Determination of Antibacterial and Antioxidant Activity of *Spondias mombin* Stem Bark Extracts

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

Department of Mathematics and Natural Sciences
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Declaration

It is hereby declared that

1. The thesis submitted is my/our original work while completing a 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 that has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. We have acknowledged all main sources of help.

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Approval

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Abstract

Plants have been used throughout history in traditional medicine, and remain a promising source of pharmaceuticals. *Spondias mombin*, an ethnomedicinal plant recognized for its efficacy in treating various diseases, is also the source of numerous therapeutic agents. Previous studies on the bark of this plant revealed its abundance of beneficial compounds along with several pharmacological properties. Although widely available in Bangladesh, limited scientific studies have been reported on this plant. Therefore, this thesis aims to investigate the antibacterial and antioxidant activity of locally sourced *S. mombin* stem bark extracts. The ethanolic bark extract was subjected to solvent partitioning with chloroform, ethyl acetate, hexane, and methanol. Then thin-layer chromatography, Kirby-Bauer disk diffusion test, and DPPH free radical scavenging assay were conducted on the solvent partitions. The ethyl acetate partition extract exhibited mild antibacterial activity against *Acinetobacter baumanni* ATCC 19606, *E. coli* ATCC 25922, *Klebsiella pneumonia* and *Salmonella typhi.* Additionally, all four partition extracts displayed very strong antioxidant activities, with the ethyl acetate extract demonstrating the strongest activity and the lowest IC50 value of 4.74 µg/mL. Visualization of the TLC plates showed the presence of several compounds in the chloroform, hexane, and ethyl acetate partitions which may be of medicinal value. Therefore, these findings underscore the importance of further research into this plant for therapeutic purposes.

Keywords: *Spondias mombin*; antibacterial; antioxidant; thin layer chromatography; disk diffusion; DPPH assay.

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List of Acronyms

S. mombin	*Spondias mombin*
TLC	Thin Layer Chromatography
DCM	Dichloromethane
NA	Nutrient Agar
MHA	Muller-Hinton Agar
UV	Ultra Violet
nm	nanometer
K	Kanamycin
CIP	Ciprofloxacin
ATCC	American Type Culture Collection
ETEC	Enterotoxigenic E. coli
DPPH	2,2-Diphenyl-1-picrylhydrazyl
IC50	Concentration that inhibits 50% of DPPH
mg	milligram
µg	microgram
mL	milliliter
µL	microliter

Chapter 1

Introduction

1.1 Background

Historically, plants have been a valuable source of various medicinal compounds. According to the World Health Organization (WHO), 80% of developing countries in the world still rely on plants as a potential source of novel drugs (Vaou et al., 2021).

With repeated use of antibiotics, the prevalence of antibiotic-resistant pathogens is escalating, making the existing drugs progressively less effective. Therefore, there is a great urgency to discover and isolate new bioactive compounds to combat this concern (Vaou et al., 2021).

To date, over 1340 plants have been reported to have antimicrobial activity, and from them, more than 30,000 antimicrobial compounds could be isolated (Vaou et al., 2021). Natural plants from medicinal plants, including extracts, fractions, essential oils, or isolated constituents offer a promising potential as an alternative strategy against pathogenic bacteria for the treatment of infectious and parasitic diseases. (de Freitas et al., 2022).

Food plants provide a rich variety of bioactive compounds, such as polyphenols, terpenes, limonoids, carotenoids, etc., each having significant biological activities. Additionally, these plants are anticipated to have lower toxicity levels compared to certain medicinal species (Sinan et al., 2021). *Spondias mombin* is one such plant, whose fruits are not only consumed, but it is also popular for its pharmacological benefits. (Omoboyowa et al., 2023). Previous research has demonstrated the medicinal properties of *Spondias mombin*, indicating its use in addressing numerous human health issues. Approximately 102 distinct compounds from various parts of the plant have been documented thus far (Ogunro et al., 2023).

1.2 Objective of the Study

The stem bark of *Spondias mombin* has been known to possess medicinally valuable compounds (de Freitas et al., 2022). An initial fundamental approach to evaluating plants' biological activity involves conducting screening tests (Ayoka et al., 2008). Hence, this study particularly aims to determine and confirm the antibacterial and antioxidant properties of different solvent partition extracts of the stem bark of local *Spondias mombin.*

1.3 Spondias mombin

1.31 Common Names

- English: Hog plum, Yellow mombin, Golden Apple, Java Plum
- Bengali: Amra
- Brazilian: Cajá
- Hindi: Junglee Aam
- Malayalam: Ambazham
- Spanish: Jobo (Bhandarkar et al., 2015), (Ogunro et al., 2023)

1.32 Taxonomy

Kingdom: Plantae

Clade: Tracheophytes

Clade: Angiosperms

Clade: Eudicots

Clade: Rosids

Order: Sapindales

Family: Anacardiaceae

Genus: *Spondias*

Species: *mombin*

1.33 Description

The *S. mombin* plant is a deciduous tree that grows as tall as 20-30 m in height, with trunk diameter varying between 60 and 75 cm (Ogunro et al., 2023). The bark is rough and thick, greyish brown; and they have blunted grey spines on the surface (Ayoka et al., 2008). The plant has compound leaves and produces ovoid fruits from small fragrant white flowers. The fruits have juicy pulp, one large seed, and a tough, thin, green skin, which turns golden-yellow upon ripening; they are edible. (Ogunro et al., 2023).

Figure 1: *Spondias mombin* Plant [Source: (Mattietto$ & Matta, 2011)]

1.4 Distribution

This plant is native to various tropical, subtropical, and temperate regions of the world. Initially only found in the Americas and West Indies, it was introduced to South Asia and parts of Africa in the 17th century where it grows naturally. It can now be found in many South Asia countries

including, India, Nepal, Bangladesh, Sri Lanka, Indonesia, etc. In some parts of the Brazilian Northeast, they are cultivated (Wikipedia, 2024).

1.5 Medicinal Uses of *Spondias Mombin*

Different parts of the *S. mombin* plant, including the leaves, bark, fruits, flowers, and seeds are of medicinal importance in tropical regions around the world (Ogunro et al., 2023). In traditional medicine, they have been used to treat various ailments (Ayoka et al., 2008). Previous studies have determined that the bark and leaves are rich in therapeutic agents such as alkaloids, flavonoids, polyphenols, saponins, and terpenes (de Freitas et al., 2022). The leaves and flowers relieve stomach pain, biliary vomiting, diarrhea, dysentery, and inflammations (Ayoka et al., 2008) (Bhandarkar et al., 2015). The bark of this plant is astringent, and its decoction is used as anti-diarrheal and emetic to treat diarrhea, dysentery, along with other diseases like malaria, hemorrhoids, gonorrhea, leucorrhea, vaginal infections (Ogunro et al., 2023). Furthermore, the bark powder is used for wound healing (Bhandarkar et al., 2015). The fruit juice is often drunk as a diuretic and febrifuge (Ayoka et al., 2008).

1.6 Thin Layer Chromatography

Thin layer chromatography (TLC) is a widely used chromatographic technique for the separation and qualitative analysis of mixtures of compounds. It involves a stationary phase, usually adsorbent material like silica gel or alumina coated onto a plate, and a solvent system which is the mobile phase (Kagan & Flythe, 2014). As the solvent migrates up the plate through capillary action, it carries the components of the sample mixture along with it. Different compounds interact with the stationary and mobile phases, causing them to separate; the degree of separation depends on many factors such as polarity, size, and affinity. This separation results in the formation of distinct spots or colored bands on the TLC plate depending on their chemical nature, which can be visualized under UV light or using appropriate chemical

reagents. TLC is the preferred technique for isolating all lipid-soluble constituents, including lipids, steroids, carotenoids, basic quinones, and chlorophylls (Harborne, 1984).

1.7 Kirby-Bauer Disk Diffusion

The Kirby-Bauer disk diffusion method is a standard laboratory technique used to determine the susceptibility of bacteria to antimicrobial agents. In this method, paper disks impregnated with specific antimicrobial agents are placed on the surface of an agar plate inoculated with the test bacteria (Hudzicki, 2009). As the antimicrobial agents diffuse into the agar, they create a concentration gradient, resulting in zones of inhibition where bacterial growth is inhibited. The diameter of these zones is measured and compared to standardized interpretive criteria (CLSI Guidelines, 2021) to determine the susceptibility of the bacteria to the tested antimicrobial agents. Currently, guidelines set by the Clinical Laboratory Standards Institute in the Performance Standards for Antimicrobial Disk Susceptibility are followed for interpretation of results (Hudzicki, 2009).

1.8 Antioxidant Activity

1.81 Free Radicals

Free radicals are highly reactive molecules containing unpaired electrons. They are generated naturally during metabolic processes or as a result of external factors like UV radiation, pollution, and smoking. Excessive production of free radicals can lead to oxidative stress, causing damage to cellular components like lipids, proteins, and DNA. This oxidative damage is associated with various diseases, including cancer, cardiovascular diseases, neurodegenerative disorders, and aging.

1.82 Antioxidants

Antioxidants are molecules that can neutralize free radicals and prevent oxidative damage. They act by donating electrons or hydrogen atoms to free radicals, thereby stabilizing them and inhibiting chain reactions of oxidative damage. Antioxidants can be enzymatic or non-enzymatic and are found in various foods. Assessing the antioxidant activity of compounds using DPPH (2,2-diphenyl-1-picrylhydrazyl) assay helps in identifying potential therapeutic agents for combating oxidative stress-related diseases and promoting overall health.

1.83 DPPH Free Radical Scavenging Activity

The DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay is used to assess the antioxidant activity of different compounds. It measures the ability of antioxidants to scavenge DPPH radicals, which are stable and purple. When antioxidants react with DPPH radicals, they donate hydrogen atoms or electrons, leading to a color change from purple to yellow, which can be quantified spectrophotometrically. The degree of color change is proportional to the antioxidant capacity of the tested compound.

1.9 Study Protocol

The focus of this study was to determine the antibacterial and antioxidant properties of the different solvent fractions of the *Spondias mombin* plant. The steps in the study protocol are the following:

- Extraction of the powdered bark of the plant
- Crude extraction and filtration
- Solvent-solvent partitioning
- Visual detection of chemicals presents
- Antibacterial activity of extract fraction on clinically significant bacteria
- Antioxidant activity of extract fraction and determination of IC50 Values

Chapter 2

Materials and Methods

2.1 Method Overview

- Collection of the plant material
- Preparation of plant sample
- Extraction and filtration
- Solvent-solvent partitioning
- Thin Layer Chromatography
- Visual analysis
- Antibacterial Activity by Kirby-Bauer disk diffusion
- Antioxidant Activity by DPPH assay

2.2 Specimen Collection

The stem bark of a *Spondias mombin* tree was collected using a clean knife from a residential area in Savar, Dhaka (Appendix A). The plant was identified by its vernacular name.

2.3 Sample Preparation

The collected bark was allowed to air dry for 4 weeks in a cool, dry environment, at room temperature (Omoboyowa et al., 2023). Then the dried bark was powdered using an electric grinder (Panasonic, Japan). 1000 g of powdered bark was weighed out and soaked in 3.5 L of 90% (v/v) ethanol, in a sealed container, for 7 days. The container was shaken every alternate day.

2.4 Extraction of Crude Ethanol Extract

After 7 days, the mixture was filtered through Whatman no.1 filter papers and a cotton sieve to remove the insolubilized solid portion. The filtrate was concentrated by evaporation of the solvent using a rotary evaporator (Heidolph, Germany) at 40-45 °C, at a speed of 80 rpm. The obtained crude extract was collected and air dried at room temperature to remove any remaining solvent, then stored at 37 °C until further use.

