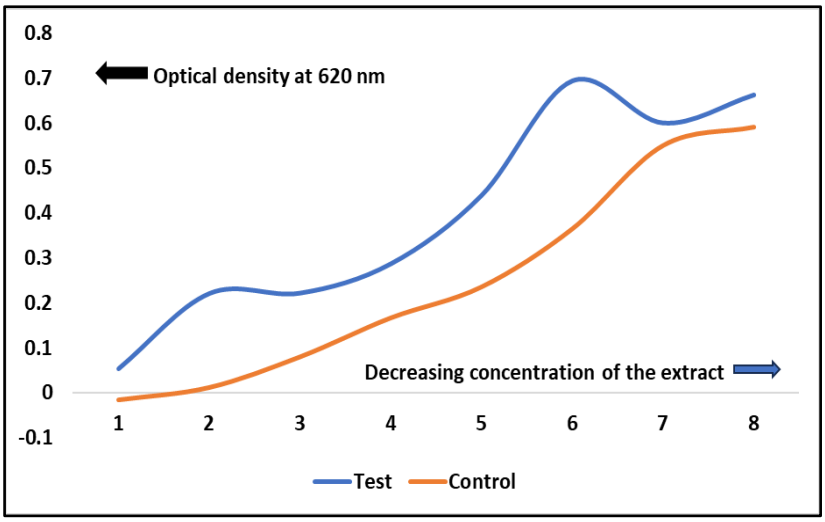

Fig 4: Effect of *Holarrhena pubescens* ethanolic extract on *Escherichia coli* (ATCC 25922) showing no antimicrobial action. Concentration of the extract 1: 100 mg/ml, 2: 50 mg/ml, 3: 25 mg/ml. 4: 12.5 mtg/ml. 5: 6.25 mg/ml, 6: 3.125 mg/ml, 7: 1.5625 mg/ml. 8: 0.78125 mg/ml

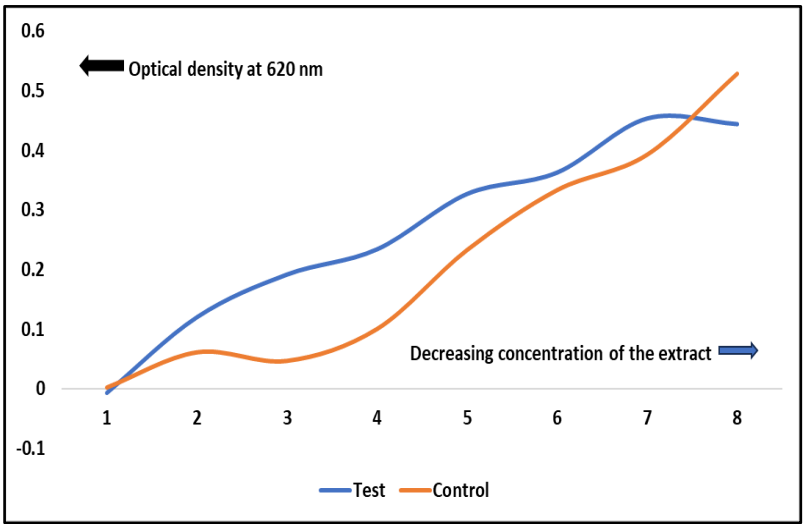

Fig 5: Effect of *Holarrhena pubescens* ethanolic extract on *Escherichia coli*(MDR) showing MIC value of 100 mg/ml. Concentration of the extract 1: 100 mg/ml, 2: 50 mg/ml, 3: 25 mg/ml. 4: 12.5 mtg/ml. 5: 6.25 mg/ml, 6: 3.125 mg/ml, 7: 1.5625 mg/ml. 8: 0.78125 mg/ml

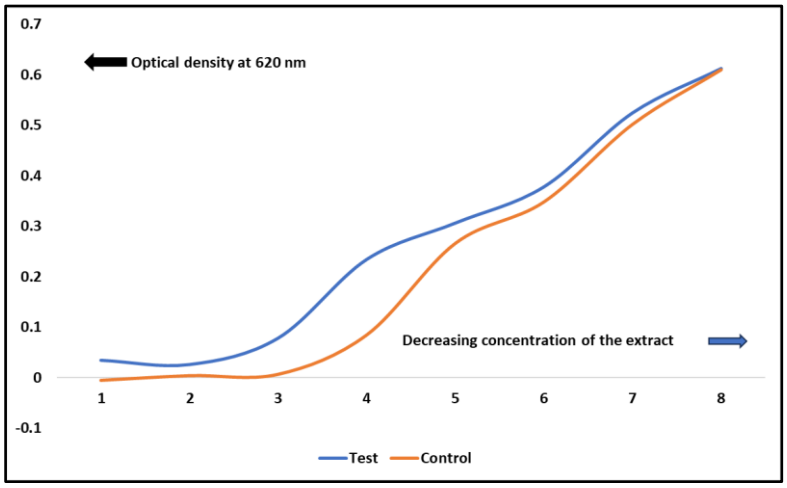

Fig 6: Effect of *Holarrhena pubescens* ethanolic extract on *Klebsiella pneumoniae*(MDR) showing no antimicrobial action. Concentration of the extract 1: 100 mg/ml, 2: 50 mg/ml, 3: 25 mg/ml. 4: 12.5 mtg/ml. 5: 6.25 mg/ml, 6: 3.125 mg/ml, 7: 1.5625 mg/ml. 8: 0.78125 mg/ml

