การศึกษาปัจจัยเสี่ยงในการเกิดโรคไตเรื้อรังในแมว ภาวะเครียดออกซิเดชั่นและผลของสารสกัดหยาบจากเม่าสร้อยต่อเซลล์เพาะเลี้ยงไตแมว

นางคคนางค์ ปิยะรังษี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาอายุรศาสตร์สัตวแพทย์ ภาควิชาอายุรศาสตร์
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2557

INVESTIGATION OF RISK FACTORS INVOLVING IN FELINE CHRONIC KIDNEY DISEASE, OXIDATIVE STRESS AND STUDY THE EFFECT OF *ANTIDESMA ACIDUM* CRUDE EXTRACT IN FELINE KIDNEY CELL LINE

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Veterinary Medicine
Department of Veterinary Medicine
Faculty of Veterinary Science
Chulalongkorn University
Academic Year 2014

Thesis Title	INVESTIGATION OF RISK FACTORS INVOLVING IN FELINE CHRONIC KIDNEY DISEASE, OXIDATIVE STRESS AND STUDY THE EFFECT OF *ANTIDESMA ACIDUM* CRUDE EXTRACT IN FELINE KIDNEY CELL LINE
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คคนางค์ ปิยะรังษี : การศึกษาปัจจัยเสี่ยงในการเกิดโรคไตเรื้อรังในแมว ภาวะเครียดออกซิเดชั่น และผลของสารสกัดหยาบจากเม่าสร้อยต่อเซลล์เพาะเลี้ยงไตแมว (INVESTIGATION OF RISK FACTORS INVOLVING IN FELINE CHRONIC KIDNEY DISEASE, OXIDATIVE STRESS AND STUDY THE EFFECT OF *ANTIDESMA ACIDUM* CRUDE EXTRACT IN FELINE KIDNEY CELL LINE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. สพ.ญ. ดร. รสมา ภู่สุนทรธรรม, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. นสพ. ดร. อนุเทพ รังสีพิพัฒน์, รศ. ภก. ดร. บุญชู ศรีตุลารักษ์, 129 หน้า.

โรคไตเรื้อรังเป็นโรคที่สำคัญในแมว วัตถุประสงค์ของการศึกษาในครั้งนี้ เพื่อหาปัจจัยเสี่ยงในแมวโรคไตเรื้อรังที่เกิดขึ้นเองตามธรรมชาติ เพื่อเปรียบเทียบค่าความเครียดออกซิเดชั่นระหว่างแมวสุขภาพดีและแมวป่วยด้วยโรคไตเรื้อรังที่เกิดขึ้นเองตามธรรมชาติ และเพื่อค้นหาผลของสารสกัดหยาบจากเม่าสร้อยต่อการแสดงออกของยีนเอ็นโดธีเลียลไนตริกออกไซด์ซินเทส (endothelial nitric oxide synthase (eNOS)) ในเซลล์เพาะเลี้ยงไตแมวที่ถูกโน้มนำด้วยดอกโซรูบิซิน (doxorubicin (DOX)) การศึกษาแบ่งออกเป็นสามส่วน ส่วนที่หนึ่งศึกษาถึงปัจจัยเสี่ยงของโรคไตเรื้อรังในแมวโดยการสัมภาษณ์เจ้าของแมวจำนวน 222 ตัว ในช่วงระหว่างเดือนมิถุนายน 2547 ถึงเดือนสิงหาคม 2556 ข้อมูลแบบสอบถามประกอบด้วย อายุ เพศ พันธุ์ น้ำหนัก ชนิดของอาหาร และอาการทางคลินิกของโรคไตเรื้อรัง ส่วนที่สองประกอบด้วยแมวที่มีอายุใกล้เคียงกันจำนวน 2 กลุ่ม คือ แมวสุขภาพดีจำนวน 13 ตัว และแมวโรคไตเรื้อรังจำนวน 23 ตัว เก็บตัวอย่างเลือดเพื่อตรวจหาค่าโลหิตวิทยา ค่าเคมีคลินิก และค่าภาวะเครียดออกซิเดชั่นประกอบด้วยค่า glutathione (GSH), oxidized glutathione (GSSG), glutathione peroxidase (GPx) และ GSH/GSSG ratio ส่วนที่สามสารสกัดหยาบจากเม่าสร้อยในขนาดที่เหมาะสมถูกบ่มในเซลล์เพาะเลี้ยงไตแมวที่ถูกโน้มนำด้วย DOX ก่อนและหลังการรักษา หาค่า cytotoxicity assay, apoptosis และ necrosis assay, oxidative stress parameters การแสดงออกของยีนและโปรตีน eNOS ผลการทดลองพบว่ารูปแบบอาหาร และการเลี้ยงดูมีความสัมพันธ์กับการเกิดโรคไตเรื้อรังในแมว โดยเฉพาะอย่างยิ่งอายุซึ่งเป็นปัจจัยเสี่ยงที่สำคัญ แมวป่วยด้วยโรคไตเรื้อรังที่เกิดขึ้นเองตามธรรมชาติมีภาวะเครียดออกซิเดชั่น และสารสกัดหยาบจากเม่าสร้อยที่ให้ก่อนการโน้มนำเซลล์ไตแมวด้วย DOX สามารถเพิ่มการแสดงออกของยีนและโปรตีน eNOS อย่างมีนัยสำคัญ เม่าสร้อยสามารถป้องกันเซลล์ไตแมวจากการเกิดโรคไตวาย ซึ่งมีกลไกในการลดภาวะ oxidative stress และเพิ่มการสร้างยีนของ eNOS

ภาควิชา อายุรศาสตร์	ลายมือชื่อนิสิต .....
สาขาวิชา อายุรศาสตร์สัตวแพทย์	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
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5475412031 : MAJOR VETERINARY MEDICINE
KEYWORDS: ANTIDESMA ACIDUM / FELINE CHRONIC KIDNEY DISEASE / FELINE KIDNEY CELL LINE / OXIDATIVE STRESS / RISK FACTORS

KAKANANG PIYARUNGSRI: INVESTIGATION OF RISK FACTORS INVOLVING IN FELINE CHRONIC KIDNEY DISEASE, OXIDATIVE STRESS AND STUDY THE EFFECT OF *ANTIDESMA ACIDUM* CRUDE EXTRACT IN FELINE KIDNEY CELL LINE. ADVISOR: ASSOC. PROF. ROSAMA PUSOONTHORNTHUM, D.V.M., M.Sc., Ph.D., CO-ADVISOR: ASSOC. PROF. ANUDEP RUNGSIPIPAT, D.V.M., Ph.D., ASSOC. PROF. BOONCHOO SRITULARAK, B.S., M.S., Ph.D., 129 pp.

Chronic kidney disease (CKD) is an important disease in feline. The purposes of the study were to determine the risk factors in cats with naturally occurring CKD, to compare the oxidative stress parameters between the clinically normal client-owned cats and cats with naturally occurring CKD and to investigate the effects of the ethanol crude extract of *Antidesma acidum* on endothelial nitric oxide synthase (eNOS) gene expression in doxorubicin (DOX)-induced feline kidney cell lines. The study was separated into three parts. In part I, risk factors in cats with CKD were determined by interviewed two hundred and twenty-two cats' owners through questionnaires during June 2004 to August 2013. Data about age, gender, breed, weight, types of food and clinical signs of CKD were obtained. In part II, thirteen clinically normal client-owned aged-matched cats and twenty-three naturally-occurring CKD cats were included. Blood collection was performed to measure completed blood count, blood urea nitrogen, creatinine, glutathione (GSH), glutathione peroxidase (GPx), oxidized glutathione (GSSG) and GSH/GSSG ratio. In part III, feline kidney cell lines were incubated with appropriate dose and time of DOX and *A. acidum* in pretreatment and post-treatment condition. Cytotoxicity assay, apoptosis and necrosis assay, oxidative stress parameters, eNOS protein and eNOS gene expression were measured. The results indicated that aging, diet and lifestyles of CKD cats were potential risk factors for CKD. Cats with naturally occurring CKD had oxidative stress when compared with age-matched control cats. Pretreatment with *A. acidum* can significantly increase eNOS gene and protein expression in DOX-induced feline kidney cell lines. *A. acidum* can protect feline kidney cells from CKD by suppressing the oxidative stress and increasing the eNOS gene production.

Department:	Veterinary Medicine	Student's Signature	.....
Field of Study:	Veterinary Medicine	Advisor's Signature	.....
Academic Year:	2014	Co-Advisor's Signature	.....
		Co-Advisor's Signature	.....

ACKNOWLEDGEMENTS

The author thanks the advisor, Associate Professor Dr. Rosama Pusoonthornthum and the co-advisors, Associate Professor Dr. Anudep Rungsipipat and Associate Professor Dr. Boonchoo Sritularak for their assistance, advice and guidance in the thesis.

She would like to thank Associate Professor Dr. Chatsri Deachapunya from Department of Physiology, Faculty of Medicine, Srinakharinwirot University for her assistance, advice and extensive help in place of study.

The author thanks Assistant Professor Dr. Somporn Techangamsuwan for her advice and assistance in part of apoptosis, necrosis assay and western blot analysis. Special thanks to Associate Professor Achara Tawatsin for her advice in statistical analysis.

She would like to thank friends and clinicians from the Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University, Suvarnachad Animal Hospital, Sukhumvit Vet Clinic, Kaithong pet care and Metta Animal Hospital for their support, encouragement and extensive help in case collection.

The author thanks Mrs. Panumas Ruantongdee, Miss Sujin Sirisawadi, Miss Noratee Bauthong, Mr. Pongpat Keiatpraseird, Mr. Yodsawee Srisomboon and Miss Waraporn Yahayo for their technical guidance.

This study was supported by grants from the Faculty of Veterinary Science and the Graduate school, Chulalongkorn University.

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CHAPTER I

INTRODUCTION

Chronic kidney disease (CKD) is the most common diseases in geriatric cats. CKD was found in 1.9% of the cat in veterinary practices in the United State (Lund et al., 1999) and 20% of the sick cats presented to the veterinary hospitals in Australia (Watson, 2001). In Thailand, the proportional morbidity ratio of cats with chronic renal failure (CRF) was 6 CRF cats per 1000 cats visited to hospitals (Pusoonthornthum et al., 2009). Previous studies reported that commercial food, which contained low potassium and high protein ingredient, caused the development of CRF in cats (Buffington et al., 1991; DiBartola et al., 1993). Hughes et al (2002) found that high fiber diet was considered as a factor to decrease risk of CRF in cats. Breed of cats was proposed to be the risk factor for CKD (Polzin et al., 2000; Pusoonthornthum et al., 2009), whereas other signalment data were not associated with CKD. Moreover, aging has been found to associate with decreased renal function (Coresh et al., 2007). Cats with CKD often present with weight loss, polyuria, polydipsia, poor body condition, dehydration, nonregenerative anemia, small and irregular kidneys. The major kidney lesion of feline CRF is tubulointerstitial nephritis (Lawler et al., 2006). Studies were reported that CKD in cats may be related to a high production of the free radicals (Viviano et al., 2009; Keegan and Webb, 2010; Krofic Zel et al., 2014). Several studies were also demonstrated that oxidative stress promotes the progression of chronic renal failure in human patient (Dounousi et al., 2006; Rutkowski et al., 2006).

Nitric oxide (NO) is produced from L-arginine by nitric oxide synthase (NOS). This enzyme is expressed in three isoforms including endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). NO plays a very important role in controlling kidney function by regulating vasodilation and sodium excretion. CRF is associated with NO deficiency by several mechanisms including reduction of L-arginine and eNOS (Chang et al., 2002). Lack of eNOS may develop progressive renal injury (Forbes et al., 2007). Other reports that the reduction of L-arginine and eNOS and accumulation of naturally occurring NOS inhibitors and oxidative stress may play role (Chang et al.,

2002). NO is related to oxidative stress through inactivation of NO by $O_2^{-}\cdot$ to create peroxynitrite ($ONOO^-$) which is a strong oxidant.

Antidesma acidum is a fruit-bearing deciduous shrub or small tree growing to between 2 – 5 meters tall. The fruit is round with dark red color. The ethanol crude extract from *A. acidum* is rich in flavonoids and stigmasterol (Thamaree et al., 2003). Previous report indicated that an extract prepared from the stem of *A. acidum* can increase the number of lymphocytes, band cells and alkaline phosphatase but decreased the level of aspartate aminotransferase, alanine aminotransferase, blood urea nitrogen (BUN) and creatinine in healthy cats (Pusoonthornthum et al., 2010). *A. acidum* was reported that it is widely used as drug supplement in cats with kidney disease (Fungbun et al., 2012; Jaimun et al., 2012) and human immunodeficiency virus(HIV) in patients (Kuman-Pawa et al., 2003). Study in moderate renal azotemia cats received the crude extract from *A. acidum* demonstrated a significantly decrease in the creatinine levels on day 28 and 42 of the study (Fungbun et al., 2012).

Up to present, the etiology of CKD is still unknown. Previous studies were concentrated on clinical signs and indicated no association between CKD and signalment data (except breed of cats). Although the crude extract from *A. acidum* has been shown to decrease creatinine levels in cats with CKD (Fungbun et al., 2012; Jaimun et al., 2012), its mechanism is unknown.

The purposes of the present study were to determine the risk factors in cats with CKD, to compare the oxidative stress parameters between the clinically normal client-owned cats and the cats with naturally occurring CKD and to investigate the effects of the ethanol crude extract of *A. acidum* on eNOS messenger ribonucleic acid (mRNA) expression in doxorubicin (DOX)-induced feline kidney cells.

Objectives of the study

1. To determine the risk factors in cats with naturally occurring CKD.

2. To compare the oxidative stress parameters [glutathione (GSH), oxidized glutathione (GSSG) and glutathione peroxidase (GPx)] between the clinically normal age-matched cats and cats with naturally occurring CKD.

3. To investigate the effects of the ethanol crude extract from *A. acidum* on eNOS mRNA expression in DOX-induced feline kidney cells.

Hypothesis

1. Gender, breed, diet and lifestyle of cats are associated with increased risk for chronic kidney disease.

2. The levels of oxidative stress parameters in the naturally occurring CKD cats are higher than the clinically normal client-owned cats.

3. The crude extract of *A. acidum* can increase eNOS mRNA expression in DOX-induced feline kidney cells.

Keywords : *Antidesma acidum*, feline chronic kidney disease, Feline Kidney Cell Line, oxidative stress, risk factors

Advantages of the study

1. To find the causes and preventive measures for cats with naturally occurring CKD in the future

2. To promote the use of local Thai herb as an alternative treatment for cats with spontaneous CKD which is one of the most common disease in geriatric cats.

3. To apply the use of natural Thai herb for the medical treatment in CKD patients.

CHAPTER II

LITERATURE REVIEW

Feline chronic kidney disease

CKD is the most common disease in geriatric cats (Lawler et al., 2006). According to the International Renal Interest Society (IRIS), there are four stages of CKD consisting of stage I (non-azotemic; creatinine < 1.6 mg/dL), stage II (mild renal azotemia; creatinine 1.6 – 2.8 mg/dL), stage III (moderate renal azotemia; creatinine 2.8 – 5.0 mg/dL), and stage IV (severe renal azotemia; creatinine > 5.0 mg/dL) (Brown, 2004). CKD is defined as an irreversible, decline in renal function and decrease in glomerular filtration rate (GFR) of more than 50% for at least 3 months duration which causes reduction of the renal excretion and homeostasis disorder (Polzin, 2010).

CKD was found in 1.9% of the cat in veterinary practices in the United State (Lund et al., 1999) and 20% of the sick cats presented to the veterinary hospitals in Australia (Watson, 2001). In Thailand, the proportional morbidity ratio of cats with CRF was 6 CRF cats per 1000 cats visit to the hospital (Pusoonthornthum et al., 2009). It was commonly found in older cats. In one previous study, 53% of 74 cats with CKD were over 7 years old and cats' age ranged between 9 months to 22 years. The study of age distribution in cats with CRF has found to be 37% less than 10 years old, 31% between 10 to 15 years old and 32% older than 15 years of age. During 1998, one study had been indicated that the mean age of 80 cats with CKD was 12.6 years. In Australia, study of 184 feline with CRF has indicated that male with CRF had median age of 12 years, whereas female with CRF had median age of 15 years (White et al., 2006). In Thailand, the mean age of CKD cats was 6 years (Pusoonthornthum et al., 2009) and 11.17 ± 0.86 years (Jaimun et al., 2012). CKD in cats is increased with increasing age.

The common clinical signs of CKD cats are weight loss, polyuria and polydipsia, poor body condition, dehydration, non-regenerative anemia, small and irregular kidneys. Metabolic acidosis in cats with CKD was found in 60 to 80% of patients. Metabolic acidosis promotes more clinical signs including anorexia, nausea, vomiting,

lethargy, weakness, muscular wasting and weight loss. Severe metabolic acidosis is occurred when blood pH values are below 7.20 resulting in reduction of cardiac output, arterial pressure, and hepatic and renal blood flows.

The major etiology of CKD in cats is still unknown. The major kidney lesion of feline CRF from renal biopsies is tubulointerstitial nephritis (Lawler et al., 2006). According to the results of renal biopsies from one study, 70% of cats with CKD were caused by tubulointerstitial nephritis, 15% were glomerulonephropathy, 11% were lymphoma and 2% were amyloidosis (Minkus et al., 1994). In one study of 676 adult domestic cats, the results showed that the aging feline kidney (older than 108 months) displayed progressive tubular deletion and peritubular interstitial fibrosis (Lawler et al., 2006). Other kidney lesions of cats with CKD are glomerulonephritis, amyloidosis, tubulonephrosis and incomplete recovery of acute renal failure (ARF) (DiBartola et al., 1987).

There were few associations between the signalment data and CKD in cats. Breed of cats was indicated as the risk factor for CKD (Polzin et al., 2000; Pusoonthornthum et al., 2009). In the United State, Maine Coon, Abyssinian, Siamese, Russian Blue and Burmese cats were frequency in CKD cats (Polzin et al., 2000). In Thailand, Siamese and Siamese-mixed breed were commonly found with CRF cats (Pusoonthornthum et al., 2009). Other signalment data were not associated with risk for CKD. Protein and phosphorous restriction can slow CKD progression (Buffington et al., 1991; DiBartola et al., 1993; Harte et al., 1994; Elliott et al., 2000; Hughes et al., 2002).

Role of Oxidative stress

Several previous studies had been reported that CKD in cats may be related to a high production of the free radicals (Viviano et al., 2009; Keegan and Webb, 2010; Krofic Zel et al., 2014). Several studies have demonstrated that oxidative stress can promote the progression of CKD in human patient (Dounousi et al., 2006; Rutkowski et al., 2006). Oxidative stress is caused by an imbalance between reactive oxygen and antioxidant mechanism resulting in tissue damage (Sies, 1997). The energy production of mitochondria in the cells uses more than 5% oxygen which produces reactive

oxygen species (ROS) as by-products (Gutteridge and Halliwell, 1994). ROS are unstable and highly reactive molecules that contain unpaired electrons. ROS with unpaired electrons usually attempt to pair all their electrons from one atom or molecule to another. The mitochondria produces ROS of mitochondrial oxidation including superoxide anion ($O_2^-\cdot$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH\cdot$) (Figure 1), which cause mitochondrial deoxyribonucleic acid (DNA) damage and aging (Schriner et al., 2005). Moreover, $O_2^-\cdot$ reacts with NO to provide $ONOO^-$, which is a strong oxidant.

$O_2 + e^-$	$\longrightarrow$	$O_2^-\cdot$	Superoxide radical
$2O_2^-\cdot + 4H^+$	$\longrightarrow$	$2H_2O_2$	Hydrogen peroxide
H_2O_2	$\longrightarrow$	$OH^- + OH\cdot$	Hydroxyl radical

Figure 1 Reactive oxygen species (ROS) include superoxide anion ($O_2^-\cdot$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH\cdot$).

Oxidative effects of ROS are maintained in stable condition controlled by endogenous enzymatic antioxidants including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) (Blokhina et al., 2003) (Figure 2) and non-enzymatic antioxidants including reduced glutathione, vitamins C and E (Sies and Stahl, 1995).

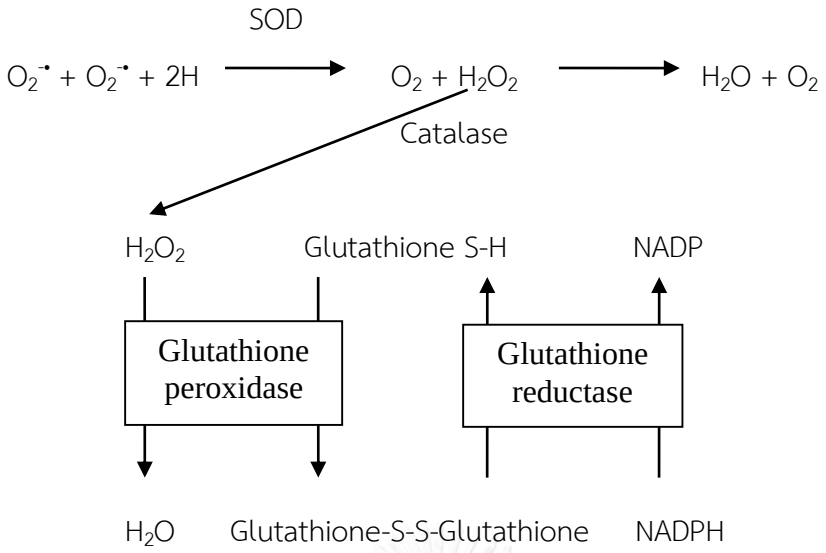


Figure 2 Endogenous enzymatic antioxidants. Superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and catalase (CAT) are the main endogenous enzymatic defense systems of all aerobic cells. They directly scavenged superoxide radicals ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), and converted them to less reactive species. SOD catalyzes the O_2^- to H_2O_2. GPx neutralizes H_2O_2 by taking hydrogens from two GSH molecules resulting in two H_2O and one GSSG. GR recycles GSH to GSSG. CAT neutralizes H_2O_2 into H_2O.
(modified from Rizvi and Maurya, 2007)

Oxidative stress in cats

Cats are known to be sensitive to oxidative injury (Webb et al., 2006). Hemoglobin molecule of cats contains 8 - 10 reactive sulfhydryl groups (R-S-H), whereas dogs and other species contain only four reactive sulfhydryl groups. An increased number of sulfhydryl groups in hemoglobin molecule in cats increases the susceptibility of the red blood cells to the oxidative damage. Hemoglobin consists of heme and protein globin (Figure 3). Sulfhydryl group can change globin conformation by producing disulfide bond. Hemoglobin, which is attached many sulfhydryl groups, changes to methemoglobin. Therefore, this hemoglobin was decreased in oxygen carrying capacity. In addition, cat's Heinz bodies are susceptibility

to the oxidant damage and cat has relatively inefficient Heinz body removal. Non-sinusoidal feline spleen does not entrap rigid Heinz body, so we can find 1 – 2% Heinz body-containing RBC in healthy cats.

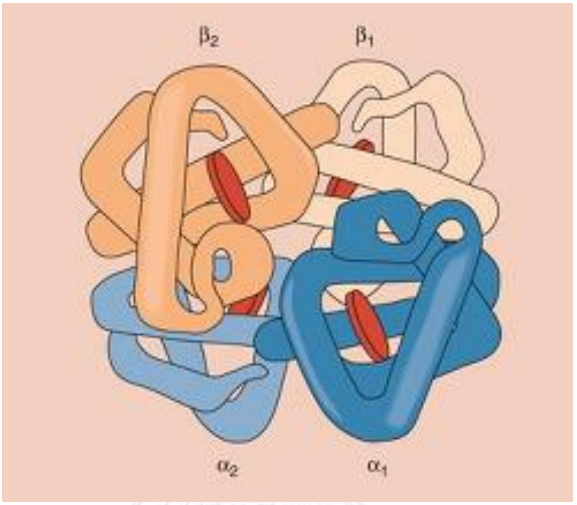


Figure 3 Hemoglobin molecule consists of four polypeptide chains: two α-chains and two β-chains.
(modified from Santiago, 2009)

Increased oxidative stress has been reported in cats with chronic disease. Increased free radical production on markers of oxidative DNA damage (8-hydroxy-2'-deoxyguanosine; 8-OHdG) has also been reported in cats with renal insufficiency (Yu and Paetau-Robinson, 2006). A study of twenty cats with diabetes mellitus (DM) indicated DM cats were significantly less plasma superoxide dismutase (SOD) levels than the control cats, resulting in the decreased in the reaction which change free radical to H_2O_2 (Webb and Falkowski, 2009). Viviano et al (2009) reported that sick cats were higher high plasma ascorbic acid than healthy cats, which may response to oxidative stress. Keegan and Webb (2010) reported that antioxidant capacity in CKD cats was significantly lower than healthy cats. Another previous study was found antioxidant mechanism was responded in CKD cats, because cats in IRIS stage IV were significantly higher activity of plasma GPx than control group (Krofic Zel et al., 2014).

Oxidative stress and chronic kidney disease

The kidney is a site of 10% of body oxygen (O_2) consumption which produces ROS as by-products. Several studies were suggested that chronic renal failure in human may be related to the high production of free radicals (Dounousi et al., 2006; Rutkowski et al., 2006; Webb et al., 2006). The role of oxidative stress and the mechanism of CKD in human patient were due to a decrease in antioxidant defenses, increased in lipid peroxidation and ROS generation (Handelman et al., 2001). Ischemic injury, nephrotoxic injury and phagocyte activation induce NADPH oxidase and xanthine oxidase to change O_2 to $O_2^{-\bullet}$ which leads to oxidative stress mechanism. Moreover, phagocyte activation induces myeloperoxidase to change H_2O_2 to hypochlorous acid (OHCl) which is a strong oxidant (Figure 4). ROS may activate the secretion of inflammatory molecules and raise the inflammatory response. The inflammatory glomerular damages caused by ROS are possible interference by antioxidants. From glomerular barrier impairment, macromolecules can appear in the urinary space because of the loss of glomerular permeability. Thus, renal tubular epithelium could be exposed to injurious chemical species. The accumulation of injurious chemical species within the interstitial space of the renal cortex plays a pathogenic role in the development of tubular injury and interstitial fibrosis in progressive chronic renal diseases in human. Increased oxidative stress also contributed to the progression stages of CKD (Dounousi et al., 2006) and uremia toxicity in human patients (Rutkowski et al., 2006). In cats, there were also found the association between oxidative stress and CKD (Webb and Falkowski, 2009; Krofic Zel et al., 2014).

In addition, CRF is known to associate with NO deficiency by several mechanisms including inactivation of NO by $O_2^{-}\cdot$ to create $ONOO^-$ (Figure 4), accumulation of naturally occurring NOS inhibitors, reduction of L-arginine and eNOS (Chang et al., 2002). One study had been demonstrated that human patients with end-stage renal disease (ESRD) have reduced L-arginine concentration because the kidney is the organ which produces the NO precursor. Braam and Koomans (1995) reported that angiotensin II (Ang II) is antagonized by NO. Ang II has also been postulated to stimulate oxidative stress (Hannken et al., 1998) which causes in renal

endothelial dysfunction, generation of hypertension and cause progression of renal damage (Agarwal et al., 2004).

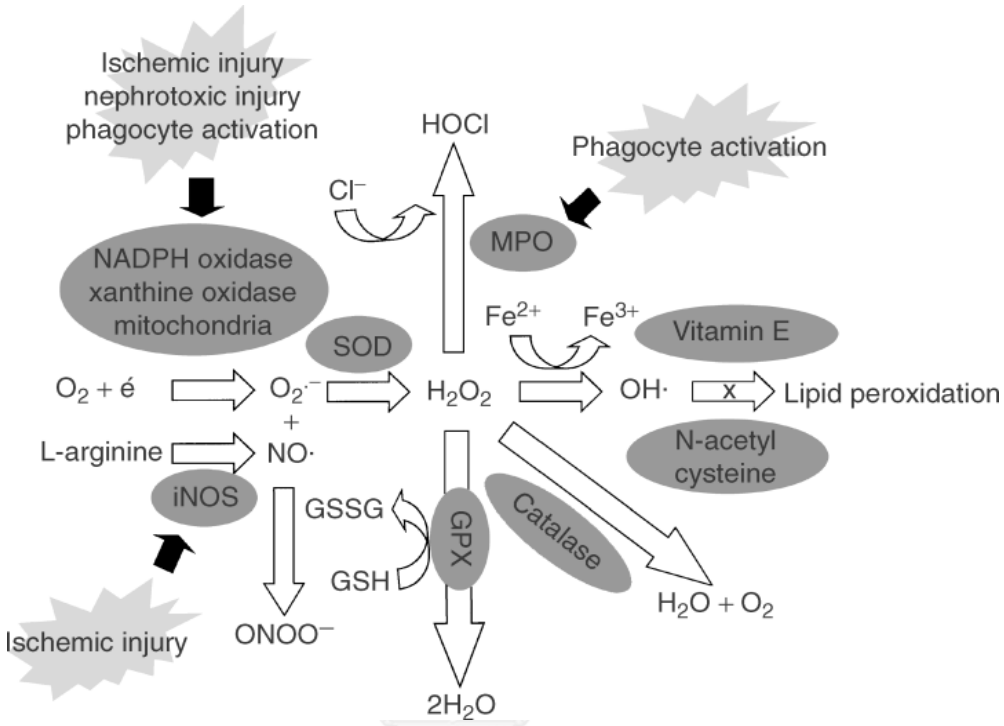


Figure 4 The generation and degradation of oxidative stress in renal failure. Ischemic injury, nephrotoxic and phagocyte activation activate NADPH oxidase and xanthine oxidase to change oxygen (O_2) to superoxide anion ($O_2^{\cdot-}$) which leads to oxidative stress mechanism. Phagocyte activation induces myeloperoxidase (MPO) to change hydrogen peroxide (H_2O_2) to hypochlorous acid (OHCl) which is a strong oxidant. Moreover, ischemic injury activates inducible nitric oxide synthase (iNOS) to create nitric oxide (NO) which is inactivated by $O_2^{\cdot-}$to generate peroxynitrite ($ONOO^-$).
(modified from Jaber et al., 2005)

Role of Nitric oxide in CKD

NO is produced from L-arginine by NOS (Figure 5). This enzyme is expressed in three isoforms including eNOS, nNOS and iNOS. The functions of NO in the kidney are dilatation of renal vascular (Majid and Navar, 2001), regulation of mitochondrial

respiration, modulation renal medullary blood flow, stimulation of fluid, sodium and bicarbonate (HCO_3^-) reabsorption in the proximal tubule, stimulation of renal acidification in proximal tubule, inhibition of Na^+, Cl^- and HCO_3^- reabsorption in the medullary thick ascending limb of loop of Henle (mTALH) (Ortiz and Garvin, 2000), inhibition of Na^+ conductance in the cortical collecting duct (CCD) and inhibition of H^+-ATPase in CCD. In summary, NO plays a very important role in controlling kidney function by regulating vasodilation and sodium excretion.

