Chapter 4

Evaluation of antimicrobial properties of *Pithecellobium dulce* pod pulp extract
INTRODUCTION

Microbial infectious diseases continue to be one of the leading causes of morbidity and mortality. It has been estimated that microbial species comprise about 60% of the Earth's biomass (Radulović et al., 2013). This, together with the fact that their genetic, metabolic and physiological diversity is extraordinary, makes them a major threat to the health and development of populations across the world. Infectious diseases caused by pathogenic microorganisms like bacteria, viruses, fungi, protozoa and multicellular parasites are also called as communicable or transmissible diseases since they can be transmitted from one person to another via a vector or replicating agent. Infectious diseases account for about half of the deaths in tropical countries (Khosravi and Behzadi, 2006).

Finding healing powers in plants is an ancient idea. People on all continents have long applied poultices and imbibed infusions of hundreds, if not thousands, of indigenous plants, dating back to prehistory. There is evidence that Neanderthals living 60,000 years ago in present-day Iraq used plants such as hollyhock; these plants are still widely used in ethnomedicine around the world. Historically, therapeutic results have been mixed; quite often cures or symptom relief resulted. Poisonings occurred at a high rate, also. Currently, of the one-quarter to one-half of all pharmaceuticals dispensed in the United States having higher-plant origins, very few are intended for use as antimicrobials, since we have relied on bacterial and fungal sources for these activities. Since the advent of antibiotics in the 1950s,
the use of plant derivatives as antimicrobials has been virtually nonexistent (Cowan, 1999).

Diseases due to pathogenic bacteria and fungi represent a critical problem to human health and they are the major cause of morbidity and mortality worldwide (Shahzad et al., 2009). In industrialized nations, despite the progress made in the understanding of microbiology and their control, incidence of epidemics due to drug resistant microorganisms and the emergence of hitherto unknown disease-causing microbes, pose enormous public health concerns.

Globally, infectious diseases are the major cause of death accounting for approximately one-half of all deaths in tropical countries. Perhaps it is not surprising to see these statistics in developing nations, but what may be remarkable is that mortality rates due to infectious diseases are actually increasing around the world. It is estimated that infectious diseases are the underlying cause of death in 8% of the deaths occurring in the United States. This is alarming given that it was once believed that we would eliminate infectious diseases by the end of the millennium. The increases are attributed to increases in respiratory tract infections and AIDS. Other contributing factors are increase in antibiotic resistance in nosocomial and community acquired infections. Furthermore, the most dramatic increases are occurring in the 25-44 year old age group (Pinner et al., 1996).
Bacterial diseases are a type of infectious diseases caused by pathogenic bacteria. It is notable that majority of bacteria are non pathogenic and are not harmful to human health. Some bacteria are even helpful and necessary for the good health. Millions of bacteria normally live in the intestine, on the skin and the genitalia. Bacterial diseases results when the harmful bacteria get into a body area, multiply their and thrash the body’s defensive mechanism. Pathogenic bacteria can invade in body through various routes like inhalation into nose and lungs, ingestion in food or through sexual contact. Once microbes enter the body, the immune system of the body recognizes the microbes, bacteria as foreign intruder and tries to kill or prevent them from multiplying. However, even a healthy immune system is not always able to prevent the bacteria from reproducing and spreading. As a result microbes thrive in the body and emit toxins which damage cells and tissues that consequently results in the symptoms of microbial diseases.

Commonly occurring pathogenic bacteria are *Staphylococcus aureus*, which can cause skin infections, *Streptococcus epidermidis*, which can cause Endocarditis, *Bacillus subtilis*, *Escherichia coli* which can cause food poisoning, *Salmonella typhi*, which can cause typhoid. General symptoms of bacterial diseases include fever, chills, headache, nausea and vomiting. Bacterial infections if untreated can lead to serious and life threatening complications such as sepsis, kidney and liver failure, toxic shock and even death. Infectious diseases are a leading cause of mortality worldwide. People who work in health centres, hospitals and pathology labs remain at a risk of
bacterial infections since they have a significant exposure to pathogenic bacteria, such as Streptococcus. Patients having compromised immune system due to diseases such as AIDS are at a high risk of bacterial diseases. People who take drugs such as corticosteroids, which suppress body's natural immunity, are also at risk of developing bacterial diseases. Other risk factors include malnutrition, high stress, and genetic predisposition to bacterial infections.

