SAFETY EFFICACY AND QUALITY ASSESSMENTS OF BEN-CHA-MOON-YAI REMEDY

Miss Rawiwan Manohan

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นางสาวระวิวรรณ์ มโนหาญ

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วิทยาลัยวิทยาศาสตร์สาธารณสุข จุฬาลงกรณ์มหาวิทยาลัย

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By	Miss Rawiwan Manohan
Field of Study	Public Health Sciences
Thesis Advisor	Associate Professor Nijsiri Ruangrungsi, Ph.D.
Thesis Co-advisor	Chanida Palanuvej, Ph.D.

Accepted by the College of Public Health Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

……..Dean of the College of Public Health Sciences
(Professor Surasak Taneepanichskul, M.D.)

THESIS COMMITTEE

……..Chairman
(Professor Surasak Taneepanichskul, M.D.)

……..Thesis Advisor
(Associate Professor Nijsiri Ruangrungsi, Ph.D.)

……..Thesis Co-advisor
(Chanida Palanuvej, Ph.D.)

……..Examiner
(Assistant Professor Naowarat Kanchanakhan, Ph.D.)

……..Examiner
(Kanchana Rungsihirunrat, Ph.D.)

……..External Examiner
(Assistant Professor Supawan Bunrathep, Ph.D.)

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ตำรับยาเบญจมูลใหญ่ เป็นยาแผนโบราณที่บรรจุอยู่ในตำราแพทย์ศาสตร์สงเคราะห์ ประกอบด้วยรากห้าชนิด ได้แก่ รากมะตูม รากเพกา รากลำไย รากแคแตร และรากคัดลิ้นในอัตราส่วนที่เท่ากัน ตำรับนี้มีการใช้เป็นยาแก้ไข้ แก้อักเสบ และแก้ปวดมาเป็นเวลานานโดยไม่มีงานวิจัยสนับสนุน ดังนั้นการศึกษาครั้งนี้จึงได้มีการประเมินมาตรฐานยาสมุนไพรประเมินลักษณะทางมหภาคและจุลภาคของเครื่องยาสมุนไพรทั้งห้าชนิด ลักษณะทางเคมี-ฟิสิกส์ และเอกลักษณ์ทางเคมีด้วยวิธีโครมาโตกราฟฟีหลายชนิด นอกจากนี้ยังได้ประเมินความปลอดภัยและประสิทธิผลของสารสกัดด้วยเอทานอลและน้ำของรากสมุนไพรทั้งห้าชนิดและตำรับยาเบญจมูลใหญ่ด้วยวิธีการทดสอบทางห้องปฏิบัติการและในสัตว์ทดลอง โดยทำการประเมินฤทธิ์ก่อกลายพันธุ์และฤทธิ์ต้านการก่อกลายพันธุ์ด้วยวิธีการทดสอบเอมส์ ในเชื้อ *Salmonella typhimurium* สายพันธุ์ TA98 และ TA100 พบว่าสารสกัดส่วนใหญ่ไม่มีฤทธิ์ก่อกลายพันธุ์ต่อเชื้อ *S. typhimurium* ทั้งสองสายพันธุ์ ยกเว้นสารสกัดด้วยน้ำของรากมะตูมมีฤทธิ์ก่อกลายพันธุ์ต่อเชื้อ *S. typhimurium* ทั้งสองสายพันธุ์ภายใต้สภาวะที่ไม่ถูกกระตุ้นด้วยไนไตรท อย่างไรก็ตามพบว่าสารสกัดโดยส่วนใหญ่มีฤทธิ์ก่อกลายพันธุ์ต่อเชื้อ *S. typhimurium* ทั้งสองสายพันธุ์หลังถูกกระตุ้นด้วยไนไตรท นอกจากนี้ยังพบว่าสารสกัดส่วนใหญ่มีฤทธิ์ต้านการก่อกลายพันธุ์จากสารก่อกลายพันธุ์ที่เกิดจากปฏิกิริยาของอะมิโนไพรีนและไนไตรทภายใต้สภาวะกรดในวิธีทดสอบเอมส์ การศึกษาพบว่าสารสกัดเอทานอลของรากมะตูมมีความเป็นพิษต่อเซลล์ต่อไรทะเล โดยมีค่า LC_{50} = 53.5 มิลลิกรัมต่อมิลลิลิตร และยังพบว่าสารสกัดด้วยน้ำและเอทานอลของรากมะตูมยังมีฤทธิ์ทำให้เกิดความเสียหายต่อดีเอ็นเอที่สกัดได้จากลิมโฟไซต์ของมนุษย์โดยมีผลรวมของคะแนนอยู่ที่ 250.50 และ 187.67ตามลำดับ สารสกัดด้วยเอทานอลของรากเพกาและรากลำไยแสดงฤทธิ์ต้านจุลชีพสูงสุดในการทดสอบกับเชื้อก่อโรคทั้ง 13 ชนิด การศึกษาพบว่าสารสกัดเอทานอลของรากลำไยมีฤทธิ์สูงสุดในการต้นอนุมูลอิสระด้วยวิธีการทดสอบทางห้องปฏิบัติการหลายวิธี นอกจากนี้ยังพบว่าสารสกัดจากตำรับยาเบญจมูลใหญ่มีฤทธิ์ลดไข้ ฤทธิ์ต้านการอักเสบและฤทธิ์ลดปวดในสัตว์ทดลองดีกว่าสารสกัดจากรากแต่ละชนิด ซึ่งอาจเนื่องมาจากการเสริมฤทธิ์กันของสารสกัดจากรากแต่ละชนิด ซึ่งเป็นเหตุผลว่าทำไมแพทย์แผนไทยจึงใช้ตำรับยาเบญจมูลใหญ่แทนการใช้รากสมุนไพรเดี่ยว

ภาควิชา วิทยาลัยวิทยาศาสตร์สาธารณสุข ลายมือชื่อนิสิต .....

สาขาวิชา วิทยาศาสตร์สาธารณสุข ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์หลัก .....

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RAWIWAN MANOHAN: SAFETY EFFICACY AND QUALITY ASSESSMENTS OF BEN-CHA-MOON-YAI REMEDY. ADVISOR: ASSCOC. PROF. NIJSIRI RUANGRUNGSI, Ph.D., CO-ADVISOR: CHANIDA PALANUVEJ, Ph.D., 313 pp.

Ben-Cha-Moon-Yai remedy is one of the Thai traditional medicines notified in Tum ra paad sard song khor. The remedy is composed of the roots of *Aegle marmelos* (L.), *Oroxylum indicum* (L.), *Dimocarpus longan* Lour., *Dolichandrone serrulata* (DC.) Seem., and *Walsura trichostemon* Miq. each in an equal part by weights. This remedy has been used as an antipyretic, anti-inflammatory and analgesic drug for a long time without scientific approved. Therefore, the pharmacognostic specifications of each root species in Ben-Cha-Moon-Yai remedy was developed in order to provided the macroscopic and microscopic specifications, physico-chemical identifications and chemical fingerprints. The ethanol and water extracts from each root species and Ben-Cha-Moon-Yai remedy were evaluated for their safety and efficacy both *in vitro* and *in vivo*. The mutagenic and antimutagenic activities of root extracts and Ben-Cha-Moon-Yai remedy were study using the pre-incubation method of Ames test. Most of the extracts exhibited non-mutagenicity without nitrite treatment in the Ames test toward *Salmonella typhimurium* strains TA98 and TA100. However, the water extract of *A. marmelos* revealed the mutagenicity on both strains in the present study. Most of the extracts were mutagenicity on both strains of *S. typhimurium* after being treated with sodium nitrite. In addition, it revealed that most of the extracts exhibited antimutagenic potential against the mutagenic reaction product produced from the reaction of 1-aminopyrene treated with nitrite under acidic condition pH 3-3.5 in the Ames test. Among five root species and Ben-Cha-Moon-Yai remedy extracts, the result demonstrated that the ethanolic extract of *A. marmelos* showed the highest toxicity against brine shrimp with LC_{50} of 53.5 µg/ml. Both water and ethanol extracts of *A. marmelos* showed the highest DNA damage in human lymphocytes with the total score of 250.50 and 187.67, respectively. The ethanol extract from the root of *O. indicum* and *D. longan* showed the highest antimicrobial activity against 13 tested microorganisms. These studies have demonstrated that the ethanol extract of *D. longan* showed the highest antioxidant capacity in various *in vitro* models. The Ben-Cha-Moon-Yai remedy extract seemed to be the most potent anti-nociceptive, anti-inflammatory and anti-pyretic activities in well-established animal models than individual components due to additive and/or synergistic effects of some herbal roots in the remedy. This might be a reason why Thai traditional doctors use Ben-Cha-Moon-Yai remedy as a therapeutic drug instead of using individual roots.

Department: College of Public Health Sciences Student's Signature .....

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LIST OF ABBREVIATIONS

BMY	=	Ben-Cha-Moon-Yai remedy
AM	=	*Aegle marmelos*
DL	=	*Dimocarpus longan*
DS	=	*Dolichandrone serrulata*
OI	=	*Oroxylum indicum*
WT	=	*Walsura trichostemon*
amu	=	Atomic Mass Unit
AOAC	=	Association of Official Analytical Chemists
°C	=	Degree Celsius
cm	=	Centimeter
ESI	=	Electron Ionization Source
ECG	=	Electrocardiography
g	=	Gram
HPLC	=	High performance liquid chromatography
hr	=	Hour
kg	=	Kilogram
LC-MS	=	Liquid Chromatography-Mass spectrometry
LD_{50}	=	Lethal dose 50%
m/z	=	Mass-to-charge ratio
mg	=	Milligram
min	=	Minute
ml	=	Microlitre
mm	=	Millimetre
mmol	=	Millimole

MS	=	Mass spectrometry
nm	=	Nanometre
PDA	=	Photo Diode Array detector
r^2	=	Correlation coefficients
rpm	=	Revolutions per minute
UV	=	Ultraviolet
α	=	Alpha
β	=	Beta

CHAPTER I
INTRODUCTION

Background and significance of the study

Herbal medicine is defined as plant derived materials or preparation with therapeutics or other human health benefits which contains either raw or processed ingredients from one or more plants [1]. It can be classified into three groups; (1) herbal materials; raw or processed herbal materials (2) traditional herbal products; decoction, tablets, pill or capsules containing crude herbal materials or crude extracts (3) standardized herbal products; formulations containing standardized extract or purified substance [2]. A wide range of conventional drugs were originally derived from plants.

Herbal medicines are becoming popular in developed countries as a result of dissatisfaction with modern medicines [3]. The reasons for increasing interest towards herbal medicines may come from their long historical usage and the claim that the preparations are “natural”, thus “safe” with no side effect [4]. However, such claims may not be true, [5] as more subtle and chronic forms of toxicity, for instance the carcinogenicity, mutagenicity, and hepatotoxicity, may have been disregarded previously by traditional practitioners. The issues related to herbal medicines are important and have to be dealt with responsibility. According to a recent WHO survey conducted from 142 member states revealed that 61% of all respondents have a registration system for herbal medicines and 12% have one thousands or more registered herbal medicines [6].

Despite a long practices of herbal medicines in a particular society, only a few herbs have been scientifically evaluated, which indicates that there are huge number of the traditional herbal medicines need to be scientifically studied [7]. Apparently, the development of evidence-based herbal medicines requires comprehensive understanding of biological, chemical, genetic, and agronomic aspects of plants. There is increasing interest in herbal medicines to give them a place in evidence-based medicine by regulated research strategies based on inventory and identification

of plants used, demonstration of pharmacological activity of an extract of the medicinal plants, bioassay guided fractionation, isolation and characterization of the active compounds and structure-activity relationship studies of the isolated compounds [8].

Herbal medicines usually consist of a mixture of active compounds which are frequently unidentified. In addition, there are several factors that impair the quality control of phytotherapeutic agents [9]. Quality control of herbal medicines is a critical and essential issue to be considered in assuring the therapeutic efficacy, safety and to rationalize the use of herbal medicinal plants in healthcare [10].

The source and quality of raw materials play a pivotal role in guaranteeing the quality and stability of herbal preparation. However, for many reasons, substitute or counterfeit herbal materials are often found in the market. The adulterations of herbal preparation are not easily distinguished from the right material using naked eyes. Even for the right species, the chemical composition and concentrations of bioactive compounds may vary dramatically with different collected seasons and regions as well as storage. Therefore, species identification and collection of the raw plant materials are considered to be the most important procedures to avoid a false influence to the quality of products or results of research [11]. Standards for minimum acceptable quality are conventionally laid down in pharmacopoeial monographs, which provide a summary of the acceptable substance and give details of relevant test to determine its identity, the presence and acceptable levels of impurities and to check that the levels of “actives” are sufficient to achieve the desired effect [10].

Requirement and methods for research and evaluation of the safety and efficacy of herbal medicines are more complex than those for modern pharmaceuticals. Therefore, WHO had published the “research guidelines for evaluating the safety and efficacy of herbal medicines” as part of effort to support the usage of a safe herbal medicines. While the guideline strengthens research in the evaluation of the safety and efficacy of herbal medicines, a number of bioassays have been carried out to explore the possible biological activities and toxicities of the

herbal medicines. Accordingly, a number of advanced biological experimental techniques have been used as standard safety tests along with the efficacy studies.

The *in vitro* bioactivities testing are usually followed by *in vivo* animal test to further confirm the functional mechanism and understand the absorption, metabolism, and toxicity of the studied herbal medicines in living organisms [12]. Results from bioassays frequently enabled those herbal medicines with acute and obvious signs of toxicity to be well recognized and their use avoided. Ideally, bioassays may also distinguish the efficacy of high quality from the adulterated or poor quality of herbal medicines. Uses of validated, reliable and relevant methods for efficacy and toxicity studies with regulatory strategies are essential to create a stronger evidence base on the safety, efficacy and quality of herbal medicines.

In Thailand, medicinal plants have been clinically used for a long time because they are easily available and inexpensive. The claims that the Thai medicinal plants are more effective and less harmful side effects than synthetic drugs also encourage the consumption among the member of society. Preparation of these plants for treatment involves preparing a tea, tincture or filtrate [13]. Moreover, consuming local medicinal plants can reduce the import of synthetic drugs from foreign countries as well [14].

Ben-Cha-Moon-Yai remedy is one of the Thai traditional medicines notified in Tumrapaadsard song khor. The remedy is composed of five roots in an equal part by weight, including the roots of *Aegle marmelos* (L.) Correa ex Roxb. (Rutaceae), *Oroxylum indicum* (L.) Kurz (Bignoniaceae), *Dimocarpus longan* Lour. subsp. *longan* var. *longan* (Sapindaceae), *Dolichandrone serrulata* (DC.) Seem. (Bignoniaceae), and *Walsura trichostemon* Miq. (Meliaceae) [15]. This remedy has been used as an antipyretic, anti-inflammatory and analgesic drug in Thai traditional medicine practice. According to herbal drug market survey, it was observed that five roots species in Ben-Cha-Moon-Yai remedy could be adulterated with upper ground parts of plants or other substances, which resulted in degrading the quality of the remedy.

Therefore, the purpose of a study is not only to explore bioactive compounds from herbal extracts, but also to standardize and control the quality of raw herbal materials and their products to ensure the safety and efficacy; and more importantly, to reveal their preventative and therapeutic effects of herbal extracts. The present study was attempted to investigate the pharmacognostic specifications, toxicities and efficacies of Ben-Cha-Moon-Yai remedy and its ingredients. The study protocol provided basic scientific evidences to answer the question of safety, efficacy and quality of Thai herbal medicines.

Objectives of the study

1. To develop the pharmacognostic specification for each plant species in Ben-Cha-Moon-Yai remedy
2. To evaluate the toxicities of the Ben-Cha-Moon-Yai remedy
3. To evaluate biological activities of the Ben-Cha-Moon-Yai remedy

Benefits of the study

1. This research provides information useful to determine the quality of Ben-Cha-Moon-Yai remedy based on pharmacognostic specification of each species in Ben-Cha-Moon-Yai remedy.
2. This research provides the scientific evidences in efficacy and safety of Ben-Cha-Moon-Yai remedy and its ingredients.
3. This research protocol can be applied to other traditional medicine formularies.

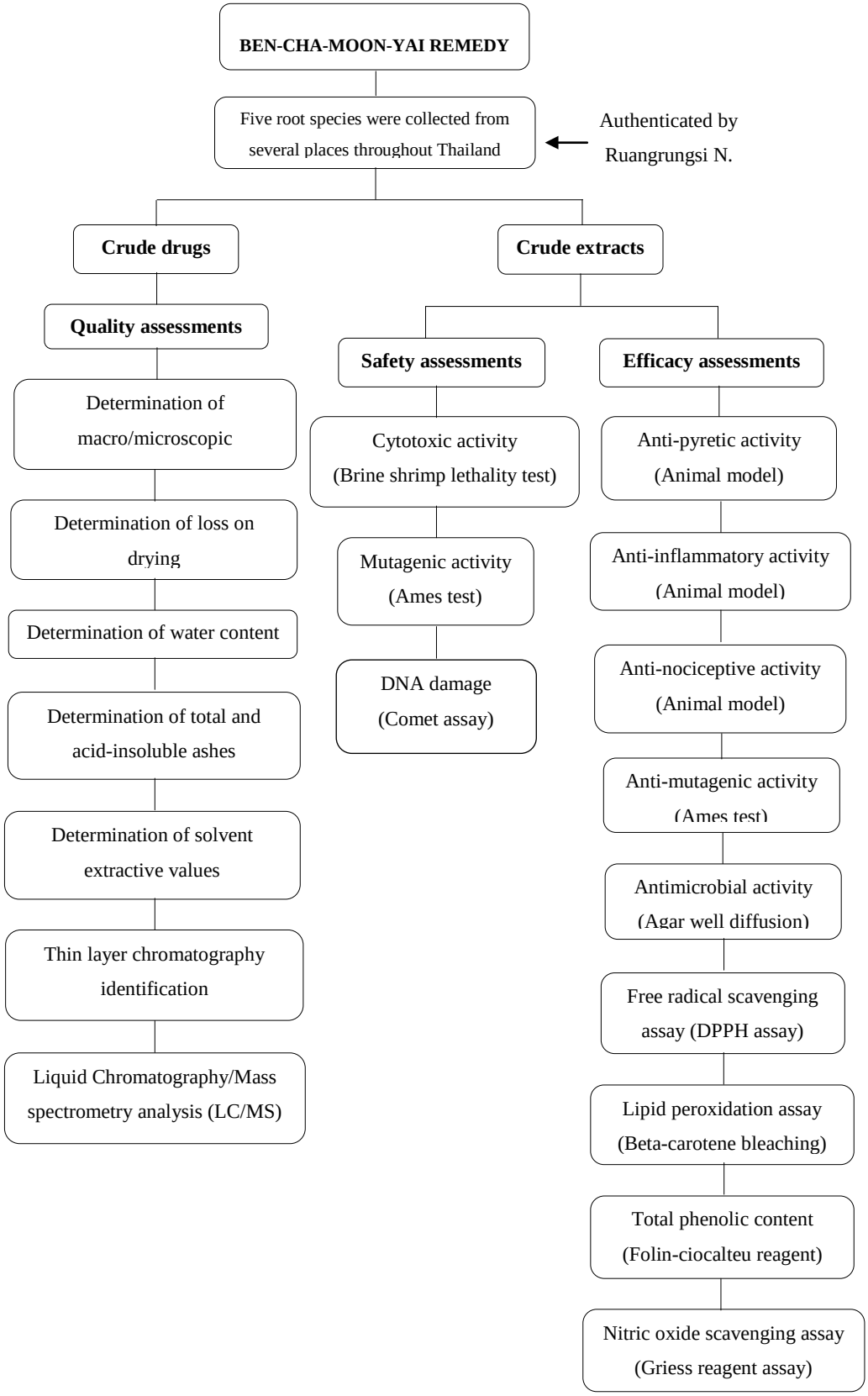


Figure 1 Scope of the study

CHAPTER II

LITERATURE REVIEWS

Quality control of herbal drugs

The quality control methods are plays an important role in traditional medicine which conserve as a tool for identification, authentication and quality control herbal drug [14]. There are numerous reports on the toxicity due to the misidentification and substitution of plant species. Pharmacognosy is the first step of quality control assessment which defined as a pharmaceutical discipline by Seider in 1815. The definition of pharmacognosy is mean “the science which has the task to learn everything about drugs originating from plants or animals in all aspects, except under the physiological effect, to describe the correctly and under a general vision connect this knowledge” [16-17]. WHO had published the *“Quality control methods for medicinal plant materials”* a recommended test procedure to evaluate the identity, purity and quality of the plant materials. The guidelines define that the quality and authenticity of the final botanical products is directly related to the proper identification and authenticity of the source materials. The majority of the information for quality assurance is based on the standardization parameters such as authentication, water content, loss on drying, total ash, acid-insoluble ash, extractive values and chemical fingerprint of medicinal plant materials. These standardization parameters are essential to publish in the pharmacopoeia [14].

Macroscopic and microscopic methods are the effective tool to establish the correct identity of the plant materials [18]. The macroscopic evaluation or the organoleptic characteristics of plant materials is based on the shape, size, colour, surface characteristics, texture, fracture and appearance of the cut surface either with the naked eye or with simple magnification such as with a hand lens or stereomicroscope. The same species of plant materials could be have similar morphological characteristics. Furthermore, the evaluation of this parameter can provide a qualitative assessment of adulterating species such as filth, organic and non-organic contaminants, and material degradation [5]. Microscopic examination gives a clear idea about the identification of crude plant material in the whole, fragmented or

powdered form. Microscopy of medicinal plants focused on the observation of the cellular structures and their content of plant material by use of a compound microscope. The powder of crude drugs can be identified the presence of absence of various cell types based on their cyto-morphological character such as parenchyma, collenchymas, fibers, stone cells, vessels, trichomes, secretary cells, epidermal cell. Botanical microscopy is an integral part of pharmacognosy training which the method requires high expertise because sometimes it may not provide unequivocal authentication based on the fact that the similar microscopic characteristics presented in related species [10].

Comparing to with other techniques, macroscopic and microscopic identification are very cost and time-efficient. The evaluations of these parameters provide the specific characteristics of crude drugs such as morphological or macroscopic characteristics, cyto-morphological and microscopic characteristic in both its entire and its powder form. Therefore, these authentication methods play an important role in the monographs on herbs in many pharmacopeias, including the Chinese, European and Thai herbal pharmacopoeia [10].

Physico-chemical parameters are important to identify the purity and quality of herbal drugs. The physico-chemical constants could be useful for detection of adulteration or improper handling of drugs, identification, authentication and also compilation of compilation of a suitable monograph [19]. The procedures normally adopted to get the qualitative information about the purity and standard of a crude drug include the determination of various parameters. The total ash is particularly important to determine the purity of crude drugs. Ash values are simply represents the total amount of material remaining after incineration which includes ash derived from the part of the plant itself and deliberately added to it crude drug as a form adulteration [19-21]. The extractive values give an idea about the chemical constituent of crude drugs which soluble in ethanol or water [22, 23]. Moisture is an unexpected component of crude drugs because an excess of moisture can result in the breakdown of important constituents by enzymatic activity and may encourage the growth of yeast and fungi during storage [24]. Methods of determination of moisture content include the loss on drying and the volumetric azeotropic distillation methods to

estimate the loss on drying after heating at 100 to 105 °C and measure of water content in crude drugs [5].

Preliminary phytochemical screening reveals the presence of a wide range of phytoconstituents from crude extract such as alkaloids, glycosides, saponins, carbohydrates, flavonoids, tannins, amino acids and steroids for supporting the reason for its wide range of biological activities [25].

The fingerprinting analysis is nowadays getting momentum for the quality control of multi-component herbal medicines and has been widely accepted as a useful tool to determine authenticity and reliability of chemical constituents of herbal drug and formulations [26]. Chromatographic techniques are the most versatile tools for the analysis of herbal medicines. A chromatographic fingerprint is a chromatogram representing the chemical characteristics of herbal materials [10]. Thin layer chromatography is the common method for herbal medicine analysis for over the past decades. Even nowadays, TLC is still frequently used to provide first characteristic fingerprints of herbal medicines and can be establish in various pharmacopoeias such as American Herbal Pharmacopoeias, Chinese drug monograph and analysis and Thai herbal pharmacopoeias [11]. The advantages of TLC method to perform the fingerprinting of herbal medicines are based on the simplicity, versatility, high velocity, specificity, sensitivity and ease to prepare the sample. High performance liquid chromatography is become popular method for the analysis of herbal medicines due to its superior precision, high resolution and capacity to separate a very wide range of organic compounds, from small-molecule drug metabolites to peptides and proteins, unlikely Gas chromatography technique which is suitable for volatile compounds [17]. The combination technique between High performance liquid chromatography and online UV spectrum detection *via* diode array detectors or tandem mass spectra to separated ingredient can be obtained simultaneously and identified these ingredients by comparing the spectra with the reference compounds [27].

Many plants contain unique sets of compounds in characteristic ratios that can allow for the differentiation of even closely related species and even different plant

part of the same part. TLC, GC, HPLC, CE and hyphenated techniques have already been used for the development of chromatographic fingerprints. The multiple chromatographic approaches can be extensively defines the authenticity and quality characteristics of a specific herbal extract containing the combination of multiple ingredients to regulated quality control of herbal medicines [17]. Several parameters could be evaluated qualitatively as well as quantitatively for determination of the quality control of medicinal plant materials such as authentication, identification, organoleptic characteristic, physicochemical analysis, chemical characterization and biological investigation as shown in figure 2.

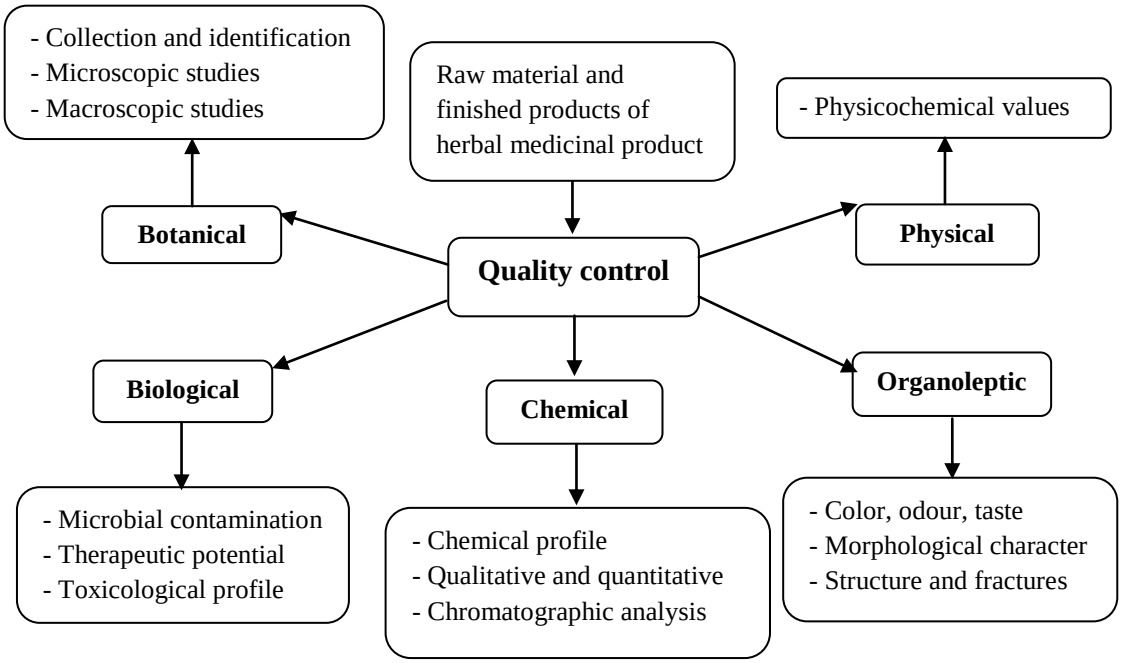


Figure 2 Quality control methods for medicinal plant materials

Safety and efficacy regulation

The safety and efficacy of herbal medicines are established through their long historical use to treat various health ailments and absence of evidence of harm, thus they are generally regarded as safe to consume. However, most herbal medicines still need to be studied scientifically in systemic toxicology and efficacy assessment. The WHO has published “Research guidelines for evaluating the safety and efficacy of herbal medicines” in order to determine the basic criteria for evaluating the safety and efficacy of herbal medicines aimed at assisting national regulatory authorities, scientific organizations and manufacturers in the particular area [1, 28]. Herbal medicine extracts contain a variety of molecules with potent biological activities which is difficult to analyze the biological activities of these extracts because of their complex nature and the possible synergistic effects of their components [29]. Numerous advanced biological experimental techniques have been used as standard safety tests along with the efficacy studies [4].

Most preclinical development studies employ rodents, primarily mice. The animal model can study effects of toxicants under well-controlled conditions in a complete mammalian system. Mammals could be share many similar aspects of anatomy, physiology and biochemistry, thus making extrapolations to humans reasonably valid scientifically and most regulatory approvals cannot take place without *in vivo* testing. However, there are some disadvantages of *in vivo* studies which are time consuming, expensive and variation in structure and function from one species to another. In addition, the used of high doses in animals may not be predictive for a target human population. Based on the rationale of animal welfare which concern about animal used for *in vivo* testing, there is much debate about the replacement of experimental animals with *in vitro* toxicology models. It is possible that a wide range of *in vitro* techniques, using both transformed and unmodified human and rodent cells, could serve as useful media in various toxicity assays.

There are many advantages of *in vitro* studies which could be reduced the number of animals used for *in vivo* tests, relatively simple to perform and quantify, using human cells is possible and less expensive than animal test. However, there are

some disadvantages of *in vitro* studies such as the route of exposure for an *in vitro* test of chemical directly to cells in culture through their culture through their culture is not comparable with an *in vivo* exposure, the results may be highly variable between laboratories or cannot repeated, difficult to relate *in vitro* dosages to those that produced toxicity to whole animals and may not satisfy regulatory demands or provide scientifically adequate information as evidence [30].

The safety of herbal medicines is a major concern for traditional medicine practitioners, pharmacists, doctors and other healthcare professional because the long historical usage of herbal medicines [31]. The importance of toxicity testing is to provide safety evaluations of these plants. The extensive traditional use of plants as medicines has enabled those medicines with acute and obvious signs of toxicity to be well recognized and their use avoided. The more subtle and chronic forms of toxicity, such as carcinogenicity, mutagenicity and hepatotoxicity, may well have been disregarded by previous generations and it is these types of toxicities that are of most concern when assessing the safety of herbal remedies [32]. However, many different side effects to herbs have been reported and recently reviewed, including effects from biologically active constituents from herbs, side effects caused by contaminants, and herb-drug interactions [33]. It is likely that many different *in vitro* cytotoxicity methods could be used to help select the *in vivo* starting dose for an acute lethality assay. Two decades of experience indicate that *in vitro* basal cytotoxicity data determined in various primary cells, as well as in various permanent non-differentiated finite or transformed cell lines, generally show comparable cytotoxic concentrations of the same xenobiotic, regardless of the type of toxic endpoints investigated [34]. The investigation of half-lethal dose (LD_{50}), the dose at which it has been proven to be lethal-causing death to 50% of the tested group of animals, has been criticized for both scientific and animal welfare reasons, and the test procedure has been modified in various ways to reduce the number of animal used [35].

Ben-Cha-Moon-Yai remedy

Ben-Cha-Moon-Yai remedy or Pikud Ben-Cha-Moon-Yai is one of the Thai ancient remedies which revealed in Tumra Paadsard Song Khor. Pikud is an herbal remedies that are compose of a set of ingredients in an equal part by weight. The components of Ben Cha Moon Yai Remedy are shown in **table 1**.

Table 1 Component of Ben Cha Moon Yai remedy [15]

Scientific name	**Thai name**	**Family name**	**Plant Part**
Oroxylum indicum (L.) Kurz	เพกา	BIGNONIACEAE	Root
Aegle marmelos (L.) Corr.	มะตูม	RUTACEAE	Root
Dimocarpus longan Lour.	ลำไย	SAPINDACEAE	Root
Walsura trichostemon Miq.	คัดลิ้น	MELIACEAE	Root
Dolichandrone serrulata (DC.) Seem.	แคแตร	BIGNONIACEAE	Root

***Aegle marmelos* (L.) Correa ex Roxb.**

Aegle marmelos (L.) Correa ex Roxb., commonly known as “Bael tree” belonging to the family Rutaceae and known in Thai as “Matum”. It is a deciduous tree with a large or medium sized tree, 12-15 m in height and has been naturalized in Thailand, India, Sri Lanka and various southeastern Asian countries [36]. The leaves, roots, bark, and fruits of this plant are widely used in Ayurvedic and ethnomedicine [37]. They are used to treat an inflammation, catarrh, diabetes, asthmatic complaints, diarrhea, dysentery, and weakness of heart [38].

Chemical constituents

The chemical literature survey of *A. marmelos* revealed that there are several chemical constituents isolated and identified from leaf, fruit, seed, bark and root such as alkaloids, coumarins, steroids and tannin.

Table 2 Chemical constituents of various parts of *A. marmelos* [39]

Part	Chemical constituent
Leaf	aegeline, skimmianine, rutin, γ-sitosterole, ß-sitosterol, lupeol, cineol, citral, *O*-isopentenyl, hallordiol, mameline, citronellal, cuminaldehyde, euginol, marmesinin, aegelin. anhydromarmeline, aegelinoside A, aegelinoside B, limonene, (Z)-*ß*-ocimene [40-43]
Fruit	marmelosin, luvangetin, aurapten, psoralen, marmelide marmesiline, 6-(4-acetoxy-3-methyl-2-butenyl)-7-hydroxycoumarin, 6-(2-hydroxymethyl-3-butenyl)-7-hydroxycoumarin, marmelonine, 8-hydroxysmyrindiol, imperatorin, valencic acid, 8-[(3”-methyl-2”oxo-3”-buten-1”-yl)oxy]-7H-furo[3,2-g]benzopyran-2-one, xanthotoxol, isogosferol, xanthotoxin, scoparone, (+)-decursinol, demethylsuberosin, 6-formylumbilliferone, (+)-marmesin, marmeline, isofraxidin, isophellodenol C, xanthoarnol [44]
Seed	imperatorin, *ß*-sitosterol, plumbagin, 1-methyl-2-(3’-methyl-but-2’-enyloxy)-anthraquinone, *ß*-sitosterol glucoside, stigmasterol, vanillin, salicin [36]

Bark	skimmianine, fagarine, marmin
Twig	marmesin, scopoletin, limonene, (Z)-ß-ocimene [43, 45]
Root	aeglemarmelosine, skimmianine, imperatorin, aurapten, epoxyauraten, marmin, xanthotoxin, aegeline, skimmianine, umbelliferone [40, 45, 46]

Pharmacological activities

Various crude extracts from various parts of this plant have shown their biological activities. The extracts from *A. marmelos* leaves showed significant analgesic activity on acetic acid-induced writhing and tail flick test in mice [47]. These extract also produced marked inhibition of the carrageenan-induced paw oedema and cotton-pellet granuloma in rats and caused a significant reduction in yeast-induced hyperpyrexia in mice [48]. Anhydroaegeline compound isolated from leaves exhibited the highest inhibitory effect on α-glucosidase inhibition assay whereas anhydromarmelin, aegelinosides A and B showed slightly weak inhibition activity [42]. Previous studies also reported that Aegeline 2, an alkaloid-amide lead, isolated from the leaves of this plant demonstrated the anti-hyperglycemic and antidyslipidemic activities in animal models. The activity may result from lowered blood glucose and decreased in lipid profile [49]. In accordance with the previous studied from different parts of this plant, the result showed that the aqueous seed extract at 250 mg/kg was also lowered total cholesterol (TC), triglyceride (TG) and low density lipoprotein (LDL) but enhanced the cardioprotective lipid (HDL) in diabetic animals [50].

A large number of compounds have been isolated from different parts of the plant and a few of them have been studied for their biological activity. A new anthraquinone, 1-methyl-2-(3'-methyl-but-2'-enyloxy)-anthraquinone, isolated from seed also displaying significant antifungal activity against *A. fumigates* and *C. albicans* by using disc diffusion assay with MIC values of 6.25 µg/disc, and MIC 31.25-62.5 µg/ml in microbroth dilution assay and 31.25 µg/ml in percent spore germination inhibition assay [36]. In addition, xanthoarnol compound isolated from

the acetone extract of the green fruits of *A. marmelos* exhibited the antibacterial activity against *E. faecalis* with the MIC values of 18. 75 µg/ml as same as vancomycin, which can be considered as a potential antibacterial agent [44].

The ethanolic extract from stem bark showed the cytotoxicity against brine shrimp lethality testing with presenting LD_{50} of 17.5±2.0 µg/ml and also possessed the cytotoxicity against leukemias and melanoma cell lines in MTT assay [51]. In addition, the stem bark extract and isolated compounds from *A. marmelos* were inhibited the *in vitro* proliferation of different human tumor cell lines and strongly inhibited human k562 cells. The result was comparable to some of the most commonly used antitumor agents such as cisplatin [52]. For *in vivo* toxicity testing, the methanolic extract from leaves indicated non-acute and subacute toxicities in rats after intraperitoneally of maximum doses up to 1000 mg/kg and 100 mg/kg with 14 consecutive days, respectively [53].

Figure 3 *Aegle marmelos* (L.) Correa. [54]

Dimocarpus longan* Lour. subsp. *longan* var. *longan

Dimocarpus longan Lour. is an evergreen tree which belongs to the Sapindaceae family. It is widely grown in China, Taiwan, and South East Asia including Thailand and Vietnam [55]. Longans commonly known as “Lamyai” in Thailand and has been widely cultivated in the northern Thailand [56]. Both longan flowers and fruits make a significant contribution in Thai food and herbal preparations. Longan fruits contain vitamins and minerals, such as iron, magnesium, phosphorus and potassium, and large amounts of vitamins A and C [57]. Previous study reported that longan fruit extract contained a significant amount of polyphenolic compounds which has been widely known for its antioxidant effects [58]. The content of polyphenolic compound is varied from different part of plant and cultivars [59]. These plant materials are considered to be a cheap source of herbal antioxidant used in neutraceutical products.

Chemical constituents

D. longan has been widely investigate, especially in the field of chemical constituents studies. Many types of chemical compounds, isolated from different parts of this plant, have been reported a significant amount of polyphenolic, polysaccharide, vitamin and essential oil compounds. The occurrences of chemical compounds in this plant were shown in table 3.

Table 3 Chemical constituents of various parts of *D. longan*

Part	Chemical constituent
Flower	(-)-epicatechin, proanthocyanidin A2 [60]
Inflorescences	caryophyllene, γ-elemene, ∂-caryophyllene, β-guaiene, Germacrene D [61]
Aril	lysophophatidyl choline, phosphatidyl choline, phosphatidyl inositol, phosphatidyl serine, phosphatidyl ethanol amine, phosphatidate, phosphatidic acid glycerol [62]
Fruit	corilagin, gallic acid, ellagic acid, ascorbic acid [63, 64]

Fruit peel	polyphenol oxidase (PPO) [65]
Fruit pericarp	acetonylgeraniin, (-)-epicatechin, 4-O-methylgallic acid, flavones glycosides, glycosides of quercetin, kaempferol, *L*-arabinofuranose, *D*-glucopyranose, *D*-galactopyranose, *D*-galacturonic acid, methylated Ara, methylated Glc, methylated Gal [66-68]
Seed	corilagin, gallic acid, ellagic acid, monogalloyl-diglucose, digalloyl-diglucose, penta-to heptagalloyl-glucose, ellagic acid-pentaose conjugate, galloyl-hexahydroxydiphenoyl-glucopyranose (HHDP), pentagalloy-HHDP, procyanidin A-type dimer, procyanidin B2, quercetin-3-*O*-rhamnoside, 1-*ß*-*O*-galloyl-*D*-glucopyranose, ethyl gallate, methyl brevifolin carboxylate, brevifolin, 4-*O*-α-*L*-rhamnopyranosyl-ellagic acid , methyl gallate, (-)-epicatechin, proanthocyanidin trimer, geraniin, isomallotinic acid, methyl brevifolin carboxylate, chebulagic acid [69, 70, 71, 75, 76]

Pharmacological activities

The biological activities of *D. longan* had been evaluated along with other plants. Phenolic compounds presented in different parts of this plant were reported to have antioxidant capacities in various models. The different extracts of *D. longan* seeds contain high amount of polyphenolic compound. The polyphenol-rich longan seed extract exhibited strong antioxidant capacities as effective as Japanese green tea extract on DPPH radical scavenging, superoxide radicals, ORAC, and anti-tyrosinase [72].

