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Assessment of the antimicrobial activity of the ethanolic extract of *Phyllanthus emblica* in combination with different classes of antibiotics against single and multi-drug resistant strains

Nisrat Jahan, Salma Akter

Abstract
Medicinal plants have been served as a great source of medicine since the ancient era. Amla is indeed, the key ingredient in the popular ayurvedic recipe, Chyavanaprasha. More than anything, it may be called as "King of Rasayana" [rejuvenation]. The fruit is rich in quercetin, phyllaemblic compounds, gallic acid, tannins, flavonoids, pectin and vitamin C and also contains various polyphenolic compounds. A wide range of phytochemical components including terpenoids, alkaloids, flavonoids, and tannins have been shown to posses' useful biological activities. Many pharmacological studies have demonstrated the ability of the fruit shows antioxidant, anticarcinogenic, antitumour, antigenotoxic, antiinflammatory activities, supporting its traditional uses. In this review, we have focused our interest on the antimicrobial potential of crude ethanolic extract of *Phyllanthus emblica* alone and in combination with 2 antibiotic drugs in 12 microorganism culture. The antimicrobial activity was evaluated by Disc diffusion method. The extract was assayed at 500µg/disc concentration with standard kanamycin disc against gram positive, gram negative and multi drug resistant strains. In view of its reported pharmacological properties and relative safety, P. emblica could be a source of therapeutically useful products.

Keywords: *Phyllanthus emblica,* Antimicrobial activity, Synergistic effect, Phytochemicals.

1. Introduction
Medicinal plants are rich sources of anti-microbial agents and its therapeutic use is becoming popular because of its lesser side effects and resistance. Nature has bestowed on us a very rich botanical wealth and a large number of diverse types of plants growing different parts of the country. Herbal medicine is still the mainstay of about 75 - 80% of the whole population, and the major part of traditional therapy involves the use of plant extract and their active constituents in the indigenous cultures higher plants have formed the basis for the treatment of the diseases since the earliest time. Accuracy in recording or observing the medical use of a plant determining whether the ethanomedicinal use can be demonstrated under the specific condition in the laboratory. Chemical characterization of the compounds and the role of the placebo effect are important issues that need to be verified in the development of drugs of plant origin [1].

The plant genus *Phyllanthus (Euphorbiaceae)* is widely distributed in most of tropical and subtropical countries. It is very large genus consisting of approximately 550 to 750 species and is subdivided in to ten or eleven subgenera.

Phyllanthus emblica L. (syn. *Emblica officinalis*) is commonly known as Indian gooseberry. In Ayurveda, *P. emblica* has been extensively used, both as edible (tonic) plants and for its therapeutic potentials. It is highly nutritious and is reported as an important dietary source of vitamin C, minerals and amino acids. All parts of the plant are used for medicinal purposes, especially the fruit, which has been used in Ayurveda [2].

The fruits of amla are widely used in the Ayurveda and are believed to increase defense against diseases. The recent study for the purpose of the research work shown that the active compounds contained by *Phyllanthus emblica* have significant medicinal value. It has its beneficial role in treatment of cancer, diabetes, liver treatment, heart trouble, ulcer, anemia and various other diseases. Similarly, it has application as antioxidant, immunomodulatory, antipyretic, analgesic, cytoprotective, diuretic, laxative, carminative and stomachic, antitussive and gastroprotective. Additionally, it is useful in memory enhancing, ophthalmic disorders and lowering cholesterollevel.It is also helpful in neutralizing snake venom and as an antimicrobial.

It is often used in the form of *triphala* which is an herbal formulation containing fruits of *Phyllanthus emblica*, *Terminalia chebula and Terminalia belerica* in equal proportions. According to ayurvedic doctors regular usage of amla will make a man live more than 100 years like a youth. Amla is supposed to rejuvenate all the organ systems of the body, provide strength and wellness. It keeps us away from all the diseases by boosting our immune system. It is believed by ayurvedic practitioners that if an individual regularly takes amla he can live up to an age of 100 without suffering from any type of ailments [3].

The aim of this review is to investigate the antimicrobial activity of *Phyllanthus emblica* in combination with different classes of antibiotics against single and multi-drug resistant strains. Further, this review will highlight the importance of *Phyllanthus emblica* and will provide baseline for future research studies.

Phyllanthus emblica: Plant review

Plants as Antimicrobials

Historically, plants have provided a source of inspiration for novel drug compounds, as plant derived medicines have made large contributions to human health and well-being. Their role is two folds in the development of new drugs: first: they may become the base for the development of a medicine, a natural blueprint for the development of new drugs, or; second: a phytomedicine to be used for the treatment of disease. There are numerous illustrations of plant derived drugs. Some selected examples, including those classified as anti-infective, are presented below. The isoquinoline alkaloid emetine obtained from the underground part of *Cephaelis ipecacuanha*, and related species, has been used for many years as and amoebicidal drug as well as for the treatment of abscesses due to the spread of Escherichia histolytica infections. Another important drug of plant origin with a long history of use, is quinine. This alkaloid occurs naturally in the bark of Cinchona tree. Apart from its continued usefulness in the treatment of malaria, it can be also used to relieve nocturnal leg cramps. Currently, the widely prescribed drugs are analogs of quinine such as chloroquine. Some strains of malarial parasites have become resistant to the quinines, therefore antimalarial drugs with novel mode of action are required. Similarly, higher plants have made important contributions in the areas beyond anti-infective, such as cancer therapies. Early examples include the antileukaemic alkaloids, vinblatine and vincristine, which were both obtained from the Madagascan periwinkle (*Catharanthus roseus* syn. Vincaroseus) [4].

Fruit of heaven

Amla is a gift of nature to mankind as well as an indispensable part of the ayurvedic and unani system with amazing remedial qualities. In Sanskrit, it is called Amalaki or Dhartiphala. Amla is perhaps the single most often mentioned herb in "Charak Samhita", the Ayurvedic medicine literature (500 BC). Amla is a wonder herb and one of the precious gifts of nature to humans. Amla is known as "Divya" and "Amrut" or Amrit Phala in Sanskrit, which literally means fruit of heaven or nectar fruit. The Sanskrit name, Amlaki, translates as the Sustainer or The Fruit where the Goddess of Prosperity Resides. In Hindu religious mythology the tree is worshipped as the Earth Mother as its fruit is considered to be so nourishing as to be the nurse of mankind [5].

Table 1: Vernacular name

1	Bengali	Amlaki
2	Hindi	Amla
3	Sanskrit	Amaliki
4	English	Emblicamyroblan
5	Italian	Mirabolanoemblica
6	German	Amla
7	French	Phyllantheemblica
8	Nepalese	Amba

Taxonomy of plant

Kingdom_- Plantae
Division- Flowering plant
Class- Magnoliopsida
Order- Malpigphiales
Family- Phyllanthaceae
Tribe- Phyllanthaceae
Sub- tribe –Fluegginae
Genus- Phyllanthus
Species- P. emblica
Zoological name- *Phyllanthus emblica*

Morphology of the plant

A small to medium sized deciduous tree, 8-18 meters height with thin light grey bark exfoliating in small thin irregular flakes, leaves are simple, subsessile, closely set along the branchlets, light green having the appearance of pinnate leaves; flowers are greenish yellow, in axillary fascicles, unisexual, males numerous on short slender pedicels, females few, subsessile, ovary 3-celled; fruits globose, fleshy, pale yellow with six obscure vertical furrows enclosing six trigonous seeds in 2-seeded 3 crustaceous cocci [6].