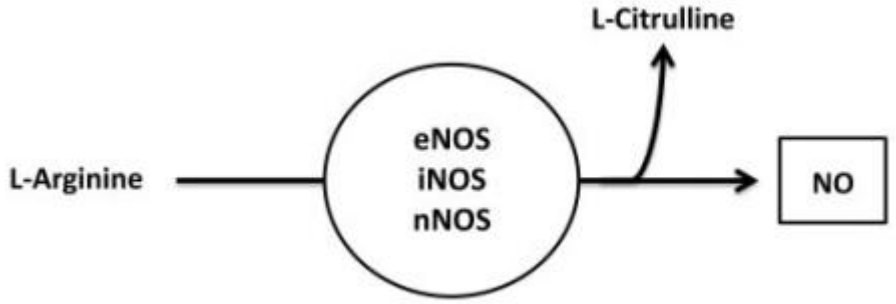


Figure 5 Nitric oxide is produced from arginine by nitric oxide synthase (NOS). NOS is divided into 3 isoform including neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS).
(modified from Moody and Calvert, 2011)

eNOS mRNA was highly expressed in endothelial cells of kidney, including glomerulus and arterioles (Ujiie et al., 1994) (Figure 6). Function of NO, which is produced from eNOS in kidney, is to regulate renal vascular tone, vascular leakage during inflammation and platelet aggregation and adhesion. Moreover, eNOS in kidney produces NO to protect renal fibrosis in rats with unilateral ureteral obstruction (Chang et al., 2002).

Decreased eNOS has been reported in rats with induced nephrotoxicity (Yanagisawa et al., 1998; Yang et al., 2010) and female aged rats (Maric et al., 2008). Although these findings were obtained using laboratory rats, several reports on human have also demonstrated decreased eNOS expression in the kidneys associated with kidney disease (Furusu et al., 1998; Albrecht et al., 2002). Glomerular eNOS expression

was decreased in human glomerulonephritis (Furusu et al., 1998) and human chronic renal transplant failure (Albrecht et al., 2002). Therefore, lack of eNOS may develop progressive renal injury (Forbes et al., 2007).

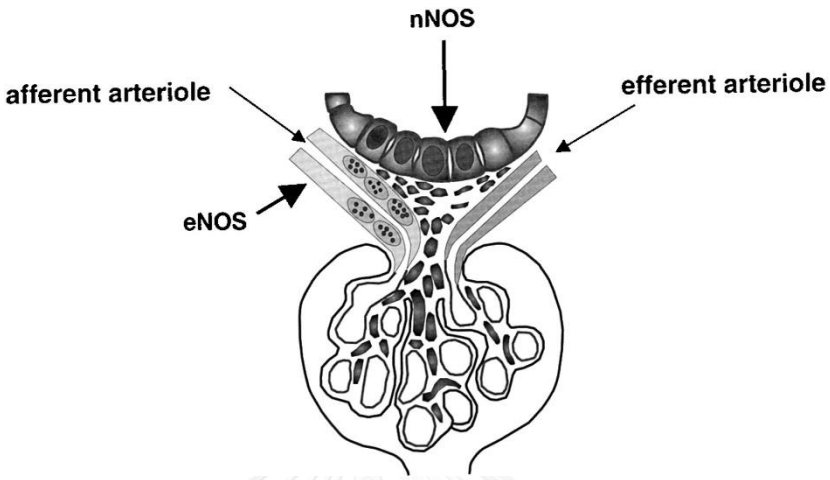


Figure 6 Production of eNOS from afferent arteriole in kidney (modified from Ujiie et al., 1994)

Role of Doxorubicin (DOX) in CKD

CKD is characterized by the development of glomerulosclerosis, tubulointerstitial inflammation and fibrosis. Doxorubicin (DOX) or anthracycline antibiotic, which is a wildly used anti-cancer drug because of cytotoxic and anti-proliferative properties, characterized by reductions in glomerular filtration rate, proteinuria, glomerulosclerosis associated with changes in the glomerular filtration barrier and tubulointerstitial fibrosis. Moreover, oxidative stress is the main factor in DOX-induced nephrotoxicity. It can activate O_2 molecule to generate ROS, including $O_2^{-}\cdot$, H_2O_2 and OH· (Muller et al., 1997), which can damage glomerular filtration membrane by inflammation and immunity. The chemical structure of DOX was shown in Figure 7.

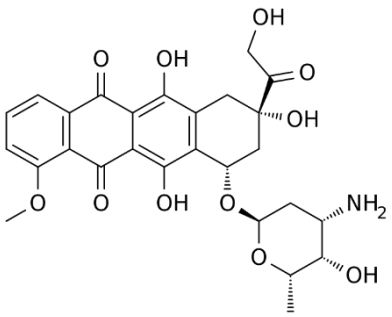


Figure 7 The chemical structure of DOX
(modified from Kalivendi et al., 2001)

A long-term study of this pathological change in rats demonstrated severe renal damage with characteristic features of chronic progressive renal diseases in human (Okuda et al., 1986). The apoptosis of renal tubular cells has been reported in DOX-treated rats (Zhang et al., 1996) and is believed to be a key feature of tubular atrophy, which is a hallmark of chronic renal disease (Schelling et al., 1998; Khan et al., 1999). This is possible that DOX has facilitated the study of the pathophysiology and therapeutics of chronic renal disease. In other animal models, DOX can induce chronic nephropathy in rats (Yoneko et al., 2007; Sarhan et al., 2014). DOX, which was injected directly into the left rat kidneys 1 week before right nephrectomy and DOX was injected again directly into the left kidneys, developed CRF within 4 weeks (Yoneko et al., 2007). Sarhan et al (2014) reported that DOX can induce chronic nephropathy in rats which are received injection of DOX via penile vein at 14 days.

Mitochondrial NADPH dehydrogenase or other reductases activate DOX to create $O_2^{-}\cdot$. Superoxide anion creates to H_2O_2 which causes an increase in eNOS expression in DOX treatment. Kalivendi et al (2001) reported that DOX can increase eNOS transcription in bovine endothelial cells. Moreover, eNOS expression increased in kidney cells of rats with DOX-induced nephropathy (You et al., 2011).

Antidesma acidum

A. acidum (Euphorbiaceae) is a fruit-bearing deciduous shrub or small tree growing to between 5 - 10 meters tall. The fruit is round with dark red color (Kwansang, 2003). In the Northern Hemisphere, the ripe fruit is typically in season from March to May. *A. acidum* has been consumed leaves and fruits in Thailand, India and Indonesia.

Figure 8 *Antidesma acidum*

The crude extract from *A. acidum* is rich of flavonoids and stigmasterol (Thamaree et al., 2003). Isolated compound from roots of *A. acidum* is rich of catechin (Kaennakam and Tip-pyang, 2011) which is a strong antioxidant in flavonoid family. *A. acidum* is widely used as drug supplement in kidney disease (Fungbun et al., 2012; Jaimun et al., 2012) and other diseases (Kuman-Pawa et al., 2003; Kaennakam and Tip-pyang, 2011). In Mauritius, leaves of *A. acidum* are a traditional medicine, used for dizziness, fever and nausea. Studies of an antibacterial, antifungal and anti-HIV activities in five Thai herbs (*A. acidum, Andrographis paniculata* Wall. ex Nees., *Cyperus rotundus* Linn., *Alternanthera bettzickiana* (Regel) Nichols. and *Lonicera japonica*

Thunb.) by Kuman-Pawa et al. (2003) provided that the *A. acidum*, *Cyperus rotundus* Linn. and *Lonicera japonica* Thunb are effectively stimulant of the immune system and control replication of HIV in patient. Two isolated compounds from roots of *A. acidum* (5,7-dihydroxy-2-eicosyl-chromone and 2,5-dimethoxy-1,4-bezoquinone) can protect cytotoxicity on HeLA cells (Kaennakam and Tip-pyang, 2011). The extract prepared from the heartwood of *A. acidum* increased lymphocytes, band cells and alkaline phosphatase levels but decreased the levels of aspartate aminotransferase, alanine aminotransferase, BUN and creatinine levels in eight healthy cats (Pusoonthornthum et al., 2010). In the moderate renal azotemia cats received the crude extract from *A. acidum*, they were also found to have significantly decreased in the creatinine levels on day 28 and 42 of the study (Fungbun et al., 2012).

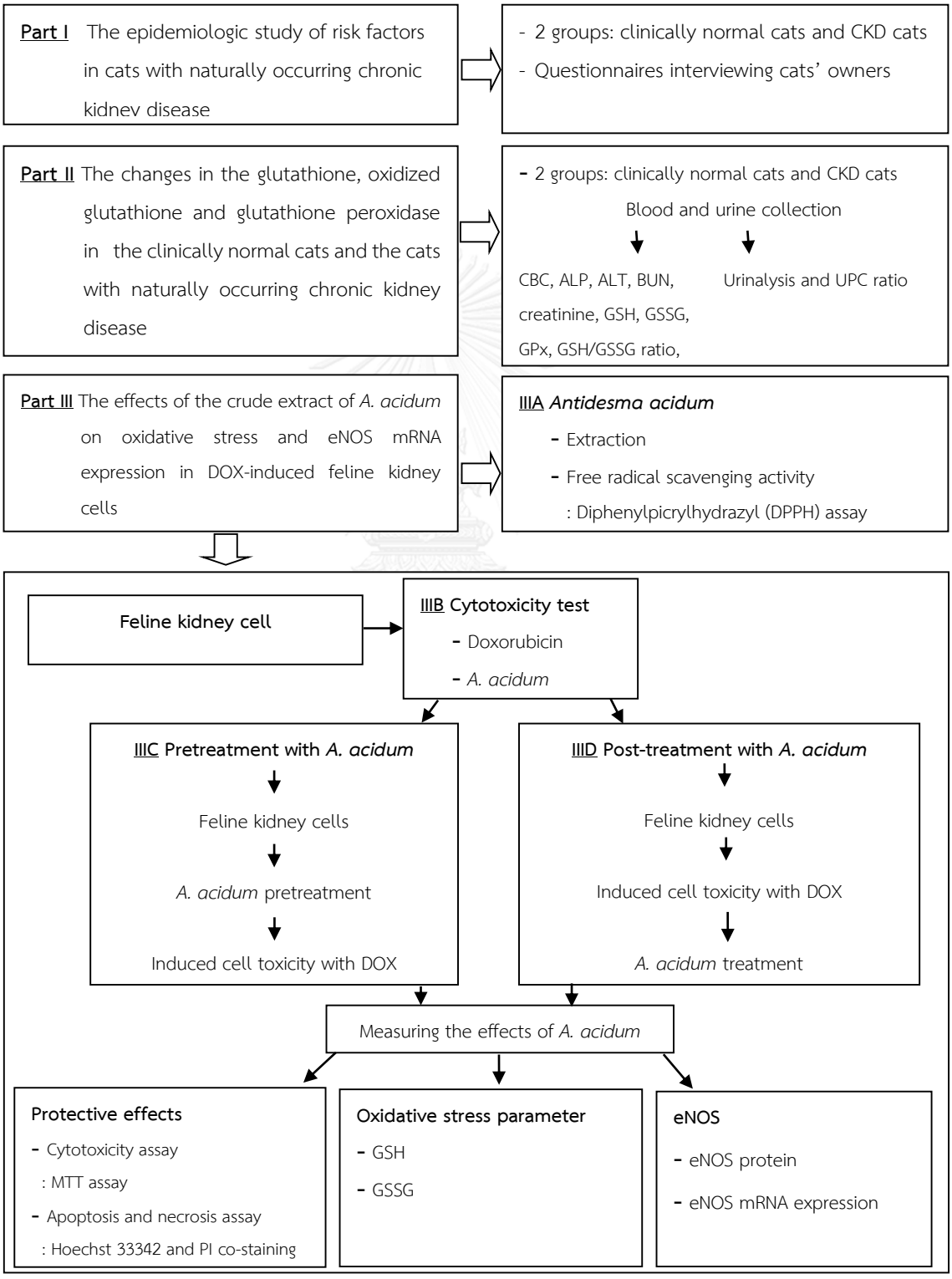
Up to present, the etiology of CKD is still unknown. The CKD risk factors from previous studies were concentrated on clinical signs and indicated no association between CKD and signalment data. From our previous studies, the crude extract of *A. acidum* has been shown to decrease creatinine levels in cats with naturally occurring CKD (Fungbun et al., 2012; Jaimun et al., 2012). However, it is unknown of how the crude extract plays a role in oxidative stress in cats with CKD. To date, there is no study to investigate the mechanism of how the crude extract of *A. acidum* can decrease creatinine levels in CKD cats.

The purposes of the present study were to determine the risk factors in cats with CKD, to compare the oxidative stress parameters between the clinically normal client-owned cats and cats with naturally occurring CKD and to investigate the effects of the ethanol crude extract of *A. acidum* on eNOS mRNA expression in DOX-induced feline kidney cell.

CHAPTER III

MATERIALS AND METHODS

Conceptual framework of this study



<u>Part I</u> The epidemiologic study of risk factors in cats with naturally occurring chronic kidney disease

A case-control study was used to identify risk factors for chronic kidney disease in cats from Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University and veterinary hospitals in Bangkok Metropolitan area. Data were collected during June 2008 to August 2013 through questionnaires interviewing cats' owners regarding the age, gender, breed, weight, family history, type of food, source of water, lifestyle, clinical signs of CKD, hematology, blood chemistry and previous illness history. Two hundred and twenty-two cats were included in this study; sixty-five clinically normal cats and one hundred and fifty-seven cats with CKD.

Animals

Clinically normal cats were cats with the followings:

1. Normal physical examination
2. Blood urea nitrogen (BUN) < 35 mg/dl
3. Creatinine < 1.6 mg/dl
4. Urine specific gravity (USG) ≥ 1.030

Cats with CKD were cats with the followings: (Mayer-Roenne et al., 2007)

1. Clinical signs including: weight loss, polyuria, polydipsia, poor body of condition, dehydration, and/or nonregenerative anemia.
2. BUN > 35 mg/dl
3. Creatinine >1.6 mg/dl
4. USG < 1.030

The CKD cat was excluded if it had lower urinary tract disease, tumor or calculi in the kidney and other severe diseases including: diabetes mellitus, lymphoma and/or heart failure.

The study protocol was approved by the Ethic Committee for the Human and/or Animal Experimentation, Faculty of Veterinary Science, Chulalongkorn University No.1431015.

Statistical analyses

Data of age and body weight were presented as mean ± standard error of mean (SEM). Means of age and body weight between groups were compared by using

Student *t*-test. Relative frequencies were used to describe breed, gender and age of cats that develop CKD. The Fisher's exact test was performed to identify variables correlated with CKD in the case of small expected frequencies. Statistic software (Epi Info™) was used to determine the associations between risk factors and CKD. This estimation of relative risk was considered significant by 95% when the confidence intervals for odds ratios do not included 1.0. P-value of less than 0.05 was considered significant.

Part II The changes in the glutathione, oxidized glutathione and glutathione peroxidase in the clinically normal cats and the cats with naturally occurring chronic kidney disease

Animals

1. Clinically normal cats

Thirteen clinically normal client-owned age-matched cats presented to the Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University and veterinary clinics/hospitals in Bangkok Metropolitan area with normal physical examination, hematology and blood chemistry results with serum creatinine of less than 1.6 mg/dl were included. The clinically normal age-matched cats were cats of seven or more years old. Cats were included without breed, age and gender preference.

2. Cats with naturally occurring CKD

Twenty-three Cats presented to the Small Animal Hospital, Chulalongkorn University and veterinary hospitals in Bangkok Metropolitan area with an increase levels of creatinine concentration (>1.6 mg/dl) and BUN (>35 mg/dl),decrease urine specific gravity (USG) of less than 1.030 (Mayer-Roenne et al., 2007), urine protein creatinine ratio (UPC) > 0.4 with clinical signs including: weight loss, polyuria, polydipsia, poor body of condition, dehydration, nonregenerative anemia and/or small, irregular kidneys were studied. Cats were included without breed, age and gender preference.

All cats with CKD were treated with the conservative medical treatment including fluid therapy and fed with the prescription diet for renal disease as calculation from daily requirement. Cats that had previously been treated with other

drugs for specific or supportive therapy for urinary tract diseases were excluded. The owners were allowed their cats to withdraw if the clinical signs worsen.

The protocol was approved by the Ethic Committee for the Human and/or Animal Experimentation, Faculty of Veterinary Science, Chulalongkorn University No.1431015.

Study designs

Blood collection from the CKD and the clinically normal client-owned cats were performed to measure complete blood count (CBC), blood chemistry (ALP, ALT, BUN, creatinine), oxidative stress parameters (GSH, GSSG and GPx) (Figure 9). Urine was collected by catheterization, cystocentesis and/or voiding of midstream. The urine specific gravity (USG) was determined by refractometer. The chemical urinalysis was measured using a commercial dipstick and examined by microscopic for the findings of casts, crystals, epithelial cells, red blood cells (RBCs) and white blood cells (WBCs).

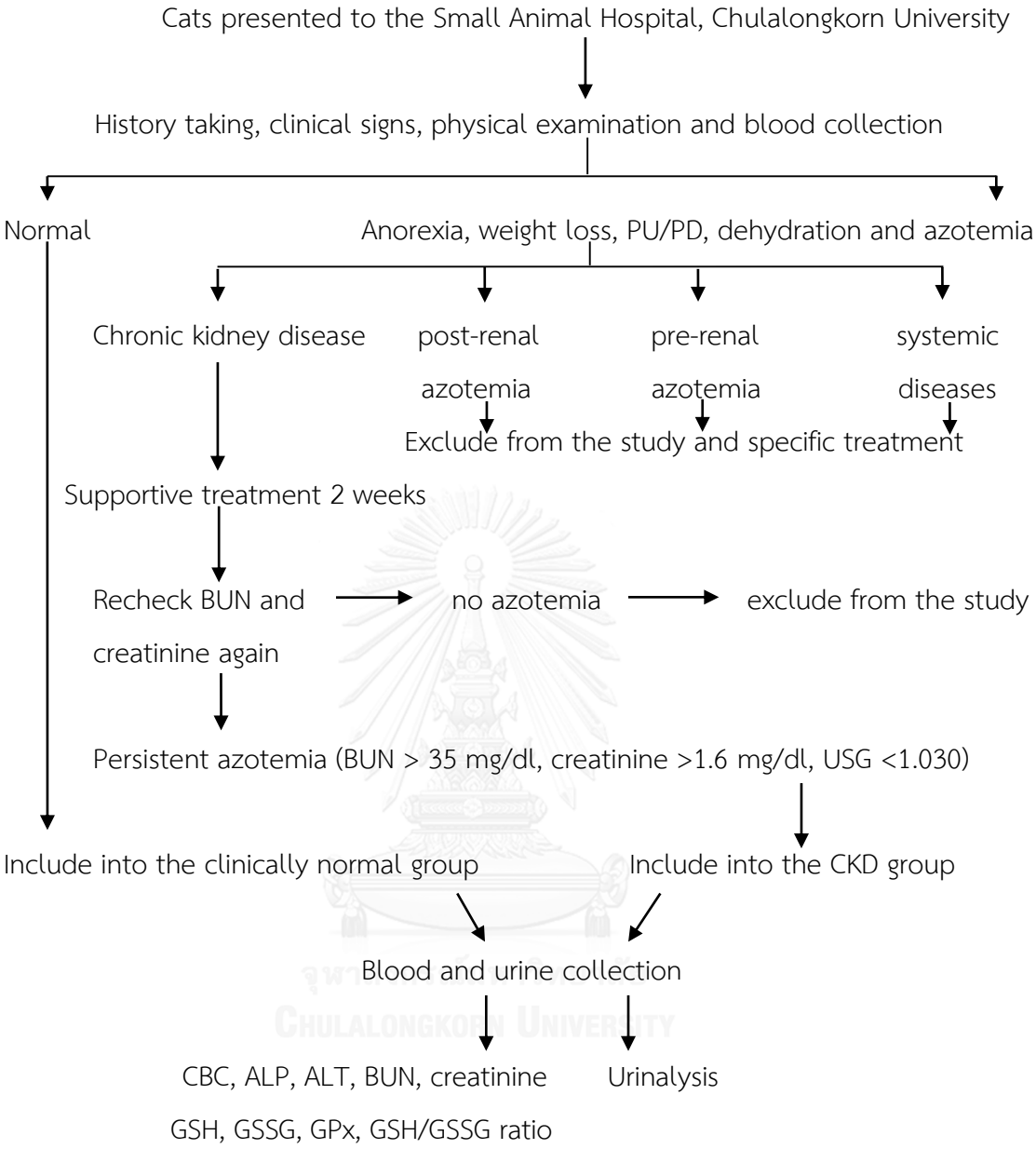


Figure 9 The diagram of the study design (part II)

Laboratory examination

Blood collection (3 ml) from cephalic or femoral vein was performed. Blood samples were divided into an ethylenediaminetetraacetic acid (EDTA)-coated tube for analysis of CBC, GPx, GSH and GSSG and heparin-coated tube for blood chemistry. Blood sample for oxidative stress parameters was immediately centrifuged at 4ºC and plasma store at - 80ºC for further analysis.

Hematocrit, white blood cell (WBC), red blood cell (RBC) and platelet counts were obtained by use of manual blood count. BUN and creatinine were determined by using enzymatic (urease) (Patton and Crouch, 1977) and Alkaline Picrate-end Point Reaction method (Toro and Ackermann, 1975), respectively.

Determination of intracellular glutathione (GSH)

Intracellular GSH was determined by using an enzymatic (glyoxalase), which was modified method from Akerboom and Sies (1981). The principle of GSH determination, GSH is converted by glyoxalase I (reaction 1).

$$\text{Methylglyoxal + GSH} \xrightarrow[\text{Glyoxalase I}]{} \text{S-Lactoglutathione} \qquad \text{(reaction 1)}$$

Distilled water (1.6 ml) was added to 0.4 ml EDTA-coated blood sample. The mixture was precipitated by 3 ml metaphosphoric acid (precipitating solution), allowed to stand for five minute at room temperature and filtered through a Whatman No.1 filter paper. It provided filtrate which was prepared from blood sample. GSH standard was prepared from different concentration: 0.625, 1.25, 2.5 and 5 mM. The reaction mixture of sample, control and standard was shown in Table 1. The control, standard and sample solution were measured by spectrophotometry at 240 nm.

Table 1 The reaction mixture of intracellular GSH determination

	Control (µl)	Sample (µl)	Standard (µl)
PBS	2,200	2,000	2,000
Filtrate	-	200	-
GSH (0.625, 1.25, 2.5 and 5 µM)	-	-	200
Glyoxalase	40	40	40
Methylglyoxal	40	40	40

PBS = Phosphate buffered saline; GSH = Reduced glutathione

Determination of intracellular oxidized glutathione (GSSG)

The concentration of GSSG was performed by enzymatic assay, which was modified method from Akerboom and Sies (1981) and Curello et al (1987). The principle of GSSG determination, the enzyme glutathione reductase (GR) recycles GSSG to GSH with the simultaneous oxidation of nicotinamide adenine dinucleotide phosphate ($NADPH_2$) (reaction 2).

$$GSSG + NADPH + H^+ \xrightarrow[\text{Glutathione reductase}]{} 2GSH + NADP^+ \text{ (reaction 2)}$$

N-ethylmaleimide (NEM) solution consists of 10 mmol of NEM, 17.5 mmol of disodium EDTA, and 100 mM of potassium phosphate per liter (pH 6.5). NEM solution 0.5 ml and 1 ml of a 200 g/L trichloroacetic acid (TCA) solution were added to 0.5 ml of the EDTA-coated blood sample. The mixture was centrifuged and the excessive of NEM was removed by 5 ml of chloroform for at least five times. It provided TCA extract. The NEM was added to scavenge GSH and to eliminate continued oxidation of GSH to GSSG. GSSG standard was prepared from different concentrations: 6.25, 12.5, 25 and 50 μM. The reaction mixture of sample, control and standard was shown in Table 2. The reaction mixture was measured by spectrophotometry at 340 nm.

Table 2 The reaction mixture of intracellular GSSG determination

	Control (μl)	Sample (μl)	Standard (μl)
PBS	2,200	2,000	2,000
NADPH	20	20	20
TCA extract	-	200	-
GSSG (6.25, 12.5, 25 and 50 μM)	-	-	200
GR	10	10	10

PBS = Phosphate buffered saline; NADPH = Nicotinamide adenine dinucleotide phosphate; TCA = Trichloroacetic acid; GSSG = Oxidized glutathione; GR = Glutathione reductase

Determination of intracellular glutathione peroxidase (GPx)

For determination of intracellular glutathione peroxidase (GPx) (modified method from Beutler, 1971), 300 µl of the mixture of Tris-HCl 1 mM and EDTA 5 mM pH 8.0, 60 µl of GSH 0.1 M, 300 µl of Glutathione reductase 10 U/ml and 300 µl of NADPH 2 mM were added to 30 µl of 1:20 hemolyzate. The mixture was divided into two tubes; added H_2O 1,005 µl into the first tube and H_2O 990 µl into the second tube. The reaction mixture in first tube was determined immediately (A_1). The reaction mixture in second tube was added with 15 µl of t-Butyl hydroperoxide 7 mM, allowed to stand for 1 minute (min) at room temperature and measured (A_2). Both reaction mixtures were measured by spectrophotometry at 340 nm. The GPx was calculated according to the equation 1.

$$\text{GPx activity (nmol/min)} = A_1\text{-}A_2\text{/min} \times 161 \quad \text{(equation 1)}$$

Statistical analysis

Data were presented as mean $\pm$ SEM. Means between groups were compared by using independent *t*-test. P-value of less than 0.05 was considered significant.

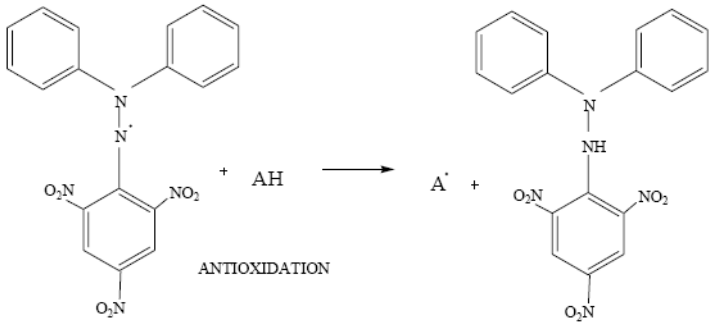
Part III **The effects of the crude extract of *A. acidum* on oxidative stress and eNOS mRNA expression in DOX-induced feline kidney cells**

Extraction of the ethanol crude extract from A. acidum

A. acidum was extracted according to Kwansang, 2003. The stem of *A. acidum* use in this study were dried at less than 50°C for 5 days and ground. The grinding *A. acidum* was macerated with 95% ethanol for one week and extracted three times with 95% ethanol. The combined extract was evaporated to obtain the crude extract. *A. acidum* was obtained from northern Thailand during January to April 2008.

Free radical scavenging of A. acidum extract

The free radical scavenging capacity of the crude extract from *A. acidum* was analyzed by diphenylpicrylhydrazyl (DPPH) assay, which is to monitor the free radical scavenging abilities of antioxidant (Molyneux, 2004; Denrungruang, 2007). The principle of DPPH assay, DPPH radical has a deep violet color due to its impaired electron, and radical scavenging could be followed spectrophotometrically by the loss of absorbance at 515 nm, as the pale yellow non-radical form (diphenylpicrylhydrazyl) is produced (reaction 3).



(reaction 3)

DPPH (violet) DPPH:H (pale yellow)

DPPH standard (6×10^{-5} M) was prepared from 0.0023 g of DPPH and 100 ml of absolute ethanol. The control solution (1 ml of absolute ethanol and 1 ml of DPPH) was measured at 515 nm by spectrophotometer. The 1 ml of standard solution (which

was different concentration of butylhydroxytoluene or 3,5-di-tert-butyl-4-hydroxytol (BHT): 1.562, 6.25, 12.5, 25, 50 and 100 µg/ml absolute ethanol) was added in 1 ml of DPPH standard, allowed to stand for fifteen minute at dark room temperature and measured at 515 nm. One ml of crude extract from *A. acidum* mixture (which was different concentration: 1.562, 6.25, 12.5, 25, 50 and 100 µg/ml absolute ethanol) was mixed with 1 ml of DPPH, allowed to stand for fifteen minute at dark room temperature, measured at 515 nm for three times and presented as mean. The free radical scavenging was calculated according to equation 2.

50% Effective concentration (EC 50) was the concentration of test reagent that decreased the concentration of DPPH to 50%

$$\% \text{ Radical scavenging} = \frac{(A_b - A_a)}{A_b} \times 100 \qquad \text{(equation 2)}$$

A_a = Absorbance value of mixture of the antioxidant substances and DPPH

A_b = Absorbance value of DPPH

Cell culture

The general information of feline kidney cells (ATCC® CCL-94™) was shown (Table 3). These cells are epithelial origin from cortex part of kidney of 12 weeks old normal female domestic cats. The culture method was followed from the manufacturer's protocol. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) and supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 µg/ml) and Non-Essential Amino Acids Solution. Then, they were cultured in TC-dish and incubated overnight at 37 °C in humidified 5% CO_2 incubator. Cells were subcultured every 2 days as follows: the culture medium was removed and the cell monolayers were washed with PBS and trypsinized with 0.25% (w/v) Trypsin-0.53 mM EDTA solution. About 7 to 9 ml of growth medium (DMEM+10% FBS) was added and homogenized. The cell suspension was dispensed to the new culture flasks and used between 185 to 203 passages. The flasks were incubated for 2 days at 37°C in humidified 5% CO_2.

Table 3 The general information of feline kidney cells

Organism	*Feliscatus*, cat
Tissue	Kidney, cortex
Morphology	Epithelial
Culture Properties	Adherent
Disease	Normal
Age	12 weeks
Gender	Female

Study designs

Cytotoxicity test of DOX and the ethanol crude extract of A. acidum in feline kidney cells

Feline kidney cells were induced cell toxicity with different concentrations of DOX (0, 1, 2, 4, 8, 10 and 100 μM) and different time periods (24 and 48 hour (h)) (Chaotham et al., 2013). DOX was obtained from Merck Millipore, Catalog No.324380. DOX was dissolved by sterile water to provide the stock solution of DOX (1, 2, 4, 8, 10 and 100 mM). Growth medium was treated with 0.1% of the stock solution of DOX. The viability of treated and non-treated (control) cells were verified using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) viability assay. DOX concentration, which can decrease 50% of cell viability, is considered an appropriate dose to be further used in the study (Figure 10).

