CHAPTER I

GENERAL BACKGROUND

1. Introduction

Polysaccharide gel (PG) isolated from dried fruit-rind of durian (*Durio zibethinus* Murr.) (Smittinand, 2001) is a water-soluble natural polymer, biocompatible and wide applicable in pharmaceutical and food industry. PG has been found to be a useful excipient for the preparation of food and pharmaceutical products such as jelly, jam, salad dressing, tablet, suspension and emulsion (Pongsamart *et al.*, 1989; Pongsamart and panmaung *et al.*, 1998; Umprayn *et al.*, 1990). Toxicity test of polysaccharide gel has also been investigated, a high oral dose (2 g/ kg) was not induced severe toxicity in male mice and rats (Pongsamart *et al.*, 2001). No toxic effect has been noted in male and female mice after 2-3 months feeding with PG 0.5 g/ kg/ d, the result has confirmed the consumptive safety of PG (Pongsamart, *et al.*, 2002).

Recently, researches on the application of natural biomaterials and biopolymers has been attracted attention by researchers because of these compounds are characterized as a high hydrolytic stability and biodegradability. The natural polysaccharide gel from dried fruit-rind of durian is a high-molecular weight polysaccharide of 500-14,000 kDa (Gerddit, 2002), build up from sugars as a heteropolysaccharide of long chains galacturonan with branches chains of neutral sugars such as glucose, rhamnose, arabinose, and fructose (Hokputsa *et al.*, 2004). PG has film-forming property similar to those of cellulose derivatives such as hydroxypropyl methylcellulose (Gerddit, 2002). Polysaccharide gel exhibited antibacterial activity against seven tested strains of bacteria. Minimal inhibitory concentration (MIC) of PG against *B. subtilis*, *M. luteus*, *S. epidermidis*, *E. coli*, and *P. vulgaris* has found at concentration 6.4 mg/ml; against *S. aureus* and *L. pentosus* at concentration 12.8 and 25.6 mg/ml, respectively (Nantawanit, 2001). Film-forming property of polysaccharide gel has been investigated to evaluate its application as a dressing for medicinal purpose. Film of durian polysaccharide gel has been prepared

by casting/solvent evaporation method from an aqueous mixture of polysaccharide gel with plasticizer (Gerddit, 2002). PG dressing film can be satisfactory used in treatment of open wound better than an ordinary treatment (Nakchat, 2002). Polysaccharide gel has expected to be useful in diet control food due to the result of PG at 2% w/v concentration has good property in trapping cholesterol and fatty acids (Tippayakul, 2002) and moderately in trapping sugars (glucose and sucrose). However, fat-soluble vitamin (vitamin A) and water-soluble vitamin (vitamin B1) has also being excluded (Piyasirananda, 2003). Polysaccharide gel has successfully used in preparation of vitamin E gel as well as a mucoadhesive film for mouth refreshing and treatment of ulcer (Lertchaiporn, 2003; Tachatawepisarn, 2003).

Many products from plants are being used in traditional medicine for treatments of bacterial infections, some of these have been subjected to *in vitro* screening for medicinal purpose but the efficacy and clinical trial of such herbal medicines has not been scientifically evaluated enough. At present people prefer to use herbal medicines instead of antibiotics even though their toxic effects have not been enough evaluated. Thus healthcare professionals should be aware of the available unexpected toxic evidence by herbal antibiotic (Karen, 2003). We are interested in the essential oils produced by herbal plants that have traditionally been used for treatment of infection. Essential oils have long been known posses antimicrobial activity of broad spectrum of a wide variety of them against pathogens (Shigeharu *et al.*, 2001).

The object of this study was to prepared antiseptic gel product that has been widely used as a hand disinfectant by using polysaccharide gel (PG) from durian fruit-rind (*Durio zibethinus* Murr.) as a major antimicrobial ingredient together with antimicrobial essential oil such as tea tree oil (*Malalueca alternifolia* Cheel) and betel oil (*Piper betle* Linn). Formulation development was performed, satisfactory products were evaluated its product stability and antimicrobial activity against bacteria and fungi.

2. Literature Review

2.1 Disinfections, Hand washing and antiseptic (G. Sykes, 1958 and Daniel K. Brannan, 1997.)

The terms antiseptic, hand washing and disinfections are described as follows,

Antiseptic: Literally interpreted from its Greek origin it means "against putrefaction" but it was now been extended to include activity against bacteria or infection. By inference, the word was meaning similar to that of "disinfectant", especially in surgery and hygiene. It can be to denote a property of inhibiting or preventing the growth of microorganisms under prescribed condition of usage. Unfortunately, its significance has been belittled in recent years by the appearance on the market of so many preparations such as mouth washes, tooth pastes, product for use on hand and the like, which are barely able even to prevent the growth of micro-organisms in normal practice. This was felt to be so important in the United States that a paragraph defining the term antiseptic was included in the Federal Food, Drug and Cosmetic Act of 1938. Which reads: "the representation of a drug, in its labeling, as an antiseptic, shall be considered purporting to be, or represented as, an antiseptic for inhibitory use as a wet dressing, ointment, dusting powder, or such other use as involves prolonged contact with the body."

Hand washing: Personnel must always wash their hands after using bathroom or whenever they are returning to the production area. The hands should be clean before working on any product or production equipment. Prominently post signs to remind employees to wash their hands after using toilet facilities and before returning to work. Proper hand washing requires readily accessible and adequate hand washing facilities that encourage hand washing. Facilities should include hot and cold running water, a pleasant it use hand cleansing agent, and single-use towels or no contact hand dryers. Use of an antimicrobial hand-cleansing agent is not important as using one that encourages the hand washing process. The agent should be pleasing. It should feel good during use. It should impart a soothing smooth texture to the hands after use and leave a pleasant odor to the hands that lingers. These signals will promote a behavior modification process that results increased hand washing. Use of an

emollient hand cleansing agent will also help prevent skin irritation, chapping and cracking that can discourage hand washing. This cracking also provides tiny foci for microbial growth. Hot air dryers also may cause excessive skin drying and cracking. If the plant uses hand-dip basins or antiseptic sprays, locate them immediately inside the re-entry site to the production area. Select the antiseptic agent used for these rinses base on both antimicrobial activity and mildness on the skin.

Disinfections: May be defined as the process of eliminating or destroying infection: it is accomplished by the use of a disinfectant. The term was introduced before the establishment of the germ theory of infection, and so, because disease was always associated with foul odors, it tended to infer primarily the destruction or masking of these odors, although often the killing of bacteria was concomitant. On this account, the term disinfections are frequently still confined to the strong smelling coal-tar fluids, whereas in fact it has a much wider application and meaning. Several authorities, with some justification prefer to confine the use of the word to the treatment of inanimate objects, and this is the generally accepted context.

2.1.1 Types of Microbial Flora

In 1938, Price divided the skin flora into two classes: residents and transients. Microorganisms that survive to multiply and colonize the skin are resident flora. Most skin antiseptics are somewhat ineffective against the resident flora. Resident flora is hardier (more resistant) and more persistent than transient flora. Different parts of the body of course have different flora, but most pervasive are the aerobic staphylococci (*S. epidermidis, S. aureus*), micrococci, diphtheroids, and *Propionibacterium acnes*. Typically the hair, face, axils, and groin harbor the highest amounts of bacteria, while the arms and hands harbor lower levels. Of the hands, the areas around and under the fingernails show the most microorganisms.

Larson has provided an extensive review of the physiologic and bacteriologic characteristics of the skin (Larson, 1985). The fingernail area is associated with a major portion of the hand flora. The subungual areas (located under the fingernail) often harbor high numbers of microorganisms, which may serve as a source of continued shedding, especially under gloves (MzGinley *et al.*, 1988).

According to Noble and Somerville (1998) other organisms may be present in small numbers. These include *Sarcina*, yeasts, and Gram-negative bacilli. *Staphylococcus aureus* colonizes intact, damaged, or abnormal skin. Only 20% of organisms are in the depths of the skin while the rest are near the surface. Microflora located in the hair follicle is especially inaccessible. Resident flora rarely causes infections unless the skin barrier is compromised. This may occur during an invasive procedure, such as surgery or catheterization. Newborn infants and patients with depressed host resistance are also susceptible.

Artificial nails (Pottinger *et al.*, 1989) and chipped nail polish (Wynd *et al.*, 1994) may be associated with a further increase in the number of bacteria on fingernails.

Transient floras, such as intestinal Gram-negative bacilli, do not survive or colonize normal skin well. Examples of transient bacteria include the *Streptococcus, E. coli* and *Pseudomonas* species. One can remove the transient bacteria more easily from the skin than the residents. Most organisms survive poorly on the surface of the skin. This selection occurs because most organisms are poorly resistant to air-drying and to the production of acidic by-products from other organisms on the skin. Some organisms can be either resident or transient flora such as *Staphylococcus aureus* and various Gram-negative organisms.

The microbial flora of the skin consists of resident (colonizing) and transient (contaminating) microorganisms. The resident microorganisms survive and multiply on the skin. Resident flora includes the coagulase-negative staphylococci, members of the genus *Corynebacterium* (diphtheroids or coryneforms), *Acinetobacter* species, and occasionally member of the Enterobacteriaceae group (Larson, 1995). Resident skin microorganisms are not usually implicated in nosocomial infections, other than minor skin infections; however, some can cause infections after invasive procedures, when the patient/client is severely immunocompromised or has an implanted device, such as a heart valve or artificial hip.

Hand washing with plain soap (detergents) is effective in removing most transient microbial flora (Reybrouck, 1986). The components of good hand washing include using an adequate amount of soap, rubbing the hands together to create some friction, and rinsing under running water. The mechanical action of washing, rinsing and drying removes most of the transient bacteria present (Gould, 1994).

In some studies, air dryers have been shown to reduce the number of organisms on hands after hand washing (Ansari *et al.*, 1991). Several studies have demonstrated that air hand dryers are unsuitable for use in critical patient care areas because of the potential for cross infection, either through airborne dissemination or contaminated personnel (Hanna *et al.*, 1996). Air dryers may be an impediment to hand drying because of the time taken to dry hands and the need to ensure that the equipment is functioning.

2.1.2 Soaps and Antiseptic Agents

The purpose of hand washing is to remove soil, organic material and transient microorganisms from the skin. Few clinical studies have defined the absolute indications for hand washing with plain soaps (detergents) versus hand antisepsis with antimicrobial products. Controlled trials have not documented decrease infection with the use of an antiseptic agent over plain soap for routine hand washing in the general health care setting. The degree of reduction in microbial numbers on the hands of health care providers necessary to protect the recipient of care has not been defined. A few studies have suggested that antiseptic agents may be preferable for the care of patients if there is a possibility of antimicrobial-resistant organisms, such as in intensive care units (Doebbeling *et al.*, 1992), in the presence of antimicrobial-resistant organisms (Webster *et al.*, 1994), and under conditions of heavy microbial soiling (e.g., in the presence of infection or a high level of contamination with organic matter such as feces) (Ehrenkran and Alfonso, 1991).