Geographical distribution

Found throughout India, the sea-coast districts and on hill slopes up to 200 meters, also cultivated in Bangladesh. The Deccan, the sea-coast districts and Kashmir [Nadkarni and Nadkarni]. It is common all over tropical and sub-tropical India and also found in Burma, it is abundant in deciduous forests of Madhya Pradesh. Grows in tropical and subtropical parts of Ceylon, Malay Peninsula and China. In Ceylon, it is very common in exposed places on patana land in the moist regions up to 4000 feet altitude

Distribution in Bangladesh

Occurs in the dry forests of Chittagong, Chittagong Hill Tracts, Cox’s Bazar, Sylhet, Dhaka-Tangail (Sal forest) and Dinajpur; also cultivated elsewhere.

Table 2: General description of *Phyllanthus emblica*

1	Habitat	Found in Bangladesh, India, Pakistan, Uzbekistan, Srilanka, South East Asia, China and Malaysia.
2	Used parts	Dried fruits, Fresh fruit, seed, leaves, root bark, flowers.
3	Fruits	Ripen from November to February Nearly spherical or globular, wider than long and with a small and slight conic depression on both apexes Fruit is 18-25mm wide and 15-20mm long Surface is smooth with 6 obscure vertical pointed furrow Mesocarp is yellow and endocarp is yellowish brown in ripened condition. In fresh fruit mesocarp is acidulous and in dried fruit it is acidulous astringent.
4	Leaves	Leaf is 8-10 mm or more long and 2-3 m broad, hairless light green outside, palegreen or often pubescent beneath. It contains gallic acid, ellagic acid, chebulic acid, chebulinic acid, chebulagic acid, a gallantonic called amlic acid, alkaloids phyllantidine And phyllantine.
5	Seeds	Four-Six, smooth, dark brown A fixed oil, phosphatides and a small quantity of essential oil. The fixed oil (yield 16% and has the following physical and chemical characteristics:- acid value12.7; saponification value 185; iodine valu 139.5; acetyl value 2.03; unsaponifiable matter 3.81%; Sterol 2.70%; saturated fatty acid 7%. linolenic acid (8.78 %), linoleic (44%). oleic (28.40%), steric (2.15%), palmitic (2.99%) and miristic acid (0.95%).
6	Bark	Thick to 12 mm, shining grayish brown or grayish green. It contains leukodelphinidin, tannin and proanthocyanidin.
7	Roots	It contains ellagic acid and lupeol

Cultivation methods

Amla can grow in light as well as the heavy soils. It is grown under the tropical conditions. The young plants are protected from the hot winds as they die easily. Amla is generally propagated through seeds. It requires proper sunlight. It is irrigated during the monsoon season. It starts bearing fruits in seven years from the day of planting.

Phytochemistry

EO primarily contains tannins, alkaloids, phenolic compounds, amino acids and carbohydrates. Its fruit juice contains the highest vitamin C (478.56 mg/100 mL). The fruit when blended with other fruits, boosted their nutritional quality in terms of vitamin C content. The other principle components are Emblicannin A, Emblicannin B, Punigluconin and Pedunculagin. Compounds isolated from EO were gallic acid, ellagic acid, 1-O-galloyl-beta-D-glucose, 3, 6-di-O-galloyl-Dglucose, chebulinic acid, quercetin, chebulagic acid, corilagin,1,6-di-O - galloyl beta D glucose, 3 Ethylgallic acid (3 ethoxy 4,5 dihydroxy benzoic acid) and isostrictiniin. Phyllanthusemblica also contains flavonoids, kaempferol 3 O alpha L (6"methyl) rhamnopyranoside and kaempferol 3 O alpha L (6"ethyl) rhamnopyranoside. A new acylated apigenin glucoside (apigenin 7 O (6" butyryl beta glucopyranoside) was isolated from the methanolic extract of the leaves of *Phyllanthus emblica* together with the known compounds; gallic acid, methyl gallate, 1,2,3,4,6-penta-O-galloylglucose. The root contains ellagic acid and lupeol and bark contains leucodelphinidin. The seeds yield a fixed oil (16%) which is brownish-yellow in color. It has the following fatty acids:
linolenic (8.8%),
linoleic (44.0%),
oleic (28.4%),
stearic (2.15%),
palmitic (3.0%) and
myristic (1.0%)
A new acylated glucoside was isolated from the methanolic extract of the leaves of P. emblica. Their structures were named as apigenin7-O-(6”-butyryl-beta) glucopyranoside, along with four known compounds gallic acid, methyl gallate, 1,2,3,4,6-penta-Ogalloylglucose and luteolin-4'Oneohesperiodoside (Desouky, 2008). The seeds of *P. emblica* contain fixed oil, phosphatides and small quantity of essential oil. In addition, the leaves contain gallic acid, ellagic acid, chebulagic acid and chebulinic acid. Phyllaemblic acid, a novel highly oxygenated norbisabolane were isolated from the roots of *P. emblica* and its structure was fully characterized by spectroscopic and chemical means Ellagic acid and lupeol are present in roots of *P. emblica* [1].

Table 3: Chemical constituents of *Phyllanthus emblica*

Sl. No	Chemical Constituents	Sl. No	Chemical Constituents
1	Tanins	11	Ellagic acid
2	Phenolic compounds	12	Chebulinic acid
3	Carbohydrate	13	Quercetin
4	Alkalods	14	Chebulagic acid
5	Amino acids	15	Emblicanin-A
6	Vitamin C (Ascorbic acid)	16	Gallic acid
7	Flavanoid	17	Emblicanin-B
8	Pedunculagin	18	Ellagotannin
9	Punigluconin	19	Trigallayl glucose
10	Citric acid	20	Pectin