Sub-toxic concentration of crude extract from *A. acidum* in feline kidney cells was first clarified for investigating the preventive effect of crude extract from *A. acidum* on DOX-induced cell toxicity. The ethanol crude extract from *A. acidum* was dissolved by dimethyl sulfide (DMSO) to provide the stock solution of *A. acidum* (0.1, 1, 10, 100 mg/ml). Growth medium was treated with 0.1% of the stock solution of *A. acidum*. After incubation with crude extract from *A. acidum* at concentrations of 0, 0.1, 1, 10, 100 μg/ml for 24 and 48 h, cell viability was determined using the MTT assay. The *A. acidum* concentration with no differences in cell viability between non-treated cells and cells treated with *A. acidum* was considered sub-toxic dose (Figure 10).

Pretreatment with A. acidum

Feline kidney cells were firstly incubated with crude extract from *A. acidum* at sub-toxic dose for 24 h and induced nephrotoxicity with a selected dose of DOX from our previous experiment. Then cytotoxicity assay, apoptosis and necrosis assay, oxidative stress parameters, eNOS protein and eNOS mRNA expression were measured (Figure 11). The cytotoxicity assay was measured by using MTT assay. The mode of cell death was analyzed by using 2'-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bi-1H-benzimidazole trihydrochloridetrihydrate (Hoechst 33342) (Sigma) and propidium iodide (PI) co-staining (Sigma) for apoptosis and necrosis, respectively. Oxidative stress parameters; GSH and GSSG were measured by using the same method as part II. eNOS protein and eNOS mRNA expression were measured by using western blot analysis and relative gene expression.

Post-treatment with A. acidum

Feline kidney cells were firstly induced nephrotoxicity with a selected dose of DOX from our previous experiment, and incubated with crude extract from *A. acidum* at sub-toxic dose. Cytotoxicity assay, apoptosis and necrosis assay, oxidative stress parameters, eNOS protein and eNOS mRNA expression were measured (Figure 11).

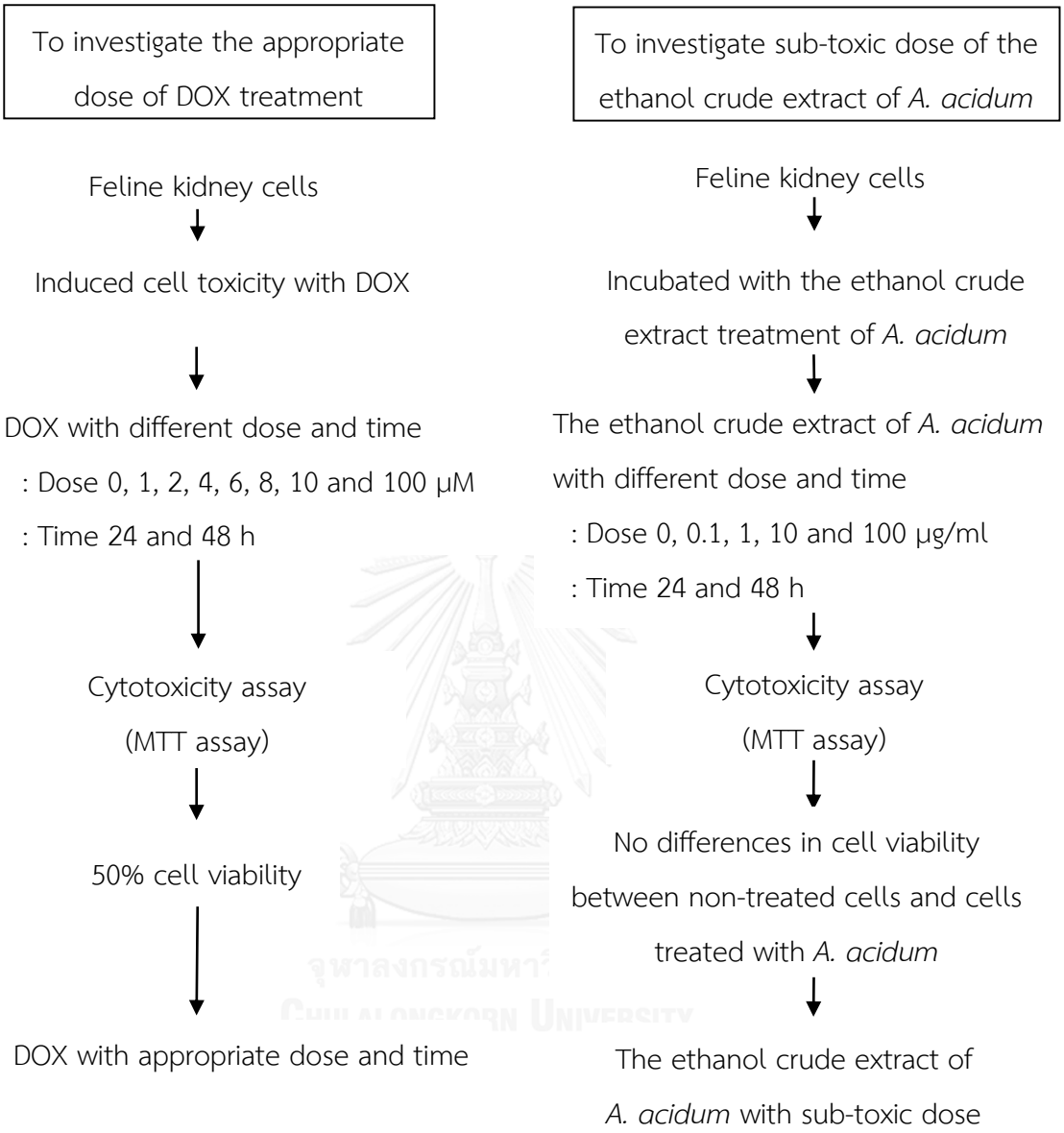


Figure 10 DOX and the ethanol crude extract of *A. acidum* cytotoxicity test in feline kidney cells

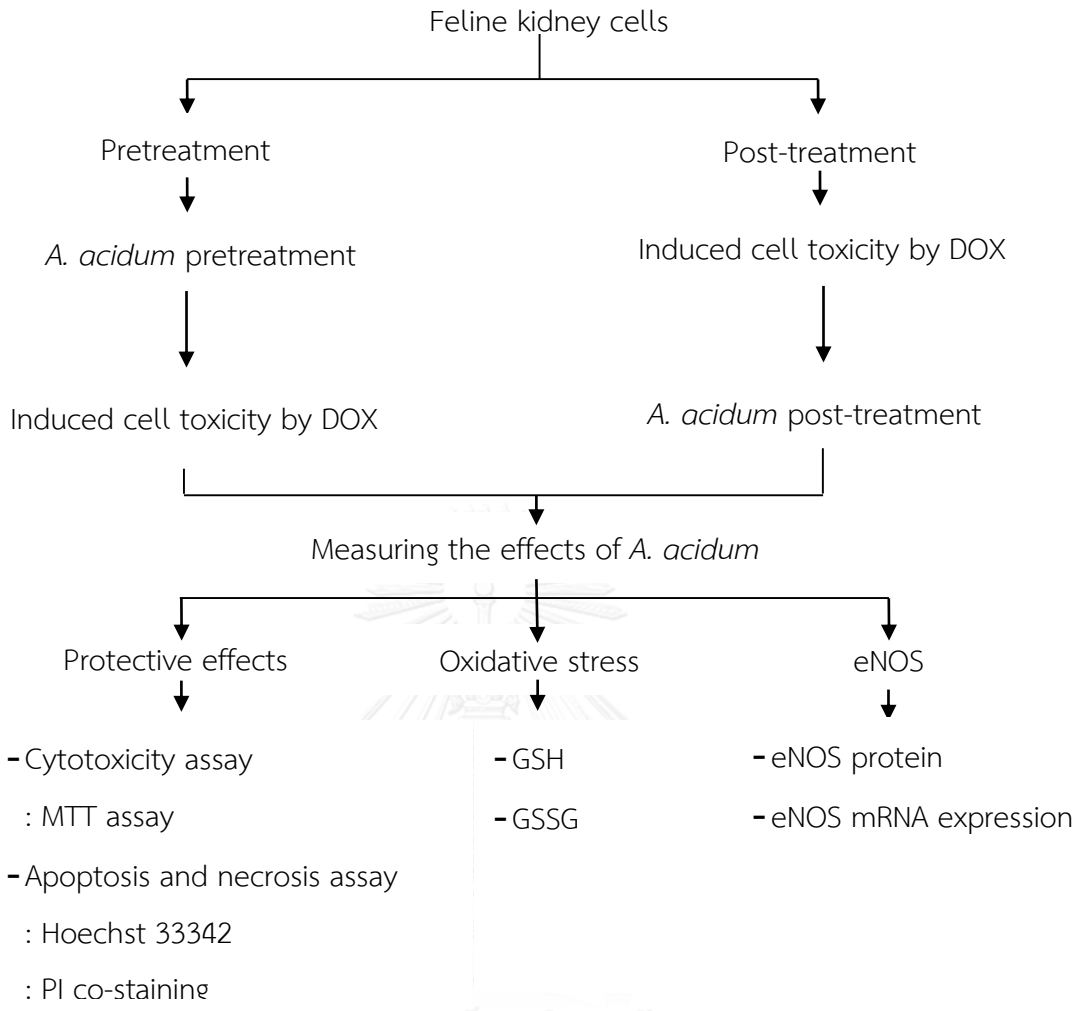


Figure 11 The ethanol crude extract of *A. acidum* pretreatment and post-treatment of DOX-induced cell toxicity in feline kidney cells

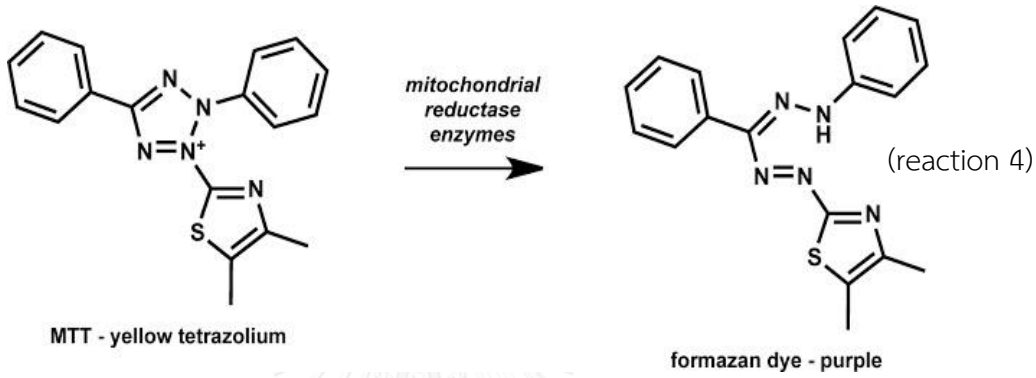
Laboratory examination

Cytotoxicity assay

Cell viability was determined by the MTT colorimetric assay using the modification of Mosmann's method (Mosmann, 1983). The principle of MTT assay, the yellow colored MTT was reduced to purple dye formazan crystals by mitochondrial succinate dehydrogenase (reaction 4).

Feline kidney cells (1×10^4 cells/well) were cultured in a 48-well plate at 37°C, and treated with the ethanol crude extract of *A. acidum* at sub-toxic concentration or DOX treatment concentration for different times of incubation (24 and 48 h). A

negative control group was the feline kidney cells without any treatments. Cells in a 48-well plate were transferred to a 96-well plate, and incubated with 5 mg/ml of MTT for 3 h at 37°C. The medium was then removed and 100 µl of dimethyl sulfoxide (DMSO) was added. The samples were measured by spectrophotometry at 570 nm. The relative cell viability (%) was presented as a percentage relative to the negative control group.



(reaction 4)

Apoptosis and necrosis assay

Cell death was detected by using Hoechst 33342 and PI co-staining. The apoptotic cells with condensed chromatin and/or fragmented nuclei were stained with Hoechst 33342 while PI positively stained necrotic cells. Hoechst 33342, which is a blue-fluorescence dye specific for DNA, can be used to identify live and apoptotic cells. PI, which is a red-fluorescence dye, can only enter to dead cells. Feline kidney cells were cultured in a 24-well plate and treated with pretreatment or post-treatment of *A. acidum* for 24 h on DOX-induced cell toxicity for 48 h. Cells were fixed with 4% formaldehyde for 20 min. at room temperature, blocked with 1% bovine serum albumin (BSA) for 1 h at room temperature, then washed with PBS 5 min. 3 times. Next, they were stained with 2 µg/ml of the Hoechst 33342 for 15 min. at dark room temperature, washed with PBS 5 min. 3 times, and stained with 2 µg/ml of the PI dye in dark room temperature for 20 min. Then they were washed with PBS 5 min 3 times, and mounted with 1:10 glycerol. Each well was visualized in 5 fields under a fluorescence microscope (Olympus) and the mean of the percentage of chromosome condensed cells (apoptotic or necrotic cells) was calculated according to equation 3.

Apoptotic or necrotic cells have DNA fragmentation and chromatin and nuclear condensation.

$$\text{Chromosome condensed cells (\%)} = \frac{\text{Number of chromosome condensed cells}}{\text{Total number of chromosome cells}} \times 100 \quad \text{(equation 3)}$$

Relative gene expression

Extraction of total RNA was performed using TRIzol® reagent, Catalog No. 15596-026 according to the manufacturer's protocol (Life Technologies). Total RNA was quantified by measuring absorbance at 260 nm in spectrophotometer. Total RNA and oligo (dT) primers were converted to complementary Deoxyribonucleic acid (cDNA) by MonsterScript reverse transcriptase, according to the manufacturer's protocol (Epicentre® Biotechnologies). Real-time polymerase chain reaction (PCR) was determined using the iQ5 real-time PCR detection system (Bio-Rad) with iQ SYBR Green Supermix (Bio-Rad). The amplification programs were 95°C for 2 min, 45 cycles (30 s 95°C for denaturation, 30 s 60°C for annealing, 30 s 72°C for extension). The cDNA in the reactions was normalized with gene β-actin, which is a housekeeping gene. Relative gene expression, which is the ratio of eNOS/β-actin, was calculated according to equation 4. The primer sequences were shown in Table 4.

$$2^{-\Delta\Delta CT} \quad \text{(equation 4)}$$

$$\Delta CT = CT_{actin} - CT_{eNOS}$$

$$\Delta\Delta CT = \Delta CT_{treatment} - \Delta CT_{control}$$

Table 4 The primer sequences

Reference seq.	Gene Name	Primer(5'- 3')	Length (bp)	%GC	Tm	Product length (bp)
XM_006929504.1 XM_003983218.2	eNOS	Forward CCTCACCCCTG TGTTCCATC-	20	60	58.8	153
		Reverse GAGATCTTCAC CGCATTGGC-	20	55	59.9	
AB051104.1	β-actin	Forward CCATCGAACAC GGCATTGT-	19	53	58.9	147
		Reverse TCTTCTCACGG TTGGCCTTG-	20	55	59.8	

Western blot analysis

Feline kidney cell lines were lysed with cytobuster protein extraction reagent (Novagen®) and quantified by bicinchoninic acid protein assay (BCA protein assay). Protein extract 60 µg/ml was added with 5% β-mercaptoethanol in loading buffer. The samples were heated with dry plate for 5 minutes at 65℃ and spun down for 5 seconds. Nanopure water 2.05 ml, resolving buffer 1.25 ml, acrylamide 1.65 ml, 10% sodium dodecyl sulfate (SDS) polyacrylamide gels 50 µl, APS (Ammonium persulfate 0.037 g with 370 µl of nanopure water) 50 µl and TEMED 10 µl were mixed for resolving gel preparation. Resolving gel 3.5 ml was added into the glass plates from the casting frame. And then 625 µl of stacking gel, which consisted of nanopure water 3.05 ml, stacking 1.25 ml, acrylamide 650 µl, 10% SDS 50 µl, APS 50 µl and TEMED 10 µl, was added on top of the solid resolving gel. Proteins were electrophoresed on SDS-PAGE and transfer onto

polyvinylidene fluoride (PVDF) membrane (Life Technologies). The PVDF membrane was soaked in 5% non-fat dried milk (blocking buffer) for 4 h, and incubated overnight with primary antibodies against endothelial nitric oxide synthase (primary goat polyclonal anti-eNOS) (R&D Systems, Inc.) and β-actin (primary rabbit polyclonal anti-β-actin) (Novus Biologicals, USA) (Table 5). Each membrane was washed 6 times for 30 min., and incubated with secondary peroxidase-linked antibodies. Secondary antibodies for eNOS and β-actin were secondary donkey anti-goat at 1:1000 dilution (R&D Systems, Inc.) and secondary goat anti-rabbit at 1:2000 dilution (R&D Systems, Inc.) (Table 5). Scion image software (Scion Corporation) was used for quantifying densitometry of proteins which was the ratio of eNOS/β-actin.

Table 5 Primary and secondary antibodies of eNOS and β-actin

Protein name	Antibodies	Dilution
eNOS	Primary goat polyclonal anti-eNOS	1:200
	Secondary donkey anti-goat	1:1000
β-actin	Primary rabbit polyclonal anti-β-actin	1:1000
	Secondary goat anti-rabbit	1:2000

Statistical analysis

Data were presented as mean ± SEM. Each experiment was at least n = 3 and n was the number of monolayers. Means ± SEM between groups were compared by one way analysis of variance (ANOVA) with Bonferroni post hoc test. P-value of less than 0.05 was considered significant.

CHAPTER IV
RESULTS

Part I The epidemiologic study of risk factors in cats with chronic kidney disease

Two hundred and twenty-two cats were studied. There were 65 clinically normal client-owned cats and 157 cats with naturally occurring CKD. Both clinically normal client-owned and CKD cats were presented at the Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University (69.2 and 84.1%, respectively) and veterinary hospitals in Bangkok Metropolitan area (30.8 and 15.9%, respectively). Mean age of CKD cats (7.41 ± 0.37 years) was significantly higher than the clinically normal cats (3.89 ± 0.46 years). Body weight of CKD cats (4.20 ± 0.13 kg) was similar to clinically normal cats (4.05 ± 0.17 kg). CKD cats were more than 7 years old (52.7 %), male (61.5%) and Siamese-mixed breed (82.6%) (Table 6). CKD cats were 41.2% male, 20.3% male castrated, 26.1% female and 12.4% female spayed (Table 6). CKD cats consumed home-made food (18.4%), fish and tuna (29.6%) and feeding twice a day (62.7%) (Table 7). The affected cats were fed with tap water (70.9%) ab libitum (100%) (Table 8). Most clinically normal and CKD cats were placed freely indoor (54.5 and 55%, respectively) (Table 8). According to the clinical sign, CKD cats were dehydration (93.6%), anorexia (82.6%), weight loss (75.8%), halitosis (71.0%), weakness (70.0%), depression (63.6%), polyuria and polydipsia (61.0%), oral ulcer (55.0%), vomit (40.7%), anemia (40.5%), pollakiuria (32%), drooping (28.1%), uremia (19.6%), hematuria (19.0%), nausea (1.9%), convulsion (1.9%) and/or coma (1.9%) (Table 9).

This study found that CKD cats with 3 to 7 years old, more than 7 to 10 years and more than 10 years were associated with increased odds ratio of CKD (OR = 2.39, 95% CI = 1.18 – 5.08, OR = 19.78, 95% CI = 3.62 – 416.92 and OR = 2.79, 95% CI = 1.25 – 6.79, respectively). Young cats aging between 1 to 3 years had lower risk than the CKD (OR = 0.055, 95% CI = 0.02 – 0.12). Siamese breed was associated with increase odds ratio for CKD (OR = 2.92, 95% CI = 1.03 – 10.21). Female spayed cats were associated with decreased risk for CKD (OR = 0.37, 95% CI = 0.17 – 0.82) (Table 6).

Feeding with commercial can and dry food was associated with decreased risk for CKD (OR = 0.12, 95% CI = 0.04 – 0.29 and OR = 0.19, 95% CI = 0.10 – 0.35, respectively). On the other hand feeding with homemade food was associated with increased risk for CKD (OR = 2.80, 95% CI = 1.05 – 7.49) (Table 7). Moreover, filter water was associated with decrease risk for CKD (OR = 0.35, 95% CI = 0.13 – 0.92) (Table 8). From logistic regression analysis using backward elimination demonstrated that cats aging between 7 to 10 years old were 21.65 times higher risk for CKD than other cats of other age groups (OR = 21.65, 95% CI = 1.22 – 382.84), while cats aging 1 to 3 years had decreased risk for CKD (OR = 0.07, 95% CI = 0.01 – 0.95) (Table 10).

Table 6 Signalment of cats presented at the Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University and veterinary hospitals in Bangkok Metropolitan area, Thailand between 2004 and 2013.

Variable	Controls n/N(%)	CKD n/N(%)	*P*	OR	95% CI
Hospital					
CU	45/65(69.2%)	132/157(84.1%)	-	-	-
Private	20/65(30.8%)	25/157(15.9%)	-	-	-
Age					
< 1 year	3/64(4.7%)	5/133(3.8%)	0.51	0.80	0.14 – 5.29
1 – 3 years	40/64(62.5%)	11/133(8.3%)	0.000	0.06**	0.02 – 0.12
> 3 – 7 years	12/64(18.8%)	47/133(35.6%)	0.007	2.39**	1.18 – 5.08
≥ 7 - 10 years	1/64(1.6%)	32/133(24.1%)	0.000	19.78**	3.62 – 416.92
≥ 10 years	8/64(12.5%)	38/133(28.6%)	0.005	2.79**	1.25 – 6.79
Sex					
Male	20/65(30.8%)	63/153(41.2%)	0.09	1.57	0.82 – 3.09
Male castrated	12/65(18.5%)	31/153(20.3%)	0.46	1.12	0.51 – 2.59
Female	15/65(23.1%)	40/153(26.1%)	0.38	1.18	0.57 – 2.51
Female spayed	18/65(27.7%)	19/153(12.4%)	0.006	0.37**	0.17 – 0.82
Breed					
Siamese	4/65(6.2%)	25/155(16.1%)	0.02	2.93*	1.03 – 8.79
Mixed Siamese	57/65(87.7%)	128/155(82.6%)	0.18	0.67	0.28 – 1.55
Burmese	1/65(1.5%)	0/155(0%)	0.29	0	0 - 16.35
Persian	3/65(4.6%)	2/155(1.3%)	0.15	0.27	0.04 – 1.66

CU = The Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University; Private = Private veterinary hospitals in Bangkok Metropolitan area; CKD = Chronic kidney disease; n = Number of cats in each categories; N = Total number of cats with CKD or clinically normal client-owned cats; OR = Odds ratio; CI = Confidence interval

*$p < 0.05$, **$p < 0.01$ when compared between the clinically normal cats and the CKD cats

Table 7 Type of diet, homemade food, seasoning and frequency of feeding of cats presented at the Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University and veterinary hospitals in Bangkok Metropolitan area, Thailand between 2004 and 2013.

Variable	Controls n/N(%)	CKD n/N(%)	*P*	OR	95% CI
Type of diet					
Commercial Can food	19/65(29.2%)	7/152(4.6%)	0.000	0.12**	0.04 – 0.29
Commercial Dry food	39/65(60.0%)	34/152(22.4%)	0.000	0.19**	0.1 – 0.35
Homemade	5/65(7.69%)	28/152(18.4%)	0.028	2.80*	1.05 – 7.49
Seasoning					
Boiled fish	1/13(7.7%)	1/27(3.7%)	0.54	0.45	0.03-7.71
Fish	8/13(61.5%)	8/27(29.6%)	0.06	0.26	0.07 - 1.06
Fish and tuna	0/13(0%)	8/27(29.6%)	0.03	Undefined*	Undefined -Undefined
Fresh fish	2/13(15.4%)	2/27(7.4%)	0.39	0.44	0.05 – 3.54
Tuna	2/13(15.4%)	5/27(18.5%)	0.59	1.25	0.21 – 7.51
Tuna and chicken	0/13(0%)	1/27(3.7%)	0.67	Undefined	Undefined –Undefined
Unknown	0/13(0%)	2/27(7.4%)	0.57	Undefined	Undefined –Undefined
Frequency of feeding					
Once a day	0/36(0%)	2/59(3.4%)	0.38	Undefined	0.11-Undefined
Twice a day	23/36(63.9%)	37/59(62.7%)	0.46	0.95	0.40 - 2.25
Three times	1/36(2.8%)	2/59(3.4%)	0.68	1.23	0.12 - 14.05
More than three times	0/36(0%)	1/59(1.7%)	0.62	Undefined	Undefined -Undefined
Ab libitum	12/36(33.3%)	17/59(28.8%)	0.32	0.81	0.33 – 1.98

CKD = Chronic kidney disease; n = Number of cats in each characteristic; N = Total number of cats with CKD or clinically normal client-owned cats; OR = Odds ratio; CI = Confidence interval

$^{*}p < 0.05$, $^{**}p < 0.01$ when compared between the clinically normal cats and the CKD cats

Table 8 Water and life style of cats presented at the Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University and veterinary hospitals in Bangkok Metropolitan area, Thailand between 2004 and 2013.

Variable	Controls n/N(%)	CKD n/N(%)	*P*	OR	95% CI
Water source					
Tap water	26/42(61.9%)	39/55(70.9%)	0.18	1.50	0.64 – 3.52
Groundwater	1/42(2.4%)	0/55(0%)	0.43	0	0 – 14.51
Filter water	15/42(35.7%)	9/55(16.4%)	0.03	0.35*	0.13 – 0.92
Boiled water	0/42(0%)	5/55(9.1%)	0.05	Undefined	0.72 - Undefined
Others	0/42(0%)	2/55(3.6%)	0.32	Undefined	0.14 - Undefined
Water frequency					
Ab libitum	40/40(100%)	61/61(100%)	-	-	-
Life style					
Cage	5/44(11.4%)	9/69(13.0%)	0.41	1.17	0.36 – 3.75
Freely outdoor	15/44(34.1%)	33/69(47.8%)	0.07	1.77	0.81 – 3.87
Freely indoor	24/44(54.5%)	27/69(39.1%)	0.06	0.54	0.25 – 1.16

CKD = Chronic kidney disease; n = Number of cats in each characteristic; N = Total number of cats with CKD or clinically normal client-owned cats; OR = Odds ratio; CI = Confidence interval

*$p < 0.05$ when compared between the clinically normal cats and the CKD cats

Table 9 Frequency of clinical signs in CKD cats at the Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University and veterinary hospitals in Bangkok Metropolitan area, Thailand between 2004 and 2013.

Variable	CKD n/N(%)
Clinical signs	
Dehydration	103/110(93.6%)
Anorexia	100/121(82.6%)
Weight loss	47/62(75.8%)
Halitosis	49/69(71.0%)
Weakness	42/60(70.0%)
Depression	56/88(63.6%)
PU/PD	47/77(61.0%)
Oral ulcer	22/63(55%)
Vomit	33/81(40.7%)
Anemia	32/79(40.5%)
Pollakiuria	8/25(32%)
Dysuria	22/73(30.1%)
Drooping	16/57(28.1%)
Uremia	11/56(19.6%)
Hematuria	12/63(19.0%)
Nausea	1/53(1.9%)
Convulsion	1/52(1.9%)
Coma	1/52(1.9%)

CKD = Chronic kidney disease; PU/PD = polyuria and polydipsia;
n = Number of cats in each clinical sign; N = Total number of cats with CKD or clinically normal cats

Table 10 Logistic regression analysis (backward) from multivariable including various ages, commercial can food, commercial dry food, homemade food, female spayed, Siamese breed and filter water in CKD cats at the Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University and veterinary hospitals in Bangkok Metropolitan area, Thailand between 2004 and 2013.

Variable	Coefficient	S.E.	*P*	OR	95% CI
1 – 3 years	-2.6618	1.3310	0.046	0.07*	0.01 – 0.95
≥ 7 - 10 years	3.0750	1.4657	0.036	21.65*	1.22 – 382.84

OR = Odds ratio; CI = Confidence interval

*$p < 0.05$ when compared between the clinically normal cats and the CKD cats

<u>Part II</u> The changes in the glutathione, oxidized glutathione and glutathione peroxidase in the clinically normal cats and the cats with naturally occurring chronic kidney disease

Thirty-six cats were studied including thirteen clinically normal client-owned cats (8 males and 5 females) and twenty-three CKD cats (13 males and 10 females) (Table 11). Twenty-three CKD cats were divided into 3 groups according to the International Renal Interest Society (IRIS): stage II (mild renal azotemia; n = 8), stage III (moderated renal azotemia; n = 11) and stage IV (severe renal azotemia; n = 4). Mean age of the clinically normal client-owned cats was 10.62 ± 0.76 years (range, 6 to 14 years). Mean age of the CKD cats was 11.04 ± 0.94 years (range, 3 to 17 years) (Table 11). Seventy percent of the CKD cats were over 10 years old. Breeds of the clinically normal cats were Siamese mixed breed (12/13; 92.3%) and Siamese (1/13; 7.69%). Breed of the CKD cats was Siamese mixed breed (22/23; 95.65%) and Siamese (1/23; 4.35%) (Table 11). Body weight was similar between the clinically normal cats (4.61 ± 0.31 kg) and the CKD cats (4.64 ± 0.31 kg) (Table 11). Clinical signs of the CKD cats were dehydration (23/23; 100%), poor hair coat (10/23; 43.47%), polyuria and polydipsia (9/23; 39.13%), pale mucous membrane (4/23; 17.39%), gingivitis (5/23; 21.74%), stomatitis (2/23; 8.69%) and chronic respiratory tract infections (2/23; 8.69%). There were one Feline Leukemia Virus (FeLV) positive cats and two Feline Immunodeficiency Virus (FIV) positive cats in the CKD group.

Red blood cell numbers of the CKD cats (6.12 ± 0.42 x 10^6 cells/ml) was significantly lower than those of the clinically normal cats (7.96 ± 0.46 x 10^6 cells/ml) ($p< 0.01$) (Table 12). The CKD cats had significantly lower PCV (33.13 ± 1.41%) than the clinically normal cats (46.85 ± 1.73%) ($p < 0.01$) (Table 11). White blood cell numbers of CKD cats (13,341.30 ± 845.70 cells/ml) were significantly higher than the clinically normal cats (8,526.92 ± 823.02 cells/ml) (Table 12). A number of neutrophils and lymphocytes of CKD cats (8,689.09 ± 768.35 cells/ml and 3,863.74 ± 545.98 cells/ml) were significantly higher than the clinically normal cats (6,425.19 ± 533.85 cells/ml and 1,515.62 ± 423.84 cells/ml) (Table 12). A number of eosinophils and basophils of CKD cats (144.07 ± 41.62 cells/ml and 7.07 ± 7.07 cells/ml) were

significantly lower than the clinically normal cats (287.46 ± 45.50 cells/ml and 41.62 ± 13.73 cells/ml) (Table 12). Monocytes were not significantly different between the clinically normal and the CKD cats. Mean ± SEM of creatinine and BUN levels of the CKD cats (3.64 ± 0.29 mg/dl and 62.01 ± 5.08 mg/dl) were significantly higher than the clinically normal cats (1.41 ± 0.03 mg/dl and 24.12 ± 1.33 mg/dl) ($p < 0.01$) (Table 12).