Understanding the distinctive ingredients and uses of the soap and antiseptic products available is important in choosing the appropriate agent for the appropriate situation. If an antiseptic product is used, it's type and spectrum of

activity, its onset and duration of activity, the application for which it will be used, its cost, allergenic potential and acceptability to the users. Whatever product is used, it should be applied at the right dilution for the recommended time with standard methods of application.

Antiseptic hand cleansers are designed to rapidly wash off the majority of the transient flora by their mechanical detergent effect and to exert an additional sustained antimicrobial activity on the resident hand flora in the following tables (Larson, 1989).

The goal of a preoperative scrub is to remove or kill as many transients and resident flora as possible. By blinding to the skin, a disinfectant may continue exerting a residual effect for some time. This is important during surgery. Under glove occlusion, the skin flora multiplies too extremely high levels. If a nick in the glove occurs, the bacteria-laden sweat can leak into the patient.

A perceived danger of repeated use of antiseptics is that they may destroy much of the normal resident flora and allow colonization with other microorganisms. This supposedly upsets the ecosystem of the skin and allows the skin's natural defenses to be ineffective against opportunistic bacteria. Although this hypothesis is intuitively logical, few studies have conclusively proven it.

A. **Soaps and Antiseptic Agents for Hand Washing** (Health Canada Laboratory Center for Disease Control, 1998)

Product	Indications	Special considerations
Plain soap, bar soap, Liquid*, granules	For routine care of patients/residents/clients (Larson, 1996). For washing hands soiled with dirt, blood or other organic material	May contain very low concentrations of antimicrobial agents to prevent microbial contamination growth in the product. Bar soap should be on racks that allow water to drain; small bars that can be changed frequently are safest.
Waterless antiseptic agents: - Rinses - Foams - Wipes - Towelettes	Demonstrated alternative to conventional agents (Butz, Laughon, Gullette, 1990) For use where hand washing facilities are inadequate, impractical or inaccessible (e.g., ambulances, home care, mass immunization) For situations in which the water supply is interrupted (e.g., planned disruptions, natural disasters)	Not effective if hands are soiled with dirt or heavily contaminated with blood or other organic material. Follow manufacturer's recommendations for use. Efficacy affected by concentration of alcohol in product. Hand creams should be readily available to protect skin integrity (France, 1968).
Antiseptic agents	May be chosen for hand scrubs prior to performance of invasive procedures (e.g., placing intravascular lines or devices) (Maki, 1994) When caring for severely immunocompromised individuals Based on risk of transmission (e.g., specific microorganisms) Critical care areas Intensive care nurseries Operating room scrub When caring for individuals with antimicrobial resistant organisms (Webster, Faogali and Cartwright, 1994).	Antiseptic agents may be chosen if it is felt important to reduce the number of resident flora or when the level of microbial contamination is high. Antiseptic agents should be chosen when persistent antimicrobial activity on the hands is desired. They are usually available in liquid formulations*. Antiseptic agents differ in activity and characteristics (Lorson, 1989). Routine use of hexachlorophene is not recommended because of neurotoxicity and potential absorption through the skin (O'Connor and Rubino, 1991). Alcohol containers should be stored in areas approved for flammable materials.

* Disposable containers are preferred for liquid products. Reusable containers should be thoroughly washed and dried before refilling, and routine maintenance schedules should be followed and documented. Liquid products should be stored in closed containers and should not be topped-up

B. Characteristics of Antiseptic Agents (Health Canada Laboratory Center for Disease Control, 1998)

Group and subgroup	Gram-positive bacteria	Gram-negative bacteria	*Mycobacterium tuberculosis*	Fungi	Virus	Speed of killing sensitive bacteria	Inactivated by mucus or proteins	Comments
Alcohols	Good	Good	Good	Good	Good	Fast	Moderate	Optimum strength 70% to 90% with added emollients (glycerin or cetyl alcohol is less drying), not recommended for physical cleaning of skin; good for hand antisepsis and for surgical site preparation.
Chlorhexidine 2% and 4% aqueous	Good	Good	Fair	Fair	Good	Intermediate	Minimal	Has persistent effect; good for both hand washing and surgical site or preoperative patient skin preparation; do not use near mucous membranes; toxic effects on ears and eyes reported; activity neutralized by nonionic surfactant.
Hexachlorophene 3% aqueous	Good	Poor	Poor	Poor	Poor	Slow	Minimal	Provides persistent, cumulative activity after repeated use (washing with alcohol reduces persistent action), can be toxic when absorbed from skin especially in premature infants; good for hand washing but not for surgical site preparation; limited spectrum of antimicrobial activity.
Iodine compounds, iodine in alcohol	Good	Good	Good	Good	Good	Fast	Marked	Causes skin "burns," but this is unusual with 1% tincture, especially if it is removed after several minutes; too irritating for hand washing but excellent for surgical site preparation.
Iodophors	Good	Good	Fair	Good	Good	Intermediate	Moderate	Less irritating to the skin than iodine; good for both hand washing and surgical site preparation; rapidly neutralized in presence of organic materials such as blood or sputum.
Para-chloro-meta-xylenol (PCMX)	Good	Fair*	Fair	Fair	Fair	Intermediate	Minimal	Activity neutralized by nonionic surfactants.
Triclosan	Good	Good	Fair	Poor	Good	Intermediate	Minimal	

Activity improved by addition of chelating agent such as EDTA

Note: Some of these agents, such as iodine or chlorhexidine, are combined with alcohol to form tinctures and are available in the combined formulation.

Table used with permission of author and publisher (Larson, 1996)

2.2 Gelling agent

The use of polysaccharide in cosmetic has been as ubiquitous as the use of cosmetic themselves. Historically, because of there ready availability from common natural sources and their varied and unique multifunctional, polysaccharides have been included in cosmetics for centuries, for example, the use of β-glucan, derived from yeast extracts, as a natural healing agent. Today, polysaccharides play as even larger role in cosmetic formulation technology. The interplay of polysaccharides with other ingredients in a formulation (e.g., actives, surfactants, salts, other polymers, etc.) and their facile chemical modification has allowed their preeminent use in cosmetics. In addition, polysaccharides of natural origin and polymeric are renewable and have safety profile not accorded synthetic-based polymers.

The classification of gel is determined by considering some characteristic of either of the two phases. Gels are divided into inorganic and organic gels on the basis of the nature of the colloidal phase. These are further subdivided according to the chemical nature of the dispersed organic molecules. Most natural gums such as acacia, carrageenan and xanthan gum are anionic polysaccharide. A number of cellulose derivatives have been synthesized and are effective gellant; among them are sodium carboxymethylcellulose, hydroxyethyl cellulose, hydroxypropyl cellulose and methylhydroxypropyl cellulose. Gelling agent are useful as binder in tablet granulation, protective colloids in suspensions, thickeners in oral liquids, and suppository bases. Cosmetically, gels have been employed in a wide variety of products, including shampoos, fragrance products, dentifrices, and skin and hair care preparation.

Polysaccharides in solution can exist as loose random coils or rigid helices. They can be anionic, cationic, nonionic, or even amphoteric depending on the chemical identity expressed on or pendant to their native backbones. They can be single coils, double coils, and even aggregates of coils the nature of which can be influenced by, among other things, temperature, concentrations, and other species such as salts.

2.2.1 **Alginic acid** (Chaplin, 2005)

Alginic acid, present in the cell wall of certain brown seaweeds, is in itself only sparingly soluble in water. As the sodium salt, however, it is very soluble in water and falls into the use category of such thickening agents and gum substitutes as CMC and methylcellulose. The calcium salt, on the other hand, is soluble in dilute alkaline, and textile fibers can be spun from it. Structurally, alginic acid resembles cellulose closely, except that a –COOH group is present in place of the primary hydroxyl group $-CH_2OH$ (Battita, 1958).

Alginic acid essentially has a very simple primary structure but its useful properties depend not only on its secondary structures but also on the manner with which small components such as water and ions interact with these polymeric structures. Its biological synthesis occurs via poly-1, 4-→D-mannuropyranosylic acid (M). This polymonosaccharide, mannuronan, then undergoes post-polymeric modification by a C5-epimerase that introduces blocks of poly-1, 4-(-L-guluropyranosylic acid (G or guluronan) and regions of mixed M and G composition between the M and G blocks. Alginates with higher content of G show a greater affinity for Ca^{2+} ions and form stronger, harder gels. Spinning oriented fibres from such gels has not been very successful but they have been shown to contain the same molecular conformations as the acid forms which can be obtained in uniaxially oriented, polycrystalline form. Purified forms of alginate are used in antacid preparations such as Bisodol®, Asilone®, and Boots Own® tablets. Alginate is used extensivly as a mold-making material in dentistry and prosthetics, and in textiles. It is also used in the food industry, for thickening soups and jellies.

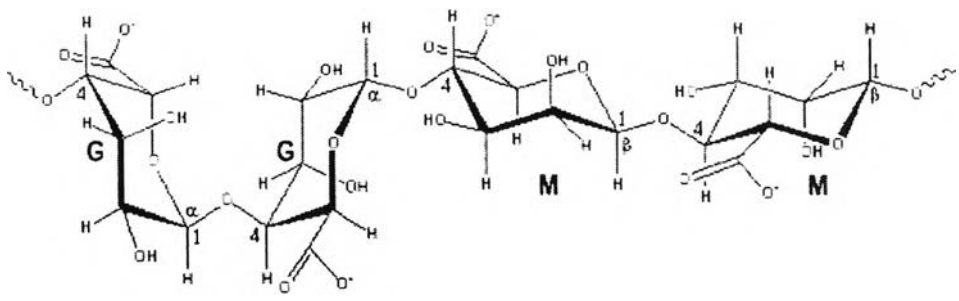

Figure 1. The structure of Alginic acid.

2.2.2 **Pectin** (Chaplin, 2004)

Pectin is a heterogeneous grouping of acidic structural polysaccharides, found in fruit and vegetables and mainly prepared from 'waste' citrus peel and apple pomace. Pectin is a complex polysaccharide consisting mainly of esterified D-galacturonic acid resides in an α-(1-4) chain. The acid groups along the chain are largely esterifed with methoxy groups in the natural product. There can also be acetyl groups present on the free hydroxy groups. The galacturonic acid main chain also has the occasional rhamnose group present which disrupts the chain helix formation. Pectin is also known to contain other neutral sugars which are present in side chains. The most common side chain sugars are xylose, galactose and arabinose. The sidechains tend to occur in groups and have led to the description of the pectin molecule as having hairy and smooth regions. Pectins are mainly used as gelling agents, but can also act as thickener, water binder and stabilizer. Low methoxyl pectins (< 50% esterified) form thermoreversible gels in the presence of calcium ions and at low pH (3 – 4.5) whereas high methoxyl pectins rapidly form thermally irreversible gels in the presence of sufficient (*e.g.* 65% by weight) sugars such as sucrose and at low pH (< 3.5); the lower the methoxyl content, the slower the set. The degree of esterification can be (incompletely) reduced using commercial pectin methylesterase, leading to a higher viscosity and firmer gelling in the presence of Ca^{2+} ions. Highly (2-O- and/or 3-O-galacturonic acid backbone) acetylated pectin from sugar beet is reported to gel poorly but have considerable emulsification ability due to its more hydrophobic nature, but this may be due to associated protein impurities.

