CHAPTER 4

DISCUSSION

1. Isolation of Limonin

Limonin has been known as a *Citrus* constituent since 1841 (Bernay, 1841). It is the first limonoid known as a bitter compound in *Citrus* fruits. Limonoid is a group of chemically related triterpene derivatives found in the *Rutaceae* and *Meliaceae* families. Bitterness in *Citrus* juices due to limonin has become an increasingly serious economic problem, which has stimulated research in this area (SBHTI-USDA, 2004) to eliminate this compound from the food.

Most work on the isolation of limonin has been done on seeds, because it is richer in seed than fruit tissues. Analytical values of dry *Citrus* seeds are presented in table 15. The nitrogen-free substances are mostly carbohydrate-containing substances and much of the crude fiber could be digested by ruminants. The oil constituents are of primary importance. *Citrus* seed oils are primarily composed of triglycerides (>95%) with lesser amounts of free fatty acids, hydrocarbons, sterols, tocopherols, phospholipids, pigments, other minor lipids and bitter nonlipid material, e.g., limonin and narigin. Analytical values show seeds to be a rich source of protein and calories for use as food (Maier *et al*, 1977).

Table 15 Composition of air dry *Citrus* seeds

Constituent	As-is Basis (%)
Nitrogen free substances	22.2-44.6
Crude fat (ether extract)	21.9-34.4
Crude fiber	5.4-17.8
Crude protein (N x 6.25)	6.9-15.9
Water	6.8-11.9

Ash	2.5-3.6

Source: Maier, Bennett, and Hasegawa, 1977.

It is reasonable to assume, if we find a use for limonin, especially to improve health, as an addition to the product, it must add economic va 90 product. Thus the purpose of this study is to increase the value of limonin, which is abundant in lime seed and lime juice. It would be augment the value or economic advantage of the abandoned seed, as well as lime juice, which is one of the food ingredients in Thailand's most popular foods, such as Tom Yum Kung, and can be used as immunomodulating agent. Although it was supposed in the past that most of the immunity-promoting effects in *Citrus* fruits was due to vitamin C. Several comparative studies suggested that *Citrus* fruits contain not one but multiple preventive agents, and limonin is one that is found in high concentration in *Citrus* fruits and *Citrus* products (Miller, *et a*l, 1994). Nevertheless one advantage of limonin as immunomodulating agent is that it occurs naturally in edible plants and is present in human foods. These data support that limonin may be a benefit to humans. Thus lime juice, especially in several kinds of Thai food, may be useful for building up immunoefficiency in the patients's daily life. However further experiments, including pre-clinic efficacy and mechanistic studies, are warranted to fully evaluate limonin for immunomodulating properties and to understand its mode of action.

There are several methods for the isolation of limonin from *Citrus* juice, peel and seeds. For actual isolation of limonoid from extracts, column chromatography is usually necessary. Pifferi *et al.* (1993) have described the most practical method which gives a higher yield and reduces losses caused by eventual reactions with other components of the seeds, such as proteins, amino acids, etc., which may occur during extraction. This method uses petroleum ether to remove fat. The final extraction was carried out using acetone in a soxhlet apparatus. After concentration in a vacuum evaporator, the acetonic extract was precipitated.

The method used for the isolation and purification of limonin in the study gives a high yield. This higher yield is due to the fact that accurately dried and previously defatted seed powder was used, thus favoring the subsequent extraction of the limonin with acetone. The accurate drying of seeds results in a considerable delay to radical emergence (germination) (Agriculture Research Service, 2000), which reduces the loss of components in the seed. Moreover, the use of this solvent, which has a lower boiling point for the extraction of the limonin, reduces loss of limonin caused by eventual reactions with other components of the seeds, which may take place during both extraction and concentration.

