ANTILITHOGENIC EFFICACY OF AN INNOVATIVE BEVERAGE HYDROZITLA: IN VITRO AND IN VIVO STUDIES

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Medical Sciences
Common Course
Faculty of Medicine
Chulalongkorn University
Academic Year 2018

ประสิทธิภาพการยับยั้งการเกิดนิ่วในปัสสาวะของนวัตกรรมเครื่องดื่มต้านนิ่วไฮโดรซิทลา: การศึกษาในหลอดทดลองและสัตว์ทดลอง

น.ส.นลินทิพย์ หล่อดำรงเกียรติ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาวิทยาศาสตร์การแพทย์ ไม่สังกัดภาควิชา/เทียบเท่า
คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2561

Thesis Title	ANTILITHOGENIC EFFICACY OF AN INNOVATIVE BEVERAGE HYDROZITLA: IN VITRO AND IN VIVO STUDIES
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นลินทิพย์ หล่อดำรงเกียรติ : ประสิทธิภาพการยับยั้งการเกิดนิ่วในปัสสาวะของนวัตกรรมเครื่องดื่มต้านนิ่วไฮโดรซิทลา: การศึกษาในหลอดทดลองและสัตว์ทดลอง. (ANTILITHOGENIC EFFICACY OF AN INNOVATIVE BEVERAGE HYDROZITLA: IN VITRO AND IN VIVO STUDIES) อ.ที่ปรึกษาหลัก : ผศ. ดร.ชาญชัย บุญหล้า, อ.ที่ปรึกษาร่วม : ผศ. ดร.เดภิชา จินดาทิพย์,ผศ. ดร.ณัฐธิดา โชติช่วง

โรคนิ่วปัสสาวะพบได้บ่อยทั่วโลก และเป็นโรคที่เกิดซ้ำได้บ่อยมาก นิ่วที่พบมากที่สุด คือ นิ่วแคลเซียมออกซาเลต (CaOx) สาเหตุหลักของการเกิดนิ่วปัสสาวะ ได้แก่ การดื่มน้ำไม่พอ การมีซิเตรทต่ำในปัสสาวะ และการมีภาวะเครียดจากออกซิเดชัน ในปัจจุบันยาที่ใช้รักษาและป้องกันการเกิดนิ่วซ้ำ คือ ยาโพแทสเซียมซิเตรท จากปัญหาดังกล่าวผู้วิจัยได้คิดค้นเครื่องดื่มที่มีฤทธิ์ต้านการเกิดนิ่วปัสสาวะ ภายใต้ชื่อ ไฮโดรซิทลา (HydroZitLa) มีลักษณะเป็นไซรับเข้มข้น สำหรับผสมน้ำดื่ม 500 มิลลิลิตร การดื่มไฮโดรซิทลาจะทำให้ผู้ป่วยได้รับน้ำเพิ่มขึ้น ส่วนประกอบสำคัญของไฮโดรซิทลาที่มีฤทธิ์ต้านการเกิดนิ่ว คือ ซิเตรท (16 mEq/ซอง) และมีสารต้านอนุมูลอิสระธรรมชาติจากสมุนไพร ผลการศึกษาในหลอดทดลองพบว่าไฮโดรซิทลาสามารถยับยั้งการเกาะกลุ่มของผลึก CaOx และลดภาวะเครียดจากออกซิเดชันในเซลล์ HK-2 ที่กระตุ้นด้วยผลึก CaOx และ H_2O_2 ทำการศึกษาในหนูทดลองเพื่อทดสอบประสิทธิภาพของไฮโดรซิทลาในการยับยั้งการเกิดนิ่วหนูที่ทำให้เกิดนิ่วด้วยสาร ethylene glycol (EG) นำหนู (Wistar rats) 20 ตัว แบ่งแบบสุ่มออกเป็น 4 กลุ่ม ได้แก่ control (n=2), EG (n=6), EG+HydroZitLa (n=6) และ EG+Uralyt-U (n=6) ผลการศึกษาพบว่าการเกาะติดของผลึก CaOx ในไตของหนูกลุ่ม EG+HydroZitLa และกลุ่ม EG+Uralyt-U น้อยกว่าหนูกลุ่ม EG อย่างมีนัยสำคัญ การอักเสบและภาวะเครียดจากออกซิเดชันในไตหนูกลุ่ม EG น้อยกว่ากลุ่ม EG+HydroZitLa และ EG+Uralyt-U อย่างชัดเจน ระดับของซิเตรทในปัสสาวะของหนูกลุ่ม EG+HydroZitLa และ EG+Uralyt-U สูงกว่ากลุ่ม EG สรุปการศึกษานี้แสดงให้เห็นว่า HydroZitLa และยา Uralyt-U สามารถยับยั้งการเกาะติดของผลึก CaOx และลดภาวะเครียดจากออกซิเดชันในไตของหนูที่ทำให้เกิดนิ่วด้วย EG ซึ่งนวัตกรรมเครื่องดื่มไฮโดรซิทลานี้มีศักยภาพที่จะใช้รักษาและป้องกันการเกิดนิ่วปัสสาวะได้ เนื่องจากมีประสิทธิภาพเทียบเท่ากับยาโพแทสเซียมซิเตรท (Uralyt-U) อย่างไรก็ตามจำเป็นต้องศึกษา clinical trials เพื่อตรวจสอบประสิทธิภาพทางคลินิกต่อไป

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2561 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5874095030 : MAJOR MEDICAL SCIENCES

KEYWORD: Nephrolithiasis, Aggregation, Crystallization, HydroZitLa

Nalinthip Lordumrongkiat : ANTILITHOGENIC EFFICACY OF AN INNOVATIVE BEVERAGE HYDROZITLA: IN VITRO AND IN VIVO STUDIES. Advisor: Asst. Prof. Chanchai Boonla, Ph. D. Co-advisor: Asst. Prof. DEPICHA JINDATIP, Ph. D. ,Asst. Prof. NATTIDA CHOTECHUANG, Ph.D.

Urinary stone disease is a common urologic disease worldwide, and it is highly recurrent. Calcium oxalate (CaOx) stone is the most common type of urinary stones. The main causes of urinary stone formation include inadequate water intake, low urinary citrate excretion and increased oxidative stress. At the present day, potassium citrate is a drug of choice for preventing stone formation and recurrence. We developed a new regimen for preventing urinary stone formation, called HydroZitLa. It was in a form of concentrate in pouch. For consumption, water was added up to 500 mL and shake well before drink. Therefore, drinking of HydroZitLa ensured that patients gained more water intake. The active antilithogenic ingredients of HydroZitLa were citrate (16 mEq/pouch) and natural antioxidants from Thai medicinal plants. In vitro study found that HydroZitLa significantly inhibited CaOx crystal aggregation. HydroZitLa had antioxidative function to reduce oxidative stress in HK-2 cells treated with CaOx and H_2O_2. In vivo experiment was performed to investigate the stone inhibitory effect of HydroZitLa in nephrolithic rats was induced by ethylene glycol (EG). Twenty male Wistar rats were randomly divided into four groups; control (n= 2) , EG (n= 6) , EG+HydroZitLa (n=6) and EG+Uralyt-U (n=6). The results showed that CaOx crystal deposits in the kidneys of EG+ HydroZitLa and EG+ Uralyt-U groups were significantly lower than the EG group. Inflammation and oxidative stress in the kidney tissues of EG group were evidently higher than the EG+ HydroZitLa and EG+ Uralyt-U groups. Urinary citrate excretion in the EG+ HydroZitLa and EG+ Uralyt-U groups were increased compared to the EG group. In conclusion, the present study demonstrated that HydroZitLa and Uralyt-U drug exhibited the ability to inhibit CaOx crystal deposition and reduce oxidative stress in the kidneys of EG-

Field of Study:	Medical Sciences	Student's Signature .....
Academic Year:	2018	Advisor's Signature .....
		Co-advisor's Signature .....
		Co-advisor's Signature .....

ACKNOWLEDGEMENTS

Firstly, I would like to express my special thanks of gratitude to my advisor Asst. Prof. Chanchai Boonla for merciful get me into membership in CB Lab 806, continual supports which enable me to achieve my master degree study and always giving me encouragement.

I also would like to thank my thesis committees Prof. Vilai Chentanez, Assoc Prof. Wilai Anomasiri, M.D., Asst. Prof. Asada Leelahavanichkul, Asst. Prof. Dapicha Jindathip, Asst. Prof. Nattida Chotechuang and Asst. Prof. Supranee kongkham for excellent suggestion about knowledge and educate more laboratory techniques and facilitate all laboratory instruments.

I also would like to my special thank Assoc Prof. Chintana Chirathawon Asst. Prof. Vivornpun Sanprasert and Dr. Arkhom Sai-ngam for support giving me encouragement all 4 years.

I also would like to special thanks Mr. Chakriwong Ma-on for all kidney tissue section, Mr. Wasin Manuprasert for excellent suggestion about knowledge and skills in histopathology. Mr. Tewarit Soongrung, Miss. Nungruthai Suntronwong and Miss Apinya suksompak waiting for help me and always support me.

I also would like to 90th Anniversary of the Chulalongkorn University Fund (RatChadaphiseksomphot endowment fund) and Research Gap fund for the financial support.

Finally, massive thanks to my teamwork family and lovely friends in CB lab including everyone always support to me for 4 years.

Nalinthip Lordumrongkiat

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CHAPTER I

INTRODUCTION

1. Background and Rationale

Urinary stone disease or urolithiasis is a common urologic disease worldwide, particularly in the tropical countries including Thailand. Most of urinary stones are found in kidneys, so called kidney stone or nephrolithiasis. In Thailand, urinary stone disease is endemic in the northeastern of Thailand with the prevalence up to 17% (1). The disease is highly recurrent with recurrence rate of 50-80%(2). Recurrent stone formation causes increases in intrarenal inflammation and renal fibrosis leading to, onset of chronic kidney disease (3). Urinary stone formation is primarily driven by supersaturation of lithogenic substances such as calcium oxalate, phosphate and uric acid leading to formatiom of lithogenic crystal, for instance, calcium oxalate (CaOx) crystals. There are several types of urinary stones classified by major mineral composition of stones. (CaOx) is the most common stone type found over 60% of all stones in all countries, including Thailand. (4, 5). The major risk factor for CaOx stone is the condition with low level of urinary citrate or hypocitraturia (6). According to this fact, the medical treatment of choice for preventing urinary stone recurrences is potassium citrate drug, e.g. Uralyt-U, Xitrin and Rowatinex. However, these medications have side effects such as diarrhea and vomiting symptoms, and also, the taste is unpleasant that lead to low compliance in the long-term treatment. Based on the guideline, the duration of medical treatment for effectively preventing stone recurrence is last for 6 months. In addition to hypocitraturia, low water intake and increased oxidative stress are important etiological factors for urinary stone development. Several lines of evidences demonstrate that insufficient water intake per day, low level of citrate in urine, high dietary oxalate intake and high oxidative stress (as well as low antioxidant level) are significant causative factors for urinary stone disease (7). In order to effectively prevent recurrent stone formation, these causative factors have to be addressed or corrected.

Citrate is a highly negatively charged molecule that can bind with many cations including calcium. It has been known as a very potent stone inhibitor, as it efficiently inhibits the formation of CaOx crystals by competitively binding to calcium to produce a water soluble salt calcium citrate. Our previous studies showed that nephrolithiasis patients had increased oxidative stress and CaOx crystals directly induced the production of reactive oxygen species (ROS) in renal tubular cells that lead to oxidative damage and injury (8). Also, many studies report that oxalate is toxic to renal epithelial cells through an induction of oxidative stress (9). Interestingly, treatment with antioxidants has been shown to inhibit kidney stone formation in experimental nephrolithic rat models(10).

Alpha-lipoic acid (ALA) is a well-known antioxidant that is proved to reduce oxidative stress and prevent cell injury and apoptosis (11). Also, it also has been shown to decrease the level of blood sugar in the diabetes patients (12). A recent study showed that ALA inhibited cystine stone formation in the mouse model of cystinuria (13) emphasizing ALA as an antioxidant remedy. Beside supplementary antioxidants, natural or dietary antioxidants have gained attention for preventing disease development. Many herbal remedies have been documented and used in India and China for treating urinary (14, 15). *Musa sapientum* L. (banana stem, Kluai Nam-Wa) is one of them that shows a great clinical potential for treating urinary stone. We are interested in this herbal plant because banana is a one of the major agricultural products in Thailand, and it is easily accessible. Banana stem crude extract had been shown to have diuretic effect and be able to decrease urinary oxalate in hyperoxaluric rats(16)

We, therefore, formulated a new regimen for preventing urinary stone formation, called HydroZitLa that contains major ingredients of citric acid, ALA and banana stem crude extract. The HydroZitLa solution was naturally colored by adding *Clitoria ternatea* L. or Blue pea (Un-Chan) and *Caesalpinia sappan* L. (Fang). It is prepared in a form of concentrate in pouch (55mL). For consumption (drink), pour concentration HydroZitLa into the bottle, add drinking water (cold is preferred) to get 500 mL, shake it and it well be ready to drink. As one HydroZitLa a pouch contains 16 mEq of citrate. Our recommendation to use in the patients is to consume twice a

day, one is the morning and the other is in the evening after meals. In this study, we aimed to test therapeutic capability of our innovative beverage HydroZitla to inhibit the formation of urinary stones both in *vitro* and in *vivo* models. An *in vitro* cell culture model was performed in HK-2 cell line. An *in vivo* nephrolithic model was conducted in Wistar rats induced by ethylene glycol (EG).

Research question

1. Does HydroZitLa have stone inhibitory action to inhibit CaOx aggregation?

2. Does HydroZitLa have antioxidative function to reduce oxidative stress in HK-2 cells exposed to H_2O_2 and CaOx crystal?

3. Can HydroZitLa inhibit CaOx deposit and reduce oxidative stress in the in the kidneys of the EG-induced nephrolithic rat?

Research Hypothesis

We hypothesized that HydroZitLa was capable of inhibiting CaOx aggregation in vitro, reducing oxidative stress in ROS-treated HK-2 cells and diminishing CaOx crystal deposits and oxidative damage in the experimental nephrolithic rats.

Research Objectives

1. To investigate the inhibitory effect of HydroZitLa on CaOx aggregation in dose-dependent manner.
2. To investigate the toxic effect of HydroZitLa in renal tubular HK-2 cells.
3. To measure total antioxidant capacity (TAC) in HydroZitLa compared to potassium citrate drug (Uralyt-U).
4. To investigate the antioxidant activity of HydroZitLa in HK-2 cells exposed to H2O2 and Calcium oxalate monohydrate (COM).

5. To examine efficacy of HydroZitLa to inhibit CaOx crystal deposit and oxidative damage in nephrolithitic rat induced by ethylene glycol (EG) compared with the treatment with Uralyt-U.
6. To investigate the effect of HydroZitLa on urinary citrate excretion in EG-induced nephrolithitic rats compared with Uralyt-U.

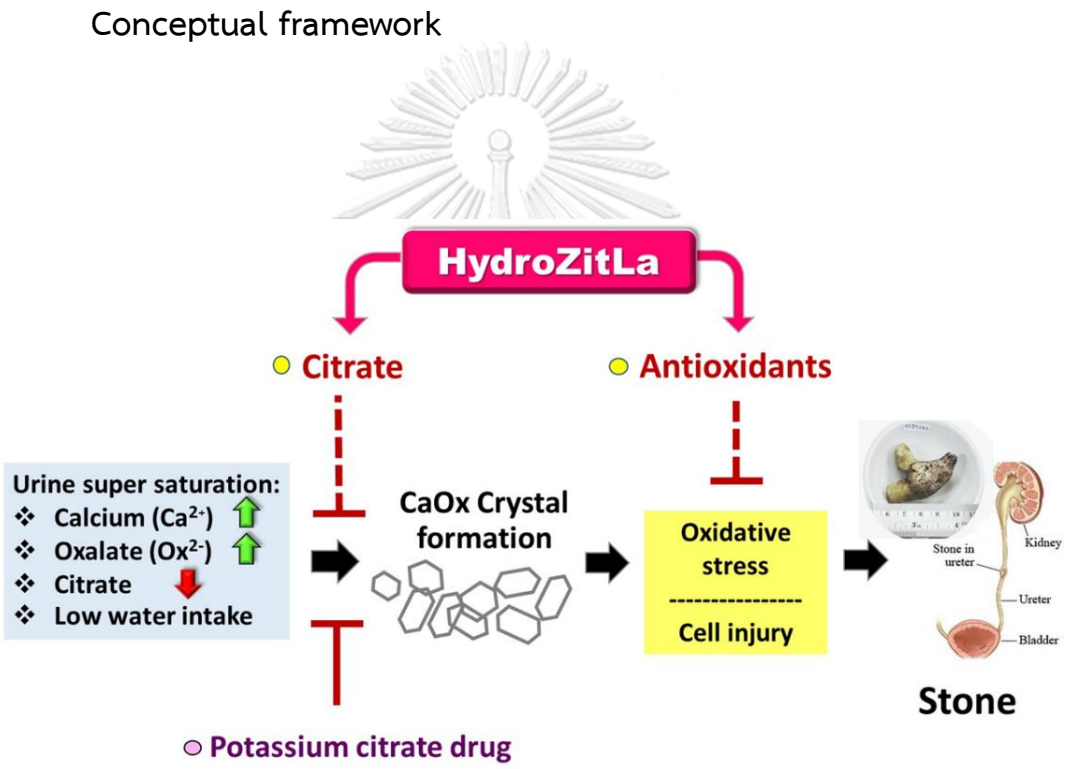


Figure 1 Conceptual framework of the present study.