The prevalence of invasive, opportunistic fungal infections has increased at an alarming rate especially in immunocompromised individuals. This trend has also been attributed to the increasing use of cytotoxic and immunosuppressive drugs to treat both malignant and non malignant diseases. Mycologists estimate that there were about one lakh validly characterized species of fungi, with at least as many species waiting to be discovered. Of those identified, nearly 150 species are recognized as pathogens of humans and animals. They cause a broad spectrum of infections ranging from systemic and potentially fatal diseases to localized cutaneous, subcutaneous or mucosal infections. Although it appears to be a great array of antifungal drugs, there is at present a quest for new generations of antifungal compounds due to the low efficacy, side effects or resistance associated to the existing drugs. Based on the knowledge that plants develop their own defense against fungal pathogens they appear as an interesting source for antifungal compounds (Gurgel et al, 2005).
Bacterial resistance to antibiotics has been a great problem for many years. The threats that bacterial resistance present today are greater than they were in the past. The first bacteria that were detected to be resistant to several antibiotics were reported in Japan during the 1950s. They reported resistance in *Shigella* species that were isolated from patients who had been on antibiotic treatment (Schlegel and Schmidt, 1985). The degree and the speed with which resistance now develops vary with different organisms and different drugs (Laxminarayan and Weitzman, 2002).

According to the World Health Organization (WHO), antibiotic resistance was first documented nearly six decades ago and became an important issue in the 1960s when resistance plasmids and plasmids transmissibility were detected (WHO, 2004a). By the beginning of the 1990s, more resistance developed in certain pathogens and most of the antibiotics were found to be ineffective against these pathogens. Antibiotic-resistant pathogens became an important and growing threat to public health which was addressed by national agencies and international bodies particularly the WHO. Resistance development is still continuing for a number of bacterial pathogens which, apart from *Mycobacterium tuberculosis* and *Streptococcus* are particularly associated with nosocomial infections such as Methicillin Resistant *Staphylococcus aureus*.

The β-lactam antibiotics are the most widely used family of antibiotics. This exposes more of the cephalosporins to microorganisms which eventually lead to antimicrobial resistance (Payne and Thomson, 1998). It is
generally accepted that infections caused by Extended Spectrum β-lactamase (ESBL) producing organisms are associated with increased risk of treatment failure with 3rd or 4th generation cephalosporins.

The prevalence of ESBLs among clinical isolates varies from country to country and from institution to institution. India is affected seriously by the emergence and spread of different ESBLs among hospital-acquired and some community-acquired pathogens. According to the WHO, resistance in micro-organisms has out-spaced the development of newer antimicrobial agents. Concerns have already been expressed in many quarters that the world may soon be heading towards a post-antibiotic era where none of the available antimicrobial agents will be effective against commonly encountered microbes. This will lead to terrible morbidity and mortality, taking the nations back to the pre-antibiotic era (WHO, 2004b).

Resistance to antibiotics and the toxicity during prolonged treatment with present-day drugs have been the reasons for an extended search for newer drugs to opportunistic microbial infections (Fostel and Larney, 2000). This situation has forced the scientists to search for new antimicrobial substances from various sources like medicinal plants (Cordell, 2000).

Medicinal plants are the ‘back bone’ of traditional remedy (Mesfin and Sebsebe 1992). In addition, the traditional medicine related to treatment of both human and animal mycoses with plant-derived preparations is considered a valuable knowledge for the discovery of new antifungal drugs.
Plants contain many biologically active ingredients with wide array of medicinal properties. India is very rich in natural resources and the knowledge of traditional medicine and the use of plants as source of new drugs is an innate and very important component of healthcare system.

