ผลของกระบวนการทางความร้อนต่อสารออกฤทธิ์ทางชีวภาพในใบพลับพลึง *Crinum asiaticum*

นางสาวสินีนาฏ เหลาคำ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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EFFECT OF THERMAL PROCESS ON BIOACTIVE COMPOUNDS IN *Crinum asiaticum* LEAVES

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A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	EFFECT OF THERMAL PROCESS ON BIOACTIVE COMPOUNDS IN *Crinum asiaticum* LEAVES
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สินีนาฏ เหลาคำ : ผลของกระบวนการทางความร้อนต่อสารออกฤทธิ์ทางชีวภาพในใบพลับพลึง *Crinum asiaticum* (EFFECT OF THERMAL PROCESS ON BIOACTIVE COMPOUNDS IN *Crinum asiaticum* LEAVES) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.นาตยา งามโรจนวณิชย์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร.นงนุช เหมืองสิน, 56 หน้า.

สารสกัดจากใบพลับพลึงที่ผ่านกระบวนการให้ความร้อน 2 รูปแบบ คือ การนึ่ง (อุณหภูมิ 95 ºC, 1 ชั่วโมง) และการอบแห้ง (160 ºC, 10 นาที) เปรียบเทียบกับสารสกัดจากใบพลับพลึงสด ใบพลับพลึงนึ่งและใบพลับพลึงอบแห้งให้ปริมาณสารไลโครีนซึ่งเป็นสารออกฤทธิ์ทางชีวภาพที่สำคัญในใบพลับพลึงเป็น 3.69±0.04 และ 4.48±0.02 mg/mL ตามลำดับ ในขณะที่ใบพลับพลึงสดให้ปริมาณไลรีน 2.35±0.09 mg/mL ซึ่งใบพลับพลึงที่ผ่านกระบวนการอบแห้งให้ปริมาณไลโครีนมากถึงเกือบ 2 เท่า ของใบพลับพลึงสด ดังนั้นใบพลับพลึงที่ผ่านการให้ความร้อนด้วยกระบวนการอบแห้งจึงถูกเลือกเพื่อศึกษาผลของอุณหภูมิ (50, 70, 100 และ 120 ºC) และเวลา (10, 30, และ 60 นาที) ต่อสารออกฤทธิ์ทางชีวภาพในใบพลับพลึง พบว่าใบพลับพลึงที่ผ่านการอบแห้งที่อุณหภูมิ 50 ºC เป็นเวลา 30 นาที ให้ปริมาณไลโครีนมากที่สุด สารสกัดทั้งหมดได้ทดสอบฤทธิ์ในการต้านแอนตี้ออกซิแดนท์พบว่าใบพลับพลึงอบแห้งที่อุณหภูมิ 50 ºC เวลา เป็นเวลา 30 นาที และ 100 ºC เป็นเวลา 10 นาที ออกฤทธิ์ต้านแอนติออกซิแดนท์ได้ดีที่สุด นอกจากนั้น สารสกัดทั้งหมดได้ทดสอบฤทธิ์ต้านมะเร็งทั้ง 5 ชนิด ได้แก่ SW620 CHAGO KATO-III Hep-G2 และ BT474 โดยใช้วิธี MTT สารสกัดจากใบพลับพลึงอบแห้งที่อุณหภูมิ 50 ºC เป็นเวลา 30 นาที มีฤทธิ์สูงในการยับยั้งมะเร็งกระเพาะอาหาร KATO-III ซึ่งมีค่า IC_{50} เป็น 1.5 ± 0.26 µg/mL นอกจากนั้นสารสกัดจากใบพลับพลึงอบแห้งที่อุณหภูมิ 50 ºC เป็นเวลา 30 นาทียังไม่มีความเป็นพิษต่อเซลล์ปกติ (WI-38) ที่ปริมาณทดสอบ 50 µg/mL อีกด้วย มากกว่านั้นเรายังได้ศึกษาผลของสารสกัดจากใบพลับพลึงต่อการยับยั้งการอักเสบ พบว่าที่ความเข้มข้นสารสกัด 0.1 µg/mL ใบพลับพลึงอบแห้งที่อุณหภูมิ 50 ºC เป็นเวลา 30 นาที และ 100 ºC เป็นเวลา 10 นาที สามารถยับยั้งการอักเสบได้ดีที่สุด โดยยับยั้งได้ดีกว่าสารประกอบพาทีโนไลด์ ในขณะที่ใบพลับพลึงสดมีฤทธิ์ต่ำที่สุด ดังนั้นใบพลับพลึงที่ผ่านการกระบวนการให้ความร้อนด้วยวิธีอบแห้ง โดยเฉพาะการอบแห้งที่อุณหภูมิ 50 ºC เป็นเวลา 30 นาที มีผลในการเพิ่มปริมาณไลโครีน ซึ่งเป็นสารประกอบที่มีศักยภาพในการต้านแอนตี้ออกซิแดนท์และมะเร็งกระเพาะอาหาร นอกจากนั้นสารสกัดจากใบพลับพลึงที่ผ่านการอบแห้งยังมีประโยชน์ในการพัฒนาต่อสำหรับต้านการอักเสบได้

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

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SINEENAT LAOKAM: EFFECT OF THERMAL PROCESS ON BIOACTIVE COMPOUNDS IN *Crinum asiaticum* LEAVES. ADVISOR: ASSOC. PROF. NATTAYA NGAMROJANAVANICH, Ph.D., CO-ADVISOR: ASSOC. PROF. NONGNUJ MUANGSIN, Ph.D., pp.

Crude extracts from heated-*Crinum asiaticum* Linn leaves by 2 processes including steaming (95 ºC, 1 h) and oven drying (160 ºC, 10 minutes) compared with crude extracts of fresh *Crinum asiaticum* Linn leavevs. Steamed leaves and oven dried leave gave the lycorine content, which is important bioactive compound in *Crinum asiatiucum* Linn leaves were 3.69±0.04 and 4.48±0.02 mg/mL, respectively. While lycorine content of the fresh leaves was 2.35±0.09 mg/mL, which oven dried leaves gave lycorine content approximately to 2 fold of fresh leaves. Therefore, heated-*Crinum asiaticum* Linn leaves by oven-drying process were chosen for study the effects of various temperatures (50, 70, 100 and 120 ºC) and times (10, 30, and 60 minutes) on bioactive compounds in *Crinum asiaticum Linn* leaves. Oven dried *Crinum asiaticum* Linn leaves of temperature at 50 ºC for 30 minutes was the highest lycorine content. All crude extracts were tested antioxidant activities that the heated leaves at 50 ºC for 30 minutes and 100ºC for 10 minutes showed the greatest antioxidant activity. Moreover, all crude extracts were tested cytotoxic activities against 5 cancer cell lines including SW620, CHAGO, KATO-III, Hep-G2 and BT474 by MTT assay. Crude extracts of oven dried-*Crinum asiaticum* Linn leaves of temperature at 50 ºC for 30 minutes showed high cytotoxic activity against gastric cancer cell line (KATO-III), IC_{50} was 1.5 ± 0.26 µg/mL. Moreover, Crude extracts of oven dried *Crinum asiaticum* Linn leaves of temperature at 50 ºC for 30 minute were non-toxic to lung normal cell line (WI-38) at 50 µg/mL. Furthermore, we studied the effect of crude extracts from *Crinum asiaticum* Linn leaves for anti-inflammatory. For 0.1 µg/mL, The heated *Crinum asiaticum* Linn leaves of 50 ºC for 30 minutes and 100ºC for 10 minutes showed the greatest inhibited inflammation, which is more than the parthenolide compound. While the fresh leaves exhibited minimal effect. Therefore, heated-*Crinum asiaticum* Linn leaves by oven-drying process especially oven dried leavevs of temperature at 50 ºC for 30 minute had effect to increased lycorine content which has efficacy for antiantioxidant and against gastric cancer cell line. Moreover, crude extracts of oven dried *Crinum asiaticum* Linn leaves can helpful to develop for anti-inflammatory.

Field of Study: Biotechnology
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Student's Signature .....
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LIST OF ABBREVIATIONS

mL	milliliter
µL	microliter
mg	milligram
g	gram
µM	micromolar
ºC	degree Celsius
min	minute
h	hour
MeOH	methanol
CD_3OD	deuterated methanol
DMSO-d_6	deuterated dimethylsulfoxide
DDW	double-deionized water
nm	nanometer
m/z	mass per charge ratio
MALDI-TOF	matrix-assisted laser desorption ionization-time of flight
MS	mass spectroscopy
NMR	nuclear magnetic resonance spectroscopy
v/v	volume/volume
w/v	weight/volume
δ_H	proton chemical shift
δ_C	carbon chemical shift

CHAPTER I
INTRODUCTION

1.1 Introduction

Thermal processing is well known that natural nutrients could be significantly lost during the thermal processing due to the fact that most of the bioactive compounds are relatively unstable to heat. However, thermal processing can found increasing of biological compounds and new compounds in plants such as ginseng, turmeric and ginger [1-4]. Moreover, thermal processing could be increased antioxidant and anticancer activities of citrus peel and ginseng, respectively [5, 6]. Ancient time, indigenous people used the heat from grilled herbal leaves to treat the aches, arthritis pain, swelling and bruising. Therefore, thermal processing can be used to enhance the biological activities of herbal plants.

Antioxidant is a molecule that inhibits the oxidation of other molecules, which the oxidation reaction can produce free radical in the body. Free radical can cause of chronic diseases such as cancer and inflammation.

Cancer is one of disease that leading cause of death worldwide. Cancer can rapidly spread to other tissues of bodies due to unregulated proliferation. There are many ways for cancer treatment, including chemotherapy, surgery, radiotherapy and combination of these treatments. Among them, chemotherapy is the common treatment by using chemotherapeutic drugs to destroy cancer cells such as doxorubicin, amonafide and cisplatin. However, these drugs also kill normal cells resulting in side-effects such as vomiting and hair loss. Thus, bioactive compounds from herbal plants was more selected for cancer treatment due to it is less toxic to normal cells than the chemical drugs.

Inflammation is processes of body tissues that response to harmful stimuli that cause tissue injury such as pathogen and dead cells from losing blood and oxygen. The initial mechanism of inflammation, white blood cell move to the inflamed area, and then free radicals are created to eliminate pathogen and tissues. Protective response of inflammation involves immune cells, blood vessels and molecular

mediators for example, pro-inflammatory cytokine, anti-inflammatory cytokine, chemokine, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). Moreover, prolonged or chronic inflammation can cause tissue destruction and tissue malfunction. The symptom is joints pain and arthritis, which is more common in the elderly such as osteoarthritis, rheumatoid, gout and myelitis. Non-steroidal anti-inflammatory drug (NSAID) widely used to treat the patients, is a good drug for pain treatment especially the pain from inflammation. However, NSAID has virulent side-effects to gastroenterology including stomach and intestine such as nausea, vomiting, abdominal pain and diarrhea [7]. Therefore, the researchers are interested in herbal plants to treat various diseases.

Due to natural product is less toxic to normal cells, thus it is the most candidates for many drugs. *Crinum asiaticum* Linn. (Crinum lily) is common plant distributed in Southeast Asia including Thailand. It is small to moderate herbal plant with greenish leaves that is famous in injury and joints pain treatment. Indonesian people used *C. asiaticum* leaves smeared with warmed oil to treat wounds by poisoned arrows [8]. Moreover, it also used for various disease treatments such as anti-inflammatory, anti-allergic and anticancer. *C. asiaticum* is source of bioactive compounds such as crinamine, ungeremine, lycoriside, criasbetaine, epilycorine and lycorine [9]. Lycorine is mainly bioactive compound of this plant. It was the first alkaloid of Amaryllidaceae from *Narcissus pseudonarcissus* that found in 1877 [10]. Previously reports, lycorine was used for anticancer, antiviral, antimalarial and anti-inflammatory [11-15].

1.2 Objectives of this research

1. To study effect of heated *Crinum asiaticum* leaves at various temperatures (50, 70, 100 and 120 ºC) and times (10, 30 and 60 minutes) on lycorine content.
2. To study antioxidant acitivties of crude extracts from the heated *Crinum asiaticum* leaves.
3. To study anticancer activities of crude extracts from the heated *Crinum asiaticum* leaves.
4. To study anti-inflammatory activities of crude extracts from the heated *Crinum asiaticum* leaves.

CHAPTER II

BLACKGROUND AND LITERATURE REVIEWS

2.1 *Crinum asiaticum* Linn (Crinum lily)

The *Crinum asiaticum* Linn (*C. asiaticum* L.) is a common herbaceous plant that widely distributed in Southeast Asia and Thailand. It has used for various treatments such as anti-inflammatory, anti-allergic and anticancer. In addition, *C. asiaticum* L. is a famous traditional medicine in Southeast Asia showing a potential treatment of injury and inflamed joints [8].

In 1956, Chopra et al. used the leaves of *C. asiaticum* L. for expectorants, against skin diseases and inflammation processes [13].

In 1995, Paul et al.[16] gave the information that people of Indonesia uses the leaves of *C. asiaticum* L. which was smeared with coconut oil. The smeary leaves was heated and then stroked on pains of the kidney part. The result showed that the pain was relieved.

Figure 1 Feature of *Crinum asiaticum* Linn (Crinum lily).

