Standardization and DNA Barcoding of Cannabis in Thailand and Pharmacological properties of Cannabis-based Thai Traditional Medicine formula extract (Kealomkeasan)

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Public Health Sciences
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มาตรฐานและดีเอ็นเอบาร์โค้ดของพืชกัญชาในประเทศไทยและฤทธิ์ทางเภสัชวิทยาของตำรับยาที่มีกัญชาปรุงผสมอยู่ (ตำรับแก้ลมแก้เส้น)

น.ส.ขนิษฐา นาคเกลี้ยง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Field of Study	Public Health Sciences
Thesis Advisor	Associate Professor KANCHANA RUNGSIHIRUNRAT, Ph.D
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ขนิษฐา นาคเกลี้ยง : มาตรฐานและดีเอ็นเอบาร์โค้ดของพืชกัญชาในประเทศไทยและฤทธิ์ทางเภสัชวิทยาของตำรับยาที่มีกัญชาปรุงผสมอยู่ (ตำรับแก้ลมแก้เส้น). (Standardization and DNA Barcoding of Cannabis in Thailand and Pharmacological properties of Cannabis-based Thai Traditional Medicine formula extract (Kealomkeasan)) อ.ที่ปรึกษาหลัก : รองศาสตราจารย์ ดร.กาญจนา รังษีหิรัญรัตน์, อ.ที่ปรึกษาร่วม : ศาสตราจารย์ ดร.จิตรลดา อารีย์สันติชัย

กัญชา (*Cannabis sativa* L. subsp. Indica) จัดอยู่ในวงศ์ Cannabaceae มีการนำมาใช้ประโยชน์เพื่อการบำบัดรักษาโรคต่างๆ กัญชาเป็นส่วนหนึ่งของการแพทย์แผนไทยมานานกว่าสามศตวรรษและเป็นส่วนผสมในตำรับยาสมุนไพรตำรับแก้ลมแก้เส้น ประกอบด้วย สมุนไพร 7 ชนิด ได้แก่ ผลเทียนขาว เมล็ดเทียนดำ ผลเทียนข้าวเปลือก เหง้าขิง รากเจตมูลเพลิงแดง ใบกัญชา และเมล็ดพริกไทยในอัตราส่วนที่ไม่เท่ากันโดยน้ำหนัก (1:2:3:4:5:20:40) ใช้เป็นยาลดอาการปวดและแก้อักเสบ เนื่องจากกัญชาเพิ่งมีการอนุญาตให้ใช้ทางการแพทย์และการวิจัย ทำให้ข้อมูลทางวิทยาศาสตร์มีค่อนข้างจำกัด ดังนั้นการศึกษาวิจัยนี้จึงแบ่งวัตถุประสงค์ออกเป็น 3 หัวข้อ 1.เพื่อกำหนดและพัฒนามาตรฐานใบกัญชาที่เก็บจากแหล่งต่างๆทั่วประเทศไทย จำนวน 12 แหล่ง ตามข้อกำหนดขององค์การอนามัยโลกซึ่งรวมถึงการตรวจสอบลักษณะทางเภสัชเวท ลักษณะทางมหทรรศน์ จุลทรรศน์ ลายพิมพ์ทางเคมีด้วยเทคนิคทินเลเยอร์โครมาโทกราฟฟี คุณสมบัติทางกายภาพเคมีและการตรวจสอบทางพฤกษเคมีเบื้องต้นด้วยวิธีมาตราฐาน 2. ตรวจสอบบริเวณของดีเอ็นเอที่มีคุณสมบัติเป็นดีเอ็นเอบาร์โค้ดที่เหมาะสมสำหรับการพิสูจน์ชนิดของพืชกัญชาจาก 20 ตัวอย่าง และ 3. ตรวจสอบฤทธิ์ทางชีวภาพในหลอดทดลองของตำรับแก้ลมแก้เส้นได้แก่ฤทธิ์ต้านปฏิกิริยาออกซิเดชัน ฤทธิ์ต้านการอักเสบ ฤทธิ์ยับยั้งเอนไซม์แอลฟากลูโคซิเดสและฤทธิ์ความเป็นพิษเฉียบพลัน ผลการศึกษา 1.ข้อกำหนดและพัฒนามาตรฐานใบกัญชา พบค่าเฉลี่ยของน้ำหนักที่หายไปเมื่อทำให้แห้ง ปริมาณความชื้น ปริมาณเถ้ารวมและปริมาณเถ้าทีไม่ละลายในกรด มีค่าร้อยละ 4.07 ± 0.09, 7.95 ± 0.12, 14.36 ± 0.13, 2.73 ± 0.09 โดยน้ำหนักแห้งตามลำดับ ขณะที่ปริมาณสิ่งสกัดในน้ำและเอทานอลมีค่าอยู่ที่ร้อยละ 11.10 ± 0.15 และ 23.04 ± 0.16 ตามลำดับ และการตรวจสอบทางพฤกษเคมีเบื้องต้นในใบกัญชาพบสารอัลคาลอยด์ ฟีนอลิก ฟลาโวนอยด์ ไตรเทอร์พีน สเตียรอยด์ และไดเทอร์พีน 2.การศึกษาดีเอ็นเอบาร์โค้ดของพืชสกุลกัญชาจาก 20 แหล่งในประเทศไทย โดยพิจารณาลำดับเบสต่างๆ ของดีเอ็นเอ ได้แก่ ITS, *mat*K, และ *rbc*L แล้วสร้างแผนภูมิวิวัฒาการของพืชและศึกษาระยะห่างระหว่างพันธุกรรมโดยใช้วิธีการ Maximum Likelihood พบว่าบริเวณส่วน ITS สามารถใช้เป็นเครื่องบ่งชี้ที่เหมาะสมในการระบุชนิดของพืชสกุลกัญชา 3.การทดสอบฤทธิ์ทางชีวภาพสารสกัดเอทานอลของตำรับแก้ลมแก้เส้นในหลอดทดลอง พบว่า สมุนไพรแต่ละชนิดในตำรับมีฤทธิ์ในการต้านออกซิเดชัน ต้านการอักเสบ และต้านเบาหวาน สารสกัดเอทานอลของตำรับแก้ลมแก้เส้นมีความเป็นพิษต่อไรทะเลในระดับต่ำ (LC50 = 503.13 มคก/มล) ในขณะที่สารสกัดเอทานอลของสมุนไพรแต่ละชนิด มีความเป็นพิษปานกลางจนถึงไม่พบความเป็นพิษต่อไรทะเล (LC50 = 150.07 - > 1,000 มคก/มล) ข้อมูลทั้งหมดจากงานวิจัยนี้สามารถนำไปใช้กำหนดมาตราฐานใบกัญชาและพิสูน์เอกลักษณ์พืชกัญชา ซึ่งก่อให้เกิดการควบคุมคุณภาพ ความปลอดภัย และประสิทธิผลในการใช้พืชกัญชาในการรักษาโรค

สาขาวิชา	วิทยาศาสตร์สาธารณสุข	ลายมือชื่อนิสิต .....
ปีการศึกษา	2565	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
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Kanittha Nakkliang : Standardization and DNA Barcoding of Cannabis in Thailand and Pharmacological properties of Cannabis-based Thai Traditional Medicine formula extract (Kealomkeasan). Advisor: Associate Professor KANCHANA RUNGSIHIRUNRAT, Ph.D Co-advisor: Professor CHITLADA AREESANTICHAI, Ph.D.

Cannabis sativa L. subsp. Indica (Cannabaceae) has been used as a medicinal plant in various aspects. *C. sativa* has been part of Thai medicine for more than three centuries and its Ingredients in herbal medicine recipe. Kealomkeasan (KLKS) remedy is one of the Thai traditional medicines, consists of seven plants with an unequal part by weight (1:2:3:4:5:20:40) as follows: *Cuminum cyminum* L. fruits, *Nigella sativa* L. seeds, *Foeniculum vulgare* Miller fruits, *Zingiber officinale* Roscoe rhizomes, *Plumbago indica* L. roots, *Cannabis sativa* L. leaves and *Piper nigrum* L. seeds for relief of muscle pain and anti-inflammation. Recently, medical cannabis was officially legalized for research and medical purpose in Thailand. Therefore, scientific evidence to support the use of medical cannabis is still limited. This study was divided the aim of the study into 3 parts; 1. To evaluate and develop a pharmacognostic standardization of *C. sativa* leaves collected from 12 various locations of Thailand according to WHO guidance including pharmacognostic specification, macroscopic-microscopic examination, TLC fingerprinting, physicochemical parameter, and preliminary phytochemical screening using standard methods. 2. To establish DNA barcoding of *C. sativa* from 20 samples and suggested the most suitable one for *C. sativa* identification. 3. To evaluate *in vitro* biological activities of ethanolic extract of KLKS remedy including antioxidant, anti-inflammatory, anti-diabetic and acute toxicity activities. The results indicated that 1. pharmacognostic standardization evaluation of the loss on drying, water content, total ash and acid insoluble ash were 4.07 ± 0.09, 7.95 ± 0.12, 14.36± 0.13, 2.73 ± 0.09% , respectively. Water and ethanol extractive vales were 11.10 ± 0.15 and 23.04 ± 0.16%, respectively. Phytochemical screening showed the present of alkaloids, phenolics, flavonoids, triterpenes, steroids and diterpenes. 2. Establishing DNA barcoding of 20 samples of *C. sativa* from three candidate DNA regions (ITS, *mat*K, and *rbc*L), the phylogenetic tree was constructed and genetic distance was investigated using a maximum likelihood and the result found that the ITS region was suitable marker for *C. sativa* species identification. 3. *In vitro* biological activities of ethanolic extract of KLKS remedy, the results indicated that KLKS remedy and its ingredients showed antioxidant, anti-inflammatory, and anti-diabetic properties. The ethanolic extract of KLKS remedy against brine shrimp lethality tasting showed week toxicity (LC50 = 503.13 µg/ml) whereas each ingredient of KLKS remedy showed median toxicity to non-toxic (LC50 = 150.07 - > 1,000 µg/ml). The finding results provide highly useful information for establishing standardization and identification of *C. sativa* leaves, and also contribute to the quality control, safety and effectiveness of medical cannabis for health benefits.

Field of Study:	Public Health Sciences	Student's Signature .....
Academic Year:	2022	Advisor's Signature .....
		Co-advisor's Signature .....

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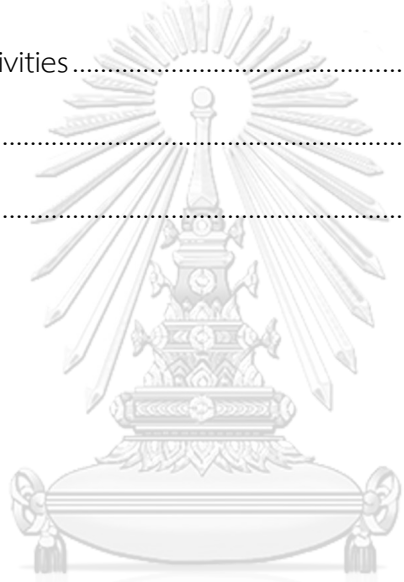

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CHAPTER I

INTRODUCTION

Background and rationale

Cannabis sativa L. subsp. Indica is a genus of annual, dioecious and flowering plants in the family Cannabaceae. The leaves are palmately compound or digitate, with serrate leaflets which usually 7-9, or maximum of 13 leaflets par leaf depending on variety and growing conditions. There are three recognized species which are *Cannabis sativa*, *Cannabis indica* and *Cannabis ruderalis*. Cannabis is indigenous to Central and Western Asia, long cultivated in Asia and Europe (Kuddus et al., 2013). People use the dry leaves, seed oil and other parts of the cannabis plant for recreational and medicinal purposed. In China and India, it has been long history used for its food, fiber and medicine. Cannabis contains at least 120 active ingredients which are known as cannabinoids. The main compounds are delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD). Besides THC and CBD, more than 100 other cannabinoids have been identified. THC is the main psychoactive compound and primarily responsible for the effects on mental state while CBD is non-psychoactive effect. In the United States, cannabis was approved widely as medicine and described in the United States Pharmacopoeia (Bridgeman and Abazia, 2017). Medical cannabis refers to the use of cannabis or cannabinoids as medical therapy. Cannabis has a history of medicinal use across many cultures. The medicinal cannabis is used to reduce nausea and vomiting during chemotherapy, chronic pain, muscle spasms (Whiting et al., 2015), analgesic, intoxicant, narcotic, anodyne, stomachic, antispasmodic, and sedative etc. (Goutopoulos and Makriyannis, 2002; Russo and Guy, 2006).

According to Austin (2019) found that cannabis (Marijuana) classified as three different types: indica, sativa, and hybrid varieties. Hybrid cannabis refers to a strain created by combining varieties ratio of both sativa and indica strains. Variations observed in the cannabis genus were largely due to man's cultivation and selection. These varieties are bred and cultivated to create specific characteristics which often reflect properties of plant including taste, color, smell, percentage of cannabinoids,

and also the medicinal effects. There are over 700 strains of cannabis have been reported. Plant species have been identified by their morphological features such as shape size and color. This type of identification often required and experienced professional taxonomy. If the plant material is damage or immature, identification was not possible thus the use of DNA for identification was developed. The only way to know the genetic composition of cannabis is to analyze its DNA. Therefore, molecular analysis play important role for identification of plant species. DNA barcoding is a taxonomic technique for species identification using a short DNA sequences marker from a specific gene in the genome (Lahaye et al., 2008). The short DNA sequence is generated from standard region of genome known as marker. Multi-locus markers commonly used region for plants are internal transcribed spacer (ITS), ribulose-1,5-bisphosphate carboxylase-oxygenase gene (*rbc*L) and maturase K (*mat*K). DNA barcoding has great applicability in medicinal plants identification. It is important role in the characterization and identification of those uncertified cannabis strains (Barcaccia et al., 2020).

Under international drug control, the use of cannabis in the world is limited to scientific and medical purposes under the Cannabis Act and the Food and Drug Act. In United States, more than 30 states have legalized medical cannabis. Thailand was classified cannabis, kratom and magic mushrooms into the least serious category five, while cannabis and kratom were a lightly addictive and psychoactive indigenous plant, which used as traditional medicine. On 18 February 2019, with the publication of the royal decree in the Royal Gazette that medical cannabis was officially legalized for research and medical purpose. On 9 June 2022, Ministry of public health was allowed to grow hemp and cannabis plants without having for the permission, as that when both plants will be remove from the “category five” narcotic list. However, extract containing more than 0.2% of THC, the primary psychoactive compound in cannabis are still classified as illegal. Extracts with more than 0.2% of THC will still be recognized as a category five substance. As Thailand is the first country in Southeast Asia to amend the narcotics laws and allow cannabis, still a narcotic drug, to be used for research and medical purposes. Narcotics Act of 2019 is a modification of the Narcotics Act of 1979, whereby cannabis was still classified as a class-5-narcotic. Long term cannabis

use adversely affects the brain that controls memory and thinking ability, as well as increases the risk for psychosis, depression and suicide. However, the legalization has been a controversial debate with a need to weigh benefits and harms like public health, misuse leading to more crimes, and a chance to be consumed by those underage. Currently, only hospitals and research facilities are allowed to apply for cannabis production and extraction licenses, but the government is reviewing regulations to enable Thai businesses to apply for permits.

As cannabis has been an important source of medicinal substances, it is necessary to develop standardization for evaluating the safety, quality and efficacy of cannabis material. Herbal medicines are natural products and their phytoconstituents depending on many factors such as time and region, processing and storage condition. These variations could impact its efficacy profile. The current standards, parameters, and protocols available to test the quality of herbal medicines were originally developed. Methods of standardization should be considered in all aspects that contribute to the quality of the herbal medicines such as correct identification, organoleptic evaluation, pharmacognostic evaluation, phytochemical evaluation, toxicity testing and biological activity.

Cannabis has been part of Thai medicine for more than three centuries. There are at least 91 formulas that have cannabis as the ingredient. The Public Health Ministry approved 16 old formulas in the pipeline that had cannabis as the main content for production and application to treat nausea, improve appetite, promoting sleep, and relieve pain and anxiety. In Thai Traditional medicine, 16 cannabis-based traditional medicine formulas have been developed for treatment of diseases or for research, one of them is Kealomkeasan remedy.

Kealomkeasan (KLKS) remedy is one of the Thai traditional medicines notified in Wechasard wannana. The remedy consists of seven plants with an unequal part by weight as follows: 1 part of *Cuminum cyminum* L. fruits, 2 parts of *Nigella sativa* L. seeds, 3 parts of *Foeniculum vulgare* Miller fruits, 4 parts of *Zingiber officinale* Roscoe rhizomes, 5 parts of *Plumbago indica* L. roots, 20 parts of *Cannabis sativa* L. leaves and 40 parts of *Piper nigrum* L. seeds. This remedy has been used as an analgesic drug for relief of muscle pain in Thai traditional medicine practice (Worakijpisan, 1917).

There are many previous reports both in vitro and in vivo pharmacological properties of individuals plant ingredient and predominantly for anti-oxidant, anti-diabetic, anti-inflammatory and analgesic activities (Bhat, Rizvi, & Kumar, 2014; Jeena et al., 2014; Onyesife et al., 2014; Shanmugapriya et al., 2012). However, there is no scientific research report on their phytoconstituents and pharmacological property of KLKS remedy extract as well as the standardization parameter and their DNA barcoding of Cannabis plant distributed in Thailand.

Therefore, this study was designed to investigate the standardization parameter and DNA barcoding of Cannabis distributed in Thailand. Together with the pharmacological properties i.e. anti-oxidant, anti-diabetic, anti-inflammatory and toxicity activities of KLKS remedy and its 7 main ingredients will also be emulated to ensure the efficacy and therapeutic effects of herbal extracts. All of the information obtained in this study provided the basic scientific evidences and support the traditional use of KLKS remedy to ensure the efficacy and quality of Thai herbal medicines.

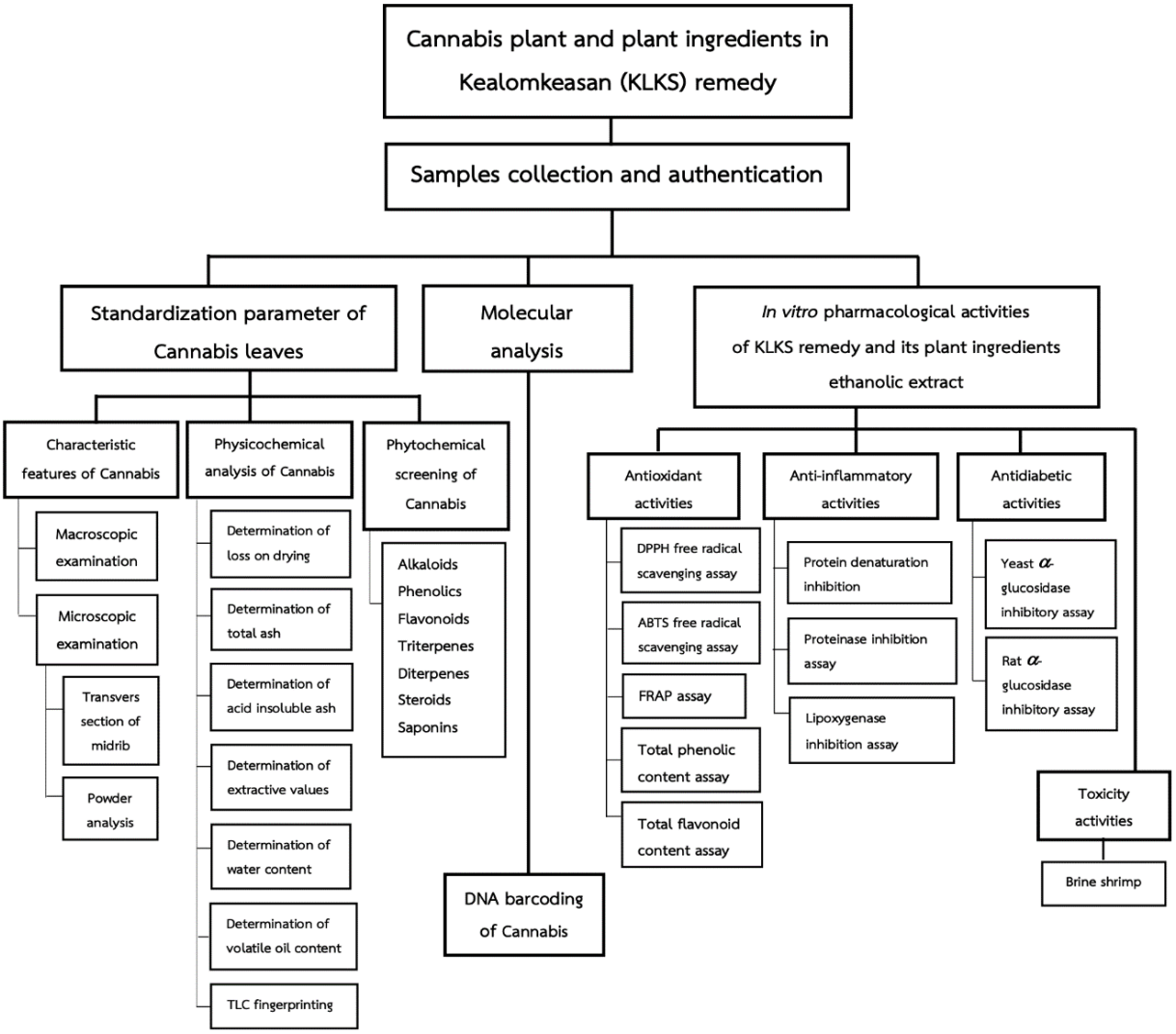
Research questions

1. How does the standardization parameters of Cannabis leaves distributed in Thailand
2. How does of the genetic diversity of cannabis plant using DNA barcoding
3. How does the pharmacological property of Kealomkeasan remedy (KLKS) ethanolic extract and its individual plant ingredients?

Objectives of the study

1. To establish the standardization parameter of Cannabis leaves distributed in Thailand
2. To characterize Cannabis plant distributed in Thailand using DNA barcoding
3. To investigate Pharmacological property of Kealomkeasan remedy (KLKS) ethanolic extract and its individual plant ingredients.

Conceptual framework



CHAPTER II

LITERATURE REVIEW

The history of Cannabis

Cannabis, also known as Marijuana, has a long history of human use. It is one of the oldest cultivated plants indigenous to Central Asia and the India subcontinent (ElSohly, 2007). The oldest known written record on cannabis use comes from the Chinese Emperor Shen Nung in 2727 BC. Ancient Greeks and Romans were also familiar with cannabis. Cannabis spread to the Western hemisphere in 1545 for its use as fiber. In Asia around 500 BC, most ancient cultures grow the plant as herbal medicine. Historically, cannabis plants used for ritual and medicinal purposes involved oral ingestion, inhaling the smoke or vapors by burning the gried plant. In Japan since the pre-Neolithic period, cannabis has been cultivated for its fibers and as a food source (Robert and Mark, 2013).

Cannabis plant in Thailand

The cannabis plant is a genus of the Cannabacaeae family. There are three species of marijuana, i.e., *Cannabis sativa*, *Cannabis indica*, and *Cannabis ruderalis*. Sativa is the most commonly grown cannabis species, and for a long time, people assumed that all marijuana was sativa. Carl Linnaeus was the first to classify the genus in 1753. He believed it was monotypic (one species), which he elected to call *C. Sativa* L. In generally find sativa plants in nations below a latitude of 30 degrees north. These countries include Thailand, Mexico, Colombia and South East Asia (Shah, 2004; Tanguay, 2021). Cannabis also called "ganja" in Thailand is a popular plant in the history of Thailand. Traditionally cannabis has been used extensively in daily life in Thailand for many purposes, particularly as a cooking and for recreational purposes. Thai cannabis is also applicable for medicinal usage even though the CBD content of Thai seeds is quite low. Traditional Thai medicine used cannabis to treat a variety of health conditions such as, sedative, analgesic, astringent and massage oils. It is also used for treatment of depression, pain and stress (Aroonsrimorakot et al., 2019). In

1979 Thailand passed the Narcotics Act, which prohibited cannabis use in all forms. In December 2018, Thailand's National Assembly unanimously voted for amending national laws in favour of medical cannabis. While Thailand became the first country in Southeast Asia to legalize medical marijuana in 2018, its use for recreational purpose is still banned. In February 2019, cannabis and hemp extracts were removed from state control and products containing hemp were reclassified in August 2019. Additional reforms a year later allowed private medical operators to grow and trade the crops. In December 2020, authorities removed additional parts of the cannabis plant from criminal statutes. In March 2021, Deputy Prime Minister and Public Health Minister announced that households could legally grow up to six cannabis plants (Tanguay, 2021)

Cannabis Plant

Cannabis is an annual, dioecious, flowering plant. The leaves are digitate or palmately compound, with serrate leaflets, the number gradually increasing up to a maximum of thirteen leaflets per leaf depending on the variety and growing conditions. The plant has been used for oil production, fiber, and traditional uses. The taxonomic classification of cannabis is as follow:

Taxonomic classification	
Kingdom	Plantae
Family	Cannabaceae
Genus	*Cannabis*
Species	*Cannabis sativa* L., *Cannabis indica* Lam., *Cannabis ruderalis* Janisch

There are 3 species that can be recognized: *Cannabis sativa* L., *Cannabis indica* Lam., and *Cannabis ruderalis* Janisch **(Figure 1 and Table 1).**

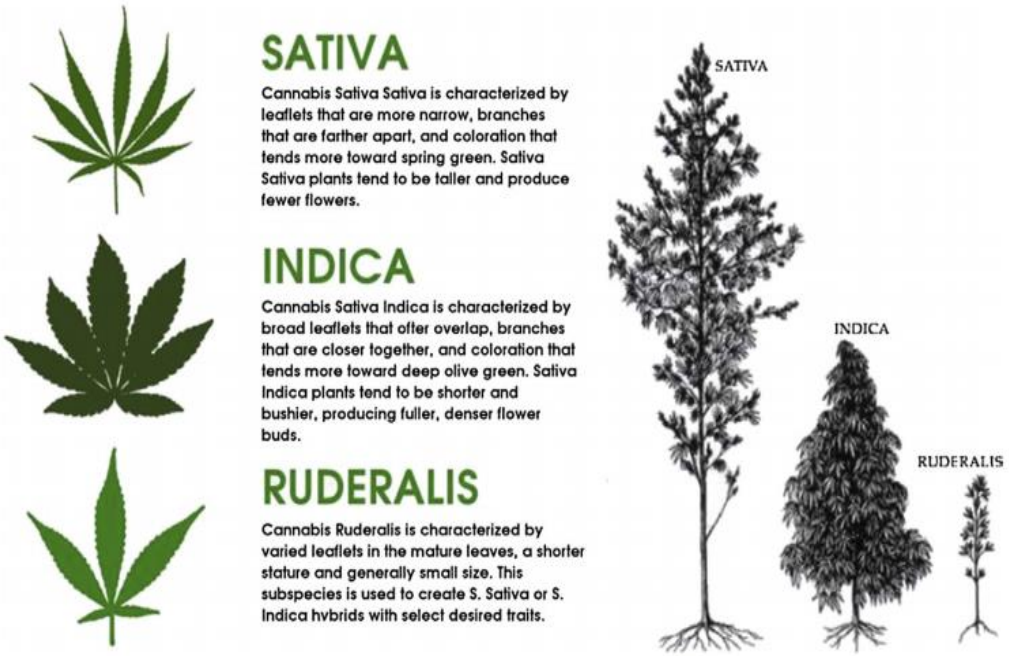


Figure 1. Morphologic differences between 3 Cannabis species (Messina et al., 2015)

Table 1 The different three species of cannabis

Characteristic of each species	***Cannabis sativa* plant**	***Cannabis indica* plant**	***Cannabis ruderalis* plant**
Origins	Asia, Africa and America	Pakistan and India	Eastern Europe and Russia
Plant characteristic	- The sativa is very tall, grow up to of 4.5 m. or more - Leaves are thin and long, long branches with large distances between nodes - Expansive root system - The flowering period usually is between 9 and 12 weeks.	- The indica is smaller than the *C. Sativa,* grow up to 1.5 m. - Leaves are wide, shot, dark green, and robust stalks - Condensed root system - The flowering period is between 7 and 9 weeks.	- The ruderalis is small in size, maximum height of 1.5 m. - Conical shape - Leaves are small, like a bush, fewer branches than Indica or Sativa plant - The flowering short period about 3 week.
Effect/use	Sativas smell sweeter than indicas and contain relatively large amounts of THC. They have a more 'high' and activating effect. Sativa plant commonly incite feelings of euphoria high when consumed. It can be used in small doses to boost energy levels, divergent thinking. and creativity	Indica plants is more intense sativa, contain higher levels of CBD and have a more relaxing effect. It is also more suitable for medical use. This plant can be used to aches, soothe muscles, and pains. It also can help induce sleep or treat insomnia.	Ruderalis usually has very low THC levels and therefore is less interesting to cultivate. However, hybrids between a ruderalis and an indica or sativa can be very useful.

Cannabis is dioecious, meaning it comes as separate female and male plants. Female plant is darker, shorter and has short hairs protruding at the end of the bracteole pods while the male plant are taller, thinner and have flower like pods which contain the fertilizing, pollen-generating anthers. Hermaphrodites are a type of plant that contains both male and female flowers. Plants may naturally become hermaphrodites or be turned into one due to stress. There are many factors that can stress out cannabis plant such as extra light, too much or not enough water, certain insects or pathogens. Characteristics of male, female, and hermaphrodite cannabis shown in **Figure 2.**

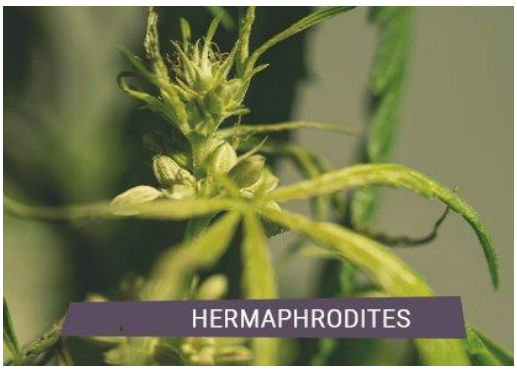


Figure 2 Characteristics of male, female, and hermaphrodite cannabis https://www.royalqueenseeds.com/blog-cannabis-plants-male-female-and-hermaphrodite-n513

Macroscopical features:

"The roots are branched taproot, generally 30–60 cm deep, up to 2.5 m in loose soils and more branched in wet soils. The stems are angular, furrowed, branched, with woody interior, sometimes hollow in the internodes, and vary from 1 to 6 m in height. The branching is either opposite or alternate. Leaves are green and palmate (seven lobes). The leaf arrangement is either opposite, or alternate or spiral. The leaflets are 6–11 cm (length) and 2–15 mm (width). Leaf margins are coarsely serrated. The adaxial and abaxial surfaces are green, with scattered, resinous trichomes. However, the size and shape of the leaflets differs markedly, according to genetic origin. Flower: Inflorescences consist of numerous flower heads that can be found on long, leafy stems from each leaf axil. The staminate (male flower) consists of five pale-green, hairy sepals about 2.5–4 mm long, and five pendulous stamens, with slender filaments and stamen. The pistillate (female flowers) are almost sessile, and are in pairs. The fruit (seed), is an achene, contains a single seed with a hard shell tightly covered by the thin wall of the ovary, and it is ellipsoid, slightly compressed, smooth, about 2–5 mm long, generally brownish and mottled" (Preedy, 2017).

Microscopical features:

Cannabis trichomes consist a nonglandular and glandular types of trichomes. Two types of the nonglandular trichome; (1) Noncystolithic trichomes: unicellular, long, curved, smooth, covering trichomes and (2) Cystolithic trichomes: unicellular, claw shape, more squat, cystolith covering trichomes, which containing calcium carbonate. Glandular trichomes are made from a series of differentiated cells with different functional properties, i.e. stalk cell and secretory cells (Kim & Mahlberg, 1991). Female plants have been described three types of glandular trichome; (1) bulbous: with smallest gland, (2) sessile: simple structure, the head of trichomes connected directly to the mesophyll cells, and (3) capitate stalked: more complex structure, and developed resin head (glandular head) (Happyana et al., 2013). Fourth type of the antherial glandular trichome have been found on male plants, which has only been found on anthers (Fairbairn, 1972). The stigma is a thick, brown structure that is part of the sexual reproductive system of the cannabis plant.

Chemical constituents of Cannabis

Cannabis contains a several of medicinally important compounds, such as cannabinoids, terpenes, flovonoids, alkaloids, and others. Cannabinoids are a unique class of terpenophenolic compounds to cannabis plants, accumulated mainly in the cavity of trichomes. Trichomes are a blanket of crystal resin coating the cannabis plant, and they contain both cannabinoids and terpenes. More than 80 cannabinoids have been isolated from *C. sativa* (Devinsky et al., 2014). The main psychoactive compound is Δ^9-tetrahydrocannabinol (THC), with well-known medicinal effects (Elbatsh et al., 2012). Cannabis is made up of more than 120 compounds, which as cannabinoids.

Tetrahydrocannabinol (THC) is a major compound known to cause psychoactive effects of cannabis plant. THC interact with brain parts which is controlled by the endogenous cannabinoid neurotransmitter called anandamide which is play role in memory, pain sensation, and sleep (**Figure 3**).

Effect: analgesic, antioxidant activity, anti-inflammatory and appetite stimulant

Cannabidiol (CBD) is a non-psychoactive compound, can be present up to 40% of the medical cannabis plants extracts**.** Rather than stimulating you emotionally like THC, CBD calms down and alleviates anxiety and pain (**Figure 3**).

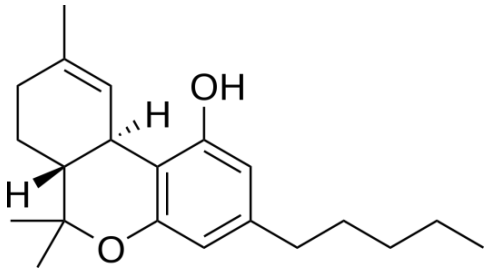
Effect: CBD has been known to relieve inflammation, convulsion, nausea, anxiety, congestion, cough, and inhibits growth of cancer cell. It use as an analgesic, antipsychotic, antioxidant and immunosuppressive**.** CBD also blocks the intoxicating effects of THC, preventing several undesirable side effects including dry mouth and it can interact with blood pressure medicines, making them less effective. Other side effects of CBD include somnolence, weakness, fatigue, diarrhoea, decreased appetite, malaise, and others

Cannabinol (CBN) is a non-psychoactive cannabinoid present only in trace amounts in Cannabis. CBN is an oxidative degradation product of THC, usually created when THC is exposed to heat and light, which breaks it down, leaving behind CBN. Because of this, no strains of cannabis contain high levels of CBN themselves. CBN acts as a weak agonist of the CB1 and CB2 receptors, with lower affinity in comparison to THC. It also possesses psychoactive properties, but not as similar as THC (**Figure 3**).

Effect: pain relief, anti-inflammatory, anti-insomnia, anti-convulsive, acts as an appetite stimulant, promoter of bone cell growth, and antibacterial effects. It is beneficial to those who are afflicted with Parkinson's and multiple sclerosis (Srivastava and VK, 2013).

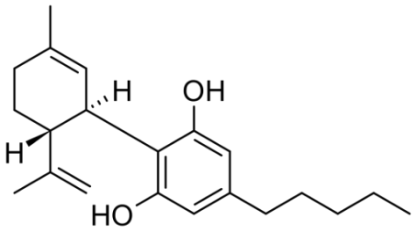
Terpenes:

Terpenes are organic aromatic compounds, commonly found in the oils of flowers including cannabis. Terpenes and cannabinoids work in harmony generating an "entourage effect" and enhance cannabis medical properties.



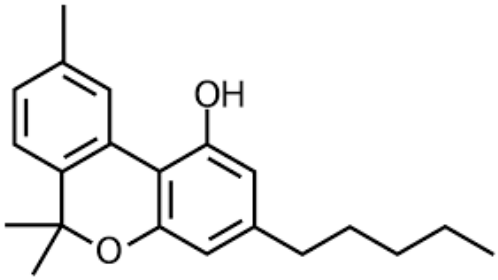
Delta-9-tetrahydrocannabinol (THC)

($C_{21}H_{30}O_2$)



Cannabidiol (CBD)

($C_{21}H_{30}O_2$)



Cannabinol (CBN)

($C_{21}H_{26}O_2$)

Figure 3 Chemical structures of cannabinoids

Chemotaxonomic classification

Recently, chemotaxonomic classification splits the phenotypes based on the quantitative differences in the cannabinoid ratio of tetrahydrocannabinolic acid (THC), cannabinol (CBN), and cannabidiol (CBD), in the ratio of ZTHC] + [CBN]/[CBD]. If the ratio exceeded 1, plants are classified as “chemo-type” otherwise as “fiber-type,” and this was the first study to differentiate between the drugand fiber-type, by Fetterman et al. (1971). Therefore, this ratio was subsequently used to discriminate chemotype, intermediate type, and fiber-type (Turner et al., 1979).

