PREVENTION AND TREATMENT OF MASTITIS
5. PREVENTION AND TREATMENT OF MASTITIS

5.1 Introduction

Eradication of mastitis is quite challenging and controlling the morbidity and mortality due to Staphylococcus aureus pathogenicity is a difficult task. Preventing mastitis is comparatively convenient task than treating. Though administering appropriate antibiotics against S. aureus infection would increase the response rate, wide usage of antibiotics resulted in resistant microbial strains leading to development of second and third generation of drugs. As an alternative medicine, various plant extracts have been explored for potential antibacterial activity (Cutler and Wilson, 2004; Fujisawa et al., 2008; Kim et al., 2005).

Treating different ailments of human and the animals using medicinal plants either in the form of whole plants or plant extracts is as old as human civilization. In India, drugs of herbal origin have been used in traditional systems of medicines such as Unani and Ayurveda. The drugs that are derived either from whole plant or from leaves, stem, bark, root, flower, seed, etc. Even the Allopathic system has adopted a number of plant derived drugs which form an important segment of the modern pharmacopoeia. Plant based drugs led strong foundation for many modern naturaceuticals that we use now-a-days for various ailments.

In India, the sacred Vedas dating back between 3500 B.C and 800 B.C give many references of medicinal plants. “Rig Veda”, one of the oldest Indian literatures written around 2000 B.C. mentions the use of Cinnamon (Cinnamomumverum Prel.), Ginger (Zingiber officinale Rose.), Sandalwood (Santalum album L.) etc. not only in religious ceremonies but also in medical preparation.
5.2.1 Review of literature

5.2.1 Microbial control and treatment with antibiotics

Chemical substances from microbial source that inhibited or killed the growth of other microorganisms are commonly known as Antibiotics. The term Antibiotic was coined by Selman Waksman in the year 1942. The properties of antibiotics are the bacterial spectrum (broad or narrow spectrum) and type of activity (bacteriostatic or bactericidal).

Discovery of antibiotics can be classified as early and modern. In early history, people from Greece, India and Russia used mould to treat wound infection. Sri Lankan used oil cake to serve as desiccant and for treating infections, less knowing about its antibacterial property. The era of modern antibiotics began with the accidental discovery of penicillin by Sir Alexander Fleming. Pharmacologist Howard Florey and biochemist Ernst Boris Chain analysed the biochemistry, proved its efficacy and delivered penicillin, a miracle drug of the 20th century, to humanity. For this notable achievement Fleming, Florey and Chain shared the Nobel Prize for Physiology and Medicine in 1945. However, within four years of penicillin introduction onto the market, resistant infections were being reported. By the 1970’s, penicillin resistant strains of Str. pneumoniae as well as many veneral diseases spread around the world. A new class of antibiotics was not developed for another 30 years. In 1999, a drug named Zyvoe was developed. A new class of antibiotic called Oxazolidinones was claimed to be effective against multi resistant strains of bacteria.

5.2.1.1 Spectrum of antibiotics in treating Mastitis

Certain antibiotics like penicillin, ampicillin and tetracycline are commonly used to treat mastitis. For severe mastitis cases, intramammary
antibiotics like cephalosporin and pirlimycin and commonly injectable rifampisin are used. Antibiotics work in either of the following two ways.

a) Bacteriostatic antibiotic inhibits the bacterial growth by acting on protein synthesis, DNA replication and other molecular aspects of bacterial cell (Fig 5.1.). Examples of such antibiotics are tetracycline, sulphonamide, chloramphenicol, macrolides, trimethoprim and lincosamides.

b) Bactericidal antibiotic kills bacteria by acting on the cell wall and its component thus disrupting the cell integrity totally. Examples of such antibiotics are penicillin, daptomycin, fluoroquinolones, metronidazole, nitrofurantoin and cotrimoxazole.

A Streptococcus strain, Streptococcus macedonicus ST91KM, obtained from Bulgarian goat yoghurt, produces macedocin ST91KM which has narrow spectrum on gram positive bacteria (Pieterse et al., 2008). It is also found to be effective on mastitis pathogens S. aureus, S. epidermidis, Str. uberis, Str. agalactiae and Str. dysgalactiae.

Fig 5.1: Mode of action of chloramphenicol, erythromycin, tetracycline and streptomycin.
Penicillin

Penicillin was the first antibiotic to be obtained as a natural product from mould Penicillium notatum. The mode of action of penicillin is to block the synthesis of cell wall by cross linking the peptides on mucosaccharide chain. At molecular level, penicillin binds to the active site of transpeptidase enzyme involved in cross linking the peptidoglycan strand. Thus improperly made cell wall allows water to enter the cell leading to cell death.

Oxacillin

Oxacillin is a derivative of penicillin obtained by addition of an acyl side chain and this prevents disruption of β-lactam ring by the enzyme penicillinase. Thus, Oxacillin belongs to the group β-lactams which are penicillinase resistant penicillin. Oxacillin binds to penicillin binding protein (PBPs) and inhibits cell wall synthesis. It mainly attacks the third and last stage of bacterial cell wall formation followed by cell lysis mediated by bacterial cell wall autolytic enzymes known as autolysins. Oxacillin is effective against β-lactamase producing bacteria like methicillin sensitive Staphylococcus aureus (MSSA) and S. epidermidis.