As with other viscous polyanions such as carrageenan, pectin may be protective towards milk casein colloids, enhancing the properties (stability, solubility, gelation and emulsification) of whey proteins whilst utilizing them as a source of calcium.

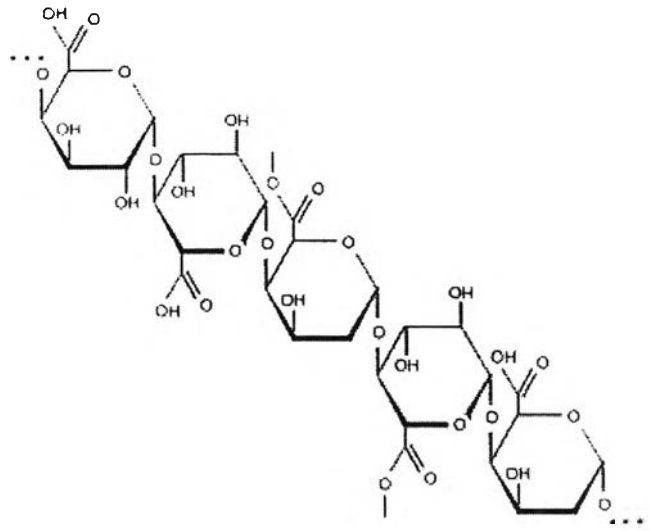

Figure 2. The structure units of pectin.

2.2.3 **Carrageenan** (Chaplin, 2005)

Carrageenan is a collective term for polysaccharides prepared by alkaline extraction (and modification) from red seaweed (*Rhodophycae*), mostly of genus *Chondrus*, *Eucheuma*, *Gigartina* and *Iridaea*. Different seaweeds produce different carrageenans. Carrageenans are linear polymers of about 25,000 galactose derivatives with regular but imprecise structures, dependent on the source and extraction conditions. Idealized structures are given below and κ-carrageenan, for example, has been found to contain a small proportion of the dimer associated with ι-carrageenan.

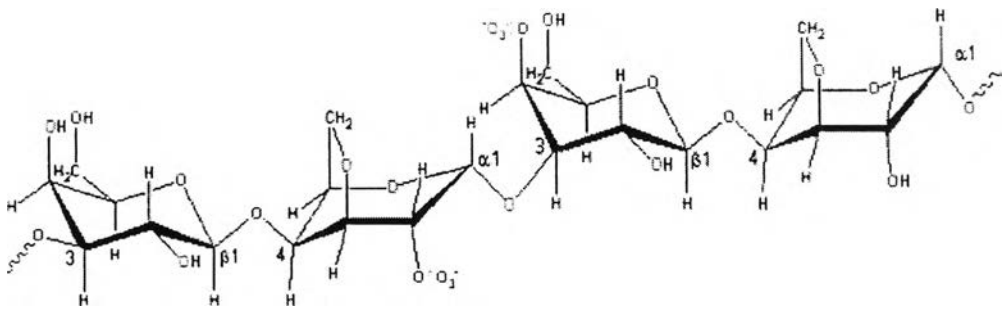

Figure 3. Carrageenan consists of alternating 3-linked-β-D-galactopyranose and 4-linked-α-D-galactopyranose units.

κ-carrageenan is produced by alkaline elimination from μ-carrageenan isolated mostly from the tropical seaweed *Kappaphycus alvarezii* (also known as *Eucheuma cottonii*).

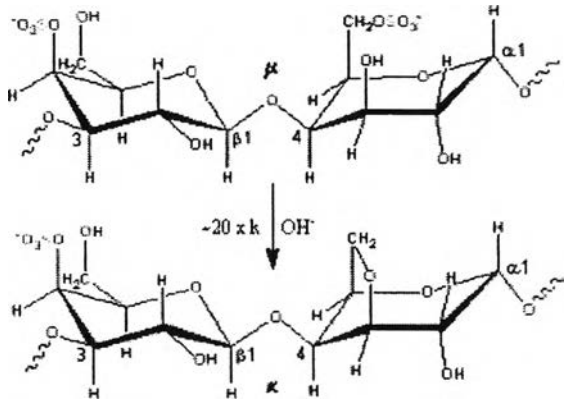

Figure 4. The structure of κ -carrageenan (kappa-carrageenan)

ι-carrageenan is produced by alkaline elimination from ν-carrageenan isolated mostly from the Philippines seaweed *Eucheuma denticulatum* (also called spinosum). The three-dimensional structure of the ι-carrageenan double helix has been determined as forming a half-staggered, parallel, threefold, right-handed double helix, stabilized by inter-chain O2-H···O-5 and O6-H···O-2 hydrogen bonds between the β-D-galactopyranose-4-sulfate units.

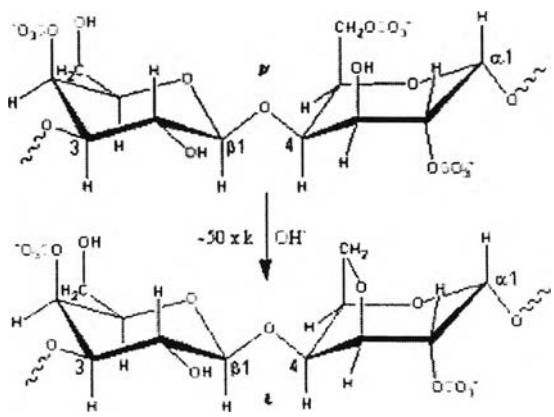

Figure 5. Structural of ι-carrageenan (iota-carrageenan)

λ-carrageenan (isolated mainly from *Gigartina pistillata* or *Chondrus crispus*) is converted into θ-carrageenan (theta-carrageenan) by alkaline elimination, but at a much slower rate than causes the production of ι-carrageenan and κ-carrageenan.

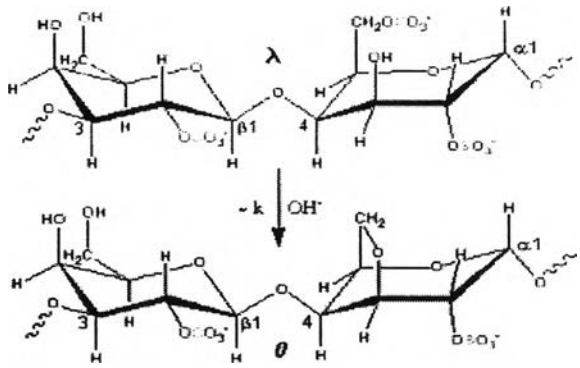

Figure 6. Structural of λ-carrageenan (lambda-carrageenan)

All carrageenans are highly flexible molecules, which at higher concentrations, wind around each other to form double-helical zones. Gel formation in κ- and ι-carrageenans involves helix formation on cooling from a hot solution together with gel-inducing and gel-strengthening K^+ or Ca^{2+} cations respectively (not Na^+, although Na^+ does take part in an aggregation process to form weak gels with κ-carrageenan due to phase separation), which not only aid helix formation but subsequently support aggregating linkages between the helices so forming the junction zones. Incomplete formation of $^1C_4$ 3, 6-anhydro-links will reduce the extent of helix formation as the unbridged a-linked galactose residues may flip to the $^4C_1$ conformation.

Note that the gelling hydrocolloid agar is produced from the same family of seaweeds, the major difference being the presence of L- rather than D-3, 6-anhydro-a-galactopyranose units but still forming double helical junction zones. Carrageenans are used mainly for thickening, suspending and gelling. κ- and ι-carrageenans form thermoreversible gels on cooling in the presence of appropriate counterions. κ-Carrageenan forms a firm clear, if brittle, gel with poor freeze-thaw stability; the coil-double helix transition being followed by a K^+ induced aggregation of the helices. κ-Carrageenan gels may be softened (and is generally regarded to be

synergistically strengthened) with locust bean gum. ι-Carrageenan has less specific ionic binding but increased ionic strength allows helices to form junction zones in soft elastic gels with good freeze-thaw stability. λ-Carrageenan is non-gelling as the lack of the $^{1}C_{4}$ 3,6-anhydro-link allows the galactose residues to revert to their $^{4}C_{1}$ conformation, which does not allow the initial double helix formation, required for gelling. Additionally, the high density of charged sulfate groups encourages an extensive conformation. λ-Carrageenan has been found to act as a cryoprotectant and improves the freeze-thaw behavior of locust bean gum. κ-Carrageenan stabilizes milk κ-casein products due to its charge interaction with the casein micelles (~200 nm diameter); their incorporation into the network preventing whey separation. Such complexes are soluble when both have same charge and are held together by counter ions or oppositely charged patches. Carrageenan is also used as a binder in cooked meats, to firm sausages and as a thickener in toothpaste and puddings.

2.2.4 Xanthan gum

Xanthan gum is a microbial desiccation-resistant polymer prepared commercially by aerobic submerged fermentation from *Xanthomonas campestris*. It is naturally produced to stick the bacteria to the leaves of cabbage-like plants. It is relatively expensive by weight but becoming rather less so. The structure of xanthan gum consists of a main chain built up of β-D-glucose units linked through the 1- and 4-position, and a side chain containing two mannose units and one glucuronic acid unit found as a mixed potassium, sodium and calcium salt. Xanthan gum is mainly considered to be non-gelling and used for the control of viscosity due to the tenuous associations endowing it with weak-gel shear-thinning properties. It hydrates rapidly in cold water without lumping to give a reliable viscosity, encouraging its use as thickener, stabilizer, and emulsifier and foaming agent. The consistent water holding ability may be used for the control of syneresis and to retard ice re-crystallization (ice crystal growth) in freeze-thaw situations; xanthan gel strength being improved on freeze-thaw. Its most important property being its very high low-shear viscosity coupled with its strongly shear-thinning character. The relatively low viscosity at high shear means that it is easy to mix, pour and swallow but its high viscosity at low shear gives good suspension and coating properties and lends stability to colloidal suspensions. Being relatively unaffected by ionic strength,

pH (1 - 13), shear or temperature it may be used in such products as salad dressings. Xanthan gum, like locust bean gum will not gel.