Additionally, the dilution of the acetonic extract with petroleum ether offers the further advantage of being able to obtain a precipitate and a solution without the formation of an emulsion as a third phase. In addition, this dilution increases the purity of the raw limonin and consequently the yield of the subsequent crystallization process. Washing of the precipitate with 95% ethanol rather than with absolute ethanol also produce pure limonin with a lower crystallization number to be obtained, i.e. one versus three. On the other hand, if washing is omitted and the precipitate is directly dissolved in methylene chloride, then at least three successive crystallizations are necessary, thus causing a considerable loss in yield.

Therefore, this method is capable of giving a high yield and also offers other advantages. Such as the use of low-toxicity solvents, ease of implementation, reproducible results, low cost reagents and raw materials as well as reduced processing time. The method, therefore, is particularly suitable for the preparation of limonin for industrial applications as a reference substance in the quality control of processed food products.

2. Identifications of Limonin

Limonin can be detected in plant extracts by thin layer chromatography (TLC). After spraying the chromatogram with Ehrlich’s

reagent (*para*-dimethylaminobenzaldehyde solution) and then exposure to hydrogen chloride gas, limonin is revealed as orange spots. Although other types of compound also react with Ehrlich's reagent, the limonin color has clear characteristics that can readily be differentiated as limonin

The most useful method for determining the structure of limonin is nuclear magnetic resonance (NMR) spectroscopy (Maier *et al.*, 1977). The characterization of limonin is performed by infrared spectroscopy (IR) analyzation. It is shown that extracted limonin contains a furan ring attached to the D-ring, as well as oxygen-containing functional groups and epoxide group. Mass spectroscopy has become an important structural tool also. The mass spectra of some *Meliaceae* limonoid, including limonin, have been published (Baldwin *et al*, 1967), and it has a molecular weight around 471.3.

NMR study has been important in the chemical structure determination of limonin for evaluation, NMR data would aid in confirming the structures of limonin, largely on the C-methyl and the furan resonances. The general features of the NMR spectra of limonin can be summarized as follows (Dreyer, 1965):

1. Bands due to the C-methyl resonances.

2. The absorption bands for the four protons at C2 and C6, α- to the carbonyl group.

3. A sharp singlet due to the proton at C15, adjacent to the epoxy group, which overlapped the band due to the proton C1.

4. A broad band due to the proton on C1 was resolved into a symmetrical triplet.

5. An AB system from the proton on C19 collapsed into a broadened singlet.

6. A very slightly broadened singlet caused by the proton at C17, adjacent to the furan ring.

7. The furan absorptions

The chemical structure of limonin was compared its NMR chemical shifts correlation with reference data to authentic standard limonin (Bennett and Hasegawa,1981)

3. Quantitative Analysis of Limonin

Quantitative analysis of limonin in the study was done by separating on a C-18 “Supelco” cartridge, which could remove any unknown substance that interfered with limonin analysis (Shaw and Wilson, 1984). So the HPLC chromatogram obtained a distinct single peak, although limonin can be quantified by direct injection of the whole solution.

4. Stability of Limonin

Limonin was not stable in basic solution of high pH, so limonin did not appear in buffered solution of pH 10, 11, and 12. It was known that the two lactone rings of limonin could be opened by treatment with base to produce a salt of limonoic acid (Figure 4). Upon acidification the D-ring carbonyl re-lactonize 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 (Figure 4). 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 (Figure 3). Treatment of limonin with strong acids causes an attack on the furan ring and a compound named limonexic acid (Figure 2) occurred.

Carboxylic acid derivatives are susceptible to attack by nucleophilic agents. Nucleophiles and bases can also act as catalysts of acyl transfers. These catalytic mechanisms could lead to chemical decomposition. Limonin was most stable in pH 5-7 buffered solution, which has an uncatalyzed reaction, indicating that the zwitterionic form is the least susceptible species with respect to hydrolysis.

