Evaluation of antimicrobial activity of certain natural products against selected human pathogens

A dissertation thesis submitted to Nirma University in

Partial fulfilment for the Degree of

MASTER OF SCIENCE

IN

MICROBIOLOGY

Submitted by:

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Krishna Patel (12MMB006)

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NIRMA UNIVERSITY, AHMEDABAD

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Under the guidance of

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This study is dedicated to God who is the head of our life, and to our family for their love, support and encouragement.

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"In order to succeed, your desire for success should be greater than

your fear of failure." -Bill Cosby

This dissertation is a milestone in our academic career.

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For any errors or inadequacies that may remain in this work, of course, the responsibility is entirely our own.

Arwa Kaizer Ali Krishna Patel Nishith Nair

Index

Abbreviations

AI- Activity index

AST -Antimicrobial susceptibility testing

CFU-Colony forming units

CV-Crystal violet

DMSO-Dimethyl sulfoxide

EPS-Exopolysaccharide

EtOH -Ethanol

HPLC- High performance liquid chromatography

MAE-Microwave assisted extraction

MBC-Minimum bactericidal concentration

MeOH-Methanol

MIC- Minimum inhibitory concentration

MTCC-Microbial type culture collection

NCCLS -National committee for clinical laboratory standards

OD- Optical density

TCP- Tissue culture plate

INTRODUCTION

1. INTRODUCTION

Nature has been a source of medicinal agents for thousands of years since it comprises of compounds that are highly diverse and often provide highly specific biological activities. This follows from the proposition that essentially all natural products have some receptor binding capacity (Verdine , 1996). Herbal medicines have been the basis of treatment for various diseases and physiological conditions in traditional methods practiced such as Ayurveda, Unani and Siddha (Kumar *et al*., 2006).To promote the proper use of herbal medicine and to determine their potential as sources for new drugs, it is essential to study medicinal plants, which have folklore reputation in a more intensified way(Ali *et al*.,2001).Over the past 20 years, there has been a lot of interest in the investigation of natural materials as sources of new antimicrobial agents (Recio, 1989). Some natural products have been approved as new antimicrobial drugs, but there is a continuous and urgent need to screen more and more plant species and discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action to combat new and re-emerging infectious diseases of today's era (Rojas *et al*., 2003). Many infectious diseases are known to be treated with herbal remedies throughout the history of mankind. Even today, plant materials continue to play a major role in primary health care as therapeutic remedies in many developing countries (Zakaria, 1991). Plant based antimicrobials represent a vast untapped source of medicines and there is a need for further exploration of plant antimicrobials. It is estimated that several plant-derived drugs prescribed in the industrialized world were discovered by studying folk knowledge. However, less than one-half of 1 percent of all plant species in the world has been studied for potential pharmacological activity (Balick, 1996). Plant based natural constituents can be derived from any part of the plant like bark, leaves, lowers, roots, fruits, seeds, etc (Gordon *et al*., 2001) i.e. any part of the plant may contain active components. Scientific analysis of plant components may give a new source of antimicrobial agents with possibly novel mechanisms of action (Motsei *et al*., 2003). World Health Organization has also approved the study of medicinal plants for the development of new drug lead (World Health Organization (WHO) 2000). In recent years, many researchers all over the world have screened plant extracts to detect and utilize secondary metabolites with relevant biological activities as medicinal agents (Parekh *et al*., 2007).

There is an evolving group of chronic infectious diseases and condition in which bacteria is present and they are difficult to culture and demonstrate consistently hence resistant to current antimicrobial and tools and often require surgical removal to resolve. Despite adequate drainage

antibiotics are not the **"silver bullet"** once envisaged for this chronic infectious condition (Hayes *et al*., 1993). The use of antimicrobials from natural sources could have been a great impact for preserving food storage from contamination, and in controlling plant and human diseases of microbial origin (Balandrin *et al*., 1985, Conner, 1993). The continued evolution of infectious disease and the resistance offered by the pathogens to the existing pharmaceuticals have led to an intensified search for the new availability of antimicrobials against fungal, bacterial and viral particles. Despite of the recent advancement through molecular modeling, and combinatorial and synthetic chemistry, natural plant derived compounds are found to be invaluable source of medicines for humans (Salim *et al*., 2008). As plants constantly interact with the rapidly changing and damaging environmental conditions. Being organism devoid of mobility plants has developed a strong immunity against the metabolites and chemical stress which come across their life. The ability of plant to carry out combinatorial chemistry by mixing matching and evolving the gene products required for certain secondary metabolite biosynthetic pathway create an unlimited pool of chemical compound, which humans have exploited to their benefit. Plants have recognised symbiotic relationship with colonizing bacteria. There may be plant based natural quorum sensing signals to control pathogens. It was observed that certain seaweed plants never became cover with bacteria or higher organism (called biofouling, which is also observed on sub surfaces of ships) because the plant produced halogenated furanones, which have quorum sensing inhibitors activity(QSIs), but these halogenated furanones contain halogen which make them restricted to human use. Therefore, large scale screening of QSIs in nature was therefore performed. In recent years multiple drug resistance occur against commercially available antimicrobials which has forced scientist to search for new antimicrobials from plants which has vast source of bioactive agents in it.

Microbes and biofilm: Free floating or planktonic describes a bacterium that acts independently, is readily mobile and often possess characteristics that allow invasion of tissues .They are the phenotypic variants associated with acute infection .Biofilm mediated infections are often chronic , are rarely resolved by host defenses, and are resistant to eradication even with directed antibiotics.

A biofilms is a matrix-enclosed microbial community attached to a surface. Because most surfaces in nature are coated with an adsorbed layer of macromolecules, the biofilm is usually attached to this layer (termed a "conditioning film") rather than directly to the surface itself.

In 1683, Antoni van Leeuwenhoek's, well quoted description of the 'tooth worm' or 'animalculi' from his own teeth represents the first description of biofilm in disease (Ring *et al*., 1971).

Morphology of biofilms have been identified in 3 billion years old sedimentary rocks (Westall F *et al*., 2001), and volcanogenic sulphur deposits (Rasmussen *et al*., 2000).The ability of bacteria or fungi to form biofilm may represent an evolutionary adaptation-

- Protection from environmental factors (moisture changes, extreme of temperatures, pH, UV light) in the harsh climate of earth, billions of years ago would provide a survival advantage (Stoodley *et al*., 2004).

- Avoidance of phagocytosis,

- The development of close cell to cell signaling and chemotactic motility (Stoodley *et al*., 2002)may also have contributed in biofilm formation.

- The microbes in biofilms are characteristically kept together by a self-produced biopolymeric matrix. The matrix contains polysaccharides, proteins and extracellular DNA originating from the microbes. Compounds from the host, e.g. immunoglobulins, may also be present and the consortium can consist of one or more species living in symbiosis (Ring *et al*., 1971). This condition is called **sociomicrobiology** (Parsek *et al*., 2005).

  Bacteria in biofilm form can express many genes which are never expressed in planktonic form. Almost 50% of the proteome is upregulated during biofilm formation. The adaptation of bacteria into biofilm is both a response to environment and genetic programming of bacteria .Thus bacteria may form different biofilms on different surfaces .The biofilm forming on mucosal surface differs from inert as it is in constant modulation by host response. The biofilm phenotype refers to a collection of bacterial phenotypes that have been influenced by osmolarity, nutrient supply, cell signaling and population density. Thus a single biofilm phenotype does not really exist. The role of planktonic cells and biofilms in causing infection is different. The role of motility and invasion properties of pseudomonal species is not expressed as in biofilm phenotype, in cystic fibrosis patient (Feltman *et al*., 2001).

Bacteria in biofilms show remarkable resistance to chemically diverse biocides during defouling in industrial processes (Costerton *et al*., 1987). Difficulty in eradicating biofilms in medical conditions, such as infected implants has proven their activity to resist against host immune response and antibacterial efforts. It is depicted that this avoidance of host mechanism was formed in the primitive earth against bacteriophages and free living amoebae (Parsek *et al*., 2005). Early research suggested that EPS could retard the penetration or diffusion of antibiotics but there are three exceptions-

- ✓ β-lactamase can accumulate in the matrix thus deactivating β-lactam antibiotics(Bagge *et al*., 2004)

- ✓ Positively charged aminoglycosides are retarded by negatively charged matrix, like alginate from *Pseudomonas* (Walters *et al*., 2005).

- ✓ The EPS from coagulase negative *Staphylococci* reduces the efficacy of glycopeptide antibiotics (Beer *et al*., 1997).

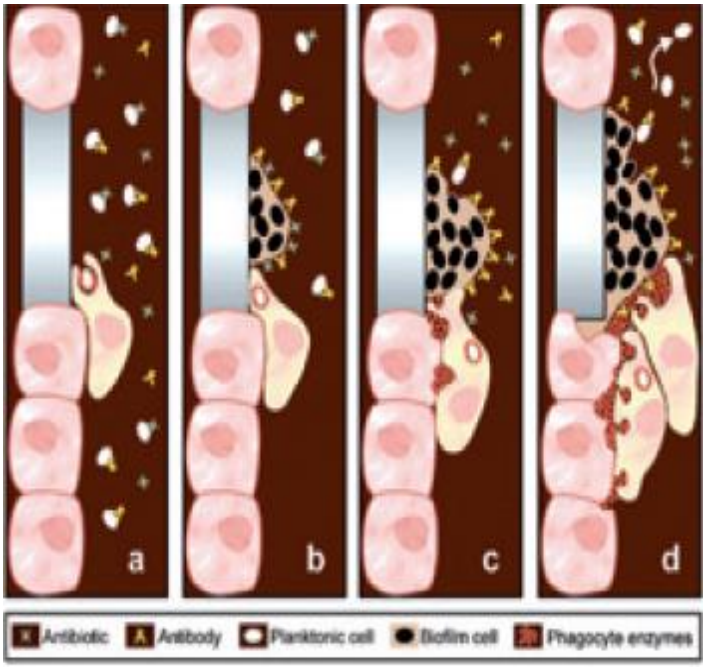


Diagram of medical biofilm. (A) Planktonic cells can be cleared by antibiotics and phagocytes, and are susceptible to antibiotics. (B) Adherent bacterial cells form biofilms on inert surfaces, and these sessile communities are antibodies, phagocytes and antibiotics. (C) Phagocytes are attracted towards biofilm. Phagocytosis are frustrated but phagocytic enzymes are released. (D) Phagocytic enzymes damage tissue around the biofilm, and planktonic bacteria are released from the film. Release may cause dissemination and acute infection in neighboring tissues. Reprinted with permission from (Costerton JW et al., 1999) Copyright 1999 AAAS.

How microrganisms form biofilms?

The first stage involve attachment. During this process, reversible attachment of bacteria takes place. Bacteria are still susceptible to antibiotics at this stage.

The second and third stage, adhesion and aggregation, involves the grouping and bonding of small numbers of bacteria (Palmer, 2006). Surface contact sensoring is used to initiate a phenotypic change and the production of quorum sensing signals (Gerke *et al*., 1998). This is facilitated by the presence of bacterial species specific characteristics such as Type IV pili of *Pseudomonas* (Davies *et al*., 1998).

The fourth stage, Growth and Maturation, allows the redistribution of bacteria away from the substratum (Whitchurch *et al*., 1996). The complex architecture of the biofilm, including micro-colonies and water channels takes place. Biofilm becomes thick and mushroom or tower-like and this is main characteristic feature of mature biofilm, It is during this stage that various microenvironments and thus metabolic niches are established. This produces a diversity of phenotypes that live within the biofilm. The final stage of detachment, or dissemination which is secondary phenomenon. This liberation process may be caused by bacteriophage activity within the biofilm. The active process of detachment can involve enzymatic degradation of matrix or modulation of surface associate adhesions (Lee *et al*., 1996). The by-products of EPS degradation may also facilitate other bacteria to be released as a planktonic form (Cramton *et al*., 1999, Baty *et al*., 2000).

Following are the plants used for testing against *S. mutans, A. hydrophila* and *A. baumannii*.

