Antibacterial and Antioxidant Properties of *Holarrhena pubescens* (Bangla Name- “Kurchi”) Bark Crude Extract

By
Harun Al Mahi
Student ID: 20126006
Hasan Samin Islam
Student ID: 20126015

A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of
Bachelors of Science in Microbiology

Department of Mathematics and Natural Sciences
Brac University
June 2024

Declaration

It is hereby declared that

1. The thesis submitted is my/our own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I/We have acknowledged all main sources of help.

Student's Full Name & Signature:

Harun Al Mahi	**Hasan Samin Islam**
Student ID: 20126006	Student ID: 20126015

Approval

The thesis/project titled "Antibacterial and Antioxidant Properties of *Holarrhena pubescens* (Bangla Name- "Kurchi") Bark Crude Extract" submitted by

1. Harun Al Mahi (20126006)
2. Hasan Samin Islam (20126015)

has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelors of Science in Microbiology on 6th June 2024.

Examining Committee:

Supervisor: _____

(Member) M. Mahboob Hossain, PhD

Professor, Department of Mathematics and Natural Sciences

Brac University

Program Coordinator: _____

(Member) Nadia Sultana Deen, PhD

Associate Professor, Department of Mathematics and Natural Sciences

Brac University

Departmental Head: _____

(Chair) Md. Firoze H. Haque

Associate Professor and Chairperson, Department of Mathematics and Natural Sciences

Brac University

Abstract

The use of plants and their derivatives for therapeutic purposes dates back to the beginning of human civilization. *Holarrhena pubescens*, commonly known as "kurchi," is a medicinal plant recognized for its diverse therapeutic properties. This study investigates the antibacterial and antioxidant potentials of *Holarrhena pubescens* bark extracts.

Antioxidant activity was evaluated using the DPPH assay. The free radical scavenging activities of the extracts ranged from very strong to strong, with chloroform extracts showing the lowest IC50 value of 15.59 µg/ml and ethyl acetate extracts showing the highest at 157.14 µg/ml. All partitioned extracts demonstrated significant potential as antioxidants, given that IC50 values below 50 indicate very strong activity.

Contrarily, the antibacterial tests indicated that *Holarrhena pubescens* lacks antibacterial properties. Despite this, the plant's potent antioxidant capabilities merit further investigation. Future research should focus on the isolation of pure compounds using column chromatography and explore the plant's potential anticancer properties.

These findings underscore the therapeutic promise of *Holarrhena pubescens*, particularly its antioxidant activity, which could lead to the development of new, effective medicinal drugs. Further in-depth studies are necessary to fully elucidate the plant's bioactive compounds and their possible applications in medicine.

Acknowledgement

We would like to express our deepest gratitude to Dr. M Mahboob Hossain, Professor in the Microbiology Program at the Brac University Department of Mathematics and Natural Sciences, for his unwavering guidance, encouragement, and continuous inspiration throughout this research project, his expertise and insights have been invaluable. We extend our sincere appreciation to all the faculty members of the Department of Mathematics and Natural Sciences at Brac University, particularly Associate Professor Md. Firoze H. Haque, the department chair and Dr. Nadia Sultana Deen. Their support and encouragement have been crucial in the successful completion of this thesis.

Special thanks are due to the esteemed Laboratory Officers of the Brac University Microbiology & Biotechnology Laboratory, whose unwavering support and cooperation were instrumental in completing the laboratory work for this research. We are also grateful to all the lab assistants for their assistance and dedication.

Additionally, we would like to thank the involved research assistants, special mention to Rifa Tamanna Subarna's support throughout our work, for which we are most grateful.

Lastly, we sincerely appreciate our well-wisher's unwavering support of our academic endeavors

Their collective efforts and contributions have been fundamental to the completion of this thesis, and we are deeply appreciative of their support.

Table of Contents

List of Tables

List of Figures

List of Acronyms

ft	Feet
cm	Centimetre
H. pubescens	*Holarrhena pubescens*
TLC	Thin layer chromatography
%	Percentage
ºC	Degree Celsius
ATCC	American Type Culture Collection
MHA	Mueller-Hinton agar
NA	Nutrient Agar
µg	Microgram
mg	Milligram
ml	Milliliter
mm	Millimeter

DCM	Dichloromethane
µl	Microliter
CFU	Colony Forming Unit
UV	Ultra-Violet
nm	Nanometer
DPPH	2,2-Diphenyl-1-picrylhydrazyl
RSA	Radical Scavenging Activity

Chapter 1

Introduction

1.1 Background

The utilization of plants and plant derivatives for therapeutic purposes has been observed since the beginning of human civilization Throughout history, numerous plants with medical characteristics have benefited humanity, making nature a valuable resource for vital medicinal plants. Medicinal plants serve as a distinct gift from nature to humanity(Sharma et al., 2017). Plants serve as an abundant source of various natural products that display a number of therapeutic characteristics and are constantly being investigated for the development of novel medicines(R. J. Singh & Jauhar, 2006). Also, natural products contain bioactive ingredients and these chemicals have biological activity against numerous disease-causing agents (Nasim et al., 2022). Approximately 40% of pharmaceutical medicines are currently derived from natural sources and traditional knowledge, including notable medications such as aspirin, artemisinin, and treatments for children cancer (World Health Organization: WHO, 2023). Upon further investigation, it became obvious that the scientists responsible for these treatments relied on traditional knowledge as a foundation to reach their groundbreaking findings. The World Health Organisation (WHO) has stated that 80% of the global population depends on traditional medicines due to their minimal adverse effects, as opposed to the chemical medicines commonly used. The traditional medicine system is depended upon by over 85-90% of the global population to combat a range of diseases(Wangchuk, 2018). So, exploring these medicinal plants and their qualities is crucial due to their potential to significantly influence the pharmaceutical industry in the future. This study on plants should be thoroughly researched in order to generate safer and more potent drugs.

1.2 Objective of the Study

The stem bark of this plant, commonly referred to as "kurchi," possesses medicinal characteristics such as antidiarrhoeal, antidysenteric, anthelmintic, stomachic, digestive, astringent, and tonic effects(Siddiqui et al., 2013). Furthermore, it has effectiveness in treating piles, toothache, chest infections, diuresis, as well as skin and spleen illnesses. Although widely utilized, there is a scarcity of scientific study on the medicinal qualities of this substance, particularly in Bangladesh. This study aims to examine the unexplored potential *of Holarrhena pubescens* bark extracts, specifically in terms of their antibacterial

and antioxidant properties. The objective of the study is to separate and examine the biologically active constituents found in the bark, with the intention of potentially creating new drugs.

1.3 *Holarrhena pubescens*

Common name:

- Bengali: Kurchi
- English: Bitter Oleander, Conessi bark, Ester tree, Indrajao, Ivory tree, Tellicherry tree
- Kannada: Alippe, Koodsaloo
- Malayalam: Kadalapala, Kudagapala
- Marathi: Kadu-indrajau
- French: Ecorce de conessie.
- Nepalese: Gnedor, Vhaate khiro, Indrajau, Kevat, Kurcii, Madise khirro.
- Portugese: Quine, Erva do Malabar.

(Source: *Holarrhena Pubescens Wall. Ex G.Don | Species*, n.d.-c & *Holarrhena, Holarrhena Pubescnes, TELLICHERRY BARK/ Alternative Medicine*, n.d.)

Synonyms:

Chonemorpha antidysenterica (Roth) G. Don, *Holarrhena antidysenterica* (Roth) Wall. ex A. DC., *Holarrhena febrifuga* Klotzsch, *Holarrhena glabra* Klotzsch, *Holarrhena villosa* Aiton ex Loudon (*Medicinal Plant Details*, n.d.)