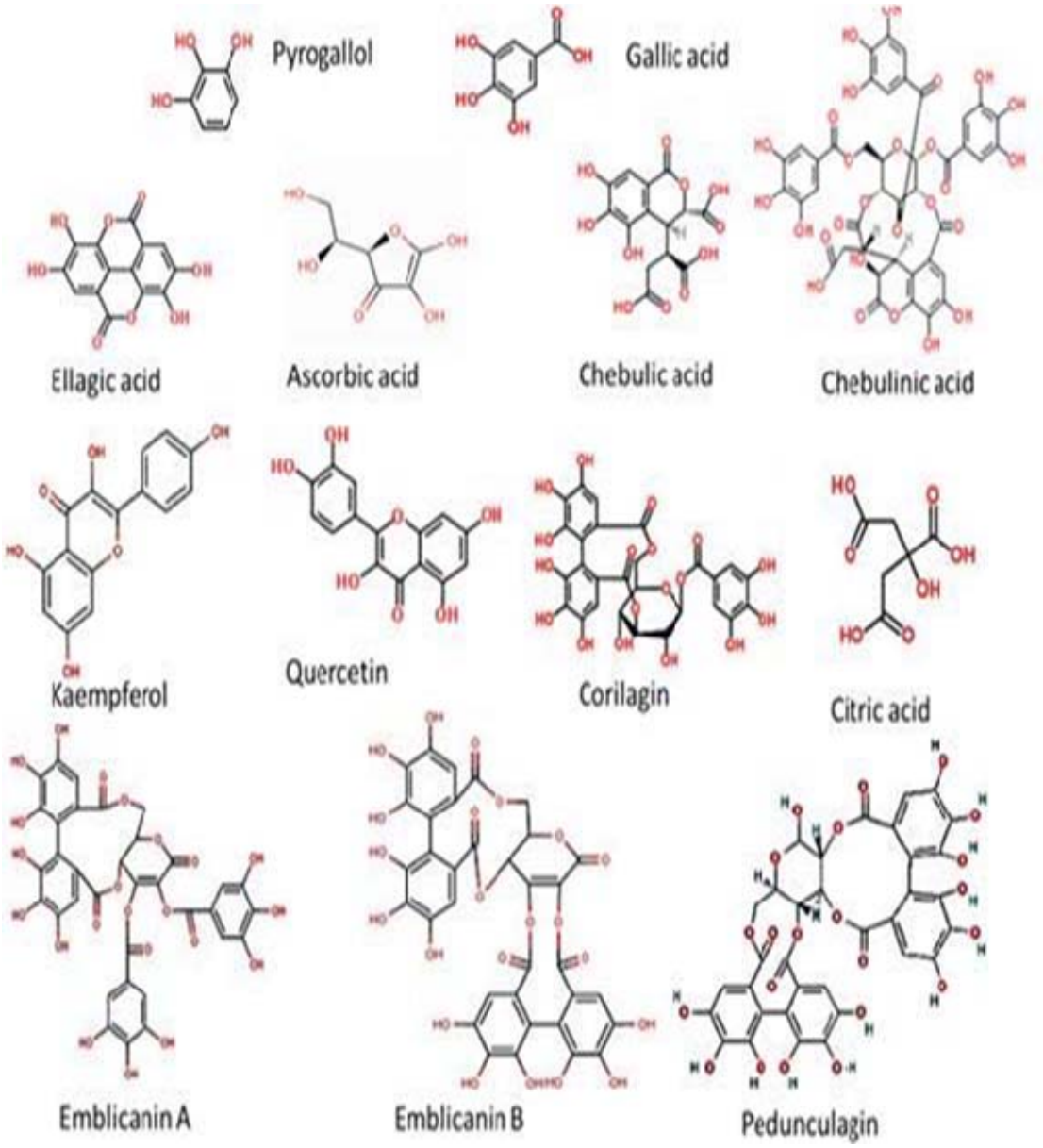


Fig 1: Structures of some chemical constituents of *Phyllanthus emblica*

Nutritional value

Amla is well known for its nutritional qualities. It is rich in polyphenols, minerals and is regarded as one of the richest source of vitamin C (200-900 mg per 100 g of edible portio

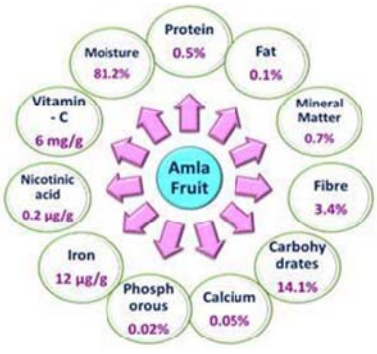


Fig 2: Average percentage composition of the fruit pulp of *Phyllanthus emblica*

Medicinal activities of *P. emblica*

Antioxidant activity

Ethanolic extract of amla fruit has antioxidant activity. It contains tannoid principles comprising of emblicanin A. emblicanin B, punigluconin and pedunculagin, have been reported to possess antioxidant activity *in vitro* and *in* vivo [15].

Antiproliferative activity

The norsesquiterpenoid glycosides phyl-laemblicins B (3) and C (4) showed significant antiprolifera-tive activity against tumor cells, even though their yields from the roots were not high. These compounds are worthy of consideration as a potential cancer chemopreventive and/or anticarcinogenic agents after additional biological evaluation in vivo [16].

Immunomodulation

Immune activation is an effective as well as protective approach against emerging infectious diseases. Albino rats

were used to assess the immunomodulatory activities of Triphala on various neutrophil functions like adherence, phagocytic index, avidity index and nitro blue tetrazolium. Oral administration of Triphala appears to stimulate the neutrophil functions in the immunized rats and stress induced suppression in the neutrophil functions were significantly prevented by Triphala.

Antipyretic and Analgesic Activities

Extracts of ethanol possess potential anti-pyretic and analgesic activities. A single oral dose of ethanolic extract and aqueous extract (500 mg/kg, i.p.) showed significant reduction in hyperthermia. This may be due to the presence of tannins, alkaloids, phenolic compounds, amino acids and carbohydrates [11].

Cytoprotective Properties

Ethanolic extract of amla fruit has been reported for its cytoprotective and immune modulating properties against chromium (VI) induced oxidative damage. It inhibited chromium induced immunosuppression and restored gamma-IFN production by macrophages and phagocytosis.

Antitussive Properties

EO (ethanolic extract) was tested for its antitussive activity in conscious cats by mechanical stimulation of the laryngopharyngeal and tracheobronchial mucous areas of airways. Antitussive activity of EO was more effective than the non-narcotic antitussive agent dropropizine but less effective than shown by the classical narcotic antitussive drug codeine. It is supposed that the dry extract of EO exhibit the antitussive activity not only due to antiphlogistic,

Gastroprotective Properties

EO (ethanolic extract) was investigated for its antisecretory and antiulcer activities using various experimental models in rats, including pylorus ligation Shay rats, indomethacin, hypothermic restraint stress induced gastric ulcer and necrotizing agents. It was then reported that Amla extract exhibits antisecretory, cytoprotective and antiulcer properties [2].

Memory Enhancing Effects

Amlachurna produced a dose-dependent improvement in memory of young and aged rats. It reversed the amnesia induced by scopolamine and diazepam. Amla churna may prove to be a useful remedy for the management of Alzheimer's disease due to its multifarious beneficial effects such as memory improvement and reversal of memory deficits.

Ophthalmic effects

Ophthacare is a herbal eye drop preparation containing basic principles of different herbs. Clinical trial was conducted in patients suffering from different ophthalmic disorders namely, conjunctivalxerosis, conjunctivitis, acute dacryocystitis, degenerative conditions and Post-operative cataract patients with a herbal eye drop preparation. In most cases improvement was observed with the treatment of the herbal eye drop. During the course of study no side effects were observed and the eye drop was well tolerated by the patients. Ophthacare exhibit beneficial role in a number of inflammatory, infective and degenerative ophthalmic disorders.

Reducing Cholesterol and Dyslipidemia

Cu^{2+} induced LDL oxidation and cholesterol-fed rats were used to investigate the effects of Amla on low-density lipoprotein (LDL) oxidation and cholesterol levels *in vitro* and *in vivo*. It was concluded that Amla may be effective for hypercholesterolemia and prevention of atherosclerosis. It contains flavonoids which reduce the levels of lipid in serum and tissues of rats induced hyperlipidemia. Both causes the degradation and elimination of cholesterol [19].

Snake Venom Neutralizer

EO explores for the first time for antisnake venom activity. *Naja kaouthia* and *Viperarussellii* venom was antagonized by the plantextracts significantly both *in vivo* and *in vitro* studies. *V.russellii* venom-induced coagulant, haemorrhage, defibrinogenating and inflammatory activities were significantly neutralized by both plant extracts. No precipitating bands were formed between the snake venom and plant extract which confirmed that the plantextracts possess potent snake venom neutralizing capacity and need further investigation.