Endocannabinoids system

The endocannabinoid system (EC) comprise two type cannabinoid (CB) receptors; CB1 and CB2 are G-protein coupled receptors, endocannabinoids or endogenous ligands are N-arachidonoylethanolamine (AEA) called anandamide, 2-arachidonoylglycerol (2-AG), and different enzymes involved in the biosynthesis and degradation of the endocannabinoids such as fatty acid amide hydrolase (FAAH) is responsible for the degradation of anandamide; monoacylglycerol lipase (MAGL) is the key enzyme involved in the hydrolysis of 2-AG; and recently- α, β-hydrolase-6 and -12 (ABHD-6 and -12), have been identified to participate in the 2-AG hydrolysis in several tissues, especially in the brain (Marrs et al., 2010; Savinainen et al.,2012). Nowadays, most drugs used therapeutically that interact with the EC system are derived from Cannabis and produce their effects by modulating cannabinoid receptors activity.

Cannabinoid receptor system

Cannabinoid receptors (CB receptor) are commonly two type receptor located throughout the body: CB1 and CB2 receptor, It is activated by three major groups: ligands, endocannabinoids (produced by the mammalian body), plant cannabinoids (such as THC), and synthetic cannabinoids

CB1 receptors are found primarily in the brain, and also expressed in several peripheral tissues, such as liver, kidney, lungs, skin gastrointestinal tract, adipocytes, bone and immune system (Pagotto et al., 2006). CB1 receptors, besides appearing to be responsible for the euphoric and anticonvulsive effects of Cannabis, It also involved in certain other biological activities such as gastrointestinal, pain, anxiety, cardiovascular, olfaction, and liver de novo lipogenesis (Cabral et al., 2008).

CB2 receptors are mainly found in the immune system (macrophages, T- and B-cells), peripheral tissues, central nervous system (CNS) in glial cells, and astrocytes (Cabral et al., 2008). CB2 receptors are involved in physio pathological conditions of ability to modulate the immune cells activity. The CB2 modulation activity is an appealing therapeutic option, as it lacks the typical psychotropic side effects linked to CB1 modulation. The relevance of an individual cannabinoid to each receptor type

determines the selectivity of the effect and there for the benefit or risk ratio of that cannabinoid. Due to the numerous activity of the CBs receptors, the EC system is becoming an interesting target for the treatment of several diseases, among the others neuroinflammation, endocrine, cancer, and motor dysfunctions (Messina et al., 2015)

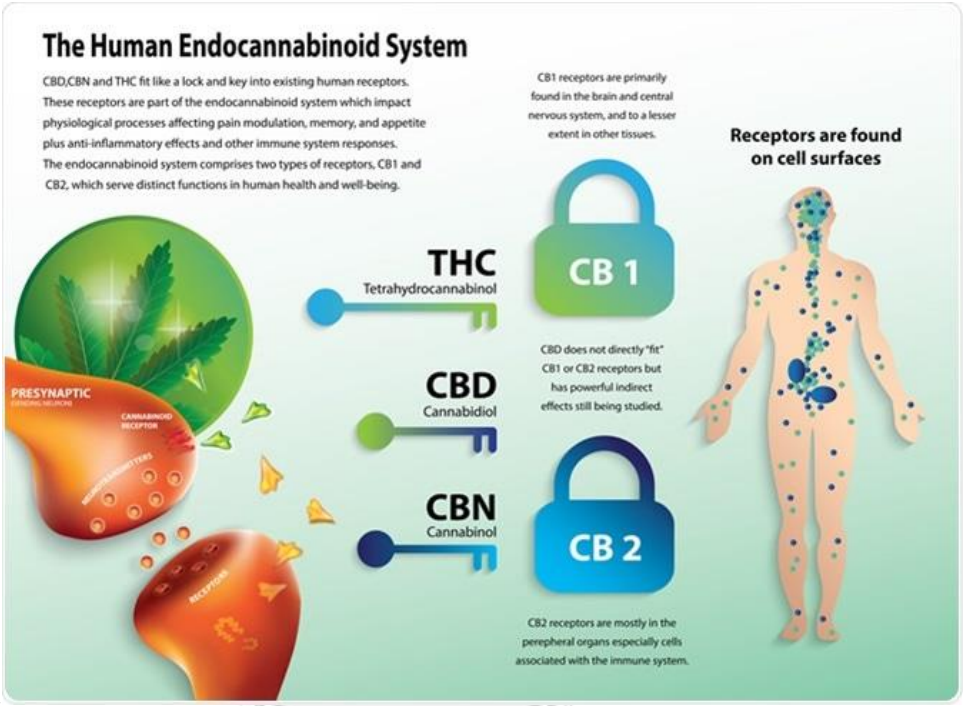


Figure 4 The Cannabinoid (CB) Receptors and Endocannabinoids (EC)
https://www.cbdoliv.com/la-cannabis/index

Molecular classification

Various molecular techniques have been applied to evaluate the genetic relationship among different varieties of Cannabis. Some recent studies have classified and identified Cannabis by several molecular techniques such as random amplified polymorphic DNA (RAPD) (Gillan et al., 1995) and restriction fragment polymorphism (RFLP) analysis (Faeti et al., 1996) Inter simple sequence repeat (ISSR) (Kojoma et al., 2002) but still a few works conducted by using DNA barcoding.

DNA barcoding of *C. sativa*

Although various authors, supporting the one species for cannabis, recommend to classify its varieties based on chemical constituent; terpenoids and cannabinoids profile (Hazekamp et al., 2016; Piomelli & Russo, 2016), a molecular analysis based on DNA barcoding could represent a time and cost-effective technique of good help in clarifying some of the taxonomic issues related to the genus Cannabis (Natasha et al, 2015).The protocol for DNA barcoding analysis shown in **Figure 5.**

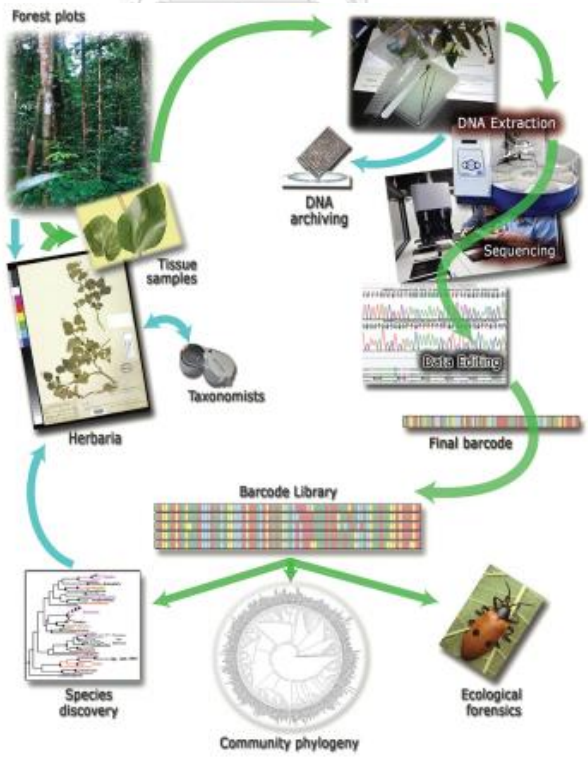


Figure 5 Protocol for DNA barcoding analysis

DNA barcoding is a technique for species identification is performed by using DNA sequences from a small fragment of the genome, with the objective of supporting to a wide range of conservation and ecological studies in which traditional taxonomic identification is not practical (Lahaye et al., 2008). DNA barcoding play important role in the characterization and identification of those uncertified cannabis strains, which are derived from black market or hybrid strains (Barcaccia et al, 2020). In other hand, cytochrome c oxidase I (coxI) mitochondrial gene has been established for animals. However, it is not suitable for plant species distinction due to the low mutation rate in the plant mitochondrial genome.

The general molecular approach for the taxonomic evaluation of a subspecies or species is applying DNA barcoding to the extra-nuclear genome. According to Asahina (2007) presented the suitable of the ribulose-bisphosphate carboxylase (*rbc*L) gene and *trn*H-*psb*A noncoding spacer region as DNA barcodes for plant categorization. Other studies confirmed that the materase (*mat*K) gene was comprised in the list of useful marker for DNA barcoding in plant (Asahina et al., 2010; de Vere, 2015). Furthermore, as the classification efficacy of these barcodes has sometimes been demonstrated to not be sufficiently informative, the use of other regions, both nuclear and plastid such as ITS1 and ITS2, and *rpo*Cl and *ycf*5 respectively, has been suggested for this purpose.

Other studies confirmed that four primary gene regions; one nuclear gene region (ITS) and three chloroplast gene (*rbc*L, *mat*K and *trn*H-*psb*A) have mostly been accepted as standard DNA barcoding for plants (CBOL Plant Working Group, 2009; China Plant BOL Group, 2011; Li et al., 2015).

Single locus DNA barcoding

Internal transcribed spacer (ITS):

Internal transcribed spacer (ITS) is the spacer DNA (non-coding DNA) located between the large-subunit rRNA genes in the chromosome and small-subunit ribosomal RNA (rRNA). Eukaryotic cells including plant has two ITS regions which is ITS1 and ITS2. The ITS-1 is situated between 18S and 5.8S rRNA genes while ITS-2 is in

between 5.8S and 25S rRNA (**Figure 6**) were analyzed to examine nuclear inheritance (White et al. 1990, Baldwin et al. 1995)

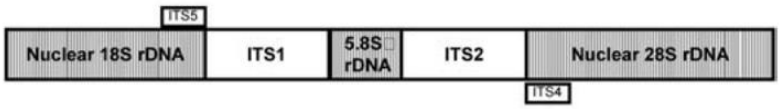


Figure 6 The internal transcribed spacer (ITS) region of nuclear rRNA genes (Sturtevant et al., 2009)

Ribosomal ITS sequences are the most commonly used sequence in studies of plant molecular phylogeny (Alvarez and Wendel, 2003). Mutations tend to accumulate in the ITS region because it does not code for either rRNA or protein (Baldwin et al. 1995). Because the ribosomal ITS sequences are nuclear-encoded they are inherited biparentally (Alvarez and Wendel, 2003), making the ribosomal ITS sequence useful in studies on hybrid speciation (Andreasen and Baldwin, 2003; Koch et al., 2003). Ribosomal ITS sequences have been successfully used to distinguish between *M. sibiricum, M. spicatum*, and the hybrid at the molecular level using the universal primers. Molecular characterization of the ribosomal ITS region has been used to identify genotypes of watermilfoil found in Wisconsin, Minnesota, Florida and Connecticut, but has only recently been used to study watermilfoil in Michigan (Moody and Les, 2002).

Maturase K (*mat*K):

Maturase K (*mat*K) gene of chloroplast is common size approximately 1500 base pairs, located within the *trn*K gene intron **(Figure 7)** and codes for maturase as protein,which is involved in Group II intron splicing. The two exons of the *trn*K gene that flank the *mat*K were lost, leaving the gene intact in the event of splicing. *mat*K has high evolutionary rate, substitution rates, suitable length and obvious interspecific divergence as well as a low transition and transversion (Selvaraj, 2008).

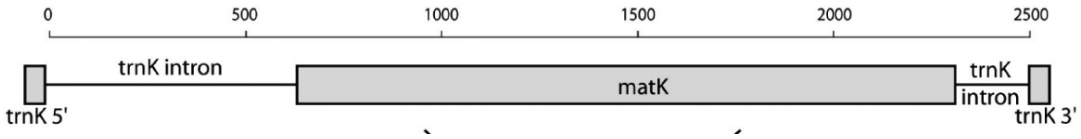


Figure 7 The *mat*K genes, diagram modified from Kim and Won (2016)

Ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbc*L):

Ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbc*L) gene namely known as the RuBisCO, with about 1430 base pairs in size is an enzyme involved in the first major step of carbon fixation, a process by which atmospheric carbon dioxide is converted by plant to energy-rich molecules such as glucose. This gene has slow substitution rate and substitutions between *rbc*L of the parent species are non-synonymous. The sequence of *rbc*L has large phylogenetic importance because of its conserved nature (Selvaraj et al.,2013). A study by de Vere et al. (2012) found that comparing the two DNA barcode markers *mat*K and *rbc*L, *rbc*L indicated better coverage than *mat*K for the native flowering plants.

Beta subunit of the ATP synthase (atpB):

According Hu et al., (2011) showed thatchloroplast gene *atp*B located near *rbc*L gene (**Figure 8**) in the large single-copy region of the plastid genome, its size approximately 1497 base pairs in plants. This gene codes for the beta subunit of the ATP synthase (Zurawski et al., 1982). Furthermore, the *atp*B gene and intergenic spacer region between *rbc*L genes have been used successfully in phylogenetic studies at higher taxonomic levels (Hoot et al., 1999).

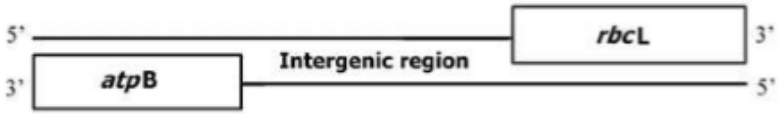


Figure 8 The *rbc*L and *atp*B genes

Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR), an in-vitro technique for the rapid synthesis of DNA sequences, was first introduced by Kary Banks Mullis in 1983. The process simply starts by splitting double-stranded DNA into single-stranded DNA, using oligonucleotide primers for reproduction, and eventually adding polymerase to replicate the DNA strands. The DNA will be doubled for each cycle, hence exponentially increasing the amount of DNA for multiple cycles. As a result of the innovative invention of PCR, Mullis was successfully co-awarded the Nobel Prize in Chemistry, 1993. Since then, PCR has become a priceless tool to today's molecular scientists, biotechnology industries and other related fields (e.g., forensics, GMO food detection, paternity testing, archaeology, disease diagnosis, etc.) (Mullis et al., 1994; Thermo Fisher Scienctific, 2012).

Chemical components of PCR include water, template DNA, primer, deoxynucleoside triphosphates (dNTPs), Mg^{2+}, Buffer and *Taq* DNA polymerase (Weising et al., 2005; Thermo Fisher Scientific, 2012). Starting with water, it is used as the medium for all components in the PCR reaction mixture. Secondly, template DNA is the DNA of sample that contains the target sequence. Larger amounts of template DNA increase the risk in generating the nonspecific PCR products, whereas smaller amounts of template DNA lower the accuracy of the amplification.

Thirdly, primer is composed of short segments of nucleotides, usually 18-30 bases long. Longer primers give more specificity but tend to anneal with lower efficiency, leading to decreased yield. However, they may be useful to distinguish genes with a high degree of sequence homology. On the other hand, shorter primers (less than 15 nucleotides long) anneal very efficiently but they may not be specific enough. The recommended concentration range of primers is 0.1-1.0 μM. Higher primer concentration may promote primer dimers, as well as the probability of mispriming, resulting in the presence of nonspecific PCR products. Lower primer concentration may be exhausted before the reaction is completed, giving lower yields of desired product (Weising et al., 2005; Thermo Fisher Scientific, 2012).

The over temperature of annealing phase may generate the failure of annealing process. In other hand, if the temperature is too high, no annealing occurs. Therefore, in most cases, annealing temperature must be determined and optimized empirically. Optimal annealing temperatures are often 5 °C to 10 °C lower than the melting temperature (T_m) of the primers (Equation 1). Primers in a set (forward and reverse primers) should have similar T_m. Besides, primers should contain no internal secondary structure, have GC content between 50-60%, and be neither complementary to each other at their 3' ends nor self-complementary to prevent primer-dimers (Weising et al., 2005; Thermo Fisher Scientific, 2012).

Equation 1. $T_m = [4\times(G+C)] + [2\times(A+T)]$

Fourthly, the recommended concentration of each dNTP is 0.2 mM. It is essential to have equal concentrations of all four nucleotides (dATP, dCTP, dGTP and dTTP). The imbalanced concentrations of dNTP may increase the PCR error rate and lower the fidelity of the thermostable DNA polymerase. If the concentration of dNTPs is increased, the concentration of Mg^{2+} ion in the reaction must also be increased. An increase in dNTP concentration reduces free Mg^{2+}, thus interfering with polymerase activity and decreasing primer annealing (Weising et al., 2005; Thermo Fisher Scientific, 2012).

Fifthly, most thermostable DNA polymerases require a source of divalent cations to function. In most cases, the divalent cation required is Mg^{2+}. Mg^{2+} influences enzyme activity and increase the T_m of double-stranded DNA. Mg^{2+} forms soluble complexes with dNTPs to produce the actual substrate that the polymerase recognizes. Some DNA polymerases use Mn^{2+} rather than Mg^{2+}. However, in general, DNA polymerase reactions in the presence of Mn^{2+} make DNA copies with significantly lower fidelity than in reactions in the presence of Mg^{2+} (Weising et al., 2005; Thermo Fisher Scientific, 2012).

The concentration of Mg^{2+} in general stabilizes primer-template complexes. Due to the binding of Mg^{2+} to dNTPs, primers and DNA templates, Mg^{2+} concentration needs to be optimized for maximal PCR yield. The recommended concentration ranges from 1-4 mM. Lower Mg^{2+} concentrations lead to specific amplification with less PCR products, while higher Mg^{2+} concentrations produce more non-specific amplification

with lower PCR fidelity. The Mg^{2+} concentration is usually the first one in consideration when comes to optimization. This component can have a huge effect on the efficiency, yield and specificity of the reaction (Weising et al., 2005; Thermo Fisher Scientific, 2012).

Next, PCR buffer is necessary to create optimal conditions for activity *of Taq* DNA polymerase. Buffers often contain Tris-HCl and KCl. PCR buffers are often available in 10X concentration and are sometimes *Taq* formulation-specific. Although most protocols recommend a final buffer concentration of 1X, increasing the concentration to 1.5X might result in increased PCR product yield (Weising et al., 2005; Thermo Fisher Scientific, 2012).

Lastly, *Taq* DNA polymerase is the most commonly used enzyme for PCR. It is suitable for most amplification reactions that do not require high fidelity enzyme or PCR products longer than 3 kb. Normally, 1-1.5 U of *Taq* DNA polymerase is recommended for a 50 µl volume of a PCR mixture. Non-specific PCR products may present at higher concentrations of the polymerase. Non-specific priming events, such as mispriming or primer-dimers, which occur at ambient temperatures, may lead to non-specific amplification products during PCR. Therefore, PCR reaction set-up should be performed on ice (Weising et al., 2005; Thermo Scientific, 2012).

To automate the PCR chemical reaction, a machine called a thermocycler jump-starts each stage of the reaction by raising and lowering the temperature of the chemical components at specific times and for a preset number of cycles. Fundamentally, PCR cycle includes three steps: denaturation, annealing and extension (**Figure 9**) (Science Info World, 2012). Sometimes, two more steps are added for advanced protocol; such steps are pre-denaturation and final extension (**Figure 10**) (Thermo Fisher Scientific, 2012).

The complete denaturation of the DNA template at the start of the PCR reaction is of key importance to make all potential primer binding sites available. Incomplete denaturation of DNA results in the inefficient utilization of template in the first amplification cycle and in a poor yield of PCR product. If the GC content is $\leq$ 50%, the pre-denaturation is performed over an interval of approximately 5 minutes at 95°C;

on the contrary, the interval should be extended up to 10 minutes if the GC content is > 50% (Thermo Fisher Scientific, 2012).

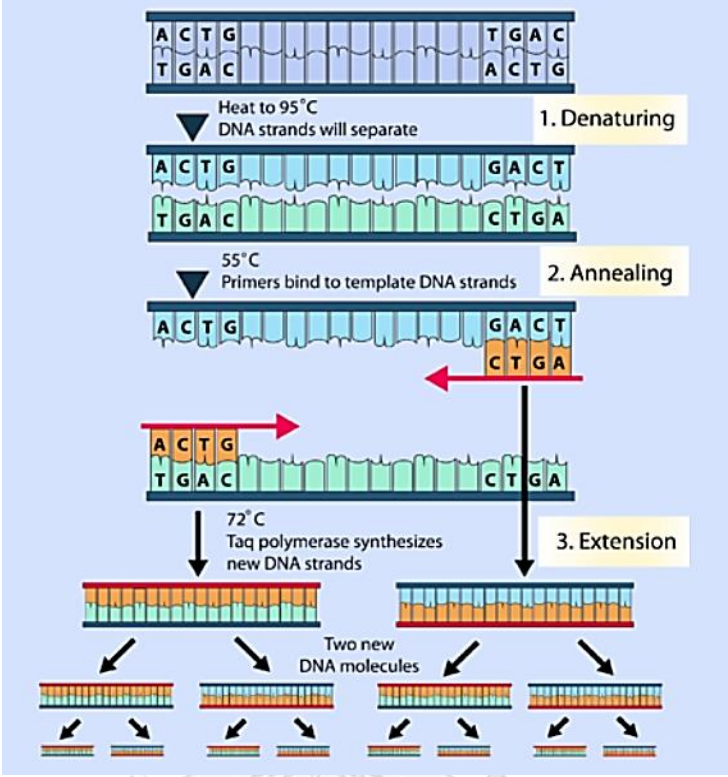


Figure 9 Fundamental PCR thermocycling steps (Science Info World, 2012).

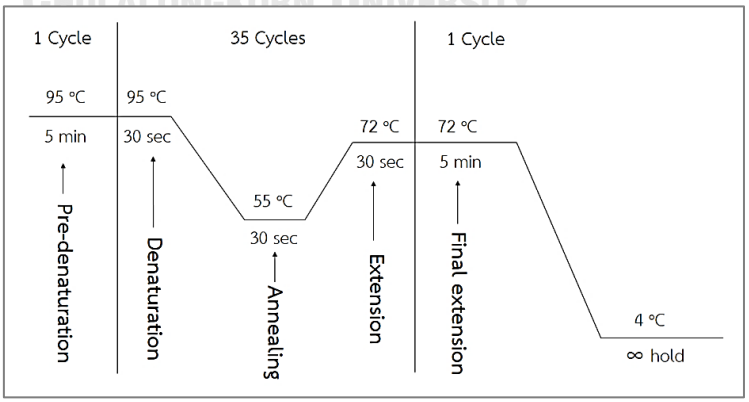


Figure 10 Advanced PCR thermocycling conditions.

Denaturation for 30-45 seconds at 94-95 °C is sufficient to completely denature the DNA synthesized in the first amplification cycle. If the amplified DNA has a very high GC content, denaturation time may be increased up to 3-4 minutes. The high temperature causes the hydrogen bonds between the bases in two strands of template DNA to break and then separate. This results in two single strands of DNA, which will act as templates for the production of the new strands of DNA (Thermo Fisher Scientific, 2012).

The annealing step lasts for approximately 30-45 seconds, which allows enough time for the primers to locate their complement and anneal to the template DNA strand (**Figure 10**). The optimal annealing temperature is commonly 5 °C lower than T_m of primer-template DNA duplex. In most cases, the annealing temperature is between 35 °C and 65 °C, depending on the primer sequence and experimental strategy. However, if non-specific PCR products are obtained in addition to the expected product, the annealing temperature should be optimized by increasing it stepwise by 1-2 °C (Thermo Fisher Scientific, 2012).

Once the primers anneal to the complementary DNA sequences, the extension step begins (Figure 10). The temperature is raised to approximately 72 °C and a thermostable polymerase (e.g., *Taq* DNA Polymerase) starts to synthesize new double-stranded DNA molecules which are identical to the original target DNA. It does this by facilitating the binding and joining of complementary nucleotides that are free in solution (dNTPs). Synthesis always begins at the 3' end of the primer and proceeds exclusively in the 5' to 3' direction.

Thus, the new synthesis effectively extends the primers, creating a complementary, and double stranded molecule from a single-stranded template. After the last cycle, the extension, the samples are usually incubated at 72 °C for 5-15 minutes to fill-in the protruding ends of newly synthesized PCR products. After the final extension, the products may be electrophoresed or stored at 4 °C (Thermo Fisher Scientific, 2012).

Chain termination DNA sequencing method:

"The key principle of this method was the use of dideoxynucleotide triphosphates (ddNTPs) as DNA chain terminators. This method is better than chemical method because of the lower of toxic chemicals and lower amount of radioactivity is used. The method requires a single-stranded DNA template, a DNA primer, a DNA polymerase, normal deoxynucleotide triphosphates (dNTPs; dATP, dGTP, dCTP and dTTP), and modified nucleotides (dideoxyNTPs; (ddATP, ddGTP, ddCTP, or ddTTP), lacking a 3'-OH group required for the formation of a phosphodiester bond between two nucleotides, thus terminating DNA strand extension and resulting in DNA fragments of varying length. These ddNTPs will also be radioactively or fluorescently labelled for detection in automated sequencing machines. The DNA sample is divided into four separate sequencing reactions, containing all four of the standard deoxynucleotides and the DNA polymerase. To each reaction is added only one of the four dideoxynucleotides. The newly synthesized and labelled DNA fragments are heat denatured, and separated by size on a denaturing polyacrylamide-urea gel electrophoresis with each of the four reactions run in individual lanes (lanes A, T, G, C); the DNA bands are then visualized by autoradiography or UV light, and the DNA sequence can be directly read off the X-ray film or gel image (**Figure 11**)"

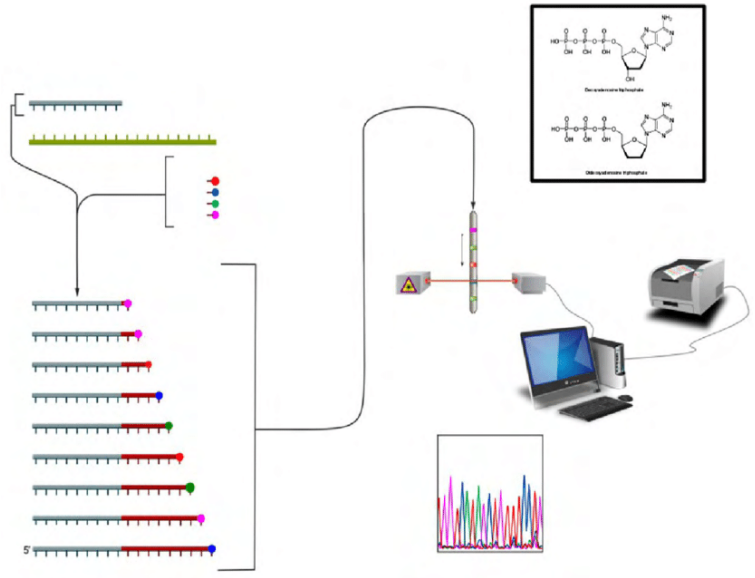
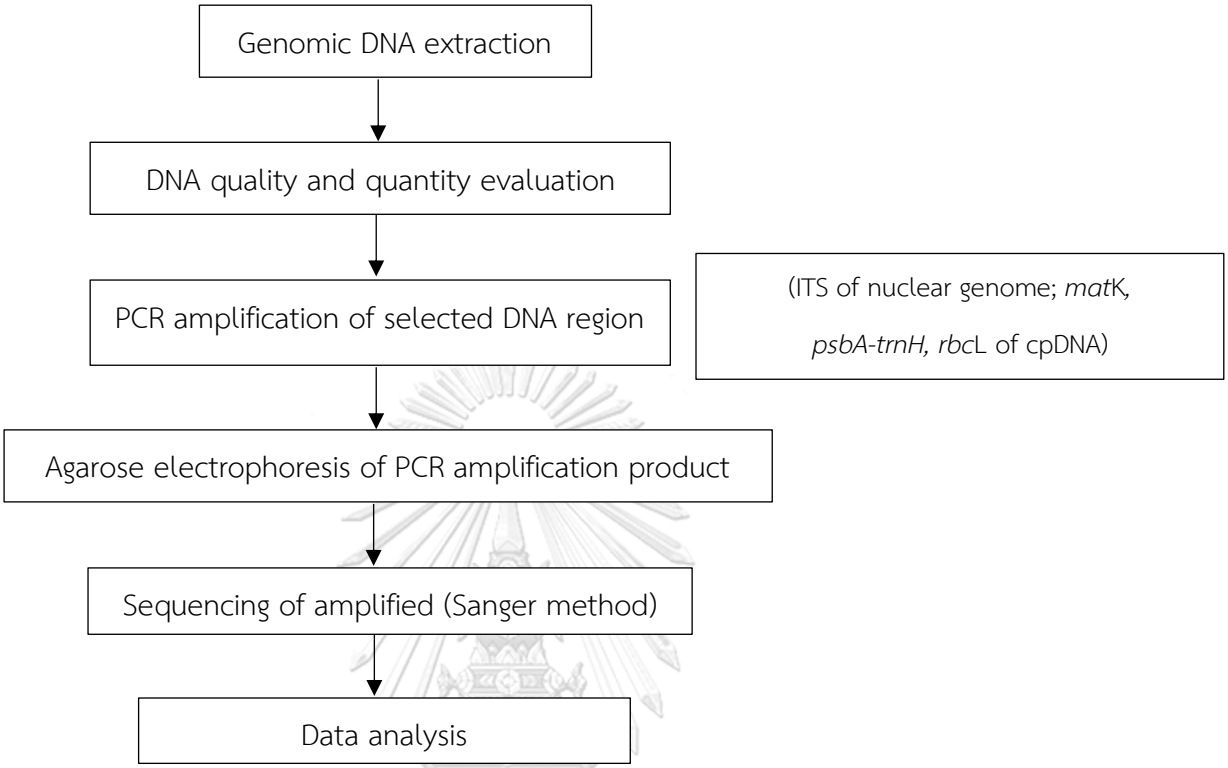


Figure 11 Chain termination method

The summarized protocol of DNA barcoding was descried below:



Phytochemical screening and extraction

Plants are the rich source of drugs of traditional medicine systems, folk medicines, modern medicines, nutraceuticals, food supplements, and pharmaceutical intermediates, it had been worldwide use for treat various disease. The medicinal properties of the plant it depend on organic compounds and the important of bioactive constituents are flavonoids, alkaloids, saponins, glycosides, steroids, tannins, anthraquinones, and terpenoids. These compounds are synthesized by primary or secondary metabolism of living organisms. Secondary metabolites are taxonomically and chemically extremely various compounds (Shrestha et al, 2015) . In plants, secondary metabolites can be separated into three groups; (Hartmann, 2007).

Terpenoids; such as sterols, carotenoids, cardiac glycosides and plant volatiles

Phenolics; such as phenolic acids, flavonoids, lignin, lignans, stilbenes, coumarins and tannins.

Alkaloids; nitrogen containing compounds

Terpenoids

Terpenoids $(C_5H_8)n$ are the largest and diverse family of natural products which are composed of isoprene units (C5) and classified by the number of isoprene unit present in the structure (Mahmoud et al. 2002), Many aromatic and flavor molecules such as linalool, menthol, caryophyllene and geraniol are formed by monoterpenes (C10) and sesquiterpenes (C15). Other bioactive compounds including diterpenes (C20), triterpenes (C30) and tetraterpenes (C40) exhibited special properties.

Phenolic compounds

Phenolic compounds are numerous secondary metabolites of plants. There are more than 8,000 phenolic structures ranging from simple molecules to highly polymerized substances. Phenolic compounds include phenolic acid, flavonoids, tannins and lignans. Phenolics are synthesized primarily from products of the shikimic acid pathway (Knaggs & Andrew, 2001). A common of phenolic compounds structure is the presence of at least one hydroxyl-substituted aromatic ring system. Major phenolic compounds belong to the flavonoids (flavones, anthocyanin, chalcons, quercetin, kaempferol). Flavonoids are commonly known for their antioxidant activity. Previous reports have shown that natural phenols have antimicrobial property (Rauha et al., 2000), antiviral (Perez, 2003), vasodilatory actions (Padilla et al., 2005) and anti-inflammatory (Santos et al., 2006)

Alkaloids

Alkaloids are commonly derived from amino acid and more than 10,000 alkaloids have been discovered from 300 plant families (Raffauf, 1996). Alkaloids largely composed of one or more carbon rings bearing nitrogen which determine the type of alkaloids. Since a long history, alkaloids have been used for medical purpose. Numerous alkaloids are still being used in medicine, usually in the form of salts. For

examples, vinblastine which has antitumor properties (Jordan & Leslie, 2004); quinine which has antipyretics and antimalarial properties (Reyburn et al, 2009); and reserpine which can be used to treat high blood pressure (Moser, 1987).

Principle of phytochemical analysis

The components contained in the extract were analyzed by their compounds by colour test (qualitative) with several reagents**.**

Detection of Terpenoids and steroid (Tiwari, 2011)

- Salkowski's Test:

The powder sample were extracted with chloroform following by the addition a few drops of conc. Sulphuric acid (H_2SO_4), shaken and allowed to stand. The presence of triterpenes are indicated by golden yellow color (Abebayehu et al., 2016).

- Libermann Burchard's test:

The powder sample is extracted with chloroform and evaluated by few drops of acetic anhydride ($C_4H_6O_3$), boiled and cooled followed by adding Conc. Sulphuric acid. The presence of phytosterols are indicated by brown ring occurrence (Linn et al., 2017). An array of colour changes into orange or purple indicated the sample containing terpenoid while a blue colour change showed the positive result of steroid.

Terpenoid/steroid screening is based on the ability of compounds to form concentrated H_2SO_4 colours in solvents of acetic acid anhydride red orange or purple for terpenoids and blue for steroids (Parbuntari et al., 2018).

Detection of phenols

Ferric chloride test

Extract are treated with 3-4 drop of Ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

Detection of flavonoids (Tiwari, 2011)

- Alkaline Reagent Test:

Extracts are evaluated with sodium hydroxide solution. Presence of flavonoids are confirmed by the occurrence of intense yellow colour which becomes colourless after adding of diluted acid.

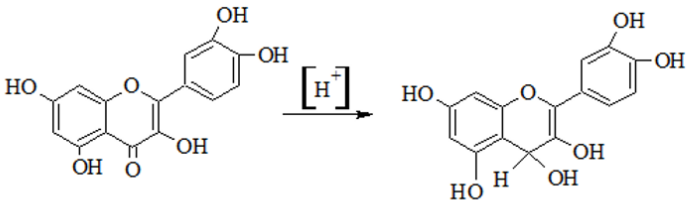
- Lead acetate Test:

Extracts are evaluated by lead acetate solution. Presence of flavonoids are indicated by the occurrence of yellow colour precipitate.

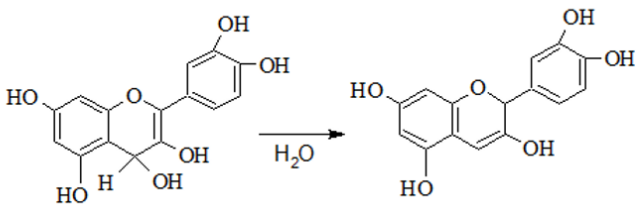
- Shinoda's test:

The alcoholic extract is treated with magnesium turning and concentrated HCl gives red colour which indicates the presence of flavonones. Orange-red color indicates the presence of flavonols.

Flavonoids have a bond with a sugar group which causes the flavonoids to be polar, so that in the flavonoid test the solvent is replaced into a more polar solvent. Methanol solvent is a solvent used in flavonoid tests which are more polar than chloroform with a polarity level of around 4.1. Addition of magnesium powder and hydrochloric acid to flavonoid testing will cause the reduction of flavonoid compounds that exist, causing a red reaction that is characteristic of flavonoids. In flavonoid testing, it is positive in this test because there is a colour changing to redish solution (Parbuntari et al., 2018). The reaction for flavonoid test was proposed as belows.



Flavonoid



Detection of alkaloids (Tiwari, 2011)

There are several chemical tests to screening the present of alkaloids as follow;

- Mayer's Test:

Extract is evaluated with Mayer's reagent (Potassium Mercuric Iodide) and the presence of alkaloids are indicated by yellow coloured precipitate.

- Wagner's Test:

Extract is evaluated with Wagner's reagent which is contained Iodine in Potassium Iodide. The presence of alkaloids is indicated by the occurrence of brown/reddish precipitate.

- Dragendroff's Test:

Extract is evaluated with Dragendroff's reagent which containing Potassium Bismuth Iodide. The presence of alkaloids is indicated by of red precipitate.

- Hager's Test:

Extract is evaluated with Hager's reagent which is contained saturated picric acid solution. The presence of alkaloids is indicated by occurrence of yellow coloured precipitate.

The precipitation in Mayer, Wagner and Dragendorff identification ensure the presence of alkaloid compounds in the ethanol extract. The aim of adding of sulphuric acid is because of the properties of alkaloid which is base. Therefore, it should be extracted in the acid solvents (Parbuntari et al., 2018). The positive result of Mayer test was confirmed by yellow precipitate. It was expected as a complex of potassium-alkaloid. In the formation of Mayer reagent, the solution of mercury (II) chloride was added by potassium iodide and produced a red precipitate of Mercury (II) iodide. The excess of potassium iodide addition introduce to potassium tetraiodomercurate (II) formation (Altemimi et al., 2017). Alkaloids consist of nitrogen atoms which have lone pair electrons. The lone pair electrons are examined to form covalent coordinate bonding with metal ion (Parbuntari et al., 2018). In alkaloid identification with Mayer reagent, the nitrogen in alkaloids was predicted to react with metal ion of potassium

(K+) from potassium tetraiodomercurte (II) producing a complex of potassium-alkaloid precipitating. The reaction Mayer test was proposed as belows.

