

Original Research Article

Antimicrobial Properties of Ethanolic Extract of *Holarrhena pubescens* Against MDR Strain of *Pseudomonas aeruginosa*

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Article History

Received: 05.06.2023

Accepted: 06.07.2023

Published: 16.07.2023

Journal homepage:

<https://www.easpublisher.com>

Quick Response Code



Abstract: In the present times the multidrug resistant (MDR) bacteria has become widespread all over the World and has increased the rate of mortality in the patients. *Pseudomonas aeruginosa* colonizes in the intensive care units (ICU's) of the hospitals and is the reason behind health care associated infections. *Pseudomonas* infection further increases the chances of morbidity and mortality in the patients. In this study we explored the antimicrobial nature of *Holarrhena pubescens* also known as Kurchi on the MDR strain of *Pseudomonas aeruginosa*. The antimicrobial nature of extract of the leaves of Kurchi was studied by Micro dilution test and then minimum inhibitory concentration (MIC) value was calculated by serial dilution in Mueller Hinton Broth. The ethanolic extract showed antimicrobial property with MIC value of 6.25mg/ml in case of MDR strain of *Pseudomonas*. Further synergy test was done using the antibiotics Meropenem and Polymyxin B to check if the ethanolic extract of the leaves could increase the inhibitory property of these antibiotics against the infection caused by *Pseudomonas aeruginosa*. It was found that there was slight increase in the efficacy of the antibiotics against the MDR strain when it was combined with the ethanolic leaf extract of *Holarrhena pubescens*. Thus this antimicrobial nature of *Holarrhena pubescens* can be further used in the field of pharmacy to develop more efficient drugs against MDR strains of *Pseudomonas aeruginosa*.

Keywords: *Holarrhena pubescens*, MIC value, MDR bacteria, antibiotics, antimicrobial action, Morbidity.

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INTRODUCTION

Since time immemorial, medicinal plants have been a great part of the healthcare domain. From the ancient times, many plants having its medicinal properties have served the humankind and hence nature itself has been a great source of various plants that are important in the field of medicine. Even in religious texts and manuscripts those were written thousands of years ago there is mention a varied number of herbs and plants of high medicinal value. These plants are potential source of therapeutics and curative aids.

Medicinal plants have given humankind a large number of potent drugs that have eradicated many serious infections. Studying these medicinal plants and their properties have become important because in the coming years they can have a huge impact in the pharma industry. They have anti-fungal, anti-bacterial, anti-cancer, anti-inflammatory, anti-diuretic properties

due to the presence of secondary metabolites like terpenoids, alkaloids, flavonoids, tannins, etc. World Health Organization (WHO) has reported that 80% of the Earth's population depends on traditional medicines because of its negligible side effects in comparison to the chemical drugs that are administered in our body on a regular basis. (Sofowora et al, 2008)

Holarrhena pubescens belongs to the family of Apocynaceae. It is native to China, Indochina, Indian Subcontinent, Africa, Nepal, and Bhutan. It is a deciduous tree having elliptic and oblong leaves with white flowers. Flowering season is April-July and fruiting season is August - October. It has been found that the presence of steroidal alkaloids, triterpenes and sterols has made this an important medicinal plant for further research. According to the studies the fruits and bark of this plant has anti-diarrhoeal properties and it is used in the Ayurvedic medicine to treat dysentery,

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stomach pain, diarrhoea, cholera, epilepsy. The bark, flowers, seeds, roots of *Holarrhena pubescens* has been reported for notable medicinal properties (Sahrawat et al, 2018). In Unani medicine the leaves of this plant is used for treating wounds, ulcers, chronic bronchitis, for muscle relaxation and to regulate menstruation. Our study is based on antimicrobial properties of the leaves of *Holarrhena pubescens* on the microorganism *Pseudomonas aeruginosa*. A study has shown that the leaves of *Holarrhena pubescens* has anti-diabetic properties. The methanolic extract of this plant has hypoglycemic properties too in vivo. (Srivastava et al, 2015)

