ผลของการเสริมกลีบเลี้ยงกระเจี๊ยบแดงเป็นสารต้านออกซิเดชั่นและสารเสริมกรดต่อคุณลักษณะการเจริญเติบโตและการย่อยได้บริเวณลำไส้เล็กส่วนท้ายในสุกรหลังหย่านม

นางสาววรรธนา อภิรักษ์ชาติสกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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THE EFFECT OF ROSELLE (*HIBICUS SABDARIFFA* LINN.) CALYX AS ANTIOXIDANT AND ACIDIFIER ON GROWTH PERFORMANCES AND ILEAL DIGESTIBILITY IN POSTWEANING PIGS

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วรรธนา อภิรักษ์ชาติสกุล : ผลของการเสริมกลีบเลี้ยงกระเจี๊ยบแดงเป็นสารต้านออกซิเดชั่นและสารเสริมกรดต่อคุณลักษณะการเจริญเติบโตและการย่อยได้บริเวณลำไส้เล็กส่วนท้ายในสุกรหลังหย่านม (THE EFFECT OF ROSELLE (*HIBICUS SABDARIFFA* LINN.) CALYX AS ANTIOXIDANT AND ACIDIFIER ON GROWTH PERFORMANCES AND ILEAL DIGESTIBILITY IN POSTWEANING PIGS) อ. ที่ปรึกษา : รศ. สุวรรณา กิจภากรณ์, อ. ที่ปรึกษาร่วม : รศ. น.สพ. ดร. กฤษ อังคนาพร, 58 หน้า. ISBN 974-14-2178-8.

การวิจัยครั้งนี้มีวัตถุประสงค์เพื่อศึกษาผลของการเสริมกลีบเลี้ยงกระเจี๊ยบแดงเป็นสารต้านออกซิเดชั่นและสารเสริมกรดต่อคุณลักษณะการเจริญเติบโตและการย่อยได้บริเวณลำไส้เล็กส่วนท้ายในสุกรหลังหย่านมแบ่งเป็น 2 การทดลอง การทดลองที่ 1 เป็นการศึกษาหาระดับที่เหมาะสมของกลีบเลี้ยงกระเจี๊ยบแดงต่อคุณลักษณะการเจริญเติบโต โดยใช้สุกรหย่านมคละเพศที่อายุ 5 สัปดาห์ จำนวน 36 ตัว แบ่งออกเป็น 6 กลุ่มดังนี้ กลุ่มที่ 1 เป็นกลุ่มควบคุม กลุ่มที่ 2 3 และ 4 เสริมกลีบเลี้ยงกระเจี๊ยบแดงในรูปผงแห้งที่ระดับ 4 8 และ 12% ในอาหาร กลุ่มที่ 5 เสริมสารเสริมกรดที่ระดับ 4 ก./กก.อาหาร และกลุ่มที่ 6 เสริมยาปฏิชีวนะ (คลอเตตร้าซัยคลิน) ที่ระดับ 100 มก./กก. อาหาร อาหารทุกสูตรคำนวณให้มีระดับโปรตีนและพลังงานใกล้เคียงกันให้มากที่สุด ชั่งน้ำหนักสุกรทดลองทุกกลุ่มพร้อมทั้งบันทึกปริมาณอาหารที่กินในสัปดาห์ที่ 7 9 และ 11 ของอายุสุกร การทดลองที่ 2 เป็นการนำกลีบเลี้ยงกระเจี๊ยบแดงในระดับที่ให้ผลต่อคุณลักษณะการเจริญเติบโตที่ดีที่สุดจากการทดลองที่ 1 มาศึกษาคุณสมบัติของการเป็นสารต้านออกซิเดชั่นและสารเสริมกรด โดยใช้สุกรหย่านมคละเพศที่อายุ 5 สัปดาห์ แบ่งออกเป็น 4 กลุ่มดังนี้ กลุ่มที่ 1 เป็นกลุ่มควบคุม กลุ่มที่ 2 เสริมกลีบเลี้ยงกระเจี๊ยบแดงในรูปผงแห้งที่ระดับ 8% ในอาหาร กลุ่มที่ 3 เสริมสารเสริมกรดที่ระดับ 4 ก./กก.อาหาร และกลุ่มที่ 4 เสริมยาปฏิชีวนะ (คลอเตตร้าซัยคลิน) ระดับ 100 มก./ กก. อาหาร อาหารทุกสูตรคำนวณให้มีระดับโปรตีนและพลังงานใกล้เคียงกันให้มากที่สุด ชั่งน้ำหนักสุกรทดลองทุกกลุ่มในสัปดาห์ที่ 7 และ 9 ของอายุสุกร และสุ่มมากลุ่มละ 4 ตัวนำมาฆ่าเพื่อวัดความเป็นกรดเป็นด่างที่บริเวณกระเพาะอาหารและลำไส้เล็กทั้ง 3 ส่วน เก็บเซลล์เยื่อบุกระเพาะอาหารวัดระดับเอนไซม์เปปซิน เก็บตับอ่อนวัดระดับเอนไซม์ทริปซิน เก็บอาหารที่ย่อยแล้วบริเวณลำไส้เล็กส่วนท้ายตรวจหาค่าการย่อยได้ของโปรตีนและไขมัน และเก็บเลือดและตับตรวจวัดปริมาณ malondialdehyde (MDA) และ glutathione ทั้งในพลาสม่าและเนื้อเยื่อตับ

จากการทดลองที่ 1 พบว่า สุกรทุกกลุ่มให้อัตราการเจริญเติบโตไม่แตกต่างกัน ($P>0.05$) แต่พบความแตกต่างของอัตราแลกเนื้อในช่วงอายุ 5-7 สัปดาห์ ($P<0.05$) กลุ่มที่ได้รับผงกระเจี๊ยบแดงระดับ 8% ให้อัตราแลกเนื้อต่ำสุด จึงถูกนำมาใช้เป็นระดับที่เหมาะสมในการทดลองที่ 2 และไม่พบความแตกต่างของอัตราการเจริญเติบโตในทุกช่วงอายุเช่นกัน ($P>0.05$) ในเรื่องคุณสมบัติการเป็นสารเสริมกรด การเสริมกลีบเลี้ยงกระเจี๊ยบแดงในระดับ 8% ช่วยลดความเป็นกรดในทางเดินอาหาร ($P>0.05$) เพิ่มการทำงานของเอนไซม์เปปซิน ($P>0.05$) เพิ่มการทำงานของเอนไซม์ทริปซินที่อายุ 7 สัปดาห์ ($P<0.05$) และเพิ่มการย่อยได้ของไขมันที่อายุ 7 สัปดาห์ ($P<0.05$) แต่ไม่มีผลต่อการย่อยได้ของโปรตีน ($P>0.05$) ส่วนในเรื่องคุณสมบัติการเป็นสารต้านออกซิเดชั่น การเสริมกระเจี๊ยบแดงช่วยลดปริมาณ MDA ทั้งในพลาสม่าและในตับ ($P>0.05$) และเพิ่มระดับ glutathione ในพลาสม่าที่อายุ 7 สัปดาห์ ($P<0.05$) และตับ ($P>0.05$) ความแตกต่างเกิดขึ้นระหว่างกลุ่มที่เสริมกระเจี๊ยบแดงกับกลุ่มควบคุมในทุกค่าสังเกตุแต่ไม่แตกต่างจากกลุ่มที่เสริมสารเสริมกรดและสารปฏิชีวนะ

สรุปได้ว่ากระเจี๊ยบแดงน่าจะมีคุณสมบัติในการเป็นสารเสริมกรดและสารต้านออกซิเดชั่น แต่การแสดงผลไม่เด่นชัดนักเนื่องจากการทดลองครั้งนี้ใช้ผงกระเจี๊ยบแดงและเลี้ยงในสภาพที่ควบคุมให้มีความเครียดต่ำ จึงควรมีการศึกษาเพิ่มเติมโดยการใช้สารสกัดของกลีบเลี้ยงกระเจี๊ยบแดงและทดลองในสภาพการเลี้ยงจริงในระบบอุตสาหกรรม

ภาควิชา	สัตวบาล	ลายมือชื่อนิสิต วรรธนา อภิรักษ์ชาติสกุล
สาขาวิชา	อาหารสัตว์	ลายมือชื่ออาจารย์ที่ปรึกษา
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KEY WORDS : ROSELLE/ ANTIOXIDANT/ ACIDIFIER/ ILEAL DIGESTIBILITY/ GROWTH PERFORMANCES/ POSTWEANING PIGS

WANTANA APHIRAKCHATSAKUN : THE EFFECT OF ROSELLE (*HIBICUS SABDARIFFA* LINN.) CALYX AS ANTIOXIDANT AND ACIDIFIER ON GROWTH PERFORMANCES AND ILEAL DIGESTIBILITY IN POSTWEANING PIGS. THESIS ADVISOR : ASSOC. PROF. SUWANNA KIJPARKORN, M.S., THESIS CO-ADVISOR : ASSOC. PROF. KRIS ANGKANAPORN,Ph.D., 58 pp. ISBN 974-14-2178-8.

The objectives of this investigation were to study the effect of Roselle (*Hibicus sabdariffa* Linn.) calyx as antioxidant and acidifier on growth performances and ileal digestibility in postweaning pigs. In experiment 1, the suitable level of Roselle calyx on growth performance was examined. Twenty four castrated male and twelve female piglets were allocated into 6 treatments : T1, basal diet; T2,T3,T4, basal diet plus crude powder of Roselle calyx at the level of 4, 8, 12 % feed; T5, basal diet plus acidifier at the level of 4 g/kg feed; T6 basal diet plus antibiotic (chlortetracycline) at the level of 100 mg/kg feed. All diet were calculated to be isocaloric and isonitrogenous. Body weight and feed intake were measured at 7, 9 and 11 weeks of age . The suitable level of Roselle calyx on growth performances from Expt 1 were used in the experiment 2 to determine the antioxidant and acidifier properties. Thirty two castrated male and female piglets were allocated into 4 treatments : T1, basal diet ; T2 basal diet plus crude powder of Roselle calyx at the level of 8 % feed; T3, basal diet plus acidifier at the level of 4 g/kg feed; T4 basal diet plus antibiotic at the level of 100 mg/kg feed. At 5-7 and 7-9 weeks of age, body weight were measured, four pigs in each group were randomly selected, exthanased and the pH of gastrointestinal tract were measured ; stomach mucosa were collected for determination of pepsin activity; pancreas for trypsin activity, ileal digesta for protein and fat digestibility, plasma and liver for malondialdehyde and glutathione concentration.

Experiment 1 showed that there was no significant difference in growth performance among groups ($P>0.05$); however, it was found that feed conversion ratio (FCR) was significantly different at 5-7 weeks of age ($P<0.05$). Pigs fed 8% Roselle calyx had the lowest FCR; therefore, 8% Roselle calyx was set to be an appropriate level in feed and was used in the experiment 2. Experiment 2 also showed no significant difference in growth performance among groups at all ages ($P>0.05$). Concerning acidifier property of Roselle calyx, the additional of 8% Roselle calyx help lower pH in gastrointestinal tract ($P>0.05$), increase activity of pepsin ($P>0.05$), increase activity of trypsin at the age of 7 weeks ($P<0.05$) and increase fat digestibility at the age of 7 weeks($P<0.05$) but there was no effect found on protein digestibility. Besides, Roselle calyx act as an antioxidant as well for the results showed that additional 8% Roselle calyx help lower MDA both in plasma and liver ($P>0.05$) and help increase glutathione in plasma at the age of 7 weeks ($P<0.05$) and in liver ($P>0.05$). Significant differences were found in every parameter between basal diet and Roselle fed groups except acidifier and antibiotic.

In conclusion, Roselle calyx is likely to have properties of being acidifier and antioxidant though the result did not show that significantly. Since this experiment used Roselle calyx in form of powder and was conducted in controlled environment to maintain low stress in pigs, there should be further research using the extraction form and conducting in commercial conditions.

Department	Animal nutrition	Student's signature.....
Field of study	Animal husbandry	Advisor's signature.....
Academic year	2005	Co-advisor's signature.....