To survive on earth, there has to be a suitable relationship between people and the environment. As the human population grows and people strive for improved living standards, this relationship is prone to many stresses and strains (Bromilow, 2001). Since the beginning of civilization, survival of the human race was dependent on plants not only as a source of food and oxygen, but also as a source of natural remedies. Books, magazines and many sources of information give a genuine tidal wave of information, declaring the merits and medicinal effectiveness of plants (Schneider, 2002).

Common sense and experience have shown that plants freshly picked, which involve a minimum amount of processing are better able to retain their active principles and therapeutic value. Antimicrobials of plant origin have an extremely large therapeutically potential. They are effective in the treatment of infectious diseases while simultaneously alleviating many of the side effects that are often encountered with synthetic antimicrobials (Iwu and Laird, 1998).

Use of medicinal plants as a source of relief and cure from variety of illnesses is as old as humankind itself. Even today, medicinal plants provide
an inexpensive source of drugs for the greater number of the world’s population. Plants have provided and will continue to provide not only directly usable drugs, but also various chemical compounds that can be used as starting points for the synthesis of new drugs with improved pharmacological properties. Many modern medicines have their origins in plants (Mukherjee, 2002).

The WHO has estimated that about 80% of the population living in the developing countries relies on traditional medicine for their health care needs (WHO, 2002) and there is estimation that about 80% of all Indians use traditional medicine derived from plant species indigenous to the region. Traditional medicine is beneficial even in developed countries and has also influenced pharmaceutical products. Extracts of plants and algae have been incorporated in the products and plants in particular are an indispensable source of pharmaceuticals (Evans et al., 2002). Recently, there has been a dramatic increase in the demand for “herbal medicines”.

Traditional medicine is readily available accessible and affordable and that enable traditional healers to treat people of all age groups, presenting with any problem. Their treatment is said to be holistic, they treat the whole person not only the symptoms of the disease (surroundings inclusive). In countries like Chile, Mexico, Peru, Philippines South Africa and Zimbabwe, traditional medicine is being actively promoted with the aim of making it part
of the national health care system. The role of traditional healers in primary health care can be formalized if the government of such countries as mentioned above could fully recognize the services that the traditional healers provide (Kamble et al., 2010).

The risk of opportunistic fungal infections greatly increased in patients who were severely immune-compromised due to cancer chemotherapy, organ or bone marrow transplantation and human immunodeficiency virus infection. Likewise, bacterial diseases accounts for high proportion of health problems in both developed and developing countries. Despite the progress made in the understanding of invasion, pathology and control, the incidence of epidemics due to drug resistant microorganisms and the emergence of hitherto unknown disease causing microbes, pose immense clinical problem in the treatment of public health concerns (Hancock et al., 2012). This situation highlights the need for advent of safe, novel and effective antimicrobial agents.

Antibiotics are one of the most important weapons in fighting bacterial infections and have greatly benefited the health-related quality of human life since their introduction. However, over the past few decades, these health benefits are under threat as many commonly used antibiotics have become less against certain illnesses not, only because many of them produce toxic reactions, but also due to emergence of drug-resistant bacteria. It is
essential to investigate newer drugs with lesser resistance. Drugs derived from natural sources play a significant role in the prevention and treatment of human diseases (Farnsworth, 1993; Houghton, 1995).

Rational drug design does not always yield effective antimicrobials. In the past, potent enzyme inhibitors have been successfully designed and synthesized but they had only modest antibacterial activity, probably owing to the complex issue of drug uptake by the cells (Mukne et al., 2011). The necessity to develop new drugs requires varied strategies, among them, the bioprospection of secondary metabolites produced by medicinal plants (Dionisi et al., 2012).

