# Antimicrobial activities of *Terminalia chebula, Terminalia arjuna* and *Swertia chirayita* against multidrug resistant *Pseudmonas aeruginosa* and comparison of their activities with locally available antibiotics.


A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN BIOTECHNOLOGY

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***Dedicated to***
***My beloved mother and to my family.***

# DECLARATION

I, D. M. Ashiquzzaman hereby solemnly declare that the research work embodying the analysis and results reported in the following thesis entitled "Antimicrobial activities of *Terminalia chebula, Terminalia arjuna* and *Swertia chirayita* against multidrug resistant *Pseudmonas aeruginosa* and comparison of their activities with locally available antibiotics", has been carried out under the supervision of Dr. Mahboob Hossain, Professor, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University, Dhaka, Bangladesh. It is further declared that the research work presented here is original and no part of this thesis has been submitted to any other institution for any degree or diploma.

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I perceive this opportunity as a big milestone in my career development. I will strive to use the gained skills and knowledge in the best way possible, and I will continue to work on their improvement, in order to attain my desired career objectives.

Sincerely,

D. M. Ashiquzzaman

## Abstract

The continuous emergence of resistant bacteria is endangering the efficacy of antibiotics across the globe. The antibiotic resistance crisis has been occurring due to the overuse and misuse of these medications. However, the lack of new drug development by the pharmaceuticals making antimicrobial resistance on of the biggest upcoming calamities. Therefore, scientists are selecting plants as a source of medication as plant extracts reportedly have shown potential antimicrobial activities against the microbes and have the potential to serve as the alternative sources to antibiotics. Traditional medicinal plants such as Haritaki (*Terminalia chebula),* Arjun *(Terminalia arjuna)* and Chirata *(Swertia chirayita)* have been used for long to treat various diseases. Thus, the ethanolic, ethyl acetate and chloroformic extracts of these plants were tested against a *Pseudomonas aeruginosa* strain which showed resistance to thirty antibiotics available in the market and only sensitive to polymyxin B. The extraction method was done via Soxhlet apparatus and Rotary evaporator. The potential antimicrobial activities of crude extract against resistant *Psedomonas aeruginosa* was obtained via agar well diffusion method. Only ethyl acetate extract of Haritaki (*Terminalia chebula),* ethyl acetate extract of Arjun (*Terminalia arjuna),* ethanolic extract of Chirata *(Swertia chirayita)* showed significant activity against resistant *Psedomonas aeruginosa.* The highest Zone of was found in ethyl acetate extract of Haritaki (*Terminalia chebula)* on resistant *Psedomonas aeruginosa* found (16 millimeter in diameter). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of ethyl acetate extract of Haritaki (*Terminalia chebula)* was found 25 milligram per milliliter (MIC) and 30 milligram per milliliter (MBC) respectively. The results thus represents a possibility of developing antimicrobial drugs from these medicinal plants.

# TABLE OF CONTEXTS

| List of abbreviation |
| --- |
| ALTA- anti-live trypomastigote antibodies |
| $BaCl_2.H_2O$- Barium chloride dehydrate |
| BHI- Brain Heart Infusion |
| CDC- Centre for Disease Control |
| CFU- Colony Forming Units |
| CLSI- Clinical and Laboratory Standards Institute |
| DDPH- 2,2-diphenyl-1-picryl-hydrazyl-hydrate |
| DMSO- Di Methyl Sulphoxide |
| et al - And others |
| Fig.- Figure |
| g- gram |
| $H_2SO_4$- Sulphuric acid |
| LPO- Lipid peroxidase |
| LV- leff ventricular |
| MBC- minimum bactericidal concentration |
| MHA- Muller Hinton Agar |
| MIC- minimum inhibitory concentration |
| m- meter |
| mg- milligram |
| mg/ml- milligram per ml |
| ml- milliliter |
| mm- millimeter |
| MPO- myelo peroxidase |
| PMN- peripheral mononuclear |
| RND- Resistance-Nodulation-Division |
| WHO- World Health Organization |

# CHAPTER 1: Introduction

## 1.1 Background

Antibiotics also known as antimicrobials are the type of drugs that are used in the treatment of bacterial infection. The antibiotics either may kill the bacteria or inhibit the growth of them. The first antibiotic Penicillin, was discovered by Alexander Fleming in 1928 and more than 100 compounds have been found since, but no new class has been found since 1987. The lack of new drugs coupled with over-prescribing has led to bacteria becoming increasingly resistant to modern medicines. The death by antimicrobial resistance will increase to ten million by 2050 according to the global report on antibiotic resistance by WHO (2014).

In Bangladesh, it is estimated that around 86 percent of antibiotics are consumed without the prescription (Morgan et al., 2011). Scientist are believing that, antimicrobial resistance to antibiotics will present a greater danger to humankind than cancer by the middle of the century unless world leaders agree international action to tackle the threat, The Guardian (2016).

Because of the rising incidence of drug resistance among infectious bacteria, new antibacterial are constantly being searched (Raghunath, 2008). Nowadays, researchers are seeking for some novel antimicrobial molecules which have a broad spectrum of activity against both Gram-negative and Gram-positive bacteria without having many or any side effects (Chandra et al., 2017).

From the ancient period of time plants have been used and considered as a biggest source of medicinal compounds. Thus in recent years, the research on plant-based drugs has increased tremendously and there is some hope seen in certain medicinal plants which can be used for the treatment of incurable diseases as they have much less or almost no side effect at all.

Bangladesh has very rich Bio-diversified collection of faunas and is famous for the wide storage of medicinal plants. More than 500 plants species have been reported to be available in Bangladesh having medicinal values (Yusuf et al., 1994).

Haritaki (*Terminalia chebula),* Arjun *(Terminalia arjuna)* and Chirata *(Swertia chirayita)* these three common medicinal plants are most frequently used to treat diseases and it is found that the crude extracts of these three medicinal plants Haritaki (*Terminalia chebula),* Arjun *(Terminalia arjuna)* and Chirata *(Swertia chirayita)* have the potential antimicrobial activity against a strain of multidrug resistance *Pseudomonas aeruginosa*.

## 1.2 Aim of the project

The project was established by focusing the following objectives:

- Isolating the crude extracts from Haritaki (*Terminalia chebula),* Arjun *(Terminalia arjuna)* and Chirata *(Swertia chirayita).*
- Discovering any potential antimicrobial activity of Haritaki (*Terminalia chebula),* Arjun *(Terminalia arjuna)* and Chirata *(Swertia chirayita).*
- Comparison of the antimicrobial activity of these plant extracts and the antibiotics that are available in the local market and their sensitivity against the multidrug resistance *Pseudomonas aeruginosa.*
- Establishing minimum inhibitory concentration (MIC) values of the plant extracts and sensitive antibiotics.
- Establishing minimum bactericidal concentration (MBC) values of the plant extracts and sensitive antibiotics.

## 1.3 *Terminalia chebula:*

**Fig. 1: Fruits of *T. chebula* (left)** **Fig. 2: Dried fruits and powder of *T. chebula* (right)**

**Image source: Google "*Terminalia chebula*"**

### 1.3.1 Taxonomy:

Current name: *Terminalia chebula,*

**Botanical description**

| | |
|---|---|
| Kingdom: | Plantae |
| Division: | Magnoliophyta |
| Class: | Magnoliopsida |
| Order: | Myrtales |
| Family: | Combretaceae |
| Genus: | *Terminalia* |
| Species: | *chebula* |

### 1.3.2 Description of *Terminalia chebula*:

*Terminalia chebula* (*T. chebula*) is a flowering evergreen tree of the family Combretaceae. It has several common names such as black myrobalan, ink tree, or chebulic myrobalan (English), haritaki (Sanskrit and Bengali), harad (Hindi), harada (Marathi and Gujrati) Karkchettu (Telgu) and Kadukkaya (Tamil). In Tibet, *T. chebula* is called as the "King of Medicine" (Aneja, 2009).

*T. chebula* is a medium to large highly branched deciduous tree with a height up to 30 m and girth 1-1.5 m. Leaves are 10-30 cm long elliptical with an acute tip and cordate base. The vasculature of the leaves consists of 6-8 pairs of veins. Flowers are short stalked, monoecious, dull white to yellow with a strong unpleasant odour and are found in simple terminal spikes or short panicles (Gupta, 2012). Fruits are 3-6 cm long and 1.3-1.5 cm broad yellowish-green ovoid drupes containing one oval seed. *T. chebula* is capable of growing in a variety of soils, clay as well as shady. The trees may grow at places up to a height of about 2000 m from the sea level, and in areas with an annual rainfall 100-150 cm and temperature 0-17° C. *T. chebula*, though, is a native of Asia, but also found in Nepal, Sri Lanka, Myanmar, Bangladesh, Egypt, Iran and Turkey and also in Pakistan and Yunnan, Tibet, Guangdong, Guangxi province of China. In India, it grows in deciduous forests of Himachal Pradesh, Tamil Nadu, Kerala, Karnataka, Uttar Pradesh, Andhra Pradesh and West Bengal (Pullaiah, 2004)

### 1.3.3 Phytochemical properties:

*T. chebula*, though, contains several phytoconstituents like tannins, flavonoids, sterols, amino acids, fructose, resin, fixed oils *etc*., however, it is fairly rich in different tannins (approximately 32% tannin content). Further, tannin content of *T. chebula* largely depends on its geographic location (Kumar, 2006). The chief components of tannin are chebulic acid, chebulinic acid, chebulagic acid, gallic acid, corilagin and ellagic acid.

Tannins of *T. chebula* are of pyrogallol (hydrolysable) type. There are about 14 hydrolysable tannins (gallic acid, chebulic acid, punicalagin, chebulanin, corilagin, neochebulinic acid, ellagic acid, chebulegic acid, chebulinic acid, 1,2,3,4,6-penta-O-galloyl-b-D-glucose, casuarinin, 3,4,6-tri-O-galloyl-D-glucose and terchebulin) which have isolated from fruits of *T. chebula* (Juang et al., 2004). Phytochemicals like anthraquinones, ethaedioic acid, sennoside, 4,2,4 chebulyl-d-glucopyranose, terpinenes and terpinenols have also been reported to be present (Pullaiah., 2004 & Srivastava, 2010). Triterpenoids and their glycosides have been isolated from stem bark of *T.*

*chebula* (Kundu et al., 1993). Recent studies showed that *T. chebula* contains more phenolics than any other plant (Saleem et al., 2002).

## 1.3.4 Pharmacological properties:

In Tibet, *T. chebula* is called as the "King of Medicine" (Aneja, 2009). It was named "Haritaki" as it carries away all the diseases. *T. chebula* has a very wide range of pharmalogical properties. They are elaborately described below.

### 1.3.4.1 Antibacterial activity:

Two antibacterial compounds, gallic acid and ethyl ester against methicillin-resistant *Staphylococcus,* have been isolated from ethyl alcohol extract of fruits of *T. chebula* (Sato et al., 1997). Various extracts of *T. chebula* exhibit antibacterial activity against a number of bacterial species (Ahmad et al., 1998). *T. chebula* is well effective against *Helicobacter pyroli*, a bacterium responsible for gastritis, ulcer and stomach cancers. The ether, alcoholic and aqueous extracts of *T. chebula* were tested against *Helicobactor pylori*, but aqueous extract of the plant, at a concentration of 1-2.5 mg/ml, inhibited urease activity of *H. pylori* (Malekzadeh et al., 2001). Tannins extracted from immature fruits of *T. chebula* inhibited *Staphylococcus aureus* and *Klebsiella Pneumonia in vitro* and promoted cutaneous wound healing in rats due to a powerful anti-bacterial and angiogenic activity of the extract (Li et al., 2011). Several biologically active components were isolated from butanol fraction of fruit extract of *T. chebula* and tested against six intestinal bacteria. Ethanedioic acid showed strong and moderate inhibitory activity against *Clostridium perfringens* and *Escherichia coli,* respectively, with no adverse effects on the growth of the four tested lactic acid-producing bacteria. Ellagic acid exerted a potent inhibitory effect against *C. perfringens* and *E. coli,* but little or no inhibition was observed for behenic acid, β-caryophyllene, eugenol, isoquercitrin, oleic acid, α-phellandrene, β-sitosterol, stearic acid, α-terpinene, terpinen-4-ol, terpinolene, or triacontanoic acid (Kim et al.,2006). The ethanolic extract of *T. chebula* fruit was found effective against both gram-positive and gram-negative bacteria such as *Salmonella typhi* SSFP 4S, *Staphylococcus epidermidis* MTCC 3615, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* MTCC 441 and *Pseudomonas aeruginosa* ATCC 27853 suggesting its broad spectrum antimicrobial activity (Kannan et al., 2009).

#### 1.3.4.2 Antifungal activity:

Aqueous extract of *T. chebula* has been reported to show antifungal activity against a number of dermatophytes (*e.g. Epidermophyton, Floccosum, Microsporum gypseum* and *Tricophyton rubrum*) and yeasts (*e.g. Candida albicans*) (Dutta et al., 1998 & Mehmood et al., 1999 & Vonshak et al., 2003). Aqueous, alcoholic and ethyl acetate extracts of leaves of *T. chebula* were also tested against five pathogenic fungi (*Aspergillus flavus*, *A. niger*, *Alternaria brassicicola*, *A. alternata* and *Helminthosporium tetramera*) using paper disc method and were found effective compared to that of the reference standard Carbendazim (Shinde et al., 2011).

#### 1.3.4.3 Antiplasmodial activity:

The water extract of *T. chebula* showed antiplasmodial activity *in vitro* by its ability to inhibit the uptake of [3H] hypoxanthine into the *Plasmodium falciparum* K1 multidrug-resistant strain and *in vivo* (Pinmai et al., 2010). Acetone seed extract of *T. chebula* was also found to have good antiplasmodial activity in a study (Bagavan et al., 2011).

#### 1.3.4.4 Molluscicidal activity:

The molluscicidal activity of ethanolic extract of *T. chebula* fruit powder was studied against the vector snail *Lymnaea acuminata* and was found time and concentration dependent. Column, thin layer and high performance liquid chromatography analyses demonstrated that the active molluscicidal component in *T. chebula* was tannic acid. Hence, *T. chebula* could be a potent source of molluscicides against the snail *L. acuminata* (Upadhyay & Singh, 2011).

#### 1.3.4.5 Anthemintic activity:

The ovicidal and larvicidal activities of ethyl acetate, acetone, and methanol extracts of dried leaves and seeds of *T. chebula* were tested *in vitro* on *Haemonchus contortus* based on egg hatch and larval development assays at 50, 25, 12.5, 6.25 and 3.13mg/ml. The extracts of leaves and seeds of *T. chebula* showed complete inhibition at 50mg/ml (Kamaraj & Rahuman, 2011).

#### 1.3.4.6 Antiviral activity:

The extract of fruits of *T. chebula* showed inhibitory effects on human immunodeficiency virus-1 reverse transcriptase (Mekkaway et al., 1995). Hot water extract of *T. chebula* showed anti-herpes

simplex virus (HSV) activity *in vivo* and anti-cytomegalovirus (CMV) activity both *in vitro* and *in vivo* in a study (Yukawa et al., 1996). A study proved that *T. chebula* fruits contain four human HIV-type 1 integrase inhibitors such as gallic acid and three galloyl glucoses, and suggested that galloyl moiety had a major role for inhibition of the 3′-processing of HIV-1 integrase by these compounds (Ahn et al., 2002). *T. chebula* can also be used in sexually transmitted diseases and AIDS (Vermani & Garg, 2002). Recently, acetone extract of *T. chebula* has emerged as a new alternative to treat pandemic swine influenza A infection due to its low cost, easy preparation and potential effect (Ma et al., 2010). Herpes simplex virus 1 (HSV-1) is the cause of lifelong latent infection of sensory neurons. Two hydrolyzable tannins, chebulagic acid and punicalagin, isolated from the dried fruits of *T. chebula* inhibited HSV-1 entry at non-cytotoxic doses in A549 human lung cells (Lin et al., 2011).

**1.3.4.7 Antimutagenic and anticarcinogenic activities:**

In all cell lines studied, the extract decreased cell viability, inhibited cell proliferation, and induced cell death in a dose dependent manner (Saleem et al., 2002). Acetone extract of *T. chebula* has been reported to contain phytochemicals with promising antimutagenic and anticarcinogenic properties (Arora et al., 2003). One of the fractionated compounds from ethanolic fruit extract of *T. chebula*, chebulagic acid, showed potent dual inhibition against COX and 5-LOX. It also showed anti-proliferative activity against HCT-15, COLO-205, MDA-MB-231, DU-145 and K562 cell lines (Reddy et al., 2009).

**1.3.4.8 Antioxidant activity:**

*T. chebula* is an excellent anti-oxidant. In a study, 6 extracts and 4 pure compounds of *T. chebula* exhibited anti-lipid peroxidation, anti-superoxide radical formation and free radical scavenging activities at different magnitudes of potency (Chen et al., 2003). The aqueous extract of *T. chebula* protected the antioxidant enzymes from reactive oxygen species (ROS) produced by gamma radiation in the rat liver microsomes and mitochondria (Naik et al., 2004). The ethanolic extract of the fruits of *T. chebula* decreased the level of lipid peroxidase in albino rats (Suchalatha et al., 2005). In histopathologic examination of the rat livers showed that *T. chebula* extract reduced the incidence of liver lesions including hepatocyte swelling and neutrophilic infiltration, and repaired necrosis induced by *t*-BHP (Lee et al., 2005). Further, a hepatoprotective compound, isolated from

the ethanolic extract of the fruits of *T. chebula*, was identified as a mixture of chebulic acid and its minor isomer, neochebulic acid that also reduced the tert-butyl hydroperoxide (t-BHP)-induced cell cytotoxicity in isolated rat hepatocyte experiment (Lee et al., 2006). An aglycone isolated from the fruits of *T. chebula*, triethylchebulate, significantly inhibited FeSO4 /Cys-induced microsomes lipid peroxidation and protected both H2O2- -induced RBCs hemolysis and RBCs auto-hemolysis in a dose-dependent manner. Furthermore, triethylchebulate demonstrated potent DPPH free-radical scavenging ability and moderately suppressed azide-induced mitochondria ROS formation, (Chen et al., 2011).

**1.3.4.9 Antianaphylactic and adaptogenic activities:**

*T. chebula* along with several other medicinal plants helps to resist against a number of stressors in different ways (Rege et al., 1999). *T. chebula,* when given following anaphylactic shock, reduces the serum histamine level showing a strong antianaphylactic activity (Shin et al, 2001).

**1.3.4.10 Antinociceptive activity:**

The petroleum ether, chloroform, ethanol and water extracts of *T. chebula* fruits were evaluated for their analgesic activity using the tail immersion model in mice. The results suggested that *T. chebula* could be a potential candidate for bioactivity-guided isolation of natural analgesic agents in the management of chronic pain (Kaur & Jaggi, 2010).

**1.3.4.11 Antiulcerogenic activity:**

The *T. chebula* extract increased mucus production in aspirin and ethanol-induced ulcer models and showed antisecretory activity in pylorus ligated model leading to a reduction in the gastric juice volume, free acidity, total acidity, and significantly increased gastric pH (Sharma et al., 2011).

**1.3.4.12 Anti-arthritic activity:**

The hydroalcoholic extract of *T. chebula* produced a significant inhibition of joint swelling as compared to control in both formaldehyde-induced and CFA-induced arthritis. *T. chebula* treatment also reduced serum TNF-α level and synovial expression of TNF-R1, IL-6 and IL-1β. The authors believed that *T. chebula* could be used as a disease-modifying agent in treatment of rheumatoid arthritis (Nair et al., 2010).

#### 1.3.4.13 Wounds healing activity:

Topical administration of alcoholic extract of the leaves of *T. chebula* caused much faster healing (Sugun et al., 2002). In another study, healing activity of ethanol extract of *T. chebula* against the indomethacin-induced stomach ulceration was reported (Bhattacharya et al., 2007). In alloxan induced diabetic rats, the hydroalcoholic extract of *T. chebula* fruit exhibited 82% reduction in the wound area due to faster epithelialization compared to controls (Singh & Sharma, 2009). The wound healing activity of ethanolic extract of fruits of *T. chebula* in the form of an ointment with two concentrations (5% and 10% w/w ointment of bark extract in simple ointment base) showed significant response in excision and incision models in albino rats compared to controls (Choudhary, 2011).

#### 1.3.4.14 Cytoprotective and antiaging activities:

Gallic acid and chebulagic acid, isolated from fruit extract of *T. chebula*, blocked cytotoxic T lymphocyte (CTL)-mediated cytotoxicity. Granule exocytosis in response to anti-CD3 stimulation was also blocked by the above phytochemicals at the equivalent concentrations (Hamada et al., 1997). The ethanol extract of the fruits of *T. chebula* inhibited oxidative stress and the age-dependent shortening of the telomeric DNA length (Na et al., 2004). The extracts of *T. chebula* gall were tested for antioxidative and tyrosinase inhibition activities as well as for proliferative and MMP-2 inhibition activities on early aging human skin fibroblasts to evaluate *in vitro* anti-aging activity (Manosroi et al., 2010).

#### 1.3.4.15 Cardioprotective activity:

Cardioprotective effect of ethanolic extract of *T. chebula* fruits (500 mg/kg body weight) was investigated in isoproterenol induced myocardial damage in rats. (Suchalatha & Devi, 2004; Shuchalatha & Devi, 2005; Suchalatha et al., 2007).

#### 1.3.4.16 Hepatoprotective activity:

The 95% ethanolic extract of *T. chebula* fruit showed hepatoprotective activity against anti-tuberculosis (anti-TB) drug-induced toxicity which could be attributed to its prominent anti-oxidative and membrane stabilizing activities (Tasduq et al., 2006).