Many chemical constituents in *C. asiaticum* including (1) ungeremine, (2) lycorine-1-0-1-ß-D-glucoside, (3) crinamine, (4) belladine, (5) lycoriside, (6) pratorimine, (7) anhydrolycorine, (8) criasbetaine, (9) epilycorine and (10) lycorine.

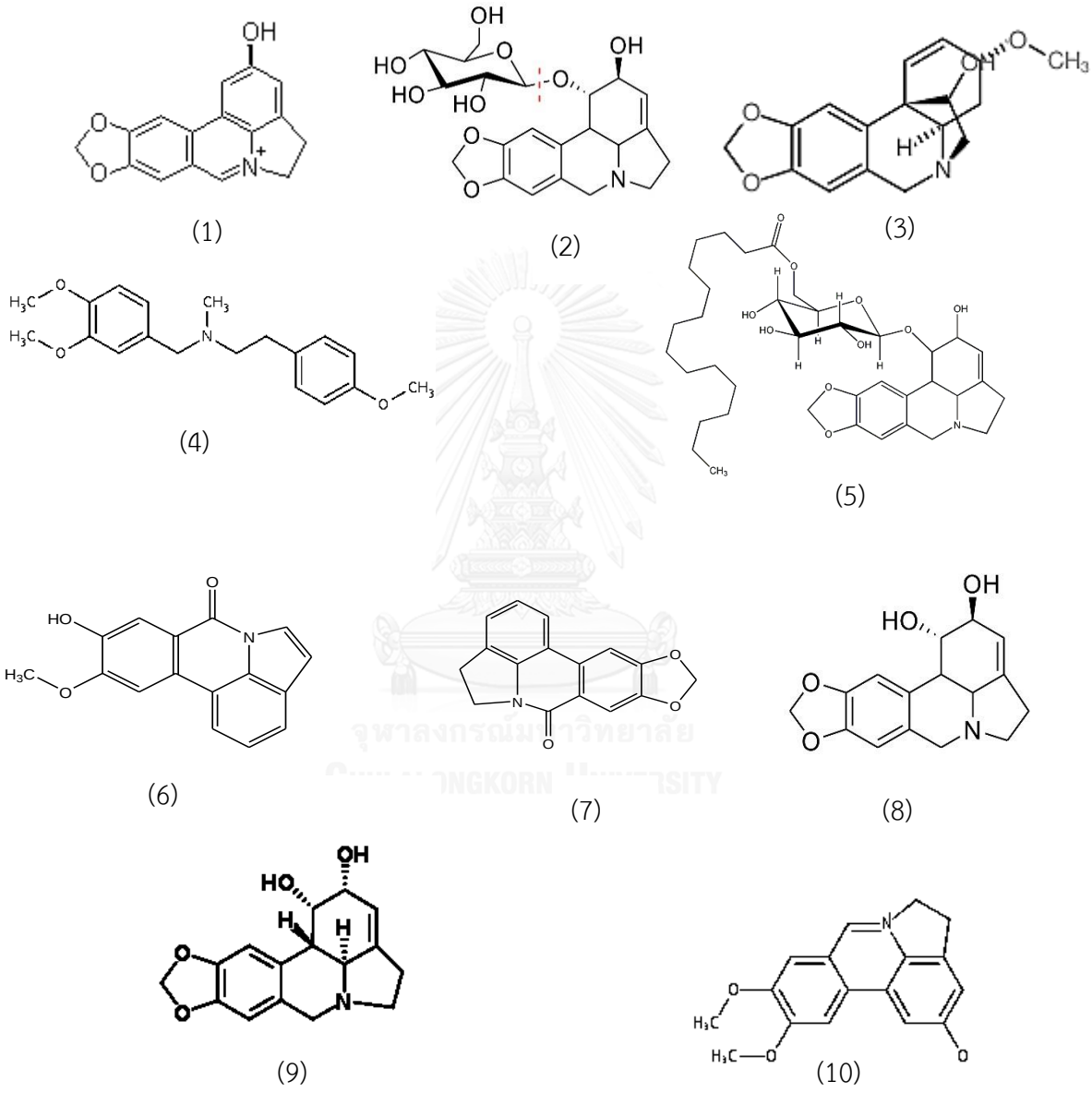

Figure 2 Chemical structure of compounds in *Crinum asiaticum* leaves.

In 1976, Ochi et al. extracted new alkaloid from fruit of *Crinum asiaticum* L. and it was called hamayne [17].

In 1986, Ghosal et al. extracted lycoriside and acylglucosyloxy alkaloid from *Crinum asiaticum* L. The lycoriside showed a good activity against Tween 80-induced degranulation, as also to sensitized mast cells challenged with an antigen (horse serum)[18] .

In 1986, Ghlsal et al. reported a good anticancer activity of lycobetaine and criasbetaine alkaoloids from *Crinum asiaticum* L. [19].

In 2008, Sun et al. found a new phenolic compound, 1-(2-hydroxy-4-hydroxymethyl) phenyl-6-0-caffeoyl-ß-D-gluco-pyranoside, in a bulb of *Crinum asiaticum* L. [20].

In 2001, Barthelmes demonstrated lycobetaine (ungeremine) that inhibited the growth of human tumour cells by reacting with topoisomerase IIß [21].

In 2006, Kim et al. extracted crinamine from aerial parts of *Crincum asiaticum* L.. The extracted product could inhibit hypoxia inducible factor-1 (HIF-1) and showed the IC_{50} of 2.7 µM [22] .

The major biological active compound of *C. asiaticum* is lycorine (8) (Figure 2). It shows various biological effect such as anti-tumor and anti-inflammatory which is reported from several researches.

In 1998, Yui et al. reported the strong inhibitory effect of lycorine on tumor cell. It could inhibit protein translation by working with polymorphonuclear leukocyte-derived calprotectin [11].

In 2010, Lamoral-Treys et al. reviewed capability of lycorine for anticancer design. Lycorine shows very promising anti-tumor activities to various cells such as leukemia, lumphoma, carcinoma, multiple mueloma and melanoma cell lines, which IC_{50} was shown in Table 1 [12].

Table 1 *In vitro* anti-tumor activities of lycorine.

Compound	*In vitro* Growth Inhibitory Concentration (IC_{50})
Lycorine	$0.5 < IC_{50} < 10$ μM on various types of leukemia and lymphoma cell line $3 < IC_{50} < 10$ μM on various types of carcinoma, multiple myeloma and melanoma cell lines.

In 2012, Kang et al. performed the effect of lycorine on anti-inflammatory and found that lycorine inhibited lipopolysaccharide-induced nitric oxide (NO), PGE_2, TNF-α, IL-6 production and iNOS expression in rat macrophage cancer cell line (RAW264.7) and the survival rate of mice after LPS stimulation was increased [15].

2.2 Thermal Processes

Thermal processing is generally used to prolong shelf life of foods. Thermal processes divide into many methodologies including;

(1) Boiling process, is well known that natural nutrients could be significantly lost during the thermal processing due to most of bioactive compounds are relatively unstable to heat. Previous reported, curcumin and piperine of turmeric and red pepper were decomposed by the boiling process [3].

(2) Roasting process, Xu and coworker suggested that the content of benzoic acids and cinnamic acids of citrus peels was significantly increased after the roasting process [5]. The oven dried process caused the loss of gingerol and shogaol from ginger [4]. However, this process is difficultly controlled temperature and time. Moreover, some biological active compounds could be destroyed after roasting process.

(3) Steaming process, many researches have been reported the steamed treatment of herbs can increase the active ingredients and hence increase the biological or pharmaceutical activities. The new biological compounds in plants such as ginseng to obtain ginsenoside (Rg3, Rh2) that showed strong anti-proliferation against cancer cells such as human colon carcinoma cell (HCT-116) and human colon adenocarcinoma cells (SW-480) [2, 23], but this process is difficultly controlled temperature and time.

(4) Oven dried process is a typical process for preserving herbs such as tea, bergamot leaves. Some works have been reported that the oven dried treatment reduced the amount of active ingredients because of decomposition. In contrast, this treatment can increase of the major active ingredients, for example increasing of flavonoids from wheatgrass [24]. Moreover, there are many advantage of this process such as prolonging storage time of products, controlling the temperature and time. Thus, it is worth to investigate the amount of lycorine after process the oven dried treatment.

2.3 Inflammation

Inflammation is one of the leading factors related in carcinogenesis and other degenerative disorders. Inflammation is a process which the body's white blood cells and substances produce the protection from infection with foreign organisms, such as bacteria and viruses as shown in Figure 3. There is a large body of evidence that nitric oxide (NO) is involved in several inflammatory disorders. Virtually every cell and many immunological parameters are modulated by NO [25].

Nitric Oxide (NO) is produced by inducible nitric oxide synthase (iNOS). In mammals including humans, NO is an important molecule involving in many physiological and pathological processes included defense various pathogen such as viruses, bacteria, fungi and parasites [26]. However, excessive production of NO effects on tissue damage related with acute and chronic inflammations.

Lipopolysaccharide (LPS) is the major constituent of outer membrane of gram-negative bacteria. LPS induces expression of pro-inflammatory cytokines such as NO produced by iNOS. Thus, the inhibition of NO on LPS-stimulated RAW 264.7 cell line is one of principles to screen drugs for anti-inflammatory.

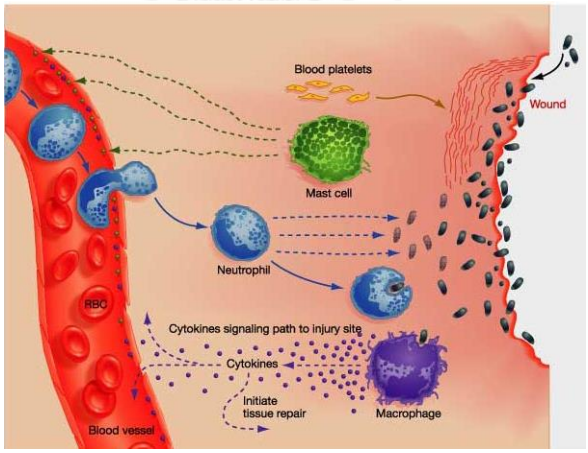

Figure 3 Inflammation process [27].

2.4 Anti-inflammatory drugs

Anti-inflammatory drugs are the property of treatment that reduces inflammation or swelling. Most of these drugs make up of analgesics. At present, considering the anti-inflammatory drugs that doctors use with patients is non-steroidal anti-inflammatory drugs)NSAIDs(which is great of analgesics drugs such as aspirin and indomethacin.

Side-effects of NSAIDs:

- Long- term use NSAIDs can cause gastric erosions.
- For extreme cases, NSAIDs cause severe haemorrhage, resulting in death.
- Other dangers are kidney damage, the risk of myocardial infarction and stroke.

2.5 Parthenolide

Parthenolide (Figure 4), is a sesquiterpene lactone of the germacranolide. Parthenolide inhibited eicosanoids and the transcription factor nuclear factor kappa-B (NF-KB) [28]. It was demonstrated good anti-inflammatory activities. However, parthenolide has affected to the smooth muscle of the body and potential thin the blood, thus it is danger for who take aspirin and blood thinners.

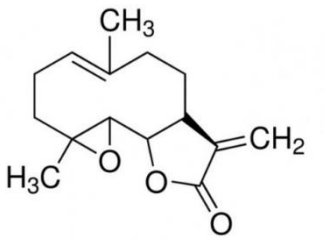

Figure 4 Structure of Parthenolide.

2.6 Cancer

Cancer is a worldwide disease, which is the leading causes of death every year. Cancer cells are abnormal of cell proliferation with the potential to invade or rapidly spread to other tissues of bodies. The cancer are caused by a series of mutation involving uncontrolled cell division as shown in Figure 5.

Around one third of cancer deaths are due to habitats and dietary risks concluding high body mass index, low fruit and vegetable intake, lack of physical activity, tobacco and alcohol. The principle cancer treatment include chemotherapy, surgery, radiotherapy and combination of these treatments, depending on stage and type of cancers.

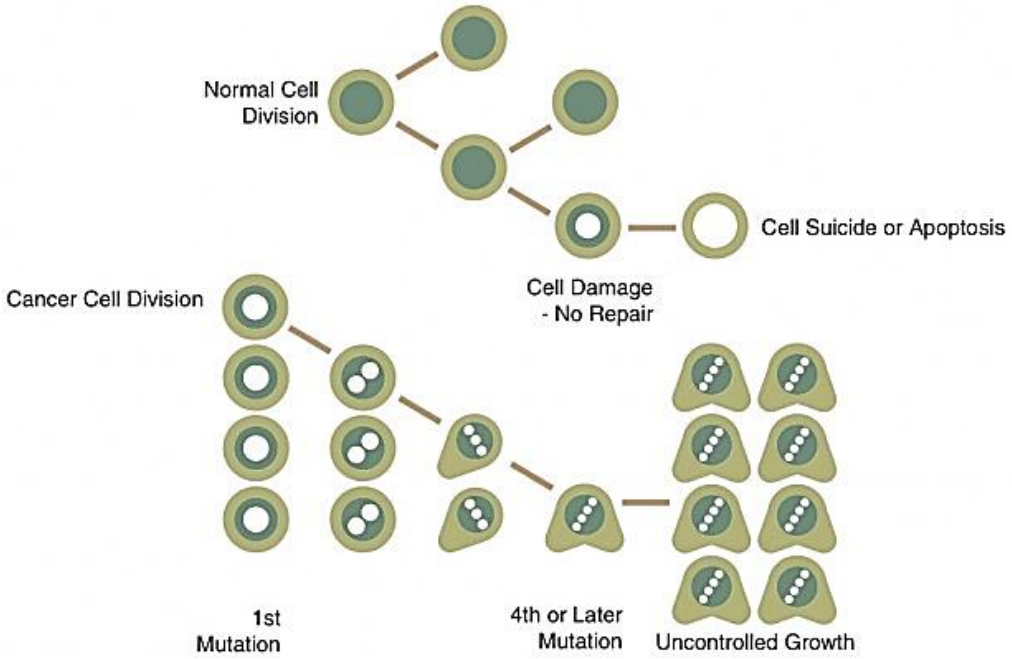

Figure 5 Cell division of normal cells and cancer cells [29].

