CHAPTER I

GENERAL BACKGROUND

1. Introduction

Natural polymers including polysaccharides are a class of biopolymers constituted with simple sugar monomers. These used in commerce and industry are isolates from terrestrial and marine plants or are principally the exogenous metabolites of some bacteria. Plant polysaccharides may correspond to storage polymers or to molecules involved in the cell-wall structure (Warran *et al.*, 2005). The gelation mechanism of polysaccharides has long been studied by many research groups not only from a scientific interest but also for this mechanism's importance in food, pharmaceutical, biomedical, cosmetic, coating, painting, and related industries (Nishinari and Takahashi, 2003).

Polysaccharide gel is a water soluble polysaccharide and isolate from fruit-hulls of durian (*Durio zibethinus* Murr.) (Smittinand, 2001). Polysaccharide gel composes of polygalacturonan with branches point sugars including arabinose 1.2%, rhamnose 4.8%, xylose 0.4% galactose 4.9%, glucose 20.9%, and galacturonic acids 67.9% (Greddit, 2002; Hokputsa *et al.*, 2004; Pongsamart and Panmaung, 1998).

Toxicity test of PG has been determined, a high oral single dose (2g/kg) has not induce severe toxicity in male mice and rats (Pongsamart *et al.*, 2001). Subchronic toxicity test indicated that PG has not induced toxic effect in male and female mice after longterm feeding at dose of 0.5 g/kg/d for 60-100 days (Pongsamart *et al.*, 1989; Pongsamart *et al.*, 2002). PG stimulates wound healing activity in pig skin (Nakchat, 2002). PG has been found to be used as a film forming agent and it has been used to prepare a satisfactory product of dressing film (Gerddit, 2002).

The polysaccharide gel (PG) isolated from dried fruit-hulls of durian (*Durio zibethinus* Murr.) has found to be useful for a wide range of application in pharmaceutical products and food industries such as tablet binder, film coat tablet, suspension, emulsion, gelling, and diet food (Pongsamart *et al.*, 1989). The formulation of PG containing vitamin E or vitamin C or mixture of E and C; and film-dressing products (Lerchiporn, 2003).

The previous study has also shown that the water soluble polysaccharide has antibacterial activities against certain strains of gram positive and negative bacteria such as *Staphylococcus aureus*, *Staphylococcus epidermidis, Micrococcus luteus, Bacillus subtilis, Lactobacillus pentosus, Escherichia coli and Proteus vulgaris* (Nantawanit, 2001). Especially, *Staphylococcus aureus* and *Staphylococcus epidermidis*, are bacteria that can cause skin infection. Normal skin has also found *Propionibacterium acnes* and *Staphylococcus epidermidis* as normal flora. *Propionibacterium acnes* is considered to play an important role in acne development by secreting inflammation-inducing factors (Paranjpe and Kulkarni, 1995). *Staphylococcus epidermidis*, an aerobic organism, usually involves in superficial infections within the sebaceous unit (Chomnawang *et al.*, 2005).

Biopolymers, including polysaccharide, have important technological applications as thickeners, gelling agents and coating (Marudova *et al.*, 2004). Xanthan gum is a natural high molecular weight polysaccharide and use as a thickener, stabilizer, and suspension (Katzbauer, 1998). Chitosan forms a gel like layer in aqueous environment, which is favorable for interpenetration of polymer and glycoprotein chains into mucous (Sinha *et al.*, 2004). The antimicrobial activity of chitosan in lipid emulsions as well as in aqueous solutions has been investigated to produce a formulation with improved activity in reducing the numbers of microorganism (Jumaa *et al.*, 2002).

Essential oil obtained from *Piper betle* Linn. has a bactericidal effect (Perry, 1980). The in vitro study of *Piper betle* has been investigested antimicrobial activity against human pathogenic bacteria and phytopathogenic fungi by comparing with standard microbial susceptibility testing biodiscs (Shitut *et al.*, 1999). Eugenol isolated from *P. betle* exhibited antifugal activity against *Aspergillus flavus* (Parmar *et*

al., 1997). There are reports about antibacterial activity for aqueous alcohol extracts against *Vibro cholerae, V. parahemolyticus, Salmonella typhosa, Shigella flexneri, E. coli, S. aureus* and *Ps. aeruginosa* (Chalermpunchai *et al.*, 1987).

The objective of the present study was to formulate antibacterial preparation of natural products that can be combined with antibacterial durian polysaccharide gel such as betel vine oil in order to develop anti-acne preparation.

2. Literature Review

Skin

The skin is the large organ of the body, accounting for more than 10% of body mass. The skin is composed of three layers: epidermis, dermis, and subcutaneous tissue (fat). The most obvious functions of the skin are to protect the body by preventing the loss of fluids and the penetration of undesirable substances or radiation, and by cushioning it against mechanical shocks. This allows the survival of humans in an environment of variable temperature and the presence of environment dangers, such as chemicals, bacteria, allergens, fungi, and radiation (Odom *et al.*, 2000)

Epidermis

The epidermis, the outermost layer, is directly contiguous with the environment. It is formed by an ordered arrangement of three basic cell types: keratinocytes, melanocytes, and Langerhans' cells. The keratinocyte is the principal cell of the epidermis. The function of keratinocyte is to synthesize keratin, a filamentous protein that serves a protective function. The epidermis can be divided into four distinct layers: stratum basale or stratum germinativum, stratum spinosum, stratum granulosum and stratum corneum.

Dermis

The dermis is the middle layer which bounded distally by its junction with the epidermis and proximally by the subcutaneous fat, contributes 15-20% of the total weight of the human body. The constituents of the dermis are mesodermal in origin except for nerves, which, like melanocytes, derive from the neural crest. The principal component of dermis is the fibrillar structural protein collagen. The dermis contains few cells: most are fibroblasts which secrete the dermal constituents: others are mast cells, histocytes or macrophages, lymphocytes or other leucocytes, and melanocytes. The dermis also has vascular beds at various levels, lymphatics, nerves and various kinds of nerve endings.

Subcutaneous tissue

Beneath the dermis lies the panniculus, lobules of fat calls or lipocytes separted by fibrous septa composed of collagen and large blood vessels. The collagen in the septa is continuous with the collagen in the dermis. Just as the epidermis and the dermis vary in thickness according to skin sites, so does the subcutaneous tissue. Certain inflammatory dermatoses, known as the panniculitides, principally affect this level of the skin, producing subcutaneous nodules. The pattern of the inflammation, specifically whether if primarily affects the septa or the fat lobules themselves, serves to distinguish various conditions which clinically may resemble one another.

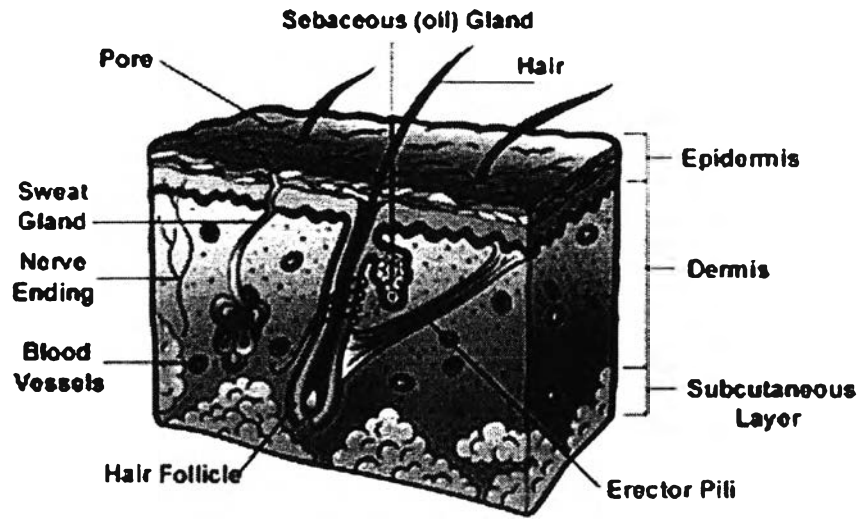


Figure 1. The skin is composed of epidermis, dermis, and subcutaneous tissue (fat).

Acne

Acne vulgaris, a chronic inflammatory disease of the sebaceous glands, is common skin disease that induces inflammation at the skin surface of the face, neck, chest, or back. Nearly everyone suffers from outbreaks of pimples at some point in life, making acne one of the most common skin disorders. Although acne remains largely a curse of adolescence, about 20 percent of all cases occur in adults. Acne commonly arises during puberty and tends to be worse in people with oily skin. It occurs in both sexes, although teenage boys tend to have the most severe cases. Women are more likely than men to have mild to moderate forms into their thirties and beyond. Acne develops mostly in young people due to several factors, namely, hormonal imbalance, bacterial infection, stress food, or cosmetic application (Kubo *et al.*, 1994). Normal skin commensals including *Propionibacterium acnes* and *Staphylococcus epidermidis*. Especially, *Propionibacterium acnes* which is one of major organism isolated from the surface of skin. *P. acnes,* an anaerobic pathogen, plays an important role in the pathogenesis of acne by inducing certain inflammatory mediators. *P. acnes* secretes lipase and degrades sebum oils into free fatty acids, which are potent acne stimuli. These free fatty acids stimulate the hair follicle, from

the comedo, and then induce the inflammation. These bacteria also secrete leukocyte chemotatic factors, infiltrating leukocytes in the hair follicle. These leukocytes stimulate and destroy the hair follicle wall. Subsequently, the contents of the hair follicle flows into the dermis. *Staphylococcus epidermidis*, an aerobic organism, usually involves in superficial infections within the sebaceous unit (Chomnawang *et al.*, 2005). Therefore, *P. acnes* is considered to play an important role in acne development by secreting inflammation-inducing factors (Paranjpe and Kulkarni, 1995).

Propionibacterium

Propionibacteria are small gram-positive bacilli that are frequently arranged in short chains or clumps. The non-spore-forming gram-positive bacilli are a diverse group of bacteria. Many members of the genus *Corynebacterium* and their anaerobic equivalents, *Propionibacterium* species, are members of the normal flora of skin and mucous members of humans. The organisms are anaerobic or aerotolerant, nonmotile, catalase-positive, and capable of fermenting carbohydrate, producing propionic acid as their major by product.

Aetiology

Four major factors are involved in the pathogenesis: increased sebum production, ductal hypercornification, bacteria and inflammation.

1. Increased sebum production

Sebum is primarily consisted of a mixture of squalene, wax and sterol esters, cholesterol, polar lipids and triglycerides (Zouboulis, 2004). Acne patients tend to have higher levels of squalene and wax esters, lower levels of fatty acid and a more frequent occurrence of particular free fatty acids. The sebaceous glands enlarge and become more active in puberty because of male sex hormones of gonadal or adrenal origin (Bensouilah, 2002). Abnormally high levels of sebum secretion could thus result from high overall androgen production or increased

availability of free androgen because of deficiency in sex-hormone-binding globulin (SHBG). Equally, they could involve an amplified target response mediated either through 5α-reduction of testosterone or the capacity of the intracellular receptor to bind the hormone (Brieva *et al.*, 1997). Androgenic hormones are also important, as they control the activity of the sebaceous gland. Elevated levels of these hormones in certain medical conditions are often associated with acne. Androgens are the main hormones to stimulate sebum excretion, although other hormones have minor effects too. It is currently assumed that intracellular conversion of testosterone into a more active metabolite, 5α-dihydrotestosterone (DHT) which the most potent androgens in tissue, takes place by 5α-reductase. Then dihydrotestosterone binds to a high-affinity specific cytoplasmic receptor protein in sebaceous glands, increasing sebum excretion that is transported to the cell nucleus. Sebaceous glands are composed of undifferentiated, differentiated and mature cells. The increased concentration of lipids in mature cells leads to cell disintegration and the formation of sebum that is eventually excreted onto the surface of skin. Of the abnormal sebaceous lipids in acne patients, linoleate levels may be relevant. Patients with acne have a significantly lower concentration of linoleic acid in their skin surface lipids than do persons without acne (Cunliffe *et al.*, 2004). A low level of linoleate also results in a decreased epidermal barrier function, which might make the comedonal wall permeable to inflammatory substances.

As the sebum moves up the duct, bacteria, especially *Propionibacterium acnes* (*P. acnes*), hydrolyse the triglycerides result in the formation of monoglycerides and diglycerides, as well as free fatty fatty acids, within the sebaceous follicle duct.

2. Ductal epidermal hyperproliferation

An abnormality in the process of desquamation of follicular corneocytes in the sebaceous follicle ducts leads to plugging of the follicular orifice forming the primary lesion of acne, the comedone. Comedones are produced by this dead skin accumulating in the follicular canal leading to distention of the follicle. Comedones are frequently seen before puberty and often precede the development of

inflammation by several months or even 1-2 years. Increased follicular keratinization results in the formation of the comedo. Comedogenesis is central to the pathogenesis of acne. Comedogenesis occurs specifically in sebaceous follicles, not in terminal or vellus follicles. The keratinization is thought to be due to hormonal changes, which result in the adherence of keratinocytes to the follicular canal (Harper and Thiboutot, 2003). The follicular canal becomes plugged with these cornified cells. The follicular canal is consisted of two portions, the more distal acroinfundibulum, contiguous with the surface epithelium, and the infrainfundibulum, or the region between the epithelium of the sebaceous duct and the follicular epithelium. Within the surface epithelium are tonofilaments, desmosomes, keratohyalin granules, and melanocytes (Cunliffe *et al.*, 2004).

