IN VITRO ANTIBACTERIAL ACTIVITY OF ETHANOL,METHANOL AND WATER EXTRACTS OF PERSIMMON FRUIT(DIOSPYROS PEREGRINE) AGAINST SOME MULTI-DRUG RESISTANT HUMAN PATHOGENS

M.S THESIS

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Dedicated to
My beloved family

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*D*iospyros peregrine is a small middle sized tree of costal Bangladesh. The fruits have ethno medicinal significance for the treatment of diarrhea, dysentery, cholera, ulcer of mouth and wounds. The world-wide increase in the prevalence of antibiotic resistant bacteria requires the rapid development of novel and more potent drugs .The unripe matured fruits are successfully used to treat worm infestations of children in Bangladesh. The fruits contain triterpenes, alkanes, flavonoids and tannins. The aim of the present study was to investigate antimicrobial activity of methanol and ethanol extract of Diospyros peregrina fruits. Persimmon fruit were examined against some selective gram positive and gram negative bacterial strains. Preliminary antimicrobial activity was evaluated by agar disc diffusion method. Minimum inhibitory concentration was determined by tube dilution (MIC) whilst Minimum Lethal Concentration concentration (MLC). Three organisms were sensitive to Methanol ethanol and water. Persimmon fruit was most effective for *Staphylococcus aureus* (MIC value 1125 µg/ml), followed by *Vibrio cholerae* non O1 (MIC value 1500 µg/ml) and *Vibrio cholerae* 569B (MIC value 2250 µg/ml).

Chapter 1

Introduction

1. INTRODUCTION

1.1 General Introduction:

Plants that possess therapeutic properties or exert beneficial pharmacological effects on the animal body are generally designated as Medicinal plant. Accordingly, the world health organization (WHO) consultative group on medicinal plants has formulated a definition of medicinal plants in the following way: "A medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes or which are precursors for synthesis of useful drugs"(Sofowora, 1982).

Nature has been a source of medicinal agents for thousands of years and since the beginning of mankind. The application of medicinal plants especially in traditional medicine is currently well acknowledged and established as a viable profession (Kafaru, 1994).Extraction of bioactive compounds from medicinal plants permits the demonstration of their physiological activity. It also facilitate pharmacological studies leading to synthesis of a more potent drug with reduce toxicity (Ebana, 1991,Manna & Abalaka, 2000).Furthermore, the active components of herbal remedies have the advantage of being combined with many other substances that appear to be inactive. However, these complementary components give the plant as a whole a safety and efficiency much superior to that of its isolated and pure active components (Shariff, 2001).

Clinical microbiologists have two reasons to be interested in the topic of antimicrobial plant extracts. First, it is very likely that these phytochemicals will find their way into the arsenal of antimicrobial drugs prescribed by physicians; several are already being tested in humans. It is reported that, on average, two or three antibiotics derived from microorganisms are launched each year (Clark, 1996). After a downturn in that pace in recent decades, the pace is again quickening as scientists realize that the effective life span of any antibiotic is limited. Worldwide spending on finding new anti-infective agents (including vaccines) is expected to increase 60% from the spending levels in 1993 (Alper, 1998). New sources,

especially plant sources, are also being investigated. Second, the public is becoming increasingly aware of problems with the over prescription and misuse of traditional antibiotics. In addition, many people are interested in having more autonomy over their medical care. A multitude of plant compounds (often of unreliable purity) is readily available over-the-counter from herbal suppliers and natural-food stores, and self-medication with these substances is common place. The use of plant extracts, as well as other alternative forms of medical treatments, is enjoying great popularity in the late 1990s. Earlier in this decade, approximately one-third of people surveyed in the United States used at least one "unconventional" therapy during the previous year (Eisenberg, *et al.*, 1993). It was reported that in 1996, sales of botanical medicines increased 37% over 1995 (Klink, 1997). It is speculated that the American public may be reacting to over prescription of sometimes toxic drugs, just as their predecessors of the 19th century reacted to the overuse of bleeding, purging and calomel (Yankauer, 1997).

Medicinal plants constitute an important natural wealth of a country. The reasons behind selecting cumin seeds (Nigella sativa) for this research were its easy availability throughout the country, and has excellent potency against pathogens. The current work aided to prepare extracts of cumin seeds having intrinsic organic and inorganic compounds from seeds using polar and non-polar solvents (chloroform, ethanol),to assess the antibacterial activity of the extracts against some pathogenic multi-drug resistant bacteria such as those causing toxin induced acute diarrhea, food poisoning and other diseases.

1.2 Significance of plants as medicine:

It is estimated that there are 250,000 to 500,000 species of plants on Earth (Borris, 1996). A relatively small percentage (1 to 10%) of these is used as foods by both humans and other animal species. Hippocrates (in the late fifth century B.C.) mentioned 300 to 400 medicinal plants (Schultes, 1978). The fall of ancient civilizations forestalled western advances in the understanding of medicinal plants, with much of the documentation of plant pharmaceuticals being destroyed or lost (Stockwell, 1988.). North America's history of plant medicinal use follows two

strands—their use by indigenous cultures (Native Americans), dating from prehistory (Weiner, 1980), and an "alternative" movement among Americans of European origin, beginning in the 19th century.

Holmes noted that medical treatments in the 1800s could be dangerous and ineffective. In 1861 Holmes wrote, "If the whole material medica as now used could be sunk to the bottom of the sea, it would be all the better for mankind—and all the worse for the fishes" (Holmes, 1861)

Mainstream medicine is increasingly receptive to the use of antimicrobial and other drugs derived from plants, as traditional antibiotics (products of microorganisms or their synthesized derivatives) become ineffective and as new, particularly viral diseases remain intractable to this type of drug. There is a feeling among natural-products chemists and microbiologists alike that the multitude of potentially useful phytochemical structures which could be synthesized chemically is at risk of being lost irretrievably (Borris, 1996). There is a scientific discipline known as ethnobotany (or ethnopharmacology), whose goal is to utilize the impressive array of knowledge assembled by indigenous peoples about the plant and animal products that they have used to maintain health (Georges, *et al.*, 1949). Lastly, the ascendancy of the human immunodeficiency virus (HIV) has spurred intensive investigation into the plant derivatives which may be effective, especially for use in underdeveloped nations with little access to expensive western medicines (De Clercq, 1995.)

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1.2.1. Medicinal plants used in traditional medicine:

Plants, plant parts and plant product of all description, particularly those with medicinal properties, are invariably used as principal components or ingredients of various traditional medicines. The number of plants with medicinal properties included in the meteria medica of traditional medicine in this subcontinent at present stands at about 2000 (R.N. Chopra *et al.*, 1958, Indigenous Drugs of India). More than 500 of such medicinal plants have so far been enlisted as growing in Bangladesh (M Yusuf *et al.*, 1994, Medicinal plants of Bangladesh).This number of the indigenous medicinal plants is in the increase with discovery and introduction of

newer plants everyday. In the traditional system at the present time almost every plants and herb growing in the country has ascribed to it some medicinal virtues and is used as either principal therapeutic agent or as necessary associate in medicinal preparations to increase the potency of the principal ingredients .Although uses of some of these plants are based on old and new experiences and clinical data, any of them no foundation whatsoever. Their introduction into traditional medicine is rather empirical and based on individual experience or isolated cases of their beneficial effect. This is how the numbers of medicinal plants have multiplied or sufficient scientific or clinical proof of their therapeutic properties. At the same time it is also true that, while the employment of a large number of the currently used medicinal plants would appear to have been based on empirical evidence handed down from generation to generation, many of these plants have been recommended as efficacious drugs after been clinically tied by the practicing physicians.

1.3. The isolated bacteria:

1.3.1. Staphylococcus aureus

S. aureus is a facultatively anaerobic, Gram-positive coccus, which appears as grape-like clusters when viewed through a microscope and has large, round, golden-yellow colonies, often with hemolysis, when grown on blood agar plates. The golden appearance is the etymological root of the bacteria's name; *aureus* means "golden" in Latin.

S. aureus is catalase positive (meaning that it can produce the enzyme "catalase") and able to convert hydrogen peroxide (H_2O_2) to water and oxygen, which makes the catalase test useful to distinguish staphylococci from enterococci and streptococci. A small percentage of *S. aureus* can be differentiated from most other staphylococci by the coagulase test: *S. aureus* is primarily coagulase-positive (meaning that it can produce "coagulase", a protein product, which is an enzyme) that causes clot formation while most other *Staphylococcus* species are coagulase-negative. However, while the majority of *S. aureus* are coagulase-positive, some may be atypical in that they do not produce coagulase (the most common organism in patients with nosocomial bacteremia is coagulase-negative staphylococcus).

Incorrect identification of an isolate can impact implementation of effective treatment and/or control measures.

S. aureus may occur as a commensal on human skin; it also occurs in the nose frequently (in about a third of the population) and throat less commonly. The occurrence of *S. aureus* under these circumstances does not always indicate infection and therefore does not always require treatment (indeed, treatment may be ineffective and re-colonisation may occur). It can survive on domesticated animals such as dogs, cats and horses, and can cause bumblefoot in chickens. It can survive for some hours on dry environmental surfaces, but the importance of the environment in spread of *S. aureus* is currently debated. It can host phages, such as the Panton-Valentine leukocidin, that increase its virulence.

S. aureus can infect other tissues when barriers have been breached (e.g., skin or mucosal lining). This leads to furuncles (boils) and carbuncles (a collection of furuncles). In infants *S. aureus* infection can cause a severe disease Staphylococcal scalded skin syndrome (SSSS).

S. aureus infections can be spread through contact with pus from an infected wound, skin-to-skin contact with an infected person by producing hyaluronidase that destroy tissues, and contact with objects such as towels, sheets, clothing, or athletic equipment used by an infected person. Deeply penetrating *S. aureus* infections can be severe. Prosthetic joints put a person at particular risk for septic arthritis, and staphylococcal endocarditis (infection of the heart valves) and pneumonia, which may be rapidly sprea.

1.3.2. Bacillus subtilis

Bacillus subtilis, known as the hay bacillus or grass bacillus, is a Gram-positive, catalase-positive bacterium commonly found in soil. A member of the genus *Bacillus*, *B. subtilis* is rod-shaped, and has the ability to form a tough, protective endospore, allowing the organism to tolerate extreme environmental conditions. Unlike several other well-known species, *B. subtilis* has historically been classified

as an obligate aerobe, though recent research has demonstrated that this is not strictly correct.

B. subtilis is not considered a human pathogen; it may contaminate food but rarely causes food poisoning B. subtilis produces the proteolytic enzyme subtilisin. B. subtilis spores can survive the extreme heating that is often used to cook food, and it is responsible for causing ropiness — a sticky, stringy consistency caused by bacterial production of long-chain polysaccharides — in spoiled bread dough.

1.3.3. Bacillus cereus

Bacillus cereus is an endemic, soil-dwelling, Gram-positive, rod-shaped, beta hemolytic bacterium. Some strains are harmful to humans and cause foodborne illness, while other strains can be beneficial as probiotics for animals. *B. cereus* bacteria are facultative aerobes, and like other members of the genus *Bacillus* can produce protective endospores.

There are two illness associated with *B. cereus*, emetic and diarrheal illness. The emetic illness is caused by the ingestion of a heat-stable toxin produced by the microorganisms in the food. The diarrheal illness is caused by the ingestion of moderate to high number of *B. cereus* and their subsequent production of toxin in the stomach.

The emetic type of food poisoning, with symptoms similar to that caused by *Staphylococcus aureus*, is characterised by nausea and vomiting. The symptoms of *B. cereus* diarrhoeal illness, similar to *Clostridium perfringens* food poisoning, include watery diarrhoea, abdominal cramps, and pain. Nausea may sometimes occur, and vomiting rarely occurs. Other *Bacillus* species will cause vomiting and diarrhoea.

A wide variety of foods including meats, milk, vegetables, and fish have been associated with the diarrheal type food poisoning. The emetic illness has generally

been associated with rice products and other starchy foods such as potato, pasta and cheese products. Food such as sauces, puddings, soups, casseroles, pastries, and salads have frequently been incriminated in food poisoning outbreaks.

1.3.4. Escherichia coli O157:H7

Escherichia coli O157:H7 is an enterohemorrhagic strain of the bacterium *Escherichia coli* and a cause of foodborne illness. Infection often leads to hemorrhagic diarrhea, and occasionally to kidney failure, especially in young children and elderly. Most illness has been associated with eating undercooked, contaminated ground beef, drinking unpasteurized milk, swimming in or drinking contaminated water, and eating contaminated vegetables.

E. coli O157:H7 was first recognized as a foodborne pathogen in 1982 during an investigation into an outbreak of hemorrhagic colitis (bloody diarrhea) associated with consumption of contaminated hamburgers (Riley, et al., 1983). The following year, Shiga toxin (Stx), produced by the then little-known *E. coli* O157:H7, was identified as the real culprit.

In the ten years following the 1982 outbreak, approximately thirty *E. coli* O157:H7 outbreaks were recorded in the United States (Griffin&Tauxe, 1991). The actual number that occurred is probably much higher because *E. coli* O157:H7 infections did not become a reportable disease (required to be reported to public health authorities) until 1987 (Keene et al., 1991 p. 60, 73). As a result, only the most geographically concentrated outbreaks would have garnered enough attention to prompt further investigation (Keene et al., 1991 p. 583). It is important to note that only about 10% of infections occur in outbreaks, the rest are sporadic.

The CDC has estimated that 85% of *E. coli* O157:H7 infections are foodborne in origin (Mead, *et al.*, 1999). In fact, consumption of any food or beverage that becomes contaminated by animal (especially cattle) manure can result in contracting the disease. Foods that have been sources of contamination include ground beef, venison, sausages, dried (non-cooked) salami, unpasteurized milk and cheese,

unpasteurized apple juice and cider (Cody, *et al.*, 1999), orange juice, alfalfa and radish sprouts (Breuer, et al., 2001), lettuce, spinach, and water (Friedman, et al., 1999).

E. coli serotype O157:H7 is a gram-negative rod-shaped bacterium. The "O" in the name refers the somatic antigen number, whereas the "H" refers the flagella antigen. Other serotypes may cause (usually less severe) illness, but only those with the specific O157:H7 combination are reviewed here. Other bacteria may be classified by "K" or capsular antigens. (The "O" stands for *ohne Hauch* [Ger. "without huff" or "without film"]; "H" for *Hauch*; and "K" for *Kapsel*.) This is one of hundreds of serotypes of the bacterium *Escherichia coli*. While most strains are harmless and normally found in the intestines of mammals, this strain may produce Shiga-like toxins, cause severe illness, and is a member of a class of pathogenic *E. coli* known as enterohemorrhagic *Escherichia coli* or EHEC. Sometimes also referred to by their toxin producing capabilities, Verocytotoxin producing *E. coli* (VTEC) or Shiga-like Toxin producing *E. coli* (STEC).

E. coli O157:H7 infection often causes severe, acute hemorrhagic diarrhea (although non-hemorrhagic diarrhea is also possible) and abdominal cramps. Usually little or no fever is present, and the illness resolves in 5 to 10 days. It can also be asymptomatic.