Gentamicin

Gentamicin belongs to the group of aminoglycosides. They are produced by bacteria of Micromonospora genus. Most of the gram negative bacteria are destroyed by displacing cell biofilm-associated Mg²⁺ and Ca²⁺, these aids in shedding of cell membrane and forms transient holes in the cell wall. The overall permeability of the cell is lost before gentamicin reaches 30S ribosome and inhibits bacterial protein synthesis.
Tetracycline

Tetracycline, as the name suggest, it is chemical substance made up of 4-ring system acting as a broad spectrum polyketide antibiotic produced by Streptomyces genus of Actinobacteria. It is used against various skin infections specially acne and recently, rosacea. Tetracycline is available in market under the brand names Sumycin, Panmycin and Tetracyclin. The mode of action of the said antibiotic is of protein inhibition where it binds to 30S subunit of charged aminoacyl tRNA. This prevents the introduction of new amino acids to the extending peptide chain. Resistance does occur by changing permeability of the microbial cell envelope.

Vancomycin

Vancomycin is a compound which is grouped as glycopeptides antibiotic used in the prophylaxis and treatment of infections caused by Gram positive bacteria. It prevents the introduction of N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG)-peptide subunits from being incorporated into the peptidoglycan layer of the cell wall. Vancomycin being a large hydrophilic molecule, it can easily react with the D-alanyl-D-alanine moieties of the NAM/NAG-peptides. This hydrogen bond interaction with D-Ala-D-Ala of Vancomycin prevents the incorporation of NAM/NAG molecule into the cell wall.

Rifampicin

Rifampicin, also known as Rifampin, belongs to bactericidal rifamycin group. It is a semisynthetic molecule derived from Amycolatopsis rifamycinica and Streptomyces mediterranei. Most clinically effective group derived is 4-methyl-1-piperazinaminyl group. Rifampicin is an intensely red solid, and the
small fraction which reaches the body fluids is known for imparting a harmless red-orange color to the urine also sweat and tears of patients. Maximal concentration in blood are decreased by about third when the antibiotic is taken with food.

Rifampicin acts on DNA transcription by stalling mRNA synthesis. It inhibits DNA dependent RNA polymerase by binding to beta- subunit thus, preventing transcription to RNA and subsequent translation to proteins. Most of the acid-fast positive bacteria’s membrane is mycolic acid complexed with peptidoglycan, this permits easy uptake of this chemical molecule into the cell.

5.2.1.2 Diagnostic tests for antibiotic susceptibility

Muller Hinton agar test

Muller Hinton Agar is used to test the growth of aerobic and facultative anaerobic bacteria in clinical samples as well as food material. The composition of the medium consists of beef infusion which is rich in amino acids and nitrogenous compounds, minerals, vitamins, carbon and other nutrients to support growth of microorganisms. Starch is incorporated to adsorb any trace toxic substances present in the medium. During autoclaving, starch breaks down and gives dextrose, which again serves as energy source. Agar is simply used as solidifying agent.

![Image](image.png)

Fig 5.2: A clear zone of inhibition is found around disc A whereas no zone of inhibition is observed around control disc.
Antibiotic discs are used where the said antibiotics are impregnated in paper discs. These discs are kept on the surface of the medium (Fig 5.2.). After incubation, zone of inhibition around discs are measured and compared to that of a standard interpretation chart used to categorize the isolate as susceptible. Incubated plates are observed for zone of inhibition around each discs and the diameter is measured. It is said that various factors such as height of agar, inoculum concentration, disc potency, medium pH and beta-lactamase production by test organisms influence zone of inhibition.

Pour plate

Pour plate is a technique used to enumerate colonies within the media instead on surface, whereas in case of streak plate technique, colonies form onto the surface of the media. Colonies observed in streak plate are larger than colonies appearing within the media in pour plate technique. The main advantage of pour plate technique is that it does not require previously prepared plates whereas streak plate technique requires such pre prepared plates. The disadvantage of pour plate is that colonies formed in such technique are smaller than that of colonies forming on surface.

Antibiotic solution diffuses from the well and inhibits the growth of microorganisms embedded in the agar. Measuring the zone of inhibition by the solution and comparing with the standard chart depicts the sensitivity or susceptibility of the microorganism.

Agar diffusion method

The antimicrobials obtained from microorganisms and plant source are allowed to diffuse into the medium. This reacts with the test organisms streaked or spread onto the medium and shows inhibition or no effect on the organisms.
Inhibition by the antimicrobials lead to the appearance of clear zone around the wells loaded with antimicrobials. This is a simple method to check the ability of antibiotics to inhibit the bacterial growth.

The colonies grown on the surface of media is inhibited by the antibiotic solution that gets diffused into the media. Thus by measuring the zone of inhibition and comparing with the standard chart will give depict the sensitivity or susceptibility of the test organisms.

5.2.1.2 Alternatives to Antibiotics

The increase in antibacterial resistant strains necessitated the development of alternative strategies to treat bacterial infections or diseases.

1) Resistance-modifying agents

Metabolic stimuli such as sugar can help eradicate certain type of antibiotic tolerant bacteria by keeping their metabolism active.

2) Phage therapy

Phage therapy is the use of viruses (called phages) that infect bacteria for the treatment of bacterial infections.

3) Bacteriocins

Bacteriocins are peptides that can be more readily engineered than small molecules. (Bacteriocins and their potential as the next generation of antimicrobials) and are possible alternatives to conventional antibacterial compounds.
4) Chelation

Chelation of micronutrients that are essential for bacterial growth to restrict pathogen spread in vivo might supplement source antibacterials.

5) Vaccines

Vaccines depend on immune modulation or augmentation and it either excites or reinforces the immune competency of a host to prevent infection, leading to activation of macrophages, production of antibodies, inflammation and other classic immune reactions.

6) Biotherapy

Biotherapy is the use of organisms such as protozoa to consume the bacterial pathogens. Maggot therapy is another such approach.

7) Probiotics

Probiotics consist of a five culture of bacteria which become competing symbionts and inhibit or interfere with colonization by microbial pathogens.