Multi Drug Resistant infections are a serious threat in recent times and will continue to be the same in the near future. As the pathogenic microorganisms are being exposed to a wide range of antibiotics with time it is developing resistance against most of them creating a difficulty in providing proper treatment to patients. According to WHO carbapenem resistant MDR *P.aeruginosa* as one of the critical pathogen that needs new therapies to combat its resistance. Centre for Disease Control and Prevention (CDC) has declared *P.aeruginosa* as a serious threat. In hospitals, especially in ICU's *P.aeruginosa* colonizes and infects the patients increasing their mortality rate (Bertrand et al,2001). . Immunocompromised patients suffering from chronic pulmonary disease admitted to ICU and have been receiving carbapenem or fluoroquinone for the last 3 months are at a very high risk of infection by MDR *P.aeruginosa* (Vincent et al, 2000). Delayed administration of anti-microbial therapy within 52hr in patients developing this kind of infection increases their mortality rate by two folds. Furthermore, the proportion of patients who received delayed appropriate therapy increased as the number of resistant classes increased. Specifically antibiotic resistance to atleast three antibiotic classes was significantly associated with delayed appropriate therapy (Mesaros et al, 2007).

In this present study, we have used to ethanolic leaf extract of *Holarrhena pubescens* to study its antibacterial properties, which can be useful in the field of medicine. Further synergy test has been performed using the extract and the commonly used antibiotics to treat MDR *P.aeruginosa* to check if this extract increases the efficacy of these antibiotics. Thus the study aims at new drug composition that can be introduced in the pharma industry to treat MDR *P.aeruginosa* associated infections.

MATERIALS AND METHODS

Collection of plant material

Sample of *Holarrhena pubescens* plant (Figure 1) was collected from a forest near Dharagiri falls in Ghatshila, which is a town on the Subarnarekha River in the east Indian state of Jharkhand. The coordinates for Ghatshila is, latitude: 22.5833751280477 and

longitude: 86.4784143282216. The date of collection of the sample was 19 December 2022.



Figure 1: Leaves of *Holarrhena pubescens*

Preparation of plant extract

The leaves were collected from the plant and then dried properly before extraction. 5gm of the dried leaves was taken and impregnated in 5μL of 70% ethanol solvent. Then the extract was mixed thoroughly by vortex for about 15-20 min and then was incubated at 37°C for 24 hrs. After 24 h of incubation, the extract was again mixed by vortex for 15min and then centrifuged at 3000rpm for 10min. The supernatant thereby obtained after centrifugation was used for the subsequent procedures.

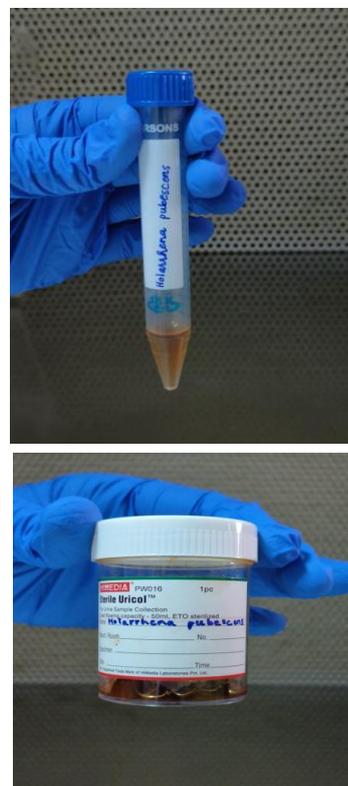


Figure 2: Plant extract after vortex

The strain of *Pseudomonas aeruginosa* (MDR strain) was used in this experiment. The antimicrobial sensitivity is shown in the following table below. The

tested organism was acquired from the Department of Microbiology, Peerless Hospital, Kolkata, West Bengal, India.