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ABBREVIATION

ADFI	=	average daily feed intake
ADG	=	average daily gain
AGPs	=	antibiotic growth promotants
AIA	=	acid insoluble ash
ALT	=	alanine aminotranferase
ANOVA	=	analysis of variance
AST	=	aspartate aminotranferase
DM	=	dry matter
DNA	=	deoxyribonucleic acid
ER	=	endoplasmic recticulum
FCR	=	feed conversion ratio
FRAP	=	ferric reducing ability of plasma
g	=	gram
GSH	=	reduce glutathione
ID	=	Ileal digestibility
Kg	=	kilogram
LDH	=	leakage dehydrogenase
MBC	=	minimal inhibitory concentration
MDA	=	malondialdehyde
ME	=	metabolite energy
mg	=	milligram
ml	=	milliliter
MIC	=	minimal bactericidal concentration
min	=	minute
mM	=	milimolar
mmole	=	milimole
N	=	number
ORAC	=	oxygen radical absorbance capacity

PCA	=	protocatechuic acid
ROS	=	reactive oxygen species
SD	=	standard deviation
TAS	=	total antioxidant status
TBARS	=	thiobarbituric acid reactive substance
t-BHP	=	tert-butyl hydroperoxide
v/v	=	volume per volume
wt	=	weight
w/v	=	weight per volume
µl	=	microliter
µmole	=	micromole

CHAPTER I

INTRODUCTION AND AIMS

During recent years, Pig raising for commerce in Thailand has been developed. The pig industry continues to move towards earlier weaning from routinely weaned at 28 days of age to 15-21 days of age. This production is driven by economic factors and trade competition. However, earlier weaning of pigs may lead to higher risk of postweaning diarrhea, which causes retarded growth, increasing of mortality and extra medication costs. At that time, weaning piglets face both nutritional and environmental stress, which often results in reducing feed intake, little or no weight gain, and in some instances morbidity and death (Partanen and Mroz,1999). This postweaning lag period is a result of a limited digestive and absorptive capacity due to insufficient production of hydrochloric acid (HCl), pancreatic enzymes and sudden changes in feed consistency and intake (Cranwell, 1995). Weaned piglets are physiologically immature and can not produce enough hydrochloric acid (HCl) to keep stomach at an optimal pH. Therefore, digestion of proteins and populations of beneficial bacteria are minimized and harmful bacteria are exhibited (Doyle, 2001). Additionally, many of the ingredients utilized in phase one of nursery rations have a high acid binding capacity which can further reduce stomach acidity (Webel et al.,2003) .

According to the mention losses, antibiotics have played an important role in curing disease in animals. Furthermore, subtherapeutic levels of antibiotics could increase feed efficiency and growth in food animals. The antibiotic growth promotants (AGPs) have been used to prevent infection of pathogenic bacteria. The addition of antibiotics to feed is positive from an economic veiwpoint (Mahady, 2002) whereas the using of growth promoting antibiotics in feed may lead to antibiotic resistance in human and animal . If pigs are fed with subtherapeutic levels of antibiotics for extended periods of time, the pig's intestinal bacteria can become resistant to the antibiotics used. At slaughter, these resistant bacteria may enter the human food chain and cause illness in

human. These infections may be more difficult to treat with antibiotics similar to those that were fed to the pig (Salyer,2000) . At present, alternative feed additives or supplements such as probiotic , prebiotic, enzyme and organic acids that have been considered to replace antibiotic. The quest for alternatives to sub-therapeutic doses of antibiotics in pig feed has recently included the testing of a number of herbs due to the rationale for using these nutural remedies. Many herbs are known to have compounds which show the anti – bacterial effects and also increase the palatability of diets and thereby increase feed intake (Doyle, 2001).

Roselle (*Hibicus sabbriffa* L.) is an interesting herb because its petals consist of anthocyanin pigment which has many properties to response for biological activities such as antioxidant activity (Ali et al.,2003; Lin et al.,2003; Liu et al.,2002; Tsai et al.,2002; Wang et al.,2000; Duh and Yen.,1997; Tseng et al., 1997,1996) , a chemopreventive agent against tumor and cancer (Tseng et al., 1998; Tanaka et al.,1993) , and inhibition of pathogenic bacteria(Kramonwan and Narumon, 2001, Thawatchai and phongjak, 1990, Chu et al.,1987). In addition, Roselle petals may play a role as an acidifier because it consists of several organic acids. These properties could probably be used as a feed additive to promote healthiness and growth performance in animals.

However, many researches involving Roselle are mostly done in in vitro and in vivo using laboratory animals but not in livestock; therefore, this study is the first to be conducted in weaned pigs. The objectives of this study are to determine the effects and suitable dose of the Roselle petals fed to weaning pig on growth performance , antioxidant activity in plasma and liver tissue, pH and activity of enzyme in gastrointestinal tract and ileal digestibility of nutrient.

CHAPTER II

BACKGROUND INFORMATIONS

2.1 *Hibiscus sabdariffa* Linn.

Hibiscus sabdariffa Linn. is the plant in Malvaceae family. It has been known by different synonyms and local names such as Roselle, Jamica sorrel, Roselle of Rama, Krachiap daeng, Karkade, Som keng khen, Som taleng khreng and Som – unu (Nanthawan and Auranut, 1998). It is extensively cultivated in India, Thailand, Senegal and Egypt. The red colour calyxes are widely used for producing jam, jelly and bottled drinks. The dried calyxs of *Hibiscus sabdariffa* Linn. have been used effectively in folk medicines against hypertension, pyrexia and liver disorders (Tseng et al.,1997) The picture of the calyx of Roselle is shown in figure1

Figure 2.1 The calyx of *Hibiscus sabdariffa* Linn.(Roselle)

(Farnworth and Bunyapraphatsara, 1992)

2.1.1 Botanical description

Hibiscus sabdariffa Linn.(Roselle) is an annual herb having cylindrical stem about 1-2 metres . Its stem and branch have light bluish purple in colour. Leaves are simple, having 3-5 lobed of petiole, the lobes serrated or obtusely toothed. Flowers are solitary , having pink colour , axially, nearly sessile, 5-7 cm in diameter, consisting of epicalyx-segments which adnate at a base of the calyx. The calyx is thick, red and fleshy. The shape of calyx is cup-like, deeply parted and prominently 10 nerved. The

petals are yellow in colour. Stamens are numerous. The filaments are united into a staminal column. The style is single, five - branched near summit and the stiga is capitate. The fruit is capsule, ovoid, pointed, 1-2 cm long and has densely sharp and stiff hairs packing a lot of seeds (Gaet, 1999 and Farnworth, 1992).

2.1.2 The nutrient composition of Roselle calyx

The nutritient composition of 100 g fresh Roselle calyxs contained 86.6 g water, 1.4 g protein, 0.3 g fat, 1.3 g fiber, 9.4 g carbohydrate, 151 mg Ca, 59 mg P, 1.0 mg Fe, 10,833 I.U. vitamin A, 0.01 mg vitamin B1, 1.24 mg vitamin B2, 1.80 mg Niacin, 18.0 mg vitamin C and 46 calories (Chaweewan and Buchsaraporn, 1989)

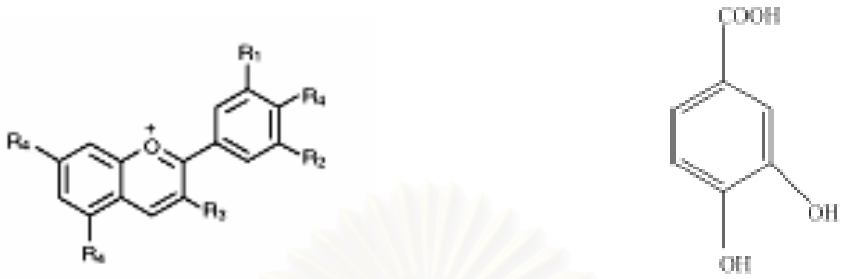
2.1.3 The chemical constituents in the Roselle caylx (Farnworth and Bunyapraphatsara, 2001)

The chemical compounds present in calyx of Roselle are as followed :

1. Plant acid are acetic acid, citric acid ,formic acid, malic acid, stearic acid, tartaric acid, (+)-allohydroxycitric acid lactone (hibiscus acid),
2. Anthocyans are anthocyanin, delphinidin, delphinidin-3-sambubioside, cyanidin-3-sambubioside, cyanin,
3. Flavone derivatives are gossypectin –3-glucoside, quercetin, myrecetin, protocatechuic acid, hibiscetrin, sabdaritin, gossypetrin,
4. Phytosterol are β-sitosterol, ergosterol,
5. Aqueous extract contains mucilage polysaccharide, pectin,
6. Others are L-ascorbic acid, β-carotene, niacin,resin , saponin.

The major flavonoid compounds in Roselle caylx are protocatechuic acid and anthocyanin. The anthocyanins , colour substance in Roselle calyx occur in the form of glycosylated polyhydroxy derivative and polymethoxy of flavylium cation. Anthocyanidins or aglycone are coloured compounds occurring in the form of glycosides (anthocyanins). The anthocyanin substance in Roselle calyx is approximately 1.5 g/100 g calyx weight. The type of anthocyanin found in Roselle

caylx are cyanidin-3-glucoside, delphinidin–3-glucoside, cyanidin-3-sambubioside and delphinidin –3 - sambubioside, respectively.



Anthocyanins

R1 = H or OH

R2 = H or OH

R3 = H or glycosyl

R4 = OH or glycosyl

Protocatechuic acid

Figure 2.2 Chemical structure of anthocyanin and protocatechuic acid in Roselle calyx (Du and Francis, 1973)

2.1.4 The toxicity of Roselle calyx

The examination of toxicity of Roselle calyx by Nanthawan and Auranut (1998) found that the LD_{50} of water extraction of dried Roselle calyx administered by gastric intubation to rabbits was 129.1 g/kg . Thawatchai and Phongjak (1991) mentioned that the crude extract with water of dried Roselle calyx administered in mice and rats showed LD_{50} higher than 4 g/kg whereas Onyenekwe et al (1999) reported that the LD_{50} in rats was higher than 5 g/kg.

2.2 Free radical

Free radicals are chemical species that have a single unpaired electron in an outer orbit. Energy created by this unstable configuration is released through reactions with adjacent molecules, such as inorganic or organic chemicals proteins, lipids, carbohydrates particularly with key molecules in membrances and nucleic acids. Moreover, free radicals initiate autocatalytic reactions whereby molecules with which

they react are themselves converted into free radicals to propogate the chain of damage. One important mechanism of membrance damage, alluded to in the disscussion of reperfusion injury, is injury induced by free radicals, particularly by activated reactive oxygen species (ROS). It contributes to such varied processes as chemical and radiation injury, oxygen and other gaseous toxicity, cellular aging, microbial killing by phagocytic cells, inflammatory damage and tumor destruction by macrophages and others. (Ramzi et al., 1999). A list of some major ROS is presented in Table 2.1(Bottjie et al., 1995).

Table 2.1 A list of some major reactive oxygen species (ROS)

Species	Abbreviation	Major cellular source(s)
Superoxide radical	O_2^-	Mitochondria, Oxidase (e.g. Xanthine oxidase)
Hydroxyl radical	OH^-	Cytochrome P 450, reaction of H_2O_2 with metals
Hydrogen peroxide	H_2O_2	Mitochondria
Peroxyl radical	ROO^-	Membranes, protaglandin metaboism
Hypochlorous acid	$HClO^-$	White blood cells

2.3 Lipid peroxidation

Free radicals in the presence of oxygen may cause peroxidation of lipids within plasma and organellar membranes. Oxidative damage is initiated when the double bonds in unsaturated fatty acids of lipids membrane are attacked by oxygen-derived free radicals, particularly by OH^-. The lipid-radical interactions yield peroxides, which are themselves unstable and reactive, and an autocatalytic chain reaction ensues (called propagation) which can result in extensive membrane, organellar, and cellular damage. Other more favorable termination options take place when the free radical is captured by a scavenger (called scavenging), such as vitamin E, embedded in the cell membrane.