1. *Pongamia pinnata* Lam.

a. A tree

b. Seeds

Family- Papilionaceae; Common name- karanj

It is a species of tree in the pea family. It is native in tropical and temperate Asia including parts of India, China, Japan, Malaysia, and Australia. It is often known by the synonym ***Pongamia pinnata*** as it was moved to the genus *Millettia* recently. It is often used for landscaping purposes as a windbreak or for shade due to the large canopy and showy fragrant flowers. The bark can be used to make twine or rope and it also yields a black gum that has historically been used to treat wounds caused by poisonous fish. The seed oil has been found to be useful in diesel generators and, along with *Jatropha* and Castor, it is being explored in hundreds of projects throughout India and the third world as feedstock for biodiesel**.** Other studies have shown some potential for biocidal activity against *V.cholera* and *E.coli*, as well an anti-inflammatory, antinociceptive (reduction in sensitivity to painful stimuli) and antipyretic (reduction in fever) properties.

2. ***Manilkara hexandra*** **Roxb.**

a. Ripped fruits

b. Seeds

Family- Sapotaceae; Common name- rayan, raina, khirni

Manilkara hexandra (Roxb.)Dubard belonging to family Sapotaceae, is a socio-economically important underutilized fruit species of western-central India. It is locally known as 'Khirni', 'Raina' and 'Rayan' by tribal people of different states of India. It is believed to be originated in India (Stewart and Brandis 1992). The tree is found as natural wild in the south, north and central India mostly in the states of Rajasthan, Gujarat, Madhya Pradesh and Maharashtra (Malik *et al*. 2010).It is a commercially and medicinally important tropical tree species and is a significant source of livelihood and nutritional support species for local tribal population. In India, this species is occasionally cultivated in backyards, homestead gardens, public parks as avenue tree and in farmer's fields near villages due to its economic importance as fruit tree having nutritional and medicinal properties. Fruits of Khirni have high economical value as mature fresh fruits which are sweet and a good source of minerals, sugars, protein, carbohydrate and vitamin A (Pareek *et a*l. 1998). Whole partially matured fruits are dried in the sunlight to reduce the chances of infection and deterioration. Tribal people sell the fresh as well as dried fruits in the local market at the cost of Rs. 30–40 per kg. Each tree provides fruits worth of Rs. 500–2,000/- to a tribal family, which is a substantial livelihood support to them. Fresh fruits are generally consumed by entire tribal family which provides good nutritional support in the form of vitamin A, hence whole fruit works as **''vitamin A capsule''** for the nutritionally deficient tribal population especially children and women. Bark and fruits are also used for several medicinal purposes like treatment of ulcers, dyspepsia, opacity of the cornea, bronchitis, urethrorrhea, leprosy, etc. (Anonymous 1962; Warrier *et al*.1995; Pareek *et al*. 1998; Hoareau 1999; Raju and Reddy 2005; Chanda and Parekh 2010). The seeds contain approximately 25 % oil, which is

used for cooking purposes (Xian-zi, 1996). The bark also contains 10 % tannin, which is used for treatment of fever and may be utilized in tanning purposes (Anonymous, 1962).The wood of this tree is very hard, tough and durable and is used for oil presses, house building and turnery.

3. Pyrus pyrifolia Burm.

a. Fruit

b. Seed

(http://www.floridata.com/ref/p/pyrus_pyrifolia.cfm)

Family-Rosaceae

Common name- nashpati, pear

The pear is native to coastal and temperate regions , from western Europe and north Africa east right across Asia. Pears are consumed fresh, canned, as juice, and dried. Fermented pear juice is called perry or pear cider. Pears are a good source of dietary fiber and vitamin C. Pear wood is one of the preferred materials in the manufacture of high-quality woodwind instruments and furniture. Pears are used to treat nausea. Stems and leaves of succulent young shoots of the Pear (Pyrus spp.) exude some substances with strong antibacterial activity when the tissues are injured. Aqueous extracts of the tissues also exhibit strong antibacterial activity. Hildebrand and Schroth (1963,1964) tried to elucidate the antibacterial activity in pear using a semi quantitative bioassay, the so-called inhibition zone method, with *Erwinia amylovora* (Burr.) Winslow et al., which is the causal agent of fire blight disease in pears

Test microorganisms-

1. ***Acinetobacter baumanii:*** It is non fermentive gram-negative bacteria that have minimal nutritional requirement and can survive on wide variety of surfaces and on aqueous environment. They rarely causes any infection in healthy person. Infection with *A. baumanii* is of serious concerned for hospitalized patients, especially for those in intensive care units

(ICU). Hospital-acquired infections with *A. baumannii* also most commonly involve the respiratory tract. *A. baumannii* also causes nosocomial urinary tract infections and wound infections, and infections may progress to septicemia.

2. ***Streptococcus mutans*:** It is facultatively anaerobic, gram-positive coccus-shaped bacterium commonly found in the human oral cavity and is a significant contributor to dental caries. It is carieogenic organism. *S. mutans* plays a major role in tooth decay, metabolizing sucrose to lactic acid using the enzyme glucansucrase. The acidic environment created in the mouth by this process is what causes the highly mineralized tooth enamel to be vulnerable to decay. Sucrose is used by *S. mutans* to produce a sticky, extracellular, dextran-based polysaccharide that allows them to adhere to tooth surface, forming plaque. *S. mutans* cells growing in a biofilm are transformed at a rate 10- to 600-fold higher than single cells growing in planktonic form.

3. ***Aeromonas hydrophila*:** *Aeromonas hydrophila* is one of the most challenging, ubiquitous and opportunistic food borne pathogens. Due to its capacity to grow even at low temperatures (temperature range: -0.1 to 37ºC) it has major role in spoilage of packaged foods (Adams and Moss, 1995). It causes various diseases like endophthalmitis, gastroenteritis, cellulitis, meningitis, diarrhoea, etc. This gram-negative rod is also resistant to many antibiotics, hence it has become one of the most notorious foodborne pathogens to handle. It is required to develop novel approaches for controlling this organism (Patel *et al*., 2010). Most significant feature with regard to any threat *A. hydrophila* may pose in foods is its ability to grow down to chill temperatures.

4. ***Chromobacterium Violaceum*:** *Chromobacterium violaceum* is a Gram-negative rod which is found in the soil and water of tropical and subtropical areas. *Chromobacterium violaceum* (Cv) appears to be an opportunistic pathogenic bacterium, which affects humans and animals in subtropical and tropical areas. The low infectious capability of Cv is evidenced by the fact that rivers such as Negro River, in the Amazon region of Brazil, where the bacteria is highly abundant, are sources of drinking water, without the occurrence of widespread infection among the local population.

Materials And Methods

Plant Materials

The plants were selected on the basis of earlier reported antimicrobial activity of their other parts (leaf, fruits, flowers etc.), and same parts against other organisms. The seeds of three plants *Pongamia pinnata*, *Pyrus pyrifolia* and *Manilkara hexandra* were collected during October 2013 to December 2013 from the fruits purchased from the local market of Ahmedabad city. They were authenticated for their unambiguous identity by Dr. Himanshu Pandya, Department of Botany, Gujarat University, Ahmedabad. They were thoroughly washed with tap water, air-dried in shade and stored in opaque air-tight containers (at room temperature) to avoid photo-oxidation (Houghton and Raman, 1998). The seeds were checked at regular intervals for any physical or biological damage. Damaged seeds were removed from the collection.

Table 1: Plant Seeds

Scientific Name	**Seed**	**Family**	**Common Name**	**Partsreportedfor antimicrobial activity**
Pongamia pinnata Lam		Papilionaceae	Karanj	Leaves(Arote *et al*., 2009), Flower extract (Kagithoju *et al*., 2012), Seeds (Kesari *et al*., 2010)
Pyrus Pyrifolia Burm		Rosaceae	Nashpati	Fruit(Cho *et al*., 2013)
Manilkara hexandra Roxb		Sapotaceae	Rayan	Leaves(Mahida and Mohan, 2007) fruit(patel and Rao, 2012)

Test Organisms

Following test organisms (Table 1) were procured from Microbial Type Culture Collection (MTCC), Chandigarh.

No.	Organisms	MTCC code	Medium	Remarks (with input from MTCC manual)
1	*Streptococcus mutans*	497	BHI (HiMedia M2101)	Isolated from carious dentine, Streptomycin resistant up to 30 µg/ml, cefaclor and cefotaxime (30µg/ml)resistant,Gentamicin sensitive at 10 µg/ml
2	*Streptococcus mutans*	890	BHI (HiMedia M2101)	Opportunistic pathogen, causative agent of dental caries, production of streptokinase in small amount, Gentamicin sensitive at 10 µg/ml, and streptomycin resistant at 30µg/ml
3	*Aeromonas hydrophila*	646	ASA	
4	*Acinetobacter baumannii*		NA	Isolated from ICU patient at Jeevraj Mehta Hospital

#Antibiotic susceptibility pattern determined by microbroth dilution assay and disk diffusion assay in our lab; BHI – Brain heart infusion; NA – Nutrient agar, ASA- Aeromonas starch agar with ampicillin.

Microwave Assisted Extraction (MAE):

Seeds were extracted by MAE method earlier published by us (Kothari et al., 2009,Kothari *et al*., 2011; Ramanuj *et al*., 2012; Darji *et al*., 2012). Seeds were crushed twice in grinder (Maharaja white line bonus grinder) to coarse powder at knob 1 for 60 sec. One gram of seed powder was soaked into 50 ml of respective solvent in brown coloured screw capped bottle (250 ml, Merck, Mumbai, India), cap was loosened slightly to avoid pressure build up. Solvents used for extraction were acetone, methanol (Merck, Mumbai) and ethanol (50%; Ureca consumers Co. Op Stores Ltd., Ahmedabad India). Then this was subjected to microwave assisted extraction (MAE). For this, bottle was kept in microwave oven (Electrolux, EM30EC90SS) for extraction

at 720W. At a time only one bottle was kept for procedure. The time for extraction of all seeds for respective solvent is reported in Table 6. The extraction was followed by macro filtration (nylon strainer), which was further subjected to centrifugation at 7,500 rpm for 20 min. Then after microfiltration was carried out using Whitman filter paper no. 1(Whatman International Ltd., England) to remove the fine particulate matter and was allowed to evaporate in pre-weighed petridishes (in weighing balance, Setra, BL-410S) a temperature below the boiling point of respective solvents. For determination of antimicrobial activity, extract were reconstituted in dimethyl Sulfoxide (DMSO, Merck, Mumbai) as it possesses the ability to dissolve polar as well as non-polar compounds. Extract was collected in sterile flat bottom glass vials (15ml, Merck, Mumbai) which were protected from light to avoid photo-oxidation of light sensitive compounds (Houghton and Raman, 1998). The internal surface of vial cap was wrapped with aluminum foil to avoid leaching of vial cap material and their absorption by extract (Houghton and Raman, 1998). They were then stored in refrigerator at 4˚C.

Table 3: Heating and cooling cycles of different solvents during MAE are as follows

Solvents	Total extraction time	Total heating time (s)	First heating time (s)	Cooling Time (s)	Reheating Time (s)
Ethanol (50%)	8 min 6 s	120	25	40	10
Methanol	12 min 16 s	90	10	40	5
Acetone	3 min 6 s	70	40	40	10

Extraction efficiency and reconstitution efficiency was calculated by using following formula:

Extraction efficiency:

$$\text{Extraction efficiency} = \frac{\text{weight extracted (mg)}}{\text{weight of initial material (mg)}} \times 100$$

Weight extracted (mg) = (Weight of petriplate after evaporation of solvent) - (weight of empty plate)

Reconstitution efficiency:

$$\text{Reconstitution efficiency} = \frac{\text{weight of extracted reconstituents (mg)}}{\text{total weight of dried material (mg)}} \times 100$$

Weight of extract reconstituted (mg) = (weight of petriplate after evaporation of solvent) - (weight of petriplate after reconstitution)

Inoculum standardization: For Bacteria 10ml sterile normal saline was taken in sterile test tube in which few (3-4) isolated colonies of 24h old culture (48h in case of *S.mutans*) were added and turbidity was visually matched with 0.5 McFarland standard. This 0.5 McFarland turbidity standard at 625 nm is equivalent to 1.5 × 108 CFU/ml.