8) Host defense peptides

An additional therapeutic agent is used in the enhancement of the multifunctional properties of natural anti infections, such as cationic host defense (antimicrobial) peptides.

9) Antimicrobial coatings

Functionalization of antimicrobial surfaces can be used for sterilization, self cleaning and surface protection.
5.2.1.3 MIC and MBC

Minimum Inhibitory Concentration (MIC) is said to be the lowest concentration of an antibacterial and antimicrobial compound that tend to inhibit or kill the growth of a microorganism over a period of time. MIC is most widely used as diagnostic test for antibiotic resistant microorganism in laboratories but also used to check invtro antimicrobial activity of many newly discovered compounds (Lambert et al., 2001).

Minimum Bactericidal Concentration (MBC) is said to be the lowest concentration of an antibacterial and antimicrobial compound that tend to prevent the growth of a microorganism after subculture onto antibiotic free medium. MBC are less frequently carried out and mostly for diagnostic purposes like in case of endocarditis.

5.2.2 Medicinal plants in controlling Mastitis

Ever since human evolution, mankind has been dependent on plants for food and health care products. Plant extracts have been used for treatment of different diseases since ancient times, all over the world. Ayurvedic medicine is an ancient science that has been practiced for over many centuries, perceived through a universal intelligence, present in every aspect of life and appreciating that very intelligence through our surroundings. Ayurvedic medicine has created a perfect balance through nutrition, environment using herbal medicines and minerals. Many of the herbs are used as spices such as turmeric, ginger, garlic, cumin, fennel, cardamom, black pepper. These herbs have been shown to harbor powerful actions against bacteria, virus, fungus as well as protection against cancers, arthritis, heart disease, hypertension and diabetes.
Medicinal preparations from combination of the different plants and identification of the specific bioactive compounds are integral part of modern medicine. Though antimicrobial activity of natural products has been known, studies on their molecular mechanism are scarce. Of a wide range of compounds are known to control the pathogens, only few have been studied in depth. Yet, the limited studies have contributed much to our knowledge.

India is one of the most well-known countries in Asia for traditional knowledge systems related to the use of plant species. Indian subcontinent has a vast repository of medicinal plants that are used in traditional medical treatments, having around 20,000 medicinal plants been recorded. About 7,000 - 7,500 plants are being used by traditional communities for curing different diseases. The medicinal plants are listed in various indigenous medicinal systems such as Siddha (600 species), Ayurveda (700 species), Amchi (600 species) and Unani (700 species). Ayurveda remains an important system of medicine and drug therapy in India. Plant alkaloids are the primary active ingredients of Ayurvedic drugs (Table 5.1). Major pharmaceutical industries depend on the plant products for the preparation of medicines. In some parts of the world medicinal plants are given during pregnancy and lactation for medicinal purposes, in order to improve immune power.
Table 5.1: Chemical constituents and therapeutic uses of some Ayurvedic crude drugs.