Steps of lipid peroxidation (Bottjie et al., 1995)

(Initiation) Lipid + $R^{\bullet}/OH^{\bullet}$ → Lipid

(Propagation) $Lipid^{\bullet} + O_2$ → $Lipid.OO^{\bullet}$

$Lipid.OO^{\bullet}$ + Lipid → Lipid-OOH + $Lipid^{\bullet}$

(Termination) $Lipid^{\bullet}$ + $lipid^{\bullet}$ → Lipid-lipid

$Lipid\text{-}OO^{\bullet}$ + $lipid^{\bullet}$ → Lipid-OO-Lipid

(Scavenging) $Lipid^{\bullet}$ + Vit E → Lipid + $Vit\ E^{\bullet}$

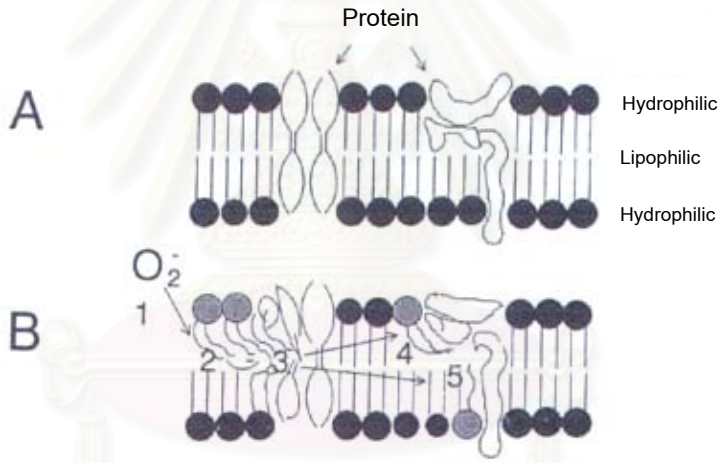


Figure 2.3 lipid peroxidation (Bottjie et al., 1995)

Picture A : Stylized drawing of the membrane showing the lipid bilayer and membrane - associated and proteins.

Picture B : Sequence of events in which reactive oxygen species can initiate lipid peroxidation.

1) A reactive compound or free radical abstracts an electron from a lipid molecule.
2) If the resulting lipid peroxyl radical is not reduced by antioxidants, the lipid radical abstracts electrons from neighboring lipid molecules.

3&4) The function of proteins associated with the membrane are disrupted by conformational damages to the membrane (physical) or by direct oxidation of proteins (chemical).

5) Radical formation can damage additional structures distal to the initial site of injury.

2.4 Proteins oxidation

Free racidals promote oxidation of amino acid residue side chains, formation of protein-protein cross-linkages (e.g., sulfhydryl mediated), and oxidation of the protein backbone resulting in protein fragmentation. Oxidative modification enhances degradation of critical enzymes by the multicatalytic proteasome complex, raising havoc throughout the cell.

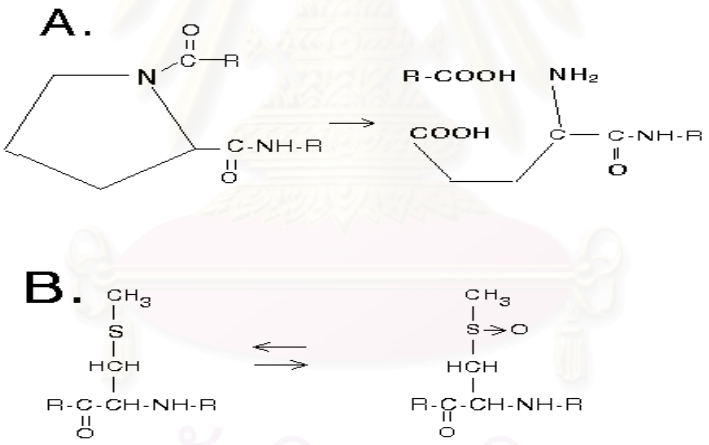


Figure 2.4 Methionine and proline oxidation (Thomas, 1999).

A) The reaction shows the irreversible oxidation of proline in a peptide breaked in the polypeptide chain .

B) The reaction demonstrates the reversible oxidation/reduction of methionine.

2.5 Lesion in DNA

Reactions with thymine in nuclear and mitochondrial DNA produce single-stranded breaks in DNA. This DNA damage has been implicated in cell aging and in malignant transformation of cells (Ramzi et al.,1999).

2.6 The cellular antioxidants

Antioxidants are compounds that prevent oxidation by donating reducing equivalents to inactivate reactive compounds. Antioxidants can be classified as either nonenzymatic, which react directly with radicals, toxins and enzymatic which donate reducing equivalents through the action of a specific enzyme. A list of major antioxidants is provided in Table 2.2 (Bottje et al., 1995)

Table 2.2 Major antioxidant protective compounds or protective systems.(Bottje et al., 1995)

Type	Tissue Location	Action
1.Nonenzymatic.		
- Vitamin E (tocopherol)	- Membranes, - Extracellular fluid	- Coverts O^-_2, OH^- and lipid peroxyl radicals to less reactive compounds - Chain breaking antioxidant.
- Vitamin A (β-carotene)	- Membranes	- Scavengers O^-_2 Interacts directly with peroxyl radicals
- Vitamin C (Ascorbic acid)	- Widely distributed in intra and extracellular fluid	- Interacts directly with O^-_2, OH^- neutralizes ROS released from WBC's - Can regenerate Vit E. from E radical form
- Glutathione(GSH)	- Mainly intracellular	- Interacts directly with O^-_2 ,OH^- and lipid hydroperoxides - Serves as substrate for GSH recycling enzymes - Binds transition metals; interacts with O^-_2 ,OH^- and peroxyl radicals
- Uric acid	- Widely distributed	- Spares or prevents oxidation of ascorbic acid
2. Enzymatic		
- Superoxide dismutase (SOD)	- Mitochondria and cytosol	- Convert O^-_2 to H_2O_2 by dismutation reaction
- GSH recycling system		
GSH peroxidase	- Cytosol and mitochondria	- Reduces H_2O_2 and other hydroperoxides, low Km, functions during 'normal' metabolism
GSH reductase	- Cytosol and mitochondria	- Reduces low molecular weight disulfides (GSSG GSH) using NAD(P)H
- Catalase (CAT)	- Peroxisome	- Reduces H_2O_2 high Km, functions mainly in disease states

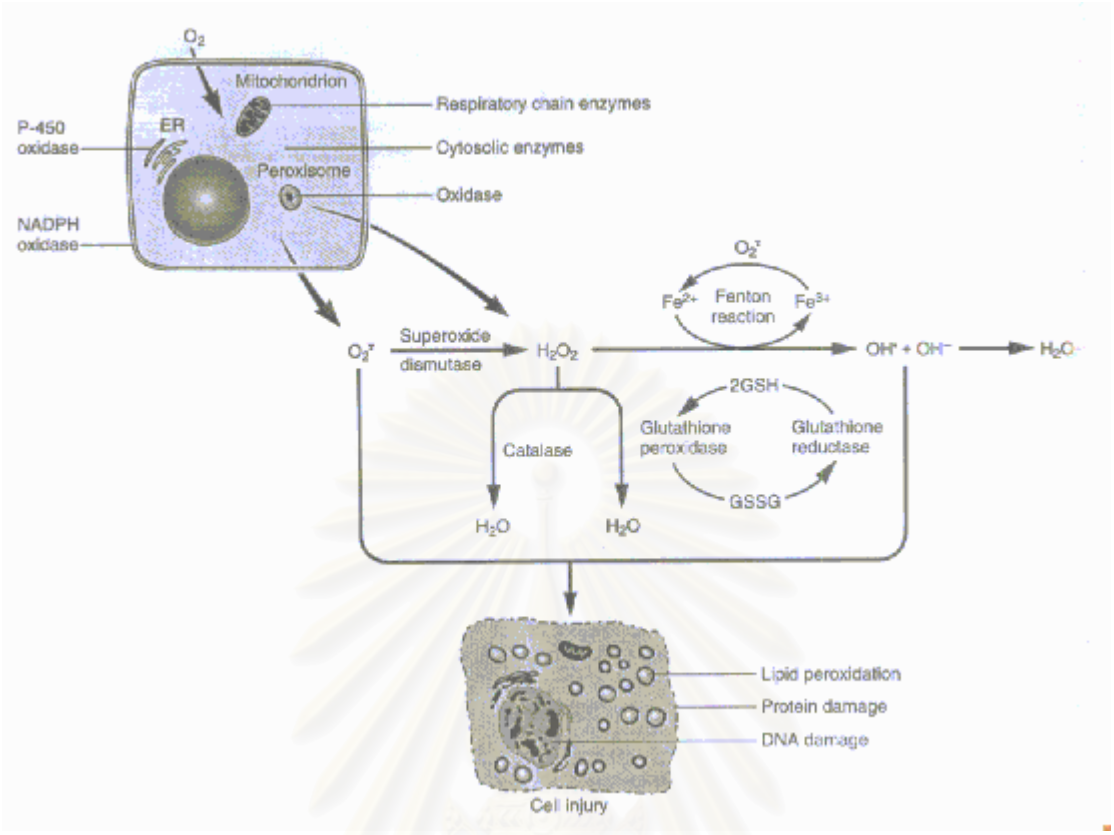
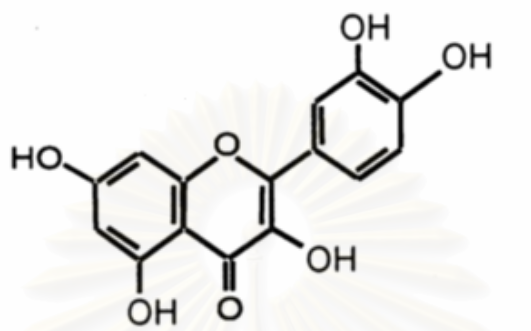


Figure 2.5 Formation of reactive oxygen species and antioxidant mechanisms in biologic systems. O_2 is converted to superoxide (O_2^-) by oxidative enzymes in the endoplasmic reticulum (ER), mitochondria, plasma membrane, peroxisomes and cytosol. O_2^- is converted to H_2O_2 by dismutation and thence to $OH^\cdot$ By the Cu^{2+}/Fe^{2+} catalyzed Fenton reaction. H_2O_2 is also derived directly from oxidases in peroxisomes. Resultant free radical damage to lipid (peroxidation), proteins and DNA leads to various forms of cell injury. The major antioxidant enzymes are superoxide dismutase, catalase, glutathione peroxidase . (Ramzi et al.,1999)

2.7 Flavonoid on antioxidants

Flavonoids are low molecular weight polyphenolic substances based on the flavan nucles. They are widespread in nature, occuring in all plant families, and are found in considerable quantities in fruits and vegetable. The major flavonoid classes

include flavonols, flavones, flavanones, flavanols (catechins), anthocyanidins, isoflavones. Representatives of major groups of flavonols were characterised as having antioxidant properties *in vitro* and *in vivo* (Surai, 2002) Flavonoids have been proposed to act as antioxidants, most probably because of their radical-scavenging abilities.



Quercetin [3,5,7,3",4" pentahydroxyflavone]

Figure 2.6 Structure of some flavonoid (Roberfroid and Calderon, 1994)

The optimal antioxidant capacity of a given substance depends on two function:

a. The catechol group in the B-ring confers high stability on the aroxyl radicals.
b. Extensive electron delocalization is ensured by conjugation of the B-ring to the 4-oxo structure via a 2,3-double bond.

Some flavonoids are strong oxygen radical scavengers and good metal chelators, effective in preventing lipid peroxidation. For instance, polyphenolic flavonoids inhibit the peroxidation of low-density lipoproteins and their subsequent cytotoxicity (Roberfroid and Calderon, 1994) . The inhibition of the cytotoxic effect correlates with that of thiobarbituric acid-reactive substance formation (Wang et al.,2000).

2.8 Roselle calyx on antioxidant activity

The effect of anthocyanin on antioxidant capacity was determined in Roselle (*Hibiscus sabdariffa* L.) extracting in boiling water by comparing with ferric reducing ability of plasma (FRAP), oxygen radical absorbance capacity (ORAC) and total antioxidant status (TAS) using antioxidation assays in vitro. The results suggest that anthocyanin is the major source of antioxidant capacity in Roselle extract (Tsai et al.,2002). Duh and Yen (1997) found that the calyx of *Hibiscus sabdariffa* L. showed marked antioxidative activity in linoleic acid and in liposome model systems, indicating that it may protect the cell from damage by lipid peroxidation. It possessed high contents of phenolic compounds and exhibited reducing power indicating showed good hydrogen donating abilities and had effective activities as radical scavengers.

Wang et al (2000) was evaluated the antioxidant bioactivity of natural pigments occurring in the dried calyx of *Hibiscus sabdariffa* L. using the model of *tert*-butyl hydroperoxide (t-BHP) induced cytotoxicity in rat primary hepatocytes and hepatotoxicity in rats. The results demonstrated that the extract at the concentrations of 0.1 and 0.2 mg/ml significantly decreased the leakage of lactate dehydrogenase and the formation of malondialdehyde(MDA) induced by a 30 min treatment of t-BHP (1.5 mM). The in vivo investigation showed that the oral pretreatment of the extract (100 and 200 mg/kg) for 5 days before a single dose of t-BHP (0.2 mmol/kg) significantly lowered the serum levels of hepatic enzyme markers (alanine and aspartate aminotransferase) and reduced oxidative liver damage by significantly decreased MDA and increased glutathione(GSH).

