CHAPTER II

LITERATURE REVIEW

1. Mangosteen Pericarp Extract and Its Therapeutic Potentials

Garcinia mangostana Linn. (family Guttiferae) or mangosteen, the queen of fruit, is commomly encountered in Southeast Asia. It is one of the most interesting medicinal plants because of its distinguished pharmacological activities. The fruit hull or pericarp of this plant has been used in as a Thai traditional medicine for the treatment of diarrhea, amoebic dysentery, skin infection, suppuration, chronic wound and Hongkong foot. (Mahabusarakum, et al., 1983)

Phytochemical studies have shown that the active ingredients from mangosteen pericarp extract belong to a group of xanthones such as α-mangostin (formerly mangostin), β-mangostin and γ-mangostin, isomangostin, garcinone A, B, C and D, gartanin, rubraxanthone, etc. (Mahabusarakam, et al., 1987) The structure of xanthone ring and its derivatives (Budavari, et al., 1996) are shown in Figure 1. Among these xanthones, α-mangostin is the major component of mangosteen pericarp. (Bennett and Lee, 1989) However, the differences in solvent and methods of extraction and purification may yield different combinations of xanthones in the extract. (Nakatani, et al., 2002b)

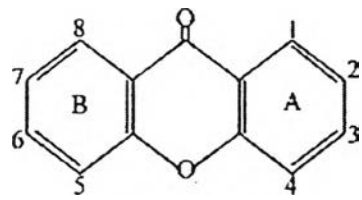
Extract from mangosteen pericarp has been shown to exert antimicrobial activities against several microorganisms including bacteria, (Iinuma, et al., 1996; Sundaram, et al., 1983) fungus (Gopalakrishnan, Banumathi and Suresh, 1997; Sundaram, et al., 1983) and virus. (Chen, Wan and Loh, 1996) It also possesses other biological activities such as anti-inflammatory, (Nakatani, et al., 2002b, Nakatani, et al., 2002a) anti-histamine, (Chairungsrilerd, et al., 1996, Nakatani, et al., 2002b) anti-oxidant, (Williams, et al., 1995) and anti-tumor (Moongkarndi, et al., 2004) properties.

A. Xanthone ring

Chemical name : Diphenylene ketone oxide

Molecule formula : $C_{18}H_8O_2$

Molecular weight : 196.26 gram

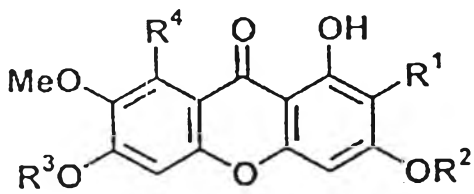


B. Xanthone derivatives

α-mangostin : R^1, R^4 = A and R^2, R^3 = H

β-mangostin : R^1, R^4 = A and R^2 = Me, R^3 = H

γ-mangostin : R^1 = A and R^2 = R^3 = R^4 = H and Me is substituted by H



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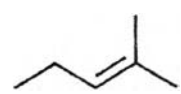

α-mangostin

Chemical name : Tetraoxygenated diprenylated xanthone

Molecule formula : $C_{24}H_{26}O_6$

Molecular weight : 410.46 gram

Melting point : 181.6-182.6 °C

Figure 1. Chemical structures of (A) xanthone ring and (B) xanthone derivatives: α-mangostin, β-mangostin and γ-mangostin. (Budavari et al., 1996)

Xanthones from mangosteen pericarp extract has broad-spectrum anti-bacterial activities against a variety of gram-positive and gram-negative bacteria including enteric pathogens, (Sindermsuk and Deekijsermphong, 1989; Sundaram, et al., 1983) both methicillin-sensitive and methicillin-resistant *S. aureus*, (Mahabusarakum, et al., 1983, 1986) and mycobacterium, (Suksamrarn, et al., 2003) etc. (Table 1). Among these xanthones, α-mangostin appears to exert the strongest anti-bacterial activity with MIC values ranging from 1 to 50 μg/ml. However, the antimicrobial effect of the extract against oral pathogens has never been demonstrated.

