CHAPTER 1

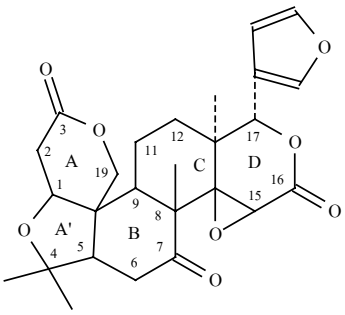
INTRODUCTION

Statement of Problem

Thailand is rich in edible fruit and vegetable (Murakami *et al.*, 1995). One of the common fruit consume in Thai daily life is *Citrus*. There are many kinds of dishes that contain *Citrus*, not only as the active ingredient but also as a food additive (e.g. as flavoring agents or preservatives). An example of Thai food containing *Citrus* is Yum Som-O, containing pummelo as the active ingredient and mixed with a sour and sweet dressing. Another example is Tom Yam Kung, a world famous and specialty order in a restaurant. This contains several Thai spices and chilies in a soured soup which is furnished by lime juice. However in recent times, this common Thai fruit has faced economic problems, such as low-prices in seasonal periods and non-availability in out-of-season periods. Therefore, currently there are many attempts to solve these problems. This includes the study of the best breed of *Citrus* (especially lime), and the study of suitable processing technique. A most recent interesting research area is the development of *Citrus* components as neutraceutical product.

A class of compound widely distributed in the *Citrus* fruit is terpenes. They occur widely in foods consumed by human. One of the terpenes (which are responsible for the bitter taste of *Citrus*) is limonoid such as limonin, nomilin, and obacunone.

Limonin has a triterpene structure, which is most prevalent in *Rutaceae* plants, such as lemon, lime, orange and grapefruit. It has been recognized as a *Citrus* constituent for several decades. It has a bitter taste with a lower solubility in water compared to other *Citrus* components. The structure of limonin contains a furan ring attached to the D-ring lactone as shown in Figure 1



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Limonin (1)

Figure 1 Characteristic structural features of limonin (1)

The most appropriate method for determining the structure of limonin is Nuclear Magnetic Resonance (NMR) spectroscopy. Column chromatography is usually necessary for the actual isolation and purification of limonin from fruit extracts. The method for the quantitative analysis of limonin is Thin-Layer Chromatography (TLC) (Maier *et al.*, 1977). Limonin can be isolated and purified from lemon seeds, consequently determined by High Performance Liquid Chromatography (HPLC) and TLC (Pifferi *et al.*, 1993).

Limonin is generally not desirable in *Citrus* food, because of its bitter taste. Therefore attempts are often made to eliminate this compound during food processing. However the Thai proverb says that “sweet is dessert, bitter is medicine” and this suggests that bitterness due to limonin might be of potential benefit to health.

The review of Lam *et al.* (1994) revealed that limonin has certain biological activities, and that limonin could possibly be used as chemopreventive agent. Other biological activities suggested for limonin include inhibition of oral carcinogenesis, amoebicidal effect and abortifacient, and reduction of chemically induced tumorigenesis. However, the immunological activity of limonin has been less well studied, and this aspect will be the main focus of this research.

Citrus fruits are generally used as vitamin and mineral supplemental foods, and in Thailand *Citrus* fruits are ingredients as flavoring agents. In recent years more studies have examined the potential dietary benefits and health promotion effects of *Citrus* fruits (Middleton and Kandaswami, 1994). The effects of naturally occurring triterpenoids such as glycyrrhizic acid, urosolic acid, oleanolic acid, and nomilin on the immune system have also been studied (Raphael and Kuttan, 2003).. Therefore *Citrus* fruits, which contain an abundance of triterpenoids, might be an excellent dietary source of immunomodulatory substances.

However it is realized that in the industrial processing of *Citrus* (storage, handling, and cooking of food) can result in active damage to limonin and other *Citrus* components. The degree of damage depends on a variety of factors, including temperature, pH, exposure to light and air, and other factors of the processing. Processing may therefore have a major effect on absorption and bioavailability of limonin (Bengmark, 1998). There are numerous research studies on the processing of *Citrus*, such as the clarification of *Citrus* juice, removing the bitterness of the juice, and stabilization of the suspended pulp of the juice. It is necessary to know whether these processes affect the active ingredients, especially the possible immunological activity of limonin. It is expected that suitable processing will promote functional activity, and stabilize the texture and appearance of the products.

Therefore, the two primary objectives of the proposed research were: (a) to study the immunomodulatory property of limonin, using a variety of *in vitro* and *in vivo* techniques described in the literature, and (b) to study the stability of limonin, especially a study of how processing of *Citrus* fruits in industry affects the concentration of limonin in the final products.

Review of Literature

It has been stated that nutritionists recommend a diet rich in fruits and vegetables as practical good health foods for reducing the risk of heart disease and certain cancers (Papas, 1999 and Craig and Bech, 1999). The beneficial effects of fruit and vegetables are very likely due to many of their components such as fiber, micronutrients, and other chemical constituents. Dietary components, which can promote good health, contain at least some phytochemicals and have a major impact on pharmacological action. Many phytochemicals have now been studied extensively for their potential role in health, especially their anti-oxidant status (Platzman, 1998). Major classes of phytochemicals with potential for antioxidant activity are listed in Table 1

Table 1 Major classes of phytochemicals with antioxidant activity

Class of phytochemicals	compounds
Carotenoids	lycopene, lutein, astaxanthin
Bioflavonoids	genistein, diadzein, quercetin
Phytosterols	sitosterol, stigmasterol, oryzanol
Tannins	catechins and other polyphenol
Chlorophylls	chlorophyll A and chlorophyllin
Terpenoids	limonin and limonene
Allylic compounds	diallyl sulfide and disulfide
Indoles	indole-3-carbinol

Many phytochemicals possess antioxidant properties. *Citrus* fruits contain several classes of phytochemicals and micronutrients such as carotenoids, flavonoids, and terpenoids, which have been reported to have biological effects *in vitro* and *in vivo* (Rouseff and Nagy, 1994, Gharagozloo *et al*., 2002). More than 1,000 individual phytochemicals in *Citrus* have been identified. One class prevalently in *Citrus* fruits is terpenoids.