2.7 Anticancer drugs

Anticancer drugs are agents for kill cancer cells or modify their growth such as doxorubicin and amonafide. It is chemotherapy may use one or several drugs at time, and the most of chemotherapeutic agents often have many side-effects to normal cells.

2.8 Doxorubicin

Doxorubicin (Figure 6) consists of four fused rings and positively charged amino sugar. It is a commercial drug that used to treat many types of cancer such as leukemias, hematological malignancies, germ cell tumors and carcinoma. It works by blocking topoisomerase II, consequently, the cancer cells cannot divide and grow. However, doxorubicin has many general side effects such as hair loss, myelosuppression, nausea and vomiting during treatment. Moreover, the dangerous effect of doxorubicin is to cause congestive heart failure.

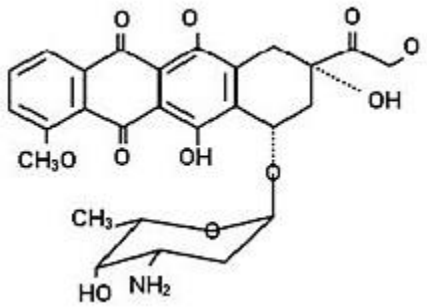

Figure 6 Structure of doxorubicin**.**

2.9 Amonafide

Amonafide (Figure 7) is anticancer agent. The amonafide analogs were the first anticancer drug that was treated in the clinical experiment and showed good anticancer activty. This drug can against cancer cells by inhibiting topoisomerase II and DNA intercalation. However, amonafide caused a high-variable and unoredictable toxicity due to its $-NH_2$ group was easily metabolized to *N*-acety-amonafide by enzyme *N*-acetyltransferase 2 (NAT2).

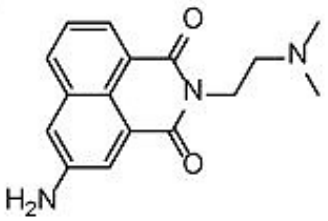

Figure 7 structure of amonafide**.**

2.10 NO determination (Griess Reaction)

The griess reagent system is used for detecting nitrite (NO_2^-) based on the chemical reaction of sulfanilamide and *N*-1-napthylethylenediamine dihydrochloride (NED) under dark condition as shown in Figure 9. Nitrite is detected and analyzed by formation of a red pink color upon treatment of a nitrite containing sample with the griess reagent. Nitrite gives a red pink color with griess reagent, which demonstrated that highly inflammatory.

The color solution is measured by UV-Vis spectrophotometer measured absorbance at 540 nm. The concentration of nitrite is determined by comparison the average absorbance of each sample to the nitrite standard reference curve.

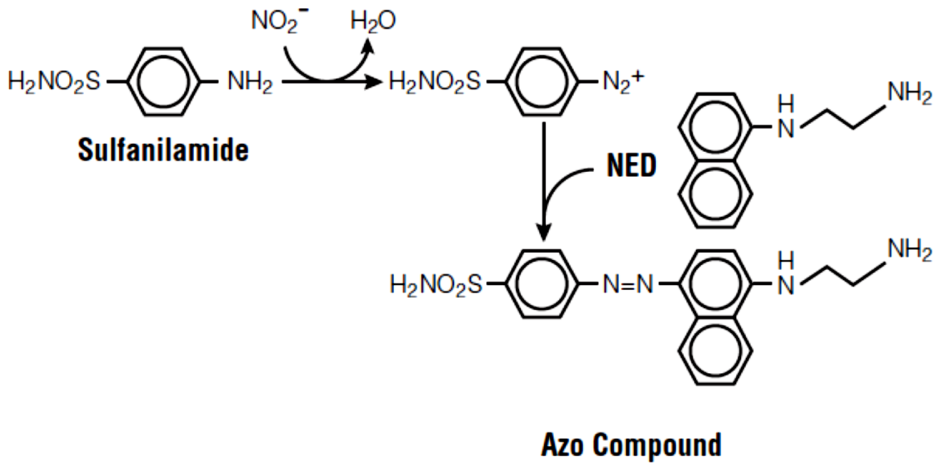

Figure 8 Chemical reactions involved in the measurement of NO_2^- using the griess reagent system [30].

2.11 MTT assay

MTT assay is colorimetric assay for assessing cell viability. The yellow tetrazole of MTT is reduced to purple formazan in living cells, which is the action of mitochondrial reductase enzymes of active cells as shown in Figure 9. Thus, died cells lost the ability to convert MTT into purple formazan.

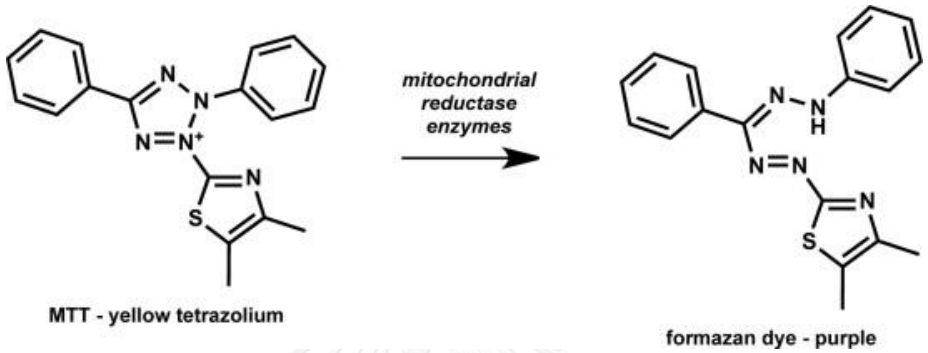

Figure 9 Reduction of MTT into formazan dye in living cells[31].

MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], terazolium reduction assay is converted into a purple colour formazan product. The colour solution is measured by UV-Vis spectrophotometer (measure absorbance at 540 nm), which the amount of survival cells are directly proportional of formazan creation. Cell viability can calculated by the following equation:

$$\% \text{ Cell viability} = \frac{\text{Absorbance of treated cell in well}}{\text{Absorbance of untreated cell in well}} \times 100$$

CHAPTER III

EXPERIMENTAL

3.1 MATERIALS AND INSTRUMENTS

3.1.1 Chemicals and solvents

The chemical reagents were used in this study purchased from Sigma-Aldrich and Fluka chemical Co., Ltd.. All solvent in analytical reagent grade (AR grade) were used for preparing compounds and the reagents of HPLC grade were used for HPLC procedures.

- Methanol (CH_3OH or MeOH)
- Acetronitrile (CH_3CN)
- Potassium dihydrogen phosphate (KH_2PO_4)
- Dimethyl sulfoxide (DMSO)
- Ethyl Acetate ($C_4H_8O_2$)

3.1.2 Extraction and Instruments

- Reflux condenser
- Round bottle flasks
- Magnetic bar
- Rotary evaporator
- Thin layer chromatography (TLC)
- Erlenmeyer flask
- Beaker

3.1.3 Cell Culture and Materials

3.1.3.1 Cell lines

- Colon carcinoma (SW620)
- Lung carcinoma (CHAGO)
- Breast carcinoma (BT474)
- Gastric carcinoma (KATO-III)
- Hepatocellular carcinoma (Hep-G2)
- Rat macrophage (Raw 264.7)
- Normal cell, human lung fibroblast cell line (WI-38)

3.1.3.2 Media and Enzyme

- Dulbecco's Phosphate-Buffered Saline (DPBS)
- Fetal Calf Serum (FCS)
- Trypsin
- Rosewell Park Memorial Institute's medium (RPMI)
- Dullbecco's MEM (DMEM)

3.1.3.3 Cell Culture Apparatus

- Laminar Flow hood
- Incubator
- Centrifuge
- Vortex
- Microscope
- Cell culture flask 25mL
- Tubes
- Micropipette
- Multi-channel pipette

- Eppendorf
- 96-well plates
- Counting Slides
- Volumetric Pipette
- Dropper
- Forceps
- Rubber bulb

3.1.4 MTT Assay Materials and Apparatus

- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)
- Normal Saline Solution (NSS)
- Dimethyl Sulfoxide (DMSO)
- Glycine
- Multi-channel pipette
- Microplate reader Spectrophotometer

3.1.5 Griess Reagent System and Reagent for anti-inflammatory assay

- Sulfanilamide solution (1% sulfanilamide in 5% phosphoric acid)
- NED solution (0.1% N-1-napthylethylenediamine dihydrochloride in water)
- Nitrite standard (10 mM sodium nitrite in double-deionized water)
- Lipopolysaccharide (LPS)
- interferon-gramma (INF-γ)
- Parthenolide

3.2 Plant materials

Crinum asiaticum leaves (crinum lily) were purchased from Pak Khlong Talad (Bangkok, Thailand) in 2013. *C. asiaticum* leaves were cleaned and sliced. It was divided into three groups for study the effect of heated processing including 1) fresh leaves (untreated), 2) steamed leaves and 3) oven dried leaves (using 1 kilogram of fresh leaves). Moreover, thirteen groups of fresh leaves were prepared for thermal treatment by using oven drying (including untreated leaves and heated leaves at temperature of 50, 70, 100 and 120 °C for 10, 30 and 60 minutes). Each group was used 100 grams of wet weight of *C. asiaticum* leaves and were done in triplicated for each groups.

3.3 Heat treatment

For preliminary study, the leaves of *C. asiaticum* were divided into three groups; in 1) fresh leaves, 2) steamed leaves, and 3) oven dried leaves. Each of group was prepared from 1 kilogram of the fresh leaves.

1) The fresh leaves (untreated) as untreated condition were used immediately without drying.

2) Steamed leaves were performed using steamer, the fresh leaves were steamed in a closed container at temperature approximately 95 ℃ for 1 h, and then the leaves were dried at room temperature.

3) Oven dried leaves were operated by drying oven at temperature 160 ℃ for 10 minutes.

Moreover, the leaves of *C. asiaticum* were subjected to heat treatment by using drying oven for various temperature at 50, 70, 100 and 120 ℃ and various times for 10, 30 and 60 minutes and the fresh leaves (untreated) as control condition. These conditions were chosen by using condition that comprehensive with phase of steaming and oven dried process for searching the optimum condition. Experimental was done in triplicate for each treatments. All heat-processed leaves were grinded with a blender and extracted in the next step as shown in section 3.4.

3.4 Extraction procedures

100 grams of the heated leaves of *C. asiaticum* (starting material) were extracted and purified from *C. asiaticum* leaves using the method modified from a standard procedure reported by [32]. Heat processed leaves were immersed and extracted with 500 mL of methanol for 24 hours and repeated extraction until the solution was cleared. The extracted solution was filtered and methanol extracts were evaporated in a rotary evaporator under reduced pressure, to give a greenish-brown oil of crude extracts. Then, the lycorine contents were detected and determined by high performance liquid chromatography (HPLC) and characterization by ^{1}H NMR, ^{13}C NMR and MS-MALDI TOF.

3.5 Analytical instruments

3.5.1 High Performance Liquid Chromatography (HPLC)

Crude extracts (10 mg) and lycorine (1 mg) were dissolved in methanol and the compounds were investigated and calculated amount of major compound by HPLC. HPLC analysis was carried out using a liquid chromatographic system (Spectra system), instated with a uv variable-wavelength detector (spectra system uv 6000LP), a quaternary pump system (spectra system P4000), vacuum degasser (spectra system SN4000), a manual injector with 100 µL loop (RESTEK SGE syringe 100R-GT-LC-SS) and a chromatographic data processing software (windows 2001). The chromatographic assay for lycorine as major biologically active compound in *Crinum asiaticum* and crude extracts were operated on PinnacleII C18 Column (250 mm × 4.6 mm; 5 µm particle size) at 290 nm. The mobile phase was 0.05 M KH_2PO_4: CH_3CN (96:4) applied at a flow rate of 0.7 mL/min, injection volume 20 µL.

Calibration curve of lycorine: A calibration curve was generated to confirm the linear relationship between the peak area (y) and the concentration of lycorine standard (x). Standard lycorine was injected to HPLC at concentrations of 0.5, 1.0, 2.0, 4.0 and 8.0 mg/mL. The sample with unknown amount of lycorine was evaluated by using a calibration curve of lycorine at concentration 0.5, 1.0, 2.0, 4.0 and 8.0 mg/mL.

3.5.2 Nuclear magnetic resonance spectroscopy (NMR)

The ^{1}H NMR spectra at 400 MHz and ^{13}C NMR spectra at 101 MHz were measured on a Varian Mercury plus spectrometer and a Bruker model ACF200 spectrometer, respectively. All chemical shifts (δ) were reported in part per million (ppm) relative to the residual proton or carbon signal in deuterated solvents peak using CD_3OD, and DMSO-d_6 solvent as internal reference. Coupling constants (*J*) were reported in Hertz (Hz). The ^{1}H NMR and ^{13}C NMR data were processed with the MestReNova software.

