การทดสอบความเป็นพิษของฟลาโวนอยด์บางชนิดและยาปฏิชีวนะ
ในกลุ่มเบตาแลคแทม ในสัตว์ทดลอง

นายอภัย ดวงคำ

IN VIVO TOXICITY TEST OF SOME FLAVONOIDS

AND β-LACTAM ANTIBIOTICS

Aphai Duangkham

A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Master of Science in Environmental Biology

Suranaree University of Technology

Academic Year 2011

IN VIVO TOXICITY TEST OF SOME FLAVONOIDS AND

β-LACTAM ANTIBIOTICS

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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อภัย ดวงคำ : การทดสอบความเป็นพิษของฟลาโวนอยด์บางชนิดและยาปฏิชีวนะ ในกลุ่มเบตาแลคแทม ในสัตว์ทดลอง (*IN VIVO* TOXICITY TEST OF SOME FLAVONOIDS AND β-LACTAM ANTIBIOTICS) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.เกรียงศักดิ์ เอื้อมเก็บ, 107 หน้า.

ฟลาโวนอยด์เป็นสารประกอบในกลุ่มโพลีฟีนอลที่เกิดขึ้นเองตามธรรมชาติซึ่งพบ ได้ในผลไม้และผัก ฟลาโวนอยด์เป็นส่วนประกอบสำคัญของอาหารที่เรารับประทานเป็นประจำ และมีฤทธิ์ทางเภสัชวิทยาที่หลากหลายโดยเฉพาะอย่างยิ่งฟลาโวนอยด์สามารถเสริมฤทธิ์กับยา ปฏิชีวนะในกลุ่มเบตาแลคแทม ต้านเชื้อแบคทีเรียดื้อยาได้ อย่างไรก็ตาม ยังไม่มีรายงานการศึกษา ความเป็นพิษของฟลาโวนอยด์ในสัตว์ทดลอง ดังนั้นวัตถุประสงค์ของการวิจัยครั้งนี้ เพื่อศึกษาพิษ กึ่งเฉียบพลันและพิษกึ่งเรื้อรังของฟลาโวนอยด์ 5 ตัวคือ เคอร์เซติน ไบคาลิน เอพิจีนิน ลูทีโอลิน และกาแลนจิน เมื่อใช้เดี่ยวและผสมกับยาคลอกซาซิลินหรือเซฟทาซิดีนในหนูถีบจักร วิธีการศึกษา พิษกึ่งเฉียบพลันโดยฉีดสารต่อไปนี้เข้าช่องท้องเป็นเวลา 14 วัน ได้แก่ เคอร์เซติน ไบคาลิน เอพิจี นิน ลูทีโอลิน และกาแลนจินเดี่ยวๆ ขนาด (ปกติและสูง) 20 และ 80, 10 และ 40, 20 และ 80, 1 และ 4, 10 และ 40 มิลลิกรัมต่อกิโลกรัม (น้ำหนักตัว) ต่อวัน ตามลำดับ และฉีดเคอร์เซตินหรือไบคาลิน ผสมกับคลอกซาซิลิน ขนาด (ปกติ, สูง) 20+150, 80+600 และ 10+150, 40+600 มิลลิกรัมต่อ กิโลกรัม (น้ำหนักตัว) ต่อวัน ตามลำดับ ส่วนเอพิจีนิน ลูทีโอลิน และกาแลนจินผสมกับเซฟทาซิดีน ขนาด (ปกติ, สูง) 20+160, 80+640; 1+160, 4+640 และ 10+160, 40+640 มิลลิกรัมต่อกิโลกรัม (น้ำหนักตัว) ต่อวัน ตามลำดับ วิธีการศึกษาพิษกึ่งเรื้อรังโดยฉีดสารต่อไปนี้เข้าช่องท้องเป็นเวลา 90 วัน ได้แก่ เคอร์เซติน ไบคาลิน เอพิจีนิน และลูทีโอลินเดี่ยวๆ ขนาด (ปกติและสูง) 20 และ 40, 10 และ 20, 20 และ 40, 1 และ 2 มิลลิกรัมต่อกิโลกรัม (น้ำหนักตัว) ต่อวัน ตามลำดับ และฉีดเคอร์เซ ตินหรือไบคาลิน ผสมกับคลอกซาซิลิน ขนาด (ปกติ, สูง) 20+150, 40+300 และ 10+150, 20+300 มิลลิกรัมต่อกิโลกรัม (น้ำหนักตัว) ต่อวัน ตามลำดับ ส่วนเอพิจีนิน หรือลูทีโอลินผสมกับเซฟทา ซิดีน ขนาด (ปกติ, สูง) 20+160, 40+320 และ 1+160, 2+320 มิลลิกรัมต่อกิโลกรัม (น้ำหนักตัว) ต่อ วัน ตามลำดับ ภายหลังสิ้นสุดการทดลองได้นำอวัยวะที่สำคัญและเลือดมาวิเคราะห์ผลทางชีวเคมี และโลหิตวิทยาทั้งพิษกึ่งเฉียบพลันและพิษกึ่งเรื้อรัง พบว่า น้ำหนักสัมพัทธ์และน้ำหนักของอวัยวะ สำคัญ (ต่อน้ำหนักตัว) ได้แก่ หัวใจ ตับ ม้าม ปอด ไต และกระเพาะอาหาร และผลการตรวจสอบ ชีววิทยาของเนื้อเยื่อของหนูทดลองไม่มีการเปลี่ยนแปลงอย่างมีนัยสำคัญทางสถิติ เมื่อเปรียบเทียบ กับกลุ่มควบคุม ส่วนผลทางชีวเคมีและโลหิตวิทยาของการทดสอบพิษกึ่งฉับพลันและกึ่งเรื้อรัง พบว่า เคอร์เซติน เอพิจีนิน และลูทีโอลีน ไม่มีพิษต่อพารามิเตอร์เหล่านี้ ยกเว้น กาแลนจินทำให้ค่า

เอชซีทีลดลง และไบคาลินทำให้ค่าเอ็มซีวีลดลง เมื่อให้สารเหล่านี้ในขนาดปกติ เป็นเวลา 14 วัน นอกจากนี้ เอพิจีนินขนาดสูงและให้เป็นเวลานานจะทำให้ค่าเม็ดเลือดขาวและเอ็มซีวีลดลง สิ่งที่ น่าสนใจอย่างยิ่งคือ สารฟลาโวนอยด์เหล่านี้สามารถลดคอเลสเตอรอลในเลือดของหนูทดลอง เมื่อ ให้ในปริมาณสูงและเป็นเวลานาน ค่าคอเลสเตอรอลที่ลดลงในหนูทดลองที่ได้รับไบคาลินนั้น สอดคล้องกับผลจากเอฟทีไออาร์ ที่องค์ประกอบของโครงสร้างทุติยภูมิของเอไมด์วันลดลง ผลที่ ได้จากการศึกษาครั้งนี้ จะเป็นข้อมูลที่เป็นประโยชน์ในการทดสอบความเป็นพิษในสัตว์เลี้ยงลูก ด้วยนมชั้นสูงรวมทั้งในคน
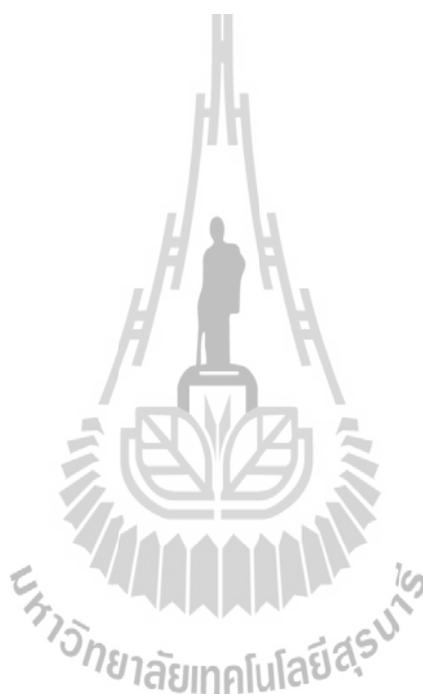
สาขาวิชาชีววิทยา   ลายมือชื่อนักศึกษา_____

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                                         ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

APHAI DUANGKHAM : *IN VIVO* TOXICITY TEST OF SOME

FLAVONOIDS AND β-LACTAM ANTIBIOTICS. THESIS ADVISOR :

ASST. PROF. GRIANGSAK EUMKEB, Ph.D. 107 PP.

FLAVONOIDS/β-LACTAM ANTIBIOTICS/SUBACUTE TOXICITY/

SUBCHRONIC TOXICITY

Flavonoids are part of a family of naturally occurring polyphenolic compounds and represent one of the most prevalent classes of compounds in vegetables and fruits. Flavonoids are important constituents of normal human diet and also have various pharmacological properties. Especially, flavonoids have synergistic activity with β-lactam antibiotic against drugs resistant bacteria. However, *in vivo* toxicity test of these flavonoids have not been studied. Thus, the purpose of this study was to investigate the subacute and subchronic toxicity of five flavonoids (quercetin, baicalein, apigenin, luteolin, and galangin) alone and in combination with cloxacillin or ceftazidime antibiotics in mice. In subacute toxicity test, the mice were administered intraperitoneally (i.p.) with the following flavonoids and/or drugs: (normal and high dose) 20 and 80 mg/kg BW/day of both quercetin and apigenin, 10 and 40 mg/kg BW/day of baicalein, 1 and 4 mg/kg BW/day of luteolin, and 10 and 20 mg/kg BW/day of galangin when used singularly. In addition, quercetin plus cloxacillin at 20+150, 80+600; baicalien plus cloxacillin at 10+150, 40+600 mg/kg BW/day, and apigenin; luteolin; or galangin in combination with ceftazidime at 20+160, 80+640; 1+160, 4+640; or 10+160, 20+640 mg/kg BW/day, respectively. In the subchronic toxicity test, the mice were injected (i.p.) with the following flavonoids and/or drugs: 20 and 40 mg/kg BW/day of quercetin or apigenin, 10 and

20 mg/kg BW/day of baicalein, and 1 and 2 mg/kg BW/day of luteolin alone. Moreover, the combinations of quercetin or baicalien plus cloxacillin at 20+150, 40+300; or 10+150, 20+300 mg/kg BW/day, respectively. Furthermore, apigenin or luteolin in combination with ceftazidime at 20+160, 40+320; or 1+160, 2+320, respectively. At the end of the experiments, blood and the selected main organs were collected for haematological and histological analyses. The results showed that there were no significant difference in either the relative growth rate of total body weight or weight of the selected main body organs of mice such as heart, liver, spleen, lung, kidney, stomach, and their histology treated with all doses of five flavonoids using singly and in combinations when compared to the control. The blood chemistry and haematological analysis of subacute toxicity and subchronic toxicity showed that quercetin, apigenin and luteolin exhibited no toxicity in these parameters except for galangin caused Hct decrease and baicalein resulted in MCV reduction when these flavonoids were administered at therapeutic dose for 14 days. Moreover, apigenin at high dose and long duration led to WBC and MCV decrease. Interestingly, These flavonoids provide evidence that its can reduce cholesterol in mice blood when administered at high dose and long duration. The lowering of cholesterol in baicalein treated mice are substantial agreement with the result from FTIR that the amide I secondary structure component was decreased. In summary, this study could be useful information for further investigation of toxicity in higher mammal animals, including human beings.

School of Biology   Student's Signature_____

Academic Year 2011   Advisor's Signature_____

                                  Co-Advisor's Signature_____

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

AST	=	aspartate aminotransferase
α	=	alpha
β	=	beta
BUN	=	blood urea nitrogen
CREnC	=	Ceftazidime Resistant *Enterobacter cloacae*
CO_2	=	carbon dioxide
DMSO	=	dimethyl sulfoxide
EDTA	=	ethylenediaminetetraacetic acid
FBS	=	fasting blood sugar
gm	=	gram
h	=	hour
Hb	=	hemoglobin
Hct	=	hematocrit
Hrs	=	hours
i.p.	=	intraperitoneal
kg	=	kilogram
L	=	liter
LPS	=	lipopolysacharide
MCV	=	mean corpuscular volume

LIST OF ABBREVIATIONS (Continued)

mg	=	milligram
mM	=	millimolar
RBC	=	red blood cell
µg/mL	=	microgram per milliliter
µL	=	microliter
µM	=	micromolar
WBC	=	white blood cell
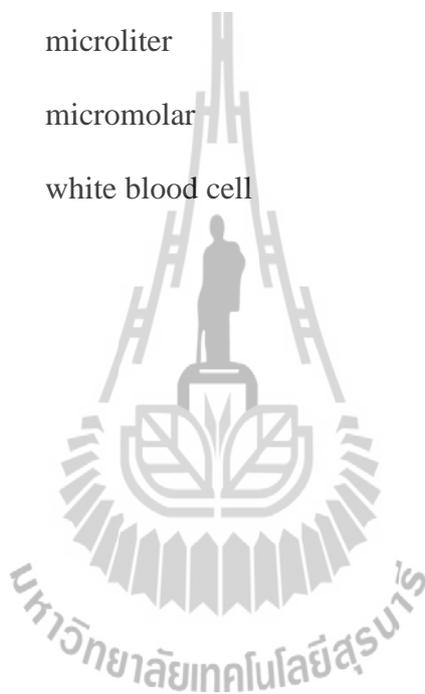
CHAPTER I

INTRODUCTION

1.1 Introduction

Bacterial resistance to antibiotics is a serious global problem. Strains of β-Lactam-resistant *Staphylococus aureus* including methicillin-resistant *S. aureus* (MRSA) and strains of ceftazidime-resistant *Enterobacter cloacae* (CREnC) now pose serious problem to hospitalized patients, and their care providers (Mulligan *et al*., 1993). New approaches to resolve this problem are needed. Active compounds from medicinal plants have long been isolated to use for multipurpose, including antibiotic purpose.

Flavonoids are polyphenolic compounds found as integral components in the human diet. They are generally found in fruit, vegetables, grains, bark, stems, roots, wine, and tea. The beneficial effects of these natural compounds have been long studied in a variety of methodologies. More than 4,000 varieties of flavonoids have been identified, many of which are responsible for attractive colors of flowers, leaves, and fruits. It is widely accepted that the high intake of foods and beverages rich in polyphenols, especially in flavonoids, has been associated with decreased risk of coronary heart disease and also inhibited the proliferation of various tumor growth. Furthermore, epidemiologic studies suggested a protective role of dietary flavonoids against cancer induction in several human tissues, including colon, lung, prostate, and urinary bladder (Lakhanpal and Rai, 2007).

Flavonoids can be divided into various classes on the basis of their molecular structure. Some major classes of flavonoids and their chemical structures are shown in Table 1.1 and Figure 1.1, respectively.

Table 1.1 Some major classes of flavonoids, examples of individual flavonoids and their food sources.

	Structure	Example	Major food sources
Basic structure of flavonoids			
Chalcone	Chalcone	Hop chalcones (xanthohumol and dehydrocyclo-xanthohumol hydrate)	Hops, beer
Flavone	Apigenin	Acacetin Apigenin Baicalein Chrysin Diosmetin Luteolin Tangeretin	Parsley, thyme, celery, sweet red peppers, honey, propolis
Flavonol	Kaempferol	Galangin Kaempferol Morin Myricetin Quercetin	Onions, kale, broccoli, apples, cherries, berries, tea, red wine
Flavanone	Naringenin	Eriodictyol Hesperetin Homoeriodictyol Naringenin	Citrus
Flavanol	Epicatechin	Catechin Epicatechin Proanthocyanidins	Cocoa, green tea, chocolate, red wine, hawthorn, bilberry, motherwort, and other herbs
Anthocyanin	Cyanidin	Cyanidins Pigmented compounds	Cherries, grapes, berries, red cabbage
Isoflavone	Genistein	Biochanin A Genistein Diadzein Equol Formononetin	Red clover, alfalfa, peas, soy and other legumes
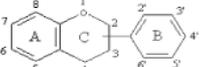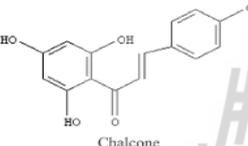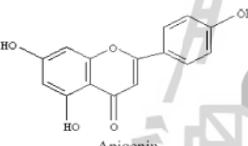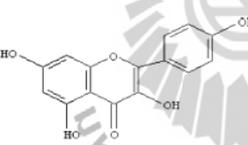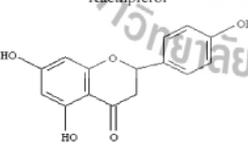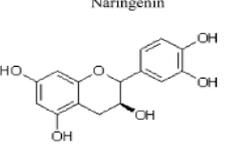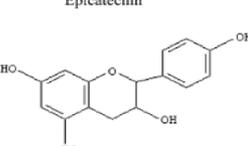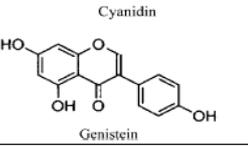
Source: Moon *et al.* (2006).
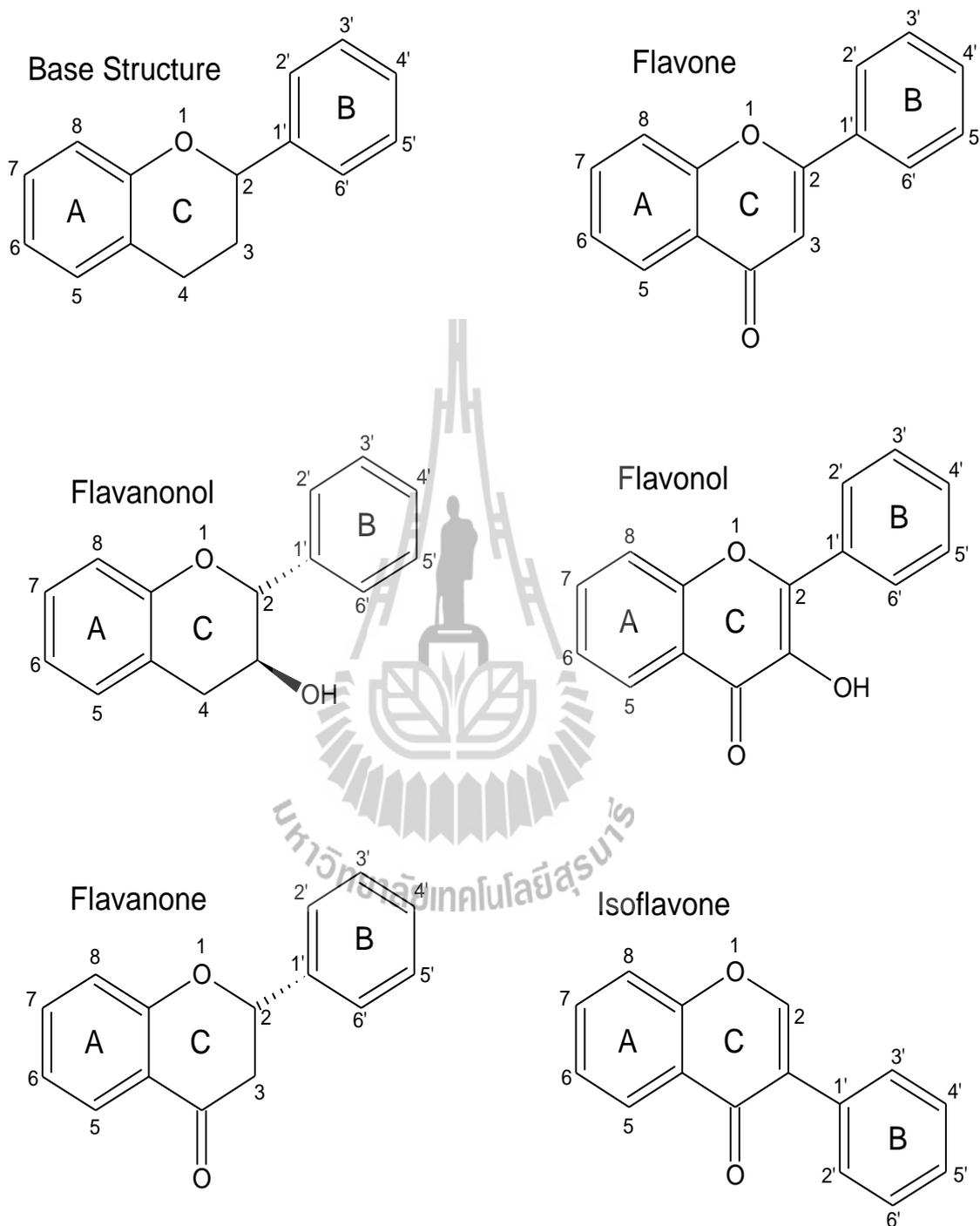
Figure 1.1 Structures of the major classes of flavonoids.

Source: Lakhanpal and Rai (2007).

The combination between antibiotics and active compounds from medicinal plants are the new approach against the resistant bacteria. Recent studies have been reported the synergistic antibacterial activity of flavonoids and antibiotics (Cushine and Lamb, 2006). There were growing evidence that flavonoids in combination with antibiotics interfere with various bacterial virulence factors, incuding enzymes, toxins and signal receptors (Cushine and Lamb, 2005; Eumkeb *et al*., 2008). The synergistic activity of galangin and ceftazidime againt ceftazidime resistant *S. aureus* may involve three mechanisms of action by galangin. The first is on the integrity of the cell wall and on septum formation prior to cell division. This implies an effect on protein synthesis including an effect on penicillin-binding protein 2a (PBP 2a). The second mechanism of galangin activity is via inhibition of the activity of certain penicillinase enzyme by interaction with this enzyme. The third is galangin causes cytoplasmic membrane damage results in potassium loss (Eumkeb *et al*., 2010). However, *in vivo* toxicity test of some potential flavonoids have not been studied. Thus, the purpose of this thesis was to investigate the toxicity of flavonoids alone and in combination with antibiotics *in vivo*. This study may help us to clearify *in vivo* toxicity of these flavonoids when used alone or in combination with antibiotics certain.

1.2 Research objectives

1.2.1 To investigate the toxicity of flavonoids in mice when used alone and in combination with β-lactams antibiotics.

1.2.2 To investigate the serum biological changes of flavonoids alone and flavonoids plus β-lactam antibiotics treated mice by using infared microspectroscopy with multivariate data analysis.

1.3   Research hypothesis

These flavonoids when administrated singly and in combination with β-lactam antibiotics in mice do not show any toxicity.

1.4   Scope and limitation of the study

1.4.1 IRC male and female mice matched for age and weight were purchased from National laboratory Animal Center, Salaya Campus, Mahidol University, Thailand.

1.4.2 All tested flavonoids (apigenin, baicalein, luteolin and quercetin) were obtained from Indofine Chemical Company, USA.

1.4.3 Ceftazidime and cloxacillin were obtained from Sigma-Aldrich, USA.

1.5   Expected results

1.5.1 Data of subacute and subchronic toxic effects of some flavanoids on mice when used alone and in combination with β-lactams antibiotics would be clarified.

1.5.2 These toxicities information would be useful for further investigation in other higher mammal animals.

CHAPTER II

LITERATURE REVIEW

2.1 Overview of toxicity and definition of key terms

2.1.1 Definition of toxicity

Toxicity is defined as "the potential of a substance to exert a harmful effect on humans or animals, and a description of the effect and the conditions or concentration under which the effect takes place" (Health and Safety, 2004). In order to support an application for a clinical trial or for the registration of a new drug, it is necessary to satisfy legislation that requires certain data produced from a variety of toxicological investigations that show the safety profile of the compound to which humans may be exposed. Therefore, in the majority of cases of evaluation of the toxicity of most substances, rodents and non-human primates are first used in preclinical animal safety studies before further studies are done in humans. These animals are mainly used because of their biological similarity to humans that allows them to be regarded as the suitable metabolic models for humans in a broad range of investigations (Loomis and Hayes, 1996; Pascoe, 1983).

In general, toxicity testing methods can be divided into two categories: The first category is designed to evaluate the overall effects of compounds on experimental animals. Individual test in this category differs from each other basically in regard to the duration of the test and the extent to which the animals are evaluated

for general toxicity. These tests are classified as acute, prolonged and chronic toxicity tests (Loomis and Hayes, 1996). The second category consists of those that are designed to evaluate specific types of toxicity in detail. The prolonged and chronic tests do not detect all forms of toxicity, but they may reveal some of the specific toxicities and indicate the need for more detailed studies. Thus, this second category of tests has been developed for determination effects of compounds on the fetus in a pregnant animal (teratogenic tests), on the reproductive capacity of the animals (reproduction tests), on the genetic system (mutagenic tests), for the determination of the ability of agents to produce tumors (tumorigenicity and carcinogenicity tests) (Timbrell, 2002).

Acute and chronic toxic effects differs principally from each other with respect to the amount of chemical compound involved and the time intervening before the effect is seen (Timbrell, 2002). Acute effects are normally observed soon after exposure and result from the uptake of large amounts of poison, generally as a single dose. On the other hand, chronic effects are often detected over an extended period of time during which exposure may be continuous or intermittent, though obviously at levels which are too low to produce an acute effect (Loomis and Hayes, 1996; Pascoe, 1983).

2.1.1.1 Acute toxicity tests

Acute toxicity is those designed to determine the effects which occur within a short period after dosing. These tests can determine a dose-response relationship and the LD_{50} value if required (Timbrell, 2002).

2.1.1.2 Chronic toxicity tests

These tests involve exposure animals with interested compounds for at least 12 months to 2 years in rodents (about 50 percent lifespan) and 6-12 months in non-rodents. Chronic toxicity tests may be combined with *in vivo* carcinogenicity tests, in which case the exposure of rodents will be lifetime, and satellite groups may be used for interim chronic toxicity information. There is currently discussion in the International Committee on Harmonization as whether chronic toxicity tests need to be as long as 2 years or whether shorter time will yield as much information (Timbrell, 2002).