#### 1.3.4.17 Chemopreventive activity:

In an investigation, *T. chebula* extract treatment prevented nickel chloride induced renal oxidative stress, toxicity and cell proliferation response in male Wistar rats (Prasad et al., 2006).

#### 1.3.4.18 Hypolipidemic and hypocholesterolemic activities:

*T. chebula* extract administration showed hypolipidaemic activity against experimentally induced atherosclerosis (Thakur et al., 1988) and hypocholesterolemic activity against cholesterol-induced hypercholesterolemia and atherosclerosis (Shaila et al., 1998).

## 1.4 *Terminalia arjuna:*

**Fig.3: *Terminalia arjuna* plant, fruit, bark and its powder**

**Image source: Google "*Terminalia arjuna*"**

### 1.4.1 Taxonomy:

Current name: *Terminalia arjuna,*

**Botanical description**

| | |
|---|---|
| Kingdom: | Plantae |
| Division: | Magnoliophyta |
| Class: | Magnoliopsida |
| Order: | Myrtales |
| Family: | Combretaceae |
| Genus: | *Terminalia* |
| Species: | *arjuna* |

### 1.4.2 Description of *Terminalia arjuna*:

*T. arjuna* (*Terminalia arjuna*) is an ayurvedic plant which has important medicinal value. It is commonly known as Arjuna, Indradru, Partha and Veeravriksha (Sharma and Yelne, 2005) which belongs to Combretaceae family and it is comprised of nearly 200 species distributed around the world. About 24 species of Terminalia have been reported from various parts of India, some selected species are *T. arjuna, Terminalia bellirica, Terminalia bialata, Terminalia catappa, Terminalia elliptica, Terminalia porphyrocarpa, Terminalia mantaly* etc. In India, *T. arjuna* is about 60-80 feet in height, buttressed trunk and horizontally spreading crown and drooping branches distributed in India, Bangladesh, Burma, Mauritius and Sri Lanka (Kapoor et al., 2014; Chopra et al 1958 & Nadkarni 1976).

*T. arjuna* is distributed throughout sub Indo-Himalayan tracts of Uttar Pradesh, Punjab, Bangladesh, South Bihar, Orissa, West Bengal and Madhya Pradesh mainly along riverside, rivulets and ponds. It is known by its various vernacular names, the most commonly used ones are Arjuna (Common Name), Arjun (Hindi, Bengali & English), Marudhu (Tamil and Malayalam), Tella Maddi (Telugu), Sadaru (Marathi), Sadado (Gujarati), Neer matti (Kannada) and some traditional formulations prescribe in the name of Arjunarishta and Arjunaghrita. Leaves of *T. arjuna* are simple, often crenulations, borne subopposite, shortly acute or obtuse at the apex, coriaceous and oblong or elliptic and their upper face is pale or dark green and the lower face is pale brown. The tree grows white sessile bisexual flowers in short auxiliary spikes or in a terminal panicle arrangement. Fruits of *T. arjuna* are drupe, ovoid, fibrous-woody and smooth-skinned with five hard wings or angles which are oblique and curved upwards. Stem bark is simple, smooth and pinkish-gray in color in external view. An internal view, the bark is soft and reddish color (Ali, 1994).

### 1.4.3 Phytochemical properties:

The analysis of the plant extracts (*T. arjuna*) revealed the presence of different phytochemicals including proteins, carbohydrates, phenols, tannins, flavonoids, saponins, glycosides, steroids, terpenoids and alkaloids. Several studies have described the antioxidant properties of different parts of various medicinal plants which are rich in phenolic compounds (Brown & Rice, 1998) & (Krings & Berger, 2001). *T. arjuna* is a widespread medicinal plant used in the pharmacological system of medicine to care for various degenerative diseases (Nema et al., 2012). Another

phytochemical analysis revealed a large amount of phytosterol, lactones, flavonoids, phenolic compounds and tannins and glycosides present in methanol extract of *T. arjuna* bark (Mandal et al., 2013).

## 1.4.4 Pharmacological properties:

*T. arjuna* is a tree having an widespread medicinal potential in most of the diseases particularly cardiovascular disorders. It is also exhibited antimicrobial, gastroprotective, antioxidant, anticarcinogenic and antimutagenic, hypocholesterolaemic activities (Amalraj & Gopi, 2016).

### 1.4.4.1 Cardioprotective activity:

It was found that triterpenoids are essentially responsible for cardiovascular properties. Alcoholic and aqueous bark extracts of *T. arjuna*, arjunic acid, arjunetin and arjungenin were evaluated for their potential to inhibit CYP3A4, CYP2D6 and CYP2C9 enzymes in human liver microsomes (Varghese et al., 2015). *T. arjuna* bark has protective effects against Dox-induced cardiotoxicity and may have potential as a cardioprotective agent (Singh et al., 2008).

The protective effect of *T. arjuna* bark extract on left ventricular (LV) and baroreflex function in chronic heart failure and to elucidate the possible mechanistic clues in its cardioprotective action. After 15 days of isoproterenol administration, rats exhibited cardiac dysfunction, hypertrophy, and LV remodeling along with reduced baroreflex sensitivity. Thr prophylactic and therapeutic treatment with *T. arjuna* improved cardiac functions and baroreflex sensitivity. It has also attenuated hypertrophy and fibrosis of the LV. *T. arjuna* exerts beneficial effect on LV functions, myocardial remodeling, and autonomic control in chronic heart failure possibly through maintaining endogenous antioxidant enzyme activities, inhibiting lipid peroxidation and cytokine levels (Parveen et al., 2012). *In vivo* ischemic-reperfusion injury induced oxidative stress, tissue injury of heart and hemodynamic effects were prevented in the *T. arjuna* treated rabbit hearts (Kumar et al., 2009).

Physicochemical property and inotropic effect of the aqueous extract of *T. arjuna* bark (TAAqE) were investigated on adult rat ventricular myocytes in comparison with extracts prepared sequentially with organic extracts. They found that TAAqE decoctions exerted positive inotropy, accelerated myocyte relaxation and increased caffeine-induced contraction concentration dependently. TAAqE-induced cardiotonic action via enhancing SR function, a unique action

minimizing the occurrence of arrhythmias, makes TAAqE a promising and relatively safe cardiotonic beneficial to the healthy heart and the treatment for chronic heart disease (Oberoi et al., 2011).

**1.4.4.2 Antioxidant activity:**

Arjungenin and its glucoside are extracted from *T. arjuna* and exhibited a moderate free radical scavenging activity on the superoxide release from PMN cells. Arjungenin also exhibited greater inhibitory action on the hypochlorous acid production from human neutrophils (Pawar & Bhutani, 2005). *T. arjuna* tree bark powder has significant antioxidant action that is comparable to vitamin E and also has a significant hypocholesterolaemic effect (Gupta et al., 2001).

The antioxidant and antimutagenic activities of alcoholic extract of TA bark. The alcoholic extract of the stem bark of *T. arjuna* (ALTA) has shown potent antioxidant activity with $EC_{50}$ in DPPH assay, superoxide radical scavenging activity and lipid peroxidation assay. In micronucleus test ALTA showed significant reduction in percentage of micronucleus in both polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) and also showed a significant reduction in P/N ratio (Viswanatha et al., 2010).

The antioxidative properties of an ethanol extract of the bark of *T. arjuna* (TAEE) against sodium fluoride (NaF)-induced oxidative stress in the murine heart. (Sinha et al., 2008).

Diethyl ether, ethyl acetate and ethanol extractions of *T. arjuna* exerted hypolipidemic and antioxidative effects at two different dose levels of 175 and 350 mg/kg body weight in Poloxamer (PX)-407 induced hyperlipidemic albino Wistar rats. The results suggested that the ethanolic fraction of *T. arjuna* possesses the potent properties of being an antioxidant and hypolipidemic than other fractions (Subramaniam et al., 2011).

The *T. arjuna* bark aqueous extract significantly prevented the isoprenaline-induced increase in oxidative stress and decline in endogenous antioxidant level and also prevented fibrosis. The oral administration of *T. arjuna* for 12 weeks in rabbits caused augmentation of myocardial antioxidants; superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) along with induction of heat shock protein72 (HSP72) (Gauthaman et al., 2005). A significant correlation was also observed between free radical scavenging activity, *in vitro* DNA damage activity and the total phenolic/flavonoid content (Kumar et al., 2013).

#### 1.4.4.3 Antibacterial activity:

The antimicrobial properties of methanolic extract of *T. arjuna* bark were investicated. The antimicrobial activity showed that higher inhibition against Gram negative bacteria than gram positive bacteria (Mandal et al., 2013).

Methanol extract from bark of *T. arjuna* exhibited medicinal as well as physiological activities. Methanol, ethanol, acetone, aqueous both hot and cold extracts from the leaves and bark of *T. arjuna* were tested for their antimicrobial activity against *Staphylococcus aureus*, *Acinetobacter* sp., *Proteus mirabilis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*, pathogens causing ear infections. Three organic solvents evaluated, acetonic leaf extract was found to be best against *S. aureus*. Organic bark extract showed almost equal inhibition of all tested Gram negative bacteria except *P. aeruginosa*. Aqueous extract of *T. arjuna* bark exhibited good activity against *S. aureus* (Aneja et al., 2012).

#### 1.4.4.4 Anticarcinogenic and antimutagenic properties:

The anticarcinogenic and antimutagenic potential of extracts of *T. arjuna* were investigated and highlighted. The role of *T. arjuna* extracts in reducing metaphase aberrations due to aflatoxin $B_1$ (AFB1) is quite significant, the reduction varying from 23.49%, 42.47%, and 59.65% down to 12.32%, 28.00%, and 36.88% respectively at the highest dose *T. arjuna* for the three different durations viz., 24, 48 and 72 h. Similarly the number of sister chromatid exchanges got reduced from a higher level of 15.00 ± 1.40 per cell to 7.70 ± 0.50 per cell with liver microsomal metabolic activation system mix at 48 h of treatment. The replication index was enhanced from 1.33 to 1.55 in the *in vitro* experiment. Similar trends were noticed in the *in vivo*experiments that are effective reductions in clastogeny ranging from 15.22% to 54.82% from the mutagen treated positive control and the total frequencies in aberrant cells got reduced from 429 due to AFB1 to 141 due to 5th concentration of *T. arjuna* extracts at 32 h of exposure (Ahmad et al., 2014).

#### 1.4.4.5 Gastroprotective properties:

The effect of methanolic extract of *T. arjuna* (100 mg/kg to 50 mg/kg body weight) on diclofenac sodium (80 mg/kg bodyweight in water, orally) induced gastric ulcer in rats. The gastroprotective effect of *T. arjuna* was assessed from volume of gastric juice, pH, free and total acidity, pepsin concentration, acid output in gastric juice, the levels of non-protein sulfhydryls (NP-SH), lipid

peroxide (LPO), reduced glutathione (GSH), and activities of enzymic antioxidants-super oxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and myeloperoxidase (MPO) in gastric mucosa. The levels of DNA, protein bound carbohydrate complexes-hexose, hexosamine, sialic acid, fucose in gastric mucosa and gastric juice and the levels of RNA in gastric mucosa were assessed. The stomach tissues were used for adherent mucus content and also for the histological examination. A significant reduction in lesion index was observed in ulcer induced animals treated with *T. arjuna* (DIC + TA) compared to ulcerated rats (DIC). A significant increase was observed at pH, NP-SH, GSH, enzymic antioxidants, protein bound carbohydrate complexes, adherent mucus content, nucleic acids with a significant decrease in volume of gastric juice, free and total acidity, pepsin concentration, acid output, LPO levels and MPO activities in DIC + TA rats compared to DIC rats. It is proved that *T. arjuna* could act as a gastroprotective agent probably due to its free radical scavenging activity and cytoprotective nature (Devi et al., 2007).

**1.4.4.6 Hypocholesterolaemic properties:**

The effect of an Ayurvedic formulation of *T. arjuna*, known as 'Arjuna Kwatha' was assessed in 36 hypertensive patients at stage III with increased LV mass. The patients were divided into two groups, one group received atenolol (50 mg twice daily) and the other group 'Arjuna Kwatha' (25 ml twice daily) along with atenolol for 6 months. A significant decrease was observed in both SBP and DBP ($P < 0.001$) in both the groups. However, LV mass index was only significantly reduced in the atenolol-plus-'Arjuna Kwatha' group as compared to atenolol alone ($P < 0.001$), due to negative chronotropic and inotropic effects of the herbal preparation (Rao et al., 2001). The administration of *T. arjuna* bark powder along with statins for 3 months to 30 patients with coronary artery disease resulted in a 16% in LDL-cholesterol, 15% decrease in total cholesterol and 11% in triglycerides, confirming its immense potential to correct dyslipidemia in conjunction with statins (Khalil, 2005).

## 1.5 *Swartia chirata:*

**Fig. 4: *Swertia chirayita* plant**

**Fig. 5: Dried *Swertia chirayita* plant**

**Image source: Google "*Swartia chirayita*"**

### 1.5.1 Taxonomy:

Current name: *Swertia chirayita,*

**Botanical description**

| | |
|---|---|
| Kingdom: | Plantae |
| Division: | Tracheophyta |
| Class: | Magnoliopsida |
| Order: | Gentianales |
| Family: | Gentianaceae |
| Genus: | *Swertia* |
| Species: | *chirayita* |

### 1.5.2 Description of *Swertia chirayita*:

*Swertia Chirayita* is also known as Haima, kirata Tikta, Nidrari, Ramasenka, kairata in Sanskrit, in urdu language it is called Chiravata, Chireta in Bengal and in Arabic and Farsi called as Qasabuzzarirah. Chiretta is its market name (Anonymous, 1982; Kirtikar and Basu, 1984). The plant Swertia chirata belongs to Gentianaceae family which is a tropical family of small trees which consists of 180 species of which 40 species exist in Indian subcontinent (Hooker, 1885; Clarke 1885; Kirtikar and Basu, 1984). This plant is indigenous to Himalayas at altitudes above 4000 ft from Kashmir, Nepal to Bhutan (Bhattacharjee, 1980).

### 1.5.3 Phytochemical properties:

The main chemical constituents of this plant are two bitter principles, *viz*, ophelic acid, an amorphous bitter principle and chiratin, a yellow bitter glucoside. The plant also contains resins, tannin, gum, carbonates, phosphates and 4-6% ash, lime and magnesia (Nadkarni, 1976; Chopra, 1982). A number of workers have shown that the plant contains bitter glucosidal components, chiratin and amarogentin, swerchirin, gentiopicrin, phytosterd and also a number of acid, yellow crystalline phenols and saccharine (Korte, 1955; Dalall and Shah, 1956; Korte and Schicke, 1956). Xanthones are another main secondary metabolites of Swertia species. Structures of xanthones are related to that of flavonoids and the chromatographic behaviors are also considered as similar. Xanthones are sometimes considered as the parent polyhydroxylated compounds but most xanthones are mono or poly methyl ethers or are found as glycosides (Hostettmann and Miura, 1977). Unlike iridoids, xanthones are apparently not present in all plant species investigated in the family Gentianaceae. This is documented by the systematic study of (Hostettmann-Kaldas et al., 1981). The natural xanthones are mainly isolated from about 150 plants associated with four families; Guttiferae, Gentianaceae, Moraceae and Polygalaceae. 278 natural xanthones were reported from total of 515 xanthones (Vieira and Kijjoa, 2005). In this period, the xanthones from higher plants appear to be associated mainly with the families Clusiaceae (55 species in 12 genera) and Gentianaceae (28 species in 8 genera). Xanthones isolated from nature are classified into six main groups; simple xanthones, xanthone glycosides, prenylated xanthones, xanthonolignoids, bis-xanthones and miscellaneous xanthones. These are further subdivided according to the degree of oxygenation into non-, mon-o, di-, tri-, tetra-, penta- and hexa-oxygenated substances (Mandal et al., 1992; Sultanbawa, 1980; Demirkiran, 2007).

Mangiferin is the most common C-glycosides in *S. chirayita*,*S. mussotii*, *S. cordata*, *S. macrosperma* and *S. connata*. Xanthone O-glycosides (swertianolin) from *S. japonica* and *S. ciliata* (Plouvier *et al*., 1967) have been reported. The first xanthone O-glycoside, norswertianin-1-O-glucosyl-3-O-glucoside has been isolated from *S. perennis* (Hostettmann and Wagner, 1977). Xanthones in *Swertia chirata, S. speciosa* and *S. paniculata* were determined by HPLC. Mineral elements, based on their concentration can play different roles in human health and plant life. Nine elements (Zn, Cu, Mn, Fe, Co Na, K, Ca and Li) in *S. chirayita* and *S. speciosa* have been analyzed by atomic absorption spectrometry (Negi et al., 2009 & 2010).

## 1.5.4 Pharmacological properties:

The genus *Swertia* exhibit variety of biological activity such as hepatoprotective, antihepatotoxic, antimicrobial, anti-inflammatory, anticarcinogenic, antileprosy, hypoglycemic, antimalarial, antioxidant, anticholinergic, CNS depressant and mutagenicity. The pharmacological properties of *Swertia* have raised great interest recently. (Negi et al., 2011).

### 1.5.4.1 Hepatoprotective and antihepatotoxic activities:

Sweroside and gentiopicroside two compounds which got hepatoprotective activities have been reported and both compounds are being used as antihepatitis drugs (Kondo *et al*., 1994). Lignan also known syringaresinol which is hepatoprotective in nature, and the ubiquitous ß-sitosterol are also present (Chatterjee and Pakrashi 1995; Rastogi and Mehrotra, 1998).

### 1.5.4.2 Antimicrobial and anti-inflammatory activities:

Antibacterial activity of rectified spirit extract of *Swertia chirayita* were tested against eight bacteria at different concentrations. At 90 µg/disc dose, the produced zone of inhibition against the same bacteria was 16mm, 13mm and 12mm respectively (Sultana et al., 2007).

Ursolic acid has anti-inflammatory and antimicrobial activities. (Chatterje and Pakrashi 1995; Rastogi and Mehrotra 1998). *Swertia hookeri* extract is used in the treatment of microbial infections (Ghosal et al., 1980).

**1.5.4.3 Antiviral and chemoprotective activities:**

Swertifrancheside isolated from *Swertia franchetiana* was found to be potent inhibitor of the DNA polymerase activity of human immunodeficiency virus-1 reverse transcriptase (HIV-1RT). (Ghosal et al., 1973; Bhattacharya et al., 1972). Oral and topical compounds containing mangiferin are useful for the treatment of diseases caused by herpes virus (Ghoshal et al., 1975).

Mengiferin compound, which is isolated from chirayita species possesses strong anti-inflammatory activity in arthritic mice, and accounted for lowering down TNF-alpha, IL-1beta, IL-6, and IFN-gamma and up regulation of IL-10 in the joint homogenates of mice (Kumar et al., 2003). It is also found to be a strong chemoprotective agent (Yoshimi et al., 2001).

**1.5.4.4 Neuroprotective and mental relaxant activities:**

Xanthones especially mangiferin are reported to give CNS (Central nervous system) stimulation (Bhattacharya et al., 1972). Over all Xanthones are important bioactive constituent present in the drug which shows CNS down regulation in mice and rats (Bhattacharya et al, 1974). *Swertia paniculata* is used in the Indian System of Medicine as a bitter tonic and in the treatment of some mental disorders (Prakash et al., 1982).

**1.5.4.5 Cardiotonic, convulsant and choleretic activities:**

Mangiferin was the first xanthone to be investigated pharmacologically and has been found to exhibit a broad spectrum of biological activities. It shows monoamine oxidase inhibition, cardiotonic, convulsant and choleretic activities (Ghosal et al., 1973; Bhattacharya et al., 1972).

It is also found that iridoids such as swertiamarin have anticholinergic propertiy (Bhattacharya et al., 1974).

**1.5.4.6 Hypoglycemic activity:**

1,8-Dihydroxy-3,5-dimethoxyxanthone (swerchirin), isolated from the hexane fraction *of Swertia chirayita,* has a very significant blood sugar lowering effect in fasted, fed, glucose loaded and tolbutamide pre-treated albino rats (Ghosal et al., 1974). A class of pentacyclic triterpenoids also belongs to this herb including ß-amyrin, friedlin, chiratenol, kairatenol, oleanolic acid, ursolic acid. Among them kairatenol is found to be hypoglycemic in nature. (Chatterjee and Pakrashi 1995;

Rastogi and Mehrotra 1998). Swertiamarin a secoiridoid glycoside obtained from *Swertia chirayita* having analgesic property (Lei et al., 1982)

#### 1.5.4.7 Gastroprotective activity:

Amaroswerin it is a Secoiridoid glycoside collected from *Swertia chirayita* and found to be gastro-shielding (Niiho et al., 2005).

#### 1.5.4.8 Other activities:

The antimalarial drug AYUSH-64 contains *S. chirayita* as one of the ingredients. Xanthones of *S. chirayita* are reported to produce CNS depression (Ghosal et al., 1973). The total extract of *S. chirayita* showed significant antifeedant activity against *Jute semilooper* (Malic et al., 1985).
Some of the Herbaceous medicaments like Ayush-64, Diabecon , Mensturyl syrup and Melicon V ointment restrain chirayita essence in variable expanse for its antipyretic, hypoglycemic, antifungal and antibacterial effects (Edwin and Chungath , 1988; Mitra et al., 1996).