The search for drugs and dietary supplements derived from plants have accelerated in recent years. Ethnopharmacologists, botanists, microbiologists, and natural-products chemists are combing the Earth for phytochemicals and “leads” which could be developed for treatment of infectious diseases. While 25 to 50% of current pharmaceuticals are derived from plants, none are used as antimicrobials. Traditional healers have long used plants to prevent or cure infectious conditions; Western medicine is trying to duplicate their successes. Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found in vitro to have antimicrobial properties. Plants are valuable sources of ecologically developed secondary metabolites which are
important for normal growth and defense against infection and injury. The earliest drug discoveries were made by presumably random sampling of higher plants. Therefore, it is of great importance to carry out a screening of traditional medicinal plants in order to validate their use in folk medicine and also to reveal the active principle by isolation and characterization of their secondary metabolites.

Approximately 20% of the plants found in the world have been subjected to pharmacological screening and a substantial number of new antibiotics introduced in the clinical use are obtained from natural or semi-synthetic resources (Mothana and Lindequist, 2005). *Pithecellobium dulce* is one such traditional medicinal plant that lacks scientific scrutiny for its antimicrobial activity.

*Pithecellobium dulce* Bentham is an evergreen medium sized, branched, spiny tree that reaches heights of about 22m. It has vernacular names including Manila tamrind, Madras thorn, Monkeypod, Vilayati babul, Black beard and Kodukkapuli. It belongs to the family *Leguminosae* and subfamily *Mimosoideae*. The generic name refers to the curly pod that mimics an ape’s earring (pithekos ellobium) and the species name “dulce” refers to the sweet pod. *Pithecellobium dulce* is the only species among 100-200 species in the genus and has become widespread outside its origin.

It has been commonly used for fencing and tanning, as fodder for feed and pods for food. It coppices readily and can be managed as a hedge.
*P. dulce* is noted for their tolerance of heat, drought, salinity and impoverished soils. The plant is well known for its edible fruits and they have been consumed for various ailments in a traditional manner. The fruits are linear, curved legumes (Pods) that range in length from 10 to 13 cm. The pod splits along both margins. The legumes may contain 5 to 12 seeds which are reddish brown to black in colour. The fruits turn from green to reddish brown when they ripen. The pod fragments can be eaten raw or made in to a drink for its nutritive as well as therapeutic values but still most of the chemical constituents of the pods are remained unexplored and underutilized (Duke and Wain 1981; Murugesan sugumaran 2008).

Various parts of the tree such as bark, leaves and seeds have been studied for their medicinal properties (Adinarayana and Ramachandraiah 1985; Saxena and Singal., 1998; Megala and Geetha, 2010). In the absence of systematic reports in the literature, the present was aimed to determine the bactericidal and fungicidal effects of *P. dulce* pod pulp using common pathogenic bacteria and fungi.
MATERIALS AND METHODS

Plant Material

The plant was taxonomically identified and authenticated by a qualified taxonomist and a voucher specimen has been deposited in our laboratory for future reference. Mature fruits of *P. dulce* were picked from the trees which are growing in the natural environment at the banks of the river “Thamirabarani” in Tirunelveli district, Tamilnadu.

Preparation of Plant extract

Seeds are removed by hand flailing and pod pulp fragments were dried in shade, pulverized by a mechanical grinder and passed through a 40 mesh size to get a fine powder and stored at 0° until further use. Known quantity of pulp powder was extracted with petroleum ether (60-80°C) to remove wax and then extracted with 80% methanol in a soxhlet apparatus. The solvent was evaporated to dryness in a rotary evaporator at reduced pressure below 40° C. The extract was used for further experiments (Yield 17.6g).

Bacterial and Fungal strains and growth medium

The bacterial and fungal strains were all standard laboratory strains obtained from the stock cultures of the Division of Microbiology, CAS in
Botany, University of Madras, and Chennai and maintained on slopes of Muller Hinton Agar (MHA) and Sabouraud Dextrose Agar (SDA) at 28°C.