3. Bacteria

Bacteria are probably not involved in the initiation of comedones. Electron microscopy of early noninflamed lesions taken from prepubertal and early pubertal individuals has demonstrated few or no bateria. Quantification of bacteria from comedones suggests that follicular colonization may be unrelated to comedogenesis. Biopsy and culture of early noninflamed lesions has shown that 30% of these are without bacteria, suggesting that ductal bacteria are not needed for the initiation of cornification in the development of comedones. However, bacteria, particularly *P. acnes,* are likely involved later in comedogenesis. *P. acnes*, a normal skin commensal, plays a pathogenic role (Harper and Alabama, 2004). It colonizes the pilosebaceous ducts, breaks down triglycerides releasing free fatty acids, produces substances chemotactic for inflammatory cells and induces the ductal epithelium to secrete pro-inflammatory cytokines, including IL-1, IL-2 and TNF-α. There is currently no evidence linking *S. epidermidis*, which resides primarily on the skin surfaces rather than within the follicles, with the pathogenesis of acne. Indeed, *S. epidermidis* counts remain at pretreatment levels, after an initial drop, during the course of successful antibiotic acne therapy (Bojar and Holland, 2004).

4. Inflammation

The inflammatory process is centered around the metabolic activity of the anaerobic bacterium, *Propionibacterium acnes*. This bacteria metabolizes sebum to produce free fatty acids (Brieva *et al.*, 1997). The free fatty acid hypothesis was one of the first explanations offered for the inflammation that accompanies acne. It was based on two findings: there is a decrease in the percentage of free fatty acids in skin surface lipids with successful antibiotic therapy, and a large proportion of those free fatty acids resulted from hydrolysis of sebaceous gland triglycerides by *P. acnes* (Leyden, 1995). *Propionibacterium acnes* has been shown to be associated with inflammatory acne through antibiotic resistance studies. However, this bacterium has not been shown to be a direct cause of the disease or to be involved in the initiation of inflammation. Numbers of viable bacteria within follicles show no correlation with severity of inflammation, and some inflamed lesions do not contain viable *P. acnes*. However, nonviable *P. acnes* cells are immunostimulatory. The inflammatory probably results from biologically active mediators which diffuse from the follicle where they are produced by *P. acnes*. The early inflammation may be due to mediators moving through the duct wall. *Propionibacterium acnes* produces many enzymes, including three proteases, lipase, phosphatases and hyaluronate lyase, all of which might in theory be involved. It is now believed that *P. acnes* is not the cause of acne, but is a significant contributing factor to the inflammatory stages of the disease (Farrar and Ingham, 2004).

Topical Antiacne Preparation

Antiacne drugs

The therapeutic modalities used today target the different pathogenic factors that produce acne. These factors include sebaceous gland activity, follicular keratinization, bacterial proliferation and inflammation. Topical therapy should be considered first in patients with comedonal or mild to moderate type acne. Management usually includes a combination of therapies depending on the severity, location and type of acne. There are numerous treatments that have been recommended for the treatment of acne. Topical treatments aimed at preventing the

formation of new comedones take several months to show maximal benefit. The most widely used topical therapies are retinoid, benzoyl peroxide, azelaic acid and antibiotic.

1. Retinoids

Retinoids are vitamin A derivatives (Gollnick and Schramm, 1998). They act directly on the keratinocytes in the epidermis and are effective in the treatment of comedones. Retinoids have been shown to expulse mature comedones (open and closed type), suppress the development of new microcomedones, inhibit inflammatory reactions and enhance the penetration of other topical anti-acne agents. They also likely help maintain some remission of acne by inhibiting microcomedo formation, thus preventing new lesions. Retinoids have been used for over 25 years in the treatment of acne vulgaris. Tretinoin (Retin A) and isotretinoin (Isotrex) are commonly used examples. The major side effects are dryness, redness and irritation of the skin. During the last years, new polyaromatic retinoids (adapalene, tazarotene) have been developed for topical treatment of acne.

- Tretinoin

Tretinoin has become a standard therapeutic agent in acne. It provides a strong anticomedogenic effect and has an indirect antimicrobiotic effect. It initiates increased cell turnover in comedones and reduces cohesion between keratinized cells. It is a highly effective comedolytic agent that normalizes desquamation of follicular epithelium, promotes drainage of comedones and inhibits the formation of new comedones. The follicle becomes less anaerobic and more accessible to topical antibiotic agents, with the resultant decrease in *P. acnes*. A common side effect of tretinoin treatment, is a low grade irritant dermatitis with erythema, scaling, burning and increased susceptibility to sunburn due to thinning of stratum corneum. Tretinoin is available as Retin-A cream (0.025%, 0.05%, 0.1%), gel (0.01%, 0.025%) and liquid (0.05%). Treatment should begin with a low-strength cream or gel applied once daily before bed to affected areas. If there is no response after 3 weeks, then an increased concentration can be used. The cream is best for patients with dry skin, and the gel is best for patients with oily skin.

- Isotretinoin

Isotretinoin decreases sebum production, abnormal keratinization, and the inflammatory process by reducing chemotaxis. Subsequently, the growth of *P. acnes*, as well as free fatty acid production, is decreased. Isotretinoin is available as a 0.05% gel and a 0.05% and 0.1% cream. Topically applied isotretinoin has similar effectiveness as tretinoin in clearing acne lesions, while causing less skin irritation. Side effects include dryness of mucous membranes and skin, dry eyes, hair loss, and decreased night vision.

- Adapalene

Adapalene, a new topical gel, is a third-generation napthoic acid derived of retinoic acid recently has become available for acne patients. This gel may be less irritating than tretinoin. It is available as a 0.1% gel, solution and cream. Additionally, adapalene has a significantly better cutaneous tolerance than tretinoin. Recently adapalene was shown to have additional anti-inflammatory mechanisms, including inhibition of polymorphonuclear granulocytes, suppression of chemotactic activity of human polymorphnuclear leukocytes, reduction of lipoxygenase activity and the resulting leukotriene.

- Tazarotene

Recently, tazarotene has been investigated in the topical treatment of acne and psoriasis. It is available as a 0.05% and 0.1% gel. In a comparative trial over 12 weeks, tazarotene 0.1% gel seemed to be more efficient than tretinoin 0.5%gel in reducing papules and open comedones, but similar in reducing closed comedones (Krautheim and Gollnick, 2004).

2. Benzoyl peroxide

Benzoyl peroxide has been known as a highly effective topical agent in acne vulgaris therapy for a long time. The strong antimicrobial effect of benzoyl peroxide acts by decreasing the numbers of colonization with *Staphylococcus*

aureus and *P. acnes* on the skin surface. Subsequently, there are less free fatty acids and other pro-inflammatory byproducts produced on the skin surface can be observed. It has comedolytic activity and is good at treating inflammatory acne, both through direct antibacterial and anti-inflammatory mechanisms. Benzoyl peroxide is marketed as gels, creams and lotions and is available in concentrations of 2.5, 5 and 10%, either alone or with a combination of sulphur, imidazole or hydroxyquinolone. Benzoyl peroxide should be applied once or twice daily. The liquid and cream are more useful for patients with dry skin, and the gel, although slightly more irritating, is effective for patients with oily skin. The common induction of an irritant dermatitis with erythema, scaling, and itching can be avoided by less frequent application (Shaw and Kennedy, 2003).

3. Azelaic aid

Azelaic acid is a naturally occurring dicarboxylic acid with moderate antibacterial and comedolytic effects. It does not reduce the sebum secretion excretion rate nor influence the size of the sebaceous gland. The antimicrobial effect of azelaic acid is based on a reduction of the colonization with *P. acnes* on the skin surface ant the pilosebaceous duct with at lest one log cycle reduction within 4 weeks. Azelaic acid also has bacteriocidal effect on *S. epidermidis*. Azelaic acid is available as a 20% cream; a gel preparation was added recently. The advantage of azelaic acid consists in the lack of prominent side effects.

4. Antibiotics

Topical antibiotics are indicated to treat mild inflammatory acne. Erythromycin and clindamycin are the most common topical antibiotics. Topical antibiotics reduce the population of *P. acnes* on the skin surface and particularly within the follicles, thereby reducing free fatty acids. They demonstrate anti-inflammatory activity by suppressing chemotaxis. Subsequently, an indirect anticomedogenic effect can be observed, which seems to be stronger with clindamycin (Baur and Butler, 1998).

- Clindamycin

Clindamycin decreases the number of *P. acnes* and also has a direct anti-inflammatory effect. It has been shown to be clinically equal to erythromycin for treating moderate facial acne. Clindamycin is available in a 1.0% solution, gel and lotion.

- Erythromycin

Erythromycin inhibits the release of lipase from *P. acnes* and suppresses neutrophil chemotaxis. It significantly decreases *P. acnes* from the duct of sebaceous glands. Erythromycin is available as a 1.0% and 2.0% solution, a 2.0% ointment, and a 2.0% and 4.0% gel.

5. Naturally anti-acne agents

For many years, antibiotics have been used to treat acne vulgaris, however, antibiotic resistance has been increasing in prevalence within the dermatologic setting. The development of antibiotic resistance is multifactorial, including the specific nature of the relationship of bacteria to antibiotics, how the antibacterial is used, host characteristics, and environmental factors. As therapeutic agents for acne, antibiotics are usually employed to inhibit inflammation or kill the bacteria. However, the antibiotics have been known to induce side effects. Medicinal plants have been extensively studied as alternative treatments for diseases. In addition, antibacterial activity of various secondary metabolites from plants against *Propionibacterium acnes* was investigated. Hence, a large number of active principles have been isolated from various plants.

Some study was conducted to evaluate antimicrobial activities of Thai medicinal plants against etiologic agents of acne vulgaris. Among those, *Senna alata, Eupatorium odoratum, Garcinia mangostana,* and *Barleria lupulina* had strong inhibitory effects. (Chomnawang *et al.*, 2005).

For example, anacardic acids isolated from the cashew, apple, nut, and nut shell oil; β-caryophyllene and δ-cadinene identified in green tea flavor; and totarol isolated from the bark of *Podocarpus nagi*, showed potent activity against *P. acne* (Kubo *et al.*, 1994).

Glycyrrhiza glabra showed a remarkable antibacterial activity against *P. acnes*, resulting in negligible induction of resistance, in comparison with a marked development of resistance in the bacteria treated with erythromycin (Nam *et al.*, 2003).

Extracts of Eucalyptus globules, E. maculate and E. viminalis significantly have antimicrobial activities against six gram-positive bacteria (*Staphylococcus aureus, MRSA, Bacillus cereus, Enterococcus faecalis, Alicyclobacillus acidoterrestis* and *Propionibacterium acne*), and of a fugus (*Trichophyton mentagrophytes*) (Takahashi *et al.*, 2004).

Hayes and Markovic, 2002 reported that lemon myrtle oil has potential usage as an antiseptic or antimicrobial agent in the treatment of skin microorganisms associated with cuts, bites, acne and tinea. For lemon myrtle oil to be used as an effective topical antimicrobial is required.

It was found that *Rubia cordifolia, Curcuma longa, Hemidesmus indicus*, and *Azadirachta indica* caused a statistically significant suppression of reactive oxygen species (ROS) from polymorphonuclear leukocytes (PMNL). In the case of proinflammatory cytokine-induced monocytes, maximum suppression was shown by *Azadirachta indica* and Sphaeranthus indicus, followed by *Hemidesmus indicus*, *Rubia cordifolia* and *Curcuma longa* (Jain and Basal, 2003).

Aloe vera gel enhanced the anti-acne properties of *Ocimum oil*; the oil or its combination with aloe vera gel is more effective than 1% clindamycin in the treatment of acne vulgaris (Orafidiya *et al.*, 2004).

Hydrogel patches were prepared using sodium polyacrylate (SPA) and carboxymethylcellulose (CMC) as matrix polymers, and Al^{3+}, produced by the reaction of dihydroxy aluminum aminoacetate (DAA) and tartaric acid, as a cross linker. The patches were well-adhesive to facial skin for the treatment acne and they could be detached from the skins without any pain (Lee *et al.*, 2003).