In some people, particularly children under 5 years of age and the elderly, the infection can cause haemolytic uremic syndrome, in which the red blood cells are destroyed and the kidneys fail. About 2–7% of infections lead to this complication. In the United States, haemolytic uremic syndrome is the principal cause of acute kidney failure in children, and most cases of haemolytic uremic syndrome are caused by *E. coli* O157:H7.

1.3.5. Shigella flexneri

Shigella flexneri is a species of Gram-negative bacteria in the genus *Shigella* that can cause diarrhea in humans. There are several different serogroups of

Shigella; *S. flexneri* belongs to group *B. S. flexneri* infections can usually be treated with antibiotics although some strains have become resistant. Less severe cases are not usually treated because they become more resistant in the future.

Shigella flexneri is a non-motile, non-spore forming, rod-shaped bacterium that is physiologically similar to *Shigella dysenteriae*, *Shigella boydii*, and *Escherichia coli*. It is important because it causes shigellosis, an acute bloody diarrhea. *Shigella flexneri* is the most common cause of the endemic form of shigellosis, and the endemic form is the cause of most Shigellosis-related deaths. While not much of a problem in developed countries, *Shigella flexneri* (specifically *Shigella flexneri* 2a) is a major public health concern in developing countries. *Shigella* was recognized as the cause of bacillary dysentery in the 1890s by Shiga, hence the genus name

In humans and other primates, *Shigella flexneri* causes an acute bloody diarrhea known as shigellosis or bacillary dysentery (Jin et al.). Aside from bloody diarrhea, other symptoms include fever and stomach cramps. The bleeding is due to destruction of the intestines. The bacteria destroy the intestinal epithelium, then continue to break down the intestinal mucosa in the cecum and rectum (Clark and Maurelli). The condition can be fatal if not treated, and early diagnosis is important to effective therapy (Nato et al.). *Shigella flexneri* is not susceptible to dapsone, but it is susceptible to ampicillin, nalidixic acid, ciprofloxacin, and trimethoprim/sulfamethoxazole (AKA Bactrim or Septra). However, antibiotics should be used only for severe cases since antibiotic resistance is on the rise (Huang and Zhou).

Infection typically occurs via ingestion. Once internalized, *Shigella flexneri* survives within human hosts by causing apoptosis (programmed cell death) in macrophages while inhibiting apoptosis in epithelial cells. A protein called IpaB activates caspase 1 in macrophages, and the caspase cascade leads to apoptosis.

Diagnosis of Shigellosis involves determining whether the Shigella bacteria is responsible for the symptoms. This is done with a series of laboratory tests identifying the bacteria in the stool of the infected person. This procedure can also isolate the species responsible for the infection, and can determine which antibiotics would be best for treatment.

1.3.6. Vibrio cholerae 569B and non O1:

Vibrio cholerae, a noninvasive gram-negative bacterium and the causative agent of the diarrheal disease cholera, is serologically classified as belonging to the O antigenic group. Strains belonging to O group 1 (O1) are responsible for cholera. Strains other than O1 are called non-O1; they can cause only sporadic infections and do not have the potential to cause epidemics. Strains of serovar O1 consist of two biotypes, classical and El Tor. Only recently, an outbreak of cholera in India and Bangladesh which subsequently spread into several parts of the subcontinent was caused by a novel non-O1 strain, O139 Bengal. *Vibrio cholerae* as a species includes both pathogenic and nonpathogenic strains that vary in their virulence gene content. This bacterium contains a wide variety of strains and biotypes, receiving and transferring genes for toxins, colonization factors, antibiotic resistance, capsular polysaccharides that provide resistance to chlorine and new surface antigens, such as the 0139 lipopolysaccharide and O antigen capsule. The lateral or horizontal transfer of these virulence genes by phage, pathogenicity islands and other accessory genetic elements provides insights into how bacterial pathogens emerge and evolve to become new strains.

A transmission electron micrograph of *Vibrio cholerae*, negatively stained to enhance contrast. (Copyright: Wadsworth Center, New York State Department of Health). Cholera is characterized by watery, mucus-flecked stools commonly referred to as "rice water stool". Fluid and electrolyte loss in cholera can be severe. *V. cholerae* thrive in marine environments in temperate or tropical areas of the world. Infection is generally acquired through ingestion of contaminated food or water

The general assumption, until quite recently, was that cholera was spread only by infected people to other susceptible individuals via fecal contamination of water and food and that global movement of populations accounted for the global movement of the disease. Recent studies of the aquatic environment, however, have shown that V cholerae, including strains of O1 and O139, are normal inhabitants of surface water, particularly brackish waters, and survive and multiply in association with zooplankton and phytoplankton quite independently of infected human beings. Because global climate changes affect the growth of plankton, growth of the vibrios associated with plankton could also be modified. The continuing presence of cholera in the Indian subcontinent and the re-emergence of cholera in other continents may be highly dependent on environmental factors. The movement of the bacteria in association with plankton has led to the suggestion that ship ballast may be a cause of its global spread

Vibrio cholerae 569B is a hypertoxinogenic strain of Vibrio cholerae that was first isolated from a patient in 1948 in India

The non-O1 serogroups of *V. cholerae* comprise a heterogeneous group of organisms whose clinical association with humans is inadequately understood. Clinically, apart from the O1, the non-O1 serogroups continue to be of negligible significance since these strains are associated with illness in only a low percentage of patients hospitalized due to acute secretory diarrhea. Nucleotide analysis of the asd genes of 45 strains of *V. cholerae* has yielded provocative evidence which indicates that the classical and El Tor biotypes and U.S. Gulf Coast strains of *V. cholerae* O1 evolved independently from environmental nontoxigenic, non-O1 strains. Therefore, it has become increasingly clear that the non-O1serogroups are involved in the emergence of newer variants of *V. cholerae.*

Vibrio cholerae Serogroup Non-Ol This bacterium infects only humans and other primates. It is related to V. cholerae Serogroup O1, the organism that causes Asiatic or epidemic cholera, but causes a disease reported to be less severe than cholera. Both pathogenic and nonpathogenic strains of the organism are normal inhabitants

of marine and estuarine environments of the United States. This organism has been referred to as non-cholera vibrio (NCV) and nonagglutinable vibrio (NAG) in the past, although at least 139 "O" serogroups have been identified.

Diarrhea, abdominal cramps, and fever are the predominant symptoms associated with this illness, with vomiting and nausea occurring in approximately 25% of infected individuals. Approximately 25% of infected individuals will have blood and mucus in their stools. Diarrhea may, in some cases, be quite severe, lasting 6-7 days. Diarrhea will usually occur within 48 hours following ingestion of the organism. It is unknown how the organism causes the illness, although an enterotoxin is suspected as well as an invasive mechanism. Disease is caused when the organism attaches itself to the small intestine of infected individuals and perhaps subsequently invades.

Diarrhea resulting from ingestion of the organism usually lasts 7 days and is self-limiting. Antibiotics such as tetracycline shorten the severity and duration of the illness. Septicemia (bacteria gaining entry into the blood stream and multiplying therein) can occur. This complication is associated with individuals with cirrhosis of the liver, or who are immunosuppressed, but this is relatively rare

1.4 Major groups of antimicrobial compounds in plants

Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives (Geissman, 1963). Useful antimicrobial phytochemicals can be divided into several categories, described below and summarized in Table 1.1

Table 1.1: Major classes of antimicrobial compounds from plants

Class	Subclass	Examples	Mechanism
	Simple phenol	Catechol, Epicatechin	Substrate deprivation, Membrane disruption
	Phenolic	Cinnamic acid	
	Quinones	Hypericine	Bind to adhesins

Phenolics			complex with cell wall
	Flavonoids	Chrysin	Bind to adhesins
	Flavones	Abyssinone	Inactivate enzymes
			Inhibit HIV reverse transcriptase
	Tannins	Ellagitannin	Bind to adhesins
			Enzyme inhibition
			Substrate deprivation
			Complex with cell wall
			Metal ion complexation
	Coumarins	Warfarin	Interaction with eukaryotic DNA (antiviral activity)
Alkaloids		Berberine,Piperine	Intercalate into cell wall and/ or DNA
Lectins and polypeptides		Mannose-specific agglutinin	Block viral fusion or adsorption

1.4.1 Phenolics and Polyphenols

. The common herbs tarragon and thyme both contain caffeic acid, which is effective against viruses (Wild, 1994), bacteria (Brantner, *et al*, 1996), and fungi (Duke, 1985).In addition, some authors have found that more highly oxidized phenols are inhibitorier (Scalbert, 1991). The mechanisms thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins (Mason and Wasswrman, 1987).

1.4.2. Quinones.

Quinones are known to complex irreversibly with nucleophilic amino acids in proteins (Stern, *et al.*, 1996), often leading to inactivation of the protein and loss of function. Hypericin, an anthraquinone from *Hypericum perforatum* has general antimicrobial properties (Duke, 1985).

1.4.3. Flavones, flavonoids, and flavonols.

Flavones are phenolic structures containing one carbonyl group (as opposed to the two carbonyls in quinones). The addition of a 3-hydroxyl group yields a flavonol (Fessenden, *et al.*, 1982). Since they are known to be synthesized by plants in response to microbial infection (Dixon, *et al.*, 1983), it should not be surprising that they have been found in vitro to be effective antimicrobial substances against a wide array of microorganisms. More lipophilic flavonoids may also disrupt microbial membranes (Tsuchiya, *et al.*, 1996).

1.4.4. Tannins.

Tannins may be formed by condensations of flavan derivatives, which have been transported to woody tissues of plants. Alternatively, tannins may be formed by polymerization of quinone units (Geissman, 1963). One of their molecular actions is to complex with proteins through so-called nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation (Stern, *et al.*, 1996). Tannins in plants inhibit insect growth (Schultz, 1988) and disrupt digestive events in ruminal animals (Butler, 1988).

1.4.5. Coumarins.

Coumarins are phenolic substances made of fused benzene and pyrone rings (O'Kennedy, *et al.*, 1997). They are responsible for the characteristic odor of hay. Their fame has come mainly from their antithrombotic (Thastrup, *et al.*, 1985), anti-inflammatory (Piller, 1975), and vasodilatory (Namba *et al.*, 1988) activities.

1.4.6. Terpenoids and Essential Oils.

Common terpenoids are methanol and camphor (monoterpenes) and farnesol and artemisin (sesquiterpenoids). Artemisin and its derivative -arteether, also known by the name qinghaosu, find current use as antimalarials (Vishwakarma, 1990). Terpenenes or terpenoids are active against bacteria (Ahmed *et al.*, 1993), fungi (Rana, *et al.*, 1997), viruses (Xu,. *et al.*, 1996), and protozoa (Ghoshal, *et al.*, 1996). A terpenoid constituent, capsaicin, has a wide range of biological activities in

humans, affecting the nervous, cardiovascular, and digestive systems (Gebhart, *et al.*, 1979) as well as finding use as an analgesic (Cordell, *et al.*, 1993).

1.4.7. Alkaloids.

Solamargine, a glycoalkaloid from the berries of *Solanum khasianum*, and other alkaloids may be useful against HIV infection (McMahon, *et al.*, 1995) as well as intestinal infections associated with AIDS (McDevitt, *et al.*, 1996). While alkaloids have been found to have microbiocidal effects (including against *Giardia* and *Entamoeba* species (Ghoshal, *et al.*, 1996).

1.4.8. Lectins and Polypeptides.

Peptides which are inhibitory to microorganisms were first reported in 1942 (Balls, *et al.*, 1942). They are often positively charged and contain disulfide bonds (Zhang, *et al*). Their mechanism of action may be the formation of ion channels in the microbial membrane (Terras, *et al.*, 1993) or competitive inhibition of adhesion of microbial proteins to host polysaccharide receptors (Sharon, *et al.*, 1986).

1.5. Biological activity of different extracts from *Diospyros* species.

Diospyros species have been summarized in Table I and Table II. The chemical constituents isolated from different plant parts of *Diospyros* species have been given in the Table 1.2.

1.2.Biological activity of different extracts from *Diospyros*

S. No	Plant species	Different extracts	Biological activity
1.	*D. tricolor*	Petroleum ether	Antimicrobial
2.	*D. Montana*	Ethanol	-
3.	*D. marrisiana*	Hexane	Cytotoxicity Antibiotic activity
4.	*D. peregrina*	Ethanol	Antiprotozoal Antiviral

			Hypoglycemic Antibacterial

1.3.Biological activity of different compounds from *Diospyros* species.

S. No	Plant species	Different compound	Biological activity
1.	*D. leucomelas*	Betulinic acid	Anti-inflammatory
		Betuline	Anti-inflammatory
		Ursolic acid	Anti-inflammatory
2.	*D. morrisiana*	Isodiospyrin	Cytotoxicity
		β-amyrin	Cytotoxicity
		Olean-12-en-3-one	Cytotoxicity
		Bi-Naphthoquinone	Cytotoxicity
3.	*D. tricolor*	Diosquinone	Antibacterial
4.	*D. mollis*	Phenolic compound	Anthelmintic
5.	*D. usambarensis*	7-methyljuglone	Molluscocidal and antifungal
		Mamegakinone	Molluscocidal and antifungal
		Isodiospyrin	Molluscocidal and antifungal

1.4.Chemical compounds of *Diospyros* species.

S. No	Plant species / Plant part	Compound
1.	*D. peregrine roots*	Dihydroflavonol glycoside
		5, 7, 3, 5' – Tetra hydroxyl – 3' – methoxy flavone
		4'–O- α-L–Rharmnopyranoside
	Leaves	Triterpenes, anthrocyanin
	Fruits	Lup-20 (29)-3n-3α, 27-diol-29
		Lup-20 (29)-3n-3β-diol-29
		Taraxerone
		Sitosterol
		Gallic acid
		Peregrinol
	Fruit Pulb	Hexacosane
		Hexacosanol
		β-sitosterol
		Monohydroxy triterpene ketone
		Betulin
		β-D-Glycoside of β–sitosterol
		Gallic acid
		Betulinic acid
		Methyl ester acetate, Methylester

		B-D-Glycoside of β-sitosterol
2.	*D. mollis* Griff	
	Fruits	Lupeol
		α-amyrine
		β-sitosterol
		Diospyrol
		1, 8 dihydroxynapthalene
		8-dihydroxy-2-acetyl-3-methyl
		naphthalene
3.	*D. Montana*	
	Leaves	Lupeul
		Sitosterol,
		Stigmasterol,
		Epi-uvaol,
		Betulin,
		Urs-12-en-3β-28-diol
		Oleanolic acid
4.	*D. melanoxlon* Roxb.	
	(Heart wood)	β-sitosterol terpenoid
		Lupeol
		Betulin
		Betulinic acid
		2-methyl-5-methoxy-1

		4-naphthaquinone, 3-methyl-8-methoxy-1, 9, naphthaquinone, 2-methl-3-hydroxy-5-methoxy, and 2- methyl 5, 6 Di methoxy-1, 4-napthaquinone.
	Leaves	β-sitosterol Monohydroxy monocarboxylic acid, Monohydroxy triterpene Bauererys acetate, Ursolic, Betulinic acid, Baurenol, ursolic Diospyric acid, Isobanerenol, Methyl betulinate
5.	*D. morrisiana* Root Stem	Isodiospyrin Betulinic acid
6.	*D. ismailli* Ng Fresh wood	Isodiospyrin β-amyrine Olean-12-en-3-one β-amrine acetate Novel napthoquinone Coumarin

		Ismallin
		4-hydroxy-5-methyl coumarins
		4-hdroxy-5-methy
7.	*D. lotus* (L.)	Taraxerol, Isodiospyrin,
		7-methyljugulone
		Betulinic acid
		Xallobetulin
		8, 8'-dihydroxy-6,
		61-dimethl binaphtho quinonyl-2,2'
8.	*D. tricolor*	Isodiospyrin
		Diosquinone
9.	*D. canaculata* De Wild	Napththoquinone
		Coumarin
		Ismailin
		Canaculation
10.	*D. mollis*	Tetra hydroxy dimethyl-2, 2'
		Binaphthyl
11.	*D. usambarensis*	
	Root bark	7-methyljuglone,
		Mamagakinone,
		Isodiospyrin,
		Diosindigo A
		7-methyluglone
		Diosindigo B

	Stem bark	Diosindigo A 7-methyljuglone
12.	*D. leucomelas* Leaves	Betulin Betulinic acid Ursolic acid
13.	*D. chloroxlon* Wood	7-methyljuglone Diospyrin Isodiospyrin Xylospyrin 2-methyl-3, 6-dihdroxy-4, 5 Dimethoxy haphthalenes 2-methyl-3, 4, 5, 6-tetra methoxy-napthalene

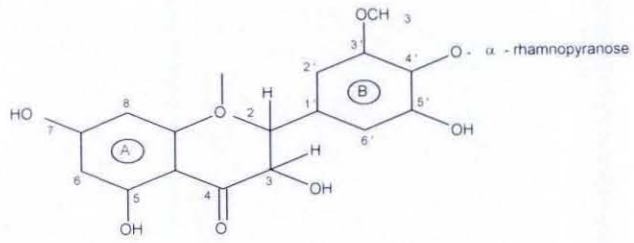
1.5.1Chemical constituents of *Diospyros* species

Different classes of compounds have been isolated from different species. They are as follows.