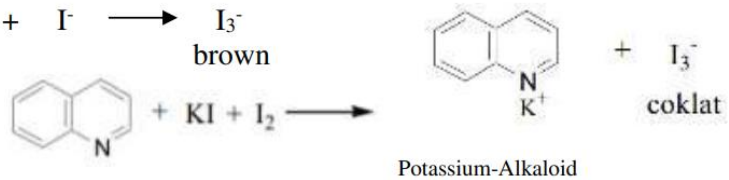
$$HgCl_2 + 2KI \longrightarrow HgI_2 + KCl$$
$$HgI_2 + 2KI \longrightarrow K_2[HgI_4]$$

Potassium-Alkaloid precipitate (orange)

The positive results of alkaloid test in Wagner test was confirmed by the presence of brownish to yellowish precipitate. The precipitate was predicted as the presence of potassium-alkaloid. In Wagner reagent preparation, iodine reacts with I- ion from potassium iodide producing I3- ion (brownish solution). In the Wagner test, the metal ion of K+ will bind as covalent coordinate bonding with nitrogen to alkaloid producing a complex precipitate of potassium-alkaloid. The reaction of Wagner test was predicted as bellows

$$I_2 + I^- \longrightarrow I_3^-$$

brown



Potassium-Alkaloid precipitate (orange)

coklat

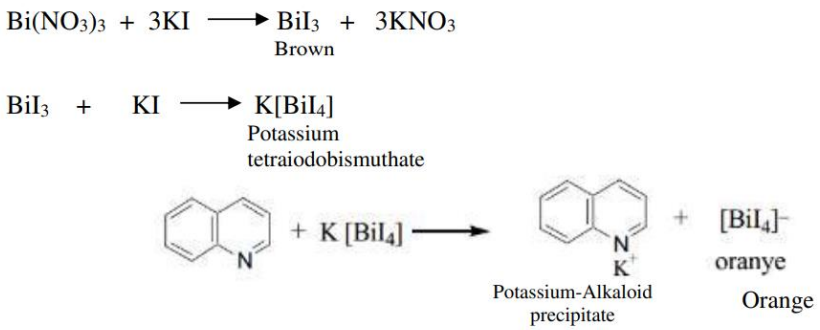
Brown

The positive result of alkaloid identification in Dragendorff test was as well identified as brownish or yellowish precipitate. The precipitate comes from complex compound of potassium-alkaloid. In Dragendorff reagent preparation, bismuth nitrate was dissolved in hydrochloric acid protecting to hydrolisis reaction because salts of bismuth are easily hydrolised producing BiO^+ ion. The reaction was proposed as follows;

$$Bi^{3+} + H_2O \longrightarrow BiO^+ + 2H^+$$

The hydrolysis reaction will control the presence of $Bi3^{+}$ ion in the solution. Therefore, the solution should be added an acid compound and moved the equilibrium to left. Moreover, Bi^{3+} ion from bismuth nitrate may react to potassium iodide producing dark brownish precipitate of Bismuth (III) iodide and dissolved in the excess of potassium iodide producing potassium tetraiodobismuthate (Altemimi et al., 2017).

In the alkaloid identification of Dragendroff test, nitrogen acted to form covalent coordination bond with K+ ion (metal ion). The reaction was proposed as follows; (Parbuntari et al., 2018).



Extraction techniques

Extraction method is the separation of medicinally active part of plant tissues using selective solvents base on standard procedures. The extract products achieved from plants are relatively complex mixtures of metabolites, in liquid or semisolid state was removed solvent in dry powder form. The common techniques of medicinal plant extraction such as infusion, maceration, percolation, decoction, soxhlet extraction, countercurrent extraction, microwave-assisted extraction, sonication (ultrasound extraction), and supercritical fluid extraction (Handa et al., 2008) have been developed. Effect of extracted plant phytochemicals depends on (Ncube, 2008).

- Its origin
- The nature of the plant material
- Particle size
- Moisture content
- Degree of processing

The different extraction methods will affect quantity and composition of secondary metabolite consisted in the extract depend upon type and time of extraction, temperature, and nature, concentration and solvent polarity (Ncube, 2008).

There are several methods of extraction for plant product commonly use

Serial exhaustive extraction or successive extraction:

It is common method of extraction with increasing polarity of solvents from a non-polar to a more polar solvent to ensure that a wide polarity range of compound could be extracted. Polarity index of the solvent shown in **Table 2.** Some researchers apply soxhlet extraction of dried plant material using organic solvent. This technique cannot be used for thermolabile compounds as prolonged heating may lead to degradation of compounds (Sharma et al., 2010).

Table 2 Polarity index of the solvent

	Solvent	Polarity index Units	Boiling point °C	Density @ 25 °C g/mL
1	Petroleum ether	0.1	35.0–60.0	0.640
2	Hexane	0.1	69.0	0.659
3	Cyclohexane	0.2	80.7	0.779
4	Isooctane	0.4	99.2	0.690
5	Toluene	2.4	110.0–111.0	0.865
6	Benzene	2.7	80.0	0.874
7	Diethyl ether	2.8	34.6	0.706
8	Dichloromethane	3.1	39.8–40.0	1.325
9	Isopropanol	3.9	82.0	0.785
10	Chloroform	4.1	60.5–61.5	1.492
11	Acetone	5.1	56.0	0.791
12	Methanol	5.1	64.7	0.792
13	Ethanol	5.2	78.0	0.789
	Water (for comparison only)	10.2	100.0	1.000

Soxhlet extraction

This technique is required where the desired compound has a limited solubility in a solvent. The advantage of this method is small solvent volume needed, that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled. Additionally, high efficiency and complete extraction can be obtained by using this technique.

Maceration extraction

Coarsely powdered or whole plant drug is kept in a stoppered container for a defined period with frequent agitation which allow the contact with the solvent in until soluble matter has dissolved. This method is best applied for the thermolabile drugs.

Decoction extraction is used of the heat stable constituents and water soluble from crude drug by boiling with water for 15 minutes, cooling, straining and passing sufficient cold water through the drug to produce the required volume.

Infusion extraction is a dilute solution of the readily soluble components of the crude drugs. Fresh infusions are prepared by macerating the solids for a short period of time with either cold or boiling water (Remington, 2005)

Digestion extraction is a kind of maceration in which gentle heat is applied During the maceration extraction process (Remington, 2005).

Percolation extraction is continuous extract compound by using percolator. The plant powder is soaked with a sufficient amount of solvent and kept for 4 h in a closed tight container followed by packing the mass and closing the top of percolator. Addition of solvent is performed to form the layer on the top of the mass then the mixture is allowed to be macerated in the percolator for further 24 h. After maceration finished, the percolator's outlet is opened, generating the slowly driping of extract liquid out of the percolator. Required solvent is added until percolate measure in approximately three quarters of product final volume needed. Expressed liquid is obtained after pressing the marc. An enough amount of solvent is added to produce the required volume then mixed liquid is filtrated followed by decanting process.

Sonication extraction involves increases the permeability of cell walls by using of ultrasound with frequencies ranging from 20 - 2000 kHz. This method is limited due to the higher costs. One disadvantage of the procedure is the occasional effect of ultrasound energy (more than 20 kHz) on the active constituents of medicinal plants through formation of free radicals and consequently undesirable changes in the drug molecules.

Solvent of extraction

The biological active compounds of plant material are mostly depending on the type of solvent used in the extraction. Effects of a good solvent in plant extractions including ease of evaporation at low heat, low toxicity or nontoxic and should not interfere with the bioassay, promotion of rapid physiologic absorption of the extract. The factors affecting the finding of solvent are quantity of phytochemicals to be extracted, variety of different compounds extracted, rate of extraction, variety of inhibitory compounds extracted, ease of subsequent handling of the extracts, toxicity of the solvent in the bioassay system and potential health hazard of the extractants (Eloff, 1998). The alternative of solvent is depend on what is intended with the extract

and the targeted compounds to be extracted. The various solvents that are used in the extraction are described in below and shown in **Table 3.**

Table 3 Summarized solvents for active component extraction (Cowan, 1999).

Water	Ethanol	Methanol	Chloroform	Ether	Acetone
Anthocyanins	Tannins	Anthocyanins	Terpenoids	Alkaloids	Phenol
Starches	Polyphenols	Terpenoids	Flavonoids	Terpenoids	Flavonols
Tannins	Polyacetylenes	Saponins		Coumarins	
Saponins	Flavonol	Tannins		Fatty acids	
Terpenoids	Terpenoids	Xanthoxyllines			
Polypeptides	Sterols	Totarol			
Lectins	Alkaloids	Quassinoids			
		Lactones			
		Flavones			
		Phenones			
		Polyphenols			

Cannabis strains

Sativa and Indica are the two common types of cannabis plant. Sativa plants have long and thin leaves that are lighter in color. Buds are long and wispy, and feature red or orange coloring. They tend to contain high THC and low CBD levels – optimal for daytime use, described as being energizing, stimulating, and creative. Indica plants have leaves that are wide, broad, and deep in color. Buds are dense and tightly packed, featuring purple coloring. Indica usually contains medium levels of THC, and a higher amount of CBD. Its effects are often described as being relaxing and calming, which is more optimal for nighttime use.

A hybrid strain combining sativa and indica bring together the best qualities of both into one plant

Hybrid cannabis plants

According to Austin (2019) found that cannabis (Marijuana) classified as three different types: indica, sativa, and hybrid. Hybrid cannabis plants represents a hybrid combination of sativa and indica plants. It is the product of cross-breeding two different

cannabis strains and two different sexes. (one female plant of one strain and one male plant). To develop a new cannabis strain with optimal characteristics and traits including taste, terpenes, smell, and also the medicinal effects. In recently, a major of the cannabis strains for smoke have been hybrid, that are indica dominant or sativa dominant and. The effect exhibited in **Table 4.** However, cross-breeding has limited the number of pure indica or sativa plant strains out there.

Effects of cannabis hybrid

Hybrid strains will give off a variety of effects because they are created by the two different parent cannabis strains, which when use cannabis regardless of it being sativa, indica, or hybrid need to look at the chemical composition of the strain and it's between THC and CBD percentage.

Table 4 The effect of sativa and indica dominant

Effects of Sativa – dominant	**Effects of Indica – dominant**
Mind Dominant	**Body Dominant**
- Uplifting	- Sedating
- Activating	- Relaxing
- Anti-depressant	- Reduce nausea
- Anti-anxiety	- Treats acute pain
- Increase Energy	- Muscle relaxant
- Increase Alertness	- Stimulate Appetite
- Enhance Creativity	- Increase Dopamine

The typical psychoactive constituent of cannabis in Tetrahydrocanabinol (THC), and it concentration is a basis of distinction between hemp and drug (cannabis or marijuana) type, with hemp considered low in concentration, 0.3% or less THC content (non-psychoactive),and cannabis containing up to 30% THC by dry weight (Barcaccia et al., 2020).

Agricultural practices

At present, cannabis breeding and cultivation are prohibited in most countries, except by the permission for research and medicinal used purposes.

Outdoor cultivation

Growing outdoors usually means more space to work with, plenty of sunshine, free rainwater, and better ventilation. Outdoor gardens are cost-effective, do not need expensive environmental controls, and require few resources to get started. However, outdoors cultivators must also battle the natural elements, which can potentially diminish the overall yield or reduce the quality of the crop. The many factors that outdoor growers must take into consideration include diminishing light on a cloudy or rainy day, the potential to be invaded by a wide variety of pests, and the limitation to one growing season per year.

Cannabis plant can be propagated from seeds, the process of germination is usually completed in 3–7 days. The seedling stage is completed within 2–3

months and the life cycle is completed within 4–6 months, depending on the time of the plantation and the variety. It can reach up to 5 m in height, in a growing season (Raman, 1998; Clarke and Watson, 2002). The vegetative growth is described by increased biomass and total growth under long day time lengths. It is easy to recognize the female and male sex at this stage. In summer, the reproductive phase of Cannabis begins when the plant is exposed to short day time lengths of 12– 14 h or less, depending on its genetic origin and latitude (Brenneisen, 1983). Once the male flowers ripened and pollinated, the female flowers died directly. The produced seeds after flowering have combinations of traits from two parents, as a result of cross fertilization (Clarke and Watson, 2002). This technique is largely used for the cultivation of Cannabis for Cannabis seed with less than 0.2% THC or hemp fiber (Farag and Kayser, 2017).

Indoor cultivation

Cannabis plant can be complete growth cycle quality, and quantity of biomass by regulated under controlled environmental conditions with in 6–8 weeks. Indoor

cannabis cultivation needs artificial light and compressed CO_2 gas for photosynthesis, for controlling flowering and plant biomass (Jones, 1997). There is a number of different techniques that have been proposed for the indoor horticulture of Cannabis, for example, nutrient film technique, the standing aerated technique, and aeroponics technique. The successful indoor system requires an effective hydroponic system to deliver nutrients and oxygen, and support the plants'growth. In hydroponic growing, the nutrient solution is best at a pH within a certain range (5.5– 6.5) for maximum uptake and good plant growth (Argo and Fischer, 2002). This method of breeding is used for increasing resin potency, and avoiding unwanted male plants (Chandra et al, 2010).

Optimal conditions for cannabis growth depending on these factors:

Light: the light (spectrum and light intensity) play important role in controlled environmental systems in cannabis cultivation, where plants capture energy from light, assimilate CO_2 and water into dry matter through photosynthesis. Plants utilize light in the visible spectrum between 400 - 700 nm, which is generally, referred to as photosynthetically active radiation. Light intensity is a close relationship between yield and photosynthetic rate (Jinet al., 2019).

Temperature: The optimum cannabis growth temperature is 25°C to 30°C for tropical varieties and 25°C for temperate varieties, whereas low temperatures slow phytosynthesis and excessive heat stops phytosynthesis (Upton et al., 2013).

Humidity: Ventilation is precious to control humidity for indoor cultivation. Vapor pressure deficit (VPD) impacts the opening of leaf stomata, which are responsible for CO_2 and water vapor exchange, thereby affecting phytosynthesis and nutrient transportation. High VPD may induce wilt and necrosis of the leaf tips. At a growing temperature of 25°C, the recommended relative humidity is 75% for young cannabis plants and 55% - 60% for vegetative growth and flowering (Chandra et al., 2013).

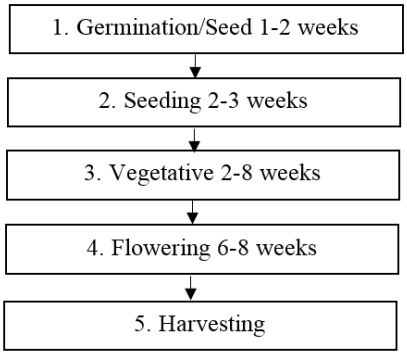
Growing medium: Soil and soilless (hydroponics) mediums are used for cannabis production and widely used for commercial cultivation. The American Herbal Pharmacopoeia recommends sandy and loamy soil with a pH of 6.5 to 7.2. Soil has a greater buffer capacity than hydroponics and is simpler to set up. While a hydroponics

system is expansive, more complex and requires constant availability of nutrients, water, electricity, and other supplies. Hydroponic systems allow for comprehensive control, enabling quicker and easier access to nutrients and water delivery. However, evidence suggested that yields and potency are not improved by hydroponics compared to soil systems (Carpentier et al., 2012).

Water: The quality of water is critical for photosynthetic and its products and nutrient transport. The water should be absent of contamination from metals, pesticides, herbicides, and toxicologically hazardous substances (Scholten, 2003). In addition, water should be tested for pH (6.5 - 7.2 for soil and 5.8 - 6.0 for hydroponics), alkalinity ($CaCO_3$ concentration 30 mg/L -100 mg/L), and electrical conductivity below 1.5 mS/cm (Swistock, 2016)

Cannabis life cycle:

Cannabis plants are usually propagated through the seed (sexual reproduction, outdoor cultivation) or by the vegetation propagation (asexual reproduction, indoor cultivation). However, both techniques have advantages and disadvantages.



Cannabis contaminations and adulterants

The common cannabis contaminants include microbes (e.g., bacteria, fungi), heavy metals and pesticides. These contaminants and are usually introduced during cultivation and storage (McLaren et al., 2008). Cannabis can also be adulteranted for marketing purposes, adding substances (e.g., lead, tiny glass beads), to increase the weight of the cannabis product or adding psychotropic substances (e.g., calamus, tobacco) and cholinergic compounds to either enhance the efficacy of low-quality

cannabis or to alleviate its side effects (Busse et al., 2008; McPartland et al., 2008; Randerson, 2007).

Insecticide: Bifenazate and abamectin are two generally identified insecticides found on Cannabis products and harmful to mammals (Radi et al., 2020). Bifenazate is not considered to be acutely toxic, though is considered to be toxic when chronically exposed to mammals (European Food Safety Authority, 201). Abamectin is comonlly considered safe with toxicity arising only after ingestion of large quantities and is approved for edible plants (Da Silva et al., 2018).

Herbicide: Paraquat is one of the most widely used herbicides in cannabis production. Some are turning to industrial-strength chemicals, raising concerns about safety. Herbicide contamination is not resistant could damage the plant, while prohibited residues could result in rejection by domestic or foreign buyers

Heavy Metal: There are varity of heavy metals found in Cannabis plants and Cannabis product (e.g. tinctures and oils), including lead, cadmium, mercury, magnesium, and copper (Busse et al., 2008; Mcpartland and Mckernan, 2017; Dryburgh et al., 2018; Gauvin et al., 2018). Most heavy metals have persisted in the body long-term causing a wide range of health problems. It has been presented to have fatal effects in humans when exposed both acutely or chronically, causing a plethora of diseases, such as, neurological disorders and cancers (Daley et al., 2013). At the higest risk for detrimental effects from heavy metal contamination are those using CBD as a medical treatment including children suffering from pediatric epilepsy, and the various conditions leading to compromised immune systems (Vardhan et al., 2019).

Standardization of Cannabis preparation

Methods for quality control of crude drugs are described in WHO guidelines (2011).The first step to categorize the herbal plant materials is the determination according to their macroscopic and microscopic characteristics for establishing the identity of herbal plant materials. Visual by eye based on the appearance of morphological characteristic provides the simplest and quickest inspection. However,

macroscopic examination is sometime inadequate. It is often necessary to combine with other methods such as microscopic, chemical constituent or molecular analysis.

Macroscopic examination

Macroscopic examination is based on their morphological features such as color, size, shape, texture and other characteristics, which always used to distinguish various species or evaluate their quality. There are conducted by observing, touching, smelling, tasting and testing by other ways. The important of macroscopic botanical characters of herb are specified to permit a clear identification.

Microscopic examination

Microscopic examination is method using microscope to identify the characters anatomical structure, histological and substances in section, powder, tissue or surface if the sample. For transvers section of midrib such as the arrangement of stomata in epidermis or the presence/absence trichomes, palisade cells, etc. Each cell type, form, size and its distribution within midrib cross section can provide distinguished identity for plant authentication.

Determination of physicochemical values

The physicochemical evaluation of the crude drug is an important parameter in detecting adulteration or improper handling of drugs. It is the methods for controlling the quality of the medicinal plants.

Determination of loss on drying

Loss on drying is method to determine the amount of water and any volatile matter in a crud drug, it is dried under specified conditions.

Determination of moisture content

A direct measurement of the water present in the herbal material being examined by the azeotropic method. The moisture content of herbal drugs should be at minimal level to discourage the growth of bacteria, yeast or fungi during storage.

Determination of total ash

Total ash measure the total amount of material remaining after ignition. This includes physiological ash which is derived from the plant tissue itself.

Determination of acid insoluble ash

Acid insoluble ash residue obtained after boiling the total ash with dilute hydrochloric acid and igniting the remaining insoluble matter. This measure the amount of silica present, especially as sand and siliceous earth.

Determination of water soluble ash is the difference in weight between the total ash and the residue after treatment of the total ash with water

Determination of extractive value

This parameter determines the amount of active constituents in a given amount of medicinal plant material when extracted with solvent (e.g. water-soluble extractive, alcohol-soluble extractive, and etc.).

Determination of volatile oil

Volatile oil are characterized by their odour, oil-like appearance and ability to volatize at room temperature. The chemical compounds are usually composed of monoterpenes, sesquitepenes and their oxygenated derivatives. Aromatic compounds predominant in certain volatile oils. Carry out the determination by steam distillation. The plant material is distilled in water using Clevenger apparatus.

Thin layer chromatography Fingerprint

Thin-layer chromatography (TLC) is a fast screening method to identify and separate compounds in herbal extracts and it can separate relate to herbal species. TLC has some advantages more than other chromatographic techniques such as inexpensive of instrumentation, short time analysis and easy to use. Sample preparation for TLC method: the solution must be enough concentrated so that analysis can be detected in the applied volume. The solvent for dissolve the sample must be proper to viscosity and volatility. Application of samples must be accurate, precise volumes and without damage the surface of TLC plate. Stationary phase: TLC plates are coated with thin layers such as alumina, silica, gypsum, cellulose, and

polyamides on glass, plastic or aluminum sheet supporter. Mobile phase is a mixture of two to five different solvents selected experimental using trial and error guided by prior personal experiences and literature reports of similar separations. Samples can be detected on TLC plate analysis under the ultraviolet light with 254 and 366 nm wavelengths (Sherma, 2005; Soponaret al., 2008; Shewiyo et al., 2012).

An important qualitative parameter, which characterizes the position of a spot onto TLC plate, is the retardation factor (R_f) value. It is defined as:

$$R_f \quad \frac{\text{Distance of the compound from original spot travelled to the developed spot}}{\text{Distance of the solvent from original line travelled to the developed line}}$$

TLC is frequently used as a qualitative and quantitative method. Qualitative method can be determined by the number of compounds in a mixture and identified substances. Whereas quantitative method is used for content determination of require testing substances (Trease and Evans, 2002)

TLC fingerprint is a method for the quality control of herbal samples that has been accepted by WHO. It's suitable for adulterations detection and plant species identification (Cieśla, 2011). Chromatographic methods consist of TLC, HPLC and GC are commonly used for fingerprint. TLC is the most method used for identify and authenticate compounds in herbal medicines and its derivative for obtains a fingerprint profile (Hajimehdipoor et al., 2009 and Rafi et al., 2011).

Pharmacological properties of ingredient of KLKS remedy

There are several methods of pharmacological properties in medicinal plant commonly use:

Tests on antioxidant activity

Antioxidant is a substance which able to prevent or decreasing the damage of cell caused by free radical, the unstable molecules generated by reaction occurred in the body in exposed of environmental and other pressure. “ Free radicals are types of Reactive Oxygen Species (ROS), which include all highly reactive, oxygen-

containing molecules. This protection may be based on several mechanisms of action, namely: inhibition of generation and scavenging capacity against ROS/RNS (Reactive Nitrogen Species); reducing capacity; metal chelating capacity; activity as antioxidative enzyme; inhibition of oxidative enzymes"

There are many tests in vitro methods regarding antioxidant activity such as 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay, ABTS radical scavenging activity, ferric reducing antioxidant power (FRAP) assay, β-carotene bleaching assay, nitric oxides scavenging assay, metal chelating assay, total phenolic and flavonoid content assays.

DPPH free radical scavenging assay

On the basis of free radical scavenging potential, DPPH radical scavenging assay is most widely used due to its rapid, simple and inexpensive method to measure the antioxidant property of compounds in reference to their ability to scavenge this free radical. DPPH• is characterized as a stable free radical because of the delocalization of the spare electron over the whole molecule. The delocalization also gives rise to the violet color, characterized by an absorption band at 517 nm. When a solution of DPPH is mixed with that of a substance that can donate an electron, the reduced form of DPPH or the non-radical 1,1-diphenyl-2-picryhydrazine (DPPHH) is then generated with the loss of the violet color to pale yellow (**Figure 12**) (Prakash, 2001; Prior et al., 2005; Shahidi and Zhong, 2015).

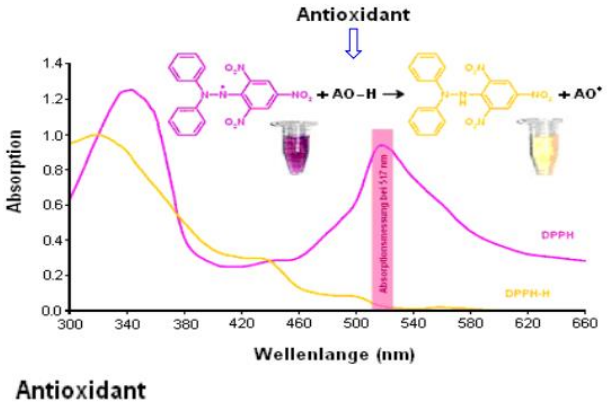


Figure 12 The structure of DPPH• radical and its reduction by an antioxidant (AO-H).

ABTS+ free radical scavenging assay

ABTS (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)) is a chemical compound used to observe the antioxidant activity. The ABTS•+ is generated by reacting a strong oxidizing agent (e.g., potassium permanganate or potassium persulfate) with the ABTS salt. The reduction of the blue-green ABTS•+ by hydrogen-donating antioxidant is measured by the suppression of its characteristic long wave absorption spectrum, during this reaction, the blue ABTS radical cation is converted back to its colorless neutral form (**Figure 13**) (Boligon et al, 2014). The assay principle is base on the ability of antioxidant to donate electron or hydrogen atom to dark green stable ABTS radical cation, causing the decolorization. The method is rapid, simple to perform, good repeatability and can be used over a wide range of pH values, which is useful to study the effect of pH on antioxidant mechanisms. It is widely reported. Moreover, the $ABTS^{\bullet+}$ radical is stable and soluble in organic solvents and water, enabling the determination of antioxidant capacity of both lipophilic and hydrophilic compounds of samples (Alam et al., 2013). However, as the results obtained for samples are related to an antioxidant standard compound that shows different kinetic behavior, the results provided by this assay are dependent of time of analysis.

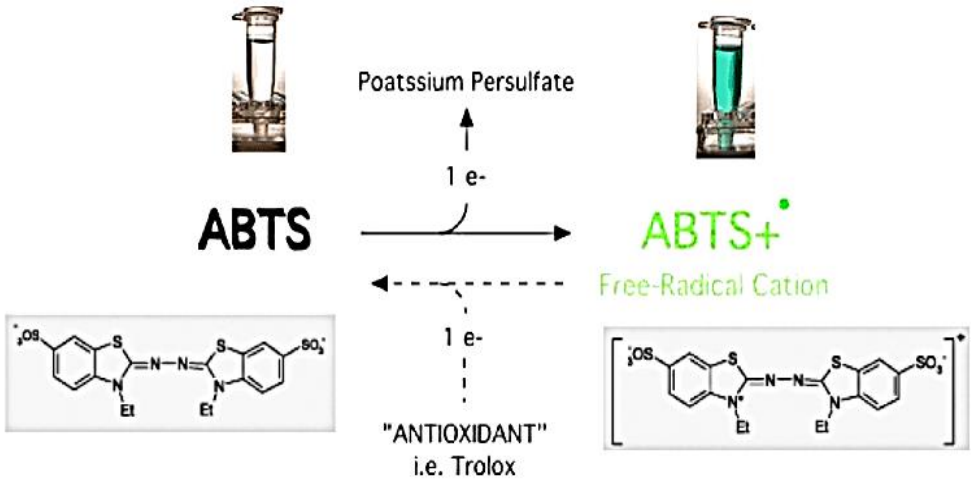


Figure 13 The ABTS chemical reaction

Ferric reducing antioxidant power (FRAP assay)

In terms of reducing power, the FRAP assay was formerly developed by Benzie and Strain, in 1996, to evaluate the reducing ability of plasma (Benzie and Strain, 1996); however, it has been applied for the tests on antioxidant activity in various fields, including medicinal plants (Benzie and Szeto, 1999). The principle of FRAP assay works upon redox reaction; that is, the ferric ion (Fe^{3+}) complex undergoes the reduction reaction while the antioxidant compound takes part in the oxidation reaction. This assay is simple a robust spectrophotometric technique and inexpensive.

The most widely-used mechanism, 2,4,6-tripyridyl-s-triazine (TPTZ) is used as an iron-binding ligand, and the assay is carried out in acidic medium (pH 3.6) to maintain the iron solubility.The ferric-TPTZ complex, $[Fe(III)\text{-}(TPTZ)_2]^{3+}$, is served as an oxidizing agent toward the antioxidant; whereas, the antioxidant itself acts as a reducing agent to reduce ferric ion in the complex to ferrous ion (Fe^{2+}), developing the intensely violet-blue colored ferrous complex, $[Fe(II)\text{-}(TPTZ)_2]^{2+}$ (**Figure 14**). The reducing power of the antioxidant is subsequently detected by measuring the increase in absorbance mediated by the $[Fe(II)\text{-}(TPTZ)_2]^{2+}$ formation at the wavelength of 593 nm, and comparing with the ferrous ion standard solution or relating to an antioxidant standard solution. The FRAP value is eventually expressed as mM Fe(II) equivalents (Henderson et al., 2015).

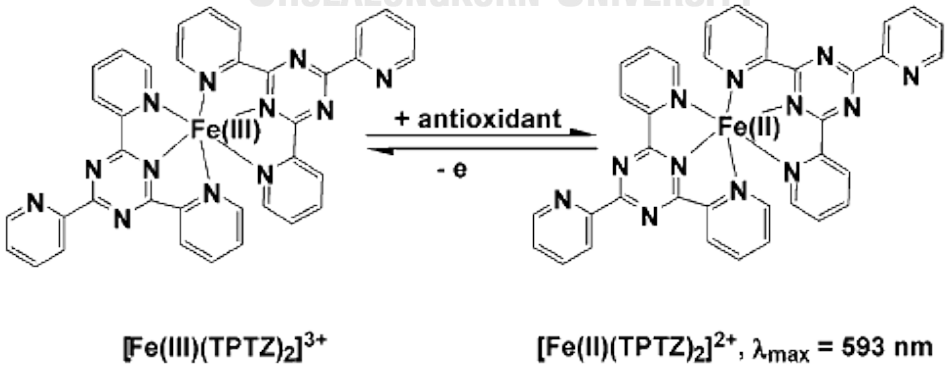


Figure 14 Basic mechanism on FRAP assay (Antioxidant Capacity, 2015).

Total phenolic and flavonoid content assay

More than eight thousand kinds of phenolic compounds, whose half of them are flavonoids, are secondary metabolites naturally found in plants (Kumar and Pandey 2013). These considerable second metabolites own a wide range of bioactive potentials. In determination of total phenolic and flavonoid contents, colorimetric assay is generally used in the UV/VIS spectrophotometric technique because of its easiness, rapidness and cost effectiveness (Pelozo et al., 2008).

For total phenolic content assay, polyphenolic compounds in plant extract react with a specific redox reagent, Folin-Ciocalteu reagent, to form a blue complex that can be quantitated by visible-light spectrophotometry (Schofield et al., 2001). The molybdenum center in the complexes act as a reduction site where Mo^{6+} ion is reduced to Mo^{5+} by accepting an electron donated by the phenolic antioxidant. In this assay, gallic acid is often used as a reference substance to set up a calibration curve.

Without interfering with phenolic compounds, the aluminium chloride colorimetric assay is widely used to evaluate the flavonoid content in plant materials (Chatatikun and Chiabchalard, 2013; Ramos et al., 2017). The carbonyl and hydroxyl groups present in flavonoids can interact with metal ions and form metal complexes. Particularly, flavones and flavonols tends to form a stable complex with $AlCl_3$, which can be quantified by visible-light spectrophotometry (Chang et al., 2002; Mabry et al., 2012). Quercetin, an example of flavonols, is said to have a strong affinity towards metal ions (i.e., Fe^{3+}, Cu^{2+} and Al^{3+}) at the o-dihydroxyl position of quercetin under alkaline condition (Mira et al., 2002; Torreggiani et al., 2005). Thereby, quercetin is usually used as a reference substance for constructing a calibration curve (Woisky and Salatino, 1998).

Tests on in vitro anti-inflammatory activity

Inflammation is a biological response to variety of stimuli and local injury (i.e. pathogens, damage cells, toxic compounds). Three major components occur during the inflammatory response: 1) Vasodilation and increased blood flow. 2) Capillary permeability is increased, extravasation and deposition of proteins and plasma fluid (that lead to edema), and 3) Emigration of the leukocytes (white blood cells) from the capillary vessels into the surrounding interstitial spaces to the site of injury or inflammation **(Figure 15)**.

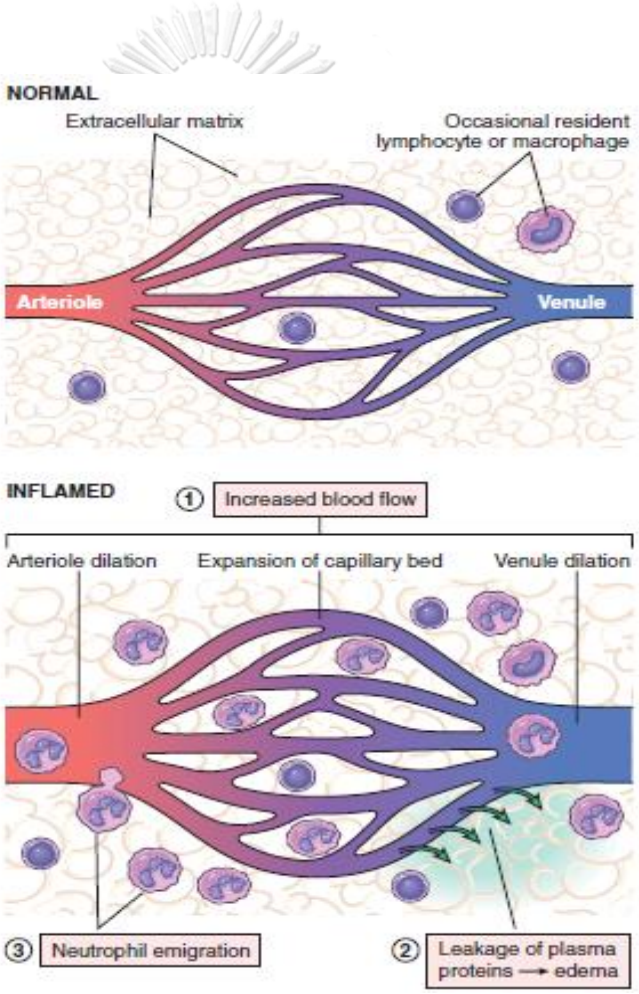


Figure 15 The major local expression of acute inflammation compared to normal. (Kumar et al., 2005)

Inflammation has been recognized by the symptoms identified as pain, redness, heat, swelling and loss of function (Egger, 2012). The inflammation are separate into two type; acute and chronic inflammation. Response of the body, when there is an initial harmful stimuli, leads to acute inflammation. It is from the increased movement of leukocytes and plasma from bloody to the site of tissue injury. This leads to inflammatory response through various biochemical events. Chronic inflammation or prolonged inflammation causes a change in the nature of cells present at the inflammatory region. This leads to a destruction of the tissue and proceeds to heal the damaged site (Medzhitov, 2018). It widely occurs in response to tissue repair the injured and it related to the release of different mediators such as bradykynin, histamine, nitric oxide (NO, cyclooxygenase (COX-2), prostaglandin (PGs), tumor necrosis factor-alpha (TNF-α) and Interlukin-6 (IL-6) (Saha et al., 2004). The inflammation response can cause different diseases such as rheumatoid arthritis, cardiovascular dysfunctions, allergies and pain (Bagad et al., 2013). There are various medicines for treatment inflammatory such as nonsteroid anti-inflammatory (NSAID) drugs, steroids drug and immunosuppressant, which involve with adverse effects like gastric irritation, ulceration, haematochezia, headache, hepatic failure, angioedema, hemolytic cinema, hyperglycemia, osteoporosis (Laine et al., 2006; Rodrigo et al., 2002). Hence natural anti-inflammatory factors are needed to apply to enhance the pharmacological response as well as reduce side effects.

There are various methods for evaluating in vitro anti-inflammatory activity such as protein denaturation inhibition, proteinase inhibition hemolysis inhibition, and lipoxygenase inhibition.

Protein denaturation inhibition

According to Narayanan and Chitra (2018), Inflammation is an adaptive response which involves an increase in the vascular permeability, causes membrane alteration and increase in the protein denaturation.

Protein denaturation assay is a process which loss of biological properties of protein molecule by function of external stress or compound such as heat, strong acid, alkalines, an organic solvent, or a concentrated inorganic salt (Kiranmayi et al., 2018). Protein denaturation has been correlated with the formation of various inflammatory disorders including rheumatoid arthritis, diabetes and cancer. Tissue injury might be attributable to denaturation of the protein components of intercellular substance or cells (Opie, 1962). Therefore, the capability of a substance to inhibit the denaturation of protein signifies apparent potential for anti-inflammatory activity (Osman et al., 2016).