CHAPTER II

Review literatures

2.1. Epidemiology and pathophysiological mechanisms of kidney stone

Nephrolithiasis or kidney stone or renal stone is a common disease worldwide and it is still an importance urologic problem (1). The prevalence of stone formation generally varies among different parts of the world. Its prevalence is greatly high in the tropical countries, for instances, 20% in Saudi Arabia, 13% in North America, 5-9% in Europe and 1-5% in Asia (17). Moreover, stone disease is found around 10 -12 % of population in the industrial areas (18, 19). In Thailand, the high prevalence of kidney stone disease is reported in the northeastern region, approximately 10-16% (20). The disease mainly affects male, and peak age risk is between 40 and 60 years old. However, incidence of renal stone in female has been progressively increasing (6).

2.2. Urinary system

The urinary systems are consist of kidney, ureter, urinary bladder and urethra (Figure 1). The main function of the system is urine formation and excretion of urine to the outside of the body (21)

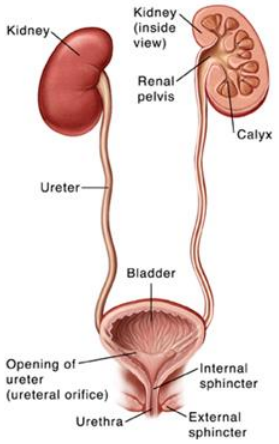


Figure 2 Urinary system

The most important part of the urinary system is kidneys (Figure 2). There are two kidneys in the human body at the retroperitoneal space cavity on both sides of vertebrae.

Also, their shape is similar to beans with brown color. The external layer of kidney is called cortex and the internal layer is called medulla. A kidney is comprising of a large number of tubules that are called nephrons which filter the blood and form urine.

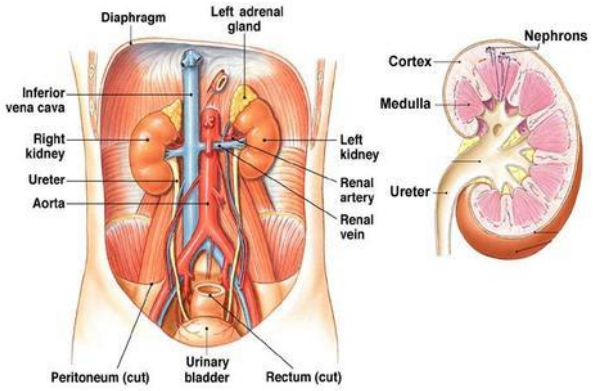


Figure 3 Structure of kidneys

Important function of the kidney

- Removing waste
- Absorption electrolyte, protein and glucose
- Water and acid base balance
- Synthesis including erythropoietin, renin and vitamin D

Nephron is a functional unit of kidneys (Figure 3). The first part of nephron is glomerulus. Next is a bowman's capsule, proximal tubule for reabsorption of substances such as Na^+, Cl^-, K^-, HCO_3^- glucose, albumin, amino acid, uric acid,

calcium, magnesium and phosphate. Loop of Henle (reducing volume of urine by absorbing and secreting NaCl) and the last part is collecting tubules that collect urine and generate the last concentration of urine before excreting out of the body.

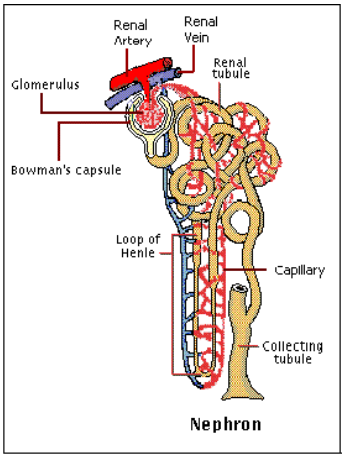


Figure 4 Nephron system

Formation of urine

- Plasma filtrate
- Selective reabsorption
- Secretion

Nephrolithiasis or kidney stone is caused by abnormality of urinary concentrations of substances. The stones were mostly located in the upper urinary system (82.9%). Nowadays, the exact cause and mechanism of stone formation are still largely unknown. The significant challenge if stone disease is that it is highly recurrent. According to data from Khon Kaen hospital the recurrence rate of kidney stones was founded at 39% within 2 years after surgery. Also, in Chulalongkorn hospital 25% of recurrent rate within 3 years after the stone treatment had been documented (20). It should be noted that kidney stone patients in Thailand had a larger stone size than those patients in the western countries (20).Therefore, Thai stone patients have higher degree of renal impairment and have more chance to develop chronic renal failure. The major common risk factor for stone disease is

insufficiency of water intake per day that leads to increased urine supersaturation, and hence lithogenic crystal formation and stone development.

The pathophysiological mechanism of calcium kidney stone formation is diverse and complex comprising of low urine volume, hypocitraturia, hyperoxaluria, hypercalciuria, hyperuricosuria and abnormal in urine pH (22). Low urinary citrate excretion is a very significant risk factor for stone development and recurrence. Low consumption of dietary citrate (citrus fruit) and high intake of oxalate-rich diets such as chocolate, spinach, brewed tea and cola drinks are major dietary causes found in stone patients (6).

2.3. Types of kidney stones

Kidney stones are originated from lithogenic crystals formed in the supersaturated urine. Types of urinary stones are classified according to major crystals or mineral component in the stones. Calcium stones are the most common stone type found up to 80%. The major one is calcium oxalate (CaOx). Calcium oxalate monohydrate (COM) is important CaOx crystal type that forms CaOx stones (Figure 4.) Uric acid stone and calcium phosphate are found at 10-20% and 14.7%, respectively (23). Uric acid stone is a major non-calcium stones. It is mainly caused by acidification of urine (Figure 5). The stone type that is associated with bacterial infection is struvite stone (Figure 6). Cystine stone is a monogenic stone that is primarily caused by genetic mutation and usually occurred in children (Figure 7) (24).

Figure 5 Calcium oxalate monohydrate stone (COM)

Figure 6 Uric acid stone

Figure 7 Struvite stone

Figure 8 Cystine stone

2.4. Mechanism of kidney stone formation

Urinary saturation is a driving force of urinary stone formation. High level of lithogenic substances such as calcium and oxalate triggers CaOx crystal formation. These crystals grow and aggregate and adhere to the renal tubular cells. Interaction of CaOx crystals to renal tubular cells leads to cell injury through oxidative stress and inflammatory reaction. The renal tubular cell injury area is served as a site for crystal adhesion and retention. Nidus or stone core is formed. It grows bigger and subsequently become a stone.

2.5. Oxidative stress and stone formation

Oxidative stress is an imbalance of oxidants and antioxidants leading to oxidative damage. The main oxidant in the body is reactive oxygen species (ROS). Evidence shows that lithogenic CaOx crystals induce ROS generation and cause oxidative stress in renal tubular HK-2 cells (25). Oxidative damage and cell injury such as lipid peroxidation is increased in the HK-2 cells exposed to COM or oxalate (9). Apoptosis is also increased in cells treated with CaOx crystals and oxalate (26). Therefore, oxidative stress and apoptosis of renal tubular cells are considered to involve in the process of urinary stone formation.

2.6. Citrate

Citrate or its acid form citric acid is an important molecule for treatment of urinary stone. Citrate have a molecular weight of 192 Da. It consists of three carboxylic groups and it is frequently called or tricarboxylic acid. It has 3 different pK_a value as shown in (Figure 8). Thus, at the body fluids pH of 7.4, it is completely dissociated (protonated) and gives rise three negative charge (Cit^{3-}). It can bind to many positively charged ions such as Ca^{2+}, Na^{+} and Mg^{2+}. One important function of citrate in urine is it inhibits CaOx crystal formation and prevents stone formation as well as stone relapse. The current drug of choice and most commonly used for urinary stone disease is potassium citrate (5). The drug can increase excretion of urinary citrate and correct hypocitraturic condition. However, adverse effect of potassium citrate drug has been reported, mostly gastrointestinal upset. Additionally, the taste of the drug is rather unacceptable that leads lo low compliance. Therefore, new regimen with a better taste and has no side effect is needed to be developed. Risk factor CaOx stone is low urinary excretion of citrate or hypocitraturia calcium will combine with oxalate to become CaOx lead to kidney stone. Citrate will catch with calcium to replace oxalate in urinary combine with calcium to dissolve in water and inhibit CaOx crystal deposit.

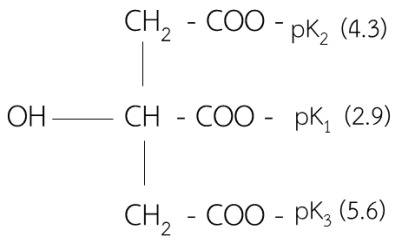


Figure 9 Citrate structure formula

2.7. Antioxidants and stone formation

Antioxidant is a molecule that inhibit oxidant activity of other molecules. There are several methods to quantitatively measure the antioxidant activity including total activity capacity (TAC) measurement. Plants are major sources of natural antioxidants. One type of phytochemicals that have very high antioxidant property is phenolic compounds (Figure 10). Flavonoids are well-known phenolic compounds found in medicinal herbs and have potential to be included in the remedy for treatment of the diseases.

Based on our research findings, we consider stone disease as an oxidative stress-medicated disease. Therefore, intervention with antioxidants may be beneficial for preventing stone formation. Previous study in animal model of cysteine stone shown that alpha-lipoic acid (ALA) can inhibit cysteine stone formation and increase the solubility of urinary cystine (27). Basically, ALA is a natural compound known as 1,2-dithiolane-3-pentanoic acid or thioctic acid (Figure 9).

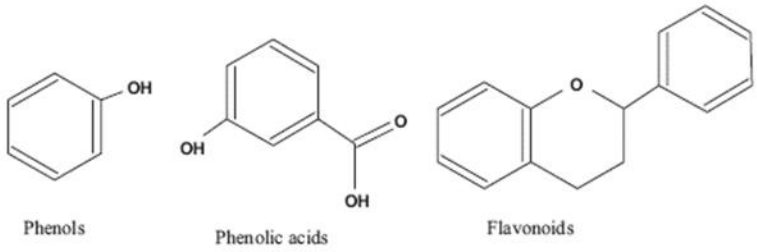


Figure 10 Structure phenolic compounds

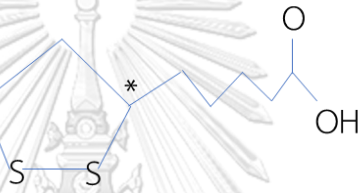


Figure 11 Chemical structure Alpha lipoic acid

2.8. Herbal alternative for stone disease

There are several herbal remedies in India and China that are documented in literature for treatment of urinary stones (Table 1). In this study, we are interested in two kinds of medicinal plants. First is *Musa sapientum* L. (banana stem, Kluai nam-wa), as it is a one of the major agriculture products in Thailand. Previous study from India treated in the rat with aqueous banana stem extract, urinary oxalate excretion was significantly reduced when compared with control, decreasing urinary oxalate, glycolic and glyoxylic acid and phosphorus excretion in the hyperoxaluric rats (16) (Figure 12). Second is *Caesalpinia sappan* (CS) heartwood (FANG) which is a plant in the Leguminosae family, commonly known as Brazil or Sappan wood. CS is distributed in Southeast Asia and its dried heartwood is traditionally used in Indian Ayurveda and Chinese folk medicine (Figure 11). In Thailand, it is mostly used as coloring agent in beverage, food, garment and cosmetics. Chemical composition

investigation of sappan wood various structural types of phenolic components include coumarin, chalcones, xanthone, flavones, homoisoflavonoids, etc (Figure 12). CS heartwood is also used to reduce pain and swell caused by external injury and improvement of complexion(28). Previous study demonstrated that a major compound in *Caesalpinia sappan* is Brazillin (Figure 13.) and it had the a potential functions of antioxidant, antibacterial, anti-inflammatory and anti-photoaging (29).

Table 1 Herbal medicine for treatment kidney stones in rats.

No.	Botanical name	Useful	References
1	*Melia azedarach*	Decrease urinary CaOx and Phosphate	(30)
2	*Bergenia ligulate*	Inhibit CaP, CaOx Diuretic, Lithotriptic	(30)
3	*Mallotus phillippensis*	Decrease urinary crystal	(31)
4	*Chenopodium Album Linn*	Decrease the deposit oxalate crystal in kidney	(32)
5	*Biophytum sensitirum*	Increase diuretic decrease concentration of urinary stone	(10)
6	*Bryophyllum pinnatum* (Lam.)	Reduce urine oxalate level significant	(33)
7	*Costus igneus*	Significantly lower of CaOx	(30)
8	*Cerasus avium stem*	Reduce the number of calcium oxalate deposits.	(34)
9	*Asparagus race mosus*	Increase Diuretic	(35)
10	*Achillea millefolium L.*	significant reduction in urinary oxalate concentration	(36)
11	*Musa sapientum* L.	reduces urinary oxalate, glycolic and glyoxylic acid and phosphorus excretion in the hyperoxaluric rats	(16)
12	*Caesalpinia sappan*	high antioxidant, antibacterial, anti-inflammatory and anti-photoaging	(29)

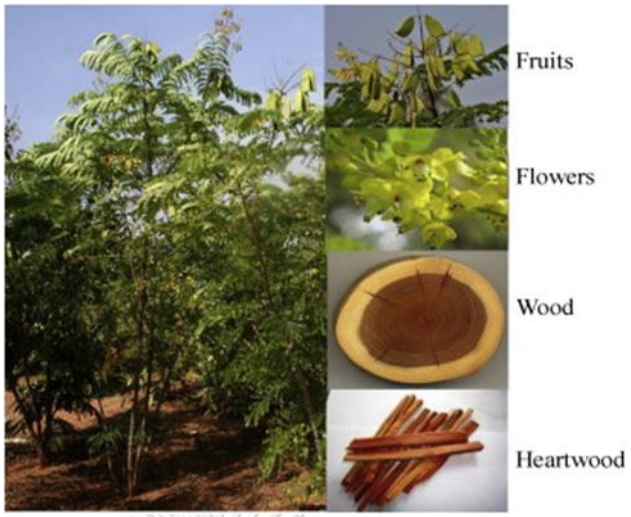
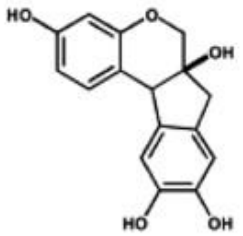


Figure 12 *Caesalpinia sappan*

Figure 13 *Musa sapientum* L. (banana stem, Kluai Nam-wa)



Brazilin

Figure 14 Chemical structure of Brazilin

2.9 Ethylene glycol induced CaOx crystal deposition

Ethylene glycol (EG) is well-known compound to generate the model of nephrocalcinosis (37). It induces CaOx crystal deposit in males Wistar rats and widely used as experimental model of nephrolithiasis. EG is converted to glycoaldehyde using alcohol dehydrogenase, and glycoaldehyde is further converted to glycolic acid using aldehyde dehydrogenase (Figure 14). Then glycolic acid is changed to glycoxylic acid using glycolic acid oxidase. Finally, oxalic acid is produced from the glycolic acid substrate. Oxalic acid binds to calcium and make CaOx crystals that can further cause cell injury (37). In this study, we will use 1 (% v/v) EG supplemented in drinking water to induce CaOx deposit in male Wistar rats (38).