Antimicrobial susceptibility testing (AST) with planktonic cells (Microbroth Dilution method):

Plant extracts: Microbroth dilution method was performed as per NCCLS guidelines in 96 well flat bottom (polystyrene) microtitre plate (TPG96, HiMedia, Mumbai). All the seed extracts were challenged against the test organisms as they have showed antimicrobial property against other organisms. Muller Hinton broth(MH), in some cases minimal media(MM) was used for antimicrobial susceptibility testing (AST) against most of the organisms. Brain heart infusion broth - BHI (Galvao et al., 2012) and minimal media (in case of precipitation) were used for testing against *S. mutans*. BHI was used for AST, because MH did not support the growth of S. mutans (All the media used were from Himedia).The inoculum was prepared in normal saline solution (0.85 % NaCl solution; Appendix C) from 24 h old culture and 48 hrs in case of *S. mutans*. Various controls and experimental wells were prepared as below and the final volume in each well taken was 200 µl. In all controls (except sterility and turbidiy) 10% v/v inoculum was added. Turbidity controls (abiotic control) were also kept to nullify the contribution of extract itself towards total turbidity.

- Sterility Control: 200 µl uninoculated sterile broth.
- Growth Control: 180 µl broth + 20 µl inoculum.
- Negative Control: 178 µl broth + 2 µl DMSO + 20 µl inoculum.
- Positive Control: 178 µl broth + 2 µl antibiotic + 20 µl inoculum
- Turbidity Control: 178 µl broth + 2 µl extract + 20 µl normal saline.
- Experimental: 178 µl broth + 2 µl extract + 20 µl inoculum.
  Normally 1% v/v extract was added in the well, but in case where the concentration of available extract was low, 2% or 3% v/v was added to achieve final concentration in well. Respective to it negative control also changed. After inoculation incubation was carried out at 30±35ºC.

For *S. mutans* with minimal media this incubation was carried out for 23-24 h, because growth rate of *S. mutans* is slower in minimal media compared to BHI. After incubation MIC values were determined on basis of visual observation of turbidity as well as optical density measurement in micro plate reader (BIO-RAD 680) at 655 nm. Before taking optical density shaking of plate (in ELISA reader) was done for 3 min to create uniform condition in well. Each extract was tested from broad to narrow range until minimal inhibitory concentration (MIC) value was determined. MIC was recorded as the lowest concentration at which no growth was observed. All MICs were determined on three independent occasions. Concentration at which growth was inhibited by 50% was recorded as IC_{50} value. Total activity (mL/g) was calculated as (Eloff, 2004): Amount extracted from 1 g (mg) / MIC (mg/mL). Activity index was calculated as ratio of MIC of antibiotic to MIC of test extract against susceptible organisms (Borgio et al., 2008).Minimal media was used with extract of *P. pinnata* and *P. pyrifolia* because it showed precipitation in BHI and MH medium against *S. mutans*. None of the extracts of *M. hexandra* were precipitating in BHI and MH medium for the respective organism.

Pure compounds

Violacein is a pigment produced by *Chromobacterium violaceum (*MTCC 2656). Violacein (molecular mass 343.3), a purplish-black needle prism, is insoluble in water, slightly soluble in ethanol, moderately soluble in dioxane and acetone, and soluble in DMSO, methanol and ethyl acetate. Its melting point is >290 ◦C (decomposition) (Duran *et al*, 2007). The UV–VIS (UV and visible absorption) spectrum exhibits maximum absorbances at 258, 372 and 575 nm (ε575 =2.97+ −0.09×10−2)ml/μg/cm)in ethanol(Newman,2000). Violacein already exhibit many biological activities like antitumoral, antioxidant, tripanocide, antimalarial, leishmanicide and antibiotic properties (Menck 2001, Duŕan *et al*.2007, Duŕan and Lopes et al. 2009). In terms of antimicrobial activity, that Violacein has activity against *Mycobacterium tuberculosis* has already been described (DeSouza et.al,1999). It exerts bactericidal action against *Staphylococcus aureus* in concentrations of 0.001 to 0.01 percent when the number of bacterial cells is approximately 5,000 per ml, and a bacteriostatic action against larger inocula (Borgio *et al*., 2008)

The organism was inoculated in 4 different 100ml nutrient broth flask at 10%v/v and kept at 35°C in continuously shaking condition in a shaker incubator. After 48hrs of incubation the medium becomes turbid and violacein was isolated from it. The liquid medium was taken in ocrage tubes and centrifuged at 7500rpm at 25°C for 30 min, pellets appear at the bottom of the centrifuge tube. Supernatant was discarded from the tube and DMSO was added to the pellet formed at the bottom of the tube. These ocrage tubes were kept still for one hour. Ocrage tubes were again centrifuged at 7500rpm for 30 min, pellets were formed at the bottom of the tube then supernatant was withdrawn from the tube and collected in sterile 15 ml vial (Merck).

Purity of violacein was confirmed by taking O.D in UV-Vis (Agilent Technologies Cary 60 UV-Vis) at 585nm.

Concentration of Violacein is determined by the molar extinction coefficient formula:

(0.05601 ml µg-1 cm-1) at 585 nm

i.e. A=εcl (Pantella et al., July 2006).

Mouthwashes

Mouthwashes are an antiseptic solution intended to reduce the microbial load in the oral cavity, although other mouthwashes might be given for other reasons such as for their analgesic, anti-inflammatory or anti-fungal action. Anti-cavity mouth rinse uses fluoride to protect against tooth decay.

Market products were as control to compare our seeds antimicrobial action-

LISTERINE®

It is a brand of antiseptic mouthwash product. It is promoted with the slogan "Kills germs that cause bad breath", and was named after Joseph Lister who advocated the idea of sterile surgery by sterilizing instruments. Originally marketed by the Lambert Pharmacal Company (which later

became Warner-Lambert), it has been manufactured and distributed by Johnson & Johnson since that company's acquisition of Pfizer's consumer healthcare division in late December 2006.

The Listerine brand name is also used in toothpaste, Listerine Whitening rinse, Listerine Fluoride rinse (Listerine Tooth Defense), Listerine Smart rinse (children's fluoride rinse), Pocket paks, and Pocket mist. In September 2007, Listerine began selling its own brand of self-dissolving teeth whitening strips. The active ingredients listed on Listerine bottles are essential oils menthol 0.042%, thymol 0.064%, methyl salicylate 0.06%, and eucalyptol 0.092%. In combination all have an antiseptic effect and there is some thought that methyl salicylate may have an anti inflammatory effect as well. Ethanol, which is toxic to bacteria at concentrations of 40%, is present in concentrations of 21.6% in the flavored product and 26.9% in the original gold Listerine Antiseptic. At this concentration, the ethanol serves to dissolve the active ingredients.

Microbroth dilution assay was performed as per NCCLS guidelines with listerine against planktonic form of both strains of *S.mutans* in 96 well flat bottom (polystyrene) microtitre plate (TPG96, HiMedia, Mumbai).Spreading was done on plates containing BHI media when there is no growth in experimentals before taking reading in microplate reader(BIO-RAD 680) at 655 nm.

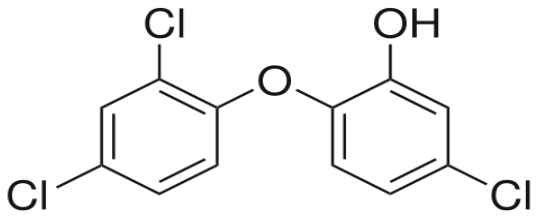
Colgate Plax®

Colgate (sub-brand of Colgate-Palmolive) is an oral hygiene product line of toothpastes, toothbrushes, mouthwashes and dental floss. Colgate toothpaste was first sold by the company in 1873, sixteen years after the death of its founder, William Colgate.

Its active Ingredients are 0.05% w/w Cetyl Pyridinium Chloride (CPC) 5.7 % v/v Ethanol (Excluded in Alcohol Free).

Microbroth dilution assay was performed as per NCCLS guidelines with colgate plax against both strains of *S.mutans* in 96 well flat bottom (polystyrene) microtitre plate (TPG96, HiMedia, Mumbai).Spreading was done on plates containing BHI media when there is no growth in experimentals before taking reading in microplate reader(BIO-RAD 680) at 655 nm.

Triclosan



Triclosan is an antibacterial agent found in numerous consumer products. It is a polychlorophenoxy phenol.

According to the United States' Food and Drug Administration (FDA), at the present time, there is no evidence that triclosan in personal care products provides extra benefits to health beyond its anti-gingivitis effect in toothpaste (Triclosan April 8 2010). The FDA does not recommend changing consumer use of triclosan-containing products one way or the other due to insufficient safety evidence (Triclosan April 8 2010) Triclosan has been used since 1972, and it is present in soaps (0.10-1.00%), shampoos, deodorants, toothpastes, mouth washes, and cleaning supplies,(Thompson A *et al*., 2005) and is incorporated into an increasing number of consumer products, such as kitchen utensils, toys, bedding, socks, and trash bags.(Crinnion *et al*., 2005)It is also found in health care settings in surgical scrubs and personnel hand washes(. Triclosan has been shown to be effective in reducing and controlling bacterial contamination on the hands and on treated products.

Macrobroth dilution assay was performed as per NCCLS guidelines with triclosan against planktonic form of *S. mutans* in screw cap tubes. Tubes were incubated for 48h in incubator and then O.D was taken in UV-spectrophotometer at 655 nm.

Chlorhexidine gluconate

Chlorhexidine is a cationic polybiguanide (bisbiguanide). It is used primarily as its salts (e.g., the dihydrochloride, diacetate and digluconate).It is on the World Health Organization's List of Essential Medicines, a list of the most important medication needed in a basic health system(."WHO Model List of Essential Medicines 2013). Chlorhexidine is used in disinfectants (disinfection of the skin and hands), cosmetics (additive to creams, toothpaste, deodorants, and antiperspirants), and pharmaceutical products (preservative in eye drops, active substance in wound dressings and antiseptic mouthwashes) (Güthner *et al*. 2007).

Chlorhexidine is active against gram-positive and gram-negative organisms, facultative anaerobes, aerobes, and yeast (Leikin *et al*., 2008). It is particularly effective against gram-positive bacteria (**in concentrations ≥ 1 µg/L). Significantly higher concentrations (10 to more than 73 µg/mL)** are required for gram-negative bacteria and fungi. In the presence of blood or protein the efficacy is reduced by a factor of 100 to 1000. Chlorhexidine is often used as an active ingredient in mouthwash designed to reduce dental plaque and oral bacteria.

Precipitation problem was there in chlorhexidine in microbroth dilution assay, chlorhexidine was directly pour with media at concentration of 1, 2.5, and 5 µg/ml in petriplates and inoculum(both strains of *S.mutans*) was spreaded on to plates. Plates were incubated for 48h at 35°C temperature. To confirm the bactericidal effect, plates were further more incubated for 72h.

Minimum bactericidal concentration (MBC):

An aliquot of 10 µl from tubes/wells showing visually complete inhibition of growth were transferred to BHI agar plate and were spread evenly on it with the help of sterilized glass spreader. Another BHI agar plate was inoculated from the tube/well which is having negative control; this plate was kept as control. The plates were kept at 30±0.5˚C and for human pathogens up to 72 hours for cidal/static/post extract effect. If the experimental plate shows presence of one or two colonies indicating 99.99% inhibition and that concentration was taken as MBC. Presence of few colonies compared to the control (but killing not amounting to 99.99%) indicates that the extract is having bactericidal effect, but not as MBC. While the presence of colonies equivalent to control indicates that the extract is bacteriostatic. If the experimental plate shows delayed appearance of colonies compared to control indicates post extract effect (Darji *et al*., 2012; Ramanuj *et al*., 2012).