## 1.6 Extraction of selected plants:

The study of medicinal plants mainly conducted with the pre-extraction and the extraction procedures and it is an important step in the processing of the bioactive constituents from plant materials. Most common traditional methods such as maceration and Soxhlet extraction are commonly used at the small research setting or at Small Manufacturing Enterprise (SME) level. The initial stage of studying medicinal plants is the preparation of plant samples to preserve the biomolecules of the plants prior to extraction. The common plant samples are mainly the leaves, barks, roots, fruits and flowers. They can be extracted from fresh or dried plants material. Both fresh and dried sample can be used in medicinal plants studies. However in most cases, dried sample is preferred as fresh samples are fragile and tend to deteriorate faster than dried samples (Azwanida, 2015).
In a study the comparison between fresh and dried *Moringa oliefera* leaves showed no significant effect in total phenolics but with higher flavonoids content in dried sample (Vongsak et al., 2013). Lowering particle size increases surface contact between samples and extraction solvents. Grinding resulted in coarse smaller samples. The powdered samples have more homogenized and smaller particles which leads to a better surface contact with extraction solvents and for that

particular pre-preparation is important for efficient extraction to occur. The solvent must make contact with the target analytes and particle size smaller than 0.5 mm which is considered ideal for efficient extraction. Investigation of nanoparticles powder of *Centella asiatica* produced by Planetary Ball Mill (PBM) showed 82.09% higher yield compared to micro powder using maceration technique in 90% methanol for 3 days (Borhan et al, 2013). Therefore because of these purposes, in this study the dried powder form of the *Terminalia chebula* (fruits), *Terminalia arjuna* (barks), *Swertia chirayita* (roots, leaves and flowers) were used.

In Soxhlet extraction method, finely ground powder of the is placed in a porous bag or "thimble" made from a strong filter paper or cellulose, which is place, is in thimble chamber of the Soxhlet apparatus (Figure 6). Extraction solvents is heated in the bottom flask, vaporizes into the sample thimble, condenses in the condenser and drip back. When the liquid content reaches the siphon arm (Figure 6), the liquid contents emptied into the bottom flask again and the process is continued (Azwanida, 2015).

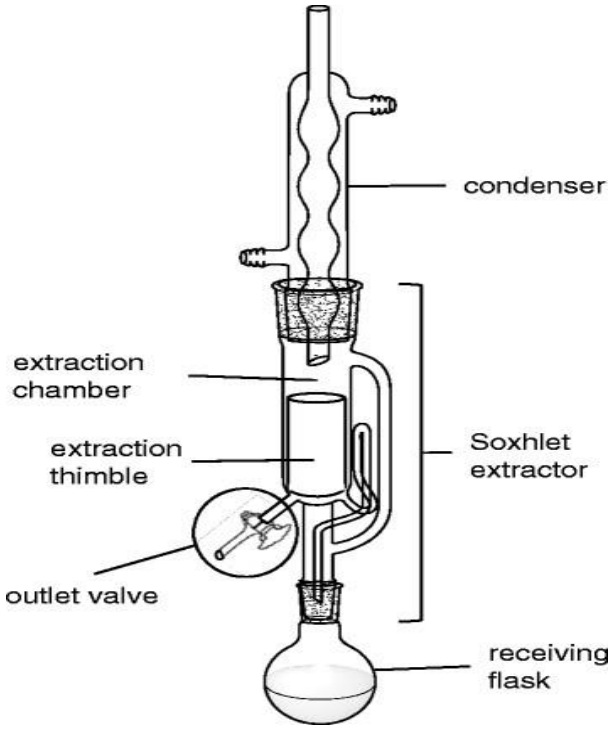



**Fig. 6: Soxhlet apparatus**

**Image source: Google "Soxhlet apparatus".**

## 1.7 Selected organism for testing antimicrobial activity:

In this study, only a highly resistant strain of *Pseudomonas aeruginosa* bacteria was selected. The bacteria were collected from the food borne bacteria samples BRAC University's biotechnology laboratory, where it had been already identified. The morphological description of *Psedomonas aeruginosa* bacteria is given below.

### 1.7.1 *Psedomonas aeruginosa:*

*Pseudomonas* genus is comprised of 120 species. They are mostly leave in moist environment such water, soil. *Pseudomonas aeruginosa,* a motile, non-fermenting Gram-negative bacterium, an opportunistic pathogen that results respiratory infections, urinary tract infections, gastrointestinal infections, keratitis, otitis media, and bacteremia in patients with compromised host defenses [e.g., cancer, burn, HIV, and cystic fibrosis (CF)]. These infections often result in significant morbidity and mortality. *P. aeruginosa* plays an increasingly prominent role in hospital infections.

The bacteria itself is a ubiquitous and metabolically versatile microbe that flourishes in many environments. It can grow under both aerobic and anaerobic conditions, and possesses several virulence factors that contribute to its pathogenesis (Schurek et al., 2012). It is found that *P. aeruginosa* possesses an intrinsic resistance to many antimicrobials because of its outer-membrane barrier, the presence of multidrug efflux transporters, and endogenous antimicrobial inactivation (Poole, 2011). Although anti-pseudomonas agents (e.g., carbapenems) have been discovered and developed, *P. aeruginosa* readily acquires resistance to individual agents via chromosomal mutations and lateral gene transfer (Poole, 2011).

It was found that *Pseudomonas aeruginosa* possesses multifactorial mechanisms of responses and resistance to antimicrobials. The antimicrobials were originally developed and used to kill bacteria, however recent work revealed that the biological functions of antibiotics are not limited to bactericidal (killing) or bacteriostatic (growth inhibition) effects. The most likely function of antibiotics in natural ecosystems is in intercellular "signaling," with specific consequences on the collective behavior of the bacterial population (Linares et al., 2006; Aminov, 2013).

## 1.8 Determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extract:

Any newly reported drugs or plant extracts potency can be determined by measuring the minimum inhibitory concentrations (MIC) value. MIC can be termed as the lowest concentration of antimicrobials that is required to inhibit the growth of a test organisms within a defined interval period of times, most commonly within 18-24 hours. MIC is the most basic laboratory measurement used in determining the activity of any antimicrobial agent against an organism (Turnidge et al., 2003).

The serial broth dilution method is most common method used in the measurement of MIC. Serial broth dilution requires various dilutions of the compound (antimicrobial) and is under tested in a suitable solvent. Selection of appropriate solvent is very important because it plays the most crucial role and has significant influences in MIC measurements in lab. Ethanol, Methanol or DMSO are the most common solvent that are used in the lab. Ethanol (Houghton and Raman, 19988).

DMSO are most preferable since they are miscible in water. DMSO is a highly polar, stable substance with exceptional solvent property (Randhawa, 2006). However, DMSO in various bioassays have been reported for their antimicrobial effect (Ansel et al., 1969). Therefore, the final concentrations of DMSO should be taken in such way which does not interfere with MIC determination. Different organisms may exhibit different susceptibility to DMSO's. Thus he concentration of the solvent (DMSO) should be ranged within 2.5% v/v. As the solvent DMSO (2.5%) would not inhibit the growth of microorganisms (Zgoda and Porter, 2001). The minimum bactericidal concentration (MBC) can be determined by spread plating a little portion of broth from MIC containing tubes.

## 1.9 Objectives:

- To test the ethanol, chloroform and ethyl acetate extracts of the *Terminalia chebula* (fruits), *Terminalia arjuna* (barks), *Swertia chirayita* (roots, leaves and flowers) against a highly resistant strain of *Psedomonas aeruginosa* bacteria.
- To identify the Minimum inhibitory concentration (MIC) and minimum bacteriocidal concentration (MBC) values of the extract which showed highest sensitivity against the resistant strain of *Psedomonas aeruginosa* bacteria.

# CHAPTER 2: Materials and methods

## 2.1 Methodology:

The experiment was carried in the biotechnology laboratory of BRAC University to observe, antimicrobial activities of *Terminalia chebula, Terminalia arjuna* and *Swertia chirayita* against multidrug resistant *Pseudmonas aeruginosa* and comparison of their activities with locally available antibiotics.The experiment process mainly involved preparing the ethyl acetate and chloroform extract of *Terminalia chebula,* the ethanol and ethyl acetate extract of *Terminalia arjuna* and finally the ethanol and ethyl acetate extract of *Swertia chirayita.* After preparing the crude extracts their potential antimicrobial activity is tested against highly multidrug resistant *Pseudmonas aeruginosa* via agar diffusion method. The antibiotic disk polymyxin B is used as a control to examine the activity index. After finding out the antimicrobial activity of crude extracts of the selected plants, the MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) is determined. To make sure whether the organism were resistant or not, the selected bacteria *Pseudmonas aeruginosa* needs to be tested via disc diffusion method at the beginning of the experiment.

## 2.2 Collection of the plants sample:

The readily made fine ground powder of *Terminalia chebula* (fruits), *Terminalia arjuna* (barks), *Swertia chirayita* (roots, leaves and flowers) bought from Gulshan DCC market, Dhaka, Bangladesh. The powder of the selected plants were tightly packed in an air tight bag (250grams). Finally, they were transferred in sterile autoclaved jars respectively and stored properly at room temperature and kept away from direct sunlight until further use.

## 2.3 Extraction process:

During extraction process, each time 75 grams of the ground powder was measured via electronic balance and then the powder was carefully placed on the extraction thimble, inside the Soxhlet apparatus. The round receiving flask of the apparatus was then filled with 250 ml of extraction solvent such as ethanol or ethyl acetate or chloroform. During extraction, the Soxhlet should be run at different temperatures. As different solvents boiling temperature varies from solvent to solvents. The boiling temperature of ethyl acetate, chloroform and ethanol are 77.1 ℃, 61.2 ℃ and 78.37 ℃ repectively. However, the Soxhlet apparatus was run at a temperature which was little lower than the exact boiling point of the solvents as it reduces the chance of bumping. The

extraction cycles were repeated for three to four cycles consecutively and it took almost three to four hours and then the cycle was stopped as the color of cotton inside the extraction thimble become colorless.

The contents of the round flask is then transferred into the receiving flask of the rotary evaporator. Plants extract contents evaporation and condensation is done into the rotary evaporator at the temperature of 80 ℃. The solvent of the plant extract contents that recovered from Soxhlet was evaporated to a point where the extract become concentrated. The extract was then scraped via spatula and collected in the sterile petri plates and stored into the incubator at 40 ℃ for a day until the extract was completely dried. Then the dried crude extract again scraped and crushed via spatula and then stored into the sterile micro-centrifuge tubes as it is easier to measure the weight of the extracts and then stored those tube inside a sterile jar for further uses. The process is then repeated for each plants using the mentioned solvents that is described above.

## 2.4 Collection of the multidrug resistant bacterial strain:

The highly multidrug resistant strain of *Pseudmonas aeruginosa* was collected from the biotechnology lab of BRAC University which was stored in the culture fridge in cetrimide agar media. Before using the bacteria it was again transferred into cetrimide agar medium and regularly maintained through subculture. The purpose of using cetrimide agar other than nutrient agar, the cetrimide agar is a selective media for *Pseudmonas aeruginosa.*

## 2.5 Preparation of media:

### 2.5.1 Preparation of Cetrimide Agar media:

Cetrimide agar is used for the selective isolation of the gram-negative bacterium, *Pseudomonas aeruginosa*. The required amount of agar at first measured in electronic balance and mixed with distilled water in a conical flask and then were heated and dissolved by heating until the agar was fully boiled and become transparent yellowish with bubbles. The mouth of the conical flask was covered with aluminum foil paper. After sealing the mouth it was then placed into an autoclave machine at 121 ℃ and 15 psi for 45 minutes. After autoclaving the mixture is then poured into the sterile medium sized petri dishes. When the agar solidifies the petri dishes were then labeled and were stored at 4 ℃ inside the refrigerator for further use.

### 2.5.2 Preparation of Muller-Hinton Agar (MHA):

Muller-Hinton Agar **(**MHA) media were mainly used to observe the antimicrobial activities of the both antibiotics and the plant extracts as Muller-Hinton Agar **(**MHA) typically used in determining antimicrobial activity of antibiotics and extracts.

The required amount of agar at first measured in electronic balance and mixed with distilled water in two conical flasks and then were heated and dissolved by heating until the agar melted. The mouth of the conical flask was covered with aluminum foil paper. After sealing the mouth it was then placed into an autoclave machine at 121 ℃ and 15 psi for 15 minutes. After autoclaving the mixture is then poured into the sterile large sized petri dishes. When the agar solidified the petri dishes were then labeled and were stored at 4 ℃ inside the refrigerator for further use.

### 2.5.3 Preparation of Brain Heart Infusion Broth:

Brain Heart infusion broth were used in serial broth dilution to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

The required amount of broth at first measured in electronic balance and mixed with distilled water and final volume of the solution was made 200 ml. Then 10 ml aliquots were added in 20 sterile 15 ml test tube. After autoclaving the tubes, they were appropriately labelled and stored in a clean beaker and stored at room temperature.

## 2.6 Subculture of the selected isolates:

Twenty four hours old sub-cultured strain of resistant *Pseudmonas aeruginosa* was used to observe antimicrobial activity. The subculture of the bacteria required a loop full of selected bacteria from stock culture and streaking it on fresh cetrimide agar media. Before streaking the loop was heat steriled and then cooled. The streaking plates were then incubated at 37 ℃ for 24 hours and after taking the samples the culture were stored at 4 ℃ for further use.

## 2.7 Preparation of physiological saline:

Physiological saline was made to prepare bacterial suspension and it was matched with McFarland standard 1 solution. At first 0. 9 g NaCl was dissolved in 80 ml deionized or distilled water in clean conical flask. Then the water was added to bring total solution volume to 100 ml. Then 10

ml aliquots were in 10 sterile 15 ml test tube. After autoclaving the tubes, they were appropriately labelled and stored in a clean beaker and stored at room temperature.

## 2.8 Preparation of McFarland standard 1 solution:

McFarland standard 1 solution were used as a reference to adjust the turbidity of bacterial suspensions so that the number of bacteria will be given within a range to standardize microbial testing. The preparation of McFarland standard 1 solution was mainly made for the antibiotic susceptibility testing by measurement of minimum inhibitory concentration. Another reason was if a suspension used is too heavy or too dilute, an error in the result (either falsely resistant or falsely susceptible) for any given antimicrobial agent could occur.

McFarland standards were made by mixing specified amounts of barium chloride and sulfuric acid together. Mixing the two compounds forms a barium sulfate precipitate, which causes turbidity in the solution. A McFarland standard 1 solution was prepared by mixing 0.10 mL of 1.175% barium chloride dihydrate ($BaCl_2 \bullet 2H_2O$), with 9.90 mL of 1% sulfuric acid ($H_2SO_4$).

The standard were visually compared to a suspension of bacteria in sterile saline when required. When the bacterial suspension was found too turbid, it was diluted with more diluent and when suspension was not turbid enough, more *Pseudmonas aeruginosa* was added. McFarland standard 1 solution had the cell density of $3.0 \times 10^{\wedge 8}$ CFU/ml (Colony Forming Units) per ml.

## 2.9 Disc diffusion method:

Agar surface of each plate Muller-Hinton Agar was streaked by a sterile cotton swab with the reference bacterial (*Pseudmonas aeruginosa)* strain from the physiological saline which was compared with McFarland standard 1 solution. Antibiotics Discs of 6 mm size were impregnated in and was placed on solidified agar plates at equal distance with control. The plates were allowed to standby for 30 min. The plates were incubated at 37 °C for 24 hours. The disc diffusion test was mainly done to re-determine the resistance against the reference antibiotic discs that were used. Around 30 different antibiotic discs were used.

## 2.10 Agar well diffusion method:

Agar surface of each plate Muller-Hinton Agar was streaked by a sterile cotton swab with the reference bacterial (*Pseudmonas aeruginosa)* strain from the physiological saline which was

compared with McFarland standard 1 solution. Agar plate was punched with a sterile cork borer of 4 mm size and 100 μL of each plant extract sample was poured with micropipette in the well. Before that the raw extracts were diluted with different solvents in distilled water so the extracts may dissolve. Three different solvents 2.5% DMSO (Di-methyl sulfoxide), 2.5% Tween R 20 and concentrated chloroform water were used to dissolve crude extracts of the plants. Along with the extracts a sensitive antibiotic disc (Polymyxin B) was used in each plates as a control. The plates were then allowed to standby for 30 min. The plates were incubated at 37°C for 24 hours.

## 2.11 Determining the Activity index of the extracts:

After 24 hours of incubation period, the clear zones around the wells (Zone of inhibition) was measured using a measuring scale and it was recorded. The activity index of each extracts was then measured by using the following formula. The formula was repeated thrice.

$$\text{Activity Index (AI)} = \frac{\text{Zone of inhibition of the plant extract (mm)}}{\text{Zone of inhibition of the control antibiotic (mm)}}$$

## 2.12 Determining the MIC and MBC of the *Terminalia chebula's* extract:

The MIC (minimum inhibitory concentration) and MBC (minimum bacteriocidal concentration) was only determined for the extract that showed the highest zone of inhibition in diameter (mm) or activity index. The ethyl acetate extract of *Termanilia chebula* showed the highest zone of inhibition in diameter (mm) and activity index too.

Determining the MIC and MBC required the different concentration of plant extract solution and undiluted volumes of Brain Heart Infusion broth (BHI) and respective solvent DMSO were mixed in different sterilized test tubes. DMSO was mainly used to dissolve the extract within the broth. The concentration of the solvent (DMSO) used were ranged within 2.5% v/v. as the solvent DMSO (2.5%) would not inhibit the growth of microorganisms. The ethyl acetate extract of *Termanilia chebula* were then dissolved into the (BHI + DMSO) solution. The (BHI + DMSO + Extract) was mixed in such way that each ml had 40 mg of the acetate extract of *Termanilia chebula* in concentration. Then the plant extract solution (BHI + DMSO + Ethyl acetate extract) of the plant of different concentrations such as 0.2 mg/ml, 0.4 mg/ml, 0.6 mg/ml, 0.8 mg/ml, 1.2 mg/ml, 1.6 mg/ml, 2 mg/ml, 3.2 mg/ml, 4 mg/ml, 6 mg/ml, 8.8 mg/ml, 10 mg/ml, 12 mg/ml, 15 mg/ml, 18

mg/ml, 20 mg/ml, 25 mg/ml and 30 mg/ml were added on different undiluted volumes of Brain Heart Infusion broth. After this 100 µl or 0.1 ml of inoculum of resistant *Pseudmonas aeruginosa* was added from the physiological saline which was compared to McFarland Standard solution 1 and via 100 µl of inoculum mainly $3\times10^7$ cell forming units were transferred into the broth. The total volume of each tube was made 10 ml. A tube containing only growth medium and 100 µl of inoculum is put as a growth control another tube containing each only extract used as positive control and a tube of only BHI broth was put as negative control.

The tubes were then incubated at 37 °C for 24 hours. After incubation period the tubes are observed carefully. The inoculated tubes with the lowest concentration of the extract which showed no turbidity were considered as the minimum inhibitory concentration containing tube. To determine MBC the 100 µl of the inoculated broth of each tubes were spread platted via sterile glass on the MHA agar plates. The concentration at which no bacterial colonies were formed in the agar plate was determined as minimum bactericidal concentration of the extract.

Same procedure was followed to determine the MIC and MBC of the control antibiotic polymyxin B (ARISTOPHARMA LTD) to set comparisons between the extract and the control antibiotic.

# CHAPTER 3: Results

The antibiotics susceptibility and zone diameter interpretation chart against the multidrug resistant *Pseudmonas aeruginosa* strain showed in table 1. The average zone of inhibition and the activity indexes of ethyl acetate and chloroform extract of the fruits of *Terminalia chebula* are summarized at at table 2 and 3. The average diameter of the zone of inhibition and the activity indexes of ethyl acetate and ethanol extract of the barks of *Terminalia arjuna* and the leaves, roots and flowers of *Swertia chirayita* on multidrug resistant *Pseudomonas aeruginosa* strain showed at table 4, 5, 6 and 7 respectively. All the extracts showed antimicrobial activity more or less except for chloroform extract of *Terminalia chebula* and ethyl acetate extract of *Swertia chirayita*. The MIC and MBC value of polymyxin B and ethyl acetate extract of *Terminalia chebula* and their comparisons are represented on figure 41 and 42 respectively.

## 3.1 Extract yield:

Percent of yield was calculated as follows:

Extract yield % = $(W_1/W_2) \times 100$

Where, $W_1$ is net weight of powder in grams after extraction and $W_2$ is total weight of wood powder in grams taken for extraction.

The yield of ethyl acetate extract of *Terminalia chebula* (fruits) was, 2.7 grams

Extract yield % = $(2.7/75) \times 100 = 3.6\%$

The yield of chloroform extract of *Terminalia chebula* (fruits) was, 0.7 grams

Extract yield % = $(0.7/75) \times 100 = 0.93\%$

The yield of ethyl acetate extract of *Terminalia arjuna* (barks) was, 1.9 grams

Extract yield % = $(1.9/75) \times 100 = 2.5\%$

The yield of ethanol extract of *Terminalia arjuna* (barks) was, 4.8 grams

Extract yield % = $(4.8/75) \times 100 = 6.4\%$

The yield of ethyl acetate extract of *Swertia chirayita* (roots, leaves and flowers) was, 1.8 grams

Extract yield % = $(1.8/75) \times 100 = 2.4\%$

The yield of ethanol extract of *Swertia chirayita* (roots, leaves and flowers) was, 2.4 grams

Extract yield % = $(2.4/75) \times 100 = 3.2\%$

## 3.2 Antibiotics susceptibility and zone diameter interpretation:

Table 1 shows that the list of 30 tested antibiotics. It was seen that, the resistant *Pseudmonas aeruginosa* strain was sensitive to polymyxin B only. The *Pseudmonas aeruginosa* strain was found resistant against other 28 tested antibiotics and intermediate to Imipenem.