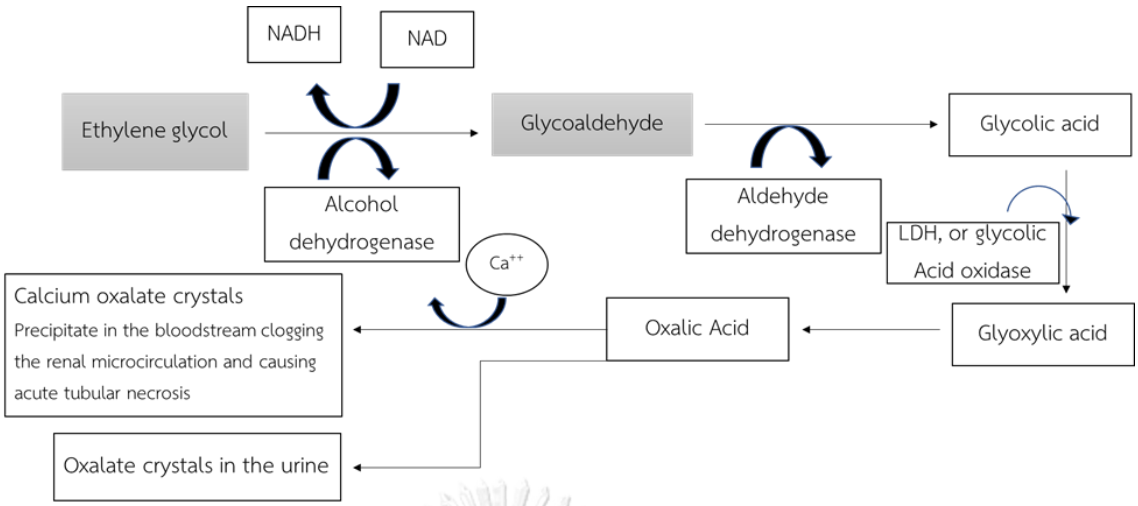


Figure 15 Pathway for metabolism of ethylene glycol to cause increased urinary oxalate excretion and CaOx crystal deposit in the kidneys modified from

Chapter III

MATERIAL AND METHOD

3.1 HydroZitLa formulation and ingredients

- Citric acid 1.024g
- Banana stem crude water extract 50g
- Butterfly pea flower 0.15g
- Caesalpinia sappan hearthwood powder 0.05g
- Stevia Artificial Sweeteners 0.03g
- Sucralose 0.03g
- ALA powder 0.025g

Banana stem crude water extract was derived from a banana tree. Banana tree was cut down, and the tree banana core was collected and chop thoroughly before boiling with water. After that, the water extract was filtered. To make HydroZitLa, the 50 g of the water extract was used to mix the other ingredients as shown above.

3.2 Aggregation assay

Seed COM crystals were prepared by mixing an equal volume of 100 mM sodium oxalate (NaOx) with 100 mM calcium chloride ($CaCl_2$). The mixture was incubated at 60°C for 1 h and then 37°C overnight. The formed crystals were filtered through 0.22 µm membrane and seed COM crystals were collected. The seed crystals were further dried at 37°C overnight. In this study, seed COM crystals at working concentration of 50 mg/mL. In 0.05 M Tris buffer was used (before use it must be mixed well). The working COM suspension (2 mL) was placed into the test tubes. Distilled water (control), 1 mg/µL bovine serum albumin and HydroZitLa (200 µl each) were added into each test tube and mixed well. Absorbance at 620 nm is measured the beginning (AT_0) and after incubated at 37°C for 10 min (AT_{10}). Aggregation coefficient (AC) of each condition is calculated as followed:

$$AC = \left(\frac{(AT_0 - AT_1)}{10} \right) x1000$$

3.3 Total antioxidant capacity (TAC) measurement by ABTS assay and DPPH method

Total phenolic content in HydroZitLa was determined with the Folin-Ciocalteu reagent according to the method described by Kim and coworker. Briefly, Folin-Ciocalteu reagent (200µL) was placed onto 96-wells plate. Distilled deionized water (blank), varied concentrations of gallic acid (as standards) and HydroZitLa (20 µL) are added, mixed well and incubated for 5 min. Na_2CO_3 7% (W/V) solution (30µL) was further added and incubated at room temperature for 2 h in dark. Absorbance at 750 nm was measured(**39**).

Total flavonoid content in HydroZitLa was determined by spectrophotometric method (**39**). Briefly, HydroZitLa or standard (catechin) (500 µL) was added to 3.2 mL of distilled water and mixed well (time 0) Sodium nitrite ($NaNO_2$, 5% (w/v)) (150 µL) was added, mixed well and incubated for 5 min. Aluminum chloride (10% (w/v) $AlCl_3$) (150 µL) was added and mixed well. After 6 min incubation, 1 mL of sodium hydroxide (NaOH, 1 M) is added and mixed. Absorbance at 510 nm was measured. The concentration of total phenolic and total flavonoid contents were calculated against the standard curves.

3.3.1 ABTS assay

ABTS assay was used for determination of TAC, and was performed according to the studies by Van den Berg et al. (**39**) and Kim *et al*. (**40**). In brieft, 2.5 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) or ABTS was mixed with 1 mM 2,2-azobis(2-amidinopropane) dihydrochloride in 10 mM phosphate buffer saline solution (PBS) pH 7.4, and heated at 68˚C in water bath for 40 min. The obtained blue green $ABTS^{+}$ solution was cooled down and then diluted with PBS buffre untill the absorbance at 734 nm was equal to 0.650 ± 0.020. For the reaction, HydroZitLa or urine or serum samples (20 µL) were added to 980 µL of ABTS solution and incubate at 37˚C for 10 min in water bath. Absorbance at 734 nm was measured. Vitamin C at various concentrations were used as standards to generate the standard curve. TAC was calculated reported as vitamin C equivalent antioxidant capacity (VCEAC).

$$\% \text{ antioxidant activity (\%AA)} = \frac{(OD_{Blank} - OD_{sample}) \times 100}{OD_{Blank}}$$

3.3.2 DPPH assay

DPPH was used for determination of TAC, and was performed according to the studies by Brand-Williams et al. DPPH was added into 96–well plate 300 µl for measure the absorbance at 517 nm was equal to 0.650 ± 0.020 dilutute with 80% methanol. After that, DPPH were added 295µL into 96-well plate and add HydroZitLa or urine 5 µL mix well, incubated at room temperature in the dark for 30 min.

3.4 MTT assay

To investigate the toxic effect of HydroZitLa in renal tubular HK-2 cells, MTT assay was carried out. The MTT assay is a colorimetric assay that relies on the enzymatic reduction of a yellow tetrazolium salt, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), which forms a purple formazan crystal in metabolically active viable cells (Figure 16.). The formazan is then solubilized and

measured its signal at 570 nm. The obtained signal is proportional to the cell number and viability.

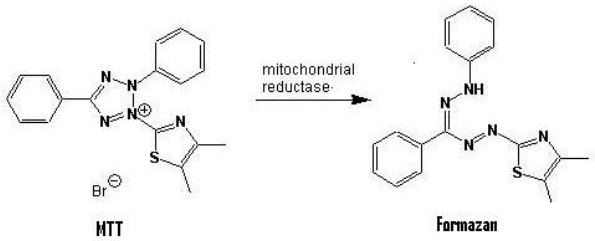


Figure 16 Basic principle of MTT assay

Renal tubular cells used in the study is HK-2 cell line which was purchased from ATCC. HK-2 cells (200 cells/well) were seeded and grew in 96-wells plate overnight at 37°C, 5% CO_2 to achieve 70-80% cell confluence. Cells were treated with various concentration of HydroZitLa (0 as control, 2.5, 5, 10, 50 and 100%) and then incubated at 37°C, 5% CO_2 for 24 hour. After discarding media, MTT solution (100 µL/well) was added and incubated for 1 hour. Finally, dimethyl sulfoxide was added and the absorbance at 570 nm was measured. Cell viability (%) was calculated according to the equation shown below.

$$\% \text{ Cell viability} = \frac{(OD_{test} \times 100)}{OD_{control}}$$

3.5 DCFH-DA assay

To investigate the antioxidative effect of HydroZitLa in H_2O_2-treated HK-2 cells dichloro-dihydrofluorescein diacetate (DCFH-DA) assay was carried out. In principle, ROS convert DCFH-DA into dichloro-dihydrofluorescin (DCF) that emits fluorescence (Figure 17). HK-2 cells were seeded into 96-wells plate and maintained at 37°C, 5%

CO_2 for 24 h. 0.1 mM DCFH-DA solution was added (100 µL/wells) and incubated at 37°C, 5% CO_2 for 30 min. After removing media and washing with PBS, cells were treated with H_2O_2 (10 µM), H_2O_2 (10 µM) + HydroZitLa (10%) with or without HydroZitLa. Fluorescent intensity (excitation at 485 nm and emission at 535 nm) was measured at the beginning (time 0) and at 60 min. Arbitrary fluorescent unit (AFU) a reflective of ROS generation was calculated as shown below. High AFU indicated high intracellular ROS generation.

$$\text{Arbitrary fluorescent unit (AFU)} = \frac{\text{Fluorescent intensity } (T_{60})}{\text{Fluorescent intensity } (T_0)}$$

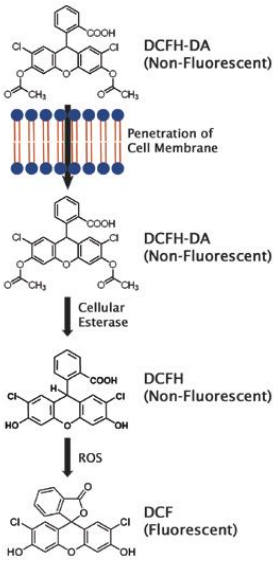


Figure 17 A principle of DCFH-DA assay

3.6 Protein carbonyl determination in cell lysate

Treated cells from each condition were washed with PBS twice, and then RIPA buffer (containing protease inhibitor) was added to disrupt cells (on ice). Cells were crushed using cell scraper, and incubated on ice for 30 min with vortex every 10 min. To collect cell lysate, the lysed cells were centrifuged at 10,000 g at 4°C for 15 min. The supernatant was collected and kept at -80°C until testing for protein concentration and protein carbonyl content.

Protein carbonyl content is used as indicator of protein oxidation occurred under oxidative stress condition. Briefly, dinitrophenylhydrazine (DNPH) solution and 2 N HCl (for reagent blank) were separately placed into test tubes. Protein sample was added into each tube and incubated for 1 h in the dark. After adding 300 µL of trichloroacetic acid (TCA) 20% (w/v), the mixture was vortexed, incubated at 4°C for 10 min, centrifuged and discard supernatant. The pellet was collected and washed with ethanol: ethyl acetate (1:1 v/v). The protein pellet was further re-dissolved with 300µl of 6 M GdmCI and incubated at 60°C for 30 min. Absorption at 375 nm was measured using the reagent blank (of the same protein sample) to set zero. The basic principle of protein carbonylation assay by DNPH reaction is shown in (Figure.18).

Protein concentration of each cell lysate sample was measured by Bradford assay. Content of protein carbonyl in each sample was normalized by total protein concentration.

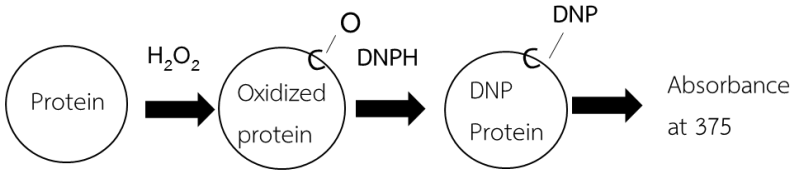


Figure 18 Protein carbonyl

3.7 Animals experiments

Twenty male Wistar rats (5 weeks old, 180-300 g) purchased from the National Laboratory Animal center, Salaya Campus Mahidol University, Nakhonpathom, Thailand, were used in the study. The experimental procedures were conducted in accordance with the guidelines for experimental animals by National Research Council of Thailand, and authorized by the committee of animal care, Faculty of Medicine, Chulalongkorn University (license number: 022/2560). Each Wistar rat lived in the stainless-steel cage were maintained at 25°C, 12:12-hour light-darkness. Before starting the experiment, rats were acclimatized and intimately monitored for two weeks.

3.7.1 Research design and method

Animals were randomly divided into four groups. In this study, 1% (v/v) EG-supplemented in drinking water was used to induce CaOx crystal deposit in the rat kidneys. Control group received normal drinking water (normal rats, Group I), while rats in groups II-IV were nephrolithic rats that received EG-containing drinking water. Group II received on EG in drinking water (EG group). HydroZitLa (Group III, EG + HydroZitLa) and Uralyte-U drug (Group IV, EG + Uralyt-U) were force-fed to the rats (at the dose of 2 mEq citrate per day) twice a day (morning and evening, 1 mEq citrate each). The treatment period was 35 days (5 weeks) (Figure 19). All of the rats were measured blood sugar by strip test (EASYMAX®).

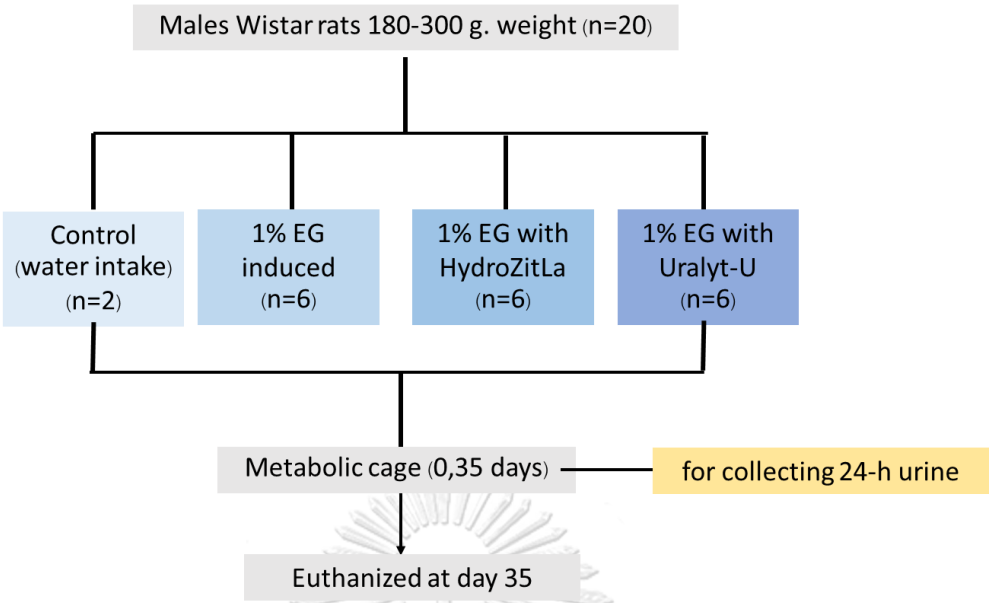


Figure 19 Study design for in vivo nephrolithic rat model. EG: ethylene glycol

3.7.2 Tissues collection and fixation

At the end of the experiment (day 35), rats were anesthetized with Isoflurane (1-4% mg/kg) of dose by inhalant. Both kidneys of the rats were collected and cut into half. One half was fixed in 10% neutral formalin buffer for 24-48 hours, and the other half kidney were soaked in liquid nitrogen and then kept at -80°C.

3.7.3 Kidney tissues processing

After formalin fixation process, the kidney tissues were subjected to automated tissue processor. The tissues were embedded in the paraffin blocks. Tissue sections were cut and prepared on slides. Hematoxilin and eosin (H&E) staining was performed for evaluating pathogenesis of the rats' kidneys. Firstly, the slides were loaded in a slides holder and deparaffined at 70°C for 20 minutes. After that, the slides were soaked into 1st xylene for 10 minutes, 2nd for xylene 10 minutes, and 3rd xylene for 10 minutes. Then the slides were sequentially dipped into absolute ethanol for 3 minutes, 95% ethanol for 3 minutes, 80% ethanol for 3 minutes, and

70% ethanol for 3 minutes. The slides were rinsed with distilled water for 5 minutes. Nucleus and cytoplasm in tissue sections were labeled by hematoxylin and eosin solutions, respectively. Lastly, the slides were mounted and evaluated under light microscopy.

3.7.4 Yasue staining for CaOx histochemistry

Before Yasue staining process, the slides of the kidney tissue section were incubated at 80 ℃ for 15 minutes to remove paraffin. Then, the slides were cooled down for 10 minutes and washed with water. Tissue sections were submerged into 1^{st} xylene 4 times, 2^{nd} xylene 3 times and 3^{rd} xylene 3 times. Then, the sections were dipped into 1^{st} 95% ethanol 10 times and 2^{nd} 95% ethanol 10 times. After that, the sections were rinsed with distilled water 10 times. Wax pen was used to cincture near the section. Next, the slides were soaked in 5% acetic acid for 30 minutes and washed with distilled water 3 minutes before incubating the sections in 5% $AgNo_3$ for 12 minutes. After washed with distilled water 3 minutes, sections were incubated in rubeanic acid with 10% ammonium for 1 minutes. Sections were then soaked in 50% ethanol and washed with distilled water. The sections were counterstained with Hematoxylin for 4 minutes and then washed with distilled water. Finally, the sections were dehydrated in 95% ethanol for 10 minutes, 3 times for each jar, then, 100% ethanol 10 minutes. Lastly, the slides were soaked in xylene for 10 minutes.