Zinc, especially in the form of zinc gluconate or zinc sulfate, can help prevent acne. Zinc helps heal blemishes, reduces inflammation, and reduces androgenic hormonal effects on the skin. Comparing zinc to the antibiotic tetracycline found zinc to be as effective as tetracycline. Another study found a mild yet definite effect of zinc. It has been reported that the combination of erythromycin and zinc reduces comedones, papules, pustules, total inflamed lesions, nodules and macules more than erythromycin alone. With zinc-erythromycin association, a significant decrease in the number of microorganisms and in the presence of free fatty acids at the surface of the skin has been observed (Cerri *et al.*, 2004). Vitamin B6 may help premenstrual or mid-cycle acne. This vitamin is essential for the proper metabolism of steroid hormones and can reduce the sensitivity of skin to the effects of testosterone.

Sulfur has antifungal, antibacterial, and keratolytic activity. Sulfur was used widespread in dermatological disorders such as acne vulgaris, rosacea, seborrheic dermatitis, dandruff, pityriasis versicolor, scabies, and warts. Adverse events associated with topically applied sulfur are rare and mainly involve mild application site reactions. Sulfur, used alone or in combination with agents such as sodium sulfacetamide or salicylic acid, has demonstrated efficacy in the treatment of many dermatological conditions (Gupta and Nicol, 2004).

Salicylic acid is a beta hydroxy acid and has multifunctional uses in the treatment of various diseases in skin such as acne, psoriasis, and photoaging (Rhein *et al.*, 2004). Both inflammatory and noninflammatory acne lesion counts were decreased in proportion to the duration of treatment. The side effects were tolerable in most cases, and all patients were pleased with their peel results. Stratum corneum hydration, skin surface lipid, skin pH, and transepidermal water loss were unchanged from baseline levels. Salicylic acid peels are an effective and safe

therapy for acne vulgaris in Asian patients (Lee and Kim, 2003). Comparative studies of salicylic acid have shown it to be superior to benzoyl peroxide in reducing the total number of acne lesions (Zander and Weisman, 1992). Some results suggest that superficial salicylic acid peels are both safe and efficacious for treatment of acne vulgaris, oily skin, textural changes, melasma, and post-inflammatory hyperpigmentation in patients (Grimes, 1999). The results demonstrated that only patients treated with an acne cleanser containing 2% salicylic acid had a significant reduction in comedones and improvement in acne, but worsened during benzoyl peroxide therapy (Shalita, 1989). Dermatologist evaluations of the salicylic acid acne treatment showed a significant reduction in inflammatory acne lesion erythema (Miller *et al.*, 2005).

Piper betle

Piper betle, belonging to the Piperaceae family, is a tropical plant that grows in Malaysia, Taiwan, and other Southeast Asian countries. Some Piper species are used in folk medicine to treat many diseases. The plant is a stout creeper, climbing by adventitious roots at the nodes, quite glabrous. Leaves are simple, alternate, broadly ovate or rounded, 5-18 by 2-10 cm, having apex acute or acuminate, unequally rounded at the base or broadly heart-shaped, coriaceous, having prominent vein beneath. Flowers are very minute, in cylindrical male or female spikes, pendulous, male spikes are 2-12 cm long, having peduncle 1.5-3 cm long, female spikes are long-peduncled, without calyx and corolla, having one small bract with each flower; ovary with one cell and one ovule. Fruit is a berry, small, round, pulpy; containing one globose seed (Mishra and Gaur, 1979).

Piper betle Linn leaves have antimicrobial activity towards bacteria in the mouth i.e. *Streptococcus viridans*, *Staphylococcus aureus* and *Streptococcus mutans*. Essential oils of the plant contained phenolic compounds such as cavicol, cavibetol, carvacrol, eugenol and allilpyrocatechol. These compounds are assumed could inhibit foodborne pathogens as well as food spoilage microorganisms (Jenie *et al.*, 2001). The aromatic leaves describes as having carminative, stimulant, corrective, prophylactic, stomachic, expectorant, tonic, astringent and sialagogue properties (Perry, 1980).

Essential oil obtained from betel leaves also showed a dose-dependent larvicidal effect on Chrysomya larvae. This natural product may be effective in the treatment of wound myiasis (Kumarasinghe *et al.*, 2002). The essential oil (mainly chavicol) has a bactericidal effect, valuable for treating affections of the mucous membrane of the nose and throat; the leaves are included in numerous native medicines. The essential oil chiefly contains two phenols, the betel-phenol isomers of eugenol and chavicol, accompanied by variously composed phenols; mention allylpyrocatechin, terpene, cineole, caryophyllene, cadinene, and menthone (Perry, 1980). The essential oil of betel leaves, at a concentration of 1.0 g/L, exhibited anthelmintic activity on earthworms (*Ascaris lumbricoides*). At a concentration of 12.5 mg/L, the essential oil decreased contractions of small intestines in rats and rabbits. Dogs were given betel essential oil intravenously at a dose of 15 mg and a transient hypotensive effect was detected. Essential oil of betel leaves at a dose of 2.5 mg/L produced a relaxation of frog rectus abdominis (Ali and Mehta, 1970).

Water, petroleum ether and ether extracts exhibited antibacterial against *Escherichia coli, Salmonella enteritidis, Shigella flexneri, Aerobacter aerogenes, Erwinia sp., Serratia marcescens, Proteus vulgaris* and *Klebsiella pneumoniae* (Silpasuwan, 1979; Lumyong and Silpasuwan, 1986). Water extract exhibited smooth muscle relaxant properties when tested on isolated rat ileums (Apisariyakul, 1984; Apisariyakul *et al.*, 1987). The aqueous extracts of *Piper betle* and *Psidium guajava* were prepared and tested for their antiadherence effect on the adhesion of early plaque settlers (*Strep. mitis, Strep. sanguinis* and *Actinomyces* sp.). Raza and Rahim, 2003 reported that the anti-adherence activities of *Piper betle* and *Psidium guajava* extracts towards the bacteria were different between the bacterial species.

The in vitro antimicrobial activity of different varieties of *Piper betle* Linn, leaf stalk extracts have been studied against human pathogenic bacteria and phytopathogenic fungi by comparing the results with standard microbial susceptibility testing biodiscs (Shitut *et al.*, 1999). Some report showed antibacterial activity for aqueous alcohol extracts against *Vibro cholerae, V. parahemolyticus, Salmonella typhosa, Shigella flexneri, E. coli, S. aureus* and *Ps. aeruginosa* (Chalermpunchai *et al.*, 1987). Eugenol isolated from *P. betle* exhibited antifugal activity against

Aspergillus flavus. Sitosterol, ursolic acid and ursolic acid-3β-acetate isolated from *P. betle* inhibited arachidonic acid-induced platelet aggregation in decreasing order of potency (Parmar *et al.*, 1997). The allyphenols from *P. betle* (leaf) have been reported earlier, this is the report which establishes that these constituents exhibit strong antimicrobial effects against obligate oral anaerobes (Ramji *et al.*, 2002).

The results show that both hot water extract and cold ethanolic extract for *Piper betle* leaves posses marked hypoglycaemic activity and antihyperglycaemic activity (Arambewela *et al.*, 2005). The presence of the betle leaf extract inhibited the radiation induced lipid peroxidation process effectively (Choudhary and Kale, 2002). The results revealed the potential, reversible male contraceptive effect of *Piper betle* leaf-stalk extrative (Sarkar *et al.*, 2000). A water extract of betel leaves exhibited slight activity on oriental fruit flies (Areekul *et al.*, 1987).

One such medicinal plant studied was *Piper betle* L. Results showed that among the 300 plant species screened, *P. betle* maintained a broad spectrum antibacterial activity against all the test pathogens, such as Ralstonia, Xanthomonas, and Erwinia. It was also revealed that the *P. betle* solvent extract had more superior action than streptomycin. Some study was carried out to investigate the hepatoprotective and antioxidant properties of *P. betle*, using ethanol intoxication as a model of hepatotoxic and oxidative damage (Saravanan *et al.*, 2002)

All patients had a history of using facial dressings with steamed leaves of piper betle. The clinical course and histopathologic findings suggest that the evolution of this pigmentary disorder finally leads to confetti-like depigmentation. It may be induced by chemicals in the betle leaves such as phenol, catechol and benzene derivates, perhaps through inhibition of melanin synthesis or melanocytotoxicity

The leaf suspension of *P. betle* 75 mg/kg body weight showed significant antioxidant effects in streptozotocin diabetic rats (Santhakumari *et al.*, 2003).

The radioprotective activity of PE could be attributed to its hydroxyl and superoxide radicals scavenging property along with its lymphoproliferative activity (Bhattachrya *et al.*, 2005)

Hydroxychavicol is the major phenolic compound in *P. betle* leaf. Some result showed that hydroxychavicol-induced copper-dependent single-strand DNA break and 8-hydroxy-2'-deoxyguanosine formation in cultured CHO-Kl and $HepG_2$ cells (Chen *et al.*, 2000). The results suggest that hydroxychavicol-induced dihydrodiol dehydrogenase (DDH) is more important than site-by-site up-regulation of cyclooxygenase-2 (COX-2) in B[a]P-induced cytotoxicity and *HPRT* gene mutation (Tang *et al.*, 2004).

Tea tree oil

Tea tree oil is the essential oil steam distilled from the Australian plant *Melaleuca alternifolia*. This species is unique to Australia and native to Northern New South Wales. Tea tree oil contains over 100 components, mostly terpines, cymones, pinines, terpineols, cineol, sesquiterpenes and sesquiterpene alcohols. Terpinen-4-ol is present at the highest levels (minimum 30%) and is responsible for most of the antimicrobial activity. In recent years tea tree oil has become increasingly popular as an antimicrobial for the treatment of conditions such as tinea pedis and acne (Koh *et al.*, 2002). The essential of *Melaleuca alternifolia* (tea tree oil) has been used medicinally and has broad-spectrum antimicrobial and anti-inflammatory activity in vitro (Messager *et al.*, 2005).

Some study supports the use of tea tree oil in the treatment of acne. The major components of tea tree oil such as terpinen-4-ol, alpha-terpineol and alpha-pinene were found to be active against *Staphylococcus aureus, Staphylococcus epidermidis* and *Propionibacterium acnes.* The MIC value of the compounds increased in the order alpha-terpineol < terpinen-4-ol < alpha-pinene for all three microorganisms. MIC values of the tea tree oils and terpinen-4-ol were lower for *P. acnes* than for *S. aureus* and *S. epidermidis* (Raman *et al.*, 1995).

The results of this study showed that both 5% tea tree oil and 5% benzoyl peroxide had a significant effect in improvement the patients' acne by reducing the number of inflamed and non-inflamed lesions, although the onset of action in the case of tea tree oil was slower (Bassett *et al.*, 1990).

Many *S. aureus* isolates (MSSA and MRSA) have been found to be susceptible to *Melaleuca alternifolia* (tea tree) essential oil (Halcon and Milkus, 2004).

Types of preparation

Suspension

Suspensions may be defined as preparations containing finely divided drug particle distributed somewhat uniformly throughout a vehicle in which the drug exhibits a minimum degree of solubility. Some suspensions are available in ready-to-use form, that is, already distributed through a liquid vehicle with or without stabilizers and other additives.

Cream

Cream are opaque, soft solids or thick liquids consisting of mediations that are dissolved or suspended in water-removable or emollient bases. Creams are usually applied to moist, weeping lesions because they have a somewhat drying effect in that the lesions' fluids are miscible with the aqueous external phase of creams (Allen *et al.*, 2004).

Lotion

Lotions are fluid emulsions or suspensions designed for external application to skin. Most lotions contain finely powdered substances that are insoluble in the dispersion medium and are suspended through the use of suspending agents and dispersing agents (Allen, 2002).

Gels

One of the most versatile delivery systems that can be compounded is the pharmaceutical gel. Gels are an excellent drug delivery system for various routes of administration and are compatible with many different drug substances. Gels containing penetration enhancers are especially popular for administering anti-inflammatory and antinouseant medications. They are relatively easy to prepare and are quite efficacious.

Definitions of gel

According to the United States Pharmacopeia (USP), gels (sometimes called Jellies) are defined as semisolid systems consisting of suspensions made up of either small inorganic particles or large organic molecules enclosing and interpenetrated by a liquid. If the gel mass consists of a network of small discrete particles, the gel is classified as phase is large, the product is referred to as a magma. Conversely, single-phase gels throughout a liquid in such a manner that no apparent boundaries exist between the dispersed macromolecules and the liquid. Single-phase gels can be made from synthetic macromolecules or from natural gums (mucilages). The continuous phase is usually aqueous but can also be alcoholic or oleaginous (Allen *et al.*, 2004; Gibson, 2001; Zatz and Kushla, 1996).

Depending on their constituents, gels may be clear or opaque, and be polar, hydroalcoholic or non-polar. The simplest gels contain water thickened with natural gums, semi-synthetic materials, synthetic materials or clays. Gel viscosity in generally a function of the amount and molecular weight of the added thickener. Gels can be used to administer medications orally, topically, intranasally, vaginally, and rectally. They can serve as ointment bases. The characteristics of the gelling agents will determine the techniques used in their preparation. To appeal to the consumer, gels should have clarity and spakle. Most gels act as absorption bases and are water washable, water soluble, water absorbing, and greaseless. Gels should maintain their viscosity and character over a wide range of temperatures. There are a variety of semisynthetic celluloses in use as thickeners in gel formulations. These

include MC, CMC, HEC, hydroxypropyl cellulose (HPC) and hydroxypropyl methyl cellulose (HPMC).