2.2.5 Chitosan

Chitosan is a high molecular weight polycationic polysaccharide derived from naturally occurring chitin by alkaline deacetylation. Chemically, it is a poly (N-glucosamine). Chitosan has favorable biological properties such as non-toxicity, biocompatibility and biodegradability. Similar to other polysaccharides it also undergoes degradation by the action of colonic micro flora and hence posses it's candidature for colon targeted drug delivery. Tozaki *et al.* (1997) developed colon-specific insulin delivery with chitosan capsules.

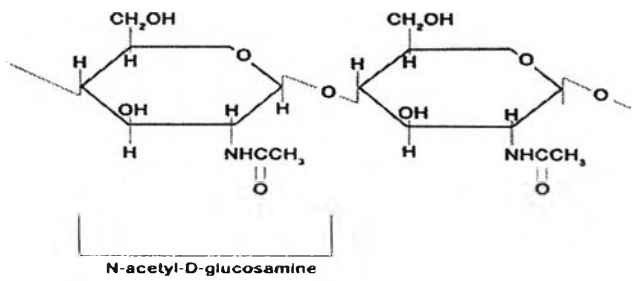

Figure 7. The structure of chitosan

2.2.6 Carboxymethylcellulose (CMC) (Battista, 1958)

Carboxymethylcellulose (CMC) is a derivative of cellulose formed by its reaction with alkali and chloroacetic acid. The CMC structure is based on the β-(1→4)-D-glucopyranose polymer of cellulose. Different preparations may have different degrees of substitution, but it is generally in the range 0.6 - 0.95 derivatives per monomer unit. CMC dissolves rapidly in cold water and mainly used for controlling viscosity without gelling CMC, at typical concentrations, does not gel even in the presence of calcium ions. As its viscosity drops during heating, it may be used to improve the volume yield during baking by encouraging gas bubble formation. Its control of viscosity allows use as thickener, phase and emulsion stabilizer (*e.g.* with milk casein), and suspending agent. CMC can be also used for its water-holding capacity, as this is high even at low viscosity, particularly

when used as the Ca^{2+} salt. Thus, it is used for retarding staling and reducing fat uptake into fried foods.

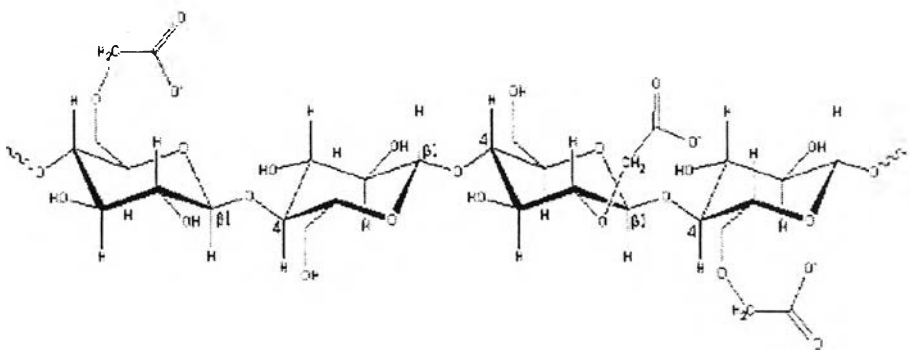

Figure 8. Structural of Carboxymethylcellulose (CMC)

2.2.7 Hydroxypropyl methycellulose (HPMC)

Hydroxypropyl methycellulose (HPMC) is nonionic cellulose ether available in a variety of type and viscosities. There are four distinct USP grades of hydroxypropyl methylcellulose with vary levels of methyl and hydroxypropyl substitution. The methyl substitution imparts to HPMC one of its unique features thermal gelation. Solutions of hydroxypropyl methylcellulose may be sterilized by autoclaving without a loss of viscosity. Hydroxypropyl methylcellulose is surface active and reduces surface tension and interfacial tension. Solutions of HPMC exhibit pseudoplastic rheology and there is no yield point.

2.2.8 Polyacrylic acid (Carbomer)

The chemical composition and the chemical and physical properties of the carbopol resins suggest that similar toxicological properties are to be expected with these polymers. The carbopol resins are cross-linked homopolymers of acrylic acid or cross-linked copolymers of acrylic acid with a minor acrylic comonomer. The molecular weight range of these polymers is estimated to be from 740,000 to 4-5 million. There are no methods available to measure the actual molecular weight of a cross-linked (i.e. 3-dimensional) polymer of this type. The backbone of the homopolymer carbopol is the same (see Figure 9). The main difference is related to cross-link density and molecular weight, rather than the cross-linker used. With very minor adjustments in the cross-linker density, one can produce

a large number of carbopol type products similar in gross molecular structure but varying in application properties, for example, viscosity. Cross-link density can be varied by minor shifts in position of the cross-linker on the acrylic backbone. The three-dimensional nature of these polymers confers some unique characteristics, such as biological inertness, not found in similar linear polymers. The carbopol resins are hydrophilic substances that are not soluble in water. Rather, these so-called "water soluble" resins swell when dispersed in water forming a colloidal, mucilage-like dispersion. Many of the carbopol resins have found diverse applications in the cosmetic, detergent and pharmaceutical industries. Due to their physical properties, inertness and low toxicity, the carbopol resins have been used in such preparations as suspending, flow control, thickening and emulsion stabilizing agents. Another family member, carbopol 934P, has a National Formulary (USP-NF) monograph, carbomer 934-P, pertaining to its use as a drug ingredient.

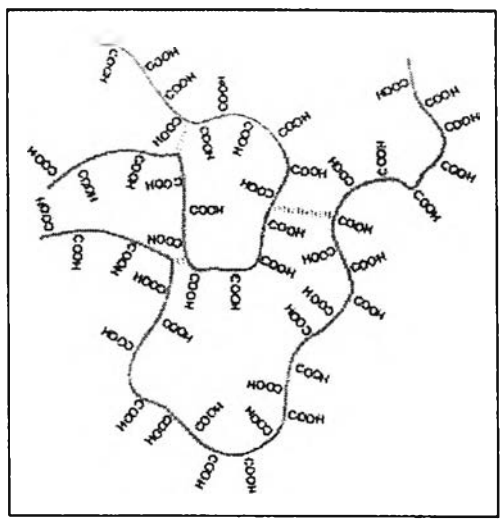

Figure 9. Schematic drawing of a molecular segment of a cross-linked polyacrylic acid polymer

2.3 Gels (Zatz and Kushla, 1996)

2.3.1 Definitions:

"Gel: a substance in a state between solid and liquid; jelly: hair gel" (Longman Dictionary of Contemporary English 2nd Ed., 1987)

The United States Pharmacopeia (USP) defines gels as semisolids, being either suspension of small inorganic particles or large organic molecules interpenetrated with liquid. Gels are transparent or translucent semi-solid or solid preparations, consisting of solutions or dispersions of one or more active ingredients in suitable hydrophilic or hydrophobic bases. They are made with the aid of a suitable gelling agent. Usually gels exhibit pseudo-plastic flow properties and those made with synthetic or semi-synthetic polymers with a high degree of cross-linking have relatively high yield values and low viscosity. Gels are often non-greasy and are generally applied externally. As vehicles for the presentation of water-soluble medicaments, gels are ideal because of their high water content. Products tend to be smooth, elegant, and produce cooling effects because of evaporation of water; they may also dry out to form films. Films adhere well to the skin and are usually easily removed by washing; gelatin-containing films may be less readily removed. For the presentation of insoluble materials hydrophilic gels have the limitation that the resultant products may lack clarity and smoothness. Ideally, gelling agents for pharmaceutical and cosmetic use should be inert, safe, and non-reactive with other formulation components. A potential incompatibility is illustrated by the combination of a cationic drug, preservative, or surfactant with an anionic gel former. Inactivation or precipitation of the cationic substance is possible. Sodium alginate has been shown to reduce the concentration of cationic preservatives in solution, as well as complex with chlorpheniramine, reducing the drug release rate from gelled formulations. Polyesters have been shown to interact with phenols and carboxylic acids, leading to reasonable solid like nature during storage that can be broken easily when subjected to the shear forces generated in shaking a bottle, squeezing a tube, or during topical application. Cost considerations require a low concentration of gallant to produce the desired characteristics.

The gel should exhibit little viscosity change under the temperature variations of normal use and storage. For example, Placitas exhibits a lesser decrease in consistency than petrolatum over the sane temperature range. This minimizes unacceptable changes in the product's characteristics. Many gels, particularly those of a polysaccharide nature are susceptible to microbial degradation. Incorporation of a suitable preservative may prevent contamination and subsequent loss of gel characteristics due to microbial attack. The gel characteristics should match the intended use. A topical gel should not be tacky. Too high a concentration of gel former or the use of an excessive molecular weight may produce a gel difficult to dispense or apply. An ophthalmic gel must be sterile. The aim is to produce a stable, elegant, economic gel product adequately suited for its intended use.

2.3.2 Swelling

Gels can swell absorbing liquid with an increase in volume. Swelling can be looked on as the initial phase of dissolution. Solvent penetrates the gel matrix. Gel-gel interactions are replaced by gel-solvent interactions. Limited swelling is usually the result of some degree of cross-linking in the gel matrix that prevents total dissolution. Such gels swell considerably when the solvent mixture possesses a solubility parameter comparable to that of the gellant.

2.3.3 Syneresis

Many gel systems undergo a contraction upon standing. The interstitial liquid is expressed, collecting at the surface of the gel. This process is referred to as syneresis. Syneresis is not limited to organic hydrogels but has been seen in organogels and inorganic hydrogels as well. Typically, syneresis becomes more pronounced as the concentration of polymer decreases.

The mechanism of concentration has been related to the relaxation of elastic stresses developed during the setting of the gel. As these stresses are relieved, the interstitial space available for solvent is reduced, forcing the expression of fluid. Osmotic effects have been implicated, as both pH and electrolyte

concentration influence syneresis from gels composed of the ionic gel formers gelatin or psyllium seed gum.

2.3.4 Structure

The long chains of organic gel formers are extended in good solvents, as would be the casein aqueous gels, due to hydrogen bond formation between water and hydroxyl groups of the gelling agent. In a poor solvent, the gel molecule would be more or less tightly coiled, preferring self-interaction to interaction with the solvent. Each segment of the dissolved molecules is in constant random motion, buffeted by the movement of solvent molecules through the bulk of the liquid. This random motion serves to entangle polymer strands. Molecular entanglement is responsible for the viscosity and structure of organic gels.

The organic polymers used in hydrogels tend to be sheathed with an envelope of water of hydration. This enables the polymer molecules to slip past each other at low concentrations due to the lubricity of the intervening water molecules. If the degree of hydration is low, then intermolecular attractive forces such as hydrogen bonding and Van der waals forces form weak secondary bonds between polymer strands. At sufficiently high concentration, a continuous network of weakly interacting chains can be formed. The association may proceed far enough to produce small local regions of crystalline nature dispersed through a bed of randomly entangled polymer strands.