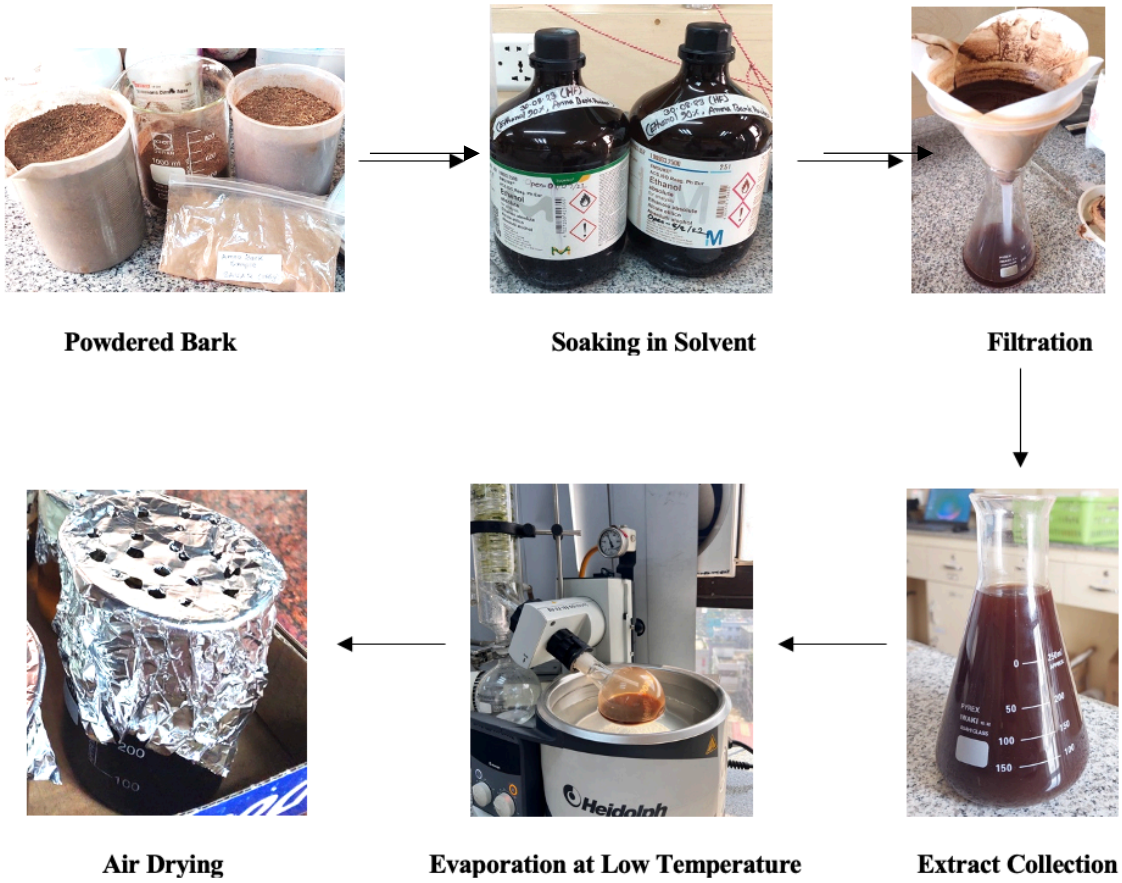


Figure 2: Plant Sample Preparation and Crude Extraction

2.5 Solvent-Solvent Extraction

Kupchan's method of partitioning (Scribd, n.d.) was followed. A 90:10 ratio methanol: water solution was prepared. 50 mL of the 90% (v/v) methanol solution was added to 5 g crude extract and the solution was transferred to a separating funnel. Following the separation of

the organic phase, the aqueous phase was extracted using a series of different organic solvents of increasing polarity (hexane, chloroform, ethyl acetate, and methanol) (Otsuka, 2006). 50 mL hexane was added to the funnel and mixed well by shaking and inversion, then left aside for layer separation. The hexane layer was collected in a beaker. 25 mL of water was added to the funnel, then 50 mL chloroform was added, mixed well, and set aside for layer separation. Afterward, separated chloroform layer was collected in a separate beaker. Next, 50 mL ethyl acetate was added to the remaining MeOH/H2O mixture in the funnel. The mixture was shaken and left aside for layer separation. The ethyl acetate layer at the bottom was collected in another separate beaker. Finally, 50 mL methanol was added and allowed to mix well by shaking. The methanol layer was also collected in a separate beaker, leaving behind an aqueous layer in the flask (Scribd, n.d.). Each partition was performed 2 times for the entire crude extract sample. All 4 collected solvent partitions were left to air dry until no solvents remained in the extracts.

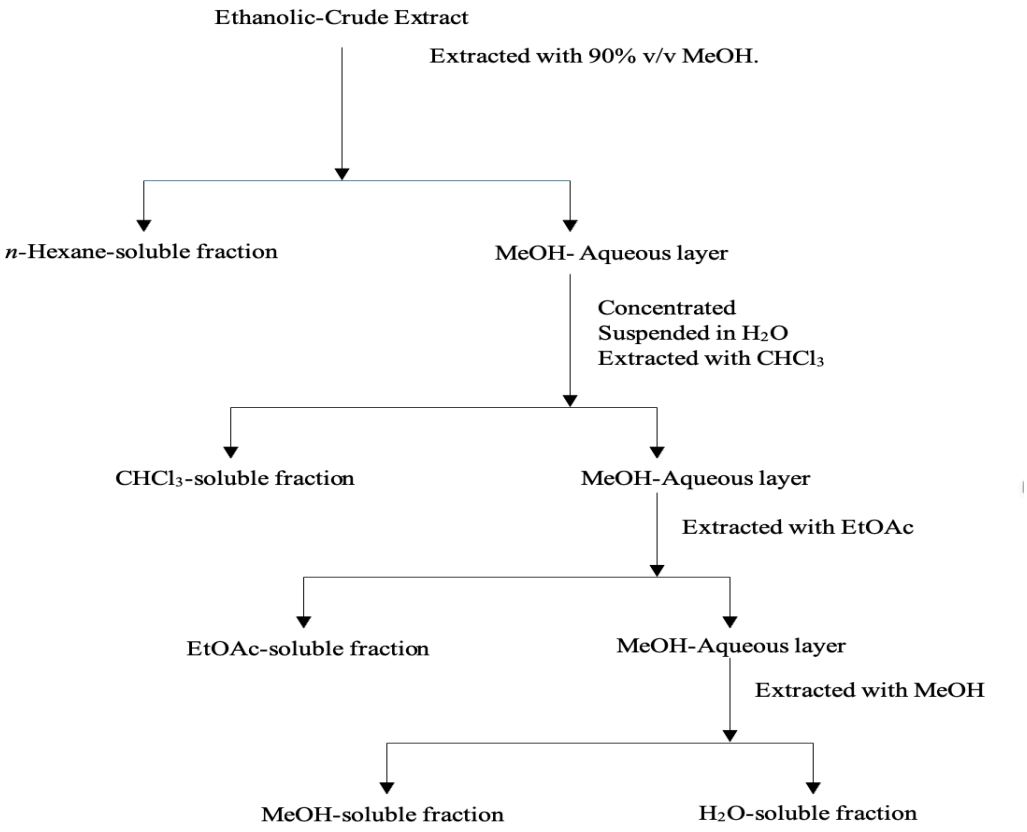


Figure 3: Schematic Diagram of Solvent Partitioning

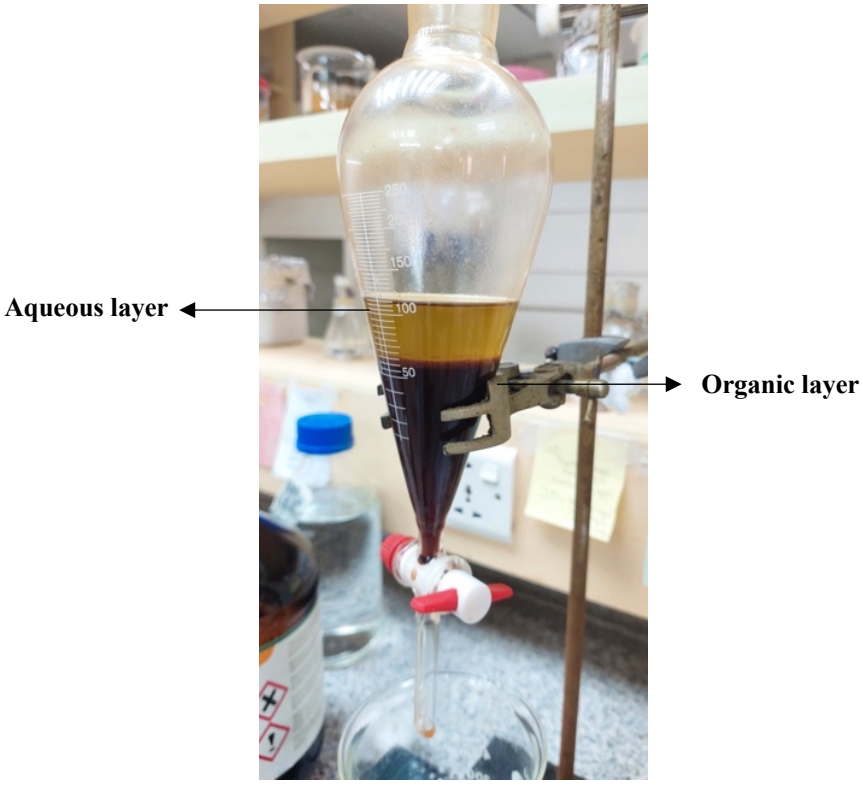


Figure 4: Solvent Partitioning of Plant Material

2.6 Thin Layer Chromatography

TLC (Thin Layer Chromatography) of the crude extract and the four solvent partition extracts (chloroform, ethyl acetate, hexane, and methanol) of *S. mombin* bark were performed. About 2 µg (Mandal et al., 2013) of each partition extract was transferred to 4 separate vials pre-washed with MeOH. 2-3 drops of suitable solvent were added to make a solution. Commercially available silica gel plates (20×20 cm) were cut to inappropriate size. A baseline was drawn near the bottom of the plates using a pencil. With clean pipette tips, a small drop of each extract solution was placed on the baseline. The plates were then placed in a closed TLC chamber (Kagan& Flythe, 2014) with forceps, and then run in 5 ml solvent of toluene and ethyl acetate in different ratios. The polarity of the solvent was gradually increased for better separation and visualization. The chromatograms were visualized under UV light using a UV torch at 365 nm (long-wave UV) for the identification of separated components.

2.7 Antibacterial Activity Analysis

Kirby-Bauer disk diffusion method (Hudzicki, 2009) was used to determine the antibacterial properties of the four solvent partition extracts (chloroform, ethyl acetate, hexane, and methanol) of *S. mombin* stem bark on 10 clinically significant bacterial strains.

2.71 Test Organisms

A total of 10 bacterial strains were collected from the Biotechnology Laboratory of BRAC University for this study. They were: *Acinetobacter baumanni* ATCC 19606, *Bacillus cereus*, *E. coli* ATCC 25922, *Enterotoxigenic E. coli* (ETEC), *Klebsiella pneumonia*, *Kocuria rhizophila* ATCC 9341, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhi, Staphylococcus aureus, and Shigella flexneri.*

Table 1: Gram-positive and Gram-negative Test Organisms

Gram-positive	**Gram-Negative**
Bacillus cereus	*Acinetobacter baumanni*
Kocuria rhizophila	*Enterotoxigenic Escheria coli*
Staphylococcus aureus	*Escheria coli*
	Klebsiella pneumonia
	Pseudomonas aeruginosa
	Salmonella typhi
	Shigella flexneri

2.72 Agar Plates Preparation

NA (Nutrient Agar) media plates were used for the subculture of the selected bacterial isolates. MHA (Mueller-Hinton Agar) media plates were used for disk diffusion assay. The required

mass of media power was weighed, dissolved, and in distilled water by boiling, then sterilized by autoclaving at 121 °C for 15 minutes. About 25 mL of this solution was poured into labeled petri dishes under laminar flow and allowed to solidify. Fresh plates were kept in refrigerator at 4°C until use.

2.73 Bacterial Subculture

Bacterial strains preserved in NA media vials were stabbed with a sterile needle and isolated on the prepared fresh NA media plates by streak plate method (Katz, 2008). The plates were then incubated at 37°C for 18-24 hours prior to lawning on MHA.

2.74 Sample Solution Preparation

Concentrations ranging from 5 mg/mL to 20 mg/mL were used for the antibacterial assay of the four solvent partition extracts. To prepare each concentration solution, 10 mg, 20 mg, 30 mg, and 40 mg of each partition extract (chloroform, ethyl acetate, hexane, and methanol) were taken in separate labeled clean sterile vials and then dissolved in 2 mL of an appropriate solvent (Castro-Ontengcoe & Capal, 2004). The solutions were then vortexed for homogenization.