1.3.1 Taxonomy

- Kingdom: Plantae
    - Phylum: Tracheophyta
        - Class: Magnoliopsida
            - Order: Gentianales
                - Family: Apocynaceae
                    - Genus: Holarrhena
                        - Species: *Holarrhena pubescens*

(Source: *Holarrhena Pubescens Wall. Ex G.Don | Species*, n.d.-b)

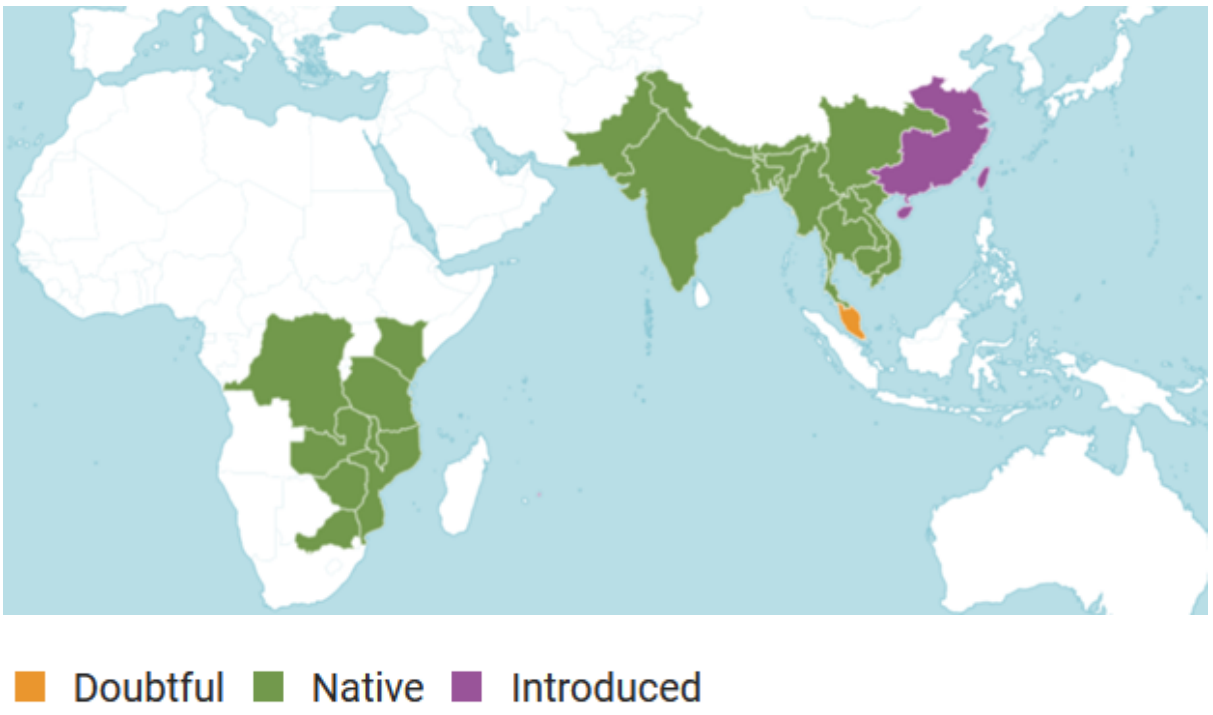
1.3.2 Description

Holarrhena Pubescens is a medicinal tree that typically reaches a height of 30-40 ft. and has a maximum width of 4 ft.(Siddiqui et al., 2013). It is a deciduous tree having elliptic and oblong leaves with white flowers. The leaves of *Holarrhena pubescens* are simple, green, and glabrous, arranged oppositely, nearly sessile, elliptic-lanceolate with pointed apices, ranging from 4 to 20 cm in length while its flowers are white, 2 to 4 cm wide, slightly tubular at the base, scented, clustered, featuring 5 sepals and 5 petals(Ali, 2020). The period of flowering occurs from April to July, while the period of fruit development takes place from August to October(Singh et al., 2023). It has rough pale brown bark along with milky white latex(*Pioneer University in Kerala*, n.d.). Also, the fruits of this tree contain two elongated follicular mericarps. Additionally, these fruits, which are measured 25 x 1 cm, contain seeds that are 8 mm in length, oblong in shape, and adorned with a tuft of silky brown hairs at the apex(*Holarrhena Pubescens Wall. Ex G.Don | Species*, n.d.).

(Figure 1.1:*Holarrhena pubescens* Source: Ali, 2020b)

1.3.3 Distribution

Holarrhena Pubescens is native to Assam, Bangladesh, Cambodia, China South-Central, East Himalaya, India, Kenya, Laos, Malawi, Mozambique, Myanmar, Nepal, Northern Provinces, Pakistan, Tanzania, Thailand, Vietnam, West Himalaya, Zambia, Zaïre and Zimbabwe. (*Holarrhena Pubescens Wall. Ex G.Don | Plants of the World Online | Kew Science*, n.d) Typically, it is found in the tropical and subtropical regions of Asia, particularly in the deciduous forests of the tropical Himalayas, at altitudes between 900 and 1250 metres (Sinha et al., 2013).



(Doubtful: Malaya, Introduced: China Southeast, Hainan, Mauritius, Taiwan- Source: *Holarrhena Pubescens Wall. Ex G.Don | Plants of the World Online | Kew Science*, n.d.-b)

1.3.4 Medicinal Properties

Holarrhena pubescens, a member of the Apocyanaceae family, is a significant natural resource with medicinal properties(Yin et al., 2022). Further study has demonstrated that *H. pubescens* possesses a variety of activities, including as antimicrobial, anti-inflammatory, analgesic, anti-amnesic, and neuroprotective properties(Jamadagni et al., 2017). Each part of *Holarrhena pubescens* serves a distinct medicinal purpose: the bark and seeds are mainly used to treat diarrhea and dysentery, the roots are effective against malaria, while the leaves help control stomachache and vomiting(Bruschi et al., 2011; Gupta et al., 2021; Koudouvo et al., 2011; Sawadogo et al., 2012). The stem, bark, leaves, and seeds of *H. pubescens* contain the majority of the identified chemical components(Sinha et al., 2013). Ayurvedic medicine utilises this plant to address many conditions such as cholera, anaemia, jaundice and epilepsy(Kadir et al., 2013). Moreover, they are utilized for the treatment of severe abscesses, gonorrhoea, ascariasis, malaria, and venereal illnesses(Basha & Sudarsanam, 2012). It is often considered the most advantageous medicine due to its numerous therapeutic advantages and absence of harmful side effects(Jamadagni et al., 2017). This plant's bark powder is utilized intreating diabetes in Nepal, even though no scientific study has been published(Bhusal et al., 2014). Additionally, in some studies, it was found that this plant is

extensively utilised in Ayurveda, traditional Chinese medicine, and other conventional medical systems without exhibiting any noticeable adverse effects(Bhowmick et al., 2023).

Chapter 2

Materials and Methods

2.1 Methods

The chemical analysis of a plant can be broken down into several critical steps:

1. **Collection and Identification of Plant Material**
2. **Preparation of Plant Sample**
3. **Extraction Process**
4. **Solvent-Solvent Partitioning**
5. **Thin Layer Chromatography (TLC)**

2.1.1 Collection and Identification of Plant Material

Initially, a thorough literature review was conducted to select an appropriate plant for investigation. *Holarrhena pubescens* was collected from National Herbarium of Dhaka in January 2024. The bark of the chosen plant was then collected from a verified source and authenticated.

2.1.2 Preparation of Plant Sample

The collected bark was initially fresh. It was air-dried over a period of 20 days to facilitate grinding. The dried bark was then processed into a coarse powder using a grinding machine. The powder was stored in an air-tight container, properly labeled, and kept in a cool, dark, and dry environment for future use. Specifically, *Holarrhena pubescens* bark was used, dried for 20 days, and ground into a coarse powder.

2.1.3 Extraction Process

A quantity of one kilogram of the powdered bark was placed in a flask and soaked in a ethanol and water mixture (90:10 ratio)(Abubakar & Haque, 2020). The flask was wrapped in foil and left undisturbed for 14 days, with occasional shaking and stirring. After the soaking period, the mixture underwent a filtration process as follows:

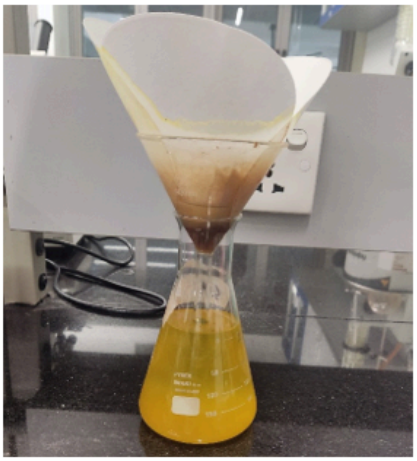
a. **Initial Filtration:** The mixture was first filtered through a clean, white, 100% cotton cloth. The filtrate was collected, and the residue on the cloth was squeezed to extract additional liquid.

b. **Secondary Filtration:** The collected filtrate was then passed through absorbent cotton.

c. **Repeated Filtration:** Step b was repeated using fresh absorbent cotton to ensure thorough filtration.