Table 1: Antibiotic sensitivity of tested *Pseudomonas aeruginosa* (MDR) in VITEK 2 automated system

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Piperacillin /Tazobactam	>=128	R	Gentamicin	>=16	R
Ceftazidime	>=64	R	Ciprofloxacin	>=4	R
Cefoperazone/Sulbactam	>=64	R	Levofloxacin	>=8	R
Cefepime	>=32	R	Tigecycline	4	R
Aztreonam	>=64	R	Fosfomycin	128	I
Doripenem	>=16	R	Colistin	1	I
Imipenem	>=16	R	Polymyxin B	1	I
Meropenem	>=16	R			
Amikacin	>=64	R			

R=resistant, I=intermediate

Micro-dilution test

MIC determination

The 96 well sterile microtiter plate was used to determine the MIC value of the ethanolic plant extract. 100 µL of Mueller Hinton (MH) broth was pipetted in each wells of the microtiter plate. Then 100 µL plant extract was added to the first well and thoroughly mixed. Further it was serially diluted in the successive wells in double dilutions. Then 10µL of 0.5McF bacterial suspension which was prepared was added in each well. The control experiment was done similarly using only 70% ethanol. The MIC values were detected at 620nm using Thermo MULTISKAN EX microplate reader at 0hr and 24hr after incubation at 37°C. The MIC value was determined as the lowest concentration of the root extract in the MH broth medium that inhibits the growth of *Pseudomonas aeruginosa* (MDR).

SYNERGISTIC ANTIMICROBIAL ASSAY

To determine if the plant extract could increase the efficacy of antibiotics

The antibiotics Meropenem and PolymyxinB were used in combination with the plant extract of *Holarrhena pubescens* to determine synergistic antimicrobial activity. Lawn culture was performed by spreading *Pseudomonas aeruginosa* on Mueller Hinton Agar (MHA) plates. Then filter paper discs were impregnated with 10-20µL plant extract and with 70%

ethanol to serve as the control disc. The antibiotic discs of Meropenem and Polymyxin B were then employed on the MHA agar plates in combination with the disc soaked in plant extract. The disc soaked in plant extract was placed beside the antibiotic disc. The zone of inhibition produced by the plant extract in combination with standard antibiotics after overnight incubation were estimated as if zones of combination treatment is greater than summation of zone of plant extract and zone of antibiotic then it is synergism, if zone of combination treatment is equal to the summation of zone of plant extract and zone of antibiotic then it is interpreted as additive, if zone of combination treatment is lesser than summation of zone of plant extract and zone of antibiotic then it is antagonism.

RESULTS

The Micro-dilution test was performed to determine the MIC value of the ethanolic extract of the leaves of *Holarrhena pubescens* against the MDR strain of *Pseudomonas aeruginosa*. The graphs depicts that the extract had inhibited the growth of *Pseudomonas aeruginosa* and the MIC was found to be around 6.25mg/ml. The following figure is of differences between the absorbance values in 0 hr and the 24 hr versus the concentration of dilution of the plant extract.

Table 2: From the Synergistic Antimicrobial Assay that was performed using the two antibiotics Meropenem and Polymyxin B are as follows the following observations were made with the diameter of the inhibition zones;

Only antibiotic disc	32mm (Meropenem)	16mm (Polymyxin B)
Only extract disc	8mm	7mm
Control disc	6mm	6mm
Antibiotic disc beside extract disc	34mm	17mm