2.75 Disk Preparation

Autoclaved Whatman filter disks were cut out under laminar flow with the sterile punch-hole machine. 6 mm diameter disks of 4 filter paper thickness were used to match the size and thickness of antibiotic disks. The blank disk was taken as a negative control. Kanamycin 30 µg and Ciprofloxacin 5 µg (Samuggam et al., 2021) were used as positive controls. Methanol and DCM were used as solvent controls.

With a micropipette, 20 µL (Castro-Ontengcoe & Capal, 2004) of each concentration solution

of each partition extract was transferred to the filter paper disks, under laminar flow and allowed to soak, and solvent to evaporate (Romero et al., 2005).

2.76 Disk Diffusion

MHA plates were lawned with each of the bacterial subcultures. Autoclaved sterile cotton swabs were used to pick up 2-3 medium-sized colonies, then lawned onto the surface of each MHA plate. The plates were labeled for each strain used.

The disks were impregnated with the partition extracts and all controls were placed on each of the lawned MHA surfaces with sterile forceps. The disks were placed about equal distances apart from one another. This process is done for all 4 partition extracts at each concentration. After disk placement, the lids were placed and the plates were allowed to stand in the refrigerator at 4°C for 2 hours to allow the extracts to diffuse. Then, the plates were shifted to an incubator, for incubation at 37 °C for 18-24 hours (Hudzicki, 2009).

After incubation, the diameter of the clear zones of inhibition around the disks was measured with a ruler in mm. Each zone was measured thrice, then their average was used for comparison and analysis.

2.8 Antioxidant Activity by DPPH Assay

DPPH scavenging activity test was done for each of the 4 partition extracts (chloroform, ethyl acetate, hexane, and methanol)

2.81 DPPH Solution Preparation

0.004% of DPPH solution (Pour et al, 2004) was prepared by dissolving 4 mg of DPPH reagent in 100 mL of MeOH. The solution was prepared in an amber reagent bottle and kept in a light-proof box until use.

2.82 Standard Solution Preparation

For standard, 1 mg/mL ascorbic acid solution was prepared by dissolving 0.01 g (10 mg) of ascorbic acid powder in 10 mL methanol in a test tube. The solution was vortexed for homogenization.

2.83 Plant Sample Solution Preparation

1 mg/mL solutions of each partition extract were prepared by dissolving 0.01 g (10 mg) of each extract in 10 mL methanol in separate labeled test tubes. The solutions were vortexed for homogenization.

2.84 Test Sample Preparation

Concentrations ranging from 5 µg/mL to 200 µg/mL were used for the DPPH assay of the four solvent partition extracts. To prepare each solution, 20 µL, 40 µL, 100 µL, 200 µL, 400 µL and 800 µL of each partition extract were transferred to separate test tubes. Then the solutions were made up to 3ml by the addition of methanol. Then, 1 mL of the 0.004% DPPH solution was added to all the test tubes, making the total sample volume 4 mL. Methanol was used as a blank. 1 mL of the DPPH solution in 3ml methanol was taken as a negative control. The test tubes were wrapped in aluminum foil and incubated in the dark for 30 min before taking absorbance readings.

2.85 DPPH Assay

A color change from purple to yellow indicates free radical scavenging activity (Figure 5).

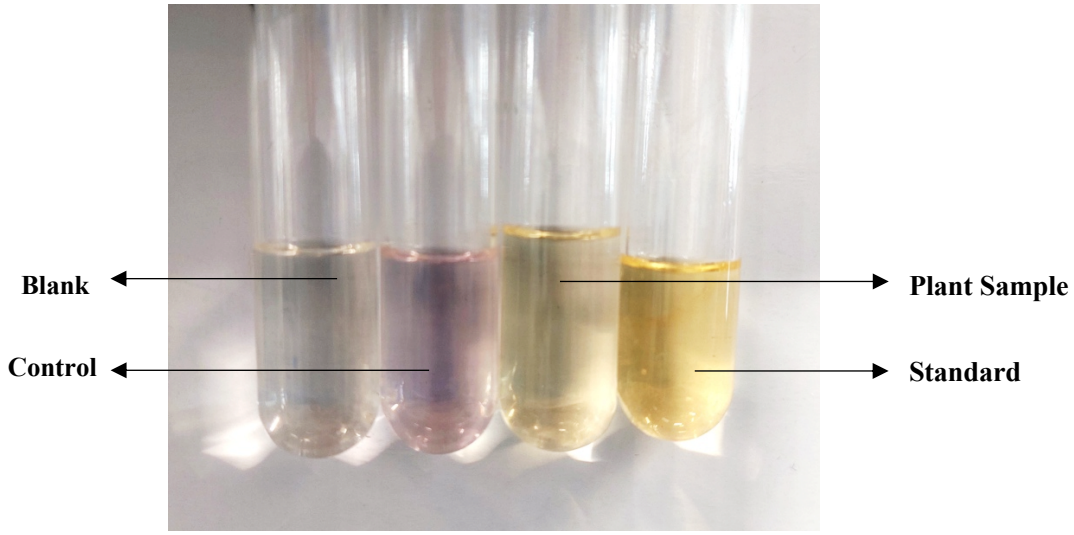


Figure 5: Color Changes After Addition of DPPH Solution

The UV Spectrophotometer (GENESYS 10S UV-VIS, Thermo Scientific) was adjusted to take readings at 517 nm. The test samples of each partition extract were transferred to cuvettes. The blank reading of methanol was taken and set to zero. The readings at each concentration for each partition extract were measured and recorded. The reading for the negative control was also measured. All measurements at each concentration were done in duplicates.

The free radical scavenging activity was calculated using the following formula (Pour et al, 2004):

$$\text{Inhibition (\%)} = \frac{\boldsymbol{Absorbance\ of\ \ Control - Absorbance\ of\ Sample}}{\boldsymbol{Absorbance\ \ of\ \ Control}} \times 100$$

The IC50 values were then calculated from the linear regression equation generated by Microsoft Excel, using the following formula:

$$\text{IC50} = \frac{\boldsymbol{50 - y\ inytercept\ value}}{\boldsymbol{Gradient\ of\ the\ line}}$$

Chapter 3

Results

3.1 Yield of Plant Extracts

About 18 g of crude ethanolic extract of *S. mombin* stem bark was obtained after rotatory evaporation of the filtrate. The weights of each partition extract after solvent-solvent extraction, along with their color and texture are shown in Table 2.

Table 2: Weight and Physical Characteristics of Different Solvent Partitions of *S. mombin*

Solvent Partition	Weight (g)	Color	Texture
Chloroform (C)	0.78 g	Dark yellowish green	Dense and sticky
Ethyl acetate (E)	0.73 g	Dark Red-Brown	Hard and firm
Hexane (H)	1.48 g	Blackish Brown	Dense and viscous
Methanol (M)	13.46 g	Reddish Brown	Powdery

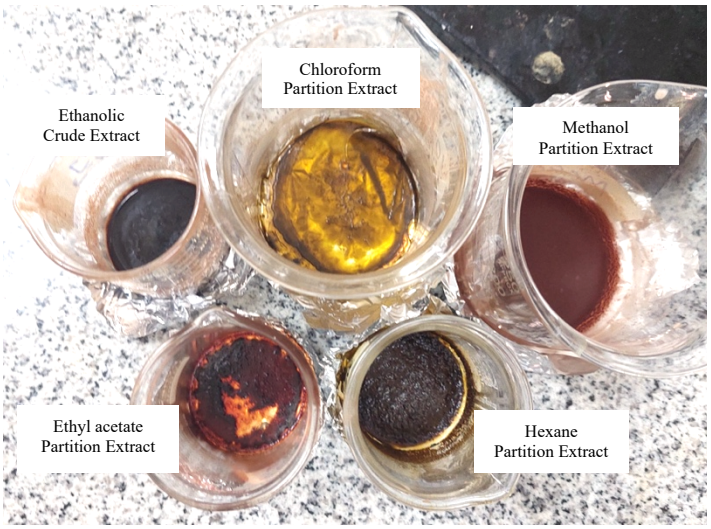


Figure 6: Different Solvent-free Extracts of *S. mombin* Stem Bark

3.2 Thin Layer Chromatography

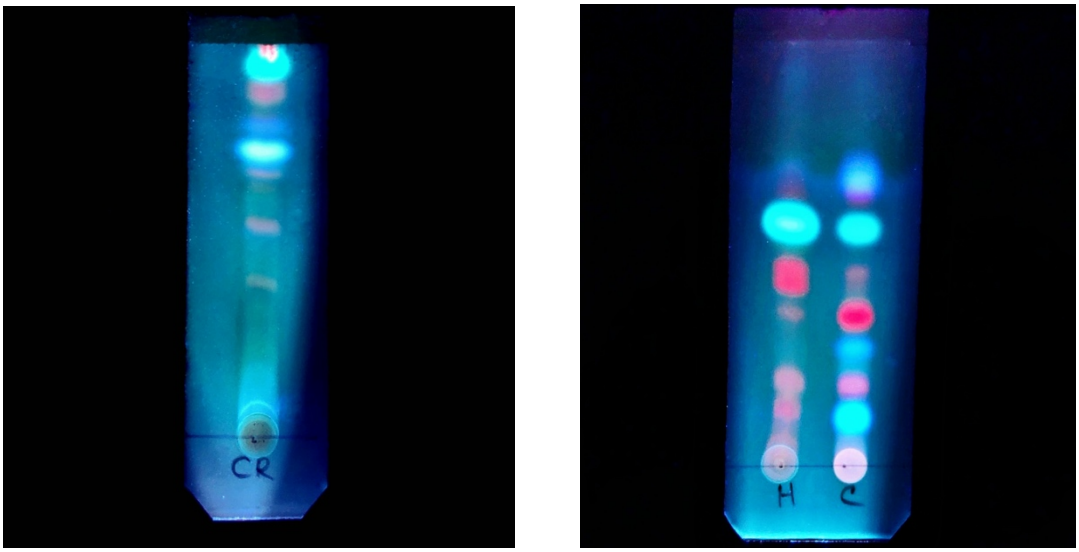
The different solvent partition extracts of *S. mombin* stem bark exhibited separation in different ratios of the two solvents- toluene and ethyl acetate, depending on their polarity.

Table 3: Solvent Ratios for TLC of *S. mombin* Extracts

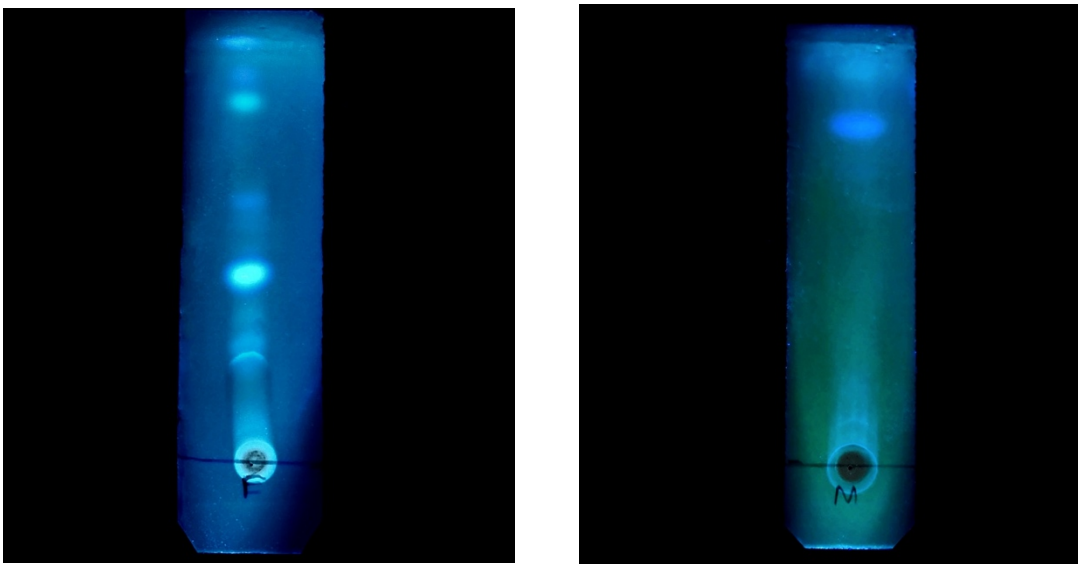
Extract	Toluene (mL)	Ethyl acetate (mL)	Solvent Ratio
Crude (CR)	2	3	4:6
Chloroform (C)	4.5	0.5	9:1
Ethyl acetate (E)	3	2	6:4
Hexane (H)	4.5	0.5	9: 1
Methanol (M)	1	4	2:8

The path traveled by the samples on the TLC plates appeared colorless with the naked eye. However, under long wavelength UV light at 365 nm, the separation of different compounds could be confirmed upon visualization of fluorescent bands of different colors. This suggests that these separated compounds have aromatic rings in their structure (Harborne, 1984). Different colors indicate the possible presence of different compounds.