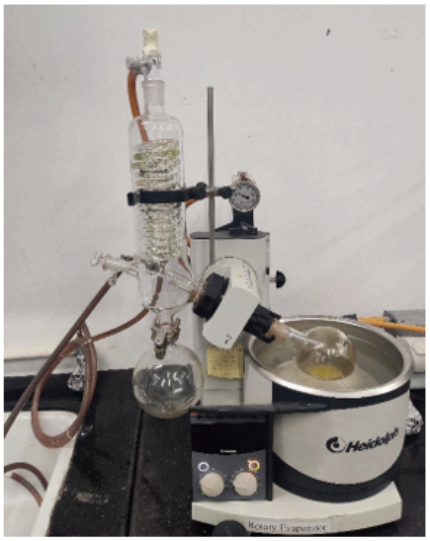
d. **Final Filtration:** The filtrate was filtered one last time using Whatman filter paper. The final filtrate was then concentrated using a rotary evaporator set at 42º C.

By following these steps, the extraction of the plant's chemical constituents was completed in a systematic manner.



Filtration

Collection of the extract



Evaporation at low temperature

Drying of the collected extract

Figure 2.1: Extraction of bark of *Holarrhena pubescens*

2.1.4 Solvent-solvent partitioning

For solvent-solvent partitioning, the kupchan partitioning method was followed (*Solvent-Solvent Partitioning Using Kupchan Scheme*, n.d.). To extend, the crude extract was diluted with a suitable organic solvent that did not mix with aqueous alcohol and was gently shaken in a separating funnel with a roughly equal volume of the solvent. To separate the organic layer from the aqueous phase, the mixture was left undisturbed for several minutes. The components of the crude extract are divided into two phases based on their affinity for the different solvents. The organic layer was then separated, and the extraction process was repeated three times to ensure maximum sample extraction. After separating the organic phase, the aqueous phase was extracted using a series of different organic solvents, typically in order of increasing polarity (such as hexane, chloroform, ethyl acetate, and methanol). Finally, the entire fraction, including both the organic and aqueous phases, was collected and evaporated to dryness separately.

Figure 2.2: Solvent-solvent partitioning of bark of *Holarrhena pubescens*

2.1.5 Thin layer chromatography (TLC)

Thin layer chromatography (TLC) is an effective method for separating and analyzing mixtures of compounds, and is particularly valuable in isolating lipid-soluble components such as lipids, steroids, carotenoids, basic quinones, and chlorophylls. The principle behind TLC is based on the differential migration of compounds as they interact with a stationary phase (often silica gel or alumina) and a mobile phase (solvent system) due to capillary

action(Kagan & Flythe, 2014). This technique is employed to separate compounds based on their polarity, size, and affinity, which results in the formation of distinct spots on a TLC plate that can be visualized under UV light or with chemical reagents.

In the case of *Holarrhena pubesens*, TLC was conducted with the crude extract along with the four solvent partition extracts (chloroform, ethyl acetate, hexane, and methanol). The process began with the preparation of the plant extract. A small amount of the extract was dissolved in an appropriate solvent to create a solution. Commercially available silica gel plates were cut to the desired size, and a baseline was drawn near the bottom of each plate with a pencil. Using clean pipette tips, a small drop of the extract solution was applied on the baseline. A TLC tank with an airtight lid was used, and the selected solvent system was poured into the tank in sufficient quantity. For hexane and chloroform, a mixture of toluene and ethyl acetate was used (4.5: 0.5=5ml) was used whereas, for ethyl acetate and methanol, mixture of chloroform and methanol (3:2=5ml) was utilized as running solvents. Then the tank was sealed and allowed to equilibrate for a few minutes to saturate the atmosphere with solvent vapor.

The prepared TLC plates were then introduced into the tank with forceps, ensuring that the spots remain above the solvent level. As the solvent migrated up the plate through capillary action, it carried the components of the extract with it, causing them to separate based on their interactions with the stationary and mobile phases. The solvent front was allowed to travel a specified distance before the plates were removed from the tank. The separated compounds were then visualized under UV light, at a long wavelength (365nm), which reveals distinct spots indicating the presence of various components. This method is essential for initial screening and assessing the purity of compounds within the plant extract.

2.2 Antibacterial Test Method

The rise of antibiotic-resistant bacteria has heightened the need for new antibacterial compounds. Investigating plant extracts for antibacterial properties is a promising avenue, and this study focuses on the antibacterial activity of *Holarrhena pubescens*. The study employs a modified Kirby-Bauer disk diffusion method to determine the efficacy of different solvent extracts of the plant against various bacterial strains.

2.2.1 Test Organisms

A total of 8 bacterial strains were utilized in this study, sourced from Molecular Laboratory of the Department of Mathematics and Natural Sciences, Brac University and characterized by specific identifiers. The strains include both Gram-positive and Gram-negative bacteria, ensuring a comprehensive evaluation of the antibacterial activity of *Holarrhena pubescens* extracts. They include: ***Klebsiella pneumoniae*, *Bacillus cereus*, *Enterococcus faecalis* ATCC: 29212, *Escherichia coli* ATCC: 25922, *Pseudomonas aeruginosa* ATCC: 27853, *Pseudomonas aeruginosa* 27853, *Shigella flexneri* (reference strain), and *Staphylococcus aureus* ATCC: 25923.**

Table 2.1: ***Gram-positive and Gram-negative bacteria selected for the antibacterial activity test***

Gram-Positive	Gram-Negative
Bacillus cereus	*Klebsiella pneumoniae*
Enterococcus faecalis	*Escherichia coli*
Staphylococcus aureus	*Pseudomonas aeruginosa*
	Shigella flexneri

2.2.2 Preparation of Culture Media

To prepare the culture media, Mueller-Hinton Agar (MHA) and Nutrient Agar (NA) were utilized. The appropriate amounts of agar were dissolved in distilled water and boiled until clear. This solution was then sterilized by autoclaving at 121°C for 15 minutes. Fresh bacterial cultures were prepared by streaking the test organisms onto NA plates and incubating them at 37°C for 18-24 hours.

2.2.3 Sterilization Techniques

All procedures were carried out in a laminar flow hood to prevent contamination. Glassware and Petri dishes were sterilized using an autoclave at 121°C for 15 minutes.

2.2.4 Preparation of Discs

Three types of discs were prepared for the antibacterial assay:

a) **Standard Discs:** Kanamycin discs (30 µg) and Ciprofloxacin (5 µg) were used as positive controls to benchmark the antibacterial activity of *Holarrhena pubescens* extracts.
b) **Blank Discs:** These discs, treated with solvents but no plant extract, served as negative controls to rule out any antibacterial effect from the solvents or filter paper.
c) **Sample Discs:** Discs were prepared by soaking them in solutions of *Holarrhena pubescens* extracts (chloroform, ethyl acetate, hexane, and methanol) at a concentration starting from 100 µg/disc. The discs were then dried and placed on MHA plates pre-inoculated with bacterial cultures.

2.2.5 Preparation of Extract Solutions

For each concentration, 10 mg, 20 mg, 30 mg, and 40 mg of each extract (chloroform, ethyl acetate, hexane, and methanol) were measured into separate sterile, labeled vials. Each sample was then dissolved in 2 ml of the appropriate solvent. The solutions were vortexed thoroughly to ensure complete mixing.

2.2.6 Disk Preparation

Autoclaved Whatman filter paper was used to prepare the disks, which were cut to 6 mm in diameter using a sterile punch-hole tool under a laminar flow hood. Discs were prepared to match the size and thickness of standard antibiotic discs. Kanamycin (30 µg) and Ciprofloxacin (5 µg) served as positive controls, while methanol and dichloromethane (DCM) were used as solvent controls. Using a micropipette, 20 µl of each concentration solution was applied to the filter paper discs. The discs were allowed to soak and the solvent to evaporate, ensuring the extract was evenly distributed.

2.2.7 McFarland Standard

McFarland Standard was used before the lawning stage to standardize bacterial suspensions to a consistent turbidity. By matching the bacterial suspension to a 0.5 McFarland standard, corresponding to 1.5 x 10^8 CFU/ml, uniform bacterial concentrations were ensured for accurate antimicrobial testing. The standard was prepared by mixing 1% solutions of barium chloride and sulfuric acid, resulting in a barium sulfate precipitate(Aryal, 2022). This preparation was vigorously agitated before use to maintain consistency.