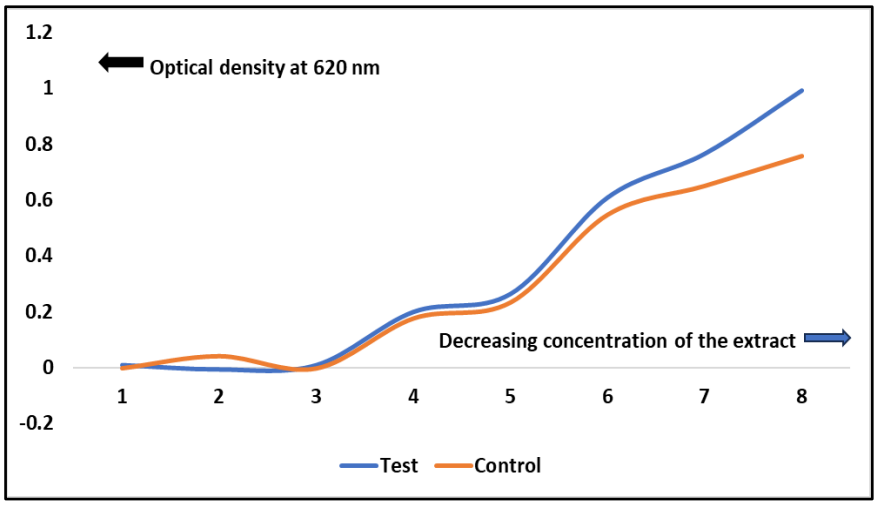

Fig 7: Effect of *Holarrhena pubescens* ethanolic extract on *Acinetobacter*(MDR) showing MIC value of 50 mg/ml. Concentration of the extract 1: 100 mg/ml, 2: 50 mg/ml, 3: 25 mg/ml. 4: 12.5 mtg/ml. 5: 6.25 mg/ml, 6: 3.125 mg/ml, 7: 1.5625 mg/ml. 8: 0.78125 mg/ml

The ethanolic extract of *Holarrhena pubescens* exhibited antibacterial activity against some of the chosen bacterial strains, namely, *Staphylococcus aureus* (ATCC 23235) having MIC value 3.125 mg/ml, *Escherichia coli* (MDR) having MIC value of 100mg/ml and *Acinetobacter* (MDR) having MIC value of 50 mg/ml.

DISCUSSION

This study revealed that the ethanolic extract of *Holarrhena pubescens* showed antibacterial properties against some of the bacterial strains such as *Staphylococcus aureus* (ATCC 23235), *Escherichia coli* (MDR), *Acinetobacter* (MDR). However, it also promoted the bacterial growth in case of *Escherichia coli* (ATCC 25922) and *Klebsiella pneumoniae* (MDR).

Studies conducted earlier revealed that other parts of *Holarrhena pubescens*such as bark, seeds and flower are antimicrobial and beneficial in nature. Its bark is widely used in Ayurvedic medicine to treat piles, diarrhoea, leprosy, biliousness, and illnesses of the spleen. Bark is used to cure headaches, piles, and heavy menstrual flow in unani medicine. There are claims that the roots are aphrodisiac and abortifacient. Additionally, they are used to treat severe abscesses, gonorrhoea, ascariasis, malaria, and sexual illnesses. They are utilised in Tibetan medicine as a cholagogue, alexipharmic, antidiarrheal, and analgesic. According to British Materia Medica, its bark can treat asthma, bronchopneumonia, stomach problems, dyspepsia, diarrhoea, and dysentery in addition to treating malaria, chest infections, asthma, and bronchitis (Zahara *et al*., 2020).

They are utilised as an astringent, anthelmintic, febrifuge, stomachic, anti-dysenteric, and anti-diarrheal in the traditional medicine of Bangladesh (Gangwar *et al*., 2010). They are apparently used in other regions of the world to treat diuresis, chronic chest infections, asthma, malaria, vaginitis, diabetes, arthritis, hematuria, epilepsy, bronchitis, diarrhoea, dermatitis, and jaundice (Kabir *et al*., 2018).

Another study was done to assess *Holarrhena pubescens'* antioxidant and anti-diabetic properties. The hexane, methanol, and water-soluble fractions contained carbohydrates, alkaloids, saponins, resins, phenols, proteins, and amino acids, according to the phytochemical screening. Oxidative stress has been determined to be primarily caused by an increase in the formation of oxygen free radicals and a significant decrease in antioxidant defences in diseases that resemble diabetes. As a result, *Holarrhena pubescens'* antioxidant activity and antihyperglycemic properties can lessen oxidative stress and decrease blood sugar (Bhusal *et al*., 2014).

CONCLUSION

It is evident from our experimental study that the ethanolic extract of *Holarrhena pubescens* leaves has some antibacterial properties against the bacterial strains of *Staphylococcus aureus* (ATCC 23235), *Escherichia coli* (MDR), *Acinetobacter* (MDR). Further studies and research is needed so that this antibacterial property can be used against disease causing resistant bacterial strains and also the compounds which are present in the leaves of this plant which attributes to this.

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