3.8 Immunohistochemistry of oxidative stress marker 4-HNE

The sections were incubated at 70 ℃ for 20 minutes for deparaffinization. After cooled down, the sections were rehydrated as described above in Yasue staining. Antigen unmasking was performed by heating in sodium citrate buffer using microwave. After cooled down (for 20 minutes), and washed with 1X PBS (for 5 minute and distilled water for 5 minutes), endogenous H_2O_2 was inactivated by incubating with 0.3% H_2O_2 for 30 minutes, and then washed with 1X PBS for 5

minutes. Blocking of nonspecific binding was performed by incubating with normal horse serum for 20 minutes at room temperature. Next, the slides were incubated with primary antibody at 4° C, overnight (4-HNE, dilution 1:1000). After washed 3 times with 1X PBS for 3 minutes each, the sections were incubated with secondary antibody (Anti-Rabbit 1:125) for 30 minutes at 37°C. Sections were washed 2 times with 1X PBS for 3 minutes each. Next, the sections were incubated with ABC kit from Vector for 30 minutes at room temperature and then washed with 1x PBS for 3 minutes, 2 times. Section were soaked in 1% DAB staining reagent for 5 minutes, washed with water for 5 minutes, and then counterstained with hematoxylin for 5 minutes. After washed with water for 5 minutes, sections were sequentially dehydrated with 70% ethanol, 80% ethanol, 95% ethanol for 10 minutes, absolute ethanol for 5 minutes and acetone for 3 minutes. At the end, the sections were soaked in 1st xylene 10 times, 2nd xylene 10 times, 3rd xylene 10 times and then mounted with mounting medium. Positive staining was evaluated under light microscope.

3.9 Urine characterization.

3.9.1 Urine collection and analysis

A 24-h urine samples were collected at Day 0 and 35 using metabolic cage. Before collecting urine, one small crystal of Thymol and 100 µL of Paraffin oil were added as preservative. 24-h urine samples were collected from individual rat using metabolic cage. Urine volume, pH, creatinine and citrate were measured. Calcium oxalate crystallization index (COCI), an estimator of stone formation risk, was determined as described in the study by Yang et al (41)

3.9.2 Quantification of citrate in rat urine by HPLC

Citric acid was determined by HPLC (Varian, USA) using ROA-organic Acid H^+ column (300x7.8 mm) (Phenomenex, USA). Mobile phase was 5 mmol/L H_2SO_4. Elution was performed at a flow rate of 0.5 mL/min. Chromatography was run at room temperature using UV detector. Citric acid standard at concentration 3 mM was prepared and used for calculation of citric acid in urine samples.

3.10 Crystalization assay

Urine sample from each rat was filtered through 0.22 µm membrane. The filtered urine samples (100 µl) were incubated at 37 °C for 10 minutes. After that, 0.1M $CaCl_2$ and 0.1 M NaOx (4 µl each) were added and incubated at 37°C for 30 minutes. The formed crystals were visualized under the light microscope. The number of CaOx crystal formed in each condition were counted by Celleste program, the micrographs were taken by EVOS cell Imaging System (Invitrogen, USA)

3.11 Statistical analysis

All data were presented as mean ± standard deviation (SD) or median (interquartile range, IQR) as appropripate. Two-sample t-test or Mann Whitney test was performed to test the difference between the two groups. One-way Analysis of variance (ANOVA) or Kruskal-Wallis test was used for more than 2 groups comparison. Paired-sample t-test or Wilcoxon-signed rank test was used for comparing numeric parameters between before and after. All data were computed and graphs were created by the Graphpad Prism Software version 6. Significant difference was set at $P < 0.05$.

CHAPTER IV

RESULTS

4.1. Total antioxidant capacities measured by total phenolics, total flavonoids content, ABTS and DPPH assay of HydroZitLa, Banana stem crude water extract, Anchan, Fang and Alpha lipoic acid.

Table 2. shows average contents of TPC (169.85±8.27 µg GAE/mL) and TFC (0.04±0.0 µg CE/mL) in HydroZitLa (n = 10). TAC determined by ABTS and DPPH assays were (201.92±11.67 VCEAC mg/L) and (188.09±4.54 VCEAC mg/L), respectively.

Table 3. shows average contents of TPC (68.28±13.56 µg GAE/mL) and TFC (0.02±0.00 µg CE/mL) in banana stem crude water extract (n = 10). TAC determined by ABTS and DPPH assays were (195.65±20.93 VCEAC mg/L) and (161.05±18.98 VCEAC mg/L), respectively

Table 4. shows average of TPC (53.98±8.89 µg/mL GAE) and TFC is (0.31±0.00 µg/mL). Thus mean total phenolic of Anchan was showed high but hardly found the total flavonoid in Anchan. In addition, antioxidant capacity by ABTS assay as (174.89±5.41 VCEAC mg/L) higher than antioxidant capacity DPPH assay (76.68±8.52 VCEAC mg/L).

Table 5. shows average contents of TPC (36.95±3.8 µg GAE/mL) and TFC (0.03±0.00 µg CE/mL) in Fang (n = 10). TAC determined by ABTS and DPPH assays were (127.43±5.68 VCEAC mg/L) and (72.55±13.32 VCEAC mg/L), respectively

Table 6. shows average contents of TPC (103.56±10.76 µg GAE/mL) and TFC is not detectable in Alpha lipoic acid (n = 10). TAC determined by ABTS and DPPH assays were (15.34±2.97 VCEAC mg/L**)** and (25.64±23.53 VCEAC mg/L), respectively.

Table 2 The levels of TPC, TFC and TAC (by ABTS and DPPH assays) of HydroZitLa.

HydroZitLa	Total phenolic (µg/mL GAE) mean ± SD	Total flavonoid (µg/mL CE) mean ± SD	ABTS (VCEAC mg/L) mean ± SD	DPPH (VCEAC mg/L) mean ± SD
1	172.3±9.0	0.05±0.01	209.5±6.62	195.1±9.51
2	159.4±22.58	0.04±0.00	201.9±10.32	193.0±9.39
3	166.6±4.65	0.04±0.00	203.2±6.54	183.8±12.87
4	175.9±13.15	0.05±0.00	199.8±14.63	181.7±15.91
5	172.1±10.30	0.04±0.00	206.7±4.67	184.2±8.10
6	170.9±10.27	0.04±0.00	207.3±8.10	190.2±6.38
7	157.4±12.93	0.04±0.00	197.2±13.82	189.6±9.70
8	168.6±16.86	0.04±0.00	199.9±10.45	188.1±5.90
9	168.5±14.60	0.04±0.00	196.5±22.03	183.8±12.31
10	186.8±25.05	0.04±0.00	197.4±15.28	191.5±5.94
AVE	169.85±15.46	0.04±0.00	201.92±11.67	188.09±9.87
%CV	9.1	12.20	5.78	5.24

CV: coefficient of variation; VCEAC: vitamin C equivalent antioxidant capacity

Each value is presented as mean ± SD

Table 3. The levels of TPC, TFC and TAC (by ABTS and DPPH assays) of Banana stem crude water extract.

Banana stem crude water extract	Total phenolic(µg/mL GAE) mean ± SD	Total flavonoid (µg/mL CE) mean ± SD	ABTS (VCEAC mg/L) mean ± SD	DPPH (VCEAC mg/L) mean ± SD
1	66.70±10.66	0.02±0.00	218.0±7.44	167.1±19.33
2	66.15±17.06	0.02±0.00	207.5±2.40	163.9±9.31
3	65.60±9.55	0.02±0.00	200.7±5.53	150.6±22.82
4	63.28±4.21	0.02±0.00	199.6±4.98	164.3±8.42
5	74.99±34.04	0.02±0.00	196.2±12.33	160.7±15.53
6	71.94±11.62	0.02±0.00	205.9±13.09	151.5±24.05
7	64.56±8.25	0.02±0.00	205.6±7.63	162.6±18.61
8	67.00±9.25	0.02±0.00	193.6±16.45	162.6±15.53
9	67.79±9.78	0.02±0.00	157.7±31.28	164.8±39.66
10	74.87±11.19	0.02±0.00	171.6±8.15	162.4±16.87
AVE	68.28±13.56	0.02±0.00	195.65±20.93	161.50±18.98
%CV	19.86	4.18	10.70	11.78

CV: coefficient of variation; VCEAC: vitamin C equivalent antioxidant capacity

Each value is presented as mean ± SD

Table 4. The levels of TPC, TFC and TAC (by ABTS and DPPH assays) of Anchan.

Anchan	Total phenolic (µg/mL GAE) mean ± SD	Total flavonoid (µg/mL CE) mean ± SD	ABTS (VCEAC mg/L) mean ± SD	DPPH (VCEAC mg/L) mean ± SD
1	49.44±4.15	0.03±0.00	179.7±4.20	88.39±13.39
2	51.03±8.36	0.03±0.00	180.5±8.42	77.70±7.66
3	51.45±5.22	0.03±0.00	177.6±1.36	80.26±4.70
4	56.88±6.51	0.03±0.00	173.6±1.67	72.13±12.09
5	55.05±11.68	0.03±0.00	175.3±1.04	72.99±6.74
6	49.75±7.75	0.02±0.00	170.3±2.46	78.34±5.97
7	51.64±5.94	0.03±0.00	174.8±3.82	74.70±7.53
8	57.00±4.95	0.03±0.00	171.9±4.87	74.06±5.27
9	59.50±18.94	0.03±0.00	168.3±6.98	75.98±7.70
10	58.16±9.44	0.03±0.00	177.0±1.82	72.35±4.69
AVE	53.98±8.89	0.03±0.00	174.89±5.41	76.68±8.52
%CV	16.48	3.59	3.09	11.11

CV: coefficient of variation; VCEAC: vitamin C equivalent antioxidant capacity

Each value is presented as mean ± SD

Table 5. The levels of TPC, TFC and TAC (by ABTS and DPPH assays) of Fang.

Fang	Total phenolic (µg/mL GAE) mean ± SD	Total flavonoid (µg/mL CE) mean ± SD	ABTS (VCEAC mg/L) mean ± SD	DPPH (VCEAC mg/L) mean ± SD
1	36.09±2.04	0.03±0.00	179.7±4.20	68.71±1.45
2	35.36±2.98	0.03±0.00	180.5±8.42	68.07±7.68
3	40.29±6.06	0.03±0.00	177.6±1.36	69.78±10.00
4	39.56±3.77	0.03±0.00	173.6±1.67	70.42±2.99
5	34.99±0.87	0.03±0.00	175.3±1.04	65.50±5.71
6	34.50±1.56	0.03±0.00	170.3±2.46	70.85±13.34
7	35.48±0.87	0.03±0.00	174.8±3.82	80.05±4.24
8	39.01±4.68	0.03±0.00	171.9±4.87	87.75±34.53
9	39.38±6.46	0.03±0.00	163.8±6.98	76.84±10.06
10	34.93±1.50	0.03±0.00	177.0±1.82	67.64±6.60
AVE	36.95±3.89	0.03±0.00	127.43±5.68	72.55±13.32
%CV	10.54	2.29	4.45	18.36

CV: coefficient of variation; VCEAC: vitamin C equivalent antioxidant capacity

Each value is presented as mean ± SD

Table 6. The levels of TPC, TFC and TAC (by ABTS and DPPH assays) of Alpha lipoic acid.

Alpha lipoic acid	Total phenolic (µg/mL GAE) mean ± SD	Total flavonoid (mg/L) mean ± SD	ABTS(VCEAC mg/L) mean ± SD	DPPH(VCEAC mg/L) mean ± SD
1	101.3±3.13	nd	17.01±2.24	27.00±18.99
2	100.5±3.08	nd	15.24±2.76	11.38±2.02
3	105.4±1.91	nd	15.59±2.58	34.70±29.86
4	103.4±12.29	nd	14.18±2.58	46.68±22.28
5	99.14±2.72	nd	14.53±3.93	43.68±35.23
6	91.70±19.38	nd	17.36±3.43	38.12±22.05
7	108.6±7.21	nd	12.41±4.33	26.57±30.82
8	110.5±3.51	nd	14.65±3.55	4.96±6.26
9	108.0±20.75	nd	15.83±1.41	11.59±2.88
10	106.9±8.75	nd	16.66±1.23	11.81±4.15
AVE	103.56±10.76	-	15.34±2.97	25.64±23.53
%CV	10.39	-	19.35	91.74

CV: coefficient of variation; VCEAC: vitamin C equivalent antioxidant capacity

Each value is presented as mean ± SD

Remark: nd is not detectable

4.2. HydroZitLa inhibited aggregation of COM crystal.

To determine the inhibition of growth and aggregation of COM crystals after treatment with HydroZitLa, aggregation assay was carried out. The COM aggregation experiment was divided into 3 groups for 3 conditions including control (200 µL of distilled water (DW), positive control (1 mg/mL of bovine serum albumin (BSA) and test (10% (v/v) HydroZitLa). The results showed that HydroZitLa significantly reduced the COM aggregation compared with DW control and also BSA (Figure 20). In addition, inhibitory effect of HydroZitLa was found in the dose-dependent manner, indicated by higher inhibition of COM aggregation in higher concentration of HydroZitLa (Figure 21).

To determine the TAC of HydroZitLa compared to the potassium citrate drug Uralyt-U, DPPH radical scavenging activity assay was performed (Figure 22). The result showed that TAC level in HydroZitLa was significantly higher than in Uralyt-U.

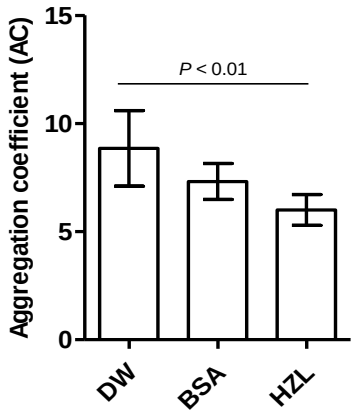


Figure 20 Comparison of the COM aggregation coefficient (AC).

Among the three different treatments, control (distilled water (DW)), bovine serum albumin (BSA) and HydroZitLa (HZL) 10 (%v/v). HydroZitLa significantly reduced AC compared to the control.

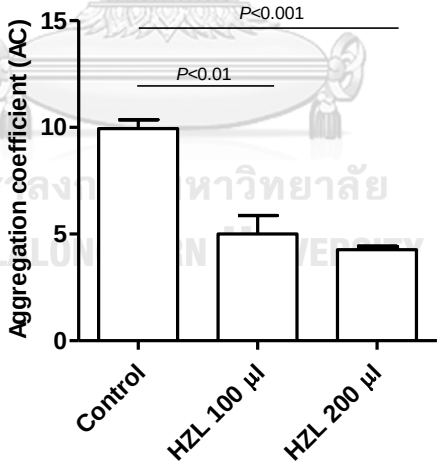


Figure 21 Comparison of seed COM aggregation activity after treatment with different concentrations of HydroZitLa.

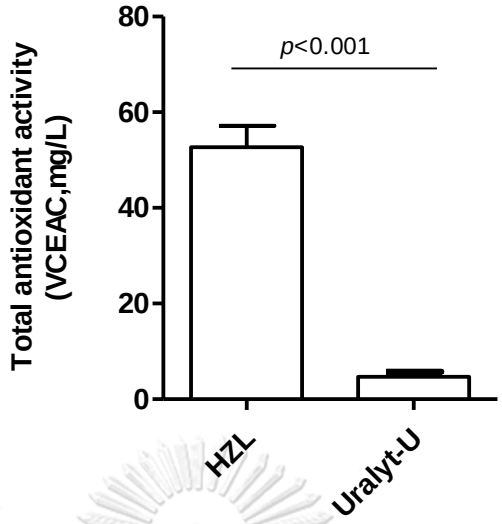


Figure 22 Level of TAC compared between HydroZitLa (HZL) and Uralyt-U.

4.3 Cytotoxicity of HydroZitLa in human renal tubular cells lines

To investigate the toxic effect of HydroZitLa in HK-2 cells, MTT assay was performed. MTT assay was performed after 24 hour treatment and, consequently, cell viability (% of control) and inhibitory concentration 50% (IC_{50}) were analyzed. Cell viability of HK-2 cells after treatment with varied HydroZitLa concentrations (from 1.25 to 100%) is shown in (Figure 23). The data showed that cell viability was starting to be significantly decreased at 10% (v/v) HydroZitLa compared with the untreated control. Log_{10} HydroZitLa concentration (% V/V). The log_{10} IC50 was 1.391 or 24.62% (v/v) (Figure 24).

In this study, we chose the concentration of HydroZitLa at 10% (v/v) for further subsequent experiments because it showed the minimal cytotoxicity.