A large number of epidemiological studies have shown that the *Citrus* plants are rich sources of various physiologically active substances, especially “health-promoting substances”. It is commonly accepted that cancer formation can be prevented by the consumption of certain foods (Kawii *et al.*, 1998). *Citrus* fruit consumption is protective in a variety of human cancers (Rouseff and Nagy, 1994). Craig and Beck (1999) have reported that *Citrus*, in addition to its ample supply of vitamin C, folic acid, potassium and pectin, contains a host of active phytochemicals that also protect human health. So it is suggested that *Citrus* fruits contain not one but multiple cancer chemo-preventive agents. There are over 60 flavonoids and 40 limonoids in *Citrus*.

Limonoids is one class of the *Citrus* terpenoids. *Citrus* contains about 40 limonoids (Craig and Beck, 1999). Gharagozloo *et al*. (2002) investigated the inhibiting effect of *Citrus* limonoids on chemically induced colon carcinoma in a dose-dependent manner accompanied by suppression of cell proliferation in a rat model. Another limonoid in *Citrus* fruits is limonin, which occurs in high concentration and is a principal component in *Citrus* juice. This provides the bitter taste in *Citrus*, and has been reported to act as an anti-tumor and anti-carcinogenesis agent in various biological systems.

Lam *et al.* (1994) reported that limonin could reduce chemically induced tumorigenesis. Miller *et al*. (1994) reported that limonin might be used as a cancer chemo-preventive agent for humans because it acts as an inhibitor of oral carcinogenesis. In addition, *Citrus* fruits had been found to be beneficial for cancer prevention in an epidemiological survey. Tanaka *et al.* (2000) examined the modifying effects of obacunone and limonin on azoxymethane-induced colon carcinogenesis in male F344 rats and their results suggested that limonin might be useful for the prevention of human colon cancers.

Limonoids exist at a very high concentration in *Citrus* juices, especially limonin which is the predominant bitter producing phytochemicals in *Citrus*, and might be one of those preventive agents. Limonin might also

occur in the waste of *Citrus* fruit, such as seeds and outer peels, and therefore a careful study of the reuse of this waste is required.

1. Limonin

Limonin is chemically related to triterpenoid derivatives found in the *Rutacea*e and *Meliaceae* families named limonoids. All naturally occurring *Citrus* limonoids contain a furan ring attached to the D-ring, at C-17, as well as oxygen-containing functional groups at C-3, C-4, C-7, C-16, and C-17. Limonin contains a C-14, 15-epoxide group (Figure 1).

Limonin was first isolated and its structure determined as a *Citrus* constituent (Maier *et al.*, 1977). Most isolation work had been done on seeds since they are richer in limonoids than the fruit tissue. However, limonin is the only limonoid present in significant amounts in *Citrus* juices.

The most useful single method for determining the structure of limonoids is nuclear magnetic resonance (NMR) spectroscopy (Maier *et al.*, 1977). The characterization of limonin is performed by the following analyze methods: The ^{1}H NMR spectrums in $CDCl_3$ with TMS as the internal standard and ^{13}C NMR spectroscopy to determine the structure.

Mass spectroscopy (MS) has also become an important structural tool in organic chemistry. However its information does not seem to be used for structure determinations (Maier *et al.*, 1977). Nevertheless, the molar activity of limonin can be determined using mass spectroscopy data. Infrared (IR) spectroscopy can be used to demonstrate the presence of hydroxyl, furan, carbonyl and olefin groups in limonoids. The IR spectrum of crystallized limonin is over a range of 4000 to 400 cm^{-1} (Pifferi *et al.*, 1993). Ultraviolet (UV) spectra of limonoids are not usually very informative. Among those functional groups which show significant absorbance, furan and conjugated acid, ester and lactone maxima are all found around 210 nm (Maier *et al.*, 1977).

Limonoids can be detected effectively in plant extracts by thin-layer chromatography (TLC) (Maier *et al.*, 1977). Spraying the chromatogram with Ehrlich's reagent (p-dimethylaminobenzaldehyde solution) and then exposing it to hydrogen chloride gas reveals the limonoids as orange spots, the limonoid color is characteristic enough to be readily differentiated. A gas chromatographic method for detection of limonin in plant extracts has also been reported (Maier *et al.*, 1977). It does not seem to have any significant advantages over the more convenient TLC method.

Limonin is responsible for the bitter taste of *Citrus* fruit juices, adversely affecting their quality even in a concentration as low as 3 to 5 ppm (Maier *et al.*, 1977). There are several methods for the isolation of limonin from *Citrus* juice, peel and seeds. For actual isolation of limonoids from extracts, column chromatography is usually necessary. This can be preceded by removal of much of the limonin by crystallization, taking advantage of the insolubility of the limonoid in most organic solvents. Separations of limonoids on silica gel TLC plates can usually be reproduced on columns of the same adsorbent.

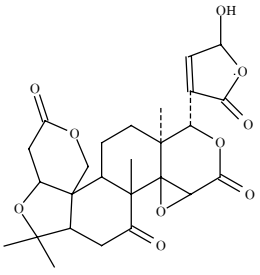
One of the methods for the isolation of limonin is the use of dried and ground *Citrus* seeds that have been in an acetone solution for 2 to 3 weeks. After this 2 to 3 weeks period, the limonin will be precipitated with petroleum ether and then re-crystallized from methylene chloride and isopropanol. Another method is slow extraction of limonin from the seeds with benzene at room temperature, after which the limonin is precipitated with petroleum ether.

Pifferi *et al.* (1993) had described the most practical method which could provide a high yield, reduces losses caused by eventual reactions with other components of the seeds, such as proteins, amino acids, etc., which may take place during extraction. This method uses petroleum ether to remove fat. The final extraction was carried out by using acetone in a soxhlet apparatus. After concentration in a vacuum evaporator the acetonic extract is re-precipitated for purification.

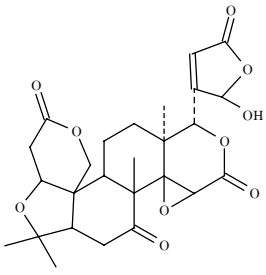
Fong *et al*. (1989) isolated limonoid glucosides in commercial *Citrus* juices by passing the extract through a C-18 reverse phase Sep-Pak washed with water and eluted with methanol. Braddock and Cadwallader (1992) suggested that recent commercialization of adsorbent resins for removing the bitterness of *Citrus* juices may allow the possibility of extending use of de-bitter juices as by products. Cationic exchange resins have been used to isolate various flavonoids and limonoids from *Citrus* juices. Reduction of limonin bitterness in *Citrus* juice using this technology has been accomplished without significantly altering most chemical components or juice properties. Ohta and Hasegawa (1995) had isolated limonoids in pummelos by passing the juice samples through a C-18 Sep-Pak washed with water and eluted with methanol. Hasegawa *et al*. (1996) used the same technique to isolate limonoid glucosides in orange molasses.