Disc diffusion assay with antibiotics:

For disc diffusion assay of antibiotics discs (Icosa universal 1TM, HiMedia, Mumbai) against *S. mutans*, sterile petriplates having diameter 150 mm was poured with 60 ml of sterile BHI to give a mean depth of 4.00 ± 0.5 mm (Darji *et al*.,2012). The inoculum was prepared in sterile normal saline (0.85% NaCl solution) from 48h old *S.mutans* and the turbidity of the inoculum was visibly adjusted using sterile normal saline solution to approximate that of 0.5 McFarland turbidity standard. Within 15 min of adjusting the inoculum turbidity to 0.5 McFarland turbidity standards, 500 µl of the suspension was taken and spread over the plate with the help of sterile glass spreader in order to get a uniform microbial growth. Immediately after spreading of organisms, discs were put on to the agar surface by applying gentle pressure with the help of sterile forcep to ensure complete contact of disc with agar. Plates were incubated at 35±0.5C for 48h. After incubation plate was observed for zone of inhibition.

Determination of time required to kill:

In order to determine the time required for a bactericidal extract to kill the susceptible test organism, the test organism was challenged with the extract at its MBC (minimum bactericidal concentration) in a test tube, from which an aliquote of 10 µl was transferred to a BHI plates(devoid of extract) at intervals of 13, 14, 15, 16 .. up to 24 h and was spread evenly. The plates were kept for incubation for 35±0.5˚C for human pathogen for 72 h. Time corresponding to the plate with no growth was taken as the time required by the extract to kill the bacteria.

Antimicrobial susceptibility testing with biofilm:

Only those plant extracts / antibiotic which showed bactericidal activity against respective test organism were use against biofilm of same organisms. Plant extracts which showed bacteriostatic effect were not used against biofilm form of organisms.

Biofilm formation by tissue culture plate (TCP) method:

Biofilm formation was initiated by addition of media and inoculum in 96-well tissue culture treated (polystyrene) plate (TPP96, Himedia). This surface treated plate allowed adhesion of organisms on well, which is prime requirement for biofilm formation. In case of BHI (Himedia) broth with 2% sucrose (HiMedia), Sucrose was autoclaved separately at 10Psi (Rukayadi and Hwang, 2006). The inoculum was prepared in sterile normal saline (0.85% NaCl solution) from 24 h old culture, 48h in case of *S.mutans* and the turbidity of the inoculum was visibly adjusted using sterile normal saline solution to approximate that of 0.5 McFarland turbidity standard, which is equivalent to 1.5×10^8 CFU/ml. Various controls and experimental wells were prepared as below, and the final

volume in each well was kept 300 µl. In all controls (except sterility and turbidity) 10% v/v inoculum was added. Turbidity controls were also kept to nullify the contribution of extract itself toward absorption of crystal violet.

- Sterility Control: 300 µl uninoculated sterile broth.
- Turbidity Control: 270 µl broth + 30 µl normal saline.
- Experimental: 270µl broth + 30µl inoculum
- Negative: 270µl broth + 30µl inoculum
- Positive: 270µlbroth + 30µl inoculum

  After addition of media and inoculum, microtiter plate was covered with parafilm to avoid evaporation of media. After inoculation, incubation was carried out at 30±35˚Cfor 48h, to allow organisms to form biofilm.

Antimicrobial susceptibility testing of organism in biofilm:

After biofilm formation media was removed by using multi-channel micropipette (apendroff) in 150mm sterile petriplates (Merck) and a gentle tap was done on it back. Then wells were supplemented with minimal media (MM) (Ramanuj *et al*., 2001). MM may not allow formation of new cells within the biofilm, but it may be expected to maintain the viable status of the preformed biofilm. As some of the extracts (all extracts of *P. pinnata* and methanolic extract of *P.pyrifolia*) get precipitated in BHI, while challenging the organism with test extract(s) MM was used to avoid precipitation. After addition of MM, various concentration according to the MIC obtained in planktonic form of the organism (viz.600, 800, 1000, 1500, 2000 µg/mL) of test extract (in DMSO) were added in different wells. Various controls and experimental wells were again added with appropriate fluids as below, and the final volume in each well was kept 300µl.

- Sterility Control: 300 µl uninoculated MM.
- Growth Control: 300 µl uninoculated MM.
- Negative Control: 297 µl MM + 3 µl DMSO.
- Positive Control: 297 µl MM + 3 µl antibiotic

  Ampicillin stock concentration (10mg/10ml) and final concentration of (10, 20, 30 40 and 50 µg/ml) and gentamicin stock concentration (10mg/10ml) (HiMedia) and final concentration of (10, 20, 30, 40 and 50 µ/ml)
- Turbidity Control: 297 µl MM + 3 µl extract.
- Experimental: 297 µl MM + 3 µl extract.

  All the controls and experimental test mix were set in six replicates i.e. in 6 different wells (of which 3 were used for crystal violet assay, and remaining 3

for viability assay). After addition of media and extract this plate was covered with parafilm and incubated for 24 h to allow extract to eradicate biofilm/kill organism within biofilm. This incubation was carried out at optimum growth temp of respective organisms.

Determination of viability by tube method:

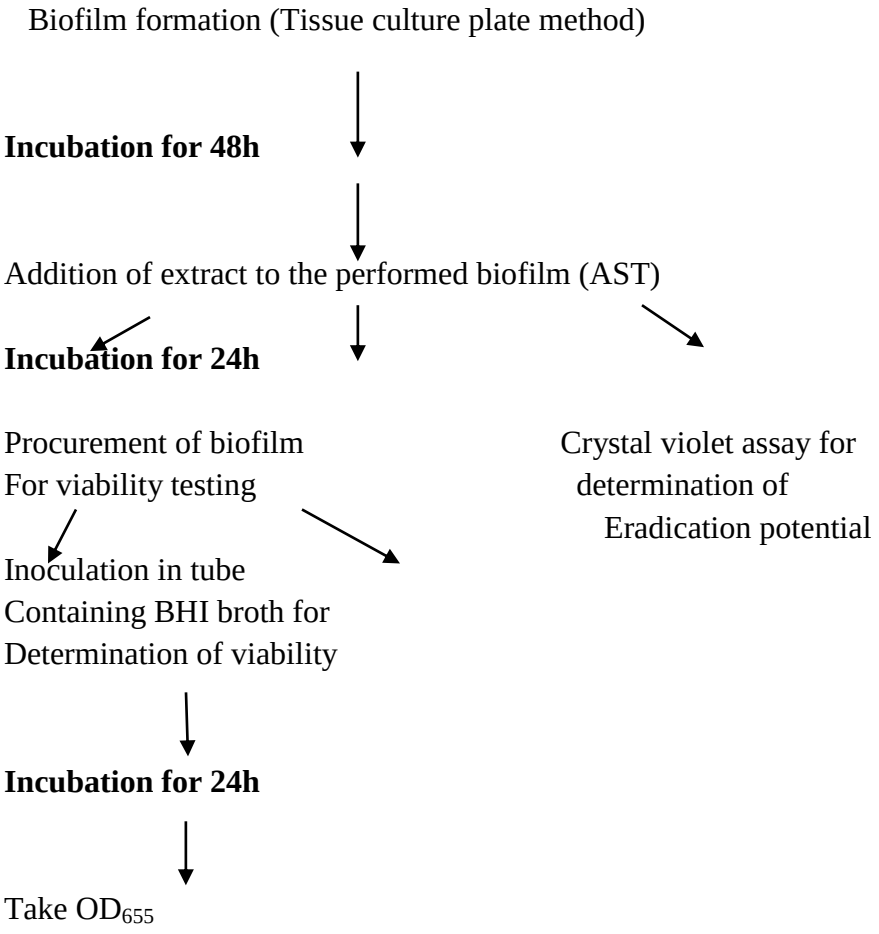
MM media is withdrawn from the plates by using multichannel pipette and plates are washed with N-saline. Again N-saline is added and 100µl is directly withdrawn into the sterile 10ml BHI broth (Himedia) tubes for measurement of the viable organism. Incubation was carried out at 35±35˚C for 24 h. After incubation optical density of this measured at 625nm in UV double beam spectrophotometer for measurement of optical density of sterile BHI broth (as a replacement of inoculum), was used as blank. All the experimental and controls were kept in triplicate. Inhibition of organism by extract/antibiotic was calculated compared to negative/growth control. In cases of high visible clarity up to 24 h, this incubation time was increased up to 48 h for confirmation of bactericidal activity. Visible clarity up to 48 h indicated bactericidal activity.

Estimation of biofilm Eradication by crystal violet assay (Rukayadi and Hwang, 2006):

After generation of the biofilm to check the eradication of cells remaining 3 wells were used for crystal violet assay. In biofilm-coated wells of micro-titre plate, 200 µl 50 mmol l-1 phosphate buffer saline (PBS, pH 7.0), was added. Plate was shaken in micro plate reader (BIO-RAD 680) for 80 s to remove all non-adherent cells. After shaking, empty the previous content and these wells again washed with 200 µl of PBS and air-dried on lab platform (at room temperature) for 45 min. After drying wells were stained with 110 µl of 0.4% aqueous crystal violet solution for 45 min. Then crystal violet was removed from wells and these wells were washed twice with sterile distilled water, followed by immediate addition of 200 µl destaining solution i.e. 95% alcohol (Ureca consumers Co. op Stores Ltd., Ahmedabad). After 45 min the destaining solutions was transferred in new microtiter plate (surface untreated). Optical density of this destaining solution was measured in micro plate reader (BIO-RAD 680) at 655 nm. Classification of bacterial adherence and degree of biofilm formation was determined on the basses of obtained optical density of

destaining solution. Eradication of biofilm by extract/ antibiotic was calculated compared to negative/growth control.

Events during biofilm assay:



Result and Discussion

Microwave Assisted Extraction: The extraction efficiency and reconstitution efficiency are shown in table:

Table 1: Extraction and reconstitution efficiency for all the seed extracts

Seed	Solvent	Extraction Efficiency (%)	Reconstitution Efficiency (%)
Pongamia pinnata	Ethanol (50%)	21.58	19.28
	Methanol	17.08	29.86
	Acetone	17.42	26.41
Manilkara hexandra	Ethanol (50%)	13.24	83.23
	Methanol	13.78	74.31
	Acetone	17.92	5.80
Pyrus Pyrifolia	Ethanol (50%)	10.40	78.85
	Methanol	9.78	77.09
	Acetone	9.32	17.59

Extraction and reconstitution efficiency of all the seeds with different solvents are recorded in the above table. Highest extraction efficiency (21.58) in case of ethanolic extracts of *P.pinnata* seeds followed by (19.28) of acetone extract of *M. hexandra* seeds. With respect to the extraction efficiency methanol and ethanol proved better than acetone in two of the seeds except in seeds of *P.pinnata.* Polar solvents are usually believed to be better for MAE than the non-polar ones because they have high dielectric constants (Proestos and Komaitis, 2007). Methanolic and ethanolic extracts of two seeds showed higher extraction efficiency compared to the other solvent, which indicates that these experimental seeds contain components which are soluble in polar solvents like methanol.

From this we can conclude that methanol seems to be better for extraction purpose. It has been also suggested that addition of some amount of water in solvent enhances the extraction efficiency. This may be due to increase in swelling of plant material by water thereby increasing the contact surface area between the plant matrix and solvent (Mandal *et al*., 2007). Extraction efficiencies of ethanolic extract of *P.pinnata* and *M.hexandra* are higher than the methanol of the same extract which shows that certain components are more soluble in ethanol than methanol. Extraction efficiency of methanol extract of *M. hexandra* showed higher extraction efficiency than ethanol of same seed which shows that certain components are soluble in methanol than

ethanol of same seed. This all results of extraction efficiency suggest that extraction efficiency depend upon the type of component present in the plant material.

Reconstitution efficiency is the % amount of material recovered form 1g of seed extract. The reconstitution efficiency is higher in case of *M. hexandra* and *P. pyrifolia* in ethanol extract as compared to the other solvents so it can be concluded that ethanol is better solvent for reconstitution. While reconstituting the dried seeds of *M. hexandra* and *P. pyrifolia* in DMSO higher reconstitution efficiency was obtained with their ethanol extracts. It should be noted that the possible advantage(s) of high extraction yield may be somewhat compromised by low reconstitution efficiency, because some of the phytoconstituents present in the original extract may be left out due to inability of the reconstituting solvent to solubilize all of them.