Anti-inflammatory drugs (eg ibufenac, indomethacin, salicylic acid and flufenamic acid) has the ability to stabilize heat treated bovine serum albumin by preventing it from denaturation, anti-denaturation effect of the drug in heat treated bovine serum albumin was used as an assay (Williams et al., 2008)

The principle effect of the underlying protein denaturation assay mentioned above using bovine serum as compound was also observed when solutions of egg albumin, and human serum albumin were used (Heendeniyaet al., 2018). The rationale behind implementing this assay is that the denaturation of albumin protein leads to formation of antigens which initiate type III hypersensitive reaction leading to inflammation (Agrawal & Paridhavi, 2007)

Proteinase inhibition activity

Proteinase have been related with the tissue damage during the inflammatory and arthritic reactions. Proteinases numerously exist in lysosomal granules of neutrophils. Proteinases of leukocytes play an important role in the development of tissue damage during inflammatory processes and significant level of protection was provided by proteinase inhibitors (Govindappa et al., 2011).

Lipoxygenase inhibition activity

Lipoxygenases (LOXs) are the key enzymes in the biosynthesis of leukotrienes. Leukotrienes play a significant role in several inflammatory diseases, such as asthma, arthritis, cancer, and allergic diseases (Rackova et al., 2007). The mechanism of anti-inflammation involve a series of events in which of the arachidonic acid metabolism. In process, arachidonic acid is cleaved from the membrane phospholipids upon appropriate stimulation of neutrophils, and can be converted to prostaglandins and leukotrienes through cyclooxygenase and lipoxygenase pathways, respectively **(Figure 16)** (Martel-Pelletier et al., 2003)**.**

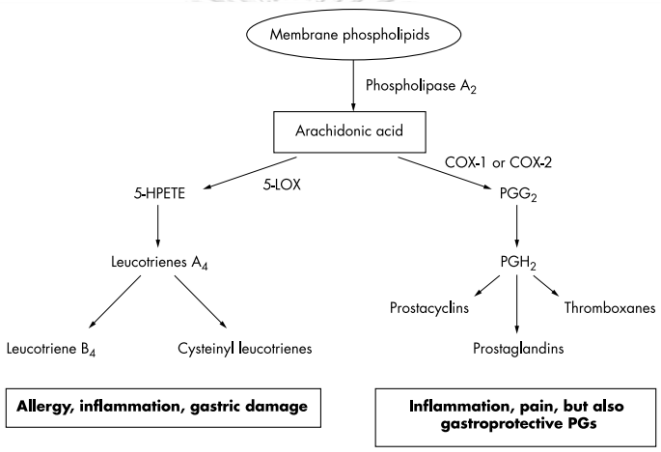
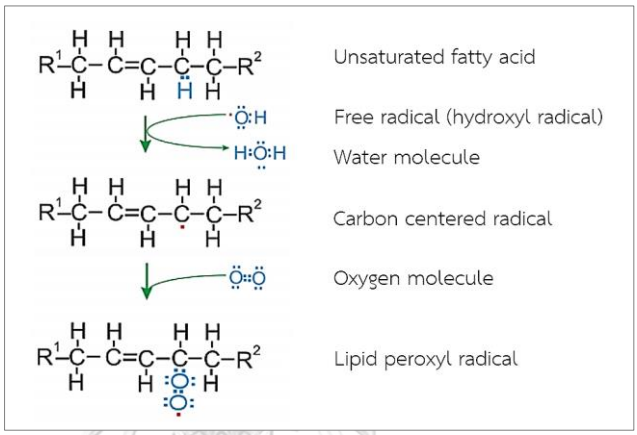


Figure 16 Products and enzymes of arachidonic acid metabolism involved in the inflammatory process

Lipoxygenase mostly found in plants, is a non-heme, non-sulphur, iron-cofactor containing protein that catalyzes the oxidation of polyunsaturated fatty acids (PUFA) as substrate (e.g. linolenic, linoleic and arachidonic acids) to yield hydroperoxides (Gardner, 1991). The soybean LOX (SLOX) has been considered as a mechanistic prototypes for studying lipoxygenation. Lipoxygenase reaction is based on the lipid peroxidation. Lipid peroxidation is one of the key indicators of oxidative stress occurred in a cell of living organisms where an unpaired-electron fatty acid captures oxygen molecule to become a lipid peroxyl radical. In general, lipoxygenase inhibitors can

bind covalently to iron or form the molecular complexes blocking access to iron (Skrzypczak-Jankun et al., 2007). The first mode to inhibit the lipoxygenase would be a direct reduction of iron to its inactive form. For soybean lipoxygenase, it has been demonstrated that nordihydroguaiaretic acid (NDGA) rapidly reduces the active ferric species (Fe^{3+}) of the enzyme to its inactive ferrous form (Fe^{2+}) , thus causing interruption of the catalytic cycle (Kemal et al., 1987).



Test on In vivo anti-inflammatory activity

Carrageenan-induced paw edema

The carrageenan-induced paw edema is one of prominent model used in the tests of natural pant for anti-inflammatory activity described by Winter et al., (1962), footpad edema or paw swelling is a suitable method for determined inflammatory responses to irritants and antigenic challenges. This model uses carrageenan as the irritant to induce paw edema (Otterness and Moore, 1988). Carrageenan is a chemical that stimulating the release of inflammatory mediators such as histamine, tachykinins, and bradykinin. Typically, this test are evaluated for acute anti-inflammatory activity base on examining their potentiality to reduce or prevent the expansion of carrageenan-induced paw swelling. The advantage inflammation-induced by carrageenan are highly sensitive, reproducible and non-immune (Sarkhel, 2016). Fort

the limitations, animals should be accommodated at least one week before beginning of an experiment to decrease the effects of stress (Whiteley and Dalrymple, 2001)

Test on in vivo analgesic activity (anti-nociceptive activity)

Analgesia or pain is defined as an unpleasant sensation to evoke by external or internal stimulus, there are various forms such as acute, chronic, inflammatory, visceral, or neuropathic (Uritu et al., 2018). Pain type are functional pain, neuropathic pain, nociceptive pain and inflammatory pain (Dongmo et al., 2003). Currently drugs which are used to relief pain are opioids, salicylates and hydrocortisone. All of these drugs have specific side and toxic effects (Tasleem et al., 2014). There are various methods for Determination of Analgesic Activity such as Acetic Acid-Induced Writhing Test, Hot Plate Test, Tail Flick Test, Tail Pressure Test, Tail Formalin Test, and Formalin Test.

Tests on antidiabetic activity

According to the World Health Organization (WHO), diabetes mellitus is considered to be a serious concern as there is an increasing number of people with diabetes year by year (WHO, 2016). Diabetes is either caused by a deficiency in insulin production by pancreas (Type 1 diabetes), or by an ineffectiveness of body's response to the insulin produced (Type 2 diabetes).

Type 1 diabetes (also known as insulin-dependent diabetes) is involved with an autoimmune condition that is often developed in kids and teenagers. In this case, the pancreatic cells that produce insulin are mistakenly attacked by the immune system, resulting in malfunction in the production of insulin.

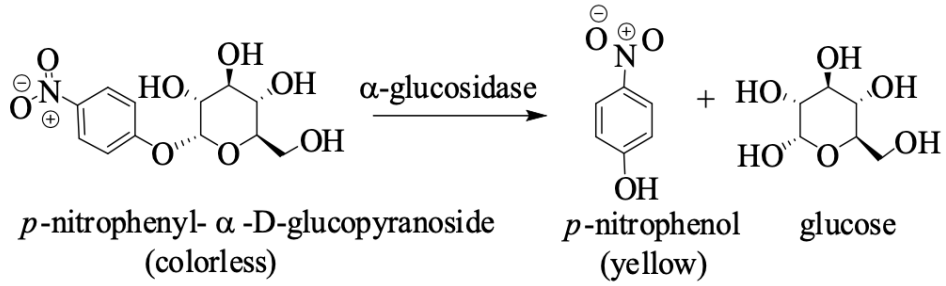
Type 2 diabetes (also known as non-insulin-dependent diabetes) is world-widely accounted for 90% of all diabetes cases. It is frequently occurred in adults but can also be developed in children. In this condition, it is resulted from the body's incapacity to use insulin properly.

α-Glucosidase and α-amylase are two important enzymes used to assess anti-diabetic activity from medicinal plants (Tadera et al., 2006). α-Glucosidase is mostly

found in the brush border of the enterocytes in small intestine, while α-amylase is plentifully located at pancreatic juice and saliva (Krentz and Sinclair, 2002). Both complex enzymes work upon the hydrolysis of carbohydrates – polysaccharides to disaccharides and monosaccharides.

Initially, α-Amylase breaks down polysaccharides to smaller units (i.e. dextrins and disaccharides) by acting upon α-1,4-glycosidic bonds. α-Glucosidase then hydrolyzes dextrins and disaccharides to monosaccharides that can be absorbed through the brush border in small intestine, hence raising the blood glucose level. Inhibitors of α-amylase and α-glucosidase slow down the cleavage of carbohydrates in the small intestine, and therefore decrease the blood glucose excursion regarding Type 2 diabetes (Kazeem, 2013). Antidiabetic drugs, as competitive inhibitors of these enzymes for Type 2 diabetes, are acarbose, miglitol, nojirimycin and 1-deoxynojirimycin (Rosa and Dias, 2014).

The principle of alpha glucosidase inhibitory assay using spectrophotometric method . The crud extracts pre-incubate with enzyme and then adding the p-nitrophenyl- **α**–glucopyranosids (pNPG) were as the substrate. The activity of this method was measure by determining the colour of the release of p-nitrophenol arising from the hydrolysis of substrate pNPG by **α**– glucopyranoside reaction at the absorbance 405 nm (Damsud et al., 2014) .



Hydrolysis of colourless p-nitrophenyl-**α**-d-glucopyranoside to coloured p-nitrophenol by **α**-glucosidase Adapted from

Tests on acute toxic activity

The Brine Shrimp Lethality Test (BSLT) is a general protocol that is used to detect a wide spectrum of bioactivity in plants extracts or compound. The toxicity of herbal extracts expressed as LC_{50} values is commonly valorized either by comparison to Meyer's or to Clarkson's toxicity index. This method is indicative of in vivo cytotoxicity and pesticidal activity of an extract. BSLT gives LC_{50} concentrations in µg/ml which when above 1000 µg/ml is considered safe but is considered toxic when below 1000 µg/ml (Meyer et al., 1982; Kamanja et al., 2018). According to Clarkson's toxicity index, the plant extracts with LC_{50} above 1000 µg/ml are non-toxic, LC_{50} of 500 - 1000 µg/ml are low toxic, extracts with LC_{50} of 100 – 500 µg/ml are medium toxic, while extracts with LC_{50} of 0 - 100 µg/ml are highly toxic (Clarkson et al., 2004). A large amount of traditional medicine research has been carried out to determine The Brine Shrimp Lethality Test (Kazeem et al., 2012; Jovanova et al.,2019). It is a primary screening, simple and inexpensive *bioassay* used for testing the efficacy of phytochemical present in the plant extracts (Waghulde et al., 2019). .

Cannabis based preparation in Thai Traditional medicine

There are sixteen cannabis-based Traditional medicine formulas have been developed mainly to treat insomnia, pain, skin disease, hemorrhoids, mental illness and appetite. The formulas were approved by a government narcotics board, the Food and Drug Administration (FDA) and the public health minister to study and research for medical purposes. The medications would be prescribed by licensed doctors of traditional medicine and folk doctors. Some of them were currently in use but without the cannabis content because it was previously banned. Sixteen cannabis-based Traditional medicine formulas list shown in **Table 5**.

Table 5 List of sixteen cannabis-based Traditional medicine formulas (ประกาศกระทรวงสาธารณสุข, 2562)

No.	Name of formulas	Thai name	Number of plant ingredients
1	Aukkineevakana	ยาอัคคินีวคณะ	10
2	Suksauyard	ยาศุขไสยาศน์	12
3	Kealomnaowanareevayoo	ยาแก้ลมเนาวนารีวาโย	12
4	Nammansanantripop	ยาน้ำมันสนั่นไตรภพ	18
5	Kealombeaungsung	ยาแก้ลมขึ้นเบื้องสูง	18
6	Faiarwut	ยำไฟอำวุธ	32
7	Keanonmailub	ยาแก้นอนไม่หลับ /ยาแก้ไข้ผอมเหลือง	8
8	Kesanthakat	ยาแก้สัณฑฆาตกล่อนแห้ง	15
9	Aummareuosod	ยาอัมฤตย์โอสถ	22
10	Apaisali	ยาอไภยสำลี	20
11	Kealomkeasan	ยาแก้ลมแก้เส้น	7
12	Kaerokjit	ยาแก้โรคจิต	14
13	Paisali	ยาไพสำลี	23
14	Redsidunglaerokpeunong	ยาทาริดสีดวงทวารหนักและโรคผิวหนัง	3
15	Tumlaiprasumain	ยาทำลายพระสุเมรุ	23
16	Tubpayathikhun	ยาทัพยาธิคุณ	16

The components of Kealomkeasan remedy

Kealomkeasan (KLKS) remedy is one of the Thai traditional medicines which revealed in Wechasard wannana for an analgesic in musculoskeletal system. The remedy consists of seven herbal an unequal part by weight, the components of KLKS remedy are shown in **Table 6** and the classification of ingredient in this remedy shown in **Figure 1**7.

Table 6 The components of Kealomkeasan remedy

No.	*Scientific name*	Thai name	Family name	Plant Part	Ratio (g)
1	*Cuminum cyminum* L.	เทียนขาว	UMBELLIFERAE	Fruit	1
2	*Nigella sativa* L.	เทียนดำ	RANUNCULACEAE	Seed	2
3	*Foeniculum vulgare Miller*	เทียนข้าวเปลือก	UMBELLIFERAE	Fruit	3
4	*Zingiber officinale* Roscoe	ขิง	ZINGIBERACEAE	Rhizome	4
5	*Plumbago indica* L.	เจตมูลเพลิงแดง	PLUMBAGINACEAE	Root	5
6	*Cannabis sativa* L.	กัญชา	CANNABACEAEC	Leave	20
7	*Piper nigrum* L.	พริกไทย	PIPERACEAE	Fruit	40

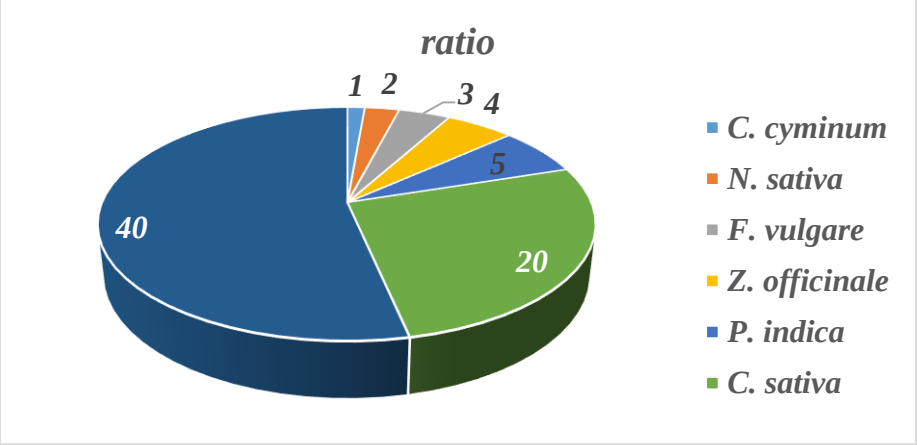


Figure 17 The classification of ingredient in this remedy

The components of Kealomkeasan remedy

***Cuminum cyminum* L.**

Scientific name: *Cuminum cyminum* L.

Family name: Apiaceae

Synonym: *Cuminum odorum* Salisb., *Cuminia cyminum* J.F.Gmel., *Cuminum sativum J.*Sm.

Common name: Cumin

Thai name: Thiankaw (เทียนขาว)

Plant description:

" The plant is a delicate, glabrous annual 10 to 50 cm high. The stem is bifurcated at the base and glabrous. The leaves are glabrous and finely pinnatifid with oblong-linear tips, of which the lower are mostly doubly trifoliate. The flowers are in umbels radiating in groups of 3 to 5. The petals are white or red, oblong and deeply bordered with a long indented tip. The involucral bracts are long and simple. The style is short and turned outward at the end. The ovary is inferior and 3-locular. The fruit is a schizocarp, about 6 mm long and 1.5 mm wide and crowned with awl-shaped calyx tips. The mericarp is almost round in transverse section, with 5 thread-like, bristly main ribs and bristly secondary ribs" The image of *C. cyminum* is shown in **Figure 18**.

Figure 18 Fruits of *C. cyminum*

Distribution: *C. cyminum* is a flowering plant native to Pakistan and India.

Traditional uses:

In traditional medicine, cumin use as medicinal plants in Western and Eastern. In Africa and America, the drugs is used as an emmenagogue and abortive. In Indonesia, it was taken orally for rheumatic ailments, used in cases of bloody diarrhea and paste is applied to the forehead for relieve headache (PDR for Herbal Medicines, 2000). In India, the fruits of *C. cyminum* were used as carminative, relieve cough, emmenagogue, ulcers, inflammation (Shivakumar et al., 2010) as well as used in chronic diarrhoea, digestive disorders and dyspepsia (Srinivasan, 2018). In Thailand, an efficient treat for indigestion, flatulence, and diarrhoea (Hays, 2014).

Chemical constituents of *C. cyminum*

Several chemical constituents of *C. cyminum* isolated and identified from fruit, leaf bark, seed, and root such as coumarins, alkaloids, steroids and tannin. The major bioactive are cuminaldehyde **(Figure 19),** cymene, terpenoids and essential oil (Srinivasan, 2018).

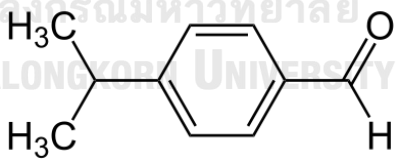


Figure 19 Chemical structure of cuminaldehyde

There have been several studies about pharmacological properties of *C. cyminum* shown in **Table 7**.

Table 7 Pharmacological activity of *C. cyminum*

pharmacological activity	Part used	Reference
Antimicrobial activity	Fruit	Bameri, 2013
Antioxidant activity	Fruit	Allahghadri et al., 2010
Antidiabetic activity	Fruit	Willatgamuwa et al, 1998 Jagtap and Patil, 2010
Anti-inflammatory and Analgesic activity	Fruit	Bhat, Rizvi & Kumar, 2014
Anticarcinogenic	Fruit	Gagandeep et al., 2003
Immunomodulatory	Fruit	Chauhan et al., 2010
Gastrointestinal effect	Leaves	Vasudevan, et al., 2000

***Nigella sativa* L.**

Scientific name: *Nigella sativa* L.

Family name: Ranunculaceae

Synonym: *Nigella truncata* Viv., *Nigella indica* Roxb. ex Flem.

Common name: Black cumin

Thai name: Thiandum **(เทียนดำ)**

Plant description:

"*The species is an erect annual herb attaining 30.0 cm to 67.6 cm at maturity. Number of primary branches per plant ranges from 4 to 10; leaf arrangement alternate, leaf phylotaxy 1-2, pinnae of leaves broad, number of pinna per rachis 5-6; total branches per plant 22.5±4.1; flower hermaphrodite with determinate flowing patterns, main axis terminate with a solitary flower, delicate; flower size 2.74 cm x 2.78 cm; color - french blue; flowers without any involucre of bracts, pedunculate; peduncle long, erect; petalloid sepals broad, ovate in a single whorl, 4-6 mostly 5 and characterized by the presence of nectaries; flower fertility 89.89%; stamens in 3 to 4 whorls, numerous (32 to 66) and shed their pollen as the filament bent outward*

during male phase; gynoecium 5, completely united follicles, each with a long indehiscent style and composed of variable number of multi ovule carpel, developing into a follicle after pollination; fruit single partially connected to form a capsule like structure (capsule 5 to 45 capsule fertility 94.5%) dehiscence through suture; fruits (length – 0.4 to 1.7 cm) with numerous seeds. seeds ovate, tetrangular, angles sharp, acute, more tapering at the end, color black ; seed size 2.33 mm ± 0.1 × 1.14 mm ± 0.02 **"** (Datta et al., 2012).The image of *N.sativa* is shown in **Figure 20.**

Figure 20 Seed of *N. sativa*

Distribution

The species is distributed all over India, Lebanon, Syria, Israel and South Europe (Paarakh, 2010), also grown in Turkey, Bangladesh, Mediterranean basin and Middle-East (Naz, 2011).

Traditional use

In traditional system, *N.sativa* seed have been used in Southeast Asian and Middle East countries including Thai traditional medicine used as an essential ingredient for are effective against asthma, bronchitis, cough, migraine, dizziness, dysmenorrheal, diabetes, obesity, paralysis, inflammation, back pain, infection, rheumatism, hypertension, and gastrointestinal diseases such as flatulence, dyspepsia, diarrhea and dysentery (Tariq, 2008; Yusuksawad & Chaiyabutr, 2011).

Chemical constituent

Many active compounds have been isolated from N. sativa seed. The most important compounds constituentare thymoquinone **(Figure 21**), thymohydroquinone, dithymoquinone p-cymene, carvacrol, 4-terpineol, trans-anethole, sesquiterpene longifolene, thymol and **α**-pinene. (Boskabady and Shirmohammadi, 2002; Ali and Blunden, 2003), Another study of N. sativa seed of reported that contains the fixed oil were oleic acid, linoleic acid, and palmitic acid, other compounds such as limonene and carvone (Nickavar et al., 2003).

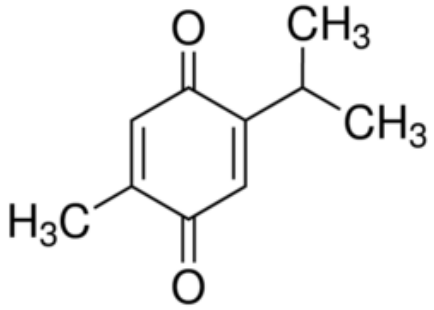


Figure 21 Chemical structure of thymoquinone

Pharmacological activity

The pharmacological properties of *N. sativa* are shown in **Table 8**.

Table 8 Pharmacological activity of *N. sativa*

Pharmacological activity	Part used	Reference
Antimicrobial activity	Seed	Hasan et al, 2013
Antioxidant activity	Seed	Bourgou et al, 2011
Antidiabetic activity	Seed	Rchid, et al., 2004
Analgesic and Anti-inflammatory	Seed	Shuid et al., 2012
Anti-cancer properties	Seed	Mahmoud and Torchilin, 2012
Gastroprotective effect	Seed	Al Mofleh et al., 2008

***Foeniculum vulgare* Miller**

Scientific name: *Foeniculum vulgare* Miller

Family name: Umbelliferae **(**Apiaceae**)**

Synonym: *Anethum foeniculum* L., *Foeniculum dulce* Mill.,
Foeniculum officinale All., *Foeniculum capillaceum* Gilib

Common name: Fennel, Sweet fennel

Thai name: Thian khao plueak (เทียนข้าวเปลือก**)**

Plant description:

"F. vulgare is an upright, branching perennial herb with soft, feathery, almost hair-like foliage growing up to 2 m tall. It is erect and cylindrical, bright green, and smooth as to seem polished, with multiple branched leaves cut into the finest of segments. The leaves grow up to 40 cm long; they are finely dissected, with the ultimate segments filiform (threadlike), about 0.5 mm wide. The bright golden flowers, produced in large, flat terminal umbels, with thirteen to twenty rays, bloom in July and August. Foliage. Stem striate, leaves 3-4 pinnate, segments filiform, up to 4 cm long; leaf bases sheathing. It has a green, sleek, and slippery stem with upright stiff branches and much divided leaves in linear segments. Rays are 5– 30 numbers with 1–6 cm long. Flowers are small, yellow, and found in large flat-topped umbels. Fruits are oblong to ovoid with 0.12–3–5 mm long and 1.5–2.0 mm broad. The fruits are elongated and have strong ribs. The most esteemed fennel seeds vary from three to five lines in length and are elliptical, slightly curved, and somewhat obtuse at the ends. They are greenish-yellow, the colour of hay, from which the term fennel is derived" (Badgujar, Patel, & Bandivdekar, 2014). The image of *F. vulgare* is shown in **Figure 22.**

Figure 22 Fruit of *F. vulgare*

Distribution

F. vulgare are native in the Mediterranean region, It was grows in nearly every country i.e Europe, India, throughout China and Thailand. (Hui xiang, 2005; Badgujar, Patel, & Bandivdekar, 2014)

Traditional use

F. vulgare seed is a well-known aromatic and medicinal herb, it is commonly use as carminative, treatment of diabetes, chronic cough, bronchitis, and kidney stones. *F. vulgare* and its herbal drug preparations are used for gastric-intestinal problem, bloating, flatulence and catarrh of the upper respiratory tract (Badgujar, Patel, & Bandivdekar, 2014). It is also have been known as a promoter of menstruation (Albert-Puleo, 1980). In traditional Chinese medicine, fruit leaves and stem are commonly used as the dietary herb to aid for digestion (Hui xiang, 2005).

Chemical constituent

This plant contains phenolic compounds such as phenolic acids, flavonoids, hydroxycinnamic acids, tannin and coumarin (Rahimi & Ardekani, 2013). The major components of *F. vulgare* seed essential oils have been reported to be estragol, trans-anethole, **α**-phellandrene and fenchone (Bilia et al., 2002).

Pharmacological activities shown in Table 9.

Table 9 Pharmacological activity of *F. vulgare*

Biological activity	Part used	Reference
Antibaterial activity	Leaves	Shahmokhtar, & Armand, 2017
Antioxidant activity	Seed	Oktay et al., 2003
Anti-diabetic activity	Fruit	Dongare et a., 2010
Anti-inflammatory and Analgesic activity	Fruit	Choi & Hwang, 2004
Anti-cancer activity	Fruit	Bogucka-Kocka et al, 2008
Gastro-protective activity	Fruit	Al-Mofleh et al,2013

***Zingiber officinale* Roscoe**

Scientific name: Zingiber officinale Roscoe

Family name: Zingiberaceae

Synonym: *Zingiber majus* Rumph, *Zingiber zingiber* (L.) H. Karst.
Zingiber missionis Wall., *Curcumia longifolia* Wall.

Common name: Ginger

Thai name: Khing

Plant *description:*

"Z officinale is herbaceous rhizomatous perennial, reaching up to 90 cm in height under cultivation. Rhizomes are aromatic, thick lobed, pale yellowish, bearing simple alternate distichous narrow oblong lanceolate leaves. The herb develops several lateral shoots in clumps, which begin to dry when the plant matures. Leaves are long and 2 - 3 cm broad with sheathing bases, the blade gradually tapering to a point. Inflorescence solitary, lateral radical pedunculate oblongcylindrical spikes. Flowers are rare, rather small, calyx superior, gamosepalous, threetoothed, open splitting on one side, corolla of three subequal oblong to lanceolate connate greenish segments" (Kawai, 1994).The image *Z. officinale* is shown in **Figure 23.**

Figure 23 Rhizome of *Z. officinale*

Distribution

Z. officinale cultivation began in South Asia and since then distribute to the Caribbean and East Africa (Rehman et al., 2011)

Traditional use

Z. officinale is traditional medicinal plant have been widely used in an Ayurvedic, Chinese, Unani and Thai to treatcarminative, nausea and vomiting, indigestion, fever, migraine and headaches symptom, menstrual periods, as well as pains, sprain, muscular aches, and rheumatic disorders (Ali et al., 2008; Ujang et al., 2015).

Chemical constituent

The major active ingredients in ginger are volatile oils, gingerol, and diarylheptanoids. Volatile oils are generally composed of terpenoids. Gingerol is the spicy component of *Z. officinale*, it which contain the 3- methoxy-4-hydroxyphenyl functional group. Gingerols can be divided into gingerols **(Figure 24),** paradols, shogaols, gingerdiones, gingerdiols, and zingerones, according to the different fatty chains connected by this functional group (Liu, Liu & Zhang, 2019)

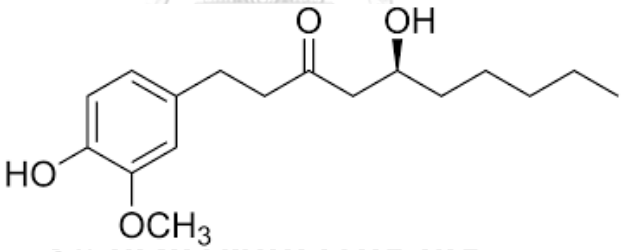


Figure 24 Structure of gingerol

Pharmacological activities of *Z. officinale* shown in Table 10.

Table 10 Pharmacological activities of *Z. officinale*

Pharmacological properties	Part use	reference
Gastroprotective activity	Rhizome	Zaman, Mirje, and Ramabhimaiah ,2014
Antioxidant activity	Rhizome	Tohma et al,2017 Li et al., 2016
Analgesic and Anti-inflammatory activities	Rhizome	Mustafa et al. 2019
Anticancer activity	Rhizome	Park et al., 1998 Ling et al., 2010
Antidiabetic activity	Rhizome	Al-Amin et al, 2006
Antimicrobial efficacy	Rhizome	Riaz et al., 2015

***Plumbago indica* L.**

Scientific name: *Plumbago indica* L.

Family name: Plumbaginaceae

Synonym: *Plumbago rosea* L., *Thela coccinea* Lour

Common name: Fire plant, Indian leadwort, Scarlet Leadwort

Thai name: Jattamoonplengdang (เจตมูลเพลิงแดง)

Plant description:

"*P. indica is a perennial herb or small shrub up to 1.5 m tall. The stems are erect, trailing or climbing, simple or branched from the base, sometimes rooting at the nodes. The leaves are oblong. The petiole is short and the auricles are absent. The blade is narrowly ovate to elliptical-ovate in shape measuring 5-15 cm x 2-8 cm. The inflorescences are elongated spike or raceme, many-flowered, measuring 10-30 cm long, glabrous. The bracts ovate in shape measuring 2-3 mm long, apex acuminate. The peduncle measures 2-10 cm long. The flowers are bisexual, regular and pentamery. The pedicel measures 0-1 mm long. The calyx is tubular in shape measuring 8-9 mm long, glandular and red in colour. The corolla tube measures 2.5-*

4.5 cm long. The lobes obovate in shape measuring 2-3 cm in diameter, apex rounded, mucronate, red in colour (Chuakul et al., 1999)." The image *P. indica* is shown in **Figure 25.**

Figure 25 Root of *P. indica*

Geographical distributions

P. indica is found scattered throughout the tropical Africa, Asia and the Pacific region. It is commonly found throughout South-East. This plant is widely cultivated in other tropical and subtropical regions (Chuakul et al., 1999).

Traditional use

P. indica is widely used in the South and Southeast Asian. The root plants are used medicine in the treatment of inflammations and fever (Padumadasa et al., 2015). In Indonesia and Malaysia, the roots and leaves are used as a remedy for paralysis, rheumatism, tumours, leprosy, swollen glands and toothache.In Thailand, the dried root have been use as emmenagogue, carminative and stomachic activities and abortifacient. It also used to stimulate digestion and. purify the blood (Chuakul et al., 1999).

Chemical constituent

The chemical constituent of roots is composed of terpenoids, tannins, flavonoids, alkaloids, and naphthoquinones (Dinda, Chel & Achari, 1994). Naphthoquinones is a major compound in the roots such as plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) **(Figure 26),** 3,3'-biplumbagin, trans-cinnamic acid, valiiniv acid, a volatile compound is prevalent (Takashi, Takada, & Ueda, 2004).

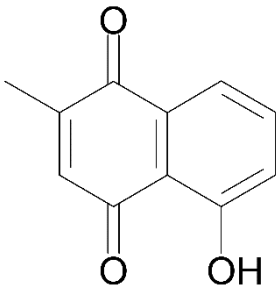


Figure 26 Chemical structure of plumbagin

P. indica has been claimed for various Pharmacological activities shown in **Table 11.**

Table 11 Pharmacological activities of *P. indica*

Pharmacological properties	**Part use**	**reference**
Antioxidant	root	Sanjana, Babita & Mishra, 2014
Anti-inflammatory and analgesic	Leaves Root	Ittiyavirah, et al, 2012 Ibrahim1 et al, 2017
Antipyretic	Root	Ibrahim1 et al, 2017
Antibacterial	Root	Jayachandran et al, 2009
Anticancer	Leaves	Hiradeve et al., 2010
Antifertility activity	Root	Devarshi et al., 1991

Piper nigrum L.

Scientific name: *Piper nigrum* L.

Family name: Piperaceae

Synonym: *Muldera multinervis* Miq. *Piper aromaticum* Lam.

Common name: Black Pepper, Pepper

Thai name: Prik-thai (พริกไทย)

Plant description:

"*P. nigrum plants easily grow in the shade on supporting trees, trellises or poles up to maximum height of 13 feets or 4 meters and roots may come out from leaf nodes if vine touch to the ground. The plants have heart shape alternate leaves with typically large size of 5-10 cm in length and 3-6 cm across, with 5 to 7 prominent palmate veins. The flowers are small, monoecious with separate male and female flowers but may be polygamous which contain both male and female flowers. The small flowers are borne on pendulous spikes at the leaf nodes that are nearly as long as the leaves. The length of spikes goes up to 7-15 cm. The black pepper's fruits are small (3 to 4 mm in diameter) called a drupe and the dried unripe fruits of. The fully mature fruits are dark red in color and approximately 5 mm in diameter. A fruit contains a single seed. The plants bear fruits from 4th or 5th year, and continue to bear fruits up to seven years. A single stem contains 20-30 spikes of fruits. The fresh harvested unripe green fruits may freeze-dry to make green pepper. The fresh harvested unripe green fruits may sun-dried to make black pepper. The red skin of the ripen fruits is removed and the stony seeds are sun-dried to make white pepper*" (Damanhouri & Ahmad**,** 2014).The image Fruit of *P. nigrum* is shown in **Figure 27.**

Figure 27 Fruit of *P. nigrum*

Geographical distributions

The primary area of *P. nigrum* are cultivated in the southern state of India. It is either native to Southeast Asia or South Asia and throughout Thailand (Takooreea et al., 2019).

Traditional uses

P. nigrum is wildly used in different traditional system of medicine, it is have been reported to treat or manage many ailments in Asia (Takooreea et al., 2019). Ayurvedic medicine, *P. nigrum* is regarded treat indigestion, stimulating expectorant and tranquilizing. In Chinese medicine, it is indicated for treat vomiting, diarrhea, and abdominal pain due to cold invading the stomach. For Thai traditional medicine, *P. nigrum* fruit is considered to be an aromatic, febrifuge, carminative. The leaves used as an antipyretic and antiseptic.

Chemical constituent

Phytochemical investigations of *P. nigrum* fruit extracts with ethanol, water, and methanol revealed the presence of secondary metabolites such as flavonoids, alkaloids, terpenoids, glycosides, steroids, anthraquinones and tannins (Nahak & Sahu, 2011). The chemical compound of *P. nigrum* are essential oil, resin and alkaloids. Piperine as its main compound, with several others including piperamine, piperyline, and piperoleines (**Figure 28**)

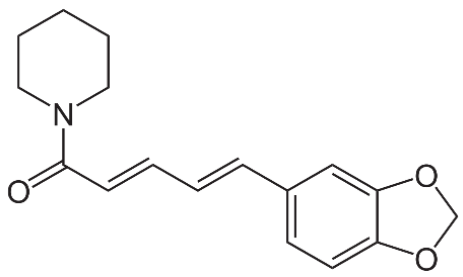


Figure 28 Chemical structure of piperine

Pharmacological activity

P. nigrum and its bioactive compounds were also found to possess important pharmacological properties shown in **Table 12.**

Table 12 Pharmacological properties of *P. nigrum*

Pharmacological properties	Part of plant	Reference
Antimicrobial activity	Fruit, seed, stem	Khan et al., 2013
Antioxidant activity	Seed leaves	Glcin, 2005 Shanmugapriya et al., 2012
Anticancer activity	Fruit, root	Sriwiriyajan et al., 2014 Prashant et al., 2017 Ee et al., 2009
Anti-inflammatory and Analgesic activities	Fruit	Tasleem et al., 2014
Antidiabetic activity	Leaves	Onyesife, Ogugua, and Anaduaka, 2014
Neuroprotective	Fruit	Hritcu et al., 2015
Toxicity	Fruit	Tasleem et al., 2014

Cannabis sativa L.

Scientific name: *Cannabis sativa* L.

Family name: Cannabaceae

Synonym: *Cannabis indica, Cannabis chinensis, Cannabis generalis*

Common name: Marijuana, Hemp

Thai name: กัญชา

Plant description:

"C. sativa is an annual, dioeciously (ie, male and female flowers are found on separate plants), pollinated plant with strong taproot, erect stems. The stems are usually angular, furrowed, branched, with woody interior, sometimes hollow in the internodes, and vary from 1 to 6 m in height. The branching is either opposite or alternate. The roots are advantageous, with branched taproot, generally 30–60 cm deep, up to 2.5 m in loose soils, very near to the surface, and more branched in wet soils. Leaves are green and palmate (seven lobes). However, the size and shape of the leaflets differs markedly, according to genetic origin. The leaf arrangement is either opposite, or alternate or spiral. The leaflets are 6–11 cm (length) and 2–15 mm (width). Leaf margins are coarsely serrated. The adaxial and abaxial surfaces are green, with scattered, resinous trichomes. Inflorescences consist of numerous flower heads that can be found on long, leafy stems from each leaf axil. The staminate (male flower) consists of five pale-green, hairy sepals about 2.5–4 mm long, and five pendulous stamens, with slender filaments and stamen. The pistillate (female flowers) are almost sessile, and are in pairs. The fruit (seed), is an achene, contains a single seed with a hard shell tightly covered by the thin wall of the ovary, and it is ellipsoid, slightly compressed, smooth, about 2–5 mm long, generally brownish and mottled" (Preedy, 2017). The image *C. sativa* is shown in **Figure 29.**

Figure 29 Leaves of *C. sativa*

Distribution

C. sativa is a native to Central and Western Asia, and long cultivated in Asia, China and Europe (Kuddus et al., 2013).

Traditional use

In China and India, it has been used long history for its food, fiber and medicine. Indian hemp (*C. sativa*) has been used for pain alleviation and inducing pleasure (Kuddus et al., 2013; Brand and Zhao, 2017). In the United States, cannabis is approved was widely as medicine and described in the United States Pharmacopoeia (Bridgeman and Abazia, 2017). The medicinal of *C. sativa* use as analgesic, intoxicant, narcotic, anodyne, stomachic, antispasmodic, and sedative etc. (Goutopoulos & Makriyannis, 2002; Russo & Guy, 2006).