The methanolic extract also inhibited the xanthine oxidase in hypoxanthine/xanthine oxidase assay [71]. In addition, a polyphenol rich longan seed extract inhibited cell proliferation against three colorectal carcinoma cell lines by blocking cell cycle progression during the DNA synthesis phase and inducing apoptotic death [73]. Therefore, it was suggested that a polyphenol rich longan extract

could be employed as a potential novel treatment agent for cancer. The seed extract also possessed strong antifungal activity toward Candida species and *Cryptococcus neoformans* [74]. In addition, *D. longan* seed extract has low acute toxicity with LD_{50} higher than 5000 mg/kg in mice and revealed no toxic effects after repeated doses by oral administration [75].

The methanolic extract of *D. longan* flower demonstrated the highest antioxidative activity followed by ethyl acetate and n-hexane extract toward *in vitro* assays such as DPPH free radical scavenging, oxygen radical absorbance capacity, and the inhibition of LDL assays. The main active components in the inhibition of LDL inhibition oxidation were characterized as (-)-epicatechin and proanthocyanidin A2 [60].

The *D. longan* fruit pericarp extract obtained from high pressure-assisted and ultra high pressure-assisted extraction methods can gave a higher potential in antioxidant and anticancer activities than conventional extraction method through various *in vitro* models such as DPPH radical scavenging, total antioxidant capacity, superoxide anion radical scavenging, lipid peroxidation, anti-tyrosianase and cytotoxicity using MTT assays. The water extract from *D. longan* twigs also showed antimutagenic, antioxidant, antityrosinase activities and decreased lipid oxidative damage. [76]

Despite polyphenolic compounds, polysaccharide was also characterized from the seed and fruit pericarp of *D. longan.* This compound demonstrated a free radical scavenging activity in DPPH assay [77] and showed a good potential in anti-glycated activity [78].

Figure 4 ***Dimocarpus longan*** **Lour.** [79]

Dolichandrone serrulata (DC.) Seem

Dolichandrone serrulata (DC.) Seem is a deciduous tree belongs to Bignoniaceae family. The plant is widely distributed in South-east Asia known in Thai as “Kae Trae” or “Kae Pa”. The flower is edible with a bitter taste and has been used as part of Thai cuisine. In addition, the bark of this plant has been used as Thai ancient remedy for antipyretic and anti-inflammatory [80].

Chemical constituents

The chemical composition of *D. serrulata* had been characterized from flower and branches.

Table 4 Chemical constituents of various parts of *D. serrulata*

Part	Chemical constituent
Flower	new cyclohexylethanoid, sitosterol-3-*O*-*β*-*D*-glucoside [81]
Branch	dolichandroside, decaffeoyl-verbascoside, verbascoside, isoverbascoside, markhamioside A, 2”-*O*-apiosylverbascoside, luteoside B, ixoside [80]

Pharmacological activities

Although this plant has been used as vegetables, only few studies on pharmacological activities of this plant were provided. Most researchers investigated the antioxidant capacities of this flower extract. The result revealed that the flower extract from this plant exhibited low antioxidant capacities in three different antioxidant assay such as DPPH free radical scavenging activity, trolox equivalent antioxidant capacity (TEAC), ferric reducing antioxidant power (FRAP) and total phenolic content [82-83]. For the isolated compound, Cyclohexylethanoid isolated from flower of *D. serrulata* demonstrated moderate anti-inflammatory activity in ethyl phenylpropiolate-induced ear edema in rats [81].

Figure 5 ***Dolichandrone serrulata*** **(DC.) Seem** [84]

***Oroxylum indicum* (L.) Kurz**

Oroxylum indicum (L.) Kurz is deciduous tree belonging to the Bignoniaceae family which commonly known as “Indian Trumpet tree”. It is an edible plant which young shoots and unripe fruits are eaten as vegetables which are widely distributed in South East Asia, South Asia and China. *O. indicum* has been used as medicinal herb for thousands of year in many Asia countries and used in folk medicine as a cure of various diseases [85]. The fruits and flowers are consumed as a common part of the diet in the north and northeast of Thailand [86]. The plant has been used as a single drug or as a component of drug formulation in India. The root is used in preparation of well known Ayurvedic formulation such as “Chyavanprash” and “Dashmularistha” which used as an anti-inflammatory, anti-helmintic, anti-rheumatic, anti-bronchitic and astringent [87]. The seeds are active in chronic cough, abdominal pain and purgative [88].

Chemical constituents

Phytochemical investigations from various parts of *O. indicum* have been presented a number of secondary metabolites such as flavonoids, glycosides, alkaloids, tannins and terpenoids [88].

Table 5 Chemical constituents of various parts of *O. indicum*

Part	Chemical constituent
Leaf	chrysin, baicalein, baicalein-7-*O*-glucoside, baicalein-7-*O*-diglucoside, chrysin-7-*O*-glucoronide, baicalein-7-*O*-glucuronide, chrysin-diglucoside [89]
Seed	chrysin 6-C-*β*-*D*-glucopyranosyl-8-*O*-*β*-*D*-glucuronopyranoside, baicalein 7-*O*-*β*-*D*-glucuronopyranosyl-(1→3)[β-*D*-glucopyranosyl-(1→6)]-*β*-*D*-glucopyranoside, scutellarein 7-*O*-*β*-*D*-glucopyranosyl-(1→6)-*β*-*D*-glucopyranoside, chrysin-7-*O*-gentiobioside, baicalein-7-*O*-diglucoside, baicalein-7-*O*-glucoside, scutellarein-7-*O*-glucopyranoside, chrysin-7-*O*-glucuronide, baicalin, chrysin-6-C-*β*-*D*-

	glucopyranosyl-8-C-α-*L*-arabinopyranoside, chrysin, baicalein, oroxylin A, pinocembrin, pinobanksin, 2-methyl-6-phenyl-4H-pyran-4-one, lupeol, 2-α-hydroxyllupeol, echinulin, adenosine, dimethyl sulfone, *β*-sitosterol, baicalein-7-*O*-*β*-gentiobiosid, chrysin-7-*O*- *β*-gentiobiosid, baicalein-7-*O*-glucosid, baicalein-7-*O*-glucuronid, chrysin-7-*O*-glucuronid, baicalein, chrysin, baicalein-7-*O*-diglucoside (Oroxylin B), chrysin-7-*O*-diglucoside, baicalein-7-*O*-glucoside, baicalein, chrysin [90-93]
Stem bark	dihydrooroxylin A-7-*O*-methyl glucuronide, 5- hydroxyl-7-methoxy-2-(2-methoxy-6-(3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yoloxy)phenyl)-4H-chromen-4-one, dihydro-iso-a-lapachone, 7-*O*-methylchrysin, 5-hydroxy- 4´,7-di methoxy flavones, dihydrooroxylin A, oroxylin A, chrysin, baicalein, 5,7-dihydroxyflavone, 5,7-dihydroxy-3-methoxyflavone, 3,5,7-trihydroxyflavone, 5,7,4´-trihydroxy-3-methoxyflavone, 3,5,7,4´-tetrahydroxyflavone, 5,7,4´-trihydroxyflavone, lapachol, [94-96]
Root	chrysin**,** oroxylin A, pthallate, lapachol, *β*-sitosterol [87, 96]
Root bark	chrysin, baicalein, ellagic acid, biochanin-A [97]

Pharmacological activities

The ethanol and hexane extracts of *O. indicum* did not show a mutagenic effect toward *S. typhimurium* strains TA98 and TA100 but after nitrosation under acidic condition these extracts become strongly mutagenic effect in both strains [98] whereas the methanolic extract from fruits exhibited the strong antimutagenic effect against the food-derived mutagen toward *S. typhimurium* strain TA98 in Ames test. The bioactive compound for antimutagenic activity was identified as baicalein [86]. In addition, among five edible plants in Thailand, the ethanolic fruit extract of *O.*

indicum exhibited strongest antioxidant scavenging activity in DPPH assay and antimutagenicity toward *S. typhimurium* strains TA98 and TA100 [99].

The total phenolic and total flavonoid contents had varied among the different part of this plant in various solvent extract. The previous studied reported that the highest amount of total phenolic and total flavonoid contents were obtained from the methanolic extract from seeds and stem bark, respectively [100]. In the stem bark, the methanolic extract contained the highest amount of both phenolic and flavonoid contents followed by dichloromethane and petroleum ether in accordance with antioxidant and antimicrobial activities whereas petroleum ether extract caused maximum cytotoxic and apoptotic activities on Hela cells [101].

Different parts of *O. indicum* have been investigated for their antioxidant potential in various *in vitro* models. The results demonstrated that the leaves and stem bark extracts exhibited the highest free radical scavenging activity in DPPH, nitric oxide, superoxide anion and hydroxyl radical scavenging assays which may be due to presence of polyphenolic compounds [102]. Seven flavonoid glycosides isolated from the seed of *O. indicum* were quantified as the main ingredient in the methanolic extract of this plant and showed the antioxidant activities in DPPH and ORAC assays [90].

The root bark extract exhibited the protective effect against ethanol-induced gastric mucosa damage and lipid peroxidation determined by malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and reduced glutathione levels (GSH) assays [103]. In addition, among crude hexane and acetone extract and nine flavonoid compounds isolated from the stem bark of *O. indicum*, chrysin displayed highest potential on gastroprotective effect against various gastric ulceration models in Wistar rats [95]. Chrysin isolated from the root of *O. indicum* produced a significant protective effect against cisplatin induced nephrotoxicity in rats and it was decreased in lipid peroxidation activity [104].

The *n*-butanol fraction from *O. indicum* root bark demonstrated the potential on immune regulation activity which might be due to the present of baicalein in root

bark. It was also presented the reduction effect in whole blood malondialdehyde (MDA) content along with a rise in the activity of superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) [105].

The ethyl acetate extracts from stem bark showed the anticancer effect to inhibited NF-KB cancer cell line and also possessed anti-inflammatory effect on LPS-induced inflammatory testing by inhibited the release of IL-1B and PGE_2. The extract also gave the highest cytotoxicity against Hela cell and antioxidant activity in DPPH and lipid peroxidation assays [106]. In addition, the low-polarity crude extract from *O. indicum* stem bark demonstrated the cytotoxicity, apoptosis inducing ability and anti-metastatic potential in both human breast carcinoma and human liver embryonic cell lines [107]. Previous studies also reported that both root bark and stem bark decoction showed anti-inflammatory activity in carrageenan induced paw edema in rats [108].

The aqueous and ethanolic extracts from *O. indicum* root at 300 and 500 mg/kg produced a significant to decrease in plasma glucose levels when compared with diabetic control group in alloxan-induced diabetes and dexamethasone-incuded insulin resistance in rats [109]. The methanolic extract from root bark of *O. indicum* produced a significant wound healing in animal model and also exhibited antimicrobial activity against various microorganisms [110] in accordance with the dichloromethane extract and all isolated compounds from the stem bark and root of *O. indicum* that also exhibited the antimicrobial activities against gram positive and negative bacteria, fungi and yeast [96].

Figure 6 *Oroxylum indicum* (L.) Kurz [111]

***Walsura trichostemon* Miq.**

Walsura trichostemon Miq is an evergreen tree belong to the family of Meliaceae. The *Walsura* genus comprises 30 to 40 species and widely distributed in China, India, Indonesia and South East Asia. The plant is naturally distributed in the evergreen forest in north, northeast and southeastern of Thailand. It is a plant with edible fruits which known as “Kad-lin”. The stem bark and root had been used in folk medicine to treat tendon disabilities and wound healing. However, there are very few information has been reported on chemical constituents of this plant.

Chemical constituents

Previous investigation on *W. trichostemon* reported that tetraacetylated apotirucallane triterpenoid compound was isolated from the root of this plant. Several tetranotriterpenoids and triterpenoids had been isolated from different species of *Walsura* genus [112].

Table 6 Chemical constituents of various parts of *W. trichostemon*

Part	**Chemical constituent**
Root	trichostemonate [113]

Pharmacological activities

Among 40 species of *Walsura* genus, there are very scarcely researches concerning the pharmacological activities of *W. trichostemon*. Trichostemonated, tetraacetylated apotirucallane triterpenoid, isolated from the root of this plant demonstrated the cytotoxicity against human cervical carcinoma (HeLa) and human epidermoid carcinoma (KB) cell lines with IC_{50} of 0.93 and 3.28 µg/ml [113].

In addition, the ethyl acetated extract from the stem bark of this plant also exhibited strongest antimycobacterial activity on *Mycobacterium tuberculosis* and cytotoxic activity against human mouth carcinoma (KB), human small cell lung cancer (NGT-H187) and breast cancer (MCF-7) cell lines whereas hexane and methanol extracts showed only cytotoxicity in those cell lines [114].

Figure 7 *Walsura trichostemon* Miq. [115]

CHAPTER III

MATERIALS AND METHODS

Materials and chemicals

1. Acetic acid (Merck, Germany)
2. Acetylsalicylic acid (aspirin) (Sigma Chemical Co., USA)
3. Agar-Agar (Merck, Darmstadt, Germany)
4. 1-aminopyrene (Aldrich, St. Louis, U.S.A.)
5. Amikacin sulfate (T.P. Drug Laboratories (1969) Co., Ltd., Thialand)
6. Ammonium sulfamate (Fluka AG, Buch, Switzerland and Sigma-Aldrich, St. Louis, U.S.A.)
7. Ampicllin sodium (T.P. Drug Laboratories (1969) Co., Ltd., Thialand)
8. Acetonitrile (Merck, Germany)
9. β-carotene (Sigma-Aldrich, St. Louis, U.S.A.)
10. λ-carrageenan (Sigma Chemical Co., USA)
11. Crystal violet indicator (Fluka AG, Buch, Switzerland and Sigma-Aldrich, St. Louis, U.S.A.)
12. D-Biotin (Sigma-Aldrich, St. Louis, U.S.A.)
13. D (+)-Glucose monohydrate (Merck, Darmstadt, Germany)
14. Dimethysulfoxide (DMSO) (Merck, Darmstadt, Germany)
15. 2, 2-Diphenyl-1-picrylhydrazyl (Sigma-Aldrich, St. Louis, U.S.A.)
16. 3, 5-Di-*tert*-4-butylhydroxytoluene (BHT) (Sigma-Aldrich, St. Louis, U.S.A.)
17. Ethylenediamineetetra acetic acid disodium salt (Ajax Finechem Pty, Ltd.)
18. Ficoll-Histopaque 1077 (Sigma-Aldrich, St. Louis, U.S.A.)
19. Filter paper Whatman No.1 and No.4
20. Folin-Ciocalteu reagent
21. Formalin (Merck, Germany)
22. HPLC guard column (5 µm, 4.0 × 10 mm)
23. HPLC column, Inersil ® ODS-3, C-18 column (particle size of the packing 5 µm, 4.6 × 250 mm)
24. Hydrogen Peroxide (QREC)
25. Indomethacin (Sigma Chemical Co., USA)

26. L-histidine monohydrochloride monohydrate (Sigma-Aldrich, St. Louis, U.S.A.)
27. Linoleic acid (Sigma-Aldrich, St. Louis, U.S.A.)
28. Lipopolysaccharide (LPS) from *E. coli* (Sigma Chemical Co., USA.)
29. Low melting point agarose (Sigma-Aldrich, St. Louis, U.S.A.)
30. Methanol HPLC grade (Merck, Germany)
31. Morphine sulfate (MO; Thai FDA)
32. Mueller Hinton agar and broth (Merck, Germany)
33. *N*-1-napthylethylenediamine dihydrochloride (NED)
34. Normal agarose (ISC, Bioexpress, Spain)
35. Normal saline solution (NSS; General Hospital Products Public Co., Thailand)
36. Oxoid nutrient broth No.2 (HiMedia Laboratories Pvt. Ltd., Mumbai, India)
37. Phosphoric acid (Merck, Germany)
38. Quercetin (Sigma-Aldrich, St. Louis, U.S.A.)
39. Sabouraud Dextrose agar and broth (Merck, Germany)
40. Sodium ammonium hydrogen phosphate tetrahydrate (Fluka AG, Buch, Switzerland and Sigma-Aldrich, St. Louis, U.S.A.)
41. 0.9% sodium chloride solution (Sigma Chemical Co., USA)
42. Sodium Hydroxide (Ajax Finechem Pty, Ltd.)
43. Sodium nitrite (Ajax Finechem Pty Ltd.)
44. Sodium nitroprusside (Sigma-Aldrich, St. Louis, U.S.A.)
45. Silica gel 60 F254 pre-coated TLC plates (Merck, Germany)
46. Sulfanilamide (Sigma-Aldrich, St. Louis, U.S.A.)
47. Trisma base (Cleveland, Ohio 44128)
48. 2% Tween 80 (Sigma Chemical Co., USA)

Instruments

1. AmaZon SL Ion Trap LC-MS (Bruker Daltonics)
2. Digital thermometer (YSI PrecisionTM model 4000A, USA)
3. Dionex C-16 Acclaim RSLC Polar Advantage column (2.1 × 100 mm, 2.2 µm, 120 °A, Dionex)
4. HPLC-PDA (SHIMADZU gradient system, Japan)
5. Hot-plate analgesiometer

6. Microscope (Zeiss Axioskop, Germany)
7. Plethysmometer
8. Rota-rod

Animals

Male Wistar rats weighing 140-180 g at age of 5 weeks obtained from National Laboratory Animal Center, Mahidol University, Salaya, Nakorn Pathom were served as experimental subjects for anti-pyretic activity testing. All animals were housed in the animal facility of the Faculty of Pharmaceutical Sciences, Chulalongkorn University and maintained under the environmentally controlled condition with temperature of 25 ± 2°C, 50-60% of humidity, 12 h light -12 h dark cycles. All animals had accessed to the standard pellet diet (Perfect Companion Group Company Limited, Thailand) and tap water *ad libitum*. The animals were allowed to acclimate to the facility for 3-5 days before starting the experiments. At the end of each experiment, the animals were sacrificed with carbon dioxide.

Male ICR mice weighing 18-25 g at age of 5 weeks obtained from National Laboratory Animal Center, Mahidol University, Salaya, Nakorn Pathom were served as experimental subjects for anti-inflammatory and anti-nociceptive activities testing. All animals were housed in the animal facility of the Faculty of Pharmaceutical Sciences, Chulalongkorn University and maintained under the environmentally controlled condition with temperature of 25 ± 2°C, 50-60% of humidity, 12 h light - 12 h dark cycles. All animals had accessed to the standard pellet diet (Perfect Companion Group Company Limited, Thailand) and tap water *ad libitum*. The animals were allowed to acclimate to the facility for 3-5 days before starting the experiments. At the end of each experiment, the animals were sacrificed with carbon dioxide.

Ethical consideration

The Institutional Animal Care and Use Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, approved all experimental protocols.

Plant materials

The roots of *A. marmelos, D. longan, D. serrulata, O. indicum,* and *W. trichostemon* were collected from different geographical areas in Thailand. All set of crude drugs were authenticated by Ruangrungsi N. and compared to the herbarium at Department of National Parks, Wildlife and Plant Conservation, Ministry of Natural Resources and Environment, Bangkok, Thailand. Voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Thailand. All samples were performed to evaluate the pharmacognostic specifications of the five root species in Ben-Cha-Moon-Yai remedy according to WHO guideline for Quality control methods for medicinal plant materials.

Crude extract preparation

Roots of *A. marmelos, D. longan, D. serrulata, O. indicum,* and *W. trichostemon* were collected from Chiang Rai, Tak, Surin and Nakhon ratchasima Provinces of Thailand. They were collected during July – December 2009. All set of crude drugs were authenticated by Ruangrungsi N. Voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Thailand. The roots were shade-dried and ground to coarse powders. The powder of each root was continuously macerated with ethanol and water respectively until exhaustion. The ethanol extracts were filtered through Whatman No.4 and evaporated under vacuum, whereas the water extracts were lyophilized to dryness. The remedy extract was prepared by mixing each extract in the quantity equivalent to the formula. The extract yields were weighed, recorded and stored at -20 °C until use to decrease the possibility of degradation of active compounds.

Quality assessments

Pharmacognostic specifications [1, 5]

The roots of five species in Ben-Cha-Moon-Yai remedy from various sources throughout Thailand were examined according to the WHO guidelines of quality control method for medicinal plant materials. Establishment of the pharmacognostic profile of five roots species in Ben-Cha-Moon-Yai remedy was done to guarantee quality, purity and identification of remedy.

Morphological identification

The macroscopic evaluation of medicinal plant materials was illustrated based on the shape, size, colour, surface characteristics, texture, fracture and appearance of the cut surface. The microscopic identification was performed under microscope to identify the structural features, cells, and ergastic substances of plant samples with application of the knowledge of plant morphology and anatomy so as to authenticate plant species [5]. Safranin was prepared as a staining solution. The drawing was made using microscope with imaging system.

Determination of loss on drying

The determination for loss on drying content was performed to estimate the loss of both water and volatile matter in crude drugs. Three grams of the air dried powdered drug was accurately weighed in a dried and pre-weighed crucible, then crude drug was directly dried at 105 °C in a hot-air oven until the constant weight was obtained. The loss of mass was expressed as per cent by weight. The test was done in triplicate.

Determination of water content

Azeotropic distillation method using a water immiscible solvent was performed to measure the water presented in the plant materials. The sample was distilled together with water saturated toluene then the water presented in the plant

materials was separated by the solvent. The volume of water completely distilled was read off and calculated as percent by weight. The test was done in triplicate.

Determination of ash values

Ash values were used to indicate presence of various impurities of crude drug such as the earthy matter or inorganic salt of carbonate, oxalate and silicate. The ashes remaining after incineration of plant material was determined by two different methods to measure the total ash and acid-insoluble ash. The total ash method was conducted by burning 3 grams of powdered drug in a pre-weighed crucible at 500 °C for 6 h to observe the carbonless ash including both “physiological ash” and “non-physiological ash” which was further weighted. After that 25 ml of 2N HCl was added into the remaining ash and gently boiled. It was filtrated and burned at 500 °C for 6 h then measured the amount of silica presented, especially from sand and siliceous earth to obtain the percent of acid insoluble ash. The test was done in triplicate.

Determination of extractive value

The determination of solvent extractive values was performed to evaluate the amount of active constituents capable to be dissolved in distinguished solvents. Five grams of powdered crude drugs was macerated in 100 ml of ethanol and water, separately. After shaking for 6 hours and standing for 18 hours, it was filtrated. The marc was rinsed and the filtrate was adjusted to 100 ml. The aliquot (20 ml) was pipetted into a pre-weighed 50 ml beaker and evaporated to dryness on a water-bath. The extract was further dried in a hot-air oven at 105 °C until the constant weight was obtained. The test was done in triplicate.

Thin-layer chromatographic identification [116]

Thin-layer chromatography is particularly valuable for the qualitative and quantitative analysis as well as purification of herbal medicines. TLC is widely used for a first screening step to analyze many different substances because it is one of the most simple, efficient, and economical methods for separation and analytical

determination of chemical compounds [117-118]. The process includes two basic steps following sample application and chromatogram developments, which may affect the sample [119].

The ethanolic extracts of each five root species were dissolved in methanol (10 mg/ml). Five microliter of each sample was applied into the TLC plate. Samples was directly compared and identified by the fluorescence and colour reaction of the developed spots. Silica gel 60 F_{254} pre-coated TLC plates 0.063–0.200 mm was used and developed over a 20 cm path. The plate was placed into a tank with sufficient suitable solvent to just wet the lower edge of the plate sorbent but not enough to wet the part of the plate where the spots were applied. The solvent front then migrates up the plate through the sorbent by capillary action, a process known as development. Remove the plate, mark the position of the solvent front and allow the solvent to evaporate at room temperature.

The spots were visualized under UV light at 254 nm and 365 nm; then spray with detecting reagent (10% sulfuric acid in methanol) and heat at 110 ◦C for 10 min. The information provided by a finished chromatogram includes the “migrating behavior” of the separated substances. After development, the plate was dried in an oven or fume hood to evaporate the solvent. Compounds were detected on thin layers by their natural colour, natural fluorescence under UV light, quenching of fluorescence on a phosphor containing layer, or as colored, UV absorbing, or fluorescent zones after reaction with an appropriate reagent.

High performance liquid chromatographic analysis

Fingerprinting spectral analysis represented by high performance liquid chromatography combines with online UV spectrum detection *via* diode array configuration has been used as a valuable tool for the quality assurance of crude drugs and compound preparations [120]. To develop the representative fingerprint of Ben-Cha-Moon-Yai remedy and five root species injection, ten milligrams of the ethanolic extract from each root species and Ben-Cha-Moon-Yai remedy extracts were dissolved in 1 ml of HPLC grade methanol then filtered through a 0.45 µm membrane

filter. HPLC-PDA analysis was performed with a SHIMADZU gradient system (Japan) equipped with LC-20AD pumps, a CTO-20AC column oven, DGU-20A3 degasser and a SPD-M20A diode array detector (DAD) set λ ranged 190-800 nm. Separation was carried out with an Inersil® ODS-3, C-18 column (particle size of the packing 5 µm, 4.6 x 250 mm) and HPLC guard column (5µm, 4.0x10 mm). The elution of mobile phase was performed by 10mM phosphoric acid-acetonitrile linear gradient (95:5) over 65 min at flow rate of 0.8 ml/min. The injection volume was 10 µl.

Liquid chromatography-mass spectrometry [121-125]

Liquid chromatography/mass spectrometry (LC-MS) is the combination of liquid chromatography and mass spectrometry. It has been grown into one of the most powerful analytical techniques currently available, because this technique can be characterized a wide variety of plant constituents ranging from small molecules to macromolecules such as peptide, proteins, carbohydrates and nucleic acids. LC-MS has provided a high level of sensitivity and selectivity and widely used in the analysis of complex mixtures in Chinese medicinal product research.

The separation can be accomplished *via* standard liquid chromatography. The mobile phase will moves the solute throughout column. The output can be directed into the mass analyzer *via* an electrospray ionization source. They deflect ions down a curved tube in a magnetic fields based on their kinetic energy determined by the mass, charge and velocity. The magnetic field is scanned to measure different ions. In a LC-MS the column flow is continuous, meaning the mass analyzer must be fast enough to thoroughly analyze one peak before the next one comes off the column.

The selection of the control substances and their fingerprints of Ben-Cha-Moon-Yai remedy and five root species extract were investigated by using Liquid chromatography-Electrospray ionization-Mass spectrometry (LC-ESI-MS). For the sample preparation, 1 mg of Ben-Cha-Moon-Yai remedy and five root species extracts were dissolved in methanol and filtered. A 100 µl of the continual filtrate was

diluted with methanol to 1 ml and filtered through a 0.45 µm of membrane filter before analysis.

The LC-ESI-MS analyses were performed with amazon SL Ion Trap LC/MS (Bruker Daltonics) instrument equipped with the standard ESI ion source (Nebulizer pressure: 25 psi; Drying gas flow rate: 8 L/min; Drying gas temperature: 280 °C). MS spectra were acquired in Ultra Scan mode between m/z 70-1500, using positive ionization. Chromatographic separations were carried out using a Dionex C16 Acclaim RSLC Polar Advantage column (2.1 x 100 mm, 2.2 µm, 120 °A) maintained at 35 °C on a Dionex Ultimate 3000 Rapid Separation LC system. The mobile phase consisted of (A) Water and (B) Acetonitrile. HPLC gradient conditions were showed in figures.

Safety assessments

Cytotoxicity in brine shrimp lethality assay

The preliminary toxicity investigation is brine shrimp lethality testing described by Meyer *et al*., 1982 and used as a "Benchtop bioassay" for natural medicine discovery [126]. Brine shrimp lethality bioassay was carried out according to the procedure described by Meyer *et al*., 1982 [127]. Brine shrimp eggs were hatched in artificial sea water. After 48 hours of incubation, ten brine shrimps were transferred to each sample vial using a Pasteur pipette and artificial sea water was added to make 5 ml. Filter papers impregnated with extracts at the concentration of 1000, 100 and 10 µg/ml in methanol were air dried before placed in vials containing the brine shrimps. Control was prepared as mentioned above using only methanol instead. Five replicates were prepared for each concentration. The vials were maintained under illumination. Twenty-four hours later, the number of survivors was counted and recorded. The concentration which caused 50% of brine shrimp lethality (LC_{50} value) was obtained from a plot of percentage of the shrimp nauplii killed against the concentrations of the extracts.

Mutagenic activity assay (Ames test)

The Ames Salmonella assay is a short-term *in vitro* testing which has gained popularity from the large number of chemical compounds to investigate their genotoxicity and modulation effect on the mutagenic response [128] toward *Salmonella typhimurium* tester strains due to a quick and relatively inexpensive assay [129]. In this study, the mutagenic and antimutagenic activity of root extracts and Ben-Cha-Moon-Yai remedy were study in the absence of enzyme activating system using the pre-incubation method of Maron and Ames in 1983 [130] to observe the response of the extracts in an acidic condition.

Preparation of the bacterial suspension

Salmonella typhimurium strain for frame-shift mutation, TA98 (*hisD3052, bio, uvrB-bio, rfa,* and *pKM101*) and strain for base-pair substitution mutation, TA100 (*hisG46, bio, uvrB-bio, rfa,* and *pKM101)* were kindly provided by the Biochemistry and Chemical Carcinogenesis Section, Research Division, National Cancer Institute, Bangkok, Thailand. All tester strains were grown in an Oxoid nutrient broth No.2 and incubated overnight in a shaking water bath at 37 °C. The culture were re-isolated by streaking the bacteria on a minimal glucose agar plates enriched with ampicillin, L-histidine HCl and biotin, then incubated at 37 °C for 48 h. After incubation, picked a well isolated colony with a sterile loop, then cultured overnight in an Oxoid nutrient broth No.2 at 37 °C in a shaking water bath. The mutant strains were confirmed for the genotypes of histidine/biotin dependence, *rfa* marker, *uvrB* deletion gene mutations and presence of plasmid *pMK101*.

Mutagenicity assay

The pre-incubation method of Ames test was employed to determine the mutagenic effect of the root extracts and BMY on both strains of *S. typhimurium* without enzyme activating system [131]. Briefly, the ethanol extracts were dissolved in DMSO, the water extracts and BMY were dissolved in water to the concentration of 25, 50, 100 and 200 mg/ml. A 200 µl of each solution was added to the tube containing 550 µl of 0.2N HCl to acidify the reaction mixture to pH 3-3.5. Adjusted

the final volume to 1 ml with 250 µl of solvent (DMSO or water). For mutagenic assay with nitrite treatment, adjusted the final volume to 1 ml with 250 µl of 2M sodium nitrite instead. Each reaction tube was shaken at 37 °C for 4 h then placed in an ice bath for 1 min to stop the reaction. Finally, added 250 µl of solvent or 2M ammonium sulfamate (for nitrite treatment) and allowed the tube to stand in an ice bath for 10 min. Mixed 100 µl of this extract mixture with 100 µl of bacterial suspension and 0.5 ml of 152mM phosphate buffer (pH 7.4), pre-incubated at 37 °C for 20 min then 2 ml of molten top agar containing 5mM L-histidine and 5mM D-biotin was added, mixed well and poured onto a minimal glucose agar plate. The final concentration of the root extracts and BMY were 0.4, 0.8, 1.6 and 3.2 mg/plate. The plates were incubated at 37 C for 48 h and the numbers of *his*$^{+}$ revertant colonies on each plate were counted. DMSO or water was used as a negative control to determine the spontaneous reversion activity. 1-Aminopyrene was used to interact with sodium nitrite in acid solution to produce a standard direct mutagen for positive control. Ten microliters (tested on TA98) or 20 µl (tested on TA100) of 1-aminopyrene (0. 0375 mg/ml) was mixed with 740 µl or 730 µl of 0.2N HCl and 250 µl of 2M sodium nitrite was added to obtain the final volume of 1 ml [132]. The results were reported as mean revertant colonies per plate ± the S.D.

The mutagenic index (MI) was calculated from the number of revertant colonies of the sample treatment divided by the number of spontaneous revertant colonies. Positive mutagenic effect was considered when the number of induced revertant colonies increase in a dose response relationship manner, at least two doses were higher than spontaneous revertants and at least one dose gave rise to twice over the spontaneous revertant (MI > 2) [133].

DNA damage assay (Comet assay)

The comet assay or single cell gel electrophoresis assay (SCGE) assay is a rapid, sensitive, reliable and relatively simple method for detecting DNA strand breaks in eukaryotic cells [134]. It has become one of the standard methods for assessing DNA damage which combines the simplicity of biochemical techniques for detecting DNA both single and double strand breaks, alkali labile sites and cross-linking, with the single cell approach typical of cytogenetic assays [135]. It is based on quantitative technique by visual evidence of the denatured DNA fragments migrating out of the cell nucleus during electrophoresis [136]. The alkaline (pH > 13) single cell gel electrophoresis assay (SCGE) assay was performed according to the procedure described by Tice *et al*., 2000 [137] with some modification. Once a suspension of human lymphocyte cells were obtained, the basic steps of the assay include (1) preparation of microscope slides layered with cells in agarose; (2) lysis of cells to liberate DNA; (3) exposure to alkali (pH 13) to obtain single-stranded DNA and to express alkalie labile site as single strand break; (4) electrophoresis under alkaline (pH 13) conditions; (5) neutralization of alkali; (6) DNA staining and comet visualization; and (7) comet scoring.

Isolated lymphocytes

Pooled blood specimens were aseptically collected in heparinized sterile tube from healthy individuals. The lymphocytes were isolated with Ficoll-Histopaque 1077 using a density gradient centrifugation technique [138]. Blood was diluted with phosphate buffer saline pH 7.4 at 1:1 in a conical centrifuge tube and gently homogenized to prepare whole blood. A 3 ml of Ficoll-Histopaque 1077 was aliquot into a conical tube and layered over with 3 ml of whole blood. The blood was centrifuged at 1800 rpm at 4 °C for 30 min. The interface of Histopaque containing lymphocytes was taken by using a pasture pipette and place in a conical 15 round bottom. Aliquot the lymphocytes to 5 ml and wash the cell with 10 ml of phosphate buffer saline pH 7.4, then dispersed with a pasture pipette and gently homogenized. The cells were centrifuged at 1600 rpm at 4 °C for 10 min. The supernatant was discarded and added 10 ml of RPMI-1640. Centrifuged for 1600 rpm at 4 °C for 10

min and removed the supernatant. The number of lymphocytes was counted using a hemocytometer and the viability of the cells was demonstrated by the trypan blue exclusion method. RPMI-1640 was added to prepare the lymphocyte suspension approximately 4×10^5 cell/ml. Aliquoted 400 µl in an eppendorf tube, preserved with fetal bovine serum containing 10% DMSO and kept in -80 °C.

Comet assay procedures

Human lymphocyte cells were treated with the ethanol and water extracts of five root species and BMY, incubated at 37 °C for 1 hour. After that, the test samples were centrifuged at 3000 rpm at 4 °C for 5 min, discarded RPMI-1640 and added 10 µl of phosphate buffer saline pH 7.4 in to adherent cells in test tubes. End frosted conventional slides were pre-coated with 1 % Normal melting agarose. Slides were dipped into molten normal meting agarose for 2/3 of their length and left to dry at room temperature. An 85 µl of treated sample was layered into the pre-coated slide, placed the cover slips over the second layer and kept on ice to solidify agarose for 10 min. After the agarose gel has solidified, the cover slips were removed. The third layer was applied by 0.5 % of low melting point agarose and allowed to solidify on ice for 10 min. The cover slips were removed and immersed the slides in freshly prepared and chilled lysis solution containing 2.5 M NaCl, 100 mM EDTA, 10 mM Tris (pH 10) with 10% of DMSO and 1% of Triton X-100 being added just before use. After that, the slides were incubated at 4 °C for 1 hour. After lysis process, the slides were drained and placed in a horizontal gel electrophoresis tank, side by side avoiding spaces and with the agarose ends nearest to the anode. Fresh and chilled electrophoresis solution containing 1 mM Na_2 EDTA and 300 mM NaOH, pH > 13 was poured in the electrophoresis tank to a level approximately 0.25 cm above the slides. The slides were left in this solution for 25 min to allow DNA unwinding and expression of alkali labile sites as DNA breaks. The electrophoresis was conducted under alkali condition at 0.7 v/cm for 25 min. After electrophoresis, the slides were placed horizontally and neutralization buffer containing 0.4 M Tris buffer, pH 7.5 was added, and allowed to sit for 5 min with three times. Each slide was stained with 75 µl of 20 µg/ml Ethidium bromide for 5 min. Hydrogen peroxide treated cell was used as a positive control whereas phosphate buffer saline was used as a negative control.

Slides were placed in a dark humidified chamber to prevent drying of the gel and analyses within 3-4 hour [137]. The DNA migration was observed under a fluorescent microscope attached to image capture device with a final magnification of 400x. The degrees of damage were classified by visual scoring technique based on the length of the comet tail visualized [139]. The five classes, from 0 (no tail) to 4 (almost all DNA tail) gave sufficient resolution. 100 comets were scored, and each comet assigned a value of 0 to 4 according to its class, the total score for the sample gel were between 0 and 400 “arbitrary units” [134].

Efficacy assessments

Anti-mutagenic activity assay (Ames test)

The antimutagenic effect of all extracts against 1-aminopyrene treated with sodium nitrite was determined by the pre-incubation method of Ames test similar to the mutagenic testing. Forty microliters (tested on TA98) or 80 µl (tested on TA100) of 0.075 mg/ml 1-aminopyrene was transferred into the sterile test tube. Then, 710 µl or 670 µl of 0.2N hydrochloric acid and 250 µl of 2M sodium nitrite were added to obtain the total volume at 1 ml. The mixtures were shaken at 37 C for 4 h. Later on, the test tubes were placed in an ice bath for 1 min to stop the reaction mixture. Two-hundred fifty microliter of 2 M ammonium sulfamate was added and allowed the test tube to stand in an ice bath for 10 min. Twenty-five microliter of the mixture above (nitrite-treated 1-aminopyrene) was transferred into the sterile test tube containing various concentrations each extract (5, 10, 15 mg/plate). Distilled water or DMSO was adjusted to the final volume of 100 µl. Then, the mixture was treated as described in the mutagenicity assay. The percent modification was calculated by the following formula: % Inhibition = [(A-B) / (A-C)] × 100. Where A is the number of histidine revertants colonies per plate induced by nitrite treated 1-Aminopyrene, B is the number of histidine revertants colonies per plate induced by nitrite treated 1-aminopyrene in the presence of each extract and C is the number of spontaneous histidine revertants colonies per plate. The percentage of inhibition is classified as strong when it is higher than 60%, moderate ranged from 60-41%, weak ranged from 40-21% and negligible effect when it was 20-0% [140].

Antimicrobial activity assay

Antimicrobial susceptibility testing is based on the growth response of various microorganisms to an antimicrobial agent [5]. In the past few decades, many research groups in the field of ethnopharmacology have been focused on determining the antimicrobial activity of plant extracts which found in folk medicine, essential oils and isolated compounds [141]. The antimicrobial agent derived from medicinal plants could be served as alternative, cheap and safe antimicrobial drugs for the treatment of common ailments [142]. Several methods have been recommended by CLSI to use as a standard protocol for detecting the in *vitro* antimicrobial susceptibility such as agar diffusion, agar dilution and broth dilution method following "Development of In *vitro* Susceptibility Testing Criteria and Quality Control Parameter" guideline. The interpretive criteria serve as zone of inhibition and the minimum inhibition concentration (MIC) values [143]. The agar diffusion method is recommended as a preliminary screening to determine the antimicrobial susceptibility of antimicrobial agents containing in the reservoir such as paper disc or well in the medium. After incubate the antimicrobial agents with an inoculated microorganism, the diameter of the zone around the reservoir is measured [144]. After preliminary screening, the effective extracts are further investigated to determine MIC by using broth dilution method. The tested samples are mixed with an inoculated microorganism. After incubation, the growth of microorganism is observed by direct visual or compares the turbidity of the test culture with a control culture. The lowest concentrations of the tested samples that prevent visible growth of a microorganism in broth dilution are considered as MIC value. The minimum bactericidal concentration (MBC) and the minimum fungicidal concentration (MFC) can be determined by plating out samples of completely inhibited dilution cultures on to solid or liquid media containing no antimicrobial agents.