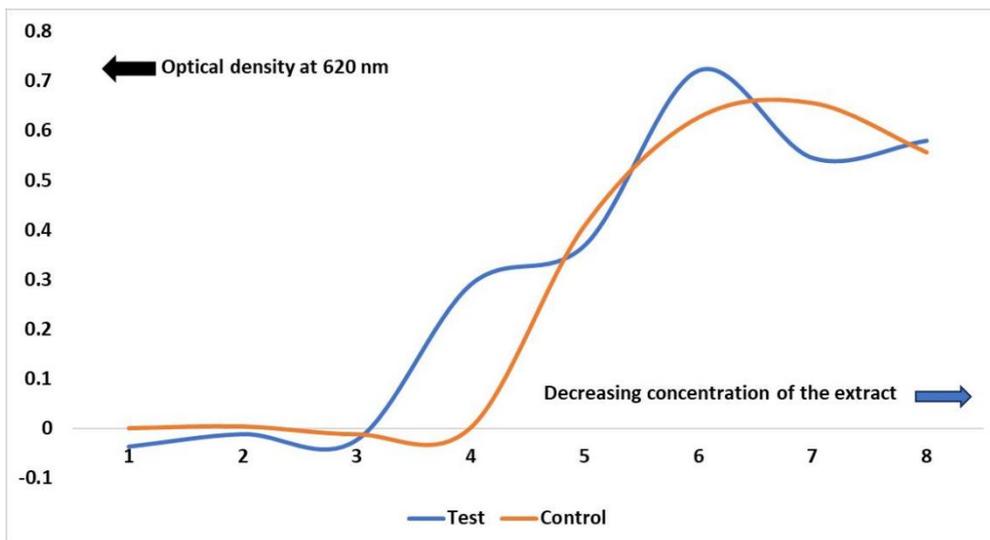


Figure 3: Effect of *Holarrhena pubescens* ethanolic extract on *Pseudomonas* (MDR) showing no antimicrobial action. Concentration of the extract 1: 100 mg/ml, 2: 50 mg/ml, 3: 25 mg/ml, 4: 12.5 mg/ml, 5: 6.25 mg/ml, 6: 3.125 mg/ml, 7: 1.5625 mg/ml, 8: 0.78125 mg/ml.



Figure 4: The inhibition zones shown by Meropenem disc, plant extract disc and the combined disc for Meropenem and plant extract on *Pseudomonas aeruginosa*

DISCUSSION

From the above experiment we thus established that *Holarrhena pubescens* leaf extract has antimicrobial properties against *Pseudomonas aeruginosa* (MDR) strain. We have obtained the MIC value of 6.25 mg/ml for the plant leaf extract where it is found that it has inhibited the growth of *Pseudomonas aeruginosa* (MDR). Further the synergy assay established that the plant extract in combination with the antibiotics Meropenem and Polymyxin B has given positive results (Saqib et al,1998.). For Meropenem it was observed that the inhibition zone was slightly extended when combined with the disc of plant extract in comparison to the diameter of the inhibition zone of the antibiotic disc only. Even for Polymyxin B it was seen that the use of the plant extract has subsequently

made the inhibition zone of the combined disc slightly enlarged in comparison to the only antibiotic disc on the MHA plate. To discuss, this plant has the potential to control infections caused by MDR *P.aeruginosa* and even it has proved to increase the potency of the antibiotics that are commonly used for the treatment of these infections (Kuok et al,2017). In future study can be done if this extract of leaves of *Holarrhena pubescens* is effective in inhibiting growth of other MDR strains (Chanda et al.,2011) .

CONCLUSION

The present study has shown that ethanolic extract of *Holarrhena pubescens* leaves has antimicrobial properties against the MDR strain of *Pseudomonas aeruginosa*. Even the plant extract can be

used in the pharmacology industry to increase the efficacy of the antibiotics used for the treatment of infections caused by MDR strain of *Pseudomonas aeruginosa*. Thus this has opened the possibility of designing new drugs with less side effects against these infections to reduce mortality rate. However, there is still a large scope of further research in this area.

Acknowledgement

The authors show sincere gratitude to the Managing Director of Peerless Hospitex Hospital & Research Centre Limited, Kolkata. The authors would also like to convey their heart felt gratitude to Mr. Arup Kumar Dawn, Senior Laboratory Executive, Peerless Hospital, Kolkata. The authors acknowledge the support and cooperation from the Department of Microbiology, West Bengal State University, Kolkata and Peerless Hospital and Research Centre Limited, Kolkata.

Authors contribution

All the authors have contributed equally.

Conflict of interest

The authors declare no conflict of interests.

Financial support

This work has not received any funds from national and international agencies.

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Cite This Article: Rittika Singh, Teesta Bhowmick, Ritojo Basu, Partha Guchhait, Bhaskar Narayan Chaudhuri, Satadal Das (2023). Antimicrobial Properties of Ethanollic Extract of *Holarrhena pubescens* Against MDR Strain of *Pseudomonas aeruginosa*. *EAS J Pharm Pharmacol*, 5(4), 87-92