Tian and Ding (2000) had utilized high-performance liquid chromatography-electrospray ionization mass spectroscopy (HPLC-ESI-MS) to rapidly screen limonoid glucosides in *Citrus tangerine (Tanaka) Tseng*. Both the UV-Vis spectrum and mass spectrum of each compound can be obtained by using two detectors (diode array detector and mass spectrometer).

Treatment of limonoids with strong acids causes decomposition to ill-defined products, due to the attack on the furan ring. This group is also responsible for the sensitivity of limonoids to oxidation. A compound named limonexic acid has been prepared by treatment of limonin with oxidizing agents and by photosensitized air oxidation.



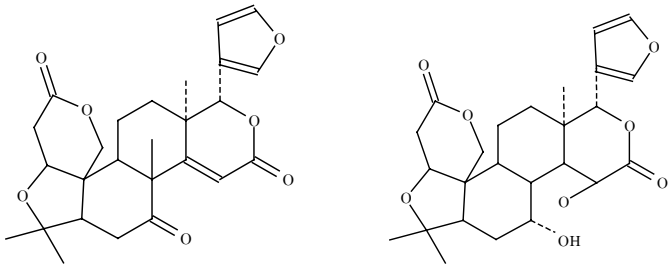
β-substituted limonexic acid (2)



α-substituted limonexic acid (3)

Figure 2 Characteristic structural features of limonexic acid isomer, β-substituted (2), α-substituted limonexic acid (3)

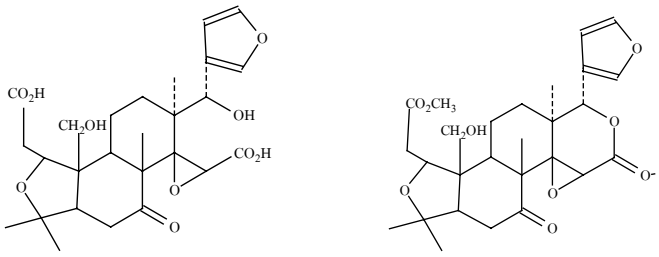
The C-14, 15-epoxide of limonoids can be reduced to the corresponding olefin by treatment with hydriodic acid or with chromous chloride. The C-7 keto group can be reduced to a 7-α-alcohol with aluminum isopropoxide in isopropanol, and to a 7-β-alcohol with sodium borohydride in dioxane.



Deoxylimonic acid (4) Limonol (5)

Figure 3 Characteristic structural features of deoxylimonic acid (4) and limonol (5)

The two lactone rings of limonin can be opened by treatment with base to produce a salt of limonoic acid. Upon acidification the D-ring carbonyl re-lactonizes so fast that it has not been possible to isolate it in the free acid form, or convert it to an ester. However closure of the A-ring lactone is much slower and the carboxyl group can be methylated to form methyl limonoate D-ring lactone. Attempts to open the D-ring lactone of deoxylimonin with base resulted in cleavage of the C7, 8-bond and shift of the double bond to produce deoxylimonic acid.



Limonoic acid (6) Methyl limonoate D-ring lactone (7)

Figure 4 Characteristic structural features of Limonoic acid (6) and Methyl limonoated-D-ring lactone (7)

2. Nutritive Aspects of Limonin

There are four chemopreventive phytochemical groups in *Citrus* fruits (Park and Pezzuto, 2002)

1. Highly methoxylated citus flavonoid, includes hesperetin, hesperidin, nobiletin, tangeretin, naringin, a cryptoxanthin and heptamethoxyflavone. These flavonoids function as antioxidants. (Bracke *et al*., 1994; Middleton and Kandaswami, 1994; Tanaka *et al*., 2000)

2. The coumarin auraptene widely occurs in *Citrus* fruits. This compound shows the chemo-preventive activity and this correlated with suppression of cell proliferation and lipid peroxidation with induction of phase II enzymes. (Murakami *et al*., 1999; Murakami *et al*., 2000)

3. The limonoids, are triterpene derivatives, present widely in the *Rutaceae* and *Meliaceae* families. Limonin, nomilin and obacunone are representative limonoids, found in *Citrus* seeds and juices, and are reported to inhibit forestomach, colon and buccal pouch carcinogenesis in rodents, and to reduce tumorigenesis. (Tanaka *et al*., 2000)

4. *Citrus* pectin, a soluble component of plant fiber, inhibited metastases in animal models of prostate cancer. (Baker, 1994)

In addition to the bitter property, limonoids possess potential biological functions. Limonoids inhibit chemically induced carcinogenesis in mice, hamsters and cultured human breast cancer cells (Lam *et al.*, 1994; Miller *et al*. 1994). Moreover, antifeedant activities against certain insects and invaders were indicated (Tian and Ding, 2000). The various biological activities of limonoids, which are abundant in *Citrus* fruits, have therefore been given recent attention. The most convenient ways for intaking limonoids is by drinking juice and eating the fruit itself, so it is easy to take limonoids as anticancer components in the diet. Because humans have being consumed *Citrus* fruits from ancient times as food, it can be said that *Citrus* limonoids are a very safe compound for human health.

The triterpene structure of limonin appears to play a part in determining the relative Glutathione-S-Transferase (GST) inducing activity. GST is a major detoxifying enzyme system that catalyzes the conjugation of glutathione with electrophiles that include activated carcinogens. An increase of GST activity by a substance, therefore, is an enhancement of the protection mechanism against the noxious effect of carcinogens. On the other hand it is interesting to determine whether the limonin enhances other preventive mechanisms, such as an immunological mechanism, so it is important to determine these aspects of nutritive limonin (Lam *et al.*, 1994)

Tanaka *et al*. (2000) examined the modifying effects of obacunone and limonin on azoxymethane-induced colon carcinogenesis in male F344 rats. The results suggested that the *Citrus* limonoids obacunone and limonin might be useful for the prevention of human colon cancers. Obacunone and limonin have been reported to enhance glutathione S-transferase (GST) activity in various organs of mice. Limonin and nomilin are reported to inhibit forestomach, buccal pouch, lung, and skin carcinogenesis in rodents. And limonin could inhibit carcinogen-DNA adduct formation. Dietary

administration of limonin increased not only GST but also quinine reductase (QR) activities in liver and colon of rats.