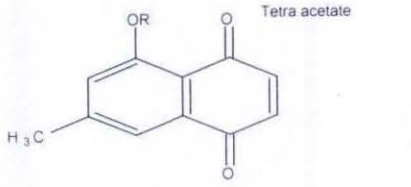
The main components isolated from the *Diospyros* species are triterpenes and their steroids compounds.

Dichloromethane extract of *D. leucomelos* Poir leaves isolated three triterpenes betulin, betulinic acid and ursolic acid were identified by ^{1}H – and ^{13}C-NMR spectra studies (Chopra *et al.*, 1956).

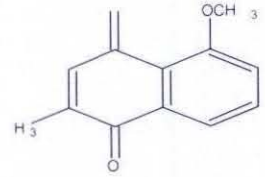
The chemical composition of the root of *D. lotus* (L.) was investigated by Yoshihira *et al.*, 1970, the chloroform extract separated in four napthoquinones, 7-methyljugulone, 150 diospyrin, and quinines besides the three tri-terpenoids, taraxerol, betulinic acid and oxallobetulin.



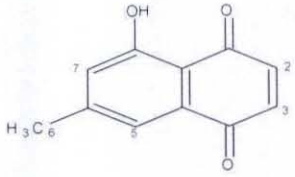
5 , 7 , 3 , 5 ' - tetra - hydroxy - 3 ' - methoxy flavanone
- 4 ' O - α - rhamnopyranoside



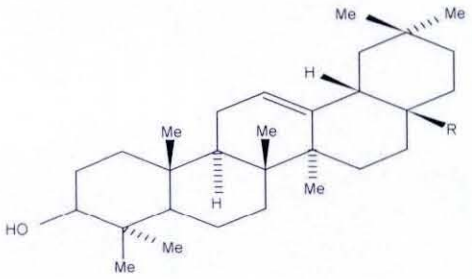
R : H-Hydroxynaphtho- 1 , 4 -quinone derivative
R : CH 3 -di methyl ether



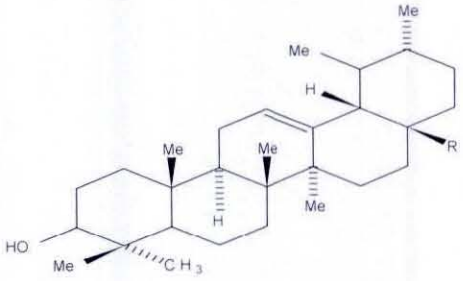
Di - O-methyl diospyroquinone



7 - methyljugulone



β - amyrin (R = Me)
Oleanolic acid (R = CO 2 H)



α - amyrin (R = Me)
Ursolic acid (R = CO 2 H)

1.6. Diospyros Peregrina

1.6.1. Botanical Classification:

Kingdom- Plantae

Division-Magnoliophyta

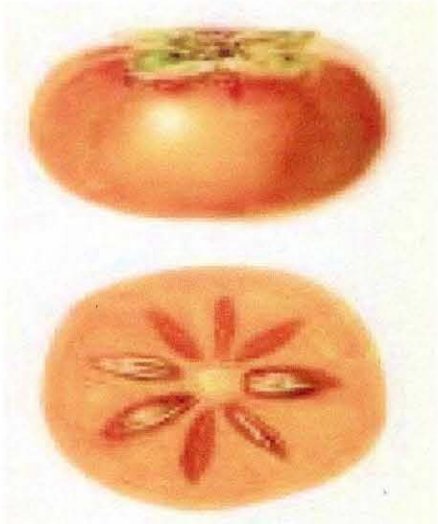
Class- Magnoliop

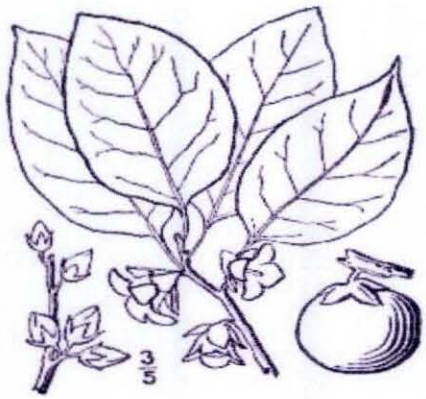
Order-Ericales

Family-ebenaceae

Genus- Diospyros

Species-*Peregrina*





1.1:Figure: *Persimmon fruit.*

1.6.2. Common name:

- English name – Indian persimmon
- Hindi name – gaabh
- Sanskrit name – tinduk
- Gujarati name – timbravon

1.6.3. Native country:

persimmon belongs to the botanical family of Ebenaceae and commonly grows in Europe, America, Japan, and south Asia.

1.6.4. Plant description:

It is a small tree usually thirty to eighty feet (ten to twenty-four meters) in height, with a short, slender trunk and spreading, often pendulous branches, which form sometimes a broad and sometimes a narrow round-topped head. The roots are thick, fleshy and stoloniferous. It is also given to shrubby growth. The tree has oval entire leaves, and unisexual flowers on short stalks. In the male flowers, which are numerous, the stamens are sixteen in number and arranged in pairs; the female flowers are solitary, with traces of stamens, and a smooth ovary with one ovule in each of the eight cells—the ovary is surmounted by four styles, which are hairy at the base. The fruit-stalk is very short, bearing a subglobose fruit an inch or rather more in diameter, of an orange-yellow color, ranging to bluish, and with a sweetish astringent pulp. It is surrounded at the base by the persistent calyx-lobes, which increase in size as the fruit ripens. The astringency renders the fruit somewhat unpalatable, but after it has been subjected to the action of frost, or has become partially rotted or "bletted" like a medlar, its flavor is improved.

- Bark: Dark brown or dark gray, deeply divided into plates whose surface is scaly. Branchlets slender, zigzag, with thick pith or large pith cavity; at first light reddish brown and pubescent. They vary in color from light brown to ashy gray and finally become reddish brown, the bark somewhat broken by longitudinal fissures. Astringent and bitter.
- Wood: Very dark; sapwood yellowish white; heavy, hard, strong and very close grained. Specific gravity, 0.7908; weight of cubic foot, 49.28 lb (22.35 kg).
- Winter buds: Ovate, acute, one-eighth of an inch long, covered with thick reddish or purple scales. These scales are sometimes persistent at the base of the branchlets.

- Leaves: Alternate, simple, four to six inches (152 mm) long, oval, narrowed or rounded or cordate at base, entire, acute or acuminate. They come out of the bud revolute, thin, pale, reddish green, downy with ciliate margins, when full grown are thick, dark green, shining above, pale and often pubescent beneath. In autumn they sometimes turn orange or scarlet, sometimes fall without change of color. Midrib broad and flat, primary veins opposite and conspicuous. Petioles stout, pubescent, one-half to an inch in length.
- Flowers: May, June, when leaves are half-grown; diœcious or rarely polygamous. Staminate flowers borne in two to three-flowered cymes; the pedicels downy and bearing two minute bracts. Pistillate flowers solitary, usually on separate trees, their pedicels short, recurved, and bearing two bractlets.
- Calyx: Usually four-lobed, accrescent under the fruit.
- Corolla: Greenish yellow or creamy white, tubular, four-lobed; lobes imbricate in bud.
- Stamens: Sixteen, inserted on the corolla, in staminate flowers in two rows. Filaments short, slender, slightly hairy; anthers oblong, introrse, two-celled, cells opening longitudinally. In pistillate flowers the stamens are eight with aborted anthers, rarely these stamens are perfect.
- Pistil: Ovary superior, conical, ultimately eight-celled; styles four, slender, spreading; stigma two-lobed.
- Fruit: A juicy berry containing one to eight seeds, crowned with the remnants of the style and seated in the enlarged calyx; depressed-globular, pale orange color, often red-cheeked; with slight bloom, turning yellowish brown after freezing. Flesh astringent while green, sweet and luscious when ripe.

1.6.5. Propagation

Diospyros Peregrina seedlings are the preferred rootstocks for persimmon cultivars. They develop long taproots with few fibrous laterals, and rootstock cultivars have been selected that produce vigorous, uniform seedlings. Rootstocks of D. virginiana

(American persimmon) and D. lotus (date plum) are known to be better for wet soils, but the former produces variable trees and excessive suckering. D. lotus is susceptible to crown gall and is incompatible with the 'Fuyu' cultivar as rootstocks or scionwood. Seeds are sown in 3-in.-deep (7.6-cm) containers. When seedlings are 3 in. (7.6 cm) high, they are transplanted to deep plastic planting bags—6 x 18 in. (15.2 x 45.7 cm)—or to nursery beds. At that time, the bottom one-fourth of the taproot is pruned to encourage lateral rooting. Grafting is done during the dormant season on rootstock stems that are at least 3/8 in. (9 mm) in diameter. Whip-grafting low on the rootstock is preferred, but chip-budding is also done. Scions with two to four buds from the previous season's growth are used. After grafting, the scion should be enclosed in a plastic bag to maintain high humidity. Large plants may be bark-grafted or cleft-grafted. In Hawaii, the three cultivars commonly grown develop very few seeds, and seed for rootstocks is usually obtained from California.

1.6.6 Soil type and location

Persimmon grows best on loamy soils, such as the Kula series. Light, sandy soils are not suitable, but it will grow on many other soil types and is tolerant of heavy clay soils if drainage is not severely impeded. Soil pH of 6.0 to 6.5 is preferred. Persimmon is grown commercially in Hawaii above elevations of 2000 ft (609 m). It is sometimes grown as a home garden fruit in cool locations at lower elevations. Most of the current production is in the Kula district of Maui, where persimmon flowers in March and April. Rainfall of at least 30 in. (762 mm) is required for good performance.

Wind damage seldom occurs in Kula, but in other areas, trees should be protected from strong winds. In the spring, the young foliage is easily damaged. In the fall, premature defoliation by wind affects fruit quality and the next year's production. Branches with heavy crop loads may be broken during windy weather. Shading by windbreak trees should be avoided. If persimmon does not receive full sun, weak growth and fruit drop may result.

1.6.7. Harvest

Persimmons are harvested when mature but still firm, with color nearly fully developed. 'Maru' fruit is greenish yellow when ripe; 'Fuyu' and 'Hachiya' fruits are orange. The fruit is removed from the tree by clipping or breaking the stems, leaving the calyx lobes attached to the fruit (Figures 2, 4, 6). Persimmons must be handled carefully to avoid damage. Rough handling causes bruising and skin discoloration.

1.6.8. Cultural practices

Tree spacing averages 15 ft to 20 ft (4.6-6.1 m) apart but varies with cultivar and soil fertility. Generally, wider spacing is used on deeper, more fertile soils. In Japan, trees are sometimes planted at close spacing and thinned after five to 10 years. Care is necessary when transplanting to the field, because persimmon roots are fragile and easily damaged by drying or rough handling. Young plants are trained to a modified central-leader structure by pruning shoots during the first few seasons, forcing growth into framework branches. The aim is to develop a pyramidal shape with from three to five main limbs at about 1-ft (30-cm) intervals on the trunk, beginning at about 3 ft (91 cm) above ground level. Staking with 5-ft (1.5-m) stakes may aid in training young trees. Pruning mature plants is done during the dormant winter months (Figure 7) to remove crossover, diseased, or broken branches. Pruning is also done to remove weak, shaded branches, open the canopy to prevent self-shading, reduce excessively vigorous shoot growth, and regulate crop load. Persimmon fruit is borne on the current season's branch growth. After three to five years, bracing may be needed to prevent the weight of the fruit from breaking branches (Figure 8). Pruning secondary branches so that bearing shoots are kept close to the main branches may help to avoid a drooping habit and reduce the need for bracing. 'Fuyu' fruit clusters are usually thinned to increase fruit size. Irrigation to supplement rainfall is desirable at times such as after transplanting, particularly when bare-rooted stock is used; during the spring growth flush; and during summer, if weather is dry or soils are shallow. Commercial growers in Hawaii use either 1616-16 or 10-20-20 N-P-K fertilizer, applied in February or March when new shoots emerge. Excessive nitrogen

fertilization will force vegetative growth, so moderate fertilizer applications are desirable.

1.6.9. Chemical composition of *D*iospyros peregrine:

A new triterpene was isolated from the fruits of *D. peregrina* and its structure elucidated as lup 20(20) – en-3α, 27 diol on the basis of spectral analysis (Jeffreys *et al.*, 1985). Maridass, 1999 analyzed the chemical composition by the fruit oil of *D. malabarica* Desr. by capillary GC and GC/MS studies. More than 35 constituents were isolated of which 29 were identified. The main constituents of trans methyl isoeugenol (31.86%), β-bisabolene (25.91).

1.7. Medicinal uses and precautions

The raw fruit is used to treat constipation and hemorrhoids, and to stop bleeding. As such, it is not a good idea to consume too many persimmons at once—they can induce diarrhea. On the other hand, the cooked fruit is used to treat diarrhea and dysentery. The fruits of some persimmon varieties contain the tannins catechin and gallocatechin (Nakatsubo et al. 2002), as well as the anti-tumor compounds betulinic acid and shibuol, although the latter may also cause gastrointestinal problems.

The soluble tannin shibuol found in unripened persimmons, upon contact with a weak acid, polymerizes in the stomach and forms a gluey coagulum that can affix with other stomach matter (Verstanding et al. 1989). The *Merck Manual of Diagnosis and Therapy* notes that consumption of persimmons has been known to cause bezoars that require surgery in over 90% of cases. Persimmon bezoars often occur in epidemics in regions where the fruit is grown (Cohen 2007). Horses may develop a taste for the fruit growing on a tree in their pasture and overindulge also, making them quite ill. It is often advised that persimmons should not be eaten with crab meat nor on an empty stomach.