<table>
<thead>
<tr>
<th>Botanical name</th>
<th>Chemical composition</th>
<th>Therapeutic uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adhatodavasica Nees</td>
<td>1.0% Vasicine, 2.0% Total alkaloids</td>
<td>Anti-asthmatic, Bronchodilator, Cold remedy</td>
</tr>
<tr>
<td>Andrographis paniculata Wallich ex Nees</td>
<td>10% Andrographolides</td>
<td>Hepatoprotectant</td>
</tr>
<tr>
<td>Boswellia serrata Roxb.</td>
<td>75% Organic Antiarthritic acids, 40% Boswellic Anti-inflammatory and acid, 20% Sennosides laxative action</td>
<td>Anti-arthritic, anti-inflammatory and acid, laxative action</td>
</tr>
<tr>
<td>Bacopamonniera (L.) Pennell.</td>
<td>20% Bacosides A&amp;B</td>
<td>Memory enhancer</td>
</tr>
<tr>
<td>Capsicum annum L.</td>
<td>40% Capsaicin, 75% Capsaicin</td>
<td>Pain reliever</td>
</tr>
<tr>
<td></td>
<td>90% Capsaicin</td>
<td></td>
</tr>
<tr>
<td>Centella asiatica Urb.</td>
<td>8% Total triterpenes</td>
<td>Skin, health, weight management</td>
</tr>
<tr>
<td>Coleus forskohlii Briq. Syn 1%</td>
<td>Forskohlin</td>
<td>Antihypertensive, weight management</td>
</tr>
<tr>
<td>Curcuma longa L.</td>
<td>Curcumin C3, 95% Curcuminoids</td>
<td>Antioxidant, anti-viral, anti-inflammatory, anticarcinogenic</td>
</tr>
<tr>
<td>Emblica officinalis Gaertn.</td>
<td>30% Tannins</td>
<td>Detoxification</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------------------------</td>
<td>------------------------------------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td><strong>Garciniacambogia Desr.</strong></td>
<td>50% (-) HCA (Ca)</td>
<td>Rejuvenating agent</td>
</tr>
<tr>
<td><strong>Garcinia indica Chois.</strong></td>
<td>Citrinò crystalline powder 10% (-) HCA</td>
<td>Weight management</td>
</tr>
<tr>
<td><strong>Gymnema sylvestre R. Br.</strong></td>
<td>Gymnema Sylvestre GS 425% 75% Gymnemic acids</td>
<td>Antidiabetic</td>
</tr>
<tr>
<td><strong>Glycyrrhiza glabra L.</strong></td>
<td>20% Glycyrrhizinic acid 5% Lutein</td>
<td>Eyesight-age related Macular degeneration</td>
</tr>
<tr>
<td><strong>Camellia sinensis (L.) Kuntze</strong></td>
<td>40% Catechins; 75% Catechins 2% Caffeine</td>
<td>Antioxidant</td>
</tr>
<tr>
<td><strong>Commiphora mukul Engl.</strong></td>
<td>Gugulipid 2.5% Guggulsterones Z&amp;E</td>
<td>Cholesterol Management</td>
</tr>
<tr>
<td><strong>Momordica charantia L.</strong></td>
<td>7% Bitter principles 0.5% Charantin</td>
<td>Antidiabetic</td>
</tr>
<tr>
<td><strong>Morinda citrifolia L.</strong></td>
<td>Fruit Powder</td>
<td>General tonic</td>
</tr>
<tr>
<td><strong>Mucuna pruriens Baker</strong></td>
<td>10% &amp; 15% L-Dopa Min. 20% Catecholamines</td>
<td>Nerve tonic Energy</td>
</tr>
<tr>
<td><strong>Melia azadirachta L.</strong></td>
<td>3% Bitter Principles</td>
<td>Anti-bacterial</td>
</tr>
<tr>
<td>Plant Name</td>
<td>Constituents</td>
<td>Use</td>
</tr>
<tr>
<td>------------------------------------------------</td>
<td>---------------------------------------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>Phyllanthus amarus L.</td>
<td>0.02% Phyllanthine &amp; Hypophyllanthine</td>
<td>Anti-hepatitis</td>
</tr>
<tr>
<td>Picrorhiza kurroa Royle 4% Kutkin ex Benth.</td>
<td></td>
<td>Hepatoprotectant</td>
</tr>
<tr>
<td>Piper nigrum L.</td>
<td>95% Piperine</td>
<td>Nutrient bio-availability, Enhancer</td>
</tr>
<tr>
<td>Piper longum L.</td>
<td>1.5% Piperine</td>
<td>Biopotentiatior, Ant-asthmatic, Thermogenic</td>
</tr>
<tr>
<td>Rubiacordifolia L.</td>
<td>4:1 Concentration</td>
<td>Skin disorders</td>
</tr>
<tr>
<td>Sidacordifolia L.</td>
<td>0.8% Ephedrine, 10% Isoflavones</td>
<td>Bronchodilator, Anti-carcinogenic</td>
</tr>
<tr>
<td>Terminalia arjuna W. &amp; A. 1% Arjunolic acid</td>
<td></td>
<td>Revitalizing, circulation</td>
</tr>
<tr>
<td>Terminaliabellerica Roxb. 35% Tannins</td>
<td></td>
<td>Rejuvenating agent</td>
</tr>
<tr>
<td>Zingiber officinale (Willd.) 5% Gingerols Rosc.</td>
<td>Gingerols</td>
<td>Digestive aid, Ginger soft extract</td>
</tr>
</tbody>
</table>
5.2.3 Phytotherapy in Mastitis

Phytotherapy is a traditional remedy for different diseases where herbs and their products are used. It is a very well applied method of therapy for both humans and animals. The various phytochemicals found in the extracts were tannins, flavonoids, saponins and alkaloids and the research on their plays an important role in the development of herbal medicines (Table 5.2). It constantly addresses a challenge because of the large number of compounds present as mixture in the extract in trace amounts. As plants consist of various phytochemicals, they are exploited for different pharmacological properties, resulting in a battery of synthetic drug discovery. Modern medicine makes use of such bioactive phytochemicals which are developed as “lead” molecule with rich medicinal values (Newman and Cragg, 2007).

The antibacterial effect of ethanolic and aqueous extracts of Ocimum gratissimum and Piper guineense on Escherichia coli and S. aureus revealed that ethanolic extract showed more antibacterial effect than aqueous extract against S. aureus. The minimum inhibitory concentration for ethanolic extract against S. aureus was 2.50 mg/ml whereas for aqueous extract was 10.00 mg/ml of Ocimum gratissimum. There was not much difference in minimum inhibitory concentration of ethanolic and aqueous extract of Piper guineense on S. aureus.

Various plants are used in Sargodha district of Pakistan to treat mastitis in bovine as well as bubaline (dairy buffalo). Few of them are Capsicum annuum L., Lepidium sativum L., Allium sativum L., Sesamum indicum L., Citrus limon (L.) Burm, Zingiber officinale Roscoe, Citrullus colocynthis (L.) Schrad, Curcuma longa L., Cuminum cyminum L., Rosa indica L., Centratherum anthelmisticum L., Triticum aestivum L., Nigella sativa L. and Peganum harmala L.
Table 5.2: Plants used in mastitis treatment

<table>
<thead>
<tr>
<th>Botanical name</th>
<th>Local name</th>
<th>Parts used</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artocarpus heterophyllus Lam.</td>
<td>Rukh Katahar</td>
<td>Part inside the fruit</td>
<td>The paste of inner part of the fruit is applied to the infected part.</td>
</tr>
<tr>
<td>Asparagus racemosus</td>
<td>Kurilo</td>
<td>Root</td>
<td>The paste prepared from the root is applied twice a day, at morning and evening.</td>
</tr>
<tr>
<td>Eclipta prostrata Bhringiraj (L.) L</td>
<td>prostrata Bhringiraj</td>
<td>Whole plant</td>
<td>The paste prepared from the whole plant is applied onto the infected part.</td>
</tr>
<tr>
<td>Solena heterophylla Gol Lour.</td>
<td>kankri</td>
<td>Root</td>
<td>The paste prepared from root is applied to the infected part twice a day for 3-4 days.</td>
</tr>
<tr>
<td>Trichosanthes anguina L.</td>
<td>Chichindo</td>
<td>Fruit</td>
<td>The powder paste of ripe fruit is applied to the infected part for 3-4 days.</td>
</tr>
</tbody>
</table>