**Table 1: Antibiotics susceptibility and zone diameter interpretation against the resistant *Pseudmonas aeruginosa* strain:**

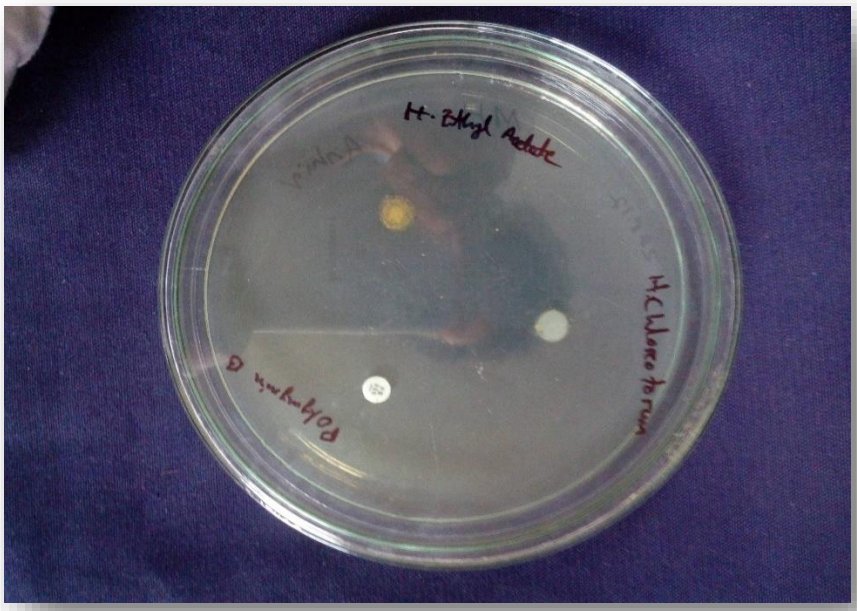
| Tested Antibiotics Against *Pseudmonas aeruginosa* | CSLI Symbol | Disc Content In (mcg) | CSLI Referred Resistant Values In (mm) Or Less | CSLI Referred Intermediate Values In (mm) Or Less | CSLI Referred Sensitive Values In (mm) Or More | Zone Of Inhibition Produced In (mm) |
|---|---|---|---|---|---|---|
| Amoxycillin | AMC | 10 | — | — | — | 0 (R) |
| Ampicillin | AMP | 10 | — | — | — | 0 (R) |
| Azithromycin | AZM | 15 | — | — | — | 0 (R) |
| Ceftazidime | CAZ | 30 | 14 | 15-17 | 18 | 0 (R) |
| Ciprofloxacin | CIP | 5 | 15 | 16-20 | 21 | 12 (R) |
| Cephalexin | CL | 30 | 14 | 15-17 | 18 | 0 (R) |
| Clindamycin | CD | 2 | — | — | — | 0 (R) |
| Ceftriaxone | CTR | 30 | 13 | 14-20 | 21 | 0 (R) |
| Chloramphenicol | C | 30 | — | — | — | 0 (R) |
| Cefoxitin | FOX | 30 | — | — | — | 0 (R) |
| Deoxycyclin | DO | 30 | — | — | — | 0 (R) |
| Erythromycin | E | 15 | — | — | — | 0 (R) |
| Gentamycin | GEN | 10 | 12 | 13-14 | 15 | 0 (R) |
| Kanamycin | K | 30 | 13 | 14-17 | 18 | 0 (R) |

| Tested Antibiotics Against *Pseudmonas aeruginosa* | CSLI Symbol | Disc Content In (mcg) | CSLI Referred Resistant Values In (mm) Or Less | CSLI Referred Intermediate Values In (mm) Or Less | CSLI Referred Sensitive Values In (mm) Or More | Zone Of Inhibition Produced In (mm) |
|---|---|---|---|---|---|---|
| Levofloxacin | LEV | 5 | 13 | 14-16 | 17 | 0 (R) |
| Imipenem | IPM | 10 | 13 | 14-15 | 16 | 14.5 (I) |
| Moxifloxacin | MXF | 5 | 14 | 15-17 | 18 | 0 (R) |
| Meropenem | MEM | 10 | 13 | 14-15 | 16 | 0 (R) |
| Metronidazole | MTZ | 50 | — | — | — | 0 (R) |
| Nalidixic Acid | NA | 30 | — | — | — | 0 (R) |
| Nitrofurantion | F | 300 | — | — | — | 0 (R) |
| Netilmicin | NET | 30 | 12 | 13-14 | 15 | 0 (R) |
| Polymyxin B | PB | 300 units | 11 | — | 12 | 17 (S) |
| Rifampicin | RD | 5 | — | — | — | 0 (R) |
| Streptomycin | S | 10 | — | — | — | 0 (R) |
| Tetracycline | TE | 30 | — | — | — | 0 (R) |
| Tobramycin | TOB | 10 | 12 | 13-14 | 15 | 0 (R) |
| Tigecycline | TGC | 15 | — | — | — | 0 (R) |
| Piperacillin | PIT | 10 | 17 | — | 18 | 0 (R) |
| Ticarcillin | TCC | 10 | 14 | — | 15 | 0 (R) |

**Key:**

**— = not found, mcg = micrograms, mm= millimeters, CLSI = Clinical and Laboratory Standards Institute, (R) = Resistant, (I) = Intermediate, (S) = Sensitive.**

### 3.3 The antibacterial activity and zone diameter interpretation of ethyl acetate and chloroform extracts of *Terminalia chebula* fruits against the multidrug resistant *Pseudmonas aeruginosa:*



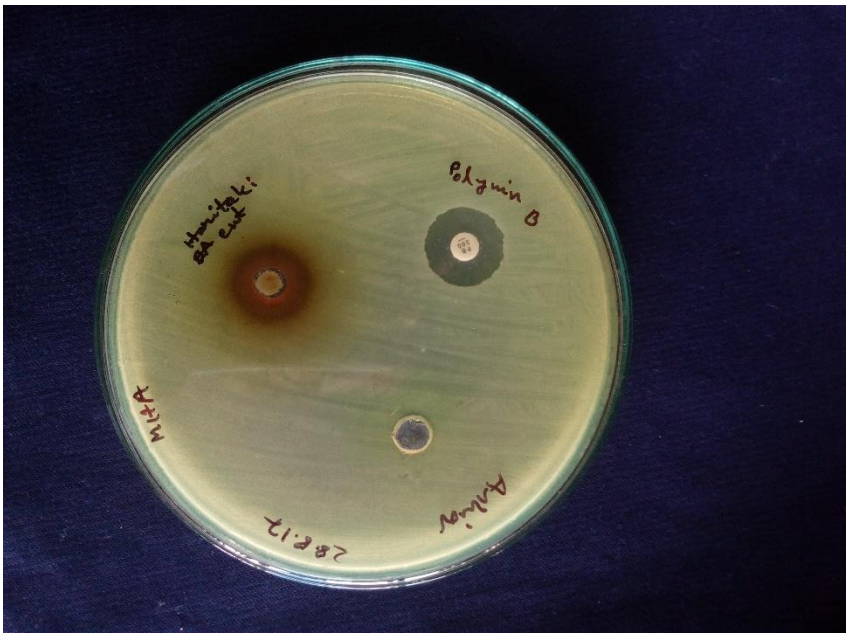




**Figure 7 and 8: Effects of ethyl acetate and chloroform extract of *T. chebula* in contrast to control Polymyxin B.**

**Table 2: Average zone of inhibition of ethyl acetate and chloroform extract of the fruits of *Terminalia chebula* on multidrug resistant *Pseudomonas aeruginosa* strain:**

| Sample | Zone of inhibition in diameter (mm) |
|---|---|
| Ethyl Acetate extract of *T. chebula* | 16 |
| Chloroform extract of *T. chebula* | No zone of inhibition found |
| Polymyxin B | 17 |

**Table 3: Average activity index of ethyl acetate and chloroform extract of the fruits of *Terminalia chebula* on multidrug resistant *Pseudomonas aeruginosa* strain:**

| Sample | Activity index |
|---|---|
| Ethyl Acetate extract of *T. chebula* | 0.94 |
| Chloroform extract of *T. chebula* | 0 |

Table 2, above showed that the ethyl acetate extract of *Terminalia chebula* got good antibacterial activity against the multidrug resistant *Pseudomonas aeruginosa*. However the chloroform extract of *Terminalia chebula* did not show any antimicrobial activity at all. The zone of inhibition in millimeter of the ethyl acetate extract of *Terminalia chebula* showed almost as similar zone of inhibition as the control antibiotic polymyxin B. The zone of inhibition of the ethyl acetate extract of *Terminalia chebula* was 16 mm in diameter and the zone of inhibition of the polymyxin B was 17 mm in diameter. In table 3, the activity index of the ethyl acetate extract of *Terminalia chebula* was found 0.94 where the activity index of the chloroform extract of *Terminalia chebula* was found 0 (zero). So the ethyl acetate extract of *Terminalia chebula* proved as good as reference antibiotic polymyxin B, which was found to be the only available antibiotic in the local market that was sensitive against the resistant *Pseudomonas aeruginosa.*

## 3.4 The antibacterial activity and zone diameter interpretation of ethyl acetate and ethanol extracts of *Terminalia arjuna* barks against the multidrug resistant *Pseudmonas aeruginosa:*

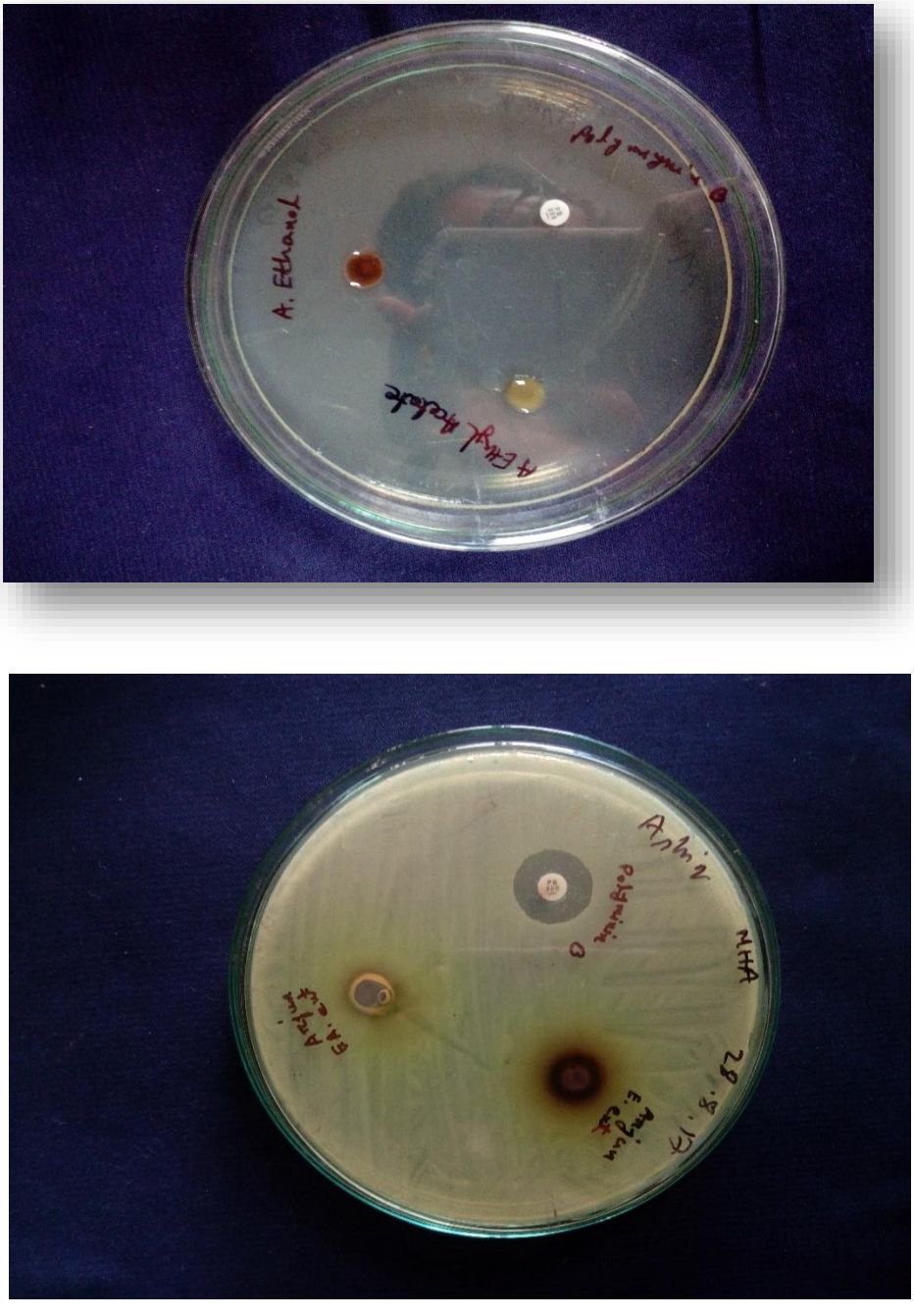



**Figure 9 and 10: Effects of ethyl acetate and ethanol extract of *T. arjuna* in contrast to control Polymyxin B.**

**Table 4: Average diameter of the zone of inhibition of ethyl acetate and ethanol extract of the barks of *Terminalia arjuna* on multidrug resistant *Pseudomonas aeruginosa* strain:**

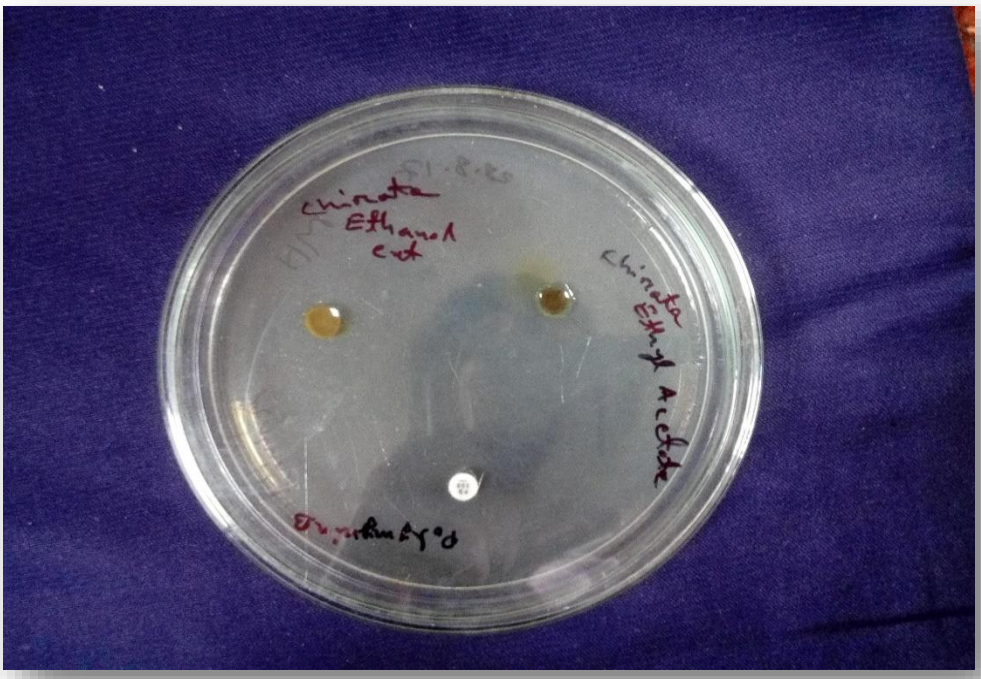
| Sample | Zone of inhibition in diameter (mm) |
|---|---|
| Ethyl Acetate extract of *T. arjuna* | 8 |
| Ethanol extract of *T. arjuna* | 11 |
| Polymyxin B | 17 |

**Table 5: Average activity index of ethyl acetate and ethanol extract of the bark of *Terminalia arjuna* on multidrug resistant *Pseudomonas aeruginosa* strain:**

| Sample | Activity index |
|---|---|
| Ethyl Acetate extract of *T. arjuna* | 0.47 |
| Ethanol extract of *T. arjuna* | 0.65 |

Table 4, showed that both the ethyl acetate and ethanol extract of *Terminalia arjuna* got lower antibacterial activity against the multidrug resistant *Pseudomonas aeruginosa*. However, the both extracts of *Terminalia arjuna* did not show as high antimicrobial activity as *Terminalia chebula's* ethyl acetate extract. The zone of inhibition in millimeter of the ethyl acetate and ethanol extracts of *Terminalia arjuna* was quite lower than the control antibiotic polymyxin B. The zone of inhibition of the ethyl acetate and ethanol extract of *Terminalia arjuna* was 8 mm and 11 mm respectively and the zone of inhibition of the polymyxin B found to be 17 mm in diameter. In the table 5, the activity index of the ethyl acetate and ethanol extract of *Terminalia arjuna* was found 0.47 and 0.65 respectively. So the ethyl acetate and ethanol extract of *Terminalia arjuna* proved to have lower antibacterial activity against the multidrug resistant *Pseudomonas aeruginosa.*

### 3.5 The antibacterial activity and zone diameter interpretation of ethyl acetate and ethanol extracts of *Swertia chirayita* roots, leaves and flower against the multidrug resistant *Pseudmonas aeruginosa:*



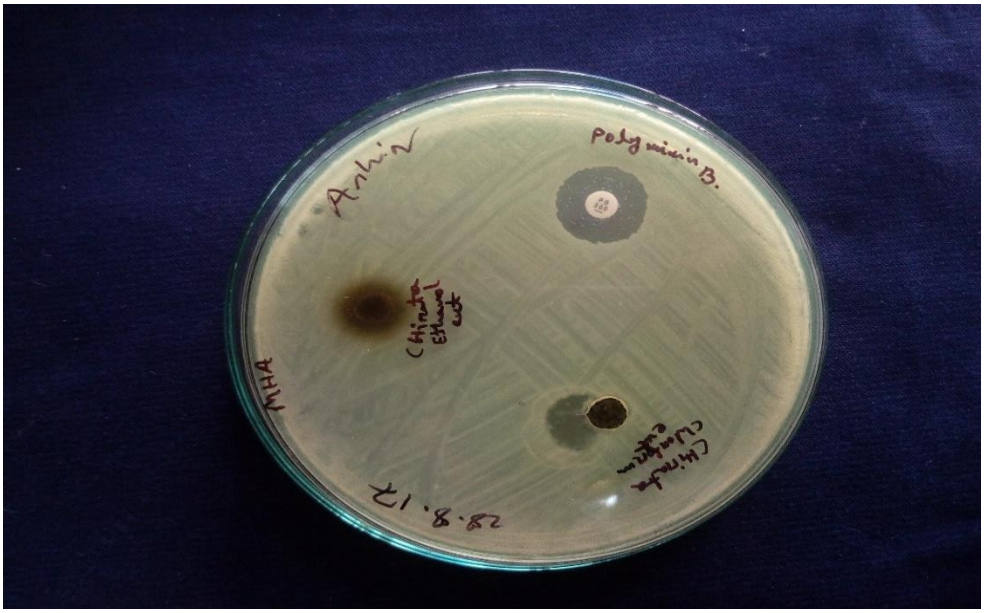




**Figure 11 and 12: Effects of ethyl acetate and ethanol extract of *S. chirayita* in contrast to control Polymyxin B.**

**Table 6: Average diameter of the zone of inhibition of ethyl acetate and ethanol extract of the leaves, roots and flowers of *S. chirayita* on multidrug resistant *Pseudomonas aeruginosa* strain:**

| Sample | Zone of inhibition in diameter (mm) |
|---|---|
| Ethyl Acetate extract of *S. chirayita* | 0 |
| Ethanol extract of *S. chirayita* | 13 |
| Polymyxin B | 17.3 |

**Table 7: Average activity index of ethyl acetate and ethanol extract of the leaves, roots and flowers of *S. chirayita* on resistant *Pseudomonas aeruginosa* strain:**

| Sample | Activity index |
|---|---|
| Ethyl Acetate extract of *S. chirayita* | 0 |
| Ethanol extract of *S. chirayita* | 0.75 |

Table 6, above showed that the ethanol extract of *Swertia chirayita* got antibacterial activity against the resistant *Pseudomonas aeruginosa*. However, the ethyl acetate extract of *Swertia chirayita* did not exhibit any antimicrobial activity. The zone of inhibition in millimeter due to the ethanol extract of *Swertia chirayita* showed found to be quite lower than the control antibiotic polymyxin B. The zone of inhibition of due to the ethanol extract of *Swertia chirayita* was found 13 mm in diameter and the zone of inhibition of the polymyxin B was 17.3 mm in diameter. The activity index of the ethanol extract of *Swertia chirayita* was 0.75 where the activity index of the ethyl acetate extract of *Swertia chirayita* was found 0 (zero). So the ethanol extract of *Swertia chirayita* showed potential moderate antibacterial activity against the multidrug resistant *Pseudomonas aeruginosa*.