1.7.1 Various Uses of Persimmon Tannin

Medical Uses

Effects	Explanation
Lowering blood pressure	Persimmon tannin has been used as folk medicine for treating stroke in Japan and as herbal medicine in China since ancient times.
Recovering from intoxication	A persimmon fruit, as an herbal medicine, is effective for recovery from intoxication. Persimmon juice lowers the density of alcohol in the blood stream.
Relieving burns	Persimmon tannin has a tendency to stick to human tissue. Since tannin prevents swelling at the level of cells and astringes cells, it suppresses the swelling and prevents blisters.
Relieving frostbite	When persimmon tannin is absorbed into tissues, it protects cells, it astringes tissues, and it suppresses the propagation of bacteria.
Relieving diarrhea	Persimmon tannin calms intestinal movements.
Treating bruises	Persimmon tannin effectively slows subcutaneous bleeding.

Equipment for sake- making	Persimmon tannin is used as paint for *sake* sacks, and wooden buckets.
Protecting fishing nets	Persimmon tannin prevents the corrosion of fishing nets made with natural materials.
Prevention of propagation of bacteria	Persimmon tannin hardens the fiber of wood and paper, prevents corrosion, and makes them waterproof. It has been used to make Japanese paper, umbrella, and wooden wear such as wooden cups.

1.7.3. Antioxidant Properties

Identification of persimmon as a source of antioxidant activity has just emerged over the past few years. A recent study of fresh and dried persimmon fruit revealed that both contain high levels of bioactive compounds and exhibit significant antioxidant activity .Earlier, a methanol extract of *D. kaki* was found to be a potent scavenger of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, demonstrating significant antioxidant potential.

Last year, a study of low-density lipoprotein (LDL) antioxidant activity in edible plants for use in preventing atherosclerosis landed persimmon extract in a group with the more heralded green tea. Extracts of both plants, along with two others from an original sample of 52 edible plants, showed the greatest ability to inhibit human LDL oxidation. Another noteworthy result of this study was that when researchers measured radical scavenging activity against DPPH, they observed that the LDL antioxidant in the most potent plant products was underestimated in the DPPH radical scavenging assay .

1.7.4. Antitumor Properties

In a study evaluating the fractionated extracts of persimmon peels for wide-ranging health benefits, significant cytotoxic activity was seen in the acetone fractions against human oral squamous cell carcinoma and human submandibular gland tumor cells. The researchers reported scavenging of the superoxide radical produced by hypoxanthine and xanthine oxidase reactions, and also found substantial reversal of multidrug resistance (MDR) activity. They concluded that such findings demonstrate the therapeutic potential of persimmon peel extract as an antitumor and MDR-reversing agent .

Previous research had suggested the antitumorigenic potential of persimmon. In a study of the effects of persimmon extract and several polyphenolic compounds on

the growth of human lymphoid leukemia Molt 4B cells, investigators found that persimmon strongly inhibited cell growth in a dose-dependent fashion as effectively as ornithine decarboxylase does. This finding, along with a morphologic study that revealed severe damage, anomalous cell shapes, and DNA fragmentation in cells treated with persimmon (as well as other polyphenols), indicated that persimmon induced apoptosis in the leukemia cells, the investigators concluded .

Persimmon extract has also been shown to strongly inhibit DNA polymerase alpha activity as well as thymidine incorporation into human peripheral lymphocyte cells stimulated by phytohemagglutinin .

Antibacterial activity has also been displayed by this astringent fruit. Persimmon exhibits rapid antibacterial effects at low concentrations against *Listeria monocytogenes* and *Vibrio parahaemolyticus,* both of which cause food-borne infections. This activity is ascribed to the polyphenolic components in the persimmon fruit .

It should be noted that the study that yielded this result was conducted using only pathogens that cause food-borne infections.

1.7.5. Other useful Properties of Persimmons:

The peculiar characteristics of its fruit have made the tree well known. This fruit is a globular berry, from an inch to an inch and a half in diameter, with variation in the number of seeds, sometimes with eight and sometimes without any. It bears at its apex the remnants of the styles and sits in the enlarged and persistent calyx. It ripens in late autumn, is pale orange with a red cheek, often covered with a slight glaucous bloom. One common joke among Southerners is to induce strangers to taste unripe persimmon fruit, as its very astringent bitterness is shocking to those unfamiliar with it. Folklore states that frost is required to make it edible, but fully-ripened fruit lightly shaken from the tree or found on the ground below the tree is sweet, juicy and delicious. The peculiar astringency of the fruit is due to the presence of a tannin similar to that of Cinchona.

The fruit is high in vitamin C. The unripe fruit is extremely astringent. The ripe fruit may be eaten raw, cooked or dried. Molasses can be made from the fruit pulp. A tea can be made from the leaves and the roasted seed is used as a coffee substitute. Other popular uses include desserts such as persimmon pie, persimmon pudding, or persimmon candy.

The fruit is also fermented with hops, cornmeal or wheat bran into a sort of beer or made into brandy. The wood is heavy, strong and very close-grained and used in woodturning.

- This fruit soothes nervous system.
- Also, persimmon increases working efficiency.
- The fruit has an antibacterial effect.
- Persimmon prevents from vascular diseases.
- Magnesium contained in persimmons lessens risk of kidney stones.
- Vitamin A protects against cancer.
- Vitamins C and P help to reduce fragility of the blood vessels.

1.8. Objectives of the study:

The emergence of multi-drug resistant bacteria has been a major problem worldwide. The pathogenic organisms causing different types of infections have become resistant to most of the commonly used antibiotics resulting in high rate of morbidity and mortality due to these infections. Widespread over prescription and misuse of antibiotics are promoting new strains of harmful bacteria that resist traditional treatments. So, attempts to find out alternative treatments for these infections are one of the vital points in current medicinal practice.

Medicinal plants are rich sources of bioreactive compounds and thus serve as important raw materials for drug production. There are many plants that have antibacterial activity. One of which is persimmon . Persimmon fruit exhibits a broad antibiotic spectrum against both gram-positive and gram-negative bacteria. So,

clinical studies can be done to assess the use of an antibiotic/persimmon fruit combination for bacteria that are difficult to eradicate.

With these points of view, the present research work has been designed with the following objectives:

1. To extract the organic and inorganic compounds from persimmon fruitwhich are soluble in different non polar and polar solvents (chloroform and 95% ethanol).
2. To assess the antibacterial activities of the extracted compounds on some multi-drug resistant (MDR) bacteria of interest.
3. To determine the minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) of the ethanol extract of for the selected bacterial isolates.

Chapter 2

Materials and Methods

2.1. Collection of plant material:

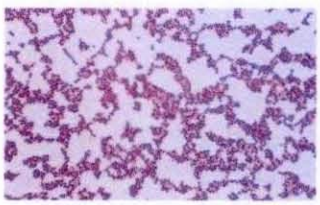
*D*iospyros peregrine (persimmon fruit) was collected from the village of Mohammedpur,Chandpur district.

2.2. Source of microorganisms:

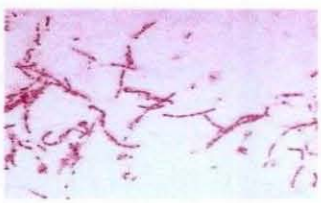
The organisms used were:

1. *Staphylococcus aureus.*
2. *Bacillus cereus.*
3. Bacillus subtilis
4. *Escherichia coli*
5. *Vibrio cholerae* 569B.
6. *Vibrio cholerae* non 01.
7. Shigella flex

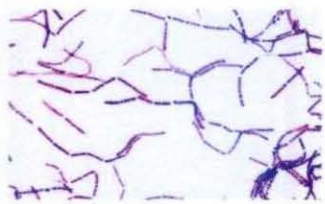
The multi drug resistant (MDR) bacteria isolates were obtained from the Department of Microbiology, University of Dhaka, and The long-term stock cultures of the test organisms in 20% Glycerol in cryogenic vials were kept at -20°C at the Department of Microbiology



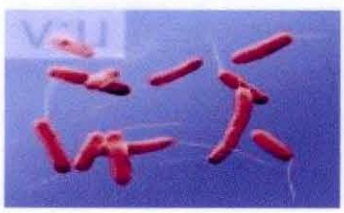
S. aureus



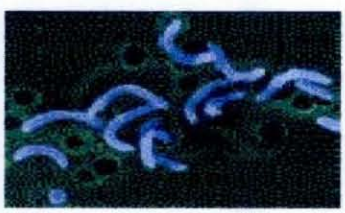
B. cereus



B. subtilis

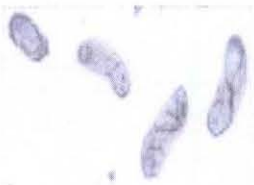


E. coli



V. Cholerae 569B

Shigella flexneri



Vibrio cholerae non 01

Fig 2.2: Representative Microbes targeted

2.3. Drug resistant pattern of the representative bacteria

The bacterial samples were tested for antibiotic sensitivity patterns against different antibiotics. Table: (2.1) show that most of the organisms were resistant to the antibiotics used. Among 7 selected microorganisms 100% organisms were resistant to Bacitracin (10µg), Streptomycin (10µg) and Spectinomycin (10µg).

About 60 to more than 70% bacteria were found resistant to ceftazimide, ceftriaxone, ampicillin, nalidixic acid and cephradin. About 20 to more than 40% bacteria were found resistant to tetracyclin, novobiocin, and Vancomycin .

Table 2.1. The resistant pattern of the MDR isolates used in this study

MDR isolates	Resistance pattern
Staphylococcus aureus	Ceftazimide; Streptomycin; Bacitracin; Spectinomycin:Amoxicillin; Vancomycin
Bacillus cereus	Streptomycin; Bacitracin; Spectinomycin; Ceftazimide; Tetracyclin; Novobiocin;
Bacillus subtilis	Streptomycin; Bacitracin; Spectinomycin; Nalidixic; Novobiocin; Cephradin.

Escherichia coli	Streptomycin; Bacitracin; Spectinomycin; Novobiocin; Vancomycin ; Tetracycline; Cephradin.
Vibrio cholerae o1	Streptomycin; Bacitracin; Spectinomycin; Vancomycin; Novobiocin
Vibrio cholerae non o1	Streptomycin;Bacitracin; Spectinomycin; Vancomycin; Novobiocin
Shigella flexi	Streptomycin;Bacitracin; Spectinomycin;Ampicillin; Tetracyclin; Cephradin.

2.4. Preparation of plant materials:

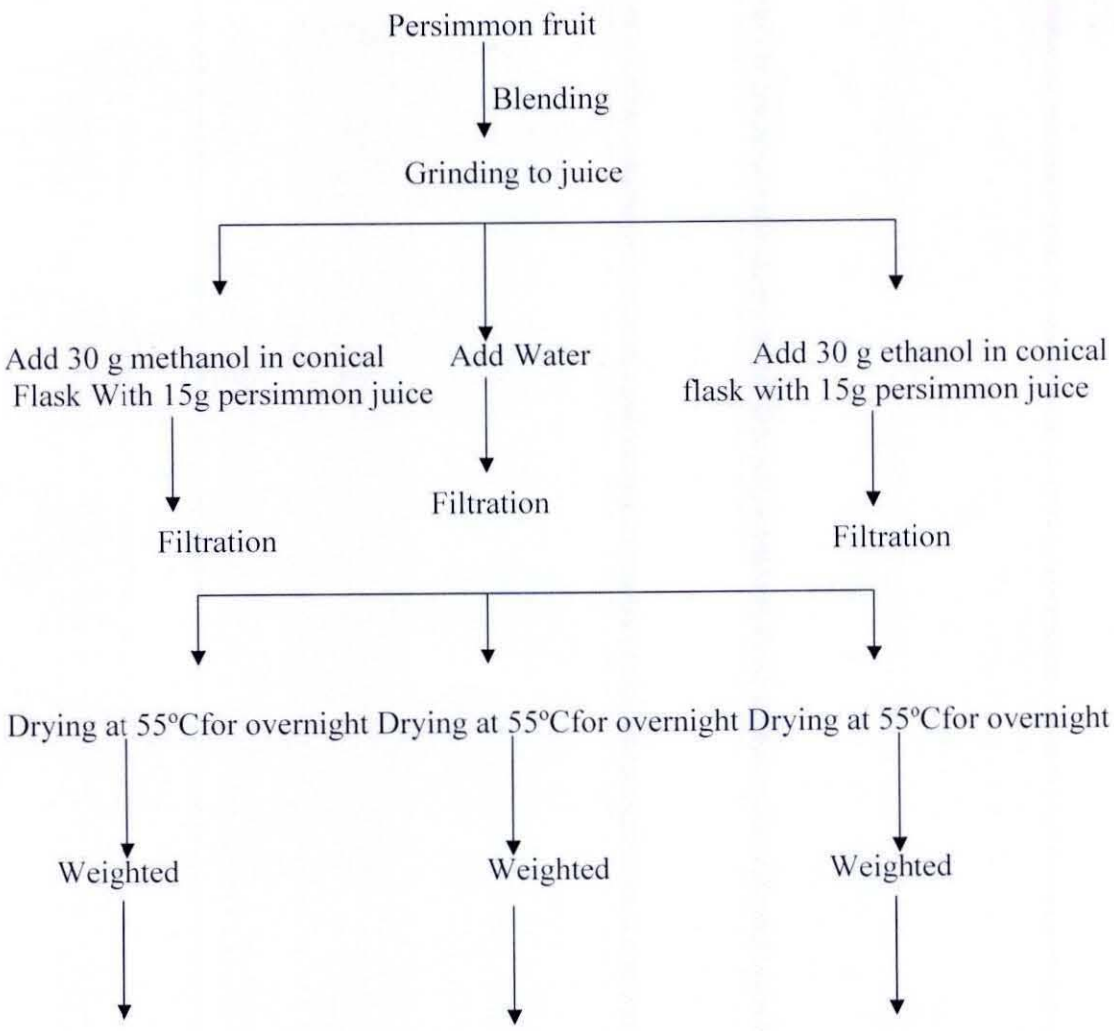
The persimmon fruit freshly collected from the tree and the fruit juice was taken by blander machine. Finally the persimmon juice is taken in the falcon tube and preserve in -20°c.

2.5. Extract preparation:

The persimmon juice was extracted by and ethanol, methanol and water by the following method:

- 15 gram of the juice was taken in a 150 ml conical flask.
- 30 ml of Methanol was added to the clove juice and was kept at 25°C for 24 hours in dark place.
- The Solution was then dried in a beaker at 55°C for two days to get the Methanol Extract.
- 15 gram of the juice was taken in a 150 ml conical flask.
- 30 ml of Water was added to the clove juice and was kept at 25°C for 24 hours in dark place.

- The Solution was then dried in a beaker at 55°C for two days to get the water Extract.
- The persimmon juice (15 g) was added to 30 ml Ethanol and was kept at 25°C for 24 hours.
- The Solution was then dried in a beaker at 55°C for two days to get the Ethanol Extract.
- The persimmon juice (15 g) was added to 30 ml Chlorororm and was kept at 25°C for 24 hours.
- The Solution was then dried in a beaker at 55°C for two days to get the Chloroform Extract.
- All the solvent extracts were weighed in a precision electronic balance
- The paste fractions were refrigerated for further study.