Aquatic plants have been explored for their antimicrobial activity. Different parts extracted in different solvents from Salvinia auriculata and Hydrocleys nymphoides were tested against bovine mastitis pathogens. Ethanolic extracts from the leaves of Hydrocleys nymphoides showed more response than hexane extracts from the roots of Salvinia auriculata. Methanolic extracts of Nigella sativa seeds were tested for in vivo and in vitro effects on C. albicans, S. aureus and P. aeruginosa.
The antimicrobial activity of Punica granatum was tested on a range of microbes affecting cattle mammary glands. The organisms screened were Str. agalactiae, S. aureus, Str. uberis, E. coli, Str. dysgalactiae and coagulase negative S. aureus. The methanol and aqueous extracts showed better effect on S. aureus with an inhibition zone of 36mm. Zingiber officinale Roscoe (red ginger) which had been used since ages to treat gastro intestinal disorders was shown to act on S aureus, S. epidermidis and Str agalactiae, concluding the traditional use of red ginger to control mastitis.

A study on the effectiveness of methanolic extracts of Spathodea campanulata and Tridax procumbens against Str. agalactiae, Str. uberis, E. coli, coagulase positive S. aureus, coagulase negative S. aureus, Klebsiella pneumonia. S. campanulata has been demonstrated to specific action against Str. agalactiae and T. procumbens against coagulase positive S. aureus.

5.2.4 Curcumin from Turmeric (Curcuma longa)

Curcumin (diferuloylmethane) 1,7-bis-(4-hydroxy-3-methoxy phenyl)-1,6-heptadiene-3,5-dione, is a polyphenol derived from the plant Curcuma longa, commonly called turmeric (Kim et al., 2005). The plant turmeric is a member of the ginger family. Its rhizomes produce a brilliant yellow dye. The primary bioactive constituents in turmeric have been found to be the phenolic curcuminooids, the most important of which is curcumin (diferuloylmethane, Fig. 5.3). The rhizomes are also used as a spice, a main ingredient in curry powder, and as a food preservative.
Traditional Chinese medicine has used turmeric for its acrid, bitter, and warming properties, both internally and externally. In Ayurvedic medicine, it is used as a stomachic, tonic, blood purifier, antiperiodic, and alterative. Both turmeric and curcumin are known for their antioxidant and anti-inflammatory activities, and may play roles in preventing atherosclerosis and cancer. Pharmacologically, turmeric has also been found to be a stimulant, a tonic, a carminative, and an anthelmintic. Curcumin has antibacterial and antifungal, anti-inflammatory, antiallergic and wound healing properties (Fig 5.4).
The antimicrobial property of turmeric has been extensively studied (Lai et al., 2004; Negi et al., 1999; Norajit et al., 2007; Park et al., 2005). Curcumin was proposed as a possible bacterial sortase A inhibitor, since it inhibited sortase A activity of S. aureus cell adhesion to fibronectin (Kim et al., 2005). Apart from sortase A activity curcumin controls the MRSA by inhibiting the MRSA invasion of HMFs (human mucosal fibroblasts) through activity of beta-lactams and alter the MRSA invasion. Gram positive bacteria especially S. aureus is sensitive to essential oil (steam distillation) derived from turmeric then other fractionations. The activities of turmeric fractions against some intestinal bacteria revealed that the inhibition of Lactobacilli growth in the presence of whole turmeric (4.5-90µl/100ml) was higher than alcoholic extract (10-200 mg/ml). Much higher concentration of Curcumin (2.5-50 mg/ml) could inhibit S. aureus. 

The effect of curcumin on the biofilm synthesis has not been fully explored especially with reference to cattle mastitis. The studies so far conducted have been on the microbial growth control, pathogenecity and cell adherence. Studying the mechanism and action of curcumin on the biofilm biosynthesis would throw more light in control and prevention of mastitis in cattle.

5.2.5 Allium sativum from Garlic

Historically, garlic has been used worldwide to fight bacterial infections (Chowdhury et al., 1991; Cutler and Wilson, 2004). Allium vegetables, particularly garlic exhibits a broad antibiotic spectrum against both gram positive and gram negative bacteria. Garlic has been reported that inhibit growth of S.aureus. Noteworthy results published include the following:
• Garlic is effective even against those strains that have become resistant to antibiotics.

• The combinations of garlic with antibiotics lead to partial or total synergism.

• Complete lack of resistance has been observed repeatedly.

• Toxin production by microorganisms is prevented by garlic.

The composition of 19 garlic natural health products (NHPs) and fresh garlic extracts were analysed for their principle active constituents and their antibacterial activity. Water extracts equivalents to fresh garlic of 200 mg/ml showed minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) against three indicator microorganisms (Neisseria gonorrhoeae, S. aureus and Enterococcus faecalis) as determined by the broth micro-dilution method. While 47% of the aqueous garlic NHP exhibited activity against N. gonorrhoeae, only 16% of the aqueous extracts inhibited S. aureus or E. faecalis. Generally products with high antimicrobial activity contained higher levels of garlic constituents with comparable activity to fresh garlic extracts (Chowdhury et al., 1991; Fujisawa et al., 2009).