Antimicrobial and Antimutagenicity Activities

EO has been reported for the antimicrobial activities. The plant have been reported to posses potent antibacterial activity against *Escherichia coli*, *K*. ozaenae, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *S. paratyphi A*, *S. paratyphi B* and *Serratia Marcescens*. Water, chloroform and acetone extracts of Triphala were investigated to evaluate an antimuta genic effect using an Ames histidine reversion assay having TA98 and TA100 tester strains of *Salmonella Typhimurium* against the direct-acting mutagens [12.13].

Supports the heart

It is hridya, which means it nurtures the heart, blood and circulation. It supports the cardiovascular system. On the other hand, it sometimes acts as a cardiac stimulant [6] Research shows that Amla helps lower cholesterol and protect from heart diseases [8].

On urinary system

Because it enhances all the thirteen agnis (digestive fires) and supports Apana Vata, Amla-Berry is especially supportive to the urinary system and can be helpful if you experience a mild burning sensation while urinating. It supports natural diuretic action, but does not force water from the body like diuretic pills. In other words, it helps eliminate waste from the body but does not over-stimulate the urinary system [7].

Ethnomedicinal Uses

P. emblica is an important herbal drug used in Unani and Ayurvedic systems of medicine. Fruits of P. emblica have been used for thousands of years in the traditional Indian medicine for the treatment of several diseases. All parts of the plant are used for medicinal purposes, especially the fruit. In traditional medicine, it is used for the treatment of diarrhea, jaundice, and inflammation. The fruits are sour, astringent, bitter, acrid,

sweet, cooling, anodyne, ophthalmic, carminative, digestive. That's why, *Phyllanthus emblica* is used in the preparation of various formulation [9].

Table 4: Formulations containing Amla are listed below [10]

Sl No	Formulation containing *P. emblica*
1.	Hajmola
2.	Chyawanprash
3.	Cinkara
4.	Carmina
5.	Kalomegh
6.	Triphala
7.	Ophthacare
8.	Pepticare
9.	Joshina Herbal
10.	Safi
11	Daburamla hair oil

Table 5: Therapeutic effects of Amla are listed below [10]

Sl No	Disease	Sl. No	Disease
1.	Cancer	11.	Lung metastasis
2.	Diabetes	12.	Healing dermal wounds
3.	Heart disease	13.	Dyslipidemia
4.	Liver treatment	14.	Pancreatitis
5.	Ulcer	15.	Atherosclerosis
6.	Anaemia	16.	Alzheimers Disease
7.	Hypercholesterolemia	17.	Fever
8.	Hyperthermia	18.	Bronchitis
9.	Ophthalmic disorder	19.	Diarrhoea
10.	Dyspepsia	20.	Jaundice

Method and Materials

Collection

For this investigation of fruit *asPhyllanthus emblica* was collected from market, in March, 2014. The plant was identified by Bangladesh National Herbarium; and the voucher was signed by Hosne Ara, Director of Bangladesh National Herbarium.

Drying and Grinding

The collected plant sample (fruit) was washed thoroughly under running tap water to remove dust and sand particles. Then it was sun-dried for 2-3 weeks, powdered and stored for further use. In order to extract the fruits were ground into a coarse powder with the help of a suitable grinder. The powder was stored in an airtight container and kept in a cool, dark and dry place until analysis commenced.

Extraction

Crude plant drugs find their way in modern medicine system through continuous extraction, followed by different isolation techniques and different pharmacological tests. Chemical constituents from crude plant can be extracted by following two extraction procedures:

A. Cold extraction

B. Hot extraction

In our current study we used cold extraction method.

Cold extraction procedure

In this process powdered fruit materials are submerged in a suitable solvent or solvent system in an air tight flat bottom container for several days, with occasional shaking and stirring. The major portion of plant materials will be dissolved in the solvent. Solvent is then separated from dispersed materials and evaporated to get desired extract.

Hot extraction procedure

In this process powdered plant materials are successively extracted to exhaustion in a Soxhlet at elevated temperature with various solvents of increasing polarity.

Individual extract is then filtered through several means. All the extracts are concentrated with rotary evaporator at low temperature (40-50 °C) and reduced pressure. Concentrated extract finally obtained is known as crude extract.

After cold or hot extraction, the extract obtained has to be stored in the refrigerator in order to avoid loss of material.

Cold extraction of *Phyllanthus emblica*:

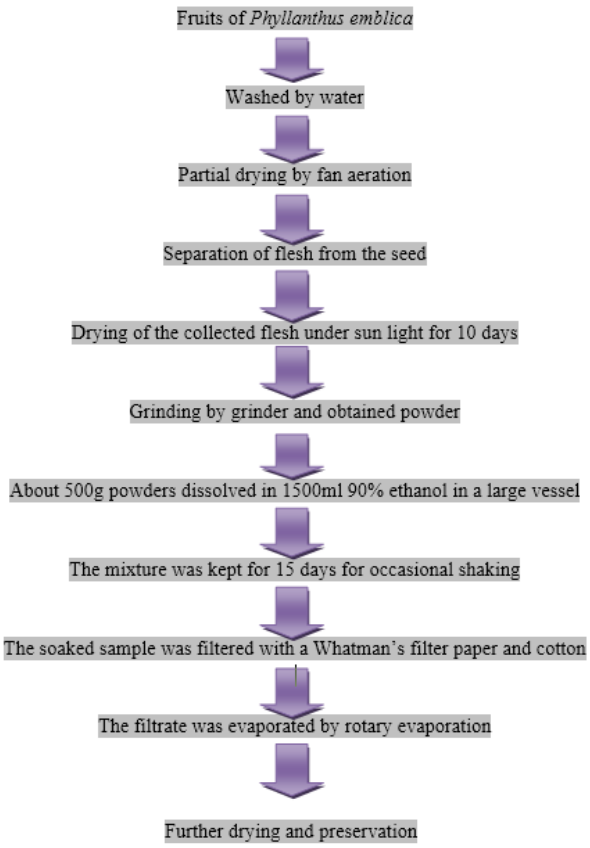
Preparation of ethanolic extracts

For each plant the dried and powdered materials (500 g for) were soaked in 1500 ml of 90% ethanol for about 15 days at room temperature with occasional stirring. After 15 days the solution was filtered using filter cloth and Whatman's filter paper.

Evaporation of the solvent

The filtrates (Ethanolic extract) obtained were evaporated under *rotary evaporator* and in a water- bath until dried. It rendered a gummy concentrates and were designated as crude extracts of Ethanol. Total amount of extract of *Phyllanthus emblica* was =207.5 gm

Extraction at a glance



Antimicrobial testing methodologies

The following requirements should be respected:

1. Bacteria subjected to test must be isolated in pure culture from the submitted sample.
2. standard reference methods should be used for identification so that the subject bacteria are consistently and correctly identified to the genus and/or species level,
3. Bacterial isolates considered to be the most important and a sampling of other isolates, should be stored for future analysis (either lyophilisation or cryogenic preservation at –70 °C to –80 °C).
4. The following factors influencing test methods should be determined, optimised, and documented in a detailed standard operating procedure:

a. Once the bacterium has been isolated in pure culture, the optimum concentration of the inoculam must be determined to obtain accurate susceptibility results. Bacteria or other organisms used in test method should be from a fresh culture,
b. The composition and preparation of the agar and broth media used (e.g. pH, cations, thymidine or thymine, use of supplemented media). Performance and sterility testing of media lots should also be determined and documented as well as employed procedures,
c. The content of antimicrobial in the carrier (antibiotics used in microtitre plates, disk, strip, tablet),
d. Composition of solvents and diluents for preparation of antimicrobial stock solutions,
e. Growth and incubation conditions (time, temperature, atmosphere e.g. CO_2), agar depth,
f. Number of concentrations tested per broth and agar dilution,
g. The test controls to be used, including the reference organisms used,
h. The subsequent interpretive criteria (clinical breakpoints, epidemiological cut-off values).