Microorganisms

The tested human pathogenic microorganisms were described in table 7 includes five gram-positive bacteria, six gram-negative bacteria and two fungal strains.

Table 7 The tested microorganisms

Tested microorganism	
Gram positive bacteria	*Staphylococcus aureus* ATCC 6538P[1]
(Non-spore forming bacteria)	*Micrococcus luteus* ATCC 9341[2]
	Staphylococcus epidermidis (Isolates)[3]
Gram positive bacteria	*Bacillus subtilis* ATCC 6633[1]
(Spore forming bacteria)	*Bacillus cereus* ATCC 11778[2]
Gram negative bacteria	*Escherichia coli* ATCC 25922[1]
(Non-spore forming bacteria)	*Enterobacter aerogenes* ATCC 13048[2]
	Pseudomonas aeruginosa ATCC 9027[1]
	Salmonella typhi (Isolates)[3]
	Salmonella typhimurium (Isolates)[3]
	Shigella spp (Isolates)[3]
Fungi	*Candida albicans* ATCC 10230[1]
	Saccharomyces cerevisiae ATCC 9763[1]

The microorganisms were obtained from the Department of Biochemistry and Microbiology[1], Faculty of Pharmaceutical Sciences the Department of Microbiology, Faculty of Sciences and Technology, Suan Sunandha Rajabhat University[2] and the Department of Microbiology, Faculty of Sciences, Chulalongkorn University[3].

Preparation on inoculums suspensions

All tested microorganisms were cultivated in a Mueller hinton agar (for bacteria) or Sabouraud dextrose agar (for fungi) then incubated overnight on agar media at 37 °C. After incubation, picked a well isolated colony with a sterile loop and suspended in 0.85% of normal saline. The turbidity of bacterial suspension was standardized by adjusting the optical density to 0.08-0.1 absorbance obtained with

spectrophotometer at 625 nm to match with the McFarland turbidity standard No. 5 (approximately 1 to 2 × 10^8 CFU/ml) [145].

Agar well diffusion assay

The antibacterial and antifungal activities testing of Ben-Cha-Moon-Yai remedy and five root species extracts were evaluated by using a slightly modified of agar well diffusion method against 13 microorganisms [146]. A 100 µl of the cell suspension of about 1 x 10^8 CFU/ml obtained from a 0.5 McFarland turbidity standard was mixed with sterile seeded agar and poured on the sterile base gar. Agar wells were cut from seeded agar plates by a cork borer with 6 mm. Twenty microliters of various plant extracts of 200 mg/ml were transferred into each well. Ampicillin and amikacin were used as a positive control whereas dimethylsulfoxide (DMSO) was used as a negative control. The plates were incubated at 37 °C for 24 h (for bacteria) or 48 h (for fungi). The antimicrobial activity was evaluated by measuring the diameters of zone inhibition surrounding the crude extracts. The zones of inhibition were measured in millimetre and reported in all cases includes the diameter of the wells. The experiment was carried out in triplicates. The antimicrobial activity was interpreted according the parameters classified by Alves *et al*., 2000 [147] as follows:

Inhibition zones < 9 mm classified as inactive
Inhibition zones between 9 – 12 mm classified as less active
Inhibition zones between 13 – 18 mm classified as active
Inhibition zones > 18 mm classified as very active.

Broth microdilution method

The MIC, MBC and MFC of the plant extracts that showed the antimicrobial activity were determined by broth dilution method described by EUCAST, 2003 [148]. A microbial suspension in broth was prepared by adding 10 µl of normal saline microbial suspensions to 1 ml of growth media, Muller-Hinton broth (for bacteria) or Sabouraud broth (for fungi). The suspension was adjusted with broth dilution and measured the absorbance of inoculums at 625 nm to obtain 0.08-0.1 which a turbidity equivalent to the 0.5 of McFarland standard. Into a sterile 96-well microplate, 50 µl of microbial suspended in broth was added to the wells containing 50 µl of each plant

extract with two fold dilution (final concentration 2000-3.9 µg/ml for crude extracts and 10-0.019 µg/ml for positive control) or control. Ampicillin and amaikacin were used as a positive control. Negative control was prepared by diluting 50 µl DMSO with broth to obtain final volume of 1 ml.

The lowest concentration of each plant extract inhibiting the growth of test microorganisms which observing the change of turbidity was defined as the MIC of an extract. The content of the known MIC wells was streaked onto fresh nutrient agar plates. The plates were further incubated at 37˚ C for 24 h. The lowest concentration of extract with no microbial growth observed on the plate after this sub-culturing was considered as the MBC or MFC values [142].

Free radical scavenging assay (DPPH assay)

The antioxidant activity of plant extracts was assessed by ability to scavenge DPPH free radical as described by Brand-William *et al*., 1995 [149]. Various concentrations of samples dissolved in methanol were added to DPPH radical methanolic solution (120 µM). After 30 min of incubation at room temperature in the dark, the absorbance was measured at 517 nm with a 96 well microplate reader. BHT and Quercetin were used as positive controls. Three replicates were made for each test sample. The scavenging activity was evaluated from the decrease in absorbance value at 517 nm and calculated using the following formula:

$$\text{DPPH radical inhibition (\%)} = [(A_{control} - A_{sample}) / A_{control}] \times 100.$$

Where $A_{control}$ is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. The activity was expressed as IC_{50} values which indicate the concentration of sample required to scavenge 50% of DPPH free radical.

Lipid peroxidation testing (β-carotene bleaching assay)

The antioxidant activity of Ben-Cha-Moon-Yai remedy and five root species extracts were evaluated by the β-carotene bleaching assay according to the method of Jayaprakasha *et al*., 2002 [150] with some modification. β-carotene in this model system, undergoes rapid discoloration in the absence of an antioxidant. This decolorization can be diminished or prevent by classic antioxidants that donate hydrogen atoms to quench radicals. Briefly, 1 mg/ml of β-carotene, 40 mg of linoleic acid and 400 mg of Tween 20 were mixed in 4 ml of chloroform. Chloroform was removed at 40 °C under vacuum using rotary evaporator. The resulting mixture was immediately diluted with 100 ml of distilled water with vigorous agitation. Aliquots (4 ml) of this emulsion were transferred into different test tubes containing 0.2 ml of test samples in ethanol or water. BHA and Quercetin were used for comparative purposes. A control, containing 0.2 ml of ethanol or water and 4 ml of the above emulsion, was prepared. The tubes were placed at 50 °C in a water bath. Absorbance of all the samples at 450 nm were taken at zero time (t=0). Measurement of absorbance was continued until the colour of β-carotene disappeared in the control reaction (t=180) at 30 min intervals. All determination was carried out in triplicate. Dose-response relationships of antioxidant activity for the extracts were determined at different concentrations. The antioxidant activity (AA) of the extracts was evaluated in the terms of bleaching of the β-carotene using the following formula:

$$\text{The antioxidant activity (\%)} = [1-(A_0-A_{180}) / (C_0-C_{180})] \times 100$$

Where, A_0 and A_{180} are the absorbance values measured at zero time and end time of the incubation for test sample, respectively. C_0 and C_{180} are the absorbance measured at the zero time and end time of the incubation for control, respectively.

Nitric oxide scavenging assay (Griess reagent assay)

Nitric oxide (NO) is a multifunctional free radical involved in the regulation of cell functions [151]. Nitric oxide (NO) is an important physiological messenger and effecter molecule in many biological systems, including immunological, neuronal and cardiovascular tissues [152]. Nitric oxide is generated by sodium nitroprusside in aqueous solution at physiological pH spontaneously which interacts with oxygen to produce nitrite ions [153]. This intermediate is then allowed to react with a coupling reagent, *N*-naphthyl-ethylenediamine (NED), (Griess reagent) to form a stable azo compound. This intense purple color of the product allows nitrite assay with high sensitivity and can be used to measure nitrite concentration as low as 0.5 µM level. The absorbance of this adduct at 540 nm is linearly proportional to the nitrite concentration in the sample [154].

Ben-Cha-Moon-Yai remedy and five root species extract in DMSO or water (8 mg/ml) were diluted with phosphate buffer saline pH 7.4 to obtain different concentrations of the extracts. The assay was carried out according to Ramli *et al*., 2011 [155] with modification. In a 1-cm path cuvette, 500 µl of the extract or control was added into 500 µl of 5mM sodium nitroprusside. The mixture was allowed to incubate at room temperature for 120 min. Then, 1 ml of Griess reagent with contains 1% sulfanilamide in 2% H_3PO_4 and 0.1% of *N*-naphthyl-ethylenediamine (NED) in distilled water was added into the test cuvette. The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and subsequent coupling with *N*-naphthyl-ethylenediamine was immediately measured at 540 nm. The nitric oxide scavenging ability of the extract was calculated as follow:

$$\text{Nitric oxide scavenging activity (\%)} = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

Where, A_{sample} is the absorbance values measured end time of the incubation for test sample. $A_{control}$ is the absorbance measured at end time of the incubation for control, respectively.

Total phenolic contents

Quantification of total phenolic content of extract was determined using Folin-Ciocalteu's phenol reagent modified from Emmy *et al*., 2009 [156]. Phenolic compounds in the extract will form a blue color complex with Folin- Ciocalteu reagent after adjusted with alkali. Briefly, 640 µl of plant extract in methanol (0.5 mg/ml) was pipetted into each vial, followed by 160 µl of 15% Folin-Ciocalteu. Distilled water was added and adjusted volume to 1600 µl. The mixture was left for 5 min. Added 800 µl Na_2CO_3 aqueous (0.106 g/ml); then incubated at room temperature for 60 min and measured the absorbance at 756 nm. Different concentrations of catechin hydrate (1, 2, 5, 10, 20, 30, 40, 50 µg/ml) were used to prepare a standard curve. The concentration of total phenolic compounds in all extract was expressed as mg of catechin hydrate equivalents per gram dry weight of extract using a linear equation.

Evaluation of antipyretic activity by animal model

Fever is a complex physiologic response of infection, tissue damage, inflammation, malignancy, graft injection and other inflammatory disease conditions. The pathogenesis of fever described a regulated rise in body temperature after an increase in the hypothalamic set point [157]. There is an increased formation of pro-inflammatory mediators which synthesize many cytokines such as interleukin 1(IL-1), interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) from infected or damaged tissues [158]. Current knowledge of the pathogenesis of fiver is based primarily upon studies of fever induced by the intravenous injection of bacterial pyrogen such as lipopolysaccharide (LPS) from gram-negative bacteria [159]. Lipopolysaccharide (LPS) can stimulate myeloid cells which further synthesize many cytokines and inducing a general homeostatic reaction, serving as the organism's first line of defense against infection and causing fever finally [160]. The present study was aimed to investigate the antipyretic activity of Ben-Cha-Moon-Yai remedy and five root species extracts in the rat hyperthermia induced by LPS.

Preparation of plant extracts

The five root species extracts was prepared from the mixture of the ethanol and water extract of each root species. A 2% Tween 80 was used as a vehicle. Various doses of *A. marmelos, O. indicum, D. longan*, *D. serrulata, W. trichostemon* (25, 50, 100, 200 and 100 mg/kg) and Ben-Cha-Moon-Yai remedy extracts (125, 250 and 500 mg/kg) and aspirin (300 mg/kg) were suspended in 2% Tween 80. LPS was dissolved in 0.9% sodium chloride solution. Aspirin was used as a standard antipyretic drug. In the control group, animals were received only the vehicle with equivalent volume in the same route.

Lipopolysaccharide-induced fever

The modification method of lipopolysaccharide-induced fever in rat described by Santos and Rao in 1998 [161] was performed to determine the antipyretic activity of Ben-Cha-Moon-Yai remedy and five root species extracts (AM, OI, DL, DS, and WT). The number of animals used in each treatment was six per group. The animals were fasted overnight before the experiments. Each animal was kept in a restrainer for 1 hr to acclimatize to its new environment. Fever was induced with 50 µg/kg of LPS injected intramuscularly into the thigh of the rat. The animals were pretreated orally with 2% Tween 80 solution (10 ml/kg), acetylsalicylic acid (ASA; 300 mg/kg), various doses of AM, OI, DL, DS, and WT (25, 50, 100, 200 and 400 mg/kg) or BMY (125, 250 and 500 mg/kg) 1 h before injection of LPS. Normal rats were received 2% Tween 80 solution (10 ml/kg) orally 1 h before 0.9% normal saline solution (NSS) injection. Rectal temperature was measured 1 h before the pretreatment of animals and at 1 h intervals for 7 h after the administration of the bacterial endotoxin (LPS) with a lubricated digital thermometer inserted 3-4 cm deep into the rectum of the rats. The rectal temperature of normal rats was also measured at 1 hr intervals for 7 hr. The control experiment involved animals treated with 2% Tween 80 plus LPS. All experiments were carried out between 08.00 h and 18.00 h in a quiet laboratory with an ambient temperature of 25 ± 2°C.

Generating experimental groups

Normal rats (n=6): Animals were pre-treated orally with 2% Tween 80 solution (10 ml/kg) 1 h before 0.9% normal saline solution (NSS) injection

Control group (n=6): Animals were pre-treated orally with 2% Tween 80 solution (10 ml/kg) 1 h before intramuscularly injected of 50 µg/kg LPS into the thigh of the rat.

LPS-Aspirin group (n=6): Animals were pre-treated orally with acetylsalicylic acid (ASA; 300 mg/kg) 1 h before intramuscularly injected of 50 µg/kg LPS into the thigh of the rat.

LPS-BMY group (n=6): Animals were pre-treated orally with BMY (125, 250 and 500 mg/kg) 1 h before intramuscularly injected of 50 µg/kg LPS into the thigh of the rat.

LPS-AM group (n=6): Animals were pre-treated orally with AM (25, 50, 100, 200 and 400 mg/kg) 1 h before intramuscularly injected of 50 µg/kg LPS into the thigh of the rat.

LPS-DL group (n=6): Animals were pre-treated orally with DL (25, 50, 100, 200 and 400 mg/kg) 1 h before intramuscularly injected of 50 µg/kg LPS into the thigh of the rat.

LPS-DS group (n=6) : Animals were pre-treated orally with DS (25, 50, 100, 200 and 400 mg/kg) 1 hr before intramuscularly injected of 50 µg/kg LPS into the thigh of the rat.

LPS-OI group (n=6): Animals were pre-treated orally with OI (25, 50, 100, 200 and 400 mg/kg) 1 h before intramuscularly injected of 50 µg/kg LPS into the thigh of the rat.

LPS-WT group (n=6): Animals were pre-treated orally with WT (25, 50, 100, 200 and 400 mg/kg) 1 h before intramuscularly injected of 50 µg/kg LPS into the thigh of the rat.

Figure 8 Digital Thermometers (YSI PrecisionTM 4000A)

Evaluation of anti-inflammatory and anti-nociceptive activities by animal model

Preparation of plant extracts

The root extract of Ben-Cha-Moon-Yai remedy (BMY; 125, 250 and 500 mg/kg) and five root species extracts: *Aegle marmelos* root extract (AM; 25, 50, 100, 200 and 400 mg/kg mg/kg), *Oroxylum indicum* root extract (OI; 25, 50, 100, 200 and 400 mg/kg), *Dimocarpus longan* root extract (DL; 25, 50, 100, 200 and 400 mg/kg), *Dolichandrone serrulata* root extract (DS; 25, 50, 100, 200 and 400 mg/kg) and *Walsura trichostemon* root extract (WT; 25, 50, 100, 200 and 400 mg/kg). Morphine sulfate, formalin, acetic acid and λ-carrageenan were dissolved in normal saline solution. Indomethacin, the root extract of Ben-Cha-Moon-Yai remedy and five root species extracts of Ben-Cha-Moon-Yai remedy were suspended in 2% Tween 80 solution. Morphine sulfate and indomethacin were used as standard analgesic drugs. Indomethacin was also used as a standard anti-inflammatory drug. The control animals were given with an equivalent volume of vehicle via the same route.

Evaluation of anti-inflammatory agents by animal model

Inflammation is a host defense mechanism living tissue in response to mechanical injury, tissue ischaemia, autoimmune processes or infectious agents caused by microbial infection and other noxious stimuli [162]. It generally caused by release of different mediators such as the prostaglandins (PGs), leukotrienes (LTs), histamine, bradykynin, platelet-activating factor (PAF) and interleukin-1 [163]. Inflammation is the central communication network and regulatory process that senses and controls threat, damage, containment, and healing, which are all critical aspects in the maintenance of organism's integrity. Inflammation is constitutive and ubiquitous, and its role in a wide spectrum of diseases and responses to diseases is increasingly recognized [164]. The inflammatory response can lead different diseases, such as rheumatoid arthritis, inflammatory bowel disease, psoriasis.[(71)] The inflammation are divided into two types including acute inflammation and chronic inflammation [5]. The four famous signs of inflammation are warmth, redness, pain and swelling [163]. Various *in vitro* and *in vivo* models have been proposed to detect anti-inflammatory effect.

The carrageenan-induced paw edema in rat, originally described by Winter *et al* in 1962 is the most common screening model to assess the anti-inflammatory effect of natural products [165]. The subcutaneous injections of carrageenan stimulate the action of pro-inflammatory agents such as bradkykinin, histamine, tachykinins, complement and reactive oxygen and nitrogen species which are caused edema, hyperalgesia, and erythema. The inflammatory response is usually quantified by increase in paw size (edema) which is maximal around 5 h post-carrageenan injections and is modulated by inhibitors of specific molecules within the inflammatory cascade [166-167].

Carrageenan-induced paw edema in mice

Carrageenan induced edema in the hind paw test was used to assess the anti-inflammatory activity of Ben-Cha-Moon-Yai remedy and five root species extracts according to the method described by Winter *et al*. in 1962 [168]. Animals were pretreated orally with 2% Tween 80 (10 ml/kg), indomethacin (IND; 10 mg/kg), various doses of Ben-Cha-Moon-Yai remedy (BMY; 125, 250 and 500 mg/kg) and five root species extracts (25, 50, 100, 200 and 400 mg/kg). After one hour, 1% carrageenan solution (50 µl) was subcutaneously injected into the plantar surface of the left hind paw of each mouse [169]. The mouse's paw was marked with black ink at the level of the lateral malleolus and immersed in saline up to this mark. The paw volume was measured before and after injection of carrageenan at 1, 2, 3, 4, 5 and 6 hr using plethysmometer (Ugo Basile, Italy). Edema was expressed as the increase in paw volume due to carrageenan injection relative to control animals [170]. The percentage of inhibition of edema was analyzed using the following formula:

$$\% \text{ Inhibition of edema} = [(V_c - V_t) / V_c] \times 100$$

Where V_c is the edema volume in control group; V_t is the edema volume in tested group.

Generating experimental groups

Control group (n=6): Animals were pretreated orally with 2% Tween 80 solution (10 ml/kg) 1 hr before intramuscularly injected of 50 µg/kg LPS into the thigh of the rat.

LPS-Aspirin group (n=6): Animals were pretreated orally with acetylsalicylic acid (ASA; 300 mg/kg) 1 hr before 1% carrageenan solution (50 µl) were injected subcutaneously into the plantar surface of the left hind paw of each mouse.

LPS-BMY group (n=6): Animals were pretreated orally with BMY (125, 250 and 500 mg/kg) 1 hr before 1% carrageenan solution (50 µl) were injected subcutaneously into the plantar surface of the left hind paw of each mouse.

LPS-AM group (n=6): Animals were pretreated orally with AM (25, 50, 100, 200 and 400 mg/kg) 1 hr before 1% carrageenan solution (50 µl) were injected subcutaneously into the plantar surface of the left hind paw of each mouse.

LPS-DL group (n=6): Animals were pretreated orally with DL (25, 50, 100, 200 and 400 mg/kg) 1 hr before 1% carrageenan solution (50 µl) were injected subcutaneously into the plantar surface of the left hind paw of each mouse.

LPS-DS group (n=6): Animals were pretreated orally with DS (25, 50, 100, 200 and 400 mg/kg) 1 hr before 1% carrageenan solution (50 µl) were injected subcutaneously into the plantar surface of the left hind paw of each mouse.

LPS-OI group (n=6): Animals were pretreated orally with OI (25, 50, 100, 200 and 400 mg/kg) 1 hr before 1% carrageenan solution (50 µl) were injected subcutaneously into the plantar surface of the left hind paw of each mouse.

LPS-WT group (n=6): Animals were pretreated orally with WT (25, 50, 100, 200 and 400 mg/kg) 1 hr before 1% carrageenan solution (50 µl) were injected subcutaneously into the plantar surface of the left hind paw of each mouse.

Figure 9 Plethysmometer. The larger one (A) is used to measure fluid displaced by the paw, a volume change that is precisely mirrored in the smaller tube (B) containing a transducer which is linked to a decoder capable of digitally displaying volumes.

Evaluation of anti-nociceptive activity by animal model

Pain is a multidimensional sensory experience that is intrinsically unpleasant and associated with hurting and soreness [171]. The International Association for the study of Pain has defines the definition of pain as "*an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage*" [172]. Pain are divided into four types includes (1) nociceptive pain, (2) neuropathic pain, (3) inflammatory pain, and (4) functional pain [171]. The nociceptive pain system is a key early warning device, an alarm system that announces the presence of a potentially damaging stimulus [171]. Nociception is the process by which intense thermal, mechanical or chemical stimuli are detected by subpopulation of peripheral nerve fibers, called nociceptors. The cell bodies of nociceptors are located in the dorsal root ganglia (DRG) for the body and the trigerminal ganglian for the face, and have both a peripheral and central axonal branch that innervates their target organ and the spinal cord, respectively [173]. The nociceptive pain pathway is start from the activation of peripheral pain receptor also called nociceptors by noxious stimuli generates signals that travel to the dorsal horn of the spinal cord. From the dorsal horn, the signals are carried along the ascending pain pathway or the spinothalamic tract to the thalamus and cortex. Pain can be controlled by pain-inhibiting and pain-facilitating neurons. Descending signals originating in supraspinal centers can modulate activity in the dorsal horn by controlling spinal pain transmission [171].

Hot-plate test

The hot-plate test is used to assess the thermal perception of the mice hind paw. The paw of mice is very sensitive to heat at temperatures which are not damaging the skin. Thermal withdrawal latency which is the duration from the start of heat stimulation until the withdrawal or licking the hind paw is obtained. The time until these responses occur is prolonged after administration of centrally acting analgesics. The hot-plate test was performed to investigate the anti-nociceptive activity according to the method described by Woolfe and MacDonald in 1948 [174]. Male ICR mice weighing 18-25 g were used in this study (N=10 per group). In these

experiments, the hot-plate (Harvard apparatus, USA) measuring 28×28 cm was maintained at 55±0.5°C and surrounded by a clear Plexiglas wall cylinder, 20 cm in diameter and 30 cm in height to confine the animal to the heated surface during testing.

Animals were randomly divided into eight groups and underwent 3 pre-drug baseline trials on the hot-plate spaced 5-10 min apart. Only those animals which had a pretreatment hot-plate latency time of less than 45 sec were used in the experiments. After pre-drug baseline trials, mice were administered various treatments and repeated. Each mouse was placed on the hot-plate from an elevation of 5 cm. The latency of nociceptive response of each mouse that was identified by the time for licking of hind paw or vigorous jumping up from the surface of the metal plate was used as the end point and recorded with a stopwatch. If this behavior was not observed within 45 sec the animal was removed from the hot-plate, given a score of 45 sec for its hot-plate latency and returned to its home cage. The average of the last two trials served as the baseline pre-drug latency. After the third baseline trial on the hot-plate was obtained, the animals in the control group were received 2% Tween 80 (10 ml/kg) while the reference groups were treated with NSS (10 ml/kg) and MO (10 mg/kg) by intraperitoneally. The animal in the test group were orally treated with different doses of Ben-Cha-Moon-Yai remedy (125, 250 and 500 mg/kg) and five root species extracts (25, 50, 100, 200 and 400 mg/kg). The post-drug latency was measured for 7 subsequent trials at 15, 30, 45, 60, 90, 120 and 240 min after drug administration. The cut-off time of observation was set at 45 sec to avoid tissue damage. The time-course of hot-plate latency was expressed as the mean percent maximum possible effect (%MPE) according to the following formula:

$$\%\ \text{MPE} = \frac{\text{(post-drug latency) - (pre-drug latency)}}{\text{(cut-off time) - (pre-drug latency)}} \times 100$$

The area of analgesia for the hot-plate assays was derived by computing the area under the corresponding 0-240 min time-course-%MPE curves; areas were calculated using the trapezoidal rule [175].

Analysis of the mechanism of antinociceptive action of herbal root extracts

The possible participation of the opioid system in the antinociceptive effect of three herbal root extracts was investigated using the model of mouse hot-plate test. Animal were pretreated with naloxone (NAL; 5 mg/kg, i.p.) 10 min before oral administration of *Aegle marmelos* root extract (AM; 400 mg/kg), *Dolichandrone serrulata* root extract (DS; 200 mg/kg) or *Walsura trichostemon* root extract (WT; 400 mg/kg).

Figure 10 Hot-Plate Analgesiometer

Formalin-test

The formalin test is a valid and reliable model of nociception which is mostly used with rats and mice for detecting a various classes of analgesic drugs involves moderate and continuous pain generated by injured tissue [176, 177]. The noxious stimulus is an injection of dilute formalin under the skin of the dorsal surface of the right hind paw. The response is the amounts of time the animal spend licking the injected paw [176]. The formalin test can be possesses two distinctive phases of licking activity which reflecting different types of pain [178]. The early phase lasting the first 5 min which is a direct effect of formalin on nociceptors (non-inflammatory

pain) and late phase lasting from 15 to 30 min which is reflects pain from inflammation [165].

Analgesic activity testing was determined using formalin-induced paw licking method as described by Hunskaar and Hole in 1987 [176]. Male ICR mice weighing 18-25 g were used (N=8 per group). Animals were randomly divided into nine treatment groups. Twenty microliter of 2.5% formalin solution was injected subcutaneously into the left hind paw of each mouse 30 min after intraperitoneal administration of NSS (10 ml/kg) and MO (10 mg/kg) or 1 hr after oral administration of 2% Tween 80 (10 mg/kg), indomethacin (IND; 10 mg/kg), various doses of Ben-Cha-Moon-Yai remedy (BMY; 125, 250 and 500 mg/kg) and five root species extracts (25, 50, 100, 200 and 400 mg/kg). Following the formalin injection, animals were immediately placed in an observation cylinder. The time that animal spent licking the injected paw in the early phase (0–5 min) and the late phase (15–30 min) after formalin injection was recorded with a stopwatch. The percentage of inhibition of early and late phases was analyzed using the following formula:

$$\% \text{ Inhibition of paw licking} = \frac{\text{Time (control) - Time (test)}}{\text{Time (control)}} \times 100$$

Where, time is meantime spent in paw licking (sec).

Figure 11 Formalin-induced paws licking in mouse

Acetic acid-induced writhing test

The writhing test or abdominal contortion test is widely used to evaluate the peripheral antinociceptive effect. Acetic acid-induced writhing test is a chemical stimulus of visceral inflammatory pain model. In this model, pain is induced by injection of acetic acid into the peritoneal cavity of mice. The acetic acid injection can produce the peritoneal inflammation which causes the response characterized by contraction of the abdominal muscle accompanied by an extension of the fore limbs and elongation of the body [178, 179].

The acetic acid-induced writhing test was carried out according to the method described by Koster *et al*., in 1959 [180]. Male ICR mice weighing 18-25 g were used (N=8 per group). Animals were randomly divided into seven treatment groups. Mice were orally administered with 2% Tween 80 (10 mg/kg), IND (10 mg/kg), various doses of Ben-Cha-Moon-Yai remedy (125, 250 and 500 mg/kg) or five root species extracts (25, 50, 100, 200 and 400 mg/kg) 1 h before intraperitoneal injection of 0.6% acetic acid (10 ml/kg). Each animal was then placed in a transparent observation cylinder. The number of writhing events, a response consisting of abdominal muscle contraction together with hind limb extension were observed and counted during continuous observation at 5 min intervals for a period of 30 min after the acetic acid administration [181]. Antinociceptive activity was reported as percentage of inhibition of the writhing response compared with the vehicle control group. The percentage of inhibition of the writhing response was calculated using the following formula:

$$\% \text{ Inhibition of writhing response} = \frac{Wr\ (control) - Wr\ (test)}{Wr\ (control)} \times 100$$

Where, Wr is the mean number of writhes.

Figure 12 Writhing response in mouse

Rota-rod performance test

The rota-rod test is used to evaluate the activity of drugs interfering with motor coordination of rodents. The typical accelerating rota-rod performance test is designed to evaluate maximal motor performance and is not optimized to detect motor skill learning. Animals were tested for their ability to remain on the revolving rod after drugs administration compared to vehicle control (cut-off time 1 min). The measured parameters can be the number of animals falling from the roller or latency which animals remained on the rota-rod [182].

The rota-rod test was performed according to the method described by Dunham and Miya in 1957. Male ICR mice weighing 18-25 g were tested on the rota-rod (N = 8 per group). Animals were placed on a horizontal rod (3.5 cm diameter) rotating at 16 rpm (Ugo Basile, Italy). Mice capable of remaining on the rotating rod for 60 sec or more in three successive trials were selected for the study. Each mouse was treated with 2% Tween 80 (10 ml/kg) or the Ben-Cha-Moon-Yai remedy (BMY; 500 mg/kg) and five root species extracts (400 mg/kg) orally and placed on the rotating rod at 30, 60, 90, 120 and 240 min after drug administration. The results were expressed as the time in second which the animal enables to remain on the rota-rod during 60 sec [183].

Figure 13 Rota-rod test in mice

Data treatment and statistical analysis

The results were expressed as mean ± S.E.M. Differences in mean values among groups were analyzed by a one-way analysis of variance (ANOVA) and Student's *t*-test followed by a post-hoc Tukey test for multiple comparisons. Statistical significance was assessed as $p<0.05$.

CHAPTER IV

RESULTS

PHARMACOGNOSTIC SPECIFICATIONS

Morphological identification

Macroscopic and microscopic specifications were illustrated in figures 14-17, 21-24, 28-31, 35-38 and 42-45. Cytological and histological characterization showed a valuable tool for the identification of each ingredient in Ben-Cha-Moon-Yai remedy.

Physiochemical identification

The physico-chemical specification (% by weight) of each root species in Ben-Cha-Moon-Yai remedy was demonstrated as the grand average and pooled standard deviation in different parameters.

Thin layer chromatographic analysis

The TLC fingerprint of the methanolic extract from five root species were performed using Silica gel F_{254}. The resolution of the separation was tried in different solvent systems and the best resolving solvent system was chosen for developing the plates. The plates were visualized under UV light at 254 nm and 365 nm and were then exposed to 10% sulfuric acid reagent. The fingerprints of each root species were provided in figure 18, 25, 32, 39 and 46.

High performance liquid chromatographic analysis

To develop the representative fingerprint, the ethanolic extract of five root species and Ben-Cha-Moon-Yai remedy were analyzed under the same HPLC condition. In order to obtain the optimal elution conditions for the separation and determination of the constituents, various linear gradients of 10 mM phosphoric acid and acetonitrile at a flow rate of 0.8 ml/min were investigated. The chromatogram

revealed a common peak of each root extract and Ben-Cha-Moon-Yai remedy within the retention time of 60 min as shown in figure 19, 26, 33, 40 and 47.

Liquid Chromatography-Mass spectrometry

LC-ESI-MS method was employed to analyze the component in Ben-Cha-Moon-Yai remedy and five root species extracts. The detected chromatographic and spectrometric data of the common peak in the HPLC chromatograms were provided in figure 20, 27, 34, 41 and 48. The mobile phase consisted of (A) Water and (B) Acetonitrile with a suitable gradient. ESI in positive modes under the optimized MS condition were used to detect the chemical constituents of the extracts. In the ESI-MS experiment, the molecular weight of each separated peak was obtained.

***Aegle marmelos* (L.) Correa ex Roxb.**

Family: Rutaceae

Vernacular names

Thailand: matum, tum (Pattani), ma pin (north). Bael or bel fruit (En). Bel Indient (Fr). Indonesia: maja, maja batu. Malaysia: bilak, bila, bel. Philippines: bael. Burma: opesheet, okshit. Cambodia: bnau. Laos: toum. Vietnam: trai mam.

Distribution

Bael grows wide in dry forest in the Indian Peninsula, Sri Lanka, Pakistan and Bangladesh. It is an old cultivated tree in that region, particularly found in temple gardens in India. It has spread to Indo-China, South-East Asia (in particular Thailand, northern Malaysia, eastern Java and north Luzon) and other parts of the tropics [184].

Description

A deciduous tree, 20 to 25 feet in height and 3 to 4 feet in grinth, with straight, sharp, axillary thorns and trifoliate aromatic leaves. The flowers are greenish white. The fruits are globose, 2 to 4 inch in diameter, gray or yellowish and with smooth, hard, aromatic rind. Seeds are numerous, oblong and compressed, and the pulp is mucilaginous, thick, orange red in color. The root bark is 3.5 mm thick, curved, with its surface cream yellow or grayish in color. The surface is rough, irregular, and shallow with ridges along the line of lenticels and ruptured all over. The stem bark is externally gray and internally cream in color. The outer surface is rough and warty. It is 4 to 8 mm thick, firm in texture and occurs as flat or channeled pieces. The fracture is tough and gritty in the outer region and fibrous in the inner; taste is sweet. The root in transection shows a pentarch to heptarch stele, the cork cambium arising in the pericycle. The cork is lignified and stratified, the phelloderm is composed of a wide zone of parenchymal cells with strands of stone cells in the mature bark. The medullary group in the inner region is uni-to triseriate, while in the outer region it is bi-to pentaseriate [185].

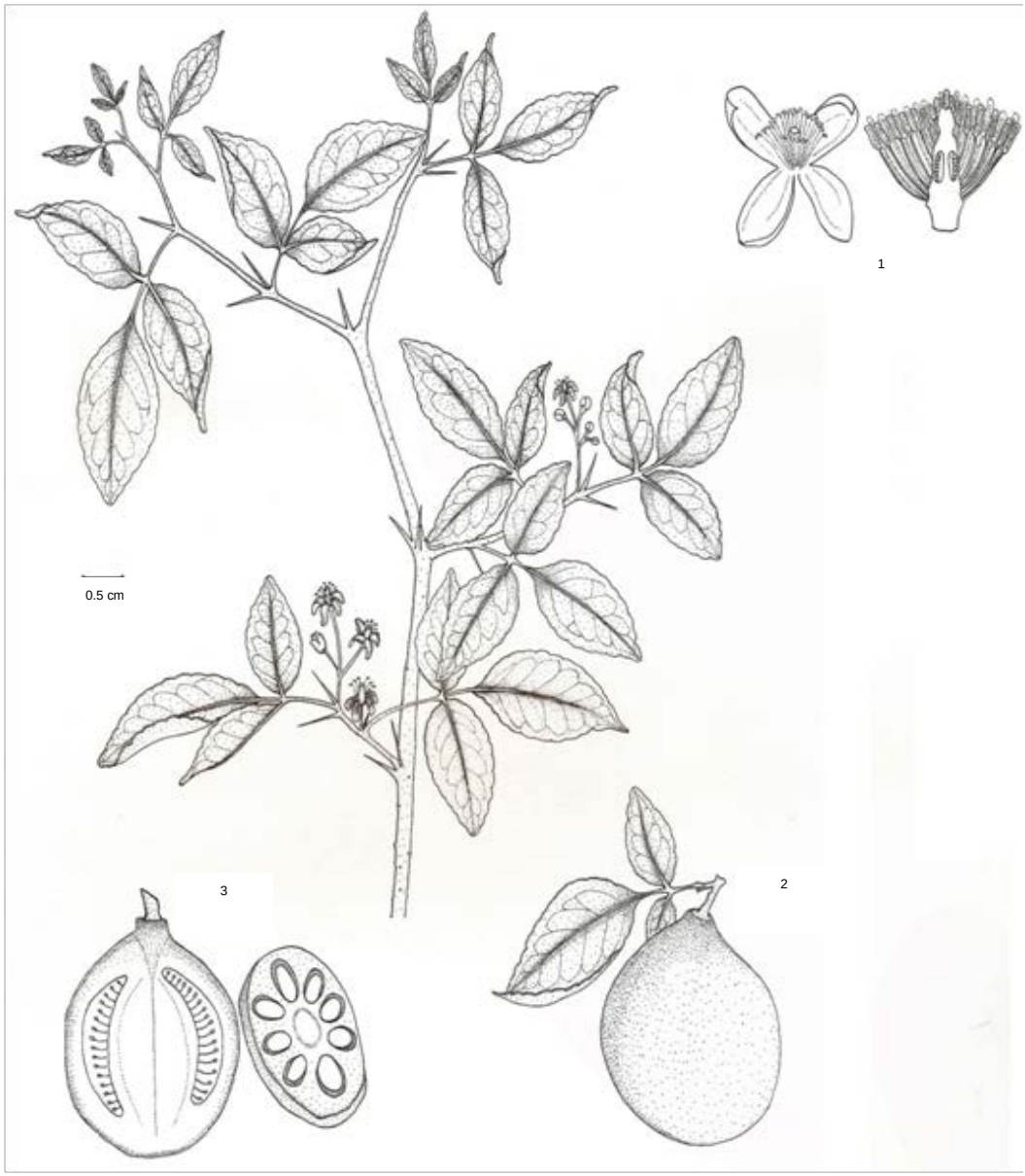


Figure 14 The flowering branch of *A. marmelos*

1. Flower 2. Fruit 3. Transverse section of fruit

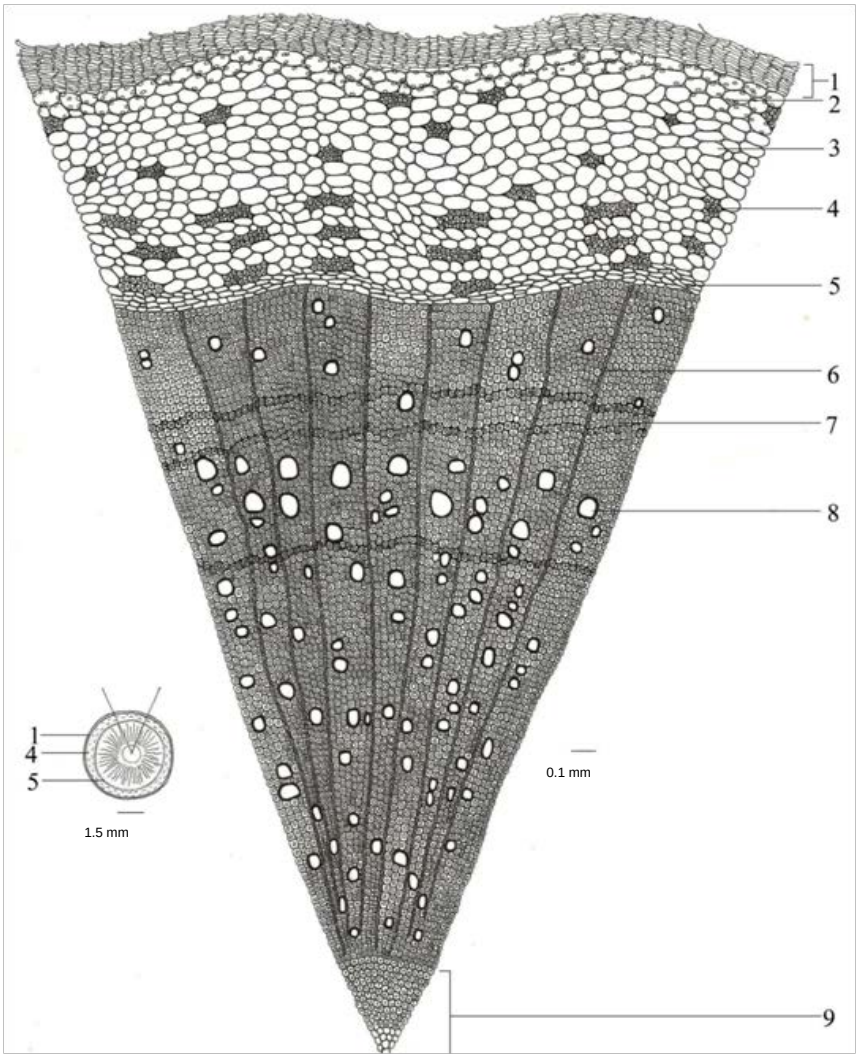


Figure 15 Transverse section of *A. marmelos* root; 1. Periderm 2. Strach granule in reserved parenchyma 3. Cortical parenchyma 4. Group of cortical fiber 5. Endodermis 6. Xylem ray with starch granule 7. Xylem fiber 8. Xylem vessel 9. Pith

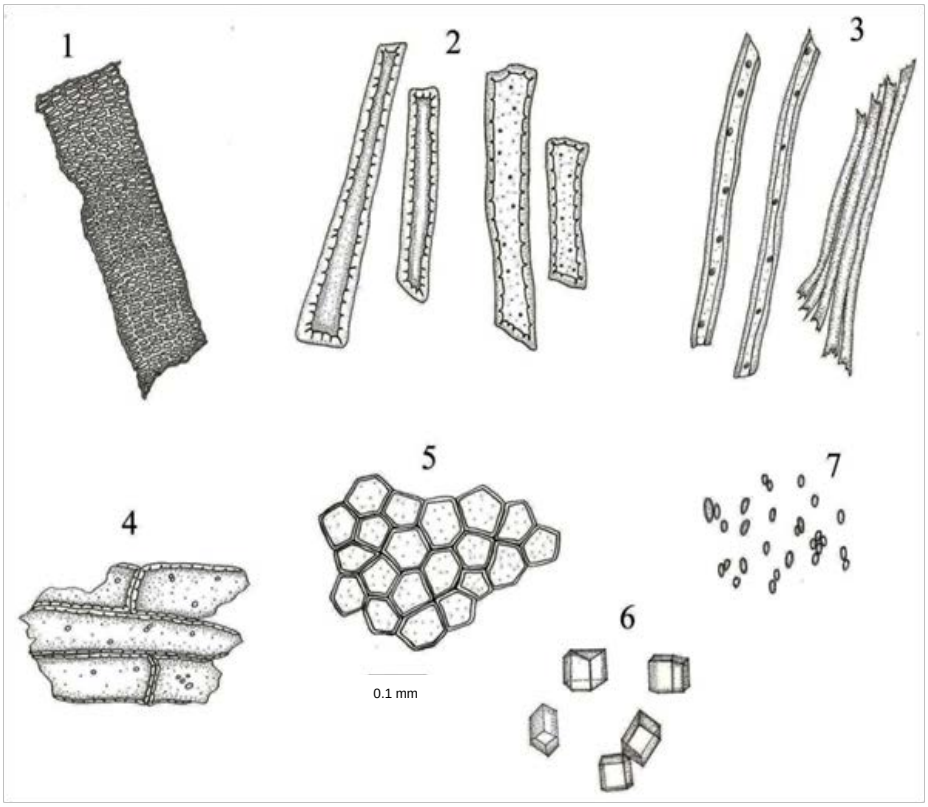


Figure 16 Powder of *A. marmelos* root ; 1. Fragment of reticulated vessel 2. Sclereid in longitudinal view 3. Fragment of fiber 4. Fragment of parenchyma in longitudinal view 5. Cork in surface view 6. Prism crystal of calcium oxalate 7. Starch granule

Figure 17 The root of *A. marmelos*

Table 8 Physico-chemical specification (% by weight) of *A. marmelos* root

Content (% by weight)	**Mean ±SD**	**Min-Max**	**n**
Loss on drying	6.97 ± 0.69	5.44-7.89	12
Total ash content	3.94 ± 0.89	2.56-5.49	12
Acid-insoluble ash content	0.70 ± 0.26	0.32-1.18	12
Water content	9.63 ± 1.24	7.94-11.58	12
Ethanol extractive values	5.19 ± 1.60	4.30-13.56	12
Water extractive values	6.48 ±2.42	1.64-7.50	12

Grand mean values were calculated from 12 sources throughout Thailand. Each source was performed in triplicate.