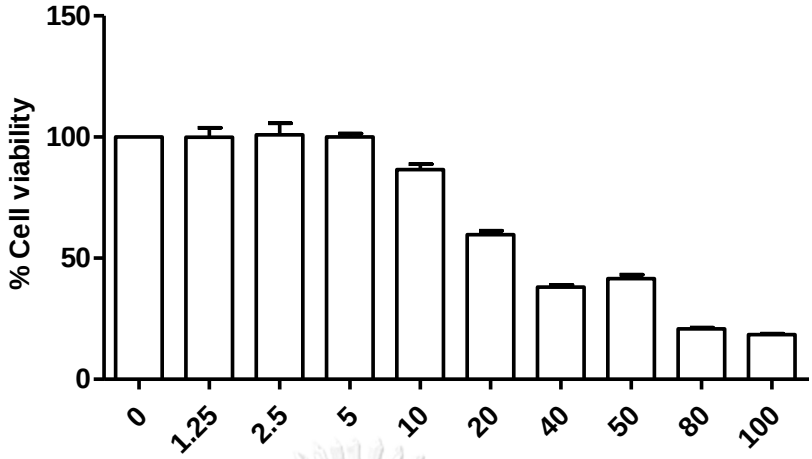


Figure 23 MTT assay result to evaluate cytotoxicity of HydroZitLa in HK-2 cells.

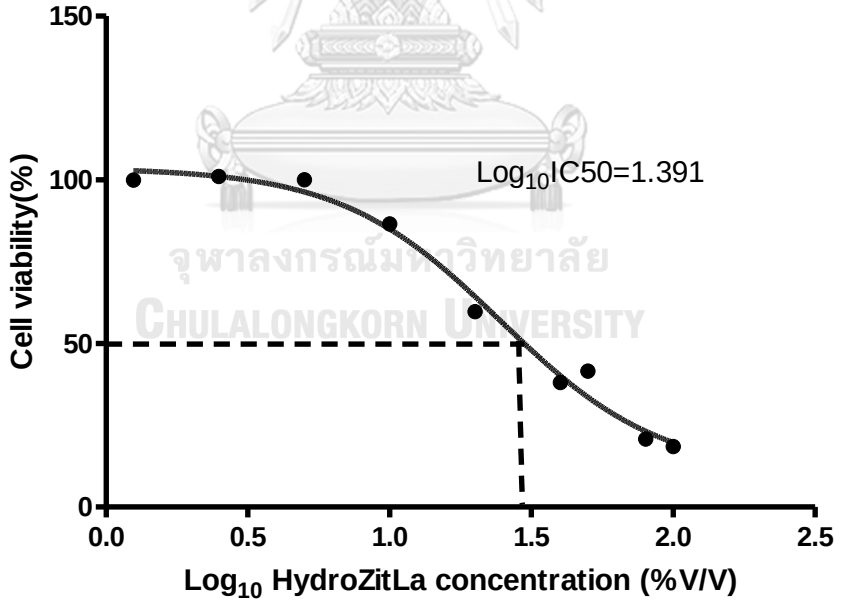


Figure 24 Log10 HydroZitLa concentration (% V/V).

The log10 IC50 was 1.391 or 24.62 10 (% v/v)

4.4 HydroZitLa reduced oxidative stress in HK-2 cells exposed to H_2O_2

H_2O_2 was used to induce the oxidative stress in renal tubular cells, and tocopheryl acetate (TA) was used as a known antioxidant. DCFH-DA assay was performed to test whether HydroZitLa had an antioxidative activity to inhibit intracellular ROS production in HK-2 cells. Cells treated with 10 μM H_2O_2 had significantly increased ROS production compared with the untreated control (Figure 25). In contrast, ROS in cells treated with 10 μM H_2O_2 together with 10% HZL were significantly decreased compared with the H_2O_2-treated cells. As expected, ROS in cells treated with 10 μM H_2O_2 and TA were significantly reduced relative to the H_2O_2-treated cells. The result demonstrated that HydroZitLa effectively delivered an antioxidant effect in attenuating intracellular ROS production in renal tubular cells.

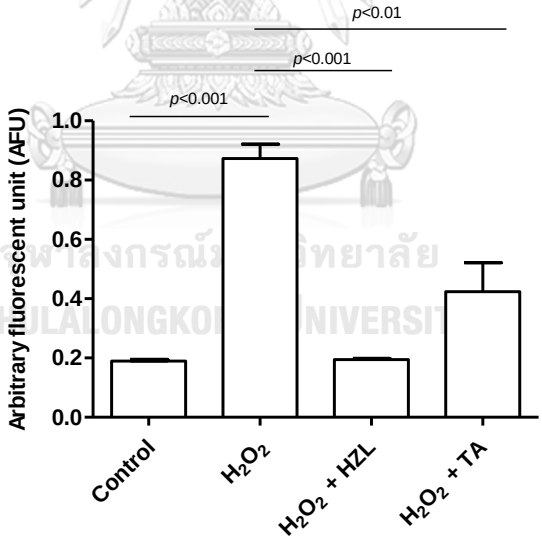


Figure 25 Intracellular ROS production in renal tubular cell lines.
After treated with different conditions; 10 μM H_20_2, 10 μM H202 with 10 (%v/v) HydroZitLa (HZL), 10 μM H_20_2 with 300 μM α-tocopheryl acetate (TA).

4.5 HydroZitLa reduced protein oxidation in HK-2 cells challenged with COM and H_2O_2

Level of protein oxidation caused by ROS is monitored by protein carbonyl assay, as carbonylated proteins are byproducts of protein oxidation. The levels of protein carbonyl content were increased in HK-2 cells treated with COM (150 μg/cm^2) and H_2O_2 (Figure 27). HydroZitLa reduced protein carbonyl content in COM-treated cells, but not significant (Figure 27). On another hand, protein carbonyl content was significantly decreased in HK-2 cell treated with 1000 μM H_2O_2 and 10% HydroZitLa compared to the 1000 μM H_2O_2-treated cells. These results indicated that HydroZitLa was capable of reducing oxidative stress in the H_2O_2-treated HK-2 cells. (Figure 26) shows the morphological change of HK-2 cells after treatments with COM, H_2O_2 and also co-treatment with HydroZitLa.

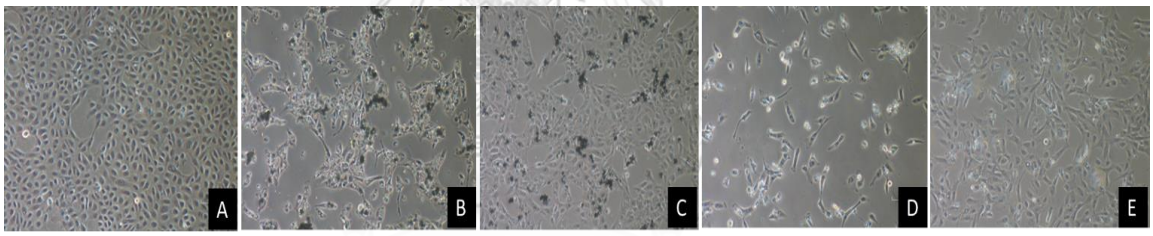


Figure 26 Comparison of change in morphology of HK-2 cells.
After 24 h treatment with different conditions. A= untreated control, B= 150 μg/cm^2 COM, C= 150 μg/cm^2 COM + 10 (%v/v) HydroZitLa, D= 1000 μM H_2O_2 and E= 1000 μM H_2O_2 + 10% HydroZitLa. Magnification: 10x

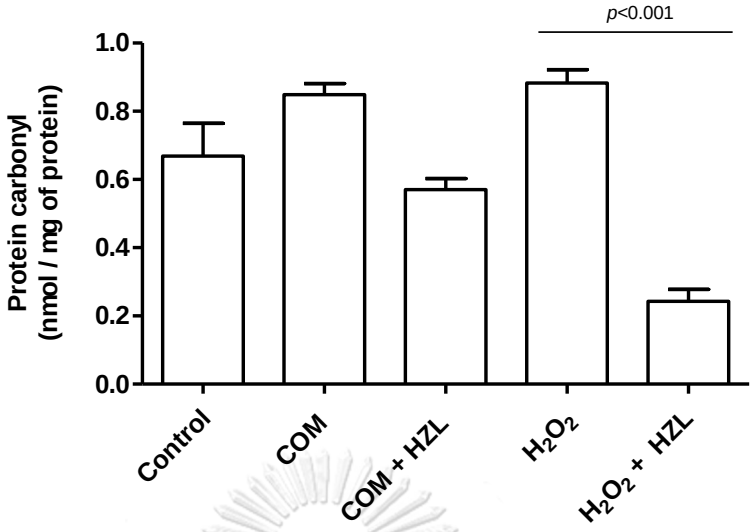


Figure 27 Level of protein carbonyl content in renal tubular cells treated with COM and H_2O_2, and co-treated with HydroZitLa

4.6 Kidney weight and gross appearance of EG-induced nephrolithic rats

All rats were sacrificed on Day 35. Kidneys both left and right sides were collected and cut in half. (Figure 28) shows the image of gross anatomy of the representatives of the right kidney obtained from each group, including control, EG, EG + HydroZitLa and EG + Uralyt-U. The kidneys of normal control rats had dark reddish color as seen in the (Figure 28 a). Kidneys of rats in EG group were much lighter color and had a bigger size than normal. Treatment with HydroZitLa and Uralyt-U effectively restored the normal appearance of the kidneys.

Kidneys from all groups were weighted and compared. Table 2 shows the weight of kidneys (left, right and total) compared among four groups. Left kidney weight of rats from EG + HydroZitLa and EG + Uralyt-U (group 3 1.18 ± 0.20 g; *P* = 0.01 and group 4, 1.2 ± 0.17 g; *P* = 0.02) was significantly lighter than the EG group (1.65 ± 0.33 g). The right kidney weight of EG group was significantly heavier than that of control (1.83 ± 0.30 g; *P* = 0.028), EG + HydroZitLa (1.21 ± 0.23 g; *P*= 0.002) and EG + Uralyt-U groups (1.25 ± 0.13 g; *P* = 0.001).

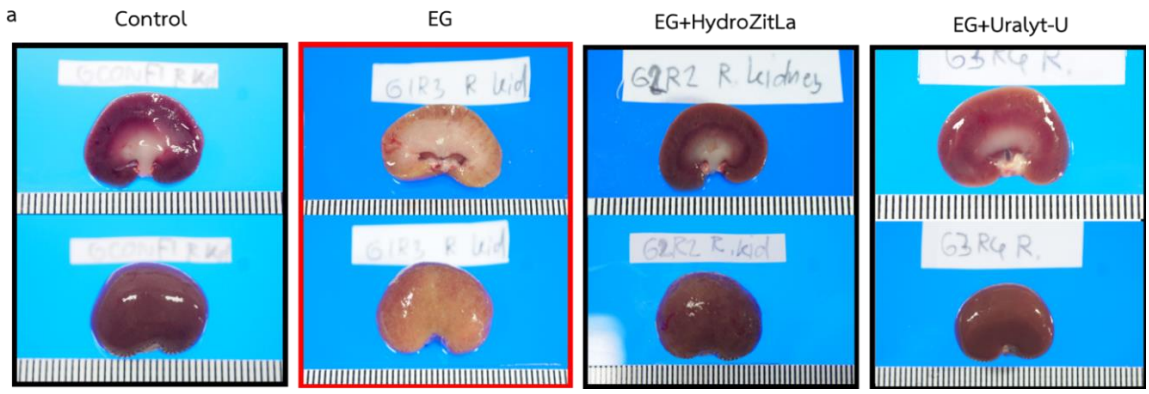


Figure 28 Representative images showing shape and color of the kidneys of the experimental rats from each group.

Table 7. The comparison of left and right kidney weight from the experimental rats.

	Group 1 Control (N=2)	Group 2 EG(N=6)	Group 3 EG+HZL (N=6)	Group 4 EG+U (N=6)
Kidney weight, left (gm)	1.1 ± 0.11	1.65 ± 0.33	1.18 ± 0.20*	1.2 ± 0.17*
Kidney weight, right (gm)	1.17 ± 0.14	1.83 ± 0.30*	1.21 ± 0.23**	1.25 ± 0.13**

Values are expressed as mean ± SD.

* $P < 0.05$ EG vs.control, EG vs EG+HydroZitLa, EG+Uralyt-U,

** $P < 0.01$ EG vs EG+HydroZitLa and EG+Uralyt-U

4.7 Histological study

H&E staining was performed to evaluate cell morphology and inflammatory lesions in the renal tissues of rats from each group. H&E micrographs of all renal sections are shown in (Figure 29). It was obvious that kidney sections of EG-fed rats in EG group had increased number of infiltrating cells relative to those sections from EG+HZL and EG+U groups. This indicated that kidneys of rats in the EG group had inflammatory reaction higher than the other groups. Inflammatory lesions in the

kidneys of rats in EG+HZL and EG+U groups were considerably lower than the EG group (Figure 29).

CaOx crystal deposits in the rat kidneys were visualized in the H&E stained sections by the polarized light microscopy. CaOx crystals are strongly birefringent under the polarized light microscope. Figure 30, 31, 32 and 33 are polarized micrographs of all rat kidney sections from control, EG, EG+HydroZitLa and EG+Uralyt-U, respectively. There was no birefringent crystal observed in the control group. In the EG group, a birefringent appearance of CaOx was clearly observed (Figure 31), both in the renal cortex and medulla. A number of birefringent CaOx crystal deposits in the EG+HydroZitLa and EG+Uralyt-U groups were markedly reduced relative to the EG group.

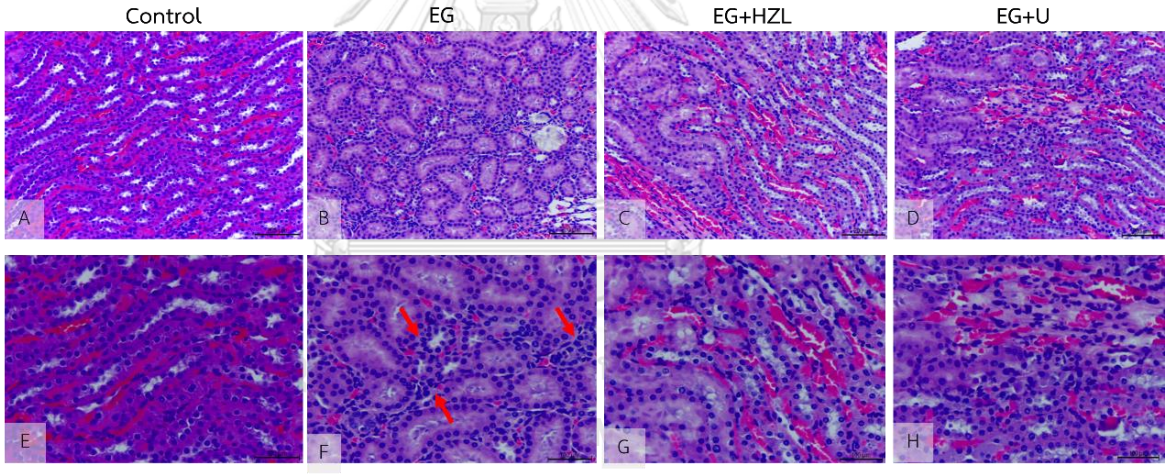


Figure 29 H&E stained histopathological images of rat kidney sections from all experimental rats.
(A, B, C and D Magnification: 10x) (E,F, G and H Magnification: 40x)

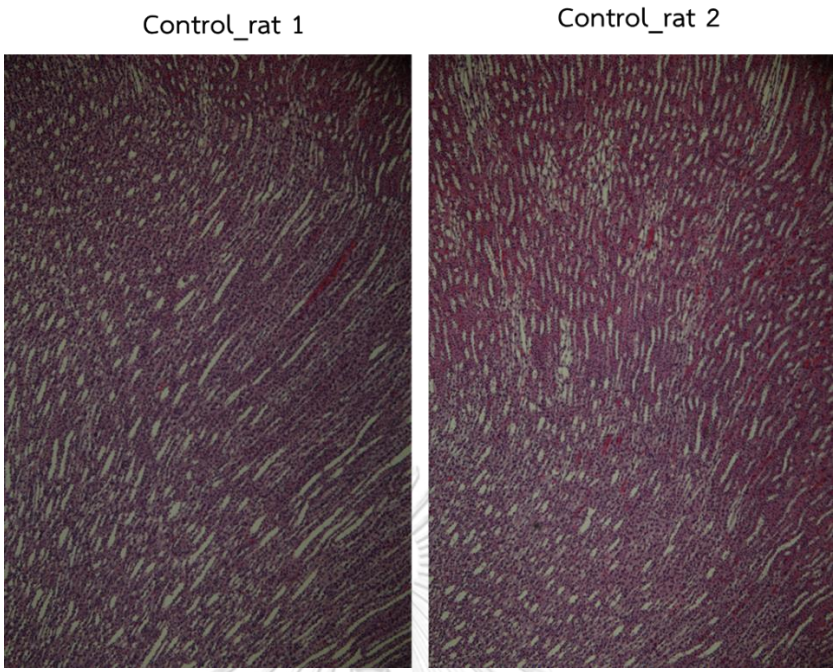


Figure 30 (H&E stain) Histopathological images of normal rat kidneys sections. Control rat 1 and 2 showing normal renal tubules from medullar cells were observed by under polarized light microscopy magnification; 100x