2.2.8 Disk Diffusion Method

MHA plates were uniformly inoculated with bacterial cultures after matching the turbidity to a 0.5 McFarland standard using sterile cotton swabs. The prepared discs were then placed on the surface of the agar plates using sterile forceps, ensuring even spacing. The plates were pre-chilled at 4°C for 2 hours to facilitate diffusion of the extracts, then incubated at 37°C for 18-24 hours.

2.2.9 Measurement of Inhibition Zones

After the incubation period, the zones of inhibition around the discs were measured in millimeters using a ruler. Each measurement was taken three times for accuracy, and the average diameter of the inhibition zones was recorded.

2.3 Antioxidant Test: DPPH assay

The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) method uses free radicals to evaluate a substance's capacity to act as a free-radical scavenger. It is a rapid method for measuring antioxidant capabilities (Mahdi-Pour et al., 2012).

2.3.1 Standard preparation

10mg/ml stock of Ascorbic acid was used as standard (Mahdi-Pour et al., 2012).

2.3.2 Sample preparation

Hexane extract, chloroform extract, ethyl acetate extract, and methanol extract of plant samples were used as test samples. The calculated number of extractives (0.1g for each sample) was measured and dissolved in methanol (10ml for each sample) to get solutions.

2.3.3 DPPH solution preparation

0.004% that is 4mg of DPPH was dissolved in 100 ml of MeOH. The solution was prepared in the amber reagent bottle and kept in a light-proof box (Mahdi-Pour et al., 2012).

2.3.4 Radical scavenging activity

Radical scavenging activity is calculated using the following formula (Mahdi-Pour et al., 2012),

%RSA = ((Absorbance of control - Absorbance of Sample) / Absorbance of Control) * 100

IC_{50} was calculated from a line chart equation.

Chapter 3

Results

3.1 Solvent-solvent partitioning

Table 3.1: Amount and physical characteristics of different fractions of *Holarrhena pubescens*

	Extract partition at different solvents			
Characteristics	Chloroform (C)	Ethyl acetate (EA)	Hexane (H)	Methanol (M)
% yield	7.066g	12.25g	1.186g	19.061g
Color	Reddish Brown	Reddish Brown	Yellowish Brown	Dark Brown
Consistency	Viscus	Sticky and hard	Grainy	Firm and dense

3.2 TLC

TLC of *Holarrhena pubescens* showed promising results, as many bands were observed under long wavelength, 365 nm to be precise. To extend, Thin Layer Chromatography (TLC) analysis revealed bands of various colors, such as blue, red, violet, and mauve, indicative of compounds like phycobilin, anthocyanins, betacyanin, chlorophylls, and carotenoids (Harborne, 1984). Observations under a 365 nm wavelength highlighted the possibility of isolating pure compounds.

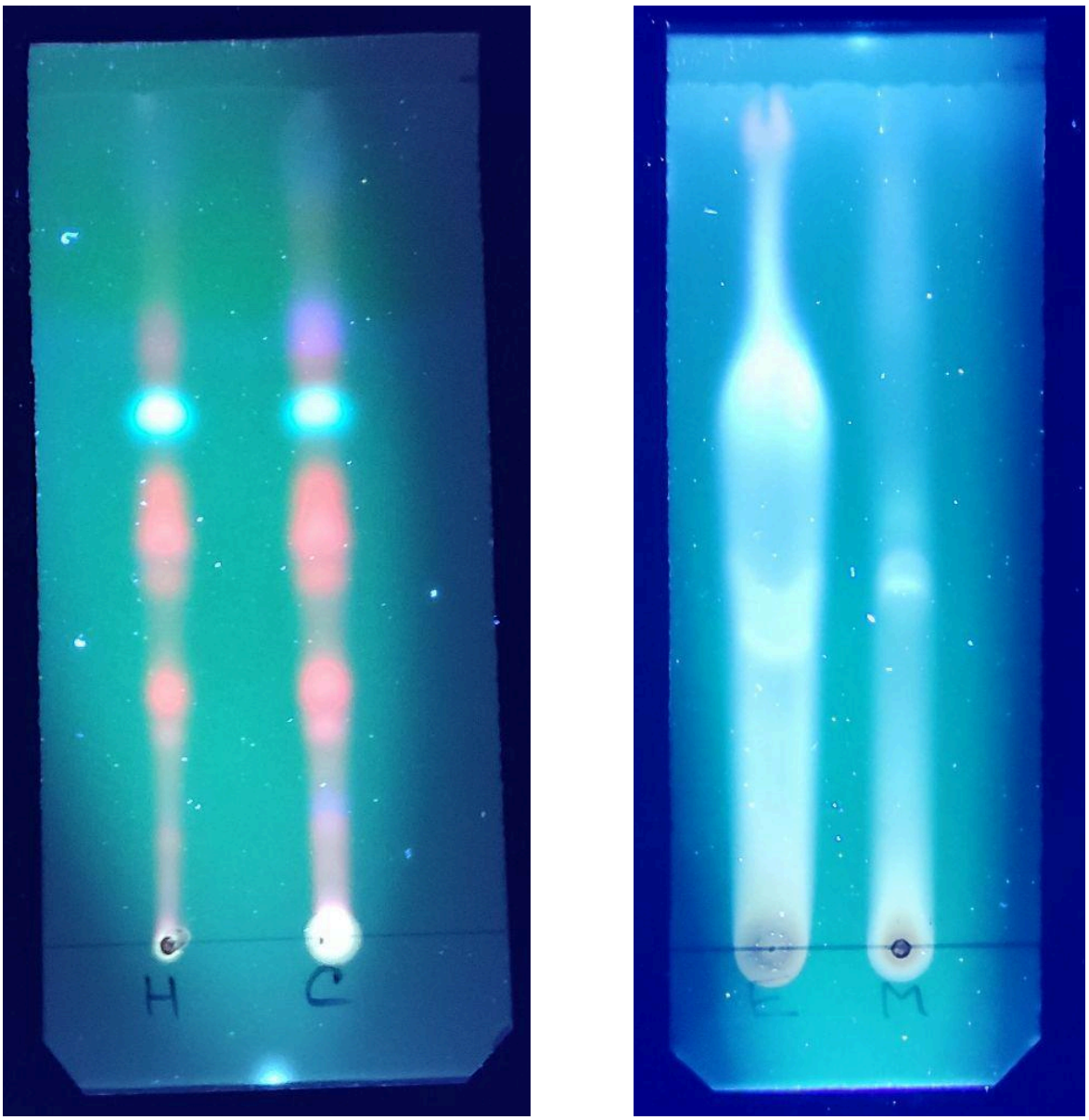


Figure 3.1: Visual detection of compounds of *Holarrhena pubescens* bark extract at long wavelength

3.3 Antioxidant Test

Results of free radical scavenging activities of *Holarrhena pubescens* extracts are shown through tables and charts below. To note, the lower the IC_{50} value the more potent the substance is at scavenging, in other words indicating higher antioxidant activity (Olugbami et al., 2014).

Table 3.2: IC_{50} value of Hexane partition extract of the bark of *Holarrhena pubescens*

Concentration µg/ml	Absorbance of Control	Absorbance of Sample	%Radical Scavenging Activity (RSA)	IC50 µg/mL
10	0.584	0.117	79.96575342	29.20930233
20	0.584	0.1135	80.56506849	
40	0.584	0.101	82.70547945	
60	0.584	0.0565	90.32534247	
80	0.584	0.046	92.12328767	
100	0.584	0.048	91.78082192	
120	0.727	0.0175	97.59284732	
150	0.727	0.0185	97.45529574	
200	0.727	0.029	96.01100413	
250	0.727	0.0305	95.80467675	
300	0.727	0.0315	95.66712517	
500	0.727	0.064	91.19669876	

From the table above, we can determine that antioxidant activity for Hexane partition extract is very strong as it is below 50 µg/ml (Jumina et al., 2019).