Four Gram positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*, *Staphylococcus epidermidis*, *Enterococcus Faecalis* and Four Gram negative bacteria (*Escherichia coli*, *Shigella dysenteriae*, *Klebsiella pneumonia*, *Salmonella typhi* were used in the present study. Fungal cultures of *Candida albicans*, *Saccharomyces cerevisiae*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus ochraceus*, *Penicillium notatum*, *Penicillium chrysogenum* were included in this study.

**Determination of antibacterial and antifungal activity**

**Preparation of inoculum**

The suspension for inoculation was prepared from the broth culture. Few colonies of similar morphology of the respective bacteria were transferred with the help of a sterile inoculating loop to a Muller-Hinton broth and were incubated until adequate growth of turbidity equivalent to McFarland 0.5 turbidity standard (10⁸ CFU/ml) were obtained.

The fungal strains were subcultured on slants of SDA at 28°C for 7 days and the colonies were suspended in 1 ml of sterile normal saline. The resulting mixture of conidia and hyphal fragments was vortexed and the turbidity of each homogenous suspension was adjusted to match that of a
0.5 McFarland standard, as read at 530 nm. At this turbidity, the fungi density was $3 \times 10^6$ to $5 \times 10^6$ CFU ml$^{-1}$.

**Preparation of the McFarland standard**

0.5 ml of 0.048M BaCl$_2$ was added to 99.5 ml of 0.18M H$_2$SO$_4$ with constant stirring. The standard was distributed in to screw cap tubes of the same size and with the same volume as those used in growing the broth culture. The tubes were sealed tightly to prevent loss by evaporation. The tubes were stored, protected from light at room temperature. The turbidity standard was agitated vigorously on a vortex mixture before use. Standards may be stored for up to 6 months, after which time they should be discarded.

Antibacterial activity of the ethanolic extract of *P. dulce* pod pulp was evaluated by agar well diffusion method (Holder and Boyce, 1994). The inocula with respective test bacteria were homogenously seeded onto the 90mm Petri dishes containing 20 ml of cooled molten MH agar medium using a sterile swab in such a way as to ensure thorough coverage of the plates and a uniform thick lawn of growth following incubation (Lall and Meyer, 2000). Wells were dug in the medium with the help of a sterile cork borer. Stock solution of the pod pulp extract (2.5 mg/ml) was prepared in sterile distilled water. Dilutions of the stock solution containing 50, 100, 150, 200 and 250 µg were also prepared in sterile distilled water. 100 µl of each dilution was added to their respective wells with a sterile pipette. Control wells received only 100 µl of sterile distilled water. The plates were kept for 1 h at room
temperature for the diffusion of the extract into the agar. Subsequently, all the plates were incubated at 37°C for 18-24 h. Following incubation the plates were examined for signs of microbial growth. Bacterial growth inhibition was determined as the diameter of the inhibition zones around the wells. Chloramphenicol (30 µg/ml) was used as positive control. Each experiment was carried out in triplicates.

Antifungal activity of the ethanolic extract of *P. dulce pod pulp* extract was evaluated by disc diffusion method. The inocula with respective fungi were homogenously seeded onto the 90mm Petri dishes containing 20 ml cooled molten SDA medium using sterile swab in such a way as to ensure thorough coverage of the plates and a uniform lawn of growth following incubation. These inoculated plates were left to dry for at least 15 min. The extract was dissolved in distilled water to obtain the different concentrations of 300, 150, 75, 37.5 and 18.75 mg ml⁻¹. Amphotericin B at concentration 10 µg/disc was used as positive control and was dissolved in dimethyl sulphoxide (DMSO). Sterile filter paper disc (6mm in diameter) were impregnated with 10 µl of each different concentration of latex extract. The discs were allowed to dry and then placed on the agar surface of each petri dish. DMSO was used as negative control. Zone of inhibitions (in mm) were measured after 48-72 h at 28°C. The complete antifungal analysis was carried out under strict aseptic conditions. Each assay was repeated three times.
Minimum inhibitory concentration (MIC) Minimum bactericidal concentration (MBC), Minimum fungicidal concentration (MFC) assays