3.5.3 Mass spectrometry (MS)

The mass spectra were recorded on a Bruker MALDI-TOF mass spectrometer and α-cyano-4-hydroxycinnamic acid (HCCA) was used as standard mass spectra. The MS data were assessed with the MestReNova software.

3.6 Biological assay

3.6.1 Antioxidant by DPPH assay

All crude extract were diluted in methanol (stock 10mg/ml), added 40 µL/well of these samples to 96 well plates. Then, added 160 µL of 0.1M DPPH solution in each well, after that incubated at room temperature for 30 minutes (dark condition). The absorbance was measured at 540 nm by a 96 well plate reader. Data was collected for three replicated and calculated as:

$$\% \text{ DPPH activity} = \frac{A - B}{A} \times 100$$

Where;

A: Absorbance of DPPH

B: Absorbance of sample contained DPPH

3.6.2 Anti-cancer assay

3.6.2.1 Cell culture

The cytotoxic activity of all compounds were evaluated by using the MTT assay against five cancer cell lines of colon cancer cell line (SW620), lung cancer cell line (CHAGO), liver cancer cell line (HepG2), gastric cancer cell line (KATO-III) and breast cancer cell line (BT474). All cancer cell line were cultured in RPMI-1640 medium supplemented with 10% (v/v) of fetal bovine serum (FCS). WI-38 was normal cell lines cultured in DMEM medium supplemented with 10% (v/v) of fetal bovine serum (FCS). Cultured Cell lines were incubated in humidified atmosphere of 5% CO_2 at 37 ºC and then it were sub cultured by trypsin enzyme every three days (72 hours).

3.6.2.2 Cytotoxicity by MTT assay

Cytotoxicity of lycorine and crude extracts were investigated against five cancer cell lines. The cancer cells were seeded into 96 well plates at density of 5×10^3 cells (180 µl/well), incubated at 37 ℃ for 24 h. Added 20 µL/well of each compound that diluted in media. After incubated 72 h, 10 µL of MTT solution (5 mg/mL) was added

to each well and the plates were incubated at 37 °C for 4 h. Then, the media containing MTT was dissolved by 150 µL of DMSO to each well. The absorbance was measured at 540 nm by a 96 well plate reader. Data was collected for three replicated and calculated as:

$$\% \text{ Cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100$$

% Cytotoxicity = 100 - percentage of cell viability

3.6.3 Anti-Inflammatory assay

3.6.3.1 Cell culture

The murine macrophage RAW 264.7 was cultured in DMEM containing 10% (v/v) of fetal bovine serum (FCS) and incubated in moisture-enriched atmosphere in 5% CO_2 at 37 °C. Cell lines were sub cultured by trypsin every three days.

3.6.3.2 Cell viability by MTT assay

Cytotoxicity of lycorine and crude extracts were investigated against RAW 264.7 cell lines. The RAW 264.7 cells were seeded into 96 well plates at density of 5×10^3 cells (180 µL/well), incubated at 37 °C for 24 h. Added 20 µL/well of each compound that diluted in media. After incubated 72 h, 10 µl of MTT solution (5 mg/mL) was added to each well and the plates were incubated at 37 °C for 4 h. Then, the media containing MTT was dissolved by 150 µL of DMSO to each well. The absorbance was measured at 540 nm by a 96 well plate reader. Data was collected for three replicated and calculated as:

$$\% \text{ Cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100$$

% Cytotoxicity = 100 - percentage of cell viability

3.6.3.3 Calibration curve of nitrite standard.

Stock solution of 10 mM sodium nitrite was prepared by using sodium nitrite in double-deionized water (DDW). Then, the concentrations of nitrite performed 6 serial dilutions (100, 50, 25, 12.5, 6.25, 3.13 and 1.56 µM) in triplicate down the 96-well plates (50 µL/well). Added 50 µL of 1% sulfanilamide and incubated at room temperature for 10 min. After that, 50 µL of 0.1% NED solution (Griess reagent, Figure 9) was added and incubated 10 minutes at dark condition. Absorbance was measured at 540 nm by UV-VIS spectrophotometry. The calibration curve of nitrite standard was shown in Figure 10.

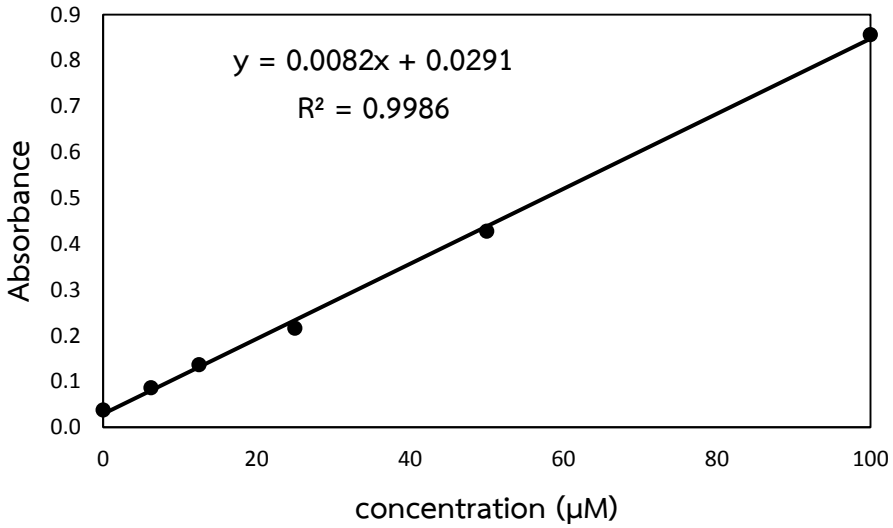

Figure 10 Calibration curve of nitrite standard.

3.6.3.4 Inhibition of nitric oxide (NO) production

RAW 264.7 cells were seeded in 96-well plates at a density of 7×10^5 cells/well and incubated at 37 ºC in 5% CO_2 for 24 h. Then, the medium was replaced with fresh medium containing concentrations at 0.01, 0.1 and 1 µg/mL of crude extracts from fresh leaves and thermal processed *C. asiaticum* leaves at various temperature 50, 70, 100 and 120 ºC for 10, 30 and 60 minutes and lycorine. After 1 h incubation, NO in the RAW264.7 cell line was stimulated with 2 µg/mL of LPS contained interferon-gramma (INF-γ) for 24 h. A volume of 50 µL of the culture supernatant was added in 96-well

plates and added 50 µL of 1% sulfanilamide, incubated at room temperature for 10 minutes. After that, 50 µL of 0.1% NED solution (Griess reagent, Figure 9) was added and incubated 10 minutes at dark condition. Absorbance was measured at 540 nm by UV-VIS spectrophotometer, and NO inhibition can be calculated concentration of NO by comparison to the calibration curve of nitrite standard.

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Thermal process on lycorine contents of *Crinum asiaticum* leaves

Since, previously reports showed the effect of thermal treatment on bioactive compounds having various pharmaceutical activities that were extracted from the fresh leaves of *C. asiaticum* L. [9], but there are no reports of bioactive compounds that extracted from heated leaves of *C. asiaticum* Moreover, ancient people have been used heated leaves of *C. asiaticum* for treatment of bruising, aches, swelling and inflammation [8]. Therefore, in this research, the leaves of *C. asiaticum* were heated by two processes including steaming and oven-drying processes, comparing with untreated leaves (fresh leaves). *C. asiaticum* leaves are divided into three groups; (1) fresh leaves were as control (untreated), (2) steamed leaves (95 °C, 1 h) and (3) oven dried leaves (160 °C, 10 min). Then, the powder of all three groups were extracted by methanol, giving a greenish-brown oil of crude extracts with weight of 17.2 g (1.72% w/w), 23.8 g (2.38% w/w) and 35.2 g (3.52% w/w), respectively (based on 1 kilogram of fresh leaves).

Lycorine, the biological active compound was identified by spectroscopic methods such as ^{1}H NMR, ^{13}C NMR, MALDI-TOF (Figure A1-A3) and HPLC analysis (Figure 11).

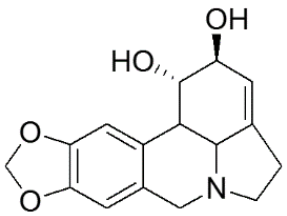

Figure 11 Chemical structure of lycorine.

The ^{1}H NMR data in $(CD_3)_2SO$ of the lycorine (1) as shown in Figure A1 demonstrated for δ (ppm) 6.80 (s, 1H), 6.65 (s, 1H), 5.95 (s, OCH2O), 5.38 (brs, 1H), 4.89 (s, 1H), 4.78 (brs, 1H), 4.26 (d, 1H), 4.02 (d, 1H), 3.23 (m, 1H), 3.18 (d, 1H), 2.60 (m, 1H), 2.40 (m, 2H) 2.17 (dd, 1H). ^{13}C NMR (101 MHz $(CD_3)_2SO$): δ_c (ppm) 145.6(C-9), 145.1 (C-10), 141.6 (C-3a), 129.7 (C-7a), 129.5 (C-12a), 118.4 (C-3), 106.9 (C-8), 105.2 (C-12), 100.5 (C-11), 71.7 (C-2), 70.1 (C-1), 60.7 (C-12c), 56.6 (C-5), 53.2 (C-7), 39.2 (C-12b), 28.1 (C-4). MS (MALDI-TOF): m/z calcd. for [$C_{16}H_{17}NO_4$] 287, found 285. The ^{1}H NMR and ^{13}C NMR data are approximately with previous report [33].

. The amount of crude extracts of heated leaves by both steaming and oven drying processes were more than the fresh leaves especially oven dried leaves were the highest crudes content. Therefore, the results indicated that *C. asiaticum* leaves were heated might be increased the kind of compounds that can soluble in methanol. Then, lycorine content in crude extracts were analyzed by HPLC.

Determination lycorine constitution by HPLC

This research, HPLC was used for determination of lycorine compound. The chromatograms of standard lycorine, fresh leaves, steamed leaves and oven dried leaves are shown in Figure 12 The peaks of lycorine from chromatograms were confirmed by comparison of retention time with lycorine standard. From the chromatogram, the retention time of lycorine standard was 6.77 minutes which is similar to the peak **b** of fresh leaves, steamed leaves and oven dried leaves were showed at the retention time of 6.79, 6.77 and 6.88 minutes, respectively. This result indicated the peak **b** from chromatograms of all crudes was lycorine compound containing in *C. asiaticum* Linn leaves.

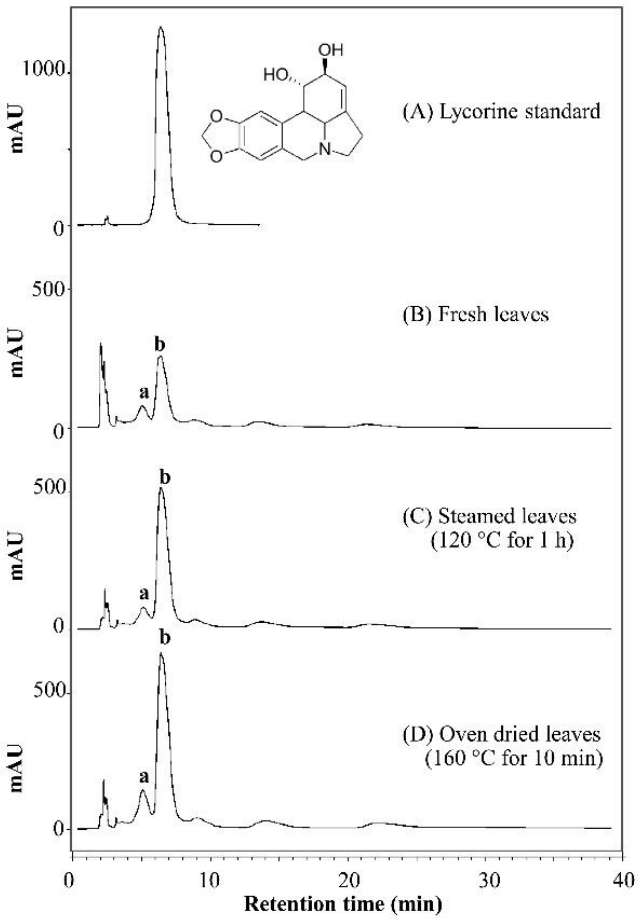

Figure 12 Chromatograms of (A) standard lycorine, (B) crude extracts of fresh leaves, (C) crude extracts of steamed leaves at 120 °C for 1 h and crude extracts of oven dried leaves at 160 °C for 10 minutes. (a) unknown compound, (b) lycorine.

The lycorine content was determined by comparison peak area with standard calibration curve of lycorine. The results found that lycorine content of fresh, steamed and oven dried leaves were 2.35±0.09, 3.69±0.04 and 4.48±0.02 mg/mL, respectively as shown in Table 2. These suggested that steamed leaves and oven dried leaves gave higher amount lycorine content than the fresh leaves to 1.57 and 1.9-fold (based on weight of fresh leaves), respectively. Thus, this experimental data was demonstrated that thermal processes can increase amount of lycorine in the *C. asiaticum* leaves. Moreover, percentage of lycorine was 23.55%, 36.94% and 44.85% in fresh leaves, steamed leaves and oven dried leaves, respectively shown in Figure 13.