5. For these reasons, special emphasis has to be placed on the use of documented procedures and validated, well documented methods, as sufficient reproducibility can be attained only through the use of such methodology [19].

Antimicrobial testing methods

The following three methods have been shown to consistently provide reproducible and repeatable results when followed correctly. They are:

1. Disk diffusion,
2. Broth dilution,
3. Agar dilution.

Disc diffusion method

Principle

When a filter paper disc impregnated with a chemical is placed on agar the chemical will diffuse from the disc into the agar. This diffusion will place the chemical in the agar only around the disc. The solubility of the chemical and its molecular size will determine the size of the area of chemical infiltration around the disc. If an organism is placed on the agar it will not grow in the area around the disc if it is susceptible to the chemical. This area of no growth around the disc is known as a "zone of inhibition".

In this method, measured amount of the test samples are dissolved in definite volumes of solvent to give solutions of known concentrations (µg/ml). Then sterile filter paper discs (5mm in diameters) are transferred in the petri dish and applied test sample in the disc by micropipette with known amounts of the test substances and dried. These plates are kept at low temperature in the refrigerator for 1-2 hours to allow maximum diffusion. The plates are then kept in an incubator (37 ºc) for 12-18 hours to allow the growth of the organisms. If the test material has antimicrobial activity, it will inhibit the growth of microorganisms, giving a clear, distinct zone is called "zone of inhibition". The antimicrobial activity of the test agents is determined in term of millimeter by measuring the diameter of the zone of inhibition. The greater the zone of inhibition, the greater is the activity of the test material against the test organism.

The principal factors which determine the size of the zone of inhibition are:

1. Intrinsic antimicrobial susceptibility of the test sample.
2. Growth rate of the test organisms.
3. Diffusion rate of the test sample which is related to its water solubility.
4. Concentration of the test organisms inoculated in the medium.
5. Concentration of the test sample per disc.
6. Thickness of the test medium in the petridishes [18].

Materials needed

Table 6: Name of test organisms

Gram positive bacteria	Gram negative bacteria	Resistant bacteria
Bacillus cereus	*Salmonella paratyphi*	*Escherichia coli*
Sarcinalutea	*Escherichia coli*	*Pseudonomous spp.*
Staphylococcus aureus	*Vibrio cholera*	*Shigellasonnie*
Bacillus subtilis	*Shigelladysenteriae*	
	Shigellaboydii	
	Salmonella typhi	

In this antimicrobial study, we referred bacteria as 1,2,3,4,6,7,8,9,10,11,12,13

Referred as	Bacteria
1.	*Bacillus cereus*
2.	*Salmonella paratyphi*
3.	*Sarcinalutea*
4.	*Escherichia coli*
6.	*Staphylococcus aureus*
7.	*Vibrio cholera*
8.	*Bacillus subtilis*
9.	*Shigelladysenteriae*
10.	*Pseudonomous spp.*
11.	*Shigellaboydii*
12.	*Shigellasonnie*
13.	*Salmonella typhi*

NB: Here *Bacillus megaterium* was referred to no.5 but due to some problem this bacteria could not be used during investigation.

Table 7: Name of antibiotics

Sl no.	Antibiotic	Reffered as
1.	Azithromycin	D1
2.	Ciprofloxacin	D2
3.	Cefuroxime	D3
4.	Nystatin	D4

List of apparatus & reagent

Sl no.	Apparatus and Reagents
1.	Filter paper discs (5mm in diameter)
2.	Petri dish
3.	Refrigerator
4.	Test tubes
5.	Sterile forceps
6.	Sterile cotton
7.	Incubating loop
8.	Bunsen burner
9.	Micropipette (10-100µL)
10.	Laminar air flow unit (Biocraft's Scientific Industries, India)
11.	Autoclave (|YX -280B 18L)
12.	Incubator (OSK-9636, Japan)
13.	Nutrient ager media (DIFCD)
14.	Ethanol
15.	Standard disc (Kanamycin 30µg/disc)
16.	Distilled water
17.	Rotary evaporator machine

Agar media

Agar is a gelatinous substance, obtained from algae and discovered in the late 1650s or early 1660s by Minoya Tarozaemon in Japan, where it is called *kanten*.

Agar is derived from the polysaccharide agarose, which forms the supporting structure in the cell walls of certain species of algae, and which is released on boiling. These algae are known as agarophytes and belong to the Rhodophyta (red algae) phylum. Agar is actually the resulting mixture of two components: the linear polysaccharide agarose, and a heterogeneous mixture of smaller molecules called agaropectin.

Throughout history into modern times, agar has been chiefly used as an ingredient in desserts throughout Asia and also as a solid substrate to contain culture media for microbiological work. Agar (agar-agar) can be used as a laxative, an appetite suppressant, a vegetarian substitute for gelatin, a thickener for soups, in fruit preserves, ice cream, and other desserts, as a clarifying agent in brewing, and for sizing paper and fabrics.

The gelling agent in agar is an unbranched polysaccharide obtained from the cell walls of some species of red algae, primarily from the genera *Gelidium* and *Gracilaria*. For commercial purposes, it is derived primarily from *Gelidium amansii*. In chemical terms, agar is a polymer made up of subunits of the sugar Galactose [20].

Table 8: Composition of Agar media

Sl no	Ingredient	%
1.	Peptone	0.5
2.	Beef extract/ Yeast extract	0.3
3.	Agar	1.5
4.	NaCl	0.5
5.	Distilled water	100ml
6.	pH	Neutral (6.8) at 25 ºC

Procedure of disc diffusion method for combination therapy

Preparation of medium

The instant nutrient agar media was accurately weighed and then reconstituted with distilled water in a conical flask. To prepare agar media solution 28grams of agar has to be mixed with 1L of water. In case of preparation of 250ml agar media solution 7grams of agar has to be mixed with 250ml distilled water. Then it is transfer to autoclave for sterilization for 20 minutes at 121 ºC.

Preparation of subculture

With the help of an inoculating loop, the test organisms were transferred from the pure culture to the agar slants under a laminar air flow unit. The inoculated slants were then incubated at 37 ºc for 18-24 hours to ensure the growth of the test organisms. The culture was used for sensitivity test. The red heat of loop is required before each and every bacteria inoculation.

Preparation of test plates

The agar media is taken in test tube after autoclaving and the organism was transferred from the subculture to the test tube containing autoclaved medium with the help of an inoculating loop in an aseptic area. The test tube was shaken by rotation to get a uniform suspension of the organism. The bacterial suspensions were immediately transferred to the sterile Petri dishes in an aseptic area. The depth of media each Petri dish was approximately 4mm [21].