A serial of 2-fold macro-broth dilution method was performed to determine the MICs and MBCs of *P. dulce pod pulp* extract for the respective tested bacterial suspensions (concentration) as recommended by the Clinical and Laboratory Standards Institute (CLSI) (Wikler, 2008). The minimum inhibitory concentration (MIC) of *P. dulce pod pulp* extract against fungal strains was determined using broth microdilution method as described by the National Committee for clinical laboratory standards for fungi (M27-A2). The stock solutions of *P. dulce pod pulp* extract was diluted suitably as required from stock solution. The ranges should be prepared one step higher than the final dilution range required that if a final dilution range of 0.5, 1, 2, 4, 8, and 16 mg/ml is required then a range of 1, 2, 4, 8, 16 and 32 mg/ml should be prepared to compensate for the addition of an equal volume of inoculums. Two rows of 12 capped test tubes were arranged in the rack. In a sterile 30 ml (universal) screw capped bottle, 8 ml of MH broth (bacteria), 8ml SD broth (fungi) containing the required concentration of *P. dulce pod pulp* extract for the first tube in each row was prepared from the appropriate stock solution already made. The contents of the universal bottle were mixed using a sterile pipette and transferred 2 ml to the first tube in each row. Using a fresh sterile pipette, 4 ml of broth was added to the remaining 4 ml in the universal bottle, mixed well and transferred 2 ml to the second tube in each
row. Dilutions were continued in this way to as many as 10 tubes. 2 ml of broth free from pod pulp extract was added to the last tube in each row. The density of the bacterial suspension was adjusted \(10^8\) CFU/ml to equal that of the 0.5 McFarland standard by adding sterile distilled water as detailed above. The bacterial suspension was suitably diluted \(10^6\) CFU/ml and added to the tubes containing MH broth. The density of the fungal suspension was adjusted \(3\times10^6\) to \(5\times10^6\) CFU ml-1 to equal that of the 0.5 McFarland standard by adding sterile distilled water as detailed above. Chloramphenicol (30 µg) was used as positive control for bacteria. After incubation at 37ºC for 24 h, turbidity of the tubes was assessed visually by comparison to uninoculated control.

Amphotericin B was included in the assays as positive control 10 µg/disc for fungi. After incubation at 28ºC for 42-78 h, turbidity of the tubes was assessed visually by comparison to uninoculated control.

The MIC is expressed as the lowest concentration of the pod pulp extract where bacterial and/or fungal growth with no visible growth after incubation. All assays were carried out in triplicates. The MBC was derived by sub-culturing 100 µl from each tube from the MIC assay onto substance free MH agar plates. The plates were incubated at 37ºC for 24 h and the MBC was defined as the lowest concentration of substance that allows no visible growth on the agar plate.
The MFC was determined by plating a 100 μl volume on SDA from the tubes showing no visible growth. The plates were incubated as described above in MIC. The MFC was defined as the lowest concentration of substance that did not allow any visible growth on the agar plate.

**DETERMINATION OF ANTIMICROBIAL ACTIVITY**

The antibacterial activity of ethanolic extract of *P. dulce* pod pulp was tested against three Gram positive and three Gram negative bacteria. The inhibitory effect was assessed by well diffusion method. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were also determined by serial dilution method.