The purpose of the chronic toxicity test is to investigate the harmful effects that foreign compounds that are introduced to animals in repeated doses or in continuous exposure over an extended period of time may produce (Poole and Leslie, 1989). As with sub-chronic toxicity tests, the chronic toxicity tests will terminate with a pathological examination and there many also be clinical chemical measurements made throughout at intervals. These clinical chemical measurements can indicate the development of pathological changes which can then be detected at post-mortem (Timbrell, 2002). Different approaches to dose ranging studies are applied depending on the species being used. The procedures used can vary, but usually involve exposing the experimental animals (in typical group sizes of two to five animals/sex/group) to various doses of the test compound, i.e. from the maximal non-lethal dose (determined in the acute studies) down to doses in the pharmacological dose range. Clinical chemistry and hematological parameters are then measured at the start of the study (i.e. within 48 hours after the first dose) and at the end of the study, along with full histopathology analysis of all abnormal tissues

plus the major organs (such as the heart, liver, kidneys, lungs and brain of the animals), at least at the end of the study (Poole and Leslie, 1989; Timbrell, 2002).

2.1.2 Toxic effects

Toxic effects are defined as "harmful responses of a biological system to a toxic compound, and death of cells or the whole organism are the major response (Timbrell, 2002).

In all the cases, the toxic effects are usually manifested either in an acute or a chronic manner, and occur mostly as a result of an acute or chronic exposure to toxic compound by oral ingestion, inhalation or absorption following skin contact (Hui *et al*., 2008). The toxic effects are seen as (1) signs or reflection of a disturbance of the normal activities of enzymes that perform essential biochemical roles in all forms of life; (2) alteration of the normal activities of plasma membrane that regulate the exchange of nutrients and metabolites between the cell and its surroundings and (3) the disturbances of other normal cell activities, e.g. RNA and DNA synthesis, growth, division and general metabolism at all levels of organization from sub-cellular to organ and organ system (Timbrell, 2002).

2.2 Routes of administration

This term refers to the way in which drugs or compounds are introduced to animals or humans. To evaluate toxicity of a compound in animals various routes may be used, but two most commonly used modes of administration for animals studies are via intraperitoneal injection or the oral route (Poole and Leslie, 1989).

2.2.1 Intra-peritoneal injection

This is one of the methods of dosing, which may occasionally provide information about local as well as systemic toxicity. To give drugs by intraperitoneal dosing, the animal is laid on its back and the abdomen shaved. This area is thoroughly cleaned and, using an appropriate syringe and needle, the abdominal wall is punctured. To ensure minimal danger of perforation of abdominal viscera, the injection should be made rostral and lateral to the bladder at an angle of about 15° to the abdomen. The depth of penetration should not exceed 5mm (Poole and Leslie, 1989; Waynforth, 1980).

2.2.2 Oral administration

The oral route is probably one of the most common means by which a chemical enters the body. In short, the oral administration is the form of administration involving the gastrointestinal tract, which may be viewed as a tube going through the body, starting at the mouth and ending at the anus. Although it is within the body, its contents are essentially exterior to the body fluids. Most orally administered chemicals can otherwise have a systemic effect on the organism only after absorption has occurred from the mouth or the gastrointestinal tract. Oral administration of chemicals that are rapidly absorbed from the gastrointestinal tract would theoretically expose the liver to concentrations of the agent that would not be obtained if other routes of administration were used (Loomis and Hayes, 1996). Furthermore, if a compound entered the enterohepatic cycle, at least a portion of the compound would be localized in the organs involved in the cycle. Compounds that are known to be toxic to the liver would be expected to be more toxic following oral

administration on repeated occasions, whereas their administration by other routes may be less hazardous (Loomis and Hayes, 1996; Waynforth, 1980).

2.3 Current use and importance of flavonoids

Flavonoids are a structurally diverse class of polyphenolic compounds ubiquitously found among plants and produced as a result of plant secondary metabolism (Tsuji and Walle, 2008). The term, phenolic compound, embraces a wide range of chemical constituents possessing an aromatic ring bearing one or more hydroxyl substituents in common. It is estimated that about 2% of all carbon photosynthesized by plants is converted into flavonoids or closely related compounds. They are virtually ubiquitous in green plants in which they are often responsible for the coloration of flowers, fruits and leaves. Their occurrence are however not restricted to the latter organs but include all parts of the plant, roots, wood, bark and nectar. As such, flavonoids constitute one of the most characteristic classes of compounds that are likely to be encountered in any work involving plant extracts (Markham, 1982; Harborne, 1973). In particular, flavonoids are becoming the subject of medical research. They have been reported to possess many useful properties, including anti-inflammatory activity, oestrogenic activity, enzyme inhibition, antimicrobial activity, antiallergic activity, antioxidant activity, vascular activity and cytotoxic antitumour activity. For a group of compounds of relatively homogeneous structure, the flavonoids inhibit a perplexing number and variety of eukaryotic enzymes and have a tremendously wide range of activities. In the case of enzyme inhibition, this has been postulated to be due to the interaction of enzymes with different parts of the flavonoid molecule, e.g. carbohydrate, phenyl ring, phenol and

benzopyrone ring (Cushinie and Lamb, 2005; 2006). Previous finding showed that galangin, quercetin and baicalein revealed the potential to reverse bacterial resistance to β-lactam antibiotics against penicillin-resistant *S. aureus* (PRSA). These could involve three mechanisms of action that galangin inhibit protein synthesis and effect on PBP 2a, interact with penicillinase and cause cytoplasmic membrane damage (Eumkeb *et al*., 2010).

2.4   Chemistry and classification

The term of flavonoids is a collective noun for plant pigments, mostly derived from benzo-γ-pyrone. Flavonoids are part of a family of naturally occurring polyphenolic compounds and represent one of the most prevalent classes of compounds in vegetables, nuts, fruits and beverages such as coffee, tea, and red wine as well as medical herbs. More than 8,000 compounds with a flavonoid structure have been identified. The classes of flavonoids include chalcones, flavones, flavonols, flavanols, anthocyanins and isoflavones (Cushinie and Lamb, 2005).

2.5   The pharmacology of flavonoids in animals

2.5.1 Biological properties of apigenin
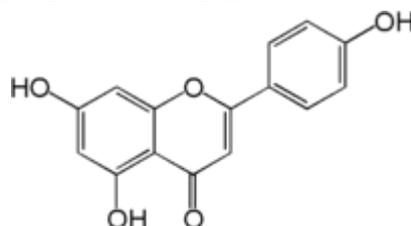
Molecular formula : $C_{15}H_{10}O_5$

Solubility : Soluble in hot alcohol and dimethylsulfoxide (DMSO), insoluble in water

Melting point : 345-350 ^{o}C

Appearance : Yellow needle

(Susan, 1996)

Apigenin is a flavonoid found in artichoke, basil, celery and other plants. It is widely used as inhibiting the prostate cancer and skin tumor promotion. Kuo, Lee and Lin (1992) reported that apigenin can inhibit the bacterial mutagenesis induced by nitropyrene and also protected against the cytotoxicity induced by 1-nitropyrenene and 1, 6-dinitropyrene and gave a significant reduction of the frequency of sister chromatid exchange. In addition, Oksus *et al.* (1984) investigated that the MIC for apiginin against *Proteus vulgaris, Pseudomonas aeruginosa, Escherichia coli* and *Klebsiella pnemoniae* ranged from 54 to 219 µg/mL and that apiginin also proved to be active against the spore-former *Bacillus subtilis*.

2.5.2 Biological properties of baicalein
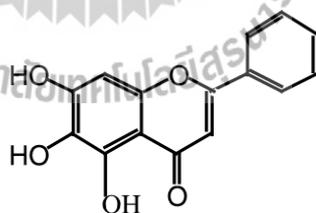
Molecular formular : $C_{15}H_{10}O_5$

Solubility : Soluble in alcohol, methanol, ether, acetone, ethyl
                 acetate, hot glacial acetic acid and DMSO

Melting Point : 264-265 ^{o}C

Appearance : Yellow needle and light yellow powder

(Susan, 1996)

The dried roots of *Scutellaria baicalensis* have been used as Chinese traditional plant for antibacterial and antivirus agents. Wang *et al*. (2004) indicated that baicalein played a protective role in β-amyloid peptide-(25-35)-induced amnesia. Furthermore, baicalein exhibited strong inhibition of transport of tetracycline with membrane vesicles prepared from *Escherichia coli* KAM32/pTZ1252 (Fujita *et al*., 2005). In addition, baicalein has been effective in anti-flammatory, anti-tumor and also induction of apoptosis in several human prostate cancer cell lines (Chan *et al*., 2000). Baicalin has the synergistic effect with benzylpenicillin which showed the killlng of MRSA and β-lactam-resistant *S. aureus* (Liu *et al*., 2000).

2.5.3 Biological properties of galangin
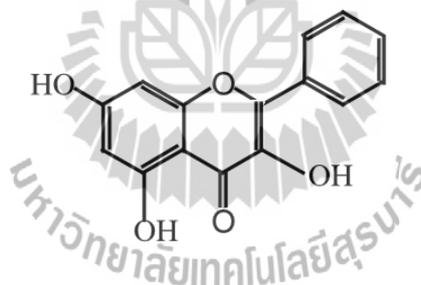
Molecular formular : $C_{15}H_{10}O_5$

Solubility : Soluble in chloroform, benzene, ethanol, ether, and

DMSO

Melting point : 214-215 ^{o}C

Appearance : Yellowish needle (Susan, 1996)

It is generally accepted that galangin is a flavonol that does not have any hydroxyl group in the B ring and has been suggested to be a substrate of cytochrome P450 which, through the hydroxyl of the B ring, could metabolise it to more

genotoxic products. Galangin have been reported to have anti-gram-positive bacteria with the minimum inhibitory concentration (MIC) ranging from 0.1 to 0.5 µg/ml, and also against *Enterobacter cloacae* which was significantly inhibited at a MIC of 0.1 mg/ml. Eumkeb and Richards (2003) reported that amoxicillin plus galangin 12.5 µg/ml can inhibit twelve clinical isolates of resistance *S. aureus* and four isolates of methicillin-resistance *S. aureus.* The combination of galangin at 5 µg/ml plus ceftazidime at 5 µg/ml showed synergistic effect by reducing the CFµ/ml of penicillin-resistant *S. aureus* (PRSA) to 1 x 10^3 over 6 and throughout 24 h. Electronmicroscopy clearly revealed that the combination of galangin and ceftazidime caused damage to the ultrastructures of the cells of this bacterial strain (Eumkeb *et al*., 2010). Galangin has the antibacterial activity against 4-quinolone resistant *S. aureus* at MICs of 50 µg/ml (Cushine and Lamb, 2006).

2.5.4 Biological properties of luteolin
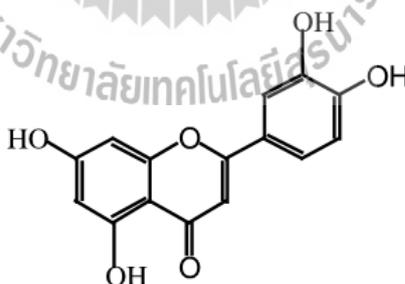
Molecular formular : $C_{15}H_{10}O_6$

Melting point : 328-330 ^{o}C

Solubility : Soluble in ethyl acetate, formic acid, water, alkaline

and DMSO

Appearance : Yellow needle (Susan, 1996)

Luteolin is a flavone that is found in foods including, parsley, artichoke leaves, celery, citrus fruits, sage, thyme and many others. There are numerous reports on biological activities of luteolin, for example, antioxidant, anti-inflammatory, anti-allergic, anticancer activities and immune-modulating properties to suppress hyperactive immune systems (Shimoi *et al*., 1998; Mino *et al*., 2004). In addition, luteolin is an antimutagenic and antitumorigenic agent, an inhibitor of protein kinase C and a potent hypoglycemic agent that improves insulin sensitivity (Shimoi *et al*., 1998). Furthermore, Chung *et al*. (2001) indicated that luteolin showed a significant inhibition in *Neisseria gonorrhoeae* and *Helicobactor pylori* N-acetytransferase activity and their growth.

2.5.5 Biological properties of quercetin
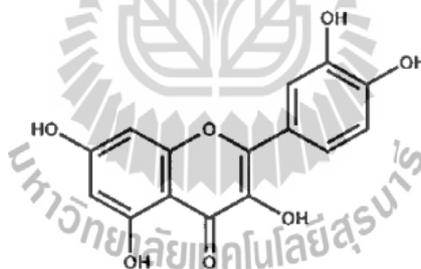
Molecular formular : $C_{15}H_{10}O$

Melting point : 314 ^{o}C

Solubility : Soluble in glacial acetic acid, aqueous

alkaline, alcohol, water and DMSO

Appearance : Yellow needle (Susan, 1996)

It is very well established that quercetin is chemically related to a class of flavonoids called anthocyanins. Pharmacological studies indicated that quercetin possesses antihistamine, anti-inflammatory, antiallergic, and antiviral properties. In

addition, Wang *et al.* (1989) reported that quercetin was active against *Bacillus cereus* when present in the amounts of 2.5 µg/ml. Furthermore, quercertin has shown to cause chromosomal mutations in certain bacteria in *in vitro* study. Kebieche *et al.* (2008) reported that quercetin at the dose of 0.33 mg/kg could protect liver cells and mitochondria from oxidative stress by maintaining normal levels of serum transaminases and preventing lipid peroxidation.

Eumkeb *et al.* (2008) reported that when combined ampicillin, cloxacillin and ceftazidime with baicalein 5 µg/ml, MICs of these drugs against clinical isolates of MRSA were reduced from 100, >1,000 and 50 µg/ml to 5, 5 and 5 µg/ml, respectively. Furthermore, clinical isolates of ceftazidime-resistant *Enterobacter cloacae* (CREnC) with MICs of ceftazidime >1,000 µg/ml had their resistance to these drugs reversed by apigenin 5 µg/ml or luteolin 5 µg/ml to MICs of ceftazidime 5 µg/ml. Viable counts showed that the killing of MRSA cells by 10 µg/ml ampicillin or cloxacillin was potentiated by 10 µg/ml baicalein. Ceftazidime 10 µg/ml in combination with 10 µg/ml of baicalein or galangin or quecertin also reduced the CFU/ml of MRSA to low levels (1×10^3 CUF/ml) over 6 h.

2.6 Antibiotics

2.6.1 Beta-lactam

The β-lactam antibiotics are useful and frequently prescribed antimicrobial agents sharing a common structure and mechanism of action-inhibition of synthesis of the bacterial peptidoglycan cell wall (Laurence *et al*., 2006).

2.6.2 Beta-lactam compounds

2.6.2.1 Penicillins

They are the most widely effective antibiotics and are among the least toxic drugs known; the major adverse reaction to penicillins is hypersensitivity. The members of this family differ from one another in the R substituent attached to the 6-aminopenicillanic acid residue. The nature of this side chain affects their antimicrobial spectrum, stability to stmach acid, and susceptibility to bacterial degradative enzymes (β-lactamases). Figure 2.1 shows the classification of agents affecting cell wall synthesis (Harvey *et al*., 2009).
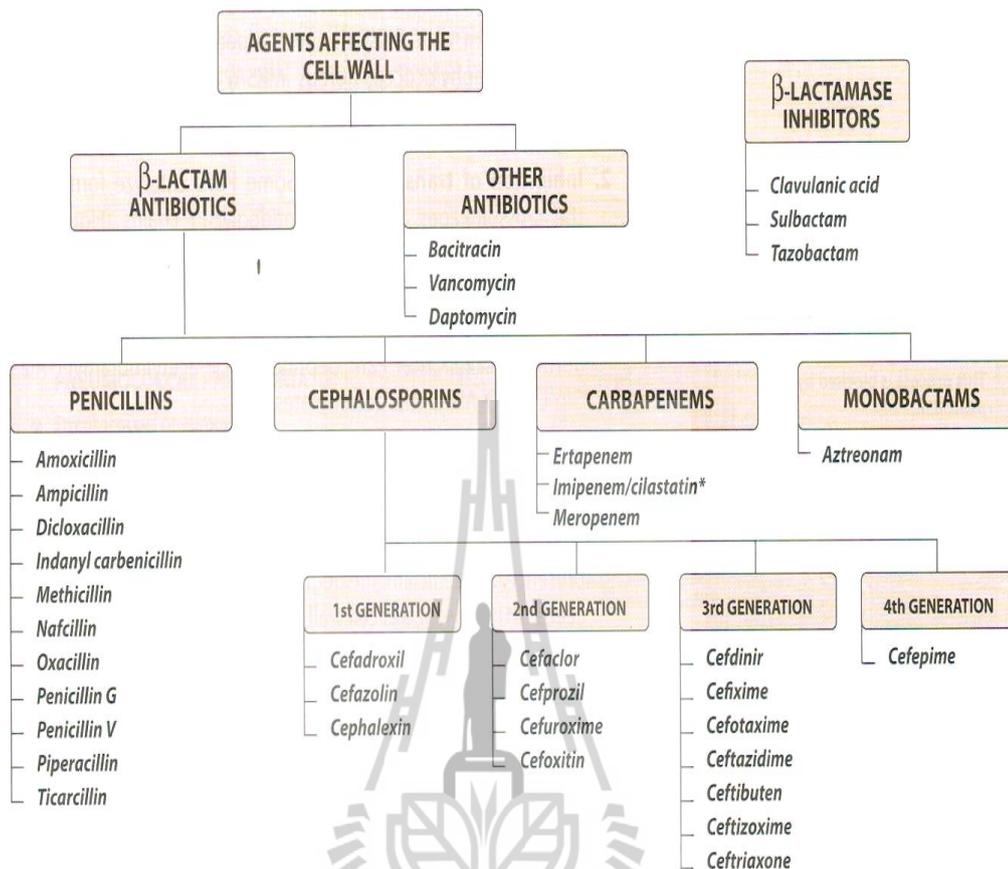
Figure 2.1 Summary of antimicrobial agents affecting cell wall synthesis.

Source: Harvey *et al.* (2009).

2.6.2.2 Cephalosporins

Cephalosporins are similar to penicillins, but more stable than many bacterial β-lactamases and therefore have a broader spectrum of activity. However, resistant strains of *E. coli* and *Klebsiella* species expressing extended-spectrum β-lactamases that can hydrolyze most cephalosporins are becoming a problem. Cephalosporins are not active against enterococci and *L. monocytogenes* (Katzung, 2007).

2.7 The use of β-lactam antibiotics

2.7.1 Cloxacillin
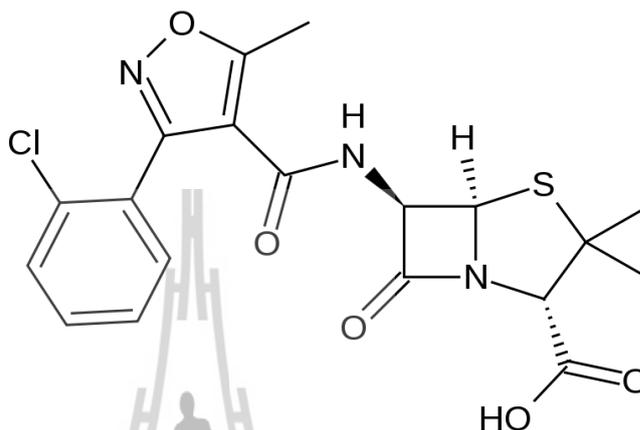
Molecular formular : $C_{19}H_{18}ClN_3O_5S$

Molecular mass : 435.882 g/mol

Solubility : Soluble in water

Appearance : white or almost white, crystalline powder

(http://en.wikipedia.org/wiki/Cloxacillin)

2.7.2 Ceftazidime
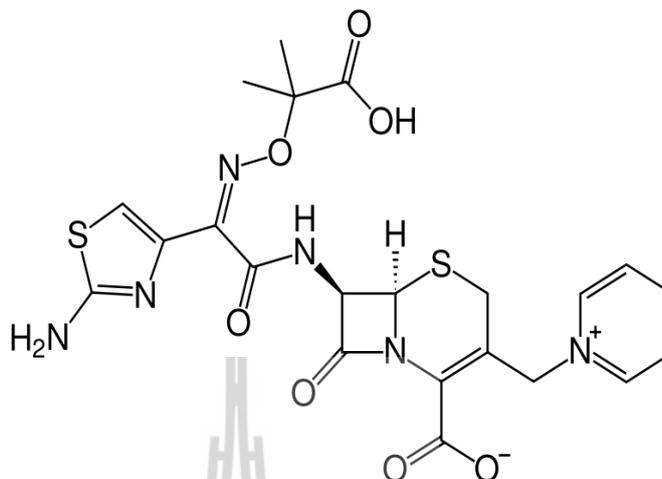
Molecular formular: $C_{22}H_{22}N_6O_7S_2$

Molecular mass : 546.58 g/mol

Solubility: Soluble in water

Appearance: white or almost white powder

(http://en.wikipedia.org/wiki/Ceftazidime)

2.8 Mechanisms of bacterial resistance to penicillins and cephalosporins

Although all bacteria with cell walls contain PBPs, β-lactam antibiotics cannot kill or even inhibit all bacteria because bacteria develop various resistance to these agents. The microorganism may be intrinsically resistant because of structural differences in the PBPs that are the targets of these drugs. Furthermore, a sensitive strain may acquire resistance by the development of high-molecular-weight PBPs that decreased affinity for the antibiotic. Because the β-lactam antibiotics inhibit many different PBPs in a single bacterium, the affinity for β-lactam antibiotics of several

PBPs must decrease for the organism to be resistant. Altered PBPs with decreased affinity for β-lactam antibiotics are acquired by homologous recombination between PBP genes of different bacterial species. Four of the five high-molecular-weight PBPs of the most highly penicillin-resistant *Streptococcus pneumoniae* isolates have decreased affinity for β-lactam antibiotics as a result of interspecies homologous recombination events (Laurence *et al*., 2006).

Bacteria can have an intrinsic immunity to a particular antibiotic because of biochemical structure and/or function. They may simply not respond to the antibiotic mechanism of action. Bacteria can also acquire resistance to a drug in one of two ways. Primary resistance occurs through spontaneous mutation, which is a rare event. Resistance is then transferred to progeny. Bacteria replicate asexually, so all offspring of a resistant bacteria will inherit the resistant gene. Secondary resistance requires a transfer of genetic material between same or different species of bacteria through transduction, transformation or conjugation (Table 2.1). Bacteria is asexual, bacteria can share genetic material by forming a conjugation bridge with bacteria from the same or different species (Figure 2.2). Genetic material is carried on plasmids or transporans. Genetic transfer may confer multi-drug resistance and cross-resistance, where by resistance to one drug in a class translates into resistance to other antimicrobial drugs in that class. The ease with which bacteria share genetic material accounts for most antimicrobial resistance (Roe *et al*., 2008).

Table 2.1 How bacteria transfer genetic material.

How Bacteria Transfer Genetic Material	
Transduction	Viruses carry genetic material from one bacterium to another
Transformation	Bacteria engulf genetic material from dead bacteria in the environment
Conjugation (most common)	Two bacteria join cells and transfer genetic material via plasmids

Source: Roe *et al*. (2008).
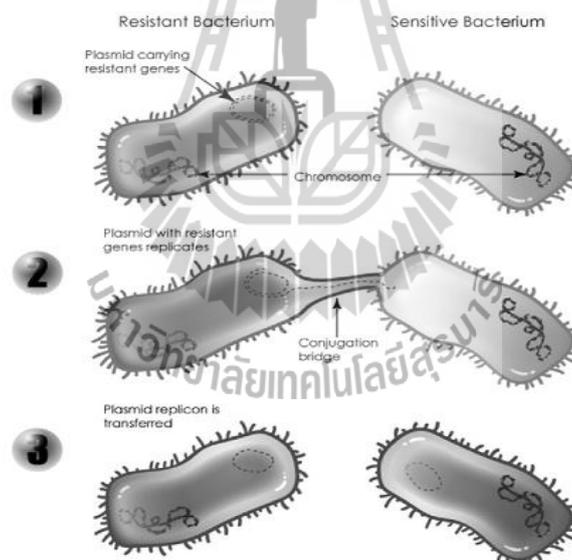
Figure 2.2 Bacterial conjugation.

Source: Roe *et al*. (2008).

Once an antimicrobial enters the bacteria, it must bind to a specific biochemical target site in order to interfere with cell metabolism. Genetically altered bacteria can combat antibiotics in several ways (Figure 2.3). They may synthesize enzymes that

destroy the drug or chemically modify it so that it becomes inactive. For example, some β-lactamase, which destroys the bacteria, synthesize β-lactam ring common to the structure of β-lactam antibiotics. Resistance to the β-lactam is widespread in both Gram-positive (Staphylococci) and Gram-negative (*Neissera gonorrhoea, Escherichia coli*, and *Haemophilus influenza*) species. Some β-lactam antimicrobials, the antistaphylococcal penicilllins (methicillin, nafcillin, oxacillin, and dicloxacillin), were developed specifically to avoid the effects of the β-lactamase producing bacteria. Dicloxacillin is the only member of this group of antibiotics available in oral form.
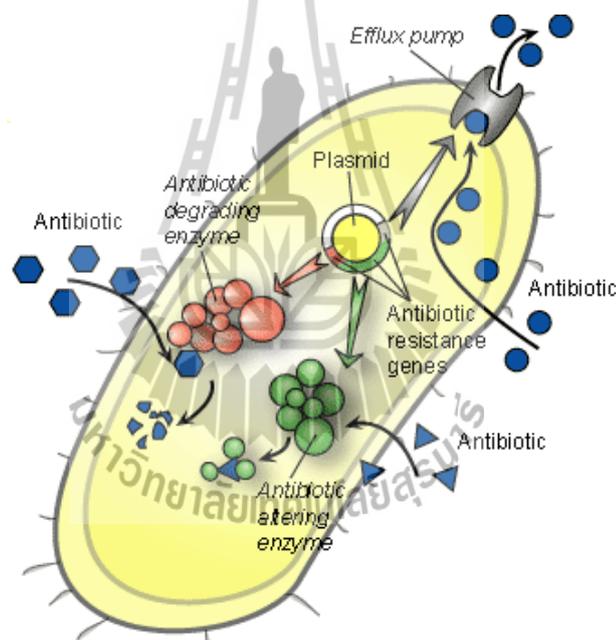
Figure 2.3 Mechanisms of antibiotic resistance.