Chemical constituents

The chemical compound of Cannabis reported various of the chemical classes, e.g.,sugars, hydrocarbons, steroids, flavonoids, terpenes, amino acid and nitrogenous compounds (Kuddus et al., 2013). The major compounds such as delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) (Atakan, 2012).

Pharmacological properties of *C. sativa* are antimicrobial activity (whole plant and seed) and other properties shown in **Table 13.**

Table 13 Pharmacological properties of *C. sativa*

Pharmacological properties	**Part of plant**	**Reference**
Antimicrobial activity	Whole Plant Root	Ali et al., 2011 Elhendawya et al., 2018
Antioxidant activity	leaves	Rastogi et al., 2018
Anticancer activity	leaves	Tariq and Reyaz., 2012
Anti-inflammatory and Analgesic activities	Seeds leaves	Musa et al., 2011
Antidiabetic activity	leaves	van de Venter et al., 2008

CHAPTER III

MATERIALS AND METHODS

Chemical and reagent

1 kb DNA Ladder	(Promega. USA)
100 kb DNA Ladder	(Promega. USA)
2,2-diphenyl-1-picrylhydrazyl (DPPH)	(Sigma-Aldrich, St. Louis, USA)
2,4,6-Tripyridyl-s-triazine (TPTZ)	(Sigma-Aldrich, St. Louis, USA)
3,5-Di-Tert-4-butylhydroxytoluene (BHT)	(Sigma-Aldrich, St. Louis, USA)
10X PCR Buffer	(Fermentas, USA)
Absolute ethanol, AR grade	(Merck, Daemstadt, Germany)
Agarose	(SeaGar, Cambrige, UK)
Alpha-glucosidase from Saccharomycess cerevisae	(Sigma-Aldrich, St. Louis, USA)
β-mercaptoethanol	(Sigma-Aldrich, St. Louis, USA)
Butylated hydroxytoluene (BHT)	(Sigma-Aldrich, St. Louis, USA)
Bovine albumin solution	Sigma-Aldrich, St. Louis, USA)
Casein	Sigma-Aldrich, St. Louis, USA)
CTAB: Hexadecyltrimethylammonium bromide	(Sigma-Aldrich, St. Louis, USA)
Chloroform, AR grade	(Merck, Darmstadt, Germany)
Dimethyle sulfoxide (DMSO)	(Merck, Darmstadt, Germany)
Forward - reverse primer primer	(Operon Biotechnologies, Germany)
Diclofenac sodium	(Sigma-Aldrich, St. Louis, USA)
Di-sodium hydrogen photphate (Na_2HPO_4)	(Sigma-Aldrich, St. Louis, USA)
EDTA: Ethylenediaminetetraacetic acid	(Merck, Darmstadt, Germany)
Ethyl acetate, AR grade	(RCl Labscan, Thailand)
Ethidium bromine	(Sigma-Aldrich, St. Louis, USA)
Fast blue b salt	(Sigma-Aldrich, St. Louis, USA)
Folin-Ciocalteu reagent	(Merck, Darmstadt, Germany)
Gallic acid	(Sigma-Aldrich, St. Louis, USA)

Hydrochloric acid 37%, AR grade	(RCl Labscan, Thailand)
Intestinal acetone power from rat	(Sigma-Aldrich, St. Louis, USA)
Iron (II) chloride tetrahydrate ($FeCl_2$•$4H_2O$)	(Sigma-Aldrich, St. Louis, USA)
Iron (III) chloride (FeCl3•$6H_2O$)	(Sigma-Aldrich, St. Louis, USA)
Lipoxygenase enzyme	(Sigma-Aldrich, St. Louis, USA)
Liquid nitrogen	
Linoleic acid	(Sigma-Aldrich, St. Louis, USA)
Loading Dye	(Fermentas, USA)
Methanol, AR grade	(RCl Labscan, Thailand)
Megnesium chloride ($MgCl_2$)	(Fermentas, USA)
Perchloric acid (70%)	(Merck, Daemstadt, Germany)
P-nitrophenyl-α-D-glucopyranoside (pNPG)	(Sigma-Aldrich, St. Louis, USA)
Potassium chloride (KCl)	(Merck, Daemstadt, Germany)
Potassium dihydrogen phosphate (KH_2PO_4)	(Merck, Daemstadt, Germany)
Quercetin hydrate	(Sigma-Aldrich, St. Louis, USA)
Isoamyl alcohol	(Sigma-Aldrich, St. Louis, USA)
Indomethacin	(Sigma-Aldrich, St. Louis, USA)
Sodium acetate	(BDH Laboratory supplies, Poole, England)
Sodium bicarbonate (Na_2HCO_3)	(Sigma-Aldrich, St. Louis, USA)
Sodium carbonate (Na_2CO_3)	(Sigma-Aldrich, St. Louis, USA)
Sodium chloride (NaCl)	(BDH Laboratory supplies, Poole, England)
Sodium diclofenac	(Sigma-Aldrich, St. Louis, USA)
Toluene	(RCl Labscan, Thailand)
Trolox	(Sigma-Aldrich, St. Louis, USA)
Trypsin	(Sigma-Aldrich, St. Louis, USA)
Tris (hydroxymethyl)-aminomethane hydrochloride	(Fluka, Biochemika, Germany)
Taq DNA polymerase	(Fermentas, USA)

Materials

Microcentrifuge tube	(Axygen, USA)
Pipet tips	(Axygen, USA)
PCR tubes	(Axygen, USA)
PCR purification kit	(QIAGEN, USA)
Filter paper No.4	(WhatmanTM paper, UK)
Filter paper No.40 ashless	(WhatmanTM paper, UK)
TLC silica gel 60 F254	(Merck, Daemstadt, Germany)

Instrument and equipment

Digital camera	(Cannon Power shot A640, Japan)
Photomicroscope	(Zeiss Image A.2 Axio, Germany)
Micropipette	(Eppendorf, Germany)
Mortar	
UV transilluminator	(AutoChem TM system, USA)
Thermal cycler	(Geneamp PCR 9700, Applied Biosystems)
Gel electrophoresis apparatus and power supply	
Centrifuge	(Biofuge Pico, Kendro, Germany)
Vertex mixer	(Scientific industries, Inc., USA)
Rotary evaporation	(Bushi, Switzerland)
Shaking incubator	(Biosan, Latvia)
Water bath	(Brinkman, USA)

Methodology

Part I: Standardization parameter of *C. sativa*

Sample collection

The *C. sativa* leaves were received from Drug Dependence Research Center, under the permission of Food and Drug Administration (FDA) (License number 4/2562), College of Public Health Sciences, Chulalongkorn University. Twelve samples of *C. sativa* leaves were collected from 12 different locations throughout Thailand. All samples were authenticated by a botanist. The voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Thailand.

Standardization parameters were determined for quality control of crude drug according to WHO guidelines standard methods (WHO, 2011).

Macroscopic examination

Leaves morphological characteristic including shape, size, color and texture of *C. sativa* was measured by visual inspection and recorded.

Microscopic examination

Microscope evaluation of plant samples were investigated using a digital camera attached above the microscope. The photograph is recorded with an attached digital camera and examining under the photomicroscope with objective lense. The images were recorded using AxioVision40 V4.6.3.0 software. AxioVision40 V4.6.3.0 software were used for images alignment and labelling. The individual sample was studied under objectives with a 10X, 20X and 40X magnifications.

Transvers section of midrib

The fresh mature leaves were cleaned and cut parallel including the midrib and lamina into pieces as thin as possible by hand-cut and transferred these tissue sections by a brush moistened with water. Selected satisfactory cross-sections were prepared and mounted onto a slide in glycerine water for microscopic examination

under photomicroscope, scaled for labelling size of each character. Transverse sections of midrib were drawn in the proportion size related to the original in drawing paper.

Powder drug examination

The dried sample was ground to fine powder, mounted with water and then determined characteristic or structure of tissue and plant cell.

Physicochemical determination

The dried leaves were ground and passed through a sieve with mesh number 20. All physicochemical parameters were performed in triplicate.

Loss on drying

The powder (3 g) was weighted in a crucible or weighing bottle and then heated at 105°C to obtain the constant weight. The percentage of loss on drying was calculated with reference to the air - dried substance.

Total ash

The powder (3 g) was placed in the previous ignited and tared crucible. The sample was spread in an even layer and ignited by gradual increasing heat at 500 - 600°C until obtain the white ash. The percentage of total ash was calculated with reference to the air - dried substance.

Acid - insoluble ash

To the crucible containing total ash, a 25.0 ml of 2N hydrochloric acid were added. The crucible then was covered with a watch - glass, and the mixture was boiled gently for 5 minutes. The watch - glass with water, and liquid was added into the crucible. The insoluble matter was collected on ashless filter paper (No.40) and washed with water. The filter paper containing the insoluble matter was transferred to the original crucible followed by drying and igniting until get the constant weight. The residue is allowed to cool in desiccators and weight without delay. The percentage of acid - insoluble ash was calculated with reference to the air - dried substance.

Extractive value

Ethanol-soluble extractive value

The ground sample (5.0 g) was macerated with absolute ethanol (100.0 ml) in a closed conical flask which is placed in the shaking bath for 6 hours and then allow to stand for 18 hours. The extract was filtered and the filtrate 20.0 ml was evaporated to dryness in an evaporating disc and then dried with the heat to obtain constant weight.

Water-soluble extractive value

The process of water-soluble extraction was proceeded as the same as ethanol-soluble extractive value method but using water in place of the ethanol.

Determination of water content (Azeotropic Distillation Method)

The dried powdered of sample (50 g) was weighted using digital balance (readability 0.01 g) into round bottom flask, the followed by addition of 200 ml of water-saturated toluene and boil by using azeotropic apparatus (**Figure 30**). Once the water completely distilled, heat was removed and allow the receiving tube to be cool in room temperature and dislodge any droplets of water adhere to the receiving tube's walls. Observation was performed to obtain the separated water and toluene layers. After that, the water's volume was measured. Water content was calculated as the percentage of dry weight.

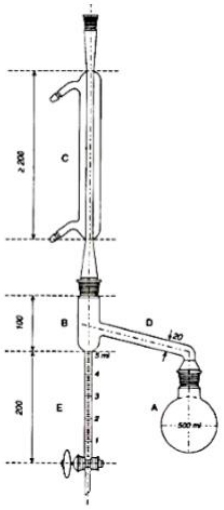


Figure 30 Azeotropic apparatus

Determination of volatile oil

Added water (200 ml) into 100 g *C. sativa* dried powder in the round bottom flask. After volatile oil completely separated from water, measured, and calculated volatile oil volume.

Thin layer chromatographic fingerprint

Transfer 20 ml of the filtrate from ethanol soluble extraction mentioned above to a small beaker, evaporate on a water-bath. Dissolve the residue in 1 ml of methanol. A Linomat IV was used for sample applications. Apply 3 µl of crude extract to the silica gel 60 F254 TLC plate and allowed to dry. The TLC plate was developed in TLC chamber saturated with a solvent system (hexane: ethyl acetate: acetic acid, ratio 4:1:0.5). After development, the plate was removed and allowed to dry and observed spot on the plate under white light, short wavelength (254 nm) ultraviolet light and sprayed the plate with 0.5 % fast blue B salt. Rf value was calculated for well using the following formula:

Rf =Distance travelled by solute/ Distance travelled by solvent

Phytochemical screening

The phytochemical screening was done qualitative method base on Hemmalakshmi et al., (2016)

Flavonoids test (alkaline reagent test)

Extract was dissolved in 10% NaOH solution followed by addition of 2 M dilute HCL. The intense yellow turn into yellow colorless indicate the present of flavonoids

Alkaloid test

10 mg/ml extract solution in chloroform was mixed with 1 ml of 25% NH_3 solution. Vortex mixture of the solution to obtain chloroform layer (lower phase). Chloroform layer then was extracted with 2 ml of 1% HCl to get alkaloidal layer (upper phase). Alkaloid layer then was test with Dragendorff and Wagner reagent.

Dragendorff's test: 1 mL of dragendorff's reagent was added into alkaloidal solution. Yellow-orange-red precipitation occurred was indicated to be alkaloids.

Wagner's test: 1 mL of Wagner's reagent was added into the alkaloidal solution. The presence of brown-red precipitate indicated the alkaloids.

Steroids and triterpenes test

1 ml extract solution in chloroform was added with few drop of concentrated sulphuric acid (H_2SO_4). The reddish brown ring was shown at the interface of the solution if the steroid moiety is present, while the yellow color at the lower phase indicated the presence of triterpenes.

Diterpenes test (Copper acetate test)

1 mL extract solution (5 mg/mL) was added with few drops of 1% copper acetate solution. The color changing to green emerald indicated the presence of diterpenes.

Saponin test (foam test)

A tiny amount of crude ethanolic extract was shake vigorously with 5 mL of water. The presence of foam on the top of solution which was stood for at least 30 minutes was indicated the presence of saponins.

Data analysis:

Macroscopic and microscopic examinations were reported by descriptive analysis. All physicochemical parameters were exhibited by grand mean ± standard error (SE). Preliminary phytochemical screening were exhibited by present or absent of phytochemical constituent.

Part II: DNA barcoding of Cannabis

DNA extraction by CTAB method

Preparation of CTAB extraction buffer

The stock of CTAB extraction buffer were prepared by mixing CTAB (2% w/v), Tris-HCl (100 mM, pH 8.0), EDTA (20 mM) and NaCl (1.4 M) were added to the CTAB stock solution to make it the ready-to-be-used CTAB extraction buffer for being used in the DNA extraction.

Using the CTAB method modified by Doyle and Doyle in 1990, the plant genomic DNA was extracted from the fresh young leaves (approximately 200 mg) of *C. sativa* plants. The young leaves of each sample were cleaned, cut into small pieces, ground to fine powder using liquid nitrogen, and transferred into 1.5-mL microcentrifuge tube filled with the ready-to-be-used CTAB extraction buffer (700 µL). The mixture was incubated in water bath at 65 °C for 1 hour, with small shake every 10 minutes. After that, the mixture was centrifuged at 10,000 rpm for 10 minutes. The supernatant was transferred into a new microcentrifuge tube. Chloroform (500 µL) was uniformly mixed with the supernatant by vortex, following with 10,000-rpm centrifuge for 10 minutes. The aqueous phase was transferred into another microcentrifuge tube, mixed with chloroform/isoamyl alcohol (24:1) solution (500 µL), and centrifuged at 10,000 rpm for 10 minutes. The obtained aqueous phase was transferred into a fresh microcentrifuge tube, and added with 1:10 volume of sodium acetate (3 M, pH 5.0). The tube was gently inverted before the addition of 2 volume of ice cold absolute ethanol (-20 °C). After being subjected to the process of gentle inversion, the tube was stored at -20°C for 1 hour, and centrifuged at 10,000 rpm for 10 minutes. The supernatant was discarded. By doing it twice, the DNA pellet was washed with cold 70% ethanol (4 °C) (1 mL), centrifuged at 10,000 rpm for 10 minutes, and the supernatant was discarded. Then, the tube was left uncovered to dry the DNA pellet. After allowing the DNA pellet to dry at room temperature, TE buffer (100 µL) was added to the tube to suspend the DNA pellet. The purity and quantity of the DNA were measured using NanoDrop One spectrophotometer. The plant genomic DNA was stored at -20 °C for further use.

PCR amplification

The 20 µL PCR reaction mixture was prepared by mixing all PCR components at final concentration in 0.2 mL PCR tube: 1X PCR buffer; 2.5 mM $MgCl_2$; 0.2 µM dNTPs; 0.4 µM of each primer; 0.5 U *Taq* DNA polymerase and 1 µl of DNA template. The primer of ITS, *rbc*L, *mat*K gene were chose for PCR amplification are shown in **Table 14,** with various PCR condition shown in **Table 15**. Five microliter of PCR product was mixed with 1 µl of loading dye and separated by 1.5% agarose gels electrophoresis in 1XTBE buffer and stained with ethidium bromide, visualized and photographed under UV illuminator.

Table 14 Detail of the primers use in PCR amplification

Primer	Sequencing (5'-3')	Reference
ITS5_F ITS4_R	GGA AGT AAAA GTC GTAA CAAG G TCC TCC GCT TAT TGA GC	White, 1990
rbcL-aF rbcL-aR	ATG TCA CCA CAA ACA GAG ACT AAA GC GTA AAA TCA AGT CCA CCR CG	Levin, 2003 Kress et al. 2009
matk-1rkim-f matk-3fkim-r,	ACC CAG TCC ATC TGG AAT CTT GGT TC CGT ACA GTA CTT TTG TGT TTA CGAG	Lee, 2007

Table 15 The PCR condition was used in PCR amplification

PCR reaction	Primer			Cycle
	ITS	matK	rbcl	
Pre- denaturation	95°C, 5 min	95°C, 5 min	95°C, 5 min	1X
Denaturation	95°C, 30 sec	95°C, 30 sec	95°C, 30 sec	35X
Annealing	50°C, 30 sec	52°C, 30 sec	55°C, 30 sec	
Extension	72°C, 30 sec	72°C, 30 sec	72°C, 30 sec	
Final extension	72°C, 5min	72°C, 5min	72°C, 5min	1X

DNA sequencing analysis:

The PCR products were purified by PCR purification kit (QIAGEN, USA). The ITS region, *mat*K, and *rbc*l sequences from both sense and antisense stand were sequenced and analyzed using BioEdit sequence alignment version 7.0. for Windows.

Data analysis:

These three barcoding regions were determined in terms of mutation, deletion and genetic distance. The phylogenetic trees were created using MEGA 11 software, and bootstrap test, with 1,000 re-samplings base on the Kimura 2-parameter (Kumar et al., 2004). Additionally, the highest log likelihood was calculated from this program to show the suitably of the tree.

Part III: In vitro pharmacological activity

3.1 Plant sample

The KLKS remedy consist of seven plants: *C. cyminum*, *N. sativa, F. vulgare, Z. officinale P. indica, C. sativa and P. nigrum* with ratio of 1 :2:3:4:5:20:40 g. The *C. sativa* leaves were received assistance from Drug Dependence Research Center, College of Public Health Sciences, Chulalongkorn University. The other sample of six plants were collected from the local market at each location. All seven samples were washed, oven drying at 45-50 °C, and then ground to coarse powders. The powder of each sample (100 g) were exhaustively macerated with 95% ethanol. The ethanol extracts were filtered through Whatman No.1 and evaporated by using rotary evaporator. The KLKS remedy extract was prepared by mixing ingredients in the quantity equivalent to the formula. The extract yields were calculated and recorded. The extract was stored at -20°C until further usage.

3.2 Test on Antioxidant activity

3.2.1 DPPH radical scavenging assay

The antioxidant property of KLKS remedy and its plant ingredient ethanolic extract were evaluated using the method from Brand-Williams et al., reported in 1995 with some modifications. Prepare stock solution 10 mg/ml. Each aliquot (20 µL) of various concentrations of the extract, standard; quercetin used as positive controls in methanol were added to DPPH• methanolic solution (120 µM, 180 µL) in 96-well microplate. The plate was incubated in darkness at room temperature for 30 minutes. Each tested sample was performed in triplicate. The ability to scavenge the DPPH• was expressed as IC_{50} value through the percent inhibition with the absorbance measured at 517 nm using the equation shown below. Regression equation was obtained from plotting between inhibition effect and concentration and IC_{50} value was calculated.

$$\% \text{ Inhibition} = \left(\frac{\text{Absorbance control} - \text{Absorbance sample}}{\text{Absorbance control}}\right) \times 100$$

3.2.2 ABTS.+ radical scavenging activity

The ABTS.+ radical scavenging activity of KLKS remedy and its plant ingredient ethanolic extract were evaluated using the method from Rachkeeree et al. (2020) with slightly modifications. The ABTS stock solution was produced by reacting ABTS aqueous solution (7 mM) and with 2.45 mM aqueous solution of potassium persulfate. The mixture was allowed in the dark at room temperature for 12–16 h; then the ABTS+ stock solution was diluted with absolute ethanol to an absorbance value of 0.7 ± 0.02, at 734 nm. 50 µL of the test sample was mixed with 100 µL of ABTS+ diluted solution, and the mixture was incubated for 15 min. And then, the absorbance was measured at 734 nm with a microplate spectrophotometer. Trolox was use as positive control. The results were expressed as IC_{50} (µg/mL) and the percentage of inhibition was calculated according to the following equation:

$$\text{ABTS Scavenging effect \%} = \left(\frac{\text{Absorbance control} - \text{Absorbance sample}}{\text{Absorbance control}}\right) \times 100$$

3.2.3 Ferric reducing antioxidant power (FRAP) assay

The reducing power of KLKS remedy and its plant ingredient ethanolic extract were assessed as described by Benzie and Strain in 1996 with some modifications. The mixture of acetate buffer (pH 3.6), containing 0.2 M acetic acid solution and 0.2 M sodium acetate, TPTZ solution (10 mM in 40 mM HCl) and ferric chloride solution (20 mM) were freshly prepared as FRAP working reagent. Then, the aliquot (10 µL) of extract, standard; BHT as positive controls, were mixed with the FRAP reagent (190 µL) in 96-well microplate, following by 30 minute incubation at room temperature. All samples were run in triplicate. The absorbance were measured at 595 nm with the use of 96-well microplate reader. The reducing antioxidant power were expressed as the FRAP value units, (mM Fe (II) per mg) of the samples with respect to the ferrous sulfate calibration curve.

$$\text{FRAF value} = \frac{C \times Vs}{D \times Cs}$$

Where; C = Concentration sample obtained from calibration curve
Vs = volume sample added in each well (ml)
D = dilution factor
Cs = concentration sample (mg/ml)

3.2.4 Total phenolic content assay

The content of phenolic compounds of KLKS remedy and its plant ingredient ethanolic extract were assessed using Folin-Ciocalteu assay in accordance with Sachindra et al. reported in 2010 with some modifications. The ethanolic extract (1mg/mL, 50 µL) and 10% Folin-Ciocalteu's phenol reagent (50 µL) were mixed in 96-well microplate. After 20 minute dark incubation, 7.5% sodium carbonate solution (50µL) were added to the mixture and allowed for 20 minute dark incubation at ambient temperature. All samples were performed in triplicate. The absorbance was measured at 765 nm by microplate reader. The total phenolic content was expressed

as gallic acid equivalents per mg extract. The total phenolic content was calculated using the formula.

C=cV/m and expressed as mg gallic acid equivalents (GAE) per g of extract in (mg/g).

Where, C = total phenolic content mg Gallic acid equivalents /g dry extract,

c = concentration of quercetin obtained from calibration curve in mg/mL,

V = volume of extract in mL, m = mass of extract in gram

3.2.5 Total flavonoid content assay

The flavonoid content of KLKS remedy and its plant ingredient ethanolic extract were evaluated using aluminium chloride colorimetric technique modified from Chang et al. in 2002. Into 96-well microplate, 50 µL of extracted sample (1 mg/mL) were mixed with aluminium chloride solution (10% w/v, 10 µL), following with ethanol (150µL) and sodium acetate solution (1 M, 10 µL), respectively. The reaction mixture was incubated for 40 minutes at room temperature, under dark condition, and then measured at 415 nm. All samples were performed in three replications. The results were expressed as quercetin equivalents per mg extract. Total flavonoid content is calculated by using the formula: C = cV/m

where, C = total flavonoid content mg QE/g dry extract,

c = concentration of quercetin obtained from calibration curve in mg/mL,

V = volume of extract in mL, m = mass of extract in gram.

3.3 Test on anti-inflammatory activity

3.3.1 Protein denaturation assay

The inhibition of protein denaturation of KLKS remedy and its plant ingredient ethanolic extract were assessed as described by Leelaprakash & Dass (2011) with some modifications. The reaction mixture was consists of 0.02 mL of sample extracts, 0.2 mL of 1% bovine albumin solution, 4.78 mL of phosphate buffered saline (pH 6.4), the mixtures were incubated in water bath at 37 °C for 15 min and then heated at 70°C for 5 min. After cooling, the turbidity was be measured by spectrometer at 660 nm.

Distilled water was instead of extracts is used as a negative control. Diclofenac was used as a positive control. Each tested sample was performed in triplicate. The Percentage inhibition of protein denaturation was calculated as follows:

$$\% \text{ Inhibition} = \left(\frac{\text{Absorbance control} - \text{Absorbance sample}}{\text{Absorbance control}}\right) \times 100$$

3.3.2 Proteinase inhibition assay

Proteinase inhibition assay of the KLKS extract and its plant ingredient ethanolic extract were performed according to method described by Sakat et al (2010) with slightly modification. The reaction mixture (5 ml) was consisting of 1 ml extract sample or positive control at various different concentrations, 0.06 mg trypsin, 1 ml 20 mM Tris HCl buffer (pH 7.4). The mixture was incubated at 37 °C for 5 min and then 1 ml of 0.8% (w/v) casein was added. The mixture was incubated for an additional 20 min, after that 2 ml of 70% perchloric acid was added to inhibit the reaction. Cloudy suspension was centrifuged, and the absorbance of the supernatant was read at 280 nm against buffer as blank. Diclofenac was used as a positive control. The experiment was performed in triplicate. The percentage inhibition of proteinase inhibitory activity was calculated as follows.

$$\% \text{ Inhibition} = \left(\frac{\text{Absorbance control} - \text{Absorbance sample}}{\text{Absorbance control}}\right) \times 100$$

3.3.3 Lipoxygenase inhibition activity

Lipoxygenase inhibition activity of the KLKS extract and its plant ingredient ethanolic extract were determined using linoleic acid as substrate and lipoxidase as enzyme follow the method described by Torres et al, 2018 with some modification. Briegly, 160 µl of borate buffer (2M, pH 9.0), mixed with 20 µl of lipoxidase enzyme solution (2,000 U/ml) was prepared in borate buffer, 10 µl of extract with various concentration, and then incubate at room temperature for 10 min. After which, 10 µl of lenoleic acid solution (0.6mM) was added, mixed well and absorbance was

measured at 234 nm. Indomethacin was used as reference standard. All tests and analyses were run in triplicate. A dose response curve was plotted to determine the IC_{50} values. IC_{50} is defined as the concentration sufficient to obtain 50% of a maximum lipoxygenase inhibition. The percent inhibition was calculated from the as following

$$\% \text{ Inhibition} = \left(\frac{\text{Absorbance control} - \text{Absorbance sample}}{\text{Absorbance control}}\right) \times 100$$

3.4 Test on antidiabetic activity

3.4.1 Yeast α-glucosidase inhibitory assay

The inhibitory activity of KLKS remedy and its plant ingredient against brewer's yeast (*Saccharomyces cerevisiae*) α-glucosidase was determined following the slightly modification with Wan et al., 2013. Each aliquot (10 µL) of the ethanolic extract with various concentrations, and positive control acabose in DMSO was mixed with α-glucosidase (0.1 U/mL, 40 µL) in 96-well microplate. After 10 minute incubation at 37°C, the substrate, p-nitrophenyl-α-D-glucopyranoside (50 µL) was added to the mixture, and then incubated further for 20 minutes, following with the addition of sodium carbonate solution (0.2 µM, 100 µL). All samples were tested in triplicate. With the use of microplate reader, the percent inhibition was calculated with the absorbance measured at 405 nm using the equation shown below.

$$\% \text{ Inhibition} = \left(\frac{\text{Absorbance control} - \text{Absorbance sample}}{\text{Absorbance control}}\right) \times 100$$

3.4.2 Rat α-glucosidase inhibitory assay

The ability of KLKS remedy and its plant ingredient ethanolic extract to inhibit rat intestinal α-glucosidase were evaluated as described by Hemalatha et al (2018) with some modifications. Each aliquot (50 µL) of the ethanolic extract at various concentrations, positive control acabose in DMSO was mixed with the substrate, p-nitrophenyl- α-D-glucopyranoside (1 mM, 100 µL) in 96-well microplate. The enzyme

solution (50 µL), containing rat intestinal acetone powder (30 mg/mL) in sodium phosphate buffer (0.1 M, pH 6.9), was added into the mixture, and then incubated at room temperature for 30 minutes. Each sample was done in triplicated. The percent inhibition was calculated with the absorbance measured at 405 nm using the following equation.

$$\% \text{ Inhibition} = \left(\frac{\text{Absorbance control} - \text{Absorbance sample}}{\text{Absorbance control}}\right) \times 100$$

3.5 Test on acute toxicity potential

The toxicity of the extracts was evaluated by Brine shrimp lethality assay (BSLA). The ability of KLKS remedy and its plant ingredient to Brine shrimp lethality was evaluated as described by Meyer et al., 1992 and Nerdy et al., 2021 with slightly modification. The extract was prepared by reconstitution of the lyophilizes with DMSO (1%) to final concentration 1 mg/mL and refrigerated until use.

Preparation of the medium and hatching of Artemia larvae:

Artemia larvae was hatched using the media composed by 38 mg/L NaCl water solution at pH 9. Brine cysts are placed in the media-mimicked artificial sea water. Constant aeration, light with a 50 Watt lamp, and room temperature (25°C) are maintained to rear the adult larvae. Brine shrimp assay was performed in the optimum age of adult brine shrimp which is using instar III of Artemia nauplii which can be obtained after 48 hours hatching.

The extracts were tested by using concentrations of 10, 100, 250, 500 and 1000 µg/mL and 10 shrimps was applied in each concentration of extract in five replicate. 1% DMSO use as negative control. Survivors was counted after 24 h using magnifying glass. The percentage lethality of brine shrimp nauplii was calculated for each concentration using the following equation;

$$\% \text{ Mortality} = N_t / N_o$$

Where N_t = Number of dead naupluii after a 24 h incubation

N_o = Number of total naupluii transferred (10)

The LC_{50} (median lethal concentration) was determined from the log concentration versus %Mortality curve

CHAPTER IV

RESULTS

Part I: Standardization parameter of *C. sativa* leaves

C. sativa leaves were collected from twelve different geographic regions throughout Thailand as showed in **Table 16**.

Table 16 The locations of 12 *C. sativa* samples collected throughout Thailand

Location	Code	Voucher	Date of collection
Northern Thailand	CM1	CSL01/2020	
	CM2	CSL02/2020	August -
	CM3	CSL03/2020	December
	LP1	CSL04/2020	2020
	PH1	CSL05/2020	
Northeastern Thailand	SK1	CSL06/2020	August -
	SK2	CSL07/2020	December
	SK3	CSL08/2020	2020
	MD1	CSL09/2020	
	NK1	CSL10/2020	
Central Thailand	SR1	CSL11/2020	August -
	SR2	CSL11/2020	December
			2020

Macroscopic examination

Morphologically, the fresh mature leaf of *C. sativa* is compound, palmate shaped, 7 - 9 linear lanceolate leaflet blades, serrate margin with acuminate apex, alternate or opposite in arrangement. The upper (adaxial) surfaces are dark green, while the lower (abaxial) are pale green with rough surface. Leaves are 0.2-2 cm wide, 3-15 cm long, 2-7 cm petiole and bitter test. The whole plant and leaves showed in **Figure 31.**

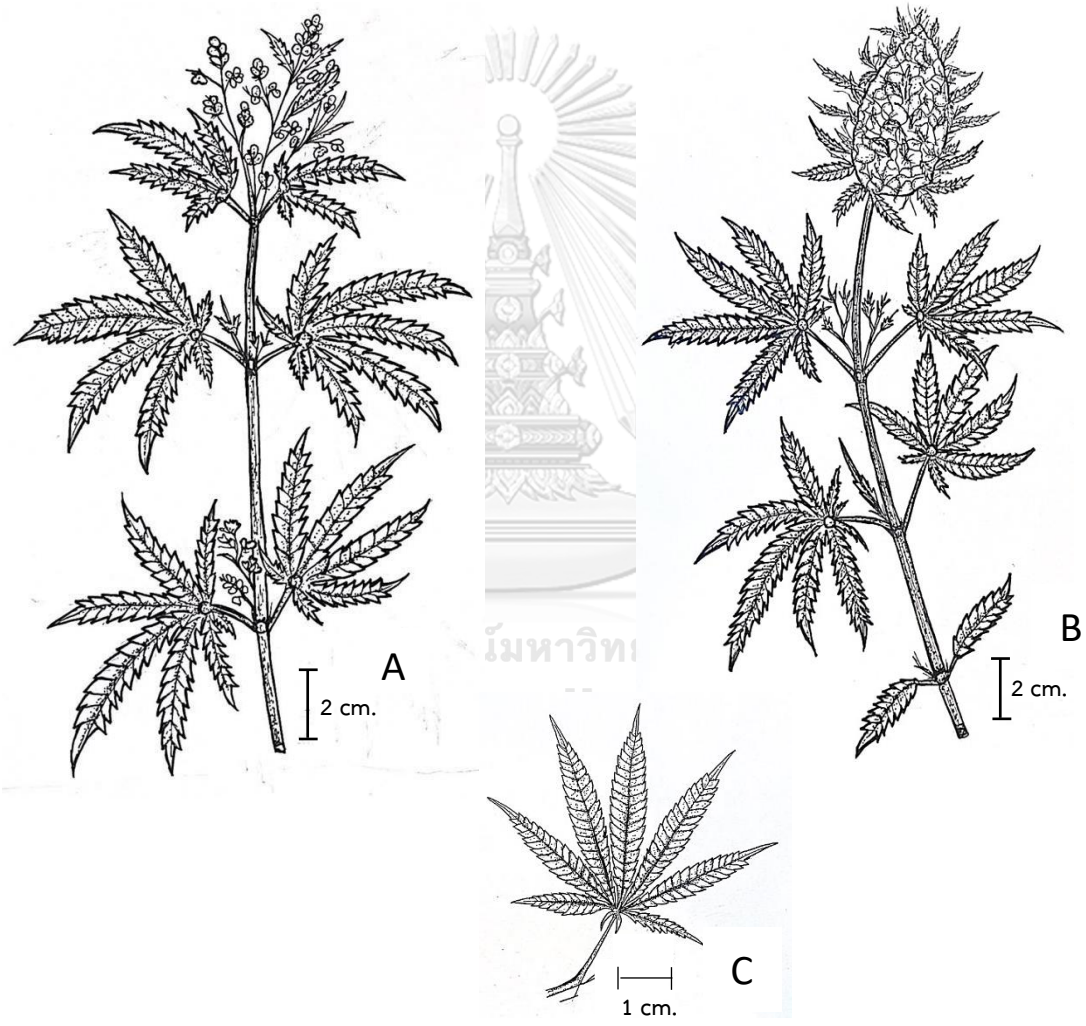


Figure 31 Whole plant of Cannabis;

; A = Cannabis male Plant,

B = Cannabis female Plant, C = Leaf of *C. sativa*

Microscopic examination

The upper epidermis surface revealed rectangular cell with striations, anomocytic stomata, palisade, and cicatrix. Trichome consist of unicellular trichome with cystolith found in the lower epidermis **(Figure 32)**.

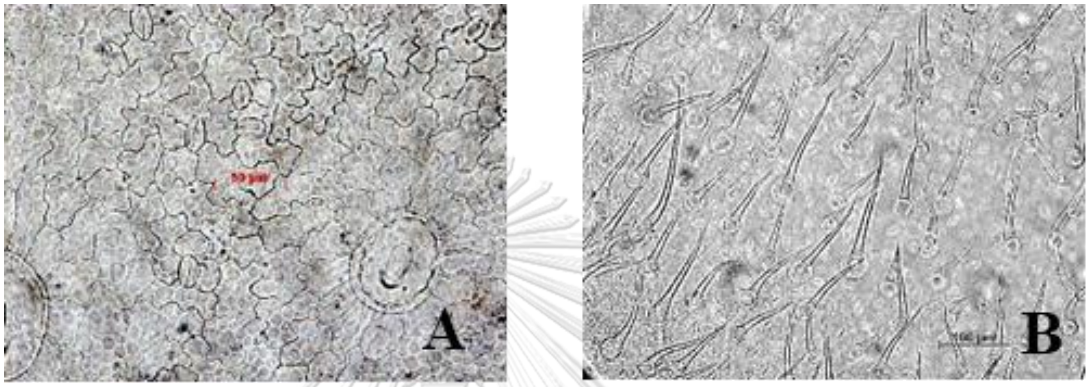


Figure 32 Upper epidermis (A), lower epidermis (B) of *C. sativa* leaf

Transverse section of midrib and lamina of *C. sativa* leaf **(Figure. 33)** showed the upper and lower epidermis surface is covered by single layer of epidermis. The epidermis is undulating, with unicellular non-glandular trichome and glandular trichome. The midrib was composed of collenchyma layer cell underneath upper and lower epidermal, parenchyma containing rosette aggregate crystal. The mesophyll showed of distinct palisade layer and spongy parenchyma. Vascular bundle was surrounded by sclerenchyma tissue.