The following figure shows the observed TLC chromatograms of the different extracts of *S. mombin* bark under UV light in the dark:



(a) **(b)**



(c) **(d)**

Figure 7: Chromatogram of (a) Crude (b) Chloroform and Hexane (c) Ethyl acetate (d) Methanol

The chloroform and hexane partition extracts had the greatest number of fluorescent bands at different lengths, in contrast to ethyl acetate and methanol partition extracts which had much fewer bands. Methanol partition extract had the least number of fluorescent bands.

3.3 Kirby-Bauer Disk Diffusion Assay

The average diameter of the zone of inhibitions for each extract at different concentrations and the positive, negative, and solvent controls are illustrated in Tables 4-7. The ethyl acetate partition extract demonstrated the highest antibacterial activity at 400 µg/disk concentration, with the average diameter of zone of inhibition ranging from 8-15 mm on 4 test organisms, followed by the chloroform partition extract with average zone of inhibition diameter of 7 mm and 9 mm on 2 test organisms. The hexane and methanol partition extracts did not have any inhibitory effect on any of the test organisms.

Table 4: Comparison of Antibacterial Activity of Different Concentrations of Chloroform Partition Extracts of *S. mombin* vs. Controls on Test Organisms

Test Organism	**Average Diameter of Zone of Inhibition (mm)**								
	Extract Concentrations (µg/disk)				**Positive Control**		**Negative Control (NC)**	**Solvent Control**	
	100	**200**	**300**	**400**	**K**	**CIP**		**DCM**	**MeOH**
Acinetobacter baumanni ATCC 19606	-	-	-	-	26	33	-	-	-
Bacillus cereus	-	-	-	-	17	24	-	-	-
E. coli ATCC 25922	-	-	-	7	19	38	-	-	-
Enterotoxigenic E. coli (ETEC)	-	-	-	-	20	30	-	-	-
Klebsiella pneumonia	-	-	-	-	18	24	-	-	-
Kocuria rhizophila ATCC 9341	-	-	-	-	21	24	-	-	-
Pseudomonas aeruginosa ATCC 27853	-	-	-	-	-	33	-	-	-
Salmonella typhi	-	-	-	9	23	30	-	-	-
Staphylococcus aureus	-	-	-	-	23	28	-	-	-
Shigella flexneri	-	-	-	-	20	36	-	-	-

"-" indicates no zone

Table 5: Comparison of Antibacterial Activity of Different Concentrations of Ethyl acetate Partition Extracts of *S. mombin* vs. Controls on Test Organisms

Test Organism	Average Diameter of Zone of Inhibition (mm)								
	Extract Concentrations (µg/disk)				Positive Control		Negative Control (NC)	Solvent Control	
	100	200	300	400	K	CIP		DCM	MeOH
Acinetobacter baumanni ATCC 19606	7	8	9	15	26	33	-	-	-
Bacillus cereus	-	-	-	-	17	24	-	-	-
E. coli ATCC 25922	-	-	8	9	19	38	-	-	-
Enterotoxigenic E. coli (ETEC)	-	-	-	-	20	30	-	-	-
Klebsiella pneumonia	-	-	-	8	18	24	-	-	-
Kocuria rhizophila ATCC 9341	-	-	-	-	21	24	-	-	-
Pseudomonas aeruginosa ATCC 27853	-	-	-	-	-	33	-	-	-
Salmonella typhi	-	7	8	11	23	30	-	-	-
Staphylococcus aureus	-	-	-	-	23	28	-	-	-
Shigella flexneri	-	-	-	-	20	36	-	-	-

"-" indicates no zone

Table 6: Comparison of Antibacterial Activity of Different Concentrations of Hexane Partition Extracts of *S. mombin* vs. Controls on Test Organisms

Test Organism	Average Diameter of Zone of Inhibition (mm)								
	Extract Concentrations (µg/disk)				Positive Control		Negative Control (NC)	Solvent Control	
	100	200	300	400	K	CIP		DCM	MeOH
Acinetobacter baumanni ATCC 19606	-	-	-	-	26	33	-	-	-
Bacillus cereus	-	-	-	-	17	24	-	-	-
E. coli ATCC 25922	-	-	-	-	19	38	-		-
Enterotoxigenic E. coli (ETEC)	-	-	-	-	20	30	-		-
Klebsiella pneumonia	-	-	-	-	18	24	-		-
Kocuria rhizophila ATCC 9341	-	-	-	-	21	24	-		-
Pseudomonas aeruginosa ATCC 27853	-	-	-	-	-	33	-	-	-
Salmonella typhi	-	-	-	-	23	30	-		-
Staphylococcus aureus	-	-	-	-	23	28	-		-
Shigella flexneri	-	-	-	-	20	36	-		-

"-" indicates no zone

Table 7: Comparison of Antibacterial Activity of Different Concentrations of Methanol Partition Extracts of *S. mombin* vs. Controls on Test Organisms

Test Organism	Average Diameter of Zone of Inhibition (mm)								
	Extract Concentrations (µg/disk)				Positive Control		Negative Control (NC)	Solvent Control	
	100	200	300	400	K	CIP		DCM	MeOH
Acinetobacter baumanni ATCC 19606	-	-	-	-	26	33	-	-	-
Bacillus cereus	-	-	-	-	17	24	-	-	-
E. coli ATCC 25922	-	-	-	-	19	38	-	-	-
Enterotoxigenic E. coli (ETEC)	-	-	-	-	20	30	-	-	-
Klebsiella pneumonia	-	-	-	-	18	24	-	-	-
Kocuria rhizophila ATCC 9341	-	-	-	-	21	24	-	-	-
Pseudomonas aeruginosa ATCC 27853	-	-	-	-	-	33	-	-	-
Salmonella typhi	-	-	-	-	23	30	-		-
Staphylococcus aureus	-	-	-	-	23	28	-		-
Shigella flexneri	-	-	-	-	20	36	-		-

"-" indicates no zone

The" antibacterial activity for each of the partition extract at each of the concentrations (100, 200, 300 and 400 µg/disk) were compared. At higher concentrations, the inhibitory effect was greater for the effective partition extracts (ethyl acetate and chloroform). *Pseudomonas aeruginosa* ATCC 27853 had no zone of inhibition, suggesting that it was resistant to the positive control Kanamycin (Figure 8).

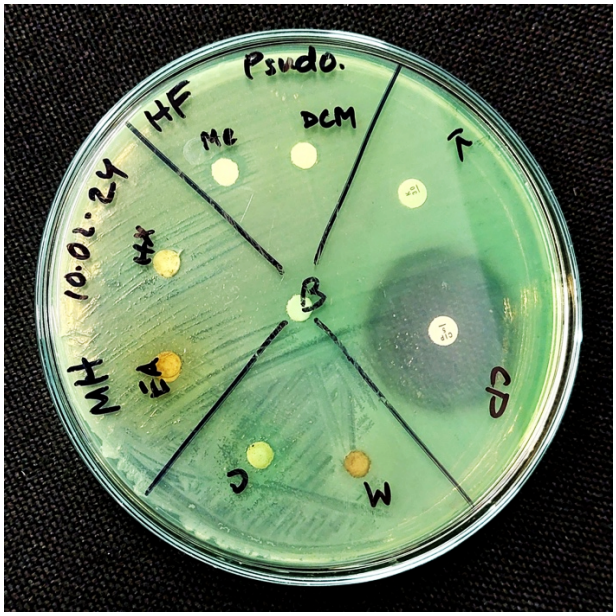


Figure 8: Disk Diffusion Assay of *Pseudomonas aeruginosa* ATCC 27853

*C=Chloroform extract; EA= Ethyl acetate extract, HX=Hexane extract, M=Methanol extract, Me=Methanol solvent, DCM=Dichloromethane Solvent, K=Kanamycin, CP=Ciprofloxacin

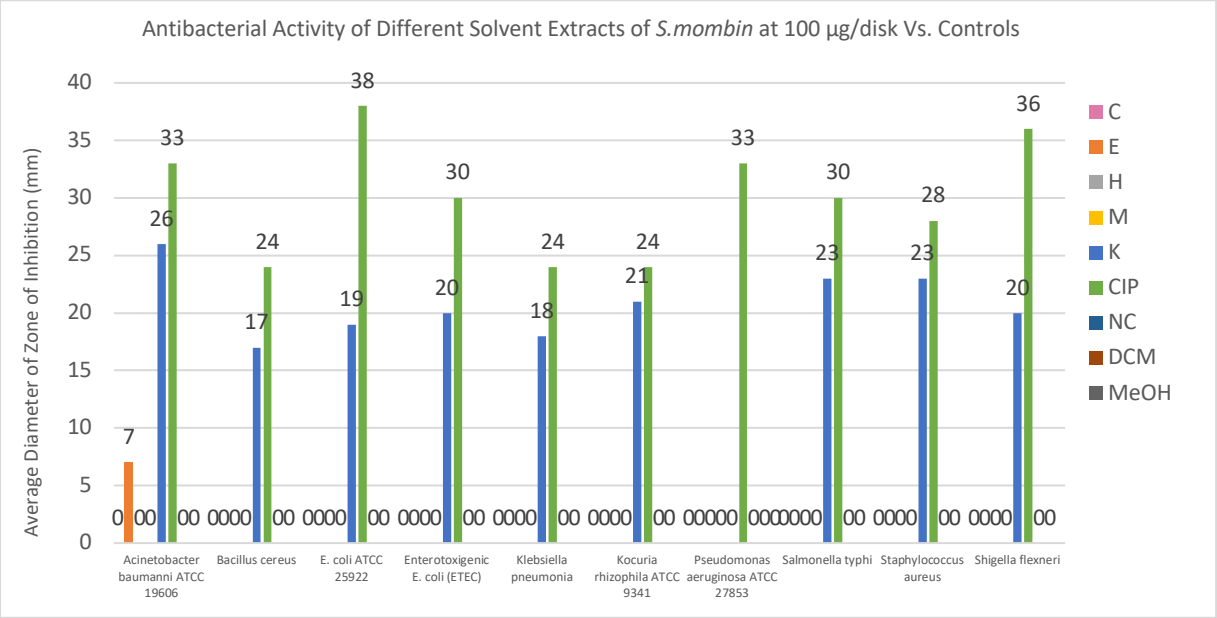


Figure 9: Graphical Representation of Antimicrobial Activity of Different Partition Extracts of *S. mombin* at 100 µg/disk Vs. Controls on the Test Organisms

At 100 µg/disk concentration, only the ethyl acetate partition extract had mild inhibitory effect on *Acinetobacter baumanni* ATCC 19606, with an average zone diameter of 7 mm (Figure 10).