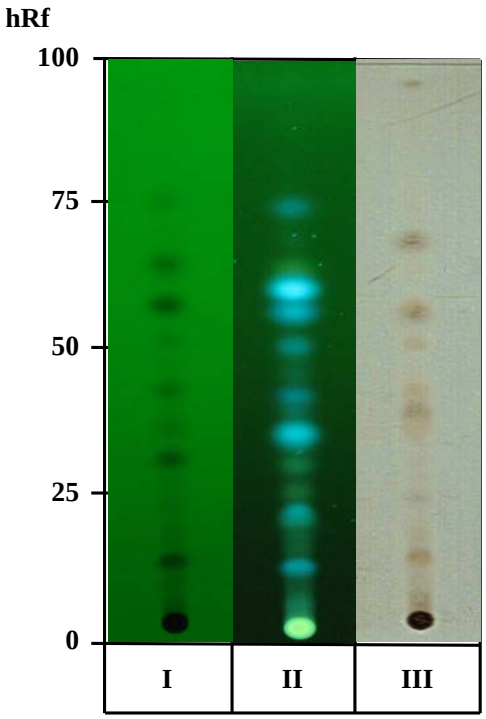


Figure 18 Thin-layer chromatogram of the methanolic extract *A. marmelos* root

Solvent system

Toluene : Ethyl acetate 3:1

Detection

I	=	detection under UV light 254 nm
II	=	detection under UV light 366 nm
III	=	detection with 10% sulfuric acid *, **

*10% sulfuric acid reagent

Preparation: conc. sulfuric acid 10 ml. in methanol 90 ml.

**Spot colour development

Heat the plate at 105 ^{o} C for 10 minutes after sprayed.

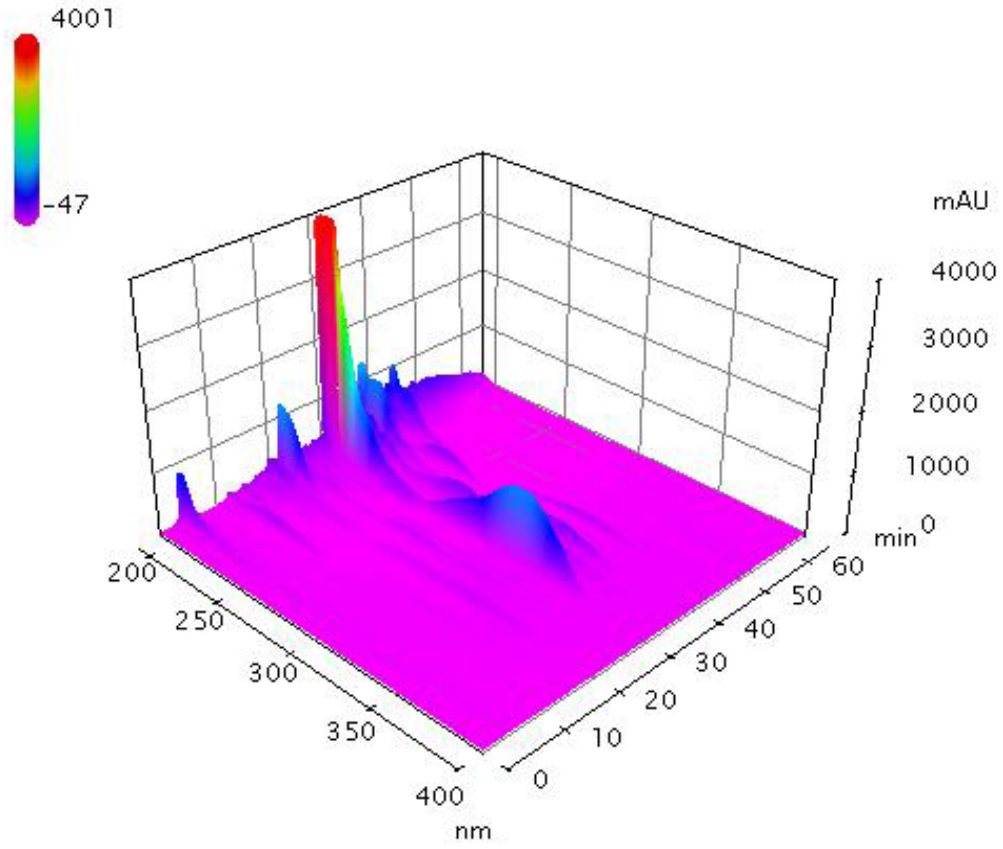


Figure 19 The 3D-HPLC profiles of ethanolic extract from *A. marmelos* root

Analysis condition

Column: Inersil ® ODS-3, C-18 column (particle size of the packing 5µm, 4.6 x 250mm)

Mobile phase: 10 mM Phosphoric acid-Acetonitrile

Linear gradient: (95:5, 65 min)

Flow rate: 0.8 mL/min

Injection volume: 10 µl

Temperature: 40°C

Wavelength: 190-400 nm.

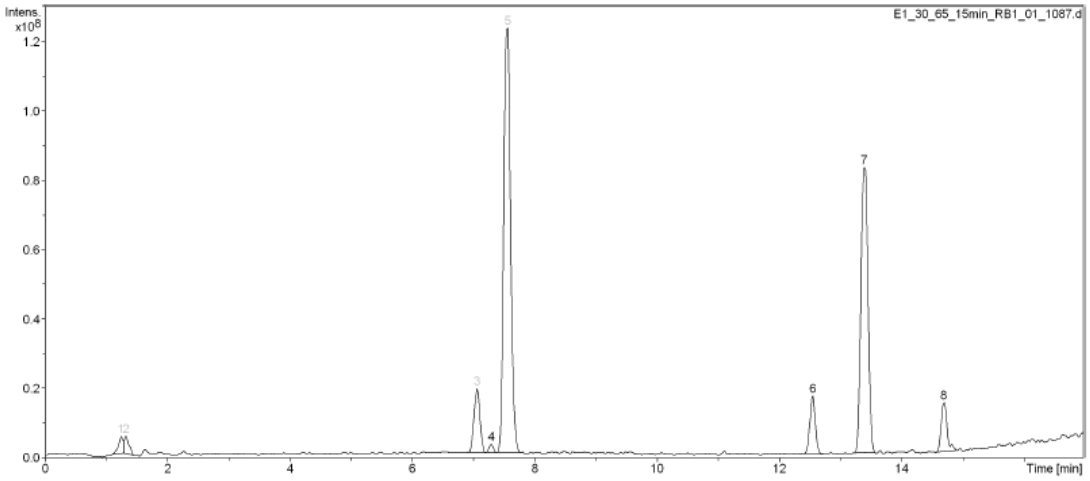


Figure 20 A representative LC/MS chromatogram from *A. marmelos* root of compound 1-8

Analysis condition

Column: Dionex C16 Acclaim RSLC PolarAdvantage column (2.1 x 100 mm, 2.2 μm,120 °A)

Mobile phase: (A) Water and (B) Acetonitrile

Linear gradient: 0-2 min (70:30%), 2-17 min (30-65%)

Flow rate: 200 μL/min

Injection volume: 5 μL of 10 ppm

Temperature: 35 °C

MS mode: UltraScan mode between m/z 70-1,500, using positive ionization

Table 9 LC- ESI-MS data and identification of constituents from the root of *A. marmelos*

Peak no.	*t*R (min)	MW	Product ions (m/z)
1	1.2	447.3	448.2, 465.1, 465.4, 470.1, 502.3
2	1.3	233.9	235.0, 252.1, 256.9, 273.0, 288.9
3	7.1	259.0	260.0, 277.1, 282.0, 297.9, 314.0
4	7.3	229.0	230.1, 247.0, 252.0, 268.0, 283.9
5	7.6	300.1	301.1, 318.0, 355.1
6	12.5	336.1	337.1
7	13.4	328.1	351.0, 383.1
8	14.7	298.1	299.1, 316.0, 321.0, 337.1, 353.0

Dimocarpus longan* Lour. Subsp. *Longan* var. *longan

Family: Sapindaceae

Synonyms

- ssp. *Longan* var. *longan*: *Dimocarpus longan* Lour. (1790), *Euphoria longana* Lamk (1792) nom. Illeg., *Nephelium longana* Cambess. (1829).
- ssp. *Longan* var. *longepetiolulatus* Leenh.: *Euphoria morigera* Gagnep. (1950) nom. Inval.
- ssp. *Longan* var. *obtusus* (Pierre) Leenh.: *Euphoria scandens* Winit & Kerr.
- ssp. *Malesianus* Leenh. Var. *malesianus*: *Nephelium malaiense* Griff. (1854), *Euphoria cinerea* Radlk. (1878) nom. Illeg., *Euphoria malaiensis* Radlk. (1879) nom. Illeq., *Euphoria gracilis* Radlk. (1913) nom. Illeg.
- ssp. *Malesianus* Leenh. Var. *echinatus* Leenh.: *Euphoria nephelioides* Radlk. (1914) nom. Illeg.

Vernacular names

- ssp. *Longan* var. *longan*: longan (En). Longanier, oeil de dragon (Fr). Indonesia, Malaysia: lengkeng. Burma: kyet mouk. Cambodia: mien. Laos: lam nhai, nam nhai. Thailand: lamyai pa. Vietnam: nhan.
- ssp. *Longan* var. *obtusus*: Thailand: lamyai khruer, lamyai tao.
- ssp. *Malesianus* var. *malesianus*: Malaysia: mata kucing (Peninsular Malaysia and Sabah), isau, sau, kakus (Sarawak). Indonesia: buku, ihau (Kalimantan), medaru (Sumatra)

Distribution

- ssp. *Longan* var. *longan*: Whereas some authors limit the area of origin to the mountain chain from Burma through southern China, others extend it to south-west India and Sri Lanka, including the lowlands. The crop is mainly grown in south China, Taiwan and north Thailand with small acreages elsewhere in Indo-China as well as Queensland (Australia) and Florida (United States) and scattered trees at higher elevations in South- East Asia.
- ssp. *Longan* var. longepetiolulatus: southern Vietnam.
- ssp. *Longan* var. *obtusus*: Indo-China, cultivated in Thailand.

- ssp. *Malesianus* var. *malesianus*: all over Indo-China and Malaysia, greatest variation found in Borneo.
- ssp. *Malesianus* var. *echinatus*: Borneo and the Philippines.

Description

Tree, up to 40 m tall and 1 m trunk diameter, sometimes buttressed, exceptionally a scandent shrub; branches terete with 5 faint grooves, sometimes warty lenticellate, rather densely ferruginous tomentose. Leaves 2-4(-6)-jugate, axial parts mostly densely hairy; petiole 1-20 cm, petiolules 0.5-35 mm long; leaflets elliptical, 3-45 cm x 1.5-20 cm, 1-5 times longer than wide, chartaceous to coriaceous, above often tomentose in basal part of midrib, beneath thinly tufted-tomentose mainly on midrib and nerves. Inflorescences usually terminal, 8-40 cm long, densely tufted-tomentose; cymules (1-)3-5-flowered; pedicels 1-4 mm; bracts patent, 1.5-5 mm long; flowers yellow-brown; calyx lobes 2-5 mm x 1-3 mm; petals 5, 1.5-6 mm x 0.6-2 mm, densely woolly to glabrous; stamens (6-)8(-10), filament 1-6 mm. Fruit drupaceous, 1-3 cm in diameter, lobe(s) broad-ellipsoid to globular, smooth to warty or sometimes up to 1 cm aculeate, sometimes granular, glabrescent, yellowbrown. Seed globular with shining blackish-brown testa; seed enveloped by a thin fleshy, translucent white arilloid [186].

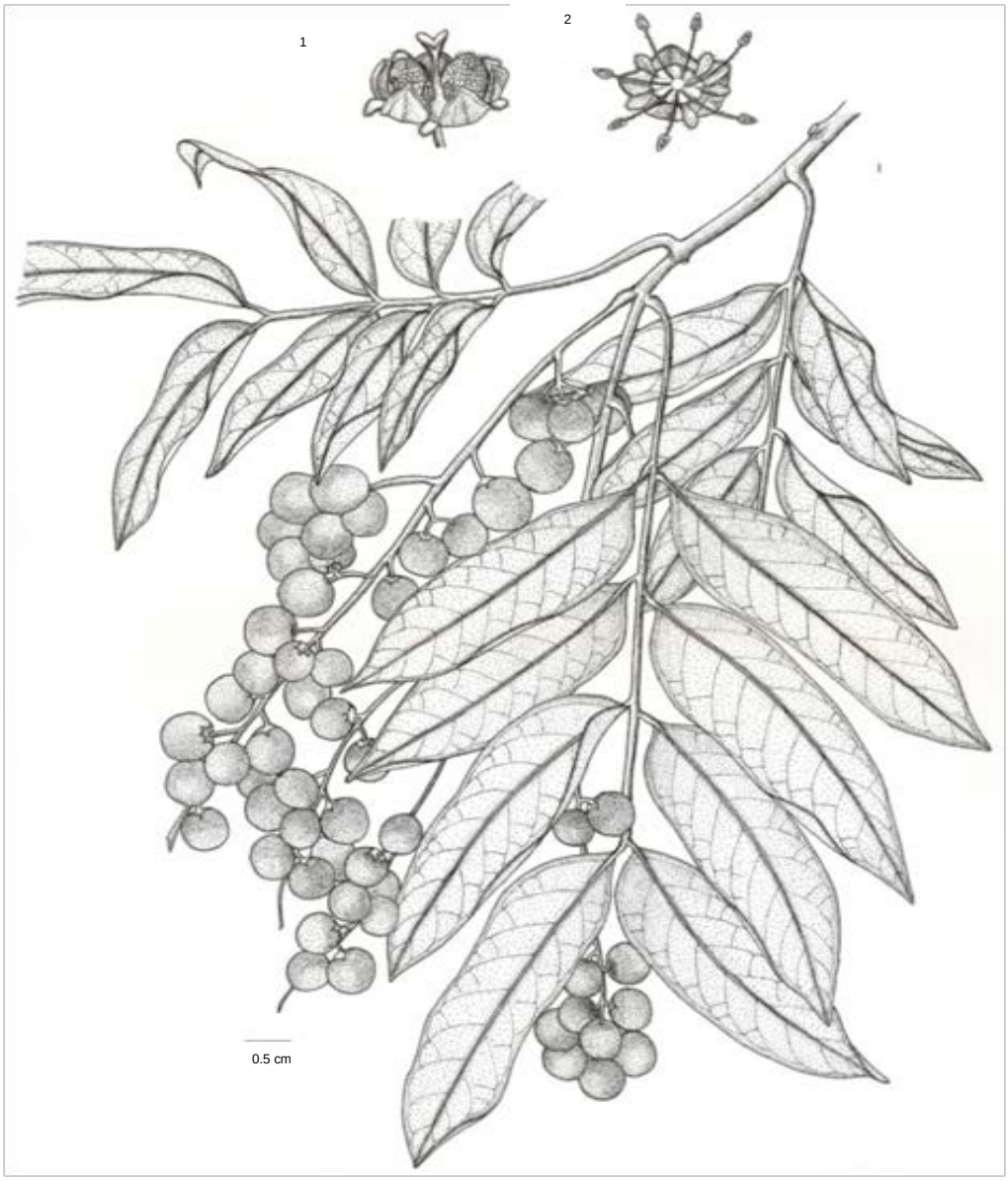


Figure 21 The fruiting branch of *D. longan*

1. Male flower 2. Female flower

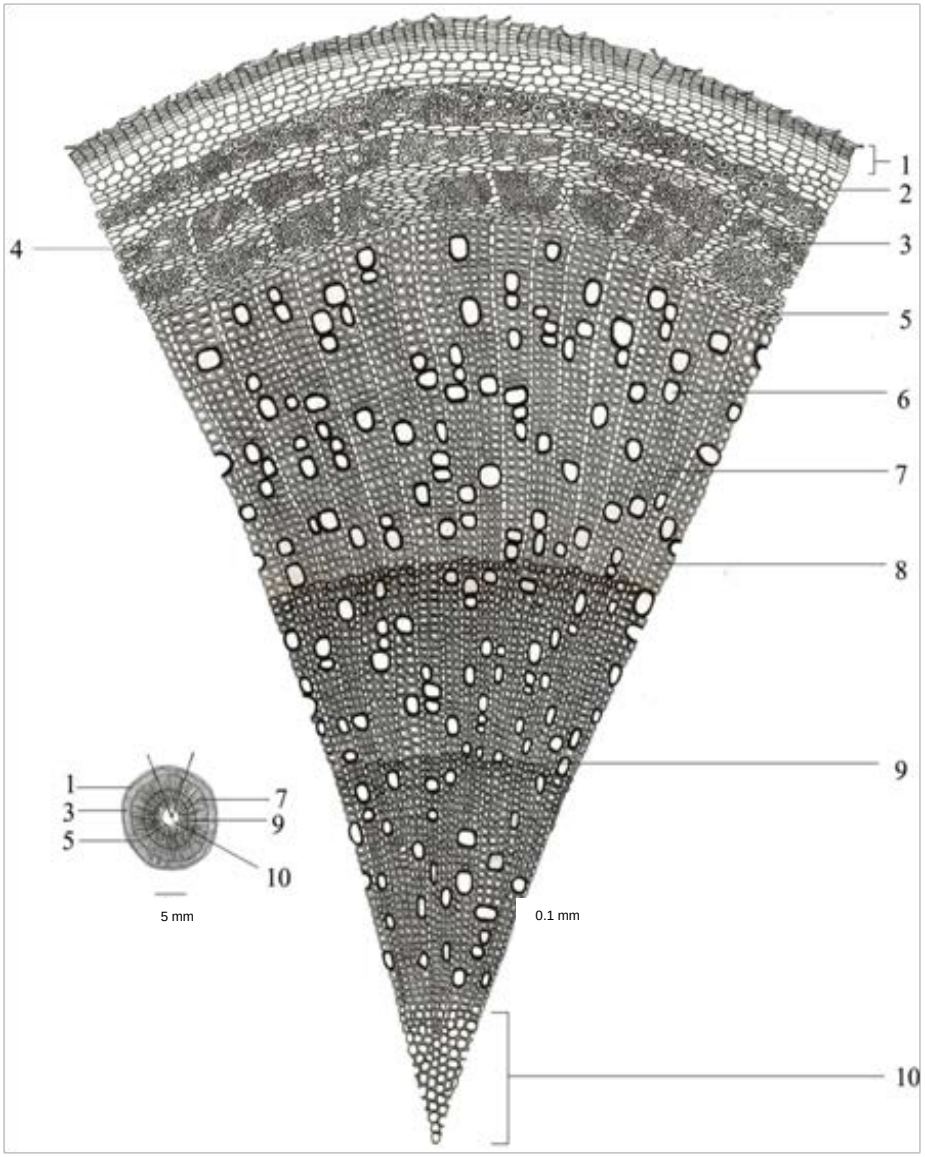


Figure 22 Transverse section of *D. longan* root ; 1. Periderm 2. Cortical parenchyma 3. Cortical fiber 4. Sclereid 5. Endodermis 6. Xylem vessel 7. Xylem ray 8. Xylem fiber 9. Annual ring 10. Pith

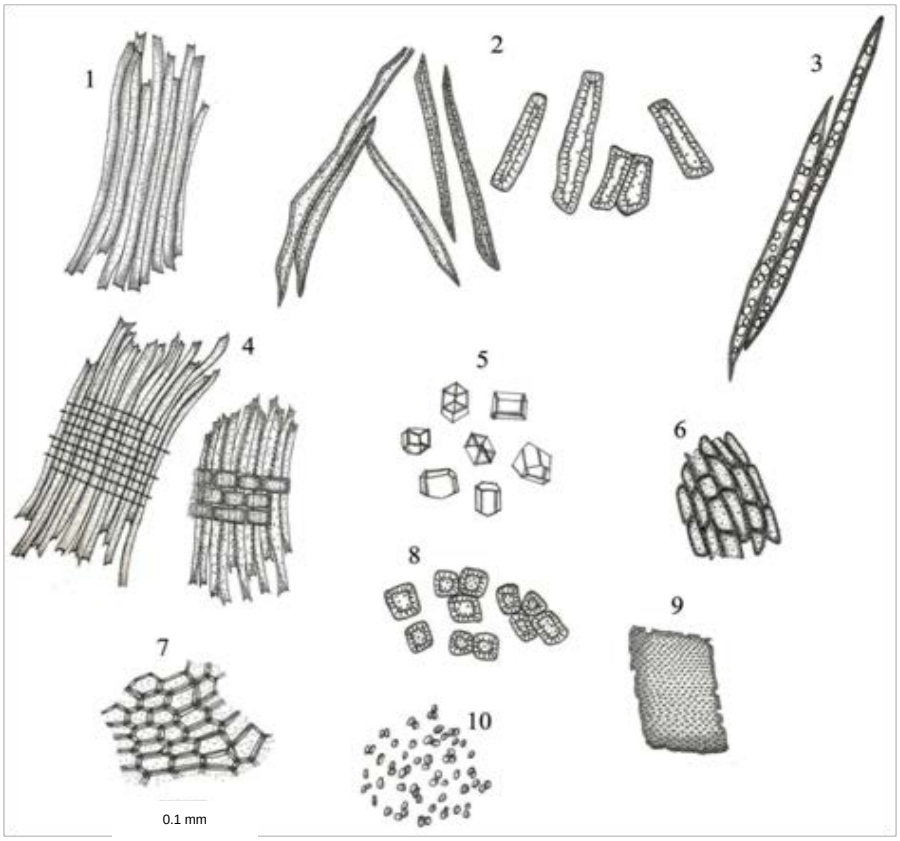


Figure 23 Powder of *D. longan* root ; 1. Fragment of fiber 2. Sclereid in longitudinal view 3. Fiber containing oil globule 4. Xylem in radial longitudinal view 5. Prism cystal of calcium oxalate 6. Parenchyma in longitudinal view 7. Cork in surface view 8. Sclereid in transverse view 9. Fragment of pitted vessel 10. Starch granule

Figure 24 The root of *D. longan*

Table 10 Physico-chemical specification (% by weight) of *D. longan* root

Content (% by weight)	**Mean ±SD**	**Min-Max**	**n**
Loss on drying	7.98 ± 0.94	6.64-10.23	13
Total ash content	3.64 ± 1.43	2.02-7.15	13
Acid-insoluble ash content	1.09 ± 1.52	0.27-5.55	13
Water content	10.57 ± 1.11	8.83-12.34	13
Ethanol extractive values	7.66 ± 1.98	3.47-6.59	13
Water extractive values	5.09 ±1.05	4.53-11.00	13

Grand mean values were calculated from 13 sources throughout Thailand. Each source was performed in triplicate.

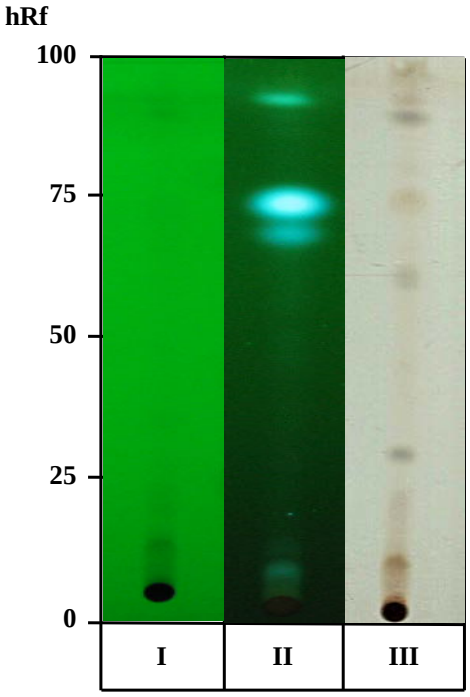


Figure 25 Thin-layer chromatogram of the methanolic extract *D. longan* root

Solvent system

Chloroform : Methanol 9 : 1

Detection

I = detection under UV light 254 nm

II = detection under UV light 366 nm

III = detection with 10% sulfuric acid*,**

*10% sulfuric acid reagent

Preparation: conc. Sulfuric acid 10 ml. in methanol 90 ml.

**Spot color Development

Heat the plate at 105 ^{o} C for 10 minutes after sprayed.

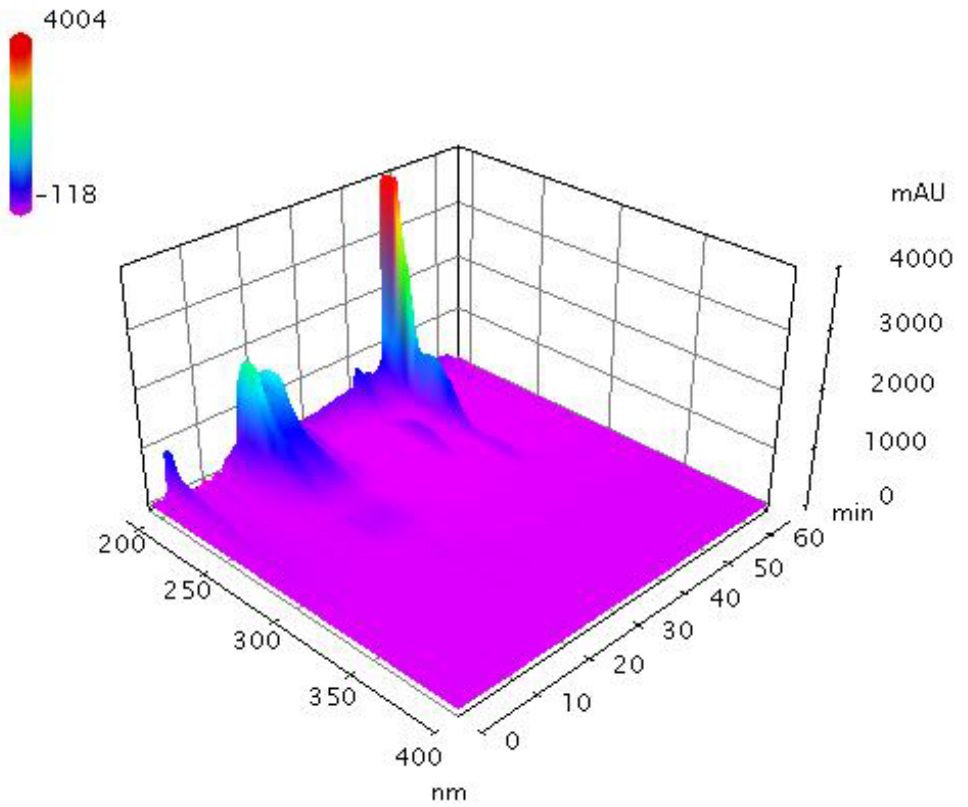


Figure 26 The 3D-HPLC profiles of ethanolic extract from *D. longan* root

Analysis condition

Column: Inersil ® ODS-3, C-18 column (particle size of the packing 5µm, 4.6 x 250mm)

Mobile phase: 10 mM Phosphoric acid-Acetonitrile

Linear gradient : (95:5, 65 min)

Flow rate : 0.8 mL/min

Injection volume : 10 µl

Temperature : 40°C

Wavelength : 190-400 nm.

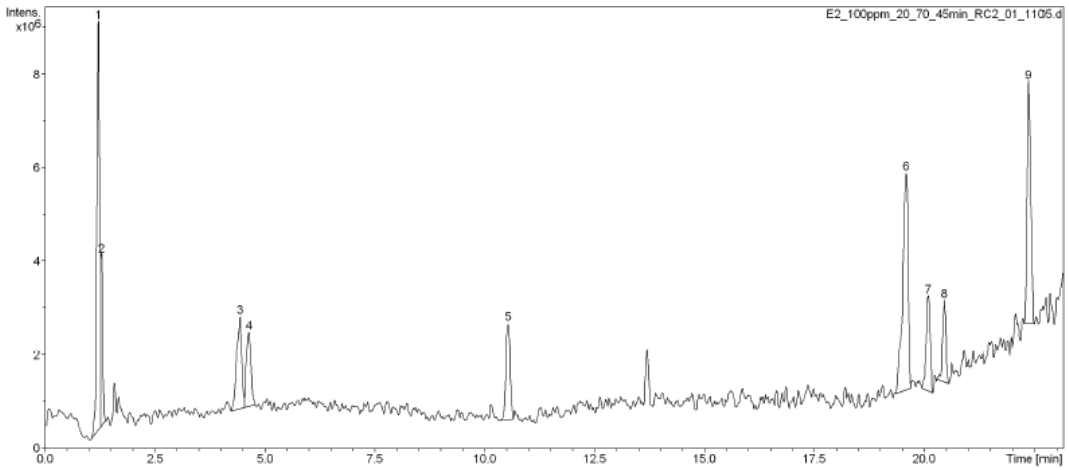


Figure 27 A representative LC/MS chromatogram from *D. longan* root of compound 1-9

Analysis condition

Column: Dionex C16 Acclaim RSLC PolarAdvantage column (2.1 x 100 mm, 2.2 μm,120 °A)

Mobile phase: (A) Water and (B) Acetonitrile

Linear gradient: 0-2 min (80:20%), 2-32 min (30-70%)

Flow rate: 200 μL/min

Injection volume: 5 μL of 100 ppm

Temperature: 35 °C

MS mode: UltraScan mode between m/z 70-1,500, using positive ionization

Table 10 LC- ESI-MS data and identification of constituents from the root of *D. longan*

Peak no.	*t*R (min)	MW	Product ions (m/z)
1	1.21	342.02	360.15, 365.01, 380.98, 397.02
2	1.28	252.06	253.13, 270.09, 275.05, 290.97
3	4.43	450.05	451.28, 489.10, 505.06
4	4.63	420.03	421.00, 438.22, 475.05
5	10.53	369.97	387.98, 393.04, 408.99, 424.96
6	19.58	312.17	313.09, 351.00, 367.19
7	20.08	354.16	355.08, 372.19, 393.02, 409.18
8	20.45	398.10	399.13, 416.19, 437.08, 453.10
9	22.36	312.17	313.05, 367.19

***Dolichandrone serrulata* (DC.) Seem**

Family : Bignoniaceae

Synonyms *Stereospermum serrulata* DC.

Description

Deciduous tree to 25 m with narrow cylindrical crown and slender branches. Bark is pale brown, smooth or slightly flaking. Leaf is to 43 cm, once-pinnate, 3-5 pairs of leaflets, 5-14 × 3-6 cm, elliptic with tapering tip and strongly asymmetric base, usually with scattered teeth. Flower is 12-21 cm, pure white, opening at night, in short unbranched clusters of 3-7 flowers at end of twigs, 2-3 cm. Fruit is up to 85×1.8 cm, pointed, spirally twisted. Seed 2.2-2.8 × 0.5-0.8 cm, rectangular, thin with transparent wing [187].

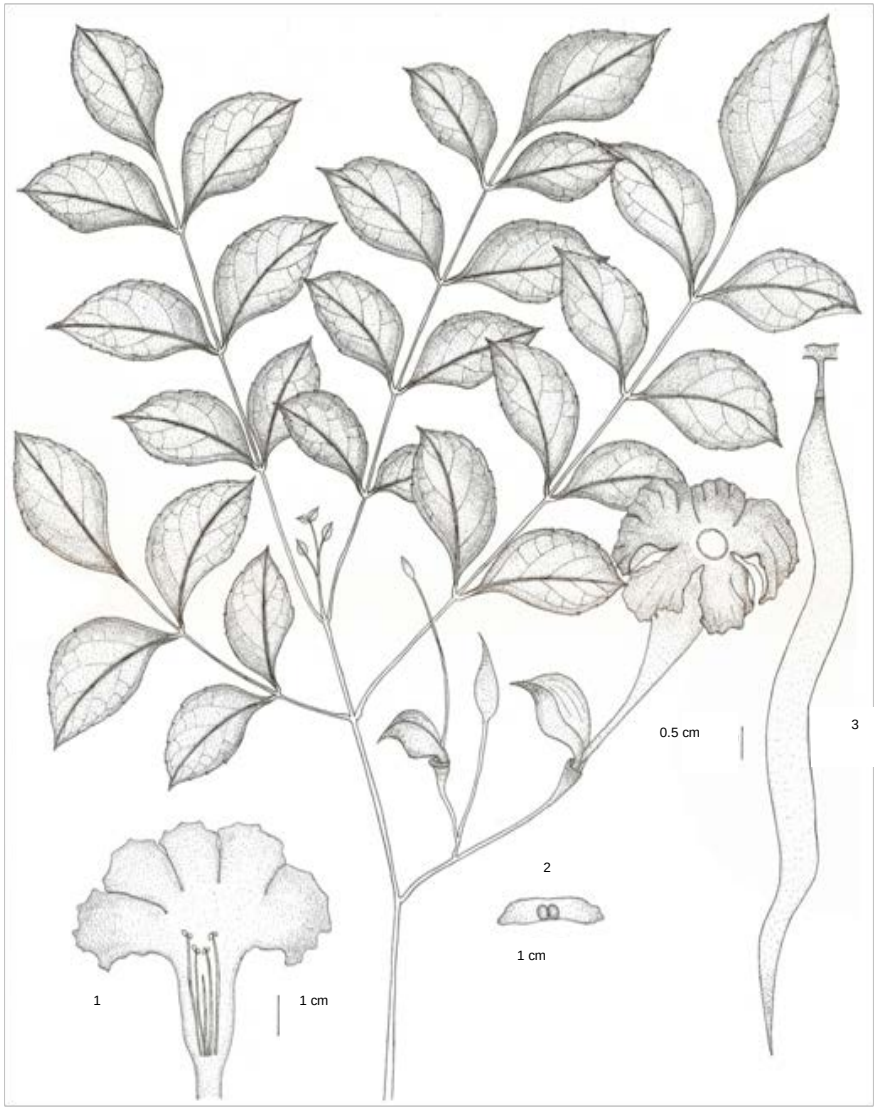


Figure 28 The flowering branch of *D. serrulata*

1. Longitudinal section of flower 2. Seed 3. Fruit

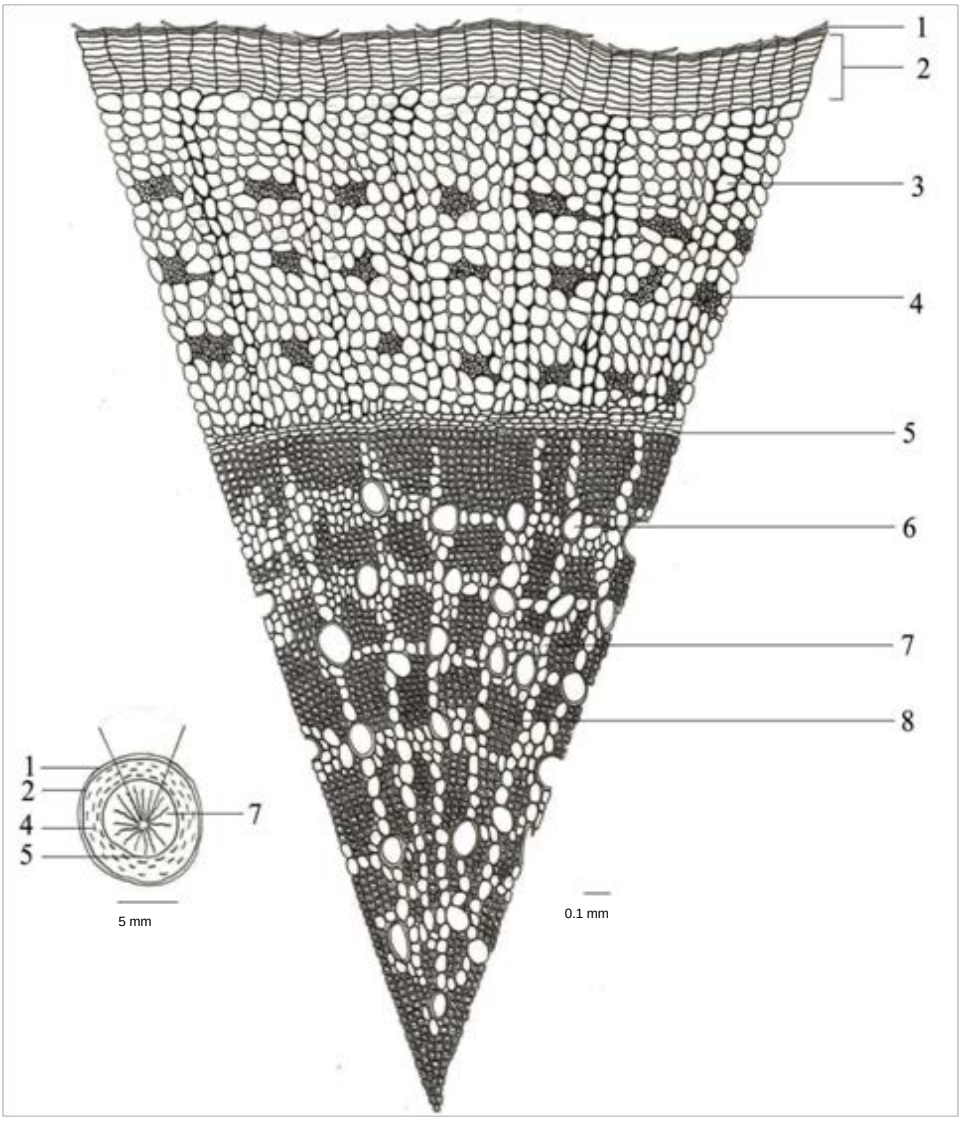


Figure 29 Transverse section of *D. serrulata* root ; 1. Epidermis 2. Periderm 3. Cortical parenchyma 4. Group of cortical fiber 5. Endodermis 6. Xylem vessel 7. Xylem ray 8. Xylem fiber

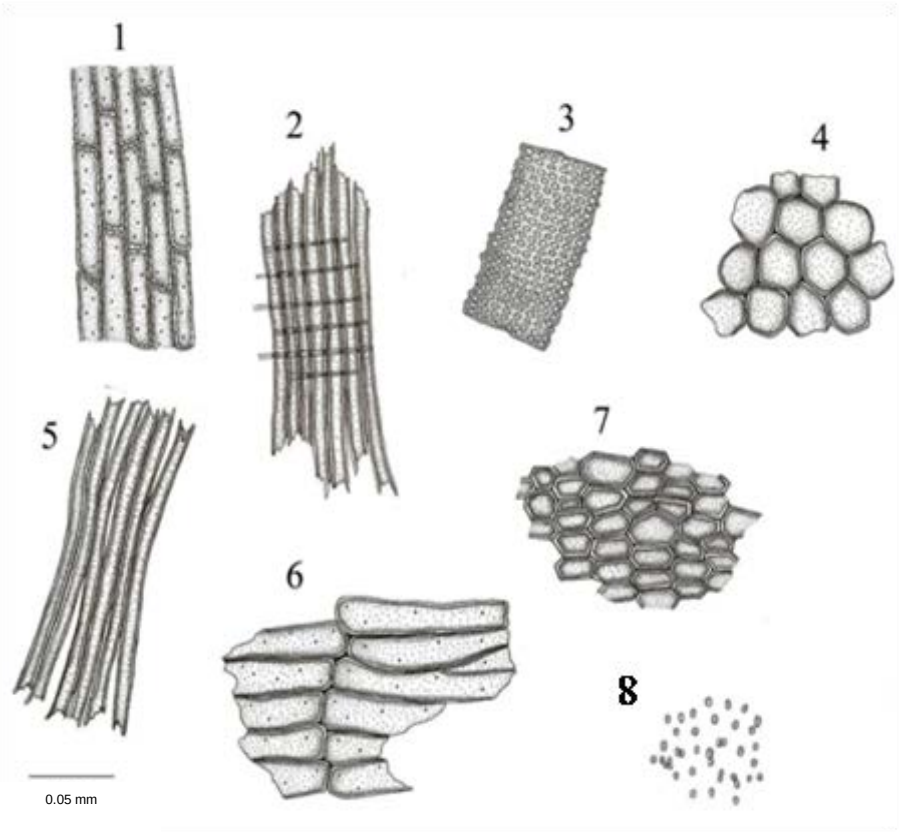


Figure 30 Powder of *D. serrulata* root; 1. Xylem parenchyma in longitudinal view 2. Xylem in radial longitudinal view 3. Fragment of pitted vessel 4. Parenchyma in transverse view 5. Fragment of fiber 6. Parenchyma in longitudinal view 7. Epidermis in surface view 8. Strach grain

Figure 31 The root of *D. serrulata*

Table 12 Physico-chemical specification (% by weight) of *D. serrulata* root

Content (% by weight)	**Mean ±SD**	**Min-Max**	**n**
Loss on drying	7.84 ± 0.75	6.69-9.31	14
Total ash content	3.63 ± 1.06	1.12-5.30	14
Acid-insoluble ash content	0.77 ± 0.69	0.37-3.62	14
Water content	12.46 ± 1.68	10.19-14.99	14
Ethanol extractive values	4.49 ± 1.70	3.64-19.67	14
Water extractive values	10.17 ± 4.28	1.12-7.50	14

Grand mean values were calculated from 14 sources throughout Thailand. Each source was performed in triplicate.