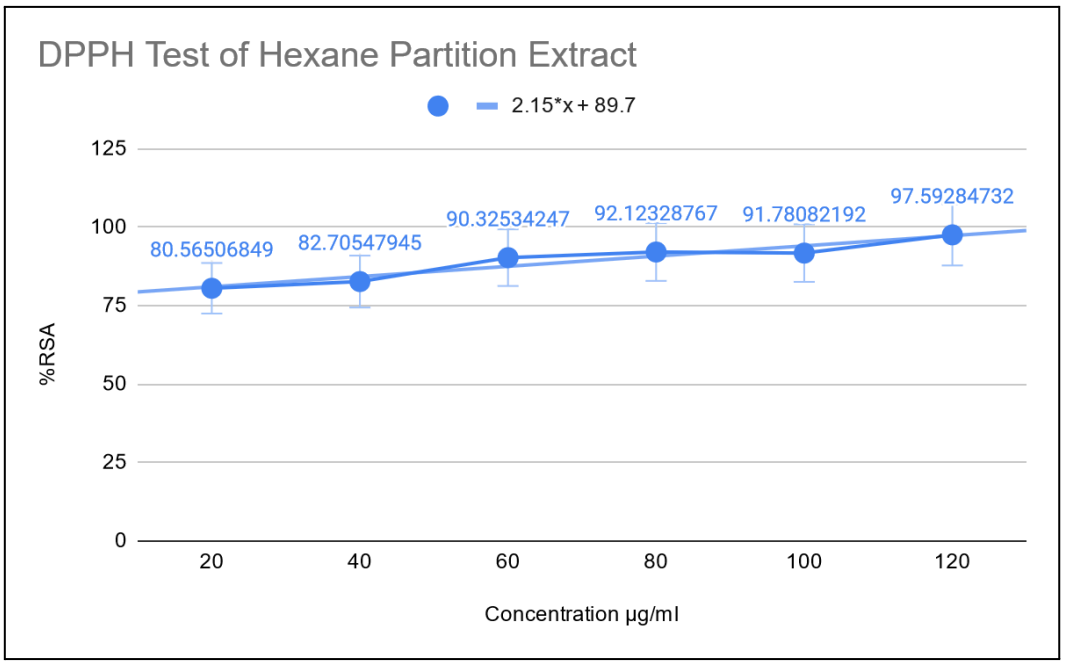


Figure 3.2: Antioxidant activity of Hexane partition extract of *Holarrhena pubescens* bark

Table 3.3: IC_{50} value of Chloroform partition extract of the bark of *Holarrhena pubescens*

Concentration µg/ml	Absorbance of Control	Absorbance of Sample	%RSA	IC50 µg/mL
10	0.584	0.1075	81.59246575	15.59090909
20	0.584	0.1115	80.90753425	
40	0.584	0.056	90.4109589	
60	0.584	0.089	84.76027397	
80	0.584	0.08	86.30136986	
100	0.584	0.0345	94.09246575	
120	0.727	0.023	96.83631362	
150	0.727	0.0235	96.76753783	
200	0.727	0.0295	95.94222834	
250	0.727	0.0395	94.56671252	
300	0.727	0.041	94.36038514	
500	0.727	0.0705	90.30261348	

From the table above, we can determine that antioxidant activity for Chloroform partition extract is very strong as it is below 50 µg/ml (Jumina et al., 2019).

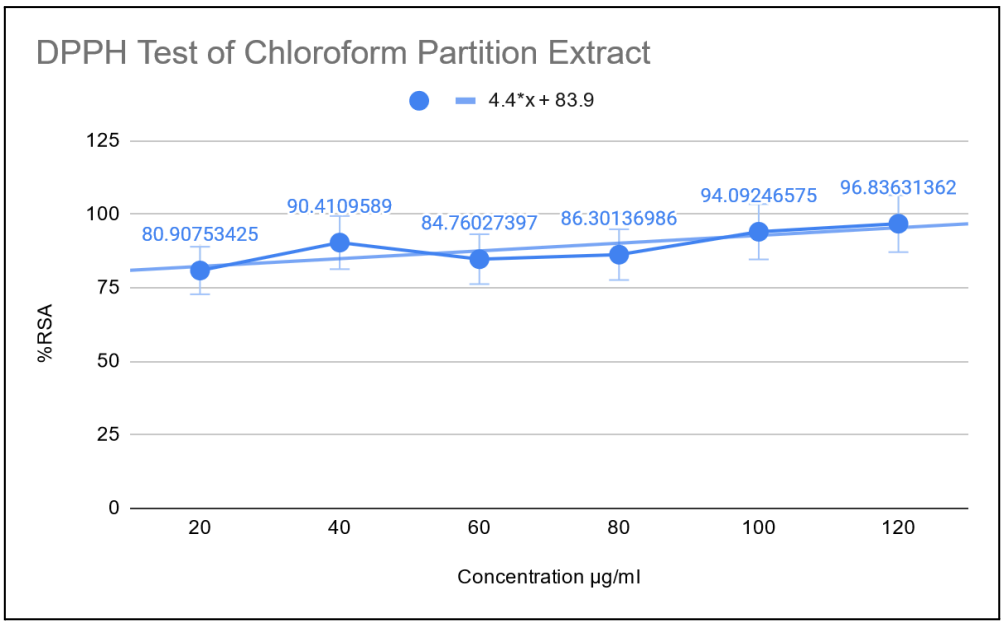


Figure 3.3: Antioxidant activity of Chloroform partition extract of *Holarrhena pubescens* bark

Table 3.4: IC_{50} value of Ethyl Acetate partition extract of the bark of *Holarrhena pubescens*

Concentration µg/ml	Absorbance of Control	Absorbance of Sample	%RSA	IC50 µg/mL
10	0.584	0.1015	82.61986301	157.1428571
20	0.584	0.0285	95.11986301	
40	0.584	0.044	92.46575342	
60	0.584	0.028	95.20547945	
80	0.584	0.023	96.06164384	
100	0.584	0.0255	95.63356164	
120	0.727	0.025	96.56121045	
150	0.727	0.029	96.01100413	
200	0.727	0.0355	95.11691884	
250	0.727	0.0655	90.99037139	
300	0.727	0.0745	89.75240715	
500	0.727	0.0765	89.47730399	

From the table above, we can determine that antioxidant activity for Ethyl Acetate partition extract is between moderate and weak as it is below ranged above 150 µg/ml but below 250 µg/ml (Jumina et al., 2019).

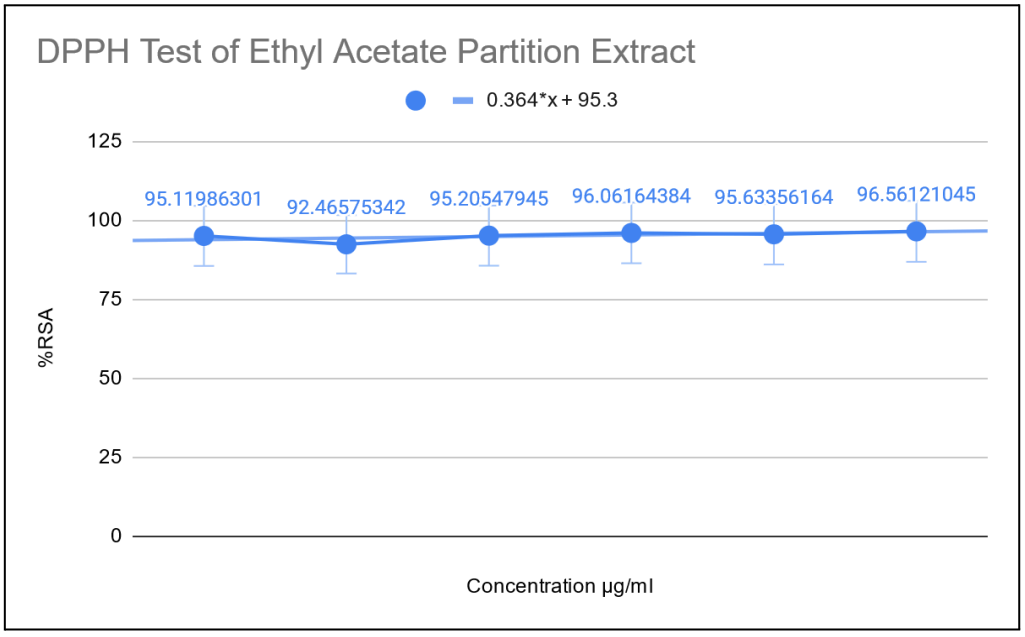


Figure 3.4: Antioxidant Activity of Ethyl Acetate partition extract of *Holarrhena pubescens* bark

Table 3.5: IC_{50} value of Methanol partition extract of the bark of *Holarrhena pubescens*

Concentration µg/ml	Absorbance of Control	Absorbance of Sample	%RSA	IC50 µg/mL
10	0.584	0.0645	88.95547945	36.19883041
20	0.584	0.0955	83.64726027	
40	0.584	0.078	86.64383562	
60	0.584	0.051	91.26712329	
80	0.584	0.053	90.92465753	
100	0.584	0.03	94.8630137	
120	0.727	0.0315	95.66712517	
150	0.727	0.0285	96.07977992	
200	0.727	0.0365	94.97936726	
250	0.727	0.04	94.49793673	
300	0.727	0.0455	93.74140303	
500	0.727	0.0685	90.57771664	

From the table above, we can determine that antioxidant activity for Methanol partition extract is very strong as it is below 50 µg/ml (Jumina et al., 2019).