Theory of gel

Gels are also defined as semirigid systems in which the movement of the dispersing medium is restricted by an interlacing three-dimensional network of particles or solvated macromolecules of the dispersed phase. A high degree of physical or chemical cross-linking can be involved. The increased viscosity caused by the interlacing and consequential internal friction is responsible for the semisolid state. A gel can consist of twisted, matted strands often wound together by stronger types of van der Waals forces to form crystalline and amorphous regions throughout the system. Some gel systems are as clear as water in appearance; others are turbid because their ingredients may not be completely molecularly dispersed or they may form aggregates, which disperse light (Pena, 1990).

The selection of formulation type for systemic transdermal products, which are designed for application to intact non-diseased skin, is guided by the requirement of the system, be it a semi-solid or patch preparation, to deliver therapeutic amounts of drug into the systemic circulation. On the other hand, the selection of formulation type for dermatological products is influenced more by the nature of the skin lesion. In general, the preparation of such formulations as poultices and pastes is extemporaneous, and it is unlikely that the industrial pharmaceutical formulator will be required to develop stable, safe and efficacious products of this type. The developed formulation will be an ointment, emulsion or a gel. Typical constituents for these types of formulations are shown in Table 1.

Table 1. Constituents of semi-solid formulations

Function	Sample Ingredients		
Polymeric thickeners	Gums		Acrylic acids
	Acaia		Carbomers
	Alginates		Polycarbophil
	Carageenan		Colloidal solids
	Chitosan		Silica
	Collagen		Clays
	Tragacanth		Microcrystalline cellulose
	Xanthan		Hydrogels
	Celluloses		Polyvinyl alcohol
	Sodium carboxymethyl		Polyvinylpyrrolidone
	Hydroxyethyl		Thermoreversible polymers
	Hydroxypropyl		Poloxamers
	Hydroxypropylmethyl		
Oil phase	Meneral oil	White soft parafin	Isopropyl palmitate
	Isopropyl myristate	Yellow soft parafin	Castor oil
	Beewax	Canola oil	Stearyl alcohol
	Cottonseed oil	Cetyl alcohol	Jojoba oil
	Cetostearyl alcohol	Arachis (peanut) oil	Stearic acid
	Lanolin (and derivaties)	Oleic acid	Silicone oils
Surfactants	Nonionic		Anionic
	Sorbitan esters		Sodium dodecyl sulphate
	Polysorbates		Cationic
	Polyoxyethylene alkyl ethers		Cetrimide
	Polyoxyethylene alkyl esters		Benzalkonium chloride
	Polyoxyethylene aryl ethers		
	Glycerol esters		
	Cholesterol		

Table 1. Constituents of semi-solid formulations (continued)

Function	Sample Ingredients		
Solvents	Polar		Nonpolar
	Water		Isopropyl alcohol
	Propylene glycol		Medium chain triglycerides
	Glycerin		
	Sorbitol		
	Ethanol		
	Industrial methylated spirit		
	Polyethylene glycols		
	Propylene carbonate		
	Triacetin		
Preservatives	Antimicrobial		Antioxidants
	Benzalkonium chloride	Benzoic acid	α-Tocopherol
	Benzyl alcohol	Bronopol	Ascorbic acid
	Chlorihexidine	Chlorocresol	Ascorbyl palmitate
	Imidazolidinyl urea	Paraben esters	Butylated hydroxyanisole
	Phenol	Phenoxyethanol	Butylated hydroxytoluene
	Potassium sorbate	Sorbic acid	Sodium ascorbate
			Sodium metabisulphite
			Chelating agents
			Citric acid
			Edetic acid
pH adjusters	Diethanolamine	Sodium hydroxide	
	Lactic acid	Sodium phosphate	
	Monoethanolamine		
	Triethanolamine		

Classification (Allen, 2002; Zatz and Kushla, 1996)

Gels are categorized according to two classification systems. One system divides gels into inorganic and organic; the other distingulishes them by the classifications hydrogels and organogels.

Inorganic gels are usually two-phase systems, whereas organic gels are generally single-phase systems. Hydrogels contain ingredients that are either dispersible as colloids or soluble in water; they include organic hydrogels, natural and synthetic gums, and inorganic hydrogels. In high concentrations, hydrophillic colloids form semisolid gels, also referred to as jellies.

Organogels include the hydrocarbons, animal/vegetable fats, soap base greases, and hydrophilic organogels. The hydrophilic organogels, or polar organogels, are soluble to about 75% in water and are completely washable.

Table 2. General classification and description of gels

Class	Description	Examples
Inorganic	Usually two-phase system	Aluminum hydroxide gel, bentonite magma
Organic	Usually single-phase system	Carbomer, tragacanth
Hydrogels (jellies)	Inorganic	Bentonite, veegum, silica, alumina
	Natural and synthetic gums	Pectin, tragacanth, sodium alginate
	Organic	Methylcellulose, sodium carboxymethylcellulose, Pluronic F-127
Organogels	Hydrocarbon type	Petrolatum, mineral oil/polyethylene gel, Plastibase/Jelene
	Animal/vegetable fats	Lard, cocoa butter
	Soap base greases	Aluminum stearate with heavy mineral oil gel
	Hydrophillic organogels	Carbowax bases (PEG ointment)

Characteristics of gels (Zatz and Kushla, 1996)

(1) Imbibition is the taking up of a certain amount of liquid by a gel without a measurable increase in volume.

(2) Swelling is the taking up of liquid by a gel with an increase in volume. Only those liquids that solvate a gel can cause swelling. The swelling of protein gels is influenced by pH and the presence of electrolytes.

(3) Syneresis is the contraction of a gel caused by the interaction between particles of the dispersed phase. This interaction becomes so great that, on standing, the dispersing medium is squeezed out in droplets, causing the gel to shrink. Syneresis is a form of instability in aqueous and nonaqueous gels. The solvent phase is thought to separate because of the elastic contraction of the polymeric molecules; as swelling increase during gel formation, the macromolecules become stretched and the elastic forces expand. At equilibrium, the restoring force of the macromolecules is balanced by the swelling forces, determined by the osmotic pressure. If the osmotic pressure decreases, such as on cooling, water can be squeezed out of the gel. The pH has a significant effect on the separation of water. At low pH, marked syneresis occurs, possibly because of suppression of ionization of the carboxylic acid groups, loss of hydrating water, and the formation of intramolecular hydrogen bonds. These conditions would reduce the attraction of the solvent for the macromolecule.

(4) Thixotropy is a reversible gel-sol formation with no change in volume or temperature. It is considered a type of non-Newtonian flow.

Stability

Gels should be observed for such physical characteristics as shrinkage, separation of liquid from the gel, discoloration and microbial contamination. Many gels will not promote bacterial/mold growth, nor will they prevent it. Consequently, they should be autoclaved or should contain preservatives. Gelling agents in the dry state are usually not a problem. The pharmacist should follow standard quality control procedures. These procedures involve checking the appearance, uniformity, weight/volume, viscosity, clarity, pH, and smell of the gels (Zatz and Kushla, 1996). Stability testing is done to ensure that a developed product will be fit for use during its expected life. Products can be placed at ambient, and elevated (e.g. 37°C and 45°C) temperatures, refrigerated and cycled through freeze/thaw cycles. Two or three months of successful elevaluated temperature testing and three or four freeze/thaw cycles will usually indicate that products will have an adequate shelf-life. It is important, however, to continue testing for longer periods at ambient temperature, to obtain an understanding of the product's ultimate shelf-life. Further testing whenever changes are made to the supply of raw materials or to the formulation is essential.

Gelling agents:

Acacia gum

Acacia gum is obtained from several species of the acacia tree, especially *Acacia Senegal* and *Acacia Arabica*. In addition acacia gum has water solubility, is insoluble in alcohols and forms colorless, tasteless solutions. It is commonly known under the name of gum Arabic. Gum arabic is consisted mainly of a (1-3)-β-D-galactan core with (1-6)-β-D-galacto-pyranosyl branches and with α-L-arabinofuranosyl-(1-3)-α-L-arabinofuranosyl and α-L-rhamnopyranosyl-(1-4)-β-D-glucopyranosyluronic acid groups attached to position 3 and 6, respectively, of the branch units (Defaye and Wong, 1986; Pinto *et al.*, 2002; Sanchez *et al.*, 2002). Gum arabic is a mixture of several related polysaccharides with a molecular weight ranging from $2.6x10^5$ to $1.2x10^6$ (Al-Assaf *et al.*, 2005).

Gum arabic is used in the food industry as a flavor fixative and emulsifier, to prevent crystallization of sugar in confections, as a stabilizer in frozen dairy products, for its viscosity and adhesive properties in bakery products, and as a foam stabilizer and clouding agent in beer. In pharmaceutics, it is used as a stabilizer for emulsions, binder and coating for tablets, and as an ingredient in cough drops and syrups. A soothing and softening agent, gum arabic is extensively employed in folk medicines. Among many other uses, it is used internally for coughs, diarrhea and externally to cover inflamed areas. Gum arabic is used in cosmetics as an adhesive for facial masks and powders, and to give a smooth feel to lotions. Solutions of gum arabic have a low viscosity and good stability over the range from pH 2 to 10. Gum arabic is a superior emulsion former that has no substitute among synthetic additives. It provides excellent shelflife stability to oil in water emulsions and does not mask flavors with a filmy texture or off-flavor on the tongue (Pinto *et al.*, 2001).

Alginate

Alginate is found in a wide variety of brown seaweeds (*Phaeophyceae*, mainly *Laminaria*) that is found throughout the world and is present as a structural polysaccharide. Alginate is made up of a linear block copolymer of 1,4 linked α-L-guluronic acid and β-D-mannuronic acid at different proportions and with different sequential occurrence. The blocks are vary composed of homopolymeric blocks MM or GG, and blocks with an alternating sequence, the MG blocks, as well as the growing conditions of the weed (Aslani, and Kennedy, 1996; Aulton, 2002). The block structure ultimately dictates the gelling properties of the alginate produced. Commercial manufacturing of alginate almost always involves a purification step where an acid treatment precipitates the alginate as alginic acid (Draget *et al.*, 2005). The prepared product of alginic acid is a tasteless, practically odorless, white to yellowish-white, fibrous powder. It is used in concentrations between 1% and 5% as a thickening agent in gels. It swells in water to about 200 to 300 times its own weight without dissolving. Cross-linking with increased viscosity occurs when adding a calcium salt, such as calcium citrate. Alginic acid can be dispersed in water that is vigorously stirred for approximately 30 minutes. Premixing with another powder or with a water-miscible liquid aids in the dispersion process.

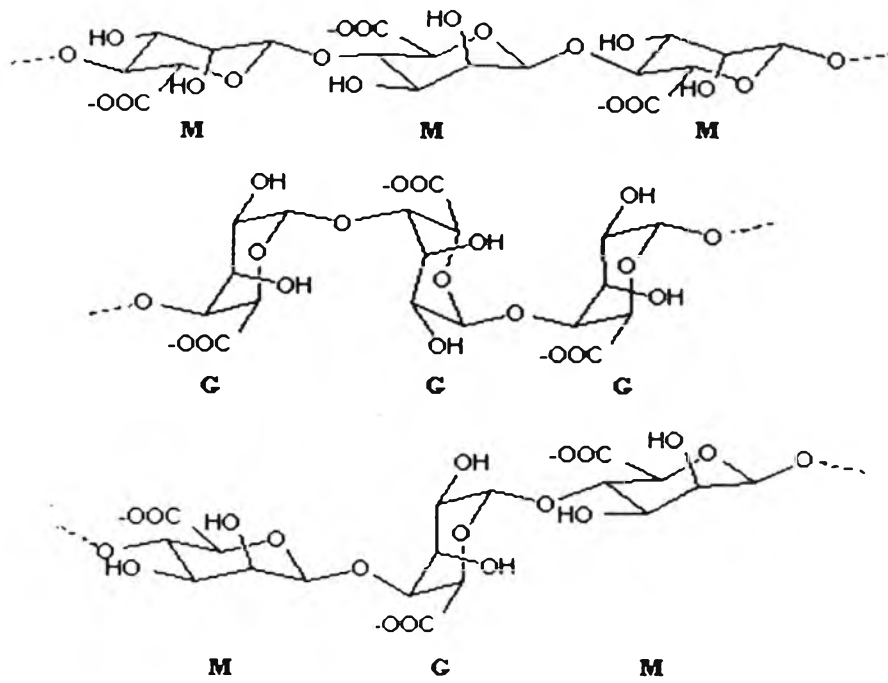


Figure 2. Structure of alginate that composed of homopolymeric blocks MM or GG, and blocks with an alternating sequence, the MG blocks.