Antimicrobial Susceptibility Testing (AST) with planktonic cells

Table 2: Results of broth dilution assay of all extracts of *P. pinnata* seed against *A. baumannii*

Conc. (µg/ml)	Methanolic extract (% inhibition)	Ethanolic extract (% inhibition)	Acetone extract (%inhibition)
250	36.53±10.64	38.41	28.57
300	35	ND	ND
400	32	ND	ND
500	**59.77±0.96**	43.67±6.93	46.80
600	62	ND	ND
750	ND	59.32	46.68
800	52	ND	ND
1000	**58.57±2.02**	**59.44±13.39**	**50.58±22.47**
1200	68	41.83	40.81
1300	47	50.78	ND
1500	56	ND	45.09
1700	ND	ND	57.84

ND = Not determined

Percent inhibition of broth dilution assay of all extracts of *P. pinnata* is reported in the above table. There was no such inhibition reported in all concentration. But in some concentration IC_{50} is obtained at the concentration range between 500µg/ml - 1000µg/ml for methanolic extract of *P. pinnata.* And the other two extract of same seed showed IC_{50} above 500µg/ml.

Table 3: Results of broth dilution assay of all extracts of *M. hexandra* seed against *A. baumanii*

Conc. (µg/ml)	Methanolic extract (% inhibition)	Ethanolic extract (% inhibition)	Acetone extract (% inhibition)
250	28.57	20.09	34.05
500	6.67	8.57	33.51
750	30.47	ND	33.51
1000	26.54	1.01	42.02
1500	33.02	45.71	ND

ND - Not determined

Percent inhibition of broth dilution assay of all extracts of *M. hexandra* seed against *A. baumannii* is reported in the above table. This report shows that there was no particular inhibition of *A. baumannii* with the all the extracts of *M. hexandra* and it also shows that gram negative bacteria cannot be easily inhibited.

Table 4: Results of broth dilution assay of all extracts of *P. pyrifolia* seed against *A. baumannii*

Conc. (µg/ml)	Methanolic extract (% inhibition)	Ethanolic extract (% inhibition)	Acetone extract (% inhibition)
250	43.33	0	50
500	60	40.74	72.72
790	ND	58.53	50
1000	66.66	48	40.90
1200	52.94	ND	ND
1500	0	ND	ND

ND - Not determined

Percent inhibition of broth dilution assay is reported in the above table. There was no such inhibition reported in all the concentration used in all extracts of *P. pyrifolia.*

Table 5: Results of broth dilution assay of all extracts of *M. hexandra* seed against *A. hydrophila* 646

Conc. (µg/ml)	Methanolic extract (% inhibition)	Ethanolic extract (% inhibition)
250	0	0
500	24.97	19.71
750	26.76	ND
1000	25.35	16.90

ND – Not determined

Percent inhibition of broth dilution assay of all extracts of *M. hexandra* against *A. hydrophila* 646. There was no such inhibition reported as IC_{50} in all concentration or any MIC against this Organism.

Table 6: Percent inhibition of *S. mutans* at various concentration of ethanolic and acetone extract of *P. Pyrifolia*

Solvent	Conc.(µg/ml)	S. mutans 497	S. mutans 890
Acetone extract	250	72.50	ND
	500	66.47	
	750	76.17	
	1000	62.50	
Ethanolic extract	250	10.52	
	500	65.63	
	750	35.71	
	1000	62.64	
	1500	41.66	

ND- Not determined

Percent inhibition of broth dilution assay of ethanolic and actone extracts of *P. pyrifolia* against *S. mutans 497*. There was no such inhibition reported as IC_{50} in all concentration or any MIC against this Organism. Methanolic extract of *P. pyrifolia* was precipitated in BHI.

Table 6: Percent inhibition of *S. mutans* at various concentration of ethanolic extract of *M. hexandra.*

Conc. µg/ml	***S. mutans* 497**	***S. mutans* 890**
250	26.47	ND
300	ND	ND
400	75.67	ND
450	ND	ND
500	65.28	ND
550	98.94	46.37
600	**97.78**	NI
650	35.78	**74.36***
700	39.28	65.25*
750	100	63.63*
800	ND	62.50*
850	ND	100
900	ND	ND
950	ND	100
1000	97.22	97.57
1200	ND	97.05
1500	ND	100

Note: *- 100% inhibition, ND- Not determined, NI- No inhibition

.Ethanolic extract of *M.hexandra* showed 97.78% inhibition against *S.mutans* 497 and was visibly clear and showed bactericidal activity while plating. So the concentration was considered as MIC. And ethanolic extract of *M. hexandra* showed 74.36% inhibition against *S. mutans* 890 but it was visibly clear and also showed bactericidal activity while plating.

Percent inhibition of various concentration of ethanolic extract of *M. hexandra* seed against *S. mutans* are reported in above table. This table shows that hydroalcoholic extract of *M. hexandra* were effective against gram positive cocci like *S.mutans.* It was not effective against gram negative bacteria *A.baumanii*.

Table 7: Percent inhibition at various concentration of methanolic extract of *M. hexandra* seed against *S. mutans*

Conc. µg/ml	***S. mutans* 497**	***S. mutans* 890**
100	NI	ND
200	18.95	ND
225	87.50	ND
250	35.69	ND
500	68.67	38.29
550	67.45	NI
575	NI	93.93
600	54.05	78.18*
625	87.31	**95.62**
650	84.12	**80.14***
700	97.88	**68.42***
750	52.77	**97.14**
800	**88.42***	**100**
900	63.27*	ND
1000	80.06*	**100**
1105	54.54*	ND
1200	ND	**96.96**
1500	ND	**93.75**

*100% inhibition, ND- Not determined, NI- No inhibition

Percent inhibition at various concentration of methanolic extract of *M. hexandra* seeds against different human pathogens are reported in table. MIC was determined in both the strains of *S. mutans*. And also showed bactericidal activity which was visibly confirmed.

Table 8: Percent inhibition at various concentration of acetone extract of *M. hexandra* seeds against *S. mutans*.

Conc. µg/ml	*S.mutans* 497	*S.mutans* 890
250	19.90	26.31
500	NI	47.36
700	42.42	100
750	**57.44***	82.35
800	80.61	**91.17***
850	45.45	ND
900	35.48	84.09*
950	NI	ND
1000	70.06*	100
1120	61.53*	ND
1200	47.36	100
1500	NI	100

*100% inhibitionND- Not determined, NI- No inhibition

Percent inhibition at various concentration of acetone extract of *M. hexandra* seeds against human pathogen *S. mutans* are reported in table. MIC was determined with both the strains of *S. mutans*. And also showed bactericidal activity which was visibly confirmed

Table 9: Final result of microbroth dilution assay of planktonic form of *S. mutans*

Seed/ Natural products and Antibiotic	Solvent	Organism *S .mutans*	IC_{50} (µg/mL)	MIC (µg/mL)	MBC (µg/mL)	Total Activity (mL/g)	Average total activity (mL/g)	Activity index		MBC/ MIC
								Gen	Str	
M. hexandra	Ethanol (50%)	MTCC 497	500	600	600	220	211.53	0.016	0.066	1
		MTCC 890	550	650	650	203.07		0.015	0.061	1
	Methanol	MTCC 497	500	800	900	152.22	185.71	0.011	**0.04**	1.12
		MTCC 890	600	625	625	219.20		0.016	0.064	1
	Acetone	MTCC 497	ND	750	750	238.66	231.20	0.013	0.053	1
		MTCC 890	500	800	800	223.75		0.012	**0.05**	1
P. pyrifolia	Ethanol (50%)	MTCC 497	>1000					NA		
	Acetone	MTCC 497	>1000					NA		
Violacein	Reconstituted in DMSO	MTCC 497	NA	1.28	1.28	NA	NA	7.812	31.25	1
		MTCC 890	NA	1.66	1.66	NA	NA	6.024	24.098	1
Colgate Plax®	% (v/v)	MTCC 497	NA	1	2	NA	NA	NA	NA	2
		MTCC 890	NA	1	1	NA	NA	NA	NA	1
Listerine®	% (v/v)	MTCC 497	4	>5	NA	NA	NA	NA	NA	NA
		MTCC 890	NA	>5	NA	NA	NA	NA	NA	NA
Chlorhexidine	Dissolved in sterile distilled water	MTCC 497	NA	2.5	2.5	NA	NA	0.25	0.00625	1
		MTCC 890	NA	1	1	NA	NA	0.1	0.025	1
Triclosan	Dissolved in sterile distilled water	MTCC 497	NA	>50	NA	NA	NA	NA	NA	NA
		MTCC 890	NA	>50	NA	NA	NA	NA	NA	NA
Gentamicin	Dissolved in sterile distilled water	MTCC 497	NA	10	10	NA	NA	NA	NA	1
Streptopmycin	Dissolved in sterile distilled water	MTCC 890	NA	40	40	NA	NA	NA	NA	1

NA- Not applicable, ND- Not determined Note- Colgate plax does not allow any other organism to grow and experimental tube was clear and there was no growth.

All extracts of *Manilkara hexandra* seeds show bactericidal effect at concentration below 1000. Violacein which was extracted from *C.violaceium2656* also shows bactericidal effect against both the strains of *S.mutans*. Colgate plax shows good effect at very low conc. of 1% v/v against both strains of *S. mutans*. Listerine and triclosan which was use in mouthwashes was not effective against both strains of *S.mutans*. Chlorhexidine was effective at very low conc. of 1 and 2.5µg/ml against *S.mutans* 890 and *S.mutans* 497 i.e. *S.mutans* 890 was more susceptible to chlorhexidine. Gentamicin was active at 10 µg/ml against both strains of *S. mutans* and streptomycin was active at 40 µg/ml against both strains of *S. mutans*.

Time required to kill with Violacein was determined with both strains of *S. mutans* and it was found that it kills S .mutans in less than one hour.

Activity index: Activity index was calculated as ratio of MIC of antibiotic to MIC of test extract against susceptible organisms (Borgio *et al*., 2008). Activity of potent extracts against susceptible microbes was compared to that of gentamicin and streptomycin and activity index was calculated. Methanolic and acetone extract of *M. hexandra* showed lowest activity index in both of the strains of *S. mutans.* The more the value of activity index, more potent is the extract.

Total Activity: It is a measure of amount of material extracted from a plant in relation to the MIC of the extract, fraction or isolated compound. Total activity is expressed in ml/g which is an indication of the degree to which active extracts of one gram can be diluted and still inhibit the growth of the test organisms (Eloff, 2004).It is found that average total activity of acetone extract of *M. hexandra* was higher than the ethanolic and methanolic extract of the same seed. Hence acetone extract of *M. hexandra* seeds even when diluted can be more potent in inhibiting the growth of the strains of *S.mutans* than diluted methanolic and ethanolic extract of the same seed. The order follows -