The dried calyx extract of Roselle also decreased the leakage dehydrogenase (LDH) and the formation of MDA induced by t-BHP (1.5mM) considerably at a concentration of 0.1 and 0.2 mg/ml in the rat primary hepatocyte cultures (Tseng et al., 1997). Moreover, the dose of 200 mg/kg of the dried calyx of Roselle decreased liver damage induced by 700 mg/kg paracetamol in rats (Ali et al.,2003).

The protocatechuic acid (PCA), a polyphenolic compound from *Hibiscus sabdariffa* Linn. possessing free radical-scavenging capacity, protected against oxidative damage induced by *tert*-butyl-hydroperoxide (t -BHP) in rat primary hepatocytes. The investigation of Liu et al (2002) showed that pretreatment with PCA (50-100 mg/kg) in rat by gavage for 5 days before induced hepatotoxicity in rats by a single dose of t-BHP (0.2 mmol/kg) significantly lowered serum levels of the hepatic enzyme markers (lactate dehydrogenase (LDH), alanine (ALT) and aspartate (AST) aminotransferase) and reduced oxidative stress of the liver by evaluating malondialdehyde (MDA) and glutathione (GSH). PCA at the concentrations of 0.05 mg/ml and 0.10 mg/ml significantly decreased the leakage of LDH ($P<0.01$) and ALT ($P<0.05$ and $P<0.01$) and the formation of malondialdehyde ($P<0.05$ and $P<0.01$)respectively, in primary cultured rat hepatocytes induced by t-BHP (1.5 mM) for 30 minutes (Tseng et al., 1996).

In addition, the calyx of Roselle performs abundantly pharmacological activities such as antibacterial activity, antiinflammation (Vlaskovska et al., 1990), chemoprevention of tumor and carcinogenesis (Tseng et al., 1998 and Tanaka et al., 1993) and effect of hypocholesterolemic (Hirunpanich ,2001) .

As an antibacterical activity, the Roselle calyx extract have an activity both in gram positive and nagative bacteria. The extract can eliminate *S. aureus, B. subtilis, E.Coli* and *P. aeruginosa* at the concentration 2,2,3 and 4 mg/ml ,respectively. The concentration of the Roselle caylx extract 100 mg/ml showed the same result as tetracycline HCl at the concentration 3.2 μg for *S. aureus* bacteria , 6 μg for *B. subtilis* bacteria and gentamycin at the concentration 540 μg for *E.Coli* and 200 μg for *P. aeruginosa* (Chu et al.,1987). Kramonwan and Narumon (2001) studied the effect of the 24 differences extracted herbs on antibacteria by agar diffusion method. Results showed that the lyophilize of Roselle calyx extract was quit active against *Salmonella typhimurium* ATCC 14028 and *Salmonella sp.*. The Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) were 3.125 μg/ml and 6.25 μg/ml, respectively. A beverage containing *Hibiscus sabdariffa* had bactericidal

activity against Escherichia coli, *Bacillus subitlis*, *Salmonella typhosa* and *Klebsiella pneumoniae* (Farnsworth and Bunyapraphatsara, 1992).

Moreover , the calyx of Roselle may act as acidifier due to the fact that it consists of several organic acids such as acetic acid, citric acid ,formic acid, malic acid, stearic acid and tartaric acid . For more than 20 years, pig producers have used acids (both organic and inorganic) to improve the performances of piglets at weaning period, both for growth promotion and diarrhea prevention (Gauthier, 2002). Acidification is crucial in young animals, which cannot secrete enough hydrochloric acid for correcting protein digestion. Numerous studies have shown that inorganic and organic based acidifiers are the most appropriate to achieve not only a regular digestion but also in health and performances . Nowadays, organic acids are used to control the growth of pathogenic microorganisms and to increase feed utilization instead of antibiotics which are baned in many countries as a growth promotor (Gauthier, 2002).

2.9 Acidifier in weaning pig.

The acidifier shows two major roles in animal, the first role one is to generate a positive effect on feed efficiency and the second one is a bacteriostatic effect (Partanen and Mroz, 1999)

2.9.1 Acidifier gives positive effect on feed utilization by

- accelerating activity of pepsinogen in the stomach
- stimulating the secretin of pancreatic enzymes
- slowing down emptying of the stomach

2.9.2 Acidifier counteracts with pathogenic bacteria by:

- decreasing the number of pathogenic bacteria in digestive tract by lowering the pH
- decreasing diarrhea by minimizing feed supply to micro-flora in the last part of the small intestine

2.10 Acidifier on digestibility

The young pigs have limited ability to produce hydrochloric acid to meet suitable pH for digestion in the stomach. The pH can regulate the movement of viable bacteria and molds from the animal's environment to the small intestine and involve in the activation of pepsin, a proteolytic enzyme. The initial proteolytic activity carried out by pepsin is necessary for the subsequent activity of trypsin in the small intestine. Thus, adding acidifier into the feed helps to maintain the optimal pH in the stomach, activates function of the proteolytic enzymes and stimulates feed consumption (Peris and Calafat, 1994). Thaela et al.(1998) reported that 2.5 % lactic acid supplementation in weaning pig significantly increased trypsin and chymotrypsin activity in pancreas ($P<0.05$).

An addtion of 0.6% of mixed acids (formic :propionic acid = 75:25) caused an improvememt of digestibility of crude protein, calcium and phosphorus in weaning pigs (Kemme ,1998).

Moreover, acidifier can improve buffering capacity of feed (Pickard et al., 2001). Most diets are composed of ingredients with high acid-binding capacities, which are potentially detrimental to maintain low gastric pH. The most active element effecting the phenomenon of acid-binding capacity is macroelement and a high concentration nutrient in pig starter diets such as crude protein which can significantly reduce post-weaning growth performance (Bolduan et al.,1988). Linderman et al (1986) reported that a low secretion of pancreatic enzymes in early-weaned pigs might be an additional factor responsible for the inefficient digestion of plant-based diets, especially if the proteolytic capacity in the stomach is limited. The efficient digestion of diets base on plant protein sources may play a major role in the mode of action of organic acids. Blank et al.(1999) studied the effect of fumaric acid (1%,2% and 3%) and dietary buffering capacity on ileal and fecal amino acid digestibilities in early-weaned pigs, the results showed that 2% fumaric acid supplemented to the diet with a low buffering capacity increased ($P<0.05$) the ileal digestibilities of crude protein, gross energy and the majority of amino acids but had no effect on the fecal digestibilities of crude protein, gross energy and amino acids. However, Gabert et al (1995) reported

that supplementation of formic acid to diets with low and high buffering capacity did not effect (P>0.05) apparent ileal digestibilities of amino acid, pH, ammonia and voluntary fatty acid concentrations, bacterial populations in ileal digesta and the incidence of diarrhea were also not effected (P>0.05).

2.11 Acidifier on antimicrobial activity

The role of acidifier is similar to antibiotic, as an antimicrobial activity. The acids can penetrate the bacterial cell wall and distrupt the normal actions of certain types of bacteria including *Salmonella* spp, *E. coli*, *Clostridia* spp, *Listeria* spp. Therefore, reductions in numbers of some species of the normal intestinal bacteria as well as pathogenic bacteria can occur in animals fed with acidifier. Acidifiers are believed to improve overall performance by reducing microbial competition with the pig for nutrients, by lowering the risk of subclinical infections by reducing the intestinal immune response and by reducing the production of harmful bacterial compounds(Dibner and Buttin,2002).

CHAPTER III

MATERIALS AND METHODS

The experiment contained 2 studies with different objectives.

(1) to find out the suitable levels of Roselle on growth performance.

(2) to determine the effects of suitable levels of Roselle on growth performance, antioxidant activity in plasma and liver tissue, pH and activity of enzyme in gastrointestinal tract and ileal digestibility of nutrient.

This study was approved by the Institutional Laboratory Animal Care and Use Committee of The Faculty of Veterinary Science, Chulalongkorn University

Animal and management

A total of 100 crossbred piglets (Hamshire x Landrace x Duroc) weaned at four week of age were used in two experiments. Total body weights of all pigs in each group were similar. All piglets were received diets and water *ad libitum* for the whole period of experiments 1 and 2.

In experiment 1 , twenty four castrated male and twelve female piglets were used in the experiment. Following an adaptation period of 7 days, all animals were randomly allocated into 6 treatments which composed of 3 replicates of 2 pigs each. Each treatment was composed of 2 castrated male and 1 female.

In experiment 2, thirty - two castrated male and female pigs were used in the experiment. Following an adaptation period of 7 days, all animals were randomly allocated into 4 treatments which comprised 4 replicates of 4 pigs each. Each treatment was composed of 2 castrated male and 2 female.

Preparation of Roselle powder

In experiments 1 and 2 ,fresh calyx of Roselle were dried, milled and passed through 2 mm screen by cutting mill machine. The petals powder were analyzed for total phenolic compound (Duh and Yen, 1997) and nutritional content by proximate analysis (AOAC, 1990). The total phenolic compound and nutritional content of Roselle

in both experiments are depicted in Table 3.1. The dried petals of Roselle were kept at –20 °C until use.

Feed and feeding

There were six treatment diets in experiment 1 and four treatment diets in experiment 2. All diets were calculated to be isocaloric and isonitrogenous according to NRC requirement (1994). The diet formula in both experiments are shown in Tables 3.2 and 3.3 . Both of experimental diets were analyzed by proximate analysis method (AOAC,1990) and the results are shown in Tables 3.4 and 3.5.

In experiment 1 , the six experimental diets were randomly assigned to three replicates of piglets according to the experimental design (Table 3.6). The pigs were fed on their diets for a period of 6 weeks.

In experiment 2, the four experimental diets were randomly assigned to four replicates of piglets according to the experimental design (Table 3.7). The pigs were fed on their diets for a period of 4 weeks. During days 10 to 14 and days 24 to 28 ,Celite, a source of acid insoluble ash (AIA) was added to all diets at the level 20 g/kg feed as an indigestible dietary marker.

Table 3.1 Chemical analysis of nutritional content and total phenolic compound of Roselle in both experiments (% DM basis).

Nutrients	Experiment 1	Experiment 2
Crude protein	10.27	10.48
Crude fat	1.98	1.84
Crude fiber	12.05	11.75
Ash	8.96	8.64
Calcium	1.29	1.22
Total phosphorus	0.39	0.38
Total phenolic compound (mg/g)	20.23	21.84

Table 3.2 The ingredient composition and calculated analysis of the experimental diets (Experiment 1).

Ingredient	Amount (kg/100kg diets)					
	T1	T2	T3	T4	T5	T6
Broken rice	38.48	33.59	28.75	23.85	38.08	38.43
Soybean meal	27.98	27.91	27.83	27.74	27.98	27.98
Fullfat soybean meal	8.00	8.00	8.00	8.00	8.00	8.00
Rice bran	12.00	12.00	12.00	12.00	12.00	12.00
Fishmeal	3.00	3.00	3.00	3.00	3.00	3.00
Whey	5.00	5.00	5.00	5.00	5.00	5.00
Coconut oil	2.90	3.97	5.03	6.09	2.90	2.90
Mono – dicalcium Phosphate	1.00	0.97	0.93	0.90	1.00	1.00
Salt	0.27	0.27	0.27	0.27	0.27	0.27
DL-methionine	0.02	0.03	0.05	0.06	0.02	0.02
Oystershell	0.86	0.76	0.65	0.55	0.86	0.86
Premix[1]	0.50	0.50	0.50	0.50	0.50	0.50
Roselle	0.00	4.00	8.00	12.00	0.00	0.00
Acidifier	0.00	0.00	0.00	0.00	0.40	0.00
Antibiotic	0.00	0.00	0.00	0.00	0.00	0.05
Calculated analysis (%)						
Crude protein	22.00	22.00	22.00	22.00	22.00	22.00
Fat	7.92	8.99	10.06	11.12	7.92	7.92
Fiber	3.97	4.36	4.75	5.14	3.97	3.97
Calcium	0.90	0.90	0.90	0.90	0.90	0.90
Total phosphorus	0.75	0.75	0.75	0.75	0.75	0.75
Lysine	1.26	1.25	1.25	1.25	1.26	1.26
Methionine	0.38	0.39	0.39	0.40	0.38	0.38
Tryptophan	0.29	0.28	0.28	0.27	0.29	0.29
Salt	0.35	0.35	0.35	0.35	0.35	0.35
ME (Kcal/kg)	3265	3265	3265	3265	3265	3265

[1]Premix /kg contained : vitamin A 4,000,000,I.U., vitamin D_3 800,000 I.U., vitamin E 8.0 g , vitamin K_3 0.8 g, vitamin B 0.4 g, vitamin B 2.0 g, vitamin B 0.6 g, vitamin B 6.0 mg, nicotinic acid 8.0 g, calcium pantothinate 4.0 g, choline choride 40.0 g, folic acid 0.4 g, biotin 20.0 mg, Fe 60.0 g, Co 0.4 g, Mn 16.0 g, Cu 80.0 g, Zn 44.8 g, I 0.4 g, Se 0.04 g, Ethoxyquin 20 g. Silicon dioxide 4.0 g

Table 3.3 The ingredients and composition of the experimental diets (Experiment 2).