The study of the stability of limonin is a powerful tool for the elucidation of mechanisms by which chemical reactions proceed, in order to determine the factors that cause the deterioration of limonin. In application to products, such information permits a rational approach to the stabilization of

products and prediction of shelf-life and optimum storage condition. On the other hand, the stability study of limonin is also useful for further approach to limonin elimination. It is simple to study the deterioration of limonin by kinetics of reaction. The kinetics of the reaction indicates how fast reactants or chemicals are converted to products by the environment.

The relative concentrations of reactants and products at equilibrium can be expressed numerically as an equilibrium constant or k_{eq}, which depends on their relative stability and is equal to products by reactants. The more stable the compound, the greater its concentration at equilibrium. Thus, if the products are more stable (have a lower free energy) than the reactants, there is higher concentration of products than reactants at equilibrium and the equilibrium constant is greater than 1. On the other hand, if the reactants are more stable than products, there are higher concentrations of reactants than products at equilibrium and the equilibrium constant is less than 1.

Kinetics is the field of chemistry that describes the rates of chemical reactions and the factors that affect the rates. The rate of a chemical reaction is the speed at which reacting substances are used up or the speed at which products are formed. The rate of the reaction is proportional to the concentration of reactants. If the rate of reaction is proportional to the concentration of only one reactant, it is called a first-order reaction. A reaction whose rate depends on the concentrations of two reactants is called a second-order reaction. Therefore the decomposition of limonin in aqueous solution is hydrolysis, of which the reactants are limonin and water, follows the second-order reaction. However, if the water concentration is held constant by having a large excess, the constant of the reactant is proportional to the limonin only. The reaction is not real first-order reaction; it is indicated as apparent first-order reaction.

Limonin, which is ester, is trend to hydrolysis. The rate of hydrolysis depends on the temperature and the pH of the solution. A rule-of-thumb is that for each 10^{o}C rise in storage temperature, the rate of reaction doubles or triples. The application of heat to increase the rate of reaction is a common

laboratory procedure. And in order to calculate activation energy for a reaction, reaction rate constant must be obtained at several temperatures (at least 3 temperatures).

If the rate of hydrolysis is studied at a constant pH in a strongly buffered solution at several different pH values in a sufficiently acid pH region, a different apparent first-order rate constant is observed for each pH value. The observed rate depends on the concentration of both reactant and hydrogen ion and actually is a second-order reaction. The observed rate constant therefore is proportional to the hydrogen-ion concentration, so $k_{observed} = k_H(H^+)$. The variation in observed rate constant with pH can be illustrated by taking logarithms as $\log k_{observed} = \log k_H + \log (H^+)$ or $\log k_{observed} = \log k_H - pH$. 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 rate constants vary with hydroxide-ion concentration. So $k_{observed} = k_{OH}(OH^-)$, and $\log k_{observed} = \log k_{OH} + \log (OH^-)$ or $\log k_{observed} = \log k_{OH} - 14 + pH$ ($\log k_w = -14$).

Thus, the graph between log k and $\log (H^+)$ or pH in extreme pH of acid or alkaline region, was plotted, with the intercept of $\log k_H$ or $\log k_{OH} - 14$ consecutively. The equation $k_{observed} = k_0 + k_H[H^+]$ or $k_{observed} = k_0 + k_{OH}[OH^-]$ can be formulated as shown in table 9, where k_0 is average k in extreme pH of acid or alkaline region.

The reaction rate of limonin in solid state is proportional to the concentration of only one reactant, so it is called a first-order reaction. It is theoretically more stable in solid state or dried substance than in aqueous form, and Arrhenius activation energy (free energy of activation is the energy barrier of a reaction) of limonin in solid state is so much more than that of limonin in aqueous phase.

It is reasonable to consider that limonin has undergone a chemical change with the increase in temperature; the most likely chemical change in the heated solutions would be hydrolysis of one or both the lactone ring to the

corresponding hydroxyacids which are so unstable (Chandler and Robertson, 1983).