Acetone>eth anol(50%)> methanol

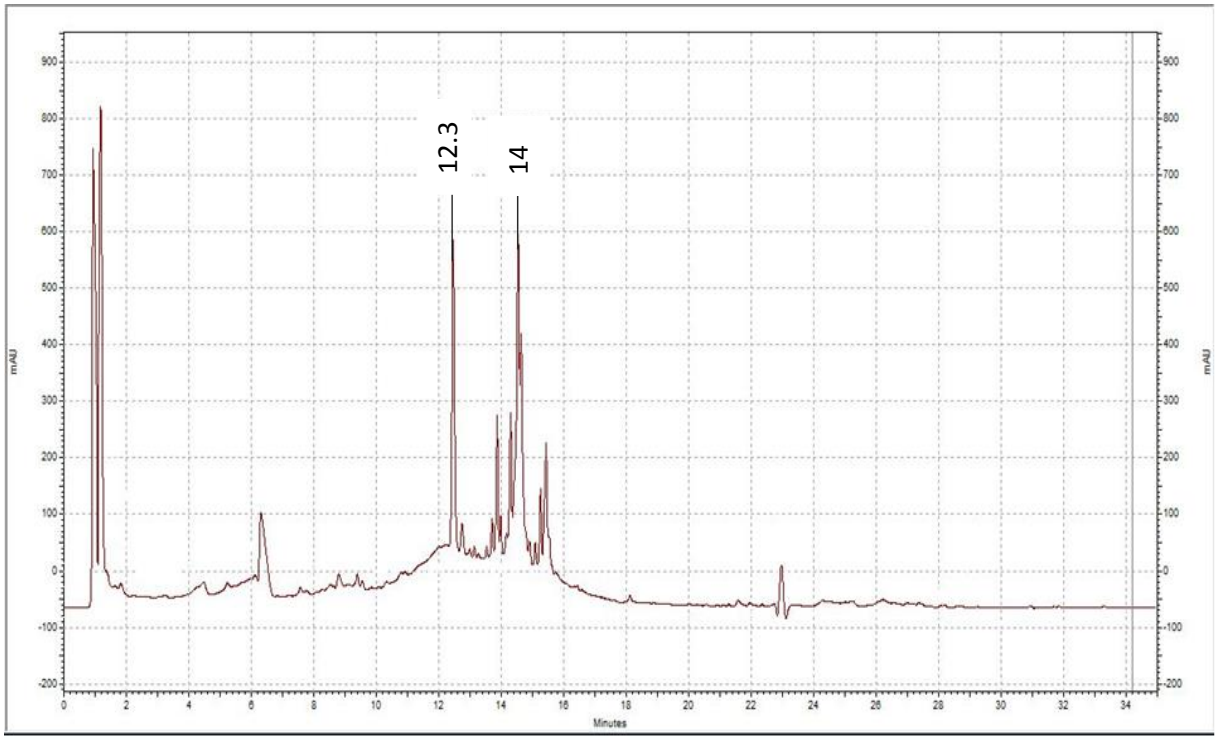
➢ High Performance Liquid Chromatography(HPLC) of *M. hexandra* seeds

 Phosphoric acid: acetonitrile (50:50)

 Extracts dissolved in their respective solvent methanol / ethanol / acetone were filtered through a PVDF hydrophilic membrane syringe filter (0.22 µm) (Himedia) and 10 µl aliquots of the filtrate were injected into HPLC system (Agilent technologies 1260 Infinity LC system, US) with a ZORBAX Eclipse Plus C18 column (4.6mm x 250 mm; 3.5 µm). This HPLC system consisted of a binary pressure pump (Agilent technologies 1260 Infinity LC

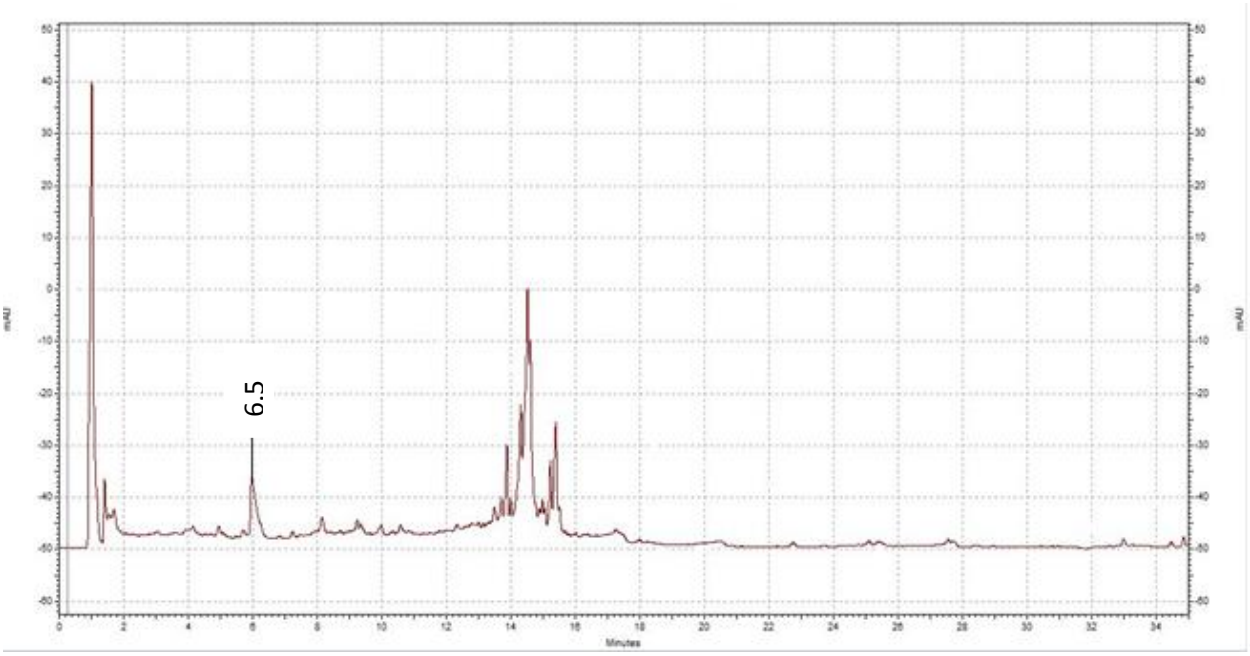
system, US), a photodiode array detector (Agilent technologies 1260 Infinity LC system, US).Mobile phase consisted of 0.05% orthophosphoric acid : acetonitrile (Merck).A gradient elution at 1 ml/min with a gradient program was performed by varying the portion of solvent A to solvent B (0–6 min 5% B, 6–15 min 15% B, 15–35 min 20% B, 35–40 min 40% B). A 0.05% H3PO4 (concentrated, 85%) in water was used as solvent A and acetronitrile as solvent B in order to resolve peaks in the sample. UV detection was carried out at 220/270 nm.

Fig 1: HPLC graph of Ethanolic extract of *M. hexandra*



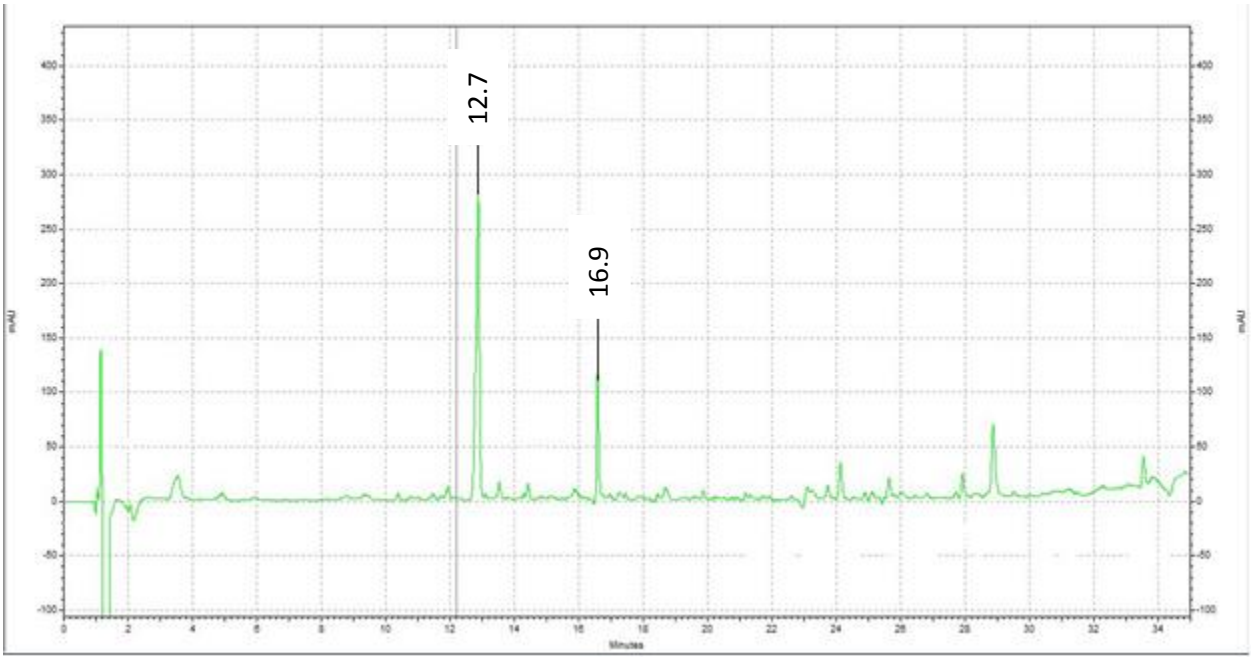
The above graph shows the ethanolic extract of *M. hexandra.* Two peaks were determined during the HPLC, the first peak has retention of 12.03min and second peak has retention time of 14.02min. It can be concluded from this profile that these peaks are might be due to the active compound present in the extract. It is possible that either single peak or synergy of multiple peaks might be responsible for antimicrobial activity of ethanolic extract.

Fig 2: HPLC graph of Methanolic extract of *M. hexandra*.



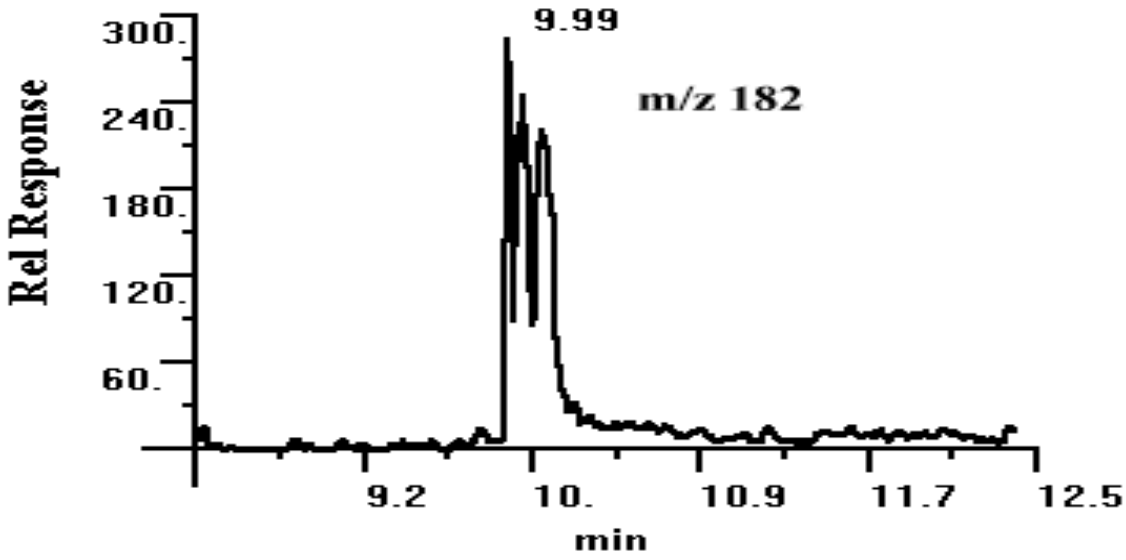
We have generated a chromatographic profile of active extract having antimicrobial activity. Methanolic extract of *M. hexandra* showed one peak which has a retention time of 6.5 min. It can be concluded from this profile that these peaks are might be due to the active compound present in the extract. It is possible that either single peak or synergy of multiple peaks might be responsible for antimicrobial activity of methanolic extract.

Fig 3: HPLC graph of Acetone extract of *M. hexandra*



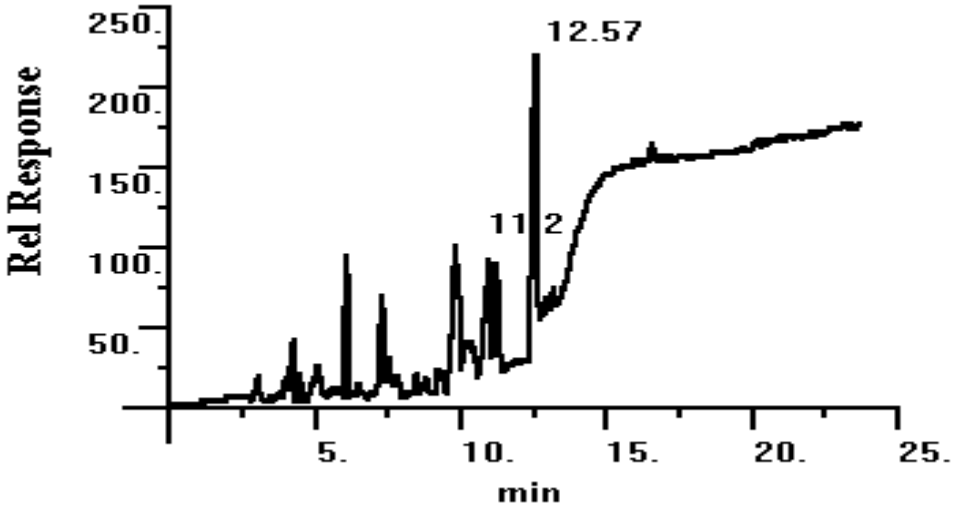
We have generated a chromatographic profile of active extract having antimicrobial activity. Acetone extract of *M. hexandra* showed two peak which has a retention time of 12.7mins and second peak showed retention time of 16.9 min. It can be concluded from this profile that these peaks are might be due to the active compound present in the extract. It is possible that either single peak or synergy of multiple peaks might be responsible for antimicrobial activity of acetone extract.