Ingredient	Amount (kg/100kg diets)			
	T1	T2	T3	T4
Broken rice	38.48	28.75	38.08	38.43
Soybean meal	27.98	27.83	27.98	27.98
Fullfat soybean meal	8.00	8.00	8.00	8.00
Rice bran	12.00	12.00	12.00	12.00
Fishmeal	3.00	3.00	3.00	3.00
Whey	5.00	5.00	5.00	5.00
Coconut oil	2.90	5.03	2.90	2.90
Mono – dicalcium Phosphate	1.00	0.93	1.00	1.00
Salt	0.27	0.27	0.27	0.27
DL-methionine	0.02	0.05	0.02	0.02
Oystershell	0.86	0.65	0.86	0.86
Premix[1]	0.50	0.50	0.50	0.50
Roselle	0.00	8.00	0.00	0.00
Acidifier	0.00	0.00	0.40	0.00
Antibiotic	0.00	0.00	0.00	0.05
Calculated analysis (%)				
Crude protein	22.00	22.00	22.00	22.00
Fat	7.92	10.06	7.92	7.92
Fiber	3.97	4.75	3.97	3.97
Calcium	0.90	0.90	0.90	0.90
Total phosphorus	0.75	0.75	0.75	0.75
Lysine	1.26	1.25	1.26	1.26
Methionine	0.38	0.39	0.38	0.38
Tryptophan	0.29	0.28	0.29	0.29
Salt	0.35	0.35	0.35	0.35
ME (Kcal/kg)	3265	3265	3265	3265

[1]Premix /kg contained : vitamin A 4,000,000,I.U., vitamin D_3 800,000 I.U., vitamin E 8.0 g , vitamin K_3 0.8 g, vitamin B 0.4 g, vitamin B 2.0 g, vitamin B 0.6 g, vitamin B 6.0 mg, nicotinic acid 8.0 g, calcium pantothinate 4.0 g, choline choride 40.0 g, folic acid 0.4 g, biotin 20.0 mg, Fe 60.0 g, Co 0.4 g, Mn 16.0 g, Cu 80.0 g, Zn 44.8 g, I 0.4 g, Se 0.04 g, Ethoxyquin 20 g. Silicon dioxide 4.0 g.

Table 3.4 Chemical analysis of the experimental diets.(experiment 1)

Nutrients (% DM basis)	Treatment					
	T1	T2	T3	T4	T5	T6
Crude protein	22.82	22.64	22.68	22.84	22.71	22.88
Crude fat	8.31	8.39	9.16	9.37	8.34	8.45
Crude fiber	2.34	2.72	3.00	3.32	2.42	2.38
Ash	6.77	6.80	7.40	7.26	7.16	6.85
Total phosphorus	0.77	0.83	0.87	0.76	0.81	0.79
Calcium	0.88	0.91	0.95	0.95	0.93	0.90

Table 3.5 Chemical analysis of the experimental diets.(experiment 2)

Nutrients (% DM basis)	Treatment			
	T1	T2	T3	T4
Crude protein	22.29	22.30	22.58	22.40
Crude fat	6.68	8.10	6.67	6.77
Crude fiber	2.05	2.22	2.00	1.96
Ash	6.72	6.46	6.15	6.44
Total phosphorus	0.83	0.86	0.82	0.79
Calcium	0.95	0.95	0.94	1.04

Table 3.6 The description of the treatments in experiment 1.

Treatment	Description
T1	Control diet
T2	Control diet + Roselle 40 g/kg diet
T3	Control diet + Roselle 80 g/kg diet
T4	Control diet + Roselle 120 g/kg diet
T5	Control diet + Acidifier 4 g/kg diet*
T6	Control diet + Antibiotic 100 mg/kg diet **

* Acidifier (Fra® Acid Dry) /Kg contained formic acid 214 g, lactic acid 184 g, citric acid 10 g,fumaric acid 100 g and carrier

**Antibiotic (Aurofac® 200 G) /kg contained chlortetracycline 200 g

Table 3.7 The description of the treatments in experiment 2.

Treatment	Description
T1	Control diet
T2	Control diet + Roselle 80 g/kg diet
T3	Control diet + Acidifier 4 g/kg*
T4	Control diet + Antibiotic 100 mg/kg diet **

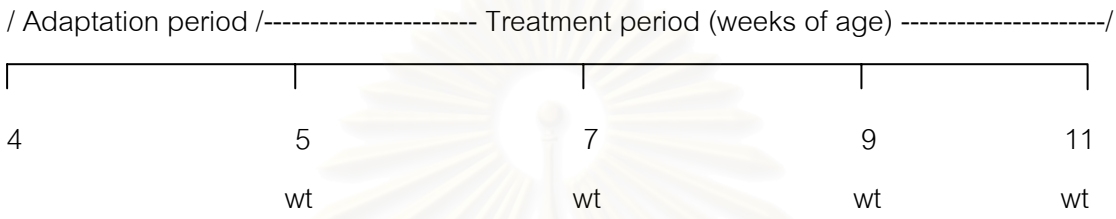
* Acidifier (Fra® Acid Dry) /Kg contained formic acid 214 g, lactic acid 184 g, citric acid 10 g,fumaric acid 100 g and carrier

**Antibiotic (Aurofac® 200 G) /kg contained chlortetracycline 200 g

Data Collection

Experiment 1

Body weight and feed intake were recorded at the 2nd , 4th and 6th week of experimental period. Number of sick and dead pigs, temperature, relative humidity and any remark environmental conditions were recorded on daily basis. The diagram of the experiment is shown in figure 7.

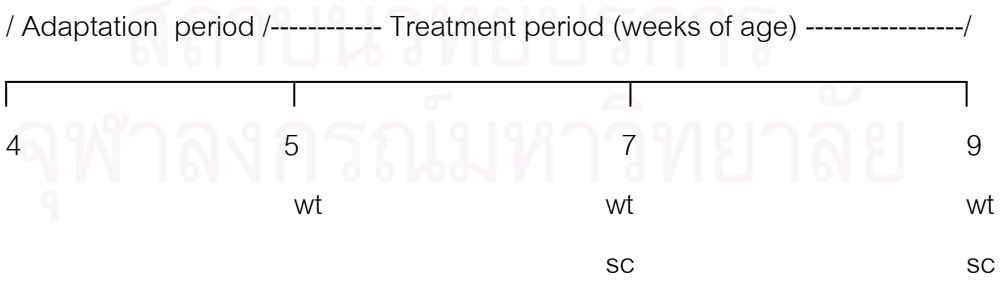


wt = Weighing (body weight and feed)

Figure 3.1 Diagram of the experiment 1.

Experiment 2

Body weight and feed intake were recorded at the 2nd and 4th week of experimental period. Number of sick and dead pigs, temperature, relative humidity and any remark environmental conditions were recorded on daily basis. The diagram of the experiment is shown in figure 8.



wt = Weighing (body weight and feed)

sc = Sample collection

Figure 3.2 Diagram of the experiment 2.

Sample collection and Tissue preparation

In experiment 1, there was no sample collected because only growth performance was studied.

In experiment 2, 16 pigs from each treatment group at 2th and 4th week of experimental period which were randomly selected and fed digestible marker for 4 days were used to collect all samples. Blood sample was collected from cranial vena cava. The pigs were euthanased by overdose intravenous injection of pentobarbital sodium. Immediately after slaughter, the abdominal cavity was opened and the entire gastrointestinal tract was removed. The pancreas and liver were carefully dissected , cleaned of extraneous tissue and kept frozen at -70 °C until analysis. The gastric pH was measured at the center of fundus or corpus area and the intestinal pH was measured at 3 points (duodenum at 1 foot from upper duodenum, jejunum at 2 feet from pancreas and ileum at 1 foot before colon) using a semisolid glass electrode probe pH meter. The stomach was opened along the greater curvature, rinsed with cold saline solution. The opened stomach was laid flat on ice. The mucosa of the fundus region was scraped from the muscular layer using a glass slide, wrapped with tin foil and freezed at -70 °C until analysis. The ileal content was collected by gentle squeezing with thumb and finger into plastic bottles. The samples of ileal content from pigs in each replicate were dried at 60°C for twenty four hours, grinded and kept frozen at - 20 °C until analysis.

Determination of pepsin activities in gastric mucosa

Sample preparation

The scraped mucosa from fundus region of pig stomach were used as the source of enzyme. Mucosa were homogenized (Homogenizer,GKH, GT MOTOR CONTROL, GLAS-COL®) with nine parts of ice-cold saline – triton solution (w/v). The homogenate was centrifuged at 4,000 x g (4°C for 10 min) to remove the large debris of the cells and aliquots of the supernatant were stored at -70 °C until analysis.

Enzyme Analysis

The homogenate of fundus mucosa was analyzed for gastric pepsin activity using the method described by Rick (1965) with slight modifications. The mucosal proteolytic zymogens were activated by adjusting the mucosal suppernatant to pH 2.0 - 2.5. The pepsin activity was measured at pH 1.5-2.5 using haemoglobin as a substrate. The 2.5 ml of substrate was added with 80 μl of homogenate and 400 μl of 0.01 N HCl and left at 25°C for 10 min. Then 5 ml of 5% Trichloroacetic acid solution was added to stop the reaction. The solution was vigorously stirred and centrifuged at 4,000 x g for 20 min and then 2.5 ml supernatant was added into 5 ml 0.5 N NaOH and 1.5 ml 1:2 Folin and Ciocalteu phenol reagent. The optical density was read at the wavelength 750 nm against blank (distilled water)using UV-VIS spectrophotometer (Shimadzu® UV 1201).

Tyrosine standard curve

The standard curve was plotted using tyrosine at 0, 0.2, 0.4, 0.6, 0.8 and 1.0 μmoles dissolved in 2.0-2.5 ml of 0.2 N HCl and 5 ml of 0.5 N NaOH. the solution was mixed before adding with 1.5 ml 1:2 Folin and Ciocalteu phenol reagent. The optical density was measured at 750 nm using a blank contained with 0.2 N HCl instead of tyrosine standard and the results were ploted against μmoles of tyrosine.

The enzyme activity were expressed as specific activity (units per mg protein). One unit (PU^{Hb}) was defined as the activity that 1 mmol tyrosine equivalent was liberated per min at 35.5°C.

Determination of pancreatic trypsin activities

Sample preparation

The pancreas was homogenized (Homogenizer,GKH, GT MOTOR CONTROL, GLAS-COL®) with 500 mM Tris-HCl buffer (1:10 w/v) containing 50 mM $CaCl_2$ at pH 8.0. The homogenate was centrifuged at 4,000 x g (4°C for 20 min) and aliquot of the supernatant was stored at -70 °C until analysis.

Enzyme Analysis

The homogenate of pancreas was analyzed for trypsin activity using the method described by Rick (1965). Activation of pancreatic trypsinogen was accomplished by a pre-incubation period with 0.1 units of enterokinase for 30 minutes. After the activation, trypsin activity was determined at 25°C pH 8.0 with *N* – (α) – Benzoyl –L-arginine ethyl ester hydrochloride as a substrate. The substrate 3 ml was added with 200 μl of enzyme preparation, mixed , started a stopwatch. The optical density was read at wavelength 254 nm using UV-VIS spectrophotometer (Shimadzu UV 1201) every 30 second for 4 minutes. Reading of the optical density were made against distilled water in silica cuvettes light path.

The activity of the enzyme can be expressed in μmoles of substrate converted/min ,since enzymes activities were expressed in specific activity (μmol/min/mg protein).