### 3.6 Comparisons among the zone of inhibitions and activity indexes of the extracts of *Terminalia chebula, Terminalia arjuna* and *Swertia chirayita:*

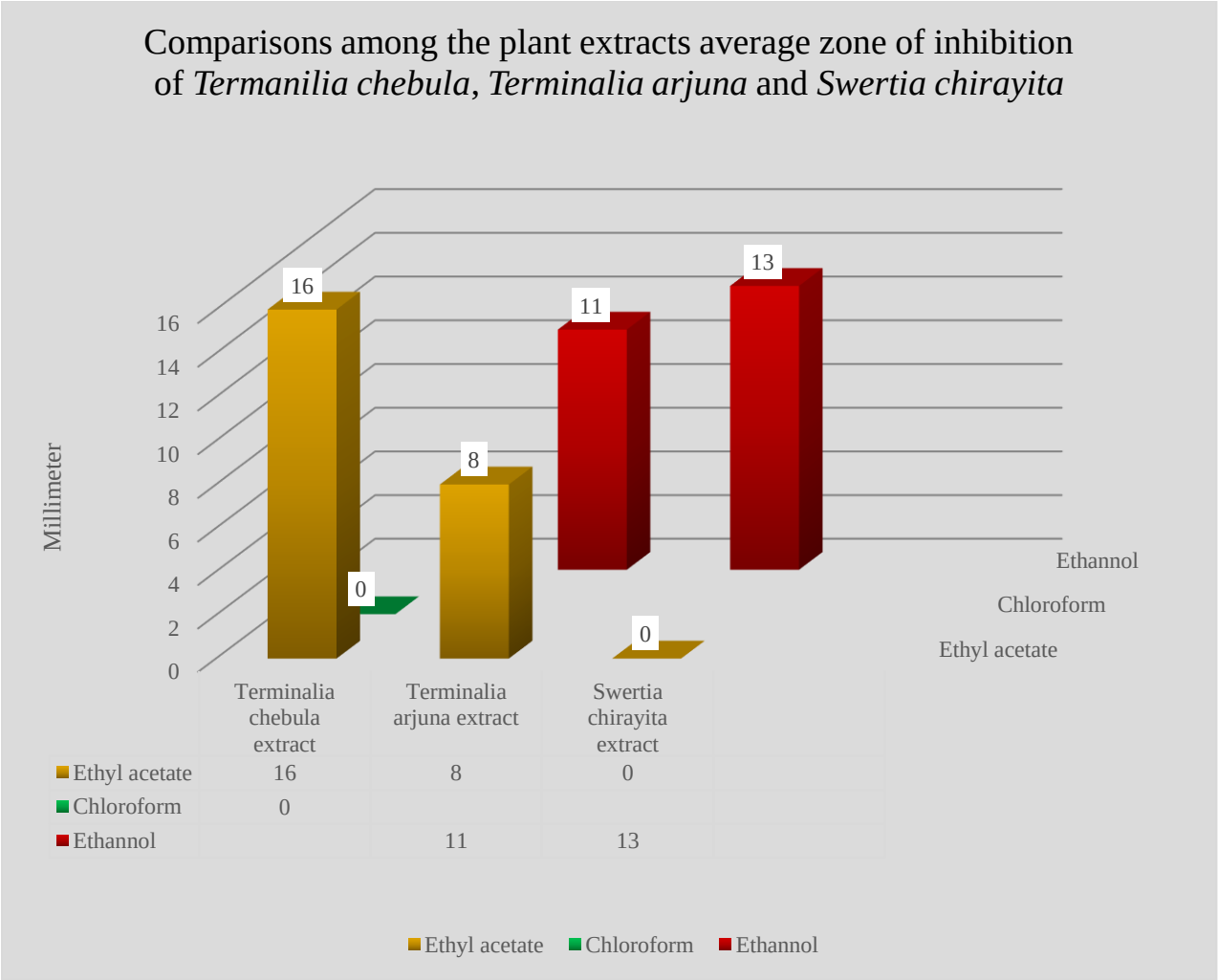



**Figure 13: Comparisons among the average diameter of the zone of inhibition in diameter of ethyl acetate, chloroform and ethanolic extract of the *Terminalia chebula, Terminalia arjuna* and *Swertia chirayita* on multidrug resistant *Pseudomonas aeruginosa* strain in (mm).**

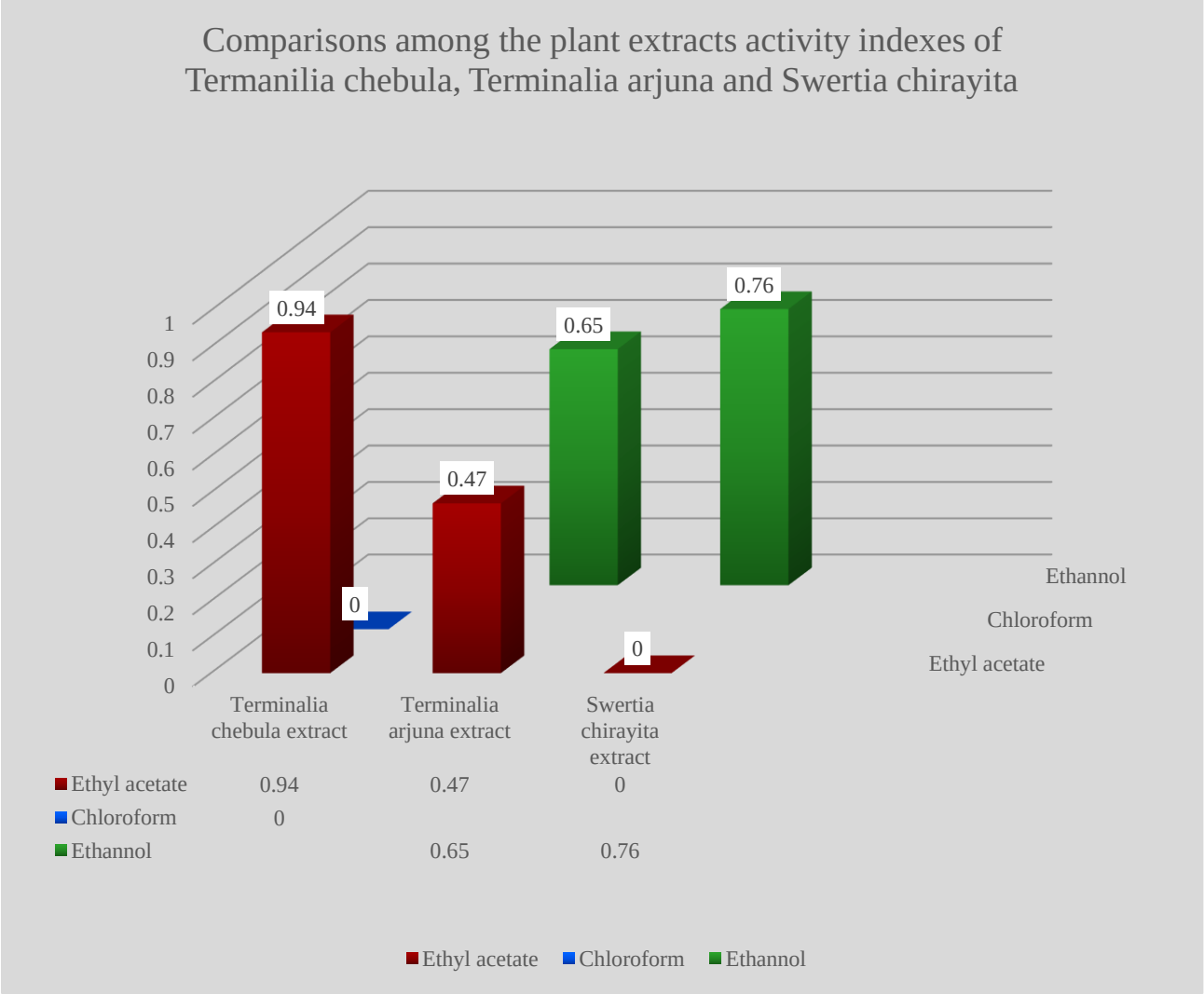



**Figure 14: Comparisons among the average activity indexes of ethyl acetate, chloroform and ethanolic extract of the *Terminalia chebula, Terminalia arjuna* and *Swertia chirayita* on multidrug resistant *Pseudomonas aeruginosa* strain.**

In table 13, most of the extracts exhibited antibacterial activities against the multidrug resistant *Pseudomonas aeruginosa*. However, the chloroform extract of *Terminalia chebula* fruits and the ethyl acetate extract of *Swertia chirayita* roots, leaves and flowers did not show any antimicrobial activity. The overall largest zone of inhibition was observed on resistant *Pseudomonas aeruginosa* by the ethyl acetate extract of *Terminalia chebula* fruits which was 16 mm in diameter. Both the ethyl acetate and ethanol extract of *Terminalia arjuna* barks showed lower antibacterial activity

against the multidrug resistant *Pseudomonas aeruginosa*. Generally the ethanol extracts consistently more effective than the other solvents.

In comparisons to the activity indexes in the figure 14, the overall highest activity was observed on resistant *Pseudomonas aeruginosa* by the ethyl acetate extract of *Terminalia chebula* fruits which was 0.94. So it can be considered that the ethyl acetate extract of *Terminalia chebula* fruits showed almost as similar activities as reference antibiotic polymyxin B.

## 3.7 Determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Polymyxin B and the ethyl acetate extract of *Terminalia chebula* fruits:

**Table 8: The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of polymyxin B on multidrug resistant *Pseudomonas aeruginosa* strain:**

| Volume Of Antibiotics (ml) | Undiluted Volume (BHI ) (ml) | Inoculum Added From Saline (ml) | Total Diluted Volume (ml) | Concentration Of Antibiotic Per ml After Dilution (mg) | Concentration Of Antibiotic Per 5 ml After Dilution (mg) | Approximate Concentration Of *Pseudomonas aeruginosa* Inoculum Transferred CFU/0.1 ml |
|---|---|---|---|---|---|---|
| 0.5 | 4.4 | 0.1 | 5 | 0.1 | 0.5 | $3\times10^7$ |
| 1 | 3.9 | 0.1 | 5 | 0.2 | 1 | $3\times10^7$ |
| 2 | 2.9 | 0.1 | 5 | 0.4 | 2 | $3\times10^7$ |
| 3 | 1.9 | 0.1 | 5 | 0.6 (MIC) | 3 | $3\times10^7$ |
| 4 | 0.9 | 0.1 | 5 | 0.8 (MBC) | 4 | $3\times10^7$ |

**Key: BHI = Brain heart infusion broth, CFU= Colony forming units.**

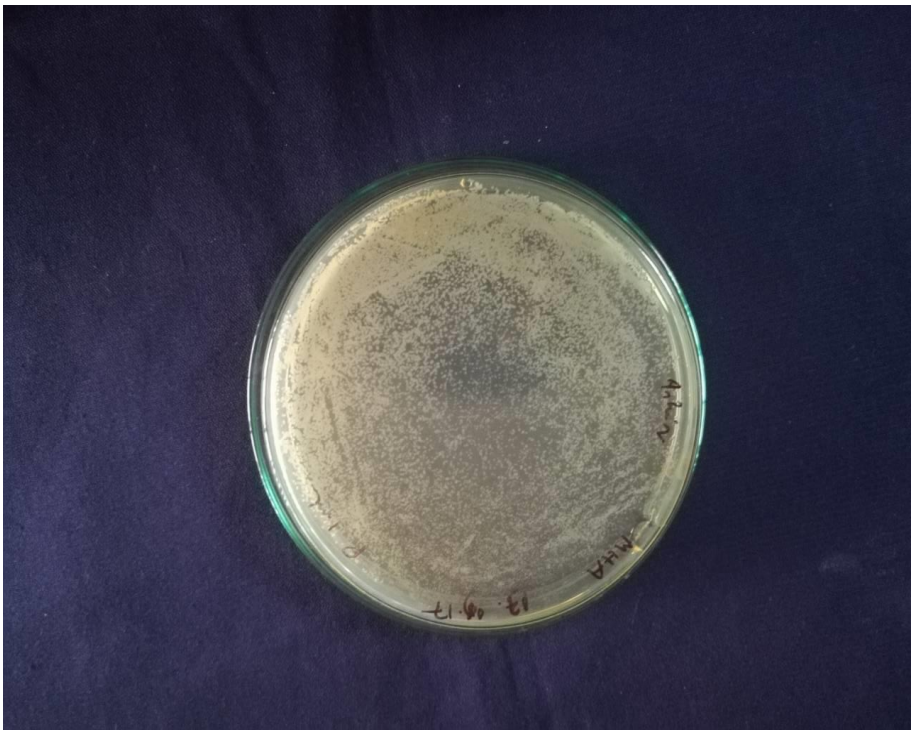
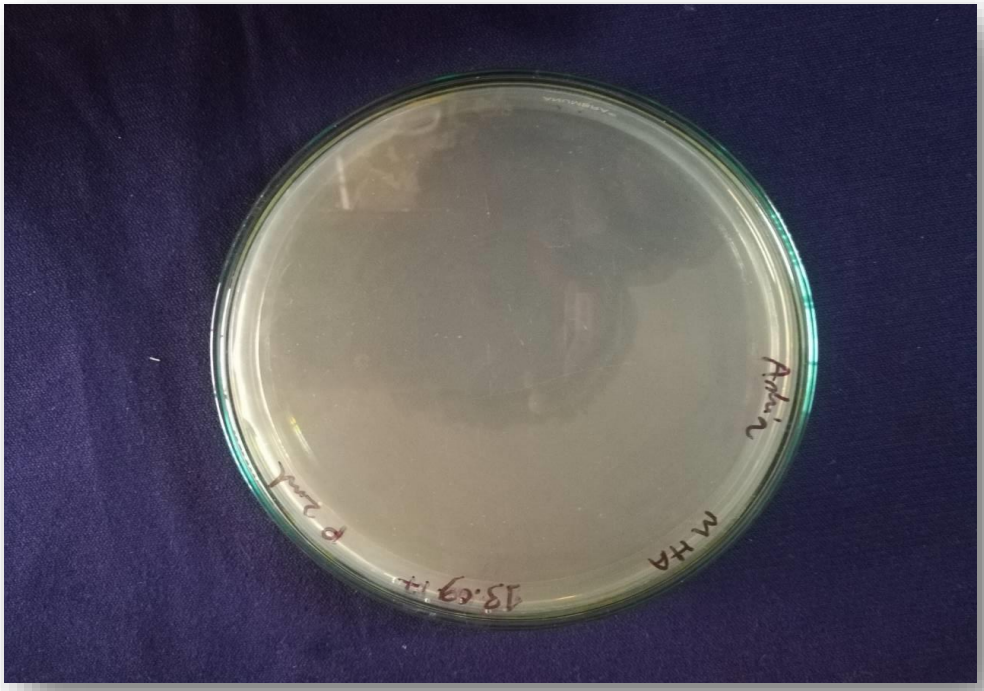


**Figure 15 and 16: The growth of resistant multidrug *Pseudomonas aeruginosa* treated with 0.1 mg per ml and 0.2 mg per ml concentration of polymyxin B when spread plated on agar plates diluted from 5 ml BHI broth.**



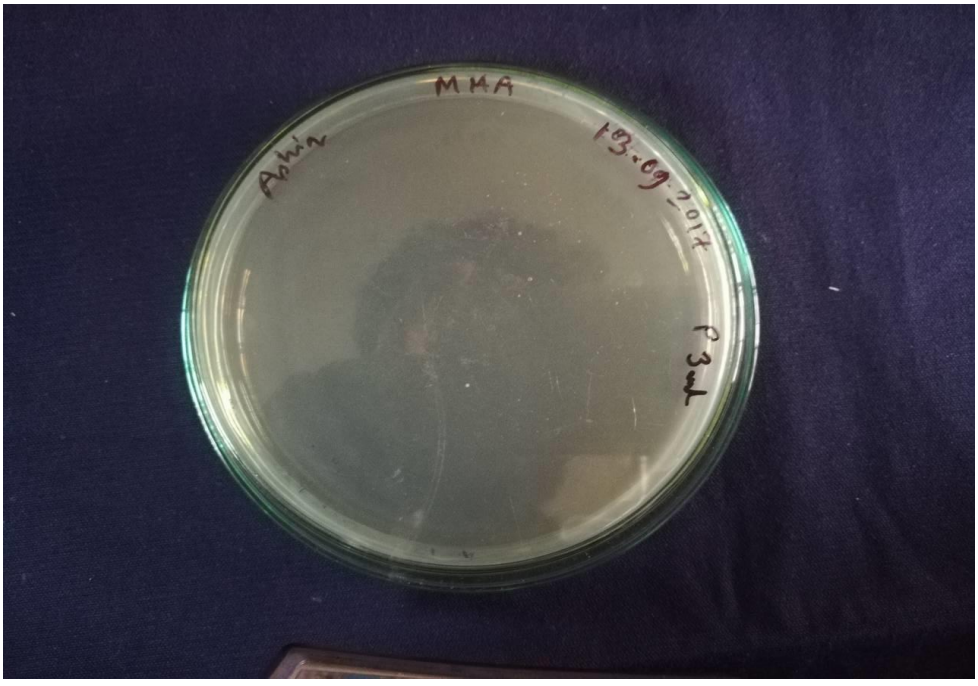




**Figure 17 and 18: The growth of multidrug resistant *Pseudomonas aeruginosa* treated with 0.4 mg per ml and 0.6 mg per ml concentration of Polymyxin B when spread plated on agar plates from diluted 5 ml BHI broth.**

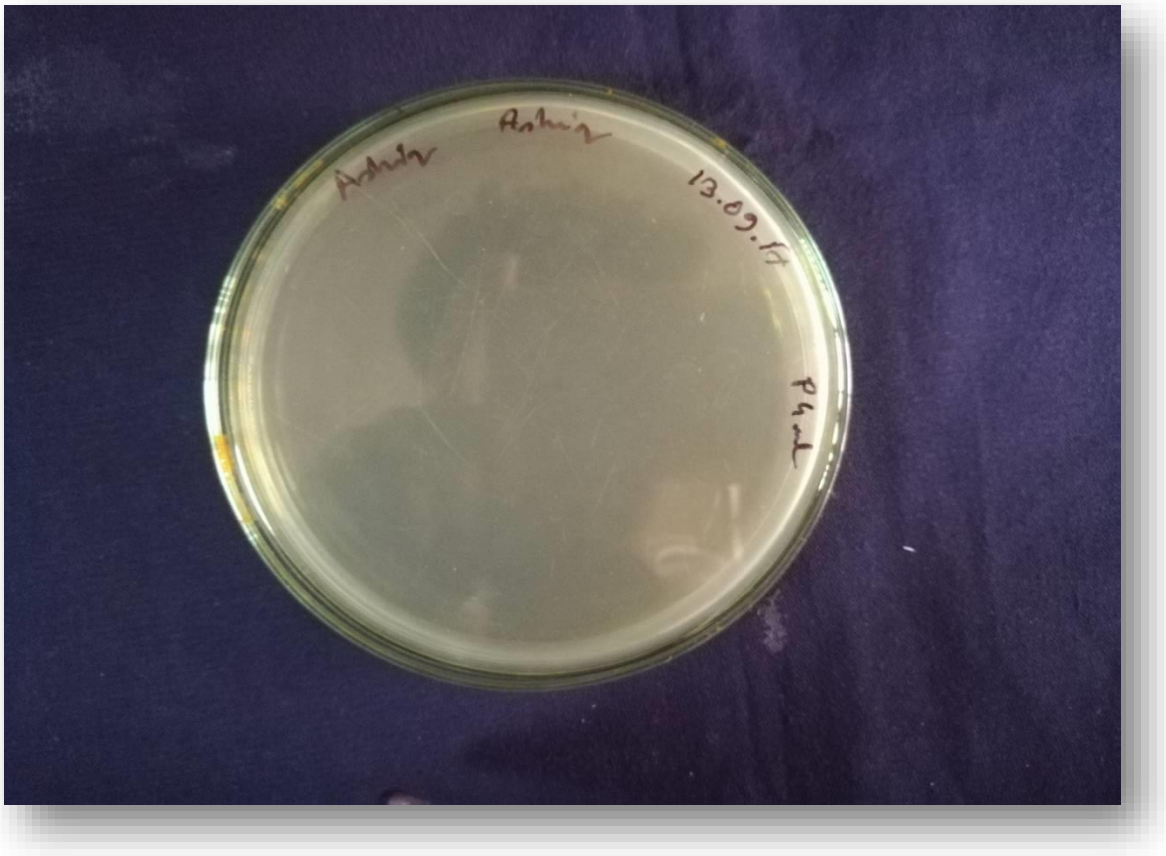


**Figure 19: The growth of multidrug resistant *Pseudomonas aeruginosa* treated with 0.8 mg per ml concentration of Polymyxin B when spread plated on agar plates from diluted 5 ml BHI broth.**

**Outcome:** The minimum inhibitory concentration (MIC) of Polymyxin B was found to be 0.6 mg per ml and when spread plated on the MHA agar media the minimum bactericidal concentration (MBC) was found to be 0.8 mg per ml.