Fig 4: LC/MS profile of Methanolic extract of *M. hexandra*



This is the LC/MS profile of methanolic extract of *M. hexandra* and the m/z ratio is 182.

Fig 4a: LC/MS profile of acetone extract of *M. hexandra*



This is the LC/MS profile of acetone extract of *M. hexandra* and the m/z ratio is yet to be analysed.

Disk Diffusion Assay:

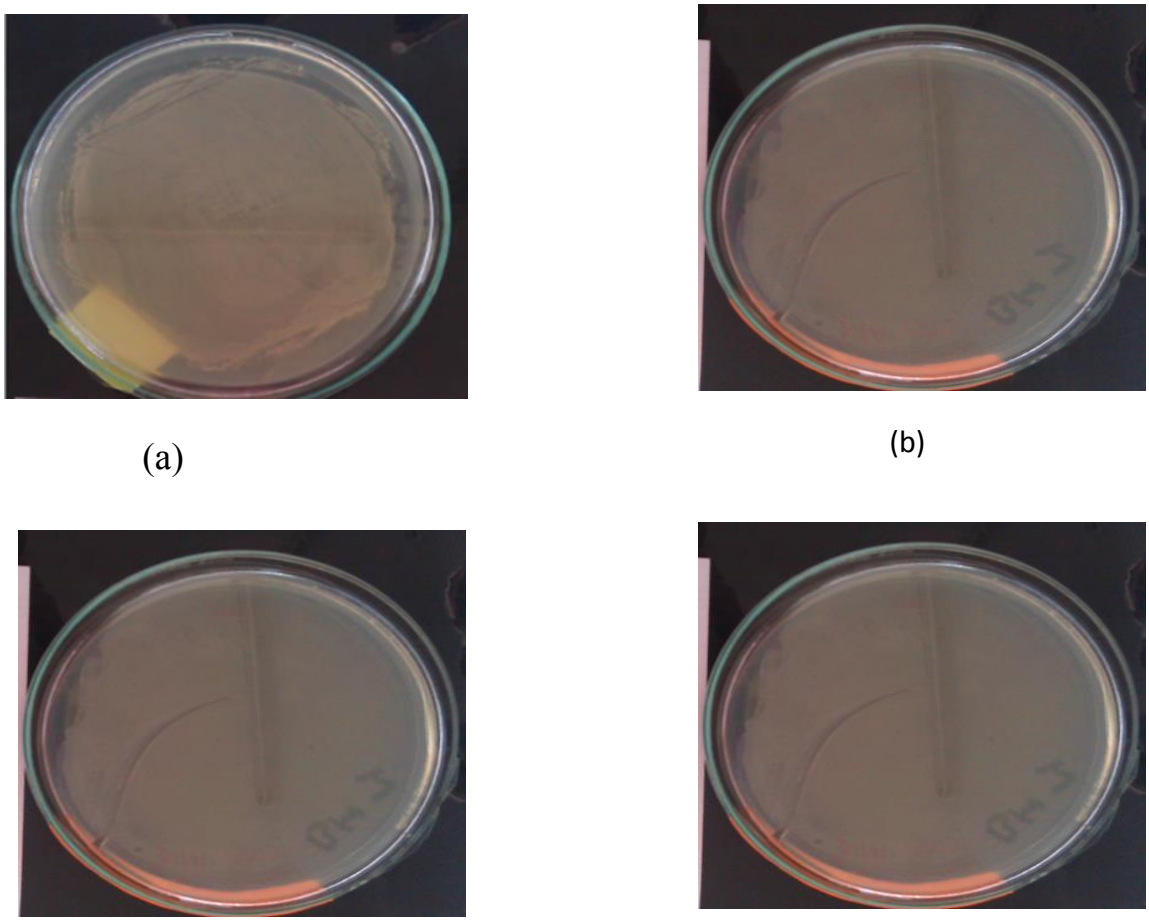
Table 11: Results of Antibiogram assay of *S. mutans* 890

Antibiotic	**Conc. of Disk (µg)**	**Interpretation**
Norfloxacin	10	Sensitive
Gentamicin	10	Sensitive
Chloramphenicol	30	Sensitive
Cefuroxime	30	Sensitive
Ciprofloxacin	5	Sensitive
Ceftazidime	30	Sensitive
Roxithromycin	30	Sensitive
Clarithromycin	15	Sensitive
Co-Trimoxazole	**25**	**Resistant**
Netillin	30	Sensitive
Cefaclor	30	Sensitive
Cefotaxime	30	Sensitive
Cefadroxil	30	Sensitive
Azthromycin	15	Sensitive
Ampicillin/Cloxacillin	10	Sensitive

S. mutans 890 strain employed in our investigation was resistant (as determined by disc diffusion assay using Icosa universal 1TM, HiMedia) to Co-Trimoxazole **(25µg/ml).** Results of microbroth dilution assay of *S. mutans* 890 was it is streptomycin resistant up to 30µg/ml, but at 40µg/ml it was able to inhibit *S.mutans 890* completely. Prevalence of drug resistance among pathogenic micro flora makes it even more important to screen natural products for novel antimicrobial compounds. The search for substances with antimicrobial activity is a continuous challenge and medicinal plants have been considered an interesting option because of their use in popular medicine to treat several infectious diseases (Silva et al., 2012).

The *average total activity* was found to have a moderately strong linear correlation (r=0.76) with the *extraction efficiency*. These two quantities were found to have a strong correlation in our previous studies too (Kothari and Sheshadri, 2010; Kothari , 2011; Patel et al., 2013). This shows the importance of selecting a proper extraction method while screening for a desired bioactivity.

Fig 5: Effect of *M. hexandra* seed extracts on planktonic form of *S. mutans*

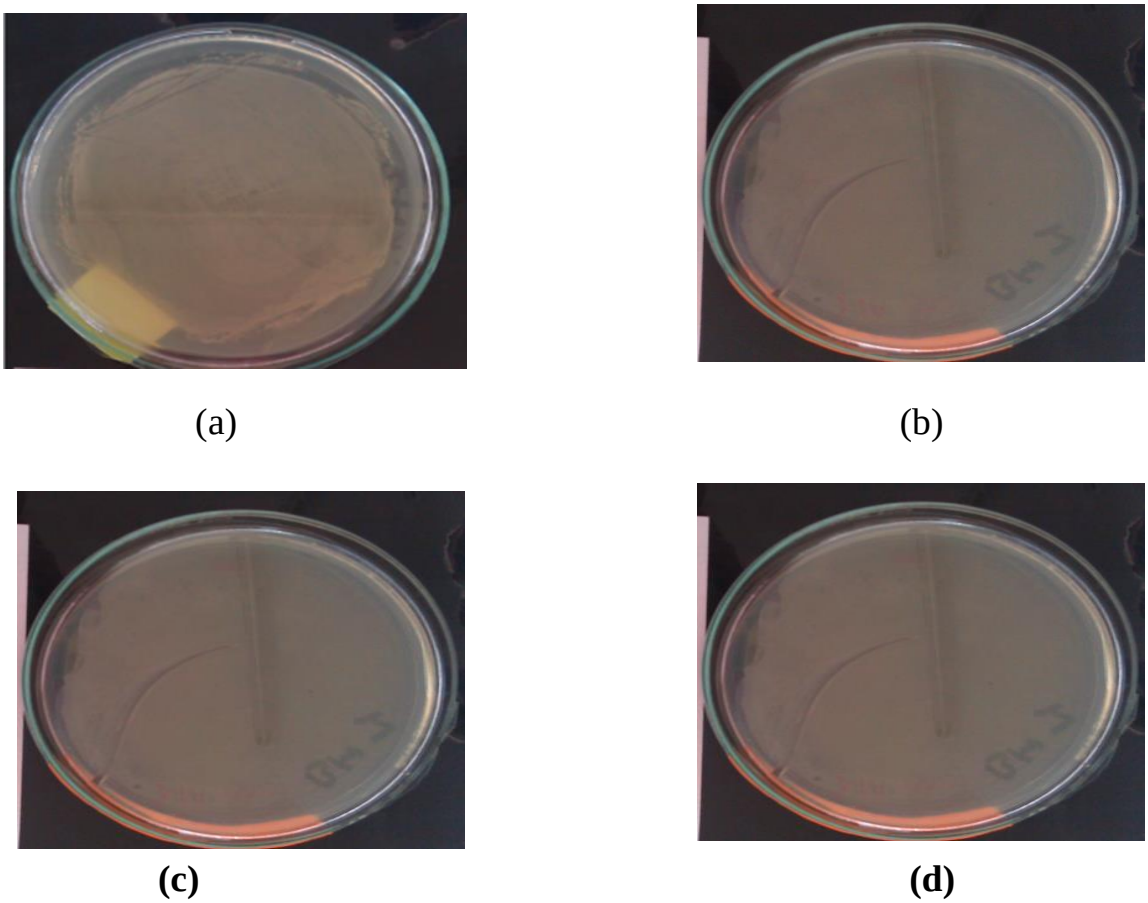


(a) (b)

(c) (d)

Figure 5: Effect of various extracts of *M.hexandra* seeds on planktonic form of *S. mutans* 497 after 72h. Figure (a): Plate corresponding to negative control well (after 72h) with DMSO; (b): Plate corresponding to experimental well (after 72h) with *M. hexandra* ethanol extract 600 µg/ml. This extract showed bactericidal activity against *S. mutans*; (c): Plate corresponding to experimental well (after 72h) with *M. hexandra* acetone extract 750 µg/ml. This extract showed bactericidal activity against S. mutans; (d): Plate

corresponding to experimental well (after 72h) with *M. hexandra* methanol extract 900 µg/ml. This extract showed bactericidal activity against *S. mutans*.