Determination of plasma and liver lipid peroxidation

Sample preparation

The liver was homogenized (Homogenizer,GKH, GT MOTOR CONTROL, GLAS-COL®) with 100 mM KCl and 0.003 M Na_2EDTA (1:10 w/v). The homogenate was centrifuged at 4,000 x g (4°C for 20 min) and aliquots of the supernatant were stored at -70 °C until analysis.

Measurement of malondialdehyde (MDA)

Lipid peroxidation, as thiobarbituric acid-reactive substances (TBARS), was determined in plasma and liver homogenated samples by method of Ohkawa et al (1979). The values of TBARS material were expressed in terms of malondialdehyde since 200 ,400 μl of plasma , supernatant of liver tissue was added to 0.2 ml 8.1 % sodium dodecyl sulfate (SDS), 1.5 ml 20 % acetic acid (pH 3.5),1.5 ml 0.8% thiobarbituric acid and volume was adjusted to 4 ml by distilled water. These solutions were heated to 95°C for 60 min and cooled down at room temperature , 1.0 ml distilled water and 5.0 of *n*-butanol-pyridine mixture (15:1 v/v) were added , mixed vigorously

and centrifuged at 3,000 g for 30 min. The absorbance of the upper layer was read at 532 nm.

Malondialdehyde standard curve

The standard curve was plotted using MDA bis-dimethyl acetal at 0, 2, 4, 6, 8 and 10 nmoles of plasma and 0, 12.5, 25, 50 and 100 of liver tissue as the external standard where Y was the value of MDA and X was the optical density at 532 nm. Results were expressed as nmol MDA/ml of plasma and nmol MDA/mg protein .

Determination of Glutathione

Sample preparation

The liver tissue was homogenized the same method as lipid peroxidation determination

Determination of plasma and liver tissue glutathione

Plasma and liver tissue glutathione content were measured by Beutler et al. (1963) as modified by Mayerly et al.(2000). 1 ml of plasma and supernatant of homogenated liver were added with1.5 ml metaphosphoric acid, and particulate debris was removed by centrifuge at 3,000 g for 10 min. Reduced GSH was measured by adding 500 μl of supernatant to 2.0 ml 0.2 M phosphate buffer and 0.25 ml 0.04% 5,5' dithio-bis 2-nitrobenzoic acid. Absorbance was read at 410 nm.

Glutathione standard curve

The standard curve was plotted using reduced glutathione at 0, 2, 4, 8, 16, 32 and 64 nmol of plasma and 0, 12.5, 25, 50, 100, 200 and 400 nmoles of liver tissue as an external standard where Y are the value of the GSH and X are the optical density at 410 nm. Results are expressed in nmol GSH/ml of plasma and nmol GSH/mg protein .

Determination of tissue protein concentration

Total tissue protein concentration included gastric mucosa cell, pancreas and liver were determined using Lowry method (1951).

The samples were homogenized with nine parts of ice-cold saline – triton solution (w/v) after diluted with distilled water 10,12 and 20 times of gastric mucosa cell, pancreas and liver respectively. The homogenated samples 100 μl was added with 3.0 ml of fresh reagent[1] and allowed to settle at room temperature for 10 min.Then 1:1 Folin and Ciocalteu phenol reagent (300 μl) was added into the solution, left for 30 min at room temperature. The optical density was read at the wavelength 650 nm against blank using UV-VIS spectrophotometer (Shimadzu® UV 1201).

Standard solution

The standard curve was plotted using the bovine serum albumin (BSA) at 0, 20, 40, 60, 80 and 100 mg%. The slope of the curve was used to calculate the concentrations of mucosal protein where Y was the value of the BSA and X was the optical density.

Determination of nutrients digestibility

Determination of acid-insoluble ash

Celite was added as a marker for the determination of ileal digestibility of nutrients. Acid-insoluble ash was measured as described by Choct and Annison (1992). The sintered glass crucibles (Pyrex® , England) were dried at 105 °C for 24 hour, and place to ash at 550°C. for 8 hour, cooled in a desicator and weighed. Two grams of diet or 1 g of dry - grinded digesta samples were weighed into sintered glass crucibles. After ashing, the crucibles were cooled and boiled slowly in 4 N HCl for 30 min on a hot plate in fume hood. The ash in crucible was washed with distilled water using suction pump, and dried at 105°C for 6 hour. The ash residues in crucible were repeatedly ashed and boiled in the same way. Finally, crucible with ash was dried at 105 °C for 6 hour, cooled in a desicator and weighed. Percentage acid-insoluble ash was calculated using the following equation;

$$\text{AIA (\%)} = \frac{\text{Wf} - \text{We} \times 100}{\text{Ws}}$$

[1]Fresh reagent consist of 50 ml of 2% Na_2CO_3 in 0.1 M NaOH ,0.5 ml of 1% $CuSO_4 .H_2O$ and 0.5 ml of 2% Na-tartrate.

Where Wf = weight of crucible with ash

We = weight of empty crucible

Ws = weight of sample (dry matter)

The percentage of ileal digestibility (ID) of nutrient (protein and fat) was calculated using the following equation ;

$$ID = 1 - \frac{\text{Ileal nutrient (\%)} / \text{Ileal acid insoluble ash (\%)}}{\text{Diet nutrient (\%)} / \text{Diet acid insoluble ash (\%)}} \times 100$$

Calculation of the growth performance

In both experiments 1 and 2 , the feed intake and individual body weight data were collected twice a week and used to calculate the average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR). The formula is shown below.

$$\text{Average daily gain (ADG,kg/day)} = \frac{\text{Final body weight} - \text{Initial body weight}}{\text{Days}}$$

$$\text{Average daily feed intake (ADFI)} = \frac{\text{Feed intake}}{\text{Days}}$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{Average daily feed intake}}{\text{Average daily gain}}$$

Statistic analysis

All data were presented as mean $\pm$ SD. The data were analyzed using one-way analysis of variance (ANOVA) to determine the effects of treatments. If there were any significant effects, Duncan's New Multiple Range Test was used to compare the individual means. The level of significant difference was set at $P < 0.05$.

CHAPTER IV
RESULTS

Experiment 1

Effect of Roselle supplementation on growth performances.

The average temperature and relative humidity of the entire experimental period were 26.36 – 30.90 °C and 60.15 – 85.17 % , respectively. At 5-7 weeks of age, it was found that there were no significant difference (P>0.05) in weight gain, feed intake and average daily gain (ADG) among treatment groups of the weaning pigs. The FCR of the piglets received 8% Roselle gave the same results which antibiotics and both groups were better than control (P<0.05) (Table 4.1). No significant difference was found among Roselle levels.

At 7-9 weeks of age, there was no difference in weight gain, feed intake , ADG and FCR among treatment groups of the weaning pigs. However, it was found that the weaning pigs received 8% Roselle tended to gave the both results when the ADG and FCR were considered together (Table 4.2).

At 9-11 weeks of age, no significant difference on weight gain, feed intake , ADG and FCR among treatment groups were found (P>0.05). However, the weaning pigs received three supplement levels of Roselle and acidifier diets showed higher ADG than control diet. The antibiotic diet tended to gave the best FCR followed by 8% Roselle (Table 4.3).

For the overall period of the experiment (5-11 weeks of age), there was no effect of any treatments on weight gain, feed intake , ADG and FCR (P>0.05) among groups fed the experimental diet. However, 8% Roselle and antibiotic tended to show better FCR compared to the others (Table 4.4).

Table 4.1 Effect of Roselle supplementation on growth performance of pigs at the 5-7 weeks of age[1] (experiment 1)

	Treatment					
Parameter	Control	4 % Roselle	8 % Roselle	12% Roselle	Acidifier	Antibiotic
Starting weight (kg/pig)	7.03 ± 0.58	7.23 ± 1.52	7.35 ± 0.53	7.14 ± 0.50	7.82 ± 1.02	7.48 ±1.03
Final weight (kg/pig)	12.13 ± 1.01	12.73 ± 2.89	13.12 ± 0.48	13.00 ± 0.79	13.90 ± 1.48	13.67 ± 1.93
Weight gain (kg/pig)	5.10 ± 0.48	5.51 ± 1.37	5.77 ± 0.13	5.86 ± 0.40	6.08 ± 5.03	6.18 ± 0.94
Feed intake (kg/pig)	6.65 ± 0.41	7.28 ± 1.80	6.72 ± 0.23	7.27 ± 0.50	7.53 ± 0.85	7.32 ± 1.24
Average Daily Gain (kg/pig/day)	0.36 ± 0.03	0.39 ± 0.10	0.41 ± 0.01	0.42 ± 0.03	0.43 ± 0.04	0.44 ± 0.07
Feed Conversion Ratio	1.31 ± 0.05^{A}	1.32 ± 0.02^{A}	1.17 ± 0.03^{B}	1.24 ± 0.06^{AB}	1.24 ± 0.04^{AB}	1.18 ± 0.09^{B}

[1]Mean ± SD.

$^{A, B}$Mean in the same row with different superscripts differed significantly ($P<0.05$)

Table 4.2 Effect of Roselle supplementation on growth performance of pigs at the 7-9 weeks of age[1] (experiment 1)

	Treatment					
Parameter	Control	4 % Roselle	8 % Roselle	12% Roselle	Acidifier	Antibiotic
Starting weight (kg/pig)	12.13 ± 1.01	12.73 ± 2.89	13.12 ± 0.48	13.00 ± 0.79	13.90 ± 1.48	13.67 ± 1.93
Final weight (kg/pig)	19.63 ± 1.75	20.73 ± 4.36	21.08 ± 1.78	20.60 ± 0.56	21.93 ± 2.49	20.90 ± 2.23
Weight gain (kg/pig)	7.50 ± 0.86	8.00 ± 1.55	7.97 ± 1.30	7.60 ± 0.28	8.03 ± 1.01	7.23 ± 0.53
Feed intake (kg/pig)	10.95 ± 1.49	12.02 ± 2.28	11.47 ± 0.76	11.56 ± 0.42	12.03 ± 1.08	10.73 ± 0.77
Average Daily Gain (kg/pig/day)	0.54 ± 0.06	0.57 ± 0.11	0.57 ± 0.10	0.54 ± 0.02	0.57 ± 0.07	0.52 ± 0.04
Feed Conversion Ratio	1.46 ± 0.06	1.50 ± 0.01	1.46 ± 0.14	1.52 ± 0.11	1.50 ± 0.08	1.49 ± 0.10

[1]Mean ± SD.

Table 4.3 Effect of Roselle supplementation on growth performance of pigs at the 9-11 weeks of age[1] (experiment 1)

	Treatment					
Parameter	Control	4 % Roselle	8 % Roselle	12% Roselle	Acidifier	Antibiotic
Starting weight (kg/pig)	19.63 ± 1.75	20.73 ± 4.36	21.08 ± 1.78	20.60 ± 0.56	21.93 ± 2.49	20.90 ± 2.23
Final weight (kg/pig)	28.67 ± 1.08	30.70 ± 4.76	31.03 ± 3.48	30.63 ± 0.32	33.07 ± 3.00	31.90 ± 2.25
Weight gain (kg/pig)	9.03 ± 1.21	9.97 ± 0.43	9.95 ± 1.87	10.03 ± 0.43	11.03 ± 0.63	11.00 ± 0.80
Feed intake (kg/pig)	14.10 ± 0.91	16.35 ± 2.08	14.93 ± 2.35	16.16 ± 0.29	17.17 ± 1.29	16.12 ± 0.74
Average Daily Gain (kg/pig/day)	0.64 ± 0.09	0.71 ± 0.03	0.71 ± 0.13	0.72 ± 0.03	0.80 ± 0.05	0.78 ± 0.06
Feed Conversion Ratio	1.57 ± 0.15	1.64 ± 0.13	1.51 ± 0.10	1.61 ± 0.08	1.54 ± 0.06	1.47 ± 0.07

[1]Mean ± SD.