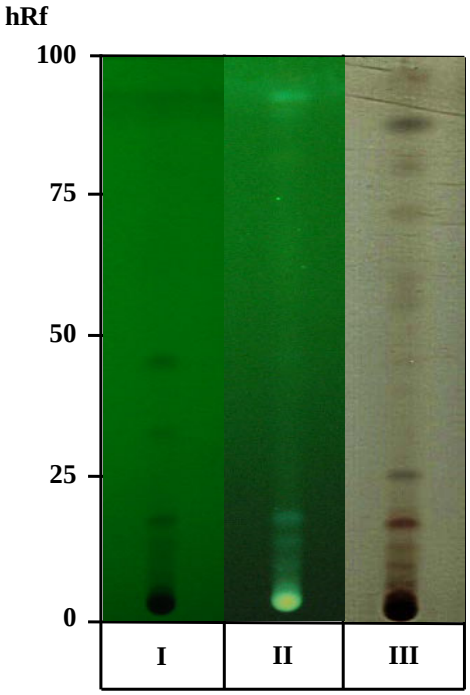


Figure 32 Thin-layer chromatogram of the methanolic extract from *D. serrulata* root

Solvent system

Chloroform : Methanol 9 : 1

Detection

I = detection under UV light 254 nm

II = detection under UV light 366 nm

III = detection with 10% sulfuric acid*,**

*10% sulfuric acid reagent

Preparation: conc. Sulfuric acid 10 ml. in methanol 90 ml.

**Spot color Development

Heat the plate at 105 ^{o} C for 10 minutes after sprayed.

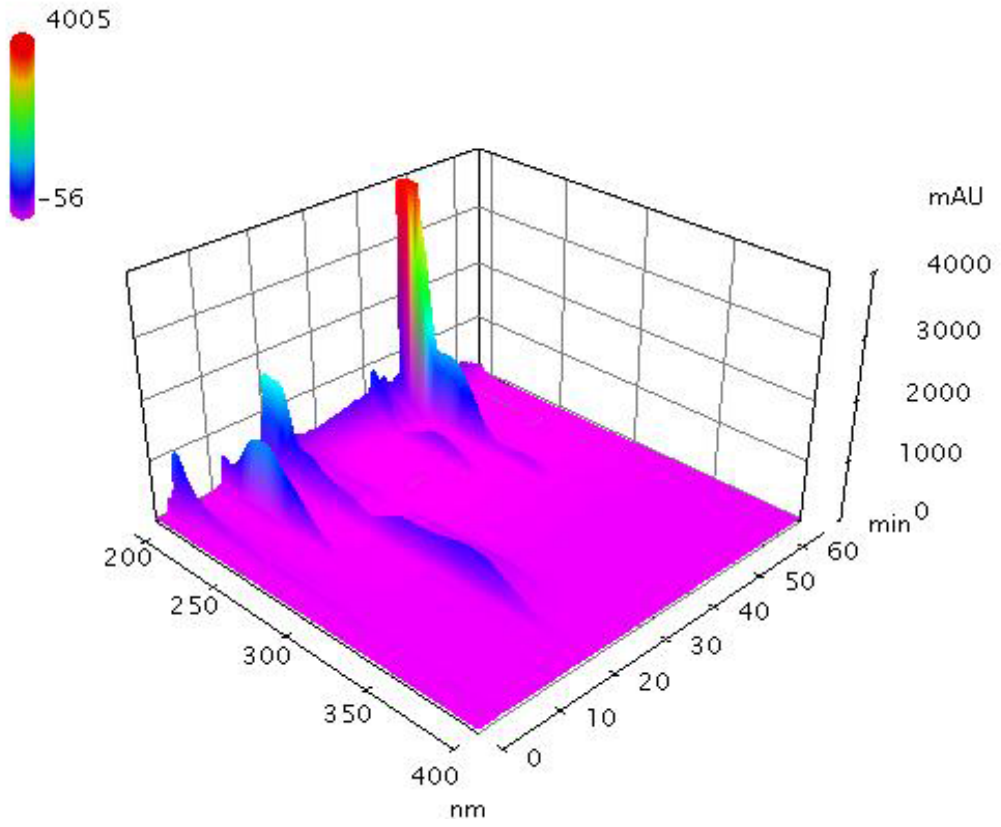


Figure 33: The 3D-HPLC profiles of ethanolic extract from *D.serrulata* root

Analysis condition

Column: Inersil ® ODS-3, C-18 column (particle size of the packing 5µm, 4.6 x 250mm)

Mobile phase: 10 mM Phosphoric acid-Acetonitrile

Linear gradient: (95:5, 65 min)

Flow rate: 0.8 mL/min

Injection volume: 10 µl

Temperature: 40°C

Wavelength: 190-400 nm.

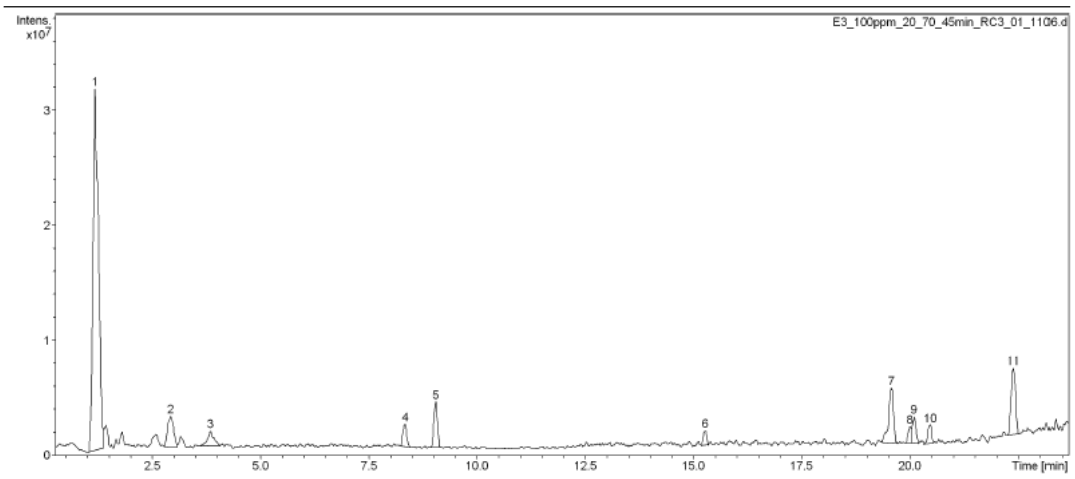


Figure 34 A representative LC/MS chromatogram from *D. serrulata* root of compound 1-11

Analysis condition

Column: Dionex C16 Acclaim RSLC PolarAdvantage column (2.1 x 100 mm, 2.2 μm,120 °A)

Mobile phase: (A) Water and (B) Acetonitrile

Linear gradient: 0-2 min (80:20%), 2-32 min (30-70%)

Flow rate: 200 μL/min

Injection volume: 5 μL of 100 ppm

Temperature: 35 °C

MS mode: UltraScan mode between m/z 70-1,500, using positive ionization

Table 13 LC- ESI-MS data and identification of constituents from the root of *D. serrulata*

Peak no.	*t*R (min)	MW	Product ions (m/z)
1	1.18	309.99	311.19, 328.15, 332.99, 349.07, 365.00
2	2.92	490.11	491.26, 508.15, 528.93, 545.13
3	3.84	646.12	647.13, 664.06, 669.11, 685.11, 701.04
4	8.33	620.16	659.26, 675.17
5	9.04	560.11	561.11, 578.08, 583.12, 599.09
6	15.26	472.27	495.45, 511.21, 527.28
7	19.56	312.16	313.13, 367.18
8	19.99	456.27	457.38, 474.32, 474.53, 495.21, 511.27
9	20.09	386.19	387.05, 404.20, 409.18, 425.16, 441.28
10	20.46	398.11	416.05, 421.00, 437.12, 453.09
11	22.37	312.17	330.34, 367.18

***Oroxylum indicum* (L.) Kurz**

Family : Bignoniaceae

Synonyms

Bignonia indica L. var. ‘ALFA’a (1753), *Bignonia pentandra* Lour. (1790), *Calosanthes indica* (L.) Blume (1826).

Vernacular names

Midnight horror (En). Indonesia: pongporang (Sundanese), kayu lanang, mungli (Javanese). Malaysia: beka, bonglai, kulai. Philippines: pingka-pingkahan (Tagalog), abong-abong (Bisaya), Kamkampilan (Iloko). Cambodia: pi ka. Laos: lin may, ung ka. Thailand: phe kaa (central), litmai (northern), lin faa (north-eastern).

Distribution

Oroxylum indicum is found from India eastward to southern China and the Philippines, and throughout South-East Asia; in Indonesia eastward to Sulawesi and the Lesser Sunda Islands. Locally cultivated near human settlements.

Description

A semi-deciduous, sparingly branched tree up to 27 m tall; trunk up to 40 cm in diameter, bark grey, with prominent leaf scars, twigs thick, pithy, later hollow, lenticellate. Leaves crowded, imparipinnate, 3-4 times pinnate, 0.5-2 m long; petiole long, rachis swollen at points of insertion; stipules absent; leaflets ovate to oblong, 4-11 (-15) cm x 3-9 cm, base cuneate or mostly oblique, apex acuminate, entire, with scattered glands on the lower surface. Inflorescence an erect raceme, terminal, 25-150 cm long, peduncle and rachis partitioned. Flowers bisexual, pedicel 2-4 cm long, bracteolate; calyx coriaceous, campanulate, containing water in bud, 2-4 cm long, 1.5-2 cm in diameter, brown or dirty violet, becoming almost woody in fruit; corolla funnel-shaped, about 10 cm long, lobes 5, subequal, margin wrinkled, reddish outside, yellowish to pinkish inside; stamens 5, inserted in the throat, hairy at the base; ovary superior, 2-celled, many-ovuled. Fruit a pendent capsule, sword-shaped, 45-120 cm x 6-10 cm, valves flat, almost woody, finally black, Seed 5-9 cm x 2.5-4 cm, including the membranous and transparent wing. Seedling with epigeal germination; 96ypocotyls elongated; cotyledons leafy [188].

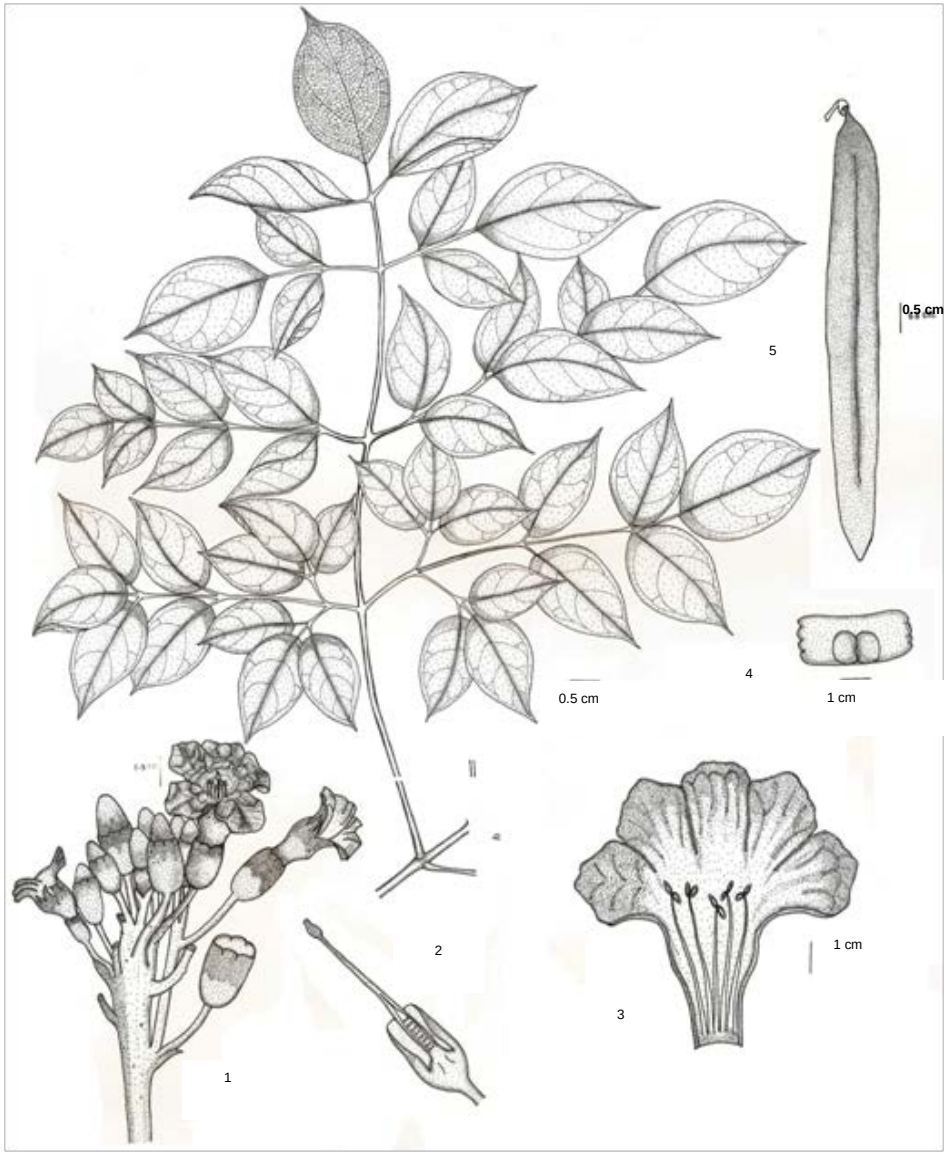


Figure 35 The flowering branch of *O. indicum*

1. Branches of flowers 2. Stamen 3. Longitudinal section of flower 4. Seed 5. Fruit

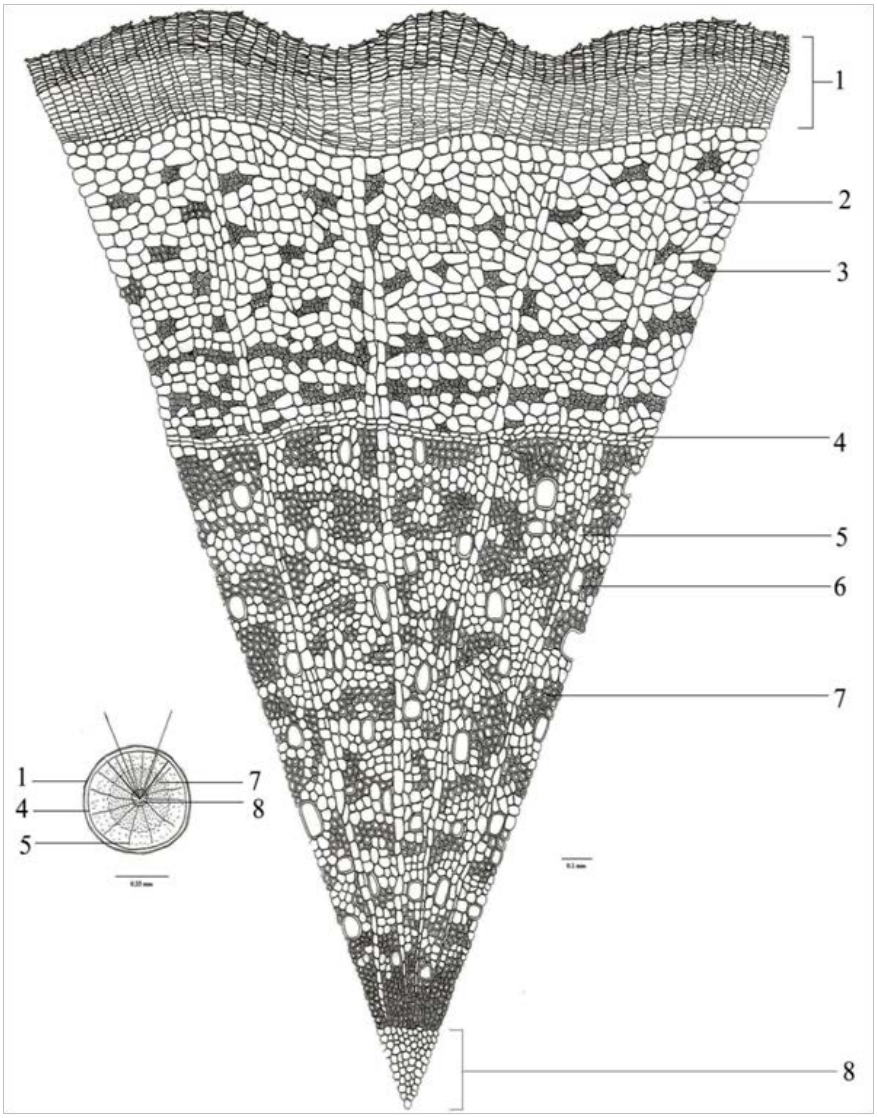


Figure 36 Transverse section of *O. indicum* root ; 1. Periderm 2. Cortical parenchyma 3. Group of cortical fiber 4. Endodermis 5. Xylem ray 6. Xylem vessel 7. Xylem fiber 8. Pith

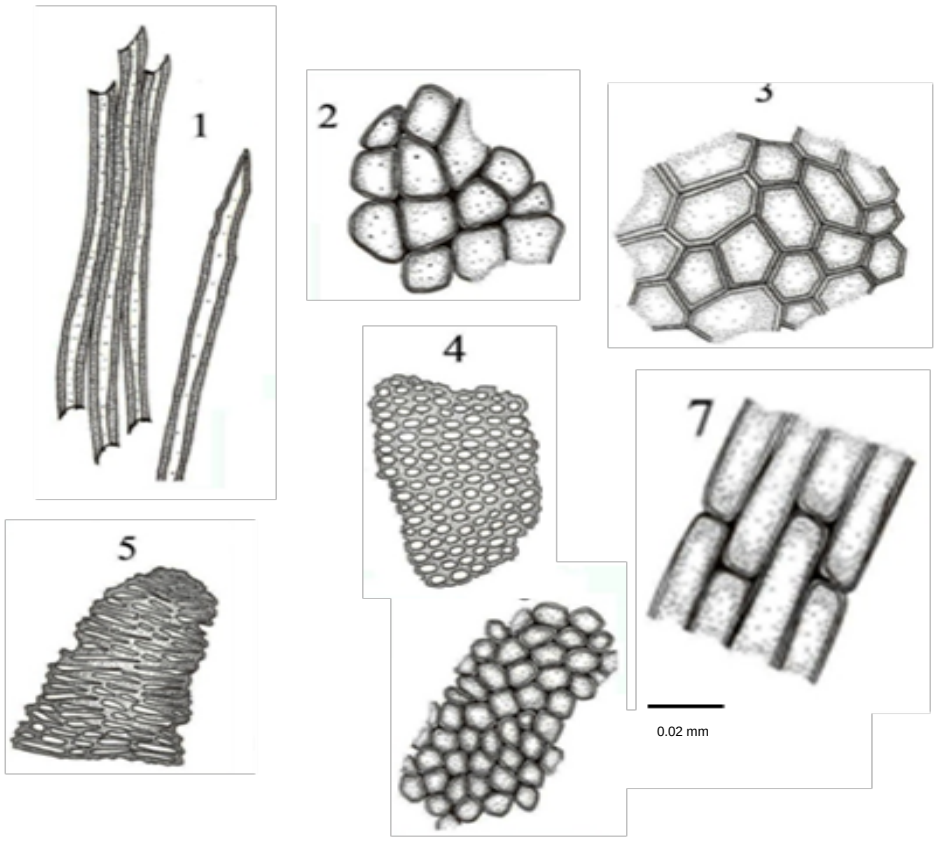


Figure 37 Powder of *O. indicum* root ; 1. Fragment of fiber 2. Epidermis in surface view 3. Cork in surface view 4. Fragment of pitted vessel 5. Fragment of reticulated vessel 6. Parenchyma in transverse view 7. Parenchyma in longitudinal view

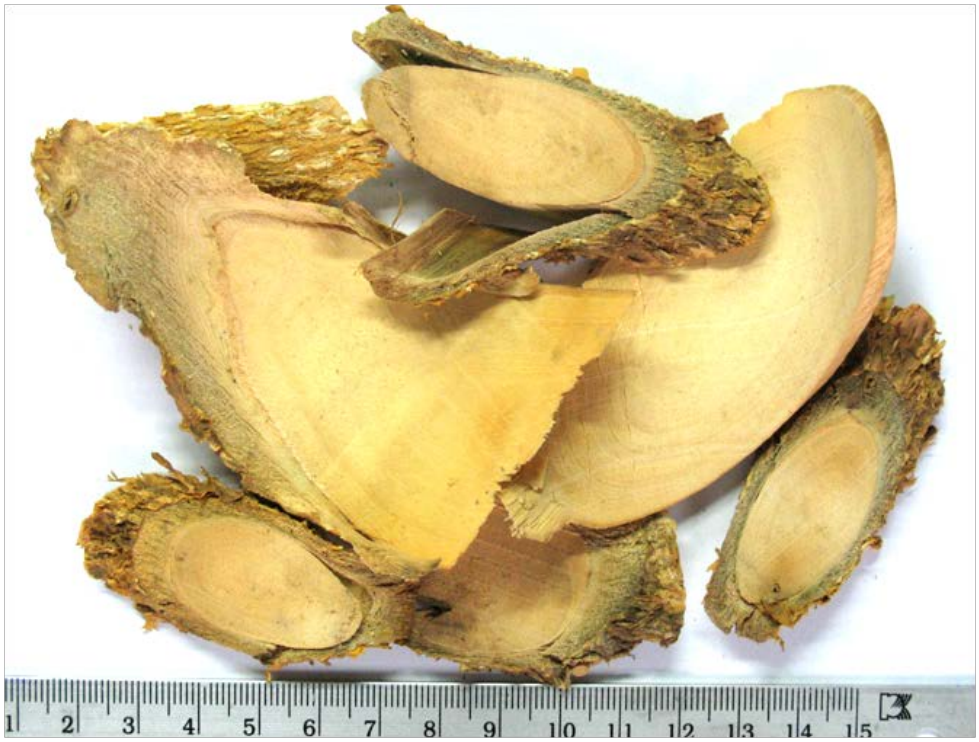


Figure 38 The root of *O. indicum*

Table 14 Physico-chemical specification (% by weight) of *O. indicum* root

Content (% by weight)	**Mean ±SD**	**Min-Max**	**n**
Loss on drying	6.95 ± 0.92	5.50-8.14	13
Total ash content	5.59 ± 1.89	2.09-8.45	13
Acid-insoluble ash content	1.29 ± 0.76	0.38-2.60	13
Water content	10.61 ± 1.29	8.55-11.99	13
Ethanol extractive values	7.93 ± 3.26	7.01-29.18	13
Water extractive values	18.50 ± 7.69	3.48-13.88	13

Grand mean values were calculated from 13 sources throughout Thailand. Each source was performed in triplicate.

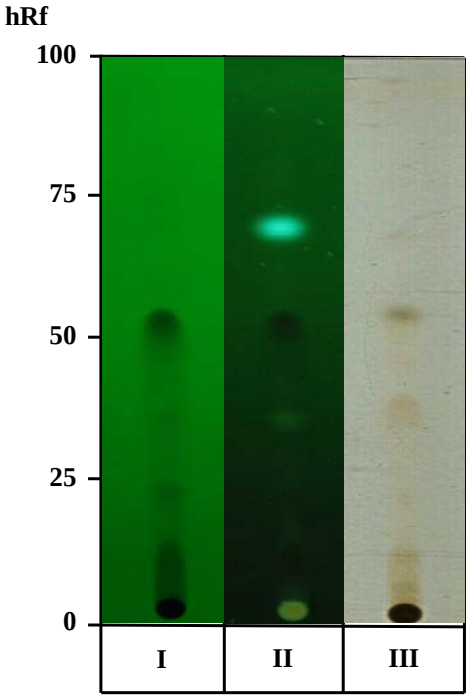


Figure 39 Thin-layer chromatogram of the methanolic extract from *O. indicum* root

Solvent system

Toluene : Ethyl acetate 3:1

Detection

I = detection under UV light 254 nm

II = detection under UV light 366 nm

III = detection with 10% sulfuric acid*, **

*10% sulfuric acid reagent

Preparation: conc. Sulfuric acid 10 ml in methanol 90 ml.

**Spot color Development

Heat the plate at 105 ^{o} C for 10 minutes after sprayed.

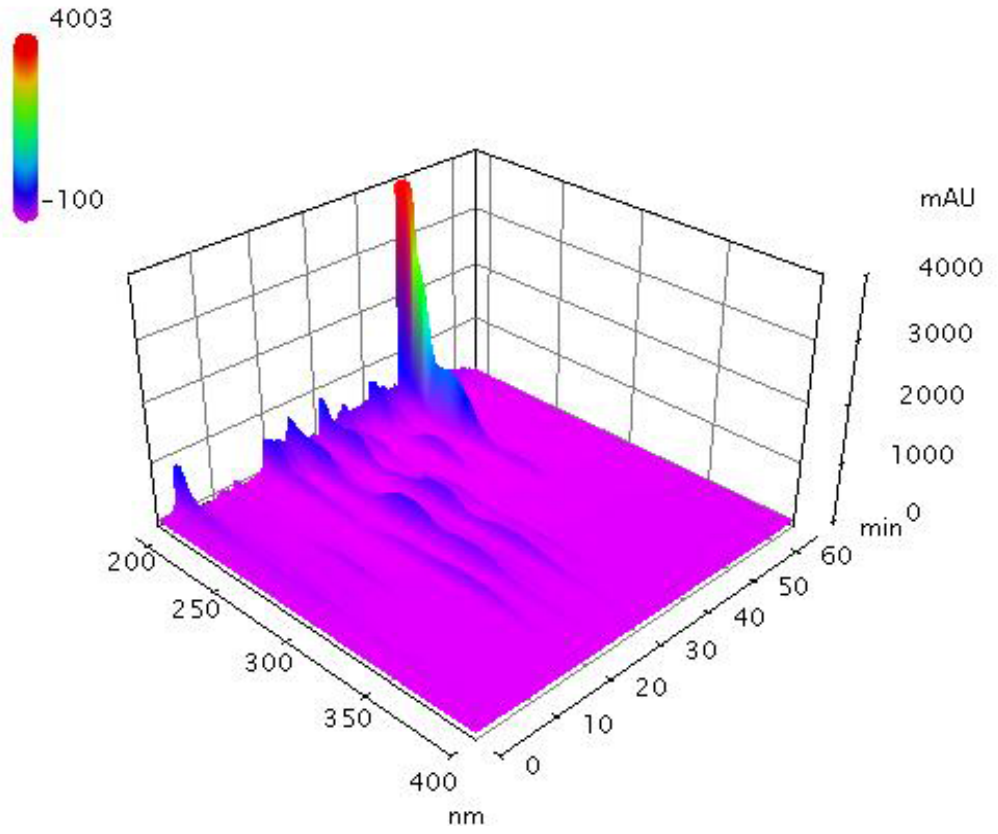


Figure 40 The 3D-HPLC profiles of ethanolic extract from *O. indicum* root

Analysis condition

Column: Inersil ® ODS-3, C-18 column (particle size of the packing 5µm, 4.6 x 250mm)

Mobile phase: 10 mM Phosphoric acid-Acetonitrile

Linear gradient: (95:5, 65 min)

Flow rate: 0.8 mL/min

Injection volume: 10 µl

Temperature: 40°C

Wavelength: 190-400 nm.

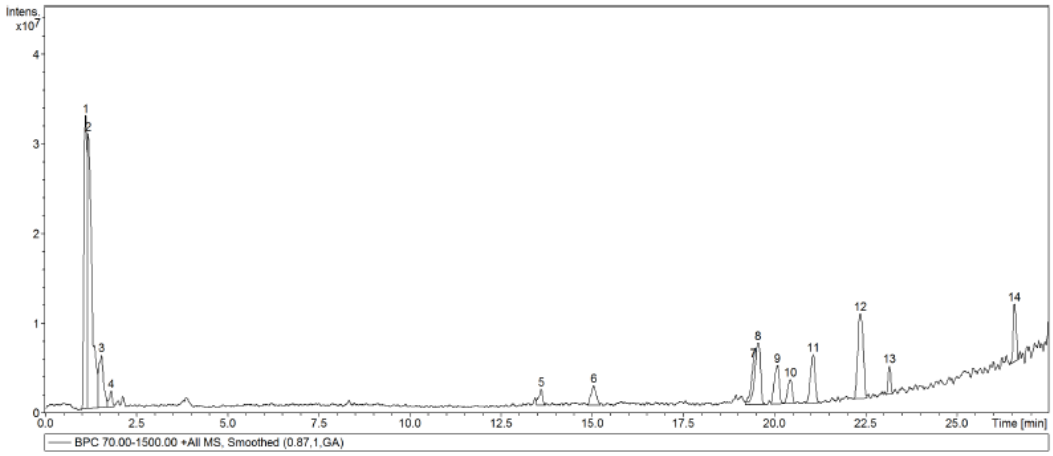


Figure 41 A representative LC/MS chromatogram from *O. indicum* root of compound 1-14

Analysis condition

Column: Dionex C16 Acclaim RSLC PolarAdvantage column (2.1 x 100 mm, 2.2 μm,120 °A)

Mobile phase: (A) Water and (B) Acetonitrile

Linear gradient: 0-2 min (80:20%), 2-32 min (30-70%)

Flow rate: 200 μL/min

Injection volume: 5 μL of 100 ppm

Temperature: 35 °C

MS mode: UltraScan mode between m/z 70-1,500, using positive ionization

Table 15 LC- ESI-MS data and identification of constituents from the root of *O. indicum*

Peak no.	*t*R (min)	MW	Product ions (m/z)
1	1.12	427.07	427.97, 472.97, 490.06, 527.09
2	1.19	427.09	473.04, 490.09, 527.12
3	1.56	176.05	177.16, 194.04, 199.06, 231.06
4	1.82	234.06	256.88, 273.03, 288.94
5	13.6	316.09	333.96, 339.07, 355.09, 371.04
6	15.04	488.07	489.06, 511.06, 527.05, 542.88
7	19.42	312.15	313.34, 335.14, 367.17
8	19.54	312.17	335.02, 351.00, 367.19
9	20.07	386.20	387.19, 404.38, 409.19, 425.16, 441.26
10	20.43	398.10	415.96, 437.10, 453.09
11	21.05	328.08	329.27, 351.19, 383.09
12	22.34	312.16	367.18
13	23.14	539.37	540.38, 557.47, 562.35, 578.35, 594.25
14	26.56	328.12	346.06, 367.00, 383.13

***Walsura trichostemon* Miq.**

Family : Meliaceae

Distribution

Walsura trichostemon Miq is a plant of family Meliaceae that has been found in evergreen forest drought throughout Southeast Asia such as Myanmar, Cambodia. Thailand found in North, Northeast and southeastern, which know the local name of Musk Mallow tree [189].

Description

Botany evergreen of briefly deciduous trees, very rarely with latex of sap. Leaves odd-pinnate, stalks swollen and jointed. Alternate, spirally arranged, leaflets usually opposite, no stipules. Flowers mostly white or yellow, regular, bisexual, in branched clusters at upper leaf axils, 4-5 free spreading petals, stamens longer than petals, style short, disc ring-like. Fruits fleshy or leathery, not splitting, 1-2 seeds with aril.

Figure 42 The fruiting branch of *W. trichostemon*

1. Branches of fruits 2. Flower 3. Stamens 4. Pistil

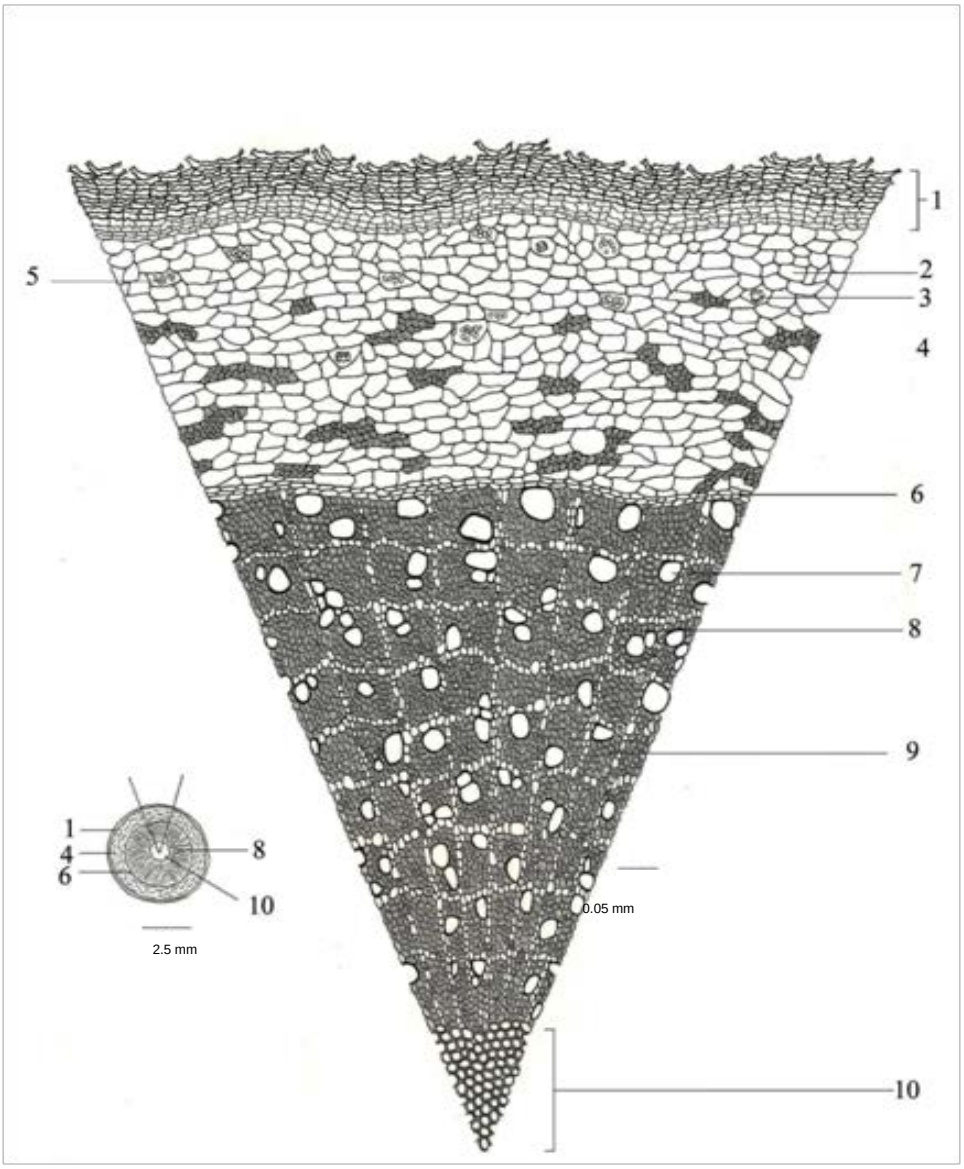


Figure 43 Transverse section of *W. trichostemon* root; 1. Periderm 2. Cortical parenchyma 3. Prismatic crystals in reserved parenchyma 4. Cortical fiber 5. Strach granule in reserved parenchyma 6. Endodermis 7. Xylem vessel 8. Xylem ray 9. Xylem fiber 10. Pith

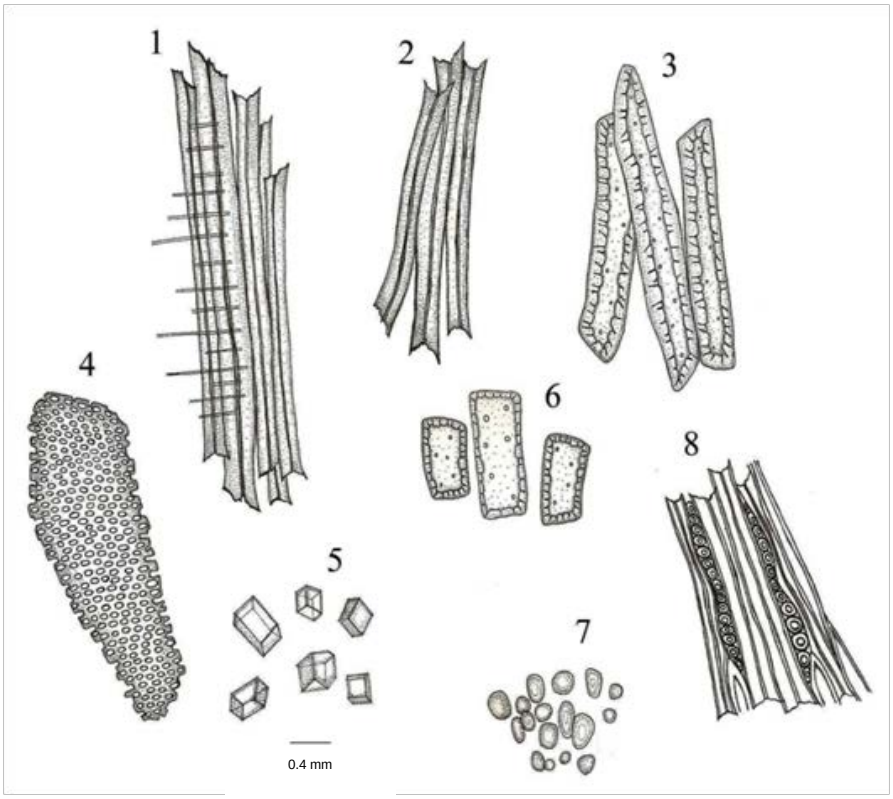


Figure 44 Powder of *W. trichostemon* root ; 1. Xylem in radial longitudinal view 2. Fragment of fiber 3. Sclereid in longitudinal view 4. Fragment of pitted vessel 5. Prism crystal of calcium oxalate 6. Sclereid in transverse view 7. Starch grain 8. Xylem in tangential longitudinal view

Figure 45 The root of *W. trichostemon*

Table 16 Physico-chemical specification (% by weight) of *W. trichostemon* root

Content (% by weight)	**Mean ±SD**	**Min-Max**	**n**
Loss on drying	7.09 ± 0.82	5.79-8.23	13
Total ash content	2.82 ± 1.13	1.60-5.80	13
Acid-insoluble ash content	0.56 ± 0.24	0.31-0.96	13
Water content	13.20 ± 1.56	11.74-16.01	13
Ethanol extractive values	6.37 ± 3.32	3.57-9.88	13
Water extractive values	5.98 ± 2.21	1.64-7.50	13

Grand mean values were calculated from 13 sources throughout Thailand. Each source was performed in triplicate.