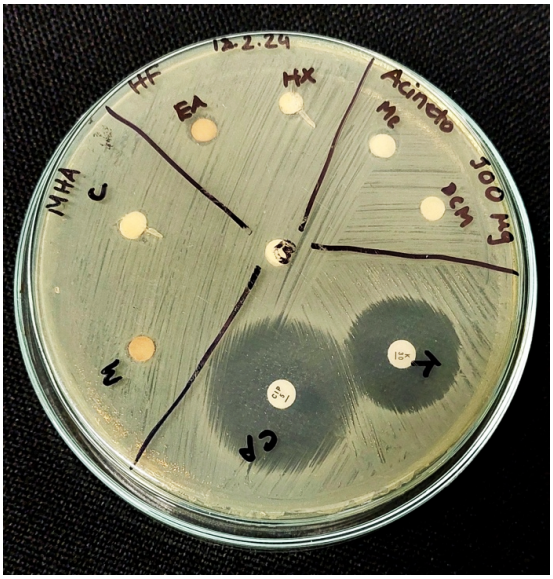


Figure 10: Antibacterial Activity of Partition Extracts at 100 µg/disk Concentration on *Acinetobacter baumanni* ATCC 19606

*C=Chloroform extract; EA= Ethyl acetate extract, HX=Hexane extract, M=Methanol extract, Me=Methanol solvent, DCM=Dichloromethane Solvent, K=Kanamycin, CP=Ciprofloxacin

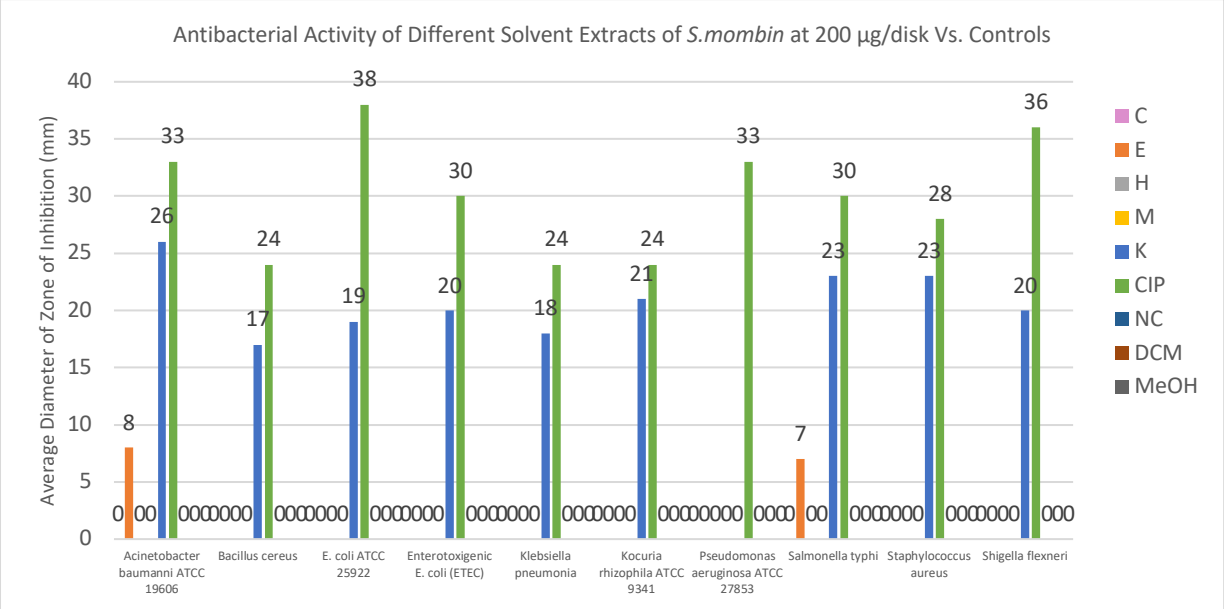
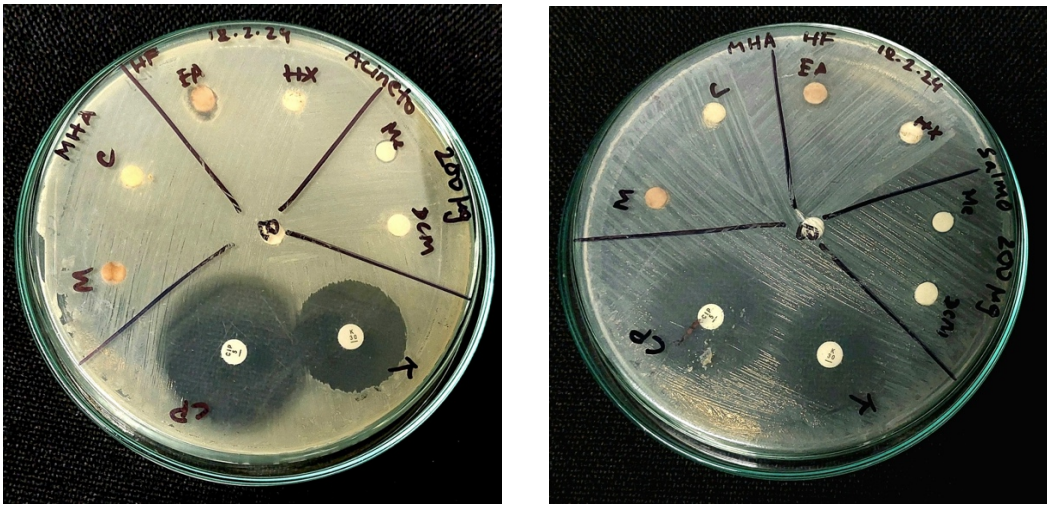


Figure 11: Graphical Representation of Antimicrobial Activity of Different Partition Extracts of *S. mombin* at 200 µg/disk Vs. Controls on the Test Organisms

At 200 µg/disk concentration, only the ethyl acetate partition extract had a mild inhibitory

effect on 2 test organisms: *Acinetobacter baumanni* ATCC 19606 and *Salmonella typhi*, with average zone diameters of 8 mm and 7 mm respectively (Figure 12).



(a) (b)

Figure 12: Antibacterial Activity of Partition Extracts at 200 µg/disk Concentration on (a) *Acinetobacter baumanni* ATCC 19606 (b) *Salmonella typhi*

*C=Chloroform extract; EA= Ethyl acetate extract, HX=Hexane extract, M=Methanol extract, Me=Methanol solvent, DCM=Dichloromethane Solvent, K=Kanamycin, CP=Ciprofloxacin

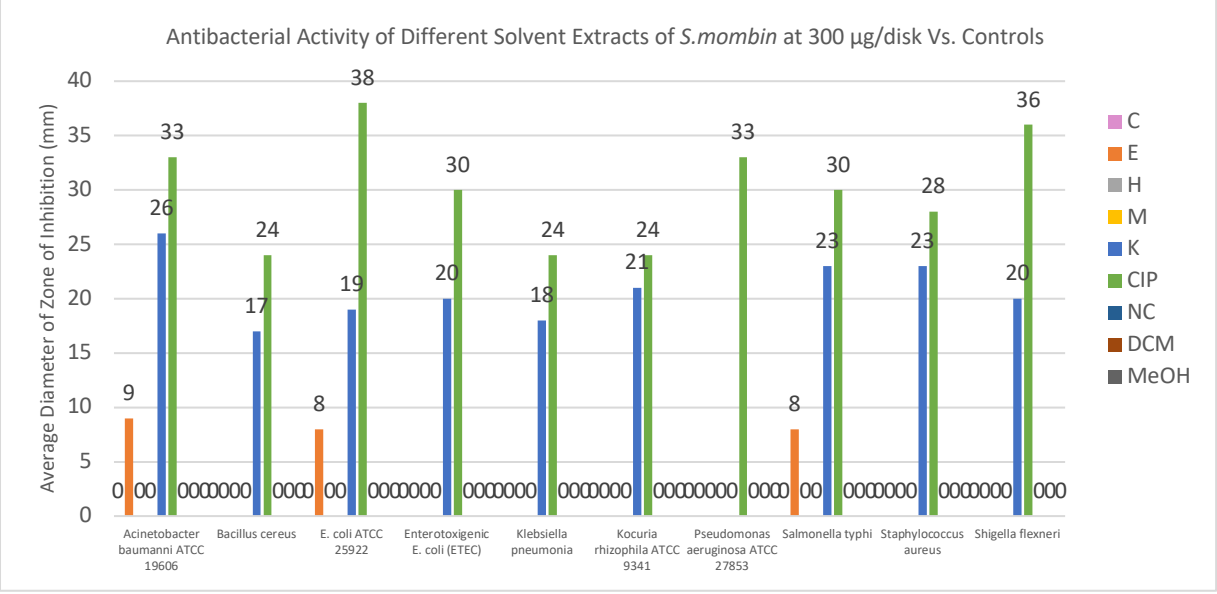
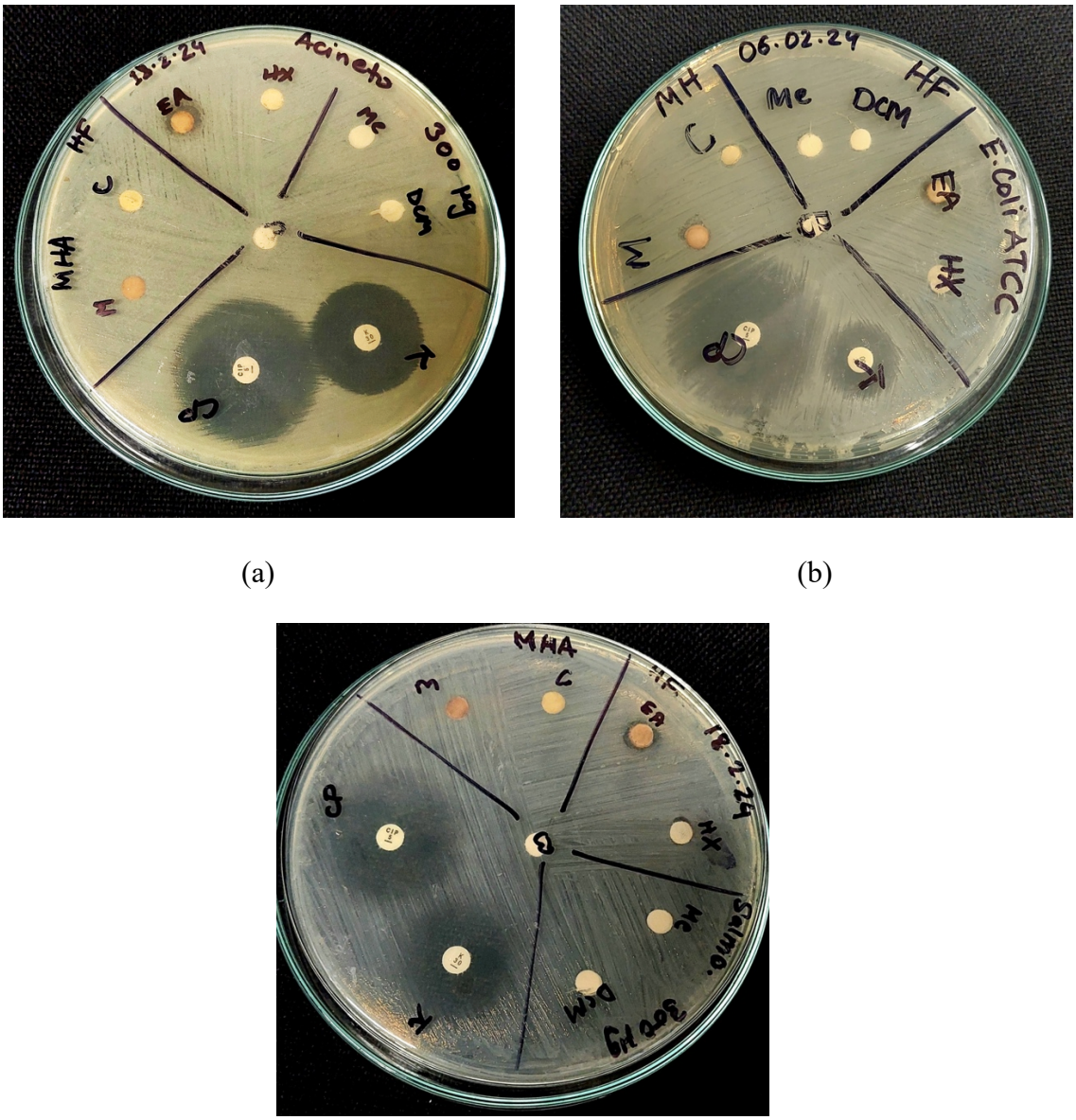


Figure 13: Graphical Representation of Antimicrobial Activity of Different Partition Extracts of *S. mombin* at 300 µg/disk Vs. Controls on the Test Organisms

At 300 µg/disk concentration, only the ethyl acetate partition extract had an inhibitory effect on 3 test organisms: *Acinetobacter baumanni* ATCC 19606, *E. coli* ATCC 25922, and *Salmonella typhi* with average zone diameters of 9 mm, 8 mm, and 8 mm respectively (Figure 14).