Preparation of Discs

In case of combination therapy we used five discs. They are-

Sl.no	Disc	Description
1.	Sample disc	Sterilized filter paper discs (5mm in diameter) were taken in a blank petridish. Sample solution of the desired concentration was applied on the discs with the help of a micropipette in an aseptic condition.
2.	Standard disc	These were used to compare the antibacterial activity of test material. In our investigation, Kanamycin (30µg/disc) standard disc was used as a reference.
3.	Blank/control disc	Only solvent was applied to the disc to determine the antimicrobial effects of the solvent. In these study blank or control disc was contain ethanol as a solvent.
4.	Drug disc	Contained the antibiotic.
5.	Combination disc	Contained combination of antibiotic and test sample of *Phyllanthus emblica.*

Preparation of Discs Containing Sample

Prepare the disc and then it transferred to the petridish by the help of forceps. Then drug or sample is transferred in the disc by micropipette.

Sample preparation

Take 500mg of *Phyllanthus emblica* and mixed it with 10ml of ethanol. Each sample disc contains 10µL solution containing 500µg of extract.

Table 9: Antibiotic sample preparation

Sl.no	Antibiotic	Sample preparation	Disc preparation
D1	Azithromycin	Take 75mg of Azithromycin and mixed it with 50ml of ethanol.	Each disc contains 10µL of solution containing 15µg of Azithromycin.
D2	Ciprofloxacin	Take 50mg of Ciprofloxacin and mixed it with 100ml of ethanol.	Each disc contains 10µL of solution containing 5µg of Ciprofloxacin.

Transfer of drug to the disc:
Here,
Drug disc=10 µl/disc
Combination disc=10µl/disc of test sample+10 µl/disc of antibiotic

Diffusion of drug and Incubation

After incorporation of drug into the disc then petridish is transferred to the refrigerator for 1-2 hours at inverted position for the diffusion of drug.

After 1-2 hours the petridish is transferred to the incubator at 37 ºC for 12-18 hours.

Measurement of Zone of Inhibition

After 18 hours of incubation, the antibacterial activity of the test samples was determined by measuring the diameter of inhibitory zones in term of millimeter [20].

Antimicrobial activity of *Phyllanthus emblica*

Results of antimicrobial activity of *Phyllanthus emblica* are listed as follows

Table 10: Antimicrobial activity of *Phyllanthus emblica*

Sl no.	Bacteria	Type of Bacteria	Diameter of zone of inhibition of extract (500µg/disc) (mm)	Diameter of Zone of inhibition of Standard (Kanamycin) (30µg/disc) (mm)
1.	*Bacillus cereus*	Gram positive	10mm	24mm
2.	*Salmonella paratyphi*	Gram negative	8mm	30mm
3.	*Sarcinalutea*	Gram positive	8mm	32mm
4.	*Escherichia coli*	Gram negative and resistant bacteria	10mm	32mm
6.	*Staphylococcus aureus*	Gram positive	20mm	31mm
7.	*Vibrio cholera*	Gram negative	12mm	37mm
8.	*Bacillus subtilis*	Gram positive	25mm	25mm
9.	*Shigelladysenteriae*	Gram negative	17mm	30mm
10.	*Pseudonomous spp.*	Resistant bacteria	8mm	40mm
11.	*Shigellaboydii*	Gram negative	10mm	27mm
12.	*Shigellasonnie*	Resistant bacteria	10mm	30mm
13.	*Salmonella typhi*	Gram negative	8mm	32mm

Discussion

In the antimicrobial study, it was observed that the extract of *Phyllanthus emblica* showed high activity against all gram-positive, gram- negative and resistant- bacteria. Especially extract of *Phyllanthus emblica* showed highest activity against *Bacillus subtilis*, and the zone of inhibition was 25mm. It showed lowest activity against *Salmonella paratyphi, Sarcina lutea, Pseudonomous spp., Salmonella typhi* and the zone of inhibition was 8mm.

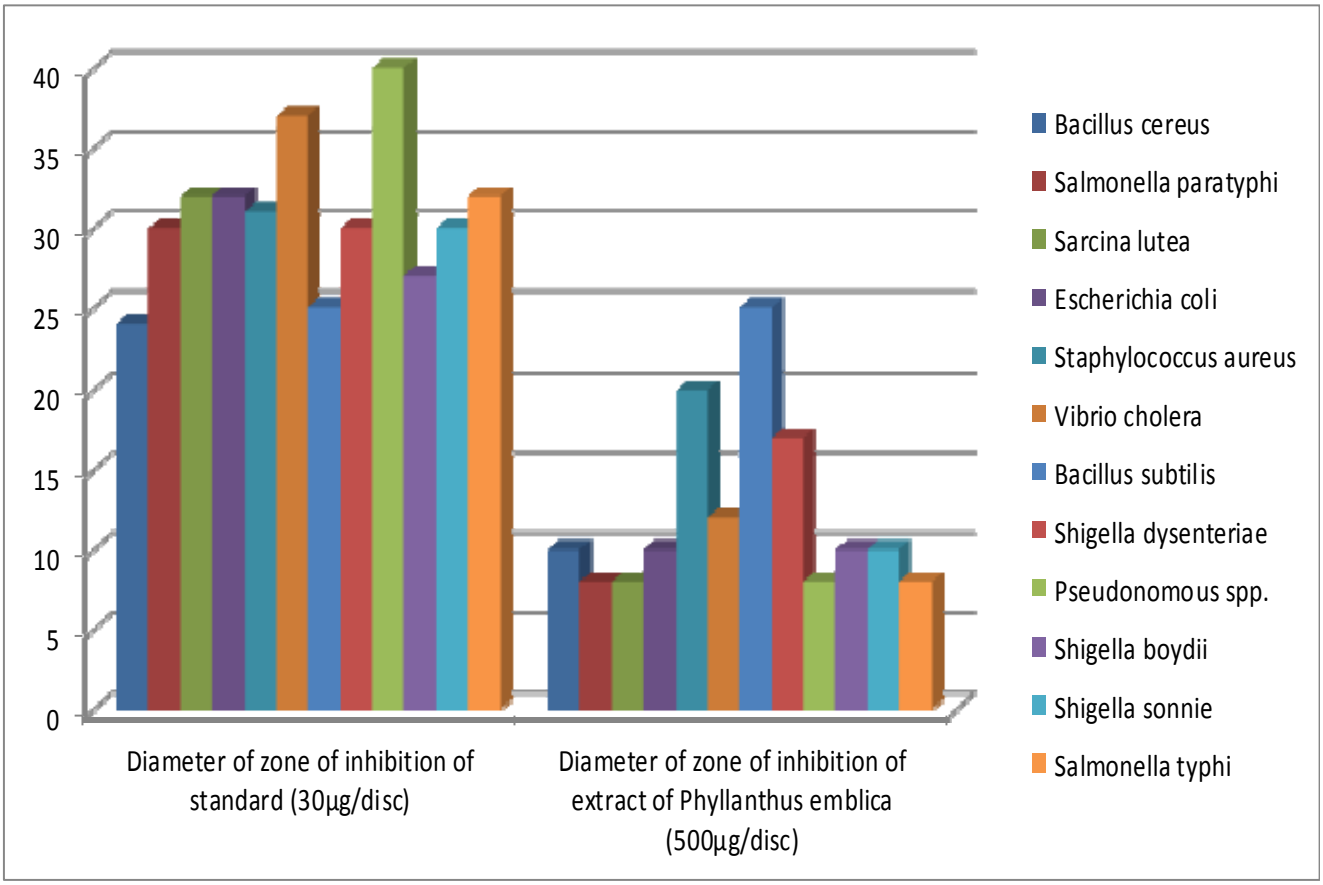


Fig 3: Graph of antimicrobial activity of *Phyllanthus emblica.*

Result for combination therapy (Azithromycin+Extract)

In combination therapy we used antibiotics and extract of *Phyllanthus emblica* combinly. Synergistic effect as well as Inhibitory effect are observed when the drug and extract are used together. Some drugs give no result when used alone.