**Table 9: The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination of the ethyl acetate extract of *Terminalia chebula* fruits for the multidrug resistant *Pseudomonas aeruginosa* strain:**

| Volume Of Ethyl Acetate Extract of Terminalia Chebula (BHI + 2.5% DMSO + Extract) (ml) | Undiluted Volume Only (BHI ) (ml) | Inoculum Added From Saline (ml) | Final Volume (ml) | Concentration Of ethyl acetate extract of *T. chebula* fruits Per ml After Dilution (mg) | Concentration Of ethyl acetate extract of *T. chebula* fruits Per 10 ml After Dilution (mg) | Approximate Concentration Of *Pseudomonas aeruginosa* Inoculum Transferred CFU/0.1 ml |
|---|---|---|---|---|---|---|
| 0.05 | 9.85 | 0.1 | 10 | 0.2 | 2 | $3\times10^7$ |
| 0.1 | 9.8 | 0.1 | 10 | 0.4 | 4 | $3\times10^7$ |
| 0.15 | 9.75 | 0.1 | 10 | 0.6 | 6 | $3\times10^7$ |
| 0.2 | 9.7 | 0.1 | 10 | 0.8 | 8 | $3\times10^7$ |
| 0.3 | 9.6 | 0.1 | 10 | 1.2 | 12 | $3\times10^7$ |
| 0.4 | 9.5 | 0.1 | 10 | 1.6 | 16 | $3\times10^7$ |
| 0.5 | 9.4 | 0.1 | 10 | 2 | 20 | $3\times10^7$ |
| 0.8 | 9.1 | 0.1 | 10 | 3.2 | 32 | $3\times10^7$ |
| 1 | 8.9 | 0.1 | 10 | 4 | 40 | $3\times10^7$ |
| 1.5 | 8.4 | 0.1 | 10 | 6 | 60 | $3\times10^7$ |
| 2.2 | 7.7 | 0.1 | 10 | 8.8 | 88 | $3\times10^7$ |
| 2.5 | 7.4 | 0.1 | 10 | 10 | 100 | $3\times10^7$ |
| 3 | 6.9 | 0.1 | 10 | 12 | 120 | $3\times10^7$ |
| 3.75 | 6.15 | 0.1 | 10 | 15 | 150 | $3\times10^7$ |
| 4.5 | 5.4 | 0.1 | 10 | 18 | 180 | $3\times10^7$ |
| 5 | 4.9 | 0.1 | 10 | 20 | 200 | $3\times10^7$ |
| 6.25 | 3.65 | 0.1 | 10 | 25 (MIC) | 250 | $3\times10^7$ |
| 7.5 | 3.4 | 0.1 | 10 | 30 (MBC) | 300 | $3\times10^7$ |

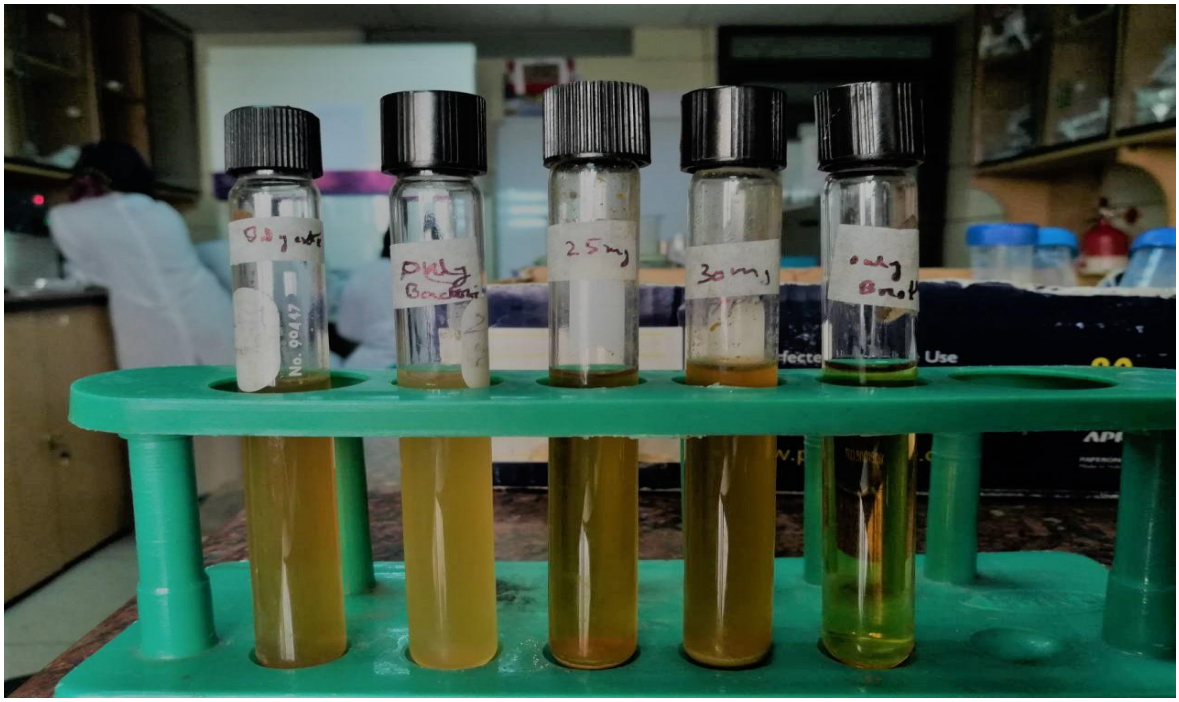



**Figure 20: Determination of the minimum inhibitory concentration (MIC) of the ethyl acetate extract of *Terminalia chebula* via serial dilution.**

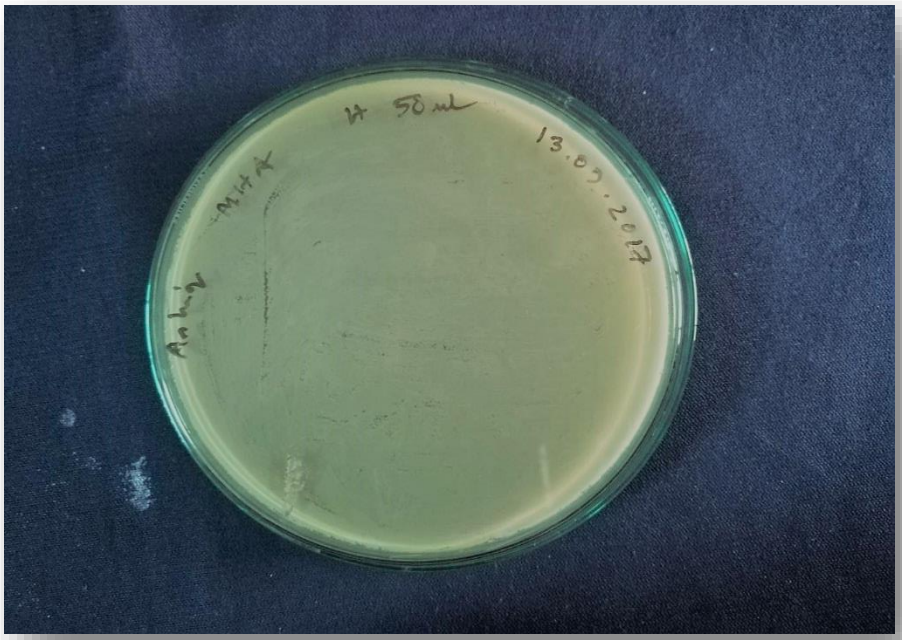
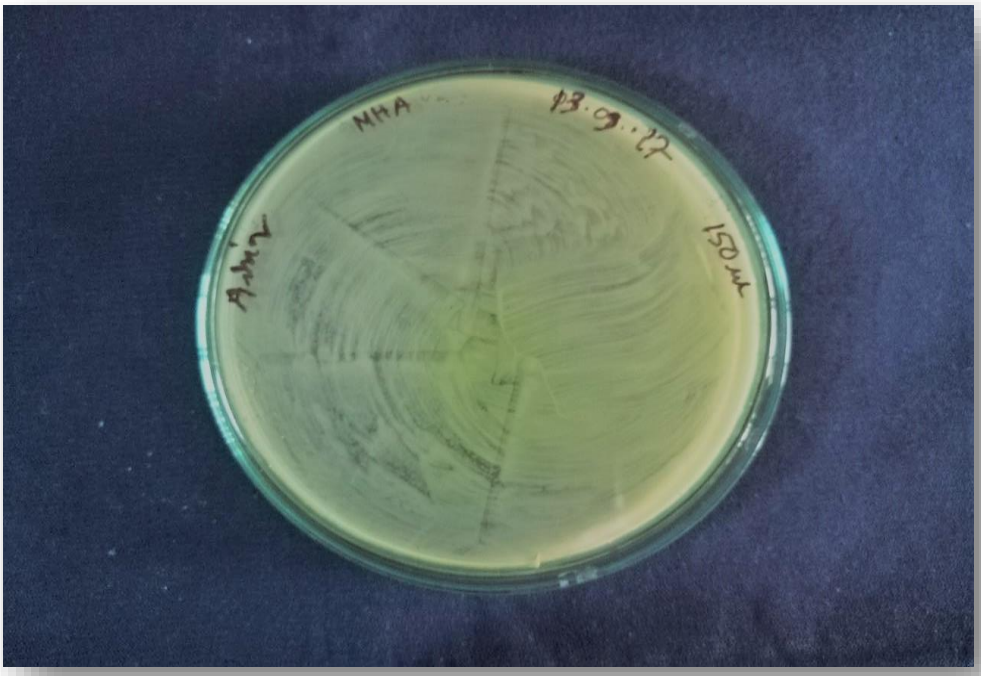



**Figure 21: The growth of multidrug resistant *Pseudomonas aeruginosa* treated with 0.2 mg per ml concentration of ethyl acetate extract of *Terminalia chebula* fruits when spread plated on agar plates from diluted 10 ml BHI broth.**



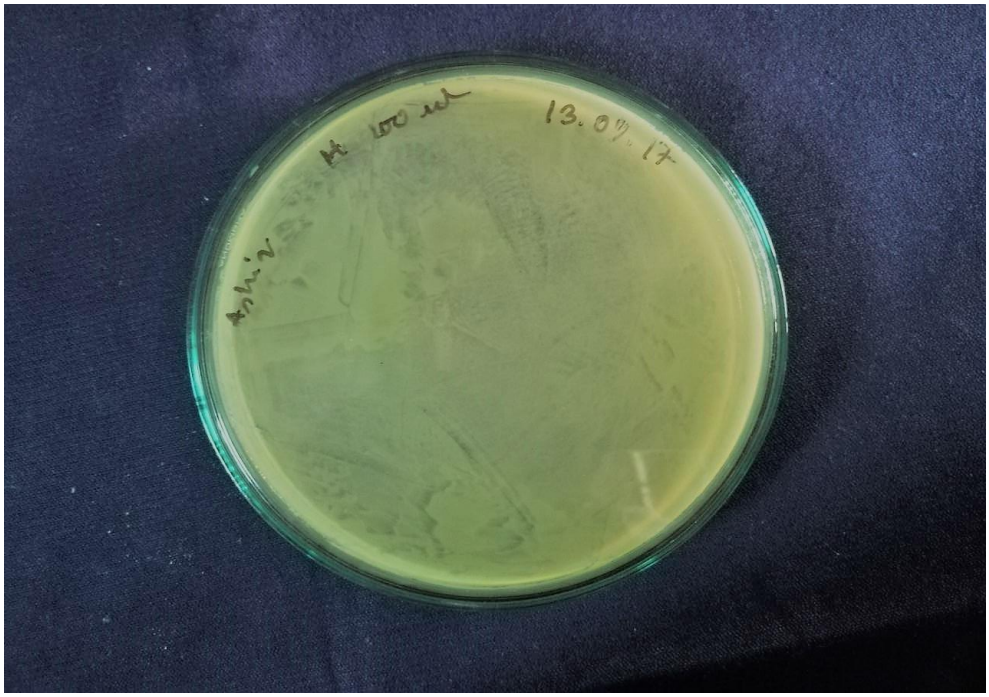




**Figure 22 and 23: The growth of multidrug resistant *Pseudomonas aeruginosa* treated with 0.4 mg (up) and 0.6 mg (down) per ml concentration of Ethyl acetate extract of *Terminalia chebula* fruits when spread plated on agar plates from diluted 10 ml BHI broth.**

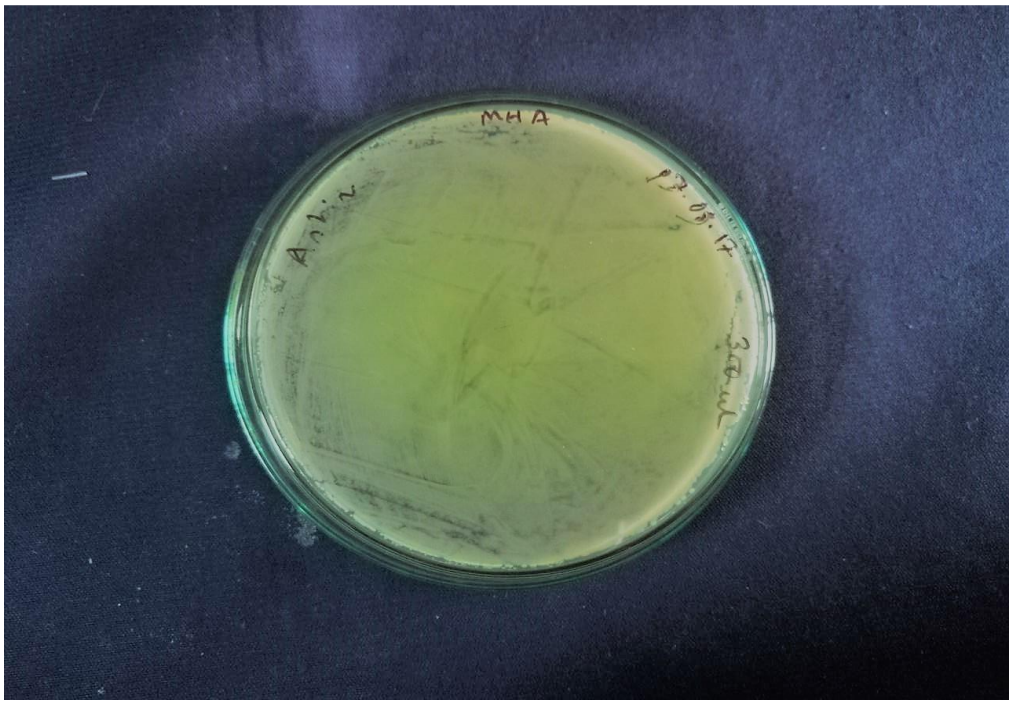
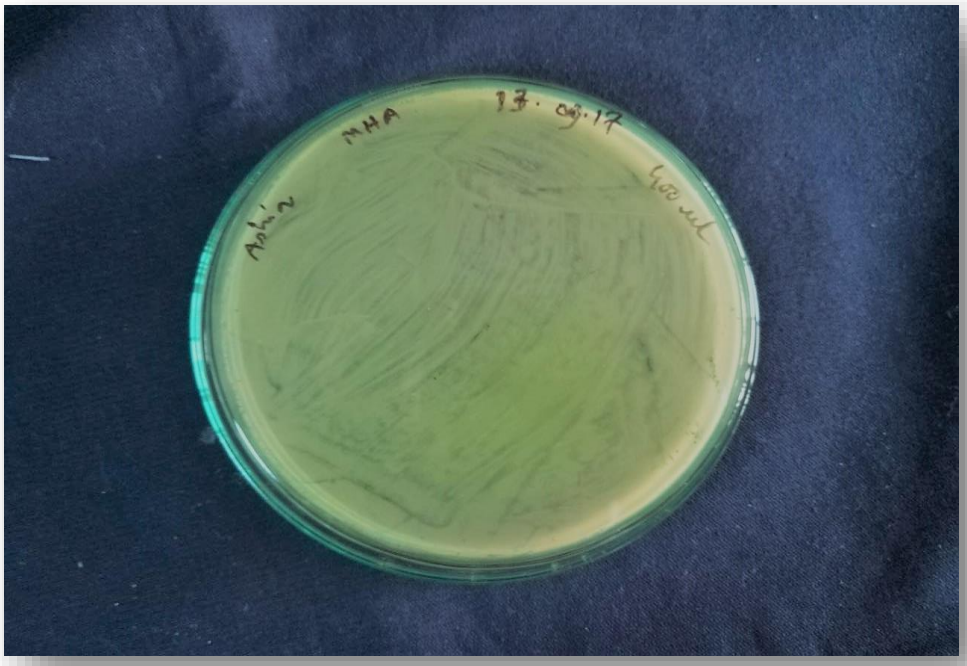




**Figure 24 and 25: The growth of multidrug resistant *Pseudomonas aeruginosa* treated with 0.8 mg (up) and 1.2 mg (down) per ml concentration of ethyl acetate extract of *Terminalia chebula* fruits when spread plated on agar plates from diluted 10 ml BHI broth.**



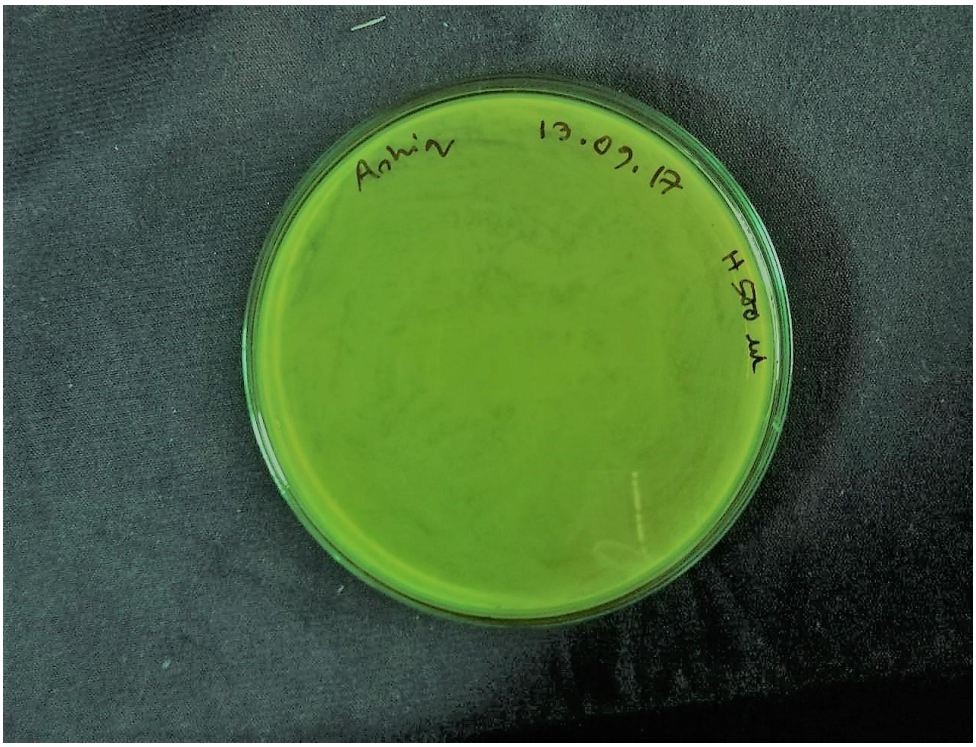
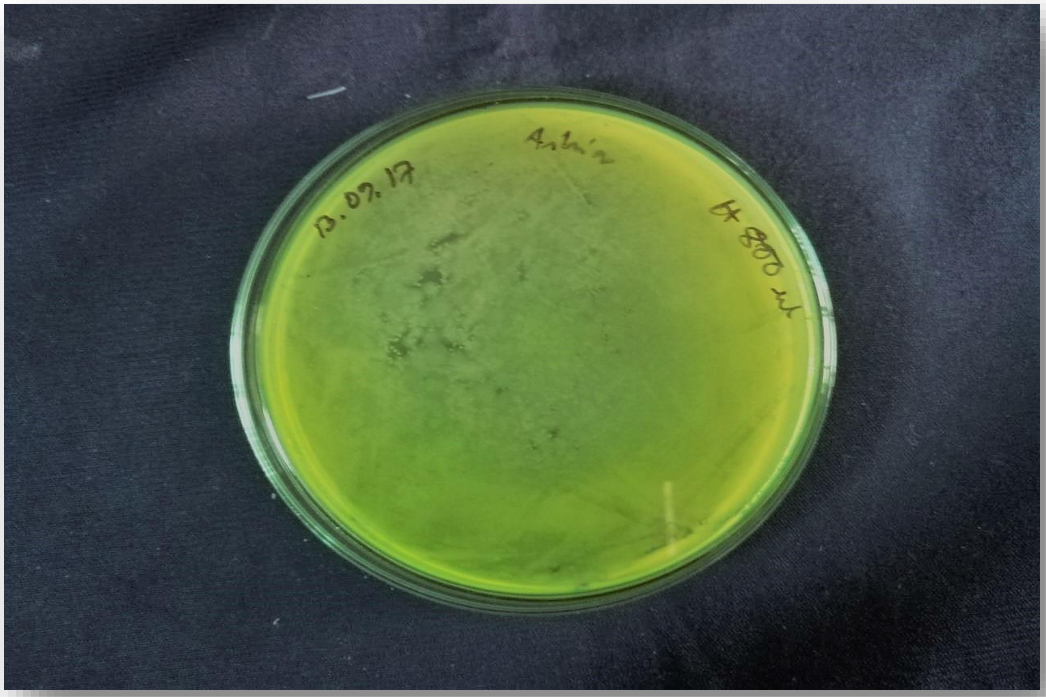




**Figure 26 and 27: The growth of multidrug resistant *Pseudomonas aeruginosa* treated with 1.6 mg (up) and 2 mg (down) per ml concentration of ethyl acetate extract of *Terminalia chebula* fruits when spread plated on agar plates from diluted 10 ml BHI broth.**



