Summary
Antioxidant activity of *P. pinnata*

Quantitative Phytochemical Analysis

- Total Phenol Content
- Total Flavonoid Content

Assay

- DPPH
- RCA
- SO
- FRAP
- ABTS

Spectral analysis

- UV
- IR
7.1 Summary

Natural products are naturally derived metabolites and/or by-products from microorganisms, plants, or animals. These products have been exploited for human use for thousands of years, and plants have been the chief source of compounds used for medicine. Since times immemorial medicinal plants have been used as a source of medicine. In ancient texts such as Vedas and Bible, the widespread use of herbal remedies and healthcare preparations has been described. It was not until Pasteur discovered that fermentation is caused by living cells that people seriously began to investigate microbes as a source for bioactive natural products. Then, scientific serendipity and the power of observation provided the impetus for research in that arena. Even with untold centuries of human experience behind us and a movement into a modern era of chemistry and automation, natural-product-based compounds have had an immense impact on modern medicine since about 40% of prescription drugs are based on them.

Natural products have been the traditional pathfinder compounds, offering an untold diversity of chemical structures unparalleled by even the largest combinatorial databases. While combinatorial synthesis produces compounds at random, secondary metabolites, defined as low-molecular-weight compounds not required for growth in pure culture, are produced as an adaptation for specific functions in nature. It appears that the search for novel secondary metabolites should center on organisms that inhabit unique biotopes. Endophytes are microbes that inhabit such biotopes, namely, higher plants, which is why they are currently considered to be a wellspring of novel secondary metabolites offering the potential for medical, agricultural, and/or industrial exploitation. Currently, endophytes are viewed as an outstanding source of bioactive natural products because there are so many of them occupying literally millions of unique biological niches (higher plants) growing in so many unusual environments.

In the battle against the ever-increasing multidrug resistance of human pathogenic bacteria, there is urgent need for new alternatives to the currently available broad-spectrum antibiotics. Bacterial species recently named as the “ESKAPE”
pathogens: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and *Enterobacter* species cause the majority of hospital infections and effectively “escape” the effects of antibacterial drugs. Resistance has increased in both Gram-positive and Gram-negative pathogens and poses a serious threat to the successful treatment of infectious diseases. Modern medicine was built on a reliance on antibiotics, but we are now heading towards a world without them, which is why there are more and more initiatives to raise awareness of this problem. So, where do we look for new antibiotics? Whenever a new niche of biodiversity is discovered and accessed, new natural products are found. The realization that there is a large, and mostly unexplored, group of bacteria living inside higher plants (endophytic bacteria) led to focused discovery efforts in both industrial and academic laboratories.

*Pongamia pinnata* L. belongs to the family *Fabaceae* and is commonly known as Karanj. It is a small evergreen tree, which is widely distributed in India, Bangladesh, China, and Australia. Different parts of this plant have been recommended in Ayurvedic literature, an alternative system of medicine in India, as a remedy for various ailments. *Pongamia pinnata* root have been described as a useful remedy for foul ulcer, fistulous sores, gonorrhea and urethritis. Seed and seed oil have been used for treating various inflammatory and infectious diseases such as leucoderma, leprosy and muscular and articular rheumatism. The leaves are digestive, laxative, anthelmintic and cure piles, wounds and other inflammations.

Healthy and asymptomatic leaf and stem of *Pongamia pinnata* L. were collected from Rajkot, Gujarat, India in the months of July and August 2012. Samples were collected, stored in polythene bags, brought to the laboratory and processed on the same day for isolation of endophytic bacteria. The plant was compared with voucher specimen (Voucher Specimen No. PSN180) deposited at Department of Biosciences, Saurashtra University, Rajkot, Gujarat, India. Surface sterilization process of leaf and stem were carried out prior to isolation of endophytic bacteria. A total of 17 morphologically distinct bacterial strains, 10 (L1-L10) from leaf and 7 (S1-S7) from stem were isolated from *P. pinnata* which demonstrates that medicinal plants hosted a diverse selection of culturable bacteria.
The isolates thus obtained were characterised on the basis of morphology, biochemical tests and 16S rRNA sequencing. The biochemical and metabolic reactions revealed the production of catalase and oxidase by 47% and 11% of isolates respectively. Isolates varied on the basis of sugar utilization and none produced gas in Durham’s tube. All the isolates were able to utilize dextrose. Based on 16S rRNA gene homology, five potential isolates; L3, L5, L8, S3 and S7 were related to their nearest homologus and the sequences were deposited in NCBI as Bacillus cereus, Bacillus licheniformis, Bacillus cereus, Bacillus megaterium and Bacillus pumilus respectively.

The endophytic bacterial isolates were preliminary screened for antimicrobial activity against a few test microorganisms by cross streak method. Among the 17 endophytic bacteria from P. pinnata plant isolated in this study, 5 showed antimicrobial activity against test microorganisms. Isolate S3 (Bacillus megaterium) was the most active endophyte which inhibited 81.8% of test bacteria. Hence S3 was then selected for optimization of antimicrobial compound production. The organism was checked for its ability to produce metabolites at different culture conditions. The isolate S3 was able to grow well in all different media, pH and temperature values tested. But the antimicrobial activity was seen best at the parameters of 37°C, pH 7, incubation for 96 h in Nutrient broth and hence these parameters were chosen for mass production of antimicrobial metabolites by S3.