Alginate, commercially available as alginic acid, sodium salt, commonly called sodium alginate. Alginic acid can be either water soluble, whereas the salts of polyvalent cations, e.g., calcium, are water insoluble, with the exception of magnesium (Jang *et al.*, 1995). Among the most versatile of the hydrocolloids, alginates are used in a wide variety of applications as thickeners, stabilizers, and gelling agents. Alginates have been used for a vast range of applications for more than 50 years. Alginates are cold-soluble and cold-setting. Further, alginate is heat and freeze/thaw stable. As a thickening agent, alginate offers a broad range of flow properties for aqueous-based systems. Alginates, especially sodium alginate, are widely used in the textile industry because they form an excellent dressing and polishing material. Calcium alginate, which is insoluble in water, has been used in the manufacture of a medical dressing very suitable for burns and extensive wounds (Borchard *et al.*, 2005; Drury, *et al.*, 2004; Richardson *et al.*, 2004). Alginate is commonly used as thickeners in foods such as ice cream and fruit-filled snacks, coffee and cheese spread. It is also used for dental impression materials, drug encapsulation,

wound dressings and as a component of the antacid. Alginate can also be produced from a bacterial source (*Azobacter Vinelandii*). However the block structure in bacterial alginate tends to give a product with poor gelling characteristics and the expense of production means the product has never been commercialized and remains of academic interest only. Alginates exhibit a maximum viscosity over a pH range of 5-9, and at low pH the acid is precipitated. Alginate mucilages must not be heated above 60°C as depolymerization occurs, with a consequent loss in viscosity.

Carrageenan

Carrageenan is a collective term for polysaccharides from red seaweed (*Rhodophycae*), mostly of genus *Chondrus, Eucheuma, Gigartina* and *Iridaea* (Bornhoft *et al.*, 2005). Carrageenans can be produced via a variety of process techniques; alcohol extraction, potassium chloride gel press or extracted with various alkalis. The process technique is important because it influences the gel characteristics. Likewise, different seaweeds also produce different carrageenans and influence gel characteristics. Carrageenan are mainly potassium, sodium, calcium, magnesium and ammonium sulfate esters of galactose and 3,6-anhydrogalactose which are alternately linked α-1,3 and β-1,4 in the polymer (Figure 3).

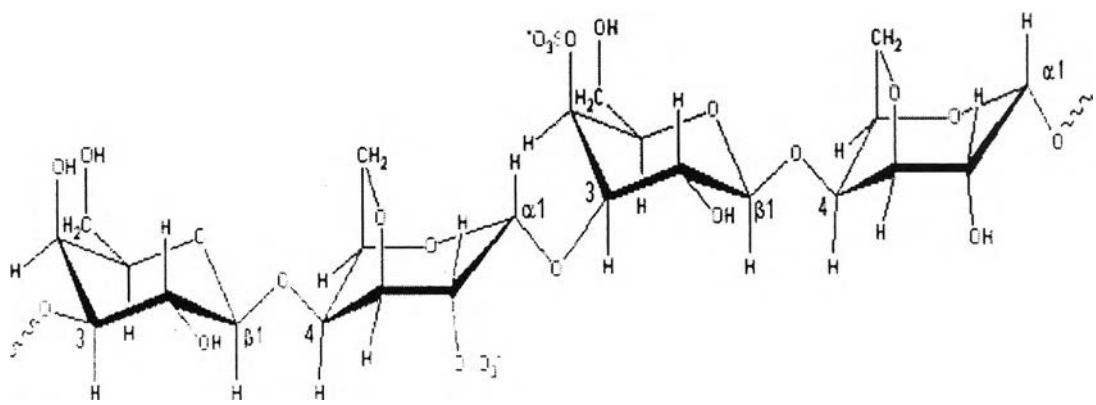
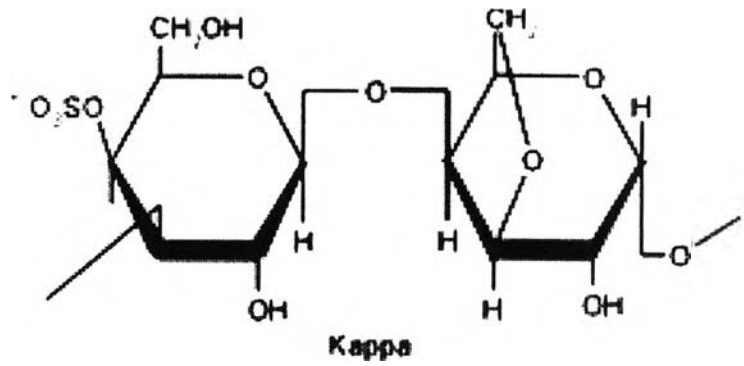


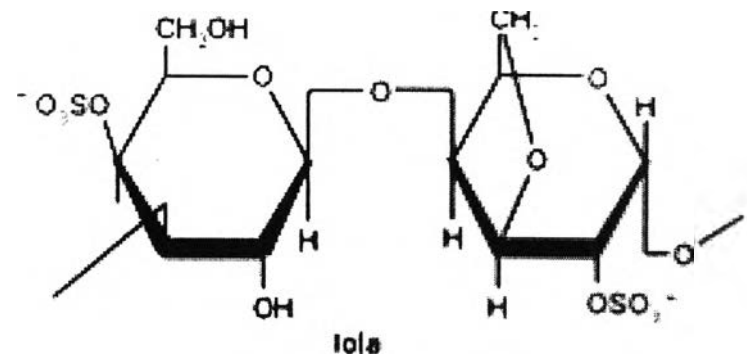
Figure 3. Structural unit of carrageenan.

There are three major types of carrageenan, kappa (κ), iota (ι) and lambda (λ)-carrageenans, which differ in the number/position of sulphate groups and the content of 3,6-anhydrogalactosyl ring per disaccharide. Kappa carrageenan consists of a repeating unit composed of the disaccharide, β-(1-3)-D-galactose-4-sulfate and α-(1-4)-3,6-anhydro-D-galactose. Iota carrageenan possesses two sulfate groups in a disaccharide repeat unit; β-(1-3)-D-galactose-4-sulfate and α-(1-4)-3,6-anhydro-D-galactose-2-sulfate. Lambda carrageenan consists of β-(1-3)-D-galactose-2-sulfate and α-(1-4)-D-galactose-2,6-disulfate including three sulfate groups. It should be noted that the represented structure is an ideal one and the real samples contain some extent of different kind of sequences (Gu *et al.*, 2005).

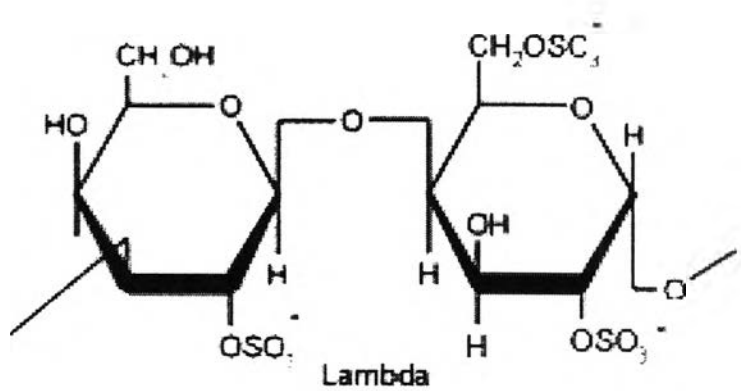
Kappa carrageenan is the most commonly used type of carrageenan. Its most important properties are its high gel strength and strong interaction with milk proteins. About 70% of the worlds carrageenan production is based on kappa carrageenan. Iota carrageenan is a type of carrageenan with a sulphate content intermediate between kappa and lambda carrageenan. Iota carrageenan forms an elastic gel with good freeze thaw and rehealing properties. Lambda carrageenan is a highly sulphated type of carrageenan mainly used for its ability to impart mouth feel and a creamy sensation to dairy products. Lambda carrgeenan does not gel. Kappa and iota types of carrageenan are not cold soluble and require heating to achieve solubility. Lambda type carrageenan is unique in that it is cold water soluble. Yuguchi *et al.,* 2002 found that aqueous solution of kappa and iota carrageenan undergoes thermoreversible sol/gel transition by cooling or increasing concentration, while no gelation takes place in lambda carrageenan having more electrolyte groups. Gels created from different carrageenan types may be fluid, elastic or rigid and are heat reversible. Gelling temperatures and gel strength are also influenced by added ingredients such as salts and proteins. Carrageenans are linear, water soluble polymers that form viscous solutions. Viscosity depends mainly on concentration of the polymer, temperature of the solution and the type of carrageenan. In hand lotions and shampoos, carrageenan not only thickens the product but also promotes healthy skin and hair. In addition, carrageenan is a natural product and can be incorporated into formulations which rely on natural ingredients for their promotion. Carrageenans are used mainly for thickening, suspending and gelling.



(A) Kappa



(B) Iota



(C) Lamda

Figure 4. There are three major types of carrageenan; (A)- kappa (κ), (B)-iota (ι) and (C)-lambda (λ)-carrageenans

Chitosan

Chitin is the second most abundant biopolymer next to cellulose. Chitin is a straight homopolymer composed of β-(1,4)-linked *N*-acetyl-glucosamine units while chitosan comprises of copolymers of glucosamine and *N*-acetyl-glucosamine. Chitosan is a useful derivative of chitin. Chitosan is a biodegradable natural polymer with great potential for pharmaceutical applications due to its biocompatibility, high charge density, non-toxicity and mucoadhesion. It is obtained by alkaline deacetylation of chitin. Chitosan is nontoxic copolymer consisting of β-(1,4)-2-acetamido-2-deoxy-D-glucose and β-(1,4)-2-amino-2-deoxy-D-glucose units. The difference between cellulose and chitosan is that the 2-hydroxy group of the cellulose has been replaced with an acetamide group. Chitin is the principal component of protective cuticles of crustaceans such as crabs, shrimps, prawns, lobsters and cell wall of some fungi such as *aspergillus* and *mucor*. It has been shown that it not only improves the dissolution of poorly soluble drugs but also exerts a significant effect on fat metabolism in the body. Chitosan has attracted considerable interest due to its antimicrobial and antitumor.

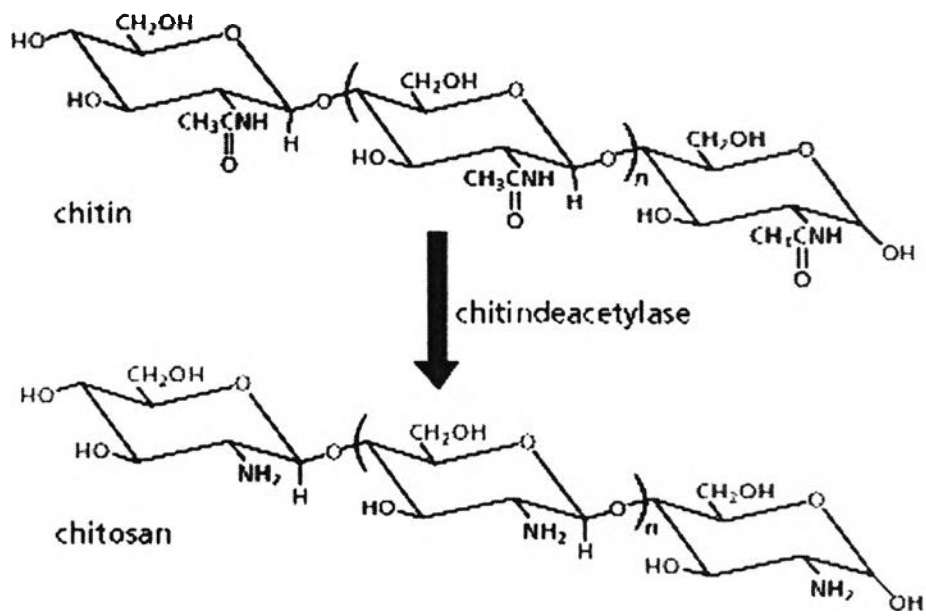


Figure 5. Deacetylation of chitin to chitosan by the chitindeacetylase

Guar gum

Guar gum is a natural nonionic polysaccharide derived from seeds of *Cyamopsis tetragonolobus* (Family: Leguminaciae). It consisted of linear chains of (1 → 4)-linked β-D mannopyranose units with α-D-galactopyranose units connected to the mannose backbone through (1 → 6) glycosidic linkages. It contains about 80% galactomannan, 12% water, 5% protein, 2% acid insoluble ash, 0.7% ash and 0.7% fat (Toti and Aminabhavi, 2004). Guar gum is made up of non-ionic polydisperse rod-shaped polymers consisting of molecules (longer than found in locust bean gum) made up of about 10,000 residues. The average molecular weight of guar gum ranges from 100,000 to 2,000,000 (Wang *et al.*, 2005). Guar gum hydrates and swells in cold water forming viscous colloidal dispersion or sols. Guar gum is a white to yellowish white powder. It is nearly odourless. Fine finished guar gum powder is available in different viscosities and different granulometries depending on the desired viscosity development and application (Ma and Pawlik, 2005). Guar gum is used as a binder (up to 10%) and disintegrating agent in solid dosage forms. It is also used as a suspending, thickening and stabilizing agent (up to 2.5%) in liquid oral and topical product (Soppimath *et al.*, 2001).