Miller *et al*. (1994) have tested *Citrus* limonoids for cancer chemo-preventive activity. They found that treatment with limonin reduced tumor burden. The results with limonin were encouraging, several properties associated with this chemical argue against the possibility that limonin might someday be used as a cancer chemopreventive agent for humans. The primary problem is that limonin is intensely bitter. The second problem is that this *Citrus* chemical is only soluble in organic solvents. These two problems would limit the types of products that limonin might be incorporated into as a possible food additive or food supplement.

Bengmark (1998) suggested that only natural food has the potential to supply most of the components needed, and thereby provide effective and successful nutrition. Kahlon and Keagy (2003) defined the functional foods as any foods, modified foods, or good ingredients that provided structural, functional, or health benefits, promoting optimal health, longevity, and quality of life. In short, functional foods were products that provided specific health benefits beyond the traditional nutrients they contained. Terms used to describe functional foods include designer, fitness, hypernutritional, longevity, medical, pharmaceutical, prescriptive, and super foods, as well as foodiceuticals and neutraceuticals. Bengmark (1998), Levy (1998) gave the term “immunonutrition” on nutrition that related to immune system. Immunonutrition is used to describe the dietary factors believed to confer an advantage to the immune system. The premise that the body is able to heal itself given the opportunity and the right foods is very attractive, as if the belief in the enhancement of natural defense through certain foods. The goal is to return the system to its natural balance by a process of re-conditioning.

Many terms are related to immunonutrition, such as immunomodulator or immunoregulator; these nutrients either enhance or suppress immunity. Immunostimulant or immunopotentiator are nutrients that enhance immunity. It is an advantage to health that the nutrient concerned

with health in most studies is immunostimulant. Immunoadjuvants are non-antigenic nutrients that stimulate antibody formation. Presently most researchers pay considerable attention to chemicals that affect the immune system and hope that those chemicals may be used to manage illness in humans. There are many levels at which the immune system could be affected by interacting nutrients such as prostaglandin synthesis by phagocytes, the production of antibodies or cytokines, the production of B-cell and T-cell, as well as antimicrobial and antitumor of macrophage. This level can be modified by nutrients that promote the synthesis of reactive oxygen and nitrogen intermediates.

Kubena and McMurray (1996) have summarized the experimental techniques commonly used to assess the functional integrity of various components of the immune system in human beings or animals (see table 2).

Table 2 Experimental measures of immune status

Immune Component	Experimental Technique
B cells	
Enumeration(b)	Surface Immunoglobulin G, CD19
Proliferation(c)	LPS(rodents); Staphylococcus protein A
Antibody production	Serum Immunoglobulin class concentrations (IgM,IgG, IgA); various assays for antigen-specific antibidies
T cells	
Enumeration(b)	CD3(all); CD4; CD6
Proliferation(c)	Mitogens(ConA,PHA); Antigens Cytokine
Cytokine Production	Biological or ELISA assay for interleukins and interferons in serum or cell culture supernatants
Cytotoxicity	Cr release from antigen-pulsed target cells
Natural killer cells	
Enumeration(b)	CD16; CD56
Cytolytic function	Cr released from natural killer target cells(eg. K562)
Macrophages	
Enumeration(b)	CD14; nonspecific esterase stain
Activation	MHC-2 expression; ROI or RNI production
Phagocytosis/Killing	Latex beads; antibody-coated bacteria

(a) Abbreviations; LPS = lipopolysaccharide; ConA = Concanavalin A; PHA = phytohemagglutinin; ELISA = enzyme-linked immunosorbent assay; MHC2 = major histocompatibility complex class 2; ROI = reactive oxygen intermediates; RNI = reactive nitrogen intermediates.

(b) Detected using monoclonal antibodies and flow cytometry

(c) Measured after 3 to 5 days of *in vitro* culture by the uptake of 3H-thymidine

Source: Kubena,K.S. and McMurray,D.N. (1996)

3. Immunological Activities of Limonin

It has been reported that certain foods possess a host defense function related to the immune system, anti-oxidation and anti-tumor activity. The immune system plays an important role in physical and chemical carcinogenesis and in tumor-bearing hosts. Tanaka *et al.* (1999) investigated the immunomodulatory action of auraptene from the peel of *Citrus* fruit (*Citrus natsudaidai* Hayata) on macrophage and cytokine production of lymphocytes in female BALB/c mice.

Raphael and Kuttan (2003) studied the effect of naturally occurring triterpenoid compounds such as glycyrrhizic acid, ursolic acid, oleanolic acid, and nomilin on the immune system. They reported the immunomodulatory activity of triterpenoids by analyzing their effects on hematological parameters, production of bone marrow cells, production of specific antibody and antibody producing cells in spleen.

The role of host immune function has become increasingly important for the mechanisms that are involved in the body's ability to prevent cancer. Although the inter-relationship between diet, immune function and carcinogenesis is unclear, there is increasing evidence that dietary alteration of the host's immune function is a key component of chemoprevention (Tanaka *et al*., 1999).

The human immune system can be divided into two classes, the first one is innate or non-specific immunity, the second one is acquired or specific immunity. Non-specific immunity is the system that moderates human resistance or susceptibility to pathology. There are many mechanisms for non-specific immunity such as mechanical barriers, physiological chemicals, microflora, phagocytes, inflammation, humoral factors, lymphocytes, and physiological factors. Innate immunity operates nonspecifically during the early phases of an immune response. Specific recognition of pathogens initially activates nonspecific cells and molecules.