5.16 g(Paste) 6.3 g (paste) 3 g (paste)

Calculation of weight of ethanol extract (paste) :

1. The weight of empty McCarty bottle including cap was 13.50
2. The weight of paste containing McCarty bottle including cap was16.50.
3. So the weight of paste (Ethanol extract) was (16.50-13.50) =3 g

Calculation of the concentration of ethanol extracts (paste):

1. 6ml Ethanol was added in 3g paste.

2. 1ml of solution was transferred into sterile eppendorf. The eppendorf was dried at 55°C for overnight.

3. The weight of empty eppendorf was 870mg. After drying the weight of eppendorf was 950mg. So the weight of dry Ethanol extract was (950-870) =80mg.

4.500µl of normal saline was added into 80mg extract containing eppendorf, so the concentration of ethanol extract was 0.16mg/ml.

Figure 2.1: Extraction procedure of different soluble compounds from persimmon fruit

Calculation of weight of methanol extract (paste) :

1. The weight of empty McCarty bottle including cap was 13.60g.
2. The weight of paste containing McCarty bottle including cap was 18.76 g.
3. So the weight of paste (Methanol extract) was (18.76-13.60) = 5.16 g

Calculation of the concentration of Methanol extracts (paste):

1.10ml **Methanol** (95%) was added in 5.16g paste.

2. 1ml of solution was transferred into sterile eppendorf. The eppendorf was dried at 55°C for overnight.

3. The weight of empty eppendorf was 990mg. After drying the weight of eppendorf was 1070mg. So the weight of dry Methanol extract was (1070-990) =80mg.

4. 500µl of normal saline was added into 80mg extract containing eppendorf, so the concentration of Methanol extract was 0.16mg/ml.

Calculation of weight of Water extract (paste) :

1. The weight of empty McCarty bottle including cap was 13.50
2. The weight of paste containing McCarty bottle including cap was 19.90
3. So the weight of paste (Water extract) was (19.90-13.50) =6.3g

Calculation of the concentration of Water extracts (paste):

1.13ml ethanol (95%) was added in 6.3g paste.

2. 1ml of solution was transferred into sterile eppendorf. The eppendorf was dried at 55°C for overnight.

3. The weight of empty eppendorf was 940mg. After drying the weight of eppendorf was 1020mg. So the weight of dry Water extract was (1070-990) =80mg. 4.

500µl of normal saline was added into 80mg extract containing eppendorf, so the concentration of Water extract was 0.16mg/ml.

Calculation of weight of Chloroform extract (paste) :

1. The weight of empty McCarty bottle including cap was 13.97
2. The weight of paste containing McCarty bottle including cap was 19.25.
3. So the weight of paste (Chloroform extract) was (19.25-13.97) = 5.28g

Calculation of the concentration of Chloroform extracts (paste):

1.10ml Chloroform (95%) was added in 5.28g paste.
2. 1ml of solution was transferred into sterile eppendorf. The eppendorf was dried at 55°C for overnight.
3. The weight of empty eppendorf was 980mg. After drying the weight of eppendorf was 1020mg. So the weight of dry Chloroform extract was (1060-990) =80mg.
4. 500µl of normal saline was added into 80mg extract containing eppendorf, so the concentration of Chloroform extract was 0.16mg/ml.

2.6. Antimicrobial sensitivity assay of antibiotics, chloroform & ethanol by Disc diffusion method:

Positive control:

Bacterial susceptibility to antimicrobial agent was determined in vitro by using the standardized agar-disc diffusion method known as the Kirby Bauer method (Barry and Thornsberry, 1985). Antibiotics and their disc potencies used were:

1. Chloramphenicol (30µg). This antibiotic disc were used here as a positive control to see antimicrobial activity.

Negative control:

Discs were soaked separately with chloroform, methanol, water and ethanol (95%) at a desirable concentration & dried two days. Dried discs were applied at plate containing test organisms to see the antimicrobial activity.

2.6.1. Preparation of inoculum:

Inocula of test organisms were prepared by suspending a small quantity of growth from an overnight subculture on nutrient agar (NA) medium in Luria burtini broth (LB broth) by adjusting the turbidity of the broth to that of McFarland standard 0.5 (approximately 10^8 CFU/mL).The suspension existed on LB broth were10^8CFU/mL.

2.6.2. Preparation of the McFarland standard:

0.05 mL (50µl) of 0.048 M $BaCl_2$ (1.17% W/V $BaCl_2.2H_2O$) was added to 9.95 mL 0f 0.18 M H_2SO_4 (1% V/V) in a test tube with constant stirring. The tube was then sealed tightly to prevent loss of evaporation. The standard may be stored for up to 6 months, after which time it should be discarded.

2.6.3. Antibacterial testing:

The antibacterial activity of Methanol ethanol ,chloroform and water extracts of Persimmon fruit sample was evaluated using agar disc-diffusion method known as the Kirby Bauer method ((Barry and Thornsberry, 1985.) against food-borne pathogens and spoilage organisms. The 7 mm in diameter discs were impregnated with 150 µl (at concentration 160mg/ml) of plant extract and were then dried two days on clean Petri dish. The inocula of the test organisms were prepared by transferring 3 to 4 colonies of the cultures (18 hours old) into 9 ml of sterile Luria-bertini broth and incubated at 37°C for 4 to 5 h. The bacterial culture was compared with McFarland (Jorgensen *et al.* 1999) turbidity standard (10^8 CFU/ml) and streaked evenly in 3 planes keeping at 60° angle onto the surface of the agar plate with sterile cotton swab. Surplus suspension was removed from the swab by being rotated against the side of the tube before the plate was seeded. Then the plates were divided into Eight parts (four for chloroform & ethanol, methanol and water extracts, three for negative control & one for positive control). After the inoculums had dried (3 to 5 min), the discs were placed on the agar using an ethanol dipped and flamed forceps and were gently pressed down to ensure contact. Plates were kept at refrigeration temperature for 30 min for better absorption, during this time microorganisms will not grow but absorption of extracts would take place. Negative controls were prepared using the same solvent without the plant extract.

The inoculated plates were incubated in an upright position at 37°C for overnight 24 h. Antibacterial activity was evaluated by measuring the inhibition zones in mm (including the 7 mm disk) with scale near the agar surface and the results were recorded. A reading of 7 mm meant no zone of inhibition. The endpoint was taken as complete inhibition of growth as determined by the naked eye.

2.7. Screening the different dilutions of ethanol extract of persimmon fruit for antibacterial activity:

2.7.1. Dilution of persimmon fruit extract:

Solutions of ethanol extract were prepared in normal saline at concentrations

d1 =0.8mg/mL, d2 = 0.4mg/mL, d3 = 0.2mg/mL, d4=Positive control

2.7.2. Preparation of inoculums:

Inoculums was prepared as described in section 2.6.1

2.7.3. Preparation of the McFarland standard:

The McFarland standard was prepared as described in section 2.6.2

2.7.4. Inoculation of plates:

Luria-bertini agar (pH 7.5) plates were inoculated as described in section 2.6.3. In this case different dilutions of ethanol extracts were soaked in paper disc (50µl) at a different concentration (0.8mg/ml, 0.4mg/ml, 0.2mg/ml). Discs were dried at 48h to remove residual ethanolic effects.

2.8. Determination of minimum inhibitory concentration (MIC) of ethanol extract of persimmon fruit:

The MIC of the persimmon fruit extract was determined by tube dilution techniques in Luria-bertini broth (Merck) according to NCCLS (2000). The range of concentration used was 17.578-4500µg/ml.The final working volume in each vial was 2 mL.

2.8.1. Preparation of inoculum:

Inocula of test organisms were prepared by suspending a small quantity of growth from an overnight subculture on nutrient agar (NA) medium in normal saline (0.85% NaCl). The turbidity of the suspension was adjusted to that of McFarland standard 0.5 (Section 2.5.2).The suspension was then diluted 1:10 in normal saline to obtain 10^7 CFU/mL.

2.8.2. Dilution of 95% ethanol extract ofpersimmon fruit:

Stock solutions of the persimmon fruit extract were prepared in normal saline at concentrations of 4500µg/mL and 3000 µg/mL. The solutions were then serially diluted two fold to yield the concentration needed. As example, two fold serial dilution of 4500 µg/mL solution would yield 2250 µg/mL, 1125 µg/mL, 562.5 µg/mL, 281.25 µg/mL, 140.625µg/mL, 70.313 µg/mL, 35.156 µg/mL and 17.578µg/mL concentration of the extract.

2.8.3. Preparation of MHB:

The Luria-bertini broth was prepared in a way that 90 mL of the broth contained media components needed to prepare 200 mL of appropriate medium; that is the strength of the medium was double.

2.8.4. Inoculation of vials:

0.9 mL of the Luria-bertini broth was taken in each of the sterile and dry glass vials appropriately labeled with concentrations of extract. Then 1.0 mL of the respective extract concentrations was dispensed into the respective vials. 100 µl of the bacterial suspension of interest were added to the vials with their names labeled to make sure that each of the organisms faced a different concentration of the extract and every bacterium faced every extract concentration. So, the final reaction volume becomes 2 mL with single strength Luria-bertini broth and an inoculum load of ~5 $\times 10^7$ cells/ mL. A positive and a negative control vial were also included. In case of positive control, there was no inoculum, but contained 1.0 mL of the exract, 0.9 mL of the Luria-bertini broth and 100µl of sterilized distilled water and in case of negative control there was no persimmon fruit extract, but contained 0.9 mL of the Luria-bertini broth, 100µl of inoculum and 1.0 mL of sterilized distilled water. The vials were then incubated at 37^0C for 24 hours. The highest concentration that exhibited no visible growth was recorded as the minimum inhibitory concentration (MIC).

2.9. Determination of minimum lethal concentrations (MLC) of extracts:

The four last vials of each bacterium with no growth from the MIC procedure were streaked onto nutrient agar (NA) plates. The plates were then incubated at 37^0C for 24 hours. The lowest concentration that killed 100% of the inoculums bacteria (no growth on plate) was recorded as minimum bactericidal concentrations (MLC).

Chapter 3

Results

3.1. Obtaining the different solvent extracts:

The polar components in persimmon fruit were extracted with methanol and ethanol and water serially to yield fractionated organic and inorganic compounds. The extracts were then dried to obtain dried organic and inorganic material. The different solvent extracts were weighed and tabulated in Table 3.1.

Table 3.1: Yields of persimmon fruit in different solvent.

Solvent	**Yield from extract**
Methanol	5.16g (paste)
Ethanol	3g (paste)
Water	6.3g (paste)

3.2. Drug sensitivity pattern of the representative bacteria: The bacterial samples were tested for antibiotic sensitivity patterns against chloramphenicol (30μg) antibiotic. All (seven) of the organisms were sensitive to this antibiotic. The zone of diameter were determined in millimeter scale & plotted on table.

Table 3.Drug sensitivity pattern of the representative bacteria

Bacteria isolate.	Antibiotic is used	Diameter of zone of inhibition in (mm).
Staphylococcus aureus		28

Vibrio cholerae non ol		40
Vibrio cholerae 569B		32
Shigella flexneri		33

.3.3. screening the different solvent extracts of Persimmon fruit for antibacterial activity against pathogens of interest:

The crude (0.16mg/ml) methanol and ethanol extracts of the persimmon fruit were tested for antibacterial activity against 24 hours old lauria Broth cultures of different pathogenic bacteria lawned on lauria Broth Agar plates. The plates were observed for zones of inhibition after 24 hours incubation at 37°C. Table 3.3 shows that all the organisms were sensitive to etanol and methnol extract except *Escherichia coli, Bacillus cereus, Bacillus subtilis & Shigella flex.*

Bacterial isolates	**Diameter of zone of inhibition in mm**		
	Methanol extract	Ethanol extract	Water extract
Staphylococcus aureus	9.0	8.0	13
Vibrio cholerae non o1	10.0	8.0	13
Vibrio cholerae 569B	12.0	10.0	12
Escherichia coli O157:H7	Resistant	Resistant	Resistant

Bacillus cereus	Resistant	Resistant	[illegible]

Table 3.3: Zones of inhibition of cultures of representative bacteria aqganist fruit extract.