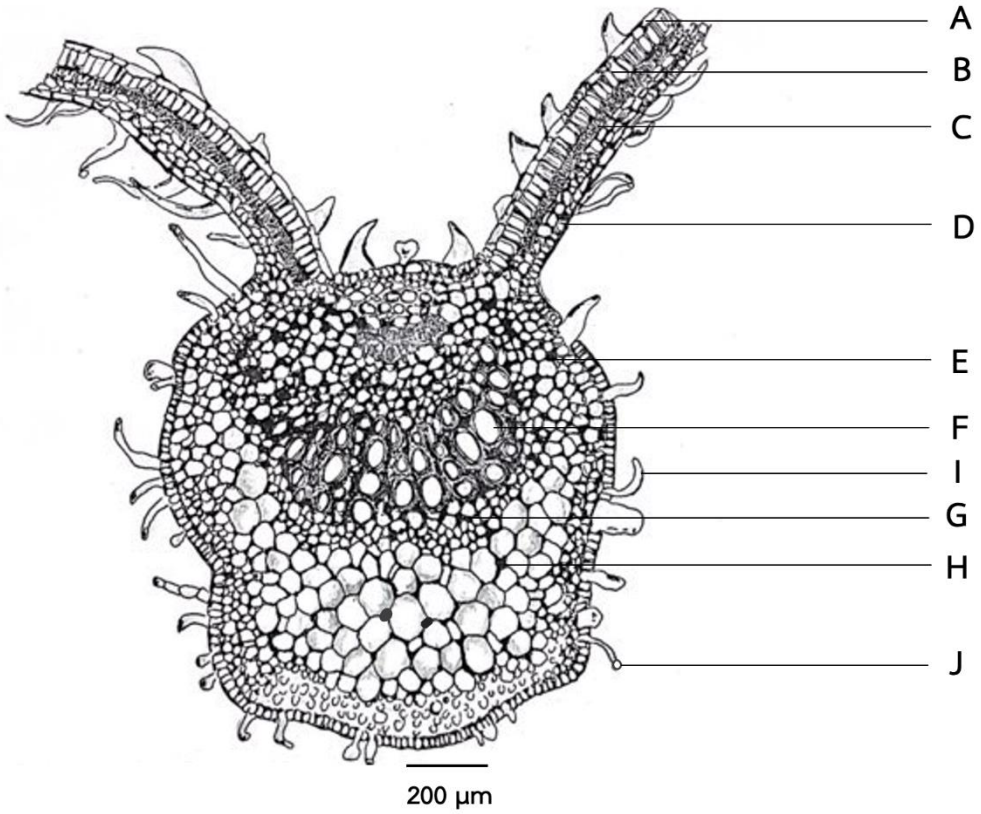


Figure 33 Transverse section of *C. sativa* leaf midrib;
(A) upper epidermis, (B) palisade cell, (C) spongy cell, (D) lower epidermis, (E) collenchyma, (F) Vascular bundle (phloem and xylem tissue), (G) Sclerenchyma tissue, (H) Parenchyma containing rosette aggregate crystal, (I) unicellular trichome, and (J) glandula trichome at 20x magnification

The powder microscopic examination of *C. sativa* leaves indicated the presence of anomocytic stoma, palisade, parenchyma cell containing rosette aggregate crystal, spiral vessels, unicellular trichomes, cystolithic, and fiber as shown in **Figure 34.**

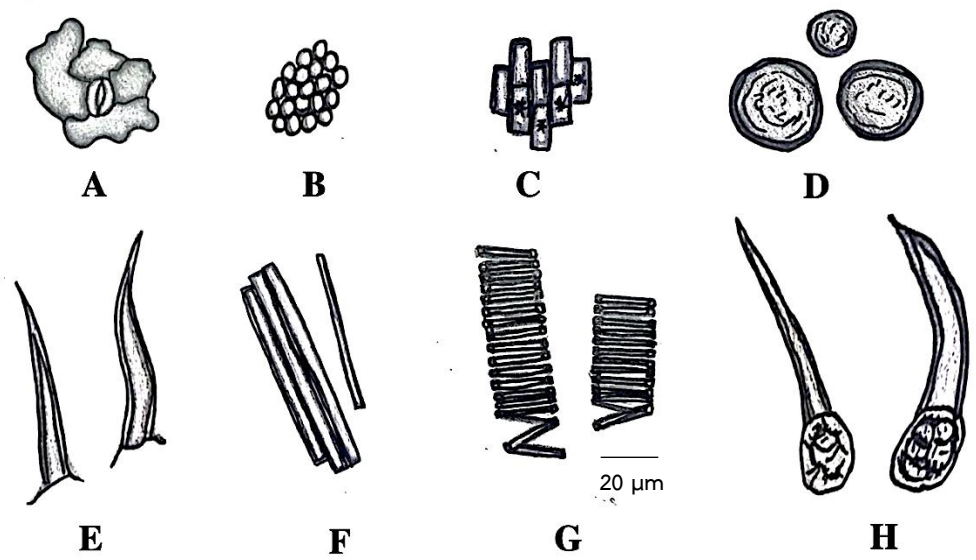


Figure 34 Powder microscopy of *C. sativa* leaf

(A) anomocytic stoma, (B) palisade cell, (C) parenchyma cell containing rosette aggregate crystal, (D) cystolith, (E) unicellular trichomes, (F) fiber, (G) spiral vessels, and (H) unicellular trichomes with cystolith

Physicochemical parameters

The physicochemical parameters of *C. sativa* leaves collected from different geographic region were found to be in an acceptable range as summarized in **Table 17.**

Table 17 Physicochemical parameters of *C. sativa* leaves

Physicochemical parameter	Value (% w/w)
Loss on drying	4.07 ± 0.09
Water content	7.95 ± 0.12
Total ash	14.36 ± 0.13
Acid insoluble ash	2.73 ± 0.09
Water-soluble extractive value	23.04 ± 0.16
Ethanol-soluble extractive value	11.10 ± 0.15
Volatile oil content	Not detected

** The value was shown as mean ± SE.

TLC fingerprinting

TLC fingerprints of this extract were showed in **Figure 35**. TLC pattern of ethanolic extract indicated nine spots with Rf value 0.14, 0.19, 0.23, 0.29, 0.32, 0.45, 0.58, 0.70, 0.76 using the solvent system n- hexane, ethyl acetate, acetic acid (4:1:0.5) with 0.5 % Fast blue B salt as staining reagent.

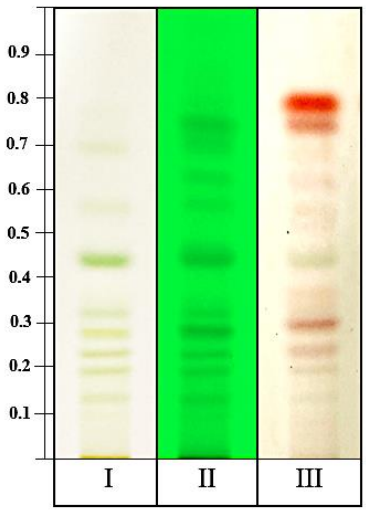


Figure 35 TLC fingerprint of *C. sativa* leaf ethanolic extract

I = detection under white light

II = detection under UV 254 nm

III = detection with 0.5 % Fast blue B salt staining reagent

Phytochemical screening

Phytochemical screening of *C. sativa* ethanolic extract showed the presence of alkaloids, flavonoids, phenolics, steroids, triterpenes, and diterpenes. However, saponin was not detected (**Table 1**8).

Table 18 Phytochemical screening of *C. sativa* leaves

Phytochemical constituents	Assay	Ethanolic extract	Color
Phenolics	Ferric chloride test	+	Green
Alkaloids	Dragendroff 's test	++	Orange precipitate
	Wagner's test	++	Reddish brown precipitate
Flavonoids	Alkaline test	++	Yellow to colorless
	Shinoda test	++	Pink
Triterpenes	Liebermann–Burchard test	+++	Reddish brown
Diterpenes	Copper acetate test	+++	Emerald green
Steroids	Salkowski test	++	Yellow colored ring
Saponin	Foam test	-	White foam layer

Note: +++ Hight presence, ++ = moderate presence, + = low presence, - = absent

Part II: DNA Barcoding of *C. sativa* L. subsp. Indica leaves

20 samples of fresh young leaves of *C. sativa* were collected throughout Thailand (**Table 19**).

Table 19 The locations of 20 *C. sativa* samples collected throughout Thailand

Location	Code	Voucher	Date of collection
Northern Thailand	N1	CM1/2020	
	N2	CM2/2020	
	N3	CM3/2020	
	N4	CM4/2020	
	N5	CM5/2020	
	N6	CM6/2020	August -
	N7	CM7/2020	December 2020
	N8	CM8/2020	
	N9	LP1/2020	
	N10	LP2/2020	
	N11	PE5/2020	
	N12	N/2020	
Northeastern Thailand	NE1	MD1/2020	
	NE2	MD2/2020	
	NE3	MD3/2020	August -
	NE4	MD4/2020	December 2020
	NE5	MD5/2020	
	NE6	H/2020	
Southern Thailand	S3	NK2/2020	August -
	S4	TR2/2020	December 2020

DNA Extraction

Genomic DNA of plant sample was extract from the fresh leaf using DNeasy Plant Mini kit (QIAGENValencia, California, USA) as shown in **Figure 36**. The obtained had no signs of degradation and the bands obtained > 2000 bp in size with a concentration approximately 40-100 ng/ul.

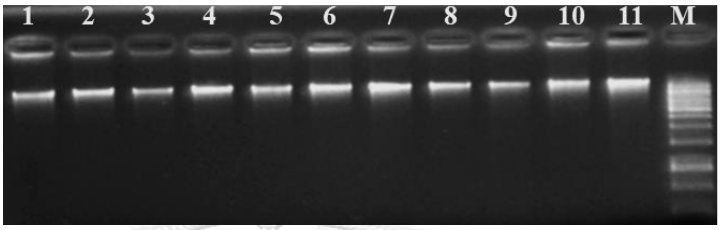


Figure 36 Genomic DNA of *C. sativa* (1-11) on 1.5% agarose gel M=1 kb DNA ladder

PCR amplification and DNA sequencing

The twenty *C. sativa* were successfully amplified using primer pair ITS4/ITS5, *rbc*L and *mat*K sequenced showed in **Table 14.**

The three loci were successfully amplified, and sequencing was done by Sanger sequencing technique. The length of the ITS, *rbc*L, and *mat*K sequence obtained from various geographical areas of *C. sativa* species were approximately 650, 1200, and 800 bp, respectively. Analyses were conducted using the Kimura 2-parameter model. This analysis involved 20 nucleotide sequences. The similarity of the ITS, *rbc*L, and *mat*K sequence among the 20 samples ranges from 91- 100%, 98 – 100%, and 99-100%, respectively. *rbc*L and *mat*K sequence was highly showed similarity index that was not suitable for DNA barcoding.

DNA barcoding of ITS region

Internal transcribed spacer (ITS) is located between the large-subunit rRNA genes. There are two ITS regions which is ITS1 (position 1-225 bp) and ITS2 (position 389-648 bp). No mutation was found in ITS2 region, but there are 46 mutations was fond in ITS1 region. The result of ITS region was indicated that there are 5 groups *of C. sativa;* group C1-C5 (**Table 20**).

Group C1: there are 2 mutations (positions 6 and 91) in ITS region. This group consists of 15 samples from Northern Thailand (N1-N10), Northeastern Thailand (NE1-2, NE5), and Southern Thailand (S3-S4).

Group C2: there are 1 deletion (position 6) in ITS region. This group consist of 2 samples from Northern Thailand (N11) and Northeastern Thailand (NE6).

Group C3: there are 21 mutations in ITS region. This group consist of 1 sample from Northeastern Thailand (NE4).

Group C4: there are 46 mutations and 1 deletion. This group consist of 1 sample from Northern Thailand (N12).

Group C5: there are 43 mutations and 1 deletion. This group consist of 1 sample from No Northeastern Thailand (NE3).

Table 20 DNA barcoding of ITS region

Code	Positions																																																		
	1	2	3	4	5	6	7	8	9	11	12	14	15	16	17	18	19	21	24	25	26	27	28	29	32	33	34	35	36	37	39	40	41	43	44	45	46	47	48	63	68	70	71	73	74	75	77	79	80	81	85	91
C1	T	C	G	A	A	A	C	C	T	C	A	C	A	G	C	A	G	A	A	C	C	C	G	T	A	C	A	C	G	T	T	T	A	A	C	A	G	C	T	G	T	C	T	C	T	T	G	C	C	C	C	T
C2	T	C	G	A	A	-	C	C	T	C	A	C	A	G	C	A	G	A	A	C	C	C	G	T	A	C	A	C	G	T	T	T	A	A	C	A	G	C	T	G	T	C	T	C	T	T	G	C	C	C	C	C
C3	T	C	A	G	C	C	A	C	G	C	T	T	C	A	C	T	A	T	A	C	G	G	A	T	A	G	G	G	T	G	T	T	A	A	C	A	G	C	T	G	T	C	T	C	T	T	G	C	C	C	C	C
C4	T	-	A	G	C	A	A	G	G	G	G	T	C	A	T	T	A	T	G	A	G	G	A	G	G	G	G	G	G	G	G	G	T	G	T	C	T	T	A	T	A	A	A	A	A	G	G	C	A	A	A	C
C5	T	-	A	A	C	A	A	G	G	G	G	T	C	A	C	T	A	T	G	A	G	G	A	G	G	G	G	G	G	G	G	G	A	G	T	C	T	T	A	T	T	A	A	A	A	G	A	A	A	A	C	C

Phylogenetic tree analysis of ITS region

The phylogenetic relationship among *C. sativa* from 20 different locations were examined in ITS using maximum likelihood (ML) method and the bootstrap analysis (1,000 replications) was done to determine the confidence of the result of ML tree. The estimation of evolutionary divergence between sequences of ITS region was showed in **Table 21**. Genetic relationship analysed by MEXA 11 using ML method presented that there are 4 groups of *C. sativa* with high bootstrap support (**Figure 37**). Group I (N1-N11, NE1-2, NE5, S3-4S) consisted of sample collected from Northern, Northeastern and Southern part of Thailand

Group II (NE4), sample collected from Northeastern Thailand

Group III (N12), sample collected from Northern Thailand

Group IV (NE3), sample collected from Northeastern Thailand

Table 21 Estimates of evolutionary divergence between sequences of ITS region

	N1	N2	N3	N4	N5	N6	N7	N8	N9	N10	N12	NE1	NE2	NE3	NE4	NE5	N11	S3	S4	NE6
N1																				
N2	0.000																			
N3	0.000	0.000																		
N4	0.000	0.000	0.000																	
N5	0.000	0.000	0.000	0.000																
N6	0.000	0.000	0.000	0.000	0.000															
N7	0.000	0.000	0.000	0.000	0.000	0.000														
N8	0.000	0.000	0.000	0.000	0.000	0.000	0.000													
N9	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002												
N10	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002											
N12	0.078	0.078	0.078	0.078	0.078	0.078	0.078	0.078	0.080	0.078										
NE1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.078									
NE2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.078	0.000								
NE3	0.071	0.071	0.071	0.071	0.071	0.071	0.071	0.071	0.073	0.071	0.014	0.071	0.071							
NE4	0.033	0.033	0.033	0.033	0.033	0.033	0.033	0.033	0.035	0.033	0.051	0.033	0.033	0.048						
NE5	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.078	0.000	0.000	0.071	0.033					
N11	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.078	0.000	0.000	0.071	0.032	0.000				
S3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.078	0.000	0.000	0.071	0.033	0.000	0.000			
S4	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.078	0.000	0.000	0.071	0.033	0.000	0.000	0.000		
NE6	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.078	0.000	0.000	0.071	0.032	0.000	0.000	0.000	0.000	

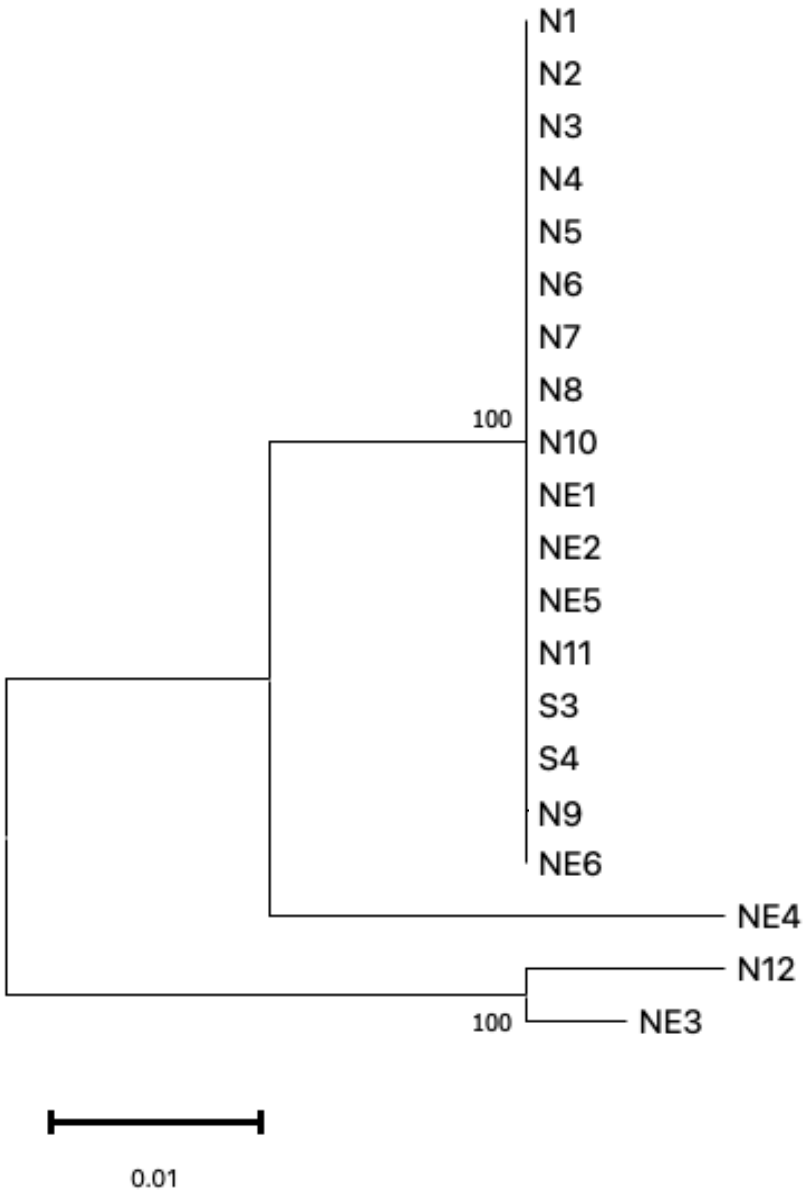


Figure 37 Phylogenetic tree using the ML method of ITS region

Part III: In vitro pharmacological activity of KLKS remedy and its plant ingredients ethanolic extract

7 samples of KLKS remedy were collected from central Thailand (**Table 22**).

Table 22 The 7 samples of KLKS remedy were collected from central Thailand

No.	samples	Locations	Date of collection
1	*C. cyminum* fruits	Central Thailand	June 2021
2	*N. sativa* seeds		
3	*F. vulgare* fruits		
4	*Z. officinale* rhizomes		
5	*P. indica* roots		
6	*C. sativa* leaves		
7	*P. nigrum* fruits		

The percentage yield of ethanolic extracts of KLKS remedy and its ingredient were showed in **Table 23.** In *N. sativa* seeds, the extract gave the highest yield (23.24%) while *F. vulgare* fruits extract gave the least yield (7.20%).

Table 23 The percent yield of ethanolic extract of KLKS remedy and its plant ingredient

Samples	The percent yield by macerate extraction (g/100g)
C. cyminum fruits	15.562
N. sativa seeds	23.245
F. vulgare fruits	7.200
Z. officinale rhizomes	15.414
P. indica roots	20.681
C. sativa leaves	13.352
P. nigrum fruits	9.993
KLKS remedy	10.468

Antioxidant activity

DPPH radical scavenging activity

The result of DPPH radical scavenging activity of extract ethanolic extract of Kalomkeasan remedy and its plant ingredient were showed in **Table 24.** Quercetin was used as positive control showed IC_{50} values of 16.43 µg/ml (**Figure 38**). The extract of *P. nigrum* fruits demonstrated the highest radical scavenging activity with IC_{50} values of 75.92 µg/ml, followed by extract of *Z. officinale* rhizomes showed the IC_{50} of 80.41 µg/ml. KLKS remedy extract showed the IC_{50} of 108.52 µg/ml, whereas the extract of *N. sativa* seeds had the lowest radical scavenging activity (IC_{50} of 254.41 µg/ml)

Table 24 The DPPH radical scavenging activity of ethanolic extracts of Kalomkeasan remedy and its plant ingredient

Samples	IC_{50} (µg/ml)
C. cyminum fruits	138.10 ± 0.57
N. sativa seeds	254.21 ± 0.13
F. vulgare fruits	126.21 ± 0.59
Z. officinale rhizomes	80.411 ± 0.56
P. indica roots	99.77 ± 0.71
C. sativa leaves	112.13 ± 0.17
P. nigrum fruits	75.92 ± 1.68
KLKS remedy	108.52 ± 0.94
Quercetin	16.43 ± 0.42

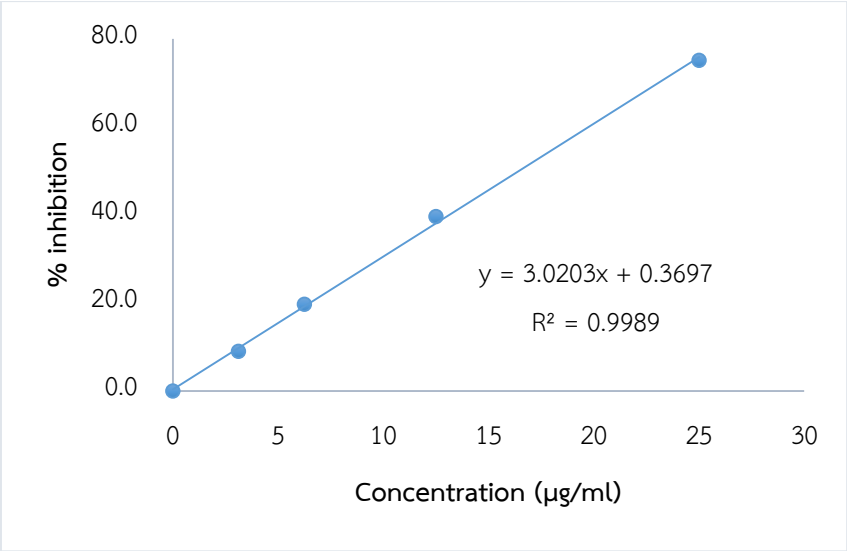


Figure 38 DPPH inhibition of Quercetin

ABTS radical scavenging activity

The result of ABTS radical scavenging activity of ethanolic extract of Kalomkeasan remedy and its plant ingredient were showed in **Table 25.** Trolox was used as positive control showed IC_{50} values of 24.19 µg/ml (**Figure 39**). The ethanolic extract of *Z. officinale* rhizomes demonstrated the highest radical scavenging activity with IC_{50} values of 93.90 µg/ml followed by *P. nigrum* fruits showed the IC_{50} of 123.53 µg/ml. KLKS remedy extract showed the IC_{50} of 223.76 µg/ml, whereas the *N. sativa* seeds had the lowest radical scavenging activity (IC_{50} of 378.34 µg/ml)

Table 25 ABTS radical scavenging activity of ethanolic extracts of KLKS remedy and its plant ingredient

Samples	IC_{50} (µg/ml)
C. cyminum fruits	179.40 ± 0.88
N. sativa seeds	378.34 ± 0.71
F. vulgare fruits	236.36 ± 1.05
Z. officinale rhizomes	93.90 ± 0.71
P. indica roots	212.91 ± 1.00
C. sativa leaves	201.11 ± 0.92
P. nigrum fruits	123.53 ± 0.99
KLKS remedy	223.76 ± 1.29
Trolox	24.19 ± 0.97

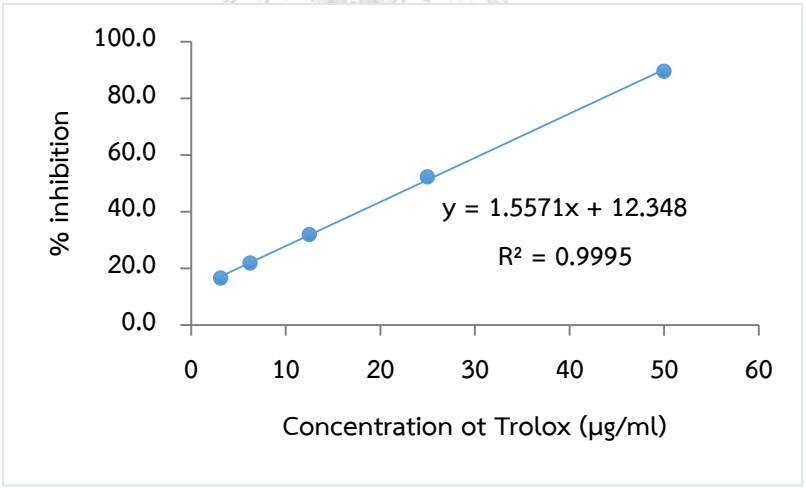


Figure 39 ABTS radical scavenging inhibition of Trolox

Ferric reducing antioxidant power (FRAP)

The ferrous sulphate ion ($FeSO_4$) concentrations was calculated according to the equation of standard curve of ferrous sulphate **(Figure 40).** The result of tested samples was presented in **Table 26.** The ethanolic extract of *Z. officinale* rhizomes demonstrated the highest reducing power ability with FRAP value of 0.72 mM Fe^{2+}/g extract, followed by *P. nigrum* fruits, KLKS remedy with FRAP value of 0.31, 0.28 mM Fe^{2+}/mg, respectively. On the other hand, the *F. vulgare* fruit had the lowest reducing power ability with FRAP value of 0.18 mM Fe^{2+}/g extract. Positive control (BHT) was showed 2.13 ± 0.83 mM Fe^{2+}/g extract.

Table 26 Table 27 FRAP value of each ethanolic extract of Kalomkeasan remedy and its plant ingredient

Samples	FRAP value (mM Fe^{2+}/g extract)
C. cyminum fruits	0.21 ± 0.73
N. sativa seeds	0.19 ± 0.29
F. vulgare fruits	0.18 ± 1.03
Z. officinale rhizomes	0.72 ± 0.43
P. indica roots	0.25 ± 0.08
C. sativa leaves	0.23 ± 0.12
P. nigrum fruits	0.31 ± 0.68
KLKS remedy	0.28 ± 0.18
BHT	2.13 ± 0.83

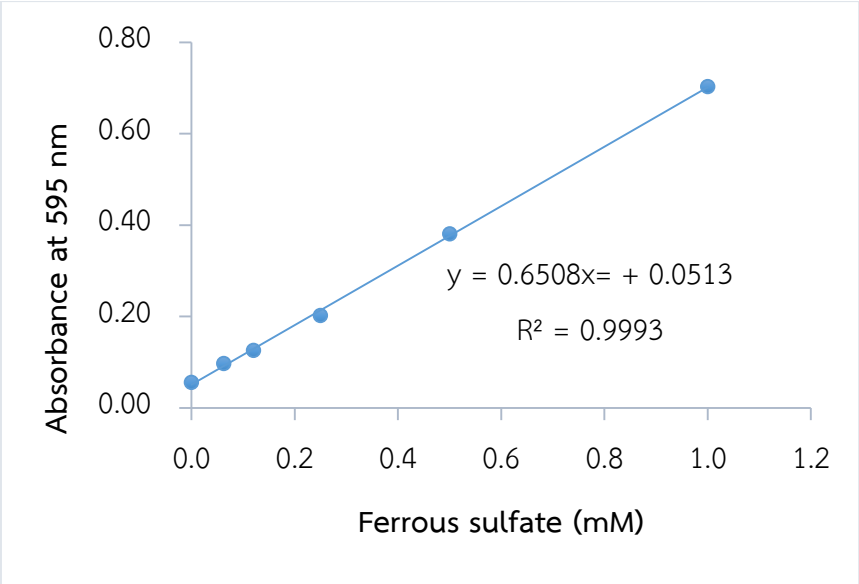


Figure 40 Standard curve of antioxidant capacity by ferric ion reducing antioxidant power

Total phenolic content

The ethanolic extract of Kalomkeasan remedy and its plant ingredient were evaluated for the total phenolic content using Folin-Ciocalteo reagent **(Table 27)**. Gallic acid was used to set up a calibration curve **(Figure 41)**. The total phenolic content of the ethanolic extract of *Z. officinale* rhizomes showed the highest total phenolic content of 36.78 mg GAE/g extract, followed by *P. indica* roots, KLKS remedy, *P. nigrum* fruits ethanolic extract with total phenolic content of 16.81, 14.61, 14.08 mg GAE/g extract, respectively

Table 27 The total phenolic content of ethanolic extracts of Kalomkeasan remedy and its plant ingredient

Samples	Total phenolic content (mg GAE/g extract)
C. cyminum fruits	6.24 ± 0.52
N. sativa seeds	7.23 ± 1.16
F. vulgare fruits	6.58 ± 0.44
Z. officinale rhizomes	36.78 ± 0.76
P. indica roots	16.81 ± 0.90
C. sativa leaves	14.08 ± 0.34
P. nigrum fruits	11.31 ± 0.80
KLKS remedy	14.61 ± 0.16

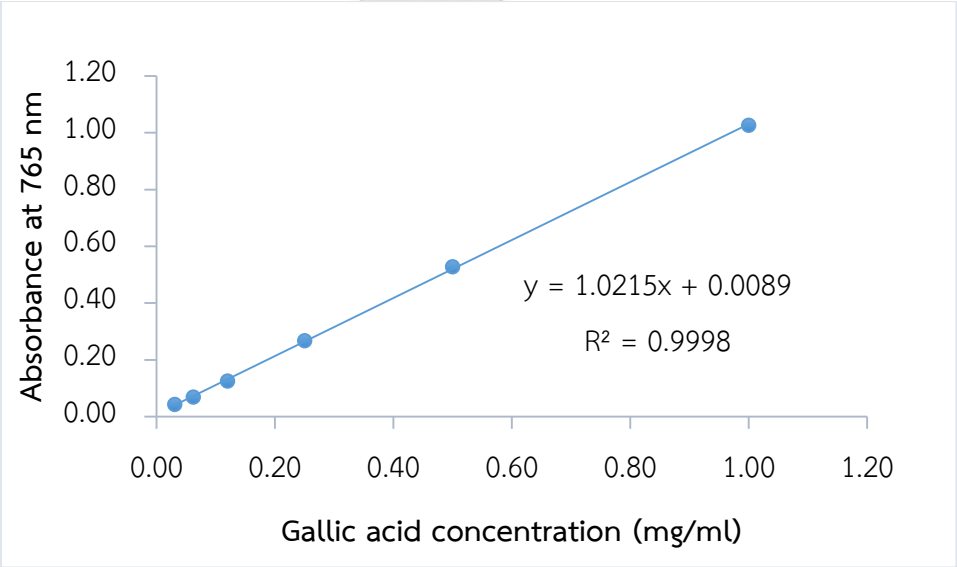


Figure 41 Gallic acid calibration curve for total phenolic quantification

Total flavonoid content assay

The ethanolic extract of Kalomkeasan remedy and its plant ingredient were evaluated for the total flavonoid content using aluminium chloride colorimetric technique **(Table 28)**. Quercetin was used to set up a calibration curve **(Figure 42)**. The total flavonoid content of the ethanolic extract of *C. sativa* leaves showed the highest total flavonoid content of 23.88 mgQE/g extract, followed by *Z. officinale* rhizomes, KLKS remedy ethanolic extract with total flavonoid content of 15.60, and 14.87 mg QE/g extract, respectively.

Table 28 The total flavonoid content of ethanolic extracts of Kalomkeasan remedy and its plant ingredient

Samples	Total flavonoid content (mgQE/g extract)
C. cyminum fruits	11.87 ± 0.86
N. sativa seeds	8.89 ± 0.45
F. vulgare fruits	9.46 ± 0.71
Z. officinale rhizomes	15.60 ± 0.23
P. indica roots	13.84 ± 0.53
C. sativa leaves	23.88 ± 0.40
P. nigrum fruits	14.32 ± 0.80
KLKS remedy	14.87 ± 0.82

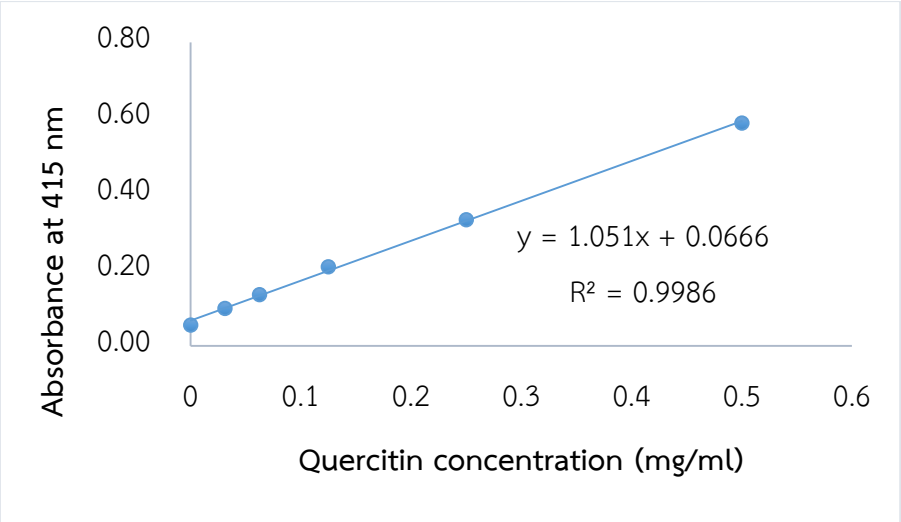


Figure 42 Quercetin concentration calibration curve for total flavonoid quantification

Anti-inflammatory activity

Anti-lipoxygenase activity

The result of anti-lipoxygenase activity of KLKS remedy and its ingredient ethanolic extract were showed in **Table 29 and Figure 44**. The ethanolic extract of KLKS remedy had higher than all its ingredient extracts inhibition of lipoxygenase with IC_{50} values of 86.37 ± 0.57 µg/ml, follow by *P. nigrum* fruits (145.89 ± 0.46 µg/ml), *Z. officinale* rhizomes (223.20 ± 0.98 µg/ml), whereas for the standard Indomethacin **(Figure 43)** was found IC50 values of 57.64 ± 0.64µg/ml, respectively.