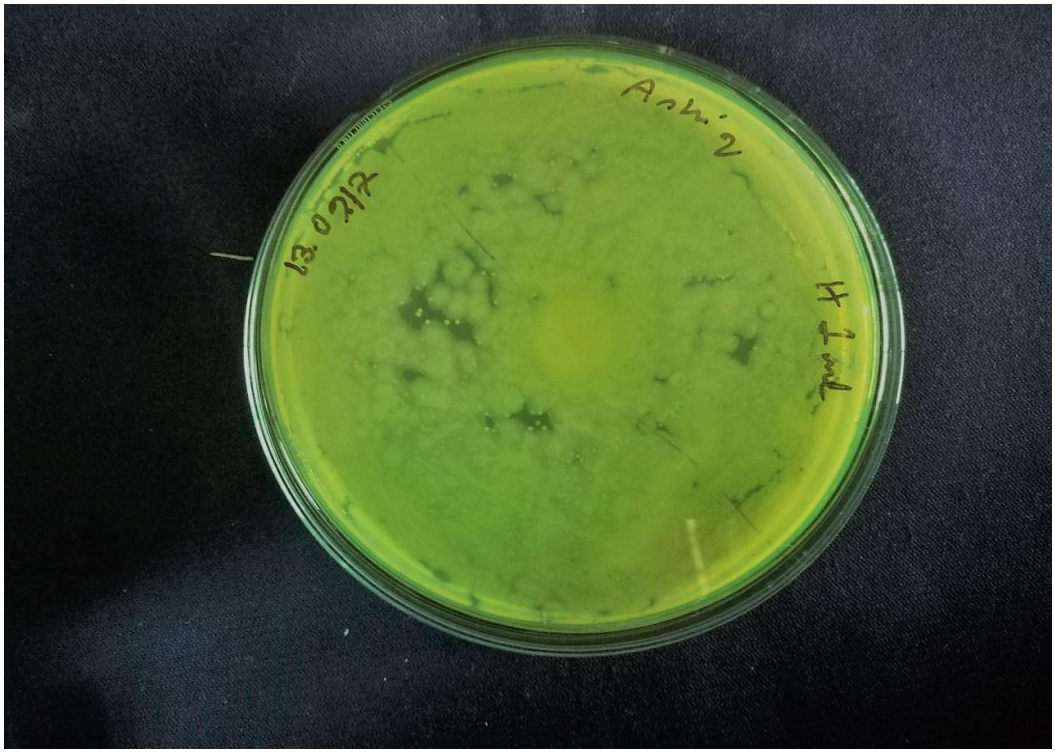


**Figure 28 and 29: The growth of multidrug resistant *Pseudomonas aeruginosa* treated with 3.2 mg (up) and 4 mg (down) per ml concentration of ethyl acetate extract of *Terminalia chebula* fruits when spread plated on agar plates from diluted 10 ml BHI broth.**

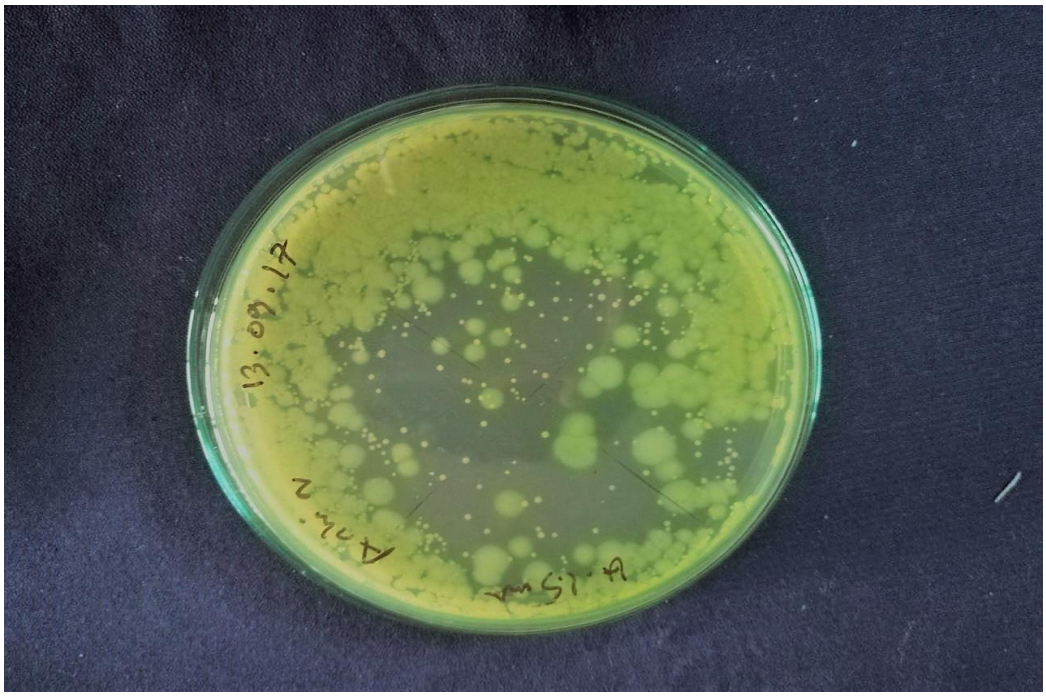
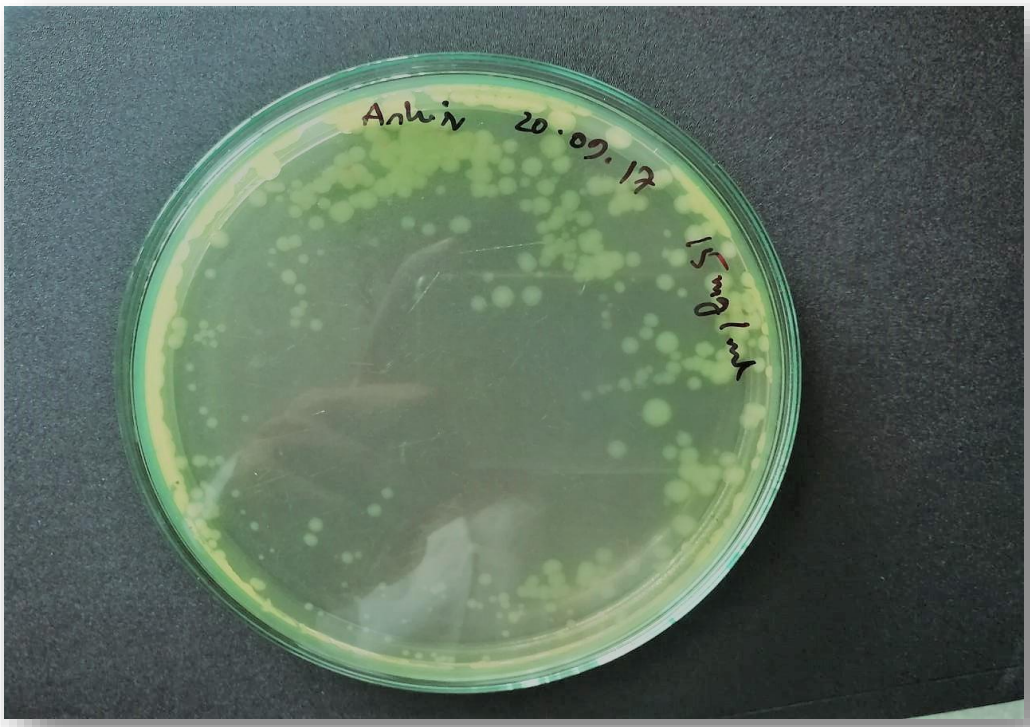


**Figure 30 and 31: The growth of multidrug resistant *Pseudomonas aeruginosa* treated with 6 mg (up) and 8.8 mg (down) per ml concentration of ethyl acetate extract of *Terminalia chebula* fruits when spread plated on agar plates from diluted 10 ml BHI broth.**

**Figure 32 and 33: The growth of multidrug resistant *Pseudomonas aeruginosa* treated with 10 mg (up) and 12 mg (down) per ml concentration of ethyl acetate extract of *Terminalia chebula* fruits when spread plated on agar plates from diluted 10 ml BHI broth.**



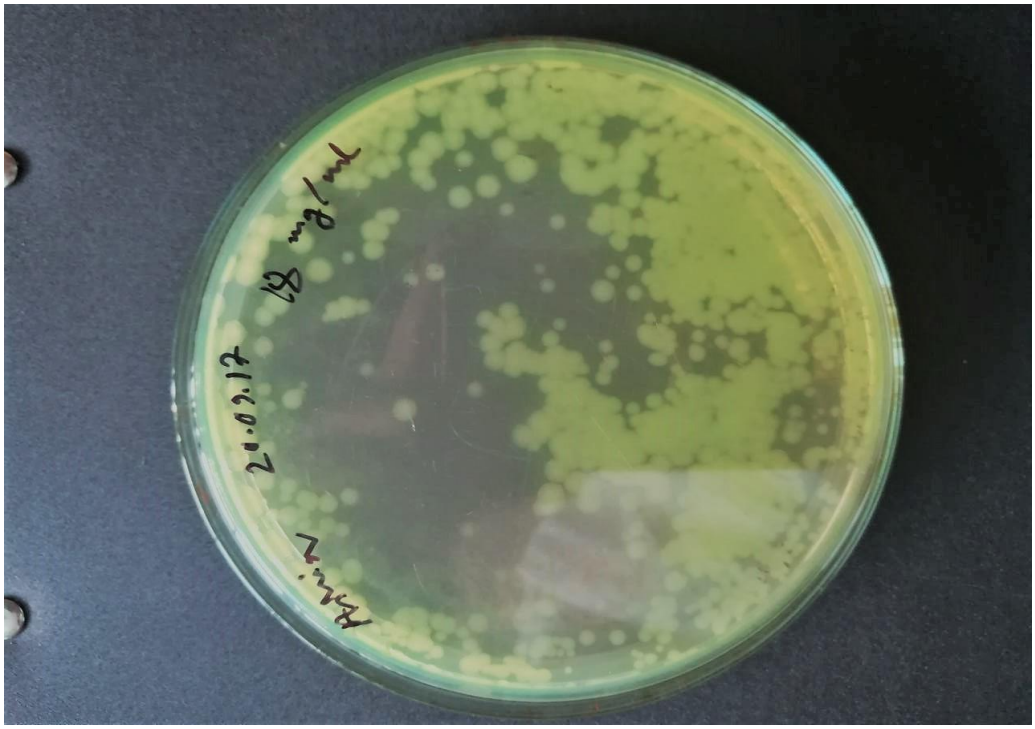




**Figure 34 and 35: The growth of multidrug resistant *Pseudomonas aeruginosa* treated with 15 mg (up) and 18 mg (down) per ml concentration of ethyl acetate extract of *Terminalia chebula* fruits when spread plated on agar plates from diluted 10 ml BHI broth.**

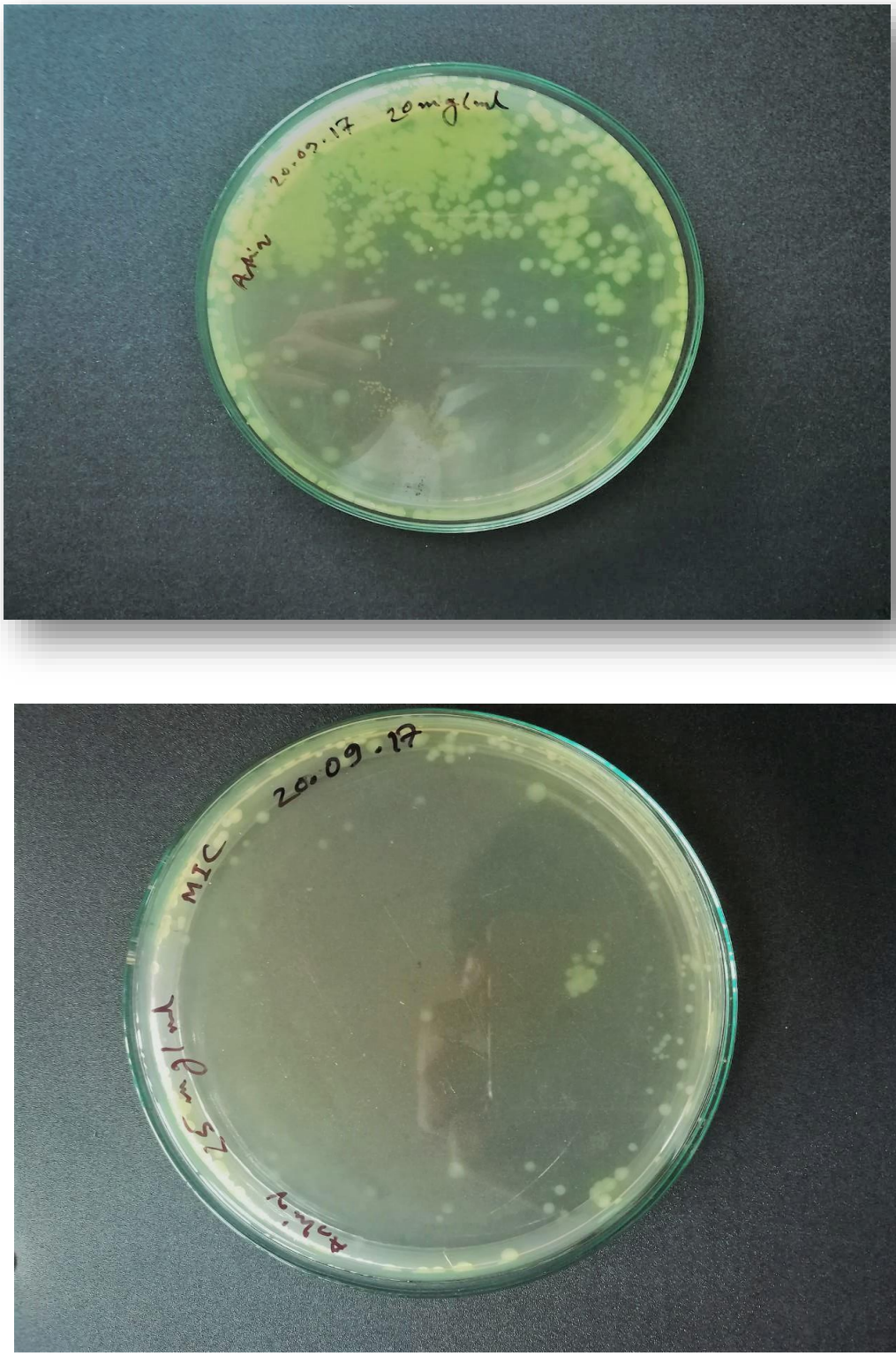



**Figure 36 and 37: The growth of multidrug resistant *Pseudomonas aeruginosa* treated with 20 mg (up) and 25 mg (down) per ml concentration of ethyl acetate extract of *Terminalia chebula* fruits when spread plated on agar plates from diluted 10 ml BHI broth.**

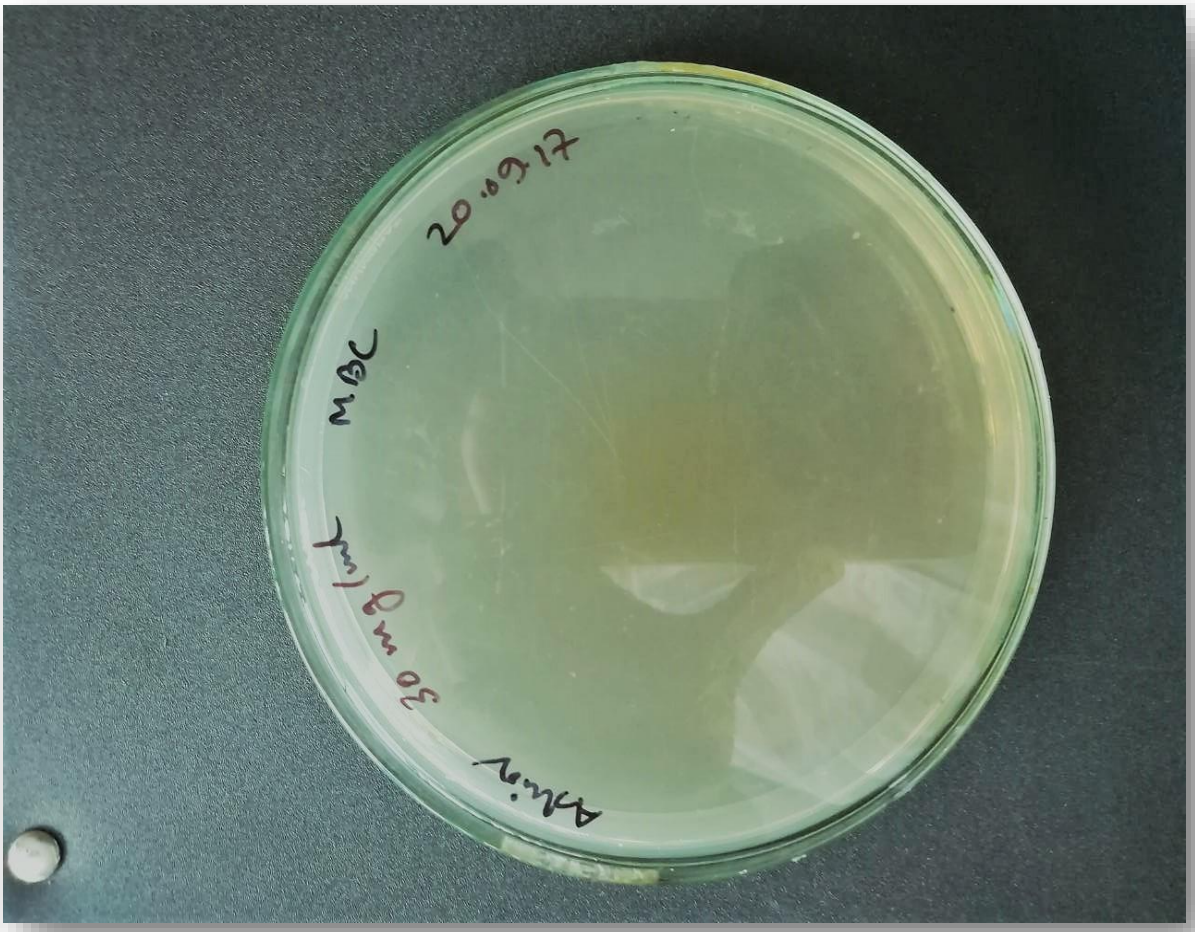
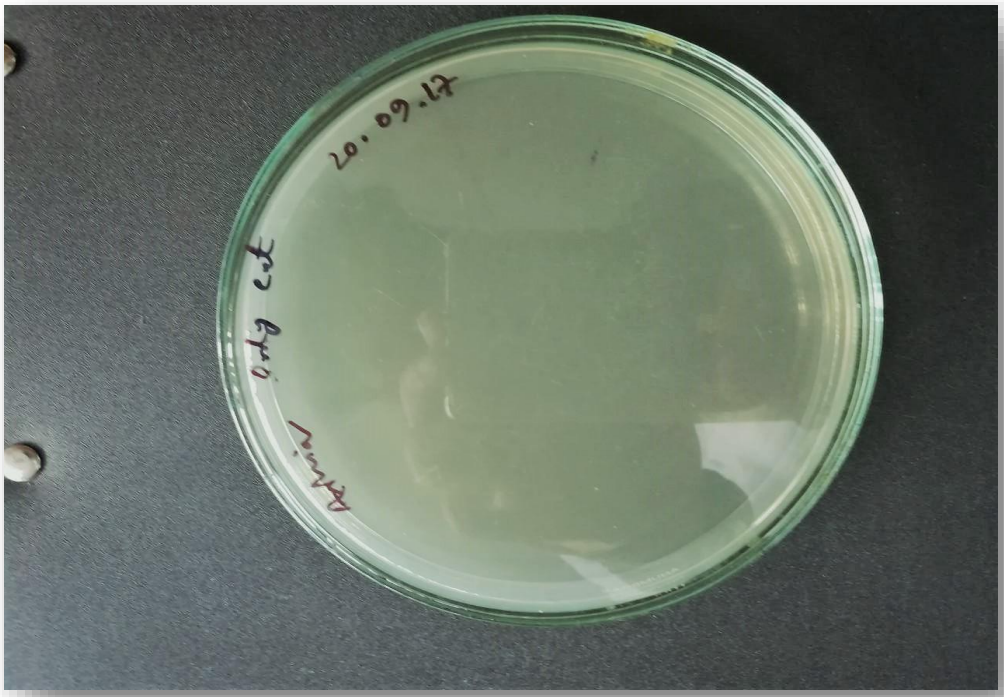



**Figure 38: The growth of multidrug resistant *Pseudomonas aeruginosa* treated with 30 mg per ml concentration of ethyl acetate extract of *Terminalia chebula* fruits when spread plated on agar plates from diluted 10 ml BHI broth.**



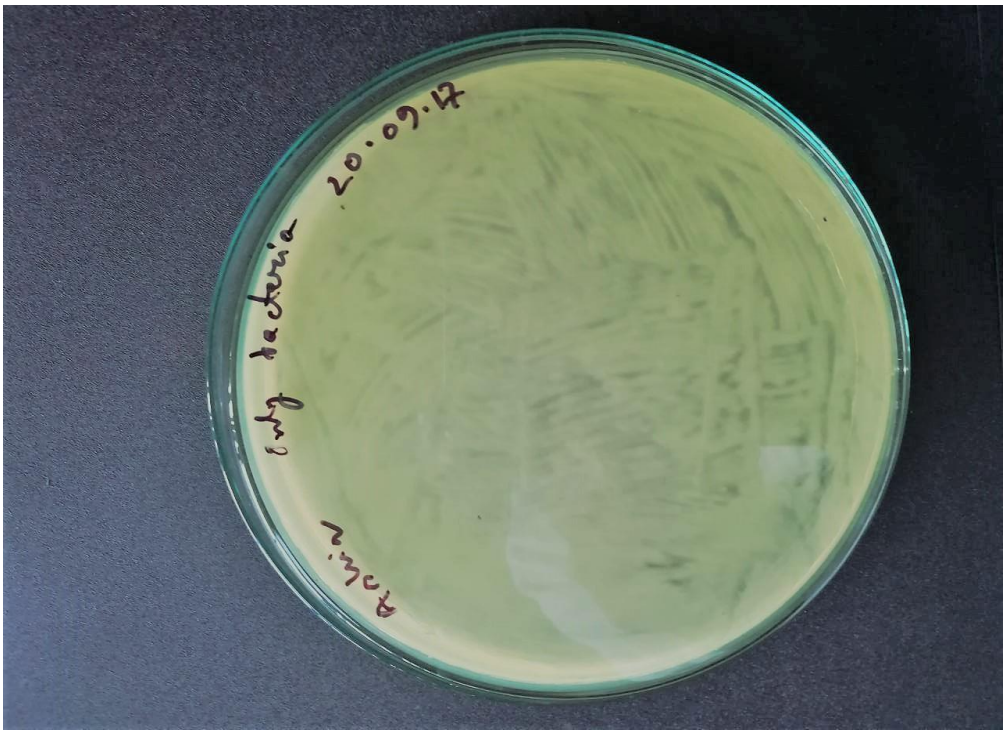




**Figure 39 and 40: The growth of multidrug resistant Pseudomonas aeruginosa treated with negative control (up) and growth control (down) tubes when spread plated on agar plates from diluted 10 ml BHI broth.**

**Outcome:** The minimum inhibitory concentration (MIC) of the ethyl acetate extract of *Terminalia chebula* fruits was found to be 25 mg per ml and when spread plated on the MHA agar media the minimum bactericidal concentration (MBC) was found found to be 30 mg per ml.