Diallyl sulfides (DAS) are organosulfur compounds obtained from garlic. The Garlic (Allium sativum) is a rich source of a wide variety of organosulfur compounds, which are considered responsible for the beneficial effects of this herb. S-allylcysteine sulfoxide (an odorless compound called alliine) in garlic is transformed enzymatically into allicin by alliinase, which is the precursor compound for different thioallyl compounds (Cutler et al., 2009; Cutler and Wilson, 2004; Fujisawa et al., 2008; Fujisawa et al., 2009).
CH - CH₂ - S - CH₂ - CH = CH₂  

Diallyl Monosulfide

CH - CH₂ - S - S - CH₂ - CH = CH₂  

Diallyl Disulfide

CH₂ = CH -CH₂ -S -S - CH₂ - CH = CH₂  

Diallyl Trisulfide

Fig 5.5: Chemical structure of diallylsulfides

Diallyl sulfide, a lipophilic thioether, is a major organosulfur derivative and is responsible for strong taste & odour. It has multiple beneficial effects such as antimicrobial, anti thrombotic, hypolipidemic, anti arthritic, hypoglycemic and anti tumor activity. It has wide range of activities including antimicrobial and anti cancer activity. The chemical analysis of garlic shows that 54.5% of total sulphides are comprised of diallyl mono-, di-, tri- and tetrasulphides (Fig 5.5).
5.3 Materials and Methods

5.3.1 Materials

Curcumin purified from Turmeric (Curcuma longa) was kindly provided by Arjuna Natural Extracts Pvt Ltd, Cochin, Kerala, India. Diallylsulfides (DAS) extracted from garlic (Allium sativum) was a kind gift from Prof. A. Banerjee, Department of Analytical Chemistry, Regional Research Lab, Government of India, Thiruvananthapuram, Kerala. The antibiotic discs were procured HiMedia, Mumbai, India.

5.3.1.1. Muller Hinton agar test

<table>
<thead>
<tr>
<th>Composition</th>
<th>gm/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef infusion solids</td>
<td>4.0</td>
</tr>
<tr>
<td>Starch</td>
<td>1.5</td>
</tr>
<tr>
<td>Casein hydrolysate</td>
<td>17.5</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0</td>
</tr>
</tbody>
</table>

Final pH 7.4 +/- 0.2 at 37°C.

5.3.1.2 Turbidity standard for inoculum

For broth based bacteriological assays, 0.5 McFarland standard (barium sulfate; BaSO₄) was prepared. To 9.95 ml of 180 mM H₂SO₄ (1%v/v), 50 µl of 48 mM BaCl₂ (1.175% W/V BaCl₂⋅2H₂O) was added. It was stirred to maintain a suspension and transferred to test tube and stored at room temperature.
5.3.1.3 Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

1. Sterile culture tubes
2. DMSO, Curcumin (250μM) and DAS (1mM)
3. Test organism

5.3.2 Methods

5.3.2.1 Muller Hinton agar test

Muller Hinton agar media was prepared by dissolving 38 gms of medium in one liter of distilled water and boiled slightly for dissolving media. Media was autoclaved at 121°C for 15 mins and poured into sterile petri dishes. Test organisms were spread onto the media and antibiotic discs were kept onto the surface and the plates were incubated at 35± 2°C in aerobic atmosphere and examined for growth at regular intervals.

Kirby-Bauer disc diffusion method was used to detect antimicrobial susceptibility for S. aureus isolates with Penicillin, Rifampicin, Gentamicin, Oxacillin, Vancomycin and Tetracycline. Staphylococcus aureus positive colonies were selected from Mannitol Salt agar plate and were inoculated onto Muller Hinton Agar plate. The antibiotic discs were placed on the S. aureus colonies inoculated onto Muller Hinton agar plate. Antibiotic susceptibility was determined by the zone of inhibition based on NCCLS guidelines.
5.3.2.2 Minimum Inhibitory Concentration (MIC) for Curcumin and Diallyl sulfide

Minimal inhibitory concentration was determined for Curcumin and DAS against S. aureus by broth dilution method, as recommended by the National Committee for Clinical Laboratory Standards. Stock solutions of Curcumin (250µM) and DAS (1mM) were prepared in DMSO from which, working solutions of different concentrations were used in the study. The inoculum, 10µl of 10^7 CFU, equivalent in turbidity to the 0.5 McFarland standard, was inoculated into the 5ml of nutrient broth and mixed thoroughly. Experimental cultures contained different concentration of study compounds and standard antibiotics in the medium. The culture tubes were kept in 37°C shaking water bath at 250 rpm for 24h and were monitored for 48 and 72 h. The lowest concentration with the absence of turbidity was considered as the minimal inhibitory concentration (MIC). The results were presented as mean ± SE of five experiments.

5.3.2.3 Minimal bactericidal concentration for Curcumin and Diallyl sulfide

The Minimal Bactericidal Concentration (MBC) was determined by sub-culturing the contents of the MIC tubes at serial dilutions (10^1 to 10^7) onto antibiotic-free nutrient agar medium. The cultures were examined for bacterial growth from lowest to highest dilution without turbidity. The concentration at which growth was observed indicated the bacterial static concentration of curcumin and DAS.
5.3.2.4 Evaluation on inhibition of ica operon genes by curcumin and DAS

The cultures analyzed for the curcumin and DAS MIC were further evaluated for ica operon. The total RNA isolated from the control and experimental culture cells was converted into cDNA and used as the template in amplifying with ica D gene specific primers. As an internal control for cellular functions, the expression of gyrB was evaluated. The action of a set of antibiotics over the biofilm expression was screened and used as positive control.
5.4 Results

5.4.1 Antibiotic sensitivity pattern of S. aureus

The S. aureus isolates obtained from the milk samples from mastitis and normal cows were tested for their sensitivity to the antibiotics used under clinical treatment. The Kirby-Bauer disc diffusion method was employed to determine the extent of sensitivity which was expressed by way zone of inhibition (Table 5.3 and Figure 5.6). In comparison with the reference range of zone of inhibition published earlier by others, the observed range in this study was encouraging.