Mean ± SEM of GSH in the CKD cats (2.77 ± 0.27 mM) was significantly lower than the clinically normal cats (4.24 ± 0.67 mM) ($p < 0.05$) (Table 13). The GPx of the CKD cats (2.32 ± 0.40 nmol/min) were significantly lower than the clinically normal cats (6.68 ± 0.79 nmol/min) ($p < 0.01$) (Table 13). The GSSG levels of the CKD cats (35.20 ± 4.37 μM) were significantly higher than the clinically normal cats (19.66 ± 2.75 μM) ($p < 0.01$) (Table 13). Moreover, the GSH/GSSG ratio of the CKD cats (148.26 ± 34.19) was significantly lower than the clinically normal cats (312.64 ± 76.80) ($p < 0.05$) (Table 13). The urinalysis results of urine samples of the CKD cats were shown in Table 14. Urine specific gravity was 1.011 to 1.024, and pH urine was 5.0 to 8.0 (Table 14).

Table 11 Signalment including age, gender, breed and body weight of the clinically normal cats and the CKD cats

Parameter	**Units**	**Clinically normal (n=13)**	**CKD (n=23)**
Age	years	10.62 ± 0.76	11.04 ± 0.94
Gender			
Male	-	1	7
Male castrated	-	7	6
Female	-	-	5
Female castrated	-	5	5
Breed			
Siamese mixed breed	-	12	22
Siamese	-	1	1
Body weight	kg	4.61 ± 0.31	4.64 ± 0.31

CKD = Chronic kidney disease

Table 12 Mean ± SEM of blood profile of the clinically normal cats and the CKD cats.

Parameter	Units	Normal value #	Clinically normal (n=13)	CKD (n=23)
RBC	$\times 10^6$cells/ml	5.24-10.89	7.96 ± 0.46	6.12 ± 0.42**
PCV	%	29.2-51.7	46.85 ± 1.73	33.13 ± 1.41**
WBC	cells/ml	4,200-17,500	8,526.92 ± 823.02	13,341.30 ± 845.70**
Neutrophils	cells/ml	1,925-14,825	6,425.19 ± 533.85	8,689.09 ± 768.35*
Lymphocytes	cells/ml	1,100-7,000	1,515.62 ± 423.84	3,863.74 ± 545.98**
Eosinophils	cells/ml	110-750	287.46 ± 45.50	144.07 ± 41.62*
Basophils	cells/ml	0-190	41.62 ± 13.73	7.07 ± 7.07*
Monocytes	cells/ml	55-780	257.04 ± 51.06	338.22 ± 59.69
BUN	mg/dl	15-35	24.12 ± 1.33	62.01 ± 5.08**
Creatinine	mg/dl	< 1.6	1.41 ± 0.03	3.64 ± 0.29**

Normal reference value from Sodikoff C.H. 1995. Serum chemical test. Laboratory profile of small animal disease. In: A guide to laboratory diagnosis 2nd (ed). Mosby-Year Book. St. Louis. 3-20p.

CKD = Chronic kidney disease; RBC = Red blood cell; PCV = Pack cell volume; WBC = White blood cell; BUN = Blood urea nitrogen

*$p < 0.05$, **$p < 0.01$ when compared to the clinically normal cats.

Table 13 Mean ± SEM of intracellular GPx, GSH, GSSG and GSH/GSSG ratio in the clinically normal cats and the CKD cats.

Parameter	Units	Clinically normal (n=13)	CKD (n=23)
GPx	nmol/min	6.68 ± 0.79	2.32 ± 0.40**
GSH	mM	4.24 ± 0.67	2.77 ± 0.27*
GSSG	μM	19.66 ± 2.75	35.20 ± 4.37**
GSH/GSSG	-	312.64 ± 76.80	148.26 ± 34.19*

CKD = Chronic kidney disease; GPx = Glutathione peroxidase; GSH = Glutathione; GSSG = Oxidized glutathione; GSH/GSSG = Glutathione to oxidized glutathione ratio

*$p < 0.05$, **$p < 0.01$ when compared to the clinically normal cats.

Table 14 The urinalysis results of urine samples collected from the cats with CKD.

Variable	CKD (n=12)
Color	Light yellow (8)
	Yellow (4)
Method of collection	Voided (7)
	Cysto (5)
Urine protein	1+ (4)
	None (8)
Urine specific gravity	1.024 (1)
	1.020 (1)
	1.018 (3)
	1.016 (1)
	1.015 (2)
	1.014 (1)
	1.012 (2)
	1.011 (1)
pH	8.0 (1)
	7.0 (6)
	6.0 (4)
	5.0 (1)
RBC count (No. of cells in sediment/hpf)	5 – 10 (4)
	None (8)
WBC count (No. of cells in sediment/hpf)	5 – 10 (7)
	3 – 5 (3)
	2 – 3 (2)
Glucose	None (12)
Ketone	None (12)
Bilirubin	None (12)
Urobilinogen	None (12)
nitrite	None (12)

CKD = Chronic kidney disease; No. = Number; hpf = High power field;

Cysto = Cystocentesis; RBC = Red blood cells; WBC = White blood cells

Part III The effects of the crude extract of *A. acidum* on oxidative stress and eNOS mRNA expression in DOX-induced feline kidney cells

Part IIIA Free radical scavenging activity and the effective concentration of *A. acidum* extract

Total stem of *A. acidum* (11.4 kg) used in this study were extracted to obtain the final ethanol crude extract of 102.612 g. The ethanol crude extract from *A. acidum* was 0.90% of the total stem.

Percent radical scavenging of BHT, quercetin and the ethanol crude extract of *A. acidum* at concentration 0 – 100 µg/ml was presented (Table 15; Figure 12). The percent radical scavenging of 3,5-di-tert-butyl-4-hydroxytol or butylhydroxytoluene (BHT) increased with increased concentration. The radical scavenging of the extract was rapidly increased with increased concentration at 0 - 25 µg/ml, and it was stable at 25 – 100 µg/ml concentration. The radical scavenging of quercetin was rapidly increased with increased concentration at 0 - 12.5 µg/ml, but it was slightly decreased at 25 – 100 µg/ml concentration. The percent radical scavenging of the extract was similar to quercetin at 25 – 100 µg/ml concentration. All substances had the same values of percent radical scavenging at 100 µg/ml concentration. Moreover, the highest percentage of free radical scavenging occurred in the ethanol crude extract from *A. acidum* at 25 – 100 µg/ml concentration (Table 15).

Table 15 Percent (%) radical scavenging by DPPH method of 3,5-di-tert-butyl-4-hydroxytol or butylhydroxytoluene (BHT), quercetinand the ethanol crude extract of *A. acidum* at various concentrations.

	Concentration (µg/ml)							
	0	**1.562**	**3.125**	**6.25**	**12.5**	**25**	**50**	**100**
BHT	0	0.77	6.67	9.23	19.35	29.87	53.46	81.15
Quercetin	0	33.33	75.51	84.62	85.25	83.59	83.84	82.82
A. acidum	0	2.82	18.72	35.90	69.35	86.67	88.84	88.72

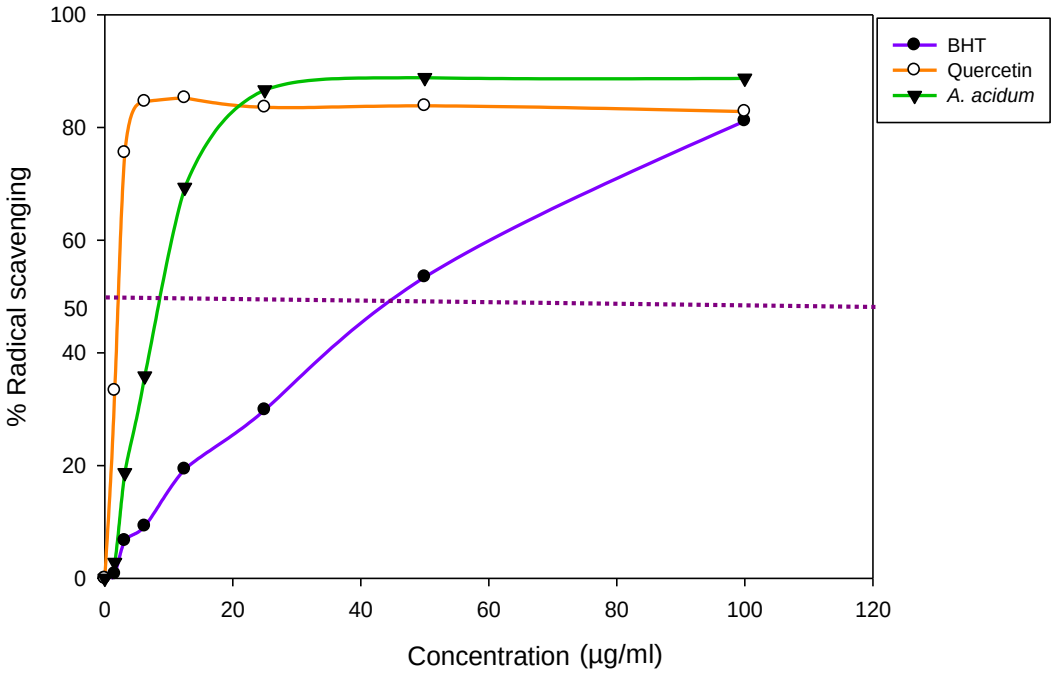


Figure 12 Percent (%) radical scavenging by DPPH method of 3,5-di-tert-butyl-4-hydroxytol or butylhydroxytoluene (BHT), quercetin and the ethanol crude extract of *A. acidum*

The effective concentration (EC_{50}) of BHT, quercetin and the ethanol crude extract of *A. acidum* was 46.43, 2.22 and 8.83 µg/ml, respectively (Table 16). The crude extract of *A. acidum* was approximately 5 times more effective than BHT, but it was less effective than quercetin at approximately 4 times.

Table 16 The effective concentration (EC_{50}) of 3,5-di-tert-butyl-4-hydroxytol or butylhydroxytoluene (BHT), quercetin and the ethanol crude extract of *A. acidum*

Type of samples	EC_{50} (µg/ml)
BHT	46.43
Quercetin	2.22
A. acidum	8.83

EC_{50}= Effective concentration of substance which can decrease 50 percent of DPPH concentration.

<u>Part IIIB</u> Cytotoxicity test of DOX and the ethanolcrude extract of *A. acidum* in feline kidney cells

To investigate the dose and time of DOX that can reduce 50% cell surviving, feline kidney cells were cultured and induced cell toxicity by various concentrations of DOX treatment and times as demonstrated in cytotoxicity test of DOX in feline kidney cells. The safety dose of the ethanol crude extract of *A. acidum* for feline kidney cells was investigated in cytotoxicity test of the ethanol crude extract of *A. acidum* in feline kidney cell.

1. Cytotoxicity test of DOX in feline kidney cells

To investigate the appropriate dose of DOX treatment to induce kidney failure, feline kidney cells were treated with various concentrations of DOX (0 to 100 μM) for 24 and 48 h. Percent of feline cell viabilities that responded to various concentrations of DOX (1, 2, 4, 8, 10 and 100 μM) at 24 h were 88.38, 90.20, 92.74, 92.48, 82.30 and 94.25% (Table 17; Figure 13). There were no significant difference in cell viability between non-treated cells and cells treated with DOX at 24 h after treatment.

After incubation with various concentrations of DOX (1, 2, 4, 8, 10 and 100 μM) for 48 h, the percent viability of feline kidney cells (87.04, 71.91, 56.45, 56.72, 43.97 and 12.45%) decreased when increased concentration of DOX (Table 17; Fig.13). Concentration-dependent reduction in cell viability was detected at 48 h after incubation. The reduction of cell surviving was significant difference at 2 – 100 μM of DOX. Percent viability at 4 and 8 μM of DOX were significantly lower than percent viability at 0 and 1 μM of DOX. DOX at 10 and 100 μM had significantly lower percent cell viability than at the concentration of 0, 1 and 2 μM. Moreover, the percent cell viability of DOX 100 μM was significantly lower than concentration at 4 μM (Table 17; Figure 13). A significant reduction of cell surviving was seen started at 2 μM DOX and cell viability was found to be 50% at 7.89 μM when plotted between percent of cell viability and concentration of DOX (Figure 14). Therefore, we used DOX at 8 μM as an appropriate dose to be used in the next experiment.

Table 17 Percent (%) viability by MTT method of cells at various concentrations of DOX treatment for 24 and 48 h.

DOX (μM)	Time (h)	
	24 (n=9)	**48 (n=9)**
0	100	100
1	88.38 ± 3.45	87.04 ± 4.97
2	90.20 ± 5.08	71.91 ±4.68*
4	92.74 ± 2.06	56.45 ± 6.2$^{*, \#}$
8	92.48 ± 3.01	56.72 ± 6.77$^{*, \#}$
10	82.30 ± 4.52	43.97 ± 6.47$^{*, \#,\$}$
100	94.25 ± 3.02	12.45 ± 2.77$^{*, \#,\$,a}$

n = Number of monolayers in each experiment

$^{*}p < 0.05$ when compared with control by ANOVA

$^{\#}p < 0.05$ when compared with concentration at 1 μM by ANOVA

$^{\$}p < 0.05$ when compared with concentration at 2 μM by ANOVA

$^{a}p < 0.05$ when compared with concentration at 4 μM by ANOVA

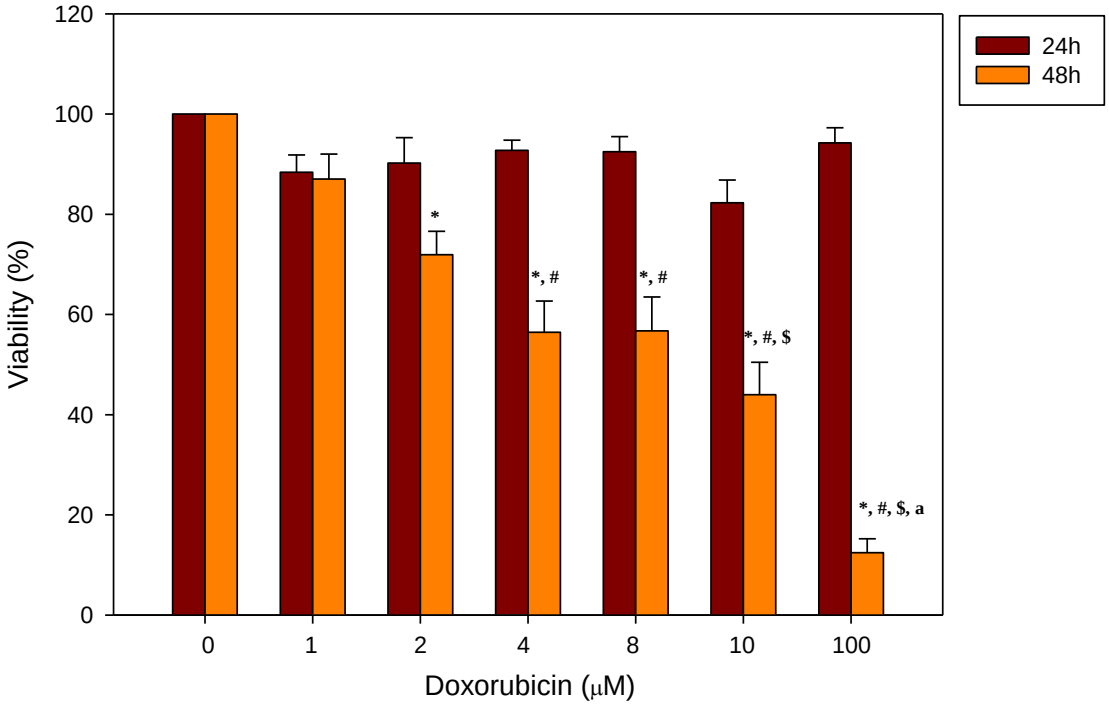


Figure 13 Cytotoxicity by MTT method of DOX treatment at 0 – 100 μM concentrations for 24 and 48 h in feline kidney cells. $^{*}p < 0.05$ when compared with control by ANOVA; $^{\#}p < 0.05$ when compared with concentration at 1 μM by ANOVA; $^{\$}p < 0.05$ when compared with concentration at 2 μM by ANOVA; $^{a}p < 0.05$ when compared with concentration at 4 μM by ANOVA.

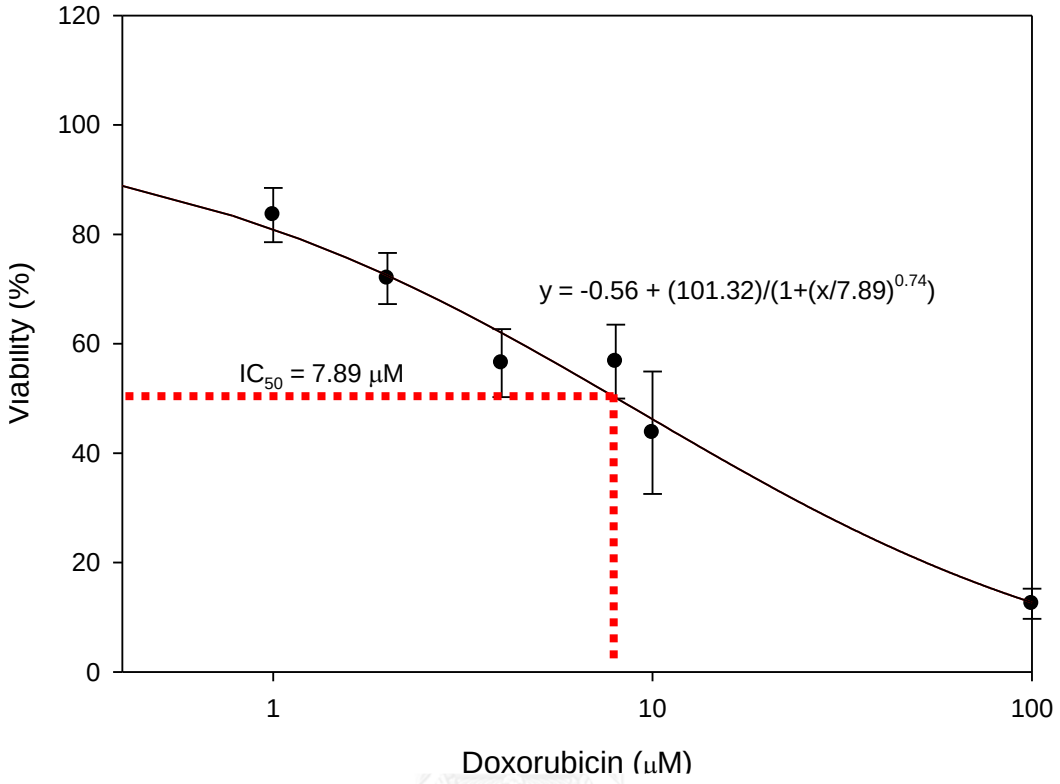


Figure 14 Cytotoxicity test by MTT method of DOX treatment at 0 to100 µM concentrations for48 h in feline kidney cells

2. Cytotoxicity test of the ethanol crude extract of A. acidum in feline kidney cells

To investigate sub-toxic dose of the ethanol crude extract of *A. acidum*, feline kidney cells were treated with the ethanol crude extract of *A. acidum* at various concentrations (0, 0.1, 1, 10 and 100 µg/ml) for 24 and 48 h. Results showed no significantly differences in cell viability between non-treated cells and cells treated with the ethanol crude extract of *A. acidum* at 24 and 48 h after treatment (Table 18; Figure 15). Therefore, *A. acidum* extract from 0.1 to 100 µg/ml concentrations were not toxic to feline kidney cell lines.

Table 18 Percent (%) viability of cells at various concentrations of *A. acidum* at 0 to 100 µg/ml treatment for 24 and 48 h.

A. acidum	**Time (h)**	
(µg/ml)	**24 (n=6)**	**48 (n=7)**
Medium	100	100
0	106.41 ± 4.40	100.36 ± 3.77
0.1	102.81 ± 3.25	89.69 ± 7.38
1	99.60 ± 5.83	93.98 ± 6.02
10	100.27 ± 4.38	92.09 ± 5.40
100	94.38 ± 4.24	97.48 ± 8.87

n = Number of monolayers in each experiment

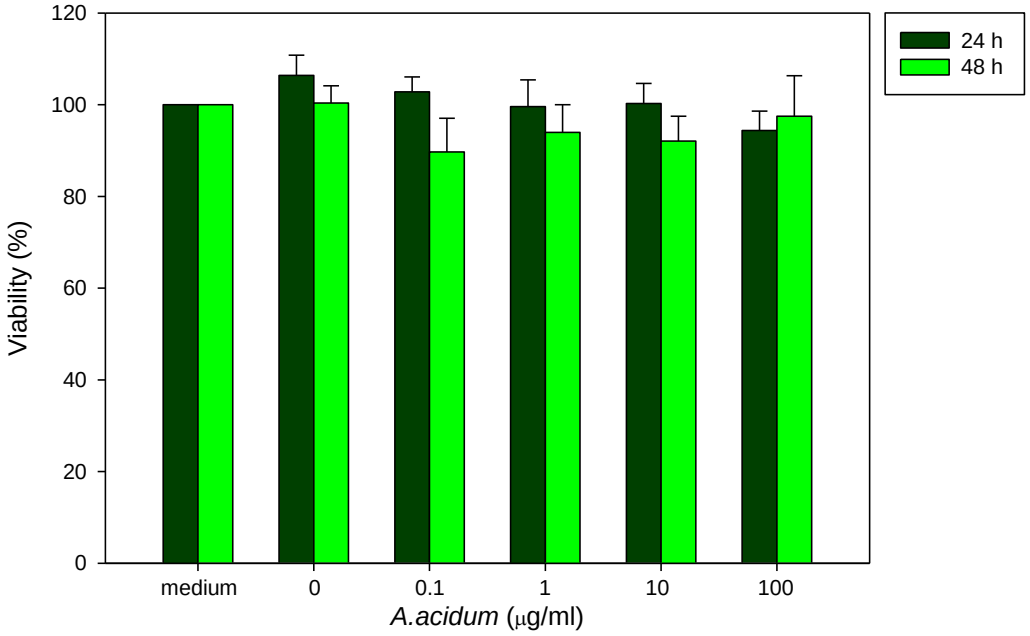


Figure 15 Cytotoxicity of the ethanol crude extract of *A. acidum* at 0 to 100 µg/ml concentrations for 48 h in feline kidney cells

<u>Part IIIC</u> Feline kidney cells pretreatment with *A. acidum* before DOX treatment

Feline kidney cells were initially treated with sub-toxic doses of *A. acidum* for 24 h. Then, kidney cell toxicity was induced with 8 μM of DOX for 48 h. The protective effects, oxidative stress parameters and eNOS gene and protein expression were determined in the end of the experiment.

1. Protective effects

The sub-toxic doses of *A. acidum* were used to treat feline kidney cells before inducing nephrotoxicity with 8 μM of DOX. The protective effects, which included cytotoxicity assay and apoptosis and necrosis assay, were measured.

1.1 Cytotoxicity assay

Feline kidney cells were pretreated with various concentrations of 0, 0.1, 1, 10, 25, 50 and 100 μg/ml ethanol crude extract of *A. acidum* for 24 h before the induction of cell toxicity using 8 μM of DOX for 48 h. The results demonstrated that the significant protective effect of pretreatment with *A. acidum* started at 0.1 to 100 μg/ml concentration (Table 19; Figure 16).

Table 19 Percent cell viability of the pretreatment with *A. acidum* for 24 h at subtoxic concentrations on DOX (8 μM) induced feline cell toxicity for 48 h

Pretreatment groups		Viability (%)
Non-treated control		$100^{\#\#}$ (n=5)
DOX-treated control		$31.03 \pm 6.51^{*}$(n=5)
A. acidum (μg/ml)	0.1	$66.07 \pm 6.12^{*, \#\#}$(n=5)
	1	$71.45 \pm 5.69^{*, \#\#}$(n=5)
	10	$68.99 \pm 2.29^{*, \#\#}$(n=5)
	25	$70.85 \pm 3.60^{*, \#\#}$(n=4)
	50	$67.53 \pm 4.41^{*, \#\#}$(n=4)
	100	$71.93 \pm 1.98^{*, \#\#}$(n=5)

DOX = Doxorubicin; n = Number of monolayers in each experiment

$^{*}p < 0.05$ when compared with non-treated control by ANOVA

$^{\#\#}p < 0.01$ when compared with DOX-treated control by ANOVA

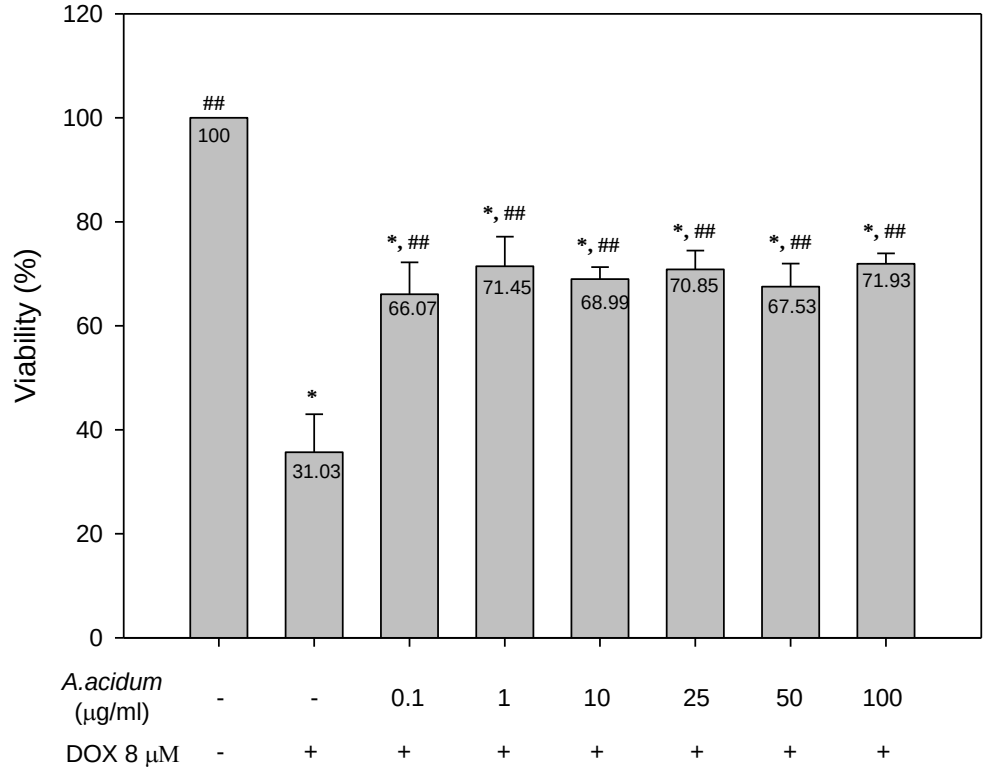


Figure 16 Cytotoxicity of pretreatment with the ethanol crude extract of *A. acidum* at 0 – 100 μg/ml concentrations for 24 h before the induction of DOX-induced feline kidney cell toxicity for 48 h. $^{*}p < 0.05$ when compared with non-treated control by ANOVA; $^{\#\#}p < 0.01$ when compared with DOX-treated control by ANOVA.

1.2 Apoptosis and necrosis assay

The results of apoptosis and necrosis assay of the pretreatment with *A. acidum* at 0 – 50 µg/ml concentrations before DOX-induced feline kidney cell were shown (Table 20; Figure 17A; Figure 17B). Apoptotic and necrotic cells, which have condensed and fragmented chromosome, are detected by staining with Hoechst 33342 and PI co-staining, respectively. Therefore, percentages of chromosome condensed cells were measured to determine apoptosis and necrosis of feline kidney cells.

Pretreatment with 25 µg/ml of *A. acidum* had significantly lower percentage of chromosome condensed cells when stained with Hoechst 33342 than DOX-treated control and other concentrations of *A. acidum*. Moreover, pretreatment with *A. acidum* at 0, 0.1, 10, 25 and 50 µg/ml were significantly higher in apoptotic cells when compared with non-treated control.