The antifungal properties of ethanolic extract of *P. dulce* pod pulp were tested against common pathogenic fungal strains. The inhibitory effect was assessed by disc diffusion method. The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were also determined by serial dilution method.
RESULTS AND DISCUSSION

The worldwide increase in resistance of pathogenic microorganisms to time-honored antibiotics necessitates the search for alternative strategies preferably from plant origin. Plants produce a variety of secondary metabolites such as flavonoids, alkaloids and tannins which have long been of interest to mankind (Lewis and Ausbel, 2006). However, very few reports are available on the pharmacological activity of medicinal plants and of the 4,000 plant species on earth, only a small percentage has been systematically studied for their antimicrobial activities (Shokeen et al., 2009). Although screening of Indian medicinal plants has revealed varying degrees of antimicrobial activity against pathogenic and opportunistic microorganisms, there is still a lack of experimental scientific studies confirming the possible antimicrobial properties of a great number of these remedies.

Antimicrobial resistance is a natural biological phenomenon of response of microbes to the selective pressure of an antimicrobial drug. Since antibiotic use became widespread 50 years ago, microorganisms have relentlessly developed resistance (Martínez and Baquero, 2002). The ultimate goal is to offer appropriate and efficient antimicrobial drugs to the patient (Nascimento et al., 2000). One way to prevent antibiotic resistance of pathogenic species is by using new compounds that are not based on existing
synthetic antimicrobial agents (Shah, 2005). This *in vitro* study demonstrated that folk medicine can be as effective as modern allopathic medicine to treat pathogenic microorganism. The use of *P. dulce* in folk medicine suggests that it represent an economic and safe alternative to treat common infectious diseases. Detailed investigations in to the active components responsible for the observed antimicrobial activity may open new avenues for drug development and control of antibiotic resistant pathogenesis (Quave, 2008). Plant based antimicrobials represent a vast untapped source for medicines and further exploration of their usefulness is necessary.

On a global basis at least 130 drugs, all single chemical entities extracted from higher plants are modified further synthetically are currently in use, though some of them were now being made synthetically for economic reasons (Newman *et al.*, 2000). Thus, it was considered worldwide to investigate the antibacterial as well as antifungal activities of *P. dulce* pod pulp, a common medicinal plant that has been widely used in traditional medicine in one form or the other for its beneficial pharmacological activity.

Table 19 shows the antibacterial activity of ethanolic extract of *P. dulce pod pulp* against four different Gram positive and Gram negative bacterial strains. The antibacterial potency of *P. dulce pod pulp* extract was evaluated by the presence or absence of inhibition zones and zone diameters (mm). The results of the present study indicate that the ethanolic extract of
*P. dulce pod pulp* showed a maximum inhibitory zone in a dose dependant manner. However, there was no significant difference between the levels of zone of inhibition at the concentration of 200 µg and 250 µg. Among the Gram positive bacteria, *B. subtilis* showed a larger diameter of clearance than that of other Gram positive bacteria used in this study. Among the Gram negative bacteria, *K. pneumoniae* than that of other Gram negative bacteria. The zone of clearance achieved by *P. dulce pod pulp* extract is comparable to that of standard antibiotic, chloramphenicol.

The minimum inhibitory concentration and minimum bactericidal concentration of *P. dulce pod pulp* extract as well as the standard antibiotic, chloramphenicol is shown in Table 20. The MIC value of *P. dulce pod pulp* extract against both Gram positive and Gram negative bacterial strains varies from 1 mg to 5 mg and the results are comparable with the standard antibiotic, chloramphenicol. The highest MIC values were shown by *Enterococcus faecalis* in Gram positive bacteria and by *Salmonella typhi* in gram negative bacteria. The lowest MIC values were displayed by *Bacillus subtilis* in Gram positive bacteria and *K.pneumoniae* in gram negative (Pradeepa et al., 2014).

The results of the study indicated that *P. dulce* pod pulp extract showed effective inhibitory activity against Gram-positive bacteria, *Bacillus subtilis* and gram negative bacteria *Klebsiella pneumonia*. *B. subtilis* showed a larger diameter of clearance than that of other Gram positive bacteria used...
in this study. Similarly, *P. dulce pod pulp* extract showed a maximum zone of clearance in the Gram negative bacteria, *K. pneumoniae* than that of other Gram negative bacteria.