The antimicrobial metabolites produced by S3 were extracted with ethyl acetate and crude extract was thus obtained. This crude extract was used to check broad spectrum antimicrobial activity against various test pathogenic organisms. B. cereus and P. putida were the most susceptible Gram positive and Gram negative bacteria respectively. The antifungal activity against the yeasts was moderate. Fractionation of this crude extract was carried out using silica gel chromatography. The antibacterial activity of the ten fractions obtained was carried out by disc diffusion method. Fraction 2 recorded best antibacterial activity inhibiting the growth of all the test bacteria and hence it was chosen for MIC study. Minimum inhibitory concentration of fraction 2 showed lowest MIC towards P. putida of 31.25 µg ml⁻¹.
IR spectroscopy and LC-MS analysis were employed to figure out the compounds present in fraction 2. The results indicated the presence of diketopiperazines (CDPs) which might be responsible for the antibacterial activity of fraction 2.

In another part of experiment, antimicrobial activity of the leaf and stem of *P. pinnata* was determined. Leaf and stem were collected, washed thoroughly with tap water, shade dried, homogenized to fine powder and stored in airtight bottles. The dried powder was extracted with organic solvents (with increasing polarity) in Soxhlet apparatus. The methanol extract of both leaf and stem was fractionated into two fractions by solvent-solvent partitioning method. The antimicrobial susceptibility of extracts and fractions was checked by agar well method. The results indicated that the leaf and stem extracts exhibited considerable antimicrobial activity against bacterial strains. As compared to leaf extracts, the stem extracts showed better antimicrobial activity. Maximum zones of inhibition were seen against *B. cereus* and *K. pneumoniae*, making them as the most susceptible Gram positive and Gram negative bacteria respectively.

The methanolic extract displayed a broad antimicrobial spectrum and exerted significant antibacterial effect against both Gram positive and Gram negative bacteria tested. Hence methanolic extracts of leaf and stem along with their fractions were selected for determining their inhibitory action by broth microdilution method. The lowest MIC values were observed against *B. cereus* and *K. pneumoniae* (MIC 78 µg ml\(^{-1}\)) by stem methanolic fraction 1 and by stem methanolic extract against *K. pneumoniae* (MIC 78 µg ml\(^{-1}\)).

An effort was also made to figure out the interactive antimicrobial activity of extracts with commercial antibiotics as well as their mixture against test microbes. The combination of leaf and stem methanolic extract with ceftazidime provided a notable interactive profile. The combination with ceftazidime displayed synergistic interactions against *B. cereus*, *L. monocytogenes* and *P. aeruginosa*. With combination of antibiotics, remarkable reductions in the MIC values were seen as compared to individual extracts.
Antioxidants are defined as compounds that protect biological systems against the potentially harmful effects caused by excessive oxidation. There are endogenous antioxidants in our body having the ability to stop formation of free radicals or to limit the damage they cause. Free radicals are generated during metabolism and are scavenged by endogenous defense system such as catalase, superoxide dismutase and peroxidase-glutathione system. But the endogenous antioxidants are either exhausted or become insufficient to scavenge these radicals generated during certain cases such as in unhealthy physical condition, ageing, or under stress environments, which result in diseases associated with oxidative stress and damage and are capable of oxidising biomolecules, resulting in cancer, coronary heart disease, hypertension, etc. Plants are repertoire of biologically active natural products having antioxidant potential.

In vitro antioxidant activity of the leaf and stem extracts of *P. pinnata* and their fractions was carried out by DPPH free radical scavenging activity, superoxide anion radical scavenging activity, ABTS radical cation scavenging activity, ferric reducing antioxidant power and reducing capacity assessment. The IC$_{50}$ value of leaf methanol extract was 78 μg ml$^{-1}$ by DPPH free radical scavenging activity. Highest superoxide anion radical scavenging activity (IC$_{50}$ 79 μg ml$^{-1}$) was shown by leaf aqueous extract which was even better than the standard gallic acid (185 μg ml$^{-1}$).

Leaf aqueous extract showed significantly more reducing capacity than the standard ascorbic acid. Leaf extracts showed better FRAP activity than stem extracts. Thus overall, leaf extracts showed better antioxidant activity than the stem extracts. There was a direct correlation between total phenol content and antioxidant activity in some of the antioxidant assays. Thus *P. pinnata* leaf extracts can be used as a natural source of antioxidants which can be used in the prevention of diseases caused by free radicals.
Graphical Summary

Isolation

Healthy and asymptomatic leaf and stem of *P. pinnata*

Isolation of endophytic bacteria from leaf and stem after surface sterilization

Pure culture of endophytic bacteria

Identification

Morphological characterization

Biochemical characterization

Molecular characterization - Phylogenetic analysis
Antimicrobial activity of endophyte

Antimicrobial screening → Extraction of antimicrobial metabolites → Antimicrobial activity of crude extract

Antibacterial activity of fractions

Fractionation of crude extract

IR → LCMS

Spectroscopic measurements
Antimicrobial activity of *P. pinnata*

Leaf and stem of *P. pinnata*

Sequential extraction by Soxhlet apparatus

Antimicrobial screening by agar well method

Fractionation of leaf methanol and stem methanol extract by solvent-solvent partition method

MIC study

Synergistic activity (1:1) (Extract + Antibiotic; Extract + Extract)