(a) (b)

(c)

Figure 14: Antibacterial Activity of Partition Extracts at 300 µg/disk Concentration on (a) *Acinetobacter baumanni* ATCC 19606 (b) *E. coli* ATCC 25922 (c) *Salmonella typhi*

*C=Chloroform extract; EA= Ethyl acetate extract, HX=Hexane extract, M=Methanol extract, Me=Methanol solvent, DCM=Dichloromethane Solvent, K=Kanamycin, CP=Ciprofloxacin

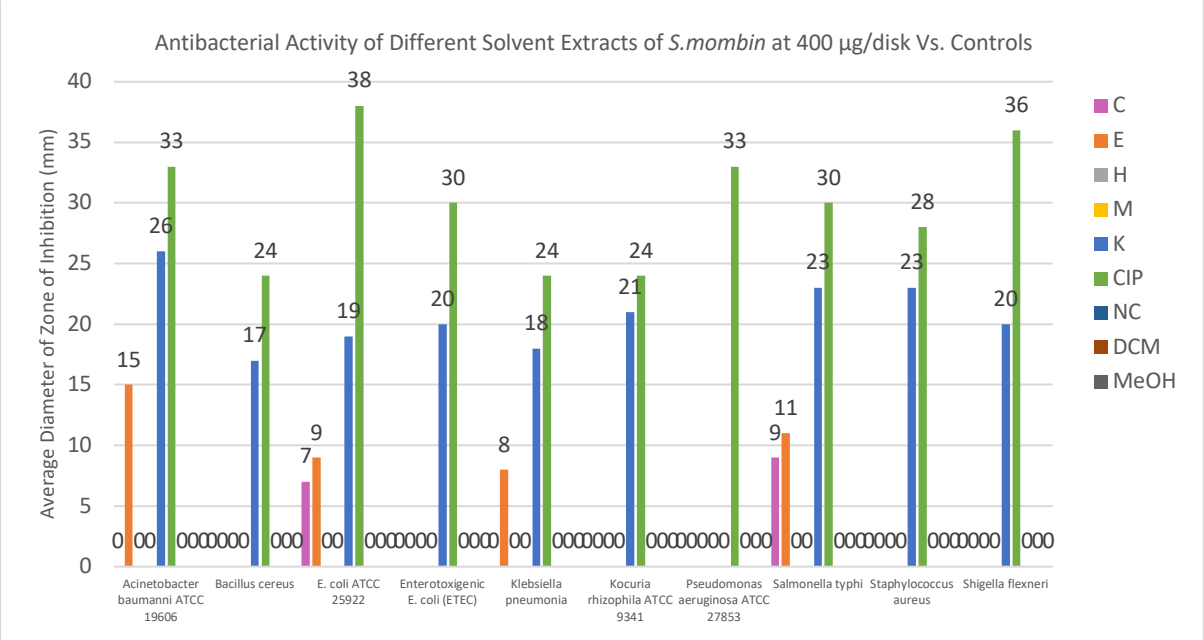
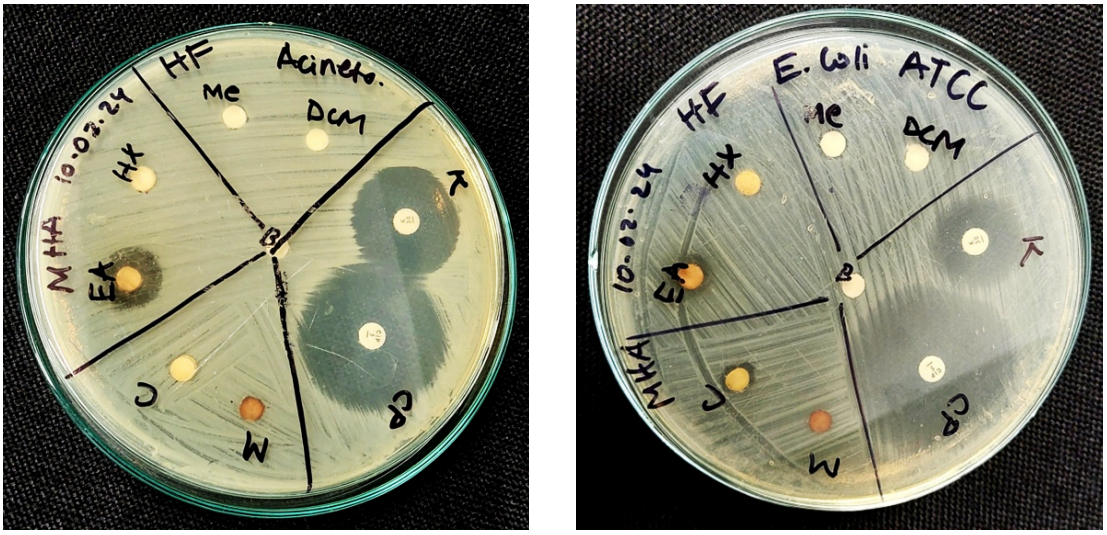


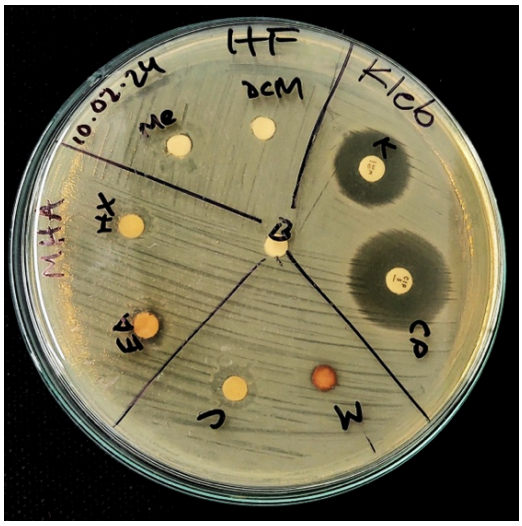
Figure 15: Graphical Representation of Antimicrobial Activity of Different Partition Extracts of *S. mombin* at 400 µg/disk Vs. Controls on the Test Organisms

At 400 µg/disk concentration, the ethyl acetate partition extract had an inhibitory effect on 4 test organisms: *Acinetobacter baumanni* ATCC 19606, *E. coli* ATCC 25922, *Klebsiella pneumonia* and *Salmonella typhi* with average zone diameters of 15 mm, 9 mm, 8 mm and 11 mm respectively (Figure 16). At this concentration, chloroform partition extract demonstrated an inhibitory effect on 2 test organisms: *E. coli* ATCC 25922 and *Salmonella typhi* with average zone diameters of 7 mm and 9 mm respectively (Figure 16).



(a)

(b)



(c)

(d)

Figure 16: Antibacterial Activity of Partition Extracts at 400 µg/disk Concentration on (a) *Acinetobacter baumanni* ATCC 19606 (b) *E. coli* ATCC 25922 (c) *Klebsiella pneumonia* (d) *Salmonella typhi*

*C=Chloroform extract; EA= Ethyl acetate extract, HX=Hexane extract, M=Methanol extract, Me=Methanol solvent, DCM=Dichloromethane Solvent, K=Kanamycin, CP=Ciprofloxacin

3.4 DPPH Free Radical Scavenging Activity

The absorbance readings for the 4 partition extracts at each concentration were measured and the percentage of DPPH inhibition along with IC50 values were calculated. The results are shown in Table 8-12. All four solvent partition extracts had significant antioxidant properties, even though they were less than the standard L-ascorbic acid. The DPPH scavenging activity for each of the extracts increased with an increase in extract concentration. At the highest concentration of 200 µg/ml, hexane partition extract had the highest percentage inhibition compared to the other 3 partition extracts. Additionally, it was found that the ethyl acetate partition extract had the lowest IC50 value among the rest.

Table 8: Effect of Chloroform Partition Extract of *S. mombin* on DPPH

Concentration (µg/ml)	Absorbance of Control	Absorbance of Sample	% Inhibition	IC50 ((µg/ml)
5	0.149	0.084	43.62	17.85
10	0.149	0.077	48.32	
25	0.149	0.069	53.69	
50	0.149	0.065	56.38	
100	0.149	0.058	61.07	
200	0.149	0.047	68.46	

*The absorbance readings were blank and corrected with methanol

The chloroform partition extract showed an increasing percentage inhibition of free radicals with an increase in concentrations, indicating antioxidant properties. 50% inhibition occurred at about 17.85 µg/ml and the highest inhibition occurred at 200 µg/ml.

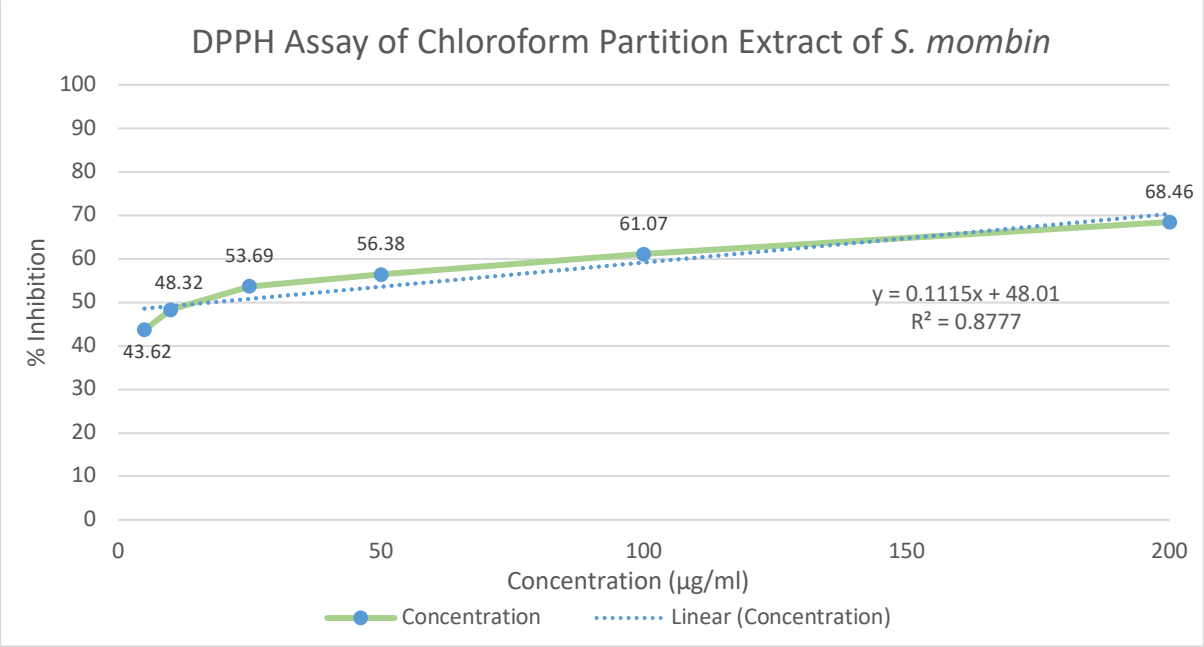


Figure 17: DPPH Scavenging Activity of Chloroform Partition Extract of *S. mombin*

Table 9: Effect of Ethyl acetate Partition Extract of *S. mombin* on DPPH

Concentration (µg/ml)	Absorbance of Control	Absorbance of Sample	% Inhibition	IC50 ((µg/ml)
5	0.149	0.079	46.98	4.74
10	0.149	0.074	50.34	
25	0.149	0.069	53.69	
50	0.149	0.060	59.73	
100	0.149	0.046	69.13	
200	0.149	0.029	80.54	

*The absorbance readings were blank and corrected with methanol

The ethyl partition extract showed an increasing percentage inhibition of free radicals with an increase in concentrations, indicating antioxidant properties. 50% inhibition occurred at about 4.74 µg/ml and the highest inhibition occurred at 200 µg/ml.