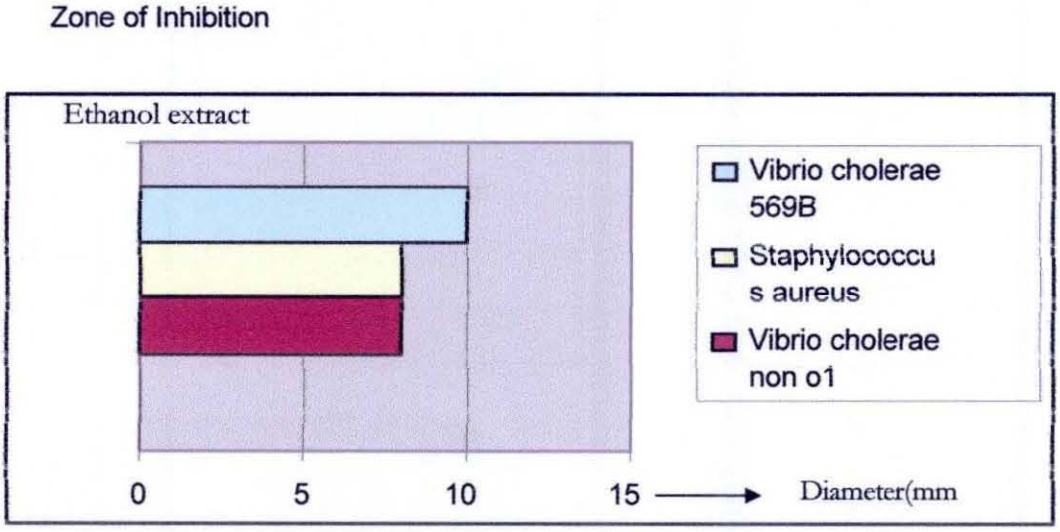


Figure 3.1: The zone of inhibition of ethanol extract of isolated bacteria

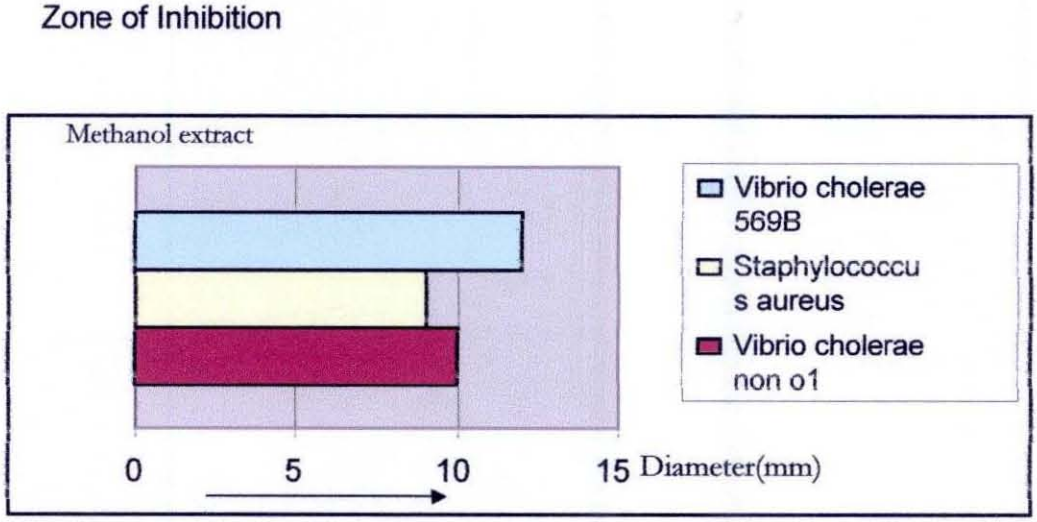


Figure 3.2: The zone of inhibition of methanol extract of isolated bacteria

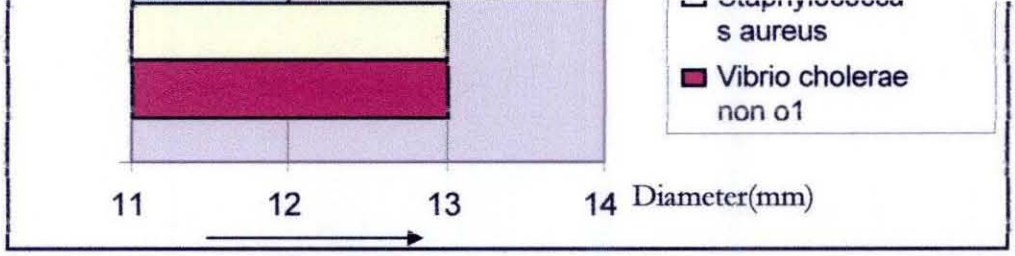
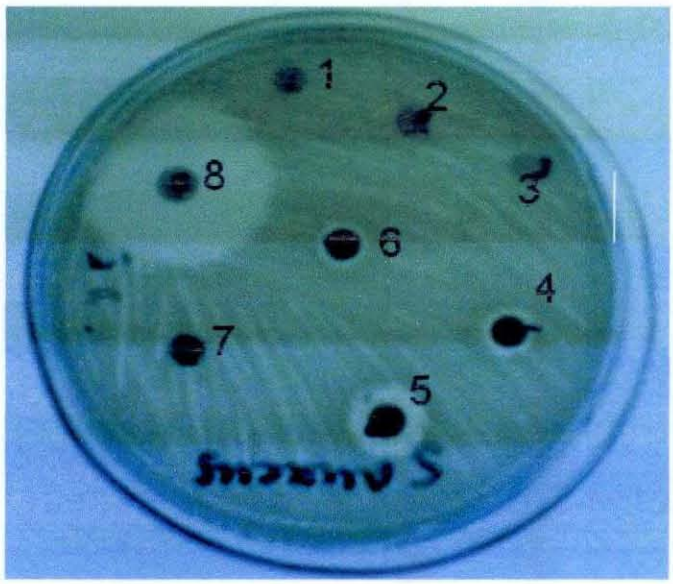
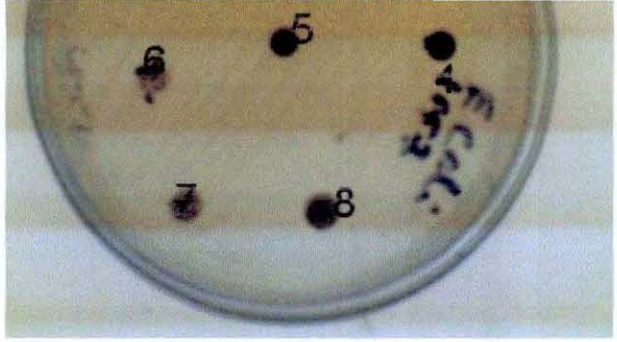


Figure 3.3: The zone of inhibition of water extract of isolated bacteria

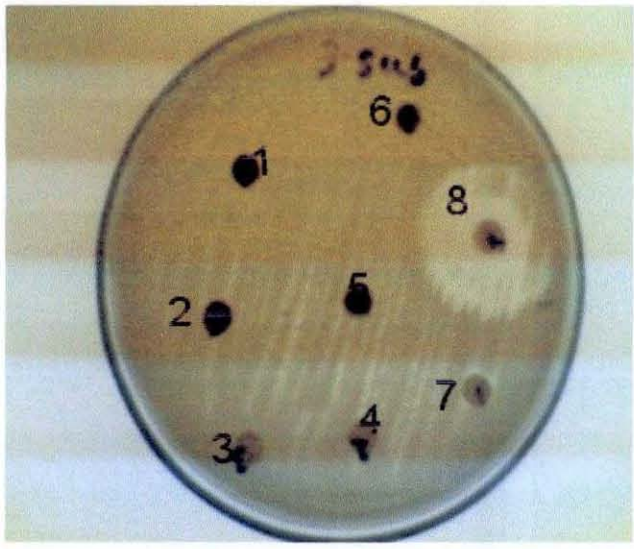
3.3(a) Zone of inhibition of representative bacteria are shown below: Here the concentration of ethanol and methanol extract was 0. 16mg/ml.



Staphylococcus aureus



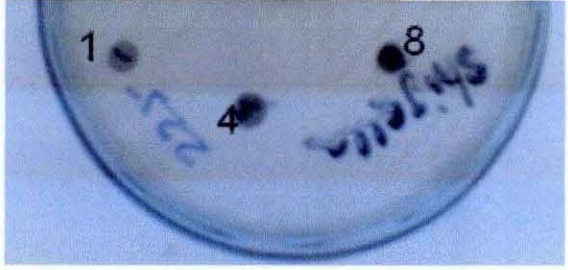
Escherichia coli



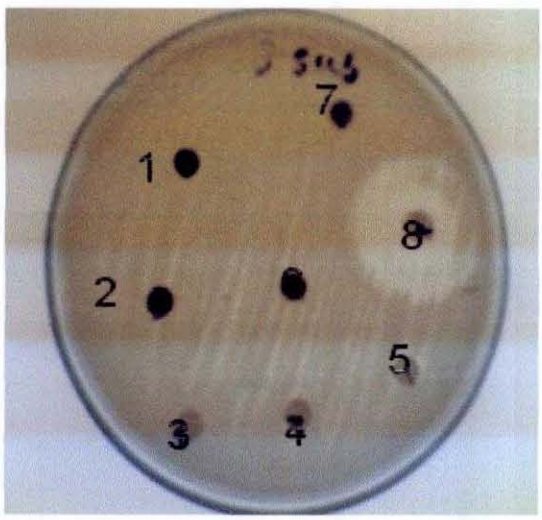
Bacillus subtilis

Figure 3.4: Antibacterial activity of persimmon fruit extracts.

1. Ethanol (negative control). 2. Methanol (negative control). 3. Chloroform (negative control). 4. Ethanol extract. 5. Water extract. 6. Chloroform extract. 7. Methanol extract 8. Antibiotic (positive control).



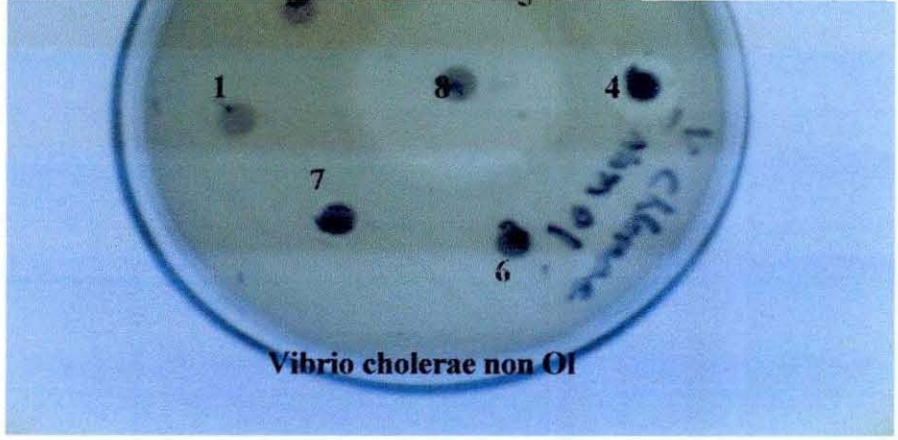
Shigella flex



Bacillus cereus

Figure 3.5: Antibacterial activity of Persimmon fruit extracts.

1. Ethanol (negative control). 2. Methanol (negative control). 3. Chloroform (negative control). 4. Ethanol extract. 5. Water extract. 6. Chloroform extract. 7. Methanol extract 8. Antibiotic (positive control).



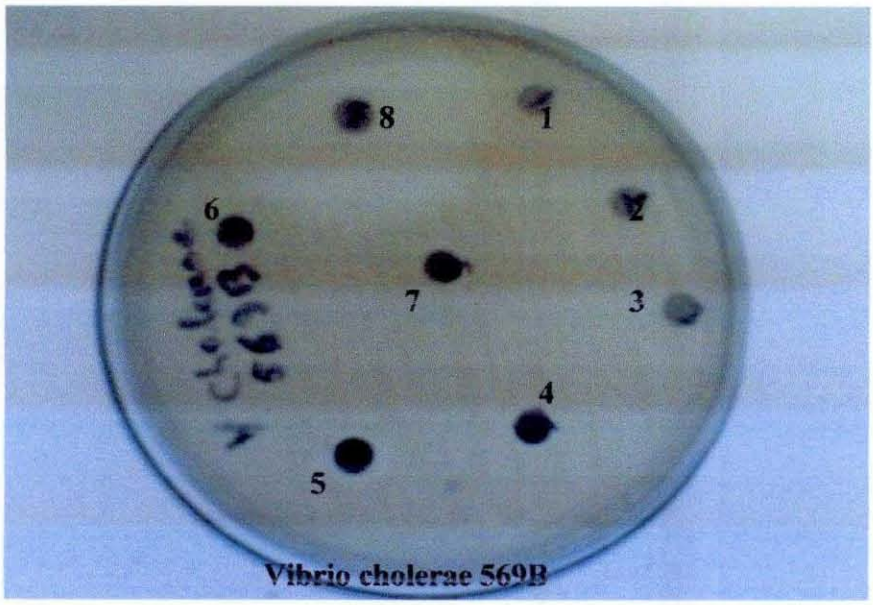


Figure 3.6: Antibacterial activity of persimmon fruit extracts.

1. Ethanol (negative control). 2. Methanol (negative control). 3. Chloroform (negative control). 4. Ethanol extract. 5. Water extract. 6. Chloroform extract. 7. Methanol extract. 8. Antibiotic (positive control).

Stock solution was used (at a concentration of 0.16mg/ml) for dilution purposes. 5 µl of the solution was put in each of the disc and applied in lb Agar plates seeded with the organisms of interest to make a comparative study of the effects of Chloramphenicol (30µg) (to which all the bacteria were sensitive) by the same upper procedure. 5µl solution of the ethanol extract at different concentrations ranging from 0.8mg/ml to 0.2mg/ml was used for the study of antibacterial activity by disc diffusion method. The results of zones of inhibition were recorded in table 3.4.

Table 3.4: Comparative study of the ethanol extract at different concentrations with Chloramphenicol (3 0µg)

Bacterial isolates	**Diameter of zone of inhibition of different dilution of ethanol extract in mm**			
	0.8mg/mL	**0.4mg/mL**	**0.2mg/mL**	Chloramphenicol(30µg)
Staphylococcus aureus	12	8	7	**28**
Vibrio cholerae non Ol	13	10	7	**40**
Vibrio cholerae 569B	12	10	7	**32**

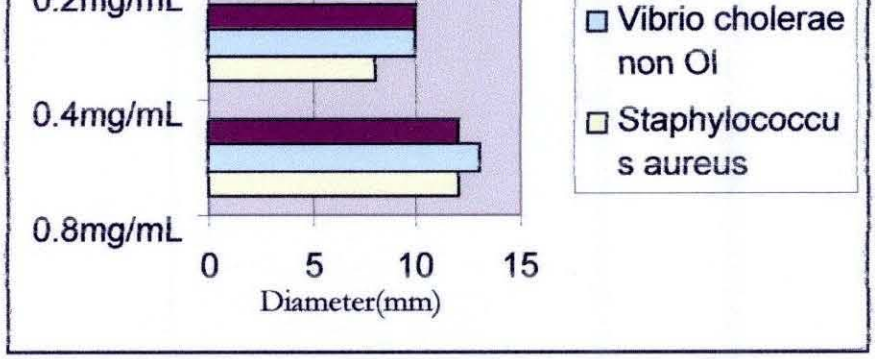


Figure 3.8: Diameter of zone of inhibition of different dilution of ethanol extract in mm

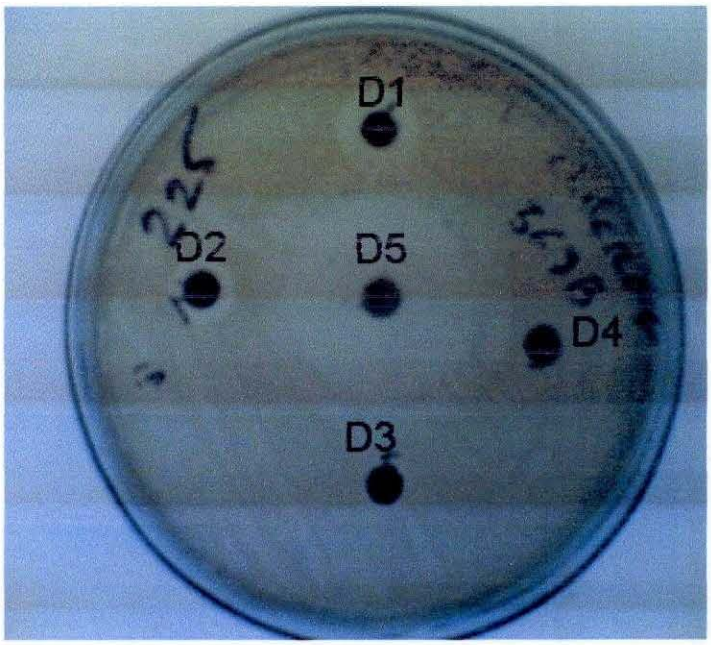


Figure ***3.9: Different dilution of ethanol extracts of persimmon fruit used for antimicrobial activity against Vibrio Cholerae 569B.***

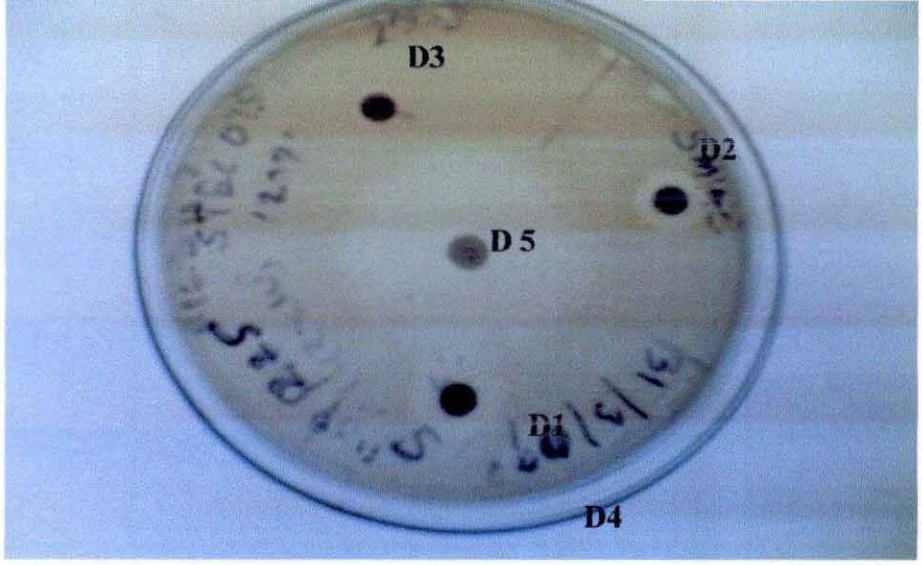


Figure 3.10: ***Different dilution of ethanol extracts of persimmon fruit used for antimicrobial activity against Staphylococcus aureus***