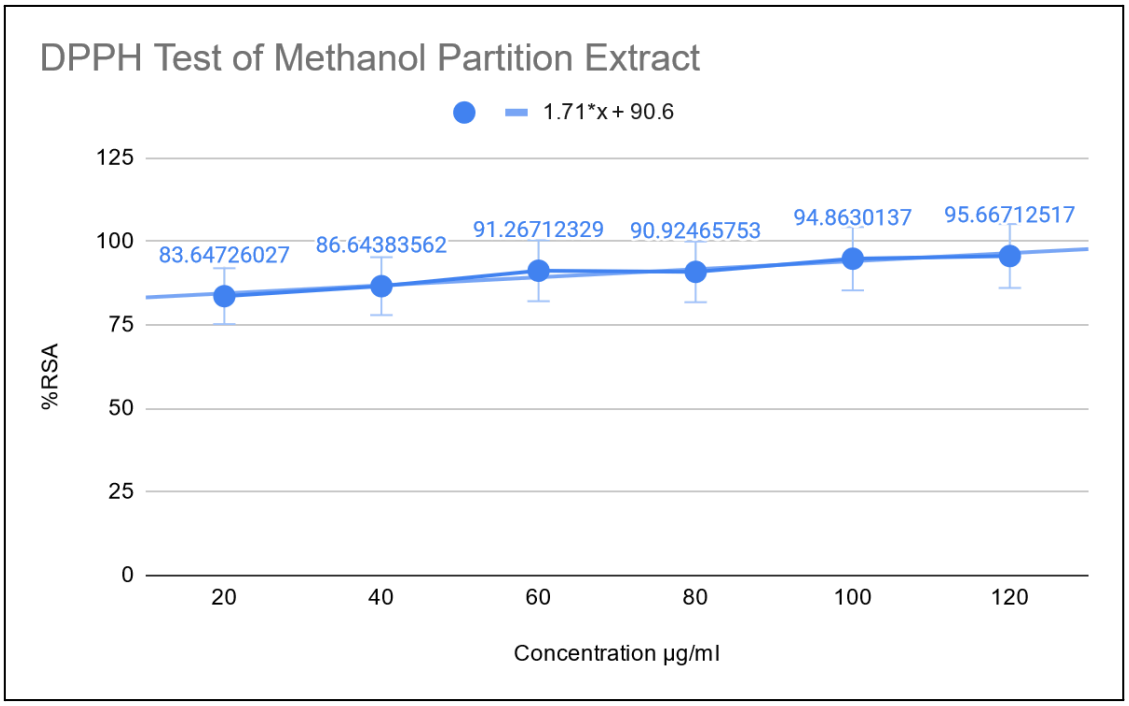


Figure 3.5: Antioxidant activity of Methanol partition extract of *Holarrhena pubescens* bark

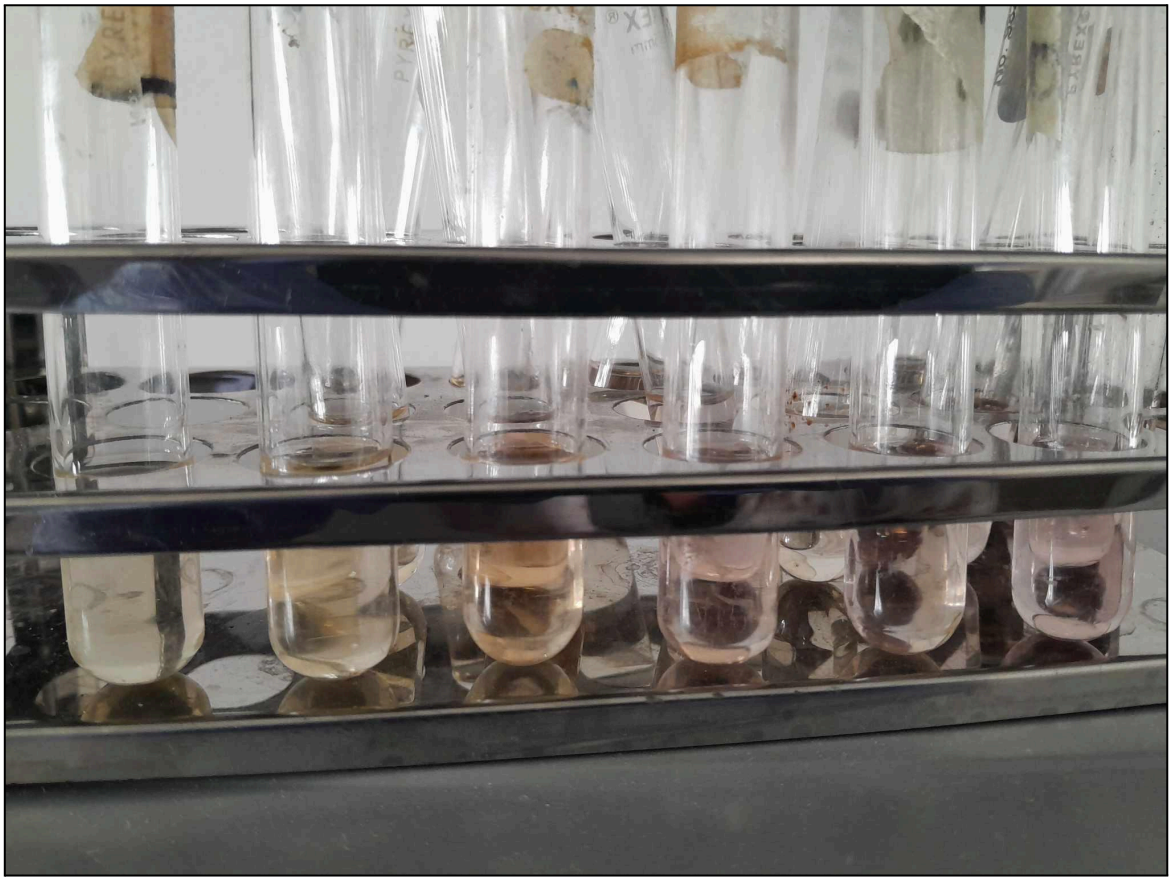


Figure 3.6: Change in color of DPPH due to antioxidant activity, purple to yellow

3.4 Antibacterial Activity Test

From the figure we can indicate that *Holarrhena pubescens* does not possess antibacterial properties.