Table 2 Lycorine content of heated *C. asiaticum* leaves by steaming and drying oven processes compared with the fresh leaves (untreated).

Type of leaves	Lycorine content (mg/mL)
Fresh leaves	2.35±0.09
Steamed leaves	3.69±0.04
Oven dried leaves	4.48±0.02

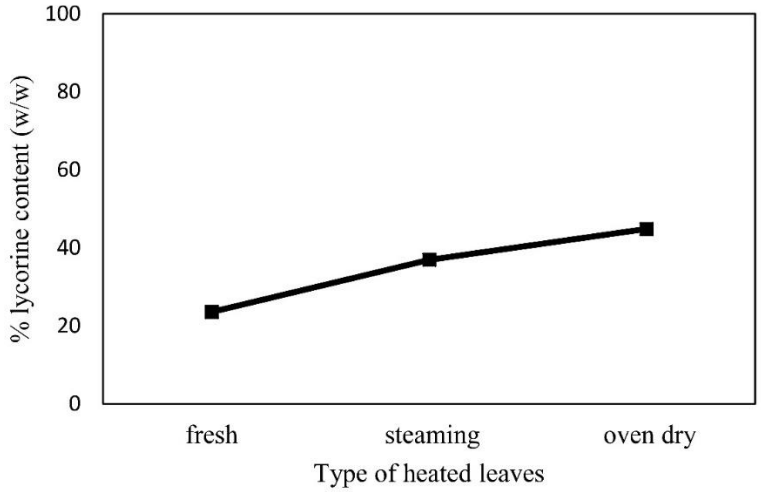

Figure 13 Percentage of lycorine content treated with difference processes (steaming and oven drying) compared with fresh leaves (untreated).

4.2 Effects of temperature on the lycorine constituents

The preliminary experiment indicated the thermal process had affect to the lycorine content on *C. asiaticum* leaves. To study influence of heating temperature on the lycorine content in *C. asiaticum* leaves, we chose drying oven for operating at various temperature of 50, 70, 100and 120 ℃ and various time for 10, 30 and 60 minutes due to this thermal process give the highest of lycorine content and avoid problem of pathogens from moisture-leaves. In this research, *C. asiaticum* leaves were heated by drying oven at various temperature of 50, 70, 100and 120 ℃ and various time for 10, 30 and 60 minutes. Figure 15 showed lycorine content of all heated leaves

were higher than the fresh leaves, which were significantly differences (p<0.05). The lycorine content of heating condition such as heated leaves of fixed time at 30 minutes for 50, 70, 100 and 120 ℃, the results found that the heated leaves at 50 ℃ was higher lycorine content in *C. asiaticum* leaves than other heating conditions. The heated leaves of higher temperature was 70, 100 and 120 ℃ that found lycorine content reduced, respectively. Hence, the result suggested that the temperature had effect to the lycorine content on *C. asiaticum* leaves.

4.3 Effects of time in thermal process on the lycorine constituent

From the preliminary study of temperature, the temperature heating at 50 ℃ showed the highest lycorine content. Moreover, we studied effect of times heating on *C. asiaticum* leaves at 10, 30 and 60 minutes. Comparison with untreated leaves, lycorine content of heated leaves at 50 ℃ was increased at 10 and 30 minutes, respectively and reduced at 60 minutes, although it was more than the fresh leaves. Moreover, the condition of heated leaves at 50 ℃ for 30 minutes to give the highest lycorine content. Thus, the optimum condition of heated leaves was temperature heating at 50 ℃ for 30 minutes, which obtained the highest lycorine content. This study concluded that the thermal treatment on *C. asiaticum* leaves extracted with methanol is effective for increasing lycorine and other bioactive compound contents.

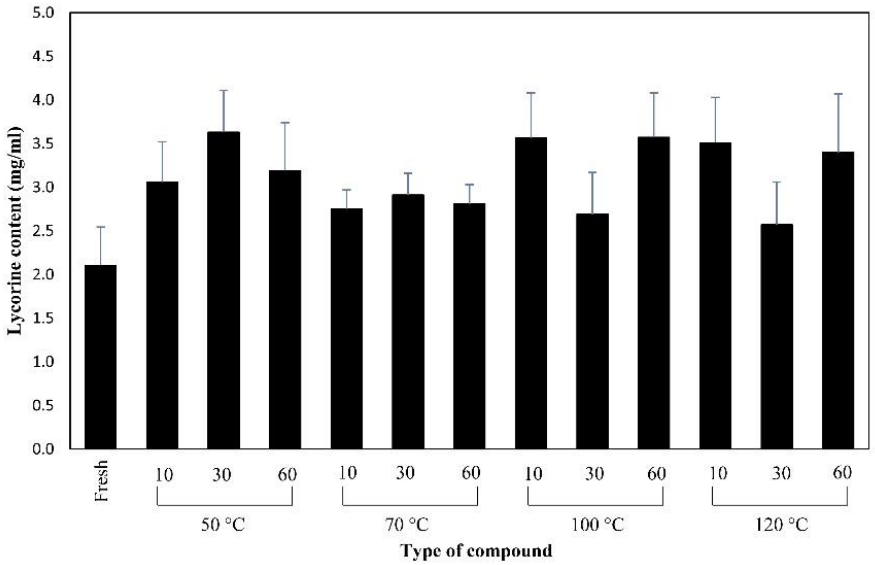

Figure 14 Lycorine content of fresh *Crinum asiaticum* leaves (untreated) and heated *C. asiaticum* leaves of temperatures at 50 ℃, 70 ℃, 100 ℃ and 120 ℃ for 10, 30 and 60 minutes.

Lycorine content was increased when the leaves were heated (Table 3). All heated leaves gave higher lycorine content than the fresh leaves (0.24%). Heated leaves of temperature at 50 ℃ for 10, 30 and 60 minutes gave high lycorine content, was 0.34, 0.54 and 0.53%, respectively (based on 100 g of fresh leaves). While, heated leaves of higher temperature at 100 and 120 ℃ for various time found reduced lycorine content when the time was higher, for example heated leaves of 120 ℃ at 10, 30 and 60 minutes, lycorine content was 0.51, 0.34, 0.34%, respectively.

Table 3 percentage of lycorine content in fresh leaves and heated leaves (temperature of 50, 70, 100 and 120 ºC at 10, 30 and 60 minutes)

condition of leaves (temperature/time)		% lycorine content (based on 100g of fresh leaves)
50 °C	10	0.34
	30	0.54
	60	0.50
70 °C	10	0.39
	30	0.44
	60	0.43
100 °C	10	0.53
	30	0.50
	60	0.46
120 °C	10	0.47
	30	0.34
	60	0.34
Fresh leaves		0.24

Due to thermal process had effect to bioactive compounds of *C. asiaticum* especially lycorine content. As we expected, increasing temperature could be lycorine-1-0-**β**-D-glucoside, on further hydrolysis gave lycorine and D-glucose was confirmed by Ghosal et al. [34]. Thus, the lycorine contents was increased significantly that shown in Figure 15.

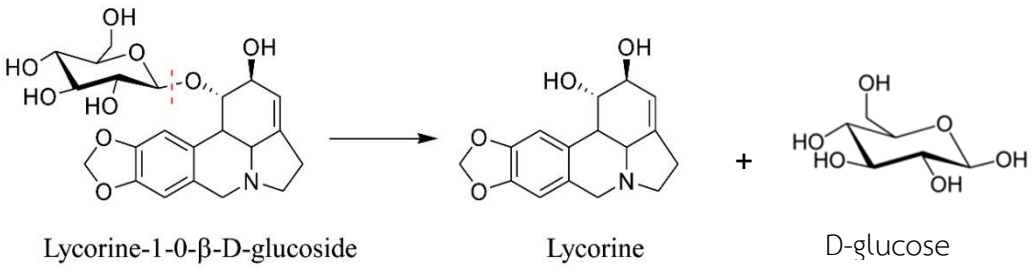

Figure 15 Proposed mechanism of lycorine was converted from lycorine-1-0-β-D-glucoside.

4.4 Antioxidant by DPPH assay

The antioxidant capacity of methanol extract of heated-*Crinum asiaticum* leaves were evaluated by DPPH assay and vitamin C was used positive control. The result are shown in figure 16, the most of heated leaves had a good antioxidant activity than the fresh leaves except the heated leaves at 50 ºC for 60 minutes, and 70 ºC for 10 minutes. The heated leaves at 50 ºC for 30 minutes, and 100 ºC for 10 minutes showed strong antioxidant activities, IC_{50} were 0.96 ± 0.06 and 1.12 ± 0.06, respectively, which is strong as purified lycorine. Therefore, these condition of heated leaves had high potency for antioxidant may be due to that give the high content of lycorine.

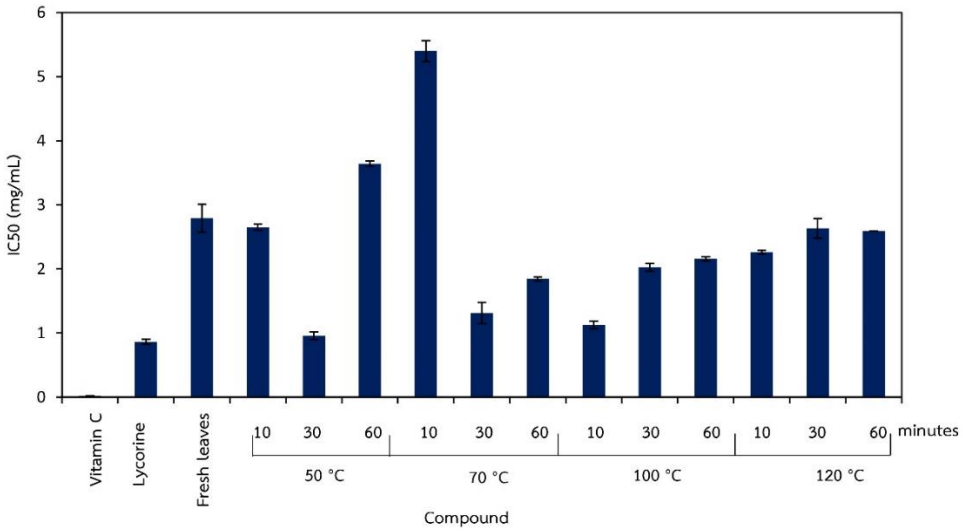

Figure 16 IC_{50} (mg/ml) of DPPH activity of vitamin C, lycorine, fresh leaves and heated leaves with different heating temperatures (50, 70, 100 and 120 ºC) and times (10, 30 and 60 minutes).

4.5 Cytotoxicity by MTT assay

All crude extracts were evaluated anticancer against five cancer cell lines including colon cancer cell line (SW620), lung cancer cell line (CHAGO), liver cancer cell line (Hep-G2), gastric cancer cell line (KATO-III) and breast cancer cell line (BT474) using MTT assays. MTT assay is based on the reduction of MTT dye by mitochondrial reductase of viable cells to give purple formazan products, which can measure by spectrophotometer at 540 nm. Preliminary study, all of crude extracts were tested at 50 µg/mL as shown figure B1, data were collected for three replications and calculated as:

$$\% \text{ Cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100$$

$$\% \text{ Cytotoxicity} = 100 - \text{percentage of cell viability}$$

The results suggested that most of crudes extracts of thermal processed leaves showed more cytotoxic activity than crudes of fresh leaves) untreated(for all types of cell lines and showed anticancer activity for gastric cancer cell line (KATO-III) more than other types of cell lines as shown in Figure 17. Consequently, all crude extracts were then investigated for the IC_{50} values against KATO-III cell line.

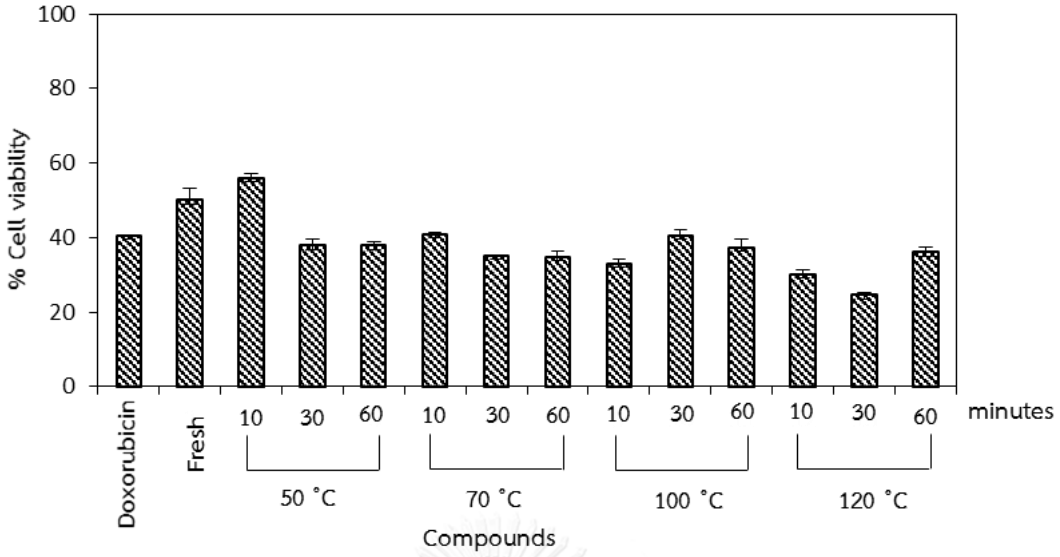

Figure 17 Preliminary screening at 50 µg/mL of fresh leaves and all heated leaves (temperature at 50, 70, 100 and 120 °C for 10, 30 and 60 minutes) against KATO-III cell line.