Table 5.3: Antibiotic Sensitivity Pattern of S. aureus

<table>
<thead>
<tr>
<th>Zone of inhibition</th>
<th>Pencillin</th>
<th>Oxacillin</th>
<th>Tetracyclin</th>
<th>Vancomycin</th>
<th>Gentamycin</th>
<th>Rifampicin</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus ref range* (dia in mm)</td>
<td>26-37</td>
<td>18-24</td>
<td>24-30</td>
<td>17-21</td>
<td>19-27</td>
<td>26-34</td>
</tr>
<tr>
<td>Sensitivity Observed (dia in mm)</td>
<td>17-32</td>
<td>14-25</td>
<td>9-45</td>
<td>14-33</td>
<td>18-40</td>
<td>19-35</td>
</tr>
</tbody>
</table>

* (NCCLS, 2000)

The percentage of isolates responded to antibiotics are presented in Table 5.4. Among the six antibiotics, Gentamicin exhibited complete inhibition while pencillin was the least effective. With higher control rate, Vancomycin (approximately 83%) followed Gentamycin. Both tetracycline and rifampicin
Table 5.4: Sensitivity pattern of S. aureus for antibiotics in mastitis treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>Breed</th>
<th>Diagnosis</th>
<th>S. aureus +ve samples*</th>
<th>Pencillin</th>
<th>Oxacillin</th>
<th>Tetracyclin</th>
<th>Vancomycin</th>
<th>Gentamycin</th>
<th>Rifampicin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>Mastitis</td>
<td>HF</td>
<td>Clinical</td>
<td>82</td>
<td>2%</td>
<td>18</td>
<td>22%</td>
<td>42</td>
<td>51%</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sub clinical</td>
<td>24</td>
<td>1%</td>
<td>5</td>
<td>21%</td>
<td>14</td>
<td>58%</td>
<td>19</td>
</tr>
<tr>
<td>Jersey</td>
<td></td>
<td>Clinical</td>
<td>48</td>
<td>3%</td>
<td>9</td>
<td>19%</td>
<td>23</td>
<td>48%</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sub clinical</td>
<td>5</td>
<td>0%</td>
<td>1</td>
<td>20%</td>
<td>2</td>
<td>40%</td>
<td>4</td>
</tr>
<tr>
<td>Kangayam</td>
<td></td>
<td>Clinical</td>
<td>19</td>
<td>1%</td>
<td>3</td>
<td>16%</td>
<td>9</td>
<td>47%</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sub clinical</td>
<td>2</td>
<td>0%</td>
<td>0</td>
<td>0%</td>
<td>1</td>
<td>50%</td>
<td>2</td>
</tr>
<tr>
<td>Normal</td>
<td>HF</td>
<td></td>
<td>2</td>
<td>0%</td>
<td>1</td>
<td>50%</td>
<td>2</td>
<td>100%</td>
<td>2</td>
</tr>
<tr>
<td>Jersey</td>
<td></td>
<td></td>
<td>1</td>
<td>0%</td>
<td>0</td>
<td>0%</td>
<td>1</td>
<td>100%</td>
<td>1</td>
</tr>
<tr>
<td>Kangayam</td>
<td></td>
<td></td>
<td>0%</td>
<td>1</td>
<td>100%</td>
<td>1</td>
<td>100%</td>
<td>1</td>
<td>100%</td>
</tr>
</tbody>
</table>

* - Number of samples positive for S. aureus as described in Table 3.3.
Legend: The antibiogram of *S. aureus* was studied against the antibiotics used in mastitis treatment as detailed under materials and methods (5.3)

R - Rifampicin, Gen - Gentamicin, P - Penicillin
Ox - Oxacillin, T - Tetracyclin, V - Vancomycin

Fig 5.6: Antibiogram of *S. aureus* against antibiotics in mastitis treatment
could inhibit 53% of the S. aureus isolates. Only about 20% of the isolates were found to be sensitive to Oxacillin.

5.4.2 MIC for Curcumin and Diallyl sulfide

The treatment of S. aureus isolates with curcumin and DAS employing broth dilution method and the minimal inhibitory concentration obtained are presented in Fig. 5.7 and 5.8. The MIC values were compared with antibiotics namely vancomycin, penicillin, tetracyclin, rifampicin, oxacillin and gentamycin. All the experiments were conducted in triplicate and the results are presented at mean ±SE. To begin with, an inoculum containing 1500CFU was plated on the nutrient agar containing 18, 36, 55, 73, 92, 110 and 147mg/ml curcumin. At 110 mg/ml concentration curcumin completely inhibited the cell growth of S. aureus (Fig 5.7). Following the NCCL standard method, an inoculum containing 10⁷ CFU cells was used to determine the MIC of curcumin. It was found that 736 mg/ml curcumin was required to inhibit the growth of both S. aureus and S. epidermidis. (Cutler et al., 2009; Cutler and Wilson, 2004; Fujisawa et al., 2008; Fujisawa et al., 2009). Interestingly, the MIC of DAS for S. aureus was 1.78 μg/ml. The results clearly indicated that curcumin and DAS requirement is at higher concentration than the commercially available synthetic antibiotics. Still, the natural products have considerable effect on the inhibition of S. aureus growth.

5.4.3 Minimal Bactericidal Concentration

The observation of culture plates inoculated with treated broth cultures exhibiting the MIC for S. aureus revealed that at the MIC concentration the study compounds were able to only inhibit the growth but not sufficient to kill the organisms. Colonies obtained from the Culture at MIC concentration indicated that these concentrations were only bacteriostatic and not bactericidal.
Legend: Minimal inhibitory concentration of Curcumin was determined on *S. aureus* as detailed under materials and methods (5.3). Curcumin was tested with μM concentration and presented as mg/ml matching the concentration of other study compounds.