Source: Roe *et al.* (2008).

2.9   Synergistic activities of flavonoid and β-lactam combinations

The synergistic effects of β-lactam and flavonoid combinations against resistant bacteria may arise from contributions of three distinct types of activities. These could involve three mechanisms of action that galangin inhibit protein synthesis and effect on PBP 2a, interact with penicillinase and cause cytoplasmic membrane damage (Eumkeb *et al*., 2010).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Animals

Male and female mice matched for age and weight were purchased from National Laboratory Animal Centre, Salaya Campus, Mahidol University, Thailand. The animals were housed in cages in the Animal Care Building at Suranaree University of Technology under a constant temperature of 25 ± 0.5 ^{o}C and hygiene conditions with 12 h. of light and dark cycle throughout the experimental period. The animals were provided free access to food and drinking water.

3.1.2 Chemicals

All chemicals used were analytical grade (AR) otherwise specified.

Dimethylsulfoxide (DMSO)

Sodium chloride

95% Ethanol   Lab grade

Formaldehyde 38% w/v   Lab grade

Cloxacillin

Ceftazidime

Apigenin

Baicalein

Galangin

Luteolin

Quercetin

3.1.3 Equipments

Experimental glassware (pipette, volumetric flasks, beaker)

Centrifuge tubes

Autoclave

Air pump; Benz aquarium pump

EDTA tubes

Sodium fluoride tubes

Haematocrit tubes

FTIR microspectroscopy

3.2 Methods

3.2.1 Subacute toxicity test in mice

The animals were divided into 24 groups. The group 1 and 12 to 14 were control groups (10 mice). The group 2 to 11 and 15 to 24 were tested groups (10 mice in each group) for each flavonoid alone and flavonoids in combination with β-lactams antibiotics. These mice were administrated by IP injection as follows:

I. The group 2 to 11 were administered only flavonoids (apigenin, baicalein, luteolin, quercetin or galangin) at therapeutic dose (4 groups) and higher dose (4 groups) (Figure 3.1).

II. The group 15 to 24 were administered flavonoids in combination with β-Lactams (baicalein + cloxacillin, quercetin + cloxacillin, apigenin + ceftazidime, luteolin + ceftazidime, galangin + ceftazidime) at therapeutic dose (4 groups) and higher dose (4 groups) (Figure 3.2).
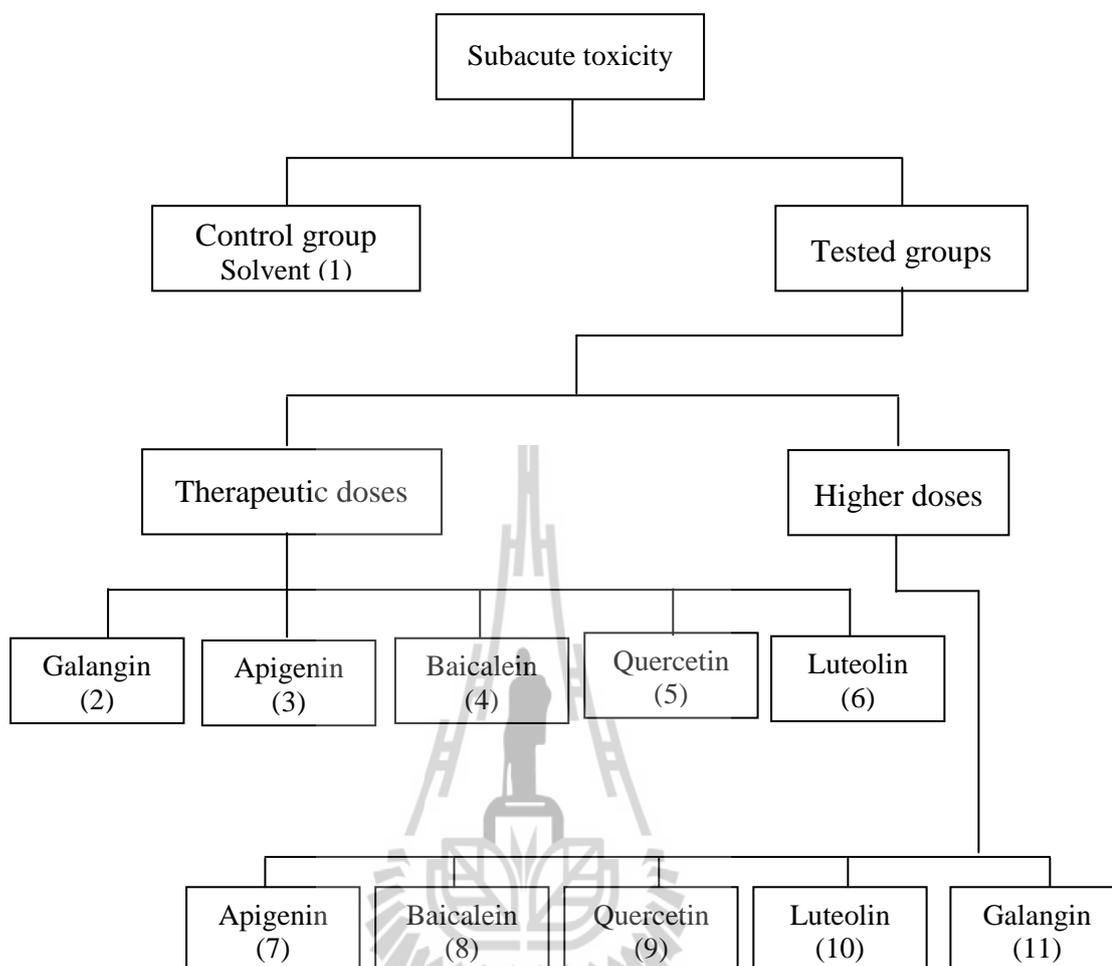
Figure 3.1   Subacute toxicity: Mice were administrated flavonoid alone.
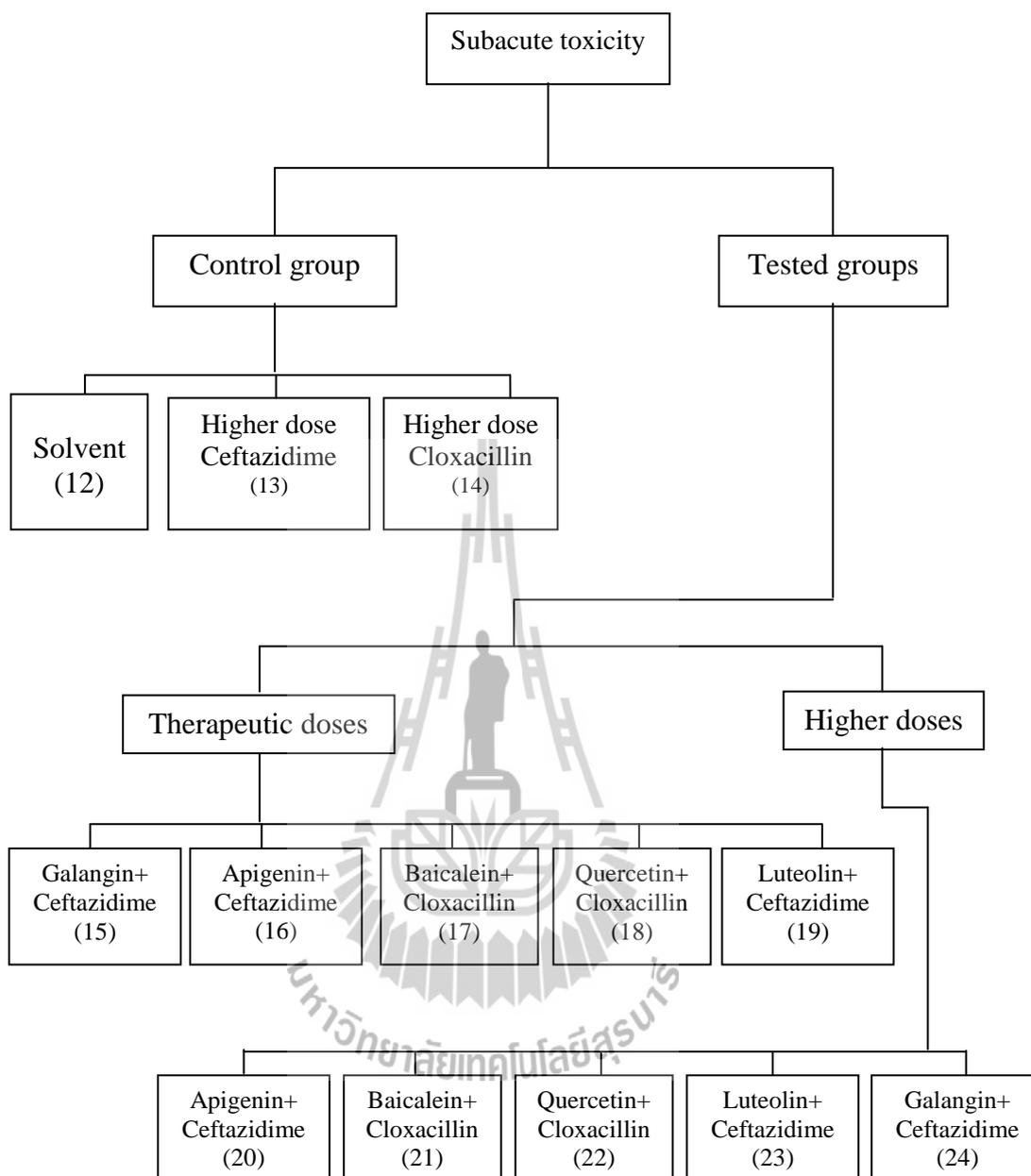
Figure 3.2   Subacute toxicity: Mice were administrated flavonoids in combination

with β-Lactams.

All these groups were administered intraperitoneally 2 times a day for 2 weeks. These mice were analyzed for the blood compositions before and after sample injection. At the end of the experiments, the animals were sacrificed under isoflurane anesthesia. The experimental protocol was followed in accordance with Breeding and Care of Laboratory Animals 1988 (Vol. 1) prepared for the World Health Organization, Health Laboratory Technology Unit, Geneva, Switzerland and the procedures of the experiment was performed in accordance with the approvement of the animal laboratory ethical committee, Suranaree University of Technology. The body weights were evaluated every weeks. At the end of exposure course, the body and organ weights (lung, liver, kidney, spleen, heart and stomach) and organ morphology were evaluated. Hematological parameter was evaluated for the hematology (Hct, RBC, WBC and MCV) and blood chemistry (FBS, AST, BUN, cholesterol and uric acid).
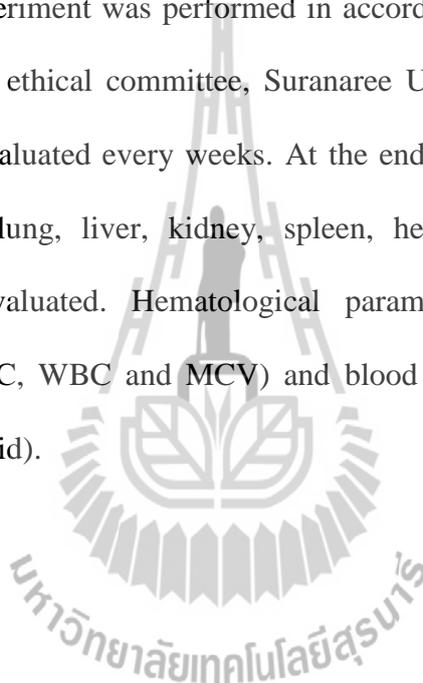
3.2.2 Subchronic toxicity study

The animals were divided into 22 groups. The group 1 and 10 to 12 were control groups (10 mice). The group 2 to 9 and 13 to 20 were tested groups (10 mice in each group) for each flavonoid alone and in combination with β-lactams antibiotics. These mice were administrated by IP injection as follows:

III. The group 2 to 9 were administrated only flavonoids (apigenin, baicalein, luteolin or quercetin) at therapeutic dose (4 groups) and high dose (4 groups) (Figure 3.3).

IV. The group 13 to 20 were administrated flavonoids in combination with β-Lactams (baicalein + cloxacillin, quercetin + cloxacillin, apigenin + ceftazidime, luteolin + ceftazidime) at therapeutic dose (4 groups) and higher dose (4 groups) higher treatment dose (Figure 3.4).
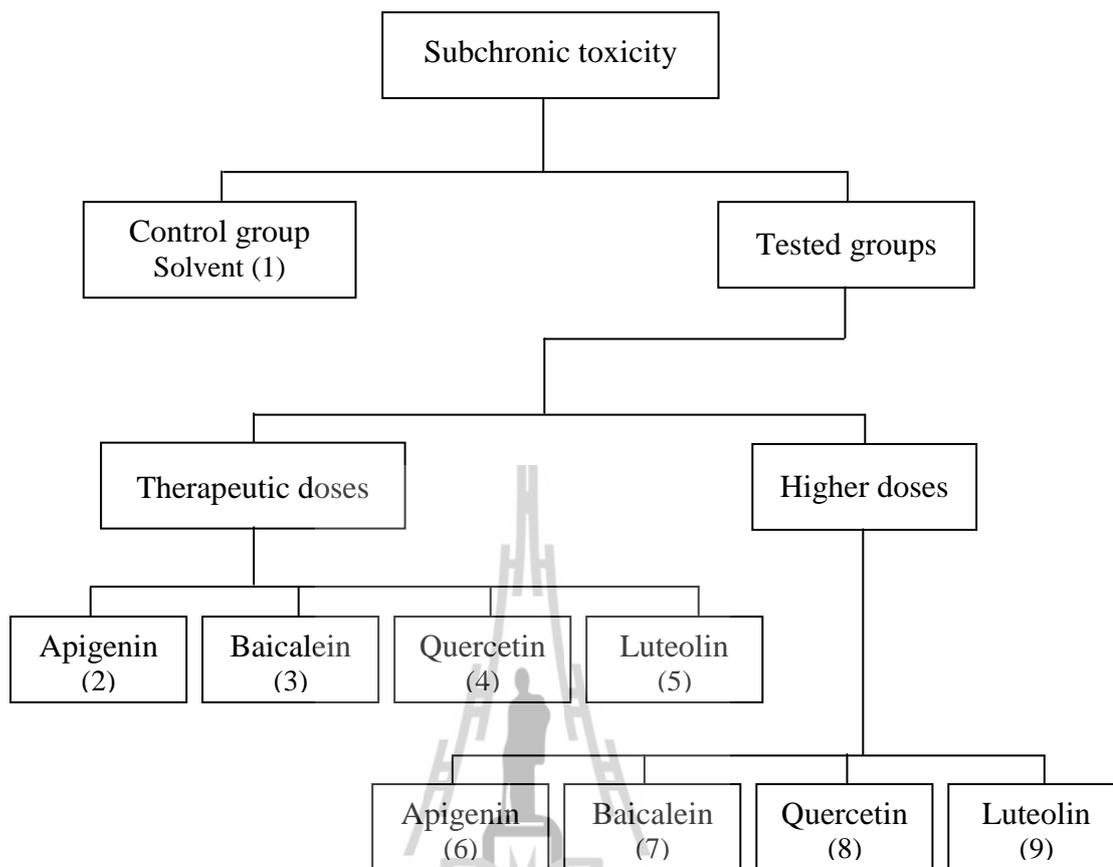
Figure 3.3 Subchronic toxicity : Mice were administrated flavonoid alone.
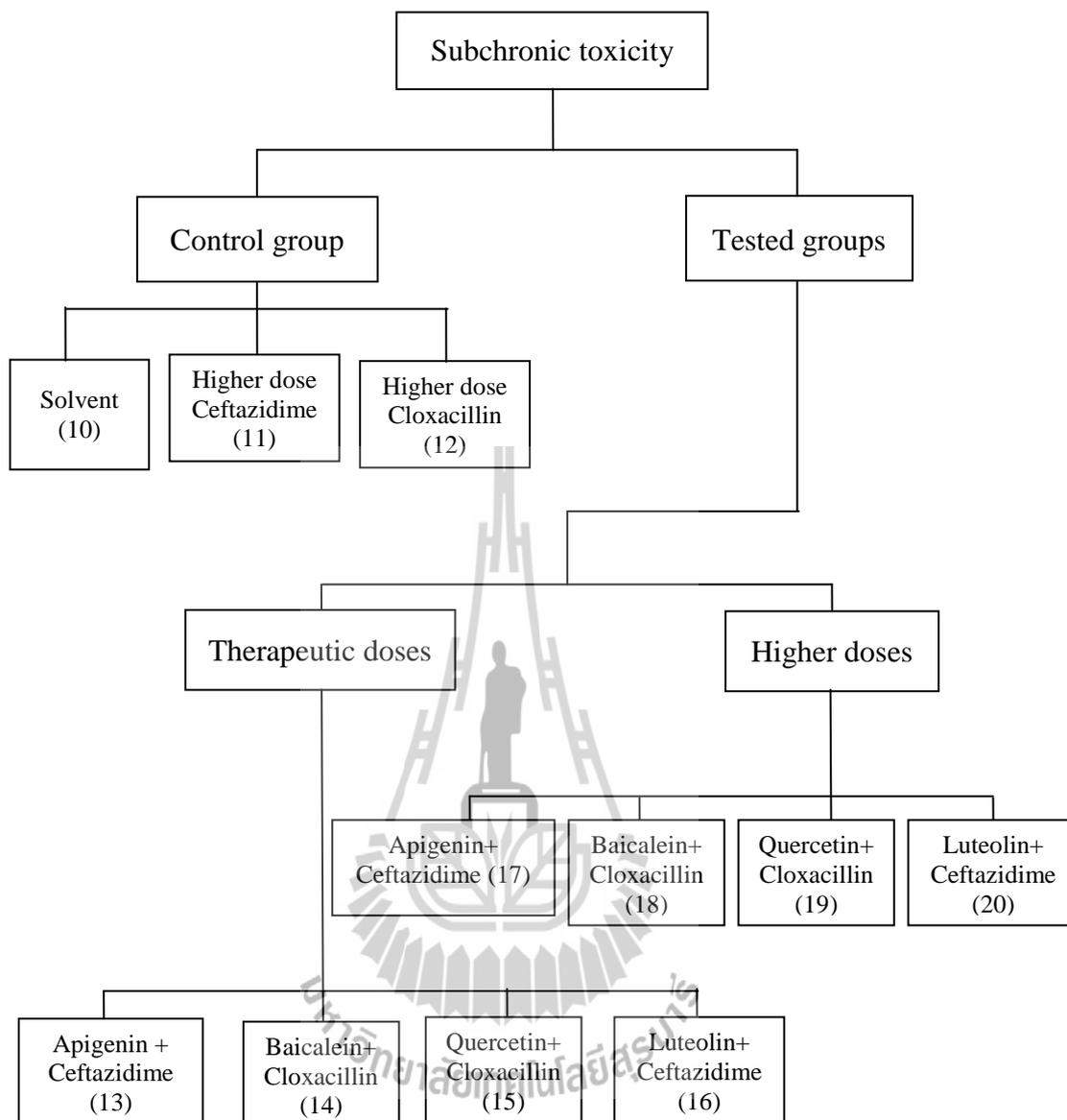
Figure 3.4   Subchronic toxicity : Mice were administrated flavonoids in combination with β-Lactams.

All these groups were administered intraperitoneally 2 times a day for 3 months (90 days). These mice were analyzed for the blood composition before and after sample injection. At the end of the experiments, the animals were sacrificed under isoflurane anesthesia. The experimental protocol was followed in accordance with Breeding and Care of Laboratory Animals 1988 (Vol. 1) prepared for the World Health Organization, Health Laboratory Technology Unit, Geneva, Switzerland and the procedures of the experiment was undertaked in accordance with the approvement of the Animal Care Building at Suranaree University of Technology. The body weights were evaluated every weeks. At the end of injection course, the result analysis was carried out by evaluating body weights, organ weights (lung, liver, kidney, spleen, heart and stomach) and organ morphology. Hematological parameter was evaluated for the hematology (Hct, RBC, WBC and MCV) and blood chemistry (FBS, AST, BUN, cholesterol and uric acid).

3.2.3 Morphological examination

At the end of the experiment, only the abnormal selected organs (lung, liver, kidney, spleen, heart or stomach) were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin for pathological examination under light microscopy. In addition, the abnormal selected organs were cut into 1 mm cubes and fixed in 2.5% glutaraldehyde for 24 h at 4 ºC, sectioned with the ultramicrotome mounting a diamond knife. Sections of about 0.8 mm were collected on copper grids, stained for 3 min in 3% glutaraldehyde and 1% osmium tetroxide, then 2% uranyl acetate. This procedure was compared with normal selected organ (Hui *et al*., 2008).

3.2.4 Blood chemistry hematological analysis

At the end of the experiment, blood samples were collected by cardiac puncture under ether anesthesia and were partly used for hematology. From the remainder blood serum was prepared by centrifugation at 1000 X g for 30 min and kept at $-20\ ^{\circ}C$ for blood chemistry analysis, including hemoglobin, WBC, RBC, HDL, serum bilirubin, blood glucose, cholesterol, triglyceride, AST, BUN and uric acid. The assays were performed with automated analytical systems.

3.2.5 Fourier Transform Infrared (FTIR) microspectroscopy

The experiments were performed at Synchrotron Light Research Institute utilizing a Tensor 27 FTIR spectrometer connected to an IR microscope (Hyperion 2000). The microscope was equipped with a Mercury Cadmium Telluride (MCT) detector, with a 15x Cassegrain objective. FTIR samples were recorded in reflection mode, 8cm^{-1} spectral resolution, 128 scans per spectrum.

3.2.5.1 Analytical potential of FTIR spectroscopy in biochemical

Characteristic frequencies, intensities, and band-widths in infrared spectra allow the identification of functional groups of molecules (Table 3.1) and the characterization of conformationally distinct structures in biological molecules (Fabian and Mantele, 2002; Naumann, 2000, 2001). IR spectroscopy was one of the earliest analytical methods identified as a powerful tool to gain information on the structural properties of proteins. As early as 1950 it was shown that there is a close correlation between the position of specific bands in the IR spectrum of polypeptides and their secondary structure. This correlation was later refined by

linking the frequencies of structure-sensitive amide bands to specific types of secondary structure such as α-helix, β-sheet, or turn (Fabian and Mantele, 2002). There are nine amide bands in the IR spectrum, called amide A, amide B and amides I–VII, according to decreasing frequency. The amide I band, in which different secondary structure elements such as α-helix, triple helix, β-sheet, β-turn, and extended coil absorb IR light of different wavelengths (Krimm and Bandekar, 1986), turned out to be the most useful band for the analysis of secondary protein structure. The amide I mode in IR spectra, which primarily represents the C=O stretching vibration of the amide groups and occurs in the region between 1600 and 1700 cm^{-1}, is particularly sensitive to β-sheet structures. The position of IR bands associated with these structures is influenced by a variety of factors, including the strength of hydrogen bonds and the packing of β-strands (Surewicz and Mantsch, 1988; Zandomeneghi *et al*., 2004) (Figure 3.5).

Table 3.1 Tentative assignment of some bands frequently found in biological

   FTIR spectra.