The IC_{50} values is the concentration which inhibited half of the maximum biological response and can be calculated by software GraphPad Prism 5. The IC_{50} values were classified into four groups as high ($IC_{50} \leq 5$ µg/mL), moderate (IC_{50} = 6-15 µg/mL), low (IC_{50} = 16-30 µg/mL), and inactive ($IC_{50} > 30$ µg/mL).

The IC_{50} were of all crude extracts was shown in Table 4, all of crude extracts showed higher cytotoxicity than fresh crude against KATO-III. Especially, the crude extracts of thermal processed leaves at 50 °C (30 minutes), 70 °C (30 and 60 minutes) and 120 °C (60 minutes) presented the high values of IC_{50} were 1.5 ± 0.26, 1.5 ± 0.14, 1.4 ± 0.15 and 1.7 ± 0.19 µg/mL, respectively approximated doxorubicin and lycorine standard, which is major compound for anticancer activity.

Table 4 The IC_{50} values of all heated *Crinum asiaticum* leaves, fresh leaves doxorubicin and lycorine against SW620, BT474, CHAGO, Hep-G2 and KATO-III.

Compounds		Cytotoxicity (IC_{50}, µg/mL)				
Temperature/time (min)		**SW620**	**BT474**	**CHAGO**	**Hep-G2**	**KATO-III**
50 °C	**10**	> 30	> 30	> 30	> 30	10.3 ± 0.19
	30	9.7 ± 0.12	> 30	> 30	9.8 ± 0.17	1.5 ± 0.26
	60	> 30	> 30	> 30	> 30	9.8 ± 0.09
70 °C	**10**	> 30	> 30	9.9 ± 0.13	> 30	9.6 ± 0.24
	30	9.7 ± 0.12	> 30	1.2 ± 0.14	9.4 ± 0.18	1.5 ± 0.14
	60	9.8 ± 0.14	> 30	9.4 ± 0.28	1.1 ± 0.19	1.4 ± 0.15
100 °C	**10**	> 30	> 30	9.8 ± 0.21	9.7 ± 0.14	9.5 ± 0.10
	30	> 30	> 30	> 30	> 30	10.1 ± 0.07
	60	> 30	> 30	> 30	> 30	9.5 ± 0.17
120 °C	**10**	>30	>30	>30	>30	10.0 ± 0.07
	30	>30	>30	>30	>30	25.1 ± 0.11
	60	>30	>30	>30	>30	1.7 ± 0.19
Fresh leaves		>30	>30	>30	>30	66.9 ± 0.17
Doxorubicin		ND	ND	ND	ND	0.71 ± 0.12
Lycorine		9.9 ± 0.12	10.1 ± 0.12	0.59 ± 0.24	0.76 ± 0.11	0.42 ± 0.27

IC_{50} = concentration of compound can inhibit the growth of 50% of cancer cells µg/mL(determined by MTT assay

ND = not determined

Moreover, the crude of heated leaves at 50 °C for 30 minutes that gave the highest lycorine content and good against KATO-III cell activity showed relatively less toxic against normal lung cell, WI-38, which was similar to doxorubicin as shown in Figure 18. Therefore, the heated leaves at 50 °C for 30 minutes had potential to be developed as an anticancer drug for treatment of gastric cancer cells.

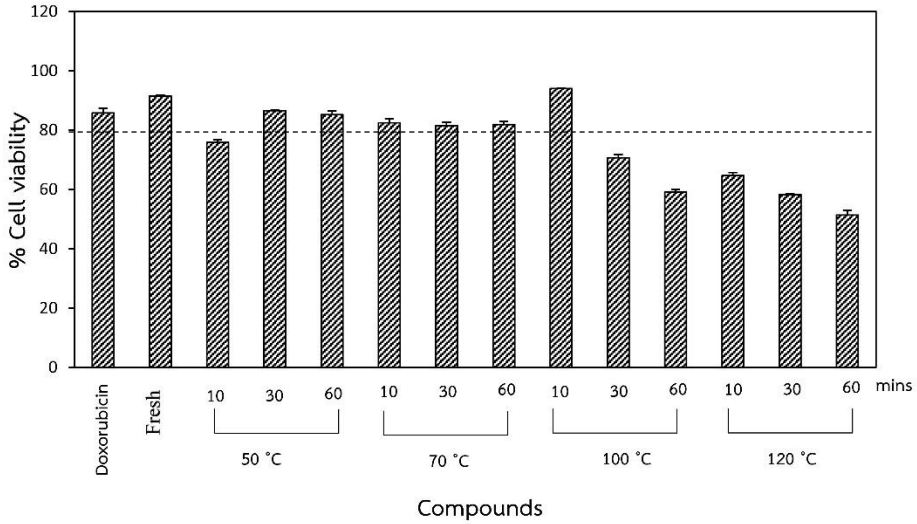

Figure 18 MTT screening test of the compounds against lung normal cell line (WI-38) at 50 µg/mL.

4.6 Effect of crude extracts on RAW 264.7 cell viability

The viability of RAW 264.7 cells in the presence of parthenolide (drug for anti-inflammatory) and crude extracts of fresh leaves at a wide range of concentrations (Figure 19). The cell viability was evaluated by MTT assay. With this result, samples with concentration of 0.01 and 0.1 µg/mL (cell viability >80%) were selected for subsequent NO inhibition experiment.

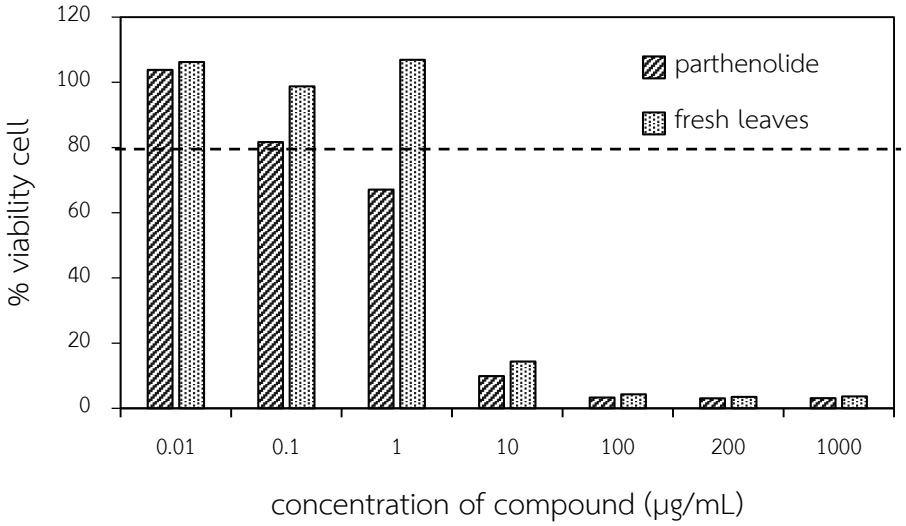

Figure 19 The cell viability of parthenolide (drug) and fresh leaves at various concentration.

4.7 Effect of crude extracts on anti-inflammatory by nitric oxide inhibition

This study, we examined the effect of crude extracts of the thermal processed *Crinum asiaticum* leaves at temperature 50, 70, 100 and 120ºC for 10, 30 and 60 minutes and lycorine on NO production from LPS -stimulated RAW 246.7 cells. Cells were treated with crude extracts and lycorine at various concentration 0.01 and 0.1 µg/mL for 1 h, and then incubated with LPS (2µg/mL) contained IFN-γ for 24h.

To investigate NO production, nitrite (NO_2^-) is measured since it is stable, novolatile breakdown product of NO. Nitrite was determined via reaction with griess reagent system. The concentration of nitrite was measured by the absorbance at 540 nm and then calculated by $NaNO_2$ standard curve, $R^2 = 0.9986$.

The nitrite accumulation was significantly increased from RAW-264.7 cell by stimulation of LPS (2 µg/mL) with IFN-γ when compared with control. In this study, nitric oxide production of LPS with IFN-γ stimulated RAW-264.7 cell line was inhibited by various crude extracts of heated *C. asiaticum* leaves, which parthenolide was used as a positive control.

From the results shown in Figure 20, crude extracts of all heated leaves significantly inhibited nitric oxide production which was more than the fresh leaves and the parthenolide (positive control). Especially, crude extracts (0.1 µg/mL) of heated leaves at temperature of 50 ºC for 30 minutes and 100 ºC for 10 minutes showed the greatest inhibition, percentage of inhibition were 53.7 ± 1.46 and 57.23 ± 0.89. Therefore, this results (Figure 19) demonstrated crude extracts of heated-*Crinum asiaticum* leaves, especially the heated leaves at 50 ºC for 30 minutes and 100 ºC for 10 minutes (the highest lycorine content) had effective to potency inhibition nitric oxide that stimulated by LPS contained IFN-γ. While, the fresh leaves showed less potency on nitric oxide inhibition. Thus, the capacity of nitric oxide inhibition might be due to the increasing lycorine content of heated leaves.

This experiment indicated crude extracts of heated-*Crincum asiaticum* leaves can be anti-inflammatory by nitric oxide inhibition that stimulated with LPS contained IFN-γ. Hence,

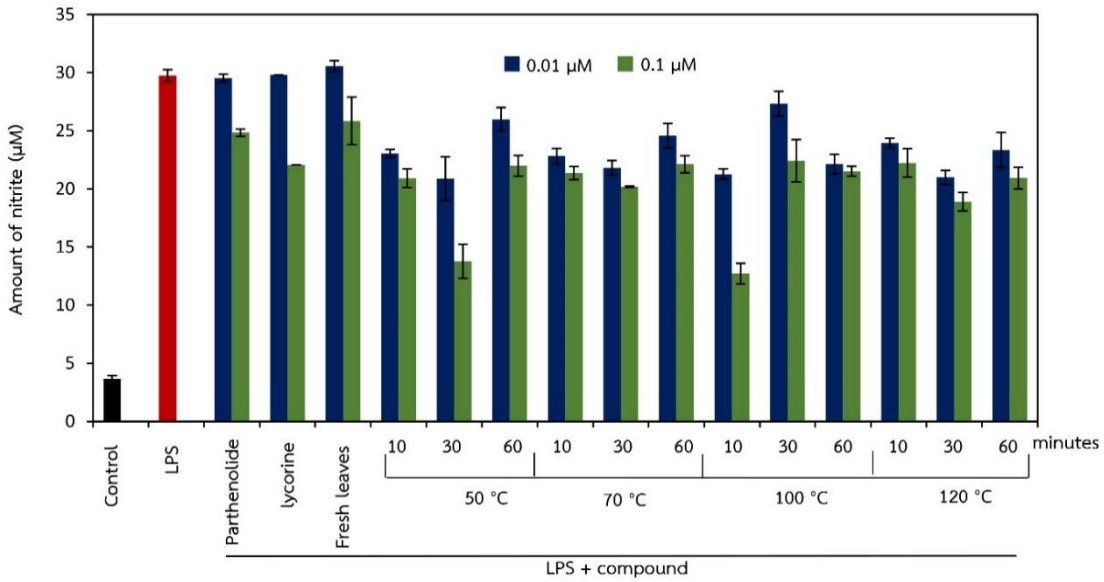

Figure 20 Nitrite content after inhibition with various compounds at concentration of ■ 0.01 and ■ 0.1 μg/mL.

CHAPTER V
CONCLUSION

In this research, heated-*Crinum asiaticum* leaves by steaming and oven-drying processes compared with the fresh leaves were designed for antioxidant, anticancer and anti-inflammatory agents.

The heated-*Crinum asiaticum* leaves by oven-drying process gave the highest lycorine content, which is more than up to 2-fold of the fresh leaves. Then, this process was selected to studied the effect of temperatures (50, 70, 100 and 120 ºC) and times (10, 30 and 60 minutes) on bioactive compounds of *Crinum asiaticum* leaves. The oven dried *Crinum asiaticum* leaves of temperature at 50 ºC for 30 minutes gave the highest lycorine content.

Crude extract of oven dried *Crinum asiaticum* leaves of temperature at 50 ºC for 30 minutes which was the highest lycorine content showed the strongest antioxidant activity.

All crude extracts of oven dried *Crinum asiaticum* leaves were evaluated cytotoxic activities against five cancer cell lines such as SW620, CHAGO, Hep-G2, KATO-III and BT474 by MTT assay. The crude extracts of oven dried leaves of temperature at 50 ºC for 30 minutes exhibited anticancer activities against KATO-III cell line with IC_{50} value of 1.5 ± 0.26 µg/mL. Moreover, the crudes extracts of oven dried leaves of temperature at 50 ºC for 30 minutes were non-toxic to lung normal cell line (WI-38) at 50 µg/mL. Thus, oven dried leaves of temperature at 50 ºC for 30 minutes were chosen as model anticancer drugs.