**Fig. 5.7:** Minimal inhibitory concentration of Curcumin on *S. aureus*
**Legend:** Minimal inhibitory concentration for Gentamycin, Rifampicin, Curcumin and DAS were determined on *S. aureus* and *S. epidermidis* as detailed under materials and methods. Curcumin and DAS were tested with μM concentration and converted to μg/ml in comparison with Gentamicin and Rifampicin.

**Fig. 5.8:** Minimal inhibitory concentration for Gentamicin, Rifampicin, Curcumin and DAS
5.4.4 Expression analysis of ica Operon.

Specific amplification of icaD mRNA by RT-PCR unraveled that only S. aureus expressed this Operon. Total RNA from the different experimental cultures was subjected to RT-PCR, confirmed our observation and in order to confirm the presence of the Operon in the genome, DNA samples from both the organisms were amplified with icaD primers. The RT-PCR analysis of the total RNA obtained from the control and different experimental cultures of S. aureus revealed that the ica Operon was more induced under increasing stress (Fig 5.9). From the data presented, it is obvious that with increasing concentration of curcumin and DAS the icaD mRNA content increased significantly. Though the cells were completely inhibited, at MIC, the expression was at the highest level.

To compare the icaD expression regulated by the antibiotics, we performed RT-PCR analysis of the total RNA obtained from the culture treated with vancomycin and gentamicin. After initiating the culture with the specific MIC concentration(s), total RNA was isolated from the cells collected at 2h intervals. After 6th h, the expression of icaD started increasing.
Legend: Total RNA was isolated from *S. aureus* cultures (from standard and milk samples) treated with curcumin and DAS. The RNA was converted into cDNA and subjected to RT-PCR analysis of icaD gene expression as detailed under materials and methods.

Lane 1: 100 bp Marker, Lane 2: Control (cells only), Lane 3: *S. aureus* DNA, Lanes 4 and above: increasing concentrations of curcumin and DAS

**Fig 5.9:** RT-PCR analysis of icaD expression in *S. aureus* treated with curcumin at increasing concentrations
5.5 Discussion

Farmers and dairy men use local treatment at most of the times for controlling mastitis along with necessary precaution of relocating the cattle to a clean surrounding. This may reduce the severity and spread of infection rather than totally eradicating the disease. At many a times, even after antibiotic treatment the condition becomes worse and with no other option the cow needs to be culled. This highly increases not only the loss of the animal but in toto economic loss to the farmer and the community. Apart from this, consumption of the abnormal milk with increased SCC by human leads to health hazards (Middleton et al., 2004).

Treatment of a disease is based on the etiology and the effective drugs. Mastitis is a complex disease with multiple causative agents inclusive of host and the pathogens. Clinically, mastitis is treated with antibiotics of which gentamycin and vancomycin are the preferred ones. Both the antibiotics have good prognostic value and the animals recover much faster. Yet, new sources of antimicrobial products required to be explored due to constant evolution of resistant microorganisms. In the process of overcoming the bacterial resistance especially multidrug resistance, phytochemicals have been identified as potential candidates (Clardy et al., 2006).

Among the causative pathogens, S. aureus has been the predominant microorganism. This pathogen is opportunistic, able to evade the host immune system, adhere to intramammary epithelial cells and colonize. Considerable percentage of S. aureus isolates express biofilm hence become untreatable. Antibiotics and host immune cells find difficult to penetrate the extracellular polysaccharide (EPS) and over a time, the bacterial cells peel off as a colony and adhere to different location (Rachid et al., 2000b). The persistence of the biofilm
and continuous episodes of the infection increase the severity of the disease. Phytochemicals could come to our rescue since they inhibit bacterial growth by different mechanisms than the presently used antibiotics. These plant bioactive compounds belong mainly to the chemical structural classes like phenolics, terpenoids and other essential oils constituents, alkaloids, lectins and polypeptides and poly-acetylenes (Gibbons et al., 2004; Stavri et al., 2007).

A wide range of activities has been discovered for curcumin including bactericidal effect. The previous reports available on curcumin to control Staphylococcal infections recommended a higher dose (2mg), which is clinically not suitable. In the present study we evaluated the MIC of curcumin and DAS for S. aureus and S. epidermidis. Albeit we observed that 0.5mg/ml of curcumin was sufficient to inhibit 10^7 CFU of both the organisms, it was still higher. At lower concentration i.e. 1500CFU aureus was inhibited at 0.1mg/ml of curcumin while S. epidermidis was inhibited at 0.14 mg/ml. This is interesting since aureus is capable of producing biofilm and required lesser concentration of curcumin then epidermidis, which lacked biofilm formation (Götz 2002).

The analysis of curcumin to control the biofilm production was performed by RT-PCR. The ica operon, comprised of four genes and responsible for the biofilm production was positively regulated when the cells were kept under stress during the treatment with antibiotics, DAS and curcumin. Our data strongly suggested that the induction of ica operon was dose dependent as clearly evidenced by RT-PCR analysis. Earlier reports have also suggested that ica Operon is positively regulated by stress inducing substances (Gotz, 2002). It is of much consideration that even the cell number decreased with the drug treatment and the biofilm production increased represented by RT-PCR based detection on microtiter plates.
The present study has evaluated the use of natural plant products for the control of mastitis. Phytochemicals behaving differently than the conventional antibiotics could be of clinical and economic value in treating resistant bacteria. The use of antibacterial phytochemicals is a highly attractive practice, particularly with respect to the emergence of MDR bacteria in both planktonic and biofilm states. The molecular evaluation of curcumin and DAS encourage further research on functional plant genomics and the discovery of biosynthetic pathways.