Frequency (cm^{-1})	Assignment
~3500	O-H str of hydroxyl groups
~3200	N-H str (amide A) of protein
2959	C-H str (asym) of –CH$_3$
2934	C-H str (asym) of >CH$_2$
2921	C-H str (asym) of >CH$_2$ in fatty acids
2898	C-H str of →C-H methine
1741-1715	>C=O str of esters, >C=O str of carbonic acids, nucleic acids
~1695,	Amide I band components resulting from antiparallel pleated
~1685, ~1675	sheets and β-turns of proteins
~1655	Amide I of α- helical structures
~1637	Amide I of β- pleated sheet structures
1548	Amide II
1515	"Tyrosine" band
1468	C-H def of >CH$_2$
~1400	C=O str (sym) of COO$^-$
1310-1240	Amide III band components of proteins
1250-1220	P=O str (asym) of >PO$_2^-$ phospodiesters
1200-900	C-O-C, C-O dominated by ring vibrations of carbohydrates C-O-P, P-O-P
1085	P=O str (sym) of >PO$_2^-$
720	C-H rocking of >CH$_2$
900-600	"Fingerprint region"

Source: Beekes *et al*. (2007).
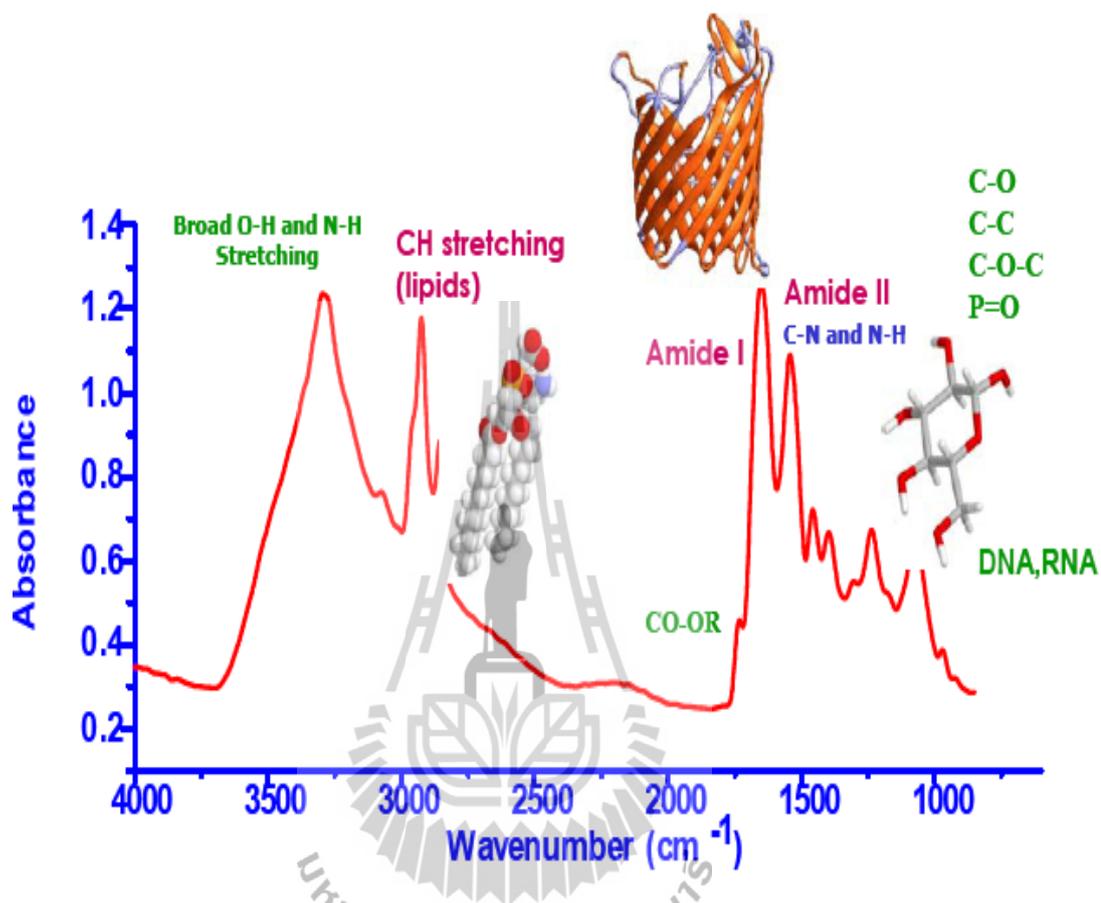
Figure 3.5   Representative spectrum in the mid-IR region. The region below 1500
cm^{-1} is defined as the fingerprint region. The region above 1500 cm^{-1} is
the functional group region.

Source: Hynes *et al*. (2005).

3.2.5.2 Serum samples analysis

A diluted serum sample, 5-10 μl, were dropped on a ZnSe window or deposited onto indium tin oxide-coated, silver-doped glass slide (low e-slides, Kelvey slide) and vacuum dried for 24 h before analysis (Figure 3.6).
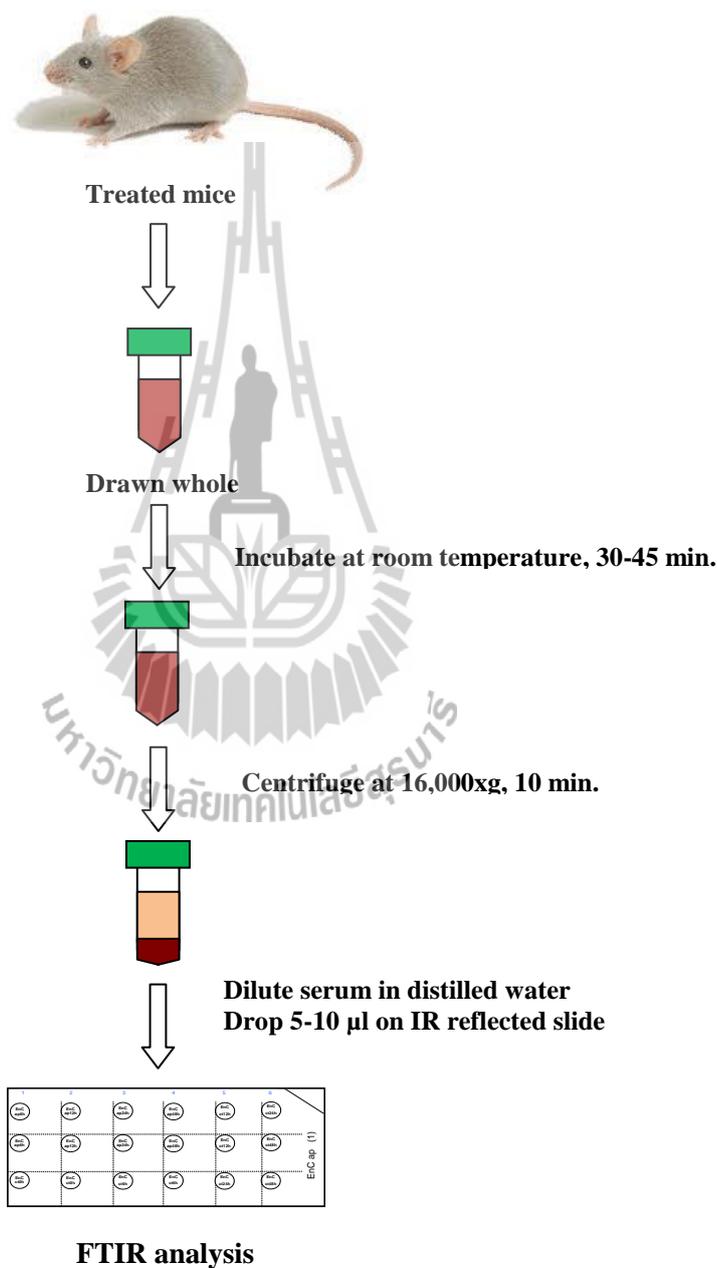
Figure 3.6   Diagram of the steps of serum preparation for FTIR microspectroscopy.

3.2.5.3 Multivariate data analysis

Individual spectra from each cluster were analyzed using Principle Component Analysis to distinguish different biochemical component between serum from treatment and control groups. The spectra were processed using $2°$ derivative, vector normalized by the Savitzky-Golay method (3rd polynomial, 9 smoothing points) using the Unscrambler 9.7 software.

3.2.6 Statistical analysis

All data were expressed as mean $\pm$ S.E.M. Significant differences between the control and treatment groups were analyzed by students t-test. P values <0.05 were considered statistically significant. The significant differences between pre- and post-treatment groups were compared using Paired-Student t-test and statistiacal difference were determined at P<0.05.
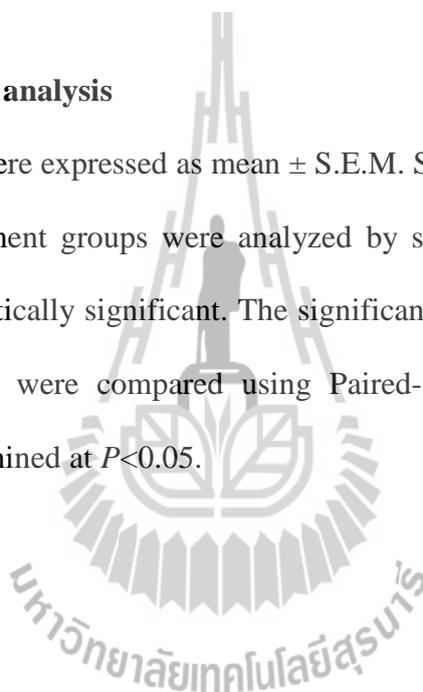
CHAPTER IV

RESULTS

The study was divided into two parts. The first part is the subacute toxicity of the flavonoids (quercetin, apigenin, baicalein, luteolin, and galangin) use singly and combination with β-Lactams antibiotics (baicalein + cloxacillin, quercetin + cloxacillin, apigenin + ceftazidime, luteolin + ceftazidime, galangin + ceftazidime). These mice were injected intraperitoneal for 14 days. The second part shows the study of subchronic toxicity of the flavonoids (quercetin, apigenin, baicalein, or luteolin) alone and in combination with β-Lactams antibiotics (baicalein + cloxacillin, quercetin + cloxacillin, apigenin + ceftazidime, luteolin + ceftazidime, galangin + ceftazidime). These mice were administered by intraperitoneal injection for 90 days.

4.1 Subacute toxicity test of some flavonoids

4.1.1 Subacute toxicity test of quercetin

4.1.1.1 Effects of the quercetin on weight changes and main organs in mice

There was no significant difference in either the relative growth rate measured by living body weight (Figure 4.1) or the relative weight of the selected main body organs of all groups of treated mice for 14 consecutive days, when compared to the control (Table 4.1). The patho-histology of all organs showed normal appearance compared to the control organs (Data not shown).
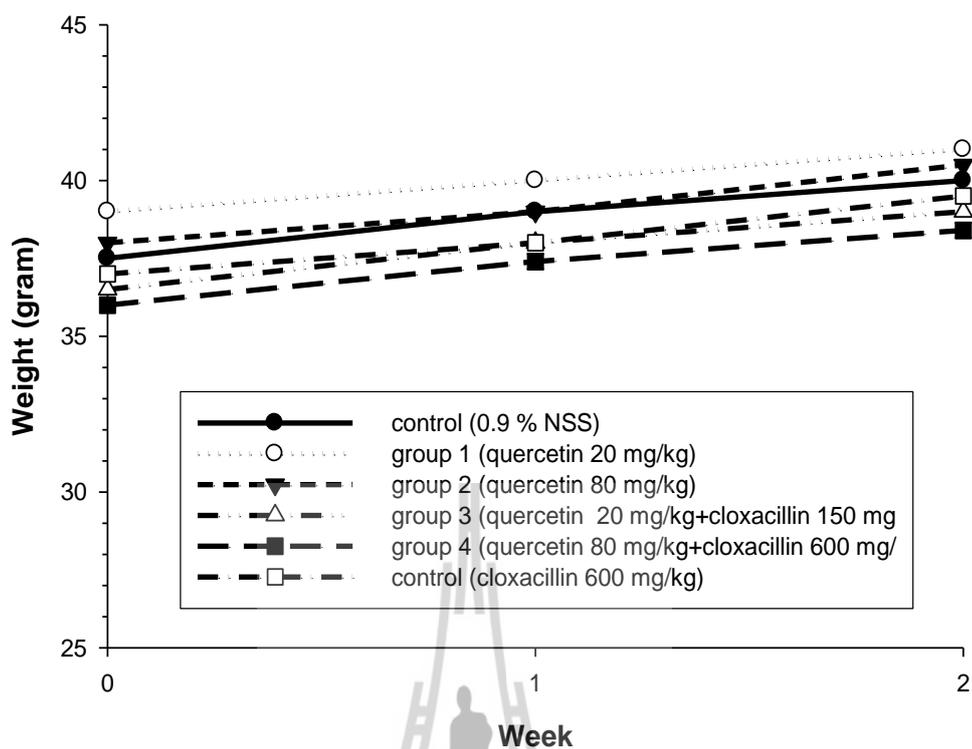
Figure 4.1 Body weights of mice treated with quercetin alone and in combination with cloxacillin for 14 consecutive days in comparison with the negative control.

Figure 4.2 Morphology of main body organs of mice treated with quercetin alone and in combination with cloxacillin for 14 consecutive days in comparison with the negative control. Control group ; a = heart; b = liver; c = spleen; d = lung; e = kidney; f = stomach. Treated group ; g = heart; h = liver; i = spleen; j = lung; k = kidney; l = stomach.

Table 4.1 Effects of intraperitoneally administered quercetin alone and in combination with cloxacillin for 14 days on relative weight of the selected main organ (per 100g body weight) in mice. Group 1 = quercetin 20 mg/kg BW/day, Group 2 = quercetin 80 mg/kg BW/day, Group 3 = quercetin 20 mg/kg BW/day plus cloxacillin 150 mg/kg BW/day, Group 4 = quercetin 80 mg/kg BW/day plus cloxacillin 600 mg/kg BW/day, Cloxa = Cloxacillin (600 mg/kg BW).

Organ (g)	Control (NSS)	Control Cloxa	Group 1	Group 2	Group 3	Group 4
Heart	0.0019 ± 0.0001	0.0018 ± 0.0002	0.0022 ± 0.0001	0.0025 ± 0.0004	0.0017 ± 0.0003	0.0017 ± 0.0001
Liver	0.0230 ± 0.0036	0.0185 ± 0.0015	0.0239 ± 0.0018	0.0212 ± 0.0026	0.0196 ± 0.0011	0.0208 ± 0.0025
Spleen	0.0053 ± 0.0017	0.0034 ± 0.0001	0.0045 ± 0.0030	0.0020 ± 0.0001	0.0047 ± 0.0008	0.0049 ± 0.0008
Lung	0.0029 ± 0.0002	0.0029 ± 0.0003	0.0035 ± 0.0002	0.0030 ± 0.0003	0.0025 ± 0.0003	0.0024 ± 0.0003
Kidney	0.0070 ± 0.0016	0.0064 ± 0.0037	0.0079 ± 0.0008	0.0068 ± 0.0013	0.0067 ± 0.0017	0.0059 ± 0.0002
Stomach	0.0092 ± 0.0010	0.0097 ± 0.0012	0.0107 ± 0.0018	0.0069 ± 0.0004	0.0127 ± 0.0036	0.0148 ± 0.0066

4.1.1.2 Effects of the quercetin for 14 days on blood chemistry and hematology in mice

The blood chemistry marker assayed indicated that there was no significant changes of AST, BUN, FBS, and cholesterol levels of all mice treated for fourteen days in comparison with pre-treatment groups (Table 4.2).

In subacute toxicity test, results of hematology exhibited that there were no significant changes of WBC and Hct in post-treatment groups compared to pre-treatment groups. RBC and Hb levels decreased significantly after treatment with 80 mg/kg BW/day quercetin compared to the controls. MCV level also reduced dramatically after treatment with 20 mg/kg BW/day quercetin plus 150 mg/kg BW/day cloxacillin compared to the controls.

Table 4.2 Blood chemistry and hematological studies on mice before and after subacute treatment with quercetin alone and in combination with cloxacillin for 14 days. Group 1 = quercetin 20 mg/kg BW/day, Group 2 = quercetin 80 mg/kg BW/day, Group 3 = quercetin 20 mg/kg BW/day plus cloxacillin 150 mg/kg BW/day, Group 4 = quercetin 80 mg/kg BW/day plus cloxacillin 600 mg/kg BW/day, Cloxa = Cloxacillin (600 mg/kg BW).

Parameter	Control (NSS)		Control Cloxa.		Group 1		Group 2		Group 3		Group 4	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
AST (U/L)	207.2±33.6	209.2±33.7	300.5±34.7	415.5±49.3	178.7±49.2	129.7±17.9	159.2±20.0	235.0±99.3	255.0±71.3	80.6±19.5	169.3±42.3	207.0±11.3
BUN (mg/dl)	23.5±0.6	23.7±1.1	25.0±1.6	25.8±4.5	32.5±3.2	34.0±4.9	15.7±1.7	23.6±7.7	25.3±2.9	44.2±17.9	47.9±14.7	29.7±1.6
FBS(mg/dl)	137.5±9.8	176.7±22.4	160.0±3.6	182.2±18.1	115.00±19.9	150.75±15.9	163.0±6.1	155.7±16.1	180.3±59.3	155.3±5.6	180.0±8.3	213.6±26.0
Cholesterol(mg/dl)	85.2±2.7	92.0±4.1	129.0±8.0	116.5±13.7	100.0±4.5	162.2±54.6	105.0±7.0	151.0±49.8	134.6±20.1	84.6±19.6	106.6±38.6	78.6±07.5
WBC ($x10^3$//µ L)	9.6±0.6	9.7±0.7	9.6±0.6	24.3±16.5	5.9±1.4	4.9±0.5	3.1±1.2	3.8±1.5	6.6±0.9	5.4±1.2	9.3±1.6	9.7±01.5
RBC ($x10^6$/µL)	9.0±0.1	8.9±0.4	9.05±0.1	8.9±0.4	7.8±0.4	8.2±0.1	8.7±0.3	6.7±0.0*	7.7±0.3	5.3±1.6	8.2±0.2	6.7±01.40
Hb (g/dl)	15.5±0.2	14.5±0.2	15.5±0.2	14.5±0.2	13.3±0.9	14.2±0.5	15.1±0.3	10.5±1.0*	14.1±0.7	9.6±1.8	14.3±1.1	11.3±01.7
Hct (%)	44.7±1.2	44.0±0.9	49.2±1.3	44.0±0.9	37.2±1.5	40.3±0.5	40.3±5.4	34.9±3.7	40.5±1.8	25.3±8.2	41.6±2.0	35.6±05.4
MCV (fL)	54.5±0.8	54.2±0.7	54.5±0.8	52.9±0.7	48.2±0.4	49.2±0.4	51.0±0.5	45.3±2.3	52.0±2.0	**47.1±0.6***	51.5±1.3	54.8±4.2

Mean ± SEM (n=5), *$p < 0.05$ (Student's t–test). Significant relative to pre-treatment value.

4.1.2 Subacute toxicity study of Baicalein

4.1.2.1 Effects of the baicalein on weight changes and main organs in mice

As demonstrated in Figure 4.3 and Table 4.3, treated mice of all groups did not significantly gain body and organ weights throughout 14 consecutive days, when compared to the control. The morphology of all selected treatment organs of mice exhibited a normal appearance in comparison with the control.
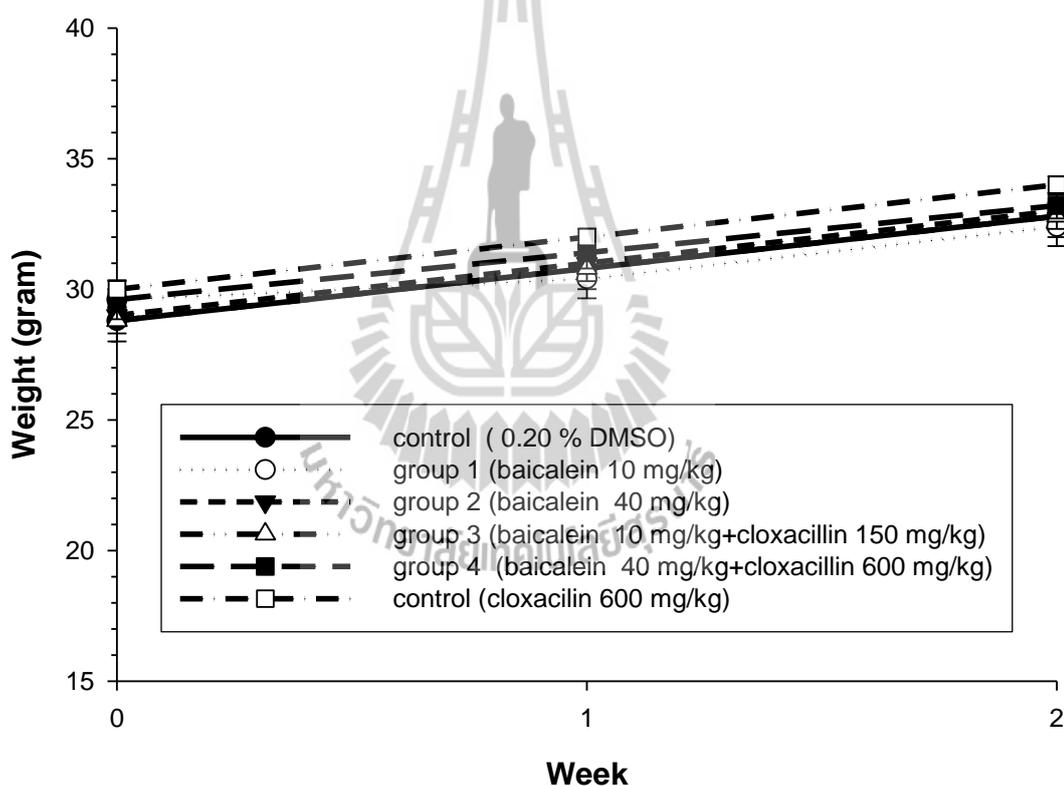
Figure 4.3 Body weights of mice treated with baicalein alone and in combination with cloxacillin for 14 consecutive days in comparison with the negative control.
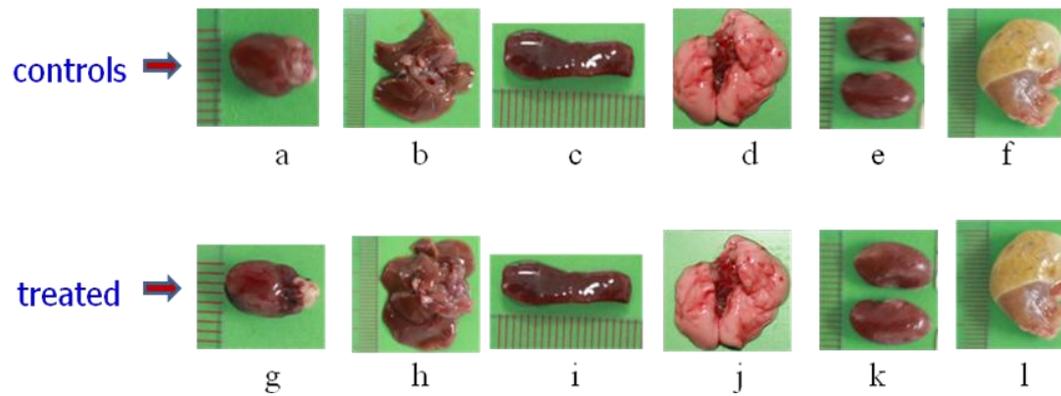
Figure 4.4 Morphology of main body organs of mice treated with baicalein alone and in combination with cloxacillin for 14 consecutive days in comparison with the negative control. Control group ; a = heart; b = liver; c = spleen; d = lung; e = kidney; f = stomach. Treated group ; g = heart; h = liver; i = spleen; j = lung; k = kidney; l = stomach.

Table 4.3 Effects of intraperitoneally administered baicalein alone and in combination with cloxacillin for 14 days on relative weight of the selected main organ (per 100g body weight) in mice. Group 1 = baicalein 10 mg/kg BW/day, Group 2 = baicalein 40 mg/kg BW/day, Group 3 = baicalein 10 mg/kg BW/day plus cloxacillin 150 mg/kg BW/day, Group 4 = baicalein 40 mg/kg BW/day plus cloxacillin 600 mg/kg BW/day, Cloxa = Cloxacillin (600 mg/kg BW).

Organ (g)	Control (0.2%DMSO)	Control Cloxa	Group 1	Group 2	Group 3	Group 4
Heart	0.0019 ± 0.0001	0.0018 ± 0.0001	0.0021 ± 0.0001	0.0019 ± 0.0001	0.0015 ± 0.0001	0.0018 ± 0.0001
Liver	0.0193 ± 0.0011	0.0178 ± 0.0010	0.0208 ± 0.0017	0.0185 ± 0.0013	0.0169 ± 0.0011	0.0198 ± 0.0008
Spleen	0.0014 ± 0.0001	0.0014 ± 0.0000	0.0017 ± 0.0001	0.0017 ± 0.0002	0.0013 ± 0.0001	0.0036 ± 0.0016
Lung	0.0025 ± 0.0001	0.0025 ± 0.0001	0.0025 ± 0.0001	0.0022 ± 0.0001	0.0028 ± 0.0003	0.0027 ± 0.0002
Kidney	0.0072 ± 0.0010	0.0060 ± 0.0006	0.0069 ± 0.0008	0.0062 ± 0.0005	0.0056 ± 0.0008	0.0057 ± 0.0007
Stomach	0.0121 ± 0.0022	0.0069 ± 0.0012	0.0179 ± 0.0025	0.0107 ± 0.0021	0.0088 ± 0.0022	0.0112 ± 0.0020

4.1.2.2 Effects of the baicalein on blood chemistry and hematology in mice

By the end of the study (14 days), blood chemistry finding were summarized in Table 4.4. There were no significant differences of AST, BUN and FBS levels in all groups of treated mice.

Hb levels decreased significantly after treatment with 40 mg/kg BW/day baicalein plus 600 mg/kg cloxacillin BW/day compared to the control. The post-treatment MCV level also decreased significantly after treatment with 10 and 40 mg/kg BW/day baicalein compared with pre-treatment.Conversely, there were no significant change of RBC and Hct levels in post-treatment groups compared to pre-treatment groups (Table 4.4).
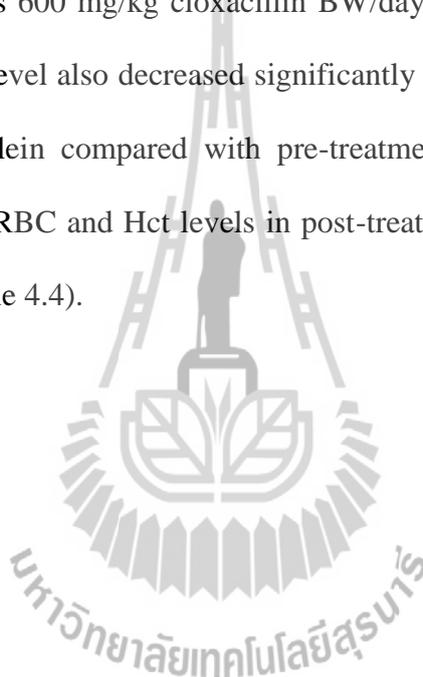
Table 4.4 Blood chemistry and hematological studies on mice before and after subacute treatment with baicalein alone and in combination with cloxacillin for 14 days. Group 1 = baicalein 10 mg/kg BW/day, Group 2 = baicalein 40 mg/kg BW/day, Group 3 = baicalein 10 mg/kg BW/day plus cloxacillin 150 mg/kg BW/day, Group 4 = baicalein 40 mg/kg BW/day plus cloxacillin 600 mg/kg BW/day, Cloxa = Cloxacillin (600 mg/kg BW).