Salts may attract part of the water of hydration of the polymer, allowing the formation of more intermolecular secondary bonds, leading to gelation and precipitation. This is known as salting out. Multivalent cations have a strong effect on the solutions of anionic polymers. Bridging of the polymers by di- or tri-valent cations, as in the addition of copper to solutions of sodium carboxymethyl cellulose or calcium to sodium alginate, leads to gel formation.

Alcohols have a similar effect. In addition, alcohols alter the solvent's characteristics, changing the solubility parameter. The addition of alcohol often brings about coacervation rather than gelation. Coacervation is the

production of a viscous, solvated, polymer-rich phase, leaving behind a phase that is mostly solvent and therefore, polymer poor.

Many gel formers are more soluble in hot than cold water. If the temperature is reduced once the gel is in solution, the degree of hydration is reduced and gelation occurs. Some polymers exhibit thermal gelation. These polymers are more soluble in cold water; solution of these materials gel on heating. The examples include methylcellulose and poloxamer.

Gelation due to changes in hydration with changes in temperature tends to be reversible; gels liquefy and set again as the temperature is cycled. Gelation due to chemical reactions as in salt bridging or cross-linking is irreversible. These gels cannot be liquefied by dilution or temperature changes.

Molecular weight is an important consideration in gel formation. Very long polymers can entangle to apreater extent, leading to higher viscosity at a given concentration. Thus, lower concentration of a high molecular weight polymer may be required to gel the solvent. This can be drawback exhibited as difficult spreading of a topical gel due to the high cohesive interactions between the gel strands. Likewise, low molecular weight polymers require a high concentration to buildup viscosity and to set to a gel, possibly increasing the cost due to the amount of gellant used and over shadowing the desired properties of the formulation.

2.4 Natural materials of antimicrobial agents

2.4.1 **Tea tree oil or Melaleuca oil** (Homer *et al.*, 2000; Russell and Southwell, 2002)

History and discovery

Tea tree oil, or melaleuca oil, is the essential oil produced by stream distillation of the leaves of the Australian native plant *Melaleuca alternifolia* or tea tree. *M. alternifolia* is in family Myrtaceae, which has single leaves, regular and bisexual flower with 4-5 petals. Several closely related species, *M. cajuputi*, *M.*

leucadendron, *M. linariifolia* and *M. quinquenervia* were used medicinally by the Aboriginals for the treatment of headaches, aches and pains, colds as an insect repellant. The tea tree oil is a paperbark tree with small narrow leaves arranged on fine branch lets. The oil is found in the leaves. Flowering occurs during spring with fluffy white flowers arranged in clusters. In most cases leaves and small braches were crushed and the vapor inhaled. Sometimes bruised leaves were soaked in water, which was then swallowed or poured over the body. The early settlers soon came to learn about the healing power of the tea tree and used it in wounds and bites. It has been used medicinally for almost 70 years and many claims have been made regarding its antimicrobial activity. There is no accurate documentation of specific medicinal application of *M. alternifolia* or the oil by Aboriginals prior to white colonization of Australia. Due to the distillation process required extracting the oil from the leaves of *M. alternifolia*. However, the Bundjabung Aborginals of Northern New South Wales who told of the healing properties of the water into which tea tree leaves had fallen and decayed may have known the medicinal properties. Regardless of the authenticity of these observations, the highly aromatic nature of the leaves of *M. alternifolia* would probably have led to there use by Aboriginals.

In 1770'S, Sir Joseph Banks, The botanist, collected samples of leaf from several species of *Melaleuca* and brewed up a native tea, giving them their common name, " Tea Tree". However extensive use of tea tree oil did not begin until its antiseptic and disinfectant properties were reported early this century.

In 1922'S, Arthur Penfold, curator and chemist, His studies determined that tea tree oil had a Ridealwalker coefficient of between 11 and 13. This means the tea tree oil is 11 to 13 times more powerful than Carbolic acid (phenol) for killing bacteria and fungi yet non-caustic the skin. Subsequent studies revealed tea tree oil as a broad-spectrum antibacterial and anti-fungal with a wide range of application.

In 1930'S, H. James, managing director, Australian Essential Oils Limited, commercial production began. The raw product his company produced was call "Ti-Trol", and in soluble from of "Melasol". The results of these investigations showed its germicidal value was far greater than anticipated. The use

of Melasol as a disinfectant and inhalant was advocated in reviews of preparations and appliances in the British Medical Journal 1933. The journal indicated that a wide range of conditions had responded to the application of tea tree including epiclesis, ringworm, tinea, throat and mouth conditions (Acute nasopharyngitis, catarrh, "apthous" atomatitis, tonsillitis, mouth ulcers and sore throat), pyorrhea and gingivitis. Melasol was used to treat large diabetic ulcer, which healed without further infection.

During 1960'S - 1970'S, tea tree oil has been experiencing a revival in popularity. The "flower power" generation of the 1960' S and 1970'S created a renewed awareness worldwide of natural products and medicines. The natural product renaissance seen in recent years has resulted in a large range of products containing tea tree oil becoming available in Australia and overseas. Tea tree oil identified chemotypes of *M. alternifolia* have three chemotypes distinguished by different levels of 1, 8-cineole. These were described as "Type" oils with a 1, 8-cineole content below 8%, "Variety A" oils with 30-45% 1, 8-cineole and "Variety B" with 54-64% 1, 8-cineole.

Today, tea tree oil has been suggested for the treatment of many common ailments, including acne, thus anti-acne activity of tea tree oil was also determined. 124 patients were randomized in clinical trial to evaluate the efficacy and skin tolerance of 5% tea tree oil gel in the treatment of mild to moderate acne when compared with 5% benzoyl peroxide lotion. The results of this study showed that both 5% tea tree oil and 5% benzoyl peroxide had a significant effect in ameliorating the acne by reducing the number of inflamed and non-inflamed lesions (open and close comedones) and fewer side effects were experienced by patients treated with tea tree oil (Bassett *et al.*, 1990).

Tea tree oil has many commercial and personal applications. Many households retain a bottle of tea tree oil for treating common ailments such as acne, minor cuts, dandruff, boils and fungal infections. Moreover, tea tree oil is also used commercially in medicated shampoos, soaps, antiseptic cream, and cosmetics, in air conditioning ducting to kill bacteria, in insecticides and in many other ways. New uses are currently in the process of development. Tea tree oil is produced by stream

distillation of freshly harvested leaves and terminal branches with water. The tea tree oil is then separated from the condensed aqueous distillate and varies from clear to pale yellow in color. The yield oil is relatively low, as it occurs at a concentration of approximately 1-2% of the wet plant material weight (Carson and Riley, 1993).

Oil components

Tea tree oil (TTO) is essential steam distilled from *Melaleuca alternifolia.* TTO contains over 100 components, the majority being monoterpene and sesquiterpene hydrocarbons and their alcohols. The Australia standard for tea tree oil (AS 2782: 1985) that the 1, 8-cineole level should be standard below 15% and the terpinen-4-ol content should be above 30%. ISO 4730 set a standard of proportional requirement of compositions as the following table. Later Williams *et al.* (1988) reported *M. alternifolia* oils could be found with a range of 0-64%, generally the 1, 8-cineloe content was in the range 6-15%. The 1, 8-cineole oil also portrayed by some workers as an undesirable characteristic of the oil, making it more irritant, and more toxic. Southwell *et al.* (1996) eventually argued against the fact that high terpinen-4-ol oils were superior antimicrobiological agents than those with higher levels of 1, 8-cineole, or that 1, 8-cineole acted as an irritant in these oils. The ρ-cymene can be formed the action of light and oxidation on α- phellandrene and γ-terpinene. As selection has results in marking of high terpinen-4-ol oils, so 1, 8-cineole levels have come down. The total monoterpene hydrocarbon levels are relatively high in tea tree oil, as the combined α-terpinene and γ-terpinene levels along can constitute over 30% of the total composition.

The Australian Standard for tea tree oil (AS: 2782: 1985)

Component	Australian Standard	
	% Min	%Max
α- pinene	1.0	6.0
Sabinene	Tr.	3.5
α- terpinene	5.0	13.0
Limonene	0.5	4.0
ρ- cymene	0.5	12.0
1,8-cineole	none	28.0
γ- terpinene	10.0	28.0
Terpinolene	1.5	28.0
Terpinen-4-ol	30.0	none
α- terpineol	1.5	8.0
Aromadendrene	Tr.	7.0
δ- cadinene	Tr.	8.0
Globulol	Tr.	3.0
Viridiflorol	Tr.	1.5

TTO purchased from Menthol Thai import export Co., Ltd., Bangkok, Thailand, which was supplied by Southern Cross Botanicals Ply, Australian & Newzealand. Batch RTTO1/4023 complied with the international standard for TTO, and contained 39.0% terpinen-4-ol, 20.6% γ-terpinine, 8.4% α-terpinene, 3.0% α-terpineol, 3.2% α-terpinolene, 3.8% 1,8- cineole, 2.7% α-pinene and ρ-cymene. Levels of components were assessed by gas chromatography, performed by Certified Botanical Extracts, Australia & Newzealand. The data were shown in Appendix D (table 1).

Antimicrobial activity

The susceptibility of 32 strains of *Propionibacterium ances* to the essential oil of *melaleuca alternifolia* has been tested by using broth micro-dilution method. As a result, the minimal bactericidal concentration (MBC) of tea tree oil for five strains have been found at 0.25%, while the remainder at 0.50% (Carson and Riley, 1994).

Tea tree oil has been examined and found to have a pH-ideal for using on human and animal skin. The pure tea tree oil is non-toxic and does not damage healthy skin around the area treated. Tea tree oil is a complex mixture of hydrocarbons and terpenes consisting of approximately 100 components. There are eight major components, which terpinen-4-ol is regarded as the main major antimicrobial component. Other major components included ρ-cymene, linalool, α- and γ-terpinene, α-terpineol, terpinolene and 1, 8-cineole. These components constitute approximately 80-90% of tea tree oil. Now, tea tree oil products are marketed widely throughout Australia and there is an increasing export market. This increasingly popular nostrum is widely available in North America and Europe in an extensive range of cosmetics and medicinal products through health food stores, alternative health practitioners and some pharmacies. These products include antiseptic creams, hair care products, acne treatments, mouthwashes, and tea tree oil-impregnated toothpicks. In addition, a wide variety of soaps and skin washes claiming antiseptic properties are also available (Carson, Riely, 1995).

Major components of tea tree oil were also determined. The disc diffusion method was used to determine the susceptibility of a range of microorganisms to 1, 8-cineole, terpinen-4-ol, ρ-cymene, linalool, α-terpinene, γ-terpinene, α-terpineol and terpinolene. Using this method, each of the individual components demonstrated some antimicrobial activity. The growth of *Pseudomonas aeruginosa* was inhibited by terpinen-4-ol only. *Bacteroides fragilis*, *Candida albicans* and *Clostidium perfringens* were inhibited by all of the components tested. Terpinen-4-ol was active against all the test organisms, while linalool and α-terpineol failed to inhibit *Pseudomonas aeruginosa* only. The least inhibitory component was

ρ-cymene, which inhibited only three of the test organisms (*Bacteroides fragilis, Candida albicans and Clostidium perfringens*) (Carson and Riley, 1995).