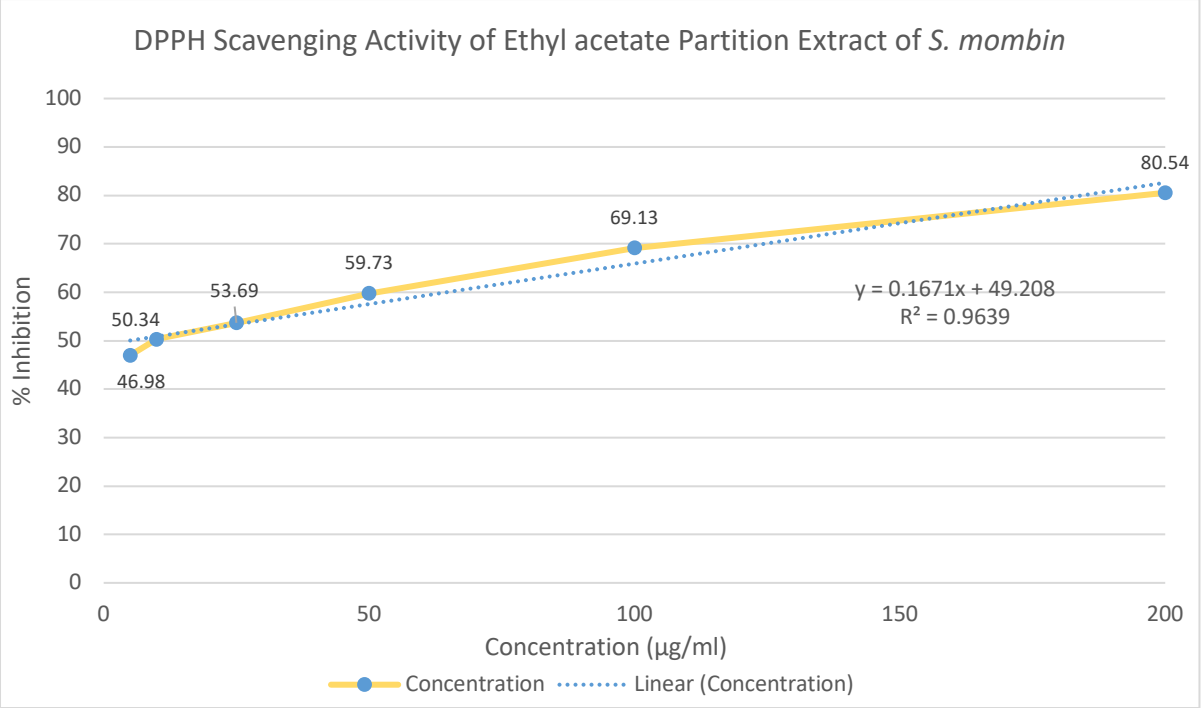


Figure 18: DPPH Scavenging Activity of Ethyl acetate Partition Extract of *S. mombin*

Table 10: Effect of Hexane Partition Extract of *S. mombin* on DPPH

Concentration (µg/ml)	Absorbance of Control	Absorbance of Sample	% Inhibition	IC50 ((µg/ml)
5	0.149	0.094	36.91	11.85
10	0.149	0.07	53.02	
25	0.149	0.066	55.70	
50	0.149	0.056	62.42	
100	0.149	0.043	71.14	
200	0.149	0.024	83.89	

*The absorbance readings were blank and corrected with methanol

The hexane partition extract showed an increasing percentage inhibition of free radicals with an increase in concentrations, indicating antioxidant properties. 50% inhibition occurred at about 11.85 µg/ml and the highest inhibition occurred at 200 µg/ml.

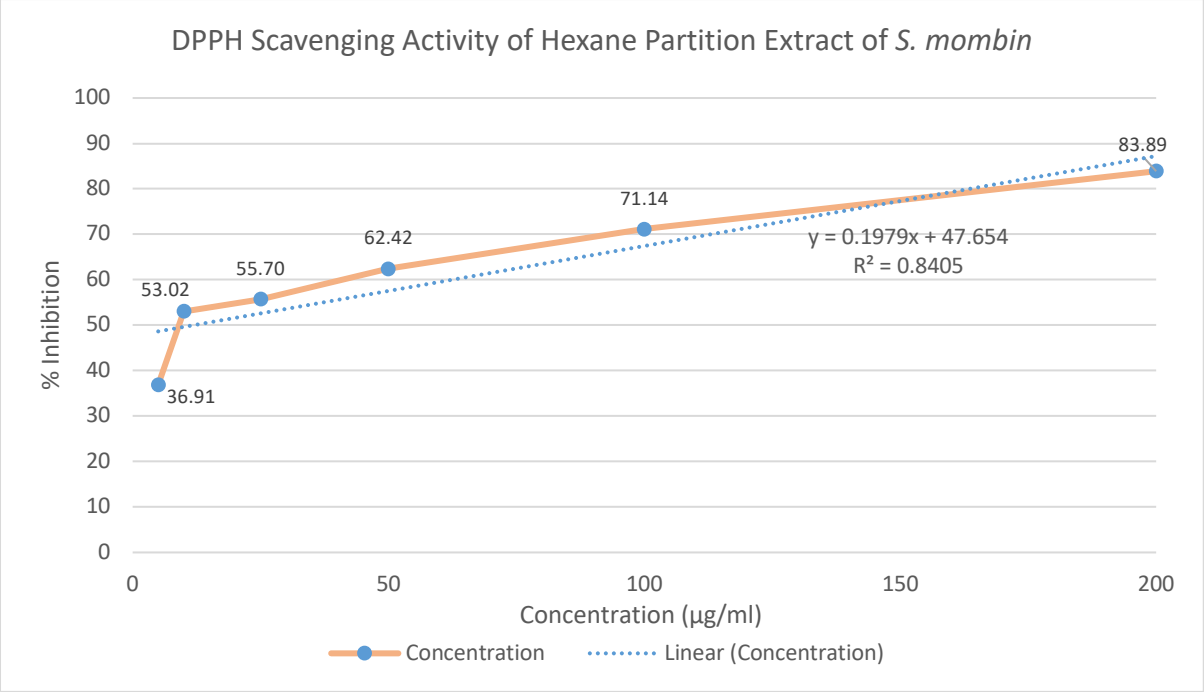


Figure 19: DPPH Scavenging Activity of Hexane Partition Extract of *S. mombin*

Table 11: Effect of Methanol Partition Extract of *S. mombin* on DPPH

Concentration (µg/ml)	Absorbance of Control	Absorbance of Sample	% Inhibition	IC50 ((µg/ml)
5	0.149	0.081	45.64	5.82
10	0.149	0.076	48.99	
25	0.149	0.068	54.36	
50	0.149	0.062	58.39	
100	0.149	0.057	61.74	
200	0.149	0.046	69.13	

*The absorbance readings were blank and corrected with methanol

The methanol partition extract showed an increasing percentage inhibition of free radicals with an increase in concentrations, indicating antioxidant properties. 50% inhibition occurred at about 5.82 µg/ml and the highest inhibition occurred at 200 µg/ml.

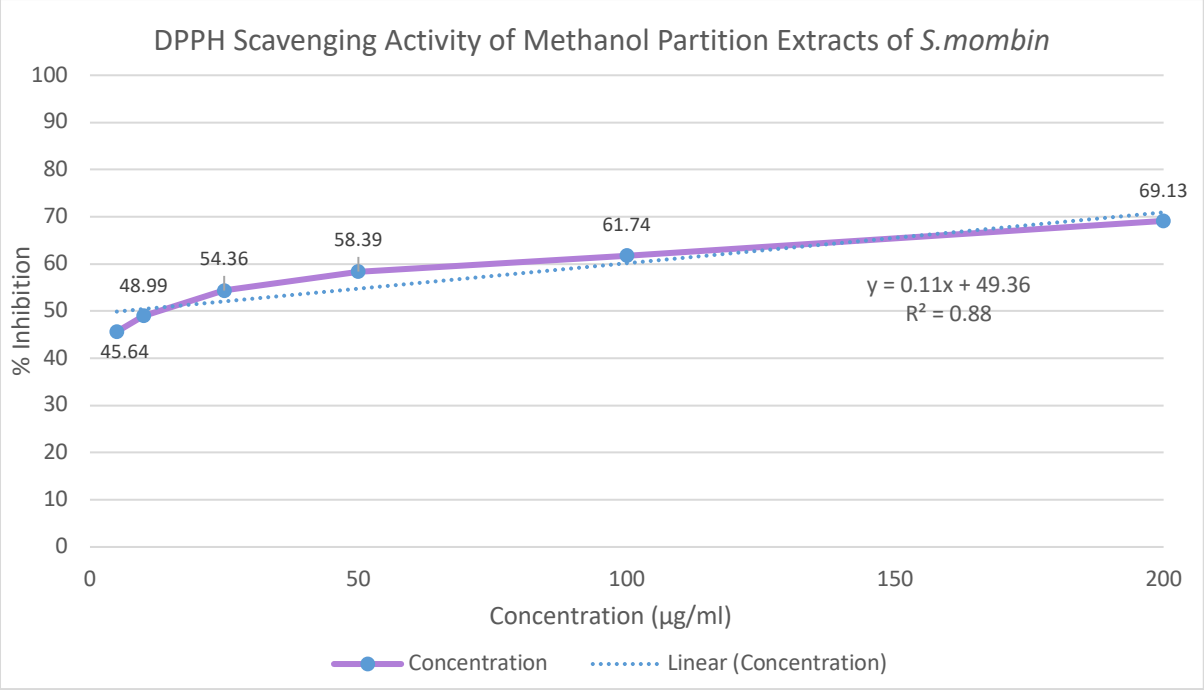


Figure 20: DPPH Scavenging Activity of Methanol Partition Extract of *S. mombin*

Table 12: Effect of Standard L-Ascorbic Acid on DPPH

Concentration (µg/ml)	Absorbance of Control	Absorbance of Sample	% Inhibition	IC50 ((µg/ml)
5	0.149	0.077	48.32	1.71
10	0.149	0.069	53.69	
25	0.149	0.067	55.03	
50	0.149	0.059	60.40	
100	0.149	0.042	71.81	
200	0.149	0.012	91.95	

*The absorbance readings were blank and corrected with methanol

L-ascorbic acid exhibited the greatest antioxidant properties. 50% inhibition occurred at about 1.71 µg/ml and the highest inhibition occurred at 200 µg/ml.