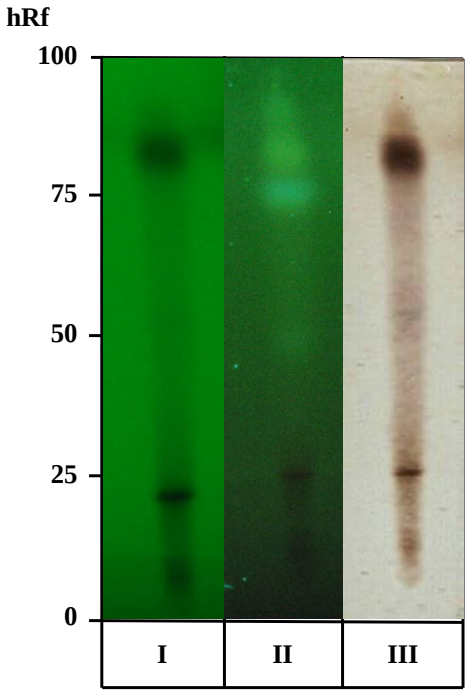


Figure 46 Thin-layer chromatogram of the methanolic extract from *W. trichostemon* root

Solvent system

n-butanol : acetic acid : water 4 : 1 : 5

Detection

I = detection under UV light 254 nm

II = detection under UV light 366 nm

III = detection with 10% sulfuric acid*,**

*10% sulfuric acid reagent

Preparation: conc. Sulfuric acid 10 ml. in methanol 90 ml.

**Spot color Development

Heat the plate at 105 ^{o} C for 10 minutes after sprayed.

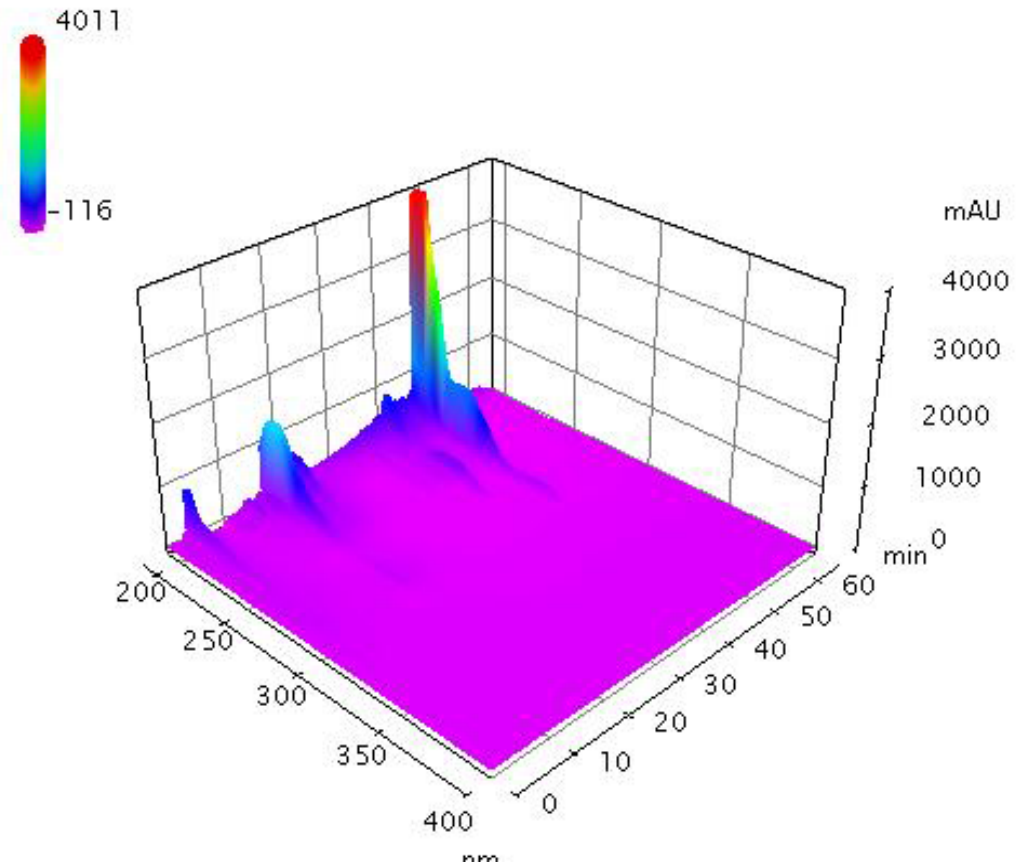


Figure 47 The 3D-HPLC profiles of ethanolic extract from *W.trichostemon* root

Analysis condition

Column: Inersil ® ODS-3, C-18 column (particle size of the packing 5µm, 4.6 x 250mm)

Mobile phase: 10 mM Phosphoric acid-Acetonitrile

Linear gradient: (95:5, 65 min)

Flow rate: 0.8 mL/min

Injection volume: 10 µl

Temperature: 40°C

Wavelength: 190-400 nm.

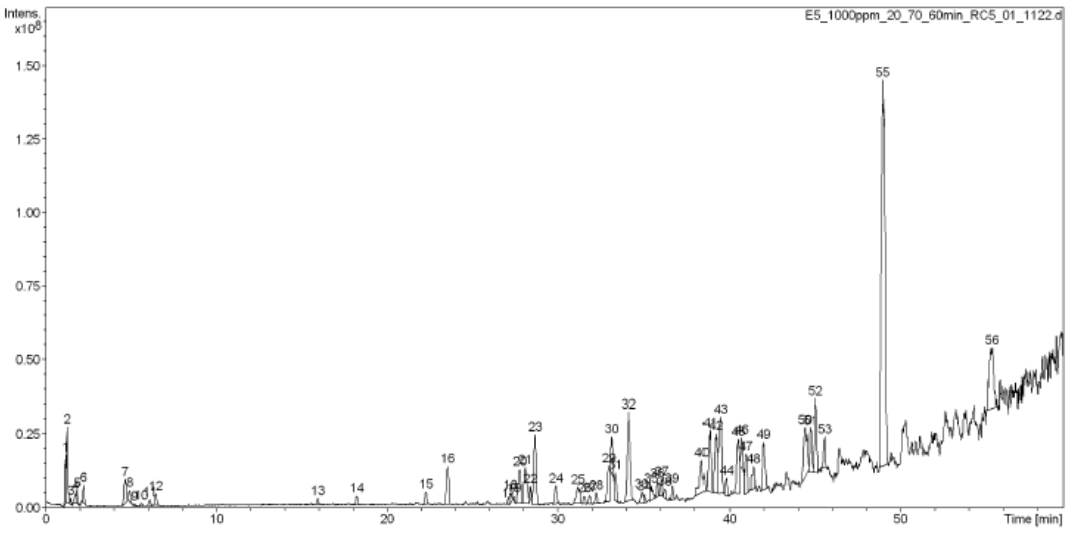


Figure 48 A representative LC/MS chromatogram from *W. trichostemon* root of compound 1-56

Analysis condition

Colum: Dionex C16 Acclaim RSLC PolarAdvantage column (2.1 x 100 mm, 2.2 μm,120 °A)

Mobile phase: (A) Water and (B) Acetonitrile

Linear gradient: 0-2 min (85:15%), 2-60 min (30-70%)

Flow rate: 200 μL/min

Injection volume: 2 μL of 1000 ppm

Temperature: 35 °C

MS mode: UltraScan mode between m/z 70-1,500, using positive ionization

Table 16 LC- ESI-MS data and identification of constituents from the root of *W. trichostemon*

Peak no.	*t*R (min)	MW	Product ions (m/z)
1	1.15	233.97	234.92, 256.79, 288.99
2	1.25	148.04	171.13, 203.05
3	1.47	344.05	344.97, 362.08, 367.05, 383.00, 398.94
4	1.72	302.02	303.01, 325.03, 340.98, 357.30
5	1.84	466.12	484.20, 489.16, 505.11, 521.08
6	2.21	278.06	279.05, 279.26, 317.05, 333.01
7	4.65	566.18	584.00, 605.19 , 621.15
8	4.9	566.20	567.07, 584.10, 605.20, 621.17
9	5.18	536.19	537.39, 559.10, 575.13, 591.24
10	5.58	356.04	357.15, 379.15, 395.06, 411.01
11	6.1	536.20	537.21, 575.18, 591.16
12	6.44	568.19	568.97, 586.00, 586.31, 607.19, 623.16
13	15.94	544.21	545.26, 562.21, 567.11, 583.20, 599.15
14	18.2	560.23	561.12, 578.14, 583.17, 599.22, 615.25
15	22.28	602.23	620.05, 641.17, 657.25
16	23.54	512.19	513.10, 551.15, 567.20
17	27.12	602.30	641.27, 657.31

Peak no.	*t*R (min)	MW	Product ions (m/z)
18	27.22	512.20	551.29, 567.21
19	27.42	558.24	581.08, 613.26
20	27.75	570.21	571.39, 588.44, 593.46, 609.36, 625.22
21	28.08	560.25	561.16, 578.01, 599.24, 615.21
22	28.37	546.31	564.31, 569.28, 585.30, 601.25
23	28.65	544.22	562.04, 567.20, 583.11, 599.24
24	29.87	632.31	633.25, 671.29, 687.25
25	31.16	558.24	559.20, 576.35, 613.26
26	31.53	468.19	486.38, 507.13, 523.20
27	31.86	554.23	555.24, 572.40, 609.24
28	32.23	670.32	671.29, 688.21, 709.31, 725.35
29	32.99	488.23	506.16, 543.24
30	33.15	670.34	671.43, 693.43, 725.36
31	22.36	496.18	551.20
32	34.14	554.21	555.28, 572.39, 593.28, 609.22
33	34.92	554.22	555.00, 572.31, 577.07, 609.24
34	35.06	586.21	604.42, 609.15, 625.13, 641.23
35	35.46	642.38	643.42, 665.22, 681.32, 697.40

Peak no.	*t*R (min)	MW	Product ions (m/z)
36	35.82	528.24	567.43, 583.25
37	36.09	644.32	645.34, 662.23, 667.13, 683.29, 699.34
38	36.29	584.34	585.20, 639.36
39	36.70	526.20	544.18, 549.19, 565.17, 581.37
40	38.39	698.38	721.50, 737.38, 753.39
41	38.84	468.21	469.07, 486.18, 491.09, 523.23
42	39.17	598.38	599.21, 637.27, 653.40
43	39.47	598.38	599.32, 616.33, 616.52, 653.40
44	39.81	656.39	659.45, 711.40
45	40.48	712.33	713.34, 730.30, 751.27, 767.35
46	40.68	712.40	735.30, 767.42
47	40.91	712.38	730.30, 751.45, 767.40
48	41.37	598.37	621.22, 621.50, 653.39
49	41.97	626.38	627.14, 644.39, 681.40
50	44.39	696.41	719.55, 735.46, 751.43
51	44.73	640.40	641.45, 663.58, 695.42
52	45.00	640.40	641.42, 663.58, 679.31, 695.42
53	45.55	652.33	670.19, 675.33, 707.34

Peak no.	*t*R (min)	MW	Product ions (m/z)
54	48.95	452.21	507.22
55	48.95	452.21	507.22
56	55.35	447.33	448.36, 470.31, 470.51, 502.34

Ben-Cha-Moon-Yai remedy

HPLC chromatogram and Mass spectrometric data of BMY extract prepared by the extracts combination according to an equal weight of each root was demonstrated in figure 49-50 and table 18.

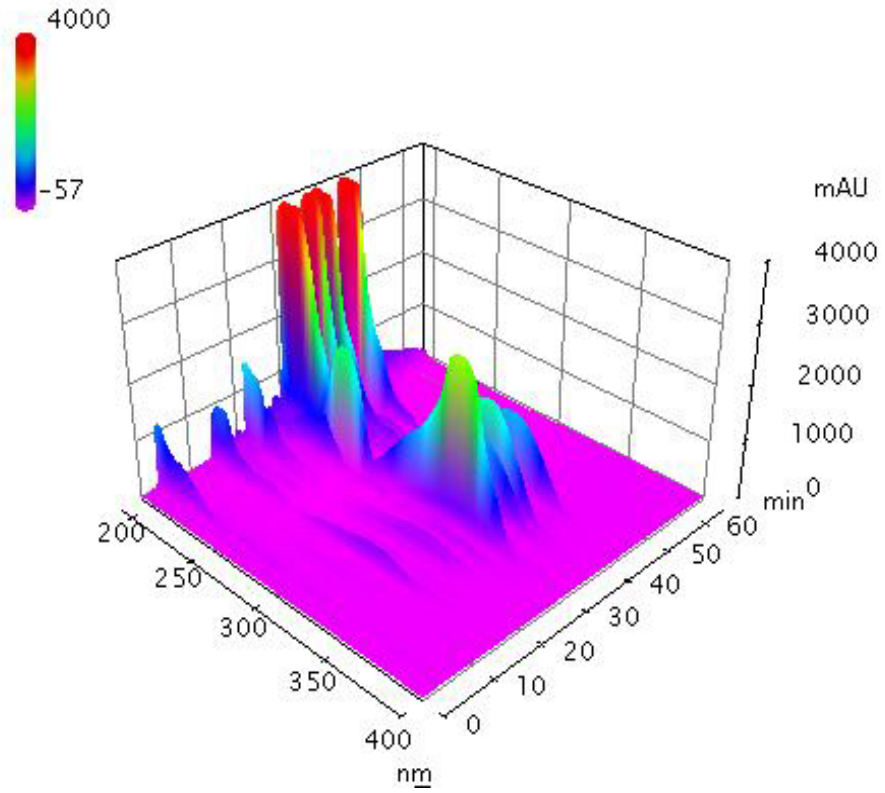


Figure 49 The 3D-HPLC profiles of ethanolic extract from Ben-Cha-Moon-Yai remedy

Analysis condition

Colum: Inersil ® ODS-3, C-18 column (particle size of the packing 5µm, 4.6 x 250mm) **Mobile phase:** 10 mM Phosphoric acid-Acetonitrile

Linear gradient: (95:5, 65 min) **Flow rate:** 0.8 mL/min **Injection volume:** 10 µl

Temperature: 40°C **Wavelength:** 190-400 nm.

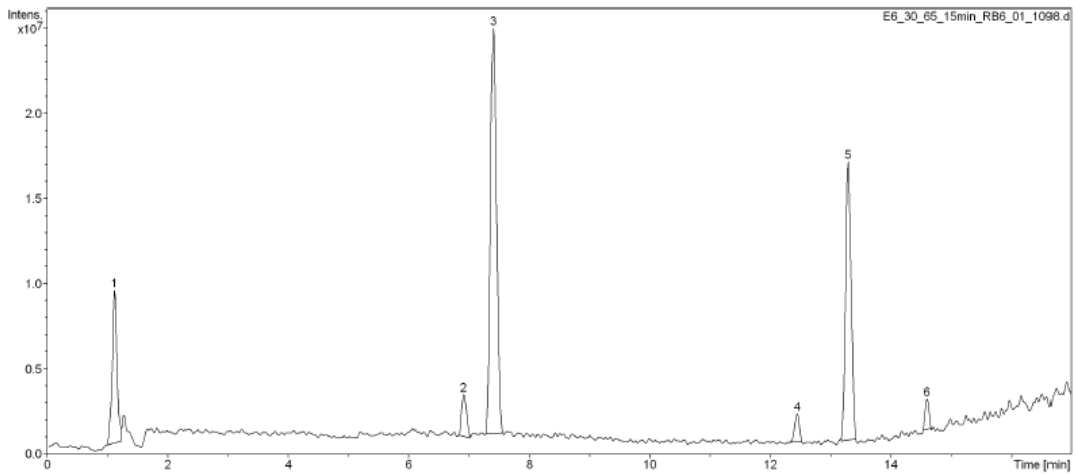


Figure 50 A representative LC/MS chromatogram from Ben-Cha-Moon-Yai remedy extract of compound 1-6

Analysis condition

Colum: Dionex C16 Acclaim RSLC PolarAdvantage column (2.1 x 100 mm, 2.2 μm,120 °A)

Mobile phase: (A) Water and (B) Acetonitrile

Linear gradient: 0-2 min (70:30%), 2-40 min (35-65%)

Flow rate: 200 μL/min

Injection volume: 5 μL of 10 ppm

Temperature: 35 °C

MS mode: UltraScan mode between m/z 70-1,500, using positive ionization

Table 18 LC- ESI-MS data and identification of constituents from the root of Ben-Cha-Moon-Yai remedy

Peak no.	*t*R (min)	MW	Product ions (m/z)
1	1.28	189.9	191.1, 213.0, 229.0, 244.9
2	6.89	259.0	260.0, 277.0, 282.0, 297.9, 314.0
3	7.54	300.1	301.1, 318.1, 323.2, 339.1, 355.1
4	12.21	298.1	299.0, 316.2, 321.0, 337.1, 353.0
5	13.35	344.1	345.1, 362.2, 367.0, 383.1, 399.1
6	14.79	298.1	299.1, 316.0, 321.0, 337.1, 353.0

Plant extraction

Table 19 showed crude extracted yields of each five root species in BMY remedy

Plant name	Yield of ethanol extract	Yield of water extract	Total yield (%)
Aegle marmelos	8.5307	5.9180	14.4487
Dolichandrone serrulata	12.4625	3.2434	15.7058
Dimocarpus longan	6.0784	3.6550	9.7334
Oroxylum indicum	16.6441	7.0639	23.7080
Walsura trichostemon	10.1250	4.6900	14.8150

Cytotoxicity assay

The results from the brine shrimp lethality testing showed in table 20. It was found that the ethanol extract of *A. marmelos* exhibited the highest toxicity against brine shrimp nauplii with LC_{50} of 53.5 µg/ml while BMY remedy showed LC_{50} of 537.3 µg/ml. The remaining extracts exhibited LC_{50} of more than 1000 µg/ml.

Table 20 Brine shrimp lethality (LC_{50}) of the extracts of Ben-Cha-Moon Yai remedy and its ingredient

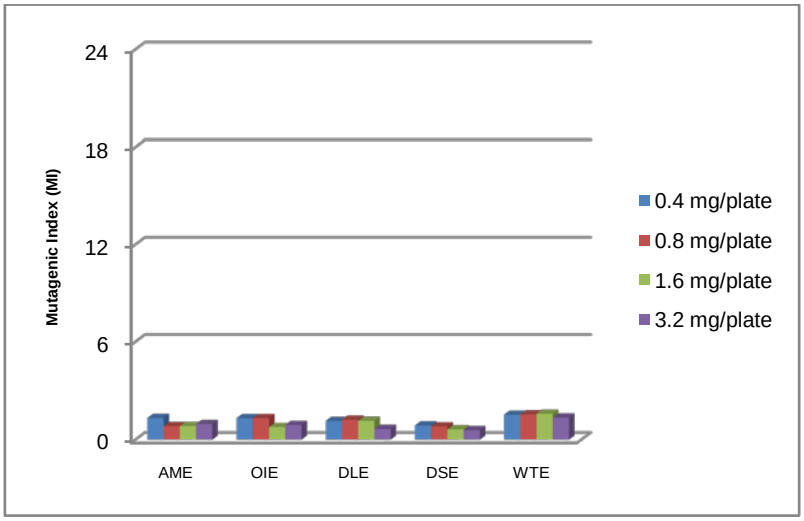
Plant name	**Brine shrimp test Mean LC_{50} (µg/ml), 24 hrs.**	
	Ethanol extract	**Water extract**
Oroxylum indicum	> 1000	> 1000
Aegle marmelos	**53.5**	> 1000
Dimocarpus longan	> 1000	> 1000
Walsura trichostemon	> 1000	> 1000
Dolichandrone serrulata	> 1000	> 1000
Ben Cha Moon Yai remedy	**537.3**	

Mutagenicity assay

MI values of BMY remedy and root extracts obtained by the Ames test were shown in figure 51-55. The result demonstrated that only the water extracts from the root of *A. marmelos* exhibited highest direct mutagenicity on both strains. The extract induced 102.33 ± 39.11 (MI=2.95) and 787.67 ± 26.84 (MI=22.72) revertant colonies at 1.6 mg/plate and at 3.2 mg/plate respectively to strain TA98 (figure 51B) and 819.5±6.36 (MI=5.17) revertant colonies at 3.2 mg/plate to strain TA100 (figure 52B).

It was observed that both ethanol and water extracts of all roots (figure 53-55) and BMY remedy exhibited their mutagenic effect after they were treated with sodium nitrite (nitrosation) under acidic condition without metabolic activation on both strains. BMY remedy extracts at all tested concentrations exhibited a positive response of mutagenicity after nitrite treated 1-aminopyrene in an acidic condition against *Salmonella typhimurium* TA98 by induced 102 ± 17.09 (MI=5.83), 191 ± 75.43 (MI=10.99), 251.67 ± 63.57 (MI=14.38) and 347 ± 18.36 (MI=19.83) revertant colonies and strain TA100 of 215 ± 35.64 (MI=3.86), 194.67 ± 24.99 (MI=4.4), 276 ± 19.52 (MI=5.56) and 392.33 ± 62.61 (MI=7.9) revertant colonies with dose-response relationship (figure 55B). However, there were no mutagenic effects exhibited by the ethanolic extract of *D. serrulata* and *D. longan* and the water extracts of *D. serrulata* to strain TA98 and the ethanolic extracts of *D. serrulata* and *W. trichostemon* towards strain TA100.

51A (TA98)



51B (TA98)

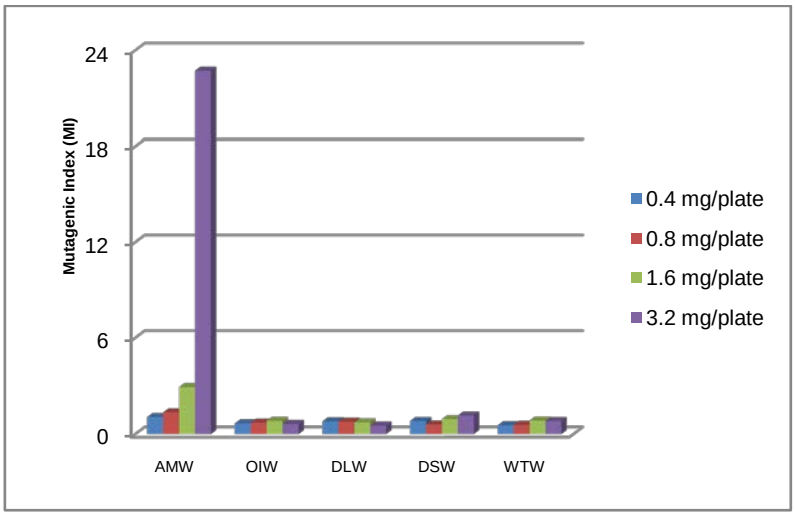
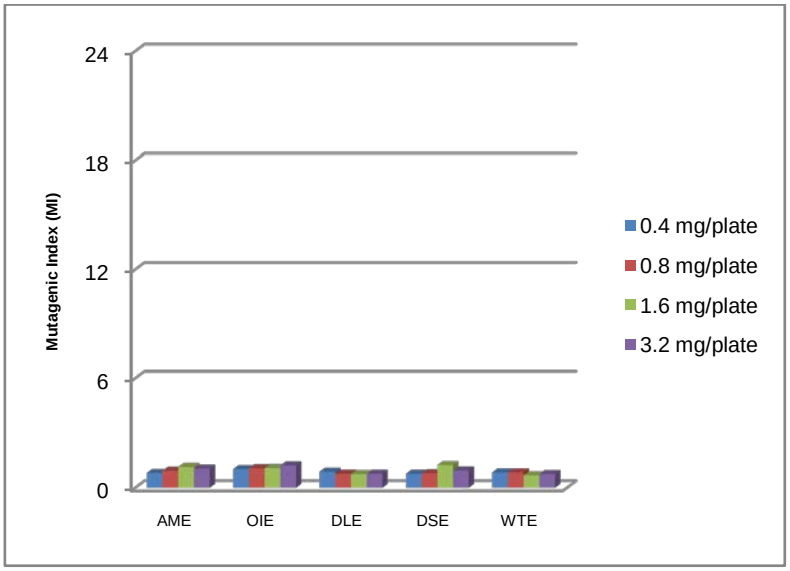


Figure 51 The mutagenic index (MI) induced by the ethanol **(51A)** and water extracts **(51B)** from each plant species of Ben-Cha-Moon-Yai remedy without nitrite treated on *Salmonella typhimurium* strains TA98 using Ames test. Abbreviations including: *Aegle marmelos*: AM, *Oroxylum indicum*: OI, *Dimocarpus longan*: DL, *Dolichandrone serrulata*: DS, *Walsura trichostemon*: WT, E: ethanol extract, W: water extract.

52A (TA100)



52B (TA100)

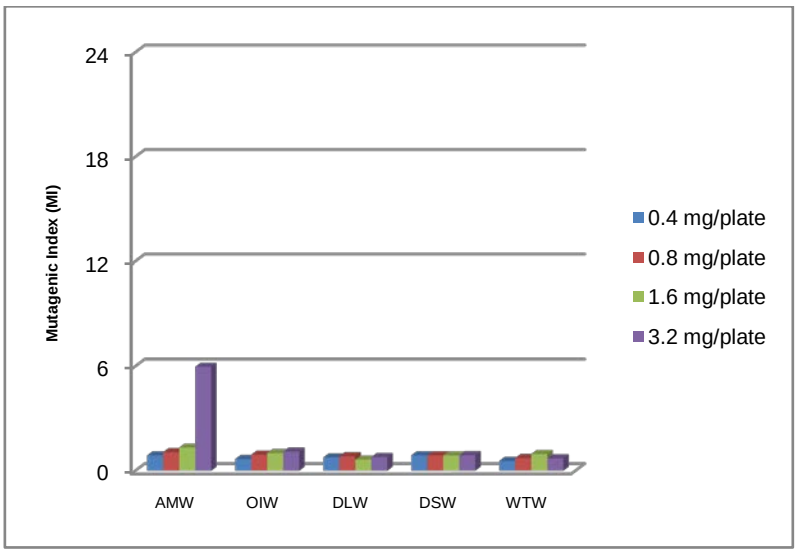
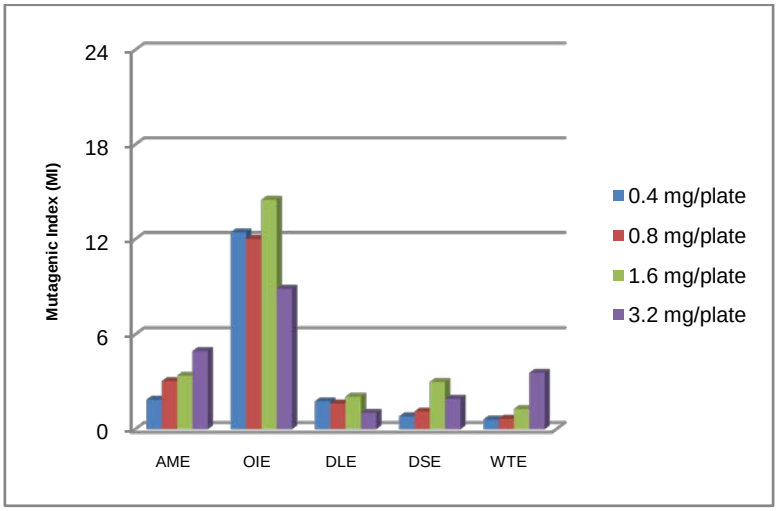


Figure 52 The mutagenic index (MI) induced by the ethanol **(2A)** and water extracts **(2B)** from each plant species of Ben-Cha-Moon-Yai remedy without nitrite treated on *Salmonella typhimurium* strains TA100 using Ames test. Abbreviations including: *Aegle marmelos*: AM, *Oroxylum indicum*: OI, *Dimocarpus longan*: DL, *Dolichandrone serrulata*: DS, *Walsura trichostemon*: WT, E: ethanol extract, W: water extract.

53A (TA98)



53B (TA98)

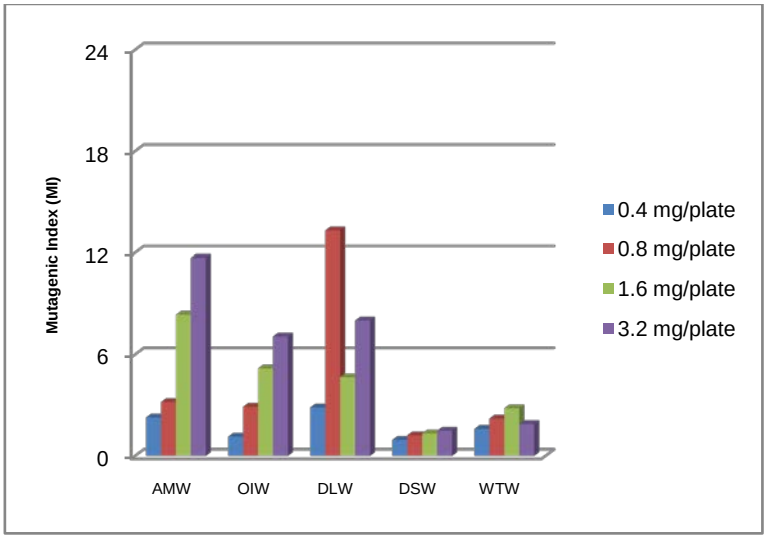
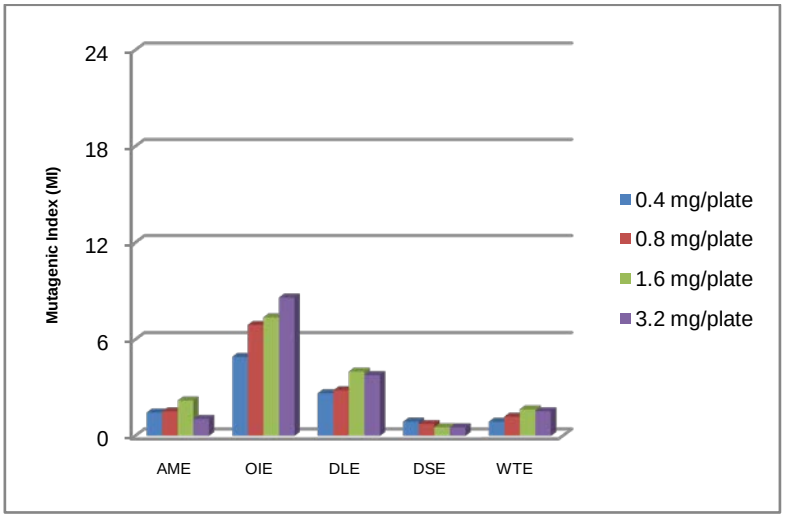


Figure 53 The mutagenic index (MI) induced by the ethanol **(3A)** and water extracts **(3B)** from each plant species of Ben-Cha-Moon-Yai remedy with nitrite treated 1-aminopyrene on *Salmonella typhimurium* strain TA98 using Ames test. Abbreviations including: *Aegle marmelos*: AM, *Oroxylum indicum*: OI, *Dimocarpus longan*: DL, *Dolichandrone serrulata*: DS, *Walsura trichostemon*: WT, E: ethanol extract, W: water extract.

54A (TA100)



54B (TA100)

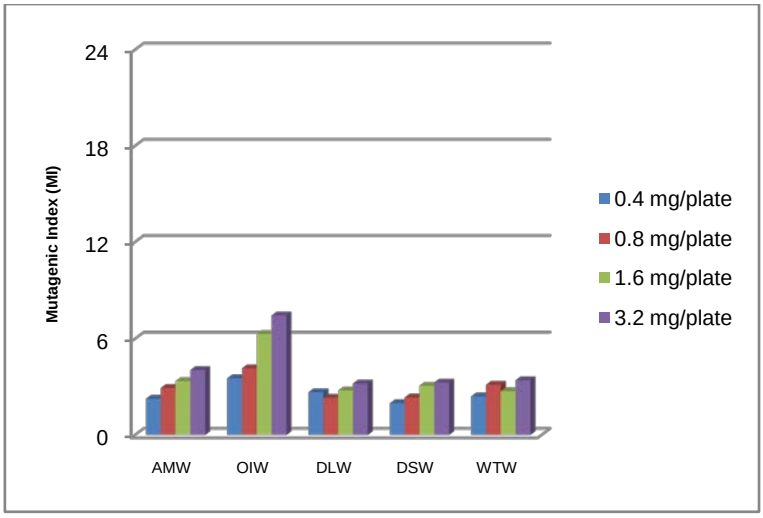
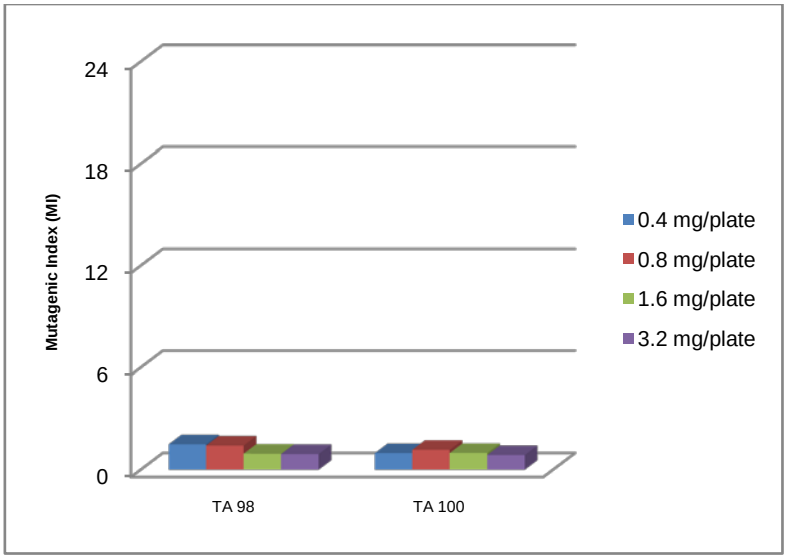


Figure 54 The mutagenic index (MI) induced by the ethanol **(4A)** and water extracts **(4B)** from each plant species of Ben-Cha-Moon-Yai remedy with nitrite treated 1-aminopyrene on *Salmonella typhimurium* strain TA100 using Ames test. Abbreviations including: *Aegle marmelos*: AM, *Oroxylum indicum*: OI, *Dimocarpus longan*: DL, *Dolichandrone serrulata*: DS, *Walsura trichostemon*: WT, E: ethanol extract, W: water extract.

55A (without nitrite treatment)



55B (with nitrite treatment)

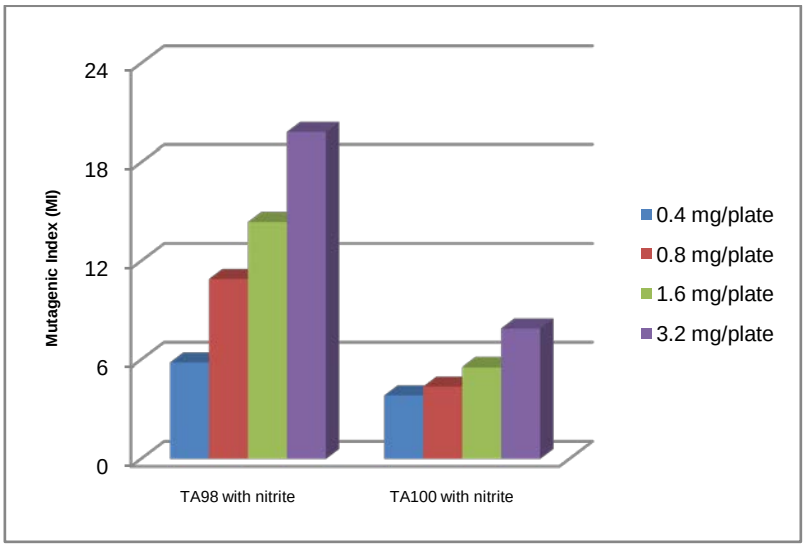


Figure 55 The mutagenic index (MI) induced by the Ben-Cha-Moon-Yai remedy extracts without (55A) and with nitrite (55B) treated 1-aminopyrene on *Salmonella typhimurium* TA98 and TA100 using Ames test.

DNA damage using Comet assay

The DNA migration of cell lymphocytes treated with Ben-Cha-Moon-Yai remedy, five root species extract, positive control and negative control were observed under a fluorescent microscope attached to image capture device with a final magnification of 400x. The degree of DNA damage from all slide were analyzed in terms of tail moment by using the five classes of visual scoring technique, from 0 (no tail) to 4 (almost all DNA tail) give sufficient resolution [134].