All crude extracts of oven dried *Crinum asiaticum* leaves were evaluated anti-inflammatory activities by inhibition NO was produced from RAW-264.7 that stimulated by LPS contained IFN-γ. All heated leaves showed good potency for NO inhibition than the fresh leaves. The heated leaves of temperature at 50 ºC for 30 minutes and 100 ºC for 10 minutes (0.1 µM) demonstrated the greatest potential for inhibition NO, which

was more than the parthenolide compounds as drug control. Therefore, thermal process on *Crinum asiaticum* leaves has potential to develop for anti-inflammatory.

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APPENDIXS

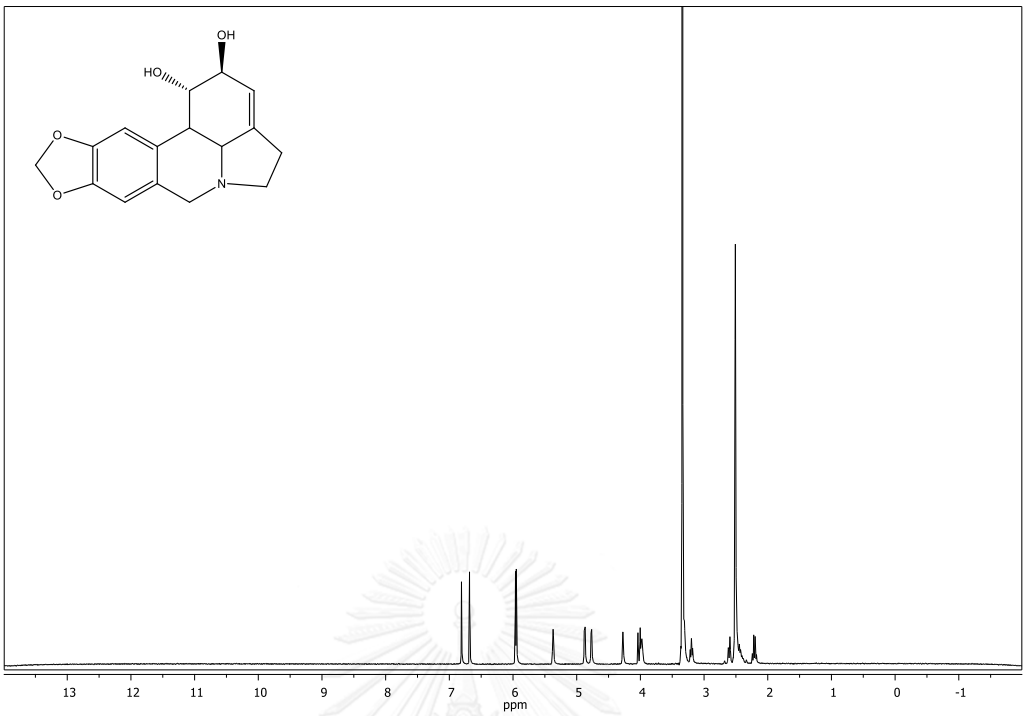


Figure A1 ^{1}H-NMR spectrum of lycorine in $(CD_3)_2SO$.

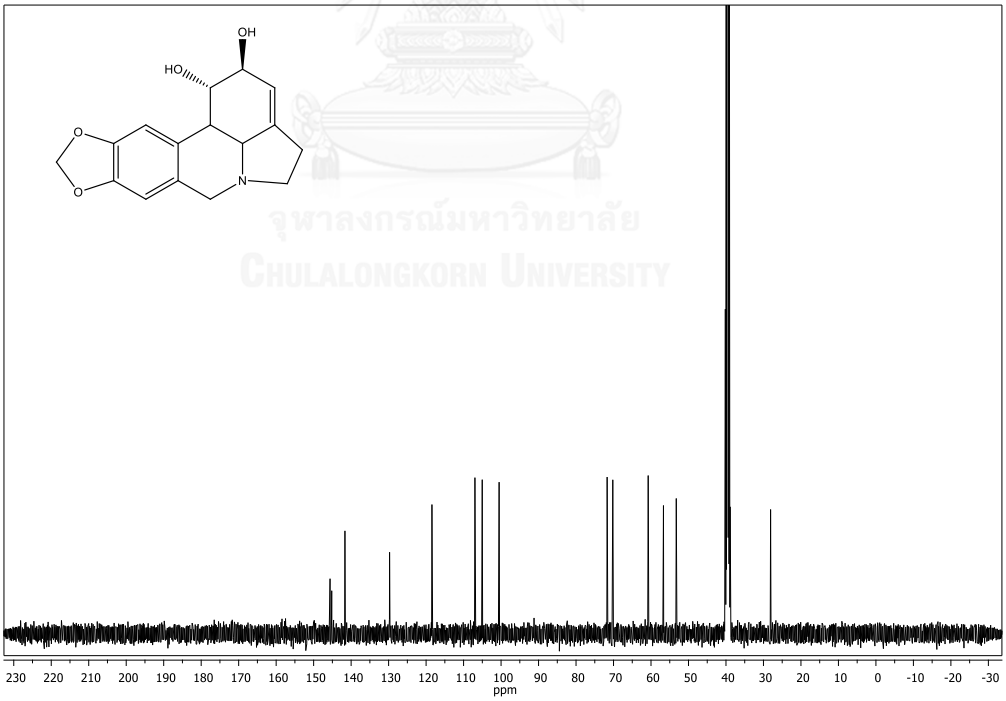


Figure A2 ^{13}C-NMR spectrum of lycorine in $(CD_3)_2SO$.

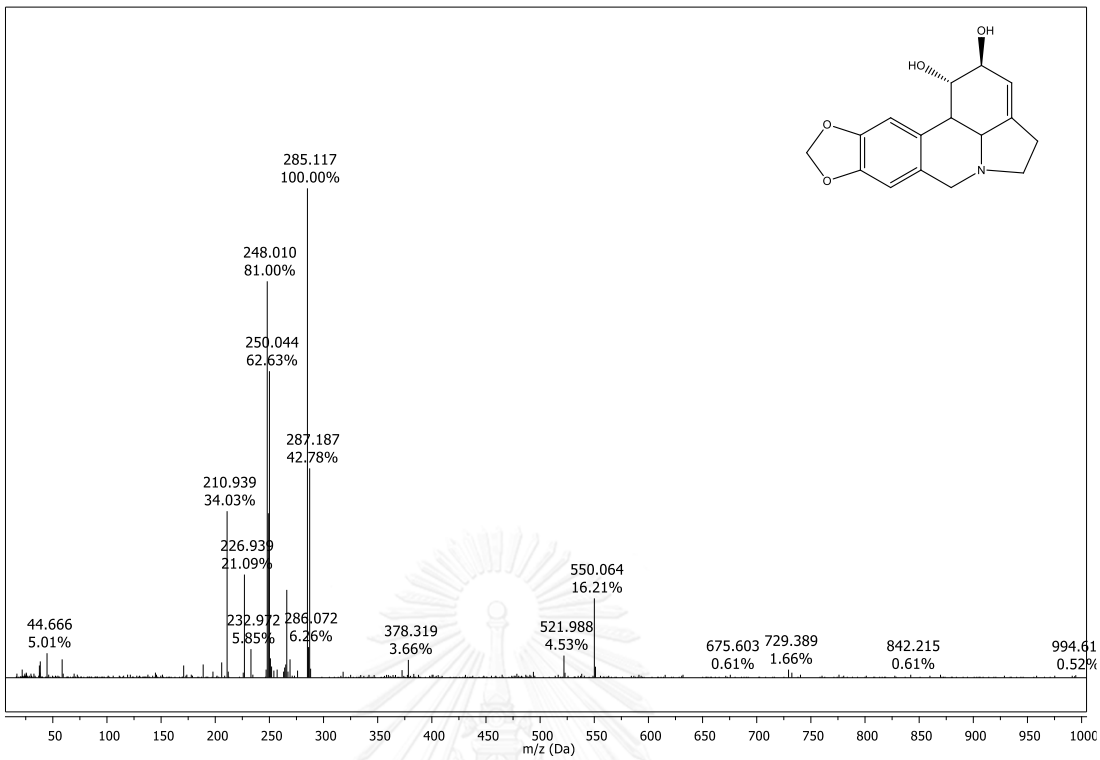


Figure A3 Mass spectrum of lycorine in MeOH

Figure A4 Stability of lycorine was determined by peak area of lycorine (0.125, 0.25, 0.50, 1.0 and 2.0 mg/ml) was investigated by HPLC at varying storage days.

	area				
	0.125mg/mL	**0.25mg/mL**	**0.5mg/mL**	**1mg/mL**	**2mg/mL**
0 day	12471730	21295583	41568691	77752761	110318178
1 day	11781593	20113111	41612295	77479380	108664428
3 day	13535389	18061896	36402912	72416892	109056604
7 day	12709831	18691083	35724768	73594885	95948395

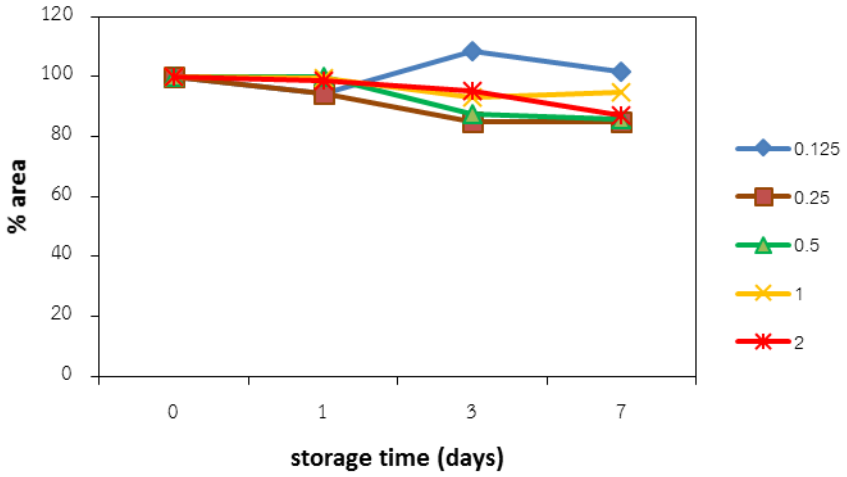


Figure B1 The preliminary screening tests of all crude extracts at the concentration of 50 µg/mL against CHAGO, KATO-III, Hep-G2, SW620 and BT474 cancer cell lines.