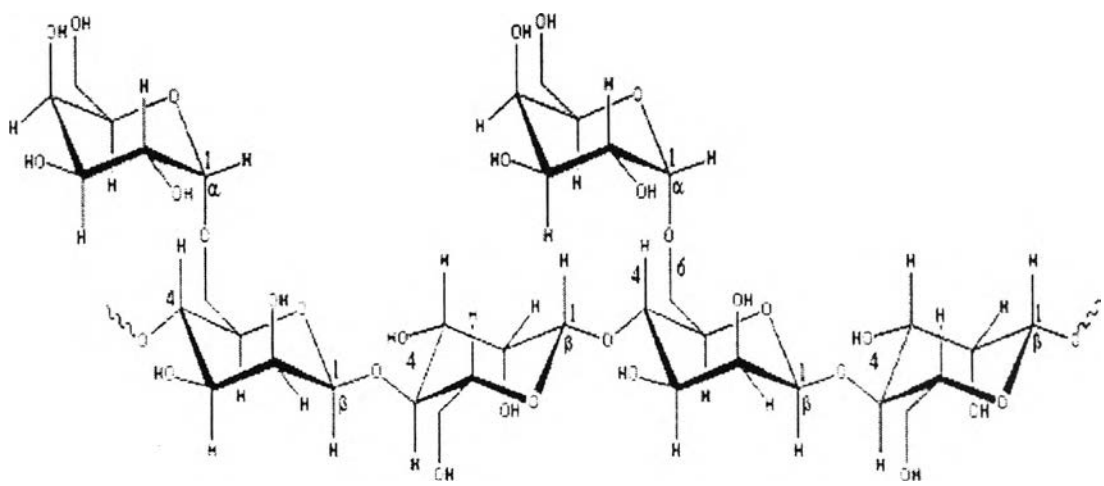


Figure 6. Structural unit of Guar gum.

Gum karaya

Gum karaya is a natural gum exudate of *Sterculia urens*, a tree native to India belongs to the family Sterculiaceae. Gum karaya is a soluble fiber which aids in the intestinal processes of the digestive system. It is widely used in food industry, as it is an approved food additive. The wider application of gum karaya due to its unique features such as high swelling and water retention capacity, high viscosity properties, inherent nature of anti-microbial activity and abundant availability. The gum karaya was used as laxative due to its high swelling ability and formation of discontinuous mucilage. Its principal food applications include ice creams and salad dressings. The pharmaceutical applications of gum karaya include medical colostomy bag fixings, dental fixatives and bulk laxatives (Cerf *et al.*, 1990).

Locust bean gum

Locust bean gum (Carob bean gum or Algaroba) is obtained from the endosperm of the Carob or locust seed (Ceratonia siliqua). Their chemical structure is a galactomannan similar to guar gum consisting of a 1,4-linked β-D-mannopyranose backbone with 1,6-linked α-D-galactose side groups (Dunstann *et al.*, 2001; Richardson *et al.*, 1998). Locust bean gum is polydisperse consisting of non-ionic molecules made up of about 2000 residues. Locust bean gum is less soluble and lower viscosity than guar gum as it has fewer galactose branchpoints. It needs heating to dissolve but is soluble in hot water. Locust bean gum differs from guar gum in that it does form thermally-irreversible weak gels by association of the galactose deficient regions and therefore has poorer freeze thaw behavior. Locust bean gum is the widely used in modern food industry (Camacho *et al.*, 2005). The most common food applications include frozen desserts, cultured dairy products, cheese products (especially spreads); sauces, dips, dough conditioner; pet food and dressings (Lundin and Hermansson, 1995; Mandala *et al.*, 2004). Industrial applications include textile warp sizing and paper fiber bonding. Locust bean gum is not capable of forming gels on its own in water systems. However, it improves gel strength when used in combination with carrageenan or xanthan gum.

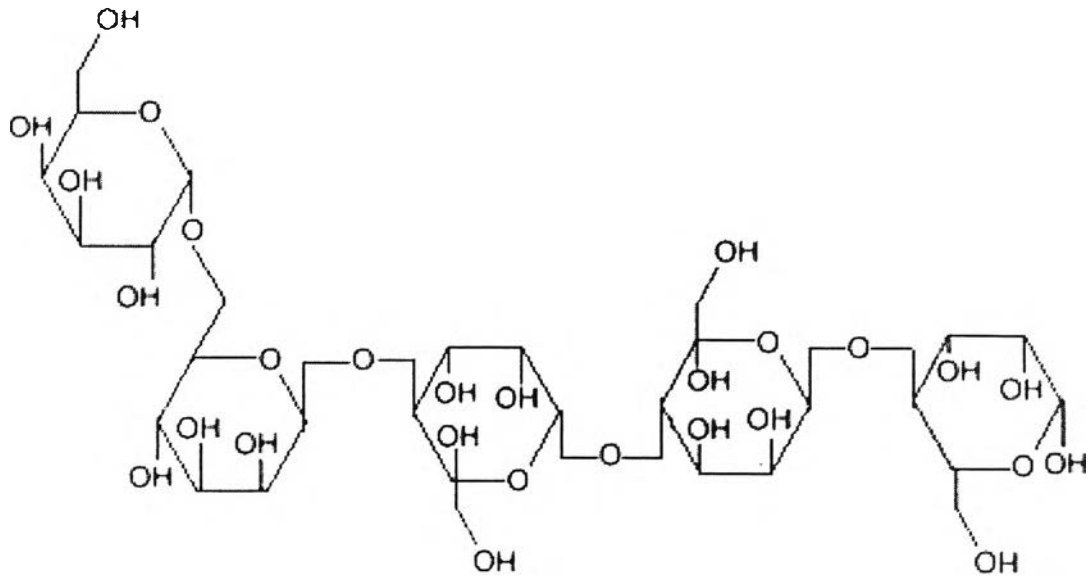


Figure 7. Structural unit of Locust bean gum.

Pectin

Pectin is an important component of the plant cell wall. It is present in the highest concentration in the middle lamella, where it acts as cementing substance between adjacent cells (Lin *et al.*, 2005; Yoo *et al.*, 2006). Pectin is a complex polysaccharide being polymolecular, polydisperse and heterogenic with respect to chemical composition and structure. Structurally they consist of a backbone of (1,4)-α-D-galacturonosyl residues interrupted with typically 10% substitution of (1,2)-α-L-rhamnopyranosyl residues. A portion of the rhamnosyl residues are branch points for neutral sugar side-chains. Chemically, pectin is a linear polysaccharide containing from about 300 to 1,000 monosaccharide units. The molecular weight of pectin ranges from 50,000 to 150,000 daltons. It is often stated that the pectic aggregates within the cell wall are of the egg-box structure, where two pectic chains in a twofold helical conformation retain calcium ions between them like eggs in a egg-box.

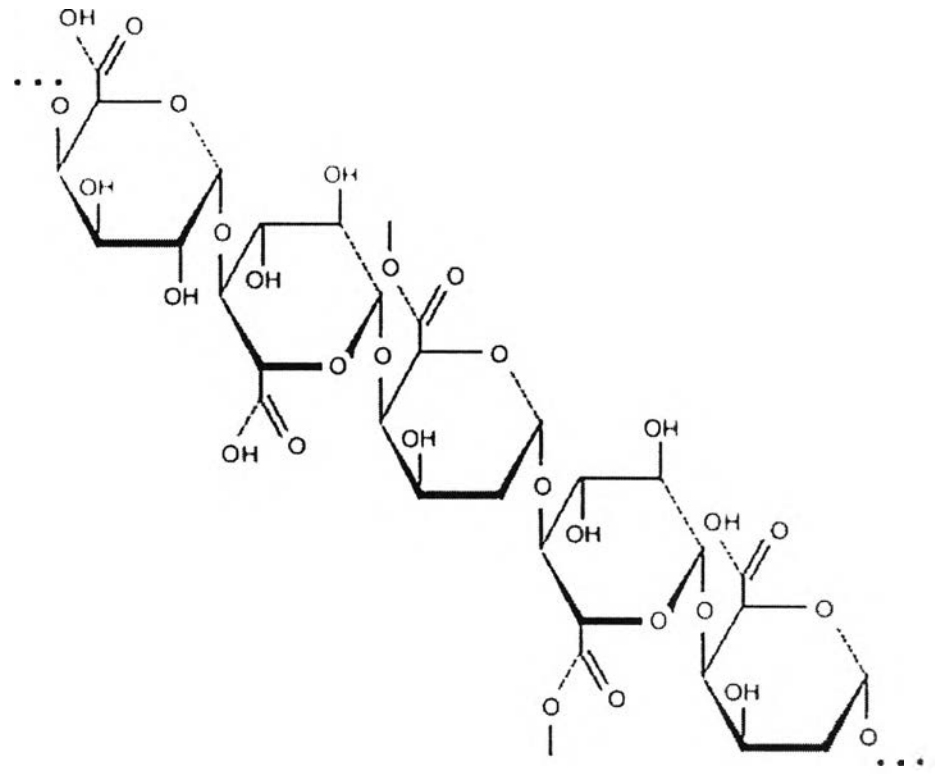


Figure 8. Structurally pectin consist of a backbone of (1,4)-α-D-galacturonosyl residues interrupted with substitution of (1,2)-α-L-rhamnopyranosyl residues.

The galacturonic acid residues in pectin may be esterified with methyl groups. There are different types of pectin. Pectin in which more than 50% of the galacturonic acid residues are esterified is called high methoxyl or HM pectin. Pectin in which less than 50% of the galacturonic acid residues are esterified is called low methoxy or LM pectin. Low methoxyl pectins 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 sugars such as sucrose and at pH (<3.5); the lower the methoxyl content, the slower the set (Ridley *et al.*, 2001). Pectin found in fruit and vegetables and mainly prepared from waste citrus peel and apple pomace. Pectin is obtained by the aqueous extraction of citrus peels and apple pulp under mildly acidic conditions. Pectin obtained from citrus peels is referred to as citrus pectin. Pectin is used in the food industry as a natural ingredient

due to its ability to form gels at low concentrations and to increase the viscosity of liquid foods. Pectin is widely applications in a variety of food formulations as a gelling and thickening agent to impart a gelled texture to foods, mainly fruit-based foods such as jams, jellies and marmalades (Gulfi *et al.*, 2005). In addition to this pectin have many other. It also has pharmaceutical industries. Pectin is used in combination with the clay kaolin (hydrated aluminum silicate) for the management of diarrhea. Pectin is also marketed as a nutritional supplement for the management of elevated cholesterol. Pectin is not digested, and is considered a beneficial dietary fiber (Evageliou *et al.*, 2000).

Tragacanth gum

Tragacanth mainly consists of polysaccharides, obtained from the secretion of tragacanth trees by incision of the stems of various species of *Astragalus* (*A. microcephalus, A. gummifier,* and *A. kurdicus*). Tragacanth consists of two polysaccharides, of which a water-soluble fraction known as tragacanthin, and a water-insoluble fraction known as bassorin. The soluble fraction is a complex mixture of acid polysaccharides with the following molecular constituents: D-galacturonic acid, D-galactose, L-fucose, D-xylose and L-arabinose (Anderson and Bridgeman, 1985; Eastwood, 1984; Tischer *et al.*, 2002). In the insoluble fraction, the carboxyl groups of the uronic acid are mainly esterified with methanol. It has been shown that the best grades of gum contain the least amount of tragacanthin. The molecular weight ranges between $8x10^5$ and $1.5x10^6$. The molecules seem to be very stretched, which is a prerequisite for solutions of high viscosity. The gum swells rapidly, in either cold or hot water, to form highly viscous dispersions, up to 4000 mPas at 1% solids, depending on the grade. Tragacanth occurs as a white to whitish powder or white to light yellowish white translucent flattened or laminar flake, and it is odorless. Since powdered tragacanth gum tends of form lumps when added to water, aqueous dispersions are prepared by adding the powder to vigorously stirred water. Tragacanth is stable over a pH range of 4-7.5 but takes several days to hydrate fully after dispersion in water. These gels must be preserved with either 0.1% benzoic acid or sodium benzoate or a combination of 0.17% methylparaben and 0.03% propylparaben. These gels can be sterilized by autoclaving. Ethanol, glycerin or propylene glycol can be used to prewet the powder. Other powder can be mixed with

the tragacanth while dry, before adding to the water. There are several grades of this material and only the best quality is suitable for uses as a pharmaceutical suspending agent. Tragacanth is used mainly in manufacturing as a thickener or stabilizer. When combined with liquids, tragacanth absorbs water and expands, forming a thick liquid or a gel, depending on how much liquid is added. This effect helps to keep the ingredients in combination products from separating. Tragacanth has many industrial uses, including cloth finishing, calico printing and waterproofing of fabrics. It has been used medicinally for thousands of years. Modern pharmaceutical uses include an adhesive agent for pills and tablets, and for emulsifying oil droplets in emulsions. Examples of products containing tragacanth include cosmetics such as body lotion, foods such as ice cream, health aids such as toothpaste, and prescription drug such as antibacterial creams (Sahin and Ozdemir, 2004).