Table 11: Antimicrobial activity of *Phyllanthus emblica* in combination with Azithromycin

Sl no.	Bacteria	Type of Bacteria	Diameter of zone of inhibition of Blank disc (10µL/ disc)	Diameter of zone of inhibition of extract (500µg/ disc)	Diameter of zone of inhibition of Azithromycin (15µg/disc)	Diameter of zone of inhibition of Extract+ Azithromycin (10µL+10µL) /disc
1.	*Bacillus cereus*	Gram positive	0	10mm	22mm	21mm
2.	*Salmonella paratyphi*	Gram negative	0	8mm	15mm	20mm
3.	*Sarcina lutea*	Gram positive	0	8mm	9mm	10mm
4.	*Escherichia coli*	Gram negative and resistant bacteria	0	10mm	9mm	15mm
6.	*Staphylococcus aureus*	Gram positive	0	20mm	29mm	35mm
7.	*Vibrio cholera*	Gram negative	0	12mm	18mm	26mm
8.	*Bacillus subtilis*	Gram positive	0	25mm	8mm	26mm
9.	*Shigella dysenteriae*	Gram negative	0	17mm	9mm	19mm
10.	*Pseudonomous spp.*	Resistant bacteria	0	8mm	0	10mm
11.	*Shigella boydii*	Gram negative	0	10mm	0	0
12.	*Shigella sonnei*	Resistant bacteria	0	10mm	26mm	25mm
13.	*Salmonella typhi*	Gram negative	0	8mm	0	11mm

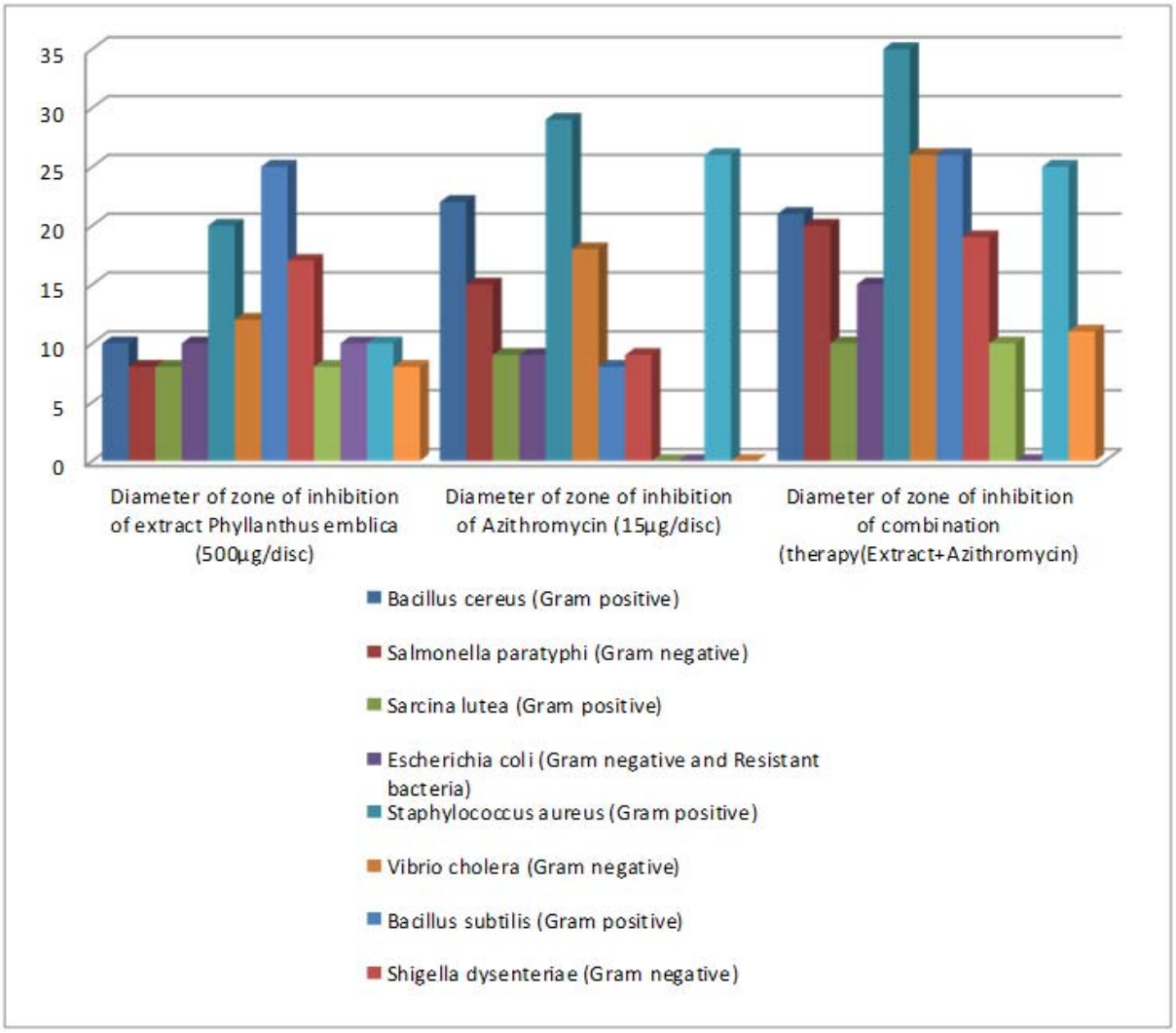
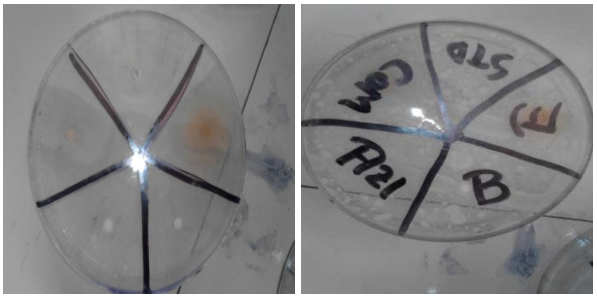


Fig 4: Graph of antimicrobial activity of combination therapy (Extract+Azithromycin) (500µg+15µg)/10µL/disc

Discussion

The antibiotic, Azithrimycin showed good activity against gram positive, gram negative, and resistant bacteria. It showed highest activity against *Staphylococcus aureus*, and the zone of inhibition was 29mm and when Azithromycin was combined with extract of *Phyllanthus emblica*, the activity was enhanced against *Staphylococcus aureus* (35mm). But Azithromycin showed no activity against some pathogens, they are *Shigella sonnie* (resistant bacteria), *Shigella boydii* and *Salmonella typhi* (gram negative). But when combination therapy was applied it showed good activity against *Shigella sonnie* and *Salmonella typhi* and the zone of inhibition was 25mm and 11mm but combination therapy of extract and azithromycin showed no activity against *Shigella boydii*.