Parameter	Control (0.20%DMSO)		Control Cloxa.		Group 1		Group 2		Group 3		Group 4	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
AST (U/L)	312.5±80.0	615.1±189.1	276.0±36.3	301.0±48.9	156.8±19.9	478.6±155.2	222.0±35.6	256.6±47.3	271.4±47.4	271.0±47.3	400.2±85.0	608.4±152.9
BUN (mg/dl)	27.2±3.2	22.5±1.6	26.2±1.8	25.7±3.4	21.0±2.8	20.5±3.5	29.1±2.6	27.52±2.53	21.8±3.0	19.82±1.16	21.48±3.20	22.26±1.36
FBS(mg/dl)	160.7±6.6	191.4±15.7	160.4±2.8	181.2±14.0	158.4±20.6	180.8±15.7	153.2±12.0	185.6±11.0	168.6±13.5	175.2±13.6	171.00±18.3	166.20±7.2
Cholesterol(mg/dl	140.3±21.6	135.7±22.3	**141.0±13.5**	**105.0±9.7***	102.6±21.8	102.6±21.6	103.0±14.4	103.0±6.9	119.0±15.0	114.8±10.6	**138.60±15.6**	**100.80±12.***
WBC (x10^3//µ L)	9.6±0.8	5.0±1.7	**9.7±0.7**	**4.5±0.64***	10.5±1.3	11.9±2.2	9.8±1.0	13.0±2.3	11.58±1.88	6.8±0.9	**12.92±0.9**	**6.0±0.7***
RBC (x10^6 /µL)	7.5±1.1	7.2±1.1	8.8±0.2	8.2±0.2	7.7±0.6	8.6±0.6	8.1±0.5	8.2±0.8	8.50±0.26	8.3±0.3	8.72±0.11	6.9±1.16
Hb (g/dl)	15.5±0.5	13.0±1.7	15.5±0.2	14.5±0.2	14.0±0.7	14.4±1.0	14.8±0.5	14.2±1.3	15.0±0.31	14.8±0.3	**15.60±0.50**	**12.0±1.5***
Hct (%)	48.0±1.7	37.2±5.8	49.2±1.3	44.0±0.9	39.4±3.2	42.3±3.3	44.1±2.8	41.4±4.0	44.8±1.06	43.4±1.3	45.80±0.91	37.4±4.6
MCV (fL)	51.5±0.2	50.7±1.0	54.5±0.8	53.0±0.9	**50.8±0.7**	**48.7±0.5***	**54.46±0.5**	**49.9±0.6***	52.9±1.38	51.9±0.4	52.50±0.78	51.1±6.0

Mean ± SEM (n=5), *p < 0.05 (Student's t–test). Significant relative to pre-treatment value.

4.1.3 Subacute toxicity study of apigenin

4.1.3.1 Effects of the apigenin on weight changes and main organs in mice

There were no significant differences in the relative body weight changes (Figure 4.5) and the relative weight changes of the selected main body organs of all groups of treated mice for 14 consecutive days, when compared to the control (Table 4.5). The morphology of all organs showed normal appearance compared to the control organs.
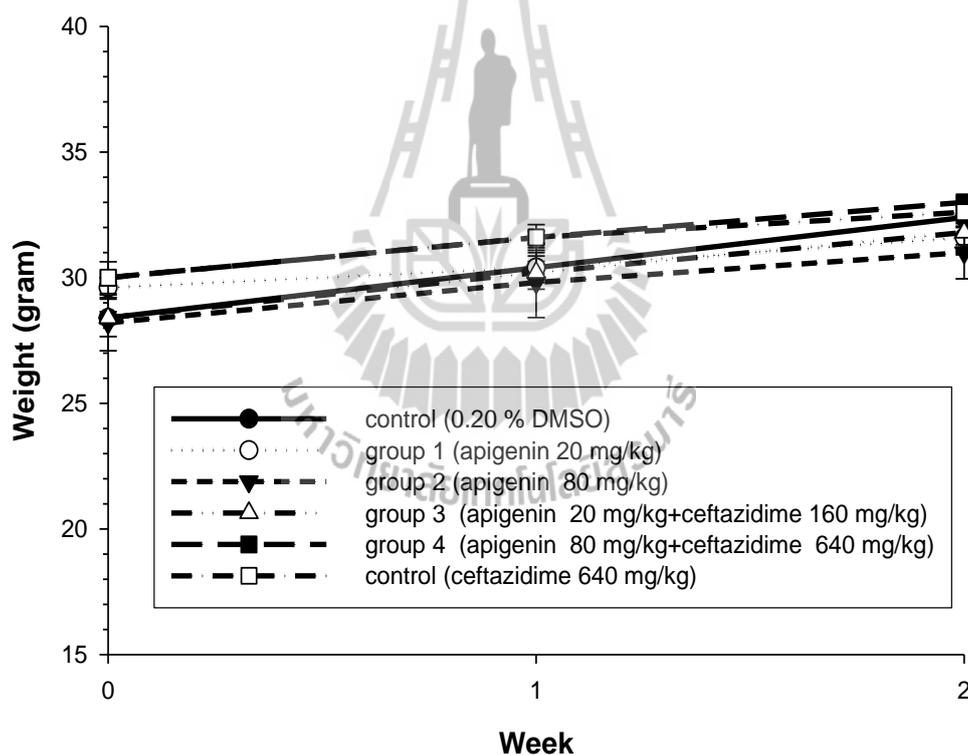

Figure 4.5 Body weights of mice treated with apigenin alone and in combination with ceftazidime for 14 consecutive days in comparison with the negative control.
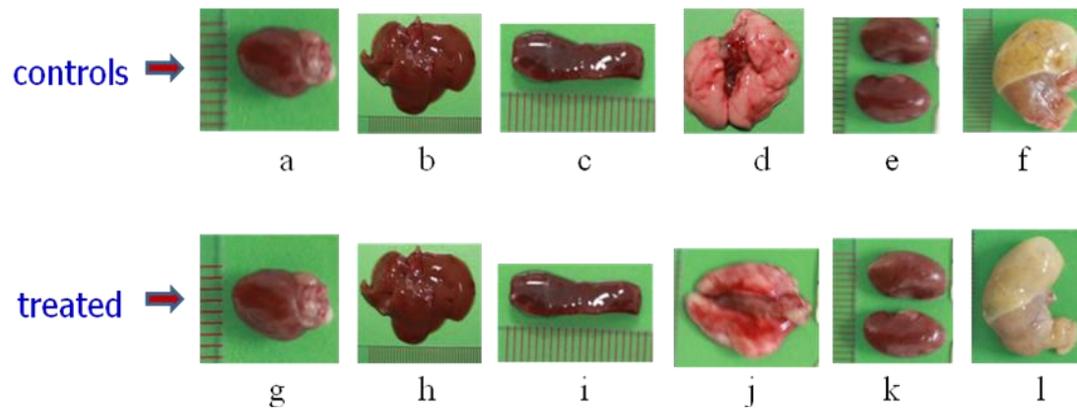
Figure 4.6 Morphology of main body organs of mice treated with apigenin alone and
in combination with ceftazidime for 14 consecutive days in comparison
with the negative control. Control group ; a = heart; b = liver; c = spleen;
d = lung; e = kidney; f = stomach. Treated group ; g = heart; h = liver; i =
spleen; j = lung; k = kidney; l = stomach.

Table 4.5 Effects of intraperitoneally administered apigenin alone and in combination with ceftazidime for 14 days on relative weight of the selected main organ (per 100g body weight) in mice. Group 1 = apigenin 20 mg/kg BW/day, Group 2 =apigenin 80 mg/kg BW/day, Group 3 = apigenin 20 mg/kg BW/day plus ceftazidime 160 mg/kg BW/day, Group 4 = apigenin 80 mg/kg BW/day plus ceftazidime 640 mg/kg BW/day, Cefta = Ceftazidime (640 mg/kg BW).

Organ (g)	Control (0.2%DMSO)	Control Cefta	Group 1	Group 2	Group 3	Group 4
Heart	0.0019 ± 0.0001	0.0018 ± 0.0001	0.0019 ± 0.0001	0.0017 ± 0.0001	0.0017 ± 0.0001	0.0094 ± 0.0049
Liver	0.0179 ± 0.0014	0.0152 ± 0.0016	0.0176 ± 0.0003	0.0187 ± 0.0006	0.0179 ± 0.0014	0.0176 ± 0.0005
Spleen	0.0015 ± 0.0001	0.0021 ± 0.0007	0.0026 ± 0.0008	0.0024 ± 0.0006	0.0020 ± 0.0005	0.0019 ± 0.0002
Lung	0.0026 ± 0.0001	0.0027 ± 0.0001	0.0028 ± 0.0003	0.0024 ± 0.0003	0.0026 ± 0.0001	0.0027 ± 0.0002
Kidney	0.0065 ± 0.0007	0.0054 ± 0.0008	0.0060 ± 0.0008	0.0064 ± 0.0006	0.0061 ± 0.0004	0.0062 ± 0.0007
Stomach	0.0084 ± 0.0016	0.0089 ± 0.0036	0.0139 ± 0.0047	0.0081 ± 0.0010	0.0083 ± 0.0012	0.0081 ± 0.0012

4.1.3.2 Subacute toxicity effects of the apigenin on blood chemistry and hematology in mice

At the end of the experiments, blood samples were collected by tail under thiopental sodium anesthesia from 9.00 to 10.00 AM. and partly used for haematology.

The blood chemistry marker assayed indicated that there were no statistical differences of all groups of mice treated with apigenin alone or in combination with ceftazidime (Table 4.6).

Results of haematology demonstrated that there were no significant changes of haematological parameters in post-treatment groups compared to pre-treatment groups.

Table 4.6 Blood chemistry and hematological studies on mice before and after subacute treatment with apigenin alone and in combination with ceftazidime for 14 days. Group 1 = apigenin 20 mg/kg BW/day, Group 2 = apigenin 80 mg/kg BW/day, Group 3 = apigenin 20 mg/kg BW/day plus ceftazidime 160 mg/kg BW/day, Group 4 = apigenin 80 mg/kg BW/day plus ceftazidime 640 mg/kg BW/day, Cefta = Ceftazidime (640 mg/kg BW).

Parameter	Control (0.20%DMSO)		Control Cefta.		Group 1		Group 2		Group 3		Group 4	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
AST (U/L)	312.5±80.0	615.1±189.1	335.0±107.0	438.7±169.0	261.0±112.5	572.0±234.7	307.2±88.3	373.2±124.5	598.0±124.5	620.0±176.4	384.0±102	525.0±100
BUN (mg/dl)	27.2±3.2	22.5±1.6	17.0±2.5	18.2±7.1	25.3±2.6	24.0±1.9	29.0±2.1	21.3±2.8	25.9±2.9	18.9±5.6	18.6±3.0	14.2±2.4
FBS(mg/dl)	160.7±6.6	191.4±15.7	138.7±20.2	193.7±5.1	138.0±9.1	230.0±31.4	144.0±13.5	165.2±15.0	173.0±16.0	179.0±16.9	144.0±16.6	169.8±18.6
Cholesterol(mg/dl)	140.3±21.6	135.7±22.3	212.5±7.5	276.2±40.2	105.0±31.3	118.0±7.0	115.8±27.9	88.6±8.5	178.0±30.0	236.2±45.0	164.0±30.4	229.0±9.1
WBC (x10^3//µ L)	9.6±0.8	8.0±1.7	9.6±0.9	4.8±1.8	10.4±0.63	5.7±1.8	12.6±1.2	12.5±1.3	9.8±0.7	8.0±0.7	7.5±0.9	5.9±0.9
RBC (x10^6 /µL)	7.5±1.1	7.2±1.1	7.7±1.2	7.2±1.1	8.5±0.0	7.3±0.5	8.4±0.5	7.8±0.1	8.0±0.6	7.9±0.6	8.9±0.3	6.6±1.0
Hb (g/dl)	15.5±0.5	13.0±1.7	16.0±0.5	11.0±2.4	14.3±0.3	12.0±1.5	14.4±0.9	13.2±0.3	16.8±0.3	14.8±0.7	16.0±0.31	14.9±0.8
Hct (%)	48.0±1.7	37.2±5.8	50.0±1.8	31.8±6.8	43.6±1.3	38.6±4.0	44.0±3.1	40.6±0.6	52.2±1.2	43.8±2.8	49.6±1.9	47.2±3.1
MCV (fL)	51.5±0.2	50.7±1.0	51.0±0.3	51.0±1.2	50.5±1.4	50.4±1.6	51.1±1.1	51.2±0.6	52.7±0.7	54.3±1.9	52.1±0.5	51.0±0.3

Mean ± SEM (n=5), *$p < 0.05$ (Student's t–test). Significant relative to pre-treatment value.

4.1.4 Subacute toxicity study of luteolin

4.1.4.1 Effects of the luteolin on weight changes and main organs in mice

There were no significant differences in the relative growth changes of body and organ weights of all treated mice in comparison with the control (Figure 4.7) (Table 4.7). In addition, the morphology of organs of treated mice exhibited normal appearance compared to the control organs.
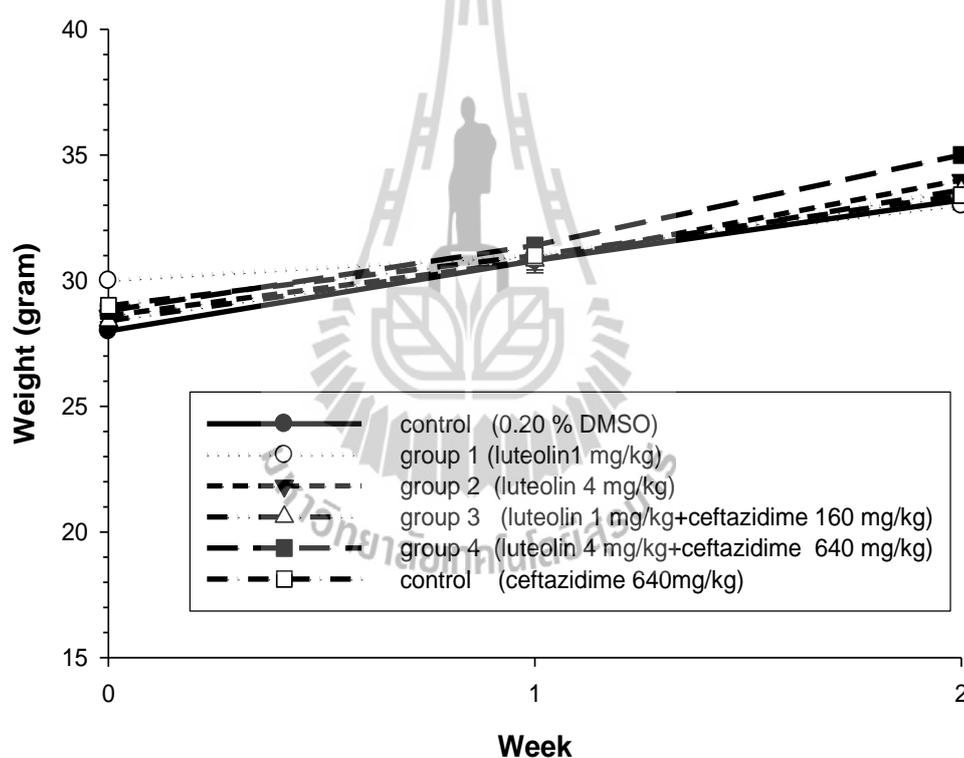
Figure 4.7 Body weights of mice treated with luteolin alone and in combination with ceftazidime for 14 consecutive days in comparison with the negative control.
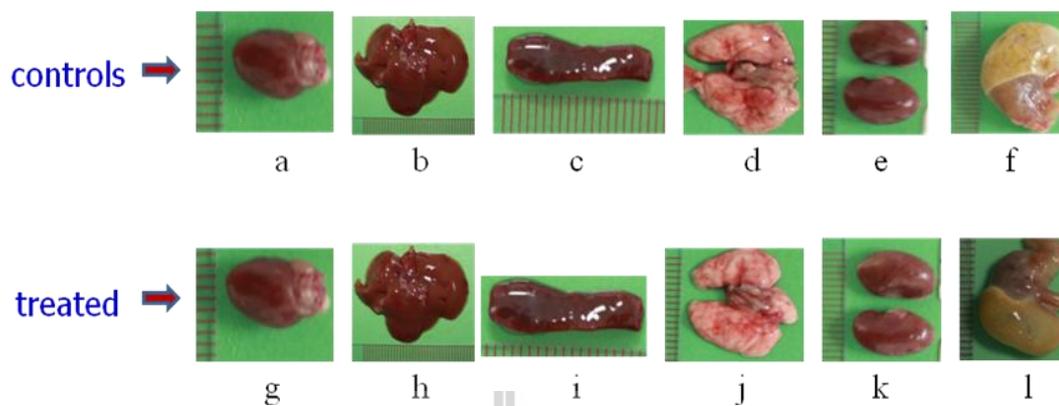
Figure 4.8 Morphology of main body organs of mice treated with luteolin alone and in combination with ceftazidime for 14 consecutive days in comparison with the negative control. Control group ; a = heart; b = liver; c = spleen; d = lung; e = kidney; f = stomach. Treated group ; g = heart; h = liver; i = spleen; j = lung; k = kidney; l = stomach.

Table 4.7 Effects of intraperitoneally administered luteolin alone and in combination with ceftazidime for 14 days on relative weight of the selected main organ (per 100g body weight) in mice. Group 1 = luteolin 1 mg/kg BW/day, Group 2 = luteolin 4 mg/kg BW/day, Group 3 = luteolin 1 mg/kg BW/day plus ceftazidime 160 mg/kg BW/day, Group 4 = luteolin 4 mg/kg BW/day plus ceftazidime 640 mg/kg BW/day, Cefta = Ceftazidime (640 mg/kg BW).

Organ (g)	Control (0.2% DMSO)	Control Cefta	Group 1	Group 2	Group 3	Group 4
Heart	0.0014 ± 0.0001	0.0014±0.0000	0.0016 ± 0.0001	0.0014 ± 0.0001	0.0015 ± 0.0001	0.0015 ± 0.0002
Liver	0.0169 ± 0.0017	0.0159±0.0012	0.0199 ± 0.0005	0.0182 ± 0.0012	0.0180 ± 0.0012	0.0168 ± 0.0017
Spleen	0.0011 ± 0.0001	0.0012±0.0001	0.0013 ± 0.0001	0.0012 ± 0.0002	0.0027 ± 0.0006	0.0013 ± 0.0000
Lung	0.0029 ± 0.0001	0.0027±0.0001	0.0025 ± 0.0001	0.0026 ± 0.0001	0.0025 ± 0.0001	0.0026 ± 0.0001
Kidney	0.0046 ± 0.0014	0.0040±0.0009	0.0054 ± 0.0004	0.0054 ± 0.0006	0.0053 ± 0.0011	0.0050 ± 0.0011
Stomach	0.0060 ± 0.0006	0.0063±0.0010	0.0209 ± 0.0059	0.0050 ±0.0004	0.0047 ± 0.0004	0.0046 ± 0.0005

4.1.4.2 Effects of the luteolin on blood chemistry and hematology in mice

The blood chemistry and heamatology marker assayed showed that there were no significant differences of all post-treatment groups compared to pre-treatment groups (Table 4.8).

Table 4.8 Blood chemistry and hematological studies on mice before and after subacute treatment with luteolin alone and in combination with ceftazidime for 14 days. Group 1 = luteolin 1 mg/kg BW/day, Group 2 = luteolin 4 mg/kg BW/day, Group 3 = luteolin 1 mg/kg BW/day plus ceftazidime 160 mg/kg BW/day, Group 4 = luteolin 4 mg/kg BW/day plus ceftazidime 640 mg/kg BW/day, Cefta = Ceftazidime (640 mg/kg BW).

Parameter	Control (0.2%DMSO)		Control Cefta.		Group 1		Group 2		Group 3		Group 4	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
AST (U/L)	207.2±33.6	209.2±33.7	207.3±18.4	209.0±19.8	369.6±108.4	452.0±61.3	349.8±77.2	731.0±377	272.0±154	703.3±109	207.3±18.4	249.3±48.4
BUN (mg/dl)	23.5±0.6	23.7±1.1	22.0±2.5	21.0±2.0	20.3±1.2	23.4±2.5	23.8±1.7	22.0±2.78	18.2±1.3	23.8±1.7	22.0±2.54	25.6±4.0
FBS(mg/dl)	137.5±9.8	176.7±22.4	175.6±3.7	176.6±4.4	157.2±9.1	175.2±15.8	194.4±7.1	199.8±4.4	133.3±50.9	190.0±8.6	154.0±11.0	176.6±4.4
Cholesterol(mg/dl)	85.2±2.7	92.0±4.1	106.6±8.8	112.3±7.2	183.6±27.1	218.4±28.0	171.0±34.1	201.8±33.9	146.6±13.7	115.0±8.6	134.0±26.6	105.0±7.6
WBC (x10^3//µ L)	9.6±0.6	9.7±0.7	9.6±0.8	5.0±1.7	14.5±1.8	13.9±1.7	12.4±0.5	10.70±1.1	9.9±0.1	6.3±1.2	10.3±0.7	8.0±2.2
RBC (x10^6 /µL)	9.0±0.1	8.9±0.4	7.5±1.2	7.2±1.1	9.0±0.2	8.4±0.3	8.7±0.1	8.0±0.2	7.8±0.7	6.6±0.8	8.5±0.7	7.1±0.6
Hb (g/dl)	15.5±0.2	14.5±0.2	15.5±0.5	13.0±1.7	15.4±0.4	14.8±0.3	15.4±0.2	13.8±0.5	15.6±1.2	12.0±1.1	15.6±0.6	12.6±1.2
Hct (%)	44.7±1.2	44.0±0.9	48.0±1.7	37.3±5.8	45.4±1.5	39.0±2.6	46.6±0.8	41.8±1.5	48.6±4.4	39.0±4.0	50.3±2.4	39.3±3.1
MCV (fL)	54.5±0.8	54.2±0.7	51.5±0.2	50.7±1.0	49.8±0.3	50.4±0.5	53.2±1.3	51.9±1.1	52.9±1.3	60.6±1.8	52.0±0.6	55.0±0.6

Mean ± SEM (n=5), *p < 0.05 (Student's t–test). Significant relative to pre-treatment value.

4.1.5 Subacute toxicity study of galangin

4.1.5.1 Effects of the galangin on weight changes and main organs in mice

There was no significant difference in either the relative growth rate measured by living body weight (Figure 4.9) or the relative weight of the selected main body organs of all groups of treated mice, when compared to the control (Table 4.9). The patho-histology of the heart, liver, spleen, kidney, and stomach all revealed normal appearance compared to the control organs.
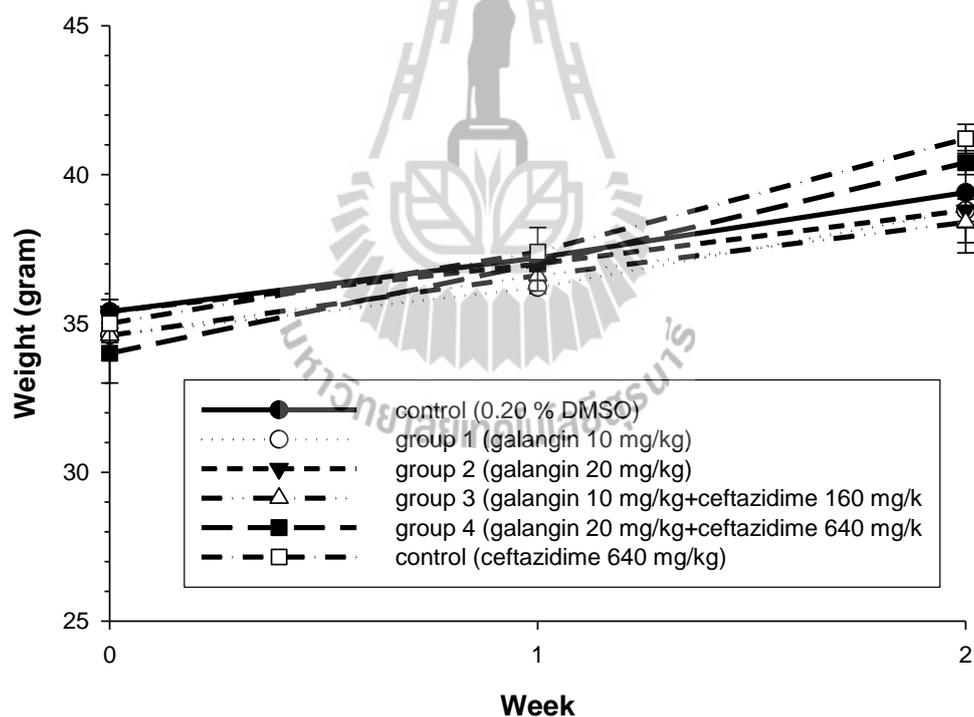
Figure 4.9 Body weights of mice treated with galangin alone and in combination with ceftazidime for 14 consecutive days in comparison with the negative control.
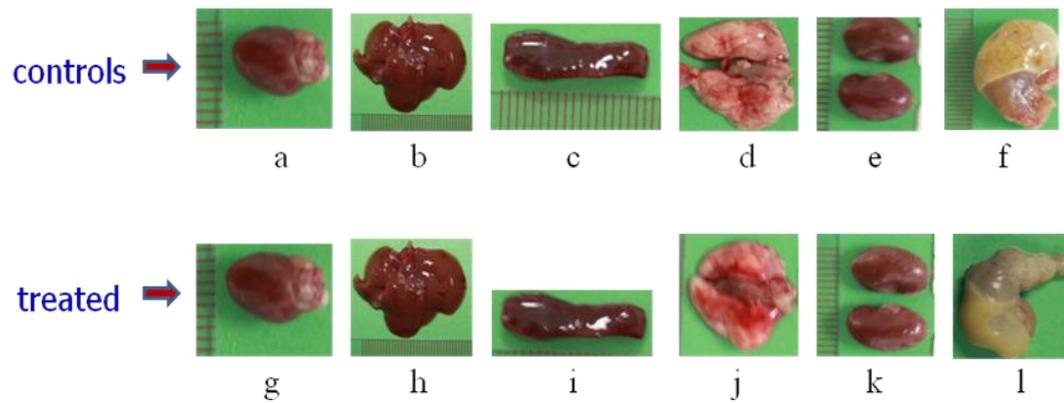
Figure 4.10 Morphology of main body organs of mice treated with galangin alone and in combination with ceftazidime for 14 consecutive days in comparison with the negative control. Control group ; a = heart; b = liver; c = spleen; d = lung; e = kidney; f = stomach. Treated group ; g = heart; h = liver; i = spleen; j = lung; k = kidney; l = stomach.