Table 20 Apoptosis and necrosis assay of pretreatment with *A. acidum* at 0 – 50 µg/ml concentrations for 24 h before DOX-induced feline kidney cell toxicity for 48 h

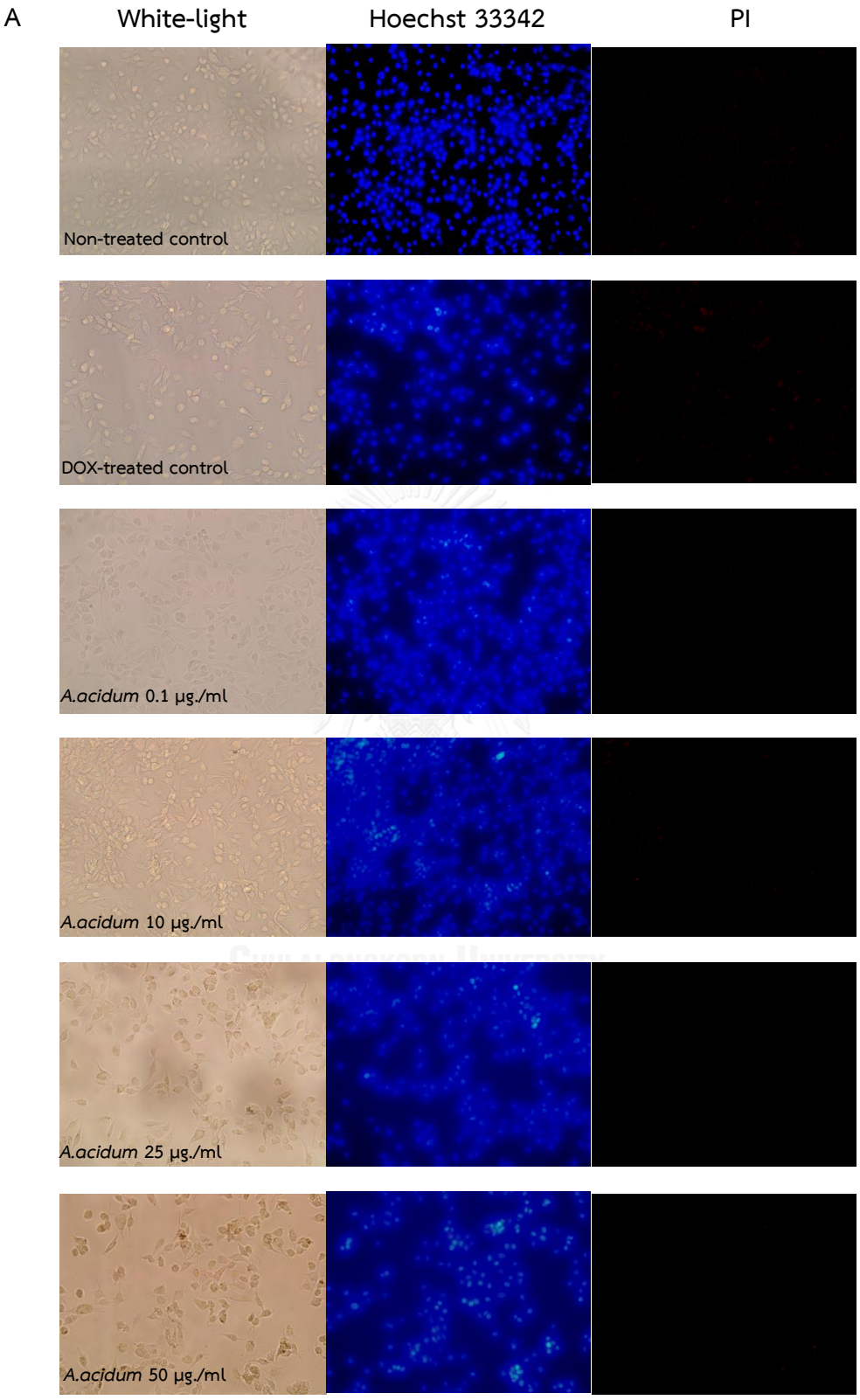
Pretreatment groups		Chromosome condensed cells (%)	
		Hoechst 33342	PI co-staining
Non-treated control		8.98 ± 0.45 (n=4)	6.96 ± 1.05 (n=4)
DOX-treated control		32.83 ± 1.42** (n=3)	23.79 ± 1.01* (n=3)
A. acidum (µg/ml)	0.1	39.33 ± 0.17** (n=3)	23.65 ± 1.74* (n=3)
	10	37.18 ± 1.36** (n=3)	24.25 ± 3.10* (n=3)
	25	19.88 ± 0.51$^{*, \#\#, aa}$ (n=3)	9.24 ± 0.43 (n=3)
	50	31.41 ± 0.84** (n=3)	23.69 ± 1.83* (n=3)

DOX = Doxorubicin; n = Number of monolayers in each experiment

$^{*}p < 0.05$, $^{**}p < 0.01$ when compared with non-treated control by ANOVA

$^{\#\#}p < 0.01$ when compared with DOX-treated control by ANOVA

$^{aa}p < 0.01$ when compared with other concentrations of *A. acidum* by ANOVA


A
White-light
Hoechst 33342
PI
Non-treated control
DOX-treated control
A.acidum 0.1 µg./ml
A.acidum 10 µg./ml
A.acidum 25 µg./ml
A.acidum 50 µg./ml

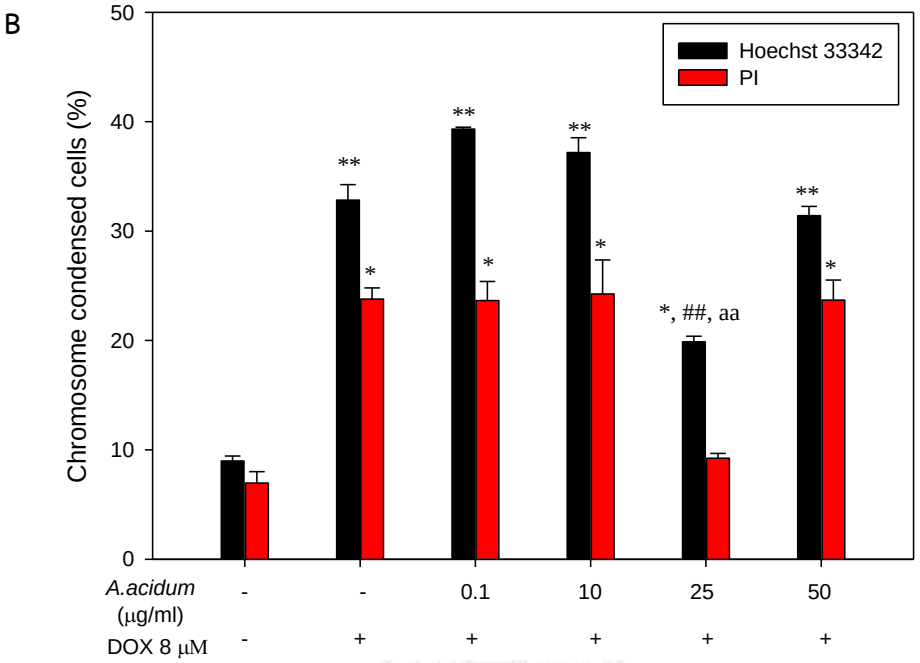


Figure 17 Feline kidney cells were treated with *A. acidum* at 0 – 50 μg/ml for 24 h and then induced cell toxicity with DOX for 48 h. They were stained with Hoechst 33342 and PI for apoptosis and necrosis assay. (A) Apoptotic cells showed bright blue nuclear condensation and necrotic cells displayed red fluorescence. (B) The percentage of chromosome condensed cells. $^{*}p < 0.05$, $^{**}p < 0.01$ when compared with non-treated control by ANOVA; $^{\#\#}p < 0.01$ when compared with DOX-treated control by ANOVA; $^{aa}p < 0.01$ when compared with other concentrations of *A. acidum* by ANOVA

2. Oxidative stress parameter

According to cytotoxic assay, pretreatment with *A. acidum* at 0.1 to 50 µg/ml concentrations can protect feline kidney cells from DOX-induced cell toxicity. These concentrations of *A. acidum* pretreatment were used to determine the oxidative stress parameters (Intracellular GSH, GSSG and GSH/GSSG ratio).

2.1 Intracellular GSH

The GSH levels of feline kidney cells with *A. acidum* pretreatment at 0, 0.1, 10, 25 and 50 µg/ml concentrations were shown (Table 21; Figure 18). Feline kidney cells of pretreatment with *A. acidum* at 50 µg/ml significantly increased GSH when compared with *A. acidum* at 10 µg/ml

Table 21 Mean ± SEM of GSH levels in pretreatment with *A. acidum* at 0 – 50 µg/ml concentrations for 24 h before DOX-induced feline kidney cell toxicity for 48 h

Pretreatment groups		GSH (mM)
Non-treated control		3.27 ± 0.08 (n=3)
DOX-treated control		2.92 ± 0.18 (n=5)
A. acidum (µg/ml)	0.1	3.50 ± 0.24 (n=3)
	10	2.41± 0.45 (n=4)
	25	2.59 ± 0.36 (n=4)
	50	3.88 ± 0.27[a] (n=4)

GSH = Glutathione; DOX = Doxorubicin; n = Number of monolayers in each experiment

[a] $p < 0.05$ when compared with *A. acidum* at 10 µg/ml by ANOVA

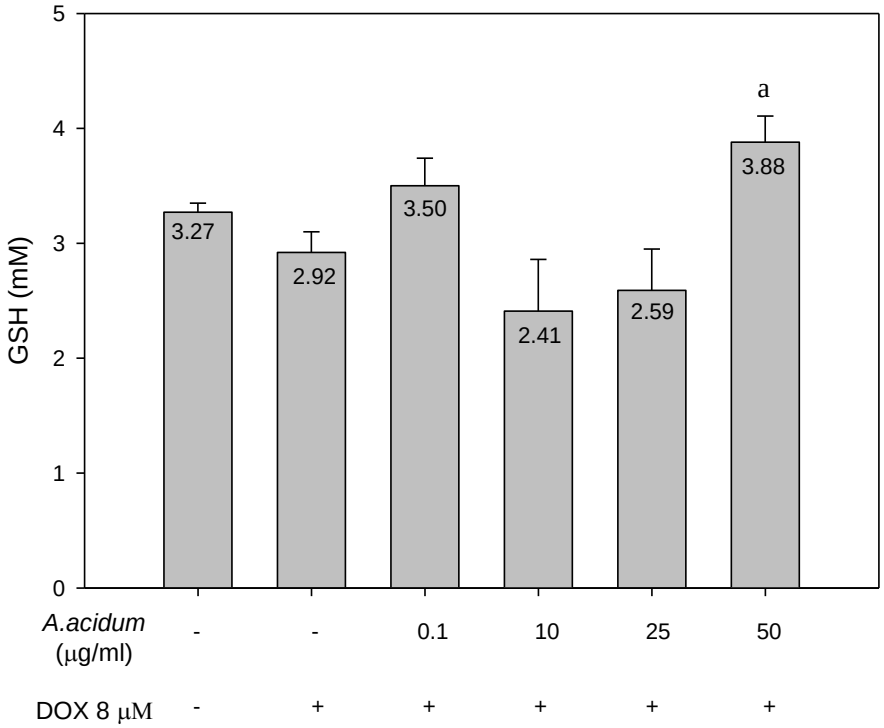


Figure 18 GSH levels in pretreatment with the ethanol crude extract of *A. acidum* at 0 – 50 μg/ml concentrations for 24 h before DOX-induced feline kidney cell toxicity for 48 h. [a]$p < 0.05$ when compared with *A. acidum* at 10 μg/ml by ANOVA.

2.2 Intracellular GSSG

The GSSG levels pretreatment with DOX-induced feline kidney cells with *A. acidum* at 0, 0.1, 10, 25 and 50 µg/ml concentrations were shown (Table 22; Figure 19). The GSSG levels in pretreatment with *A. acidum* at 50 µg/ml were significantly lower than DOX-treated control and *A. acidum* pretreatment at 10 and 25 µg/ml.

Table 22 Mean ± SEM of GSSG in pretreatment with *A. acidum* at 0 – 50 µg/ml concentrations for 24 h on DOX-induced feline kidney cell toxicity for 48 h

Pretreatment groups		**GSSG (µM)**
Non-treated control		22.31 ± 1.85 (n=3)
DOX-treated control		30.73 ± 4.07 (n=5)
A .acidum (µg/ml)	0.1	27.25 ± 1.10 (n=3)
	10	36.20 ± 3.49 (n=4)
	25	34.54 ± 3.87 (n=4)
	50	13.60 ± 1.9$^{*, aa}$ (n=4)

DOX = Doxorubicin; n = Number of monolayers in each experiment

$^{*}p < 0.05$ when compared with DOX-treated control by ANOVA

$^{aa}p < 0.01$ when compared with *A. acidum* at 10, 25 µg/ml by ANOVA

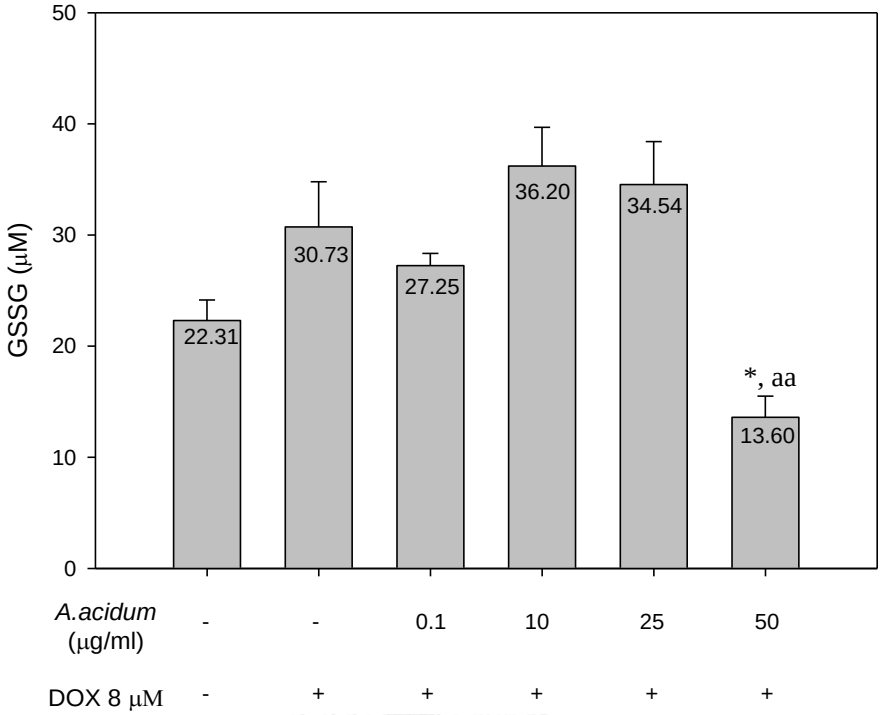


Figure 19 The GSSG levels of pretreatment with *A. acidum* at 0 – 50 μg/ml concentrations for 24 h before DOX-induced feline kidney cell toxicity for 48 h. $^{*}p < 0.05$ when compared with DOX-treated control by ANOVA; $^{aa}p < 0.01$ when compared with *A. acidum* at 10, 25 μg/ml by ANOVA.

2.3 Intracellular GSH/GSSG ratio

The GSH/GSSG ratio levels of pretreatment DOX-induced feline kidney cells with *A. acidum* at 0, 0.1, 10, 25 and 50 µg/ml concentrations were shown (Table 23; Figure 20). The results showed that pretreatment with *A. acidum* at 50 µg/ml had significantly increased in GSH/GSSG ratio when compared with other groups.

Table 23 Mean ± SEM of GSH/GSSG ratio in pretreatment with *A. acidum* at 0 – 50 µg/ml concentrations for 24 h before DOX-induced feline kidney cell toxicity for 48 h

Pretreatment groups		GSH/GSSG ratio
Non-treated control		148.20 ± 11.13 (n=3)
DOX-treated control		104.24 ± 18.95 (n=5)
A. acidum (µg/ml)	0.1	131.03 ± 12.85 (n=3)
	10	67.89 ± 11.99 (n=4)
	25	76.98 ± 12.99 (n=4)
	50	309.48 ± 46.52$^{*, \#\#, aa}$ (n=4)

DOX= Doxorubicin; n = Number of monolayers in each experiment

$^{*}p < 0.05$ when compared with non-treated control by ANOVA

$^{\#\#}p < 0.01$ when compared with DOX-treated control by ANOVA

$^{aa}p < 0.01$ when compared with *A. acidum* at 0.1, 10, 25 µg/ml by ANOVA

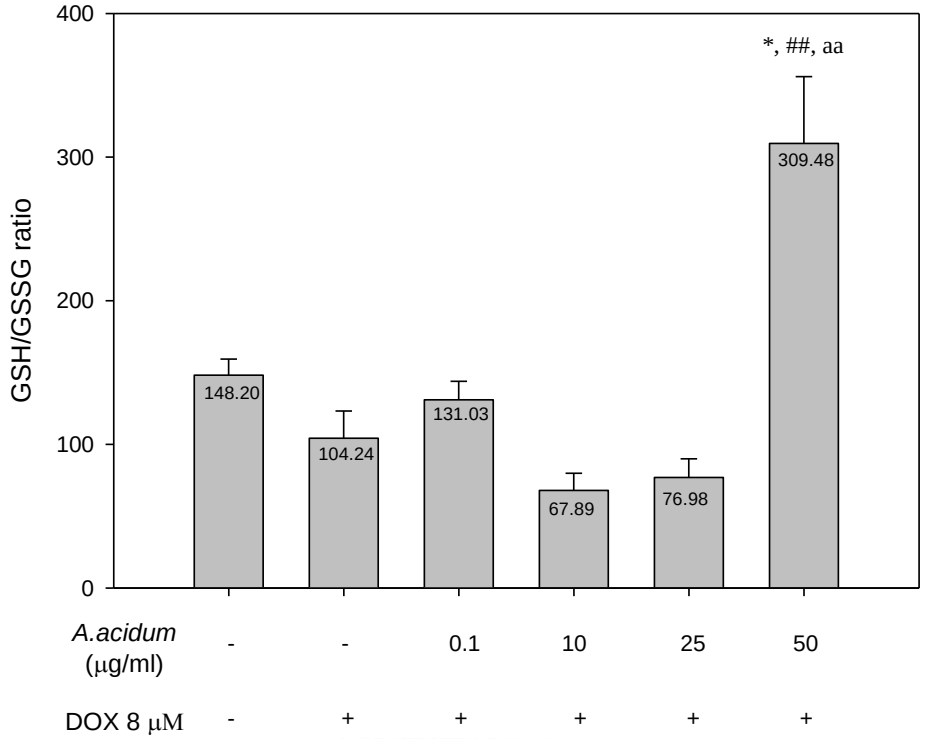


Figure 20 The GSH/GSSG ratio of the pretreatment with the ethanol crude extract of *A. acidum* at 0 – 50 μg/ml concentrations for 24 h before DOX-induced feline kidney cell toxicity for 48 h. $^{*}p < 0.05$ when compared with non-treated control by ANOVA; $^{\#\#}p < 0.01$ when compared with DOX-treated control by ANOVA; $^{aa}p < 0.01$ when compared with *A. acidum* at 0.1, 10, 25 μg/ml by ANOVA.

3. eNOS measurements

eNOS mRNA and protein expression were measured in pretreatment with *A. acidum* on DOX-induced feline kidney cell toxicity.

3.1 eNOS mRNA expression

The eNOS mRNA expression in the pretreatment with *A. acidum* at concentrations of 0, 0.1, 10, 25 and 50 µg/ml on DOX-induced feline nephrotoxicity was shown (Table 24; Figure 21). Pretreatment with *A. acidum* at 25 µg/ml (163.33 ± 58.00) had significantly higher in eNOS up-regulation when compared with non-treated control (3.81 ± 3.02) and DOX-treated control (0.15 ± 0.04).

Table 24 Relative eNOS mRNA expression in pretreatment with *A. acidum* at 0 – 50 µg/ml for 24 h before DOX-induced feline kidney cell toxicity for 48 h

Pretreatment groups		eNOS mRNA expression
Non-treated control		3.81 ± 3.02 (n=5)
DOX-treated control		0.15 ± 0.04 (n=3)
A. acidum (µg/ml)	0.1	0.05 ± 0.05 (n=3)
	10	0.06 ± 0.03 (n=4)
	25	163.33 ± 58.00$^{**, \#\#}$ (n=5)
	50	25.12 ± 14.28 (n=3)

DOX = Doxorubicin; n = Number of monolayers in each experiment

$^{**}p < 0.01$ when compared with non-treated control by ANOVA

$^{\#\#}p < 0.01$ when compared with DOX-treated control by ANOVA

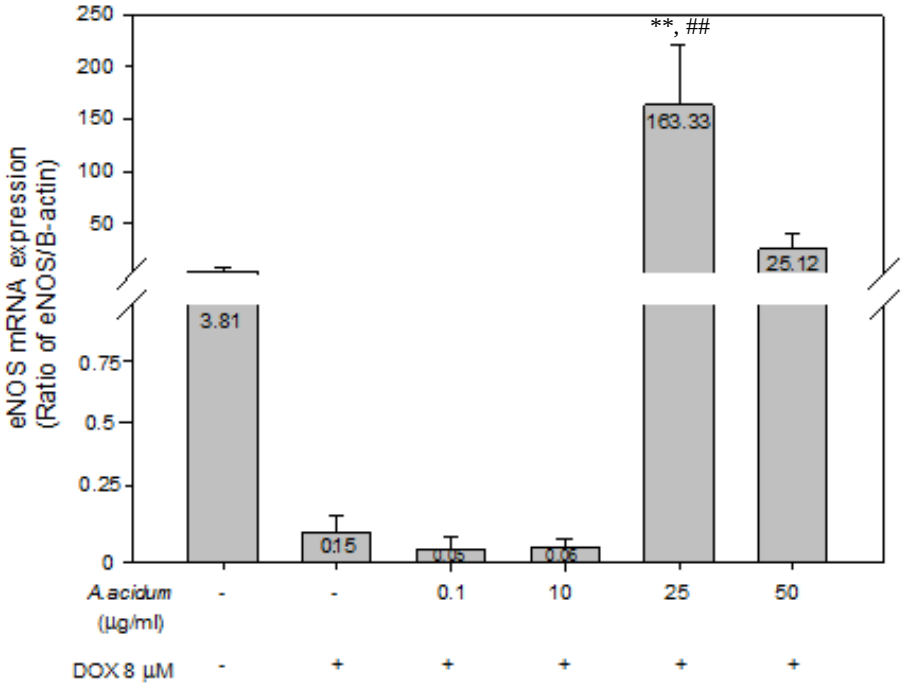


Figure 21 eNOS gene expression of the pretreatment with the ethanol crude extract of *A. acidum* at 0 – 50 µg/ml for 24 h before DOX-induced feline kidney cell toxicity for 48 h. $^{**}p < 0.01$ when compared with non-treated control by ANOVA; $^{\#\#}p < 0.01$ when compared with DOX-treated control by ANOVA.

3.2 eNOS protein expression

The eNOS protein expression in pretreatment with *A. acidum* at concentrations of 0, 0.1, 10, 25 and 50 µg/ml on DOX-induced feline nephrotoxicity was shown (Table 25; Figure 22). The present study found the protein bands of eNOS and β-actin which were 37 and 42 kDa, respectively. Pretreatment with *A. acidum* at concentration of 0.1 µg/ml (3.42 ± 1.16) had significantly increased eNOS protein expression when compared with DOX-treated control (0.58 ± 0.13).

Table 25 eNOS protein expression in pretreatment with *A. acidum* at 0 – 50 µg/ml for 24 h before DOX-induced feline kidney cell toxicity for 48 h

Pretreatment groups		**eNOS protein expression (Ratio of eNOS/β-actin)**
Non-treated control		0.88 ± 0.33 (n=3)
DOX-treated control		0.58 ± 0.13 (n=3)
A. acidum (µg/ml)	0.1	3.42 ± 1.16# (n=3)
	10	0.95 ± 0.34 (n=3)
	25	0.82 ± 0.19 (n=3)
	50	0.95 ± 0.42 (n=3)

DOX = Doxorubicin; n = Number of monolayers in each experiment

#$p < 0.05$ when compared with DOX-treated control by ANOVA

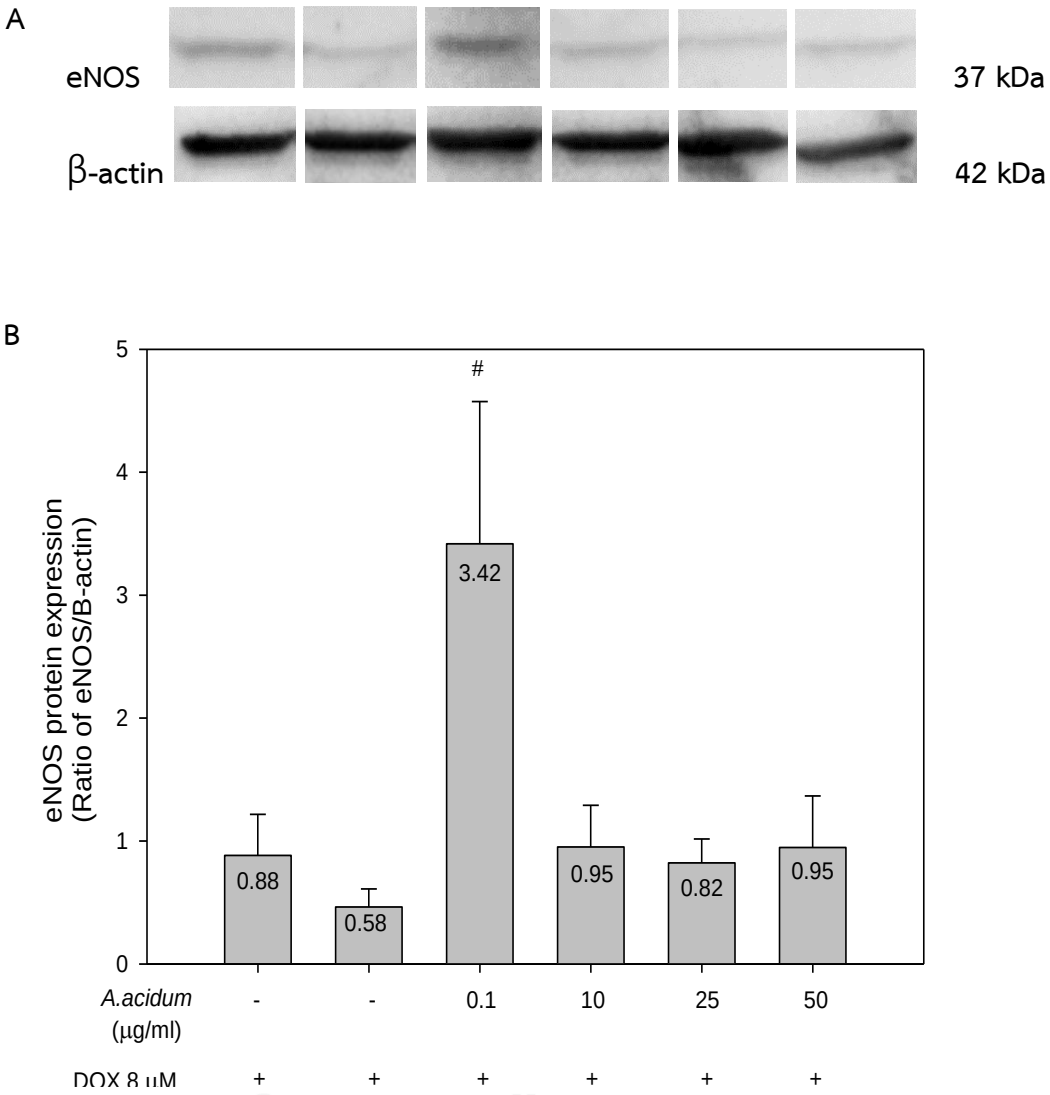


Figure 22 (A) eNOS protein expression of pretreatment with the ethanol crude extract of *A. acidum* at 0 – 50 µg/ml for 24 h on DOX-induced feline kidney cell toxicity for 48 h. (B) Quantitative densitometry analysis of eNOS protein expression. $^{\#}p < 0.05$ when compared with DOX-treated control by ANOVA.

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<u>Part IIID</u> Feline kidney cells post-treatment with *A. acidum* after DOX treatment

Renal cell toxicity were induced with 8 μM of DOX for 48 h. Then they were incubated with sub-toxic dose of *A. acidum* for 24 h. Then the protective effects, oxidative stress parameters and eNOS measurements were determined afterward.

1. Protective effects

The protective effects which consist of cytotoxicity assay, apoptosis and necrotic assay were determined after post-treatment with various concentrations of *A. acidum*.

1.1 Cytotoxicity assay

Feline kidney cells were firstly induced cell toxicity by using 8 μM DOX for 48 h and then treated with various concentrations of *A. acidum* 0 – 100 μg/ml for 24 h (Table 26; Figure 23). Post-treatment of feline kidney cells with *A. acidum* at 100 μg/ml concentration had significantly higher live cells than DOX-treated control and other various concentrations of *A. acidum*.

Table 26 Percent cell viability of the post-treatment with *A. acidum* at subtoxic concentrations for 24 h after DOX-induced feline cell toxicity for 48 h

Post-treatment groups		Viability (%)
Non-treated control		100 (n=5)
DOX-treated control		31.03 ± 6.51 (n=5)
A. acidum (µg/ml)	0.1	18.08 ± 1.57 (n=5)
	1	20.28 ± 2.18 (n=5)
	10	23.80 ± 2.34 (n=5)
	25	21.29 ± 1.76 (n=4)
	50	34.32 ± 0.97 (n=4)
	100	62.53 ± 1.40$^{\#\#, a, aa}$ (n=5)

DOX= Doxorubicin; n = Number of monolayers in each experiment

$^{\#\#}p < 0.01$ when compared with DOX-treated control by ANOVA

$^{a}p < 0.05$ when compared with *A. acidum* at 50 µg/ml by ANOVA

$^{aa}p < 0.01$ when compared with *A. acidum* at 0.1, 1, 10 and 25 µg/ml by ANOVA

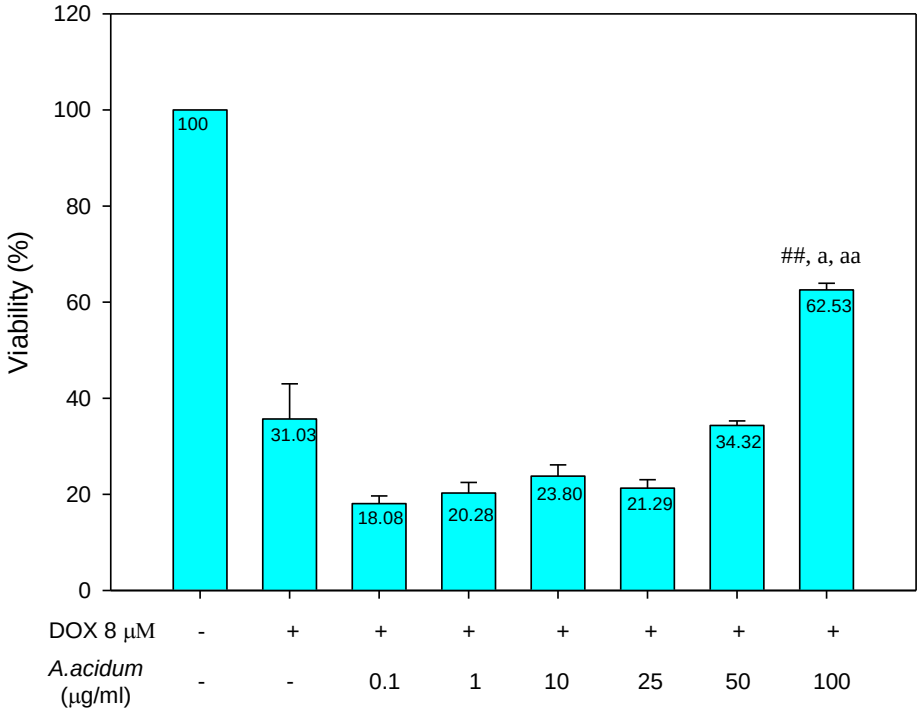


Figure 23 Cytotoxicity of the post-treatment with the ethanol crude extract from *A. acidum* at 0 – 100 µg/ml for 24 h after DOX-induced feline kidney cell toxicity for 48 h. $^{\#\#}p < 0.01$ when compared with DOX-treated control by ANOVA; $^{a}p < 0.05$ when compared with *A. acidum* at 50 µg/ml by ANOVA; $^{aa}p < 0.01$ when compared with *A. acidum* at 0.1, 1, 10 and 25 µg/ml by ANOVA.

1.2 Apoptosis and necrosis assay

The results of apoptosis and necrosis assay of pretreatment with *A. acidum* at 0 – 50 µg/ml concentrations before DOX-induced feline kidney cell were shown (Table 27; Figure 24A; Figure 24B). Post-treatment with all concentrations of *A. acidum* had significantly higher percentage of chromosome condensed cells which can be shown when stained with Hoechst 33342 than non-treated control. Post-treatment with 25µg/ml of *A. acidum* had significantly lower than DOX-treated control.