When limonin decomposition is the result of a hydrolysis reaction, an obvious and effective means of stabilization is to limit access of the limonin to water. This is simply accomplished by using a solid dosage form. Other means is to control pH. In order to select the optimum pH, it usually has available pH-rate profile, which shows the pH for maximum stability. However, it may not be possible to buffer the formulation or product to the pH of maximum stability, now that the solubility of limonin which is pH-dependent and the pH of maximum stability may lie outside the physiologically acceptable range. Thus the last means that stability of limonin might be increased is lowering the temperature, which should be not detrimental.

5. The Immunomodulating Effects of Limonin

Bengmark (1998) reviewed that natural food has the potential to supply most of the components needed, and thereby provides effective and successful nutrition. Recently, it has been shown that certain foods have a host defense function related to the immune system (Tanaka, *et al*, 1999). Most researchers pay serious attention to chemicals that affect the immune system, now that the premise that the body is able to heal itself if given the opportunity and the right foods to enhance natural defense through certain foods.

There are many levels at which the immune system could be affected by interacting nutrients such as cell proliferation, Kubena and McMurray (1996) summarized the experimental techniques commonly used to assess the functional integrity of various components of the immune system in human being or animals. One of the suggested experimental techniques, which was used in this study, is detecting the number of macrophage by using the latex-beads phagocytes and the flowcytometry, which is a precise and rapid

method. One of the most important functions of macrophages is phagocytosis, which is a cellular defense mechanism against invading microorganisms. Several methods for quantitative phagocytosis are available. The most frequently used method is direct visual counting of the number of ingested particles per 100 macrophages under a microscope (Ichinose, *et al*., 1994). However, this procedure is tedious, laborious, and time consuming. The flowcytometric assay in the present study was used to quantify phagocytic activity both percentage of phagocytic cells and the phagocytic index.

The macrophage-stimulating activity of limonin was evaluated from the number of exudated cells in the peritoneal cavity after limonin stimulation was calculated as a percentage, so it was called the percentage of phagocytic cells or PP which was defined as the percentage of macrophages with one or more ingested particles within the total macrophage population (10,000 cells). In addition, the phagocytic index (PI) which was defined as the average number of ingested particles per macrophage was calculated to indicate phagocytic activity. The total number of ingested beads is obtained by summing (number of one-bead-ingested cells) x 1, (number of two-bead-ingested cells) x 2, (number of three-bead-ingested cells) x 3, (number of four-bead-ingested cells) x 4, and (number of more than four-bead-ingested cells) x (mean number of ingested beads in more than four-bead-ingested cells population).

In the present study, the dose of 0.5 ml/day/mouse of limonin of 5 and 10 ppm did not significantly increase the PEC number, because the PEC number in mice fed with this dose was not different from PEC number in PBS-treated or untreated mice with 95% confident level. Whereas 0.5 ml/day/mouse limonin administration to mice of 20 ppm or more (50, 100, and 200 ppm) significantly increased PEC number. The more limonin concentration administer to mice, the more increasing PEC number. In addition, the more days of limonin administration, the more increasing PEC number in every groups of limonin treated mice with confident level of 95%. But limonin of every concentration affected the phagocytic activity, both PP

and PI. And the more limonin concentration administer to mice, the more increasing PP and PI. This indicate that limonin stimulate the activity of ingesting foreign substances present in the tissues, so limonin of any concentration increase the PP and PI, although limonin of concentration only of 20 ppm or more affects the PEC number. It is assumed that enhancement in PEC number by administration of limonin due to the effect of limonin on the hematopoiesis or the stem cell proliferation and differentiation, which has to be more investigated. This response stimulates the cytokine profile which activates mainly macrophages. The overall effect of this cytokine is to draw macrophages into the area and activate them, promoting increased phagocytic activity and more effective killing (Mota, 1980).