Table 29 Effect of KLKS remedy and its ingredient on inhibition of lipoxygenase

Samples test	Lipoxygenase inhibition IC_{50} (µg/ml)
C. cyminum fruits	505.59 ± 0.21
N. sativa seeds	372.63 ± 1.39
F. vulgare fruits	428.88 ± 0.58
Z. officinale rhizomes	223.20 ± 0.98
P. indica roots	289.07 ± 0.69
C. sativa leaves	270.67 ± 0.39
P. nigrum fruits	145.89 ± 0.46
KLKS remedy	86.37 ± 0.57
Indomethacin	57.64 ± 0.64

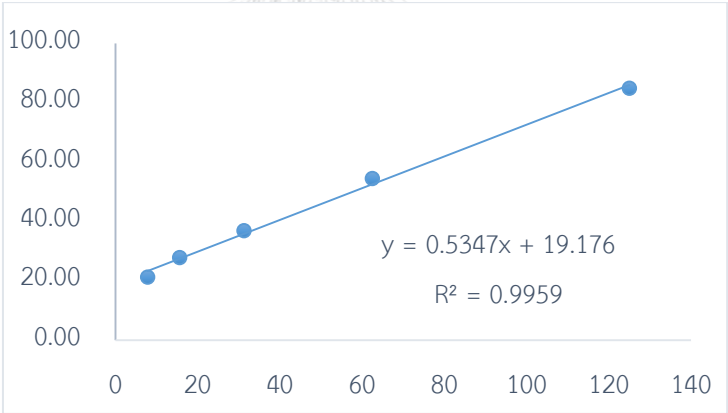


Figure 43 Lipoxygenase inhibition of Indomethacin

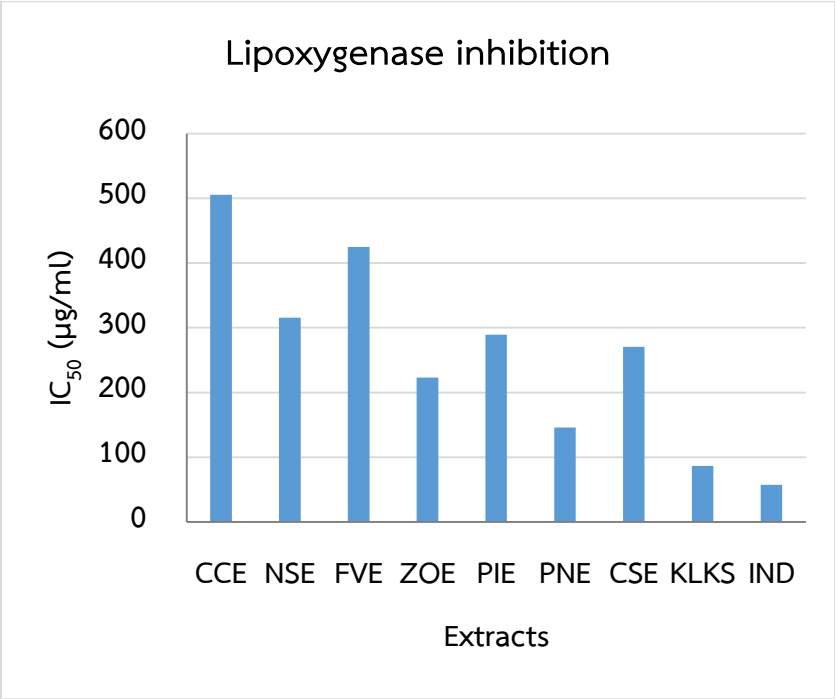


Figure 44 Effect of ethanolic extracts KLKS remedy and its ingredient on inhibition of lipoxygenas;

CCE = *C. cyminum,* NSE = *N. sativa*, FVE = *F. vulgare*, ZOE = *Z. officinale,* PIE = *P. indica*, PNE = *P. nigrum*, CSE = *C. sativa*, KLKS = Kealomkeasan remedy, IND = Indomethacin; results presented as the mean ± SD (n=3)

Protein denaturation assay

The result of protein denaturation activity of KLKS remedy and its ingredient ethanolic extract were showed in **Table 30 and Figure 45**. The ethanolic extract of KLKS remedy had higher than all its ingredient extracts inhibition of protein denaturation with IC_{50} values of 182.63 ± 0.55 µg/ml, follow by *P. nigrum* fruits (298.87 ± 0.44 µg/ml), whereas for the standard diclofenac were found IC_{50} values of 94.86 ± 0.73 µg/ml.

Table 30 Effect of KLKS remedy and its ingredient on inhibition of protein denaturation

Sample test	Protein denaturation inhibition IC_{50} (µg/ml)
C. cyminum fruits	821.21 ± 1.15
N. sativa seeds	518.33 ± 0.61
F. vulgare fruits	759.10 ± 0.89
Z. officinale rhizomes	396.59 ± 0.35
P. indica roots	511.24 ± 0.26
C. sativa leaves	401.14 ± 0.76
P. nigrum fruits	298.87 ± 0.44
KLKS remedy	182.63 ± 0.55
Diclofenac sodium	94.86 ± 0.73

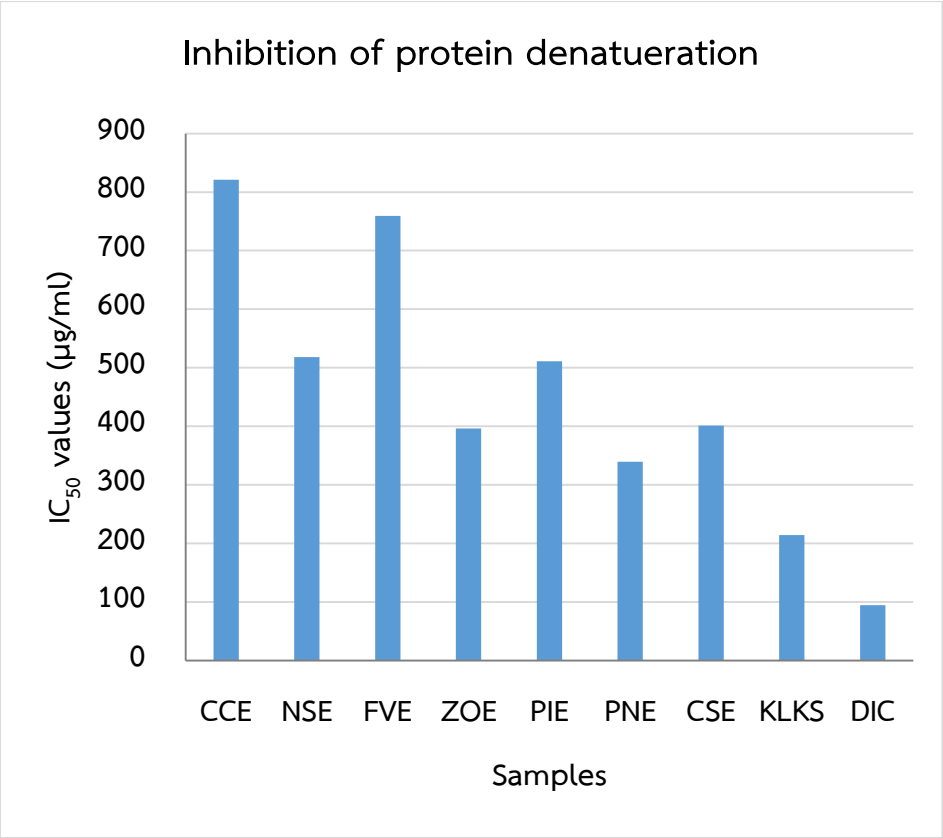


Figure 45 Effect of ethanolic extracts KLKS remedy and its ingredient on inhibition of protein denaturation;

CCE = *C. cyminum*, NSE = *N. sativa*, FVE = *F. vulgare*,

ZOE = *Z. officinale*, PIE = *P. indica,* PNE = *P. nigrum,* CSE = *C. sativa,*

KLKS = Kealomkeasan remedy, DIC = diclofenac sodium;

results presented as the mean ± SD (n=3)

Proteinase inhibition activity

The result of proteinase inhibition activity of KLKS remedy and its ingredient ethanolic extract were showed in **Table 31 and Figure 46**. The ethanolic extract of KLKS remedy had higher than all its ingredient inhibition of proteinase with IC50 values of 250.63 ± 0.82 µg/ml, follow by *P. nigrum* fruits (411.40 ± 1.03 µg/ml), *Z. officinale* rhizomes (469.54 ± 1.39 µg/ml), whereas for the standard diclofenac were found IC_{50} values of 124.90 ± 0.98 µg/ml.

Table 31 Effect of KLKS remedy and its ingredient on proteinase inhibition

Samples test	**Proteinase inhibition IC_{50} (µg/ml)**
C. cyminum fruits	> 1000
N. sativa seeds	930.07 ± 1.43
F. vulgare fruits	689.87 ± 1.08
Z. officinale rhizomes	469.54 ± 1.39
P. indica roots	579.31 ± 1.03
C. sativa leaves	639.93 ± 0.86
P. nigrum fruits	411.40 ± 1.03
KLKS remedy	250.63 ± 0.82
Diclofenac sodium	124.90 ± 0.98

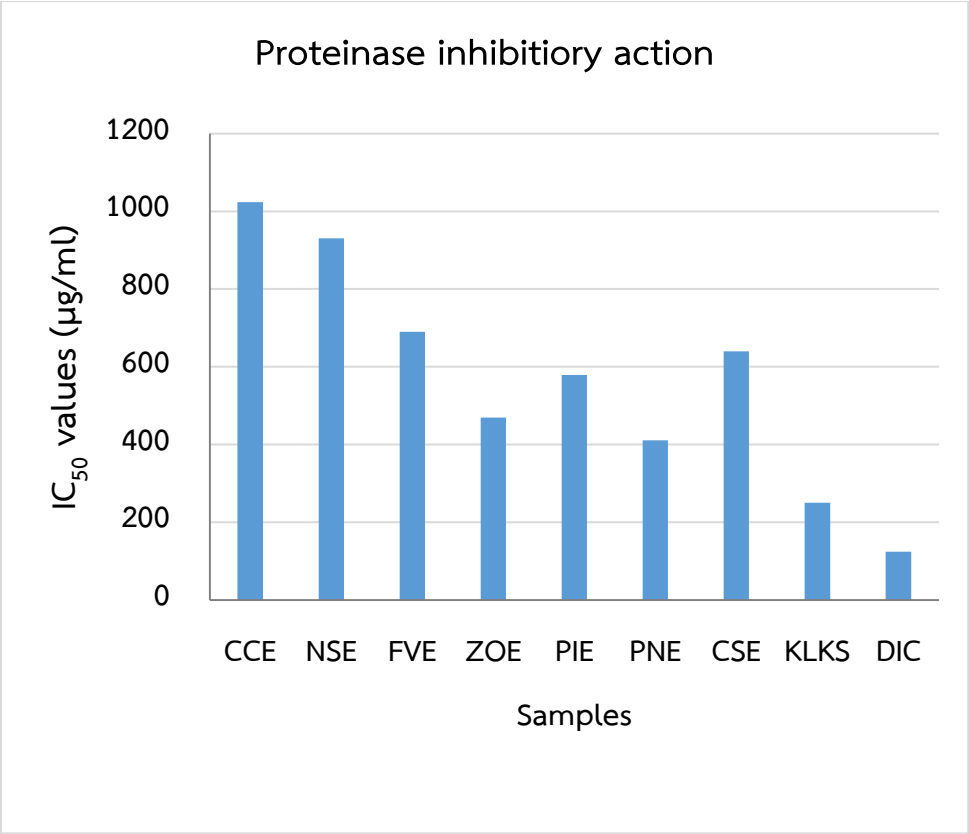


Figure 46 Effect of ethanolic extracts KLKS remedy and its ingredient on proteinase inhibition;

CCE = *C. cyminum*, NSE = *N. sativa*, FVE = *F. vulgare*, ZOE = *Z. officinale*, PIE = *P. indica*, PNE = *P. nigrum*, CSE = *C. sativa*, KLKS = Kealomkeasan remedy, DIC = diclofenac sodium; results presented as the mean ± SD (n=3)

Anti-diabetic activity

The result of antidiabetic activities using yeast alpha-glucosidase and rat alpha-glucosidase inhibitory assay of the ethanolic extract of KLKS remedy and its plant ingredient were shown in **Table 32** and **Figure 47-48.** Yeast **α**-glucosidase inhibitory assay exhibited that *C. sativa* leaves and KLKS remedy ethanol extract revealed greater potential effect than an acarbose (positive control) on brewer's yeast **α**-glucosidase inhibition with IC_{50} value of 0.06 ± 0.90, 0.10 ± 1.10, and 0.19 ± 1.06 mg/ml, respectively. *F. vulgare* fruits and *Z. officinale* rhizomes ethanolic extract were showed lowest activity on **α**-glucosidase enzyme inhibitors with IC_{50} more than 1 mg/ml. For rat **α**-glucosidase inhibitory assay, the ethanolic extracts of *C. sativa* leaves showed the highest rat **α**-glucosidase inhibitory activity with IC_{50} value of 1.05 mg/ml, *F. vulgare* fruit ethanolic extract exhibited the lowest activity on rat-glucosidase inhibition with more than 5 mg/ml. While positive control, acarbose showed IC_{50} 0.35 ± 0.68 mg/ml. Positive control acarbose show in **Figure 49.**

Table 32 Anti-diabetic activities of KLKS remedy and its plant ingredient, standard compounds and positive control

Samples test	**IC_{50} (mg/ml)**	
	Yeast α-glucosidase inhibitory	**rat α-glucosidase inhibitory**
C. cyminum fruits	0.72 ± 1.10	2.04 ± 0.57
N. sativa seeds	0.78 ± 0.74	2.88 ±0.82
F. vulgare fruits	> 1	> 5
Z. officinale rhizomes	> 1	5 ± 0.95
P. indica roots	0.62 ± 1.02	3.44 ± 0.59
C. sativa leaves	0.06 ± 0.90	1.05 ± 0.64
P. nigrum fruits	0.38 ± 1.24	2.32 ± 0.44
KLKS remedy	0.10 ± 1.10	1.29 ± 0.53
Acarbose	0.19 ± 1.06	0.35 ± 0.68

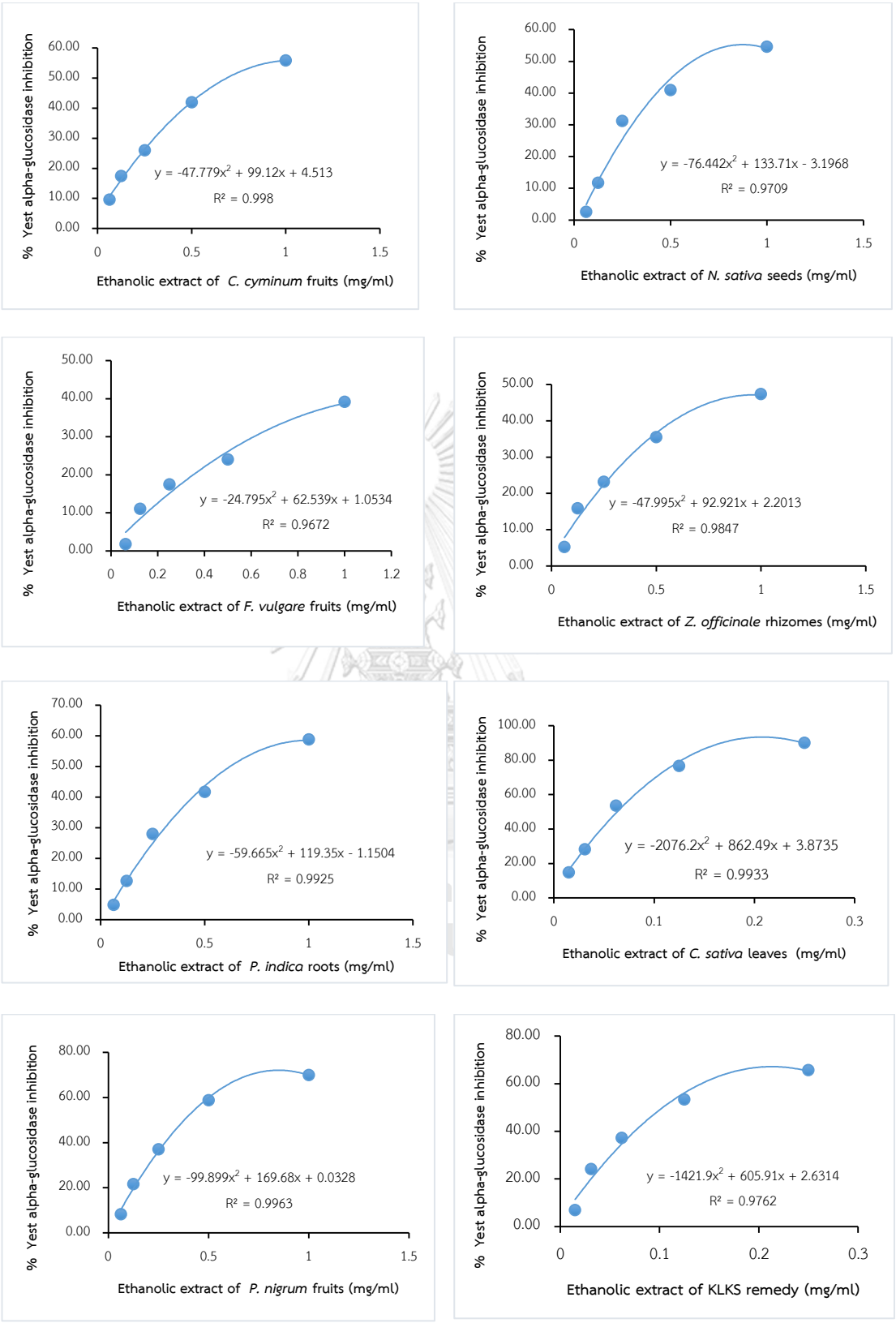


Figure 47 Yeast intestinal α-glucosidase inhibition of test samples

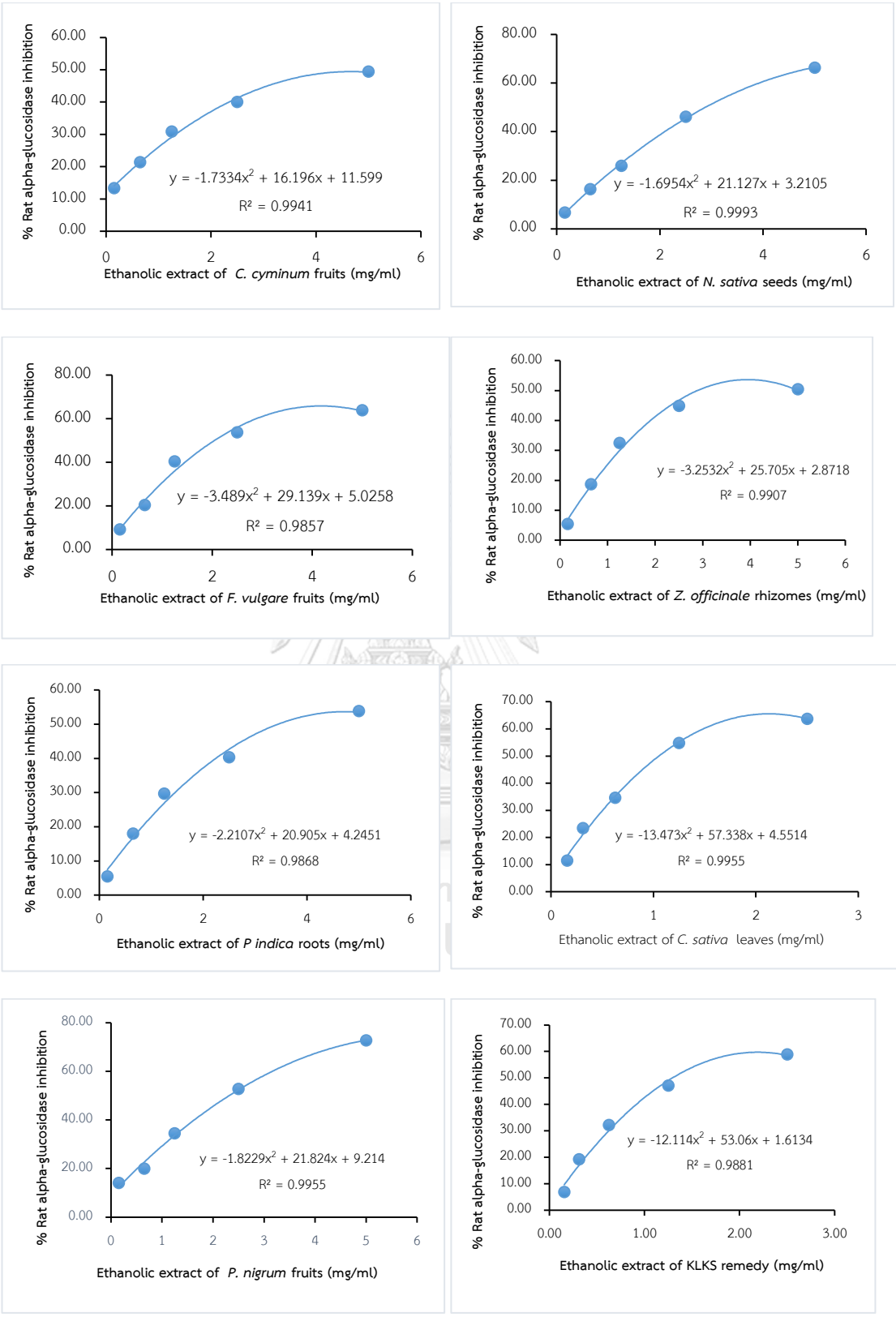


Figure 48 Rat intestinal α-glucosidase inhibition of test samples

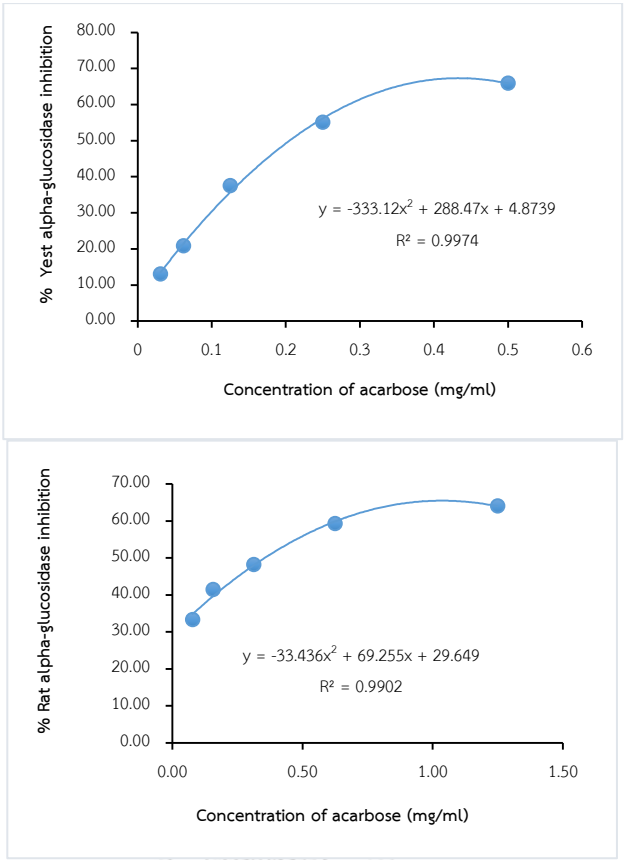


Figure 49 Yesat α-glucosidase inhibition and rat intestinal α-glucosidase inhibition of acarbose (positive control)

Toxicity activity

Brine shrimp lethality activity

The results of brine shrimp lethality testing of KLKS remedy and its plant ingredient were shown in **Table 33** and expressed as LC_{50} values: LC_{50} values > 1000 µg/ml (non-toxic), 500 - 1000 (low-toxic), 100-500 (median-toxic), and 0- 100 µg/ml (highly-toxic) (Clarkson et al.,2014), It was found that the ethanolic extract of *P. nigrum* fruits exhibited the highest toxicity against brine shrimp nauplii with LC_{50} of 150.07 whereas the ethanolic extract of *C. cyminum* fruits and *P. indica* roots ethanolic extract exhibited LC_{50} of > 1000 µg/ml

Table 33 Brine shrimp lethality (LC_{50}) of the ethanolic extract of KLKS remedy and its plant ingredient

Samples	Brine shrimp lethality LC_{50} (μg/ml)	Toxicity
C. cyminum fruits	> 1,000	non - toxic
N. sativa seeds	740.27	low-toxic
F. vulgare fruits	575.04	low-toxic
Z. officinale rhizomes	601.62	low-toxic
P. indica roots	> 1,000	non - toxic
C. sativa leaves	438.47	median-toxic
P. nigrum fruits	150.07	median-toxic
KLKS remedy	503.125	low-toxic

CHAPTER V

DISCUSSION AND CONCLUSION

Part I: Standardization of *C. sativa* leaves

Presently, standardization of medicinal plants is necessary for quality control evaluation. According to WHO, investigation of the macroscopic and microscopic characteristics are the starting step towards identification and determination of the purity (WHO, 2011).

Microscopic characterization is a reliable tool for identification of medicinal plant, a small fragment or broke of crude drugs, for detection of adulterants, substituents, and authentic plant. Epidermal cell type and stomata type are also wildly used in identification at genus and species levels. The powdered drug was assessed for its structural cell and physicochemical analysis are beneficial investigating the quality and purity of crude drugs. (Adom et al., 2022). Loss on drying measures the amount of water and volatile oil containing in the plant material. The water content of the crud drugs should be minimized (10-20%) which is an ideal range for minimum fungal and bacterial growth (Chumbhale and Upasani, 2012). For *C. sativa* powdered leaves was showed to be 4.07 ± 0.09 %w/w of loss on drying. The result of water content should not be higher than 7.95 ± 0.12 %w/w. Total ash values of crud drug give concept of inorganic matter and other impurities; phosphates, carbonates, silica and silicates (Upadhyaym et al., 2019; Pandiyan et al., 2022). Ash values of samples were found to be 14.36 ± 0.17 %w/w, while acid insoluble ash was found to be 2.73 ± 0.09 % w/w.

The result of water extractive value was higher than the ethanol extractive values, exhibit the present of water-soluble compounds contained in plant materials and their different solubility property in different solvents (Hanani et al., 2019). In

study, the volatile oil content was not detected from dry leave powder with hydro distillation method when compared to previous studied using fresh leaves, this may be happened due to the volatility of essential oil component which may be decreased upon the drying processing. Based on the taxonomic approach, plant in the same genus will contain the same chemical compounds. However, the yield and quality of essential oil are affected by a variety of environmental factors, including geographical cultivation, climatic conditions, and the extraction process (Naz et al., 2017). The phytochemical screening is a qualitative determination the class of compounds contained in the plant. Phenolics, flavonoids, steroids, triterpenoids, diterpenes, and alkaloids were presented in *C. sativa* leaves which may be responsible for some medicinal properties. However, saponins was undetectable in the crude ethanolic extract of *C. sativa* leaves. The findings in accordance with the previous study of *C. sativa* leaves cultivars from India (Kumari et al., 2017; Ahmed et al., 2019). Phytochemical determinations are helpful in quality assessed and drug discovery. In this study, present work may be successfully used for quality evaluation and could be applied as a quality control standard reference, monograph preparation, and assurance of crude drug.

Part II: DNA barcode

Recently, DNA barcoding is also used to establish species identification and taxonomic classification. This approach was successfully in medicinal plant using one or a few standard loci that can be sequenced and compared from diverse medicinal plants due to its accuracy, repeatability and rapidly. Sometime multiple markers will be required for species discrimination. DNA barcoding is particularly powerful tool for identification as it is not influenced by the morphological diversity of species, growth phases, and environmental factors (Cahyaningsih et al., 2022) unlike chemical composition. However, some previous report recommended cannabinoids and

terpenoids profile for cannabis varieties classification (Hazekamp et al., 2016; Piomelli and Russo, 2016).

However, a molecular system based on DNA barcoding could represent a cost- and time-effective technique of great help in clarifying some of the taxonomic issues related to the genus Cannabis. DNA barcoding could also play a crucial role in the identification and characterization of those uncertified cannabis strains, which are mainly derived from black market. (Barcaccia et al., 2020). According to Kress and Erickson (2007), demonstrated the suitability of the *rbc*L gene and *trn*H-*psb*A noncoding spacer region as DNA barcodes for plant classification. The *mat*K gene was also included in the list of exploitable markers for DNA barcoding in land plants (Asahina et al., 2010; de Vere et al., 2015). Not only plastid markers but also nuclear markers were also widely used in DNA barcoding (Chen et al., 2010; Wang et al., 2014).

Due to the highly conserve of *rbc*L and *mat*K sequence of *C. sativa* in this study, these regions could not be suitable for identification. Among these three regions, ITS was be selected for barcoding analysis. Using ITS region, especially ITS1, as a DNA barcoding, *C. sativa* collected from different areas of Thailand can be classified into 5 groups according to the mutations and deletion position in this ITS region. The phylogenetic tree of ITS region was also constructed, and the genetic distance were examined using maximum likelihood method. The obtained phylogenetic tree also similar with the DNA barcoding. The ITS region is the most widely sequenced DNA region in molecular ecology of plants and has been recommended among the universal barcode sequences (Kress et al., 2006; Yao et al., 2010). It has typically been most useful for molecular systematics at the species level, and even within species (e.g., to identify geographic races, varieties and ancestoral types). The standard ITS5 and ITS4 primers are used by most labs (White et al., 1990; Lightfoot et al., 2016). Currently, Thailand has legalized only the use of cannabis for medicinal and research

purposes. This also brings to increase in scientific studies on various aspects of cannabis in Thailand.

Part III: Biological activity

Kealomkeasan (KLKS) remedy had been used for relieving pain and analgesic in masculosckeletal system in Thai traditional medicine. Thai National list of essential herbal medicines also specific KLKS as a remedy for relieve pain and analgesic in masculosckeletal system. It consists of seven plants with an unequal part by weight as follows: *C. cyminum* fruits, *N. sativa* seeds, *F. vulgare* fruits, *Z. officinale* rhizomes, *P. indica* roots, *C. sativa* leaves and *P. nigrum* seeds with ratio of 1:2:3:4:5:20:40 g. This remedy is no previous report on biological activity of KLKS extract. Its effectiveness has never been determined by scientific method which is the most important for authorized safety and efficacy in clinical use according to Thai traditional medicine. Therefore, this study aimed to investigate necessary procedures for quality control of plant materials*, in vitro* antioxidant activity was assessed by five related assays including inhibitory effect on (2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2 - azinobis (3-ethylbenzthiazolinesulfonic acid) diammonium salt (ABTS), ferric reducing antioxidant power (FRAP), total phenolic content and total flavonoids content, anti-inflammatory assay by inhibition on Lipoxygenase, protein denaturature and inhibition of proteinase, anti-diabetic assay by inhibition yeast alpha glycosidase and rat alpha glycosidase and *in vitro* toxicity assay by brine shrimp lethality of KLKS extract.

KLKS and its ingredient plant extracts were done by polar aprotic (95% ethanol) solvents with maceration methods. The percentage of yields are shown as percentage weight for weight. For KLKS ethanolic extract was showed 10.47%. Each of the ingredient plants ethanolic extract, *N. sativa* seeds showed highest percentage of yield (23.25%) followed by the *P. indica* roots (20.68%) and *C. cyminum* fruits (15.56%). The

three least percentage of yields are KLKS remedy (10.47%), *P. nigrum* fruits (9.99%) and *F. vulgare* fruits (7.20%), respectively.

Antioxidant activity

It has been known that overproduction of free radicals, such as reactive oxygen species (ROS), plays an important part in the development of many chronic diseases (Mbah et al., 2019; Poprac et al., 2017). Such diseases including rheumatoid arthritis, cardiovascular disease, diabetes, neurodegenerative diseases, and cancer (Mbah et al., 2019). It has been reported that a variety of natural products possess antioxidant potential, such as cereal grains, vegetables, edible flowers, fruits, herbal infusions, and medicinal plants (Mao et al., 2019).

The DPPH assay for quantification of radical-scavenging activity in vitro is extensively used worldwide (Li et al., 2016). This method is rapid, simple, and inexpensive method. DPPH• is characterized as a stable free radical because of the delocalization of the spare electron over the whole molecule. To measure the antioxidant property of compounds in reference to their ability to scavenge DPPH. When DPPH solution is mixed with a solution of the substance (antioxidant) that can donate an electron, the reduced form of DPPH or the non-radical 1,1 diphenyl 2 picryhydrazine (DPPH-H) is then generated with the loss of the violet color to pale yellow in absorbance at 517 nm. In this study, the ethanolic extract of *P. nigrum* fruits demonstrated the highest radical scavenging activity with IC_{50} values of 75.92 µg/ml, it was exhibited strong total antioxidant activity, higher than previous studied (243.15 µg/ml) by Akbar et al (2014), followed by ethanolic extract of *Z. officinale* rhizomes was found the IC_{50} of 80.41 µg/ml higher than previously reported (IC_{50} 28.6 µg/ml) (Avci et al., 2020). The ethanolic extract of KLKS remedy possessed a potent inhibition effect on DPPH scavenging with IC_{50} value 108.52 µg/ml. However, KLKS exhibited less antioxidant activity than Quercetin (IC_{50} value 16.43 µg/ml). Quercetin is the most abundant flavonol which exhibited a good antioxidant property.

For ABTS radical cation decolorization assay, uses a diode-array spectrophotometer to measure the loss of color when an antioxidant is added to the blue–green chromophore ABTS•+. The antioxidant reduces ABTS•+ to ABTS and decolorize it. ABTS•+ is a stable radical not found in the human body (Alam et al., 2013). Investigation of the inhibitory effect on ABTS scavenging among plant ingredients showed that the most extracts possessed antioxidant activity.

The ethanolic extract of *Z. officinale* rhizomes showed the most effective on ABTS scavenging effect (IC_{50} value 93.90 ± 0.71 µg/ml), higher than previously reported of the *Z. officinale* rhizomes petroleum ether extract (250.33 ± 13.6 µg/ml) and chloroform: methanol extracts (IC_{50} value 334.86 ± 6.97 µg /mL) (Ali et al., 2020), while methanol and water extract showed IC_{50} value 6.85 ± 0.25 and 145.90 ± 0.33 respectively (Mapekaet al., 2020). The high scavenging activity of ginger rhizome against ABTS and DPPH radicals, was due to the presence polyphenolic compounds, including gingerols, shogaols, paradols and gingerdions (Mošovská et al., 2015). KLKS remedy extract showed the IC_{50} value of 223.76 µg/ml, whereas the ethanolic extract of *N. sativa* seed had the lowest radical scavenging activity (IC_{50} value of 378.34 µg/ml).

This study used the DPPH and $ABTS^{+}$ assays to evaluate antioxidant activity of our extracts. Although they share the same mechanism of antioxidant action i.e. electron transfer, DPPH radicals are more suitable for lipophilic antioxidants while ABTS+ radicals can react with both hydrophilic and lipophilic antioxidants.

The ferric reducing power (FRAP) assay represents a non-radical redox potential-based method. The reducing power of *Z. officinale* rhizomes was shown at a level of 0.718 ± 0.05 mMFe (II)/g extract, which was higher than the reducing power of KLKS and other its ingredients, it was showed at a level similar conducted by Sunthrarak, and Oonsrivilai (2022) which indicated 0.714 ± 0.01 mmol Fe (II) Equiv./g extract.

Phenolic and flavonoids are from nature are secondary plant metabolites that important antioxidants and make up a large percentage of plants. As antioxidant potential depends upon the presence of phenolic and flavonoids compounds. Phenolic compounds may directly contribute to antioxidant activity, stimulating the synthesis of endogenous oxidant molecules in the cell. Several reports exhibit that phenolic compounds could inhibit free radicals, prevent oxidative diseases and decompose peroxides (Phong et al., 2022). The total phenolic content was measured using the colorimetric assay of Folin-Ciocalture, which is a rapid, simple, low-cost procedure and reproducible (Derouich et al., 2020). According to published research by Marja et al., (1999), plants with polyphenol content greater than 20 mg GAE/g have strong antioxidant activity.

The analysis results showed that the total phenolic content of the ethanolic extract of *P. nigrum* showed the highest total phenolic content (41.31± 0.80 mg GAE/g extract), followed by *Z. officinale* rhizomes (36.78± 0.76 mg GAE/g extract), but lower than reported by Erdoğan and Erbas (2021) (48.56 ± 1.64mg GAE/g extract)

In contrast, *C. cyminum* fruits ethanolic extract was showed lowest of the total phenolic content 6.24mg GAE/g extract but higher than previously reported (3.70 ± 0.25 mg GAE/g extract) ,which was extracted by maceration in 80% methanol (Demir and Korukluoglu, 2020). According to El-Serehy et al., (2016) reported the total phenolic content of *C. cyminum* fruits aqueous extract was indicated 22.08 ± 0.72 µg/mg gallic acid equivalent of phenols/mg dried extract.

Flavonoids possess a numeral of medicinal benefits, including antioxidant, anti-inflammatory, anticancer, and antiviral properties (Ullah et al., 2022). Total flavonoids content was measured by a mixture of reactants including aluminium chloride, which bind to flavonoids thus from aluminium-flavonoid complexes having maximum

turbidity at 415- 430 nm (Derouich et al., 2020). In this study, total flavonoids content in all analyzed plants extracts varied largely and ranged from 8.89 to 23.88 mgQE/g extract. *C. sativa* leaves was found highest flavonoids level (23.88±0.40 mg QE/g extract) and higher than study published in 2019 by Ojezele et al. (12.66±2.31 mg QE/g extract), but lower than reported by Ahmed (56.001±1.85mg QE/g). These differences can be explained by plant genotype, solvent, extraction procedure and environmental conditions (Derouich et al., 2020).*N. sativa* seeds ethanolic extract was indicated showed the lowest flavonoid content (8.89 ± 0.45mgQE/g extract), while *N. sativa* oil seed using n-hexane extract by soxhlet extraction was revealed 8.94 ± 0.45 mgQE/100g extract) (Kadam and Lele, 2017).

For all antioxidant activity, KLKS remedy ethanol extract was revealed low antioxidant when compare with positive control, this may be due to antagonistic effects between its ingredient (seven herbs) and other phytochemicals, such as flavonoids and terpenoids in the crude ethanol extracts.