D1=0.8mg/ml D2=0.4mg/ml. D3=0.2mg/ml. D4= Negative Control D5=Chloramphenicol (Positive Control)

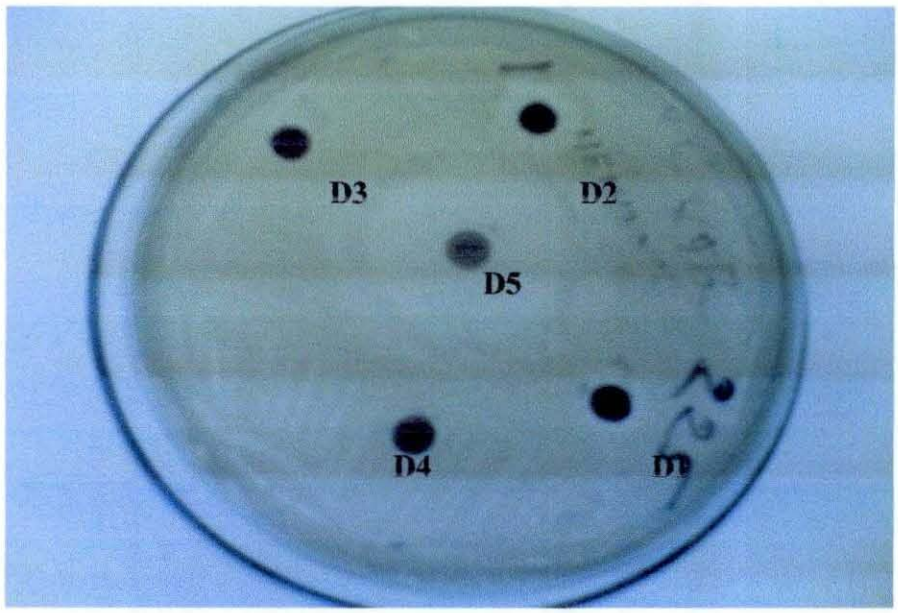


Figure *3.11: Different dilution of ethanol extracts of persimmon fruit used for antimicrobial activity against Vibrio Cholerae non O1*

organisms of interest to make a comparative study of the effects of Chloramphenicol (30µg) (to which all the bacteria were sensitive) by the same upper procedure. 5µl solution of the ethanol methanol and water extract at different concentrations ranging from 0.3mg/ml to 0.15mg/ml was used for the study of antibacterial activity by disc diffusion method. The results of zones of inhibition were recorded in table 3.4.

3.5.Comparative study of the ethanol methanol and water extract at different concentrations with Chloramphenicol (3 0µg)

Bacterial isolates	Diameter of zone of inhibition of different Concentration of ethanol methanol and water extract in mm												
	Ethanol				Methnol				Water				Chloramphenicol(
	0..266 mg/ mL	0.20 mg/mL	0.16 mg/mL	0.133 mg/mL	0..266 mg/Ml	0.20 mg/mL	0.16 mg/mL	0.133 mg/mL	0..266 mg/mL	0.20 mg/mL	0.16 mg/mL	0.133 mg/mL	30µg)
Staphylococcus aureus	13	12	12	10	12	10	11	8	14	12	10	none	**28**
Vibrio cholerae non ol	12	14	12	12	12	12	13	13	14	13	11	12	**40**
Vibrio cholerae 569B	10	11	12	08	12	11	11	10	14	12	12	10	**32**

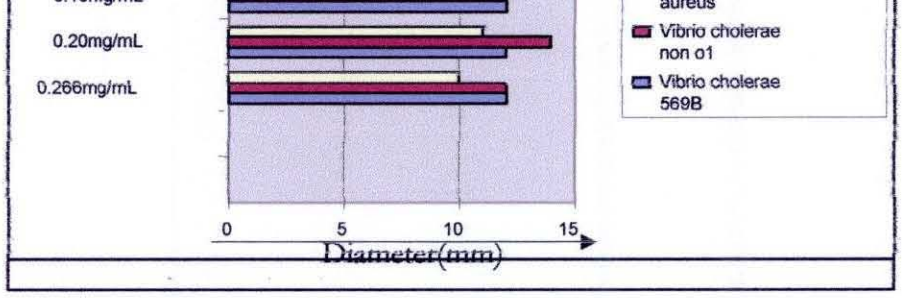


Figure 3.12: Diameter of zone of inhibition of different dilution of ethanol extract in mm

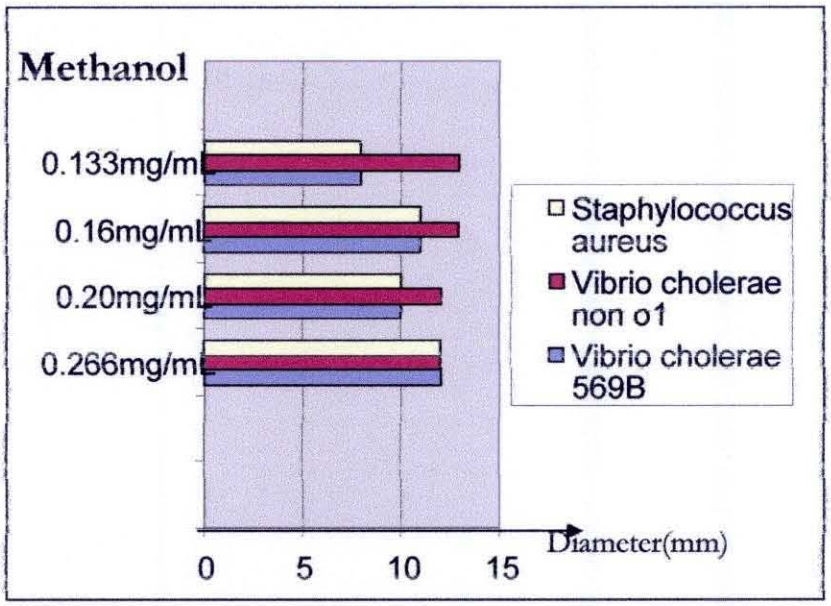


Figure 3.13: Diameter of zone of inhibition of different dilution of Methanol extract in mm

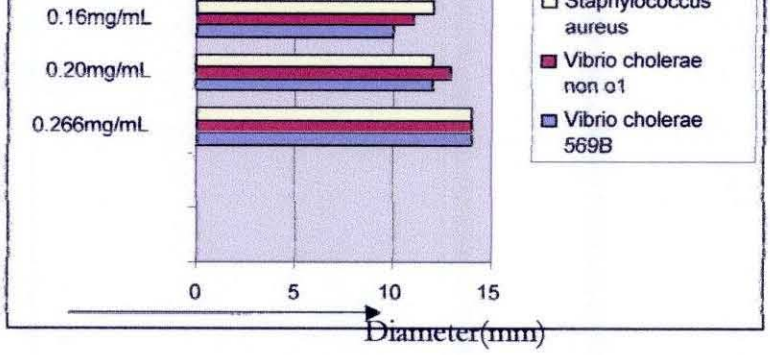


Figure 3.14: Diameter of zone of inhibition of different dilution of water extract in mm

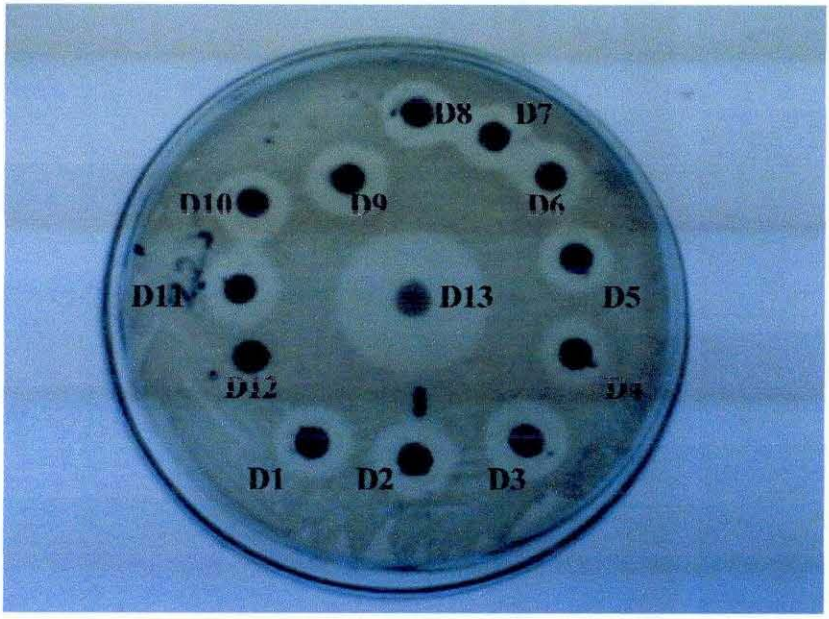


Figure 3.5 (a): ***Different dilution of ethanol extracts of persimmon fruit used for antimicrobial activity against Staphylococcus aureus***

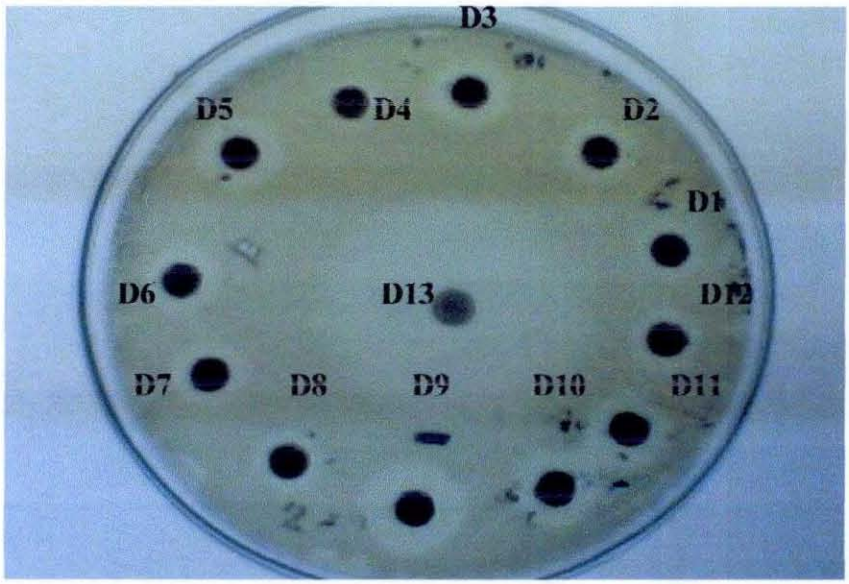


Figure 3.15 : ***Different dilution of ethanol extracts of persimmon fruit used for antimicrobial activity against*** *Vibrio cholerae 569B*

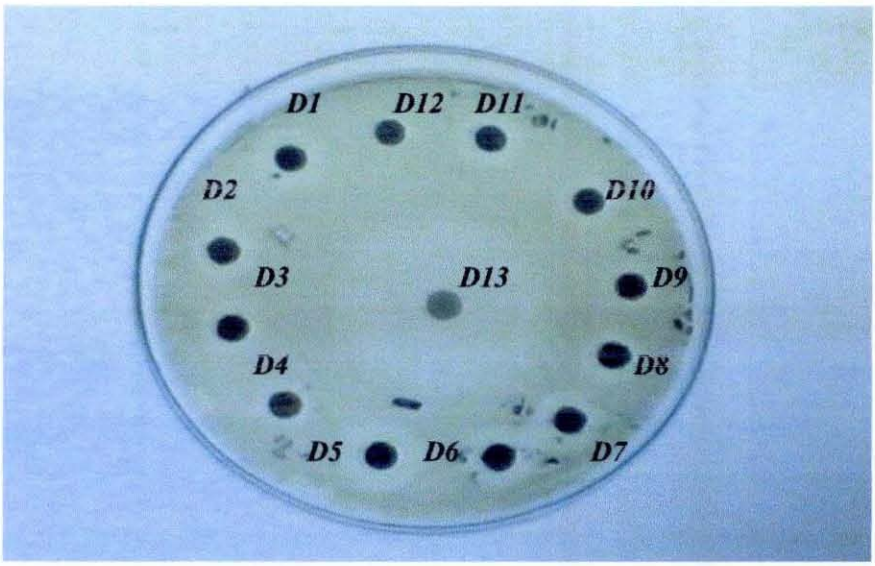


Figure 3.16 : ***Different dilution of ethanol extracts of persimmon fruit used for antimicrobial activity against*** *Vibrio cholerae non O1*

D5=0.3mg/ml (Methanol) *D6=0.225mg/ml. (Methanol)* *D7=0.18mg/ml. (Methanol)* *D8=0.15 mg/ml. (Methanol)*

D9=0.3mg/ml (Water) *D10=0.225mg/ml.(Water)* *D11=0.18mg/ml. (Water)* *D12=0.15 mg/ml. (Water)*

D13= Chloramphenicol(30µg)

3.6. Determination of Minimum Inhibitory Concentration and Minimum Lethal Concentration of ethanol extracts of Persimmon fruit extracts:

1. The minimum inhibitory concentration and minimum lethal concentration for the organisms of interest were determined by using tube dilution method.

2. The test was done in glass vials in LB broth with ethanol extracts at concentrations ranging from 1500µg/ml to140.65µg/ml.

3. The MIC and MLC of the ethanol extract for different pathogenic bacteria under investigation were determined by Tube- dilution method.

4. The tube with lowest concentration yielding no visible growth in tube but giving colonies on media is taken as MIC.

5. The lowest concentration of extract that did not yield bacterial growth both in tube and on NA plate is taken as MLC.

Table 3.5 shows the MIC and MLC values of Persimmon fruit extract for 3 bacteria, namely, *Staphylococcus aureus, Vibrio cholerae* 569B *and Vibrio cholerae* non ol.