Several studies have demonstrated low toxicity of mangosteen pericarp extract. Xanthones isolated from mangosteen pericarps were not toxic to rats when given orally at a dose of 100 mg/kg body weight/day for up to 7 days. (Sapwarobol, 1997) When α-mangostin, a major component of the extract, was administered orally to rats at a high dose (1.5 g/kg body weight). The finding indicated the low hepatotoxicity by slightly increases in serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) activities after 12 hours. These increases were much less than those of paracetamol given at the same dose, and no change in total liver proteins was observed. (Somprasit, et al., 1987) In a human clinical trial, 1.5% α-mangostin cream was locally applied on skin of patients with chronic ulcers for up to 3 weeks. No local irritation or side effects were observed. (Kusuma, 2003)

Based on its various biological activities and low toxicity, mangosteen pericarp extract has potentials for wide pharmacological applications. It has been developed in the forms of topical cream or gel for chronic skin ulcers, (Kamjanachotdamrong, 2000; Kusuma, 2003) throat spray for upper respiratory tract infection, (Kongchunmitkul, 2002) and buccal mucoadhesive film for oral ulcers. (Hiranras, 2001) This study will focus on its antimicrobial activity against oral pathogens in dental plaque. Based on this knowledge, we can further develop this extract in the forms of mouthrinse or local delivery drugs for chemical plaque control.

Table 1. Antimicrobial activity of xanthones from mangosteen pericarp extract

Bacteria	References	Active components	MICs
Pseudomonas aeruginosa *Salmonella typhimurium* *Bacillus subtilis*	Sundaram et al., 1983	α-mangostin	12.5-50 μg/ml.
Escherichia Coli *Salmonella* species *Shigella species*	Sindermsuk et al., 1989	boiled crude extract	6.3-100 mg/ml
Staphylococcus aureus	Mahabusarakum et al., 1983; 1986	α-mangostin γ-mangostin 1-isomangostin 3-isomangostin gartinin	15.6 μg/ml 31.2 μg/ml 62.5 μg/ml 125 μg/ml 250 μg/ml
Methicillin –resistance S. aureus	Mahabusarakum et al., 1983; 1986	α-mangostin 1-isomangostin γ-mangostin 3-isomangostin gartinin	1.6-12.5 μg/ml 125 μg/ml 250 μg/ml 250 μg/ml 250 μg/ml
	Iinuma et al., 1996	α-mangostin	1.6-12.5 μg/ml
Mycobacterium tuberculosis	Suksamrarn et al., 2003	α-mangostin β-mangostin γ-mangostin	6.3 μg/ml 6.3 μg/ml 25 μg/ml

2. Cariogenic Bacteria

Dental caries development involves demineralization of tooth structure by a high concentration of organic acids produced by specific bacteria in dental plaque. One of the bacteria that has been implicated in the etiology of both coronal and root caries is *Streptococcus mutans*. (Loesche, 1986; Zambon and Kasprzak, 1995) It is gram-positive, facultative anaerobic cocci. Its colony has yellowish-white color, pinpoint to medium size, irregular margin with rough and shiny surface on trypticase soy agar.

Streptococcus mutans possesses several cariogenic properties. It is able to synthesize extracellular polysaccharides including insoluble glucan and fructan from dietary sucrose. This process enhances plaque mass, promotes bacterial colonization and changes diffusion properties of the plaque matrix. In addition, this bacteria is acidogenic and aciduric. It is capable of forming acids in the presence of sugars, and maintaining sugar metabolism under extreme acidic environment such as in carious lesions. (van Houte, 1994; Zambon and Kasprzak, 1995)

3. Periodontopathic Bacteria

Periodontal disease is caused by a group of anaerobic bacteria in dental plaque, which induce a release of inflammatory cytokines from periodontal tissues, leading to periodontal destruction. The World Workshop on Clinical Periodontics in 1996 implicated 3 bacterial species including *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *Tannerella forsythia* as etiologic agents for periodontal disease. (Zambon, 1996)

These bacteria fulfill the criteria for implicating them as the etiology of periodontal disease. They are found in high numbers in the diseased sites, higher than in the healthy sites of the same subjects. Subjects with periodontal disease make elevated antibody levels to the antigens from these bacteria. They also produce a number of virulence factors, which can directly damage periodontal tissues or immune cells, or indirectly

damage the tissues by cytokine induction. Eliminating or reducing the number of these bacteria is important for the success of periodontal therapy. There is also data demonstrating that these bacteria can induce periodontal disease in animal models. (Zambon, 1996)