Innate immunity serves as the first line of defense and includes both external and internal non-specific responses. External defenses occur in those areas of the body exposed to the outside environment (the contact areas for pathogens: skin, tears, mucous and ciliated epithelium of the respiratory tract, and low pH of the stomach). Internal defenses (inflammation and phagocytosis) come into play when the pathogen has penetrated the external defenses. Inflammation involves increased blood flow to the site of injury and increased permeability of the vascular endothelium to allow access of white blood cells and serum components to the tissues. Phagocytosis, which can trigger inflammation, uses professional phagocytes (monocytes, macrophages, and polymorphonuclear leukocytes) to remove foreign materials that have been introduced into the body. Phagocytes show no specificity, and the kind of protection they provide is different from acquired immunity. Taken together, the components of innate immunity are (Elgert, 1996):

1. Performed (the components are present before challenge).
2. Standardized (the response magnitude is consistent).
3. Without memory (the host does not realize it has been re-exposed to the same invader).
4. Nonspecific (innate immunity does not distinguish between invaders).

A more effective innate internal defense is phagocytosis. This involves the engulfment and destruction of pathogens and particulate matter by cells of the mononuclear phagocyte system. The cells that make up this network are the mononuclear phagocytic cells (monocytes and macrophages). These cells also provide help during acquired immunity. Monocytes and macrophages are called professional phagocytes (professional in the sense that their primary role is phagocytosis. Phagocytic cells, the first line of internal defense, respond immediately and without specificity. Macrophages ingest and digest whole bacteria and even injured and dead host cells. Macrophages constitutively express receptors for polysaccharides found on

bacteria; these receptors facilitate phagocytosis. Also during phagocytosis, macrophages release powerful chemical molecules, called monokines, such as interleukin-1, interleukin-6, and tumor necrosis factor-α that activate many non-specific protective effects through the inflammatory response. However, phagocytosis against soluble antigens (such as toxins) is poor.

Another important group of phagocytic cells, filled with granules containing potent digestive chemicals, not included in the mononuclear phagocytes system are the polymorphonuclear neutrophil leukocytes, or neutrophils. These cells are arbitrarily excluded from the mononuclear phagocyte system because they are not participants in normal specific immune induction reaction; they only internalize microorganisms for digestion, not for subsequent activity on other immune cells. Along with their phagocytic activity, neutrophils are the main source for small peptides called defensins. Defensins have a broad antimicrobial spectrum and exert non-specific cytotoxic activity against a wide range of normal and malignant targets. Another group of granule-filled cells called natural killer (NK) cells are not phagocytic but contribute to nonspecific defense against infected body cells and tumor cells.

Acquired immunity develops during a host's lifetime and is based partly on the host's experiences. Acquired immunity is the surveillance mechanism of vertebrates that specifically recognizes foreign antigens and selectively eliminates them, and on reencountering the antigens has an enhanced response. Once a host has been exposed to a specific disease, the host will develop specific immunity and will probably not catch the disease again. Taken together, six major characteristics of acquired immunity are specificity, inducibility, diversity, memory, distinguishing self from non-self, and self-limiting.

Vertebrates possess two types of acquired immunity based on the components the immune system uses to mediate immunity.

1. Humoral immunity is mediated by antigen specific blood proteins called antibodies. Antibodies are secreted only by

plasma cells (the daughter cells of bone marrow or bursa-derived, or B-lymphocytes). This immunity protects against circulating extracellular antigens such as bacteria, microbial exotoxins, and viruses in their extracellular phase, that is, antibodies normally interact with circulating antigens but are unable to penetrate living cells.

2. Cell-mediated immunity, humoral immunity's concomitant counterpart during an immune response. This immunity is mediated by antigen-specific cells called thymus-derived, of T-lymphocytes. Cell-mediated immunity protects against intracellular parasites, such as viruses, and is important in the rejection of organ transplants and tumor cells.

Humoral immunity involves a class of immunoglobulin called antibodies. (Antibodies belong to a family of globular proteins called immunoglobulins. The terms antibody and immunoglobulin are used interchangeably). Antibodies are recognized proteins found in the serum and other fluids of vertebrates that react specifically with the antigens that induced their formation. Antibody-containing serum is called antiserum, in contrast to normal serum, the clear yellowish fluid collected when whole blood is separated into its solid and liquid parts that does not contain antibody to a specific antigen.

Antibodies are divided, based on their heavy chain structure, into six chemically distinct classes, four kinds of immunoglobulin G (IgG) and two kinds of immunoglobulin A (IgA), plus immunoglobulin M (IgM), immunoglobulin D (IgD), and immunoglobulin E (IgE). Each class plays a different role in the immune system's defense strategy. IgG, the major antibody in the blood, coats microorganisms to speed up their uptake by phagocytic cells. The dimeric IgA concentrates in body fluids to guard the entrances of the body. IgM, a pentamer, is the most effective activator of complement, and therefore, is the best indirect killer of blood-borne bacteria. IgD is found almost exclusively on the surface of B cells, where it may

regulate the cell's activation. IgE, found in trace amounts in the blood, attaches itself to specialized cells, where it triggers the symptoms of allergies.

Antibodies have many biological activities. They can prevent toxins and viruses from entering cells, whereas other antibodies coat bacteria or target cells to make them more palatable or vulnerable to attack by certain immune cells. Antigen-antibody complexes also can activate complement. Some antibodies, like IgG, cross the placenta and provide protection to the fetus. IgE can promote allergies. IgG-containing antigen-antibody complexes attach to Fc receptors on B-cells, and inhibit B-cell activation.

Interaction between antigen and antibody leads to the formation of an immune complex. Non-covalent interactions between antigens and antibodies involve weak chemical forces. No covalent bonds are formed. Various procedures are used to determine the presence and quantity of antigen and antibody, such as precipitation and agglutination. Agglutination is the combination of a particulate (insoluble) antigen with specific antibody, which leads to a clumping of particles. The agglutination of red blood cells (RBC) is called hem-agglutination, which is read by the sedimentation patterns of the RBC observed at the bottom of a tube or well. All antigen-antibody reactions are reversible and the law of mass has been applied, from which antibody affinity (the strength of which a single antigen-antibody bond is formed) given by the equation constant (K) at equilibrium stated and can be calculated as follow:

$$Ab + Ag \leftrightarrow AbAg$$

$$K = \frac{[AbAg]}{[Ab][Ag]}$$

3.1 Cells of the Immune System

The human body's immune system stockpiles a formidable arsenal of lymphocytes and other immune cells. Approximately 1% of the body's

weight is made up of lymphocytes (roughly 2 trillion cells). When exposed to an antigen, those cells of the correct specificity multiply to the numbers needed to counteract the antigen. To meet and destroy all the antigen that the body encounters, lymphocytes must remain a dynamic cell group; their turnover per year can be equivalent to a person's body weight. Maintaining the immune system may be nature's ultimate balancing act. The immune cell repertoires have stable general properties, and continuous static levels are maintained for particular immune cell subsets. The high turnover of immune elements continually provides the host with a functional immune system (Elgert, 1996).