Table 4.9 Effects of intraperitoneally administered galangin alone and in combination with ceftazidime for 14 days on relative weight of the selected main organ (per 100g body weight) in mice. Group 1 = galangin 10 mg/kg BW/day, Group 2 = galangin 20 mg/kg BW/day, Group 3 = galangin 10 mg/kg BW/day plus ceftazidime 160 mg/kg BW/day, Group 4 = galangin 20 mg/kg BW/day plus ceftazidime 320 mg/kg BW/day, Cefta = Ceftazidime (320 mg/kg BW).

Organ (g)	Control (0.2%DMSO)	Control Cefta	Group 1	Group 2	Group 3	Group 4
Heart	0.0049 ± 0.0005	0.0045 ± 0.0004	0.0050 ± 0.0002	0.0052 ± 0.0002	0.0046 ± 0.0001	0.0045 ± 0.0004
Liver	0.0514 ± 0.0029	0.0512 ± 0.0048	0.0498 ± 0.0032	0.0514 ± 0.0048	0.0474 ± 0.0012	0.0523 ± 0.0035
Spleen	0.0041 ± 0.0005	0.0041 ± 0.0005	0.0045 ± 0.0004	0.0063 ± 0.0011	0.0040 ± 0.0005	0.0042 ± 0.0006
Lung	0.0062 ± 0.0008	0.0061 ± 0.0007	0.0062 ± 0.0001	0.0062 ± 0.0003	0.0061 ± 0.0001	0.0062 ± 0.0003
Kidney	0.0172 ± 0.0033	0.0170 ± 0.0034	0.0170 ± 0.0014	0.0163 ± 0.0023	0.0165 ± 0.0023	0.0177 ± 0.0027
Stomach	0.0199 ± 0.0035	0.0199 ± 0.00*5	0.0133 ± 0.0010	0.0223 ± 0.0037	0.0286 ± 0.0043	0.0231 ± 0.0042

4.1.5.2 Effects of the galangin on blood chemistry and hematology in mice

The blood chemistry marker assayed exhibited that there was a small decrease of AST and cholesterol level after treatment with galangin alone or in combination with ceftazidime compared to pre-treatment groups. However, the AST and cholesterol reduction were not significant difference at $p < 0.05$. There was no clear dose-dependent or significant changes ($p < 0.05$) of BUN and FBS between pre- and post-treatment in all groups (Table 4.10).

Results of haematology demonstrated that there were no significant change of WBC, RBC, Hb, and MCV in post-treatment groups compared to pre-treatment groups. Conversely, the post-treatment Hct level exhibited clear dose-dependent and significant reduction ($p < 0.05$) with increasing galangin and ceftazidime doses in comparison with pre-treatment in all groups (Table 4.10).

Table 4.10 Blood chemistry and hematological studies on mice before and after subacute treatment with galangin alone and in combination with ceftazidime for 14 days. Group 1 = galangin 10 mg/kg BW/day, Group 2 = galangin 20 mg/kg BW/day, Group 3 = galangin 10 mg/kg BW/day plus ceftazidime 160 mg/kg BW/day, Group 4 = galangin 20 mg/kg BW/day plus ceftazidime 320 mg/kg BW/day, Cefta = Ceftazidime (320 mg/kg BW).

Parameter	Control (0.2% DMSO)		Control Cefta.		Group 1		Group 2		Group 3		Group 4	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
AST (U/L)	207.2±33.6	209.2±33.7	118.4±13.3	118.2±13.4	150.8±4.0	149.4±3.9	139.2±8.2	109.4±12.3	153.0±24.2	111.4±5.9	118.4±13.3	97.2±4.4
BUN (mg/dl)	23.5±0.6	23.7±1.1	20.4±2.1	16.7±0.4	21.4±1.4	15.8±2.1	16.5±1.0	22.0±4.1	25.3±1.4	24.4±1.2	22.4±2.1	16.7±0.4
FBS(mg/dl)	137.5±9.8	176.7±22.4	103.2±25.9	103.8±3.4	108.4±19.13	154.0±8.2	109.6±22.7	87.2±10.1	114.8±20.9	115.8±5.0	103.2±25.9	139.8±3.4
Cholesterol(mg/dl)	85.2±2.7	92.0±4.1	185.2±12.5	145.2±9.5	166.4±2.23	164.2±1.5	187.2±16.4	141.0±7.7	182.0±14.8	174.0±14.7	185.2±12.5	145.2±9.5
WBC (x10^3//µ L)	9.6±0.6	9.7±0.7	6.5±0.3	5.6±0.9	8.0±0.5	7.1±0.7	7.2±1.1	7.60±1.7	5.9±0.5	4.7±0.3	6.5±0.3	5.6±0.9
RBC (x10^6/µL)	9.0±0.1	8.9±0.4	8.2±0.2	7.2±0.3	8.9±0.7	7.7±0.1	8.5±0.2	8.40±0.2	8.4±0.4	7.4±0.2	8.2±0.2	7.2±0.3
Hb (g/dl)	15.5±0.2	14.5±0.2	14.5±0.8	12.2±0.6	13.9±0.6	12.6±0.2	12.7±0.2	12.0±0.2	14.0±0.7	12.6±0.6	14.5±0.8	12.2±0.6
Hct (%)	44.7±1.2	44.0±0.9	53.1±1.7	53.2±2.4	**54.8±1.3**	**45.5±0.6***	**51.5±2.4**	**33.2±1.6***	**51.3±0.6**	**38.8±2.0***	**53.1±1.7**	**36.7±2.4***
MCV (fL)	54.5±0.8	54.2±0.7	53.1±1.7	53.2±0.5	50.7±5.7	57.4±0.6	53.5±1.3	50.4±1.4	53.7±2.0	51.8±1.6	53.1±1.7	49.6±0.5

Mean ± SEM (n=5), *$p < 0.05$ (Student's t–test). Significant relative to pre-treatment value.

4.2 Subchronic toxicity test of some flavonoids

4.2.1 Subchronic toxicity test of quercetin

4.2.1.1 Effects of the quercetin on weight changes and main organs in mice

There was no significant difference in either the relative growth rate measured by living body weight (Figure 4.11) or the relative weight of the selected main body organs of all groups of treated mice for 90 consecutive days , when compared to the control (Table 4.11). The patho-histology of all organs showed normal appearance compared to the control organs.
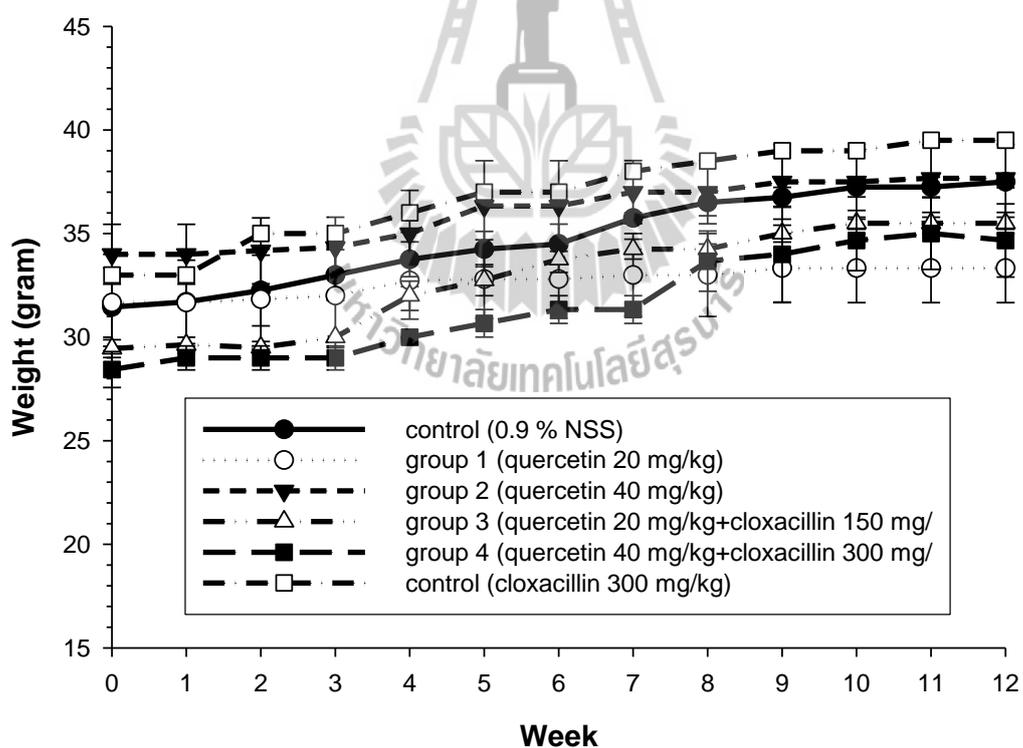
Figure 4.11 Body weights of mice treated with quercetin alone and in combination with cloxacillin for 90 consecutive days in comparison with the negative control.
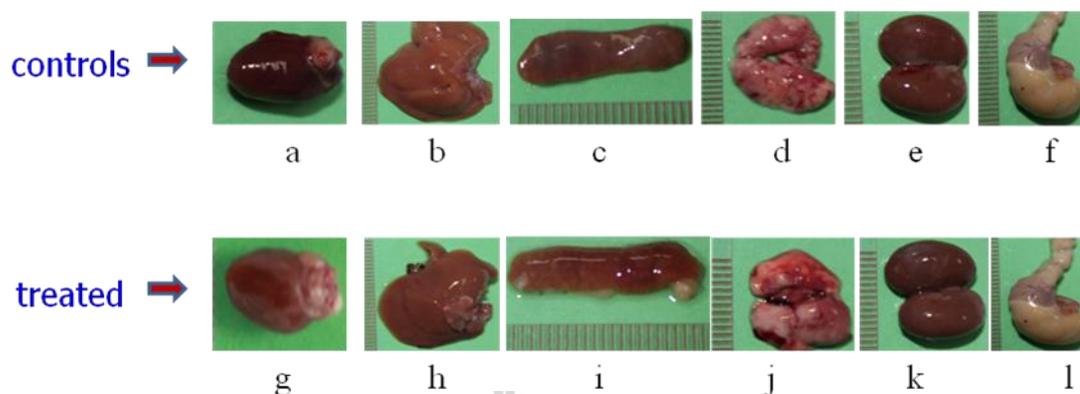
Figure 4.12 Morphology of main body organs of mice treated with quercetin alone and in combination with cloxacillin for 90 consecutive days in comparison with the negative control. Control group ; a = heart; b = liver; c = spleen; d = lung; e = kidney; f = stomach. Treated group ; g = heart; h = liver; i = spleen; j = lung; k = kidney; l = stomach.
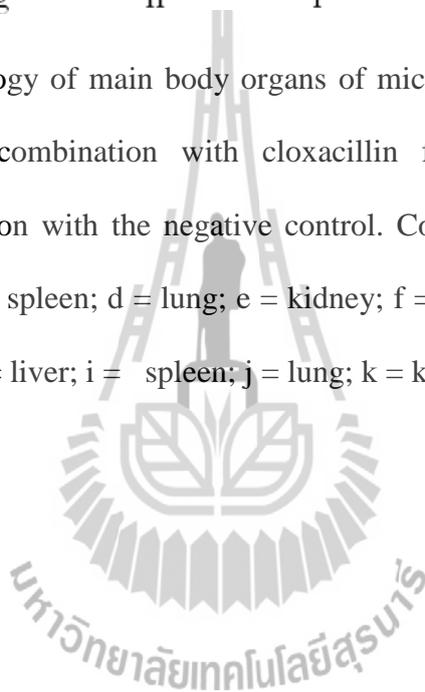
Table 4.11 Effects of intraperitoneally administered quercetin alone and in combination with cloxacillin for 90 days on relative weight of the selected main organ (per 100g body weight) in mice. Group 1 = quercetin 20 mg/kg BW/day, Group 2 = quercetin 40 mg/kg BW/day, Group 3 = quercetin 20 mg/kg BW/day plus cloxacillin 150 mg/kg BW/day, Group 4 = quercetin 40 mg/kg BW/day plus cloxacillin 300 mg/kg BW/day, Cloxa = Cloxacillin (300 mg/kg BW).

Organ (g)	Control (NSS)	Control Cloxa	Group 1	Group 2	Group 3	Group 4
Heart	0.0019 ± 0.0001	0.0016±0.0001	0.0017 ± 0.0002	0.0018 ± 0.0002	0.0019 ± 0.0001	0.0017 ± 0.0000
Liver	0.0189 ± 0.0011	0.0173±0.0012	0.0162 ± 0.0019	0.0118 ± 0.0047	0.0213 ± 0.0010	0.0196 ± 0.0011
Spleen	0.0017 ± 0.0001	0.0018±0.0001	0.0020 ± 0.0002	0.0063 ± 0.0034	0.0016 ± 0.0001	0.0051 ± 0.0034
Lung	0.0027 ± 0.0001	0.0024±0.0001	0.0029 ± 0.0002	0.0025 ± 0.0001	0.0022 ± 0.0003	0.0023 ± 0.0001
Kidney	0.0063 ± 0.0006	0.0055±0.0006	0.0068 ± 0.0022	0.0054 ± 0.0004	0.0317 ± 0.0254	0.0060 ± 0.0008
Stomach	0.0063 ± 0.0013	0.0071±0.0009	0.0066 ± 0.0013	0.0086 ± 0.0024	0.0083 ± 0.0008	0.0094 ± 0.0022

4.2.1.2 Subchronic toxicity effects of the quercetin on blood chemistry and hematology in mice

Subchronic toxicity study indicated that AST and BUN levels slightly decreased except for the group treated with 40 mg/kg BW/day quercetin plus 300 mg/kg BW/day cloxacillin, whereas FBS level decreased in all post-treatment groups compared to pre-treatment groups. However, there was no significant changes ($p<0.05$) of the levels between pre- and post-treatment in all groups (Table 4.12). Cholesterol level in post-treatment group exhibited a significant reduction in the group treated with 20 mg/kg BW/day quercetin plus 150 mg/kg BW/day cloxacillin compared to pre-treatment group.

In subchronic toxicity test, results of hematology demonstrated that there were significant reductions of Hb (groups 1 and 2), Hct (groups 2 and 3) levels in post-treatment groups compared to pre-treatment groups. Conversely, MCV and RBC levels did not change in all groups. Also, the post-treatment WBC level exhibited no significant increase in comparison with pre-treatment in all groups (Table 4.12).

Table 4.12 Blood chemistry and hematological studies on mice before and after subchronic treatment with quercetin alone and in combination with cloxacillin for 90 days. Group 1 = quercetin 20 mg/kg BW/day, Group 2 = quercetin 40 mg/kg BW/day, Group 3 = quercetin 20 mg/kg BW/day plus cloxacillin 150 mg/kg BW/day, Group 4 = quercetin 40 mg/kg BW/day plus cloxacillin 300 mg/kg BW/day.BW/day, Cloxa = Cloxacillin (300 mg/kg BW).

Parameter	Control (NSS)		Control Cloxa.		Group 1		Group 2		Group 3		Group 4	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
AST (U/L)	235.2±2.9	235.5±3.3	133.5±7.3	173.2±31.8	190.2±49.2	128.5±7.5	181.7±29.2	147.0±16.6	257.7±92.0	249.0±45.4	185.6±52.2	361.3±118
BUN (mg/dl)	23.5±0.6	23.7±0.6	26.5±3.3	25.5±1.0	33.7±2.5	33.0±2.6	33.0±1.9	27.9±1.9	37.2±10.8	20.4±2.9	19.0±1.8	23.5±3.1
FBS(mg/dl)	138.0±9.3	142.2±7.6	186.0±13.6	216.7±3.0	151.7±7.1	153.0±9.1	180.0±22.4	180.5±40.9	168.5±4.9	224.2±22.2	195.3±27.1	224.3±41.3
Cholesterol(mg/dl)	86.5±4.8	91.5±4.6	111.7±9.5	93.2±9.9	100.7±5.0	85.5±6.1	101.2±6.3	88.0±11.5	**110.2±8.8**	**90.7±7.9***	107.0±20.8	100.0±5.2
WBC (x10^3//µ L)	9.0±0.8	9.2±0.2	7.9±0.5	5.9±1.2	5.60±0.4	6.3±0.9	4.6±0.1	5.8±0.6	5.5±1.4	6.8±0.4	8.2±0.3	8.4±0.2
RBC (x10^6 /µL)	9.0±0.1	9.2±0.2	7.3±0.3	6.2±0.4	5.6±04	6.3±0.9	5.8±04	6.0±0.9	5.5±1.4	6.8±0.4	8.2±0.3	7.9±2.0
Hb (g/dl)	15.5±0.2	15.5±0.6	12.5±0.6	11.7±1.3	**15.5±0.7**	**12.7±0.6***	**13.5±0.5**	**11.0±0.0***	11.5±0.9	11.7±0.6	12.0±1.0	12.3±1.6
Hct (%)	44.7±1.2	44.0±0.8	37.7±1.3	30.0±0.5	43.6±2.0	43.7±2.4	**40.2±1.9**	**34.2±1.0***	**38.2±1.7**	**34.0±2.6***	37.3±1.7	35.6±6.3
MCV (fL)	53.2±0.4	53.5±0.2	51.5±1.1	47.5±0.6	50.5±0.6	50.0±1.0	49.9±0.3	53.6±3.2	47.4±1.9	48.9±0.5	51.4±0.4	49.2±1.5

Mean ± SEM (n=5), **p* < 0.05 (Student's *t*–test). Significant relative to pre-treatment value.

4.2.2 Subchronic toxicity test of baicalein

4.2.2.1 Effects of the baicalein on weight changes and main organs in mice

No significant difference was found in the relative growth rate and the relative weight of selected body organs of treated mice in all groups (Figure 4.13 and Table 4.13). The morphology of all organs revealed normal appearance compared to the control groups.
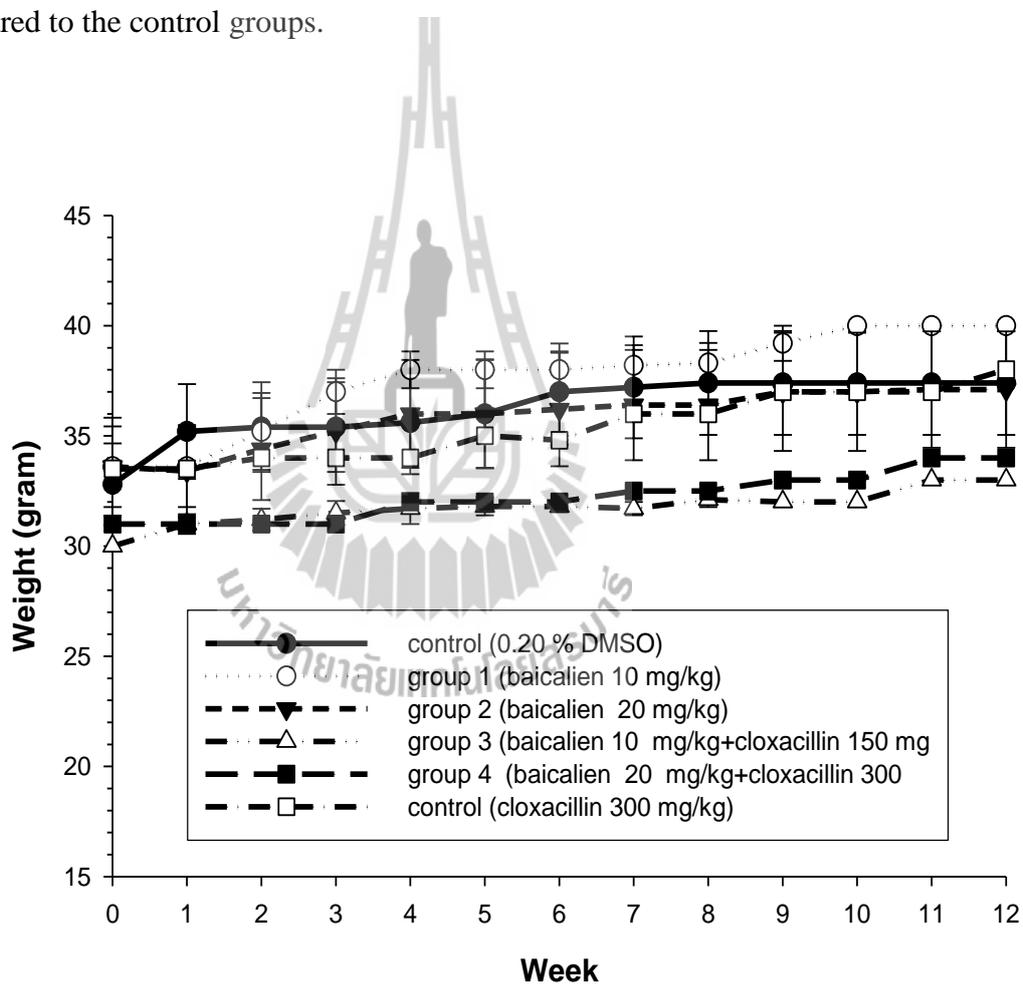

Figure 4.13 Body weights of mice treated with baicalein alone and in combination with cloxacillin for 90 consecutive days in comparison with the negative control.
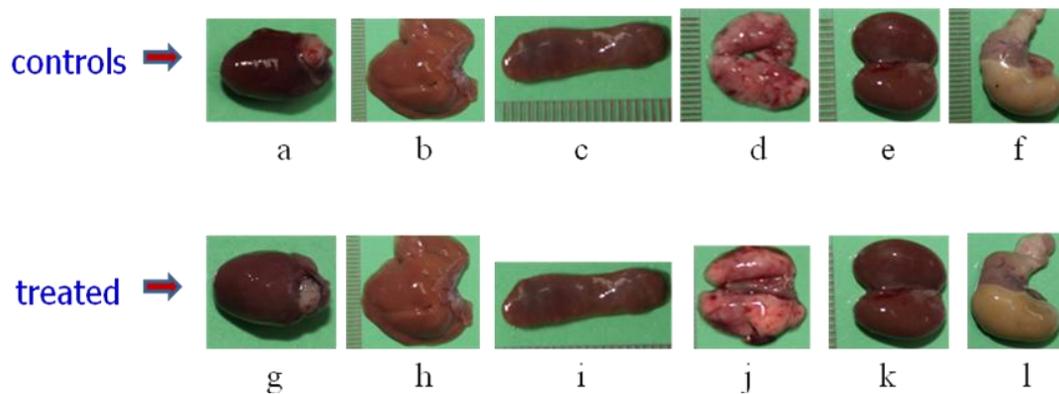
Figure 4.14 Morphology of main body organs of mice treated with baicalein alone and in combination with cloxacillin for 90 consecutive days in comparison with the negative control. Control group ; a = heart; b = liver; c = spleen; d = lung; e = kidney; f = stomach. Treated group ; g = heart; h = liver; i = spleen; j = lung; k = kidney; l = stomach.

Table 4.13 Effects of intraperitoneally administered baicalein alone and in combination with cloxacillin for 90 days on relative weight of the selected main organ (per 100g body weight) in mice. Group 1 = baicalein 10 mg/kg BW/day, Group 2 = baicalein 20 mg/kg BW/day, Group 3 = baicalein 10 mg/kg BW/day plus cloxacillin 150 mg/kg BW/day, Group 4 = baicalein 20 mg/kg BW/day plus cloxacillin 300 mg/kg BW/day, Cloxa = Cloxacillin (300 mg/kg BW).

Organ (g)	Control (0.2%DMSO)	Control Cloxa	Group 1	Group 2	Group 3	Group 4
Heart	0.0017 ± 0.0001	0.0017 ± 0.0000	0.0016 ± 0.0001	0.0018 ± 0.0001	0.0018 ± 0.0003	0.0016 ± 0.0001
Liver	0.0187 ± 0.0007	0.0207 ± 0.0021	0.0203 ± 0.0021	0.0200 ± 0.0018	0.0193 ± 0.0026	0.0160 ± 0.0017
Spleen	0.0016 ± 0.0001	0.0046 ± 0.0029	0.0046 ± 0.0029	0.0019 ± 0.0001	0.0022 ± 0.0005	0.0019 ± 0.0001
Lung	0.0029 ± 0.0001	0.0033 ± 0.0002	0.0033 ± 0.0002	0.0032 ± 0.0002	0.0029 ± 0.0004	0.0026 ± 0.0003
Kidney	0.0060 ± 0.0005	0.0058 ± 0.0007	0.0060 ± 0.0006	0.0062 ± 0.0011	0.0047 ± 0.0003	0.0055 ± 0.0006
Stomach	0.0130 ± 0.0012	0.0138 ± 0.0019	0.0135 ± 0.0022	0.0121 ± 0.0015	0.0137 ± 0.0017	0.0146 ± 0.0023

4.2.2.2 Effects of the baicalein on blood chemistry and hematology in mice

The significant increase of BUN level was found in mice treated with 20 mg/kg BW/day baicalein alone or plus 300 mg/kg BW/day cloxacillin, whereas cholesterol level significantly decreased after treatment with 20 mg/kg BW/day baicalein alone and in combination with cloxacillin 300 mg/kg BW/day when compared to the pre-treatment group. However, there was no significant change of FBS and AST between pre-and post-treatment in all groups.