Xanthan gum

Xanthan gum is a high molecular weight extracellular heteropolysaccharide, produced by fermentation with the gram-negative bacterium *Xanthamonas campestris* (Rodd *et al*, 2000; Su *et al*, 2003). Xanthan gum consists of 1,4-linked β-D-glucose residues, having a trisaccharide side chain attached to alternate D-glucosyl residues. The backbone of the polymer is similar to that of cellulose. The side chains are β-D-mannose-1,4-β-D-glucuronic acid-1,2-α-D-mannose, where the internal mannose is mostly *O*-acetylated and the terminal mannose may be substituted by a 4,6-linked pyruvic acid ketal. The molecular weight of xanthan gum distribution ranges from 2×10^6 to 20×10^6 Da. This molecular weight distribution depends on the association between chains, forming aggregates of several individual chains. The variations of the fermentation conditions used in production are factors that can influence the molecular weight of xanthan. (Garcia-Ochoa *et al.*, 2000; Rodd *et al*, 2000; Talukdar and Kinget, 1995).

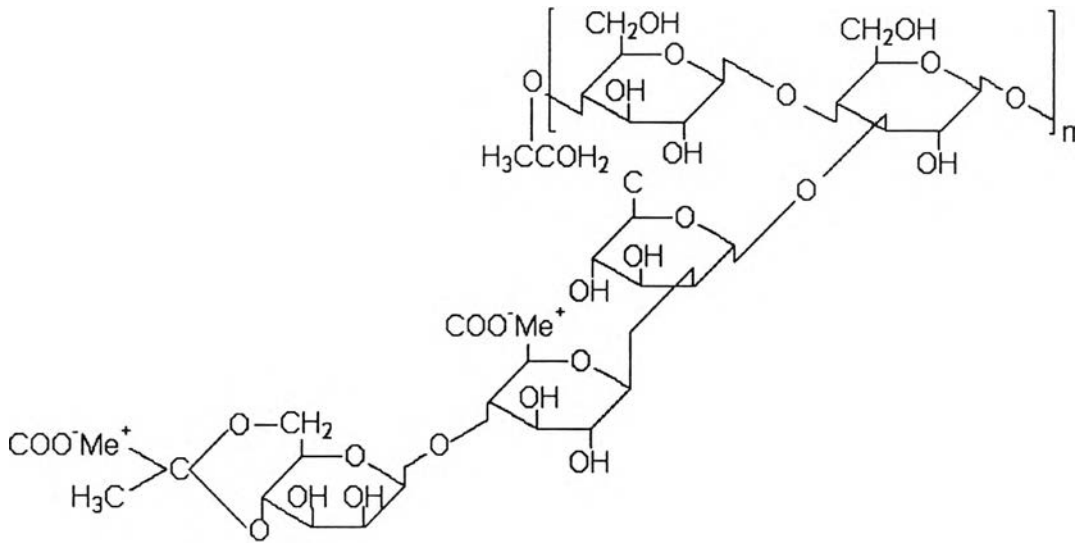


Figure 9. Molecular structure of xanthan gum

Xanthan gum is soluble in both hot and cold water. Solutions of xanthan gum are not generally affected by changes in pH value. Xanthan gum will dissolve in most acids or bases. The viscosity of xanthan gum is stable at low pH values and at high temperatures for a long period of time, whereas other hydrocolloids lose their viscosity under the same conditions. Xanthan gum solutions are very pseudoplastic; as shear stress is increased solution viscosity decreases progressively—as shear stress is reduced, solution viscosity returns almost instantaneously to its starting viscosity. Xanthan gum functionality is not influenced by salts, pH, acids, alkalis or enzymes and is compatible with most synthetic and natural hydrocolloids (Miladinov and Hanna, 1995). The effect of salts on viscosity depends on the concentration of the xanthan gum in solution. At low gum concentrations, monovalent salts such as NaCl cause a slight decrease in viscosity. Conversely, NaCl addition at higher gum concentrations increases solution viscosity. The same effects occur with most divalent metals salts. Xanthan gum used as a food additive and rheology modifier. In emulsions or suspensions for pharmaceutical use xanthan gum prevents the separation of insoluble ingredients (Santos *et al.*, 2004). Xanthan gum is used as a thickener and stabilizer in personal care products like creams, eye conture gels and the like. Typical xanthan gels feel very gentle and soft due to their shear

thinning flow behavior. The most important xanthan gum application in the field of cosmetics is in toothpastes. The shear thinning flow behavior of xanthan gum allows easy extrusion from the tube or from the pump dispenser. It also ensures a toothpaste that will keep a stable stand on the brush. The shear thinning characteristics also improve the dispersion on and the rinsing from the teeth. Toothpastes thickened with xanthan gum have a bright, shiny cord with short flow behavior (Talukdar and Kinget, 1995).

Methycellulose

Methycellulose is a long-chain substituted cellulose that can be used to form gels in concentrations up to about 5%. Because methylcellulose hydrates slowly in hot water, the powder is dispersed with high shear in about one third of the required amount of water at 80°C to 90°C. Once the powders is finely dispersed, the rest of the water is added, with moderate stirring to cause prompt dissolution. Cold water or ice should be used at this point. Anhydrous alcohol or propylene glycol can be used to help prewet the powders.

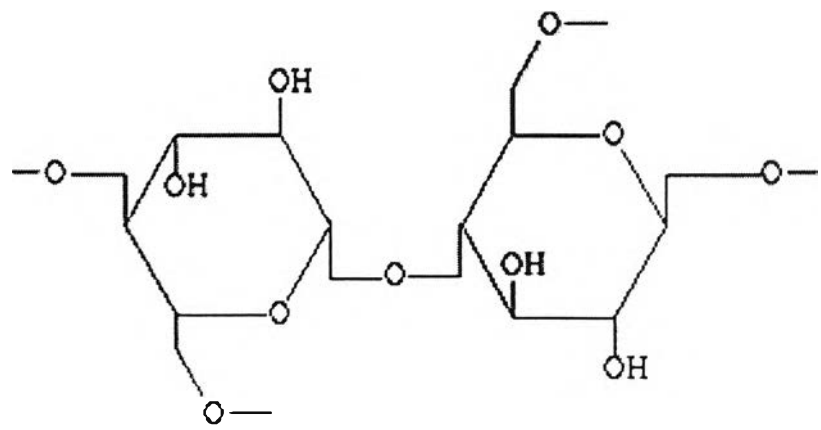


Figure 10. Structural unit of methycellulose.

Hydroxypropylmethylcellulose

Hydroxypropylmethylcellulose (HPMC) is obtained by the addition of methyl and hydroxypropyl groups to the cellulose chain, leading to a polymer with a high surface activity and unique properties regarding its hydration-dehydration characteristics in the solution state and during temperature changes. In addition, despite the presence of hydrophobic groups in the HPMC chain, this polymer partially maintains the hydrophilic properties of the cellulose (Sarkar and Walker, 1995). The rheology of an aqueous solution of HPMC is affected by the temperature, pH, and the presence of other solutes. Because HPMC is nonionic, the viscosity of its solution is generally stable over a wide pH range. Those properties allow the HPMC acting as emulsifier, strengthener of the crumb grain and increase the moisture content of the crumb (Gubbins *et al.*, 2003). It occurs as a white fibrous granular powder. Hydroxypropylmethylcellulose is employed as a suspending or a thickening agent, tablet excipient and surface-active agents. Solutions of this hydrophilic polymer are used as topical protectants or as artificial tears for contact lenses.

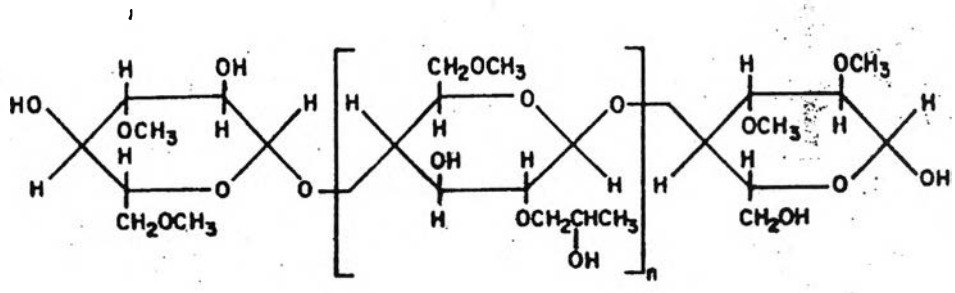


Figure 11. Structural unit of Hydroxypropylmethylcellulose

Sodium carboxymethylcellulose

Carboxymethylcellulose (CMC), an anionic polymer, is a long-chain cellulose ether prepared by reacting alkali and cellulose with sodium

monochloroacetate. It is available in low-, medium-, or high-viscosity grades. It dissolves rapidly in hot or cold water, giving a clear solution, but forms aggregates when first wet with water. Its solution characteristics depend on the average chain length and degree of substitution. As the molecular weight of CMC increases, the viscosity of its solutions increases. The viscosity of CMC solution is influenced by the temperature and pH. CMC solutions exhibit maximum viscosity and stability at pH 7-9. At a pH above 10, there is an increase in viscosity and at pH below 4, the viscosity decreases significantly due to hydrolysis of the polymer. CMC solutions have poor tolerance for electrolytes, especially di- and trivalent cations as they precipitat the polymer. CMC is compatible with other nonionic cellulose derivatives, clays, and commonly used preservatives, but is incompatible with cationic drugs.

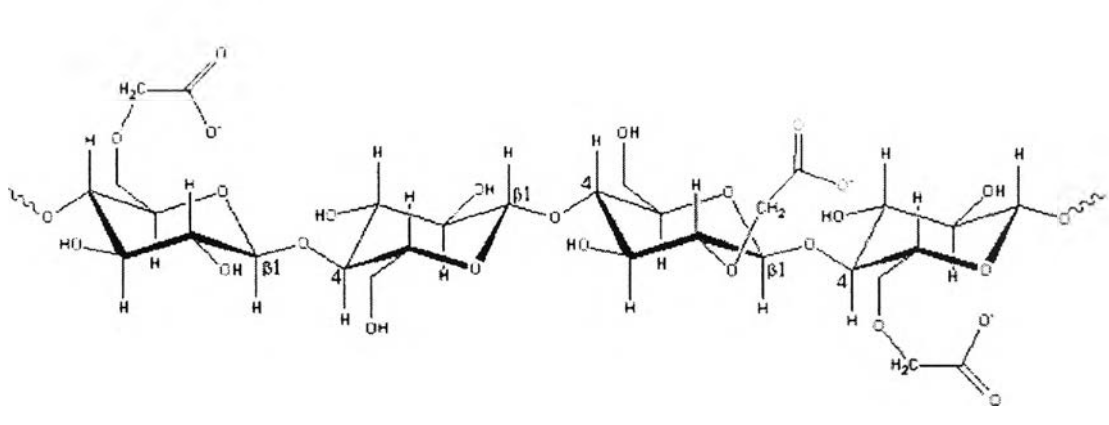


Figure 12. Structural unit of Sodium carboxymethylcellulose.

Carbomer

This homopolymer of acrylic acid is crosslinked with an allyl ether pentaerythritol, an allyl ether of sucrose, or an allyl ether of propylene. The viscosity of carbomer is influenced by temperature and pH. At low concentration, carbomer solutions are pseudoplastic, becoming plastic at higher concentrations. It is available in several grades. The choice of the proper carbomer may require evaluation of all grades since formulations must often meet several requirements. It is classified as a synthetic polymer and used as an emulsion stabilizer as well as an aqueous viscosity-increasing agent. The function of a carbomer is to act as an

emulsion stabilizer and to adjust viscosity. It can therefore also be classed as a thickening agent. Although it can be used in any type of cosmetic product, it is very often found in gel-like formulas. There are no known side effects to this chemical compound.

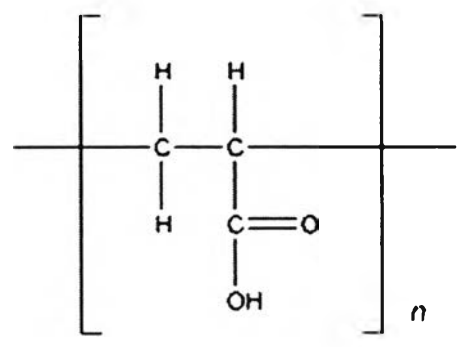


Figure 13. Acrylic acid monomer unit in carbomer resins.