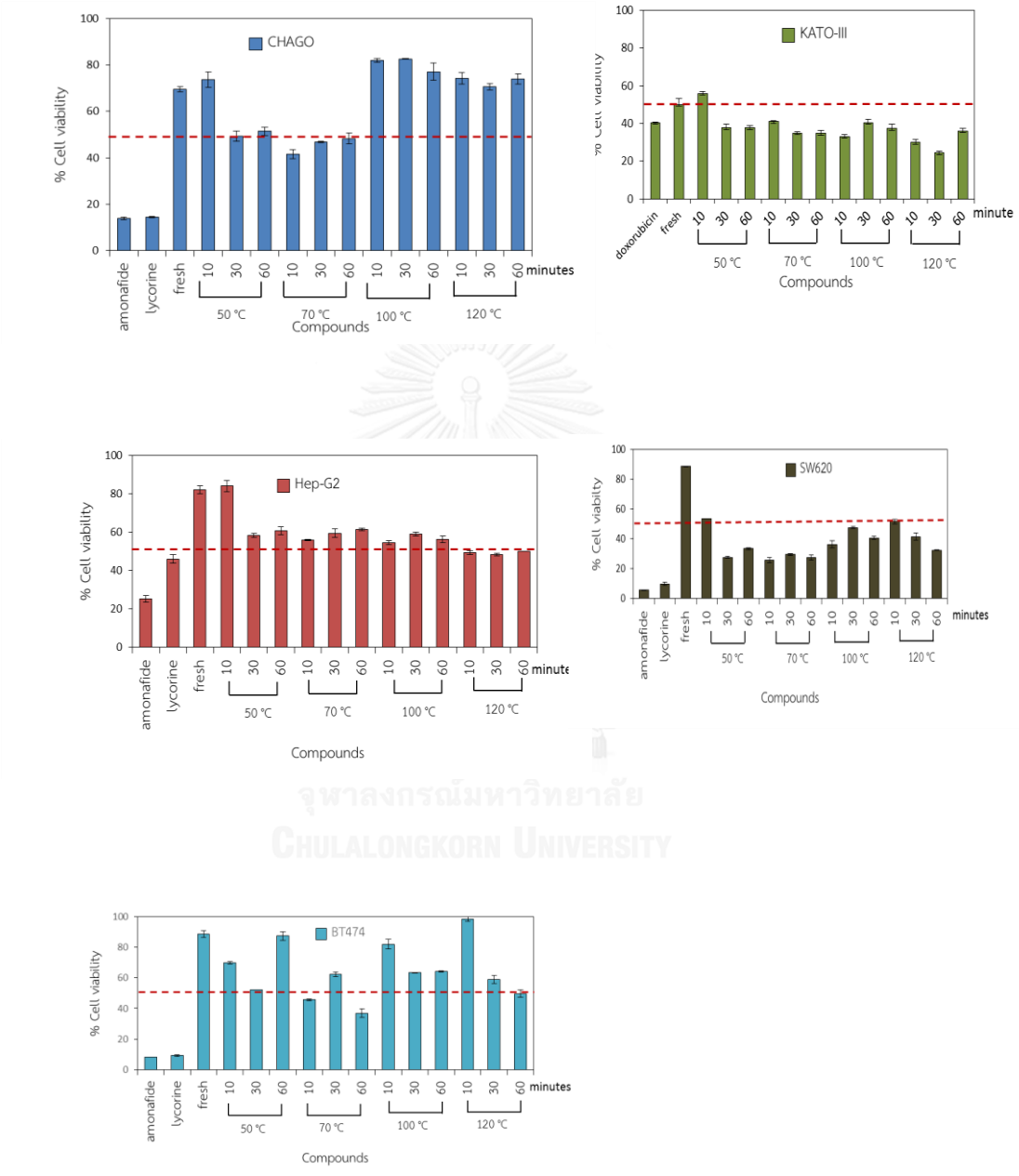
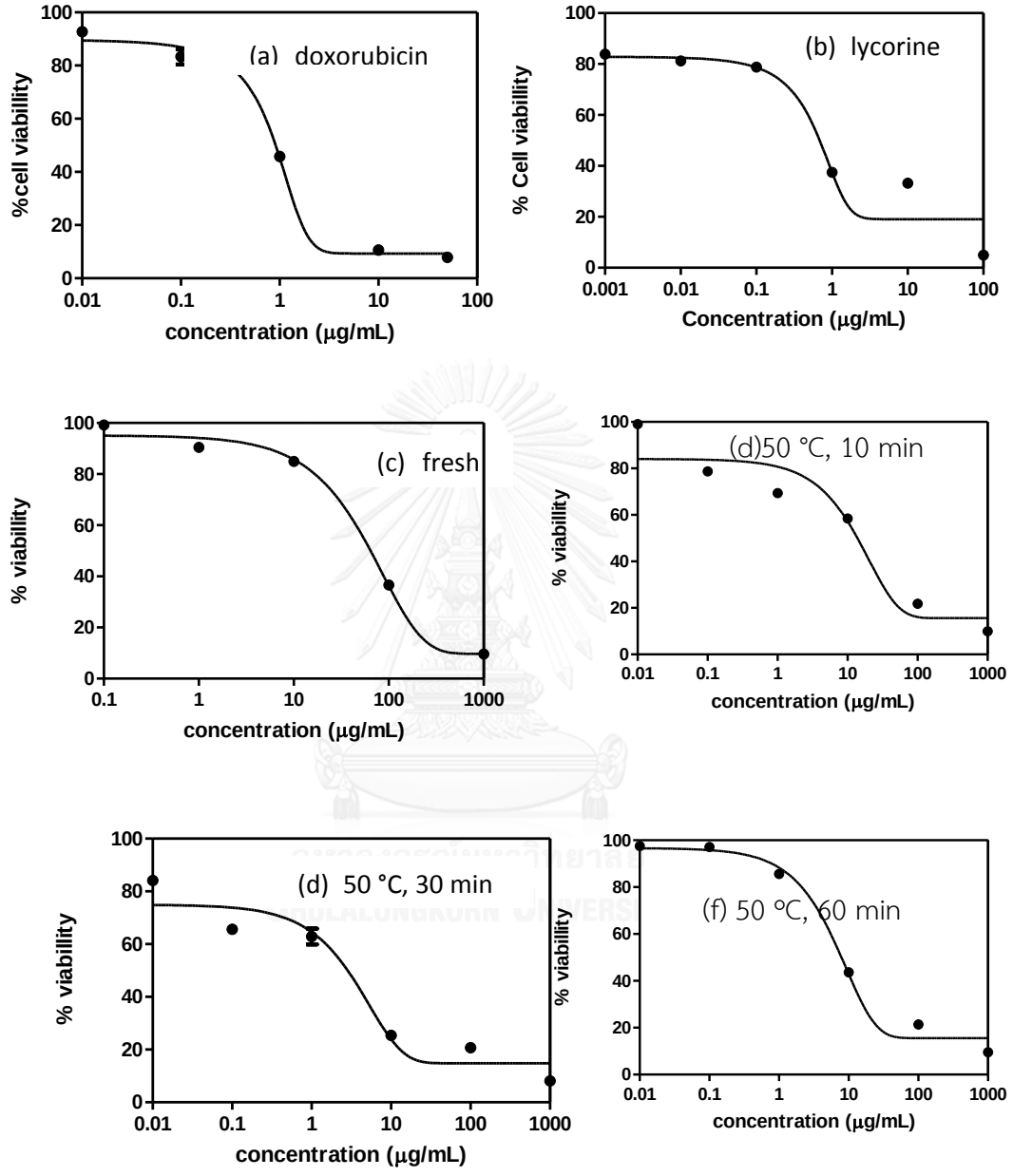
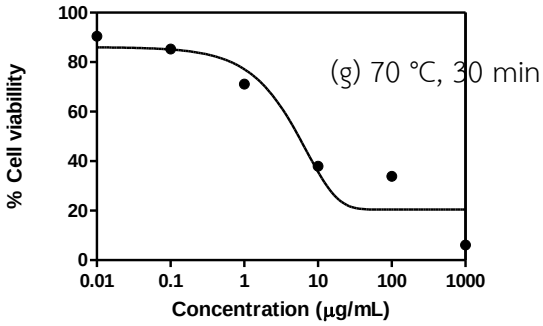


Figure B2 Cell inhibition of all crude extracts at different concentration against KATO-III)gastric cancer cell line (cancer cell lines.




(g) 70 °C, 30 min
% Cell viability
Concentration (μg/mL)
0
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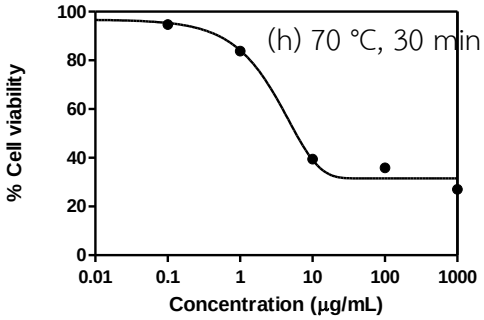
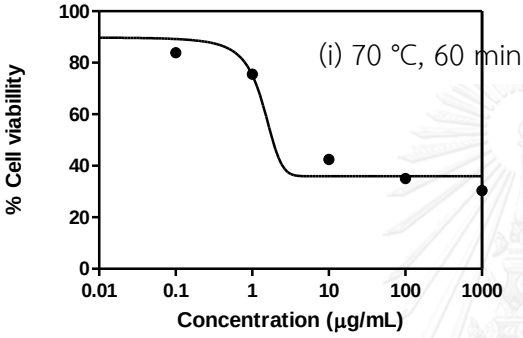
40
60
80
100
0.01
0.1
1
10
100
1000


(h) 70 °C, 30 min
% Cell viability
Concentration (μg/mL)
0
20
40
60
80
100
0.01
0.1
1
10
100
1000


(i) 70 °C, 60 min
% Cell viabillity
Concentration (μg/mL)
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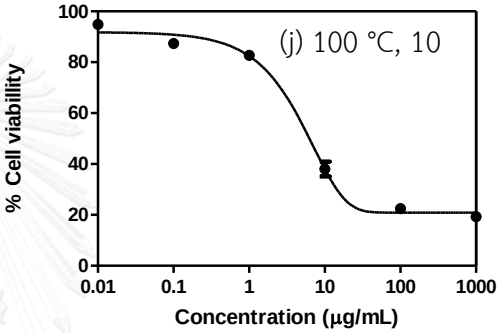
80
100
0.01
0.1
1
10
100
1000


(j) 100 °C, 10
% Cell viabillity
Concentration (μg/mL)
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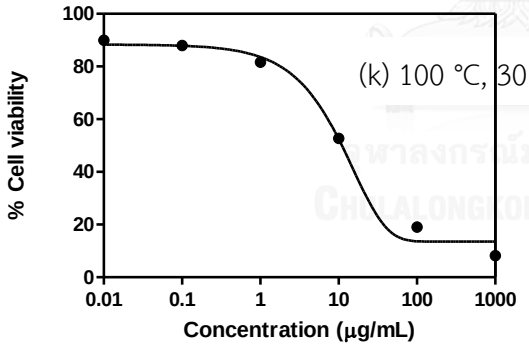
80
100
0.01
0.1
1
10
100
1000


(k) 100 °C, 30
% Cell viability
Concentration (μg/mL)
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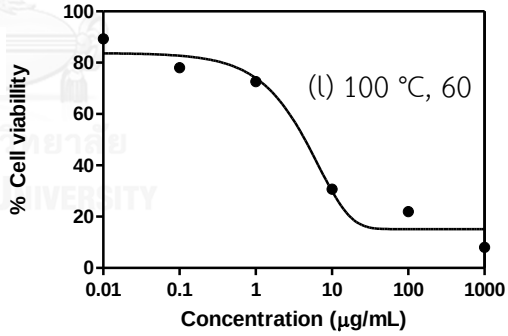
40
60
80
100
0.01
0.1
1
10
100
1000


(l) 100 °C, 60
% Cell viabillity
Concentration (μg/mL)
0
20
40
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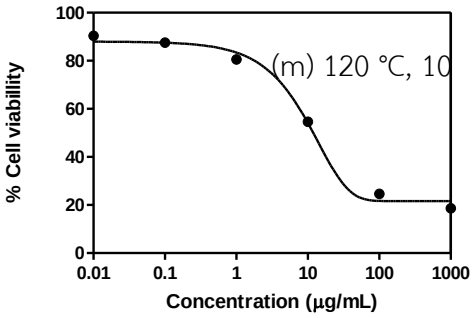
80
100
0.01
0.1
1
10
100
1000



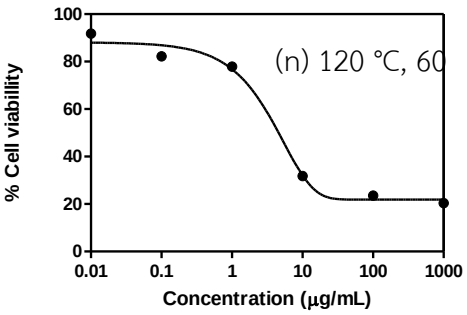
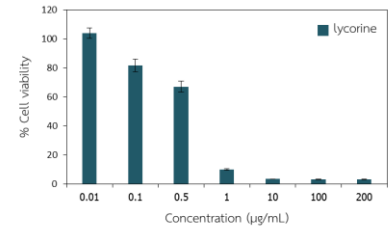
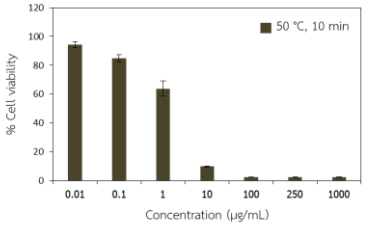
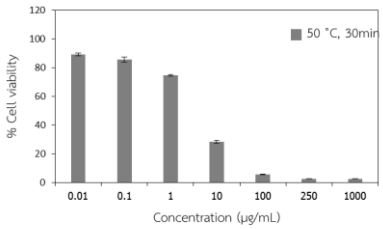
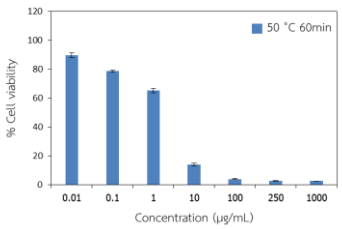


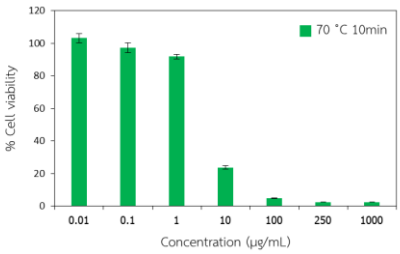
Figure C1 Cell viability of RAW 264.7 cell lines from crude extracts at different concentration.

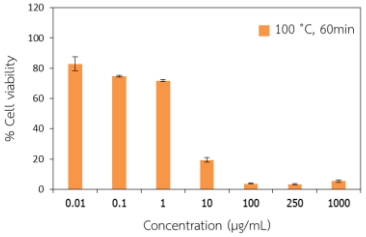


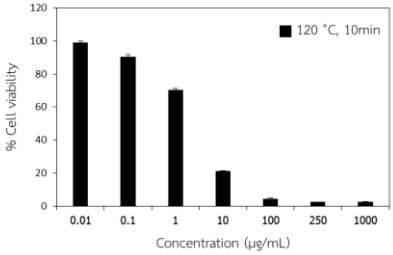


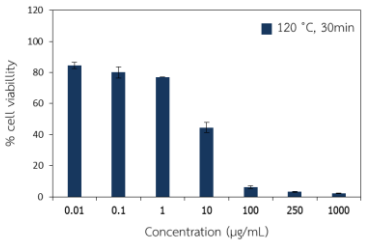


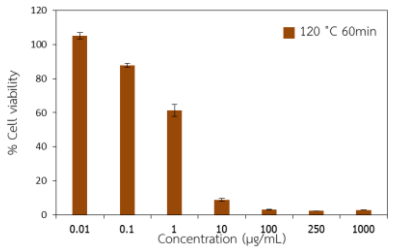












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Laokam S., Ngamrojanavanich N., Wejsuthanon S., Muangsin N. "Thermal Treatment Processes for Increase Lycorine Active Ingredient in Crinum asiaticum Leaves" Proceeding of International Conference on Biotechnology, Nanotechnology and Environmental Engineering (ICBNE'15) IAAST 2015, Bangkok, Thailand, April, 22-23, 2015, pp 10-13.