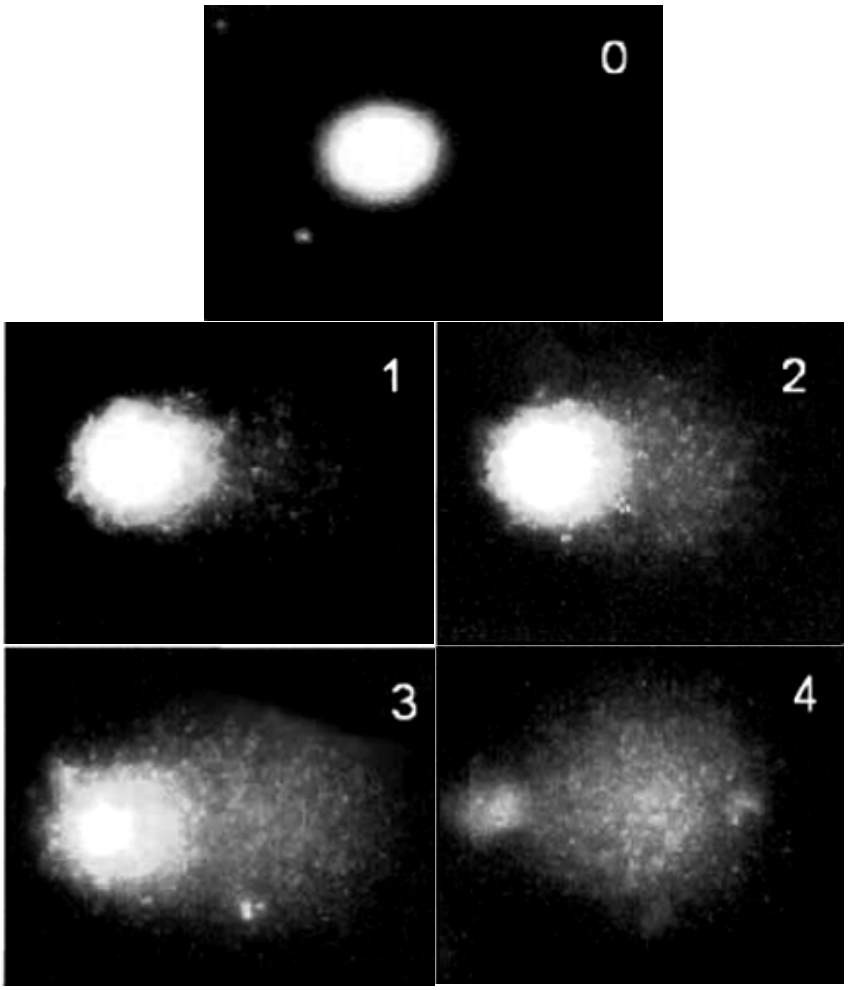


Figure 56 Image of comets from human lymphocytes stained with DAPI with 5 classes of DNA damage used for visual scoring classified by Andrew R. Collin, 2004 [134].

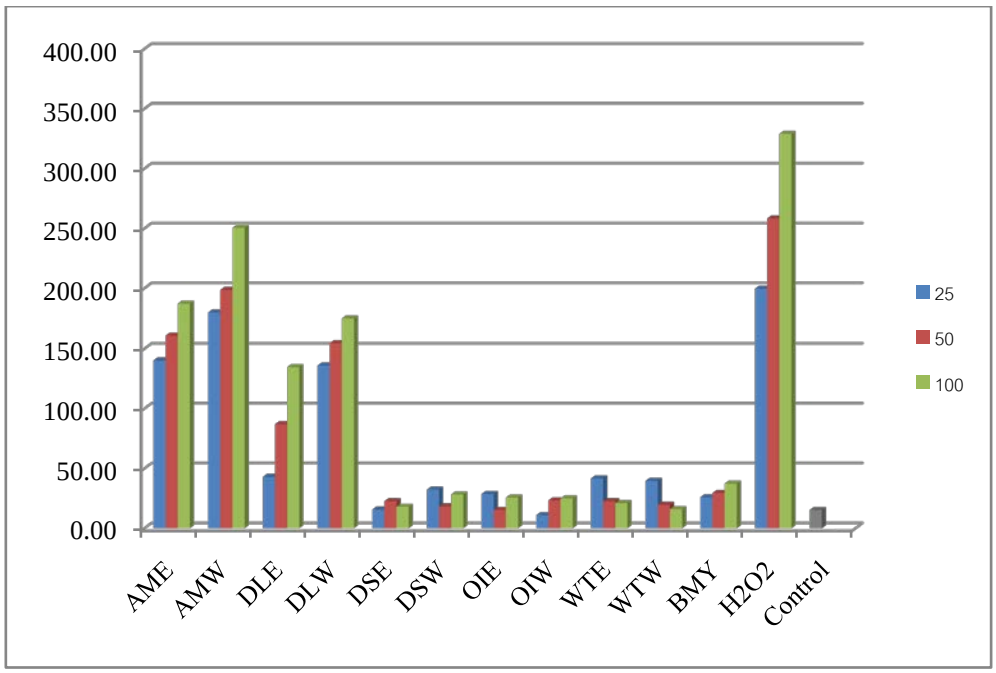


Figure 57 The DNA damage in lymphocytes treated with different concentrations of Ben-Cha-Moon-Yai remedy and five root species or control. The total summing values of the number of comet classification which obtained from the ethanol and water extract of five root species Ben-Cha-Moon-Yai remedy. Abbreviations including: *Aegle marmelos*: AM, *Oroxylum indicum*: OI, *Dimocarpus longan*: DL, *Dolichandrone serrulata*: DS, *Walsura trichostemon*: WT, E: ethanol extract, W: water extract, Ben-Cha-Moon-Yai remedy: BMY.

When 100 comets from each slide were scored, and each comet assigned a value of 0 to 4 according to its class, the total score for the sample gel was between 0 and 400 “arbitrary units”. Among five root species and Ben-Cha-Moon-Yai remedy extracts, the result demonstrated that both water and ethanol extract of *A. marmelos* at concentration of 100 µg/ml showed the highest DNA damage in human lymphocytes with the total score of 250.50 and 187.67, respectively. As followed by the human lymphocytes treated with the water and ethanol extract of *D. longan* with presented the total score of 175.33 and 134.67, respectively. Human lymphocytes treated with H_2O_2 showed the highest DNA damage whereas cell treated with phosphase buffer saline showed the lowest DNA damage effects. All samples which induced the DNA damaged in human lymphocytes revealed a dose-dependent relationship between the degree of DNA damage and concentration of sample.

Figure 58 The DNA damage in lymphocytes treated different concentration of Ben-Cha-Moon-Yai remedy and five root species extracts or control.

Figure 58.1 *Aegle marmelos* (Ethanolic extract)

(1A) 25 µg/ml (1B) 50 µg/ml (1C) 100 µg/ml

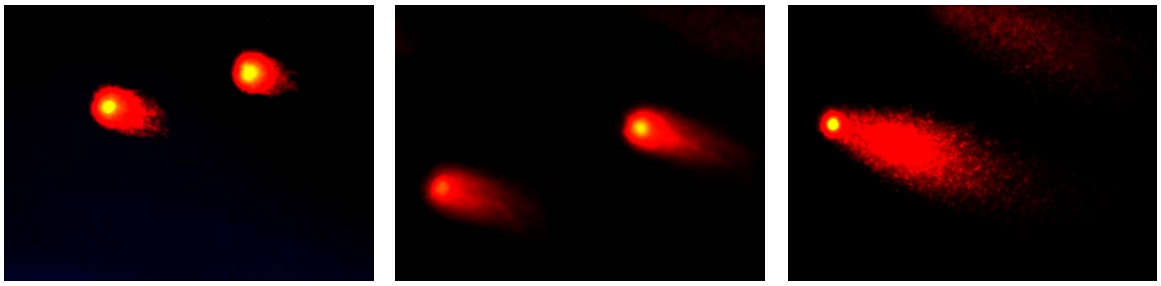


Figure 58.2 *Aegle marmelos* (Water extract)

(2A) 25 µg/ml (2B) 50 µg/ml (2C) 100 µg/ml

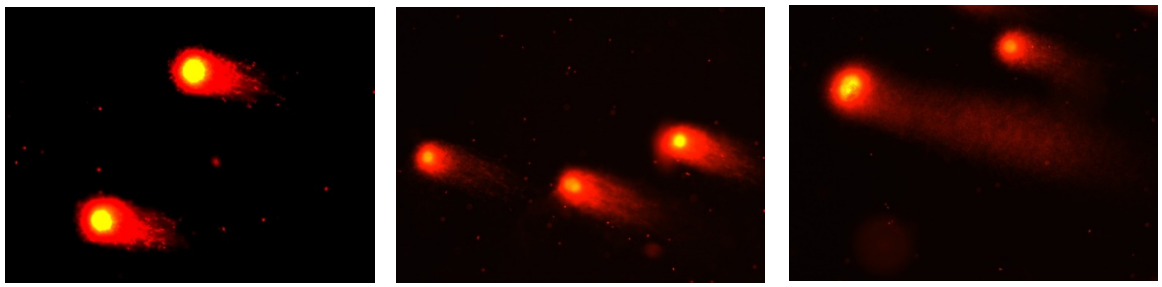


Figure 58.3 *Dimocarpus longan* (Ethanolic extract)

(3A) 25 µg/ml (3B) 50 µg/ml (3C) 100 µg/ml

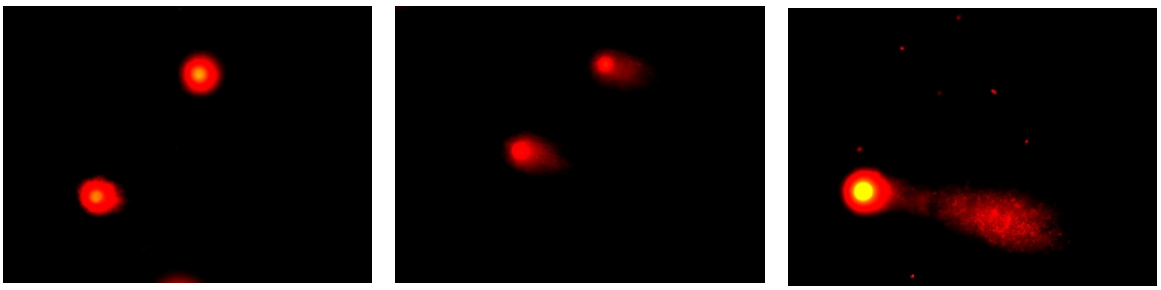


Figure 58.4 *Dimocarpus longan* (Water extract)

(4A) 25 µg/ml (4B) 50 µg/ml (4C) 100 µg/ml

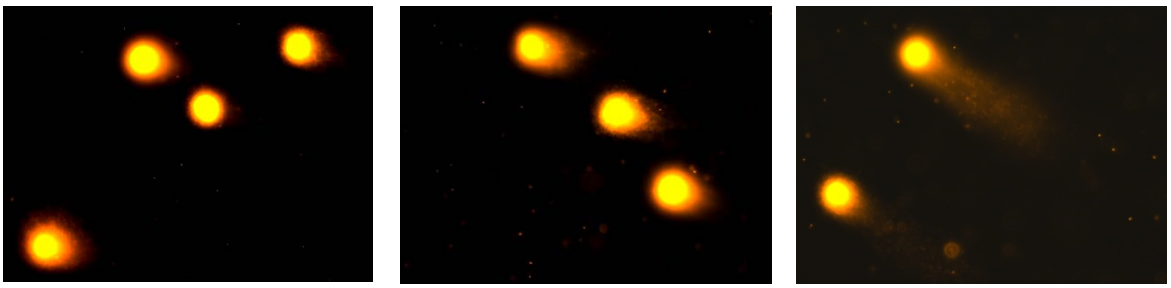


Figure 58.5 *Dolichandrone serrulata* (Ethanolic extract)

(5A) 25 µg/ml (5B) 50 µg/ml (5C) 100 µg/ml

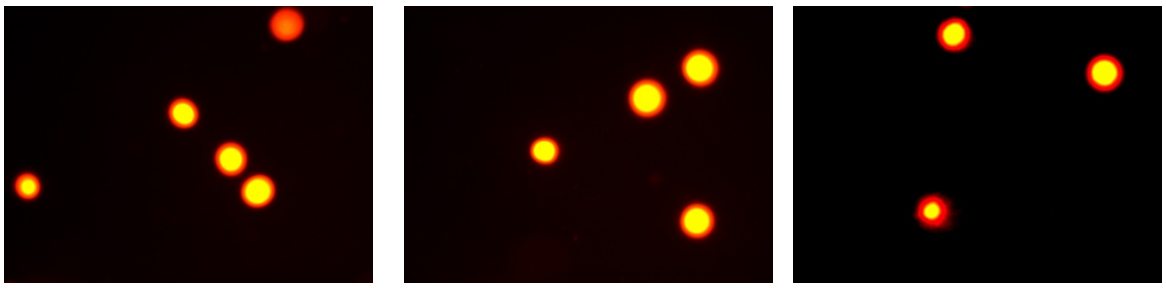


Figure 58.6 *Dolichandrone serrulata* (Water extract)

(6A) 25 µg/ml (6B) 50 µg/ml (6C) 100 µg/ml

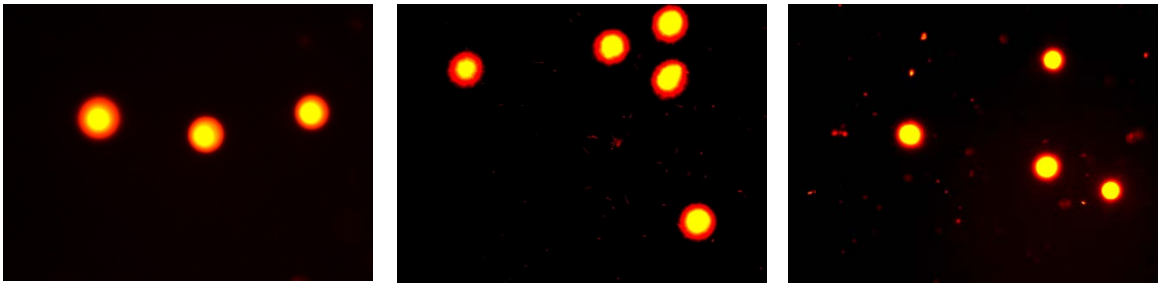


Figure 58.7 *Oroxylum indicum* (Ethanolic extract)

(7A) 25 µg/ml (7B) 50 µg/ml (7C) 100 µg/ml

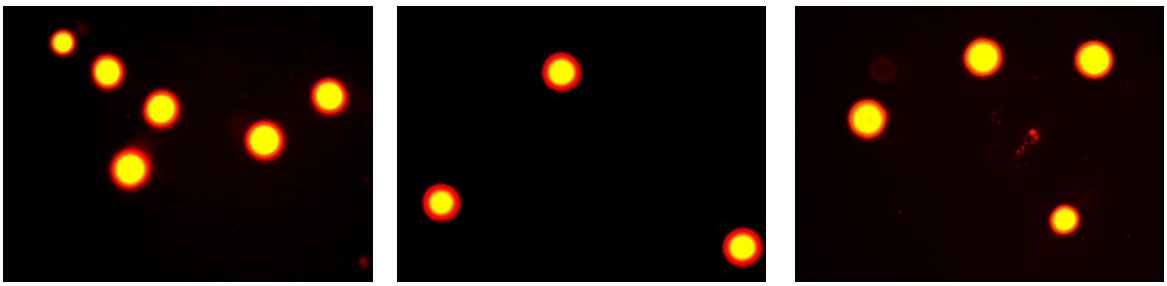


Figure 58.8 *Oroxylum indicum* (Water extract)

(8A) 25 µg/ml (8B) 50 µg/ml (8C) 100 µg/ml

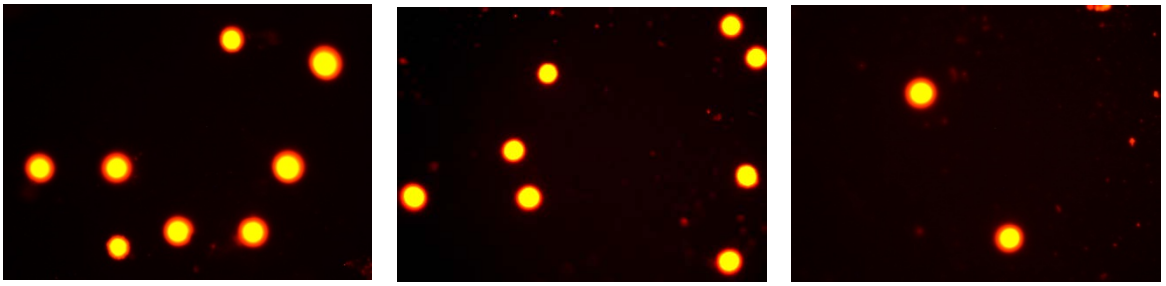


Figure 58.9 *Walsura trichostemon* (Ethanolic extract)

(9A) 25 µg/ml (9B) 50 µg/ml (9C) 100 µg/ml

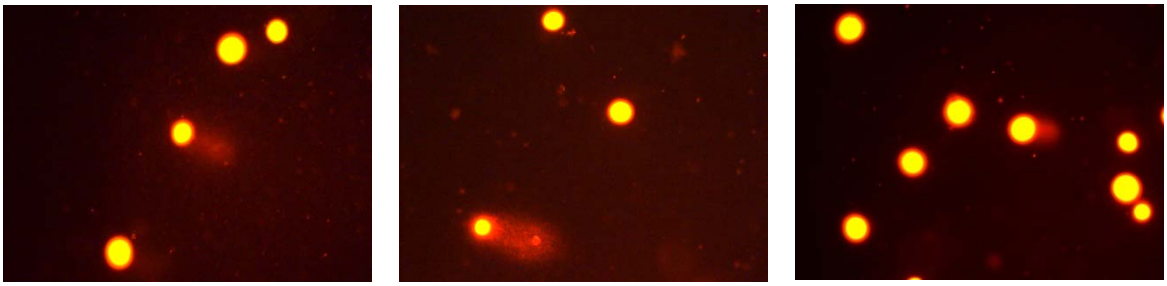


Figure 58.10 *Walsura trichostemon* (Water extract)

(10A) 25 µg/ml (10B) 50 µg/ml (10C)

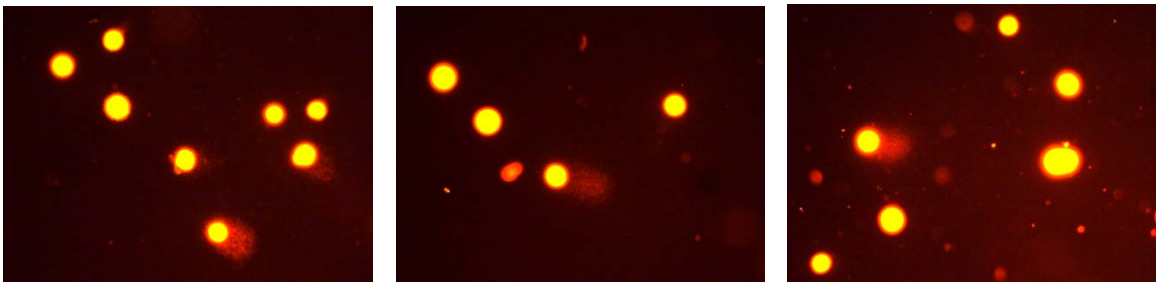


Figure 58.11 Ben-Cha-Moon-Yai remedy

(11A) 25 µg/ml (11B) 50 µg/ml (11C)

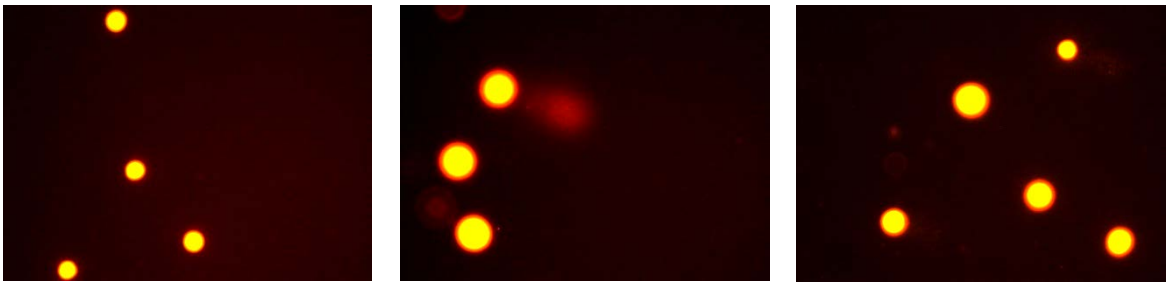


Figure 58.12 Hydrogen Peroxide

(12A) 25 µM (12B) 50 µM (12C) 100 µM

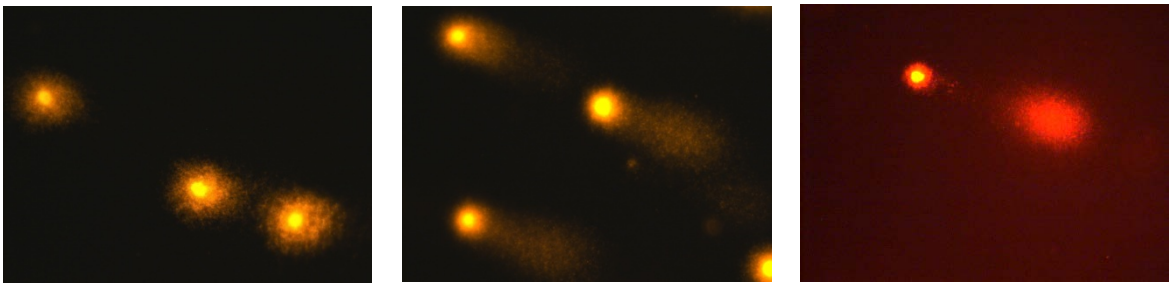
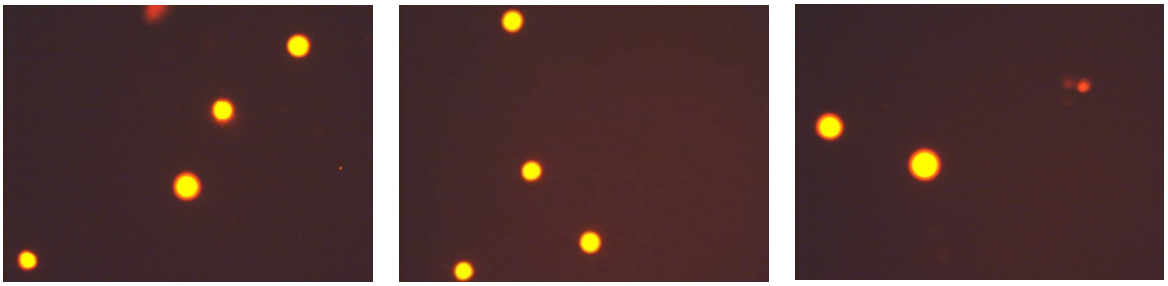


Figure 58.13 Negative Control

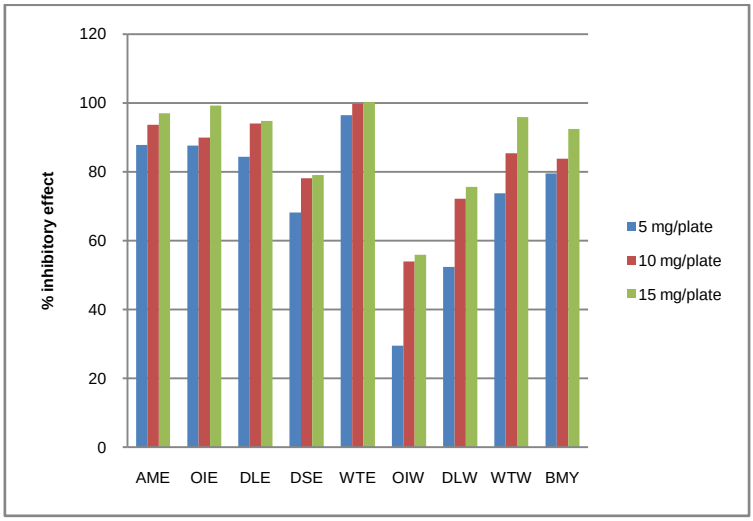
(13A) (13B) (13C)



Antimutagenicity assay

For antimutagenicity assay, all extracts inhibited mutagenicity effect towards *Salmonella typhimurium* strains TA98 and TA100 (figure 59A-59B). The effects were ranged from negligible (0–20% inhibition) to strongly active (>60% inhibition). Only the water extracts of *A. marmelos* and *D. serrulata* were not inhibited the mutagenicity on both strains of *Salmonella typhimurium*. However, the BMY remedy exhibited strong antimutagenicity on both strains of *Salmonella typhimurium*. The percentage of inhibition was increased when the doses were increased. Almost all the roots extract and BMY remedy expressed negligible to strong inhibitory effect (> 60%) on both tester strains. On the other hand, the ethanol extracts of *A. marmelos* (5 mg/plate) had negligible effect (19.5%) on *Salmonella typhimurium* TA 100. The moderate antimutagenic activity was observed on 5, 10 and 15 mg/plate of *O. indicum* water extract on *Salmenella typhimurium* TA98 and 5 mg/plate towards TA100. Whereas, the concentration of 5 mg/plate of the water extract from *D. longan* exhibited the moderate effect on both strains. The moderate (41-60%) to strong antimutagenic (> 60%) effect was observed from the water and ethanol extracts of *O. indicum* toward both strains of *Salmonella typhimurium*, however the water extract at 5 mg/plate exhibited weak inhibitory effect against mutagenicity on strain TA98. All dose of the ethanol extract from *D. serrulata* demonstrated strong antimutagenic effect on both strains, while 5 mg/plate toward strain TA100 exhibited the moderate effect. The strong inhibitory effect was expressed at all concentrations by ethanol extract of *W. trichostemon*, while the weak 20.6% and moderate 51.1% effect were demonstrated when the concentrations of 5 or 10 mg/plate were added to strains TA100, respectively. All of the extracts have dose-related inhibition effect to their mutagenicity of nitrite treated 1-aminopyrene toward *Salmonella typhimurium* strains TA98 and TA100 in the absence of enzyme activating system.

59A (TA98)



59B (TA100)

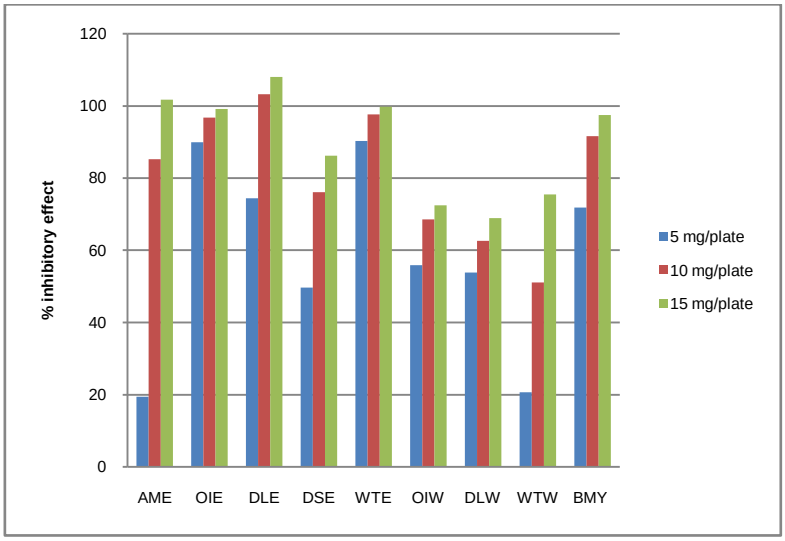


Figure 59 Inhibitory effect of Ben-Cha-Moon-Yai remedy and its components extracts on the mutagenicity of sodium nitrite-treated 1-aminopyrene on *Salmonella typhimurium* strains TA 98 (59A) and TA100 (59B) using Ames test. Abbreviations including: *Aegle marmelos*: AM, *Oroxylum indicum*: OI, *Dimocarpus longan*: DL, *Dolichandrone serrulata*: DS, *Walsura trichostemon*: WT, Ben-Cha-Moon-Yai remedy: BMY, E: ethanol extract, W: water extract.

Antimicrobial activity

The ability of Ben-Cha-Moon-Yai remedy and five root species extracts to inhibit the growth of selected microorganisms were evaluated by agar-well diffusion method and broth microdilution method. The agar-well diffusion test was performed to 13 microorganisms including Gram-positive and negative bacteria and fungi. The active extract which showed the zone inhibition were further investigated in broth microdilution method to obtain MIC, MBC and MFC. The results of the antimicrobial activity have been summarized in table 21-33 showed that the crude ethanol and water extract from five root species and Ben-Cha-Moon-Yai remedy showed the selective activity to prevent the growth of microorganisms.

Table 21 The antimicrobial activities of Ben-Cha-Moon-Yai remedy and five root species extracts at 4 mg/ml against *Staphylococcus aureus*.

Plants/positive controls/ negative control	Solvent extracts	*Staphylococcus aureus*		
		Inhibition zone (mm)	MIC (µg/ml)	MBC (µg/ml)
Aegle marmelos	Ethanol	9.33±0.58	>2000	>2000
	Water	NA	NA	NA
Dimocarpus longan	Ethanol	13.67±0.58	500	1000
	Water	10.67±0.58	>2000	>2000
Dolichandrone serrulata	Ethanol	8.33±0.58	>2000	>2000
	Water	8.00±0.00	>2000	>2000
Oroxylum indicum	Ethanol	13.00±0.00	1000	>2000
	Water	NA	NA	NA
Walsura trichostemon	Ethanol	NA	NA	NA
	Water	7.00±0.00	>2000	>2000
BMY remedy		11.67±0.58	>2000	>2000
Ampicllin		42.00±1.00	0.078	0.156
Amikacin		22.67±0.58	1.25	2.5
DMSO		NA	NA	NA

Mean ± SD, NA = no activity, each experiment was done in triplicate.

The results reported in table 1 found that the ethanol extract from the root of *D. longan* showed the highest activity to prevent growth of *Staphylococcus aureus* which exhibited the largest of zone inhibition of 13.67±0.58 mm and the lowest MIC and MBC of 500 and 1000 µg/ml, respectively. The ethanolic extract from *O. indicum* also possessed a large of zone inhibition of 13±0.00, MIC and MBC values of 1000 and > 2000 µg/ml, respectively.

Table 22 The antimicrobial activities of Ben-Cha-Moon-Yai remedy and five root species extracts at 4 mg/ml against *Staphylococcus epidermidis*.

Plants/positive controls/ negative control	Solvent extracts	*Staphylococcus epidermidis*		
		Inhibition zone (mm)	MIC (µg/ml)	MBC (µg/ml)
Aegle marmelos	Ethanol	NA	NA	NA
	Water	NA	NA	NA
Dimocarpus longan	Ethanol	NA	NA	NA
	Water	13.67±0.58	1000	>2000
Dolichandrone serrulata	Ethanol	8.67±0.58	>2000	>2000
	Water	NA	NA	NA
Oroxylum indicum	Ethanol	15.00±0.00	1000	>2000
	Water	NA	NA	NA
Walsura trichostemon	Ethanol	NA	NA	NA
	Water	7.00±0.00	>2000	>2000
BMY remedy		12.33±0.58	1000	>2000
Ampicllin		23.67±0.58	0.312	0.312
Amikacin		25.67±1.53	1.25	2.5
DMSO		NA	NA	NA

Mean ± SD, NA = no activity, each experiment was done in triplicate.

The ethanolic extract of *O. indicum* showed the highest inhibitory effect against *S. epidermidis* which the zone inhibition of 15.00±0.00 mm, followed by the water extract from *D. longan* which provided the zone inhibition of 13.67±0.58 mm. Both active extracts showed the same MIC and MBC values of 1000 and >2000 µg/ml, respectively.

Table 23 The antimicrobial activities of Ben-Cha-Moon-Yai remedy and five root species extracts at 4 mg/ml against *Bacillus cereus*.

Plants/positive controls/ negative control	Solvent extracts	*Bacillus cereus*		
		Inhibition zone (mm)	MIC (µg/ml)	MBC (µg/ml)
Aegle marmelos	Ethanol	9.33±0.58	>2000	>2000
	Water	NA	NA	NA
Dimocarpus longan	Ethanol	NA	NA	NA
	Water	NA	NA	NA
Dolichandrone serrulata	Ethanol	10.33±0.58	>2000	>2000
	Water	NA	NA	NA
Oroxylum indicum	Ethanol	12.00±0.00	2000	>2000
	Water	NA	NA	NA
Walsura trichostemon	Ethanol	NA	NA	NA
	Water	NA	NA	NA
BMY remedy		10.67±0.58	>2000	>2000
Ampicllin		35.00±1.73	0.039	0.039
Amikacin		28.67±0.58	0.312	0.625
DMSO		NA	NA	NA

Mean ± SD, NA = no activity, each experiment was done in triplicate.

The ethanolic extract from the root of *O. indicum* showed the largest of zone inhibition of 12.00±0.00 mm against *B. cereus* and the lowest MIC and MBC of 2000 and >2000 µg/ml, respectively.

Table 24 The antimicrobial activities of Ben-Cha-Moon-Yai remedy and five root species extracts at 4 mg/ml against *Bacillus subtilis*.

Plants/positive controls/ negative control	Solvent extracts	*Bacillus subtilis*		
		Inhibition zone (mm)	MIC (µg/ml)	MBC (µg/ml)
Aegle marmelos	Ethanol	7.67±0.58	>2000	>2000
	Water	NA	NA	NA
Dimocarpus longan	Ethanol	14.33±0.58	>2000	>2000
	Water	12.67±0.58	>2000	>2000
Dolichandrone serrulata	Ethanol	NA	NA	NA
	Water	NA	NA	NA
Oroxylum indicum	Ethanol	11.67±0.58	1000	>2000
	Water	NA	NA	NA
Walsura trichostemon	Ethanol	NA	NA	NA
	Water	NA	NA	NA
BMY remedy		9.67±0.58	>2000	>2000
Ampicllin		17.00±0.00	5	10
Amikacin		24.67±1.53	0.625	0.625
DMSO		NA	NA	NA

Mean ± SD, NA = no activity, each experiment was done in triplicate.

The ethanolic extract from *D. longan* root exhibited the largest on zone inhibition of 14.33±0.58 against *B. subtilis* whereas the lowest MIC was found in the ethanolic extract of *O. indicum* which MIC and MBC values of 1000 and >2000 µg/ml, respectively.

Table 25 The antimicrobial activities of Ben-Cha-Moon-Yai remedy and five root species extracts at 4 mg/ml against *Micrococcus luteus*.

Plants/positive controls/ negative control	Solvent extracts	*Micrococcus luteus*		
		Inhibition zone (mm)	MIC (µg/ml)	MBC (µg/ml)
Aegle marmelos	Ethanol	11.00±1.00	>2000	>2000
	Water	9.67±0.58	>2000	>2000
Dimocarpus loDngan	Ethanol	15.00±1.00	>2000	>2000
	Water	11.00±0.00	>2000	>2000
Dolichandrone serrulata	Ethanol	9.33±0.58	>2000	>2000
	Water	12.33±0.58	>2000	>2000
Oroxylum indicum	Ethanol	19.00±0.00	1000	2000
	Water	NA	NA	NA
Walsura trichostemon	Ethanol	10.00±0.00	>2000	>2000
	Water	10.00±0.00	>2000	>2000
BMY remedy		15.00±0.00	1000	2000
Ampicllin		49.00±1.00	0.156	0.312
Amikacin		29.00±1.00	1.25	2.5
DMSO		NA	NA	NA

Mean ± SD, NA = no activity, each experiment was done in triplicate.

The ethanolic extract from the root of *O. indicum* showed the largest of zone inhibition of 19.00±0.00 mm followed by the and the MIC, MBC values of 1000 and 2000 µg/ml. Ben-Cha-Moon-Yai remedy reported the zone inhibition of 15.00±0.00 mm and the MIC, MBC values of 1000 and 2000 µg/ml, respectively.

Table 26 The antimicrobial activities of Ben-Cha-Moon-Yai remedy and five root species extracts at 4 mg/ml against *Enterobacter aerogenes*.

Plants/positive controls/ negative control	Solvent extracts	*Enterobacter aerogenes*		
		Inhibition zone (mm)	MIC (µg/ml)	MBC (µg/ml)
Aegle marmelos	Ethanol	NA	NA	NA
	Water	NA	NA	NA
Dimocarpus longan	Ethanol	NA	NA	NA
	Water	NA	NA	NA
Dolichandrone serrulata	Ethanol	NA	NA	NA
	Water	NA	NA	NA
Oroxylum indicum	Ethanol	NA	NA	NA
	Water	NA	NA	NA
Walsura trichostemon	Ethanol	NA	NA	NA
	Water	NA	NA	NA
BMY remedy		NA	NA	NA
Ampicllin		11.00±0.00	>10	>10
Amikacin		16.33±0.58	1.25	5
DMSO		NA	NA	NA

Mean ± SD, NA = no activity, each experiment was done in triplicate.

All crude extracts were not inhibited the growth of *E. aerogenes* in the agar-well diffusion method. The lowest of inhibition concentration and the lowest of bactericidal concentration was found in Amikacin of 1.25 and 5 µg/ml.

Table 27 The antimicrobial activities of Ben-Cha-Moon-Yai remedy and five root species extracts at 4 mg/ml against *Escherichia coli*.

Plants/positive controls/ negative control	Solvent extracts	*Escherichia coli*		
		Inhibition zone (mm)	MIC (µg/ml)	MBC (µg/ml)
Aegle marmelos	Ethanol	NA	NA	NA
	Water	NA	NA	NA
Dimocarpus longan	Ethanol	NA	NA	NA
	Water	NA	NA	NA
Dolichandrone serrulata	Ethanol	NA	NA	NA
	Water	NA	NA	NA
Oroxylum indicum	Ethanol	NA	NA	NA
	Water	NA	NA	NA
Walsura trichostemon	Ethanol	NA	NA	NA
	Water	NA	NA	NA
BMY remedy		NA	NA	NA
Ampicllin		23.00±0.00	5	5
Amikacin		19.00±0.00	1.25	1.25
DMSO		NA	NA	NA

Mean ± SD, NA = no activity, each experiment was done in triplicate.

All crude extracts were not inhibited the growth of *E. coli* in the agar-well diffusion method. The lowest of inhibition concentration and the lowest of bactericidal concentration was found in Amikacin of 1.25 and 1.25 µg/ml.

Table 28 The antimicrobial activities of Ben-Cha-Moon-Yai remedy and five root species extracts at 4 mg/ml against *Pseudomonas aeruginosa*.

Plants/positive controls/ negative control	Solvent extracts	*Pseudomonas aeruginosa*		
		Inhibition zone (mm)	MIC (µg/ml)	MBC (µg/ml)
Aegle marmelos	Ethanol	NA	NA	NA
	Water	NA	NA	NA
Dimocarpus longan	Ethanol	NA	NA	NA
	Water	NA	NA	NA
Dolichandrone serrulata	Ethanol	NA	NA	NA
	Water	NA	NA	NA
Oroxylum indicum	Ethanol	10.67±0.58	>2000	>2000
	Water	NA	NA	NA
Walsura trichostemon	Ethanol	NA	NA	NA
	Water	NA	NA	NA
BMY remedy		NA	NA	NA
Ampicllin		NA	NA	NA
Amikacin		17.33±0.58	1.25	1.25
DMSO		NA	NA	NA

Mean ± SD, NA = no activity, each experiment was done in triplicate.

The ethanolic extract of *O. indicum* showed the largest of zone inhibition of 10.67±0.58 mm against *P. aeruginosa* and the MIC and MBC values of >2000 µg/ml.

Table 29 The antimicrobial activities of Ben-Cha-Moon-Yai remedy and five root species extracts at 4 mg/ml against *Salmonella typhi*.

Plants/positive controls/ negative control	Solvent extracts	*Salmonella typhi*		
		Inhibition zone (mm)	MIC (µg/ml)	MBC (µg/ml)
Aegle marmelos	Ethanol	NA	NA	NA
	Water	NA	NA	NA
Dimocarpus longan	Ethanol	NA	NA	NA
	Water	NA	NA	NA
Dolichandrone serrulata	Ethanol	NA	NA	NA
	Water	NA	NA	NA
Oroxylum indicum	Ethanol	10.33±0.58	>2000	>2000
	Water	NA	NA	NA
Walsura trichostemon	Ethanol	NA	NA	NA
	Water	NA	NA	NA
BMY remedy		7.33±0.58	>2000	>2000
Ampicllin		27.00±0.00	1.25	1.25
Amikacin		20.33±0.58	1.25	2.5
DMSO		NA	NA	NA

Mean ± SD, NA = no activity, each experiment was done in triplicate.

The ethanolic extract of *O. indicum* showed the largest of zone inhibition of 10.33±0.58 mm against *S. typhi* and the MIC and MBC values of >2000 µg/ml.