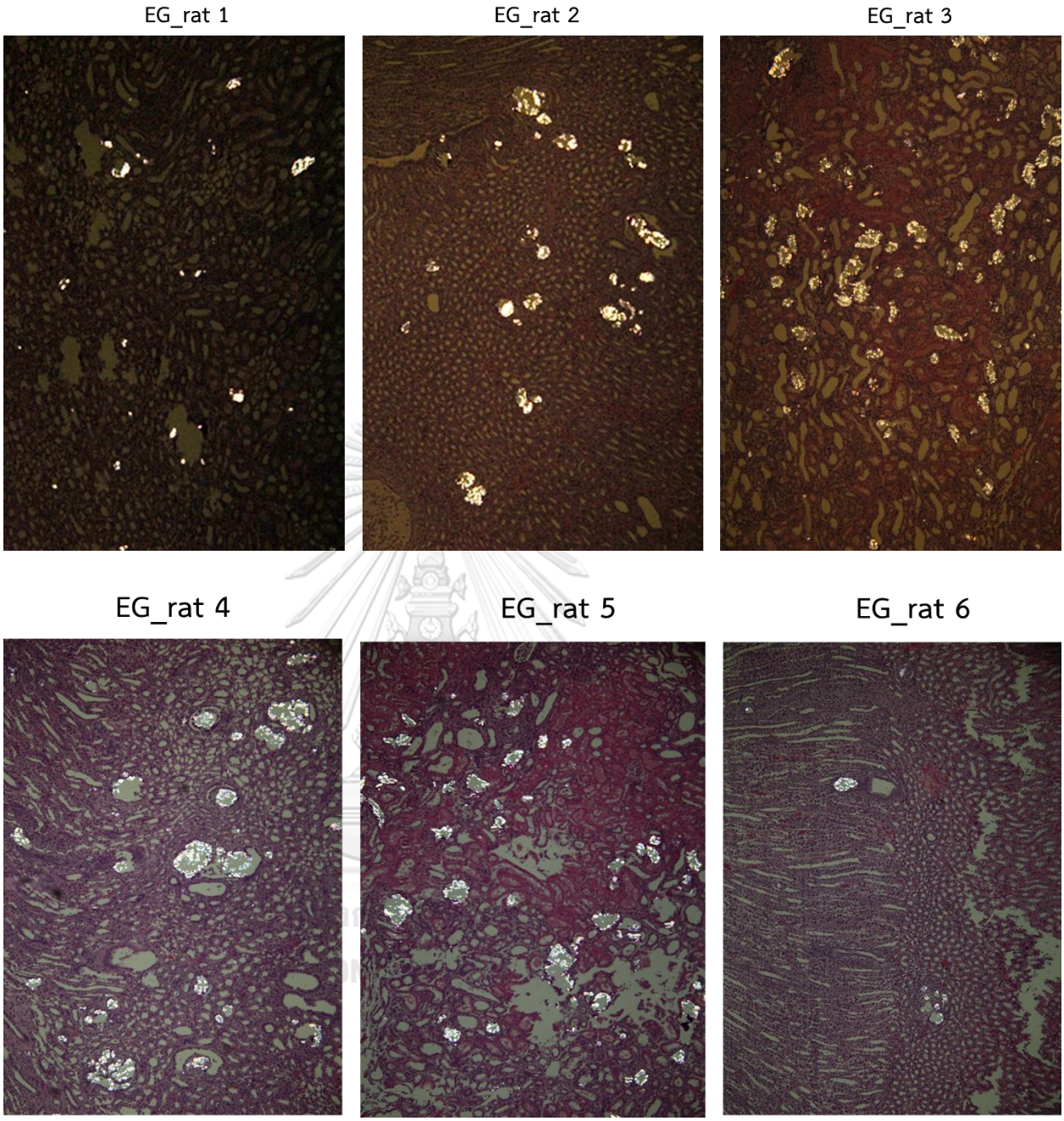


Figure 31 (H&E stain) Histopathological images of EG rats kidneys sections.
EG rat no. 1 to no. 6 kidneys showing deposition of numerous birefringent calcium oxalate crystals in renal tubules. Observe by under polarized light microscopy magnification: 100x

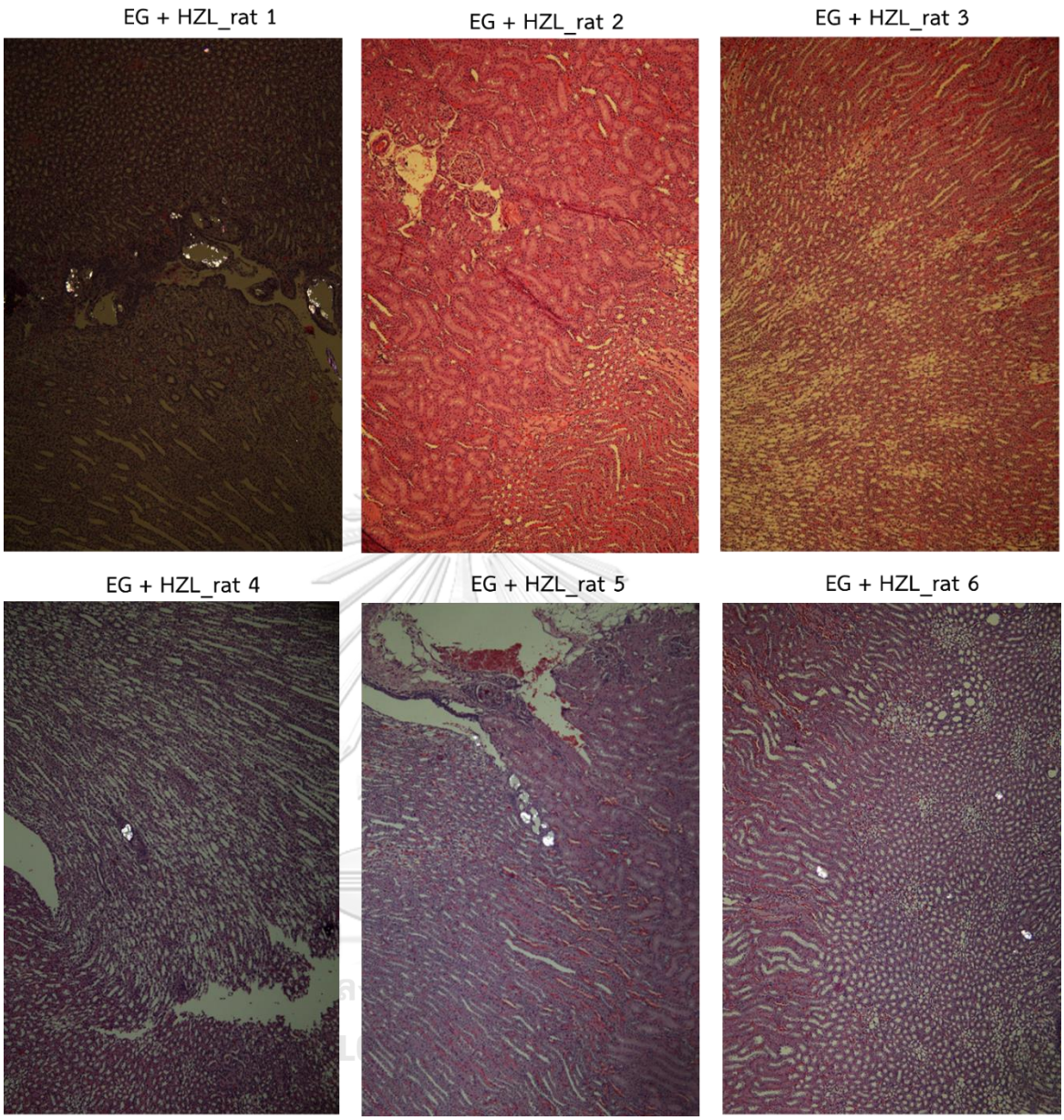


Figure 32 (H&E stain) Histopathological images of EG with HydroZitLa rats kidney section.

EG + HZL no. 1 to rat no. 6 showing only few birefringent Calcium oxalate deposit were examined by polarized light microscopy magnification: 100x

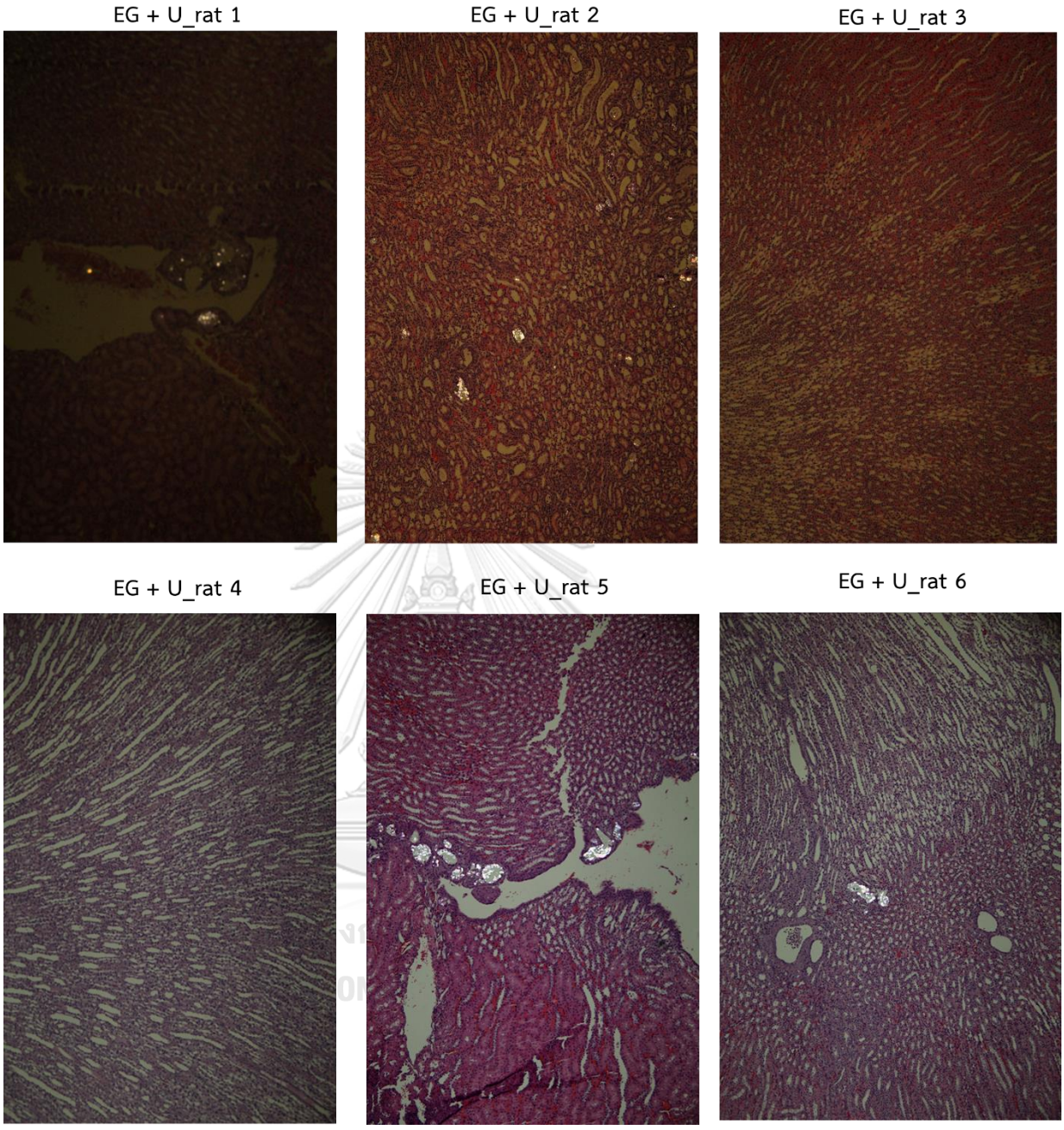


Figure 33 (H&E stain) Histopathological images of EG with Uralyt-U rats.
no. 1 to no. 6 kidney sections showing only few Calcium oxalate deposit were examined by polarized light microscopy magnification: 100x

4.8 CaOx crystal deposits in the kidneys detected by Yasue staining

Yasue staining was additionally performed to identify and count the number of CaOx crystal deposit in rat kidneys. (Figure 34) shows all micrographs of Yasue staining of all experimental rats. CaOx crystals were stained in black. Similar to the results of polarized light microscopy, number of CaOx crystals in the rat kidneys of EG group was much higher than the other groups. After quantification, the total number of crystal deposits counted in the EG+HdroZitLa and EG+Uralyt-U groups were significantly lower than the EG group (Figure 35 and Figure 36).

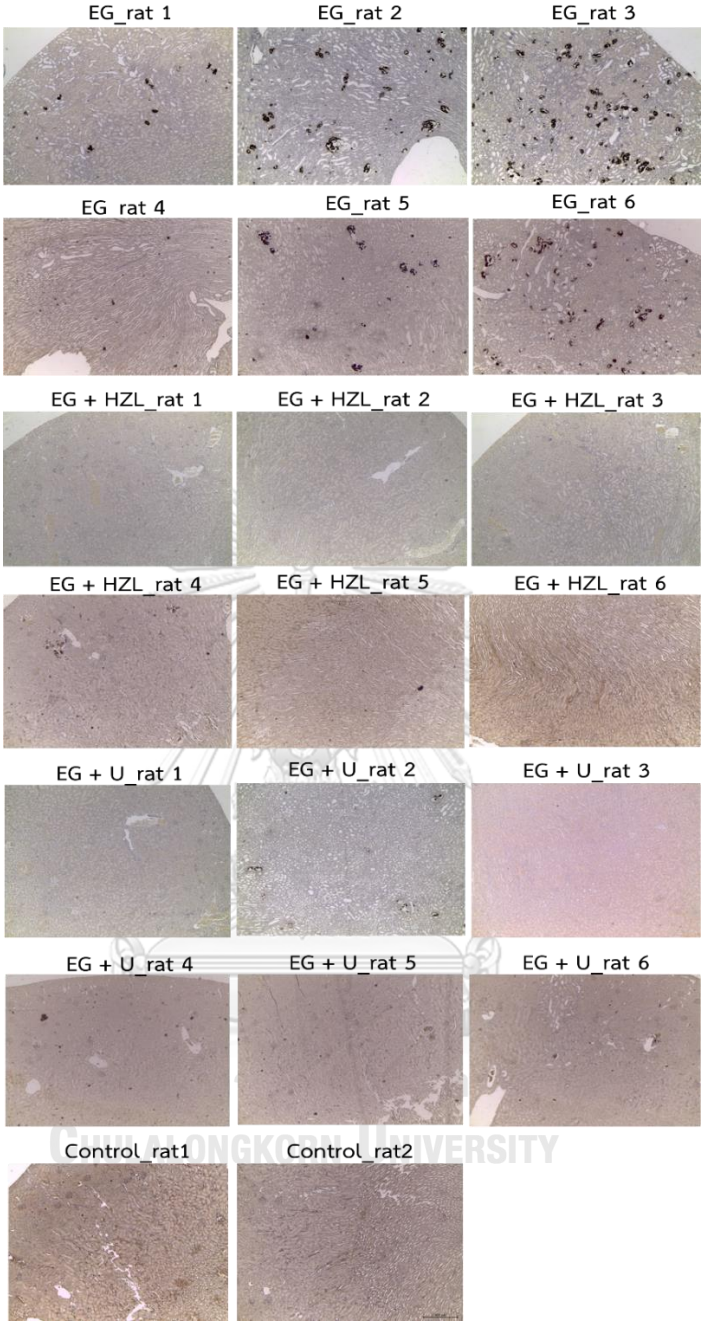


Figure 34 Micrographs of all Yasue stained renal sections.

CaOx crystals were stained in black. EG group shows higher CaOx positivity higher than control, EG+ HydroZitLa and EG+Uralyt-U groups. Magnification: x100

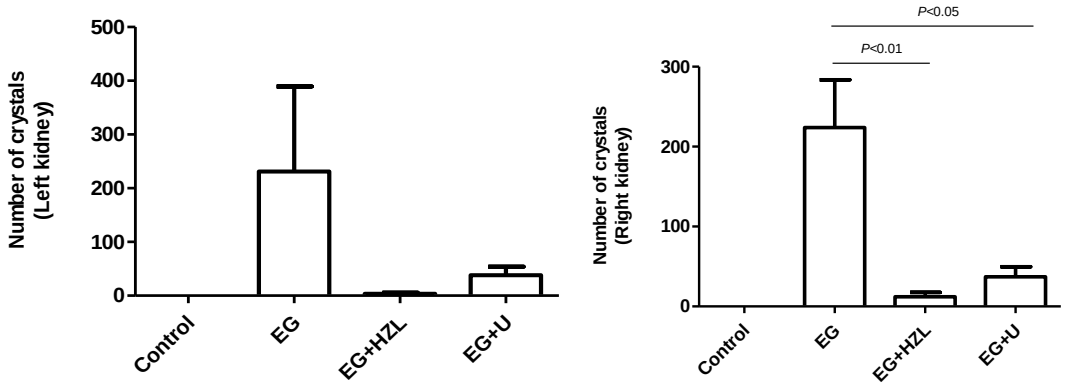


Figure 35 Comparison of the number of CaOx crystal deposits in the left and right kidneys.