The values of WBC and Hb in group treated with 20 mg/kg BW/day baicalein plus 300 mg/kg BW/day cloxacillin were significantly lower than those of the pre-treatment groups. RBC count decreased significantly in most all groups except in group that treated with 300 mg/kg BW cloxacillin alone and 10 mg/kg BW/day baicalein plus 150 mg/kg BW/day cloxacillin, when compared to the pre-treatment groups. Hct level reduced in mice treated with 0.2% DMSO (control group), 20 mg/kg BW/day baicalein, and 10 mg/kg BW/day baicalein plus 150 mg/kg BW/day cloxacillin compare to pre-treatment group. Moreover, the post-treatment MCV level of mice treated with 10 and 20 mg/kg BW/day baicalein exhibited significant increase in comparison with pre-treatment group (Table 4.14).

Table 4.14 Blood chemistry and hematological studies on mice before and after subchronic treatment with baicalein alone and in combination with cloxacillin for90days. Group1=baicalein10mg/kgBW/day,Group2= baicalein 20 mg/kg BW/day, Group 3 = baicalein 10 mg/kg BW/day plus cloxacillin 150 mg/kg BW/day, Group 4 = baicalein 20 mg/kg BW/day plus cloxacillin 300 mg/kg BW/day, Cloxa = Cloxacillin (300 mg/kg BW).

Parameter	Control (0.20%DMSO)		Control Cloxa.		Group 1		Group 2		Group 3		Group 4	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
AST (U/L)	411.8±86.0	552.4±73.7	300.5±34.7	415.5±49.3	416.5±61.5	416.0±61.6	509.8±86.4	525.0±89.0	513.3±35.2	528.6±8.8	528.0±113.9	583.2±66.4
BUN (mg/dl)	28.4±2.1	23.1±3.2	25.0±1.6	25.8±4.5	24.2±2.6	40.25±7.6	**22.8±2.0**	**27.0±2.6***	12.6±1.2	13.0±1.0	**15.6±2.2**	**28.5±2.4***
FBS(mg/dl)	162.2±9.4	157.2±10.2	160.0±3.6	182.2±18.1	118.0±10.5	136.2±13.9	176.8±19.8	165.0±12.9	210.0±10.0	144.3±8.7	172.0±18.5	146.4±13.4
Cholesterol(mg/dl)	140.1±20.6	96.0±11.9	129.0±8.0	116.5±13.7	110.5±19.9	100.0±27.1	**200.2±28.5**	**95.0±4.7***	190.0±15.2	190.3±15.0	**156.0±19.3**	**92.8±9.3***
WBC (x10^3//µ L)	9.7±0.9	9.0±0.8	9.6±0.6	24.3±16.5	8.55±1.33	10.2±2.8	7.8±1.4	6.5±1.7	4.8±1.0	4.8±1.0	**12.9±1.9**	**7.8±1.7***
RBC (x10^6 /µL)	**9.2±0.1**	**7.8±0.2***	9.05±0.1	8.9±0.4	**7.4±1.0**	**5.5±0.9***	**8.8±0.1**	**7.6±0.2***	8.5±0.1	7.7±0.3	**9.0±0.2**	**6.7±0.1***
Hb (g/dl)	16.2±0.2	15.4±0.3	15.5±0.2	14.5±0.2	15.2±0.8	10.2±1.2	13.7±0.6	13.8±0.3	15.0±0.0	14.6±0.3	**16.0±0.5**	**13.2±0.4***
Hct (%)	**49.1±1.6**	**39.9±1.1***	49.2±1.3	44.0±0.9	45.0±2.1	45.0±2.0	**44.4±0.3**	**40.2±1.0***	46.0±1.0	39.6±4.2	**47.6±1.2**	**36.2±1.6***
MCV (fL)	52.0±0.6	51.7±0.3	54.5±0.8	52.9±0.7	**49.9±0.5**	**52.1±0.8***	**49.8±0.7**	**52.2±0.3***	53.8±0.7	53.6±1.2	53.0±1.1	53.3±1.5

Mean ± SEM (n=5), *p < 0.05 (Student's t–test). Significant relative to pre-treatment value.

4.2.3 Subchronic toxicity test of apigenin

4.2.3.1 Effects of the apigenin on weight changes and main organs in mice

There were no remarkable change in the relative growth rate measured by body weight and the relative weight of the selected organs of all groups of treated mice, compared to the control (Figure 4.15 and Table 4.15). Their morphology appearances were found normal compared to the control.
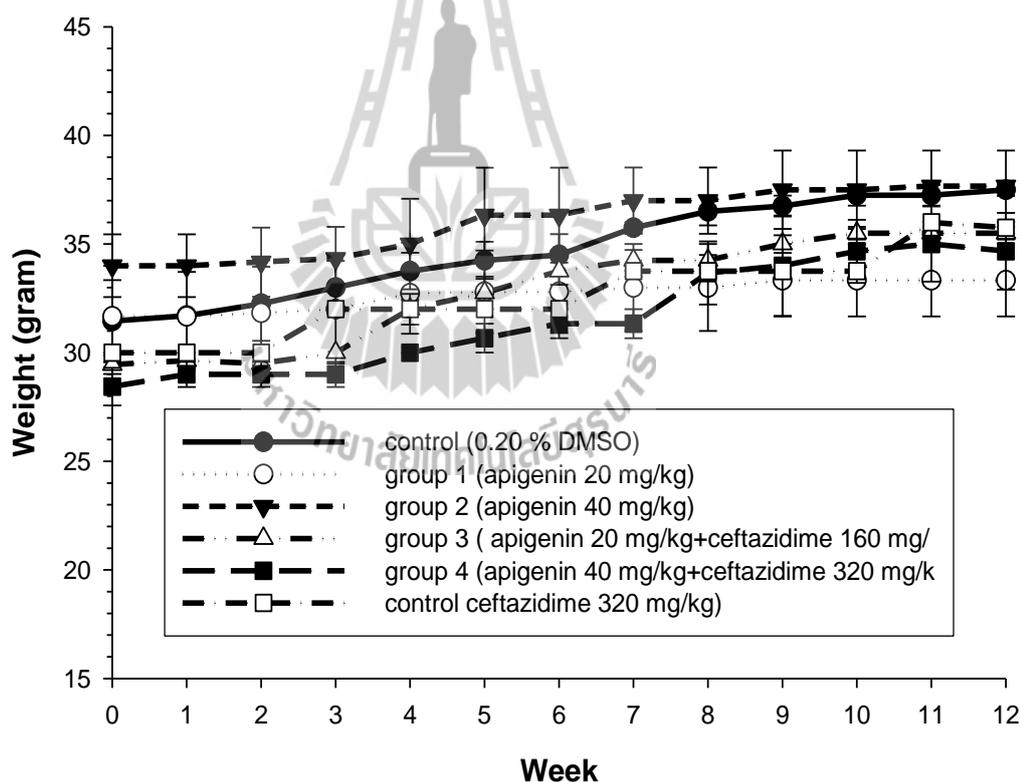
Figure 4.15 Body weights of mice treated with apigenin alone and in combination with ceftazidime for 90 consecutive days in comparison with the negative control.
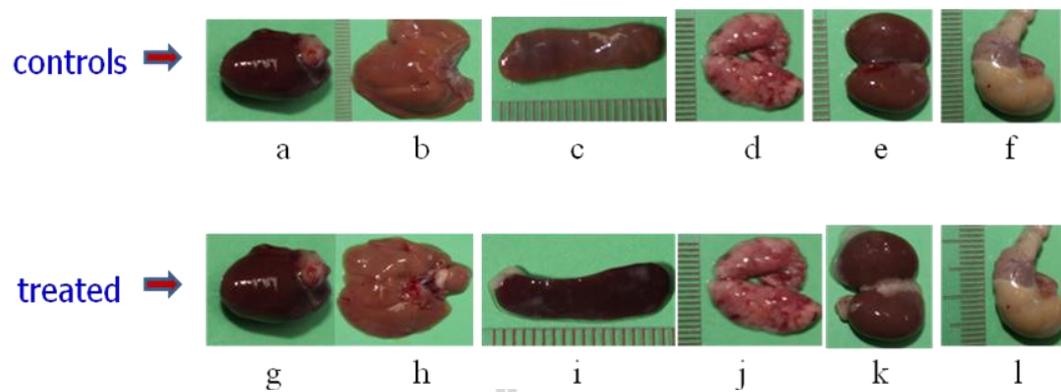
Figure 4.16   Morphology of main body organs of mice treated with quercetin alone and in combination with cloxacillin for 90 consecutive days in comparison with the negative control. Control group ; a = heart; b = liver; c = spleen; d = lung; e = kidney; f = stomach. Treated group ; g = heart; h = liver; i = spleen; j = lung; k = kidney; l = stomach.
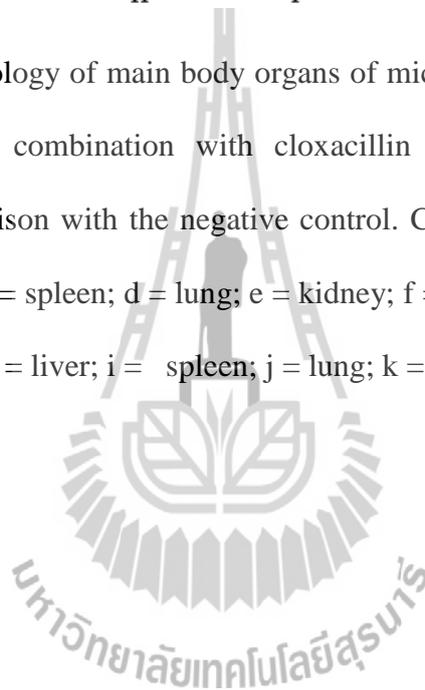
Table 4.15 Effects of intraperitoneally administered apigenin alone and in combination with ceftazidime for 90 days on relative weight of the selected main organ (per 100g body weight) in mice. Group 1 = apigenin 20 mg/kg BW/day, Group 2 = apigenin 40 mg/kg BW/day, Group 3 = apigenin 20 mg/kg BW/day plus ceftazidime 160 mg/kg BW/day, Group 4 = apigenin 40 mg/kg BW/day plus ceftazidime 320 mg/kg BW/day, Cefta = Ceftazidime (320 mg/kg BW).

Organ (g)	Control (0.2%DMSO)	Control Cefta	Group 1	Group 2	Group 3	Group 4
Heart	0.0017 ± 0.0001	0.0018 ± 0.0001	0.0017 ± 0.0001	0.0018 ± 0.0002	0.0015 ± 0.0001	0.0016 ± 0.0001
Liver	0.0207 ± 0.0005	0.0202 ± 0.0012	0.0188 ± 0.0017	0.0232 ± 0.0013	0.0207 ± 0.0002	0.0217 ± 0.0018
Spleen	0.0015 ± 0.0001	0.0020 ± 0.0003	0.0020 ± 0.0002	0.0019 ± 0.0001	0.0015 ± 0.0002	0.0018 ± 0.0001
Lung	0.0027 ± 0.0001	0.0027 ± 0.0002	0.0023 ± 0.0001	0.0030 ± 0.0002	0.0030 ± 0.0001	0.0028 ± 0.0002
Kidney	0.0077 ± 0.0007	0.0072 ± 0.0008	0.0060 ± 0.0009	0.0068 ± 0.0007	0.0062 ± 0.0000	0.0080 ± 0.0006
Stomach	0.0150 ± 0.0025	0.0164 ± 0.0049	0.0205 ± 0.0032	0.0114 ± 0.0023	0.0078 ± 0.0021	0.0116 ± 0.0020

4.2.3.2 Effects of the apigenin on blood chemistry and hematology in mice

The results of blood chemistry analysis revealed that there was a significant increase of AST level after treatment with 320 mg/kg BW ceftazidime, 40 mg/kg BW/day apigenin alone, or plus 320 mg/kg BW/day ceftazidime compared with pre-treatment. Cholesterol level decreased significantly in mice treated with 40 mg/kg BW/day apigenin compared to pre-treatment. However, there were no significant change of BUN and FBS between pre- and post-treatment in all groups of subchronic toxicity test.

Hematology data demonstrated that there was significantly reduced of WBC level after treatment with 40 mg/kg BW/day apigenin alone or plus 320 mg/kg BW/day ceftazidime compared to pre-treatment groups. The RBC level of post-treatment in 0.2%DMSO (control), 40 mg/kg BW/day apigenin alone or plus 320 mg/kg BW/day ceftazidime were significantly reduced compared with pre-treatment. In addition, The Hct level of post-treatment in 0.2% DMSO (control), 320 mg/kg BW ceftazidime and 40 mg/kg BW apigenin plus 320 mg/kg BW/kg ceftazidime were significantly reduced compared to pre-treatment. Hb levels decreased significantly in mice treated with 40 mg/kg BW/day apigenin plus 320 mg/kg BW/kg ceftazidime compared to pre-treatment groups. MCV level was also significantly reduced in group of mice treated with 40 mg/kg BW/day apigenin alone compared to the pre-treatment group (Table 4.16).

Table 4.16 Chemistry and hematological studies on mice before and after subchronic treatment with apigenin alone and in combination with ceftazidime for 90 days. Group 1 = apigenin 20 mg/kg BW/day, Group 2 = apigenin 40 mg/kg BW/day, Group 3 = apigenin 20 mg/kg BW/day plus ceftazidime 160 mg/kg BW/day, Group 4 = apigenin 40 mg/kg BW/day plus ceftazidime 320 mg/kg BW/day, Cefta = Ceftazidime (320 mg/kg BW).

Parameter	Control (0.20%DMSO)		Control Cefta.		Group 1		Group 2		Group 3		Group 4	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
AST (U/L)	411.8±86.0	552.4±73.7	**248.7±43.6**	**450.0±63.5***	385.0±82.8	581.6±89.3	**268.2±44.5**	**669.0±59.1***	450.0±80.1	532.8±80.9	**301.0±33.5**	**526.8±53.5***
BUN (mg/dl)	28.4±2.1	23.1±3.2	30.6±5.5	26.3±4.8	19.4±3.0	35.4±3.9	26.8±2.0	28.5±2.1	25.4±3.7	21.6±2.3	24.7±2.6	24.5±2.4
FBS(mg/dl)	162.2±9.4	157.2±10.2	105.0±2.8	149.7±21.1	165.0±7.5	158.6±11.7	141.6±6.3	147.6±8.7	182.0±16.0	165.2±20.7	137.0±9.0	147.6±6.8
Cholesterol(mg/dl)	140.1±20.6	96.0±11.9	141.3±33.9	169.7±40.9	177.0±26.7	147.2±36.5	**282.2±29.4**	**86.0±5.9***	127.0±18.3	120.4±17.0	91.0±9.2	85.4±4.4
WBC (x10^3//µ L)	9.7±0.9	9.0±0.8	8.1±1.9	5.4±0.5	16.0±1.3	16.0±1.2	**8.4±0.9**	**3.7±0.6***	14.9±2.7	12.5±3.4	**9.2±1.0**	**4.2±0.8***
RBC (x10^6 /µL)	**9.2±0.1**	**7.8±0.2***	8.8±0.4	7.2±0.5	9.0±0.3	7.6±0.7	**7.95±1.0**	**6.5±1.1***	9.0±0.2	7.7±0.8	**8.8±0.2**	**7.3±0.3***
Hb (g/dl)	16.2±0.2	15.4±0.3	15.5±0.8	13.3±0.6	16.0±0.3	15.7±0.3	15.4±0.5	11.2±1.5	16.0±0.5	15.0±0.9	**15.6±0.5**	**13.4±0.6***
Hct (%)	**49.1±1.6**	**39.9±1.1***	**49.5±1.4**	**39.3±1.5***	49.2±0.9	48.7±0.9	47.4±1.6	32.8±5.5	48.8±2.1	41.5±4.8	**45.6±1.5**	**38.2±2.1***
MCV (fL)	52.0±0.6	51.7±0.3	55.9±1.2	54.5±1.5	51.7±0.3	49.6±0.6	**51.3±0.2**	**49.7±0.3***	54.0±1.0	53.2±0.8	51.4±0.5	51.4±0.4

Mean ± SEM (n=5), **p < 0.05 (Student's t–test). Significant relative to pre-treatment value.

4.2.4 Subchronic toxicity test of luteolin

4.2.4.1 Effects of the luteolin on weight changes and main organs in mice

Body and organ weights of all treatment groups did not change for 90 days (Figure 4.17 and Table 4.17), which were similar to morphology appearance of treated mice.
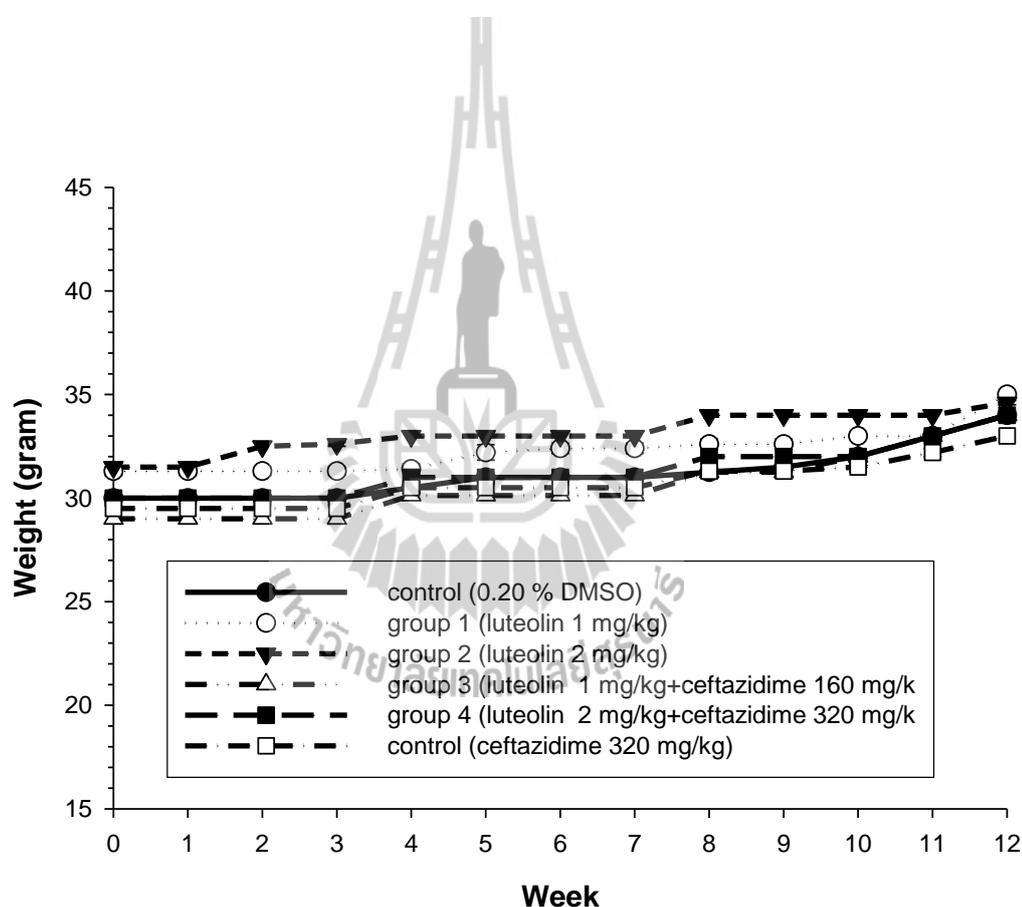

Figure 4.17 Body weights of mice treated with luteolin alone and in combination with ceftazidime for 90 consecutive days in comparison with the negative control.
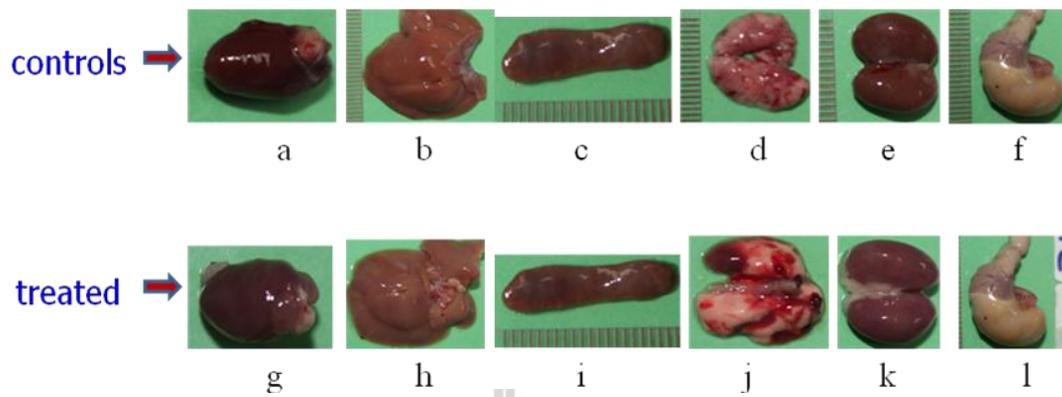
Figure 4.18 Morphology of main body organs of mice treated with luteolin alone and in combination with ceftazidime for 90 consecutive days in comparison with the negative control. Control group ; a = heart; b = liver; c = spleen; d = lung; e = kidney; f = stomach. Treated group ; g = heart; h = liver; i = spleen; j = lung; k = kidney; l = stomach.

Table 4.17 Effects of intraperitoneally administered luteolin alone and in combination with ceftazidime for 90 days on relative weight of the selected main organ (per 100g body weight) in mice. Group 1 = luteolin 1 mg/kg BW/day, Group 2 = luteolin 2 mg/kg BW/day, Group 3 = luteolin 1 mg/kg BW/day plus ceftazidime 160 mg/kg BW/day, Group 4 = luteolin 2 mg/kg BW/day plus ceftazidime 320 mg/kg BW/day, Cefta = Ceftazidime (320 mg/kg BW).

Organ (g)	Control (0.2%DMSO)	Control Cefta	Group 1	Group 2	Group 3	Group 4
Heart	0.0017 ± 0.0001	0.0018 ± 0.0001	0.0015 ± 0.0001	0.0016 ± 0.0001	0.0015 ± 0.0001	0.0016 ± 0.0001
Liver	0.0154 ± 0.0012	0.0202 ± 0.0012	0.0195 ± 0.0021	0.0186 ± 0.0011	0.0187 ± 0.0018	0.0185 ± 0.0011
Spleen	0.0012 ± 0.0001	0.0012 ± 0.0001	0.0014 ± 0.0001	0.0013 ± 0.001	0.0013 ± 0.0001	0.0014 ± 0.0001
Lung	0.0027 ± 0.0001	0.0027 ± 0.0002	0.0028 ± 0.0001	0.0026 ± 0.0001	0.0028 ± 0.0001	0.0026 ± 0.0001
Kidney	0.0066 ± 0.0006	0.0072 ± 0.0008	0.0061 ± 0.0007	0.059 ± 0.0007	0.0061 ± 0.0007	0.0059 ± 0.0008
Stomach	0.0151 ± 0.0025	0.0164 ± 0.0049	0.0172 ± 0.0115	0.0057 ± 0.006	0.0056 ± 0.0004	0.0059 ± 0.0006

4.2.4.2 Subchronic toxicity effects of the luteolin for 90 days on blood chemistry and hematology in mice

Cholesterol, WBC, Hb, and MCV levels showed a significant reduction after treatment with 1 mg/kg BW/day luteolin plus 160 mg/kg BW/day ceftazidime and 2 mg/kg BW/day luteolin plus 320 mg/kg BW/day ceftazidime, compared to the pre-treatment groups. The Hct level of post-treatment in 0.2% DMSO (control), 1 mg/kg BW/day luteolin plus 160 mg/kg BW/day ceftazidime and 2 mg/kg BW/day luteolin plus 320 mg/kg BW/day ceftazidime were significantly reduced compared to pre-treatment. In addition, BUN level decreased dramatically in mice treated with 2 mg/kg BW/day luteolin plus 320 mg/kg BW/day ceftazidime compared to pre-treatment. Whereas, RBC count reduced significantly in the group treated with 1 mg/kg BW/day luteolin plus 160 mg/kg WB/day ceftazidime compared to pre-treatment groups. However, there was no significant differences ($p<0.05$) of AST and FBS levels between pre- and post-treatment in all groups of subchronic toxicity test (Table 4.18).

Table 4.18   Chemistry and hematological studies on mice before and after subchronic treatment with luteolin alone and in combination with ceftazidime for 90 days. Group 1 = luteolin 1 mg/kg BW/day, Group 2 = luteolin 2 mg/kg BW/day, Group 3 = luteolin 1 mg/kg BW/day plus ceftazidime 160 mg/kg BW/day, Group 4 = luteolin 2 mg/kg BW/day plus ceftazidime 320 mg/kg BW/day, Cefta = Ceftazidime (320 mg/kg BW).