Actinobacillus actinomycetemcomitans is gram-negative, facultative anaerobic bacteria. It is a small, non-motile, saccharolytic, round-end rod. It appears as a yellowish-white small, smooth colony with a star-shaped inner structure. (Olsen, Shah and Gharbia, 1999) This bacterium produces a number of virulence factors such as leukotoxin and lipopolysaccharide. Leukotoxin has been shown to kill polymorphonuclear leukocyte, T cells and B cells. Lipopolysaccharide can stimulate macrophages to release inflammatory cytokines including interleukin-1, prostaglandin E_2 and tumor necrosis factor-α. In addition, the ability of this microorganism to invade host epithelium and gingival connective tissue makes it survive from host immune defense and mechanical periodontal therapy. (Fives-Taylor, et al., 1999)

Porphyromonas gingivalis is gram-negative, obligate anaerobic bacteria. It is a non-motile, asaccharolytic, short bacilli. It belongs to a black-pigmented *Bacteroides* group, in which colonies are brown to black, smooth, shiny and exhibit complete hemolysis on blood agar. (Olsen, et al., 1999) This organism has a large array of virulence factors such as lipopolysaccharide, fimbriae and gingipain proteases. Lipopolysaccharide is capable of inducing bone resorption in mice. Fimbriae mediates adherence to specific receptors on host cells, and induces bacterial internalization. It is also capable of inducing cytokine production. *P. gingivalis* proteinases have been shown to interact with the cytokine networking systems, leading to dysregulation of the local inflammatory reaction. (Holt, et al., 1999)

4. Chlorhexidine

Chlorhexidine is considered one of the most effective antiseptics. It has broad-spectrum antimicrobial activity against a variety of both gram-positive and gram-negative

bacteria including those causing dental caries and periodontal disease. Its effectiveness also contributes to its high substantivity to oral tissues. The mechanism of action is mainly on the rupturing of bacterial cell wall and precipitation of the cytoplasmic content. (Ciancio, 2000; Jones, 1997)

Chlorhexidine has been incorporated into various formulations including mouthrinse and local delivery drugs. Its efficacy in reducing plaque and dental caries and in periodontal treatment has been clinically proven. (Overholser, 1988; Twetman, 2004) However, it has some unwanted side effects including bad taste, tooth and tongue staining and taste alteration. (Ciancio, 2000; Jones, 1997)

5. Anaerobic Bacterial Culture

There are two systems used to cultivate anaerobic bacteria: anaerobic chamber and chemically generated anaerobic systems such as the BBL GasPak system (BBL Microbiology Systems, Cockeysville, MD, USA). The anaerobic chamber provides a convenient culture system for large-scale studies and exhibits good recovery for most anaerobic organisms. However, it is expensive to purchase and maintain. The GasPak system is limited to processing a few bacterial plates at a time but costs a lot less. (Doan, et al., 1999) It consists of a polycarbonate anaerobic jar, a disposable hydrogen- and carbon dioxide-generating envelope and a catalyst chamber containing 2.5 $\pm$ 0.5 g of palladium catalyst pellets. When water is added to the envelope, hydrogen gas and carbon dioxide are generated. In the presence of a catalyst, the resulting hydrogen combines with oxygen inside the jar to produce water, thus establishing an anaerobic environment. Sixty minutes after adding water, the atmosphere inside the jar contains approximately 4 to 7 % carbon dioxide and 25 to 30% hydrogen, while the oxygen is decreased to less than 1%. (Seip and Evans, 1980)

Anaerobic organisms differ in oxygen sensitivity. Anaerobic chamber is essential for work with strict anaerobes, which are incapable of growing at an oxygen concentration of greater than 0.5%. *P gingivalis* belongs to a group of moderate anaerobes, which are

capable of growth in the presence of oxygen levels as high as 2-8%. (Loesche, 1969) When the Gaspak system was compared to the anaerobic chamber for the abilities to support the growth of anaerobic periodontal pathogens, they were equally effective in isolating *P. gingivalis*, but the GasPak system demonstrated higher proportional recoveries of *T. Forsythia* . (Doan, et al., 1999) Comparison between the 2 systems was set up to test 11 antimicrobial agents regarding antimicrobial susceptibility against 38 anaerobesd. The MIC results were comparable (94% aggreement) within 1 twofold dilution for organisms incubated in both systems (Murray and Niles, 1982)

6. Measuring Bacterial Growth

Many methods are available. We'll summarize the two methods used in this study.