Terpinen-4-ol, α-terpineol and α-pinene were found to be active against *Staphylococcus aureus*, *Staphlococcus epidermidis* and *Propionibacterium acnes* whereas 1, 8 cineole was inactive against these organisms. The MIC values of the three active components increased in the order α-terpineol< terpinen-4-ol< α-pinene for all three organisms. MIC values of tea tree oil component were lower for *P.ances* than for two *Staphylococci*. This study supports the use of tea tree oil in the treatment of acnes (Raman *et al.*, 1995).

Many scientists have determined antimicrobial activity of tea tree oil. In 1995, the test antimicrobial activity by used a broth micro-dilution method to examine the susceptibility of *Escherichia coli* and *Staphylococcus aureus* to essential oil of *Melaleuca alternifolia* (Carson *et al.*, 1995).

In addition, susceptibility of methicilin-resistant *S. aureus* to tea tree oil was determined. Using disc diffusion method, 64 isolates were methicilin-resistant *S. aureus* (MRSA) and 33 were mupirocin-resistant *S. aureus*. The MIC and MBC were 0.25% and 0.50%, respectively. These results suggest tea tree oil may be useful in the treatment of MRSA carriage (Carson *et al.*, 1995).

The susceptibility of transient and commensally skin flora to the essential oil of *Melaleuca alternifolia* (tea tree oil) was results. *S. aureus* and most of the gram-negative bacteria tested were more susceptibility to tea tree oil than the coagulase-negative Staphylococci and Micrococci. These results suggest that tea tree oil may be useful in removing transient skin flora while suppressing but maintain resident flora (Hammer *et al.*, 1996).

The concentration of each component can vary widely depending on the oil sample and the combination of oil components, which optimizes antimicrobial activity, has yet to be determined. The oil has gained considerable popularity as a topical antimicrobial agent in recent years. It is reputed to have

several medicinal properties including antibacterial, antifungal, antiviral, anti-inflammatory and analgesic properties but the need for appropriate clinical data on the efficacy of tea tree oil products, data on safety are also required. However, some components responsible for adverse reactions to tea tree oil have been identified, including 1, 8-cineole, limonene, α-terpinene, ρ-cymene, aromadendrene, α-phellandrene, α-pinene, terpinolene and α-terpenine. For many years, 1, 8-cineole was regarded as an undesirable constituent in tea tree oil due to its reputation as a skin and mucous membrane irritant although a number of national and international standards for tea tree oil have been developed and implemented over the years. These have been largely superceded by the most recent international standard for tea tree oil. For tea tree to be sold commercially for medicinal purposes in Australia, it must meet the Australia Standard for " oil of Melaleuca Terpinen-4-ol type", which requires the terpinen-4-ol content to exceed 30% and the 1, 8-cineole content to be less than 15%. Terpinen-4-ol is the active antimicrobial constituent and that 1, 8-cineole is potentially a skin irritant (Carson *et al.*, 1998).

The susceptibility of tea tree oil against 58 clinical isolates *Candida albicans*, *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Trichophyton tonsurans*, *Aspergillus niger*, *Penicillium species*, *Epidermophyton floccosum* and *Microsporum gypsum*. Tea tree oil showed inhibitory activity against all isolates tested except one strain of *E. floccosum*. *In vitro* results suggest that tea tree oil may be useful in the treatment of yeast and fungal mucosal and skin infections (Concha *et al.*, 1998).

Tea tree oil was investigated for activity against 81 *C. albicans* isolates and 33 non- *Canida albicans* isolates. By the broth micro-dilution method, the minimal concentration of tea tree oil inhibiting 90% of isolates for both *C. albicans* and non- *Canida albicans* species was 0.25% (v/v). The minimal concentration of oil killing 90% of isolates was 0.25% for *C. albicans* and 0.5% for non-*Candida albicans* species. Fifty-seven *Candida spp.* isolates were tested for sensitivity to tea tree oil by the agar dilution method; the minimal concentration of oil inhibiting 90% of isolates was 0.5%. The tests on three intra-vaginal tea tree oil products showed these products to have MICs and minimal fungicidal concentrations

comparable to those of non-formulated tea tree oil, indicating that the tea tree oil contained in these products has retained its anticandidal activity. These data indicate that this essential oil is against *Canida spp.*, suggesting that it may be useful in the topical treatment of superficial candida infections. (Hammer *et al.*, 1998).

The detergent Tween 80 was used successfully to enhance the solubility of tea tree oil in the test medium. The test broth did not result in the formation of a completely homogeneous solution, but Tween 80 did enhance the solubility of tea tree oil in broth to the point where consistent results could be obtained thereby allowing the antimicrobial activity to be evaluated. *E. coli* was more susceptible to tea tree oil than *S. aureus* with all isolates having minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of $\leq$ 0.25%. In contrast, 77% of the *S. aureus* tested had an MIC of $\leq$ 0.25% while the MBCs spanned the range 0.25-2.0%. Concentration of tea tree oil, which inhibited growth of *E. coli* also inhibited glucose-dependent respiration of *E. coli* and stimulated the leakage of intracellular Potassium ion. Moreover, stationary phase cells had greater tolerance to tea tree oil when compared to exponentially grown cells (Cox *et al.*, 1998 and Gustafson *et. al.*, 1998)

The activities of ketoconazole, econazole, miconazole and tea tree oil against 54 Malassezia isolates were determined by agar and broth dilution methods. Ketoconazole was more active than both econazole and miconazole, which showed very similar activities. *M. furfur* was the least susceptible species. *M. sympodialis*, *M. slooffiae*, *M. globosa* and *M. obtusa* showed similar susceptibilities to the four agents. (Hammer *et al.*, 2000).

The combination of a 4% tea tree oil nasal ointment and 5% tea tree oil body wash was compared with a standard 2%mupirocin nasal ointment and triclosan body wash for the eradication of methicilin-resistant *Staphylococcus aureus* carriage. Tea tree oil combination appeared to perform better than the standard combination (Caelli *et. al.*, 2000).

The antimicrobial effectiveness of tea tree oil is determined in terms of MIC, MBC and minimal fungicidal concentration (MFC). The isolates include methicilin-resistant *Staphylococcus aureus* (MRSA), *S.aureus*, beta-haemolytic *streptococci*, coagulase-negative *staphylococci* and *Pseudomanas spp.*, eleven *Candida spp.* isolates from skin vaginal swabs also are tested. Using an agar dilution assay, The MICs of tea tree oil was 0.5- 1.0% (v/v), whilst with *Ps. aeruginosa* it was > 2% (v/v). The broth micro-dilution method was used to determine MICs and minimal cidal concentration (MCC) of 80 isolates. In 64 isolates, tea tree oil produced an inhibitory and cidal effect at 3% and 4% (v/v), respectively. *S. aureus* and *Candida spp.* were most susceptible to tea tree oil, with MICs and MBCs of 0.5% and 1%, respectively. *Ps. aeruginosa* and the *faecal streptococci* isolates, with MICs and MBCs of > 8%, were resistant to tea tree oil (Banes-Marshall *et al.*, 2001).

The essential oil of *Melaleuca alternifolia* has broad-spectrum antimicrobial activity. The mechanisms of action of tea tree oil and three of its components 1, 8-cineole, terpinen-4-ol and α-terpineol, against *Staphylococcus aureus* ATCC 9144 were investigated. Treatment with these agents at their MICs and two times their MICs, particularly treatment with terpinen-4-ol and α-terpineol, reduced the viability of *S. aureus*. None of the agents caused lysis, as determined by measurement of the optical density at 620 nm, although cells became disproportionately sensitive to subsequent autolysis. Loss of 260 nm absorbing material occurred after treatment with concentration equivalent to the MIC, particularly after treatment with 1, 8-cineole and α-terpineol. *S. aureus* organisms treated eighth tea tree oil or its components at the MIC or two times the MIC showed a significant loss of tolerance to NaCl. When the agents were tested at one-half the MIC, only 1, 8-cineole significantly reduced the tolerance of *S. aureus* to NaCl. Electron microscopy of terpinen-4-ol treated cells showed the formation of mesosomes and the loss of cytoplasmic contents. The predisposition to lysis, the loss of 260 nm absorbing material, the loss of tolerance to NaCl, and the altered morphology seen by electron microscopy all suggest that tea tree oil and its components compromise the cytoplasmic membrane (Carson *et al.*, 2002).

The *in vitro* activity of tea tree oil against dermatophytes and filamentous fungi was determined. Tea tree oil MICs for fungi ranged from 0.004% to 0.25% and minimal fungicidal concentrations (MFCs) ranged from < 0.03% to 8.0%. Time-kill experiments with 1-4 x MFC demonstrated that three of the four test organisms were still detected after 8 hours of treatment, but not after 24 hours. Comparison of the susceptibility to tea tree oil of germinated and non-germinated *Aspergillus niger* conidia showed germinated conidia to be, or susceptibility than non-germinated conidia. These data demonstrate that tea tree oil has both inhibitory and fungicidal activity (Hammer *et al.*, 2002).

2.4.2 **Betel oil** (Remington *et al.*, 1918)

History and discovery

Betel consists of the fresh or dried leaves of *Piper Betle* L., the leaves of which are used by the Malays as a masticatory. The leaves are usually admixed with scrapings of the Areca nut and shell lime. The Betel plants are indigenous throughout the entire Indian-Malay region and cultivated in Madagascar, Bourbon and West Indies. The plants are climbing shrubs and trained upon trellises and poles in shady but not places. The leaves are picked while green, pressed together by means of stones and dried, when they become brown in color and brittle. They generally occur in commerce tied up in small packets. The Betel leaves of the British pharmacopoeia are described as follow: 'about fifteen centimeters long, broadly ovate; they are unequally cordate at the base and acuminate at the apex. They have five to seven well-marked lateral veins, which curve round to the apex. Examined under the microscope they exhibit a multitude of rounded oil cells filled with a dark brown secretion. Certain of the epidermal and hypodermal cells contain colorless siliceous deposits, while the walls of many epidermal and other cells are impregnated with silica. Mesophyll contains abundant oil-cells filled with brown oleoresin. Taste warm, aromatic, bitter. As found in commerce the leaves are frequently tied up or stitched together into packets.