Several types of cells participate in the defense system of organisms. In adults, they almost all originate, multiply and mature in the bone marrow, and are found in the mature stages in the blood, in which they either stay or from where they migrate into other tissues. The different cell types are:

Polymorphonuclear cells (PMN) or granulocytes, the least specifically reacting cells in a defense reaction but able to phagocytose; they form the first line of defense against intruders. Cells present in blood divide into Leukocytes or white blood cells and Erythrocytes or red blood cells. White blood cells can be divided into polymorphonuclear cells and mononuclear cells.

The monocytes are endowed with the capacity of phagocytosis, processing, and presenting antigenic material in a manner recognizable by specific immune cells, and are able to bind specifically reacting receptors, forming the second line of immunity.

The lymphocytes, the immune cells, are endowed with the capacity to recognize as well as react specifically with foreign (antigen) material *via* specific receptors. B-lymphcytes are derived from bone marrow stem cells and develop within the bone marrow. Mature B-cells express surface antibodies that act as receptors for specific antigens. All antibodies on a B-

cell, irrespective of the classes or combinations of classes, have the same antigen specificity.

A fourth lineage of cell assumes only a peripheral role in the immune system but nevertheless an important one: the thrombocytes or platelets.

3.2 Macrophages

The term macrophage is generally applied to phagocytic cells encountered in the connective tissue that are capable of ingesting bacteria, cellular remains, and foreign substances generally present in the tissues. The prefix "macro-" distinguishes these cells from other smaller phagocytic cells existing in the blood and tissues, the polymorphonuclear neutrophils.

The form of the macrophage depends upon its functional status and localization. Fixed macrophages are star-shaped or spindle-shaped, possessing nuclei of delicate chromatin with one or two nucleoli and slightly basophilic cytoplasm. The mobile macrophage is usually round or oval, with a kidney-shaped nucleus. Both types of macrophages possess irregular cytoplasmic membranes with numerous prolongations and entry points that are related to the mechanism of phagocytosis.

Macrophages originate from precursor cells in the bone marrow and pass into the blood as monocytes. The monocytes remain in the circulation for some hours after which they migrate into the tissues, and transform to macrophages. The macrophages customarily migrate to the peritoneal cavity and to other serous cavities, the red pulp of the spleen and the lymph node medullae.

Tissue macrophages are extremely important to the immune response in the degradation of infectious agents, in the processing and preserving of antigenic determinants in specific immune responses, and in non-specific cellular immunity. This last condition is related to the enhanced destructive capacities of activated macrophages, a reserve or new population

of macrophages, which is summoned into action by exposure of an animal to an infectious condition or exposure to a phagocytosable particle. The activated macrophages are extremely active metabolically, including an improved phagocytic capacity toward all particles regardless of the antigenic nature of the stimulus that called forth their production.

3.3 Measuring Phagocytosis (Barrett, 1970)

To study phagocytosis one must first decide what will be used as the subject particle. A wide choice of materials is available, including living or dead bacteria, living or dead yeasts, foreign erythrocytes, lipid emulsions, colloidal silver, colloidal carbon, iron oxide particles, spheres of polystyrene, polyacrylamide or methyl methacrylate, thorotrast, and others. These cells or particles may be radiolabeled to aid in tracting them *in vivo* behavior or to case quantitation. Non-antigenic materials are preferred, since they avoid any possible contribution by the immune system. The particles should have a known and reasonable uniform size distribution, since within limits; larger particles are more easily ingested by phagocytic cells. The most widely chosen material is colloidal carbon in the form of washed India ink.

Since two classes of cells, the circulating cells and the fixed cells, are involved in phagocytosis, two separate procedures have been developed to measure their activity. Phagocytosis of circulating cells is most commonly studied *in vitro*. In this method whole blood or peritoneal washing treated with an anticoagulant is incubated with a suspension of bacterial cells or other particles in 37^{o}C water bath for a measured time interval, usually 30 minutes. At the end of this time, blood films are prepared and stained with appropriate stain and are examined microscopically. The average number of bacteria (or particles) per phagocytic cell is determined and reported as the phagocytic index.

An *in vivo* phagocytic index, which measures primarily phagocytosis in the liver and spleen, may also be calculated. This is usually

done by intravenous injection of an inert particle such as carbon or metal. Periodically thereafter blood samples are removed and the concentration of the residual, free particle is determined. The phagocytic index is expressed as K where

$$K = \frac{\log \text{concentration a} - \log \text{concentration b}}{Tb - Ta}$$

Tb and Ta are the times at which the concentrations b and a are determined. Corrections may be inserted for the body weights of different animals if comparative studied are desired.

Where as Ichinose *et al.* (1994) definited the phagocytic index as the average number of ingested particles per macrophage, so the phagocytic index is expressed as PI as

$$PI = \frac{\text{The number of total ingested beads}}{\text{The number of total macrophages analyzed}}$$

4. Processing of *Citrus* Fruits

Citrus juices are among the most universally accepted and desirable food throughout the world (Berry and Veldhuis, 1977). This widespread popularity probably also explains the fact that manufacture of *Citrus* products is one of the largest food processing operations in the world. For many years *Citrus* juices and fruit parts were only available in locations near areas where they could be grown. The advent of food processing made these products available in areas remote from *Citrus*-growing regions and brought about the tremendous growth of *Citrus* production over the past 20-30 years.

The fruit is hauled to *Citrus* plants in long semi-trailer trucks that are usually backed down a ramp so that the fruit rolls to conveyer belts by gravity. The fruits are transported *via* conveyor belts to holding bins. The

fruits may be stored in bins for 12 to 72 hours before processing. In most modern plants, bins are designed with screen or mesh ends and sides to allow adequate ventilation, thus discouraging mold and fungal growth. Usually they have built-in alternating supports to partially relieve gravitational pressure so that no fruit undergoes excess pressure during storage in bins prior to processing. From the bins, the fruits are conveyed to a brush washer. An approved detergent is applied in the brush washer, and the fruits are scrubbed by revolving brushes and rinsed with a water spray. The fruit is usually separated automatically into sizes and directed to the juice extractors. The juice is sent to a packaging unit.