Parameter	Control (0.20%DMSO)		Control Cefta.		Group 1		Group 2		Group 3		Group 4	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
AST (U/L)	411.8±86.0	552.4±73.7	253.7±16.3	375.0±98.3	358.6±81.9	360.0±81.4	467.0±85.0	469.4±86.3	284.0±24.4	284.0±20.7	296.0±76.0	296.0±75.0
BUN (mg/dl)	28.4±2.1	23.1±3.2	31.3±8.8	18.0±2.6	21.6±1.9	20.8±1.9	15.4±1.0	15.6±1.0	32.2±1.3	23.7±2.6	27.4±1.4	15.4±1.0*
FBS(mg/dl)	162.2±9.4	157.2±10.2	126.0±21.2	161.0±12.1	177.0±8.6	177.0±7.4	156.0±11.3	156.8±11.4	314.0±16.3	178.0±8.4	332.0±12.4	156.0±11.3
Cholesterol(mg/dl)	140.1±20.6	96.0±11.9	191.6±109.2	93.0±16.5	130.0±20.3	128.6±20.6	100.0±9.6	99.0±9.2	414.0±13.6	190.0±12.9*	424.0±12.8	100.0±9.6*
WBC (x103//µ L)	9.7±0.9	9.0±0.8	6.9±2.2	6.9±1.8	4.1±0.5	4.4±0.5	4.6±0.6	4.4±0.5	12.3±1.1	4.7±1.3*	13.5±1.0	4.4±0.7*
RBC (x106 /µL)	9.2±0.1	7.8±0.2*	8.8±0.3	7.3±0.7	8.1±0.3	7.9±0.7	7.8±0.2	8.0±0.3	9.0±0.1	8.0±0.3*	8.7±0.2	7.7±0.1
Hb (g/dl)	16.2±0.2	15.4±0.3	14.6±0.3	14.3±1.2	13.4±0.6	13.0±0.8	12.8±0.2	12.2±0.2	16.2±0.3	13.6±0.9*	15.6±0.6	12.8±0.2*
Hct (%)	49.1±1.6	39.9±1.1*	47.6±0.8	40.3±2.7	41.4±1.5	41.6±1.6	40.2±0.5	40.4±0.2	48.8±0.9	42.0±2.2*	48.6±1.5	40.0±0.6*
MCV (fL)	52.0±0.6	51.7±0.3	53.4±0.8	54.9±1.9	51.8±0.2	51.8±0.1	51.8±0.6	51.6±0.6	54.1±0.5	52.2±1.0*	55.7±0.9	51.7±0.7*

Mean ± SEM (n=5), $*p < 0.05$ (Student's t–test). Significant relative to pre-treatment value.

4.3 Spectral differences between serums of normal and baicalein-treated mice (90 days)

Serum samples of baicalein-treated mice was probed the spectral distribution compare to the control group by FTIR microspectroscopy combined with multivariate data analysis. Figures 4.19 and 4.20 demonstrated the two dimensional PCA clustering from FTIR data for serum samples of mice after treatment with 10 and 20 mg/kg BW/day baicalein alone for 90 consecutive days, respectively. It was found that serum samples of treated and normal mice were dramatically separated. The multivariate statistical analysis techniques based on PCA were used to statistical analyze the significant spectral data of serum samples of mice after treatment with 10 and 20 mg/kg BW/day baicalein alone for 90 consecutive days, respectively. The loading from PC1 and PC2 of serum samples of mice after treatment with 10 mg/kg BW/day baicalein alone were accounted for 98% of the total variability. Whereas, the loading from PC1 and PC2 of serum samples of mice after treatment with 20 mg/kg BW/day baicalein alone were accounted for 92% of the total variability (PC1 69% and PC2 23%).
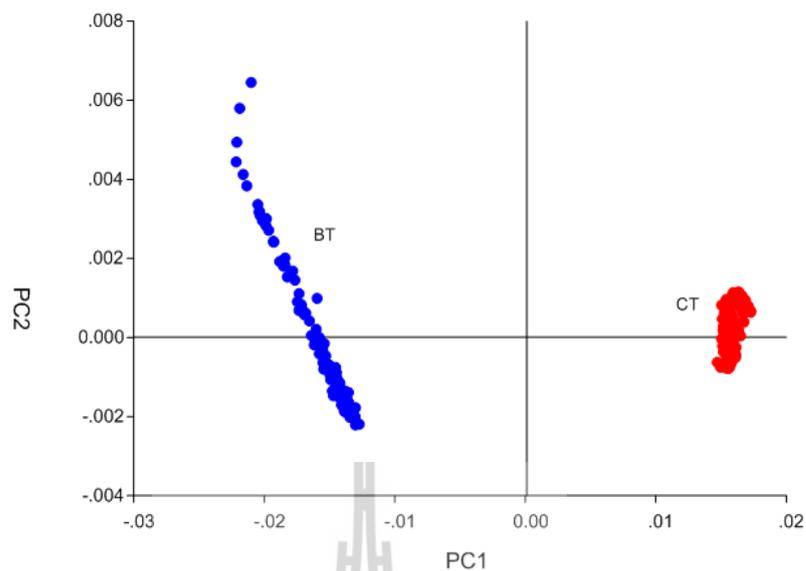
Figure 4.19 PCA analysis of spectra of serum samples of mice after treatment with 10 mg/kg BW/day baicalein alone for 90 consecutive days. BT = treatment group, CT = control group.
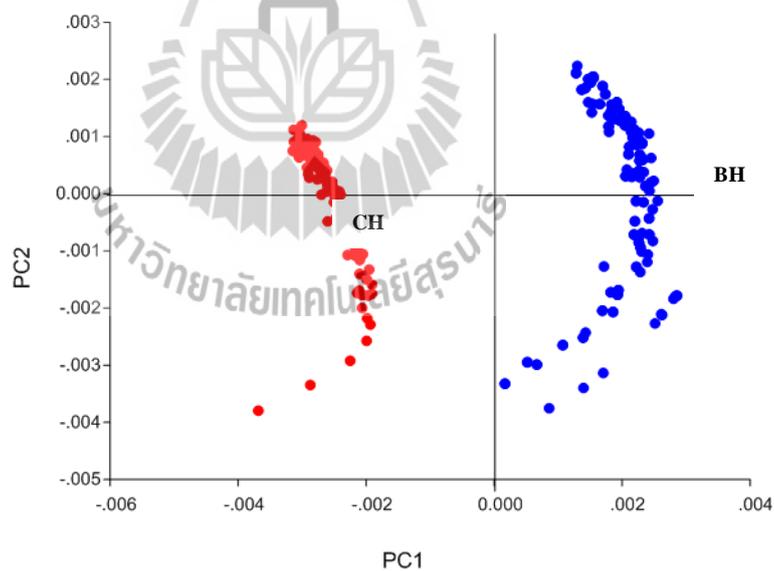
Figure 4.20 PCA analysis of spectra of serum samples of mice after treatment with 20 mg/kg BW/day baicalein alone for 90 consecutive days. BH = treatment group, CH = control group.

The second derivative procedure in the 1800-1000 and 3050-2800 cm^{-1} regions (Figures 4.21 and 4.22) gave more insight into serum samples altered by the treatment compared to the control. The amide I and II bands showed a complex profile and their absorption are widely used to estimate the protein secondary structure. The presence of an amide I maximum near 1655 cm^{-1} in the two spectra indicated that the proteins adopt a predominantly α-helical configuration. Figure 4.21a exhibited that there was a change of the 10 mg/kg BW/day baicalein serum samples in the band of amide I near 1655 cm^{-1}, when compared to control spectrum. Indeed, this band was shift significantly from 1658 cm^{-1} to 1650 cm^{-1}. The results imply that proteins in the serum samples including RBC were decreased.

Figure 4.22a showed that there was the reduction of the amide I secondary structure component in sample serums treated with 20 mg/kg BW/day baicalein. The results seem consistent with previous results that RBC and Hct were decreased. The band of α-helix was shift to 1648 cm^{-1} compared to the control centered at 1650 cm^{-1}. In addition, Figure 4.22b displayed the band centered at 2960 cm^{-1} and 2850 cm^{-1} corresponding to stretching mode of asymmetric CH_3, CH_2 vibration provide evidence that cholesterol was significantly decreased.
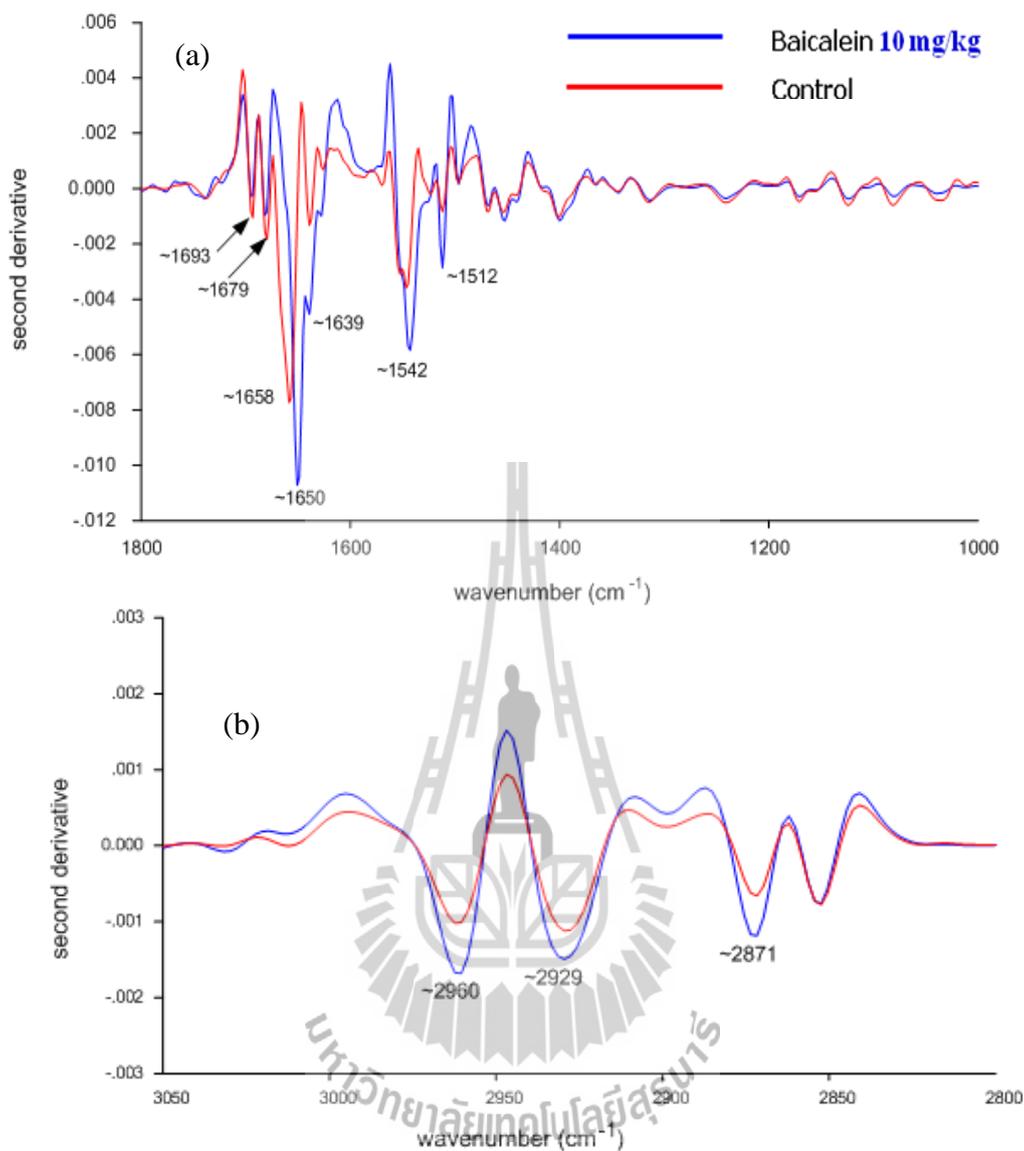
Figure 4.21 Representative 2nd derivative transformation spectra ($\sim$1800-1000 cm^{-1}) of serum samples of mice treated with 10 mg/kg BW/day baicalein alone for 90 days (a) and 2^{nd} derivative transformation spectra ($\sim$3050-2800 cm^{-1}) of serum samples of mice treated with 10 mg/kg BW/day baicalein alone for 90 days (b).
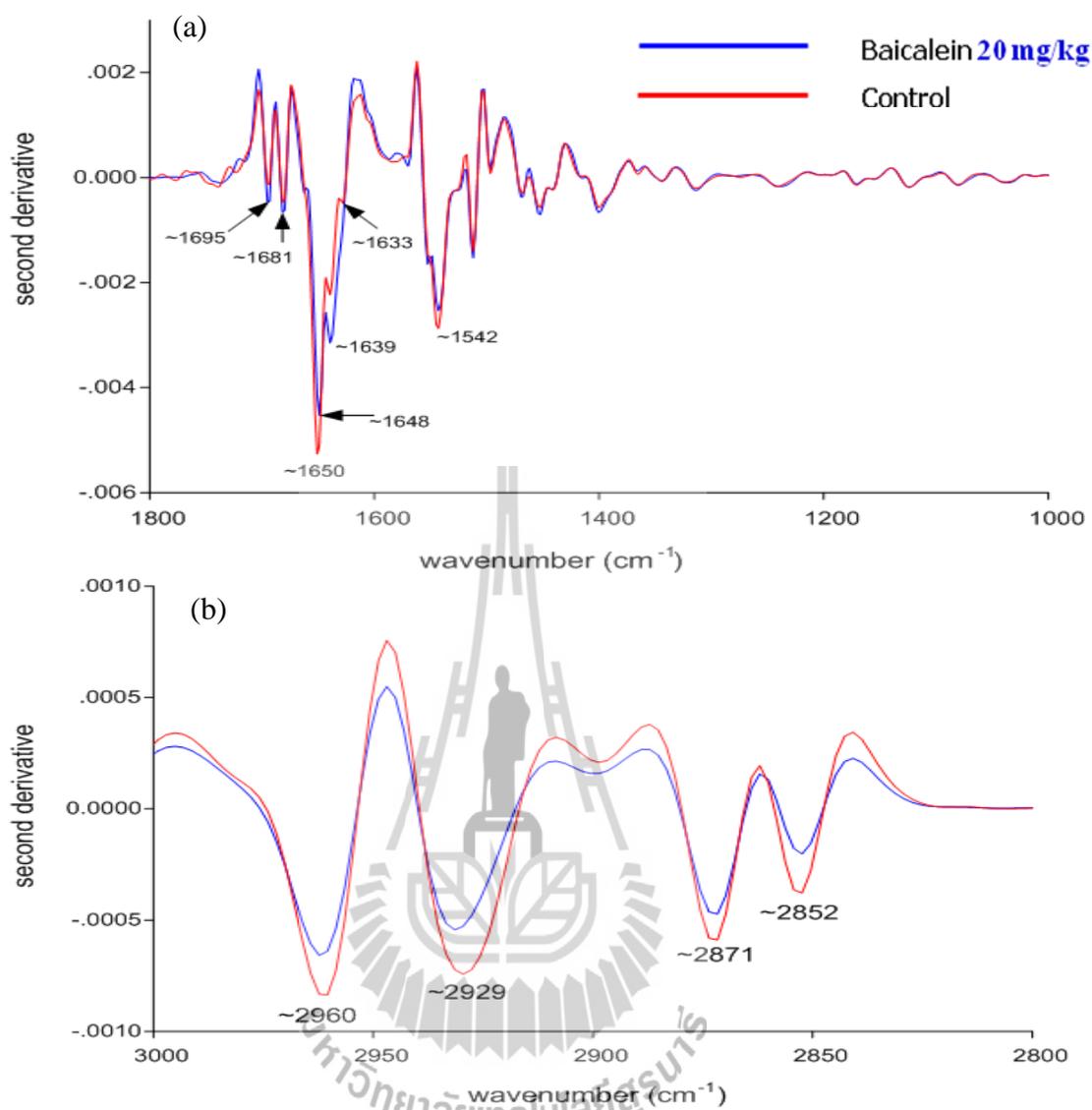
**Figure 4.22** Representative 2nd derivative transformation spectra ($\sim$1800-1000 cm^{-1}) of serum samples of mice treated with 20 mg/kg BW/day baicalein alone for 90 days (a) and 2^{nd} derivative transformation spectra ($\sim$3050-2800 cm^{-1}) of serum samples of mice treated with 20 mg/kg BW/day baicalein alone for 90 days (b).

CHAPTER V

DISCUSSION AND CONCLUSIONS

The present studies investigated the toxicity effect of flavonoids on mice when use alone or in combination with β-lactams antibiotics including cloxacillin and ceftazidime. Flavonoids used in these studies are classified into 2 major subgroups (Flavone and flavonol) according to their chemical structures and major food sources (Moon *et al*., 2006). Flavone consists of apigenin, baicalein, and luteolin, whereas, galagin and quercetin are in flavonol group.

In this study, quercetin was dissolved in 0.9% NaCl but apigenin, baicalein, luteolin, and galangin were dissolved in 0.20% DMSO for toxicity test. The test was included subacute and subchronic toxicity. Results clearly showed that there was no significant difference in either the relative growth rate measured by living body weight or the relative weight of the selected main body organs of mice treated with all doses of flavonoids alone or in combination with β-lactams antibiotics for 14 and 90 days, when compared to the control. In subacute toxicity test, quercetin revealed significant reduction ($p<0.05$) of RBC and Hb in mice group treated with 80 mg/kg BW/day i.p. injection. Similarly, quercetin at 20 mg/kg BW/day plus cloxacillin 150 mg/kg BW/day significantly reduced MCV level compared to pre-treatment group. Although a previous study indicated a relatively low-oral LD_{50} value for quercetin (i.e., in the range of 160 mg/kg body weight), the purity of the quercetin product administered was not specified, and subsequent longer-term animal toxicity showed

that quercetin was tolerated at oral dose levels exceeding this LD_{50} value by several fold. Moreover, no symptoms of toxicity were reported in rabbits receiving single intravenous injections of quercetin dose levels of 100-500 mg/kg body weight or two injections of up to 136 mg/kg body weight (Harwood *et al.*, 2007). The MCV level significantly decreased after treatment with 10 and 40 mg/kg BW/day baicalein when compared to pre-treatment group. Moreover, this flavonoid at 40 mg/kg plus cloxacillin at 600 mg/kg significantly decreased cholesterol, WBC, and Hb level. These results can be explained by assuming that synergistic effect of baicalein and cloxacillin was occurred. The results of baicalein 10 mg/kg plus cloxacillin 150 mg/kg not exhibited blood chemistry and hematological alteration on treated mice.

From the results, it can be concluded that 10 and 20 mg/kg BW/day galangin i.p. injection showed discernable adverse effect upon mice relative growth rates and the selected main body organs changes in all mice. In addition, the results from blood chemistry exhibited no hazard on these mice groups. Similary, the toxicity of galangin at these concentration on WBC, RBC, Hb and MCV were not concern. The results of galangin on WBC presumably understand with those of Heo *et al.*, 1994 who reported that galagin suppressed the induction of lymphocytes chromosome aberrations by bleomycin in a dose-dependent manner. In contrast, galangin revealed significant reduction ($p<0.05$) of Hct after treatment in all mice groups as dose-dependent way (except ceftazidime treatment group). These results seem consistent with previous research that galangin in the lower doses of treatment (0.1 mg/kg) showed more potent anticlastogenic effect than in higher dose (10 mg/kg) from both single and multiple pre-treatment (Su *et al.*, 1998). These findings provide evidence that high dose and long duration administration of galangin may result in Hct reduction.

Moreover, apigenin and luteolin in combination with ceftazidime at all doses revealed no toxicity on selected organs and blood parameters in mice when compared to pre-treament group. Interestingly, luteolin has been investigated *in vivo* and *in vitro* with the focus on its potential adverse effects. It is said to act as potent inhibitor of aromatase and β-hydroxysteroid oxidoreductase, which are enzymes involved in estrogen metabolism (Skibola and Smith, 2000). In addition, it was shown that luteolin-induced cytotoxicity in isolated rat hepatocytes correlated with mitochondrial membrane potential, and that it was also slightly more toxic than the polyphenol chrysin towards the HeLa tumor cells (Moridani *et al.*, 2002). Furthermore, Kotanidou *et al.* (2002) reported that the intra-peritoneal administration of a 50 mg/kg dose of luteolin was lethal to three of three mice receiving it.

In subchronic toxicity test, results of haematology demonstrated that there were significant reductions of Hb in mice after treatment with quercetin 20 and 40 mg/kg BW/day. In particular, Hct levels significantly declined in post-treatment groups with quercetin 40 mg/kg BW/day compared to pre-treatment groups. These results indicated that quercetin-treated mice may have anemia. Also, the post-treatment cholesterol and hematocrit exhibited significant reduction in the group treated with 20 mg/kg BW/day quercetin plus 150 mg/kg BW/day cloxacillin. These results are in substantial agreement with those of Ruiz *et al.* (2006) that no variations were observed in a number of other standard toxicological parameters including body weights, food and water consumption, clinical chemistry, and organ weights in male and female Swiss mice administered quercetin in the diet at doses of 30, 300, or 3,000 mg/kg BW/day for a period of 28 days in comparison to a control group. However,

our study conducted for 90 days may affect the toxicological parameters of treated mice.

Apigenin, one of the most common flavonoids, is found in a variety of fruits and vegetables, including onions, parsley, and oranges as well as chamomile tea, wheat sprouts, and certain seasonings. Apigenin has demonstrated anti-inflammatory, anticarcinogenic, and free radial-scavenging activities in a variery of *in vitro* systems. It has been shown using animal models that allergic airway inflammation is increased by Th2 cytokine production and decreased Th1 cytokine production (Choi *et al*., 2009). The results from subchronic toxicity test of apigenin showed that a significant reduction of cholesterol, WBC, RBC and MCV levels but significantly increased in AST in mice after treatment with 40 mg/kg BW/day apigenin when compared to the pre-treatment group, Furthermore, Mice receiving 40 mg/kg BW/day apigenin plus 320 mg/kg BW/day ceftazidime exhibited significantly decreased in RBC, Hb, Hct and WBC, Whereas AST was significantly increased. The RBC and Hct 0.2%DMSO post-treatment groups were also significantly decreased compared to pre-treatment groups. For this reason, RBC and Hct were remarkably decreased in the groups treatment with 40 mg/kg BW apigenin, and 40 mg/kg BW apigenin plus 320 mg/kg BW ceftazidime respectively, Never the less, there was prominently increased in AST levels after treatment with 320 mg/kg BW ceftazidime, 40 mg/kg BW apigenin and 40 mg/kg BW apigenin plus 320 mg/kg BW ceftazidime. The reduction of cholesterol, WBC, and MCV after treatment with 40 mg/kg BW apigenin may cause by high dose and long duration of apigenin. The reduction of RBC and Hct level in mice treatment with 1 mg/kg BW luteolin plus 160 mg/kg BW ceftazidime and 2 mg/kg, BW luteolin plus 320 mg/kg BW ceftazidime may due to 0.2%DMSO that be used as solvent and

also resulted in RBC and Hct decrease. Baicalein has been reported to have beneficial effects against benzo[α]pyrene-, aflatoxin (AF)-induced hepatic toxicity. It also has protective property against the cytotoxicity and genotoxicity of rat hepatocytes induced by *tert*-butyl hydroperoxide (*t*-BHP) due to its ability to quench free radicals (Hwang *et al*., 2005). The decrease of RBC and Hct levels in 20 mg/kg BW baicalein and 20 mg/kg BW baicalein plus 300 mg/kg BW cloxacillin treated mice may cause by 0.2%DMSO using as solvent and this also reduced RBC and Hct levels.

The lowering of RBC, Hct and cholesterol in baicalein treated mice are in substantial agreement with the results from FTIR that the amide I secondary structure component was decreased. These results provide evidence that the amide I secondary structure, protein composition, is the component of RBC, Hct and cholesterol.

In subchronic toxicity test, results of cholesterol level demonstrated that there were significant reductions in mice after treatment with 20 plus 150 mg/kg BW/day of quercetin plus cloxacillin, 20 mg/kg BW/day of baicalein alone, 20 plus 300 mg/kg BW/day of baicalein plus cloxacillin, 40 mg/kg BW/day of apigenin alone, 1 plus 160, and 2 plus 300 mg/kg BW/day of luteolin plus ceftazidime, respectively.

These finding lead us to believe that the high dose and long duration administration of these flavonoids result in blood cholesterol reduction. These results seem consistent with previous researches that flavonoids showed active hypoglycemic and hypolipidemic principle in many medicinal plants with blood glucose and lipids-lowering attributes (Oladele *et al*., 1995). In addition, Birari *et al*. (2011) indicated the potential of the chalcone scaffold as a source of PL (triacylgycerol acyl hydrolase), inhibitors for preventing obesity. Dietary catechins

and anthocyanins significantly decrease the weight of abdominal adipose tissues (Murase *et al*., 2002; Tsuda, 2008).

In conclusion, these findings lead us to believe that quercetin, apigenin and luteolin show no toxicity in blood chemistry, hematology and main organ in mice except for galangin causes Hct decrease and baicalein results in MCV reduction when these are administered at therapeutic dose for short duration. Interestingly, These flavonoids provide evidence that its can reduce cholesterol in mice blood when these are taken at hight dose for long duration. This study provides essential information for further investigation in other higher mammal, including human.
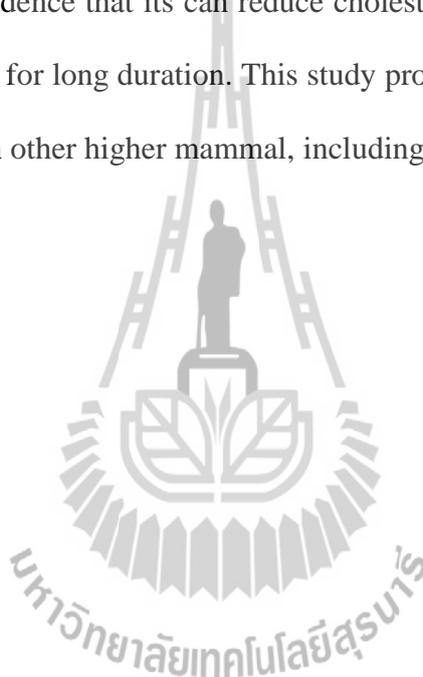
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