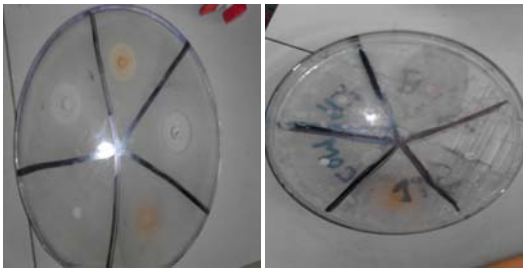


Fig 5: Image of zone of inhibition of Azithromycin and combination therapy (Extract+Azithromycin)

Result for combination therapy (Ciprofloxacin+Extract)

In case of combination therapy we used Ciprofloxacin and extract of *Phyllanthus emblica* combinedly.

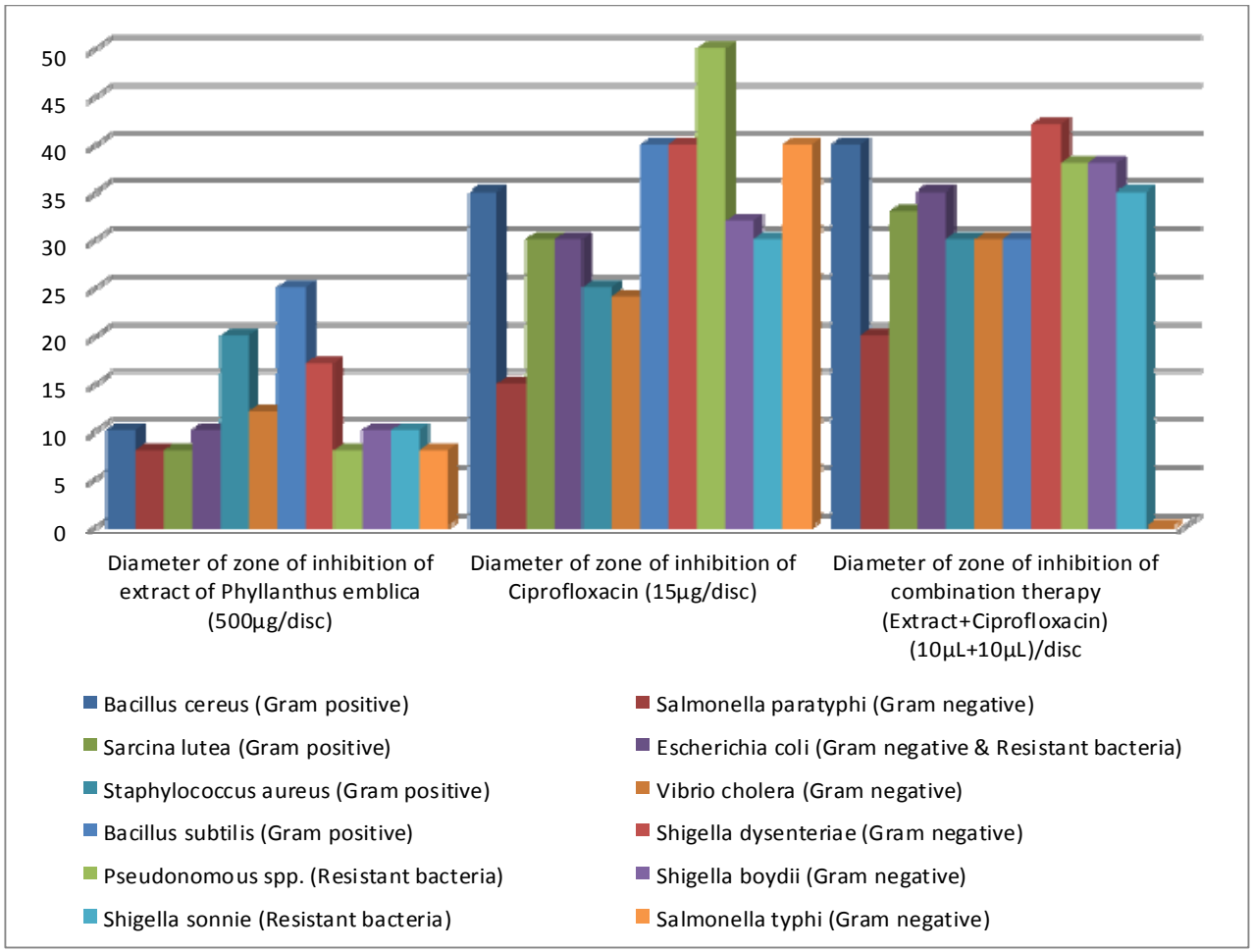


Fig 6: Graph of antimicrobial activity of combination therapy (Extract+Ciprofloxacin) (500µg+5µg)/disc

Table 12: Antimicrobial activity of *Phyllanthus emblica* combination with Ciprofloxacin

Sl no.	Bacteria	Type of Bacteria	Diameter of zone of inhibition of Blank disc (10µL/disc)	Diameter of zone of inhibition of extract (500µg/disc)	Diameter of zone of inhibition of Ciprofloxacin (15µg/disc)	Diameter of zone of inhibition of Extract+ Ciprofloxacin (10µL+10µL) /disc
1.	*Bacillus cereus*	Gram positive	0	10mm	35mm	40mm
2.	*Salmonella paratyphi*	Gram negative	0	8mm	15mm	20mm
3.	*Sarcinal utea*	Gram positive	0	8mm	30mm	33mm
4.	*Escherichia coli*	Gram negative and resistant bacteria	0	10mm	30mm	35mm
6.	*Staphylococcus aureus*	Gram positive	0	20mm	25mm	30mm
7.	*Vibrio cholera*	Gram negative	0	12mm	24mm	30mm
8.	*Bacillus subtilis*	Gram positive	0	25mm	40mm	30mm
9.	*Shigelladysenteriae*	Gram negative	0	17mm	40mm	42mm
10.	*Pseudonomous spp.*	Resistant bacteria	0	8mm	50mm	38mm
11.	*Shigella boydii*	Gram negative	0	10mm	32mm	38mm
12.	*Shigella sonnie*	Resistant bacteria	0	10mm	30mm	35mm
13.	*Salmonella typhi*	Gram negative	0	8mm	40mm	11mm

Discussion

The antibiotic Ciprofloxacin showed good antimicrobial activity against all the gram positive, gram negative, and resistant bacteria. Ciprofloxacin showed highest antimicrobial activity against *Pseudonomous spp.* (resistant bacteria) and the zone of inhibition is 50mm but combination therapy not enhanced the antimicrobial activity against this pathogen. Combination therapy enhanced antimicrobial activity against most of the bacteria except *Bacillus subtilis, Pseudonomous spp* and *Salmonella typhi.*

Fig 7: Image of zone of inhibition of Ciprofloxacin and combination therapy (Extract+ Ciprofloxacin)

Conclusion

Nature is blessed with variety of roots having medicinal properties. One of which is *Phyllanthus emblica*. On the basis of the findings of the present study it can be assumed that the fruit extract of *Phyllanthus emblica* has strong antimicrobial activity and it shows good result in combination therapy with different antibiotics. In this study the extract of *Phyllanthus emblica* give highest antimicrobial activity against some microorganisms especially, *Bacillus subtilis (25mm), Staphylococcus aureus (20mm)* and *Shigella dysenteriae (17mm)*. In case of combination therapy, significant synergistic activity was noticed along with Azithromycin against *Staphylococcus aureus (35mm),* it potentiates the activity of Ciprofloxacin against multi-drug resistant *Escherichia coli (35mm)*. Different constituent in *Phyllanthus emblica* shows antimicrobial activity for the treatment of different infection.

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