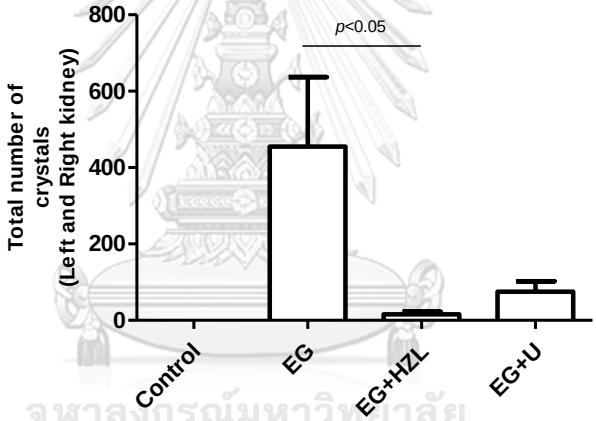


Figure 36 Comparison of the total number of CaOx crystal deposits in the experimental rat kidneys.

4.9 Oxidative stress expression in rat kidney tissue

To investigate level of oxidative stress in the rat kidney tissues, immunohistochemical staining with 4-hydroxynoneol (4-HNE) (by product from lipid peroxidation) was carried out. Figure 37 shows a negative control of staining without 4-HNE antibody. 4-HNE expression was low or even none in the control group (Figure 38). In EG group, 4-HNE expression was evidently high relative to control (Figure 39). 4-HNE expression was attenuated in the EG+HydroZitLa and EG+Uralyte-U groups (Figure 40 and 41). The number of 4-HNE positive cells (%) among the 4 groups were not statistically significant (Table 3, Figure 38). However, there was a clear trend of decrease in 4-HNE expression in the EG-HydroZitLa group.

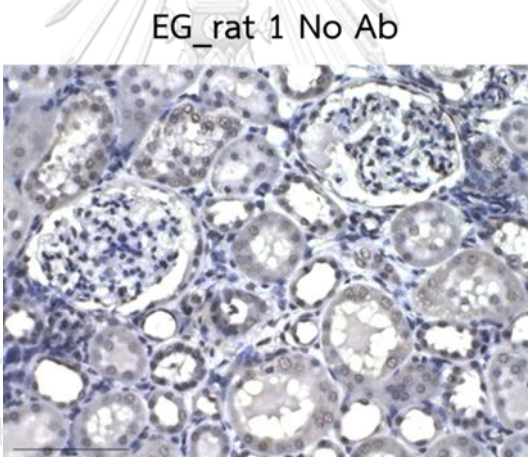


Figure 37 4-HNE unexpressed in EG rat 1 is Negative control

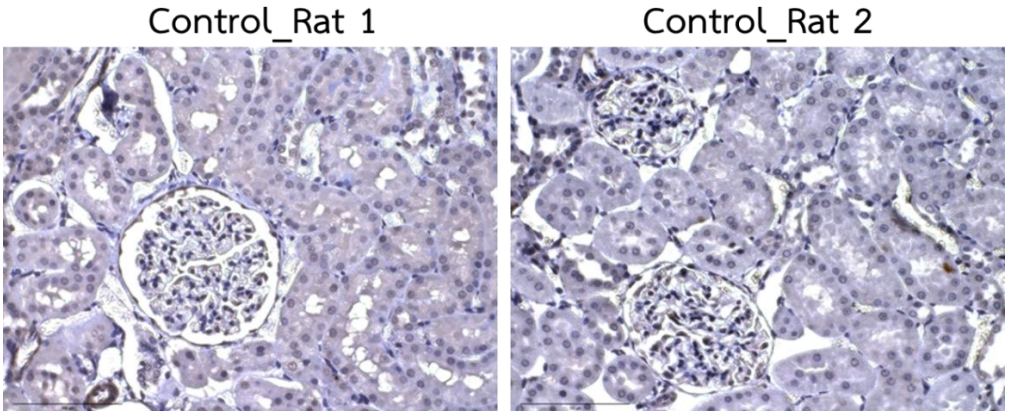


Figure 38 4-HNE expression in in the rat kidney tissues of control group.

4-HNE was unexpressed or slightly expressed in the normal rat renal tissues (Control Rat 1 and 2) Magnification: x400 (R)

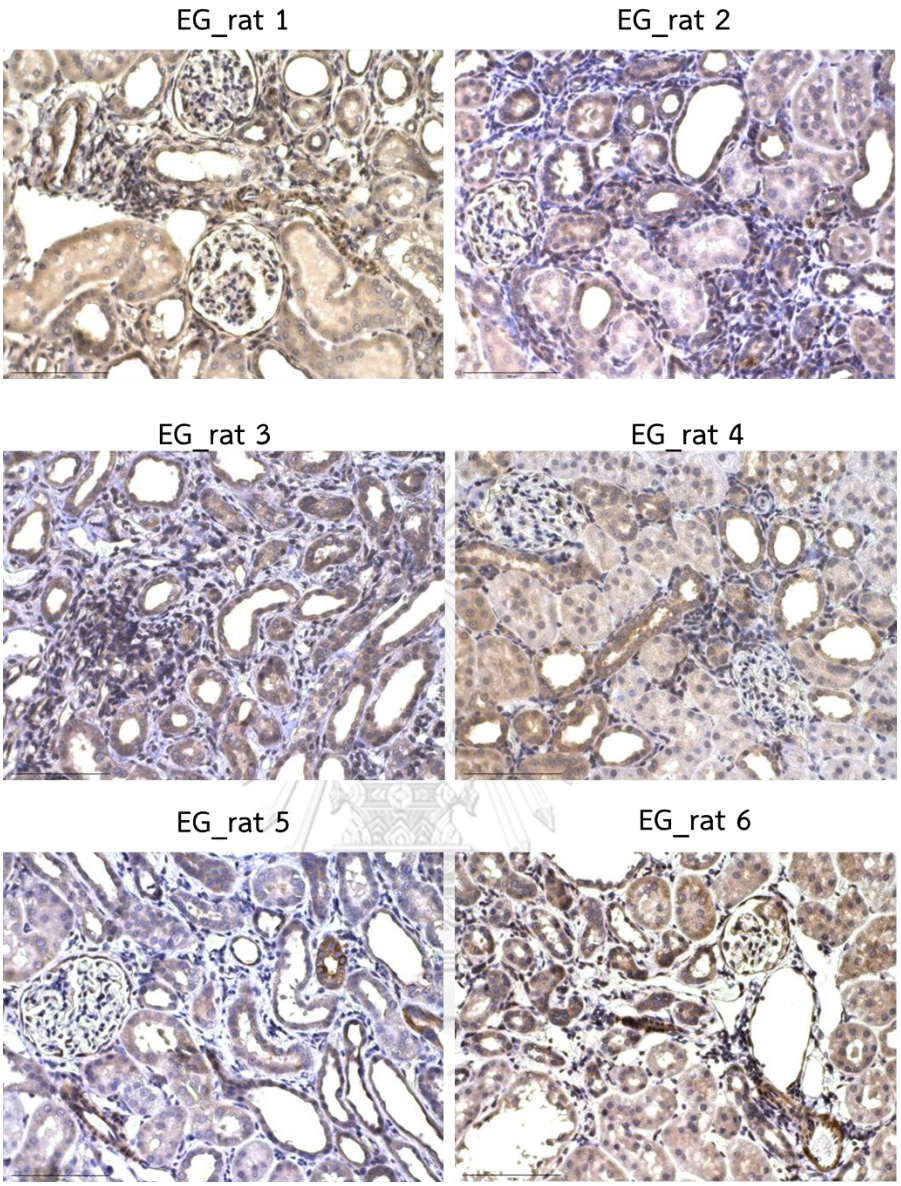


Figure 39 Oxidative stress in rat kidney tissue.

4-HNE expression in EG group; EG rat no. 1 to rat no. 6 were higher than control group magnification: 400x

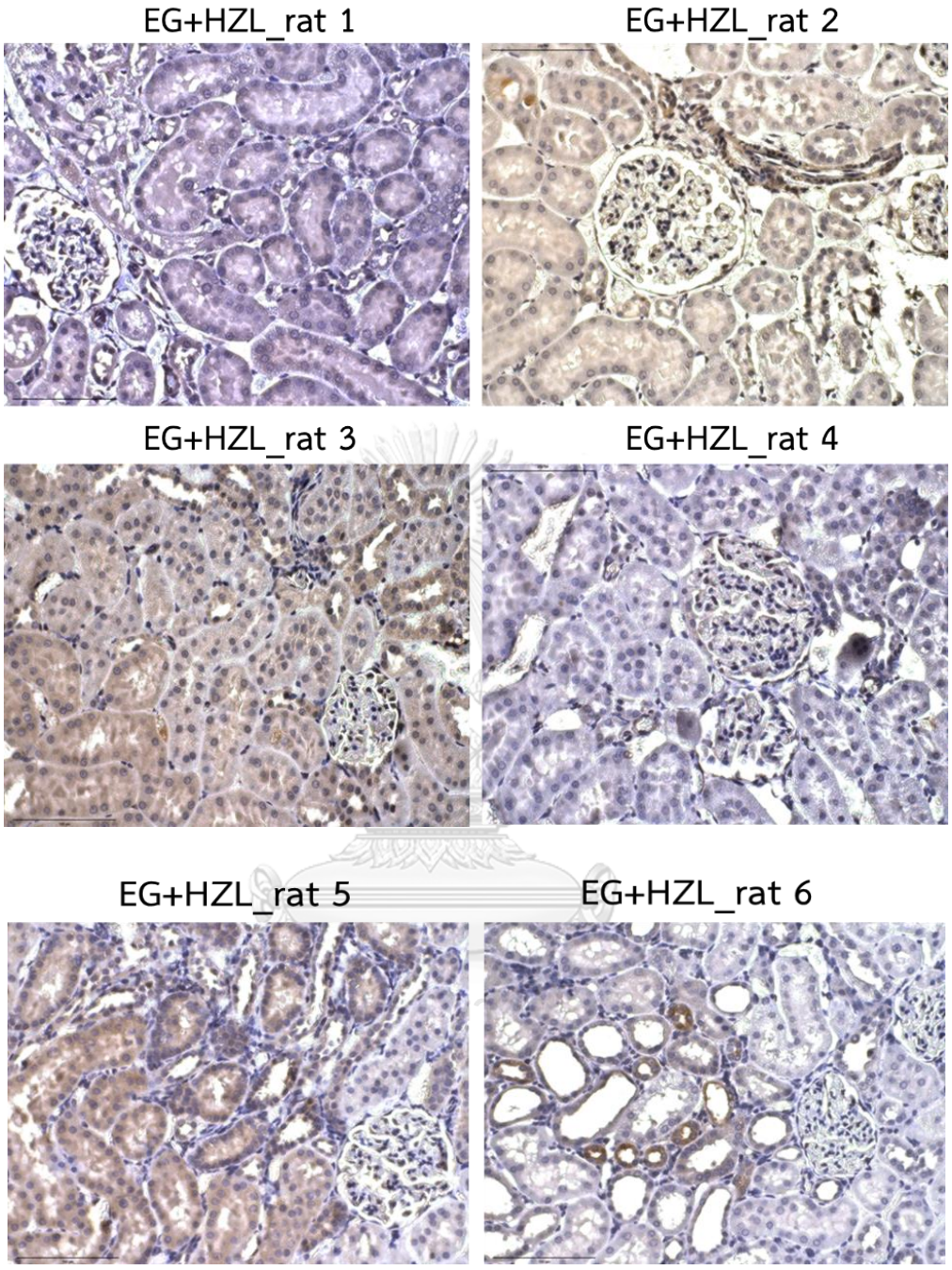


Figure 40 Oxidative stress in rat kidney tissue.

4-HNE expression in EG with HydroZitLa group; EG+ HydroZitLa rat no. 1 to rat no.6 were lower than EG group magnification: 400x

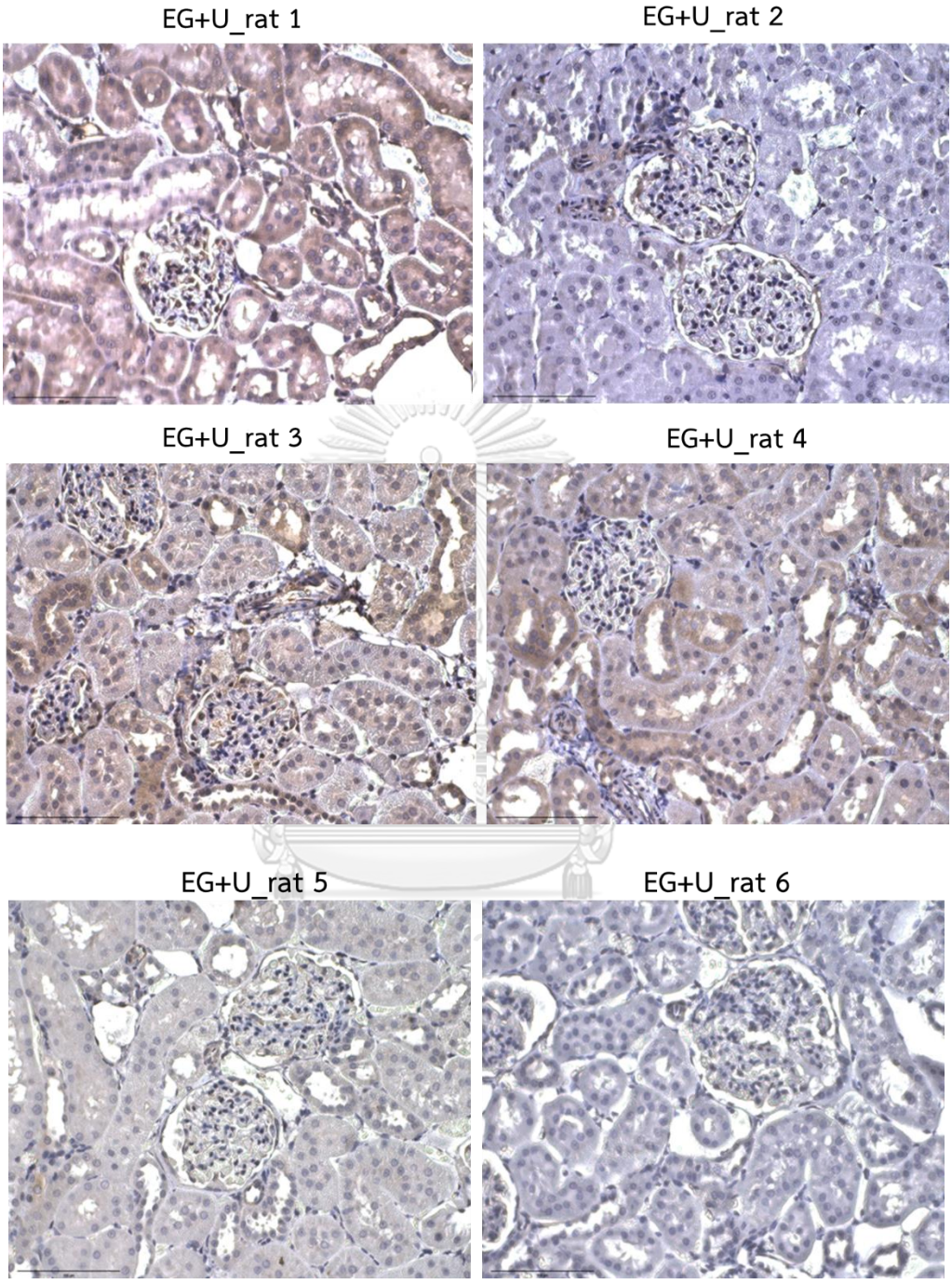


Figure 41 Oxidative stress in rat kidney tissue.

4-HNE expression in EG with HydroZitLa group; EG+ HydroZitLa rat no. 1 to rat no. 6 were lower than EG group magnification: 400x

Table 8. Comparison of percentage of cell positively stained with 4-HNE in each group.

Group	Treatment	Number of rats	% positive cell (mean ± SD)
Group 1	Normal water	2	13.56 ± 12.20
Group 2	EG	6	31.08 ± 34.91
Group 3	EG + HydroZitLa	6	12.85 ± 7.762
Group 4	EG+ Uralyt-U	6	22.78 ± 34.17

Data presented as mean ± SD.