(a) (b)

(c) **(d)**

Fig 6: Effect of various extracts of *M.hexandra* seeds on planktonic form of *S. mutans* 890 after 72h. Figure (a): Plate corresponding to negative control well (after 72h) with DMSO; (b): Plate corresponding to experimental well (after 72h) with *M.hexandra* ethanol extract 650 µg/ml. This extract showed bactericidal activity against *S. mutans*; (c): Plate corresponding to experimental well (after 72h) with *M.hexandra* acetone extract 800 µg/ml. This extract showed bactericidal activity against S. mutans; (d): Plate corresponding to experimental well (after 72h) with *M. hexandra* methanol extract 625 µg/ml. This extract showed bactericidal activity against *S. mutans*

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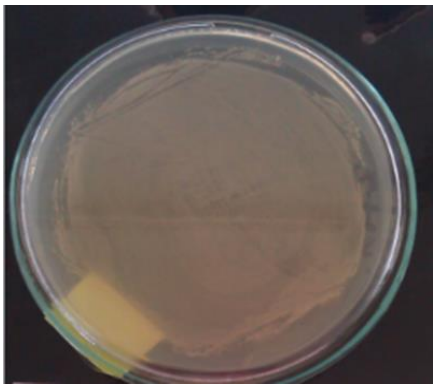
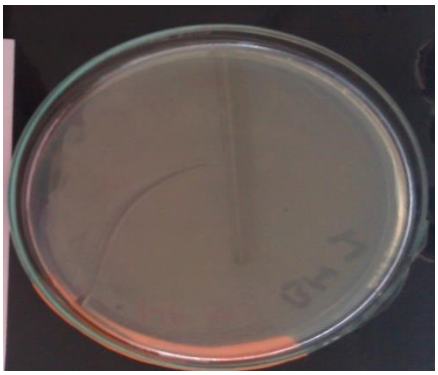
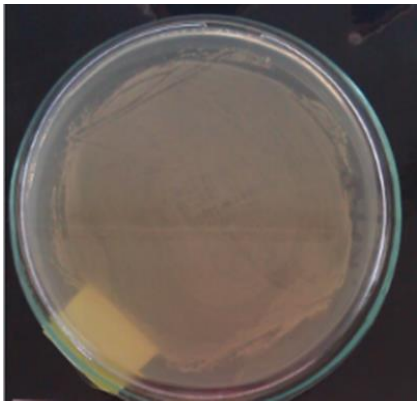


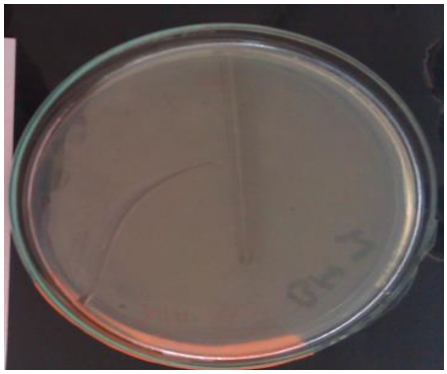
(a)



(b)

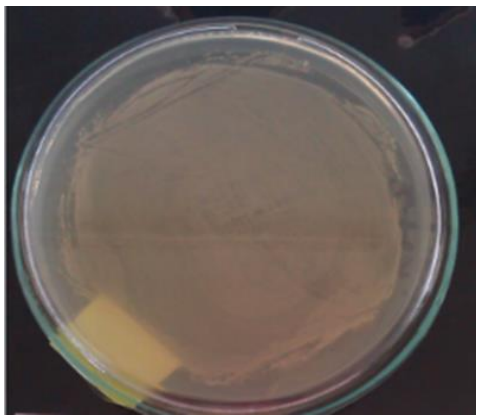


(c)

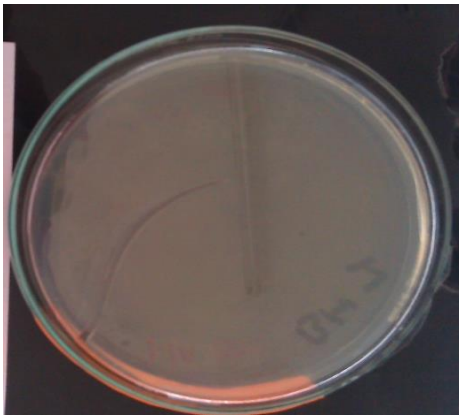


(d)

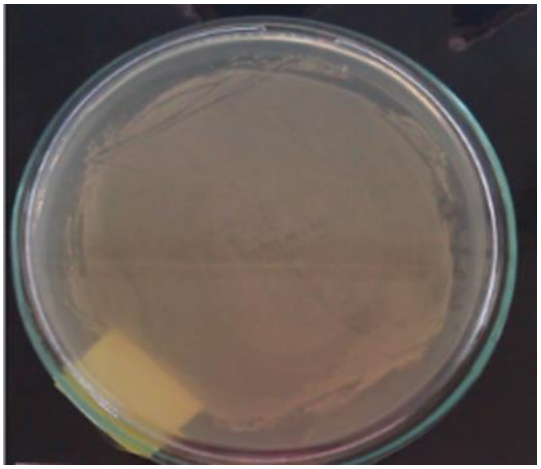
Fig 7: Effect of Chlorhexidine and Violacein on planktonic form of *S. mutans* 497 after 72h. Figure (a): Plate corresponding to growth control well (after 72h); (b): Plate corresponding to experimental well (after 72h) with chlorhexidine at 2.5µg/ml. Chlorhexidine showed bactericidal activity against *S. mutans*; (c): Plate corresponding to negative control well (after 72h); (d): Plate corresponding to experimental well (after 72h) with violacein 1.28 µg/ml. violacein showed bactericidal activity against *S. mutans*.



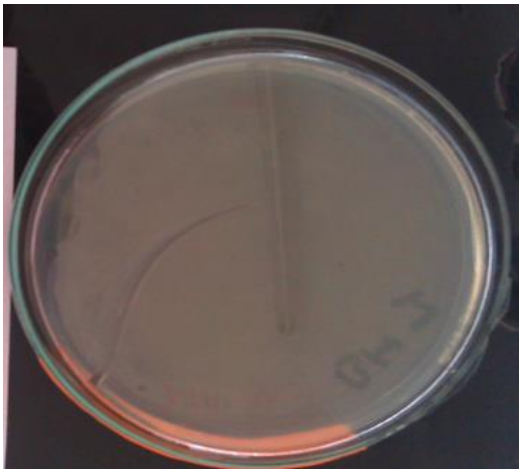
(a)



(b)



(c)



(d)

Fig 8: Effect of Chlorhexidine and Violacein on planktonic form of *S.mutans* 890 after 72h. Figure (a): Plate corresponding to growth control well (after 72h); (b): Plate corresponding to experimental well (after 72h) with chlorhexidine at 1 µg/ml. Chlorhexidine showed bactericidal activity against *S. mutans*; (c): Plate corresponding to negative control well (after 72h) with DMSO; (d): Plate corresponding to experimental well (after 72h) with violacein 1.66 µg/ml. Violacein showed bactericidal activity against *S. mutans*. *S.mutans* 890 was more susceptible to chlorhexidine.

Antimicrobial susceptibility testing with biofilm:

Table 12: Results of crystal violet assay, tube method of *Colgate Plax*® and antibiotic against biofilm of *S. mutans* 497 and *S.mutans* 890

Organism	Test product	Conc. (µg/ml)	% Eradication	% Killing
S.mutans 497	Gentamicin	10	NE	61.96±155.20
		20	23.45±0.93	17.91±6.99*
		30	NE	39.35±9.68*
		40	NE	54.69±20.77
		50	27.92±0.91	45.11±5.54
	Colgate Plax®	1	62.5±17.67	NK
		2	93.75±8.83*	33.63±9.00
		3	58.80±31.74	NK
		4	41.25±6.92	100
		5	43.55±2.67	100
S.mutans 890	Ampicillin	10	18.18±25.71*	14.69±19.44
		20	2.27±3.21	4.12±5.83
		30	NE	NK
		40	NE	4.37±6.18
		50	15.75±0.52	2.48±1.81*
	Streptomycin	40	29.15±3.17	12.45±4.60
		50	20.45±4.33	16.22±22.94
	Colgate Plax®	1	80.19±0.92*	6.52±0.33
		2	31.29±8.89	5.74±1.44
		3	78.56±10.10*	NK
		4	62.49±27.78*	100
		5	16.31±3.71	100
		10	24.73±5.21	100

**All values are highly significant with $p<0.01$, except those marked with *$p<0.05$ which is significant.

No inhibition was reported with all three extracts against biofilm of *S. mutans*. Meanwhile colgateplax was more effective than seed extracts against biofilm of *S. mutans.*

Final Comments

- This work is to attempted at evaluation of antimicrobial activity of three different plant seed belonging to different families and a natural product Violacein.
- Microwave assisted extraction was employed for preparing extracts of seeds in solvent of varying polarity. Among all the seeds highest extraction efficiency (21.58%) was achieved in *P. pinnata* in ethanol. Whereas minimum extraction efficiency was obtained with acetone extract of *P. pyrifolia* which was only 9.32%
- All the seeds were tested against different human pathogens for antimicrobial activity. From *M. hexandra* was effective against *S.mutans*. And rest other extracts showed no inhibition or it showed precipitation in the growth medium.
- *M. hexandra* was tested for their antibacterial activity against *S. mutans* from them ethanol extract of *M. hexandra* was effective upto 600µg/ml against planktonic form of *S. mutans*
- From three extracts which were tested against *S. mutans*, only one was effective. This is significant for the development of novel antibacterial agents which can inhibit the growth of pathogenic microbes and help to maintain oral hygiene.
- Highest Total activity (238.66 ml/g) was exerted by acetone extract of *M. hexandra* against *S. mutans.*
- This study identified various seed extracts with potent antimicrobial action. Further HPLC and LC/MS was done for isolation and identification of the phytoconstituents responsible for antimicrobial activity of seeds. Active constituents once isolated and purified, can be subjected to structural studies.
- Although several antiplaque agents are available in the market, the search for more effective agent still continues (Prabu *et al*., 2006). Plant products effective against oral pathogens like *S. mutans* can find use as ingredients in chewing sticks, toothpastes or other dentifrice products.

Appendices

Appendix A: Preparation of McFarland standard

McFarland Standards

McFarland turbidity standards are used to standardize the approximate number of bacteria in a liquid suspension by visually comparing the turbidity of a test suspension with the turbidity of a McFarland standard. The most commonly used standard for inoculums is 0.5 McFarland turbidity standard, representing approximate 1.5 x 108 CFU/ml.

Preparation of a 0.5 McFarland standard:

Approximately 85 ml of 1% H_2SO_4 was added to a 100 ml volumetric flask. To that flask, 0.5 ml 1.175% $BaCl_2$ was added drop wise with constant swirling to the flask. The volume was made up to 100 ml with 1% H_2SO_4 optical density of the solution was set between 0.08 to 0.1 OD at 625 nm. The solution should be stored in a dark bottle at room temp for a month

Appendix B: Media composition

1) Modified lactose minimal media (Darji et al. 2012)

Sucrose (RM134; HiMedia)	15.00 g
K_2HPO_4 (RM1045; HiMedia)	5.00 g
NH_4Cl (10017.00; CDH)	2.00 g
NaCl (Merck, Mumbai)	1.00 g
$MgSo_4$ (RM1281; HiMedia)	0.10 g
Yeast extract (RM027; HiMedia)	0.10 g
Distilled water	1000 ml
pH	7.40±0.2

2) Brain Heart Infusion Broth (M210, HiMedia, Mumbai)

Per liter	
Calf brain infusion	200.00 g
Beef heart infusion	250.00 g
Protease peptone	10.00 g
Dextrose	2.00 g
Sodium chloride	5.00 g
Disodium phosphate	2.50 g
pH	7.4±0.2

3) Muller Hinton HivegTM broth (MV391, HiMedia)

Per liter	
HiVeg infusion	2.00 g
Hiveg acid hydrolysate	17.50 g
Starch	1.50 g
pH	7.40±0.2

Appendix C: Reagent preparation

1) Crystal violet (0.4%)

Methyl violet (RM961, HiMedia)	4.0g
95% ethanol	20ml
Distilled water	80ml

2) Phosphate buffer saline (Buffer)

NaCl (Merck)	40g
KCl (HiMedia)	1.0g
Na2HPo4 (HiMedia)	7.2g
KH2Po4 (HiMedia)	1.2g
Distilled water	1000ml
pH	7.0

3) Normal saline

	100 ml
NaCl (Merck, Mumbai)	0.85g
Distilled water	100ml

Appendix D: Arrangement of different controls in tissue culture treated plate for biofilm formation

	1	2	3	4	5	6	7	8	9	10	11	12
A	S	G	N	P	E1	T1	E2	T2	E3	T3	E4	T4
B	S	G	N	P	E1	T1	E2	T2	E3	T3	E4	T4
C	S	G	N	P	E1	T1	E2	T2	E3	T3	E4	T4
D	S	G	N	P	E1	T1	E2	T2	E3	T3	E4	T4
E	S	G	N	P	E1	T1	E2	T2	E3	T3	E4	T4
F	S	G	N	P	E1	T1	E2	T2	E3	T3	E4	T4
G	E5	E5	E5	E5	E5	E5	E6	E6	E6	E6	E6	E6
H	T5	T5	T5	T5	T5	T5	T6	T6	T6	T6	T6	T6

S - Sterility control; G - Growth control; N - Negative control; P - Positive control; E - Experimental; T - Turbidity control

Note:

- *S. mutans* became inactive at one point, so to revive it was activated from lyophilized powder.
- Biofilm of *S. mutans* did not grow so we incubate with music in hybridization incubator.
- It was observed that at 2 %v/v of DMSO did not eradicate the biofilm of *S. mutans* which means attachment was highest at 2%v/v of DMSO.

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