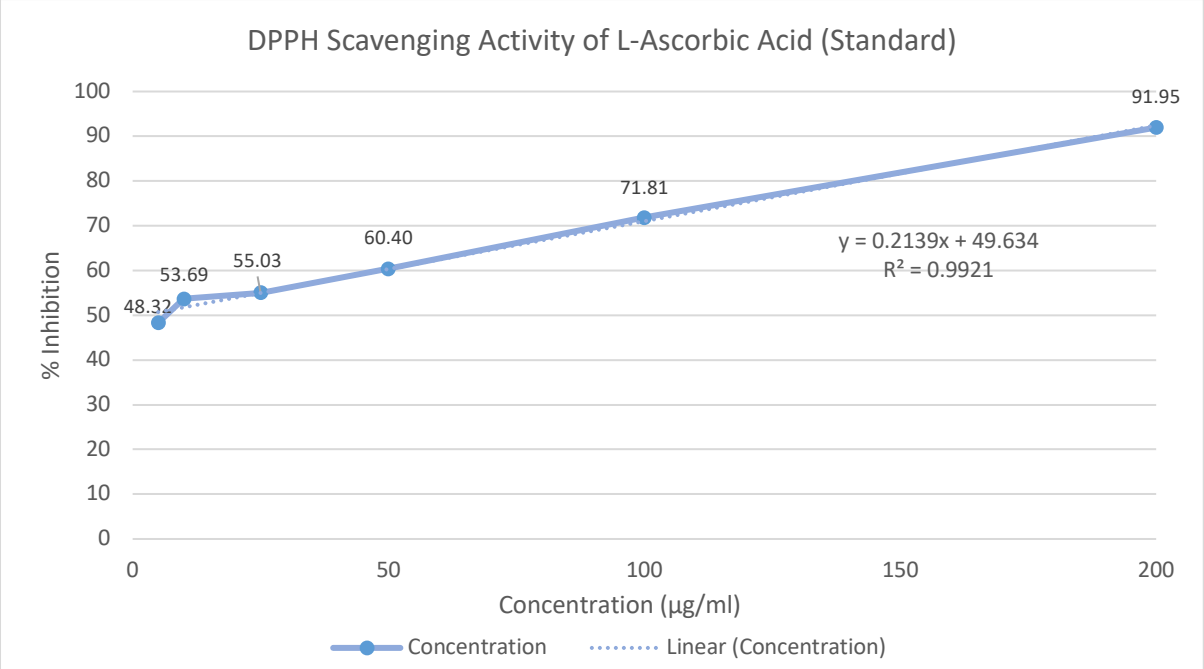


Figure 21: DPPH Scavenging Activity of Standard L-Ascorbic Acid

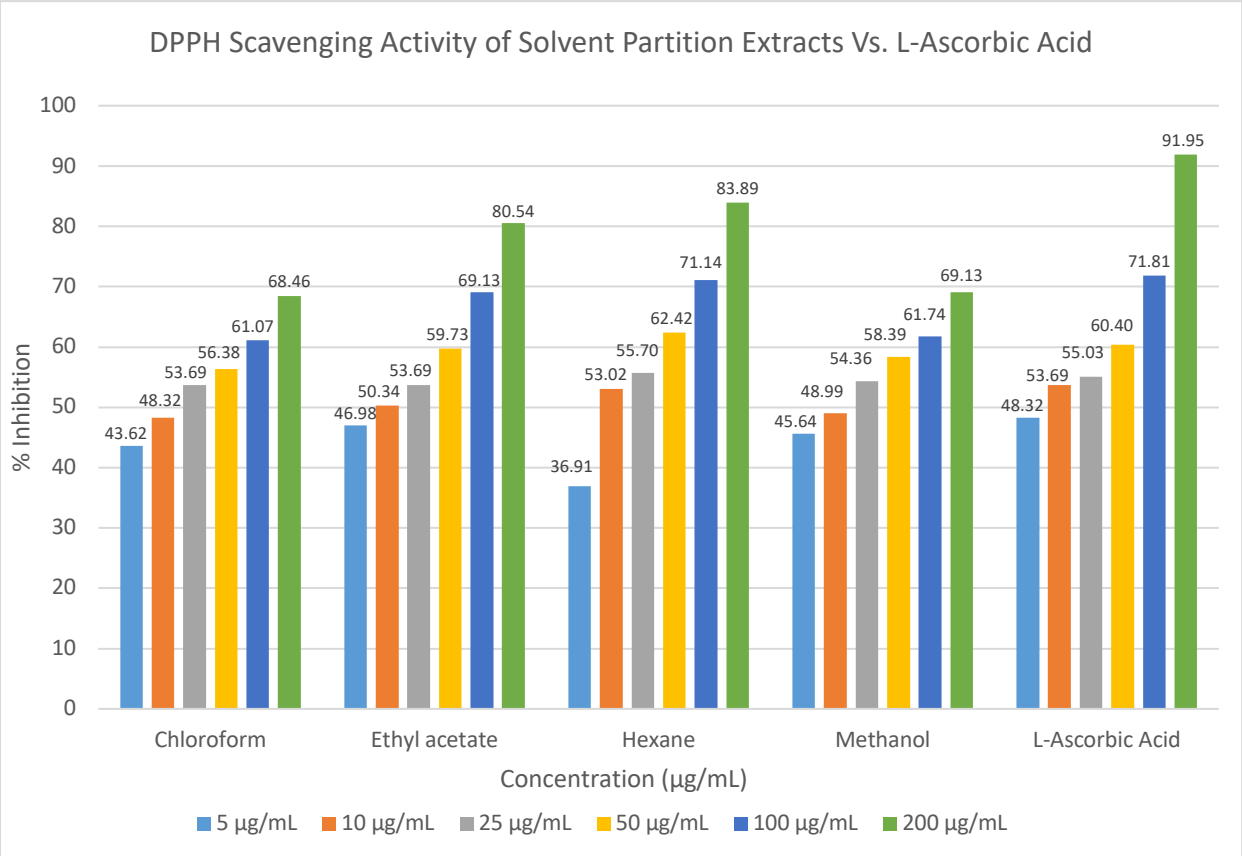


Figure 22: Graphical Representation of DPPH Scavenging Activity of Solvent Partition Extracts Vs. Standard L-Ascorbic Acid at Different Concentrations

Figure 22 illustrates that as concentration increases, the percentage inhibition also increases for all the extracts. At the highest concentration, hexane and ethyl acetate had the highest percentage inhibitions of 83.89% and 80.54% respectively. However, both were less than that of L-ascorbic acid, which had 91.95% inhibition at that concentration.

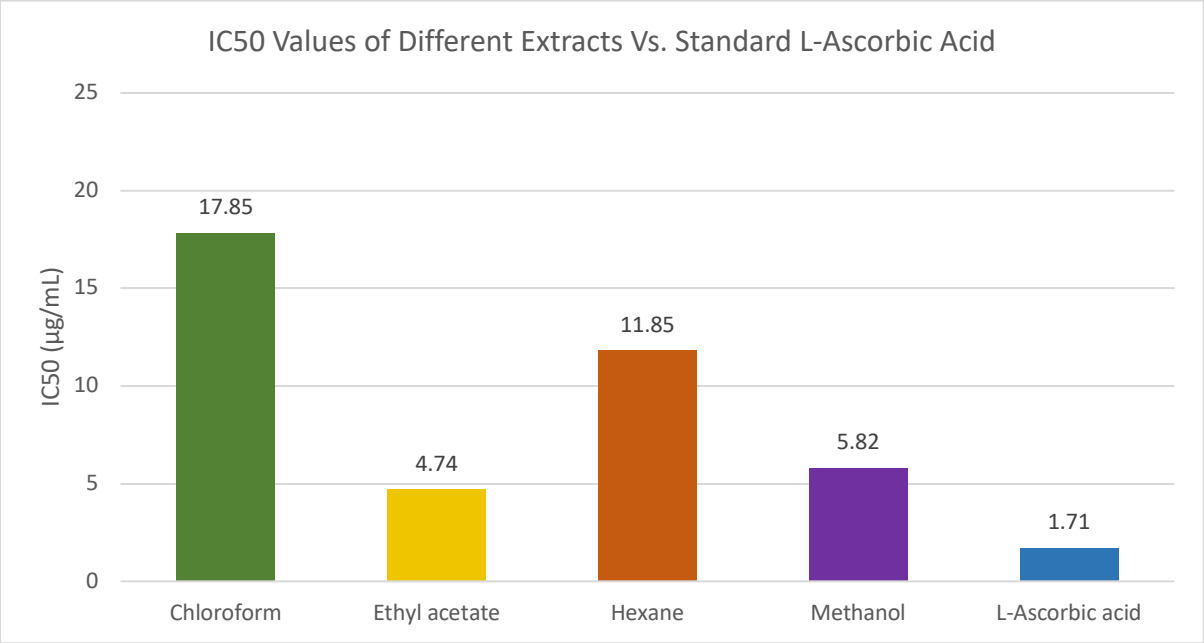


Figure 23: IC50 Values of Solvent Partition Extracts Vs. Standard L-Ascorbic Acid

Figure 23 shows that the ethyl acetate partition extract had the lowest IC50 value of 4.74 µg/mL among the extracts, followed by methanol and hexane partitions. The chloroform partition extract had the highest IC50 value, indicating weaker antioxidant properties compared to the other extracts. The standard L-ascorbic acid had the lowest value of 1.71 µg/ml, signifying the strongest antioxidant properties.

Chapter 4

Discussion

In this study, thin-layer chromatography (TLC), Kirby-Bauer disk diffusion, and DPPH free radical scavenging assay were employed to preliminarily assess the antibacterial and antioxidant properties of four solvents (chloroform, ethyl acetate, hexane, and methanol) partition extracts of *S. mombin* stem bark.

The results of TLC revealed that the chloroform and hexane partition extracts of *S. mombin* had the highest number of fluorescent bands upon visualization under 365 nm UV light and were most soluble in the used solvent system. The higher number of bands indicates the presence of a greater number of compounds. Furthermore, the different colors of the fluorescent bands indicate the presence of different phenolic substances in the plant extract (Harborne, 1984). These compounds may be flavonoids, flavones, flavonols (various colors depending on the structure), hydroxycinnamic acids such as ferulic acid, caffeic acid, p-coumaric acids (different shades of blue and green fluorescence), furanocoumarins (blue, violet, brown, green, or yellow fluorescence), chalcones, aurones, and anthraquinones (yellow fluorescence), chlorophyll (red fluorescence), beta-carotene (orange fluorescence), etc. in long-wavelength UV light (Harborne, 1984). In another study, UHPLC-HRMS (Ultra-high-performance liquid chromatography–high-resolution mass spectrometry) analysis of the bark extract of *S. mombin* (de Freitas et al., 2022) detected the presence of 98 compounds, which included hydroxycinnamic, flavonoids, flavanones, and flavonols, while qualitative analysis also found the presence of phenols and flavonoids, supporting the findings of this study.

The results of the Kirby-Bauer disk diffusion assay of the four partition extracts suggest that the solvent partition extracts have low to mild antibacterial properties at the tested concentrations compared to the positive controls - standard disks of Kanamycin and

Ciprofloxacin. All the tested bacterial strains except for *Pseudomonas aeruginosa* ATCC 27853 were sensitive to the standard Kanamycin; while they were all sensitive to standard Ciprofloxacin (Clinical & Laboratory Standards Institute, 2021). The ethyl acetate partition extract was the most effective antibacterial among all the other extracts. It showed the most efficacy against the Gram-negative bacteria *Acinetobacter baumanni* ATCC 19606. As the concentration of the extract was increased from 100 µg/disk to 400 µg/disk, the zone of inhibition diameter for this strain gradually increased from 7 mm to 15 mm. This extract at 400 µg/disk concentration also mildly inhibited *E. coli* ATCC 25922, *Klebsiella pneumonia*, and *Salmonella typhi*, all of which were Gram-negative bacteria. Furthermore, at this concentration, the chloroform extract was also slightly effective at inhibiting *E. coli* ATCC 25922 and *Salmonella typhi* but had a much milder effect than the ethyl acetate extract. These findings were in accordance with another study (de Freitas et al., 2022) which found the stem bark extracts of *S. mombin* to be only effective against Gram-negative bacteria. The hexane and methanol partition extracts did not show any antibacterial activity at the used concentrations on the test organisms.

DPPH free radical scavenging activity test determined that all four solvent partition extracts possess significant antioxidant properties, with ethyl acetate partition being the most effective antioxidant among them. At the highest concentration, hexane exhibited the highest scavenging activity of 83.89%, followed by ethyl acetate at 80.54%. Even though higher than the standard, the ethyl acetate extract had the lowest IC50 value of 4.74 µg/mL followed by methanol with a value of 5.82 µg/mL. These values indicate that they have very strong antioxidant properties (Jumina et al., 2018). Nonetheless, the IC50 values for the chloroform and hexane extracts were also below 50 µg/mL, thus it can be said that all the partition extracts of *S. mombin* are strong antioxidants (Jumina et al., 2018). The presence of various components including phenols, anthraquinones, flavonoids, etc. may be associated with this property of the extracts (Ayoka et

al., 2008). Furthermore, a study found that only the bark of *S. mombin* specifically has catechins (de Freitas et al., 2022), which may also contribute to its antioxidant properties. Previous studies have also revealed that the ethyl acetate (Omoboyowa et al., 2023) and methanolic (de Freitas et al., 2022) extracts of *S. mombin* stem bark have strong free radical scavenging potential.

Comparing the findings of these studies with those obtained in this research is challenging due to variations in experimental factors such as extraction techniques, choice of solvents, calculation methods, and the presence of genetic and environmental factors. The choice of extraction method and solvents can significantly influence the yield, composition, potency, and overall bioactivity of the plant extracts. Additionally, the limited concentration ranges, chosen test organisms, as well as the method of inoculation, may have been insufficient and could have led to biased results in terms of the extracts' antibacterial activity. Furthermore, the results are inconclusive in the sense that, in this study, the specific active compounds were not isolated. Thus, determining which specific compounds were responsible for the observed antibacterial and antioxidant effects is challenging. Without thorough chemical analysis, it is difficult to elucidate the mechanisms underlying the observed biological activities. Hence, further advanced analyses such as column chromatography and NMR (Nuclear Magnetic Resonance) can be done to evaluate the composition of the extracts.

Conclusion

The results of this study successfully revealed that all the solvent partition extracts of *S. mombin* bark possess strong antioxidant properties. Therefore, it can potentially be considered as a natural, inexpensive phytotherapeutic agent in treating health conditions caused by physiological oxidative stress, as well as be used for cosmeceutical applications. This study particularly found the ethyl acetate partition extract of *S. mombin* to have mild antibacterial activity against several Gram-negative bacteria. Thus, isolation of the bioactive compounds from this extract may lead to the discovery of novel antibacterial agents. Furthermore, the synergistic activity of these extracts in combination with available antibiotics needs to be studied to determine optimum antibacterial efficacy. It can be concluded that further analytical studies on the stem bark extracts of *Spondias mombin* could lead the creation of a promising pharmaceutical agent, thereby making a substantial contribution to in the progression of medicine and healthcare.

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