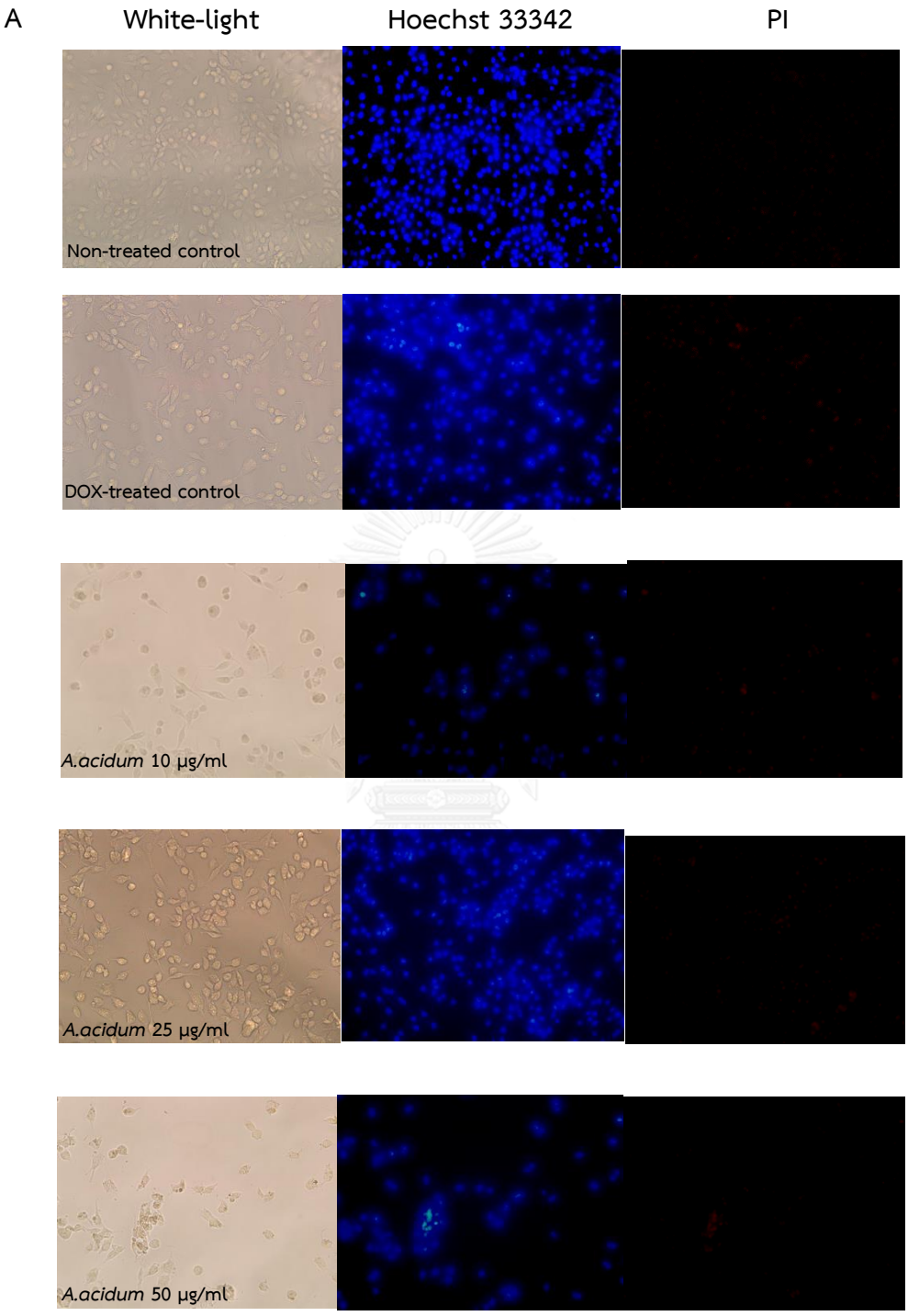
Table 27 Apoptosis and necrosis assay of the post-treatment with *A. acidum* at 0 – 50 µg/ml concentrations for 24 h after DOX-induced feline kidney cell toxicity for 48 h

Pretreatment groups		Chromosome condensed cells (%)	
		Hoechst 33342	PI co-staining
Non-treated control		8.98 ± 0.45 (n=4)	6.96 ± 1.05 (n=4)
DOX-treated control		43.18 ± 3.90** (n=3)	25.60 ± 5.54** (n=3)
A. acidum (µg/ml)	10	36.76 ± 0.45** (n=3)	25.10 ± 2.21** (n=3)
	25	31.48 ± 2.25**, ## (n=3)	19.69 ± 3.91 (n=3)
	50	35.99 ± 1.75** (n=3)	21.52 ± 2.33 (n=3)

DOX = Doxorubicin; n = Number of monolayers in each experiment

** $p < 0.01$ when compared with non-treated control by ANOVA

$p < 0.01$ when compared with DOX-treated control by ANOVA


A
White-light
Hoechst 33342
PI
Non-treated control
DOX-treated control
A.acidum 10 µg/ml
A.acidum 25 µg/ml
A.acidum 50 µg/ml

B

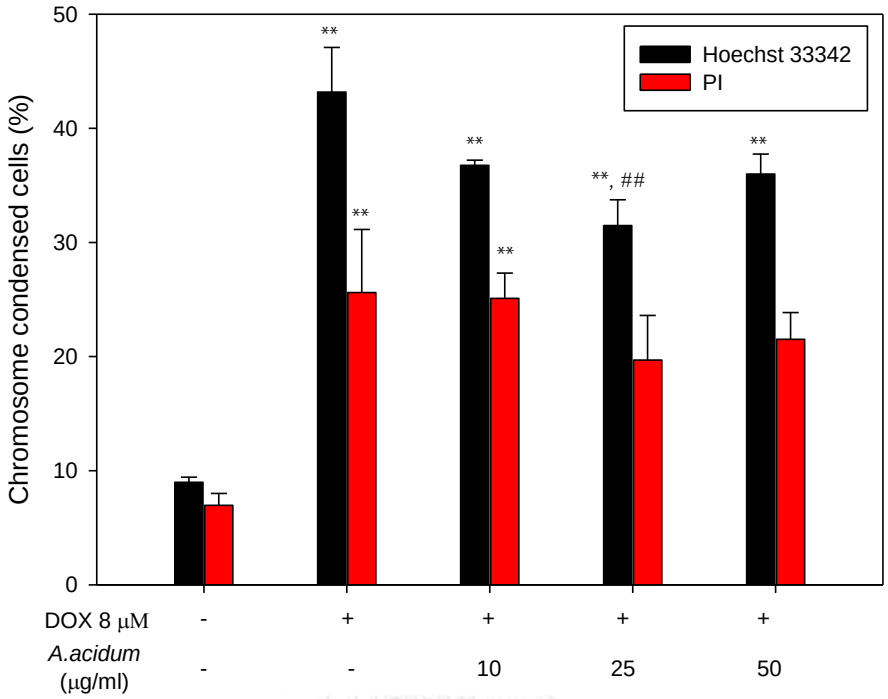


Figure 24 Feline kidney cells were induced cell toxicity with DOX for 48 h and then treated with *A. acidum* at 0 – 50 μg/ml for 24 h. They were stained with Hoechst 33342 and PI for apoptosis and necrosis assay. (A) Apoptotic cells showed bright blue nuclear condensation and necrotic cells displayed red fluorescence. (B) The percentage of chromosome condensed cells. $^{**}p < 0.01$ when compared with non-treated control by ANOVA; $^{\#\#}p < 0.01$ when compared with DOX-treated control by ANOVA.

2. Oxidative stress parameters

According to cytotoxic assay, post-treatment with *A. acidum* only at 100 µg/ml concentration can protect feline kidney cells from DOX-induced cell toxicity. However, *A. acidum* at 100 µg/ml concentration did not completely dissolve. Therefore, we investigated the oxidative stress parameters of DOX-induced feline cell toxicity and post-treatment with *A. acidum* at 0, 10, 25 and 50 µg/ml concentrations.

2.1 Intracellular GSH

The GSH levels of feline kidney cells with *A. acidum* post-treatment at 0, 10, 25 and 50 µg/ml concentrations were shown (Table 28; Figure 25). Post-treatment with *A. acidum* at 50 µg/ml had significantly lowered intracellular GSH levels when compared with the concentration at 10 and 25 µg/ml.

Table 28 Mean ± SEM of GSH in post-treatment with *A. acidum* at 0 – 50 µg/ml for 24 h after DOX-induced feline kidney cell toxicity for 48 h

Post-treatment groups		GSH (mM)
Non-treated control		3.27 ± 0.08 (n=3)
DOX-treated control		2.92 ± 0.18 (n=5)
A. acidum (µg/ml)	10	3.40 ± 0.56 (n=4)
	25	3.84 ± 0.57 (n=3)
	50	1.52 ± 0.38[a] (n=4)

DOX= Doxorubicin; n = Number of monolayers in each experiment

[a] $p < 0.05$ when compared with *A. acidum* at 10 and 25 µg/ml by ANOVA

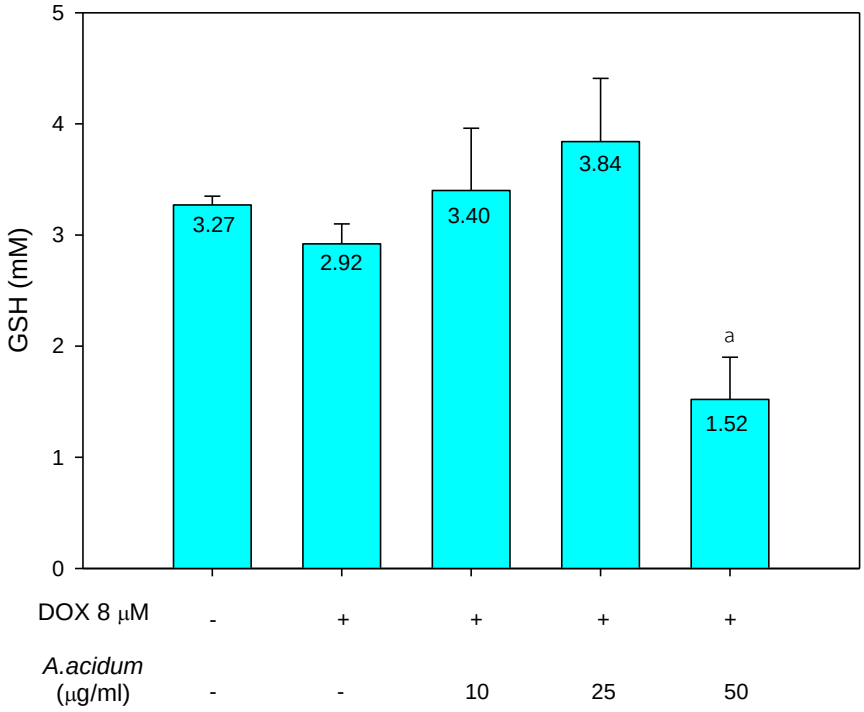


Figure 25 The GSH levels of post-treatment with *A. acidum* at 0 – 50 μg/ml concentrations for 24 h after DOX-induced feline kidney cell toxicity for 48 h. [a]*p< 0.05* when compared with *A. acidum* at 10 and 25 μg/ml by ANOVA

2.2 Intracellular GSSG

The GSSG levels of post-treatment with *A. acidum* at 0, 10, 25 and 50 µg/ml concentrations on DOX-induced feline kidney cells were shown (Table 29; Figure 26). The GSSG levels in post-treatment with *A. acidum* at various concentrations were lower than DOX-treated control, but it was not significantly difference when compared with DOX-treated control.

Table 29 Mean ± SEM of GSSG in post-treatment with *A. acidum* at 0 – 50 µg/ml for 24 h on DOX-induced feline kidney cell toxicity for 48 h

Post-treatment groups		GSSG (µM)
Non-treated control		22.31 ± 1.85 (n=3)
DOX-treated control		30.79 ± 4.07 (n=5)
A. acidum (µg/ml)	10	26.38 ± 1.46 (n=4)
	25	17.64 ± 2.45 (n=3)
	50	29.21 ± .91 (n=4)

DOX = Doxorubicin; n = Number of monolayers in each experiment

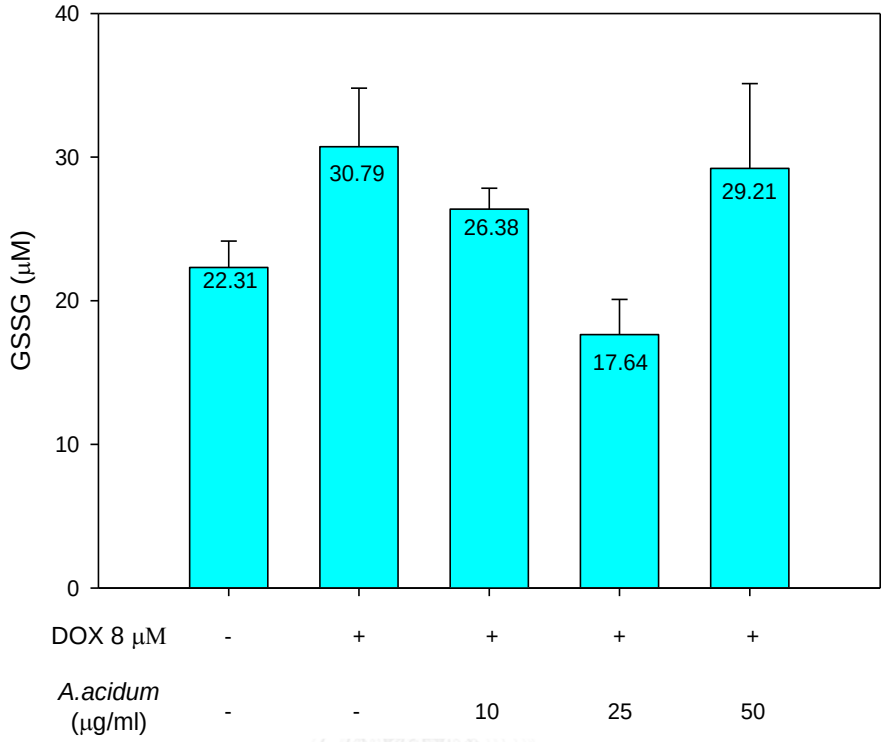


Figure 26 The GSSG levels of post-treatment with *A. acidum* at 0 – 50 μg/ml concentrations for 24 h on DOX-induced feline kidney cell toxicity for 48 h

2.3 Intracellular GSH/GSSG ratio

The GSH/GSSG ratio levels of post-treatment with *A. acidum* at 0, 10, 25 and 50 µg/ml concentrations on DOX-induced feline kidney cells were shown (Table 30 and Figure 27). Post-treatment with *A. acidum* at 25 µg/ml was significantly increased when compared with DOX-treated control and *A. acidum* at 50 µg/ml.

Table 30 Mean ± SEM of GSH/GSSG ratio in post-treatment with *A. acidum* at 0 – 50 µg/ml concentrations for 24 h on DOX-induced feline kidney cell toxicity for 48 h

Post-treatment groups		GSH/GSSG ratio
Non-treated control		148.20 ± 11.13 (n=3)
DOX-treated control		104.24 ± 18.95 (n=5)
A. acidum (µg/ml)	10	119.81 ± 23.82 (n=4)
	25	217.19 ± 41.47$^{\#, aa}$ (n=3)
	50	57.39 ± 12.86 (n=4)

DOX= Doxorubicin; n = Number of monolayers in each experiment

$^{\#}p < 0.05$ when compared with DOX-treated control by ANOVA

$^{aa}p< 0.01$ when compared with *A. acidum* at 50 µg/ml by ANOVA

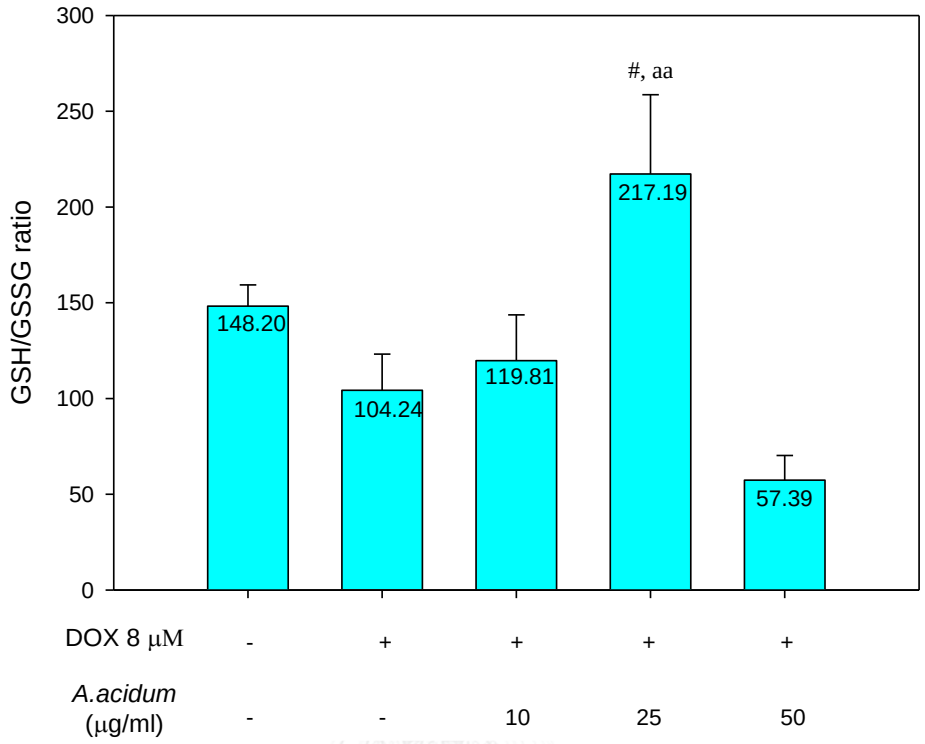


Figure 27 The GSH/GSSG ratio of post-treatment with the ethanol crude extract from *A. acidum* at 0 – 50 μg/ml concentrations for 24 h on DOX-induced feline kidney cell toxicity for 48 h. $^{\#}p < 0.05$ when compared with DOX-treated control by ANOVA; $^{aa}p < 0.01$ when compared with *A. acidum* at 50 μg/ml by ANOVA.

3. eNOS measurements

3.1 eNOS mRNA expression

The eNOS mRNA expression of post-treatment with *A. acidum* at 0, 10, 25 and 50 µg/ml concentrations on DOX-induced feline kidney cells were shown (Table 31; Figure 28). Post-treatment with *A. acidum* at 50 µg/ml (122.57 ± 47.74) had significantly increased in eNOS up-regulation when compared with non-treated control (3.81 ± 3.02) and DOX-treated control (0.12 ± 0.06).

Table 31 Relative eNOS mRNA expression in post-treatment with *A. acidum* at 0, 10, 25 and 50 µg/ml for 24 h on DOX-induced feline cell toxicity for 48 h

Post-treatment groups		eNOS mRNA expression
Non-treated control		3.81 ± 3.02 (n=5)
DOX-treated control		0.12 ± 0.06 (n=6)
A. acidum (µg/ml)	10	49.19 ± 32.30 (n=3)
	25	47.76 ± 17.02 (n=3)
	50	122.57 ± 47.74$^{**, \#\#}$ (n=4)

DOX= Doxorubicin; n = Number of monolayers in each experiment

$^{**}p < 0.01$ when compared with non-treated control by ANOVA

$^{\#\#}p < 0.01$ when compared with DOX-treated control by ANOVA

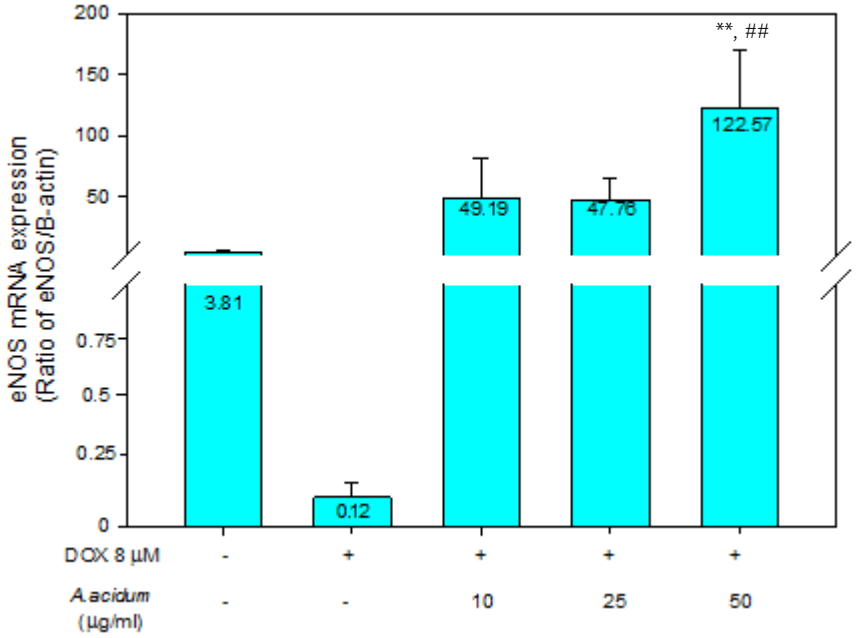


Figure 28 eNOS gene expression of post-treatment with the ethanol crude extract of *A. acidum* at 0, 10, 25 and 50 µg/ml for 24 h on DOX-induced feline cell toxicity for 48 h. $^{**}p < 0.01$ when compared with non-treated control by ANOVA; $^{\#\#}p < 0.01$ when compared with DOX-treated control by ANOVA.

3.2 eNOS protein expression

The eNOS protein expression of post-treatment with *A. acidum* at 0, 10, 25 and 50 µg/ml concentrations on DOX-induced feline kidney cells were shown (Table 32; Figure 29A; Figure 29B). Our study found that protein bands of eNOS and β-actin were 37 and 42 kDa, respectively (Figure 29A).

Table 32 eNOS protein expression in post-treatment with *A.acidum* at 0, 10, 25 and 50 µg/ml for 24 h on DOX-induced feline cell toxicity for 48 h

Post-treatment groups		**eNOS protein expression (Ratio of eNOS/β-actin)**
Non-treated control		0.88 ± 0.33 (n=3)
DOX-treated control		0.41 ± 0.15 (n=3)
A. acidum (µg/ml)	10	0.29 ± 0.03 (n=3)
	25	1.16 ± 0.83 (n=3)
	50	0.29 ± 0.19 (n=3)

DOX = Doxorubicin; n = Number of monolayers in each experiment

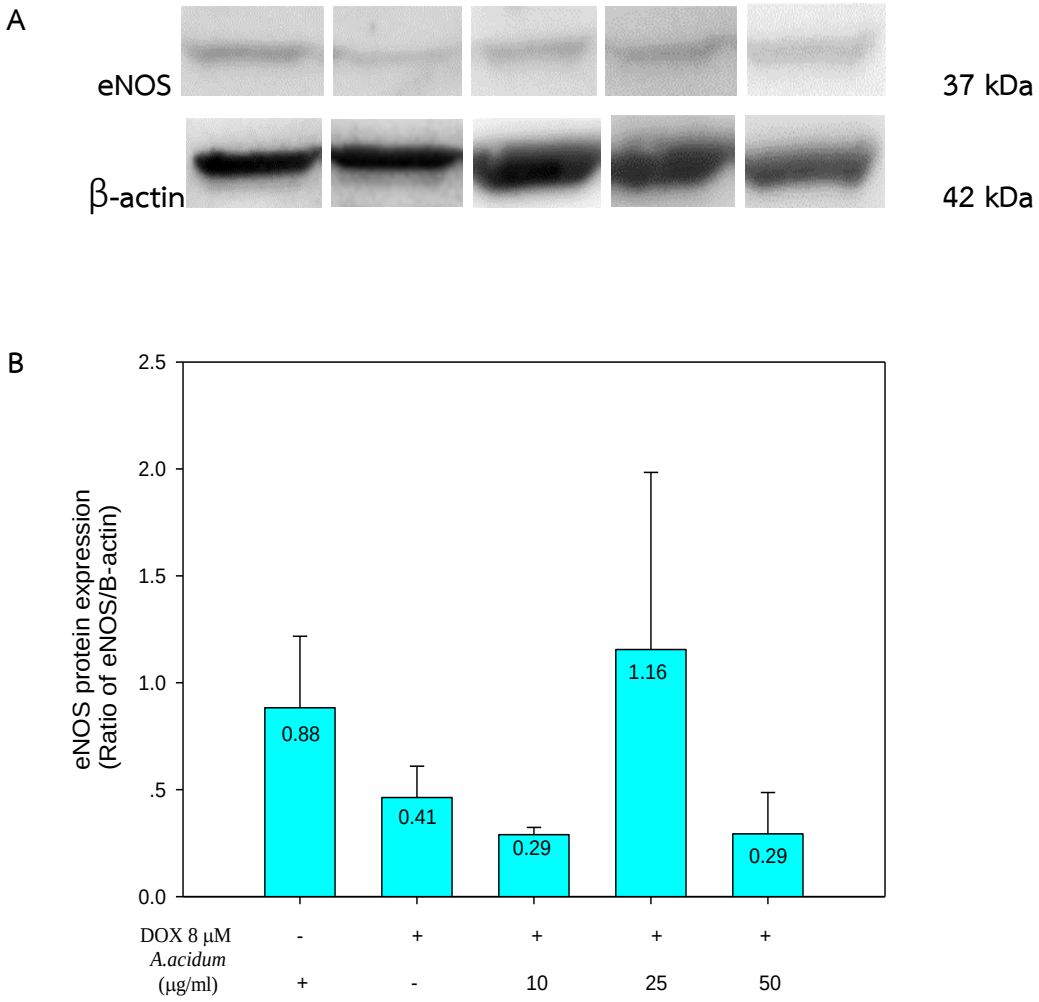


Figure 29 (A) eNOS protein expression of post-treatment with the ethanol crude extract of *A. acidum* at 0, 10, 25 and 50 µg/ml for 24 h on DOX-induced feline kidney cell toxicity for 48 h. (B) Quantitative densitometry analysis of eNOS protein expression.

CHAPTER V
DISCUSSION

Part I The epidemiologic study of risk factors in cats with chronic kidney disease

Cats of more than three years old were associated with increased risk for CKD. Cats aging between 1 to 3 years old were associated with decreased risk for CKD. The results of the present study were interesting because cats in this study developed CKD earlier than CKD cats in other previous reports (DiBartola et al., 1987; Elliot and Barber, 1998; Lawler et al., 2006; White et al., 2006). Most CKD cats in the present study were Siamese and Siamese mixed breed. Siamese breed was associated with increased risk for CKD. According to one previous study, breed of cat was associated with increased plasma creatinine (Reynolds et al., 2010). Creatinine is used in clinical practice to evaluate renal function in cats. One previous study also indicated that Siamese cats were associated with increased frequency in cats (Polzin et al., 2000). Pusoonthornthum et al (2009) demonstrated that most CRF cats in Thailand were Siamese and Siamese mixed breed. Both studies demonstrated that Siamese is one of the most prevalence breed for CKD (Polzin et al., 2000; Pusoonthornthum et al., 2009). In human, there is an association between race and end stage renal disease (ESRD). Black Americans are known to have a high risk for ESRD (Freedman et al., 1993).

Cats between 3 to 7 years old had 2.39 times increased risk for CKD more than other age groups. The results were in the same trend with several previous studies that the mean age of CKD cats is more than 7 years old (DiBartola et al., 1987; Elliot and Barber, 1998; Lawler et al., 2006; White et al., 2006). Cats of more than 7 to 10 years of age had 19.78 times higher than normal cats, and the cats of more than 10 years old were 2.79 times more at risk for developing CKD compared with other age groups. Moreover, logistic regression analysis using backward elimination indicated that aging cats were potential risk for CKD. Aging has been found to association with decreased renal function (Coresh et al., 2007). It is possible that aging process may be

one of an important cause of CKD in cats. Old cats were reported to have progressive tubular deletion and peritubular interstitial fibrosis in the kidneys (Lawler et al., 2006).

Our study found most CKD cats were more likely to have polyuria and polydipsia. In agreement with one previous study, CKD cats were increased with frequency in polyuria and polydipsia (Bartlett et al., 2010). CKD cats were presented with weight loss, whereas patients affected by ESRD always occur with overweight (Kramer et al., 2006).

Few studies demonstrated the association between cat's gender and CKD. The present study demonstrated that female spayed cats were associated with decrease risk for CKD. Previous study in Australia suggested that male cats develop CKD earlier than female cats (White et al., 2006). One study indicated that 17β-estradiol can protect female rats from hypertension which causes renal disease progression by inhibiting superoxide production (Ji et al., 2007). Human study supports that women have the trend of protection from developing ESRD (Iseki et al., 1996).

The present study found that cats fed with commercial can and dry food were associated with decreased odds for CKD, while homemade food was associated with increased odds. However, one previous study was reported no significant association between type of food and life span of CKD cats (Bartlett et al., 2010). Previous studies reported that nutritional variables are risk factor for CKD in cats (DiBartola et al., 1993). Hughes et al (2002) found that high fiber diet decreased the risk of chronic renal failure (CRF) in cats. Commercial food with low potassium (Buffington et al., 1991) and high protein ingredient (DiBartola et al., 1993) caused the development of CRF in cats. In the same trend Harte et al (1994) reported that lower protein diet can slow the rate of clinical signs in CKD cats. CKD cats given protein and phosphorous restricted diet had longer survival time than unrestricted group (Elliott et al., 2000). Spontaneous CKD cats feeding renal diet had significantly lower serum creatinine and higher blood bicarbonate concentrations than maintenance diet group (Ross et al., 2006).

Our results also demonstrated that filter water had lowered the risk for CKD. There were no studies on the association between water sources and CKD in cats. Water source was studied in cats with feline lower urinary tract disease (FLUTD) and found that there were no associated between FLUTD and water sources (Osborne et

al., 2000; Pusoonthornthum et al., 2012). However, one previous study in human was found that drinking water containing fluoride of more than 2.0 mg/l can cause kidney damage in children (Xiong et al., 2007).

In conclusion, aging, Siamese breed and home food were associated with increased risk for CKD. While, female spayed, canned and dry commercial diets were associated with decreased risk for CKD. In addition, aging was the potential risk factor for CKD in cats. Further study is needed to prove the association between these risk and protective factors and the cause of CKD in cats.