In this study, the feeding dose for immunomodulating study therefore is 100 ppm and 200 ppm, likewise the study of Tanaka, *et al* (2000) that limonin acts as a chemopreventive agent with the doses of 200 ppm and 500 ppm, which are likely above the threshold level for the effect. Limonin has been reported to enhance glutathione S-transferase (GST) activity in various organs of mice (Lam, Li, and Hasegawa, 1989) with a dose dependent manner (Kelly, Jewell, and O'Brien, 2003), and limonin could inhibit carcinogen-DNA adduct formation. Recently limonin was found to increase quinidine reductase (QR) activity in the liver and colon of rats (Tanaka, *et al*, 2000).

Another experiment used to investigate immunity modulating activity of limonin in this study is the count of white blood cells, which determines the number of white blood cells and the percentage of each type of white blood cell in animals' blood. This test provides a clue to the presence of illness. White blood cells protect the body by fighting infection and attacking foreign material. When extra white blood cells are needed, the bone marrow is increased.

The study shows that limonin significantly affected the total WBC count only at a concentration of 100 ppm or more with 95% confidence level, which corresponds to the PEC number. It is recognized that there is a group of cells which play a major role in the defense of the organism against a great

variety of extraneous invaders, named the white cells of the blood (now known as polymorphonuclear cells), and derived from the stem cell in the bone marrow under the influence of colony stimulating factor (CSF).

The polymorphonuclear (PMN) leukocytes are provide natural immunity which is in the system without the stimulation of micro-organisms, and generally are considered to be the first line to fight against bacterial invasion. This type of immunity varies according to genetics, nutrition, hormones, age and temperature. If the bacterial invasion is sufficiently strong so that the PMN are unable to cope with the swarms of invaders, the second line, the macrophages, takes over, giving rise to the condition of chronic inflammation. The third line of defense, the adaptive immunity, becomes established only after lymphocytes have been stimulated and activated by activated and antigen-presenting macrophage. So macrophages are important cells for the immune system.

The increasing number of neutrophil in a differential count after limonin administration shows that there is some tissue-inflammation. The proposed reason is limonin may cause toxicity to the organ of the animal. But the percentage of neutrophil in blood has decreased showing that toxicity due to limonin has less effect. So it is proved that limonin in 100 ppm and 200 ppm doses is safe in animals, because limonin is a naturally edible ompound in several *Citrus* fruits. The increasing number and percentage of lymphocytes and monocytes in the blood can explain that limonin enhances the proliferation of lymphocytes and monocytes, and it is co-related with the production of specific antibody titer and the phagocytic activity.

The last experiment used to investigate immunomodulating activity on limonin in this study is the measurement of the specific antibody titer, which studied the effect of antiserum produced by the animal treated with limonin against specific antigen; SRBC contains antigenic substances which is cell specific.

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 re-encountering the antigens has an enhanced response. Once a host has been exposed to a specific disease, the host develops a specific immunity and does probably not catch the disease again. Vertebrates possess two types of acquired immunity based on the components the immune system uses to mediate immunity; humoral immunity and cell-mediated immunity. Humoral immunity is mediated by antigen specific blood proteins called antibodies. Antibodies are secreted only by plasma cells and normally interact with circulating antigens but are unable to penetrate living cells. The humoral immune response in the present study was analyzed by the specific antibody production. The circulating antibody titer with hemagglutination assay was significantly enhanced in the limonin treated mice, showing its modulatory effect on the humoral immune system.

The present data show that the effect of limonin on the production of specific antibody is increasing and dose-dependent, the reason for this is unknown, but it can be assumed that the increased macrophage plays a key role in the cytokine production and antigenic presentation and recognition. Thus the mode of the mechanism of the activity has to be examined in the future.

The effect of limonin on immunological function was not fully understood. But one possible mechanism might be through the control of cell proliferation both PEC macrophage and lymphocyte. Increased cell proliferation is suggested to play an important role in immune system generation both innate and humoral immunity. However it was observed that there was no significant different on PP in the day immediately after limonin administration, but there is a significant different on the last day following limonin administration (Appendix 1, Table 11A). The reason for this was unknown, but it is presumed due to the induction phase, which was the interval during the stimulation of synthesizing activity, and due to the time for systemic absorption of limonin. So it would be interesting to investigate the long term study on dose relationship and bio-availability study.