Surfactant

Surfactants are often used in dosage forms as emulsifying agents, solubilizing agents, suspension stabilizers or wetting agents. However, surfactants in general cannot be assumed to be inert excipients as they have been shown to be capable of either increasing, decreasing or exerting no effect on the transfer of drugs across biological membranes. The number of surfactants a available for the formulations is so huge that even a cursory description is impossible.

Surfactant monomers can potentially disrupt the integrity and function of biological membrane. Such an effect would tend to enhance drug penetration and hence absorption across the gastrointestinal barrier, but may also result in toxic side-effects. Inhibition of absorption may occur as a consequence of a drug being incorporated into surfactant micelles. If such surfactant micelles are not absorbed, which appears usually to be the case, the solubilization of a drug may result in a reduction of the concentration of free drug in solution in the gastrointestinal fluids that is available for absorption. Inhibiton of drug absorption in the presence of

micellar concentrations of surfactant would be expected to occur in the case of drugs that are normally soluble in the gastrointestinal fluids, in the absence of surfactant.

Conversely, in the case of poorly soluble drugs whose absorption is dissolution-rate limited, the increase in saturation solubility of the drug by solubilization in surfactant micelles could result in more rapid rates of dissolution and hence absorption. The ability of a surfactant to influence drug absorption will also depend on the physico-chemical characteristics and concentration of the surfactant, the nature of the drug and the type of biological membrane involved.

Type of surfactants

Amphoteric (ampholytic) surfactants may be either anionic or cationic, depending on pH. They are useful because of their wide compatibility with builders, acids, and alkalis.

Anionic surfactants are those whose properties depend in part on the negatively charged ion of the molecule. This property gives us the name anionic. The detergent industry uses a wide range of anionic surfactants that are highly sudsing. Excess foaming is undesirable for surface cleaning. It leaves a residue from excess foam. This residue produces a tacky surface that presents a high resoiling problem.

Cationic surfactants have a positively charged ionic group. Quaternary ammonium compounds are the most widely used cationic surfactants. Their uses include sanitizers and disinfectants, fabric softeners, and static electricity dissipaters. They are not typically cleaning agents by themselves.

Nonionic surfactants contain neither positively nor negatively charged functional groups. They are particularly effective in removing oily soil and many are low sudsing. They do not ionize in water as do anionic and cationic surfactants.

Table 3. Classification of surfactants

Surfactants	Typical Representatives
Anionic Group	
Carboxylic acids	Soap
	Lactylates
	Polypeptide condensates
Sulfuric acid esters	Sulfacted monoglycerides
	Alkyl sulfates
Alkyl and alkyl-aryl sulfonates	Dodecylbenzene sulfonates
Phosphoric acid esters	Trioleyl phosphate
Substituted alkyl amides	Sarcosinates
	Taurates
Hemiesters	Sulfosuccinates
Cationic Group	
Amines	Alkoxylkylamines
Quaternaries	Benzalkonium chloried
Amphoteric Group	
Ammonium carboxylates	N-alkylaminoacids
Ammonium phosphates	Lecithin
Nonionic Group	
Polyalkoxyethers	Polyoxyethylene alkyl/aryl ethers
	Polyoxyethylene polyoxypropylene block polymers
Polyalkoxyesters	Polyoxyethylene fatty acid esters
	Polyoxyethylene sorbitan acid esters
Polyalkoxyamides	
Fatty acid esters of polyhydric alcohols	Sorbitan esters
	Glyceryl
	Sucrose
Fatty alcohols	Lauryl alcohol

Humectants

Humectants are used as formula adjuvants in oral as well as in formulations. Humectants such as propylene glycol, glycerin, sorbitol and polyethylene glycol are suitable humectants that can be incorporated at concentrations of about 5% into aqueous suspension for external application. They are used to prevent the product from drying out after application to the skin. They impart desired consistency, good taste, and help prevent cap locking. They can also be added to a formulation in order to reduce the evaporation of the water, either from the packaged product when the closure is removed or from the surface of the skin after application. High concentrations, if used topically, may actually remove moisture from the skin, thereby dehydrating it. Humectants are also important in moisturizing products.

Lanolin derivatives

Lanolin or wool wax obtained from sheep wool is a mixture of different esters from higher alcohols, mainly cholesterol, with higher fatty acids. The alcohol fraction contains linear and iso-C_{16-30} alcohols, while the fatty acids are mainly linear and hydroxyl fatty acids with carbon-chain lengths between 10 and 29. Lanolin can bind 200-300% of water in the form of a W/O emulsion. Since lanolin can cause allergies, lanolin alcohols extracted after saponification of the wool wax are now mainly used. Due to the high amount of cholesterol, they are better emulsifiers than lanolin itself.

Preservative

Formulations often contain a number of ingredients which readily support the growth of a variety of microorganisms. As a result, the inclusion of a preservative is a necessary part of the formulation process. Several points must be kept in mind in selecting a preservative. Microbial contamination may occur during the development or production of an emulsion or during its use. Frequently, the microbial contamination can arise from the use of impure raw materials of from poor sanitation during preparation. Prevention of contamination is recommended, and certain cardinal rules must be observed. The most important one is the use of

uncontaminated raw materials, including the water. A second precaution is meticulous housekeeping and careful cleaning of equipment. Once a microbiologically uncontaminated product has been prepared, a relatively mild antimicrobial agent suffices to protect the product against chance contamination by microorganisms. It is also desirable that the preservative system be effective against invasion by a variety of pathogenic organisms and be adequate to protect the product during use by the consumer.

Antimicrobial susceptibility tests

1. Agar diffusion method

The agar diffusion method is most useful in determining the approximate antimicrobial sensitivity of a microbe. The agar diffusion test has several advantages. It is technically simple to perform and very reproducible. The reagents are relatively inexpensive. It does not require any special equipment. It is flexible regarding the selection of antimicrobial agents used for testing (Edwin *et al.*, 1985; Leonardo *et al.*, 2000; Lorian, 1996)

Agar medium

The recommended medium for agar diffusion testing in the United States is Mueller-Hinton agar. This unsupplemented medium has been selected by NCCLS for several reason such as it demonstrates good batch-to-batch reproducibility for susceptibility testing, it is low in sulfonamide, trimethoprim, and tetracycline inhibitors, it supports the growth of most nonfastidious bacterial pathogens and years of data and clinical experience regarding its performance have been accrued. The prepared medium is autoclaved and is immediately placed in a 45 to 50°C water bath. When cool, it is poured into plastic or glass flat-bottom Petri dishes on a level surface to give a uniform depth of about 3 to 4 mm (60 to 70 ml of medium for 150 mm plates, 20 to 25 ml for agar per 100 mm round plate and 30 ml for square plates) and is allowed to cool to room temperature. Agar deeper than 4 mm may cause false resistance (excessively small zones), whereas agar less than 4 mm deep may be associated with excessively large zones and false susceptibility.

Inoculation procedures

The suspension is diluted until its turbidity matches the turbidity of a barium sulfate or equivalent 0.5 McFarland turbidity standard (ca. 10^8 CFU/ml). The Mueller-Hinton agar plate should be inoculated within 15 min after the inoculum suspension has been adjusted. The seeded agar is quickly mixed by gentle inversion and spread evenly over the surface of a plate containing Mueller-Hinton agar. The lid may be left ajar for 3 to 5 min but for no longer than 15 min to allow any excess surface moisture to be absorbed before the durg-impregnated disks are applied.

Within 15 min after the plates are inoculated, selected antimicrobial agent disks are distributed evenly on the surface, with at least 24 mm (center to center) between them. The disks are placed individually with sterile forceps or with a mechanical dispensing apparatus and are then gently pressed down onto the agar surface to provide uniform contact. Some of the antimicrobial agent in the disk diffuses almost immediately; therefore, once a disk contacts the agar surface, the disk should not be moved.

Incubation

Inoculated agar plates are allowed to stand undisturbed until the inoculum spots are completely absorbed and are then incubated at 35°C for 16 to 20 hour. No longer than 15 min after the disks are applied. A delay of more than 15 min before incubation permits excess prediffusion of the antimicrobial agents. Incubation in an atmosphere with CO_2 is not recommended because of the influence of surface pH on various antimicrobial agents. The interpretive standards for nonfastidious bacteria are based on the results of tests with plates incubated in ambient air, and the zone of inhibition diameters for some drugs, such as the aminoglycosides, macrolides, and tetracyclines, are significantly altered by CO_2; therefore, plates should not be incubated in the presence of increased level of CO_2. Testing of isolates of some fastidious bacteria, however, requires incubation in 5% CO_2, and zone diameter criteria have been established on that basis. For anaerobic bacteria, such as

Propionibacterium acnes, inoculated plates are incubated in a GasPak jar or a similar anaerobic environment at 35°C for 48 hours (Lorian, 1996).

Interpretation and reporting of results

Each plat is examined after incubation for 16 to 18 hour for all nonfastidious bacterial isolates except staphylococci and enterococci, which must be incubated for a full 24 hour to allow the detection of resistance to oxacillin and vancomycin. The endpoint by all reading systems is complete inhibition of growth, disregarding tiny colonies which can be detected only by very close scrutiny or by using transmitted light or mechanical enlargers. Large colonies growing with in the clear zone of inhibition may represent resistant variants or a mixed inoculum and may require reidentification and retesting. The drug in the disk diffuses through the agar. As the distance from the disk increases, the concentration of the antimicrobial agent decreases logarithmically, creating a gradient of drug concentrations in the agar medium surrounding each disk. In areas where the concentration of drug is inhibitory, no growth occurs, forming a zone of inhibiton around each disk. The diameter of the zone of inhibition is influenced by the rate of diffusion of the antimicrobial agent through the agar, which may vary among different drugs.

2. Dilution method

Dilution antimicrobial susceptibility tests in a broth medium have been used for decades and represent only a minor modification of the agar-based method. However, some characteristics are unique to the broth tests, and these will be discussed with regard to their variability from other methods or their contribution to test results.

2.1 Broth macrodilution method

This method is a well-standardized and reliable reference method that is useful for research purpose. The agar diffusion test is not used to determined whether a chemical is bactericidal (kills bacteria) or bacteriostatic

(inhibits bacteria) instead this characteristic is determined by the broth macrodilution method (Lorian, 1991).

Inoculation procedures

The recommended final inoculum is 5 x 10^5 CFU/ml. Isolates are inoculated into a broth that will support good growth and are incubated until they are turbid. The turbidity is adjusted to match that of a 0.5 MaFarland standard (approximately 10^8 CFU/ml). Alternatively, four or five colonies from overnight growth on a nonselective agar plate may be directly suspended in broth so that the turbidity matches the turbidity of the McFarland standard. A portion of the standardized suspension is diluted 1:100 with broth or saline. When 1 ml of this dilution is added to each tube containing 1 ml of the drug diluted, a final inoculum of 5 x 10^5 CFU/ml is achieved.

Incubation

In this method the bacterium of interest is placed in a tube containing the chemical which is being tested. Tubes are inoculated in air at 35°C for 16 to 20 hour before the MICs are determined. Incubation should be extended to a full 24 hour for the detection of vancomycin-resistant enterococci or methicillin-resistant or vancomycin-intermediate staphylococci. The use of increased levels of CO_2 is not recommended.

Interpretation and reporting of results

The bacterium is then added (sub cultured) onto a nutrient agar plate. If the bacterium grows on the nutrient agar the chemical is bacteriostatic; if not, it was killed by the chemical which is then termed bactericidal. The lowest concentration that completely inhibits visible growth of the organism as detected by the unaided eye is recorded as the MIC. The MBC is the lowest concentration resulting in no visible colonial growth.

2.2 Broth microdilution method

The use of broth microdilution trays prepared in-house provides a reliable standardized reference method for susceptibility testing. Inoculation and reading procedures allow relatively convenient simultaneous testing of several antimicrobial agents against individual organisms. However, the versatility of the antimicrobial agent selection available with commercial broth microdilution trays is limited compared with the selection available by preparing panels in-house.

Inoculation procedures

Microdilution trays with antimicrobial agents should ideally be prepared each day they are to be used. Multipoint plastic or metal inoculum replicatros are used in several commercial systems. Tray wells are usually filled with 100 μl of antimicrobial agent and inoculated with approximately 1 to 5 μl of a standardized inoculum. .With such small volumes being added, the dilution of the antimicrobial agent is not enough to require correction of the concentration.

Incubation

After the microdilution trays are inoculated, they should be covered with sealing tape to minimize evaporation. Excessive humidity should be avoided so that moisture does not condense on the surfaces of the trays, possibly contributing to contamination. Trays are incubated in air at 35°C for 16 to 20 hours before being read and should not be incubated in stacks of more the four trays.

Interpretation and reporting of results

The endpoint (MIC) is taken as the lowest concentration of drug at which the microorganism tested does not show visible growth. The simplest and most reliable method may be use of a parabolic magnifying mirror and tray stand that allows clear visual inspection of the underside of the broth microdilution trays. In judging the endpoint, it is important to consider the growth (or lack of growth) in

the test well in comparison with growth characteristics of the microorganism in the growth control well (with no antimicrobial agent) used in each tray.