## 3.8 Comparisons among minimum inhibitory concentration and minimum bactericidal concentration between polymyxin B and the ethyl acetate extract of *Terminalia chebula:*

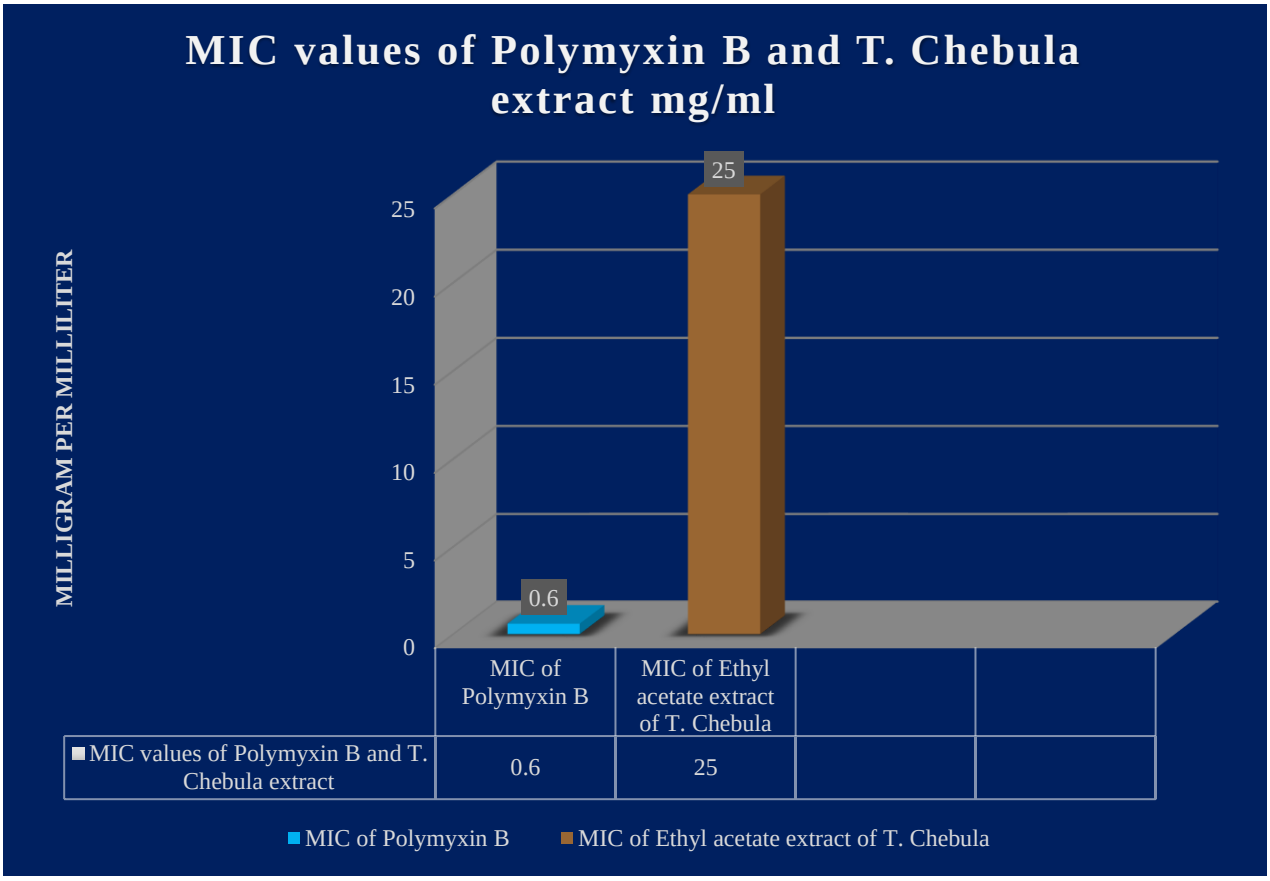



**Figure 41: Comparisons among minimum inhibitory concentrations between polymyxin B and the ethyl acetate extract of *Terminalia chebula* fruits.**

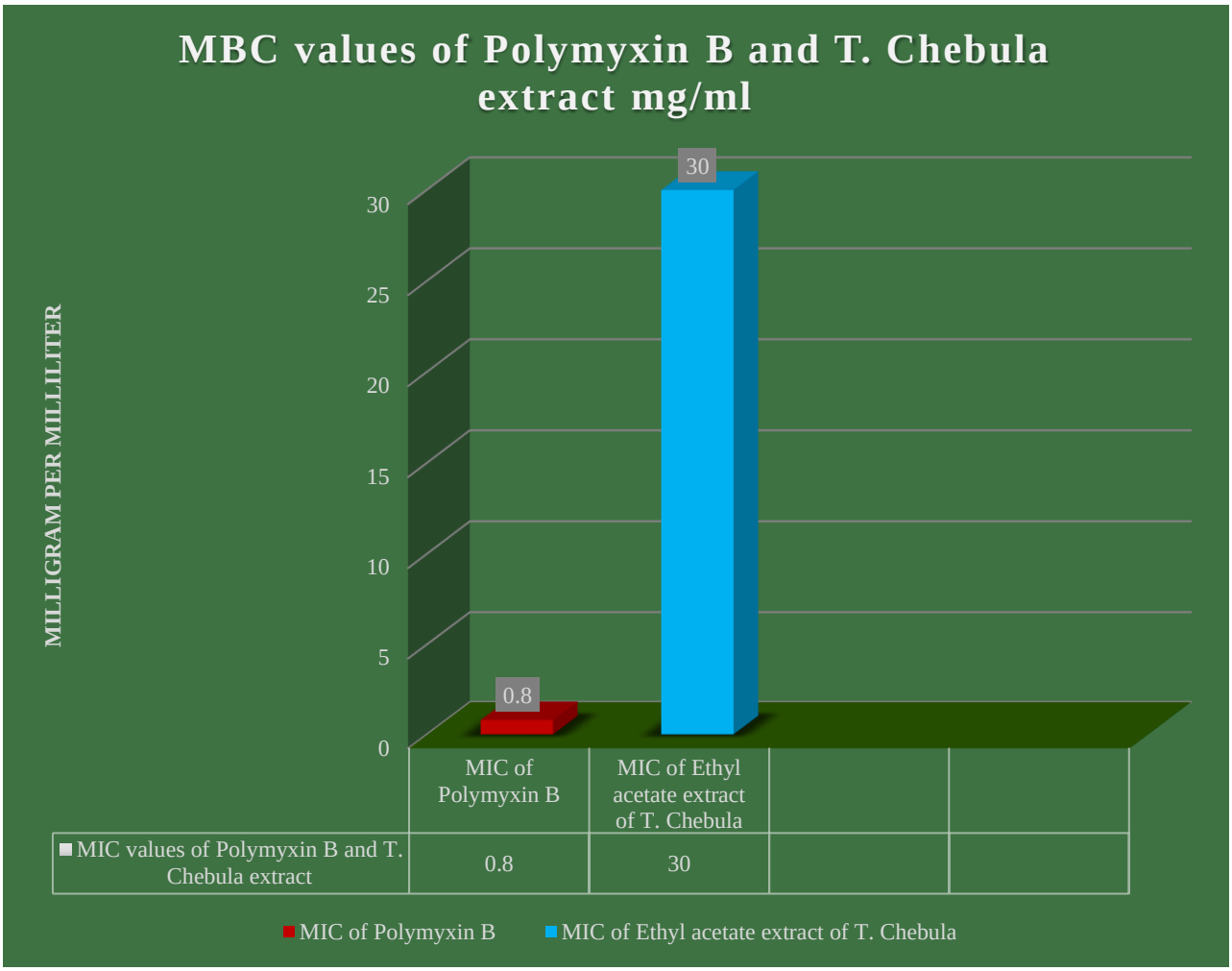



**Figure 42: Comparisons among minimum bactericidal concentrations between polymyxin B and the ethyl acetate extract of *Terminalia chebula* fruits.**

From figure 41, the minimum inhibitory concentration (MIC) of polymyxin B and the ethyl acetate extract of *Terminalia chebula* fruits was found to be 0.6 and 25 mg per ml respectively against the multidrug resistant *Pseudomonas aeruginosa*. The MIC value of ethyl acetate extract of *Terminalia chebula* fruits was found almost 40 times higher than the polymyxin B this is because the antibacterial compound within the ethyl acetate extract of *Terminalia chebula* fruits was mixed with other phytochemical compounds and it was not as purified as polymyxin B.

Figure 42 showed that, the minimum bactericidal concentration (MIC) of polymyxin B and the ethyl acetate extract of *Terminalia chebula* fruits was found to be 0.8 and 30 mg per ml respectively against the multidrug resistant *Pseudomonas aeruginosa* while spread platting on the agar plates.

# CHAPTER 4: Discussion

Incurable bacteria that resist most of the antibiotics are continuing to spread worldwide and it is becoming an alarming incident for us. The antibiotic resistance among the bacteria are increasing and getting worse day to day. Recently it was found that, a woman who died in September, 2016 at Nevada, USA last August was infected with *Klebsiella* bacteria which was resistant to 26 different antibiotics, the bacteria was resistant to all available antimicrobial drugs in the USA reported by The US Centre for Disease Control and Prevention (New Scientist magazine, 2017). They further reported in USA alone 23000 deaths occur due to the direct result antibiotic resistance to infections (CDC, 2013).

The antibiotic resistance among South Asian countries is increasing. In a study it was found that one of the major factor of increasing antibiotic resistance is the overuse and misuse of antibiotics which is common practice in the countries like India, Pakistan, Bangladesh and Sri Lanka (Bajwa, 2015). In Bangladesh, antibiotic usage stemming from local pharmacies is high and approximately 90% of antibiotic are sold without Bangladesh (Morgan et al., 2011).

*Pseudomonas aeruginosa* is a motile, non-fermenting Gram-negative bacterium, an opportunistic pathogen that can cause respiratory infections, urinary tract infections, gastrointestinal infections, keratitis, otitis media, and bacteremia in patients with compromised host defenses [e.g., cancer, burn, HIV, and cystic fibrosis (CF)]. *Pseudomonas aeruginosa* has multifactorial mechanisms of responses and resistance to antimicrobials and to develop novel class antimicrobials is very difficult for *P. aeruginosa* because of the presence of low membrane permeability and the RND (Resistance-Nodulation-Division) multidrug efflux pumps. In addition this organism has the ability to adapt to various stresses, including sub-inhibitory antimicrobial exposure, by recruiting antimicrobial resistance mechanisms, notably that of RND efflux systems such as the MexXY system (Morita et al., 2010; Morita et al., 2012a).

In this study, it was found that *Pseudomonas aeruginosa* showed resistance to 28 antibiotics. Previously it was found that the same organism showed resistance against 20 tested antibiotics (Azam, 2017). There are several reports of increasing resistance pattern of *Pseudomonas aeruginosa* which was found on different papers and they were mainly the clinical and livestock isolates. In Nepal, One hundred ninety four isolates were identified as *P. aeruginosa* which was isolated from pus/wound, sputum, urine, tracheal aspirates, central venous catheter tip, broncho-alveolar lavage fluid, catheters and vaginal swab involving 917 patients. Among 194 isolates

resistance to Chloramphenicol 144 (74.23%), Ceftriaxone 135 (69.56%), Cefepime 111 (57.22%), Cefoperazone-Salbactum 105 (54.12%) and Co-trimoxazole 103 (53.02%) was observed and another 48 (24.74%) of *P. aeruginosa* isolates were multi-drug resistant to >3rd generation classes of antibiotics (Khan et al, 2014). In another study in Bangladesh it was shown that, the resistance pattern of *Pseudomonas aeruginosa* constantly increasing. The study suggested that, the rate of resistance (R%) for azithromicin was 100%, cefixime 93.3%, cefrtiaxone and ceftazidime was 86%, ciprofloxacin 75.5% and amikacin 22.7% (Rashid et al., 2007). In previous study prior to this study during (2000-2001), in Bangladesh, it was found that, (R%) of *P. aeruginosa* to co-trimoxazole was 92%, ciprofloxacin 62.5%, cephalexin 100%, ceftriaxone 75% and ceftazidime 37% (Kawser et al., 2002) and the resistance to amikacin was 2%, ceftriaxone 43%, ceftazidime 25% and ciprofloxacin 50% (Wadud et al., 2004). The *Pseudomonas aeruginosa* that isolated from cattle in Bangladesh was found highly resistant to Ampicillin and Oxytetracycline followed by Tetracycline and Amoxicillin (Hossain et al., 2013).

So all these studies suggest that the resistance among the different strains of *Pseudomonas aeruginosa.* However in this study, we have found that the resistant pattern of the reference multidrug resistant *Pseudomonas aeruginosa* has surpassed all the previous records.

Plants are mainly rich in secondary metabolites. The most common metabolites are tannins, flavonoids, tarpenoids and alkaloids which has broad spectrum of antimicrobial activities. (WHO) World Health Organization found around 80% of the population of the globe relies on traditional herbal medicines as part of healthcare (WHO, 1993).

In several articles it was found that Haritaki (*Terminalia chebula),* Arjun *(Terminalia arjuna)* and Chirata *(Swertia Chirayita)* extracts have the potential antimicrobial activity against different bacterial strains. However, in this study the extracts of Haritaki fruits (*Terminalia chebula),* Arjun barks *(Terminalia arjuna)* and Chirata roots, leaves and flowers *(Swertia chirayita)* showed the potential antimicrobial activity against a multidrug resistant *Peudomonas aeruginosa* strain.

In the study of the antibacterial activity of *Terminalia chebula,* it was found that the gallic acid and ethyl ester against methicillin-resistant *Staphylococcus,* have been isolated from ethyl alcohol extract of fruits of *Terminalia chebula* (Sato et al., 1997). Ahmad et al., 1998, found that several extracts of *Terminalia chebula* exhibit antibacterial activity against a number of bacterial species. *Terminalia chebula* is well effective against *Helicobacter pyroli* (Malekzadeh et al., 2001).

Terpenoides from *T. avicennioides* showed antibacterial activity against *S. aureus, E. coli* and *P. aeruginosa* (Mann et al., 2009). Different chemical constituent of *Terminalia chebula* such as ellagic acid exerted a potent inhibitory effect against *C. perfringens* and *E. coli,* but little or no inhibition was observed for behenic acid, β-caryophyllene, eugenol, isoquercitrin, oleic acid, α-phellandrene, β-sitosterol, stearic acid, α-terpinene, terpinen-4-ol, terpinolene, or triacontanoic acid 23. The ethanolic extract of *Terminalia chebula* fruits was found to be effective against both gram-positive and gram-negative bacteria such as *Salmonella typhi* SSFP 4S, *Staphylococcus epidermidis* MTCC 3615, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* MTCC 441 and *Pseudomonas aeruginosa* ATCC 27853 suggesting its broad spectrum antimicrobial activity (Kannan et al., 2009). A recent study suggested that the alcoholic extract of *Terminalia chebula* fruit inhibit the growth of eight pathogenic bacterias including *Pseudomonas aeruginosa* and the zone of inhibition produced by ethanol and methanol extract was 23.67 and 20.67 in mm in diameter and the activity index was found 0.82 and 0.71 (Jahan, 2017). However, the used strain was not resistant.

In this present study, it was found that the ethyl acetate extract of *Terminalia chebula* fruits exhibit antimicrobial activity against a highly multidrug resistant *Pseudomonas aeruginosa* strain. The zone of inhibition produced by the ethyl acetate extract of *Terminalia chebula* was 17 mm and the activity index was found to be 0.94 which showed greater result compared to the other previous studies. Though the chloroform extract of *Terminalia chebula* fruits did not show any antimicrobial activity this probably happened because the chloroform solvent itself deactivated the potential antibacterial active compounds of *Terminalia chebula.*

Some studies involved in determining the MIC and the MBC values of the different extracts of *Terminalia chebula* against different organisms. (Nayak et al., 2014) found the minimum inhibitory concentration of hydro ethanolic extract was determined to be 2.5% and the minimum inhibitory concentration of the aqueous extract was determined to be 10% which was tested against *Streptococcus* mutans in vitro. In another study, (Kannan et al., 2009) showed that in 1 mg/ml concentration 70% of growth inhibition occurs in *B. subtilis* MTCC 441 and 100% of growth inhibition occurs in *S. typhi* MTCC 3615 and 79% of growth inhibition occurs in *P. aeruginosa* ATCC 27853 strain. However in this study, it was found that in 25 mg/ml concentration 99.4% and in 30 mg/ml 100% of growth inhibition occurs in highly multidrug resistant *P. aeruginosa.*

Many research reveals the conducting of a few studies on antibacterial activities of *Terminalia arjuna*. In addition to these phytocompounds bark of *T. arjuna* which contains triterpenoids responsible mainly for cardio protective and antibacterial (arjunic acid, arjungenin and, arjunetin) effect suggested by (Dwivedi, 2007; Jain et al., 2009 and Singh et al., 2008). (Aneja et al., 2012) demonstrated that the antimicrobial potential of *T. arjuna* leaves and bark extract which involved five bacteria namely *Staphylococcus aureus* (HM626197)* (Gram-positive), *Acinetobacter* sp. (HM626198), *Proteus mirabilis* (HM626199), *Escherchia coli* (HM626200), *Pseudomonas aeruginosa* (HM626201) (Gram-negative) and one yeast, *Candida albicans.* In another study, methanol and ethanol extracts of *T. arjuna* bark showed antimicrobial activity at 1000 µg/mL and 1500 µg/mL concentrations against 3 different gram positive and 4 different gram negative bacterial strains and the zone of inhibition ranged from 9-32 mm in diameter (Akhter et al., 2012). In this study it was observed that the ethanol and ethyl acetate extract of *T. arjuna* barks also have lower antimicrobial activity against a highly multidrug resistant *Pseudomonas aeruginosa* where the zone of inhibition was found to be 11 and 8 mm in diameter and the activity indexes was found 0.65 and 0.47 to be respectively.

In many studies, the antimicrobial activity of the extract of the roots and leaves of *Swertia chirayita* was demonstrated. Antibacterial activity of rectified spirit extract of *Swertia chirayita* were tested against eight bacteria at concentrations of 30µg/disc and 90µg/disc. Standard antibiotic disc of chloramphenicol (30µg/disc) was used for comparison. The produced zone of inhibition for rectified spirit extract against *Staphylococcus aureus, Bacillus megaterium* and *Escherichia coli* were 9mm, 8mm and 8mm at 30µg/disc dose respectively. At 90 µg/disc dose, the produced zone of inhibition against the same bacteria was 16mm, 13mm and 12mm respectively (Sultana et al., 2007). In another study, the ethanolic extract of *S. chirayita* was tested against 8 pathogenic bacteria 4 gram positive and 4 gram negative and they found potential antimicrobial activities and the zone of inhibition in diameter ranged between 8-15 mm in diameter (Rishikesh et al., 2012). Kotnala et al., 2008, demonstrated, the potential antimicrobial activities of *S. chirayita* which involved three strains of gram-positive bacteria *Staphylococcus epidermidis* (ATCC 3615), *Staphylococcus aureus* (ATCC 25983), *Bacillus subtilis* (MTCC 441) and six strains of gramnegative *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Proteus vulgaris* (MTCC 1771), *Enterobacter aerognes* (ATCC 111) *Klebsiella pneumonia* (ATCC 15380), *Salmonella typhi* (ATCC 43579).

In this present investigation, the moderate antimicrobial activity of the ethanolic extract of *S. chirayita* roots, leaves and flowers which was found against a highly multidrug resistant *Psedomonas aeruginosa* and the produced zone of inhibition was ranged 13 mm in diameter and the activity index was found to be 0.76. However the ethyl acetate ethanolic extract of *S. chirayita* roots, leaves and flowers did not show any legitimate activity against the reference multidrug resistant *Psedomonas aeruginosa.*

Though most of the extracts showed potential activities, there were some limitations to this research as well. For example, only ethyl acetate, ethanol and chloroform were used as solvents but there are other solvents such as acetone, hexane, ethyl ester, methanol which could be used in the future for further studies. The phytochemical assay of the plants could have been explored which would give the idea about the total chemical constituents of the respective plants. In this study, only one reference multidrug resistant organism were used. In the future, the same extract should be used against other multidrug resistant strains. Conducting HPLC on ethyl acetate extract of *T. chebula* fruits could specifically confirm and identify the potential compounds which showed the most antibacterial activity.

## Conclusion:

Even though the ethyl acetate extract of *T. chebula* produced the most acceptable result compared to the reference antibiotic Polymyxin B. However, the result obtained from the extract of *T. arjuna* and *S. chirayita* was no less insignificant. Since we are gradually entering into the era of multi drug resistance and no new class of antibiotics have been discovered since 1986. These medicinal plants extracts however proved to be valuable in the development of new antibiotics. They can be further useful in food preservation and topical use. But further studies are required.

We are currently living in the world of modern technologies. But yet we are largely relying on medicinal plants and return to them when we failed to cure any untreatable diseases. This study shows how unknown, elongated and rich the plant world is. Further researches should be done to identify the potential activity to cure any diseases and infections.

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