Among the *Citrus* varieties, the lemon is undoubtedly the most ubiquitous in its areas of application and quite appropriately, has been called "the fruit of many uses". Although not edible in the same manner as other *Citrus*, the lemon and its juices probably have had a greater variety of culinary, beverage, industrial and medicinal uses than any other fruit (Swisher *et al.*, 1977).

Most juice goes into concentrated juices. Today, however, frozen juice products, which are manufactured from a major outlet juice, is especially important to give due consideration to the fruit used in order to control flavor and other properties of this product effectively for a long periods.

Since juice contains many constituents important for their effect on flavor, storage, stability and health value, the proper choice of processing methods and storage conditions is important. Considerable detail covering composition and nutritional value of *Citrus* juice is a matter of concern. The method of analyzing method of the compound found in the juice should be discussed. Limonin is probably important from the viewpoint of processing and general use, as it is generally responsible for the unpleasant bitter flavor in *Citrus* juice. Limonin levels in the range of 4.2 to 14.2 ppm are reported in commercial juice (Swisher *et al.*, 1977). Limonin levels above about 7 ppm will definitely be expected to contribute to the bitter taste of the juices.

Our ancestors ate foods much richer in key nutrients and antioxidants than the food normally eaten today, because neither nutritionist not the food industry have fully taken into account the fact that many important food ingredients are chemically unstable and do not tolerate preservation by drying heating, freezing or other processing. In summary some food processing can destroy key nutrients (Bengmark, 1998).

However, there are many processes for preservation and stabilization of *Citrus* containing foods, such as cold process preservation of frozen orange juice concentrate products, food dehydration and concentration of lime juice, heat preservation and processing of powdered lime, filtration of orange juice with pulp, food irradiation and fermentation (Berry *et al.*, 1997; Swisher *et al.*, 1997). During the processing of food, the nutritive immunologic activity may be altered. The immunologic activity may actually remain unchanged, or decreased or even be increased by food processing. Since no studies have explained how the nutritive immunologic activity of *Citrus* foods changes in food processing, the study of the effect of processing on the immunological properties of limonin is great of interest and remains to be challenged.

5. Deteriorative Factors of Limonin

Bengmak (1998) has stated that 68% of all fruits and 73% of all vegetables have strong antimutagenic effects, which are reduced by cooking. It is known that the major causes of food deterioration are the following: growth and activities of microorganisms (principally bacteria, yeast and molds); activities of natural food enzymes; insects, parasites and rodents, temperature (both heat and cold); moisture and dryness; air and more particularly oxygen; and light. All these factors can affect stability of lime, and it is suspected that some of these deteriorative factors may also affect the activity and stability of limonin. It is therefore important to study how these factors affect limonin content and activity, and to develop appropriate food processing techniques to minimize loss of limonin.

In the study of deterioration, it is simple to study by kinetics of the reaction, which is the study of chemical change and the way in which this rate is influenced by the conditions of the concentration of reactants, products and other chemical species which may be present, and by factors such as solvent, pressure and temperature. Reaction kinetics is a powerful tool for the elucidation of mechanisms by which chemical reactions proceed. Such information permits a rational approach to the stabilization of chemicals and prediction of the shelf-life and optimum storage condition.

5.1 Reaction Rate and Reaction Order

The rate of a reaction is the velocity with which a reactant, or reactants, undergoes a chemical change. If two molecules, D and W, collide and rearrange to form one or more product molecules, it is natural to propose that the rate of the chemical reaction is proportional to the number of collisions which is proportional to the product of the concentrations of the two species. The equation for the overall reaction is

$$D + W \text{ --------} \rightarrow \text{ product}$$

Then the rate is α [D][W]

The rate is the rate of loss of the chemical D and is denoted –(d [D]/dt), hence

$$-d[D]/dt \ \alpha \ [D][W]$$

If let k_2 be the rate constant (proportionality constant), so

$$-d[D]/dt = k_2 [D][W]$$

This is an example of a second-order reaction, since the definition of overall order is the sum of the exponents of the concentration terms in the rate equation. Many decomposition reactions of chemical molecules are second-order. However, if water concentration [W] is held constant then:

$$-d[D]/dt = k_1 [D]$$

Where: $k_1 = k_2 [W]$

And the reaction is apparent first-order or pseudo first-order reaction. Another example is a reaction that depends on the hydrogen ion concentration with the solution being buffered, thus fixing $[H^+]$.

In addition to setting the water concentration or $[H^+]$ constant, the chemical concentration is fixed, the equation becomes

$$-d[D]/dt = k_0$$

Where: $k_0 = k_1 [D] = k_2 [D][W]$

And the reaction is apparent zero-order reaction. Note that the units of the respective rate constant are following: (a) second-order, $[\text{concentration}]^{-1}$ time^{-1}, (b) first-order, time^{-1}; and (c) zero-order, $[\text{concentration}]$ time^{-1}. Hence, the order of a kinetic reaction is apparent from the units used for the rate constant.

Overall zero-, first-, and second-order reaction kinetics are the most frequently encountered kinetics. More complex reactions are also observed. However, for shelf-life calculations in which the concentration change is generally no more than 10% to 20%, the distinction between orders is relatively unimportant. This can simplify data treatment.

A typical first-order reaction may be written as

$$D \dashrightarrow \text{products}$$

And its corresponding rate equation as

$$-d[D]/dt = k_1 [D]$$

This expression defines the rate of the reaction, whereas the concentration-time profile is obtained by integrating the rate from t = 0 to t = t, where [D] at t = 0 is $[D]_0$.

$$\ln[D] = \ln[D]_0 - k_1 t$$

$$\log[D] = \log[D]_0 - k_1 t/2.303$$

$$k_1 = (2.303/t)\log([D]_0/[D])$$

Thus a plot of log[chemical concentration] against time will be linear with the slope equal to - $k_1/2.303$, yielding the rate constant. The half-life ($t_{½}$) is the time for [D] to become $[D]_0/2$, that is one-half the original concentration, yield $0.693/k_1$. The shelf-life (t90) is the time for [D] to become $0.90[D]_0$, that is 10% decomposition, yield $0.105/ k_1$.