Organism	Diameter of Zone of Inhibition (mm)								
	Extract Concentrations (ug/disk)				Positive Control		Negative Control	Solvent Control	
	100	200	300	400	K	CIP		DCM	MeOH
Klebsiella pneumoniae	-	-	-	-	11	28	-	-	-
Bacillus cereus	-	-	-	-	17	13	-	-	-
Shigella flexneri	-	-	-	-	22	32	-	-	-
Pseudomonas aeruginosa (ATCC: 27853) 2019	-	-	-	-	-	26	-	-	-
Pseudomonas aeruginosa (ATCC: 27853) 2023	-	-	-	-	-	27	-	-	-
Enterococcus faecalis (ATCC: 29212)	-	-	-	-	19	30	-	-	-
Escherichia coli (ATCC: 25922)	-	-	-	-	20	25	-	-	-
Staphylococcus aureus (ATCC: 25923)	-	-	-	-	21	24	-	-	-

'-' indicates no sensitivity

Figure 3.7: Antibacterial activity of *Holarrhena pubescens* bark extracts

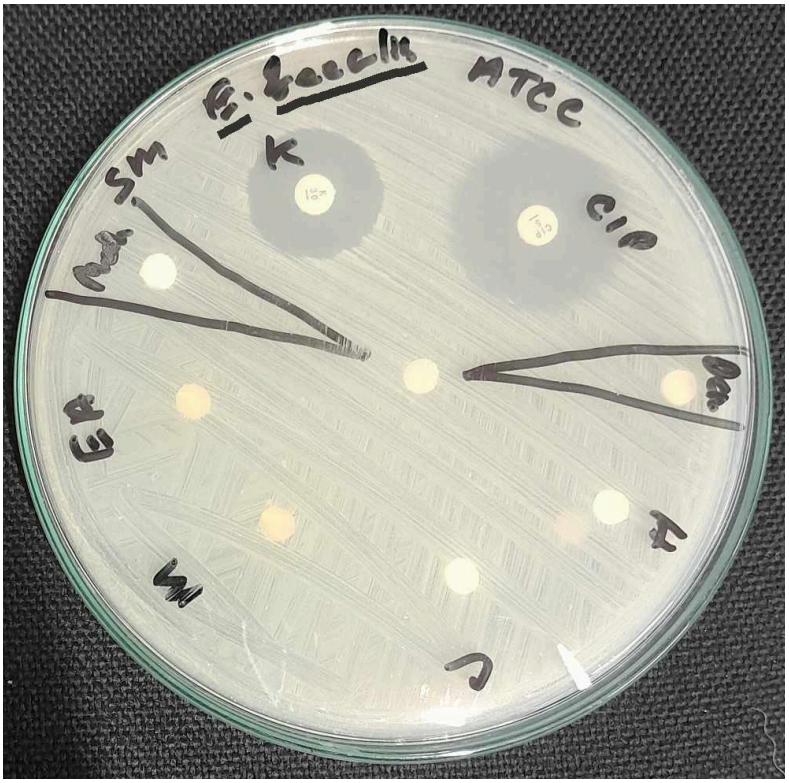


Figure 3.8: Antibacterial activity of *Holarrhena pubescens* bark extract including blank, DCM and MeOH

Chapter 4

Discussion

The investigation into *Holarrhena pubescens* yielded promising insights, particularly in terms of its chemical composition and antioxidant properties, while its antibacterial efficacy was less encouraging.

To begin with, the TLC analysis of *Holarrhena pubescens* extracts revealed a variety of colorful bands under a 365 nm wavelength, indicating the presence of multiple compounds. The colors observed—blue, red, violet, and mauve—suggest the presence of diverse phytochemicals, including phycobilins, anthocyanins, betacyanins, chlorophylls, and carotenoids (Harborne, 1984). The distinct bands, despite some commonality, indicate potential for isolating pure compounds.

Moreover, the antioxidant activity of *Holarrhena pubescens* was assessed using the DPPH free radical scavenging assay, revealing notable efficacy. For reference, IC50 values below 50 µg/ml indicate very strong antioxidant activity, values between 50-100 µg/ml denote strong activity, 101-150 µg/ml indicate moderate activity, and 250-500 µg/ml suggest weak activity (Jumina et al., 2019). The chloroform extract exhibited the most potent antioxidant activity with an IC50 of 15.59 µg/ml, closely followed by the hexane and methanol extracts, all displaying very strong activity. The ethyl acetate extract, although exhibiting the highest IC50 at 157.14 µg/ml, demonstrated moderate antioxidant activity. However, when compared to a study published in International Journal of Pharmacy, they found their Ethyl Acetate fraction to be most active in scavenging DPPH, whereas in our case it was the least (Siddiqui et al., 2013). To point out, ethyl acetate was their most polar solvent, whereas we also used methanol, being the most polar in our case, which showed very strong properties. Nonetheless, these findings suggest that *Holarrhena pubescens* is a rich source of antioxidants, which could be beneficial in combating oxidative stress-related diseases. The variation in antioxidant activity across different solvent partitions underscores the importance of solvent selection in the extraction process, as different solvents can isolate different sets of bioactive compounds.

Conversely, the antibacterial activity tests revealed no significant activity against the tested bacterial strains. This finding is particularly intriguing given the historical use of *Holarrhena pubescens* in traditional medicine for treating infections. To extend, in the study published in

EAS Journal of Pharmacy and Pharmacology, it was found that the leaf extract of *Holarrhena pubescens* has antibacterial properties against *Pseudomona aeruginosa* (Singh et al., 2023). For the record, *Pseudomona aeruginosa* is among our test organisms and our findings are not satisfactory in case of antibacterial. To extend further, the absence of antibacterial activity in this study could be due to several factors, including the possibility that the active antibacterial compounds are present in concentrations too low to be effective in the tested extracts or that they require a specific extraction method or solvent to be isolated. Additionally, the tested bacterial strains might not be susceptible to the compounds present in *Holarrhena pubescens* bark extract, suggesting a need for broader-spectrum testing against other bacterial species or strains. Last but not least, the tested bacterial strains are highly likely resistant to most antibacterials, as they also showed high levels of resistance on the tested broad spectrum antibiotics.

To emphasize, the lack of antibacterial activity, despite promising antioxidant results, highlights the complexity of plant-based bioactive compounds. It also underscores the necessity for further investigation. Future studies should focus on the isolation and characterization of individual compounds through advanced techniques such as column chromatography and nuclear magnetic resonance (NMR) spectroscopy. These pure compounds could then be tested for specific bioactivities, including anticancer properties, which were not covered in this study but represent a significant area of interest given the growing body of evidence supporting the anticancer potential of plant-derived compounds.

In conclusion, while *Holarrhena pubescens* shows strong potential as an antioxidant source, its antibacterial properties remain unsubstantiated in this study. Continued research into the isolation of pure compounds and a broader range of bioactivities is warranted. Such efforts could uncover additional therapeutic uses of this plant, ultimately contributing to the development of novel treatments for various diseases.

Bibliography

Abubakar, A. R., & Haque, M. (2020). Preparation of medicinal plants: Basic extraction and fractionation procedures for experimental purposes. *Journal of Pharmacy and Bioallied Sciences*, *12*(1), 1. https://doi.org/10.4103/jpbs.jpbs_175_19

Ali, M. (2020, January 8). Kurchi or Girimollika, Holarrhena antidysenterica. *Kurchi or Girimollika, Holarrhena antidysenterica*. http://www.floraofbangladesh.com/2018/09/kurchi-or-girimollika-holarrhena.html

Aryal, S. (2022, April 20). *McFarland Standards- Principle, preparation, uses, limitations*. Microbe Notes. https://microbenotes.com/mcfarland-standards/?fbclid=IwZXh0bgNhZW0CMTAAAR2RfIQnExRrRgXMrMBTjMpkTfgOCOD1oAvmPPCGA93cUmB-aIIpDBcl_Kw_aem_AUMM_I32wpcQXwdwUt4MLLbs0zb4X9PEZw-4Oz6DQ0M7N1dP1aU7Ppdv50RgGNOnE13MY0xY7VMoU7I8EiFmgRHK#preparation-of-mcfarland-standards

Baliyan, S., Mukherjee, R., Priyadarshini, A., Vibhuti, A., Gupta, A., Pandey, R. P., & Chang, C.-M. (2022). Determination of Antioxidants by DPPH Radical Scavenging Activity and Quantitative Phytochemical Analysis of Ficus religiosa. *Molecules*, *27*(4), Article 4. https://doi.org/10.3390/molecules27041326

Basha, S. K., & Sudarsanam, G. (2012). Traditional Use of Plants against Snakebite in Sugali tribes of Yerramalais of Kurnool district, Andhra Pradesh, India. *Asian Pacific Journal of Tropical Biomedicine/Asian Pacific Journal of Tropical Biomedicine*, *2*(2), S575–S579. https://doi.org/10.1016/s2221-1691(12)60276-7

Bhowmick, T., Chaudhuri, B. N., Guchhait, P., & Das, S. (2023). Antifungal Properties of Ethanolic Extract of Holarrhena Pubescens against Candida Species. *South Asian Research Journal of Biology and Applied Biosciences*, *5*(04), 68–74. https://doi.org/10.36346/sarjbab.2023.v05i04.002

Bhusal, A., Jamarkattel, N., Shrestha, A., Lamsal, N. K., Shakya, S., & Rajbhandari, S. (2014). Evaluation of antioxidative and antidiabetic activity of bark of Holarrhena pubescens wall. *Journal of Clinical and Diagnostic Research*. https://doi.org/10.7860/jcdr/2014/7803.4863