6.1 *Viable cell count using a spread plate method*

The viable cell count is a measure of the concentration of cells present in a broth culture that are alive and able to grow and produce colonies on the agar surface. A sample of broth culture is serially diluted, usually ten fold at each dilution, and then a small amount of each dilution is spread on a plate. After visible colonies have formed, plates from the dilutions that contain separated colonies are selected. Choosing plates with between 30 and 300 colonies offers a good compromise between speed and accuracy. Plates with fewer than 30 colonies may give exaggerated counts due to sampling errors, while plates with more than 300 colonies may be too crowded to count and to allow all the bacteria to form distinct colonies. The viable bacterial count is expressed in terms of colony-forming units per ml (CFU/ml). CFU/ml is calculated by dividing the average number of colonies with the plating volume (ml) and then multiplying with the dilution factor. (Ingraham and Ingraham, 2004)

If the number of viable bacteria (CFU/ml) is plotted against time, the curve will get steeper and steeper and finally shoots out of the range of the plot. Therefore, microbial growth is conveniently graphed on a logarithmic scale. The growth curve is plotted as the logarithm of the number of viable cells ($\log_{10}$CFU/ml). The growth curve of a typical

bacterial culture is divided into 4 phases: a lag phase, bacteria prepare to grow ; a log or exponential growth phase, cell numbers double at regular intervals ; a stationary phase, bacterial growth ceases ; and a death phase, number of live cells decline (Figure 2). (Chynoweth, 2004)

6.2 *Degree of turbidity*

The degree of turbidity or cloudiness by a broth culture can give an estimate of the number of organisms present, both dead and live. Turbidity is measured using a spectrophotometer. This device measures how much light can pass through bacterial culture (Figure 3). As the mass of cells increases, turbidity increases, less light passes through the culture, the reading on the spectrophotometer is higher. The degree of turbidity is measured in terms of the absorbance (also called optical density or OD) at 600 nm. This wavelength is selected to minimize the absorbance by the broth media because most media are brown or brownish-yellow. (Ingraham and Ingraham, 2004) The growth curve can be plotted between the absorbance at 600 nm and time in a similar fashion as $\log_{10}$CFU/ml (Figure 2).

Because the mass of the culture and number of cells are related, turbidity can also be used to determine the cell number. To convert spectrophotometer reading to the number of bacteria, it is necessary to prepare a standard curve. The graph is drawn by plotting $\log_{10}$CFU/ml of bacteria against the absorbance at 600 nm (Figure 4). Only the values from the exponential phase of growth (mostly live cells) are used to plot the standard curve. (Ingraham and Ingraham, 2004) Linear regression analysis is used to fit a straight line to the data. The equation for this straight line is :

$$Y = aX + b$$

Where Y = $\log_{10}$CFU/ml that corresponds to the absorbance value.