Oil components

The warm aromatic taste of the betel leaves is due to an essential oil known as betel oil. The chief constituent of the leaves is the volatile oil, of which they contain between 0.2 and 1.0 percent. This is of color varying from clear yellow to dark brown and of aromatic, somewhat creosote-like, odor and burning sharp taste. The specific gravity ranges from 0.958 to 1.044, the lighter oil being that obtained from the fresh leaves. This oil varied in the leaves from different countries. Two phenols, betel-phenol (chavibetol) and chavicol, have been isolated, the former of which has been found in all betel oils, and may therefore be taken as characteristic. The betel oil from Siam (Thailand) oil contains cadinene and a characteristic phenol named betel-phenol, isomeric with eugenol; the oil from Java (Indonesia) oil contains in addition to the betel-phenol, chavicol and sesquiterpene; while the Manila (Philippine) oil contains betel-phenol as the sole phenolic constituent (Gildemeister and Hoffmann, 1899). The active ingredients of betel oil, which is obtains from the leaves, are betel-phenol (or chavibetol or 3-hydroxy-4-methoxyalkybenzene, which gives a smoky aroma), chavicol and cadinene. Examination Of the oxidation products, acetyl compound and methyl ether showed that this compound was not eugenol, but an isomer, the composition of the new compound and of eugenol being represented as follows: The second constituent of betel oil boiled practically between 250° and 275°C., had a very agreeable tea-like odor, and consisted for the greater part of a sesquiterpene ($C_{15}H_{24}$), cubebene, which is characterized by its dihydrochlorate melting at 117-118°C. This composition differs considerably from that given by Professor Eykman, but how far the difference may depend upon the oil examined by Professor Eykman having been distilled from fresh leaves, whilst that examined by Messrs. Schimmel was distilled from dried leaves has not been determined (Cassaday, 1889).

Medicinal action and Use

The essential oil of betel oil is an active local stimulant and has been used in doses of one to two drops (0.06-0.12 ml) in the treatment of various respiration *catarrhs*, and as a local application, either by gargle or inhalation, in diphtheria. Fresh betel leaves are used in India as a masticatiory for their antiseptic,

stimulant and carminative properties. It is said that the juice of four leaves is equivalent in power to one drop of the oil. In Malaysia they used to treat headaches, arthritis and joint pain. In Thailand and China they are used to relieve toothache. In Indonesia they are drunk as an infusion and used as an antibiotic. They are also used in an infusion to cure indigestion, as an ointment or inhalant to cure headache, as a topical cure for constipation, as a decongestant and as aid to lactation.

2.4.3 Antimicrobial polysaccharide

Structure and properties of polysaccharide

Carbohydrates are essential components of all living organisms and are the most abundant class of biological molecules. Many of natural polysaccharides participate in a variety of biochemical reactions. Current applications used many kinds of nature polysaccharide and their derivative in the pharmaceutical applications such as, chitin, pectin, gum and cellulose. Polysaccharide may be subdivided into two main categories. The first main group is termed homopolysaccharide. Members of this group are defined as containing only a single type of monosaccharide although linkage may vary. Probably satisfactory examples are chitin, starch, amylo-pectin and cellulose (Davidson, 1967). Chitin is one of the abundant organic materials, being second only to cellulose in the amount produced annually by biosynthesis. It occurs in animals, particularly in crustacea, molluscs and insects, where it is a major constituent of the exoskeleton, and in certain fungi, where it is the principal fibrillar polymer in the cell wall. Chitin has a crystalline structure and it constitutes a network of organized fibers, this structure confers rigidity and resistance to organisms that contain it (Robert, 1992). Chitin is [poly (1→4)-2-acetamido-2-deoxy-D-glucopyranose], and its idealized structure is shown in following.

Cellulose is the most abundant polysaccharide on earth. Cellulose is a linear polymer of β- (1→4)-D-glucopyranose units in conformation. The fully equatorial conformation of β-linked glucopyranose residues stabilizes the chair structure, minimizing its flexibility (*e.g.* relative to the slightly more flexible a-

linked glucopyranose residues in amylose). Cellulose has many uses as an anticake agent, emulsifier, stabilizer, dispersing agent, thickener, and gelling agent but these are generally subsidiary to its most important use of holding on to water.

Pectin is a natural constituent of all terrestrial plants. It is a heterogonous complex of polysaccharide its composition varies with the source and the conditions applied during isolation. Galacturonic acid is the major constituent of all natural pectin and polymerized by 1, 4 bonds into linear chain. Pectin also contain varying quantities of neutral sugars, mainly arabinose, galactose, and rhamnose. The major outlet for pectin is food applications (i.e. as thickening and gelling agent in jams and jellies or as stabilizer in fruit and milk beverages) and pharmaceutical applications (Dumitriu, 1998).

Antimicrobial activity of polysaccharide

Chitosan has attracted much research attention in antifungal, antiviral and antibacterial properties in the last 20 years. Chitosan had inhibitory effects on Escherichia coli activity (100% activity vs1978%, P< 0/05). Our results show that higher temperature and acidic pH increased the antibacterial effects of chitosan. Sodium ions might complex with chitosan and accordingly reduce chitosan activity against Escherichia Coli. Divalent cations were reduced the antibacterial activity of chitosan (Qujeq, 1998). Wang *et al.* (1992) showed that gram-positive bacteria were more sensitive to chitosan than gram-negative bacteria with MIC of 1 mg/ml, whereas the MIC for gram-negative bacteria varied from 1 to 2 mg/ml. Chitosan may induce severe morphological alterations in gram-positive bacterium, while in gram-negative bacteria only retraction of the plasma-membrane was evident. Furthermore, the *in vitro* evaluation of Felt et al. (2000) showed that concentrations of chitosan as low as 0.0375% still exert a bacteriostatic effect against *E. coli*. Minimal inhibitory concentration (MIC) values of chitosan were calculated to be as low as 0.375 mg/ml for *E. coli* and 0.15 mg/ml for *S. aureus*.

A water-soluble chitosan hydrolysate with high activity against *Escherichia coli* was obtained during cellulase digestion of chitosan for 18 h. This 18-h hydrolysate is composed of low-molecular-weight chitosan (LMWC), with a

molecular weight of 12.0 kDa, and chitooligosaccharides, which are composed of sugars with a degree of polymerization of 1 to 8. LMWC has a strong activity at 100 ppm against many pathogens and yeast species, including *Bacillus cereus*, *E. coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella enterica* serovar Typhi, and *Saccharomyces cerevisiae*, while the chitooligosaccharides have much weaker antimicrobial activity than does LMWC. Accordingly, the antimicrobial activity against *E. coli* in the 18-h hydrolysate proved to come mainly from the presence of LMWC(Tsai *et al.*, 2004).

Chitosan derivatives with quaternary ammonium salt, such as *N,N,N*-trimethyl chitosan, *N–N*-propyl-*N,N*-dimethyl chitosan and *N*-furfuryl-*N,N*-dimethyl chitosan were prepared using different 96% deacetylated chitosan of M_v 2.14×10^5, 1.9×10^4, 7.8×10^3. Amino groups on chitosan react with aldehydes to from a Schiff base intermediate. Quaternized chitosan were obtained by reaction of a Schiff base with methyl iodide. The yields, degree of quaternization and water-solubility of quaternized chitosan were influenced by the molecular weight of the chitosan sample. The antibacterial activities of quaternized chitosan against *Escherichia coli* were explored by calculation of the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) in water, 0.25 and 0.50% acetic acid medium. Results show the antibacterial activities of quaternized chitosan against *E. coli* are related to its molecular weight. Antibacterial activities of quaternized chitosan in acetic acid medium are stronger than that in water. Their antibacterial activities are increased as the concentration of acetic acid is increased. It was also found that the antibacterial activity of quaternized chitosan against *E. coli* is stronger than that of chitosan (Zhishen *et al.*, 2001).

Antimicrobial susceptibility test of chitosan polymerized with xylan was determined by a dilution method, using Mueller hinton broth for the bacteria and sabouraud dextrose broth for yeast. The inhibitory activities of xylan polymerized chitosan against bacteria, *S. aureus*, *E. coli*, *B. subtilis*, *Enterococcus faecalis* and *Ps. aeruginosa*, and yeast, *Saccharomyces cerevisiae* were very high potency. The mechanism of antimicrobial action of polymerized chitosan is probably very complex. However, due to the cationic nature of derivatives, it is likely that they can interact and form polyelectrolyte complexes with acidic polymer present in the

microbial cell wall surface, as do other cationic antimicrobial agents. Also the neutral or acidic glycosyl side chains of quaternized xylan polymer may be involved in the mechanism, contributing to the intermolecular interactions (Ebringerova *et al.*, 1994).

Polysaccharide gel (PG) isolated from dried fruit-rind of durian (*Durio zibethinus* Murr.) (Pongsamart and panmaung *et al.*, 1998). The elemental compositions of PG extracts from dried fruit-rind of durian are carbon, hydrogen and oxygen in molar ratio of 2.9:5.7:3.2 according the study of Gerddit, 2001. The natural polysaccharide gel from dried fruit-rind of durian is a high-molecular weight polysaccharide of 500-14,000 kDa (Gerddit, 2002), build up from sugars as a heteropolysaccharide of long chain galacturonan with branch chain of neutral sugars such as glucose, rhamnose, arabinose, and fructose (Hokputsa *et al.*, 2004). Polysaccharide gel exhibited antibacterial activity against 7 tested bacteria. Minimal inhibitory concentration (MIC) of PG against *B. subtilis*, *M. luteus*, *S. epidermidis*, *E. coli*, and *P. vulgaris* is 6.4 mg/ml; against *S. aureus* and *L. pentosus* have found at 12.8 and 25.6 mg/ml, respectively (Nantawanit, 2001).

2.5 Antimicrobial susceptibility tests

The primary purpose of antimicrobial susceptibility test is to guide the clinician the choice of appropriate agents for therapy. The test is also provided accumulating data from which information on the suitable agents for empirical use can be derived. Antimicrobial susceptibility tests are use to evaluate an *in vi*tro activity of new agents (Collin, 1995).

In vitro antimicrobial susceptibility test are depended on two roles, diffusion and dilution. Laboratory procedures involving diffusion susceptibility tests are commonly performed in agar media called agar diffusion technique.

2.5.1 Agar diffusion susceptibility test

In general, agar diffusion tests are performed by inoculating a nutrient agar medium in a standardized manner and then applying the drug to be studied to the agar surface in some type of reservoir. The drug is allowed to diffuse

into the surrounding medium. This exposes the test organism to a continuous gradient of drug concentrations, with concentration diminishing as distance from the reservoir increase. After an appropriate period of incubation, there should be a zone of inhibited growth around the reservoir. The size of zone may be measured to determine the degree of susceptibility of test organisms (Lorian, 1991).

These tests depend on the ability of the antimicrobial agents to diffuse at predictable rates through the agar gel. The experiment work of Cooper (1964) provided a number of theoretical concepts that have led to a better understanding of variables that influence the formation of a zone inhibition in an agar medium. The more important variable influence diffusion test is described below.