Minimum inhibitory concentrations are considered the “gold standard” for determining the susceptibility of microorganisms to antimicrobials and are therefore used to judge the performance of all other methods of susceptibility testing (Andrews, 2001). A lower MIC value indicates that less drug is required for inhibiting growth of the organism; therefore, antimicrobials with lower MIC values are more effective antimicrobial agents. The highest MIC and MBC values were shown by *Enterococcus faecalis* in Gram positive bacteria and by *Salmonella typhi* in gram negative bacteria. The lowest MIC and MBC values were displayed by *Bacillus subtilis* in Gram positive bacteria and *K. pneumoniae* in gram negative (Pradeepa et al., 2014).

Table 21 shows the antifungal activity of ethanolic extract of *P. dulce pod pulp* against eight different fungal species. The antifungal potency of *P. dulce pod pulp* extract was evaluated by the presence or absence of inhibition zones and zone diameters (mm). It is evident that the ethanolic extract of *P. dulce pod pulp* showed a maximum inhibitory zone in a dose dependant manner. However, there was no significant difference between the levels of zone of inhibition at the concentration of 1.5 mg and 3 mg/disc. The
antifungal potency of *P. dulce pod pulp* on the *C. albicans* showed a larger diameter of clearance than that of other strains. Moreover, the zone of clearance achieved by *P. dulce pod pulp* extract is comparable to that of standard drug, Amphotericin B.

The minimum inhibitory concentration and minimum fungicidal concentration of *P. dulce pod pulp* extract as well as the standard antifungal drug, Amphotericin B is depicted in Table 22. The MIC value of *P. dulce pod pulp* extract against fungal strains varies from 1 mg to 7 mg and the results are comparable with the standard antifungal agent, Amphotericin B. The lowest MIC was shown by *Candida albicans* and the highest MIC values by *S. cerevisiae*.

Fungal diseases represent a critical problem to health and they are one of the main causes of morbidity and mortality worldwide. Human infections, particularly those involving the skin and mucosal surfaces, constitute a serious problem, especially in tropical and subtropical developing countries (Portillo et al., 2001). In humans, fungal infections range from superficial to deeply invasive or disseminated, and have increased dramatically in recent years. Although new drugs have been introduced to combat this problem, the development of resistance to antifungal drugs has become increasingly apparent, especially in patients who require long-term treatment or who are
receiving antifungal prophylaxis, and there is growing awareness of shifts of flora to more-resistant species.

The fungal strains used in the present study were selected on the basis of their clinical importance. Agar disc diffusion method was performed in the present study to investigate the antifungal activity of *P. dulce* pod pulp extract. The highest activity (diameter of zone of inhibition 25 mm) was demonstrated by the ethanolic extract of *P. dulce pod pulp* against *C. albicans* while the lowest activity was observed against *S. cervisiae*. The results of the *in vitro* antifungal assay revealed that the growths of fungal strains were affected by the *P. dulce pod pulp* extract by forming clear inhibition zones.

The MICs and MFCs showed that *S. cerevisiae* has the highest MIC (7mg/ml) and MFC (7mg/ml) while the lowest MIC of 2 mg/ml was demonstrated by *C. albicans*. The fungistatic or fungicidal effect of natural products and the mechanisms involved are cytoplasm granulation, cytoplasmic membrane rupture and inactivation and/or inhibition of intracellular and extracellular enzymes. These biological events could take place separately or concomitantly culminating with mycelium germination inhibition and it is also reported that plant lytic enzyme act in the fungal cell wall causing breakage of β-1,3 glycan, β-1,6, glycan and chitin polymer (Brull, 1999). The observed antifungal effect of the extract might be due to
the presence of biologically important ingredients present in the pod pulp (Pradeepa et al., 2014).

The remarkable bactericidal, fungicidal effects of *P. dulce* pod pulp extract suggest that the pod pulp may be a useful source for the development of novel antibacterial, antifungal agent against pathogenic bacteria and fungi.