Table 4.4 Effect of Roselle supplementation on growth performance of pigs at the 5-11 weeks of age[1] (experiment 1)

	Treatment					
Parameter	Control	4 % Roselle	8 % Roselle	12% Roselle	Acidifier	Antibiotic
Starting weight (kg/pig)	7.03 ± 0.58	7.23 ± 1.52	7.35 ± 0.53	7.14 ± 0.50	7.82 ± 1.02	7.48 ± 1.03
Final weight (kg/pig)	28.67 ± 1.08	30.70 ± 4.76	31.03 ± 3.48	30.63 ± 0.32	33.07 ± 3.00	31.90 ± 2.25
Weight gain (kg/pig)	21.67 ± 0.71	23.50 ± 3.24	23.70 ± 2.97	23.50 ± 0.30	25.27 ± 2.06	24.43 ± 1.33
Feed intake (kg/pig)	31.70 ± 2.01	35.67 ± 6.06	33.17 ± 3.25	35.03 ± 0.80	36.77 ± 2.48	34.17 ± 2.42
Average Daily Gain (kg/pig/day)	0.52 ± 0.02	0.56 ± 0.08	0.56 ± 0.07	0.56 ± 0.01	0.60 ± 0.04	0.58 ± 0.04
Feed Conversion Ratio	1.46 ± 0.07	1.51 ± 0.04	1.40 ± 0.04	1.49 ± 0.06	1.46 ± 0.04	1.40 ± 0.04

[1]Mean ± SD.

Experiment 2

Effect of Roselle supplementation on growth performance.

The average temperature and relative humidity of the entire experimental period were 25.20 – 31.94 °C and 51.32 – 85.71 % , respectively. At 5 -7 weeks of age , there were no significant difference on feed intake and ADG in all treatment groups. However, it was found that the weaning pig received 8 % Roselle diet had slightly more feed intake and ADG than control , acidifier and antibiotic diet.(Table 4.5)

At 7-9 weeks of age, feed intake and ADG were not significant different among all treatment groups. The weaning pigs received antibiotic diet had slightly more ADG than other treatment (P>0.05) (table 4.6). For the overall of 5-9 weeks of age of experiment, the piglets received 8% Roselle diet had most weight gain and ADG compared to the other groups (P>0.05) (Table 4.7).

Table 4.5 Effect of Roselle supplemention on growth performance of pigs at the 5-7 weeks of age[1] (experiment 2)

Parameter	Treatment			
	Control	8% Roselle	Acidifier	Antibiotic
Starting weight (kg/pig)	7.61 ± 0.04	7.62 ± 0.07	7.58 ± 0.06	7.58 ± 0.07
Final weight (kg/pig)	11.86 ± 0.49	12.22 ± 0.31	11.66 ± 0.54	11.82 ± 0.41
Weight gain (kg/pig)	4.25 ± 0.52	4.61 ± 0.27	4.08 ± 0.56	4.24 ± 0.35
ADG (kg/pig/day)	0.30 ± 0.04	0.33 ± 0.02	0.29 ± 0.04	0.30 ± 0.03

[1]Mean ± SD.

Table 4.6 Effect of Roselle supplemention on growth performance of pigs at the 7-9 weeks of age[1] (experiment 2)

Parameter	Treatment			
	Control	8% Roselle	Acidifier	Antibiotic
Starting weight (kg/pig)	11.95 ± 0.81	12.29 ± 0.81	11.56 ± 0.35	11.90 ± 0.73
Final weight (kg/pig)	20.84 ± 0.60	21.38 ± 1.67	20.58 ± 1.51	21.14 ± 1.11
Weight gain (kg/pig)	8.89 ± 0.48	9.10 ± 0.91	9.02 ± 1.47	9.24 ± 0.69
ADG (kg/pig/day)	0.64 ± 0.03	0.65 ± 0.06	0.64 ± 0.11	0.66 ± 0.05

[1]Mean ± SD.

Table 4.7 Effect of Roselle supplemention on growth performance of pigs at the 5-9 weeks of age[1] (experiment 2)

Parameter	Treatment			
	Control	8% Roselle	Acidifier	Antibiotic
Starting weight (kg/pig)	7.61 ± 0.04	7.62 ± 0.07	7.58 ± 0.06	7.60 ± 0.04
Final weight (kg/pig)	20.84 ± 0.60	21.38 ± 1.67	20.58 ± 1.51	21.14 ± 1.11
Weight gain (kg/pig)	13.24 ± 0.63	13.77 ± 1.64	13.00 ± 1.51	13.57 ± 1.06
ADG (kg/pig/day)	0.68 ± 0.02	0.70 ± 0.05	0.67 ± 0.03	0.68 ± 0.03

[1]Mean ± SD.

Effect of Roselle supplementation on pH of the gastrointestinal tract.

The pH level in various sections of the gastrointestinal tract in all treatment groups are depicted in Tables 4.8-4.9. At 7th week of age, there were no significant difference on pH of the whole gastrointestinal tract among treatment groups .The weaning pigs received 8 % Roselle diet had lower pH in stomach than control, acidifier and antibiotic diet (P>0.05). There were no change in the pH of duodenum, jejunum and ileum among treatment groups.

At 9th week of age, The weaning pigs received 8 % Roselle diet had lower pH in stomach than control, acidifier and antibiotic diet (P>0.05). Likewise, pH of duodenum and jejunum of piglet received 8% Roselle tended to be lower than other treatment groups (P>0.05).

Table 4.8 Effect of Roselle supplementation on pH of the gastrointestinal tracts at 7th week of age[1]

	Treatment			
Gastrointestinal tracts	Control	8% Roselle	Acidifier	Antibiotic
Stomach	5.21 ± 0.25	4.02 ± 0.88	4.54 ± 0.55	4.66 ± 0.40
Duodenum	5.83 ± 0.32	5.97 ± 0.39	5.98 ± 0.43	5.87 ± 0.28
Jejunum	5.56 ± 0.42	5.62 ± 0.54	5.88 ± 0.44	5.33 ± 0.51
Ileum	6.68 ± 0.25	6.91 ± 0.17	6.72 ± 0.39	6.84 ± 0.31

[1]Mean± SD.

Table 4.9 Effect of Roselle supplementation on pH of the gastrointestinal tracts at 9th week of age[1]

	Treatment			
Gastrointestinal tracts	Control	8% Roselle	Acidifier	Antibiotic
Stomach	3.65 ± 1.03	3.04 ± 0.31	4.11 ± 0.48	3.97 ± 0.25
Duodenum	5.81 ± 0.47	5.16 ± 0.19	5.96 ± 0.28	5.42 ± 0.53
Jejunum	5.58 ± 0.59	5.30 ± 0.29	5.65 ± 0.45	5.85 ± 0.45
Ileum	6.89 ± 0.27	6.79 ± 0.14	6.82 ± 0.11	6.56 ± 0.10

[1]Mean± SD.

Effect of Roselle supplementation on pepsin activity of gastric mucosa.

The pepsin activity of cardiac mucosa is shown in Table 4.10. At 7th week of age, there was no significant difference among treatment diets. Pig recieved the 8 % Roselle diet had tend slightly higher pepsin activity than pigs received other treatment diets while at 9th week of age, it was found that the weaning pigs received antibiotic diet had the highest pepsin activity compared to control, 8% Roselle and acidifier diet respectively (P>0.05).

Table 4.10 Effect of Roselle supplementation on mucosal cell stomach pepsin activity (PU^{Hb} /mg protein) of the weaning pigs[1] (experiment 2).

Week of age	Treatment			
	Control	8% Roselle	Acidifier	Antibiotic
7th wk	0.48 ±0.10	0.79 ± 0.15	0.63 ± 0.25	0.57 ± 0.11
9th wk	0.69 ±0.05	0.74 ± 0.10	0.90 ± 0.15	0.80 ± 0.21

[1]Mean ± SD.

$PU^{Hb} \times 10^{-3}$ = 1 mmol tyrosine – equivalents liberated / minute at 35.5 °C

Effect of Roselle supplementation on pancreatic trypsin activity.

The pancreatic trypsin activity in piglets is depicted in Table 4.11. At 7th week of age, The pancreatic trypsin activity in the weaning pigs received 8 % Roselle diet increased trypsin activity (P<0.05) compared to control diet but there was no significant difference with acidifier and antibiotic supplemented diets (P>0.05). At 9th week of age, there was no significant difference in all treatment groups.

Table 4.11 Effect of Roselle supplementation on pancreatic trypsin activity (U /mg protein) of the weaning pigs[1] (experiment 2).

	Treatment			
Week of age	Control	8% Roselle	Acidifier	Antibiotic
7th week of age	1.43 ± 0.28[B]	2.21 ± 0.35[A]	2.36 ± 0.35[A]	2.22 ± 0.37[A]
9th week of age	2.14 ± 0.36	2.04 ± 0.35	2.26 ± 0.34	2.38 ± 0.23

[1]Mean ± SD.

[A, B]Mean in the same row with different superscripts differed significantly (P<0.05)

U = The amount of 1 μmol hydrolysed substrate per minute

Effect of Roselle supplementation on ileal digestibility of nutrients.

Ileal digestibility of protein and fat

Ileal digestibility of protein and fat of the weaning piglets are shown in the table 4.12. All treatments have no significant effect on ileal digestibility of protein at 7th and 9th weeks of age, there was no significantly difference among all treatments but the weaning pigs received antibiotic diet had slightly higher protein digestibility than acidifier, 8 % Roselle and control group (P>0.05) respectively.

For digestibility of fat at 7th week of age, it was found that the ileal digestibility of fat in 8% Roselle diet group was greater than the control group (P<0.05) but there was no difference compared to acidifier and antibiotic group. At 9th week of age the weaning pigs received 8% Roselle diet had slightly higher fat digestibility than other treatment group (P>0.05)

Table 4.12 Effect of Roselle supplementation on ileal digestibility (DM) of nutritiens (%) of the weaning pigs[1].

	Treatment							
Nutritions	Control	N	8% Roselle	N	Acidifier	N	Antibiotic	N
Protein								
7th wk of age	81.39 ± 3.04	3	81.47 ± 1.53	2	82.20 ± 0.91	4	82.44 ± 0.48	2
9th wk of age	74.90 ± 12.37	2	78.68 ± 5.93	2	85.91 ± 7.36	4	88.13	1
Fat								
7th wk of age	81.02 ± 1.89^{B}	4	85.83 ± 0.73^{A}	2	83.18 ± 1.66^{AB}	4	85.30 ± 0.46^{A}	2
9th wk of age	85.67 ± 5.60	2	88.62 ± 5.89	2	76.95 ± 14.13	4	83.86	1

[1]Mean ± SD.

$^{A, B}$Mean in the same row with different superscripts differed significantly (P<0.05)

Effect of Roselle supplementation on plasma malondialdehyde

The plasma malondialdehyde of the weaning pigs received treatment diet at 7th and 9th week of age is shown in table 4.13. It was found that 8% Roselle diet had the lowest malondialdehyde compared with control, acidifier and antibiotic groups (P>0.05) .

Effect of Roselle supplementation on liver malondialdehyde

The liver malondialdehyde is depicted in Table 4.14. At 7th week of age, there was no significant difference in liver malondialdehyde of all treatment groups. The result showed that malondialdehyde in the piglets received control diet was slightly lower (P>0.05) than other treatments. At 9th week of age , the piglets received 8 %Roselle diet had the lower malondialdehyde than control, acidifier and antibiotic groups, respectively (P>0.05).

Table 4.13 Effect of Roselle supplementation on plasma malondialdehyde (nmol/ml) of the weaning pigs (experiment 2).

	Treatment			
Week of age	Control	8% Roselle	Acidifier	Antibiotic
7th week of age	4.57 ± 0.33	3.64 ± 0.97	4.07 ± 0.57	3.71 ± 1.98
9th week of age	4.28 ± 1.21	3.91 ± 0.77	4.66 ± 1.23	4.12 ± 0.83

[1]Mean ± SD.

Table4.14 Effect of Roselle supplementation on liver tissue malondialdehyde (nmol/mg protein) of the weaning pigs (experiment 2).

	Treatment			
Week of age	Control	8% Roselle	Acidifier	Antibiotic
7th week of age	3.20 ± 1.33	2.15 ± 1.14	3.07 ± 1.26	3.60 ± 2.26
9th week of age	3.36 ± 1.40	2.69 ± 0.79	3.34 ± 2.44	3.35 ± 1.83

[1]Mean ± SD.

Effect of Roselle supplementation on plasma glutathione

The effect of treatment on plasma glutathione is depicted in Table 4.15. At 7th week of age, the weaning pigs recieved antibiotic diet had significantly higher glutathione than control diet ($P<0.05$) but was not different from 8 % Roselle and acidifier diets ($P>0.05$). At 9th week of age, there was no significant difference in plasma glutathione of all treatment diets ($P>0.05$) while plasma glutathione in antibiotic diet was slightly higer than 8% roselle and acidifier diet .