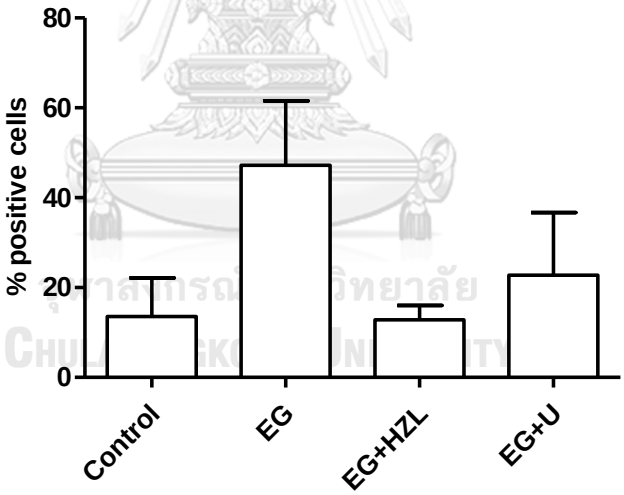


Figure 42 The comparison of 4-HNE positive cells among the 4 experimental groups.

4.10 Urinary measurement

Twenty-four hour urine specimens were collected from all 4 groups of rats at the beginning (day 0) and the end (day 35) of the experiment. Body weight and basic urinary parameters were measured and displayed in (Table 4). The result showed on day 0 and day 35 all of rats group no significant differences in body weight, 24-hour urine volume, urine pH, Urine specific gravity, urine creatinine and urine total protein.

Table 9. Body weight and basic urinary parameters of the experimental rats.

	Day 0				Day 35			
	Group 1 Control (N=2)	Group 2 EG (N=6)	Group 3 EG+HZL (N=6)	Group 4 EG+U (N=6)	Group 1 Control (N=2)	Group 2 EG(N=6)	Group 3 EG+HZL(N=6)	Group 4 EG+U (N=6)
Body-weight (gm) D0	223.04 ± 7.7	195.38 ± 21.28	214.13 ± 36.25	203.93 ± 25.42	371.47 ± 15.76	328.21 ± 30.37	352.83 ± 39.86	349.1 ± 36.14
24-hour urine Volume (ml/day)	20.27 ± 12.28	14.9 ± 6.8	17.2 ± 7.8	15.2 ± 4.9	23.68 ± 3.2	41.2 ± 18.6	34.1 ± 12.8	39.6 ± 20.9
Urine pH	8.0 ± 0.4	8.6 ± 0.3	8.5 ± 0.4	9.0 ± 0	8.0 ± 0.5	7.7 ± 0.8	7.4 ± 0.6	8.0 ± 0.6
Urine Specific gravity	1.031 ± 0	1.038 ± 0	1.032 ± 0	1.039 ± 0	1.03 ± 0	1.170 ± 0.3	1.024 ± 0	1.027± 0
Urine Creatinine (mg/day)	5.3 ± 2	2.6 ± 1.7	3.0 ± 1.8	2.7±2.1	9.3 ± 5	5.1 ± 2	7.0 ± 4.1	5.9 ± 5
Urine total Protein (mg/day)	9.66 ± 2.1	5.96 ± 3	6.30±2.4	7.44± 2.7	10.46 ± 1.2	12.44 ± 3.9	13.56 ± 2.6	16.26 ± 3.4

Data presented as means ± SD.

4.11 Urinary citrate excretion in experimental rats

Urinary citrate after the treatment (day 35) in each group of rats were determined by high performance liquid chromatography (HPLC). The urinary citrate levels at Day 35 in control, EG, EG+HydroZitLa and EG+Uralyt-U were 92.92 ± 50.62, 43.98 ± 20.13, 94.02 ± 86.96 and 02.4 ± 100.1, respectively (Figure 43). Based on one-way ANOVA test, there were no statistical significance of urinary citrate levels among the 4 groups. The baseline of urinary citrate at Day 0 were 118.4 ±24.95, 76.46±53.94, 81.63±53.09 and 60.14±44.85 in control, EG, EG+HydroZitLa and EG+Uralyt-U groups, respectively.

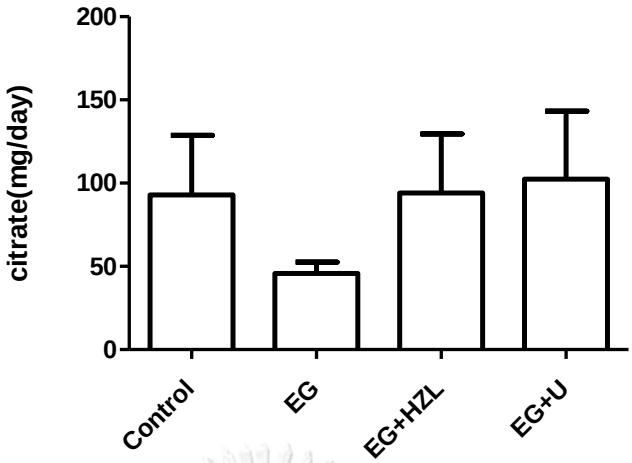


Figure 43 The comparison of urinary citrate level at Day 35
Among control (n=2), EG (n=6), EG+ HydroZitLa (n=6) and EG+ Uralyt-U (n=6) groups

4.12 Urinary CaOx crystallization of experimental rats

To investigate CaOx crystal formation in 24-h urine samples from rats (Day 35) of all four groups, crystallization assay was performed. Representatives of micrographs of crystals formed the urine of each group are shown in Figure 44. After counted, the number of crystals formed among the four groups were not statistically significant (Figure 45).

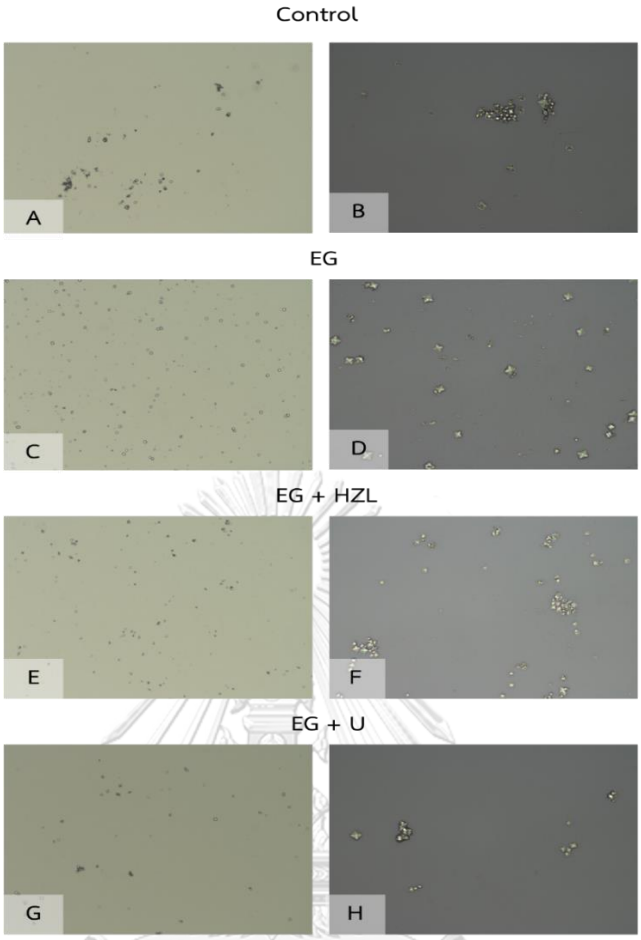


Figure 44 CaOx crystals observed in urine rat 24 hr.

After crystallization assay by light microscope magnification: 100x (A, C, E and G) and 400x (B, D, F and H)

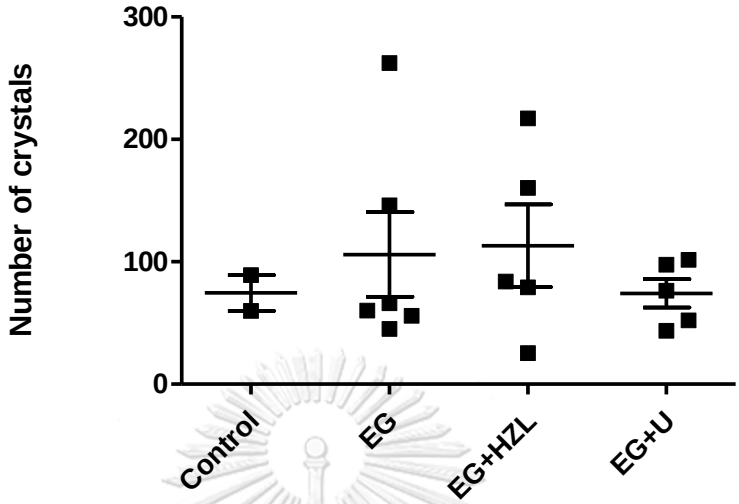


Figure 45 Comparison of number of CaOx crystal formation in 24-h urine of all groups of experimental rats.

CHAPTER V

DISSCUSSION AND CONCLUSION

Potassium citrate drugs for instance Uralyt-U have long been used as a first line drug for treating urinary stone disease. The main active ingredient is citrate that is a vital inhibitor for CaOx crystal formation, growth and aggregation. The mechanism of action of the drug is simple, as it increases urinary excretion of citrate. Although the efficacy of potassium citrate remains clinically sufficient, the major problematic issue is that it has an adverse effect in some cases leading to a low compliance rate(42). As stated in the stone management guideline, an effective period of potassium citrate treatment for prevention of recurrent stone is 6 months(43) Therefore, it is still in need to develop an innovative regimen that has clinically antilithogenic potential with a lesser adverse effect and higher long-term compliance.

As insufficient water intake, low urinary citrate and high oxidative stress (or low antioxidant capacity) are well-known risk factors for urinary stone formation, we had developed a new medicinal beverage called HydroZitLa to correct these three risk factors. A pre-clinical antilithogenic efficacy of HydroZitla was investigated in this study. In vitro experiments demonstrated that HydroZitLa effectively inhibited aggregation of CaOx crystals. The previous studies reported that citrate directly inhibits the natural nucleation of CaOx and also inhibits the crystal growth (44) (45). Although it is not definitely proved in this study, we think that inhibition of COM aggregation by HydroZitLa is mediated through the action of citrate existed in the HydroZitLa.

With its antioxidative property, HydroZitLa attenuated oxidative stress induced COM and H_2O_2 in HK-2 cells. This antioxidative action of HydroZitLa might be a beneficial advantage over the potassium citrate drug. Several studies have suggested that herbal medicine or many medicinal plants have a potential to prevent or treat urolithiasis (46). Many possible pharmacological actions of these herbal extracts have been proposed such as increased urinary citrate excretion, decreased excretion of calcium and oxalate, diuretic effect and antioxidant property. HydroZitLa contained banana stem water extract. Banana

stem juice has been used in India for treating kidney stone, and it has been shown to have an antilithogenic effect in many studies.

Experimental nephrolithiasis has been performed in several models including rat, mouse and fly (47). However, rats induced by administration of EG in drinking water is the most well-established and relatively economical model. In the present study, we used this rat model to investigate the stone inhibitory effect of HydroZitLa compared with the standard potassium citrate drug, Uralyt-U. The results showed that we successfully induced crystal formation and deposits in the kidneys of the experimental rats. Rats in EG+HydroZitLa and EG+Uralyt-U groups had significantly lower amount of CaOx crystals deposits in the kidneys relative to the rats in EG group. Oxidative stress in the kidneys of rats in EG+HydroZitLa and EG+Uralyt-U was also significantly diminished compared to the EG groups. In addition, a trend of increased urinary citrate excretion was observed in the EG+HydroZitLa and EG+Uralyt-U groups. Based on these in vivo findings, we concluded that HydroZitLa effectively inhibited development of nephrolithiasis in the experimental rat model. We believed that the medicinal action of HydroZitLa was mediated through delivering of citrate and antioxidants.

Limitations of the study have to be mentioned. Very small sample size (n=2) of the control rats were presented. However, the main purpose of using normal control rats was only to be a reference of normal histological appearance of the kidneys. Urinary excretion of calcium and oxalate did not measure and compared among groups of experimental rats. Due to the limit of number of metabolic cages (we have only 2 cages), collection of 24-h urine specimens might be problematic, and urine specimens of all rats were not collected at the same days. There has been a critique on EG-induced rat model, the EG may also induce the metabolic acidosis. However, Green et al. stated that metabolic acidosis does not always occurred in EG-treated rats if their renal function is preserved (48).

In conclusion, we successfully induced renal CaOx crystallization and deposits in EG-induced nephrolithic rat model. We demonstrated an antilithogenic activity of HydroZitLa in reducing the number of CaOx crystal deposition in rat kidney, and also attenuating oxidative damage in the rat kidneys. HydroZitLa showed the similar antilithic potential with the

currently used medicine, Uralyt-U. Although the mechanism of HydroZitLa to prevent nephrolithiasis development is not fully understood, citrate and naturally-occurring antioxidants as active ingredients in HydroZitLa might be responsible for the observed antilithogenic action.

APPENDIX

Reagent used in this study

MTT reagent

- 5 mg/mL MTT in PBS
- Store at -20°C
- Working MTT: 0.5 mg/mL

DFCH-DA reagent

- 50 mM DCFH-DA in DMSO
- Store at -20°C
- Working DCFH-DA: 0.5 mM DCFH-DA in serum-free media

RIPA buffer, pH 7.4

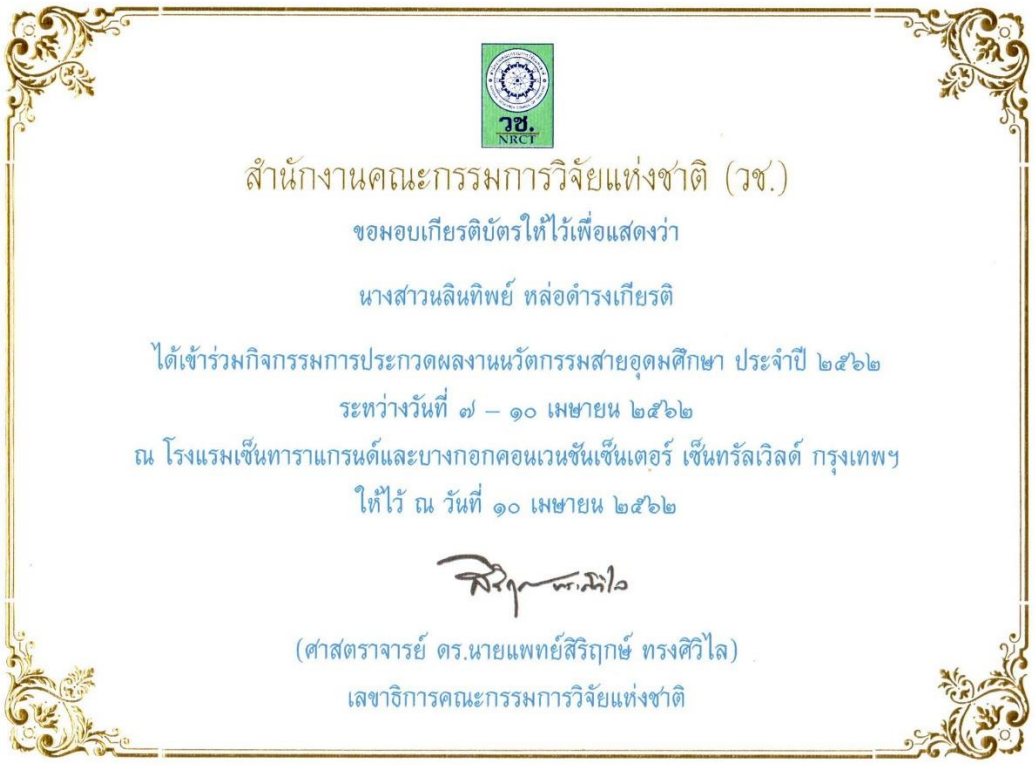
- 6 g/L Tris
- 8.7 g/L NaCl
- 1 g/L SDS
- 1% Triton X-100
- Store at 4°C

Reagent for protein carbonyl assay

- 2 N HCl
- 10 mM DNPH in 2 N HCl
- 20% w/v Trichloroacetic acid (TAC)
- 6 M Guanidine hydrochloride in 0.5 M potassium phosphate, pH 2.5

10X sodium citrate buffer (0.1 M), pH 6.0

- 29.41 g/L Sodium citrate dehydrate ($C_6H_3Na_3O_7 \bullet 2H_2O$
- Store at 4°C
- Working citrate buffer: 1X sodium citrate buffer + 0.05% Tween 20



สำนักงานคณะกรรมการวิจัยแห่งชาติ (วช.)

ขอมอบเกียรติบัตรให้ไว้เพื่อแสดงว่า

นางสาวนลินทิพย์ หล่อตำรงเกียรติ

ได้เข้าร่วมกิจกรรมการประกวดผลงานนวัตกรรมสายอุดมศึกษา ประจำปี ๒๕๖๒

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