Part II The changes in the glutathione, oxidized glutathione and glutathione peroxidase in the clinically normal cats and the cats with naturally occurring chronic kidney disease

Thirteen clinically normal client-owned cats and twenty-three cats with naturally occurring CKD were studied. The mean age of the cats with CKD (11.04 ± 0.94 years) was similarly to the previous reports in Thailand (Pusoonthornthum and Pusoonthornthum, 2004; Pusoonthornthum et al., 2009). During 1998, a study in UK indicated that the mean age of 80 cats with CKD in the UK was 12.6 years (Elliot and Barber, 1998). In a previous study, 53% of 74 cats with CKD were over 7 years old and cats' age ranged between 9 months to 22 years (DiBartola et al., 1987). The study of age distribution in cats with CRF were found to be less than 10 years old (37%), between 10 to 15 years old (31%) and older than 15 years of age (32%) (Lulich et al., 1992). In Australia, a study of 184 feline with CRF indicated that male with CRF had median age of 12 years, whereas female with CRF had median age of 15 years (White et al., 2006). There was no association between age and the oxidative stress parameters in the present study. In a study of clinically ill cats, there was no significant correlation between age and oxidative stress parameters (GSH, cystein and ascorbate concentration) (Viviano et al., 2009). The most common breeds of the cats with CKD in the present study included Siamese-mixed and Siamese breed cats. In agreement with previous study, feline CRF was also observed in Siamese and Siamese-mixed breed cats (Pusoonthornthum et al., 2009). There were one FeLV positive cats and two FIV positive cats in the CKD group. One previous study has been found oxidative stress during acute FIV infection in cats (Webb et al., 2008).

The CKD cats had significantly lower PCV and RBC levels than the clinically normal cats. However, the mean PCV and RBC in the CKD group in this study were within the normal reference range. The kidney produces an important hormone called erythropoietin (EPO). EPO deficiency is one of the important causes for anemia in CRF (Cowgill et al., 1998). The anemia in the CRF was characterized as normochromic, normocytic and hypo-proliferation (Eschach, 1989). Cats with anemia present with pale mucous membrane, poor appetite, lethargy, depression, and decreased activity. Cats

are susceptible to oxidative stress (Webb et al., 2006) and the feline erythrocytes have greater Heinz bodies as a result of oxidant damage compared with the other species (Christopher et al., 1990). However, abnormality of RBC in the CKD cats was not observed in this study.

The mean WBCs, neutrophils and lymphocytes in CKD cats were significantly higher than clinically normal cats. In agreement with previous study, increased phagocyte activation was significantly higher in CRF cats than control cats (Keegan and Webb, 2010). However, the mean WBCs, neutrophils and lymphocytes in both groups were within the normal reference range.

The results of the present study showed that cats with naturally occurring CKD were significantly lower concentration of GSH when compared with the clinically normal cats. Glutathione (GSH) is a tripeptide including glycine, cysteine and glutamine. Glutathione (GSH) and oxidized glutathione (GSSG) concentrations in the plasma have been considered as the index of the whole body glutathione and indicators of a disease risk (Jones et al., 2000). During severe oxidative stress, the concentration of GSH may decrease and the concentration of GSSG may increase in the affected cells (Sakhi et al., 2006). Erythrocyte glutathione concentration in the CRF patients were reported as lower than the clinically normal age-matched (Rutkowski et al., 2006; Pedram et al., 2009). Studies in cats with liver disease, acetaminophen toxicity and acute experimented FIV infections were demonstrated the decrease in GSH concentration (Allison et al., 2000; Center et al., 2002; Webb et al., 2003). The study using HPLC for the plasma GSH determination in twenty-three healthy cats had been reported that mean concentrations of plasma GSH were 4.51 ± 1 µmol (Denzoin et al., 2008). Another study was reported that GSH in cats, dog and human were 1.97 ± 0.07, 1.98 ± 0.15 and 2.26 ± 0.09 mmol/L RBC, respectively (Harvey and Kaneko, 1976). The study in clinically ill dogs was also showed a decrease in erythrocyte glutathione concentration (Viviano et al., 2009).

GPx in the CKD cats was significantly lower than in the clinically normal cats. GPx has biological role in protecting organs from oxidative injury by reducing lipid hydroperoxides to alcohols and cutting free hydrogen peroxide (H_2O_2) to water. The previous studies have also demonstrated that the plasma GPx concentration in CKD

patients was lower than normal age-matched (Harvey and Kaneko, 1976; Pedram et al., 2009). A study of glutathione and glutathione related enzymes in patients on maintenance dialysis found that plasma GPx activity was significantly lower in the dialysis group than in the healthy control group (Pedram et al., 2009). A study of oxidative stress in cats with diabetes mellitus (DM) was also reported that plasma GPx levels in the DM cats were less than in the control cats (Webb and Falkowski, 2009).

According to the present study, the CKD cats had significantly higher GSSG than in the clinically normal cats. The study by Denzoin et al (2008) showed that mean concentrations of GSSG in healthy cats were 19.44 ± 3.79 μmol. An increase in plasma GSSG and a decrease in GSH/GSSG ratio were observed in the CKD cats in the present study. This correlation is also found in studies with other subjects and diseases. Oxidative stress has been postulated to play a role in chronic disease as reported in the sputum supernatant of GSSG in patients with asthma (5.9 μM) was significantly higher than control (2.6 μM) (Wood et al., 2008). In hypertension pregnant patients, the GSSG levels were significantly elevated as compared with the normotensive pregnant women and the nonpregnant controls (Nemeth et al., 2001). Sakhi et al (2006) reported an increase in plasma GSSG and a decrease in GSH/GSSG ratio when cells were exposed to oxidative stress. The study in cats with liver disease also demonstrated the decrease in GSH/GSSG ratio (Center et al., 2002). In conclusion, the present study demonstrated that the oxidative stress in cats with CKD is significantly greater than the clinically normal cats. Further study is required to examine the benefit of antioxidant in cats with naturally occurring CKD.

<u>Part III</u> The effects of the crude extract of *A. acidum* on oxidative stress and eNOS mRNA expression in DOX-induced feline kidney cells

The total stem of *A. acidum* gave 0.90% of the ethanol crude extract from *A. acidum*. The final crude extract amount of *A. acidum* in this study was less than the previous study (3.17%) at approximately 3 times (Fungbun et al., 2012), but it was similar to our previous study (0.91%) (Jaimun et al., 2012). This may be due to seasonal variation and harvesting time (Jalal et al., 1982; Fang et al., 2010).

The present study used DPPH assay for determining the radical scavenging of the ethanol crude extract of *A. acidum*. The DPPH assay is a direct and reliable method for determining the radical scavenging activity. Free radical scavenging of the ethanol crude extract of *A. acidum* was compared to BHT and bioflavonoid (quercetin) which are widely used as antioxidants. Quercetin, which is a member of the class of flavonoids called flavonoles, is mostly found in onions, broccoli, apples and berries. Quercetin is considered as the most powerful flavonoids for protecting the body against reactive oxygen species which is produced during the normal oxygen metabolism of mitochondria or is induced by exogenous damage (de Groot, 1994). The radical scavenging of the extract was rapidly increased when increased the concentration at 0 - 25 µg/ml, but it was stable at 25 – 100 µg/ml concentration. The percent radical scavenging of the *A. acidum* ethanol crude extract did not depend on concentration, but it had high efficiency at 25 – 50 µg/ml as previous study (Fungbun et al., 2012). However, Jaimun et al (2012) reported that percent radical scavenging of the ethanol crude extract of *A. acidum* increase as the concentration of substances increase. The percent radical scavenging of the extract was similar to quercetin at 25 – 100 µg/ml concentration. In agreement with previous study, the percent radical scavenging of *A. acidum* ethanol crude extract at 100 µg/ml concentration was as high as quercetin (Fungbun et al., 2012; Jaimun et al., 2012), the most powerful flavonoids for protecting the body against reactive oxygen species (de Groot, 1994).

The ethanol crude extract from *A. acidum* (EC_{50} = 8.83 µg/ml) was more effective than BHT (EC_{50} = 46.43 µg/ml) at approximately 5 times, but it was less effective than quercetin (EC_{50} = 2.22 µg/ml) at approximately 4 times. One previous

study reported that *A. acidum* (EC_{50} = 5.47 µg/ml) was more effective than BHT (EC_{50} = 44.12 µg/ml) at approximately 8 times, but it was similar to quercetin (EC_{50} = 3.13 µg/ml) (Fungbun et al., 2012). In contrast with another study, EC_{50} of the ethanol crude extract of *A. acidum* (42.21 µg/ml) was similar to BHT (42.24 µg/ml), but it was less effective than quercetin (EC_{50} = 2.19 µg/ml) at approximately 19 (Jaimun et al., 2012). In another study, EC_{50} of ethanol crude extract from the bark and wood of *A. acidum* was 3.55 – 4.43 µg/ml (Tuy-on and Itharat, 2011).

Antioxidant activity of plant extracts is mainly due to the radical scavenging activity of phenolic compounds such as flavonoids, polyphenols, tannins, and phenolic terpenes (Rahman and Moon, 2007). The ethanol crude extract from *A. acidum* is rich in flavonoids, anthocyanins, tannins, β-sitosterol and stigmasterol (Thamaree et al., 2003). Isolated-compound from roots of *A. acidum* is rich of catechin (Kaennakam and Tip-pyang, 2011) which is a strong antioxidant in flavonoid family. These compositions may contribute to the antioxidant property of *A. acidum* extract. The present study found that the crude extract of *A. acidum* was more effective than BHT at approximately 5 times. Moreover, the highest percentage of free radical scavenging was occurred in the ethanol crude extract of *A. acidum* at 25 – 100 µg/ml concentration. Therefore, the present study indicated that the ethanol crude extract of *A. acidum* has a high antioxidant activity (EC_{50} = 8.83 µg/ml).

The results of the present study indicated that a reduction of 50% cell surviving was detected in feline kidney cell lines after using 8 µM of DOX for 48 h. Doxorubicin causes chronic nephrotoxicity through oxidative stress mechanism which has been suggested to play an important role in DOX nephropathy. The production of oxidative stress by DOX contains four steps. Firstly, mitochondrial NADPH dehydrogenase or other reductases activate DOX to create $O_2^{-}\cdot$ which leads to oxidative stress mechanism (Kimura et al., 2005; Chen et al., 2007). Secondly, doxorubicin activates NADPH oxidase to generate $O_2^{-}\cdot$ (Kimura et al., 2005). Thirdly, DOX can promote iron metabolism to produce OH^{-} (Chen et al., 2007). Fourthly, the metabolism of DOX such as doxorubicinol which can produce OH^{-} through iron metabolism. Moreover, the apoptosis of renal tubular cells has been reported in DOX-treated rats (Zhang et al., 1996) and is believed to be a key feature of tubular atrophy, which is a hallmark of

chronic renal diseases (Schelling et al., 1998; Khan et al., 1999). DOX at concentrations of 1 to 10 μM could induce cytotoxicity to primary human renal proximal tubular epithelial cells (hRPTEC) in dose-dependent manner at 24 h with 50 – 70% viability (Li et al., 2006). Doxorubicin can cause cytotoxicity and apoptosis in human tubular renal cells (HK-2) at concentrations of 1-8 μM which demonstrated dose and time-dependence (Chaotham et al., 2013). The appropriate dose and time of DOX treatment in human renal proximal tubular were 4 μM and 12 h (Chaotham et al., 2013). However, cells from different species may respond differently to a drug such as DOX (Dietrich et al., 2001).

The present study showed that the cytotoxicity assay of DOX correlated with apoptosis assay. DOX-treated control had significantly increased cell death and Hoechst 33342-positive apoptotic cells when compared to non-treated control. Although DOX-treated control had significantly increased apoptotic and necrotic cells when compared to non-treated control, the present study indicated that feline kidney cell damage was mainly due to DOX action through apoptosis mechanism. In agreement with previous study, DOX can predominately induce human proximal tubule cell death through apoptosis pathway, while necrotic cells were rarely observed (Chaotham et al., 2013).

This study demonstrated that DOX-treated control had lower GSH and higher GSSG levels than non-treated control. GSH levels often decrease in oxidative stress (Sakhi et al., 2006). Kimura et al (2005) and Chen et al (2007) reported that induction of oxidative stress is one of an important role in DOX-induced nephropathy. Several studies suggested that oxidative stress may involve in DOX-induced nephrotoxicity (Deman et al., 2001; Ayla et al., 2011). Deman et al (2001) reported the change of the antioxidant enzyme including GSH, GPx, CAT and SOD in DOX-induced mouse glomerulosclerosis. DOX also can decrease the GSH, GPx and catalase (CAT) levels in rat kidney tissues (Ayla et al., 2011). The results were similar to the previous studies in naturally occurring CKD which may be related to a high production of the free radicals in cats (Viviano et al., 2009; Keegan and Webb, 2010; Krofic Zel et al., 2014) and human patient (Dounousi et al., 2006; Rutkowski et al., 2006). Therefore, DOX can induce nephrotoxicity in feline kidney cells through the production of oxidative stress.

For eNOS protein synthesis, the present study showed that eNOS proteins (37 kDa) were expressed at lower expected bands (130-140 kDa). However, the antibody use in the present study is recommended for detecting human eNOS. It is possible that cells form different species may respond differently to antibodies. Moreover, our protein of interest is generated through alternative splicing or cleavage of the protein post-translation. The present study indicated the eNOS gene and protein expression in DOX-treated control decreased when compared with non-treated control. However, decreased eNOS gene and protein expression in DOX-treated control in this study may cause by different periods of time of DOX treatment and cells from different species. Duration of DOX treatment in the study (48 h) was longer than previous study in bovine endothelial cells (12 h) (Kalivendi et al., 2001). In addition, the possible reasons of decreased eNOS expression in DOX-induced nephropathy were as follows; activates NADPH dehydrogenase to create $O_2^{-}\cdot$ which provide ROS such as OH^{-}, H_2O_2 and $ONOO^{-}$ (Kimura et al., 2005; Chen et al., 2007), excess NO production or $ONOO^{-}$ could inhibit eNOS production (Griscavage et al., 1995). DOX may decrease eNOS expression from excess NO or $ONOO^{-}$ production which can be generated through mitochondrial NADPH dehydrogenase or other reductases. However, decreased eNOS in DOX-induced feline kidney cell death was similar to several reports on human with naturally occurring kidney disease (Furusu et al., 1998; Albrecht et al., 2002), in rats with induced nephrotoxicity (Yanagisawa et al., 1998; Yang et al., 2010) and female aged rats (Maric et al., 2008). eNOS deficiency is associated with CKD (Chang et al., 2002) and developed progressive renal injury (Forbes et al., 2007). Therefore, DOX may induce kidney cell toxicity in feline kidney cells through oxidative stress mechanisms which cause the increased in free radicals and the decreased in eNOS expression.

A. acidum at 0.1 to 100 µg/ml concentration was considered as a sub-toxic dose to feline kidney cell lines. In agreement with the study in normal human lung cell line (MRC-5), *A. acidum* was non-toxic to normal cell (Tuy-on and Itharat, 2011). Previous studies in animal models had been reported that the ethanol crude extract of *A. acidum* was safe for healthy cats (Pusoonthornthum et al., 2010), moderate CKD cats (Fungbun et al., 2012; Jaimun et al., 2012) and rats (Sireeratawong et al., 2012). Moreover, the appropriate dose of the ethanol crude extract of *A. acidum* in CKD cats

was 120 mg/kg (Pusoonthornthum et al., 2010; Fungbun et al., 2012; Jaimun et al., 2012).

The significant effects of *A. acidum* on DOX kidney cell toxicity were shown when pretreatment with *A. acidum* started at 0.1 µg/ml concentration. Pretreatment with *A. acidum* at 25 µg/ml concentration had significantly decreased apoptotis cells when compared to DOX-treated control and other concentrations of *A. acidum*. Two isolated compounds from roots of *A. acidum* (5,7-dihydroxy-2-eicosyl-chromone and 2,5-dimethoxy-1,4-bezoquinone) can protect cytotoxicity on HeLA cells (Kaennakam and Tip-pyang, 2011). Other antioxidant plants such as *Croton stellatopilosus* Ohba, which is rich of flavonoid and phenolic compound, had the protective effects on DOX-induced human proximal tubule cell death (Chaotham et al., 2013). However, the apoptosis and necrotic assays do not correlate with cytotoxicity assay. The significant decreased in apoptotic cells was only observed when pretreatment with *A. acidum* at 25 µg/ml. Cytotoxicity assay does not separate between apoptotic and necrotic cells. It is possible that apoptotic cells may remain in cell population which causes increased cell viability in cytotoxicity assay.

Pretreatment with *A. acidum* at 50 µg/ml had significantly increased the GSH/GSSG ratio when compared with other groups, because pretreatment with *A. acidum* at 50 µg/ml concentration to feline kidney cells were significantly lower in GSSG levels than DOX-treated control. According to Sakhi et al (2006), the GSH levels may decrease and the GSSG levels may increase in the cells which affect severe oxidative stress. The ethanol crude extract of *A. acidum* with a high antioxidant activity is rich of flavonoids (Thamaree et al., 2003) and catechin (Kaennakam and Tip-pyang, 2011). Our previous study in CKD cats reported that moderate CKD cats given the ethanol crude extract of *A. acidum* had significantly increased GPx after 2 months of treatment supports the mechanism that *A. acidum* can decrease oxidative stress in cats with CKD (Jaimun et al., 2012).

When *A. acidum* was given to feline kidney cells before the induction of kidney cell toxicity by DOX, *A. acidum* at 0.1 µg/ml concentration significantly increased in eNOS protein expression and increased in eNOS mRNA expression at 25 µg/ml concentration when compared with both non-treated and DOX-treated control. This

action may be due to the antioxidant property of *A. acidum*. The ethanol crude extract from *A. acidum* is rich in flavonoids, stigmasterol (Thamaree et al., 2003) and catechin (Kaennakam and Tip-pyang, 2011)which is one of the antioxidants in the flavonoid family. Report on antioxidants from natural plants indicated that *Morinda citrifolia* Linn. (Indian Mulberry) which is rich in flavonoids, can stimulate the release of NO (Hirazumi and Furusawa, 1999). French red wine with high polyphenol levels (Corder et al., 2001) can stimulate the expression of the eNOS mRNA in human endothelial cell (Wallerath et al., 2003). The mechanism of action of *A. acidum* in the present study has shown as the protective effects of *A. acidum* against kidney cell toxicity in feline kidney cells. This information can explain to us why the ethanol crude extract of *A. acidum* can decrease serum creatinine levels in healthy cats (Pusoonthornthum et al., 2010) and cats with natural CKD in moderate IRIS stage (Fungbun et al., 2012; Jaimun et al., 2012). CKD cats given *A. acidum* had significantly decreased serum creatinine, good quality of life through an up-regulation of eNOS expression because *A. acidum* by inhibit $ONOO^-$ (Griscavage et al., 1995). The anti-oxidative stress effects of pretreatment with *A. acidum* were effective when pretreatment with high dosage of *A. acidum*.

On the contrary, *A. acidum* given to feline kidney cells after the induction of nephrotoxicity by DOX, post-treatment with high dosage of *A. acidum* could protect feline kidney cells from doxorubicin. Post-treatment with *A. acidum* at 25 µg/ml had significantly increased the GSH/GSSG ratio when compared with other groups. Moreover, post-treatment with *A. acidum* at 50 µg/ml concentration significantly increased eNOS mRNA expression. The results of post-treatment indicated the protective and antioxidant effects of post-treatment with *A. acidum* were shown when post-treatment with high dosage of *A. acidum*. *A. acidum* can decrease oxidative stress and increase eNOS expression by inhibiting iron metabolism and scavenging free radicals rapidly. Therefore, *A. acidum* extract can be used as both pre and post-treatment for cats with nephropathy.

In conclusion, aging is the potential risk factor for CKD in cats. Cats with naturally occurring CKD had oxidative stress because GSH, GSSG, GPx and GSH/GSSG ratio were significantly different from the clinically normal age-matched cats. Pretreatment with

A. acidum (25 µg/ml) and post-treatment with higher dosage of *A. acidum* (50 µg/ml) protected feline kidney cells from DOX-induced cell toxicity by decreasing oxidative stress and increasing eNOS mRNA expression.

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APPENDIX

Appendix A Questionnaire data

ชื่อเจ้าของสัตว์_____ที่อยู่_____

เบอร์โทรศัพท์_____

ชื่อแมว_____สีขน_____ตำหนิ

จุดเด่น_____

พันธุ์ ( ) ไทย ( ) ไทยผสม ( ) Abyssinian ( ) Russian Blue

( ) Burmese ( ) Himalayan ( ) Persia ( ) Domestic short hair

อายุ_____ปี _____เดือน

เพศ ( ) ผู้ ( ) ผู้ตอนแล้ว ( ) เมีย ( ) เมียตอนแล้ว

น้ำหนักตัว_____kg

เข้ามารับการรักษาด้วยอาการ ( ) ปัสสาวะมาก/กินน้ำมาก (PU/PD)

( ) ปัสสาวะมีเลือดปน (hematuria)

( ) ปัสสาวะลำบาก (dysuria)

( ) ปัสสาวะบ่อย และกระปริดกระปรอย (pollakiuria)

( ) อ่อนแรง ( ) น้ำหนักลด ( ) เบื่ออาหาร ( ) ซึมเศร้า

( ) ภาวะร่างกายขาดน้ำ (dehydration)

( ) มีกลิ่นปาก ( ) มีแผลในช่องปาก

( ) สีเยื่อเมือกซีด ( ) น้ำลายไหลยืด ( ) อื่นๆ

เคยป่วยด้วยโรคนี้มาก่อน ( ) เคย ( ) ไม่เคย

ประวัติการรักษาโรนี้_____

โรคที่เคยเป็นมาก่อน_____

ประวัติการเลี้ยงดู

ชนิดอาหาร ( ) อาหารกระป๋องยี่ห้อ _____

( ) อาหารเม็ดยี่ห้อ_____

( ) เนื้อหมู ( ) เนื้อไก่ ( ) เนื้อวัว ( ) เครื่องใน

( ) ปลาน้ำจืด ( ) ปลาทู ( ) กุ้ง ( ) ปลาหมึก

( ) ผสมข้าว ( ) อื่นๆ

การปรุงอาหาร ( ) ไม่ได้ปรุง ( ) ปรุงด้วย_____

ความถี่ในการให้อาหาร ( ) วันละ 1 มื้อ ( ) 2 มื้อ ( ) 3 มื้อ ( ) มากกว่า 3 มื้อ

( ) ให้กินตลอดเวลา

อาหารที่ให้ ให้เป็นเวลานาน_____ ปี_____เดือน_____วัน ( ) จำไม่ได้

ชนิดของน้ำที่กิน ( ) ประปา ( ) บาดาล ( ) กรอง ( ) ต้ม ( ) กลั่น ( ) อื่นๆ

การให้น้ำ ( ) เป็นเวลาวันละ_____ครั้ง ( ) ตลอดเวลา

การคุมกำเนิด ( ) ฉีดยาคุม ( ) ทำหมัน ( ) กักบริเวณ ( ) อื่นๆ

ประวัติการทำวัคซีน ( ) โรคพิษสุนัขบ้า ( ) โรคไข้หัดแมว, โรคหลอดลมอักเสบและหวัดติดต่อ

( ) โรคลิวคิเมีย ( ) ไม่เคยฉีดวัคซีน ( ) ไม่มีประวัติ

ถ่ายพยาธิ ( ) เคย ( ) ไม่เคย

ประวัติสายพันธุ์ - มีพี่น้องจำนวน_____ตัว

- มีญาติ พี่น้องหรือพ่อแม่เคยป่วยเป็นโรคไตเรื้อรัง

( ) เคยเป็นจำนวน_____ตัว

( ) ไม่เคยเป็น

ที่บ้านมีแมวตัวอื่นป่วยด้วยโรคไตเรื้อรังหรือไม่ ( ) มีตัวอื่นป่วย มีจำนวน_____ตัว ( ) ไม่มี

ลักษณะสถานที่เลี้ยงสัตว์ ( ) บ้าน ( ) เทาว์เฮาส์ ( ) ตึกแถว ( ) คอนโด

( ) ใกล้โรงงาน ( ) ใกล้โรงพยาบาล

( ) ใกล้ปั๊มน้ำมัน ( ) มีที่เก็บสารเคมี สีทาบ้าน และสิ่งแปลกปลอม

ลักษณะการเลี้ยงสัตว์ ( ) เลี้ยงปล่อย ( ) ขังกรง ( ) อยู่แต่ในบ้าน ( ) อื่นๆ

ค่าทางโลหิตวิทยา

Parameter		Units
RBC		$\times 10^6$ cells/ml
PCV		%
WBC		cells/ml
Neutrophils		cells/ml
Lymphocytes		cells/ml
Eosinophils		cells/ml
Basophils		cells/ml
Monocytes		cells/ml
ALT		IU/L
ALP		IU/L
BUN		mg/dl
Creatinine		mg/dl

เก็บตัวอย่างปัสสาวะครั้งแรก

เก็บโดยวิธี ( ) เจาะกระเพาะปัสสาวะ ( ) สวนปัสสาวะ

( ) รองรับปัสสาวะที่แมวขับถ่ายออกมาเอง (Voiding)

( ) ใช้แรงกดดันน้ำปัสสาวะออกมา (compresasion)

สีปัสสาวะ ( ) ใส ไม่มีสี ( ) มีสี

กลิ่น ( ) ไม่มี ( ) มีกลิ่น

ความขุ่น ( ) ขุ่น ( ) ไม่ขุ่น

ความถ่วงจำเพาะ = 1.0_____

Appendix B Signalment and places of study in the clinically normal cats (part II)

Group / Name	Age (years)	Gender	Weight (kg)	Breed	Places of study	
					CU	Private
Clinically normal						
ขาว	7	M	6	Siamese	/	
โอวันติล	13	M	6	Siamese-mixed	/	
ตัวเล็ก	14	M	6.38	Siamese-mixed	/	
ดุ๊กดิ๊ก	12	F	5	Siamese-mixed	/	
แป๋วแหว๋ว	12	F	4.66	Siamese-mixed	/	
น้ำส้ม	12	F	4.42	Siamese-mixed	/	
บังเอิญ	12	M	2.76	Siamese-mixed	/	
การ์ตูน	12	F	3.26	Siamese-mixed	/	
แพนด้า	12	M	3.6	Siamese-mixed	/	
ตุ้ยตุ่ย	12	F	3.62	Siamese-mixed	/	
การ์ฟิลด์	7	M	5	Siamese-mixed	/	
จั๊กแหล่น	7	M	4.5	Siamese-mixed	/	
ข้าวโอ๊ต	6	M	4.8	Siamese-mixed	/	

CU = The Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University

Private = Private animal hospitals

Appendix C Signalment and places of study in the CKD cats (part II)

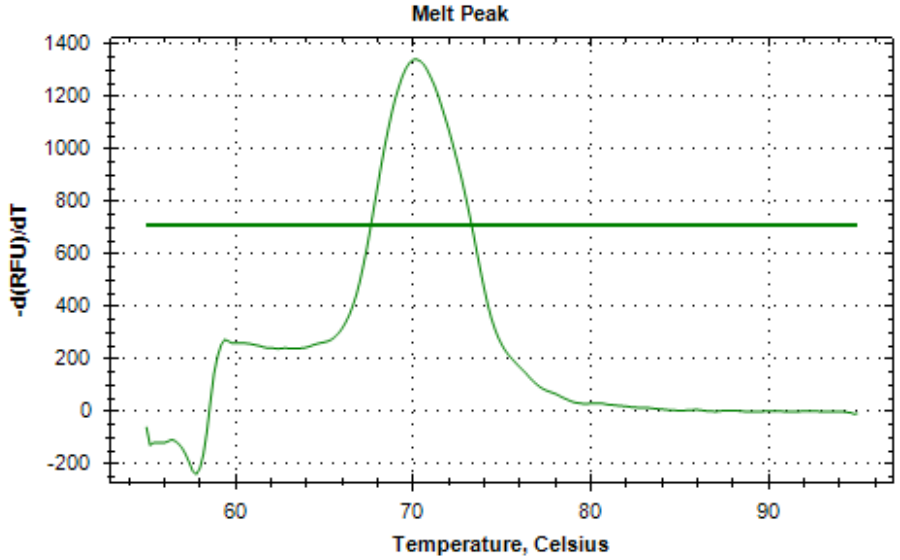
Group / Name	Age (years)	Gender	Weight (kg)	Breed	Places of study	
					CU	Private
Clinically normal						
สุขสันต์	13	M	4.42	Siamese-mixed	/	
ปุ๊กกี้	15	F	3.2	Siamese-mixed	/	
หมวย	8	F	2.9	Siamese-mixed		/
พริกขี้หนู	10	F	4	Siamese-mixed		/
ม่อน	13	M	4.3	Siamese-mixed		/
กัปตัน	7	M	3.8	Siamese-mixed		/
Nut	5	M	3.4	Siamese-mixed		/
ชาบิ	16	M	2.8	Siamese-mixed	/	
สามปอย	14	F	4.4	Siamese-mixed		/
หนำเลี๊ยบ	13	F	4.8	Siamese-mixed		/
อัมพะวา	12	F	4.5	Siamese-mixed		/
Malee	16	F	3.45	Siamese-mixed		/
แตงแว	15	F	4.4	Siamese-mixed		/
รูปหล่อ	6	M	5.6	Siamese-mixed		/
เกาลัด	13	M	4	Siamese-mixed		/
ตี๋น้อย	17	M	6	Siamese-mixed		/
ฟักทอง	16	M	6	Siamese-mixed		/
โอเลี้ยง	8	M	7	Siamese-mixed	/	
ดิกกี้	6	M	5.8	Siamese-mixed		/
หนูนา	3	F	2.4	Siamese-mixed		/
ลาเต้	3	M	5.7	Siamese-mixed		/
ก๋วยเจ๋ง	9	M	9	Siamese	/	
เฉาก๋วย	16	F	4.9	Siamese-mixed		/