Anti-inflammatory activity

This study investigated the inhibitory effected of KLKS remedy and its ingredient on lipoxygenase (LOXs), protein denaturation and proteinase. LOXs are key enzymes involved in the regulation of inflammatory responses by the generation of pro-inflammatory mediators known as leukotrienes or anti-inflammatory mediators known as lipoxins (Wisastra and Dekker, 2014). In general, lipoxygenases are classified as 5–, 8–, 12–, or 15-lipoxygenases according to their selectivity in oxygenating fatty acids in a specific position (Brash AR, 1999). 5-LOX and 15-LOX are involved in the synthesis of lipoxins (LXs), specifically lipoxin A4 (LXA4) (Shahid et al., 2021). Soybean 15-LOX is the most easily accessible than human 5-LOX and therefore it was used for *in vitro* studies so that its results could be generalized and extendable to human 5-LOX

(Shahid et al., 2021). The IC_{50} values of KLKS remedy and its ingredient extracts ranged from 141.38 to ≥ 505.59 µg/ml. KLKS remedy and *P. nigrum* fruits ethanolic crude extracts had very good activity against the 15-lipoxygenase enzyme, with IC_{50} values of 86.37 ±0.57 and 145.89 ± 0.46 µg/ml respectively. Indomethacin showed IC_{50} values of 57.64 µg/ml, it closely with previous study by Kumaraswamy and Satish (2008) and Truong et al., (2021). The standard drug indomethacin and diclofenac were inhibited inflammation. Indomethacin is nonsteroidal anti-inflammatory drug (NSAID) that has anti-inflammatory, analgesic, and antipyretic properties, and has been shown to be effective in treating a variety of acute and chronic pain and inflammatory conditions. As with all NSAIDs exerts its action via inhibition of prostaglandin synthesis by inhibiting cyclooxygenase-1 (COX-1) and cyclooxygenase- 2 (COX-2) with relative equipotency, which can inhibit the thromboxane-prostanoid receptor, affect arachidonic acid release and uptake, and inhibit lipoxygenase enzymes (Gan, 2010). However, KLKS remedy had a maximum inhibition of lipoxygenase and the highest protein denaturation inhibition. *P. nigrum* is major ingredient of KLKS remedy, it contains an alkaloid piperine which is known to possess many pharmacological actions and anti-inflammation (Damanhouri, 2014). Tasleem et al., (2014) reported that the anti-inflammatory activity of *P. nigrum* and its active compounds based on carrageenan-induced paw edema using a plethysmometer; piperine indicated an anti-inflammatory effect at doses of 10 and 15 mg/kg after 30 min, which lasted for 60 min, while ethanolic and hexane fruit extracts also had activity at a lower dose of 10 mg/kg which lasted for 120 min, and Pei et al., (2020) reports study alkaloids from *P. nigrum* that exhibit anti-inflammatory activity in murine macrophages by inhibiting activation of the NF-kB path- way. The *Z. officinale* rhizomes ethanolic extract (223.20 ±0.98 µg/ml) in this study was found to be lower than previously reported which the aqueous extract strongest anti-lipoxygenase with 58%, while positive control diclofenac showed 52% at the same concentration125 µg/ml (Ezzat et al., 2018).

For *C. sativa* leaves extract, results are in line with previous reports (Rastogi et al., 2018), the ethanol can extract flavonoid from *C. sativa* leaves, that flavonoids have anti-inflammatory activity (Lazarjaniet al., 2021; Akimat, 2021)

The Protein denaturation is one of the causes of rheumatoid arthritis. This study for determination anti-arthritis activity was performed using bovine serum albumin (BSA). When BSA is heated, it undergoes denaturation and antigens are expressed which are associated with type-III hypersensitivity reaction, which in turn is related to diseases such as serum sickness, rheumatoid arthritis, glomerulonephritis, and systemic lupus erythematosus (Gunathilake et al., 2018). The mechanism of denaturation probably involves alteration of electrostatic, hydrogen, hydrophobic and disulphide bonds (Arya et al., 2014). One of the main mechanisms of action of NSAIDs is the protection against protein denaturation as mentioned by Mizushima (1964). Inhibition of protein denaturation may play an important role in the antirheumatic activity of NSAIDs (Umapathy et al., 2010). All the extracts had a dose-dependent response in the in *vitro* anti-arthritic test. The IC_{50} ranged from 414.53 to 821.21 µg/ml. KLKS remedy, *C. sativa* and *P. nigrum* had good anti-denaturation activity, with IC_{50} values of 214.53, 339.72 and 401.14 µg/ml, respectively. The positive control, diclofenac sodium (IC_{50} 94.86 µg/ml). The promising activities of the extracts support the traditional claims of use as remedies for arthritis, rheumatism and other chronic inflammatory conditions (Elisha et al., 2016). Previous study reported of *C. sativa* leaves methanolic and aqueous extracts showed good potential protein (albumin) denaturation higher than positive control (aspirin) at 100 µg/ml. (Rastogi et al., 2018). The ethanolic extract *Z. officinale* also good effect protein denaturation inhibition (Ezzat et al., 2018).

The present results revealed that KLKS remedy on lipoxygenase inhibition and protein denaturation were showed higher than its ingredient and positive control.

These types of pharmaceutical actions, derived from the combination of multiple herbs in a formula, are called ‘chemical combination effects’ that the pharmacological due to synergistic effect of multi-herbal formulas.

Leukocyte proteinases cause tissue damage when secreted in excessive amounts; therefor inhibition of proteinases can provide significant protection against harmful inflammatory response. (Akimat et al., 2021). It was previously reported that leukocytes proteinase play important role in the development of tissue damage during in inflammatory reactions and significant level of protection was provided by Proteinase inhibitors (Thakur et al., 2013). The anti-inflammatory impact of KLKS remedy and its ingredient extract was test *in vitro* against the proteinase inhibition of trypsin in current study. The IC_{50} values of KLKS remedy and its ingredient extracts ranged from 250.90 to more than 1,000 µg/ml, whereas positive control (Diclofenac sodium) showed IC_{50} values 124.90 µg/ml. All extracts were exhibited low efficacy against trypsin inhibitory activity when compared with lipoxygenase and protein denaturation inhibition activity. This means that the potency of the extract might be through the alteration of other pathways of inflammation.

In conclusion, the present study indicates that KLKS remedy possesses high anti-inflammation potential by anti-lipoxygenase and protein denaturation activities. Our finding supports the ethnomedicinal use of KLKS remedy in the management of anti-inflammation activity. However, the further investigation is needed to detect the specific active compound in the plant extract and remedy that are responsible for these biological and their bioavailability and efficacy in vivo test.

Anti-diabetic activity

The objective of this present study is to examine inhibitory effected of the ethanolic extract of KLKS remedy and its plant ingredient, which would be applied to treat postprandial hyperglycemia, especially in patients with type 2 diabetes or impaired glucose tolerance. The enzyme alpha-glucosidase lies in the mucosal border of the small intestine and helps in catalysis of the last step of digestion of starch and disaccharides that are abundant in human diet. α-glucosidase speeds up conversion of the disaccharides to monosaccharaides, which leads to increase in glucose levels especially postprandial hyperglycemia.

Alpha-glucosidase are enzyme in the digestive tract that hydrolyte carbohydrate into glucose. Acarbose, voglibose and miglitol are currently commercialized anti-glucosidase drugs again type 2 diabetes (Assefa et al., 2020). In this study, the highest inhibitory activity on yeast and rat intestinal α-glucosidase were found in *C. sativa* leaves ethanolic extract. It was found that *C. sativa* leaves ethanolic extract demonstrated on yeast α-glucosidase with IC_{50} of 0.06 ± 0.90 mg/ml. This result showed that lower than previous study by Suttithumsatida et al., (2022); showed the strongest inhibitory activity on yeast α–glucosidase *(Saccharomyces cerevisiae)* of *C. sativa* leaves ethanolic extract by a microwave extraction showed IC_{50} value of 1.23 µg/ml, while THC showed IC_{50} value of 3.0 µg/ml and CBD 5.5 µg/ml, which were all markedly stronger than the standard drug, acarbose (IC_{50} of 488.6 µg/ml). This finding the *C. sativa* leaves extracts have greater potential to relieve diabetes. The phytochemical composition cannabis leaves has been reported contained lower levels of approximately 1-2 % cannabinoids, 0.1–0.2% terpenoids, but higher level of 0.3–0.4% flavonoids, while cannabis inflorescences contained cannabinoids (15–20%), terpenoids (1–2%), and flavonoids (0.1%) (Jin et al., 2020). This may be due to synergistic effects between the cannabinoids or entourage effects with the other

phytochemicals, such as flavonoids and terpenoids in the crude ethanol extracts. Proença et al. (2017), reported the most abundance of flavonoids in cannabis were quercetin, kaempferol, luteolin and apigenin, which have been indicated to possess good α-glucosidase inhibitor activity, with the IC_{50} values of 15, 32, 46, and 82 μM, respectively. In summary, *C. sativa* leaf with reference to flavonoid demonstrated in vitro inhibitory potential on starch digestive enzymes. Flavonoids exert their α-glucosidase inhibitory activities by forming complexes with enzymes through non-covalent interactions. Furthermore, KLKS remedy is also good effect inhibition on α-glucosidase was found IC_{50} 0.10 ± 1.10 mg/ml, while acarbose showed IC_{50} of 0.19± 1.06 mg/ml. The *Z. officinale* rhizome extract has been reported to be effective of hyperglycemia (IC_{50} 1129.72 μg/ml) (Adeyeoluwa et al., 2020). However, *P. nigrum* fruit was found to be lowest on yeast α-glucosidase inhibitory effect in this our study, with correlated reported by Magana-Barajasa et al., (2021)

For rat alpha glucosidae, all extracts revealed the potential effect on rat alpha glucosidae inhibition less effect that less effective than acarbose (positive control). The IC_{50} values resulting from KLKS remedy and its ingredient extract range from 1.05 mg/ml to more than 5 mg/ml, the highest inhibitory activity on rat intestinal α-glucosidase showed IC_{50} 1.05 mg/ml of *C. sativa* leaves extract, whereas acarbose showed IC_{50} values 0.35 mg/ml. It would appear that inhibition of yeast α-glucosidase does not translate into comparable inhibition of the mammalian version of the enzyme. This is supported by the finding that plant extracts that exerted inhibition on yeast enzyme failed to inhibit mammalian enzyme from rat intestinal acetone powders. These findings are in agreement with a study conducted by Shai et al., (2011), who observed that crude methanol extracts of several plants exerted superior inhibition on yeast α-glucosidase than the mammalian equivalent.

Brine shrimp lethality activity (BSLA)

Brine shrimp lethality assay is a preliminary assessment of toxicity of the plant extracts. This assay is very useful, simplicity, rapidness, low requirements are several. The acute toxicity of herbal extracts using this assay has been determined in a concentration range of 10 - 1000 µg/ml of the examined herbal extract. BSLA examine the toxicity after 24 hours of exposure to the tested sample (Hamidi et al., 2014). The BSLA result was measured using the median lethal concentration (LC_{50}) value that is good correlation with the in vivo test. For the result, KLKS remedy extract was revealed that low toxicity to *A. salina*, and the toxicity may be affected from its ingredient; *P. nigrum* and *C. sativa.* These results were in accordance with previous study of Aye et al. (2018), with also found that ethanolic extract of *P. nigrum* fruits showed cytotoxicity with LD_{50} 90.4 µg/ml. In contrary with Chunlaratthanaphorn et al., (2007) who has reported the different result in animal model that the water extract from dried fruits of *P. nigrum* showed does not cause acute or sub chronic toxicities in either male or female rats. According to Paarakh et al., (2015) study vitro cytotoxic and in silico activity of piperine isolated from *P. nigrum* fruits that found the IC_{50} value of 61.94 ± 0.05 µg/ml in in vitro cytotoxic activity in HeLa Cell lines.

Whereas the ethanolic extract of *C. cyminum* fruits and *P. indica* roots showed non toxicity with LD_{50} values of more than 1,000 µg/ml, which collated that the methanolic extract study of Saha and Paul (2012). For *N. sativa* seeds, *F. vulgare* fruits and *Z. officinale* rhizomes were reveled low toxicity with LD_{50} value range 575.04 - 740.27 µg/ml in accordance with study by Sharififar et al., (2017); Tofighi et al., (2020), and Rahayu et al., (2021).

In conclusion, the result obtained from this study provides useful information and also play a significant in establishing standardization and identification *C. sativa* and preparation of a monograph for this medicinal plant. The DNA barcoding for the barcoding sequence presented in this study will be of great use for researcher in selecting correct herbal specimens. In addition, this present study projected the scientific efficacy evaluation including in *vitro* antioxidant, anti-diabetic, anti-inflammation, and toxicity activities of Kealomkeasan remedy and its ingredients.

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ประกาศกระทรวงสาธารณสุข เรื่อง กำหนดตำรับยาเสพติดให้โทษในประเภท 5 ที่มีกัญชาปรุงผสมอยู่ที่ให้เสพ เพื่อรักษาโรคหรือการศึกษาวิจัยได้ พ.ศ. 2562 ลงวันที่ 29 มีนาคม 2562. ราชกิจจานุเบกษา เล่ม136 ตอนพิเศษ 94 ง วันที่ 11 เมษายน 2562

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Appendix A

Physicochemical parameter

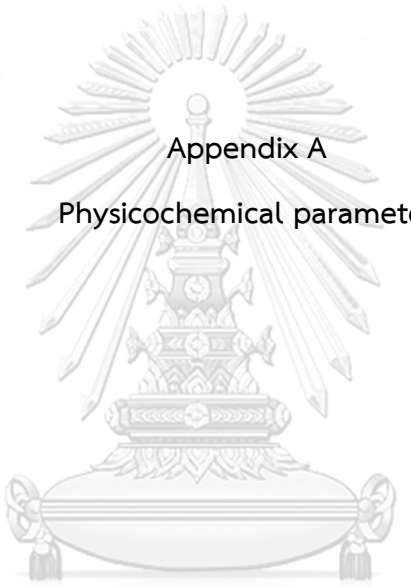


Table 34 Physicochemical values of *C. sativa* from 12 difference sources

Sources	No.	%by weight					
		Loss on drying	Water content	Total ash	Acid-insoluble ash	Ethanol extractive value	Water extractive value
SR1	1	4.485	8.000	15.054	3.815	10.641	24.677
	2	4.456	7.333	15.217	3.546	11.260	24.640
	3	4.378	8.000	15.238	3.911	10.830	24.050
SK1	1	4.096	6.667	13.407	2.249	8.968	22.817
	2	4.193	9.000	13.398	2.690	10.795	23.771
	3	4.224	10.333	13.439	2.165	8.829	23.800
MD	1	3.760	7.333	15.468	3.370	10.608	22.013
	2	3.991	9.333	15.698	3.432	10.707	22.362
	3	4.029	10.333	15.636	3.426	10.459	22.143
PR	1	4.078	6.667	14.027	3.342	6.785	22.071
	2	4.086	9.000	15.217	2.864	8.608	22.233
	3	4.256	7.667	15.461	3.409	9.729	22.038
SK2	1	4.246	9.333	13.153	2.289	8.638	22.119
	2	4.285	7.000	13.989	2.499	8.910	22.096
	3	4.240	7.667	13.853	2.493	10.289	22.816
SK3	1	4.201	7.000	14.337	3.755	7.673	22.561
	2	4.173	8.667	14.469	3.012	8.420	23.957
	3	4.051	8.000	14.291	3.905	8.876	23.022
CM1	1	4.311	7.667	14.040	3.152	10.429	22.757
	2	4.135	8.333	14.160	3.394	10.474	22.351
	3	4.392	8.333	14.205	3.466	6.949	22.641

Table 33 Physicochemical values of *C. sativa* from 12 difference sources (Cont.)

Sources	No.	%by weight					
		Loss on drying	Water content	Total ash	Acid-insoluble ash	Ethanol extractive value	Water extractive value
CM2	1	3.063	6.667	15.862	3.077	12.190	22.994
	2	3.467	10.000	15.849	3.047	11.603	22.238
	3	3.307	8.333	15.781	2.847	10.942	22.039
LP	1	2.802	8.333	15.517	2.868	11.056	24.498
	2	2.981	7.000	15.253	3.752	10.058	24.649
	3	3.266	8.333	15.681	2.780	11.675	24.390
SR2	1	3.086	6.667	15.272	3.910	11.372	22.362
	2	3.123	10.000	15.356	2.909	11.464	23.195
	3	3.108	8.333	14.919	2.761	12.302	22.366
CM3	1	3.378	7.000	14.773	3.728	7.845	24.336
	2	3.136	8.333	14.679	3.829	7.619	24.736
	3	3.630	8.000	14.643	3.943	8.527	24.516
NR	1	3.422	7.333	15.848	2.310	12.045	22.374
	2	3.391	6.000	15.384	2.225	10.574	22.634
	3	3.334	9.333	14.487	2.398	12.570	22.823

Appendix B

Antioxidant activities

Table 35 The percentage of DPPH scavenging activity of quercetin (positive control)

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
7.812	21.24 ± 0.65
15.625	27.80 ± 0.79
31.25	36.92 ± 0.65
62.5	54.53 ± 0.63
125	84.88 ± 0.49

Table 36 The percentage of DPPH scavenging activity of *C. cyminum* fruits extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
10	11.79 ± 0.76
50	28.00 ± 0.97
100	39.84 ± 0.59
150	55.36 ± 0.59
200	67.54 ± 0.29

Table 37 The percentage of DPPH scavenging activity of *N. sativa* seeds extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
100	9.80 ± 1.25
150	23.82 ± 0.44
200	36.73 ± 0.59
250	50.27 ± 0.92
300	60.31 ± 0.73

Table 38 The percentage of DPPH scavenging activity of *F. vulgare* fruits extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
10	11.79 ± 0.76
50	28.00 ± 0.97
100	39.84 ± 0.59
150	55.36 ± 0.59
200	67.54 ± 0.29

Table 39 The percentage of DPPH scavenging activity of *Z. officinale* rhizomes extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
10	17.81 ± 0.20
25	25.13 ± 0.35
50	37.76 ± 0.26
100	62.37 ± 0.31
150	77.67 ± 1.87

Table 40 The percentage of DPPH scavenging activity of *P. indica* roots extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
20	8.23 ± 0.39
40	14.99 ± 0.54
60	28.77 ± 0.31
80	37.81 ± 0.23
100	51.50 ± 0.54

Table 41 The percentage of DPPH scavenging activity of *C. sativa* leaves extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
10	15.20 ± 0.15
50	31.30 ± 0.08
100	48.10 ± 0.38
150	63.70 ± 0.08
200	75.60 ± 0.16

Table 42 The percentage of DPPH scavenging activity of *P. nigrum* fruits extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
20	30.83 ± 0.21
40	38.26 ± 1.76
60	41.83 ± 0.21
80	51.81 ± 1.19
100	63.33 ± 1.36

Table 43 The percentage of DPPH scavenging activity of KLKS remedy extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
10	23.20 ± 0.42
50	35.22 ± 1.18
100	50.96 ± 0.62
150	61.05 ± 0.13
200	71.32 ± 0.70

Table 44 The percentage of ABTS scavenging activity of Trolox (positive control)

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
3.125	23.10 ± 0.44
6.25	28.20 ± 1.35
12.5	36.71 ± 0.65
25	54.43 ± 0.25
50	78.88 ± 2.14

Table 45 The percentage of ABTS scavenging activity of *C. cyminum* fruits extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
31.25	4.64 ± 0.31
62.5	12.61 ± 0.41
125	22.52 ± 0.41
250	40.60 ± 0.59
500	57.86 ± 0.09

Table 46 The percentage of ABTS scavenging activity of *N. sativa* seeds extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
31.25	13.38 ± 0.03
62.5	23.10 ± 0.78
125	29.95 ± 0.46
250	43.30 ± 0.75
500	59.08 ± 0.30

Table 47 The percentage of ABTS scavenging activity of *F. vulgare* fruits extract

Concentration (µg/ml)	Percentage of inhibition (mean ± SD, n=3)
31.25	2.43 ± 0.23
62.5	7.43 ± 0.68
125	20.36 ± 1.10
250	33.33 ± 0.34
500	56.26 ± 0.55

Table 48 The percentage of ABTS scavenging activity of *Z. officinale* rhizomes extract

Concentration (µg/ml)	Percentage of inhibition (mean ± SD, n=3)
31.25	25.11 ± 0.54
62.5	32.92 ± 1.45
125	42.67 ± 1.59
250	55.85 ± 0.35
500	79.26 ± 1.00

Table 49 The percentage of ABTS scavenging activity of *P. indica* roots extract

Concentration (µg/ml)	Percentage of inhibition (mean ± SD, n=3)
31.25	14.70 ± 1.33
62.5	19.48 ± 0.65
125	37.87 ± 0.58
250	50.89 ± 0.45
500	68.31 ± 0.45

Table 50 The percentage of ABTS scavenging activity of *C. sativa* leaves extract

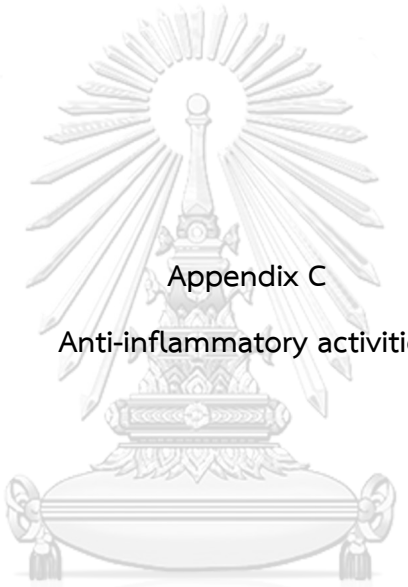
Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
31.25	20.11 ± 0.08
62.5	20.90 ± 0.38
125	37.59 ± 0.90
250	55.12 ± 0.38
500	65.99 ± 0.25

Table 51 The percentage of ABTS scavenging activity of *P. nigrum* fruits extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
31.25	35.18 ± 0.54
62.5	39.92 ± 0.88
125	49.96 ± 0.45
250	66.69 ± 0.32
500	82.06 ± 0.27

Table 52 The percentage of ABTS scavenging activity of KLKS remedy extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
31.25	42.14 ± 0.24
62.5	46.14 ± 0.86
125	55.37 ± 0.82
250	71.28 ± 0.41
500	90.24 ± 0.52



Appendix C

Anti-inflammatory activities

Table 53 The percentage of lipoxygenase inhibition of Indomethacin (positive control)

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
7.812	21.24 ± 0.65
15.625	27.80 ± 0.79
31.25	36.92 ± 0.65
62.5	54.53 ± 0.63
125	84.88 ± 0.49

Table 54 The percentage of lipoxygenase inhibition of *C. cyminum* fruits extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
31.25	4.64 ± 0.31
62.5	12.61 ± 0.41
125	22.52 ± 0.41
250	40.60 ± 0.59
500	57.86 ± 0.09

Table 55 The percentage of lipoxygenase inhibition of *N. sativa* seeds extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
31.25	13.38 ± 0.03
62.5	23.10 ± 0.78
125	29.95 ± 0.46
250	43.30 ± 0.75
500	59.08 ± 0.30

Table 56 The percentage of lipoxygenase inhibition of *F. vulgare* fruits extract

Concentration (μg/ml)	**Percentage of inhibition (mean ± SD, n=3)**
31.25	2.43 ± 0.23
62.5	7.43 ± 0.68
125	20.36 ± 1.10
250	33.33 ± 0.34
500	56.26 ± 0.55

Table 57 The percentage of lipoxygenase inhibition of *Z. officinale* rhizomes extract

Concentration (μg/ml)	**Percentage of inhibition (mean ± SD, n=3)**
31.25	25.11 ± 0.54
62.5	32.92 ± 1.45
125	42.67 ± 1.59
250	55.85 ± 0.35
500	79.26 ± 1.00

Table 58 The percentage of lipoxygenase inhibition of *P. indica* roots extract

Concentration (μg/ml)	**Percentage of inhibition (mean ± SD, n=3)**
31.25	14.70 ± 1.33
62.5	19.48 ± 0.65
125	37.87 ± 0.58
250	50.89 ± 0.45
500	68.31 ± 0.45

Table 59 The percentage of lipoxygenase inhibition of *C. sativa* leaves extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
31.25	20.11 ± 0.08
62.5	20.90 ± 0.38
125	37.59 ± 0.90
250	55.12 ± 0.38
500	65.99 ± 0.25

Table 60 The percentage of lipoxygenase inhibition of *P. nigrum* fruits extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
31.25	35.18 ± 0.54
62.5	39.92 ± 0.88
125	49.96 ± 0.45
250	66.69 ± 0.32
500	82.06 ± 0.27

Table 61 The percentage of lipoxygenase inhibition of KLKS remedy extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
31.25	42.14 ± 0.24
62.5	46.14 ± 0.86
125	55.37 ± 0.82
250	71.28 ± 0.41
500	90.24 ± 0.52

Table 62 The percentage of protein denaturation inhibition of Diclofenac (positive control)

Concentration (μg/ml)	**Percentage of inhibition (mean ± SD, n=3)**
62.5	5.26 ± 0.24
125	11.12 ± 1.29
250	21.26 ± 0.40
500	35.10 ± 0.98
1000	63.84 ± 0.27

Table 63 The percentage of protein denaturation inhibition *C. cyminum* fruits extract

Concentration (μg/ml)	**Percentage of inhibition (mean ± SD, n=3)**
62.5	3.29 ± 0.50
125	9.75 ± 1.32
250	21.62 ± 0.47
500	31.32 ± 1.75
1000	59.31 ± 1.07

Table 64 The percentage of protein denaturation inhibition of *N. sativa* seeds extract

Concentration (μg/ml)	**Percentage of inhibition (mean ± SD, n=3)**
62.5	23.98 ± 0.89
125	28.03 ± 0.56
250	37.47 ± 0.70
500	53.55 ± 0.60
1000	72.87 ± 0.31

Table 65 The percentage of protein denaturation inhibition of *F. vulgare* fruits extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
62.5	11.72 ± 0.53
125	17.65 ± 0.99
250	28.42 ± 0.97
500	42.61 ± 1.71
1000	58.74 ± 0.27

Table 66 The percentage of protein denaturation inhibition of *Z. officinale* rhizomes extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
62.5	31.59 ± 0.30
125	38.37 ± 0.56
250	46.26 ± 0.01
500	56.78 ± 0.24
1000	75.04 ± 0.67

Table 67 The percentage of protein denaturation inhibition of *P. indica* roots extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
62.5	20.77 ± 0.46
125	24.40 ± 0.20
250	40.47 ± 0.30
500	52.95 ± 0.26
1000	75.73 ± 0.07

Table 68 The percentage of protein denaturation inhibition of *C. sativa* leaves extract

Concentration (µg/ml)	Percentage of inhibition (mean ± SD, n=3)
62.5	27.33 ± 0.38
125	38.70 ± 0.76
250	44.59 ± 0.14
500	61.49 ± 1.82
1000	74.72 ± 0.61

Table 69 The percentage of protein denaturation inhibition of *P. nigrum* fruits extract

Concentration (µg/ml)	Percentage of inhibition (mean ± SD, n=3)
62.5	35.45 ± 0.44
125	41.93 ± 0.47
250	52.18 ± 0.33
500	62.43 ± 0.31
1000	80.19 ± 0.64

Table 70 The percentage of protein denaturation inhibition of KLKS remedy extract

Concentration (µg/ml)	Percentage of inhibition (mean ± SD, n=3)
62.5	40.01 ± 0.79
125	45.77 ± 0.07
250	56.40 ± 0.78
500	70.79 ± 0.77
1000	83.73 ± 0.36

Table 71 The percentage of proteinase inhibition of Diclofenac (positive control)

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
15.62	25.11 ± 0.75
31.25	33.16 ± 1.52
62.5	41.73 ± 1.08
125	51.77 ± 1.05
250	71.97 ± 0.48

Table 72 The percentage of proteinase inhibition *C. cyminum* fruits extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
62.5	8.29 ± 1.29
125	13.58 ± 1.62
250	21.54 ± 1.59
500	34.47 ± 1.83
1000	46.34 ± 1.76

Table 73 The percentage of proteinase inhibition of *N. sativa* seeds extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
62.5	2.82 ± 1.18
125	7.78 ± 2.06
250	16.24 ± 0.65
500	30.17 ± 1.26
1000	53.08 ± 0.51

Table 74 The percentage of proteinase inhibition of *F. vulgare* fruits extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
62.5	16.32 ± 0.39
125	22.82 ± 0.26
250	32.39 ± 1.82
500	43.68 ± 1.48
1000	62.91 ± 1.46

T**able 75** The percentage of proteinase inhibition of *Z. officinale* rhizomes extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
62.5	35.96 ± 0.84
125	40.96 ± 1.20
250	44.94 ± 0.59
500	51.47 ± 1.68
1000	764.29 ± 1.72

Table 76 The percentage of proteinase inhibition of *P. indica* roots extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
62.5	10.69 ± 1.53
125	19.00 ± 0.89
250	33.84 ± 1.53
500	49.53 ± 0.64
1000	74.25 ± 0.55

Table 77 The percentage of proteinase inhibition of *C. sativa* leaves extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
62.5	31.74 ± 1.48
125	38.77 ± 1.30
250	49.10 ± 0.52
500	59.52 ± 0.83
1000	76.61 ± 0.17

Table 78 The percentage of proteinase inhibition of *P. nigrum* fruits extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
62.5	26.92 ± 0.71
125	35.66 ± 0.68
250	46.54 ± 1.36
500	58.24 ± 0.71
1000	76.67 ± 0.61

Table 79 The percentage of proteinase inhibition of KLKS remedy extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
62.5	41.79 ± 0.66
125	43.57 ± 0.90
250	51.99 ± 1.22
500	78.56 ± 0.28
1000	83.73 ± 1.02

Appendix D

Anti-diabetic activities

Table 80 The percentage of yeast alpha-glucosidase inhibition of Acarbose

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
31.25	13.11 ± 0.96
62.5	20.90 ± 2.01
125	37.59 ± 1.16
250	55.12 ± 0.12
500	65.99 ± 1.04

Table 81 The percentage of yeast alpha-glucosidase inhibition of *C. cyminum* fruits extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
62.5	9.60 ± 0.31
125	17.49 ± 0.20
250	25.98 ± 0.94
500	42.00 ± 0.61
1000	55.89 ± 0.41

Table 82 The percentage of yeast alpha-glucosidase inhibition of *N. sativa* seeds extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
62.5	2.63 ± 1.19
125	11.80 ± 1.38
250	31.25 ± 0.39
500	40.95 ± 0.39
1000	54.62 ± 0.34

Table 83 The percentage of yeast alpha-glucosidase inhibition of *F. vulgare* fruits extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
62.5	2.63 ± 1.19
125	11.80 ± 1.38
250	31.25 ± 0.39
500	40.95 ± 0.39
1000	54.62 ± 0.34

Table 84 The percentage of yeast alpha-glucosidase inhibition of *Z. officinale* rhizomes extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
62.5	5.23 ± 1.20
125	15.86 ± 1.46
250	23.20 ± 0.54
500	35.47 ± 1.37
1000	47.34 ± 1.56

Table 85 The percentage of yeast alpha-glucosidase inhibition of *P. indica* roots extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
62.5	4.84 ± 0.41
125	12.66 ± 0.54
250	27.97 ± 1.18
500	41.72 ± 0.89
1000	58.83 ± 2.10

Table 86 The percentage of yeast alpha-glucosidase inhbition of *C. sativa* leaves extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
15.625	14.79 ± 1.76
31.25	28.17 ± 1.15
62.5	53.54 ± 0.03
125	76.68 ± 1.14
250	65.99 ± 0.43

Table 87 The percentage of yeast alpha-glucosidase inhibition of *P. nigrum* fruits extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
62.5	8.28 ± 1.43
125	21.63 ± 1.78
250	37.59 ± 0.59
500	58.83 ± 1.37
1000	70.00 ± 1.02

Table 88 The percentage of yeast alpha-glucosidase inhibition of KLKS remedy extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
15.625	6.92 ± 1.13
31.25	24.17 ± 0.65
62.5	37.34 ± 1.36
125	53.43 ± 0.62
250	65.72 ± 1.73

Table 89 The percentage of rat alpha-glucosidase inhibition of Acarbose

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
78.125	33.28 ± 0.27
156.25	41.49 ± 1.01
312.5	48.22 ± 0.23
625	59.29 ± 0.58
1250	64.09 ± 1.32

Table 90 The percentage of rat alpha-glucosidase inhibition of *C. cyminum* fruits extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
312.5	13.37 ± 0.31
625	21.40 ± 0.51
1250	30.84 ± 0.26
2500	40.06 ± 0.83
5000	49.44 ± 0.37

Table 91 The percentage of rat alpha-glucosidase inhibition of *N. sativa* seeds extract

Concentration (μg/ml)	Percentage of inhibition (mean ± SD, n=3)
312.5	6.78 ± 0.66
625	16.38 ± 0.50
1250	25.95 ± 0.84
2500	46.09 ± 1.23
5000	66.36 ± 0.86

Table 92 The percentage of rat alpha-glucosidase inhibition of *F. vulgare* fruits extract

Concentration (mg/ml)	Percentage of inhibition (mean ± SD, n=3)
0.312	9.15 ± 1.08
0.625	20.40 ± 1.17
1.25	40.41 ± 0.58
2.5	53.75 ± 0.25
5	63.84 ± 1.25

Table 93 The percentage of rat alpha-glucosidase inhibition of *Z. officinale* rhizomes extract

Concentration (mg/ml)	Percentage of inhibition (mean ± SD, n=3)
0.312	8.20 ± 0.76
0.625	18.71 ± 0.35
1.25	31.80 ± 0.85
2.5	45.39 ± 0.61
5	50.39 ± 0.97

Table 94 The percentage of rat alpha-glucosidase inhibition of *P. indica* root extract

Concentration (mg/ml)	Percentage of inhibition (mean ± SD, n=3)
0.312	7.81 ± 0.78
0.625	18.03 ± 0.40
1.25	29.73 ± 0.46
2.5	40.31 ± 0.63

5	53.90 ± 0.89

Table 95 The percentage of rat alpha-glucosidase inhibtion of *C. sativa* leaves extract

Concentration (mg/ml)	Percentage of inhibition (mean ± SD, n=3)
0.156	11.52 ± 0.89
0.312	23.48 ± 0.45
0.625	34.66 ± 1.15
1.25	54.89 ± 0.26
2.5	63.76 ± 0.45

Table 96 The percentage of rat alpha-glucosidase inhibition of *P. nigrum* fruits extract

Concentration (mg/ml)	Percentage of inhibition (mean ± SD, n=3)
0.312	14.11 ± 0.53
0.625	19.96 ± 0.92
1.25	34.51 ± 0.17
2.5	52.76 ± 0.25
5	73.66 ± 0.34

Table 97 The percentage of rat alpha-glucosidase inhibition of KLKS remedy extract

Concentration (mg/ml)	Percentage of inhibition (mean ± SD, n=3)
0.156	6.85 ± 0.19
0.312	19.21 ± 1.15
0.625	32.19 ± 0.42
1.25	47.08 ± 0.45

2.5	58.88 ± 0.45

Appendix E

Toxicity activities

Table 98 The percentage lethality of Brine shrimp of *C. cyminum* fruit ethanolic extract

Concentration (µg/ml)	Percentage of lethality (mean ± SD, n=5)
10	7.0 ± 0.47
100	13.0 ± 0.47
250	27.0 ± 0.47
500	40.0 ± 1.00
1000	50.0 ± 1.00

Table 99 The percentage lethality of Brine shrimp of *N. sativa* seed extract

Concentration (µg/ml)	Percentage of inhibition (mean ± SD, n=5)
10	20.0 ± 0.82
100	33.0 ± 0.47
250	37.0 ± 0.47
500	43.0 ± 0.58
1000	57.0 ± 0.58

Table 100 The percentage lethality of Brine shrimp of *F. vulgare* fruit extract

Concentration (µg/ml)	Percentage of inhibition (mean ± SD, n=5)
10	27.0 ± 0.47
100	37.0 ± 0.94
250	43.0 ± 0.47
500	47.0 ± 0.58
1000	63.0 ± 0.58

Table 101 The percentage lethality of Brine shrimp of *Z. officinale* rhizomes extract

Concentration (µg/ml)	Percentage of inhibition (mean ± SD, n=5)
10	13.0 ± 0.47
100	23.0 ± 0.94
250	37.0 ± 0.47
500	45.39 ± 1.00
1000	50.39 ± 0.58

Table 102 The percentage lethality of Brine shrimp of *P. indica* root extract

Concentration (µg/ml)	Percentage of inhibition (mean ± SD, n=5)
10	17.0 ± 0.47
100	27.0 ± 0.47
250	37.0 ± 0.47
500	43.0 ± 0.58
1000	47.0 ± 0.58

Table 103 The percentage lethality of Brine shrimp of *C. sativa* leaves extract

Concentration (µg/ml)	Percentage of inhibition (mean ± SD, n=5)
10	27.0 ± 0.47
100	37.0 ± 0.47
250	50.0 ± 0.82
500	53.0 ± 0.58
1000	70.0 ± 1.00

Table 104 The percentage lethality of Brine shrimp of *P. nigrum* fruits extract

Concentration (µg/ml)	Percentage of inhibition (mean ± SD, n=5)
10	14.11 ± 0.53
100	19.96 ± 0.92
250	34.51 ± 0.17
500	52.76 ± 0.25
1000	73.66 ± 0.34

Table 105 The percentage lethality of Brine shrimp of KLKS remedy extract

Concentration (µg/ml)	Percentage of inhibition (mean ± SD, n=5)
10	23.0 ± 0.47
100	33.0 ± 0.94
250	40.0 ± 0.82
500	47.0 ± 0.58
1000	77.0 ± 0.58

REFERENCES

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