Effect of Roselle supplementation on liver tissue glutathione

At 7th week of age, there was no significant difference in liver tissue glutathione among all treatment diets ($P>0.05$). However, pigs recieved 8 % Roselle diet had highest glutathione when compared with control, acidifier and antibiotic diet. At 9th

week of age, The liver tissue glutathione of the acidifier diet was slightly higher than control, 8% Roselle and antibiotic diet treatments (Table 4.16).

Table 4.15 Effect of Roselle supplementation on plasma glutathione (nmol/ml) of the weaning pigs (experiment 2).

	Treatment			
Week of age	Control	8% Roselle	Acidifier	Antibiotic
7th week of age	6.59 ± 2.31[B]	9.42 ± 2.31[AB]	10.60 ± 4.38[AB]	13.42 ± 1.61[A]
9th week of age	6.12 ± 2.93	10.60 ± 2.81	11.30 ± 2.43	12.25 ± 3.69

[1]Mean ± SD.

[A, B]Mean in the same row with different superscripts differed significantly ($P<0.05$)

Table 4.16 Effect of Roselle supplementation on liver tissue glutathione (nmol/mg protein) of the weaning pigs (experiment 2).

	Treatment			
Week of age	Control	8% Roselle	Acidifier	Antibiotic
7th week of age	5.58 ± 0.97	7.15 ± 1.81	6.32 ± 1.22	5.90 ± 2.03
9th week of age	5.35 ± 1.49	7.45 ± 2.92	8.95 ± 0.85	6.58 ± 0.74

[1]Mean ± SD.

CHAPTER V

DISCUSSION

In the present study, the experiments were divided into 2 separated sets of experiment. The first set was designed only to find out an appropriate level of Roselle in promoting growth performances in weaning pigs since there has never been any report concerning the determination of suitable level of Roselle in weaning pigs; while the second set was designed to examine the effects of Roselle on growth performances, physiological status and its antioxidant properties in weaning pigs.

Experiment 1

Effect of Roselle supplementation on growth performance

In the experiment 1, the result showed that supplementation of Roselle to the weaning pigs at 5-7 weeks of age significantly affected ($P<0.05$) growth performance especially feed conversion ratio (FCR). If three levels of Roselle were considered, it was found that 8% Roselle had the best FCR when compared to other levels and had the same results with acidifier and antibiotic. In addition, it was found that feed intake in 8% Roselle was lower than acidifier and antibiotic.

At 7-9 and 9 -11 weeks of age, the result showed that supplementation of Roselle did not significantly affect growth performance in both experimental periods. However, at 7-9 weeks of age, the effect of 8% Roselle tended to improve ADG and FCR and gave similar result in weaning pigs at 5-7 weeks of age . At the overall period, the result showed that weaning pig received 8% Roselle tended to have the most preferable growth performance. Thus, the supplementation of Roselle at the level 8% will be used in the second experiment.

Experiment 2

Effect of Roselle supplementation on pH of the gastrointestinal tracts, gastrointestinal enzyme activities and nutrients digestibility

There are several physiological restrictions on weaning pigs when switching from the sow's milk to the new feeding regimen. After weaning, the digestive system of the pig has to adapt to solid feed with respect to pH, enzyme secretion and gut motility (Owsley,1986). One of the most important factors in causing digestive disturbance is the low activity of proteolytic enzymes (Wilson et al,1981). There may be hypothesized that hydrochloric acid production by the weanling pig may be inadequate to activate much of the pepsinogen produced by the gastric mucosa. The low gastric pH is essential for pepsin activity to digestion of proteins. Wilson (1981) reported that the development of the gastric pH of the weaning pig is low in acidity for the first 40 days. A high gastric pH would cause a reduction in the activation of pepsin. The optimal pH for pepsin activity is 2-3.5 (Austin et al, 2000) . Pepsin activity declines rapidly when pH rises above 3-6 and remains inactive at pH 6 (Partanen and Mroz,1999). The elevation in pH would lead to a low proteolytic activity in the stomach and dietary proteins may enter the small intestine essentially intact, possibly resulting in a reduction in the efficiency of protein digestion.

In the present experiment, the addition of 8% Roselle showed lower gastric pH of stomach at 7^{th} and 9^{th} week of age and showed result similar to the addition of acidifier group. The gastric pH was reduced in weaning pigs received 8% Roselle due to the action of organic acids in calyx Roselle (Nanthawan and Auranut,1997). This reduction in pH in this experiment may decrease the pH value of stomach contents and increased pepsin activity. The low pH of digesta and the end product of pepsin digestion entering the duodenum are involved in the stimulation of the pancreatic secretion of enzymes and bicarbonate and they also play a minor role in regulation of gastric emptying (Partanen and Mroz,1999). Furthermore, the low gastric pH condition also needs to prevent passage of potentially harmful microbes to the small intestine (Austin et al, 2000). However, the

experiment showed no significant decrease in stomach and all part of intestinal pH in Roselle fed group compared to other groups. Similar results were found by Scipioni et al (1978) who reported a nonsignificant reduction in the stomach and jejunal pH of weanling pigs by feeding diets containing 1%citric acid. Burnell et al (1988) observed a nonsignificant decrease in the pH of the small intestine from 6.76 to 6.19 in 7-wk-old weanling pigs fed a starter diet with 1% sodium citrate. In addition, there was no effect of 1.8% K-diformate ($P>0.05$) on pH along the gastrointestinal tract of piglets at 7^{th} and 26^{th} day postweaning.

According to the present study, activities of proteolytic enzyme including pepsin in stomach and trypsin in pancreas were examined. At 7^{th} week of age, supplementation of 8% Roselle decreased gastric pH and effectively promoted pepsin activity. Consequently, the low pH of digesta and the end products of pepsin digestion entering the duodenum are involed in the stimulation of the pancreatic secretion of enzymes such as trypsin. This result especially trypsin was silimar to the result reported by Thaela et al (1998) that 2.5% lactic acid supplementation in weaning pig increased trypsin and chymotrypsin activity in pancreas. However, 8% Roselle addition failed to response to proteolytic enzyme in 9^{th} week of age because pigs can produce HCl adequately to maintain stomach pH due to the gastric pH at 9^{th} week of age is lower than 7^{th} week of age in all groups.

The effect of Roselle supplementation on the ileal digestibilities of nutrients included protein and fat was examined . Jongbloed et al (2000) reported that protein digestibility as well as the digestibility of other nutrients were reported to be improved by the addition of organic acids. In this study, there were no positive effect of 8% Roselle on ileal digestibility of protein at both 7^{th} and 9^{th} week of age. Similar result was found in the supplementation of 1% , 2% fumaric and citric acids in the diet which had no effect on apparent digestibility of protein and dry matter on weaning pig at 4^{th} week (Falkowski and Aherne,1984). This results support the conclusion of Giesting et al (1991) who reported that 2% fumaric acid supplementation did not affect nutrient digestibilities. These results contradict the findings of Kirchgessner and Roth (1982), who reported significant improvements in nitrogen and dry matter digestibilities when the diets contained 1.5 to 2% fumaric acid. Similary, Kemme (1998) investigated that a combination 0.6% formic and propionic acids (75:25) caused an improvement of digestibility of crude protein.

Basically organic acids increase digestibility of nutrients by means of modifying the pH of the digesta, thus stimulating endogenous proteinase. In addition organic acids can control pathogenic bacterial population and favour beneficial bacterial growth. Effect of organic acids on ileal digestibility was equivocal. In this experiment, changes in intestinal pH was not marked, possibly because organic acids in the crude powder of Roselle may not dissociate well in the intestine. Moreover, increased trypsin activity in pigs receiving Roselle and organic acid did not indicate that overall protein digestibility would improve because there were many luminal and brush border enzymes responsible for protein digestion.

For fat digestibility, improved digestibility in Roselle may be due to stimulation of bile and pancreatic lipase secretion in response to acidic pH from the stomach as seen previously. The effect of both Roselle and antibiotic on fat digestibility was clearly improved in 7^{th} week pig compared to 9^{th} week pig because both additive can facilitate on the immature function of gastrointestinal tract found 7^{th} week of age.

Due to the fact that ileal content samples could be collected in very small amount and were mostly in liquid or high in water content. The samples are inadequate to be analyzed with too many parameters. Thus, the data were too insufficient to draw the statistical conclusion. Further investigation is needed to confirm the nutrient digestibility results.

Effect of Roselle supplementation on lipid peroxidation

Reactive oxygen species (ROS) can be originated from several internal and external sources, such as metabolic reactions, dietary intake and stress . Excessive formation of oxidants in biological systems and the consequent oxidative damage are probably contribute to several diseases. (Tseng et al.,1997). Although the body possesses defence mechanisms to reduce the oxidative damage, such as using enzymes and antioxidant nutrients to arrest the damaging properties of excited oxygen species, continuous exposure to chemicals and contaminants may lead to an increase in the amount of free radicals in the body beyond control, and cause irreversible oxidative damage. Free radicals have been implicated in the development of seizures. When the production of free radicals increases, they cause cellular dysfunction by attacking at the polyunsaturated sites of the biological

membranes leading to lipid peroxidation that has been recognized as being a potential mechanism for cell injury (Gupta et al., 2003). The increase in the levels of malondialdehyde (MDA), a marker of lipid peroxidation indicates increasing free radical generation.

Phenolic compounds which are widely distributed in plants were considered to play an important role as dietary antioxidants for the prevention of oxidative damage in living systems (Wang et al., 2000). The calyx of Roselle consists of several phenolic compound and their antioxidative effects were determined in previous studies. Wang et al (2000) reported that the extract of calyx Roselle at the concentrations of 0.1 and 0.2 mg/ml in rat primary hepatocytes and 100 and 200 mg/kg in hepatotoxicity in rat induced cytotoxicity by *tert*-butyl hydroperoxide (t-BHP) significantly decreased the formation of malondiladehyde (MDA). This was similar to the report of Tseng et al (1997) who showed that the dried calyx extract of Roselle decreased significantly the formation of MDA induced by t-BHP considerably at a concentration of 0.1 and 0.2 mg/ml in the rat primary hepatocyte cultures. In this study, it was found that the 8% calyx Roselle exhibited antioxidant bioactivity by decreasing formation of MDA in plasma and liver tissue. However,there was no significant change in malondialdehyde when 8% Roselle was fed. It is possible that the observed antioxidant effect was limited because the calyx of Roselle used in this experiment were in crude powder form. However, The biological effects of phenolic compounds in calyx of Roselle have not so far been well elucidated. The present investigation is the first report showing the antioxidant properties of Roselle in intact cell systems in weaning pig, further researches should be investigated to confirm this result.

Glutathione (GSH) is an endogenous antioxidant protein existing mainly in the reduced form within the cells. It reacts with the free radicals and prevents the generation of hydroxyl radicals, the most toxic form of free radicals. This defensive process, reduced GSH is converted to its oxidized form with the help of the enzyme glutathione peroxidase. The decreased level of reduced glutathione indicates that there was an increasing generation of free radicals and the reduced glutathione was depleted during the process of combating oxidative stress (Gupta et al., 2003). The concentration of reduce glutathione in plasma and liver tissue has been determined in this experiment . The results found that the 8%

Roselle showed a slight increase in level of GSH compared to other treatment groups, the mode of action of Roselle on GSH needs further investigation.

Effect of Roselle supplementation on growth performance

At the overall period, the result showed that supplementation of Roselle to the weaning pigs tended to improve weight gain and average daily gain in 5-7 week of age. In addition, in the overall period (5-9 weeks of age) it was found that 8% Roselle had effect on growth performance similar to acidifier and antibiotic although the difference was not statistically significance. This result can demonstrate that reduction in pH in this experiment may have increased proteolytic enzyme activity, decreased MDA formation and also increased GSH to promote the positive performance of weaning pigs received Roselle.

In conclusion, the present study demonstrated that supplementation of Roselle did promote the reduction of gastric pH and the increase of proteolytic enzyme from pancreas. According to the result, the calyx of Roselle may have acidifier properties. Also, the calyx of Roselle in weaning pigs may have antioxidant properties due to the fact that it tended to decrease MDA formation and increase GSH although it had no distinct difference compared to other groups. It is also interested whether Roselle will be beneficial in weaning pigs as in acidifier and antibiotic. Further studies on the use Roselle in the form of crude extract as an antioxidant and as a promoter of nutrient digestibility in weaning pigs are needed. It is suggest that further studies on the use of Roselle as an antioxidant and as a promoter of nutrient digestibility in weaning pigs should use Roselle in the form of crude extract rather than powder and raise in commercial condition.

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