a = the slope of the line

X = the absorbance value

b = the Y-intercept of the line

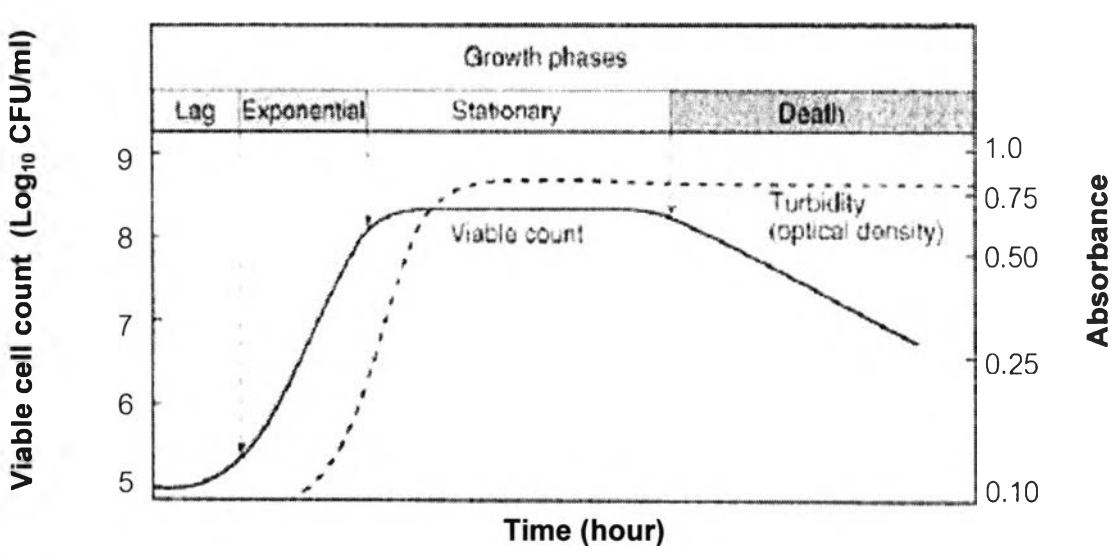

Figure 2. **Growth curve of a typical bacterial culture.** (Chynoweth, 2004)

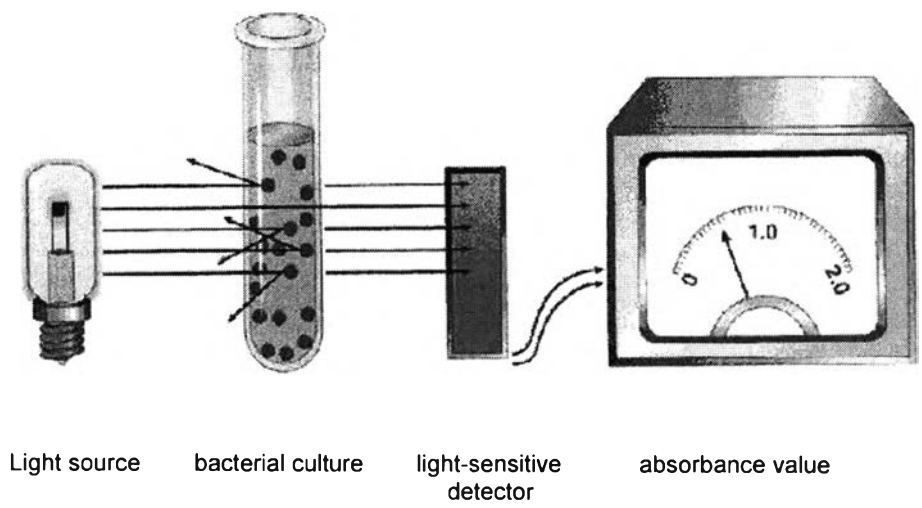

Figure 3. Components of a spectrophotometer. The amount of light striking the light-sensitive detector is inversely proportional to the number of bacteria. The higher number of bacteria, the less light transmitted, the higher the absorbance reading. (Ingraham and Ingraham, 2004)

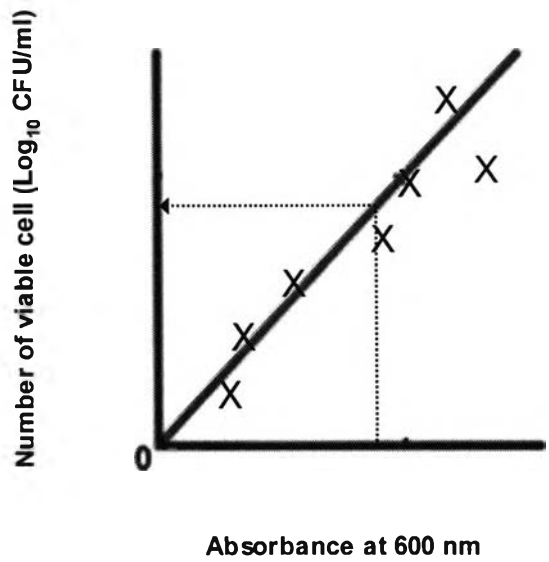

Figure 4. Standard curve relating the absorbance at 600 nm to the number of viable bacteria. Once the absorbance reading is obtained, the standard curve can be used to determine the corresponding number of bacteria.

The regression line also gives a value called correlation coefficient (r). This number ranges from 0 to 1. The closer r to 1, the more likely the data are to forming a straight line. From this linear equation, an estimate of viable bacterial count can be predicted from the absorbance readings.