Solution of antimicrobial agents may be applied to surface of a seeded agar medium in several different ways as described by Lorian, 1991. The convenient method uses filter paper disk that has been moistening with the drug solution and the applied directly to the agar while still wet. Disks may be prepared more accurately if a micropipette is use to load each disk with a measured volume of drug solution. Alternatively, glass or metal cylinder may be applied to the surface of the seeded agar medium to facilitate application of drug solutions. Agar well may be cut from the seeded agar medium by using a hollow tube (4 to 6 mm in diameters) and then be fill with the drug solutions.

About diffusion, the drug diffusion in a two-dimensional manner. In the relatively thin layer of agar normally poured into an agar plate, downward diffusion is quite limited and the concentration in the depth of medium soon approached that near the surface. The amount of drug available to diffuse outward is greater and consequently the zone of inhibition is likely to be larger (Barry and Fay, 1973). In a very thick pour plate, the three dimensional diffusion can result in a semicircular zone of inhibition, being small at the bottom and larger at the surface.

2.5.1.1 Composition of the agar medium

The agar medium itself profoundly influences the zone sizes in three ways:

(1.) It affects the activity of different antimicrobial agents

(2.) It influences the rate of diffusion of the antimicrobial agents.

(3.) It affects the growth rate of the test organism.

For susceptibility testing, the composition of test medium must be carefully standardized because the activity of antimicrobial agent is markedly influenced by many factors such as cation in the medium, pH of the medium and presence of various antagonistic materials.

The ideal medium should have sufficient nutrients to support growth of organism normally tested. Mueller hinton Agar (MHA) medium is the most widely recommended for bacterial susceptibility test (Barry *et al.*, 1978; Modugno *et al.*, 1997; Zarazaga *et al.*, 1999). Recently, Mueller hinton medium has shown to be acceptable for a wide variety of antimicrobial susceptibility test because of the relatively good reproducibility and simplicity being regulated to a reference medium (NCCLS, 1986). MHA medium is well suited for standard rapid growing pathogen such as enteric gram-negative bacilli, *Pseudomonas* spp., *Staphylococci* spp. and *Enterococcus* spp. For bacteria that do not grow readily on this medium other supplement or alternative media may be required.

2.5.1.2 Agar depth

The depth of agar medium is critically important in test systems that use very thin (2-3 mm) agar layers (e.g. bioassay system) (Lorian, 1996). In susceptibility testing, the agar medium is usually poured to a depth of about 4 mm (18-25 ml in a 9 cm petri-dish), and at that level fairly major variation in agar depth do not significantly affect the test results (Barry, 1976). With diffusion tests the size of the zone increase as the depth of the agar decrease but this effect is most marked with very thin plates. When working with very thin agar layers, extreme caution must

be taken to avoid very minor variations in the depth of the agar medium in different areas of the same plate.

2.5.1.3 Inoculum density

Inocula are prepared by adding cells from four to five isolated colonies of similar colony morphology to a broth medium and then allowing them to grow to the log phase. Four or five colonies, rather than a single colony, are selected to minimize the possibility of testing a colony that might have been derived from a susceptible mutant. Inocula may also be prepared directly by inoculating colonies grown overnight on agar plate or slant into broth or saline. This direct inoculum suspension preparation technique, which does not require incubation, but the use of freshly prepared (Mahon and Manuselis, 2000).

The numbers of organism tested must be standardizing regardless of the method used. The most widely use method of inoculum standardization involves McFarland turbidity standard and 0.5 McFarland standard (10^8 CFU/ml) is the most commonly used (Lorian, 1991). False susceptible results may be occur if too few or too many organisms. Increasing inoculums sizes reduce the susceptibility to agents in broth diffusion and dilution test (Collin *et al.*, 1995). In diffusion method, heavy inocula tend to give small zone of inhibition and when inoculum is light, organism often appear to be susceptible and give larger zone (Cooper *et al.*, 1958). Consequently, minor changes in inoculum density result in extreme change in the result of susceptibility test.

2.5.1.4 Visualization of the zone edge

Barry *et al.* (1997) demonstrated that zone of inhibition on susceptibility test plates cannot be measured with extreme degrees of precision. In all situations it is very important to standardize the intensity and angle of light used to illuminate test plates when zone measurements are being determined.

2.5.1.5 Growth characteristics of the test strain

The rate of growth on the test medium obviously affects the end result. In susceptibility testing, some strain-to-strain variability in growth rate is avoidable. Growth conditions have been standardized for optimal results with most the common rapid-growing microorganisms. Microorganisms that demonstrate delayed growth under standardized test conditions cannot be test reliably. With inch slow-growing strains, fairly large zones of inhibition may indicate susceptibility may simply represent the effect of a delayed growth rate. On the other hand, a very small zone of all can be interpreted reliably as indicating resistance (Lorian, 1991).

2.5.1.6 Incubation

Susceptibility tests are normally incubated at 35-37°C. For optimal growth of the common human pathogens. Use alternative conditions only, if essential for growth of the organisms such as, most fungi can grow well at temperature range 30-35°C. Woolfrey, *et al.* (1998) showed that exponentially growing cultures of *Staphylococcus aureus* in a few degrees above that required for maximum exponential growth and that cell death will occur with further increase in temperature. The rate of growth will be prolonged at lower temperatures and thus the critical concentration of the antimicrobial agent has more time to diffuse further. To further complicate the situation, most antimicrobial agents diffuse more slowly at lower temperature, partially because of the increased viscosity of the agar medium (Lorian, 1996). Consequently, low temperature during the critically important early hours of incubation have two effects, one tending to produce larger zones of incubation (decrease growth rate) and the other tending to produce smaller zones of incubation decrease diffusion rate. Normally, the effect of decrease growth rate predominates and the zone increases with lower temperatures.

Most of *in vitro* tests with antimicrobial agents have been standardized with and "over night" incubation period, usually defined as 16-18 hr (Isenberg, 1998). With the exception of some organisms, anaerobic bacteria and yeast most commonly incubated for 48 hr (Chin and Neu, 1984; Lorian, 1991). The incubation zone may be come smaller with further incubation because of changes in

the character of the growth at the edge of the zone. The character of the zone edge will changes as a result of (a) the appearance of delayed growth, (b) better visualization of practically inhibited growth or (c) delayed appearance of the resistant variants. Occasionally, the zones appear to increase in size because of change in character of the growth at the zone edge or because of actual lyses of the initial growth within the inner ring of the zone.

2.5.2 Broth macrodilution susceptibility

Dilution antimicrobial susceptibility test methods are used to determine quantitatively in term of the minimal inhibitory concentration (MIC), the lowest concentration of antimicrobial agent required to inhibit the growth of an organism isolate or that which kill it, the minimal bactericidal (fungicidal) concentration (MBC, MFC) (Mahon *et al.*, 2000).

Broth macrodilution tests preformed in test tube are referred to as broth macrodilution or tube dilution susceptibility tests. The macrodilution method is most suitable for small number of tests (Collin *et al.*, 1995). It is impractical for use as a routine method when several antimicrobial agents must be tested on an isolate or if several isolates must be tested. Some laboratories use broth macrodilution (Jackson and Finland, 1951; Washington and Sutter, 1980) when it is necessary to test drugs not include in their routine system of fastidious bacteria that require special growth media. Additionally, this method is often used when minimal bactericidal concentration (MBC) endpoints are to be subsequently determined.

Generally, a twofold serial dilution is prepared. Mueller hinton broth is medium most commonly used for MIC test of fastidious bacteria and sabouraud dextrose broth is commonly used for fungi. A standardize suspension of test bacteria and yeast are added to each dilution to obtain final concentration of $5x10^5$ CFU/ml (Mahon *et al.*, 2000) and 10^5 CFU/ml (Lorian, 1996), respectively. A growth control tube (broth plus inoculum) and an uninoculated control tube (broth only) are used in each test. After incubation, the MIC is determined visually as the lowest concentration that inhibits growth, as demonstrate by the absence of turbidity (NCCLS, 1997).

Minimal inhibitory concentration (MIC) test and minimal bactericidal concentration (MBC) test

Minimal inhibitory concentration (MIC) tests identify the amount of antimicrobial agent required to inhibit the growth of multiplication of as organism isolate. Minimal bactericidal concentration (MBC) test can be used for this purpose. The method is usually as extension of the broth dilution MIC. After reading the MIC, organisms are subculture from tubes of wells showing no growth to antimicrobial-free agar medium. After incubating the plates the proportion of non-viable organism, compared with origin inoculums, is assessed.

MBC tests are subject to more technical pitfalls than MIC tests, and several variables must be rigidly controlled during MBC testing. The first involves inoculums. Because of many antimicrobial agents exert a bactericidal effect only on growing cells, falsely elevated MBCs. The inoculums preparation methods described for MIC test that use stationary phase growth are unacceptable for MBC tests (Lorian, 1996). Secondly, during inoculation for MIC tests care must be taken to ensure that all bacteria in the test inoculums are deposited directly into the antimicrobial solution. If this is not done, bacteria may stick to the wall of the tube or well above the meniscus of the antimicrobial solution and may remain viable during incubation of the MIC portion of the test. These cells (which have not been exposed to antimicrobial agent) may then be inadvertently transferred during the subculture step, ultimately resulting in falsely elevated MBCs. Third, the volume subculture following reading of the MIC test must be large enough to contain sufficient inoculums but small enough to prevent carry over of large amounts of antimicrobial agent to continue to exert a bacterial effect. Usually, 10 µl (0.01 ml) is recommended (Mahon *et al.*, 2000).

For fungi strain, MFC determination was tested mostly by the same procedure as described for bacteria (Bartizal *et al.*, 1997). The method may be adapted but have recommended by reference method, NCCLS (1995).

2.5.3 Time-kill analysis

Time-kill assay is method for measures of the killing rate of microorganism by antimicrobial agent as determine by examining the number of viable organism remaining at time intervals after exposure to the test agent. It is an extension of the MBC or MIC test. Test organism in mid-logarithmic growth phase is inoculated into the tubes of broth medium containing varying concentrations of antimicrobial agent and a growth control tube without drug. Most experiments are performed with final inoculums of 10^5 to 10^7 CFU/ml. It is usually convenient first to adjust the overnight culture to match the 0.5 McFarland standards (NCCLS, 1997). These tubes are incubated. Then small aliquots are removed at specific time intervals (e.g. at 0, 4, 8, 12 and 24 hours) diluted to obtain countable number of colonies, and plated to agar for colony count determination. The number of organisms remaining in each sample is plotted over time to determine the rate of antimicrobial agent killing (Bartizal *et al.*, 1997; Hoelloman *et al.*, 1998; Nilius *et al.*, 2000). Generally, three or more $\log_{10}$ reduction in organism count and the antimicrobial suspension as compared with growth control indicates and adequate bactericidal or fungicidal response (Hoelloman *et al.*, 1998).