6. The Effect of Processing Techniques on Limonin in Lime Juices

As discussed above that limonin has been known as a *Citrus* constituent. Limonin is always thought of presenting in *Citrus* juices, such as lime juice or orange juice, which are a favorite food for human beings. Thus the experiment is designed to use lime juice as representative of limonin-containing food. Now that lime juice could be obtained by various processes, which affect the limonin.

Conditions of juice expression influence the limonin content of the juice. This stems from the fact that limonoic acid A-ring lactone is located in the fruit tissues. Lower limonin contents in the juice of oranges were reported when extracted using low rather than high pressure (Maier, Bennett, and Hasegawa, 1977). The high pressure liquid extract had considerably higher glycosides and moderately higher amino acid. This experiment illustrates that the readily accessible liquid of the vacuoles released at lower pressures is quite different from the more inaccessible liquid of the cytoplasm.

It is recognized that the pressure of hand expression is lower than machine expression, so the method of expression influences the limonin content of lime juice. Moreover, the stability study of limonin shows that temperature affects the limonin content, so heating and freezing influents the limonin content of lime juice as well.

The present study of the immunomodulating effects of limonin showed that the content of limonin had a different modulating activity on PEC number, total WBC count, and the production of specific antibody titer, with the concentration of 100 to 200 ppm. Although the present study indicated that limonin content in lime juice was around 10-17 ppm, which was much lower than the tested effective immunomodulating concentration, but lime juice extracted by various processing still affected the total WBC count and specific antibody titer of the lime-administrated mice. This indicates that not only limonin, but also other biological metabolites, in lime juice affect the immunopotency in mice.

Several *Citrus* juices and seeds contain 1-35 ppm of limonin (Maier, *et al*, 1977 and Ohta and hasegawa, 1995). Based on studies of seed limonoids, Bennett has shown the presence of the following limonoids in the *Citrus* juice: deacetylnomilin, nomilin, obacunone, deacetylnomilinic acid and nomilinic acid (Hsu, *et al*, 1973). All had been identified as constituents of *Citrus* seeds, which contain two forms of limonin, namely limonin and limonoate A-ring lactone, a non-bitter hydroxyl acid form of limonin which converts to limonin after juice extraction. Furthermore, flavonoids are widely present in lime juice, and have been shown to be powerful antioxidants and free radical scavengers (Benavente-Garcia, *et al*, 1997). It is indicated that flavonoids affected several cells in immunity and inflammation, such as lymphocytes, macrophages, and neutrophil (Middleton and Kandaswami, 1994). Whereas lime has historically been recognized as an excellent source of vitamin C, and a significant source for folic acid, and β-carotene, which have been discussed as important health-promoting and disease-preventive properties (Rouseff and Nagy, 1994).

Table 16 Phytochemicals in lime juice with antioxidative activity

Phytochemicals	Example compounds	Reference
Carotenoids	Carotene	Yokoyama and Vandercook,1967
Flavonoids	naringin, hesperidin, luteolin, nobiletin, tangeritin	Horowitz,1957
Organic acids	citric, malic, oxalic, succinic, quinic, ascorbic, folic	Vandercook *et al*, 1963
Terpenoids	limonin, nomilin, ichangin, obacunone	Dreyer, 1965
Coumarin	Coumarin	Shaw and Coleman, 1971

Thus the results in the current study clearly indicate that administration of lime juice effectively enhances both total WBC count and specific antibody titer in mice. However, the immunomodulating activities of

lime juice might not be totally affected by limonin, because the limonin content is so low that there is no significant difference in the total WBC count and specific antibody titer. So a detailed analysis of the activity of limonin in lime juice and its future development has to be further studied.