A zero-order reaction is one having a rate equation with no concentration dependence. So its corresponding rate equation is

$$-d[D]/dt = k_0$$

Integrating the rate from t = 0 to t = t, where [D] at t = 0 is $[D]_0$.

$$[D] = [D]_0 - k_0t$$

$$k_0 = ([D]_0 - [D])/t$$

Hence for a zero-order reaction, a plot of concentration against time is linear with a slope of k_0. The half-life is $0.5[D]_0/ k_0$, and shelf-life is $0.1[D]_0/ k_0$.

5.2 Effects on Reaction Rate

5.2.1. Temperature. The application of heat to increase the rate of a chemical reaction is a common laboratory procedure. The rate of most solvolytic reactions of chemicals is increased roughly 2- to 3- fold by 10^oC increase in temperature in the vicinity of room temperature. Arrhenius noted that the variation with temperature of the rate constant of chemical reactions could be express by

$$k = se^{-Ea/RT}$$

Where Ea is the Arrhenius activation energy (the difference between the average energy of reactive molecules and the average energy of all molecules), s is a constant called the frequency factor, R is the gas constant (1.987 cal/^{o}K-mole), T is the absolute temperature, and $e^{-Ea/RT}$ is the Boltzmann factor which represents the fraction of molecules having the energy Ea. In logarithmic form the Arrhenius equation becomes

$$\log k = \log s - Ea/2.303RT$$

Thus a plot of log k against the reciprocal of the absolute temperature will give a straight line with a slope of –Ea/2.303R. And the value of Ea may be calculated from the slope of the line. The intercept on the log k axis is log s.

Differentiationg the natural logarithm form with respect to temperature gives

$$d \ln k/dt = Ea/RT^2$$

And on integration between the limit k_2 and k_1 at temperature T_2 and T_1 yields

$$\log k_2/ k_1 = (Ea/2.303R)(T_2 - T_1)/ T_2T_1$$

This equation makes it possible to calculate Ea for a reaction when the rate constants are known at two temperatures, or to calculate the rate constant at one temperature, if Ea and the rate constant at another temperature are known.

5.2.2 Specific acid and specific base catalysis. The term specific acid catalysis refers to catalysis by the hydrated proton or hydrogen ion, and specific base catalysis refers to catalysis by the hydroxide ion.

If the rate of hydrolysis of an ester is studied at a constant pH in a strongly buffered solution, the rate of disappearance of intact ester will be an apparent first-order reaction. If the reaction is studied in solutions buffered at several different pH values in a sufficiently acid pH region, a different apparent first-order rate constant will be observed for each pH value.

The observed rate depends on the concentration of both the ester and hydrogen ion and actually is a second-order reaction, although at a constant hydrogen ion concentration, it is an apparent first-order reaction.

$$k_{observed} = k_1(H^+)$$

The observed apparent first-order rate constant determined in buffered solution, therefore, is proportional to the hydrogen-ion concentration. The variation in observed rate constant with pH can be illustrated by taking logarithms as

$$\log k_{observed} = \log k_1 + \log (H^+)$$

$$\log k_{observed} = \log k_1 - pH$$

Thus, a plot of logarithm $k_{observed}$ versus pH should be linear with a slope of -1.

Similarly, if the same hydrolysis reaction is studied in buffered solution at several pH values in a sufficiently alkaline region of the pH scale, the observed apparent first-order rate constants will be found to vary with hydroxide-ion concentration

$$k_{observed} = k_2(OH^-)$$

$$\log k_{observed} = \log k_2 + \log (OH^-)$$

$$\log k_{observed} = \log k_2 + \log \{Kw/(H^+)\}$$

$$\log k_{observed} = \log k_1 + \log Kw + pH$$

A plot of logarithm $k_{observed}$ versus pH would be a straight line with a slope of +1

The complete rate expression for hydrolysis of the compound described above at all pH values, therefore, would be

$$-d[D]/dt = [k_1(H^+) + k_2(OH^-)][D]$$

At any specified pH value

$$k_{observed} = k_1(H^+) + k_2(OH^-)$$

The complete logarithm $k_{observed}$ versus pH profile would be V-shape graph. The pH which the minimum rate of hydrolysis is observed is a function of the relative magnitude of the specific rate constants k_1 and k_2.

If for a specific chemical species $k_1 = k_2$, the expected minimum rate of reaction for that species would be expected to occur at pH 7 (for a temperature of 25°C, at which pKw = 14, and $(H^+) = (OH^-)$ at pH 7.0)

At pH value below the minimum, in the plot of logarithm $k_{observed}$ versus pH, the hydrogen-ion catalyzed reaction is much more significant than the hydroxide-ion catalyzed reaction, and the plot has a slope of -1. At pH values above the minimum in the plot, the hydroxide-ion catalized reaction is the much more important reaction and the plot has a slope of +1.

If a reaction is catalyzed not only by hydrogen-ion and hydroxide-ion, but also by the solvent (also called the uncatalyzed reaction), a plot of the logarithm $k_{observed}$ versus pH might appear a flat region where the rate of reaction apparently is not pH-dependent. In this region the solvent reaction to much more important than that of either the hydrogen ion or hydroxide ion. The apparent first-order rate constant for such a reaction is

$$k_{observed} = k_0 + k_1(H^+) + k_2(OH^-)$$

5.2.3 General acid or base catalysis. Acid or base catalysis is not restricted to the effect of hydrogen or hydroxide ion. Undissociated acids and bases can often be demonstrated to produce a catalytic effect and, in some instances metal ions and various anions can serve as catalysts.

Objectives

1. To analyze the quantity of limonin extracted from lime commonly used in Thailand.
2. To study the immunological action of limonin extracted from lime seeds.
3. To study the chemical stability of limonin.

Scope of Research Work

The chemical stability of limonin from lime seeds, under different pH and temperature, will be studied. Macrophage proliferation and phagocytosis activity, white blood cell counts, and the production of a specific antibody will be determined. The effects of processing techniques used commercially on limonin concentration and immune action will be investigated.

Thesis Advantages

1 To gain fundamental information on possible neutraceuticals from lime, particularly the immunostimulating properties of limonin.

2 To add value to the Thai common *Citrus* fruit, especially lime, as potential immunological relevant nutrients.

3 To provide a possible use for the waste of lime in industry as profitable sources of limonin.