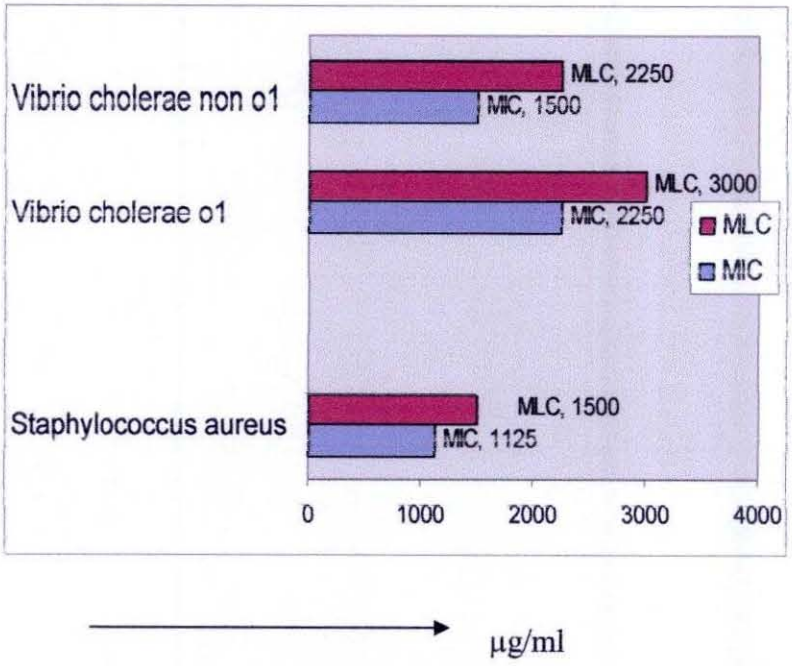
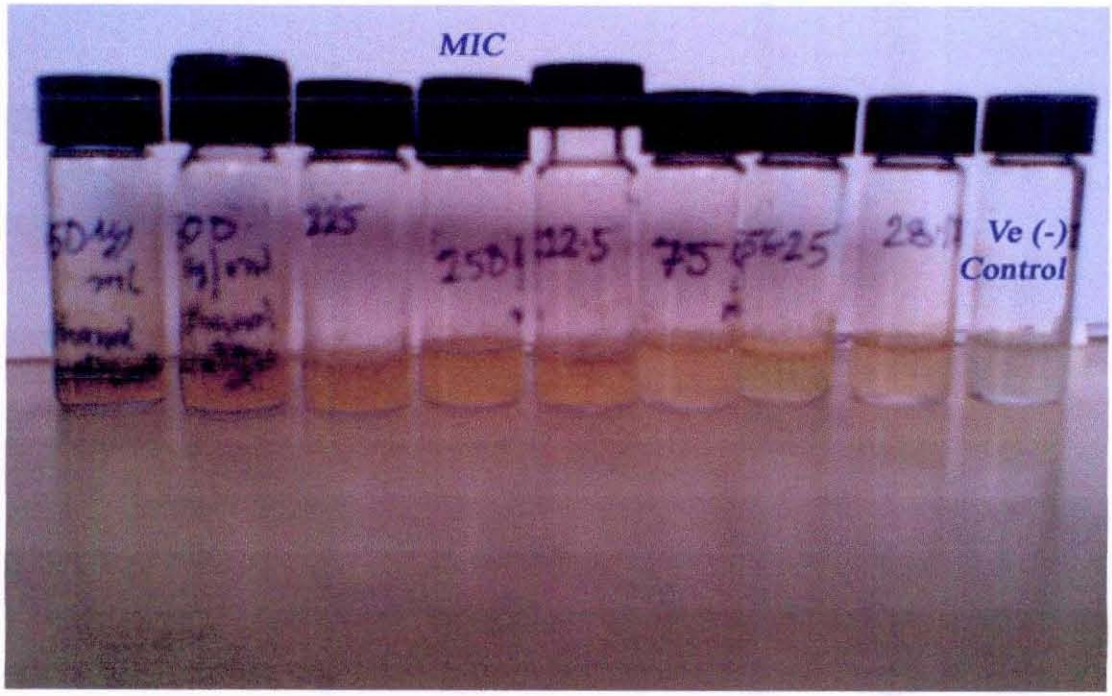


Figure 3.17 : MIC and MLC of Persimmon fruit extracts.

Vibrio cholerae 569B



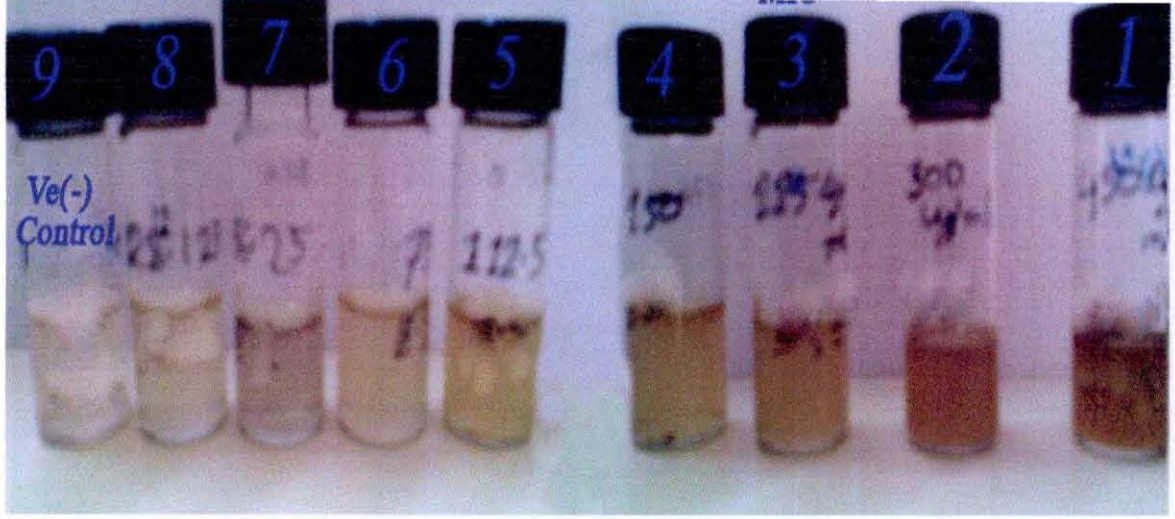


Figure 3.19 : MIC of persimmon fruit extracts.

Staphylococcus aureus

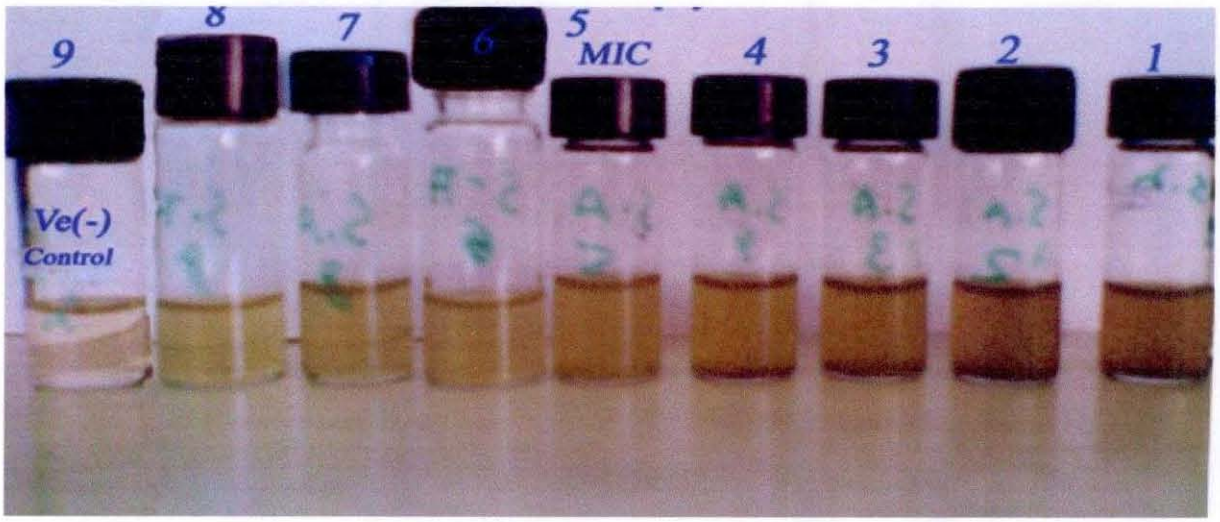


Figure 3.20 : MIC of persimmon fruit extracts.

1) = 4500µg/ml of ethanol extracts. 2) =3000µg/ml of ethanol extracts. 3) =2250µg/ml of ethanol extracts.4) =1500µg/ml of ethanol extracts. 5) =1125 µg/ml of ethanol extracts. 6) =750µg/ml of ethanol extracts. 7) =562.5µg/ml of ethanol extracts. 8) =375µg/ml of ethanol extracts. 9) = negative control.

acterial olates	Ethanol extract concentration in µg/ml														
	4500	**3000**	**2250**	**1500**	**1125**	**750**	**562.6**	**375**	**281.3**	**187.5**	**140.625**	**93.5**	**70.313**	**46.75**	**35.156**
aphylococcus ureus	-	-	-	MLC	MIC	-	-	-	-	-	-	-	-	-	-
ibrio cholerae on O1	-	-	MLC	MIC	-	-	-	-	-	-	-	-	-	-	-
ibrio cholerae 69B	-	MLC	MIC	-	-	-	-	-	-	-	-	-	-	-	-

Chapter 4

Discussion

This study showed that, using the disc diffusion assay, persimmon fruit extracts were found to inhibit both Gram-positive rarely and Gram-negative bacteria strongly. However, they were unable to inhibit *Escherichia coli* O157:H7, *Shigella Flexner*, .*Bacillus cereus*, *Bacillus subtilis*. Persimmon fruit extracts had more tendencies to inhibit Gram-positive bacteria than Gram-negative bacteria. Both methanol ethanol and Water extract of persimmon fruit was found active against *Vibrio cholerae* .However Chloroform extract doesn't show any activity.

Compounds from persimmon fruit extracts showed considerable activity against Gram-negative bacteria than Gram-positive bacteria This could to be expected because the outer membrane of Gram-negative bacteria is known to contain a barrier to penetration of numerous antimicrobial molecules, and the periplasmic space contains enzymes, which may be have ability to break down antimicrobials introduced from outside (Duffy and Power, 2001).

Being a developing country, in Bangladesh the rate of mortality due to infectious diseases is very high. One of the major reasons for this is the antimicrobial resistance of the variable drugs. Inappropriate use of readily available antibiotics, prolonged hospitalization, and poor implementation of infection control measures is the main causes of drug resistance. Moreover, powerful drugs against which antimicrobial resistance has not yet been developed are unavailable and costly. So, poor people of our country cannot afford this. In this situation, there is a crying need for an alternative treatment for gut infection, which is both effective and inexpensive. One of the strong solutions of this situation is the use of medicinal plant in healing this type of infection. Persimmon fruit, one of the easily available herbs in our country with its known antibacterial activity might be a simple and cost effective treatment. With this perspective, an attempt was made to evaluate the antimicrobial activity of Persimmon fruit on these drug resistant organisms.

extraction procedures. Among 7 isolates of bacteria, all the isolates were sensitive to ethanol extract except *Ecoli, shigella flexi. Bacillus cereus, Bacillus subtilis*.. The ethanol extract of persimmon was most effective against *Staphylococcus aureus ,Bacillus subtilis Vibrio cholerae 569B and Vibrio cholerae non o1* also showed sensitivity on this extract.

MIC of ethanol extract of persimmon fruit against 3 bacterial isolates (*Staphylococcus aureus, , Vibrio cholerae 569B, Vibrio cholerae non Ol*) were determined. Variable MIC values were found with the isolates. Among the test organisms *Staphylococcus aureus*, was most sensitive to persimmon fruit extract (MIC value 1125 µg/ml), followed by *Vibrio cholerae non ol* MIC value 1500 µg/ml), and *Vibrio cholerae 569b* ((MIC value 2250µg/ml).

It is interesting to note that the extract could be used against the *Vibrio cholerae* and showed that *Vibrio cholerae* was highly susceptible to the effects of this extracts. Such inhibitory property would prevent the organism producing the cholera toxin and this would imply that when administered to cholerae patients it should invariably lower the morbidity and mortality rate, especially in children in remote places without hospital facilities.

The present study also showed that *Staphylococcus aureus*, a common food poisoning organism, was also inhibited. Since *Staphylococcus aureus* are commonly implicated in pus causing, it would be interesting to hazard a guess that the extract could be used as an alternative in the treatment of wounds infected with this multi-resistant bacterium.

It is not always possible to isolate the bioactive agent in a plant and cases are known where attempts at such isolation have proved fruitless, even though an extract of the plant may be active, for example, a plant containing highly unstable compounds (Harborne, 1992). Nevertheless, such attempts should continue as characterization of the active agent enables structure related activity studies to be carried out, leading to

In conclusion, multi-drug resistance has become a common feature most of the organisms associated with diarrhea and other enteric diseases (Mamun *et al*, 2004; Jahan *et al*, 1997; Dahar *et al* 1996), urinary tract infection (Haque *et al*, 2001;Chowdhury *et al*, 1994), neonatal infection (Bakht, *et al*,2000; Saha, *et al*,2003) and wound Infection(Ahmed *et al*, 1999; Ahmed, *et al*, 2004; Jahan, *et al*, 2004; Rahaman MM, et al, 1997) In most of the cases, resistance was found against all commonly prescribed drugs, which is very alarming particularly for a country like Bangladesh where majority of the population even cannot afford appropriate treatment.

Therefore, persimmon fruit extract, which was shown to have antibacterial activity against some multi-drug resistant bacteria, can be used as an available and cheap medicine. More detailed work is needed to improve the extraction procedure of persimmon fruit and to separate the components of persimmon which is responsible for its antibacterial activity.

Concluding Remarks and Future Directions

The above comprehensive review almost covered what is actually known to date about the persimmon fruit and its constituents. It is clear that most of the potent and fruitful activity resides in its triterpene and aliphatic ketol and onadecan-7-ol-2-one.

Scientists from divergent fields are investigating plants a new with an eye to their antimicrobial usefulness. A sense of urgency accompanies the search as the pace of species extinction continues. Laboratories of the world have found literally thousands of phytochemicals which have inhibitory effects on all types of microorganisms *in vitro*. More of these compounds should be subjected to animal and human studies to determine their effectiveness in whole-organism systems, including in particular, toxicity studies as well as examination of their effects on beneficial normal microbiota. It would be advantageous to standardize the methods of extraction and *in*

one example of an anti-infection activity, not commonly screened for currently. Attention to these issues could usher in a badly needed new era of chemotherapeutic treatment of infection by using plant-derived principles.

In that respect, the plant product persimmon fruit from *Diospyros peregrine* are a prospective area to work on. The unavailability of sophisticated and advanced technologies like HPLC, NMR and AAS has limited the research on any plant material in the department and the laboratory where I worked in. Due to lack of these instruments, the isolation, purification and identification of an active organic/inorganic compound is not being carried out. To carry on such research work in the future, we have to equip the respective laboratories with appropriate instruments and reagents when needed. Then, and only then we can expect something new and exciting coming out from such researches.

Chapter 5

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Appendix I

Luria-Bertini Broth **pH 7.4**

Ingredients	Amount/ 1000 mL medium
Tryptone	10g
Yeast	5g
NaCl	10g

Luria-Bertini Agar **pH 7.4**

Ingredients	Amount/ 1000 mL medium
Agar	15g
Tryptone	10g
Yeast	5 g
NaCl	10 g

Appendix II

Composition of chemicals used:

McFarland standard 0.5

Ingredients	Amount/ 100 mL medium
1% Sulfuric acid	99.5 mL
1% $BaCl_2$	0.5 mL

Normal saline

Ingredients	Amount/ 100 mL medium
Sodium Chloride (Table salt)	8.5 g

Appendix I

Luria-Bertini Broth **pH 7.4**

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Yeast	5g
NaCl	10g

Luria-Bertini Agar **pH 7.4**

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