Bruschi, P., Morganti, M., Mancini, M., & Signorini, M. A. (2011). Traditional healers and laypeople: A qualitative and quantitative approach to local knowledge on medicinal plants in Muda (Mozambique). *Journal of Ethnopharmacology*, *138*(2), 543–563. https://doi.org/10.1016/j.jep.2011.09.055

Gupta, N., Choudhary, S. K., Bhagat, N., Karthikeyan, M., & Chaturvedi, A. (2021). In Silico Prediction, Molecular Docking and Dynamics Studies of Steroidal Alkaloids of Holarrhena pubescens Wall. ex G. Don to Guanylyl Cyclase C: Implications in Designing of Novel Antidiarrheal Therapeutic Strategies. *Molecules/Molecules Online/Molecules Annual*, *26*(14), 4147. https://doi.org/10.3390/molecules26144147

Harborne, J. B. (1984). Phytochemical Methods. Springer Netherlands. https://doi.org/10.1007/978-94-009-5570-7

Holarrhena pubescens Wall. ex G.Don | Plants of the World Online | Kew Science. (n.d.). Plants of the World Online. https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:79318-1

Holarrhena pubescens Wall. ex G.Don | Species. (n.d.). India Biodiversity Portal. https://indiabiodiversity.org/species/show/229948

Holarrhena pubescens Wall. ex G.Don | Species. (n.d.-b). Bhutan Biodiversity Portal. https://biodiversity.bt/species/show/1323

Holarrhena, Holarrhena pubescnes, TELLICHERRY BARK/ Alternative Medicine. (n.d.). http://www.stuartxchange.org/Holarrhena

Jamadagni, P. S., Pawar, S. D., Jamadagni, S. B., Chougule, S., Gaidhani, S. N., & Murthy, S. N. (2017). Review of Holarrhena antidysenterica (L.) Wall. ex A. DC.: Pharmacognostic, pharmacological, and toxicological perspective. *Pharmacognosy Reviews/Bioinformatics Trends/Pharmacognosy Review*, *11*(22), 141. https://doi.org/10.4103/phrev.phrev_31_16

Jumina, J., Siswanta, D., Zulkarnain, K., Triono, S., Priatmoko, P., Yuanita, E., Fatmasari, N., & Nursalim, I. (2019). Development of C-Arylcalix[4]resorcinarenes and C-Arylcalix[4]pyrogallolarenes as Antioxidant and UV-B Protector. *Indonesian Journal of Chemistry*, *19*, 273. https://doi.org/10.22146/ijc.26868

Kadir, M. F., Sayeed, M. S. B., & Mia, M. (2013). Ethnopharmacological survey of medicinal plants used by traditional healers in Bangladesh for gastrointestinal disorders. *Journal of Ethnopharmacology*, *147*(1), 148–156. https://doi.org/10.1016/j.jep.2013.02.023

Kagan, I. A., & Flythe, M. D. (2014). Thin-layer chromatographic (TLC) separations and bioassays of plant extracts to identify antimicrobial compounds. *Journal of Visualized Experiments*, *85*. https://doi.org/10.3791/51411

Koudouvo, K., Karou, D., Kokou, K., Essien, K., Aklikokou, K., Glitho, I., Simpore, J., Sanogo, R., De Souza, C., & Gbeassor, M. (2011). An ethnobotanical study of

antimalarial plants in Togo Maritime Region. *Journal of Ethnopharmacology*, *134*(1), 183–190. https://doi.org/10.1016/j.jep.2010.12.011

Mahdi-Pour, B., Jothy, S. L., Latha, L. Y., Chen, Y., & Sasidharan, S. (2012). Antioxidant activity of methanol extracts of different parts of Lantana camara. *Asian Pacific Journal of Tropical Biomedicine*, *2*(12), 960–965. https://doi.org/10.1016/S2221-1691(13)60007-6

Medicinal plant details. (n.d.). https://thinkinglaymen.org.in/plant_details.php?id=467

Nasim, N., Sandeep, I. S., & Mohanty, S. (2022). Plant-derived natural products for drug discovery: current approaches and prospects. *Nucleus/the Nucleus*, *65*(3), 399–411. https://doi.org/10.1007/s13237-022-00405-3

Olugbami, J., Gbadegesin, M., & Odunola, O. (2014). In vitro evaluation of the antioxidant potential, phenolic and flavonoid contents of the stem bark ethanol extract of Anogeissus leiocarpus. *African Journal of Medicine and Medical Sciences*, *43*(Suppl 1), 101–109.

Pioneer University in Kerala. (n.d.). https://www.keralauniversity.ac.in/digital_garden/145.html

Sawadogo, W. R., Schumacher, M., Teiten, M., Dicato, M., & Diederich, M. (2012). Traditional West African pharmacopeia, plants and derived compounds for cancer therapy. *Biochemical Pharmacology*, *84*(10), 1225–1240. https://doi.org/10.1016/j.bcp.2012.07.021

Sharma, A. K., Chauhan, S., Upadhyaya, S. K., & Sharma, S. (2017). A comparative phytochemical analysis of Wrightia species of family apocynaceae by spot tests. *International Journal of Phytopharmacy*, *7*(2), 14–17. https://doi.org/10.7439/ijpp.v7i2.4115

Siddiqui, B. S., Ali, S. T., Tauseef, S., Kamal, S., Rizwani, G. H., Begum, S., & Aqeel. (2013). EVALUATION OF ANTIOXIDANT ACTIVITY OF METHANOLIC EXTRACT OF THE BARK OF HOLARRHENA PUBESCENS, ITS FRACTIONS AND CONESSINE. *International Journal of Pharmacy*, *3*(9), 462–464. https://www.pharmascholars.com/abstractview/evaluation-of-antioxidant-activity-of-methanolic-extract-of-the-bark-of-holarrhena-pubescens-its-fractions-and-conessine

Singh, R. J., & Jauhar, P. P. (2006). Genetic resources, chromosome engineering, and crop improvement. In *CRC Press eBooks*. https://doi.org/10.1201/9780203489260

Singh, R., Bhowmick, T., Basu, R., Guchhait, P., Chaudhuri, B. N., & Das, S. (2023). Antimicrobial Properties of Ethanolic Extract of Holarrhena pubescens Against MDR Strain of Pseudomonas aeruginosa. *EAS Journal of Pharmacy and Pharmacology*, *5*(04), 87–92. https://doi.org/10.36349/easjpp.2023.v05i04.001

Sinha, S., Sharma, A., Reddy, P. H., Rathi, B., Prasad, N., & Vashishtha, A. (2013). Evaluation of phytochemical and pharmacological aspects of Holarrhena antidysenterica (Wall.): A comprehensive review. *Journal of Pharmacy Research*, *6*(4), 488–492. https://doi.org/10.1016/j.jopr.2013.04.004

Sinha, S., Sharma, A., Reddy, P. H., Rathi, B., Prasad, N., & Vashishtha, A. (2013b). Evaluation of phytochemical and pharmacological aspects of Holarrhena antidysenterica (Wall.): A comprehensive review. *Journal of Pharmacy Research*, *6*(4), 488–492. https://doi.org/10.1016/j.jopr.2013.04.004

Solvent-Solvent Partitioning using Kupchan Scheme. (n.d.). Retrieved May 22, 2024, from https://www.linkedin.com/pulse/solvent-solvent-partitioning-using-kupchan-scheme-hettiarachchi

Wangchuk, P. (2018). Therapeutic applications of natural products in herbal medicines, biodiscovery programs, and biomedicine. *Journal of Biologically Active Products From Nature*, *8*(1), 1–20. https://doi.org/10.1080/22311866.2018.1426495

World Health Organization: WHO. (2023, August 10). *Traditional medicine has a long history of contributing to conventional medicine and continues to hold promise*. https://www.who.int/news-room/feature-stories/detail/traditional-medicine-has-a-long-history-of-contributing-to-conventional-medicine-and-continues-to-hold-promise

Yin, Y., Zhou, B., Bai, L., Feng, D., Qin, T., & Li, C. (2022). Holarrhena pubescens Wall. ex G. Don Extracts Inhibit LPS-Irritated Oxidative Stress in Dendritic Cells. *Applied Sciences*, *12*(22), 11343. https://doi.org/10.3390/app122211343