CU = The Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University

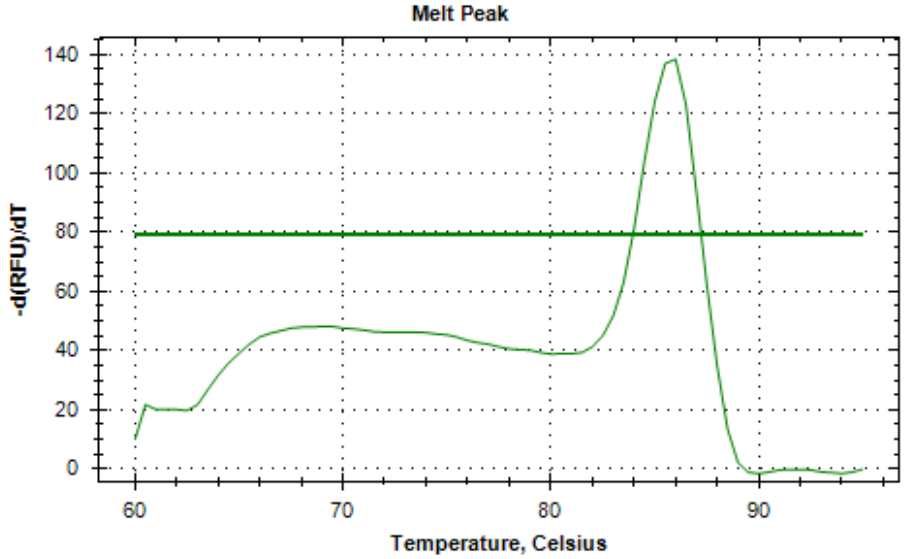
Private = Private animal hospitals

Appendix D eNOS (A) and β-actin (B) melt temperatures of non-treated control

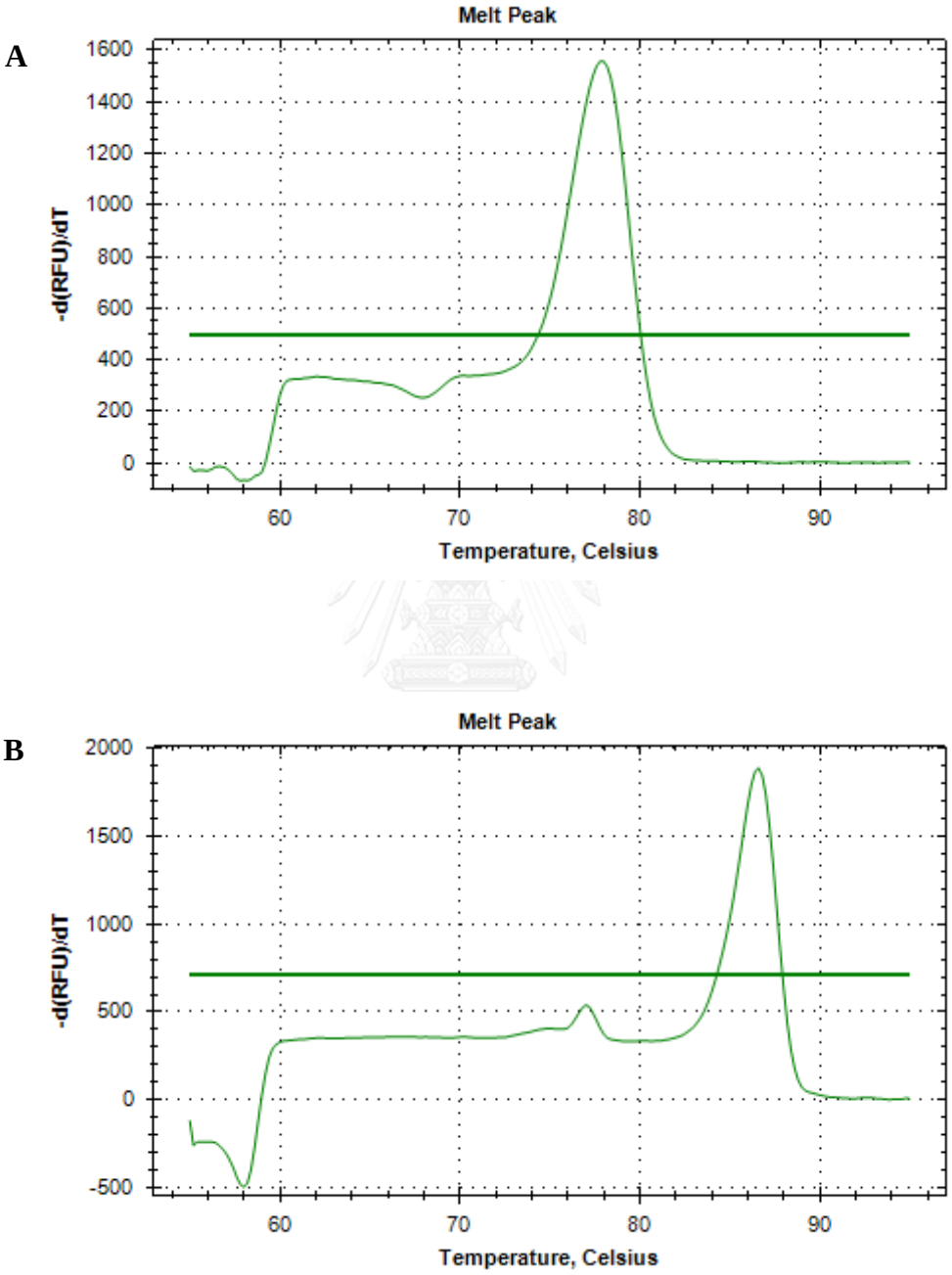
A



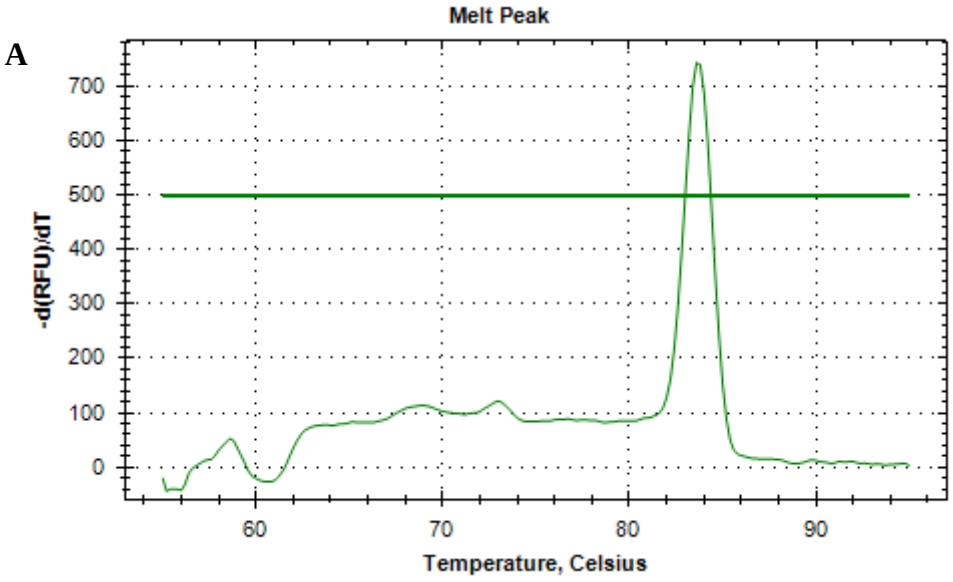
B

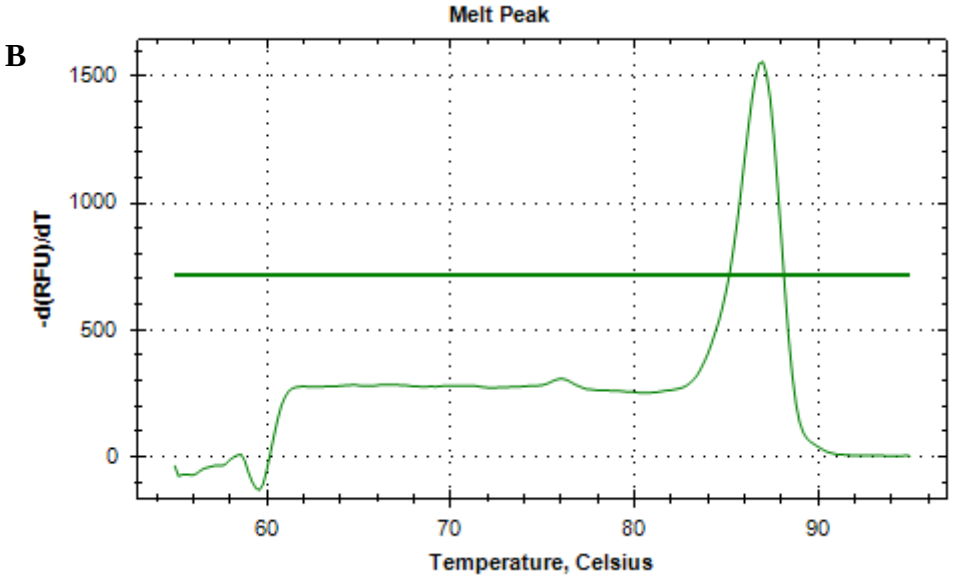


Appendix E eNOS (A) and β-actin (B) melt temperatures of DOX-treated control

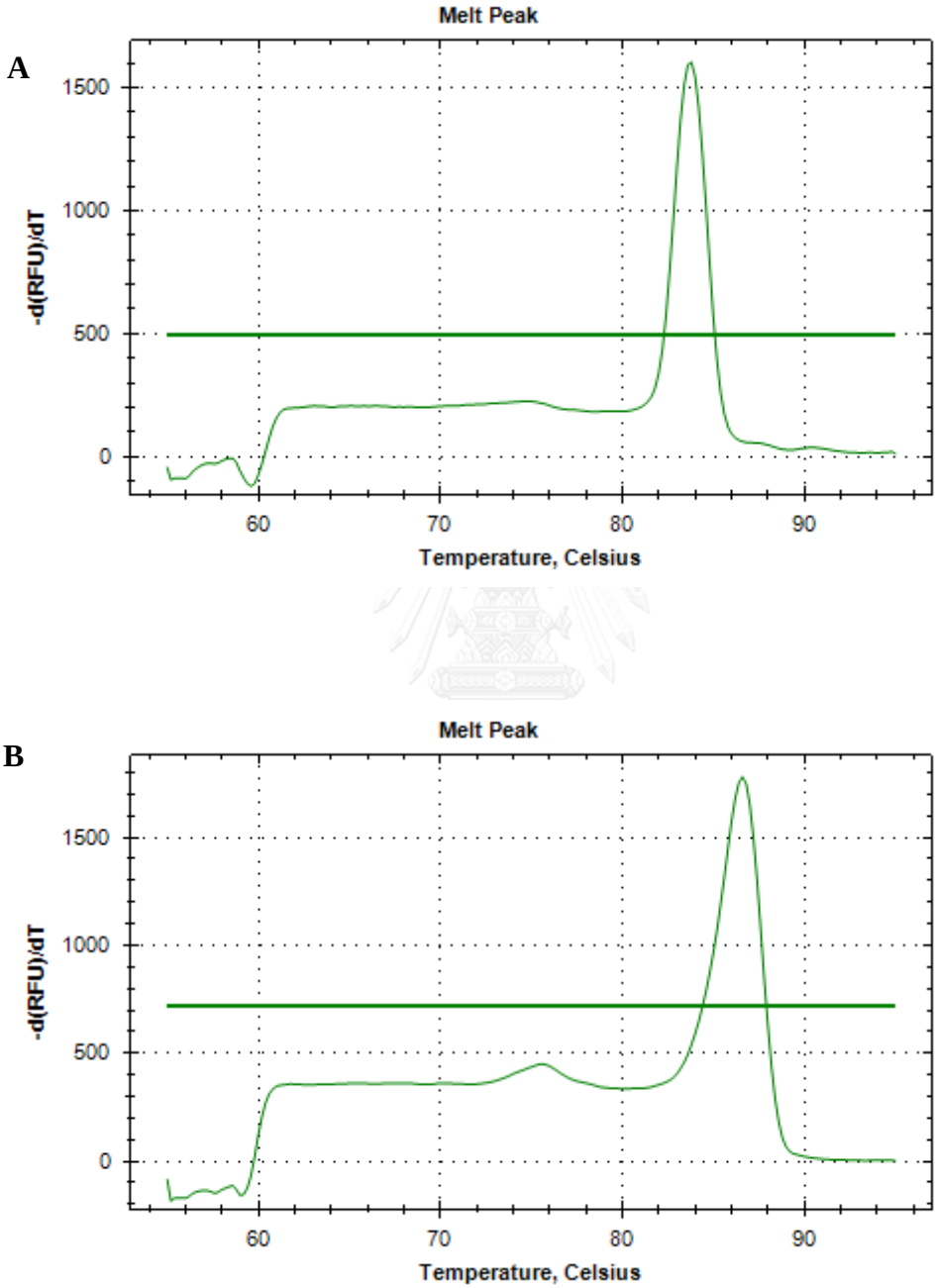


Appendix F eNOS (A) and β-actin (B) melt temperatures of pretreatment with *A. acidum* at 0.1 µg/ml



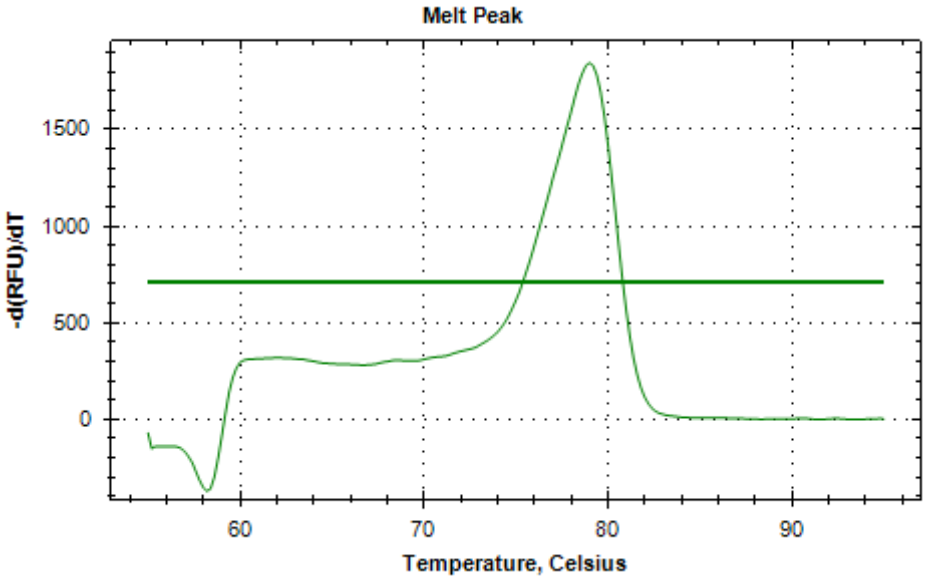


Appendix G eNOS (A) and β-actin (B) melt temperatures of pretreatment with *A. acidum* at 10 µg/ml

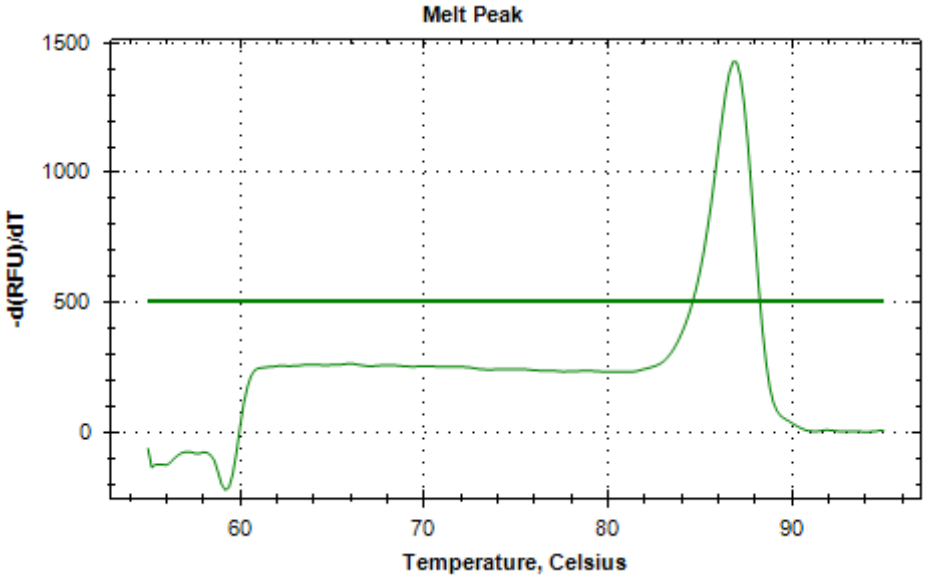


Appendix H eNOS (A) and β-actin (B) melt temperatures of pretreatment with *A. acidum* at 25 µg/ml

A

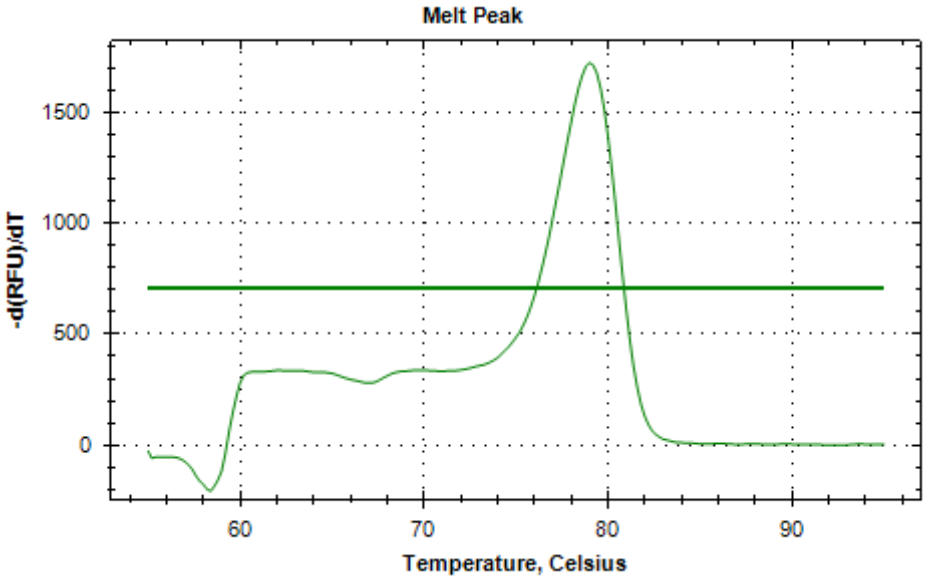


B

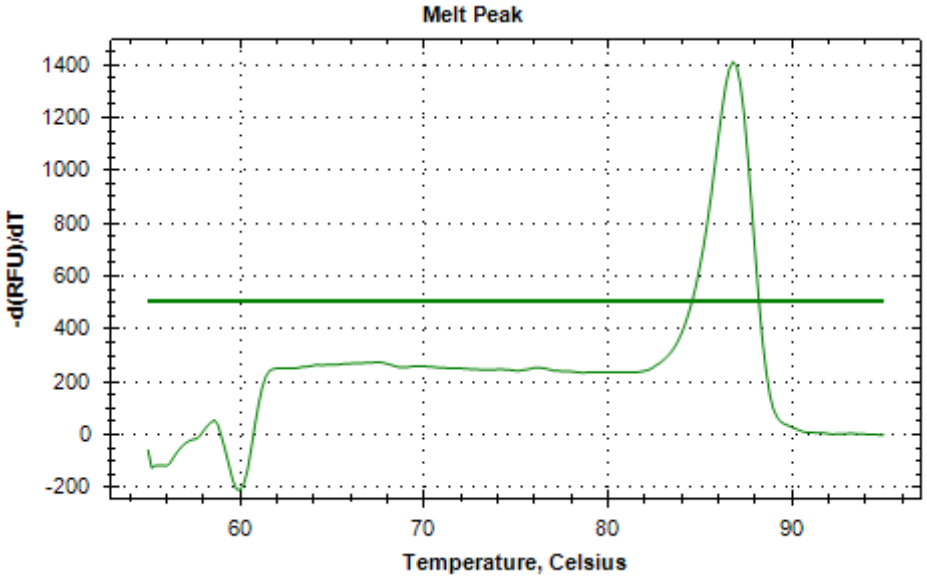


Appendix I eNOS (A) and β-actin (B) melt temperatures of pretreatment with *A. acidum* at 50 µg/ml

A

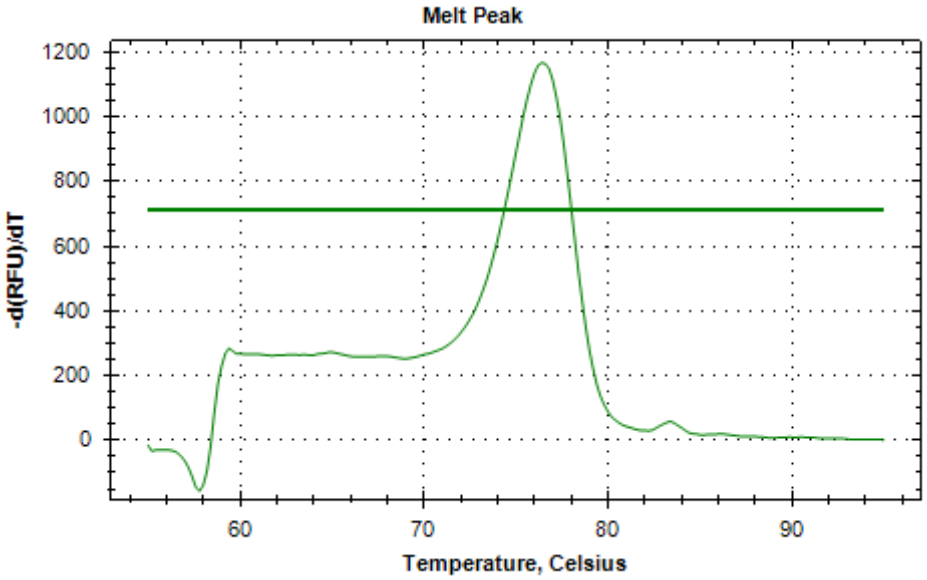


B

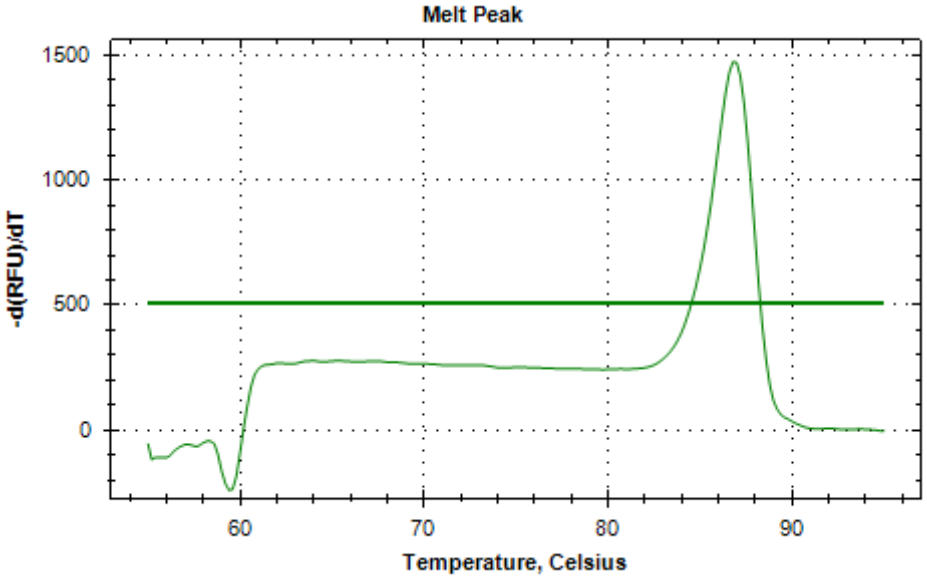


Appendix J eNOS (A) and β-actin (B) melt temperatures of post-treatment with *A. acidum* at 10 µg/ml

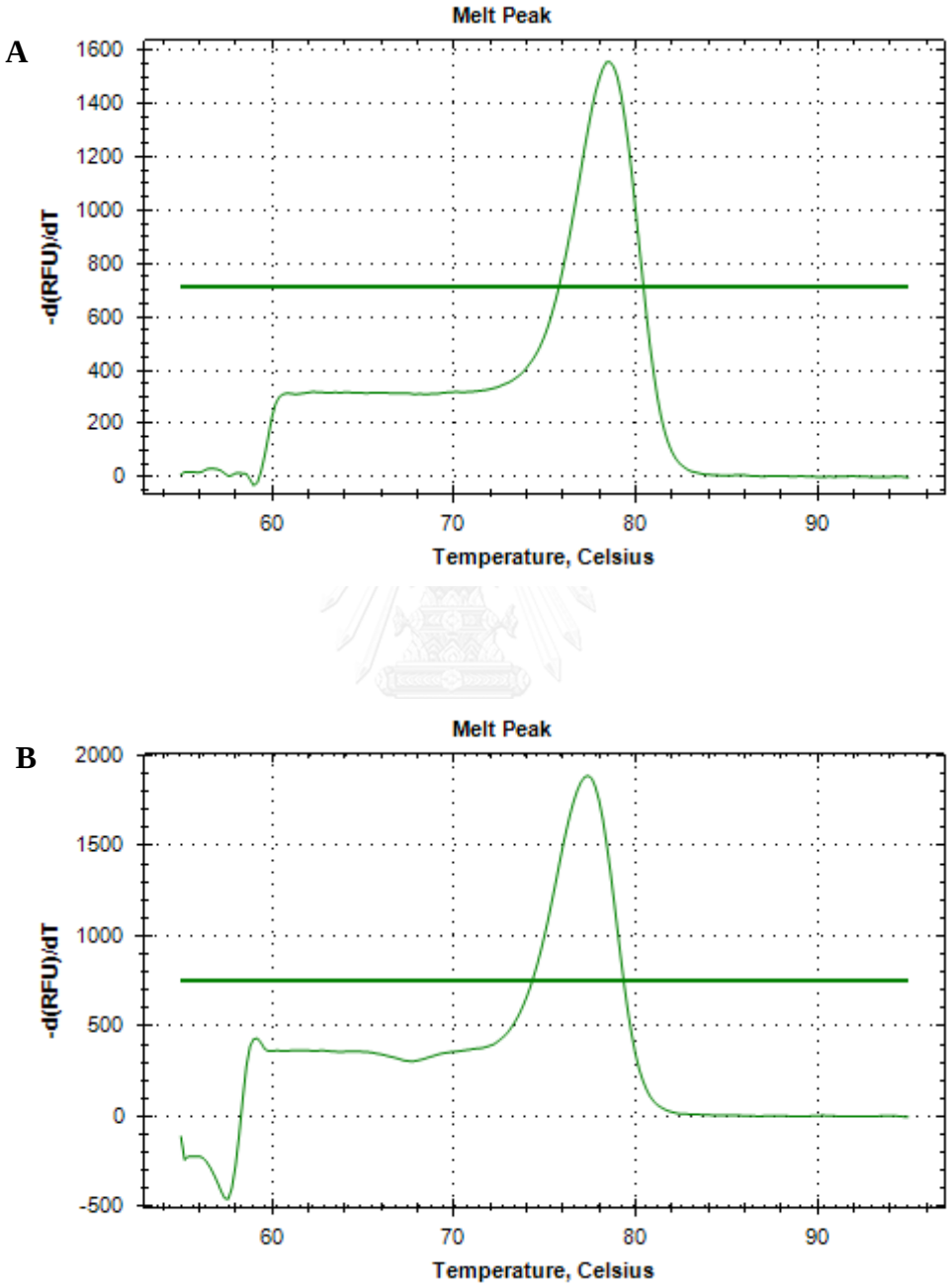
A



B

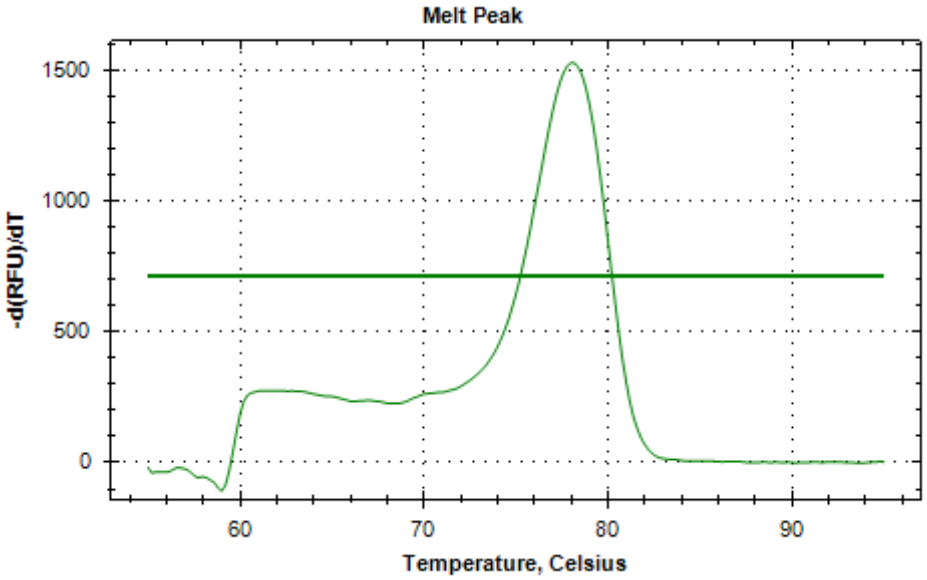


Appendix K eNOS (A) and β-actin (B) melt temperatures of post-treatment with *A. acidum* at 25 µg/ml

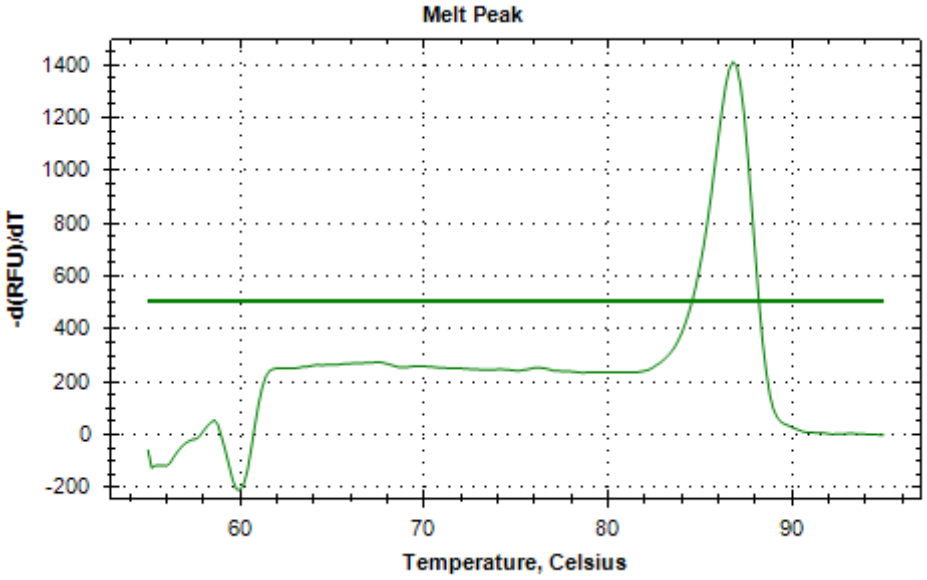


Appendix L eNOS (A) and β-actin (B) melt temperatures of post-treatment with *A. acidum* at 50 µg/ml

A



B



VITA

Mrs. Kakanang Piyarungsri was born on August 12, 1984 in Chiang Mai, Thailand. She finished her high school from the Prince Royal's College, Chiang Mai and graduated with Doctor of Veterinary Medicine (second class honor) from the Faculty of Veterinary Medicine, Chiang Mai University in 2008. She completed her Master's Degree in Veterinary Medicine at Faculty of Veterinary Science, Chulalongkorn University in 2011. Her interested is in feline medicine, antioxidants and kidney disease. Moreover, she would like to promote the researches which will be useful for animals and human.