Table 30 The antimicrobial activities of Ben-Cha-Moon-Yai remedy and five root species extracts at 4 mg/ml against *Salmonella typhimurium*.

Plants/positive controls/ negative control	Solvent extracts	*Salmonella typhimurium*		
		Inhibition zone (mm)	MIC (µg/ml)	MBC (µg/ml)
Aegle marmelos	Ethanol	NA	NA	NA
	Water	NA	NA	NA
Dimocarpus longan	Ethanol	NA	NA	NA
	Water	NA	NA	NA
Dolichandrone serrulata	Ethanol	NA	NA	NA
	Water	9.00±0.00	>2000	>2000
Oroxylum indicum	Ethanol	NA	NA	NA
	Water	NA	NA	NA
Walsura trichostemon	Ethanol	NA	NA	NA
	Water	NA	NA	NA
BMY remedy		10.00±0.00	>2000	>2000
Ampicllin		31.67±1.15	0.625	0.625
Amikacin		23.67±1.53	0.625	0.625
DMSO		NA	NA	NA

Mean ± SD, NA = no activity, each experiment was done in triplicate.

The largest of zone inhibition was found in the Ben-Cha-Moon-Yai remedy of 10.00±0.00 mm and MIC, MBC values of > 2000 µg/ml.

Table 31 The antimicrobial activities of Ben-Cha-Moon-Yai remedy and five root species extracts at 4 mg/ml against *Shigella spp*.

Plants/positive controls/ negative control	Solvent extracts	*Shigella spp*.		
		Inhibition zone (mm)	MIC (µg/ml)	MBC (µg/ml)
Aegle marmelos	Ethanol	NA	NA	NA
	Water	NA	NA	NA
Dimocarpus longan	Ethanol	NA	NA	NA
	Water	NA	NA	NA
Dolichandrone serrulata	Ethanol	8.00±0.00	>2000	>2000
	Water	9.33±0.58	>2000	>2000
Oroxylum indicum	Ethanol	9.00±0.00	>2000	>2000
	Water	NA	NA	NA
Walsura trichostemon	Ethanol	NA	NA	NA
	Water	NA	NA	NA
BMY remedy		11.33±0.58	>2000	>2000
Ampicllin		24.00±2.65	2.5	5
Amikacin		22.00±1.00	1.25	2.5
DMSO		NA	NA	NA

Mean ± SD, NA = no activity, each experiment was done in triplicate.

Ben-Cha-Moon-Yai remedy showed the largest of zone inhibition of 11.33±0.58 mm and MIC, MBC values of > 2000 µg/ml.

Table 32 The antimicrobial activities of Ben-Cha-Moon-Yai remedy and five root species extracts at 4 mg/ml against *Candida albicans.*

Plants/positive controls/ negative control	Solvent extracts	*Candida albicans*		
		Inhibition zone (mm)	MIC (µg/ml)	MFC (µg/ml)
Aegle marmelos	Ethanol	NA	NA	NA
	Water	NA	NA	NA
Dimocarpus longan	Ethanol	NA	NA	NA
	Water	NA	NA	NA
Dolichandrone serrulata	Ethanol	NA	NA	NA
	Water	8.33±0.58	500	500
Oroxylum indicum	Ethanol	16.33±0.58	>2000	>2000
	Water	NA	NA	NA
Walsura trichostemon	Ethanol	NA	NA	NA
	Water	NA	NA	NA
BMY remedy		NA	NA	NA
Ampicllin		NA	NA	NA
Amikacin		NA	NA	NA
DMSO		NA	NA	NA

Mean ± SD, NA = no activity, each experiment was done in triplicate.

The ethanolic extract of *O. indicum* showed the largest of zone inhibition of 16.33±0.58 whereas the lowest inhibition concentration and the lowest fungicidal concentration was found in the water extract of *D. serrulata* of 500 µg/ml

Table 33 The antimicrobial activities of Ben-Cha-Moon-Yai remedy and five root species extracts at 4 mg/ml against *Saccharomyces cerevisiae*.

Plants/positive controls/ negative control	Solvent extracts	*Saccharomyces cerevisiae*		
		Inhibition zone (mm)	MIC (µg/ml)	MBC (µg/ml)
Aegle marmelos	Ethanol	NA	NA	NA
	Water	NA	NA	NA
Dimocarpus longan	Ethanol	NA	NA	NA
	Water	NA	NA	NA
Dolichandrone serrulata	Ethanol	NA	NA	NA
	Water	NA	NA	NA
Oroxylum indicum	Ethanol	11.67±0.58	500	2000
	Water	NA	NA	NA
Walsura trichostemon	Ethanol	NA	NA	NA
	Water	NA	NA	NA
BMY remedy		NA	NA	NA
Ampicllin		NA	NA	NA
Amikacin		NA	NA	NA
DMSO		NA	NA	NA

Mean ± SD, NA = no activity, each experiment was done in triplicate.

Only the ethanolic extract from the root of *O. indicum* showed the inhibitory effect against the growth of *S. cerevisiae* which provided the zone inhibition of 11.67±0.58 mm and the MIC and MBC of 500 and 2000 µg/ml.

Free radical scavenging assay (DPPH assay)

Results demonstrated that the ethanolic extract of the *Dimocarpus longan* root showed the highest radical scavenging activity with IC_{50} of 9.3 µg/ml, followed by water extract of *Walsura trichostemon* showed IC_{50} of 16.1 µg/ml. Ben-Cha-Moon-Yai remedy extract showed the IC_{50} of 81.9 µg/ml. The water extract of *Oroxylum indicum* had the lowest radical-scavenging activity (IC_{50} 409.31 µg/ml).

Table 34 Inhibition concentration of DPPH scavenging activity (IC_{50}) of the extracts of Ben-Cha-Moon-Yai remedy and five root species extracts.

Plant name	**DPPH scavenging activity Mean IC_{50} (µg/ml)**	
	Ethanol extract	**Water extract**
Aegle marmelos	61.3	380.0
Dolichandrone serrulata	87.8	338.5
Dimocarpus longan	**9.3**	40.8
Oroxylum indicum	61.3	380.0
Walsura trichostemon	23.8	**16.1**
Ben-Cha-Moon-Yai remedy	81.9	
BHT	22.3	
Quercetin	9.8	

Lipid peroxidation testing using β-carotene bleaching assay

The ethanolic extract of *D. longan* showed highest ability to prevent the bleaching of β-carotene followed by the ethanolic extract of *A. marmelos*, Ben-Cha-Moon-Yai remedy, *W. trichostemon*, *D. serrulata*, and *O. indicum*, respectively. While, the water extract of *D. longan* showed higher ability to prevent the bleaching of β-carotene followed by the water extract of *W. trichostemon*, *O. indicum*, *D. serrulata*, and *A. marmelos*, respectively. All extracts had lower antioxidant activities than BHT and quercetin.

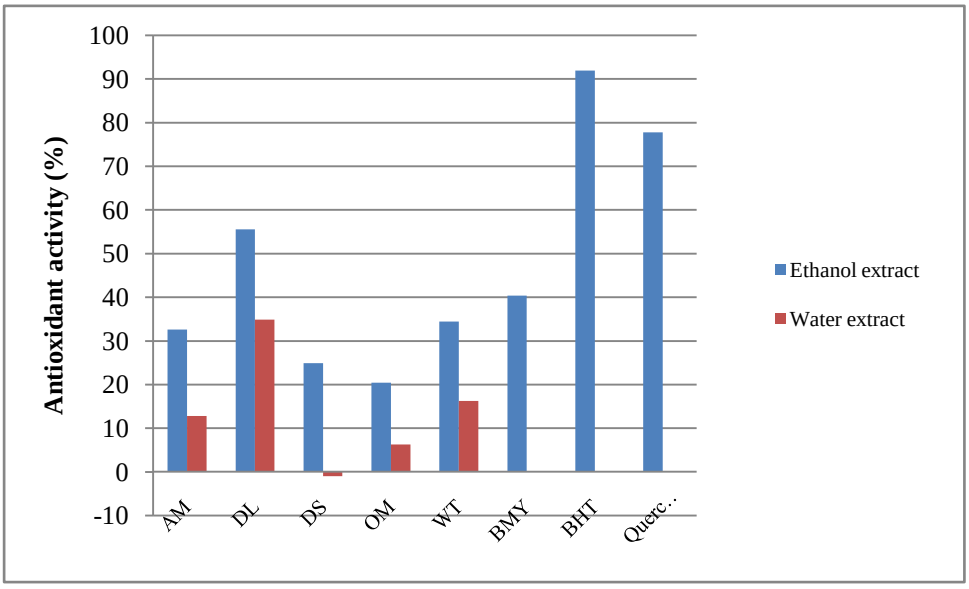


Figure 60 The antioxidant activity of five root species and Ben-Cha-Moon-Yai remedy extract against β-carotene bleaching assay. Abbreviations including: *Aegle marmelos*: AM, *Oroxylum indicum*: OI, *Dimocarpus longan*: DL, *Dolichandrone serrulata*: DS, *Walsura trichostemon*: WT, Ben-Cha-Moon-Yai remedy: BMY.

Figure 60A

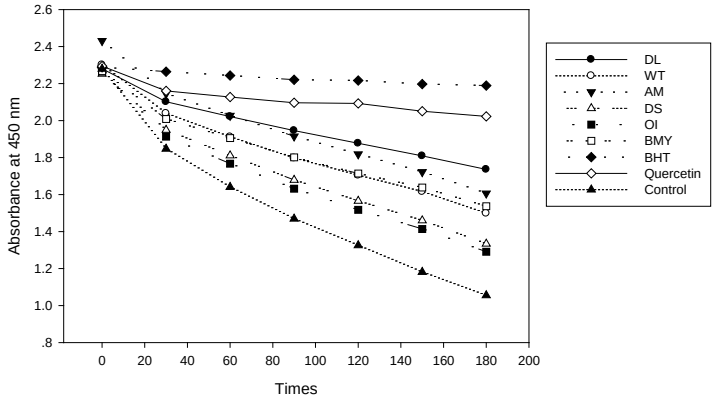


Figure 60B

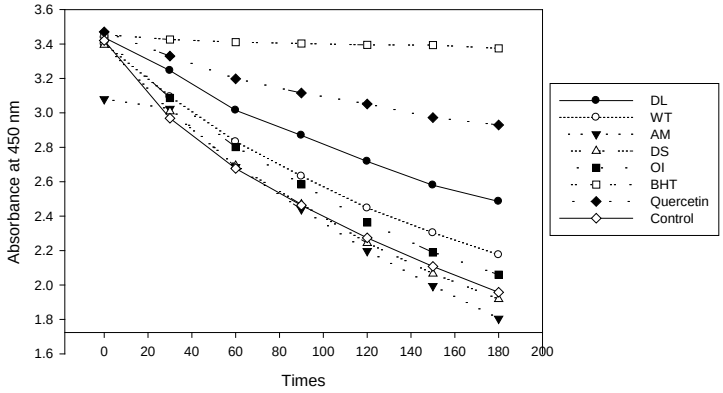


Figure 60 Changes of the absorbance at 450 nm with time for the ethanol (60A) and water (60B) extracts (1 mg/ml) in β -carotene bleaching assay. Abbreviations including: *Aegle marmelos*: AM, *Oroxylum indicum*: OI, *Dimocarpus longan*: DL, *Dolichandrone serrulata*: DS, *Walsura trichostemon*: WT, Ben-Cha-Moon-Yai remedy: BMY.

Nitric oxide scavenging assay using Griess reagent assay

Results demonstrated that the ethanolic extract from *D. longan* expressed the high potential on nitric oxide scavenging activity with IC_{50} of 23 µg/ml, followed by the ethanolic extract of *W. trichostemon* with IC_{50} of 25 µg/ml, while the Ben-Cha-Moon-Yai remedy extract exhibited the IC_{50} of 657 µg/ml.

Table 35 Inhibition concentration of nitric oxide scavenging activity (IC_{50}) of Ben-Cha-Moon Yai remedy and five root species extracts.

Plant name	**NO scavenging activity Mean IC_{50} (µg/ml)**	
	Ethanol extract	**Water extract**
Oroxylum indicum	716	3521
Aegle marmelos	883	1531
Dimocarpus longan	**23**	367
Walsura trichostemon	**25**	933
Dolichandrone serrulata	161	3577
Ben Cha Moon Yai remedy	**657**	
Quercetin	9.17	

Total phenolic contents

The quantification of total phenolic content by Folin-Ciocalteu reagent demonstrated that the water extract of *D. longan* showed the highest of total phenolic content resulting of 10.033±0.218 mg catechin hydrate/ 500 mg extract, followed by the water extract of *W. trichostemon* which resulting 8.864±0.348 mg catechin hydrate/ 500 mg extract calculated from the calibration curve of catechin dydrate equation: $y = 0.007x + 0.022$ ($R^2 = 0.989$).

Table 36 Total phenolic content from the water extract of five root species in Ben-Cha-Moon-Yai remedy at 500 mg/ml.

Plant name	**Total phenolic contents (µg CE/ 500 mg/ml of the extract)**	
	Mean	**SD**
Oroxylum indicum	5.55	0.12
Aegle marmelos	6.06	1.17
Dimocarpus longan	**10.03**	0.22
Walsura trichostemon	**8.86**	0.35
Dolichandrone serrulata	5.82	0.02

Antipyretic activity

Effects of an extract from Ben-Cha-Moon-Yai remedy on LPS-induce fever

Lipopolysaccharide (LPS; 50 µg/kg) injected intramuscularly significantly (p<0.001) produced a time-dependent increase in rectal temperature in vehicle pretreated rats starting from 1 hr and this effect was maintained for 7 hr after LPS injection. The maximum increase in rectal temperature was reached at 2 hr (0.89°C) giving a maximum observed mean rectal temperature of 38.69 ± 0.14°C after which there was a decrease (Figure 61). At the same time, the mean rectal temperature of normothermic rats was 37.80 ± 0.15°C. Thus, LPS significantly (p<0.001) increased the rectal temperature (Figure 61).

Acetylsalicylic acid (ASA; 300 mg/kg) significantly (p<0.05) reduced the increased rectal temperature produced by LPS over a period of 7 hr with a maximum reduction at 2 hr. The mean rectal temperature produced by LPS in the presence of ASA was reduced to 37.68 ± 0.23°C (Figure 61).

BMY at the dose of 125 mg/kg significantly (p<0.01) attenuated the increase in rectal temperature produced by LPS starting at 2 hr and the effect was maintained for the full 7 hr with a maximum reduction at 3 hr after LPS injection. BMY at the dose of 250 mg/kg significantly reduced LPS-induced increase in rectal temperature at 2 and 3 hr (p<0.05 and p<0.01, respectively) with a maximum reduction at 3 hr after LPS injection. BMY at the dose of 500 mg/kg significantly (p<0.01) attenuated the increase in rectal temperature produced by LPS starting at 1 hr and the effect was maintained for the full 7 hr with a maximum reduction at 3 hr after LPS injection (Figure 62).

Effects of an extracts from five root species in Ben-Cha-Moon-Yai remedy on LPS-induce fever

1. The root extract of *Aegle marmelos* (AM)

AM at the doses of 25 and 50 mg/kg significantly (p<0.05) reduced LPS-induced increase in rectal temperature at 2 and 3 hr after LPS injection and both doses showed a maximum reduction at 3 hr. AM at the dose of 200 mg/kg significantly (p<0.05) reduced LPS-induced increase in rectal temperature at 3 hr after LPS injection. AM at the dose of 400 mg/kg significantly (p<0.01) reduced the increased rectal temperature produced by LPS over a period of 2-7 hr with a maximum reduction at 3 hr (Figure 63).

2. The root extract of *Dolichandrone serrulata* (DS)

All doses of DS could not reduce LPS-induced increase in rectal temperature (Figure 16). All doses of DS did not show antipyretic effect (Figure 64).

3. The root extract of *Dimocarpus longan (*DL)

DL at the doses of 50,100 and 200 mg/kg significantly (p<0.05) reduced LPS-induced increase in rectal temperature over a period of 2-3 hr with a maximum reduction at 2, 2 and 2 hr, respectively (Figure 65) . All doses of DL did not show antipyretic effect.

4. The root extract of *Oroxylum indicum* (OI)

OI at the dose of 25 mg/kg significantly (p<0.05) reduced LPS-induced increase in rectal temperature at 2 and 3 hr with a maximum reduction at 2 hr. OI at the dose of 50 mg/kg significantly (p<0.05) reduced LPS induced increase in rectal temperature at 3 hr. OI at the dose of 100 mg/kg significantly reduced LPS-induced increase in rectal temperature at 5, 6 and 7 hr (p<0.05, p<0.05 and p<0.01) with a maximum reduction at 7 hr. OI at the dose of 200 mg/kg significantly reduced LPS induced increase in rectal temperature at 2 and 3 hr after LPS injection (p<0.01 and p<0.05) with a maximum reduction at 2 hr. OI at the dose of 400 mg/kg significantly

reduced LPS induced increase in rectal temperature at 2, 3, 5, 6, and 7 hr after LPS injection ($p<0.01$, $p<0.01$, $p<0.05$, $p<0.01$ and $p<0.01$, respectively) with a maximum reduction at 3 hr (Figure 66). OI at the dose of 400 mg/kg seemed to have the highest antipyretic efficacy.

5. The root extract of *Walsura trichostemon* (WT)

WT at the dose of 25 mg/kg significantly ($p<0.05$) reduced LPS-induced increase in rectal temperature at 4-7 hr after LPS injection with a maximum reduction at 7 hr. WT at the dose of 50 mg/kg significantly ($p<0.05$) reduced LPS-induced increase in rectal temperature at 4 and 7 hr. WT at the dose of 100 mg/kg significantly ($p<0.05$) reduced LPS-induced increase in rectal temperature at 2, 3, 4, 6 and 7 hr with a maximum reduction at 4 hr. WT at the dose of 200 mg/kg significantly ($p<0.05$) reduced LPS-induced increase in rectal temperature at 4-7 hr with a maximum reduction at 5 hr (Figure 67).

Lipopolysaccharide-induced Fever in Rats

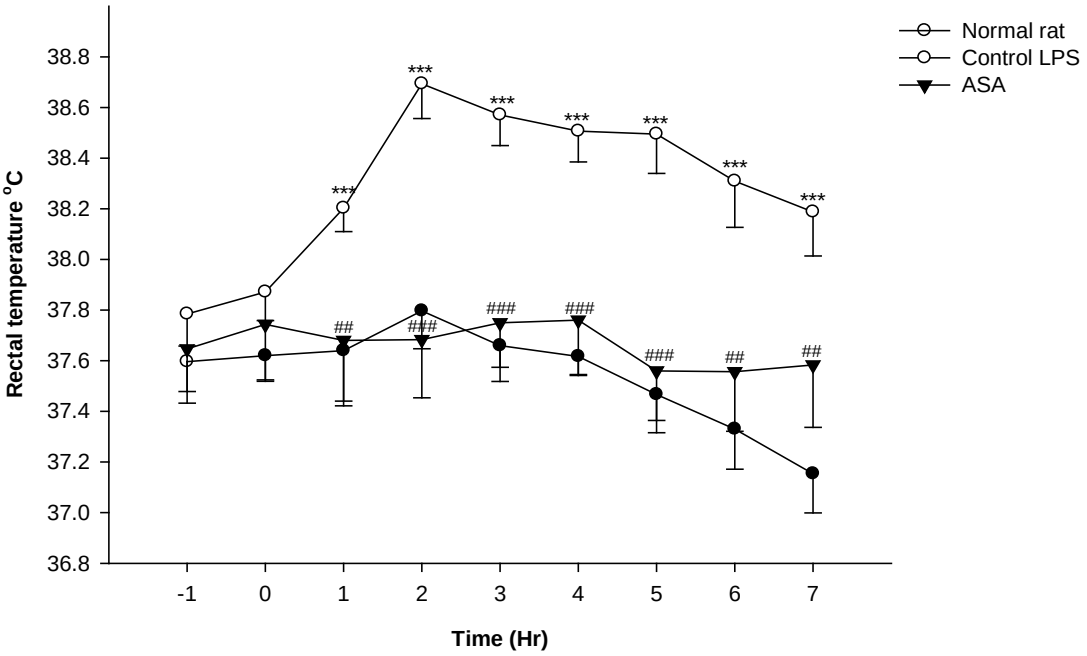


Figure 61 Changes in rectal temperature after oral administration of 2% Tween 80, acetylsalicylic acid (ASA; 300 mg/kg). Fever was induced by intramuscular injection of lipopolysaccharide (LPS; 50 µg/ml) at 0 hr. All drugs were administered 1 hr before LPS. Normal rats were received 0.9% NSS injection instead of LPS. N=6 for all groups. $^{***}p<0.001$ significantly different compared to normal rat values for the corresponding hour. $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ significantly different compared to control LPS values at the corresponding hour.

The Root Extracts of Ben-Cha-Moon-Yai Remedy (BMY)

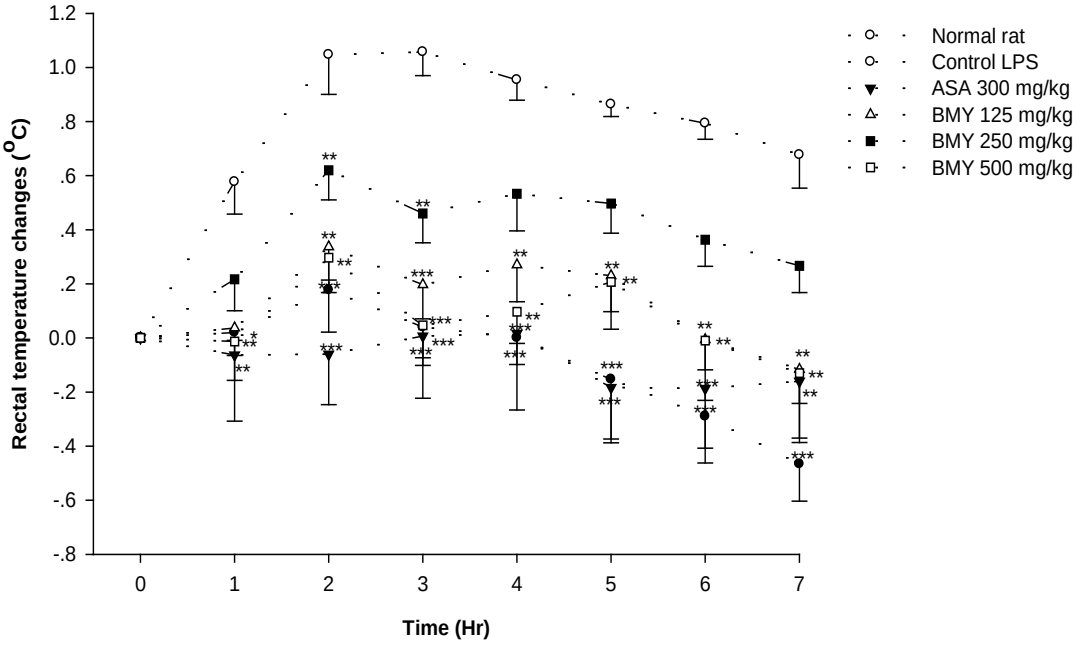


Figure 62 Changes in rectal temperature from baseline on lipopolysaccharide-induced fever after oral administration of 2% Tween 80, acetylsalicylic acid (ASA; 300 mg/kg) and various doses of the root extract of Ben-Cha-Moon-Yai remedy (BMY; 125-500 mg/kg) to febrile rats. Fever was induced by intramuscular injection of lipopolysaccharide (LPS; 50 µg/ml) at 0 hr. All drugs were administered 1 hr before LPS. N=6 for all groups. *p<0.05, **p<0.01 and ***p<0.001 significantly different compared to control LPS values at the corresponding hour.

The root extract of *Aegle marmelos* (AM)

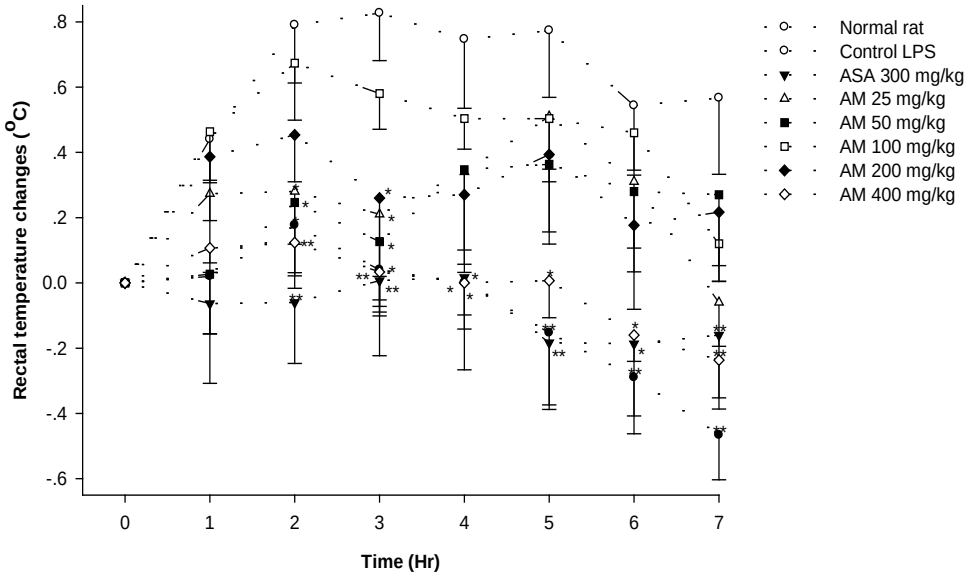


Figure 63 Changes in rectal temperature from baseline on lipopolysaccharide-induced fever after oral administration of 2% Tween 80, acetylsalicylic acid (ASA; 300 mg/kg) and various doses of the root extract of *Aegle marmelos* (AM; 25-400 mg/kg) to febrile rats. Fever was induced by intramuscular injection of lipopolysaccharide (LPS; 50 µg/ml) at 0 hr. All drugs were administered 1 hr before LPS. N=6 for all groups. *p<0.05 and **p<0.01 significantly different compared to control LPS values at the corresponding hour.

The root extract of *Dolichandrone serrulata* (DS)

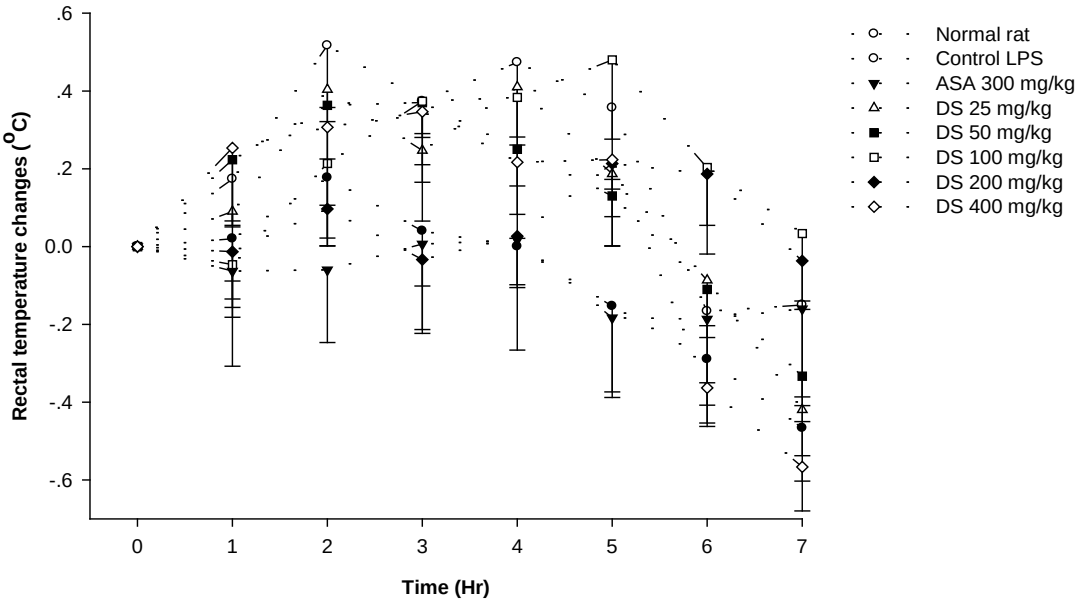


Figure 64 Changes in rectal temperature from baseline on lipopolysaccharide-induced fever after oral administration of 2% Tween 80, acetylsalicylic acid (ASA; 300 mg/kg) and various doses of the root extract of *Dolichandrone serrulata* (DS; 25-400 mg/kg) to febrile rats. Fever was induced by intramuscular injection of lipopolysaccharide (LPS; 50 µg/ml) at 0 hr. All drugs were administered 1 hr before LPS. N=6 for all groups.

The root extract of *Dimocarpus longan* (DL)

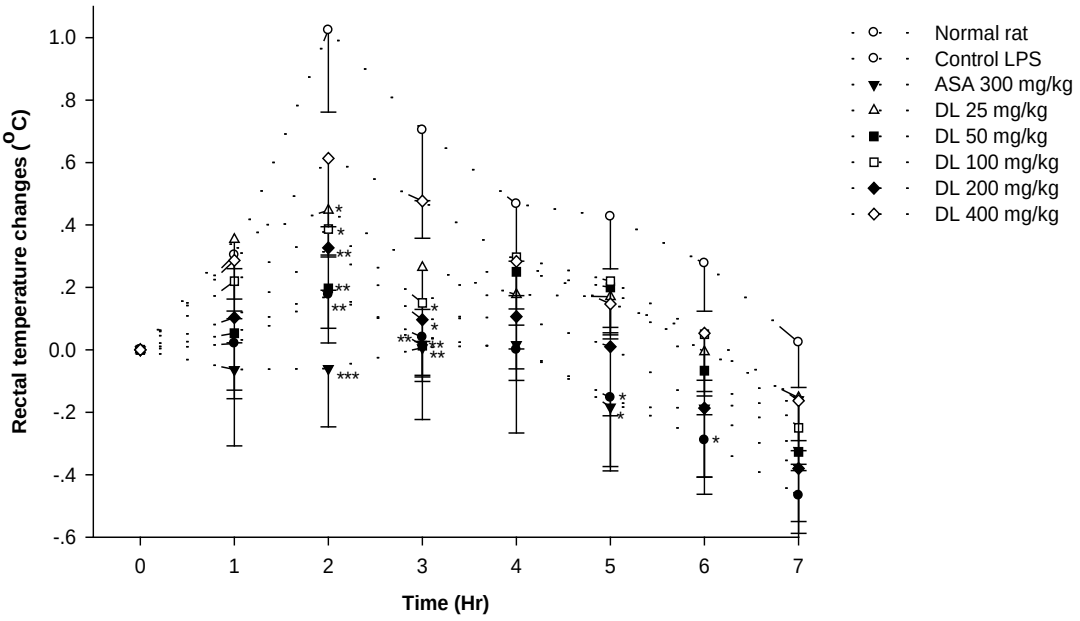


Figure 65 Changes in rectal temperature from baseline on lipopolysaccharide-induced fever after oral administration of 2% Tween 80, acetylsalicylic acid (ASA; 300 mg/kg) and various doses of the root extract *of Dimocarpus longan* (DL; 25-400 mg/kg) to febrile rats. Fever was induced by intramuscular injection of lipopolysaccharide (LPS; 50 µg/ml) at 0 hr. All drugs were administered 1 hr before LPS. N=6 for all groups. *p<0.05, **p<0.01 and ***p<0.001 significantly different compared to control LPS values at the corresponding hour.

The root extract of *Oroxylum indicum* (OI)

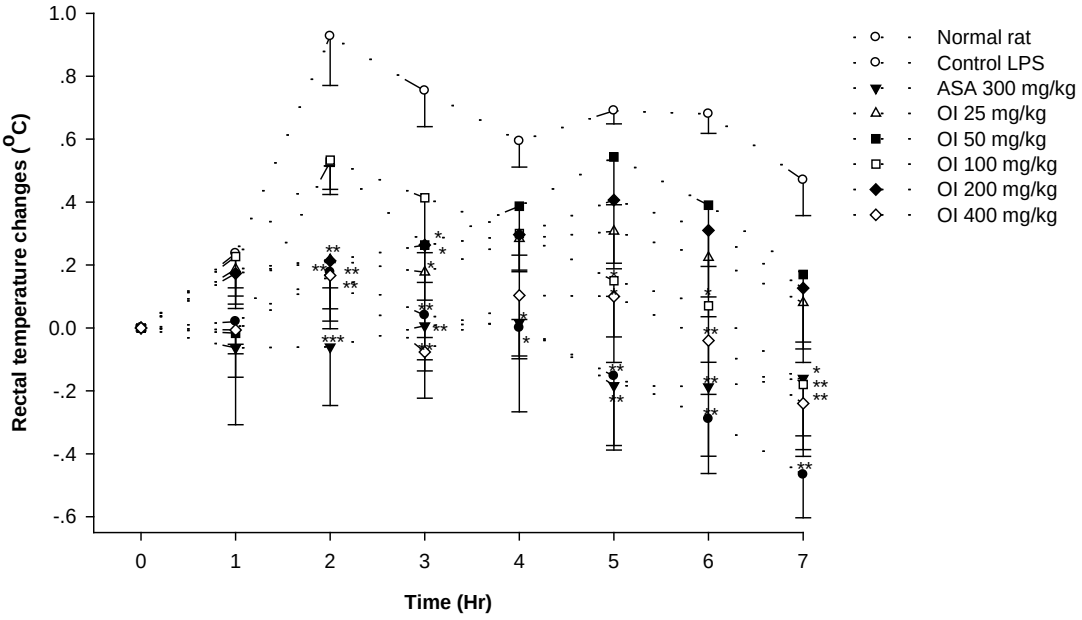


Figure 66 Changes in rectal temperature from baseline on lipopolysaccharide-induced fever after oral administration of 2% Tween 80, acetylsalicylic acid (ASA; 300 mg/kg) and various doses of the root extract of *Oroxylum indicum* (OI; 25-400 mg/kg) to febrile rats. Fever was induced by intramuscular injection of lipopolysaccharide (LPS; 50 µg/ml) at 0 hr. All drugs were administered 1 hr before LPS. N=6 for all groups. *p<0.05, **p<0.01 and ***p<0.001 significantly different compared to control LPS values at the corresponding hour.

The root extract of *Walsura trichostemon* (WT)

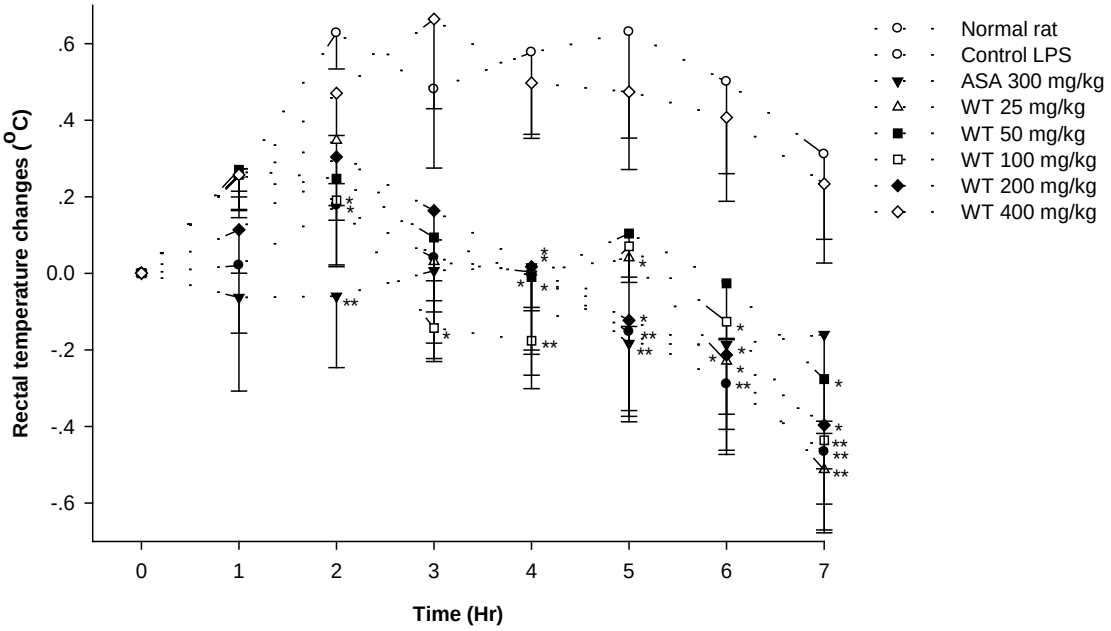


Figure 67 Changes in rectal temperature from baseline on lipopolysaccharide-induced fever after oral administration of 2% Tween 80, acetylsalicylic acid (ASA; 300 mg/kg) and various doses of the root extract of *Walsura trichostemon* (WT; 25-400 mg/kg) to febrile rats. Fever was induced by intramuscular injection of lipopolysaccharide (LPS; 50 µg/ml) at 0 hr. All drugs were administered 1 hr before LPS. N=6 for all groups. *p<0.05, and **p<0.01 significantly different compared to control LPS values at the corresponding hour.

Anti-inflammatory activity

Carrageenan-induced paw edema in mice

The anti-inflammatory effects of Ben-Cha-Moon-Yai remedy and five root species extracts were initially evaluated in the carrageenan-induced paw edema in mice. Each mouse was orally pre-treated with 2% Tween 80, indomethacin (IND; 10 mg/kg), various doses of BMY (125, 250, 500 mg/kg) or AM, OI, DL, DS, WT (25, 50, 100, 200, 400 mg/kg). IND 10 mg/kg was used as a positive control. The result demonstrated that IND was the most potent drug with significantly decreased paw edema after carrageenan administration by 40.48%, 56.16%, 69.35% and 72.22% at 2, 3, 4 and 5 hr, respectively when compared to 2% Tween 80.

BMY 125 mg/kg significantly (p<0.05) decreased paw edema and produced an inhibition of paw edema of 32.91% at 3 hr after carrageenan administration compared with that of 2% Tween 80. BMY at the dose of 250 mg/kg significantly (p<0.001) decreased paw edema at 3 and 4 hr compared with that of 2% Tween 80 and produced a maximum inhibition of paw edema of 51.61% at 4 hr. The highest dose of BMY (500 mg/kg) significantly (p<0.001, p<0.001 and p<0.05, respectively) decreased paw edema at 3, 4 and 5 hr compared to 2% Tween 80 and produced a maximum inhibition of paw edema of 65.77% at 3 hr. The extracts demonstrated a dose-dependent by inhibitory effect against paw edema in mice at 3 hr. BMY at 500 mg/kg produced high anti-inflammatory effect than IND (Table 37).

AM at the dose of 400 mg/kg significantly (p<0.05) decreased paw edema at 3 and 4 hr after carrageenan administration compared to 2% Tween 80 and produced an inhibition of paw edema of 23.93% at 4 hr. All doses of AM (25-400 mg/kg) produced less anti-inflammatory effect than IND (Table 386).

DL at the dose of 200 mg/kg significantly (p<0.05) decreased paw edema at 3, 4 and 5 hr after carrageenan administration compared to 2% Tween 80 and produced a maximum inhibition of paw edema of 34.85% at 4 hr. DL at the dose of 400 mg/kg significantly (p<0.05, p<0.01 and p<0.05, respectively) decreased paw edema at 3, 4 and 5 hr compared to 2% Tween 80 and produced a maximum inhibition of paw

edema of 39.39% at 4 hr. All doses of DL (25-400 mg/kg) produced less anti-inflammatory effect than IND (Table 39).

DS at the dose of 200 mg/kg significantly ($p<0.05$) decreased paw edema at 4 hr after carrageenan administration compared to 2% Tween 80 and produced an inhibition of paw edema of 29.69%. DS at the dose of 400 mg/kg significantly ($p<0.05$) decreased paw edema at 3 and 4 hr after carrageenan administration compared to 2% Tween 80 and produced a maximum inhibition of paw edema of 34.38% at 4 hr. All doses of DS (25-400 mg/kg) produced less anti-inflammatory effect than IND (Table 40).

OI at the dose of 200 mg/kg significantly ($p<0.05$) decreased paw edema at 3 and 4 hr after carrageenan administration compared to 2% Tween 80 and produced a maximum inhibition of paw edema of 26.18% at 4 hr. OI at the dose of 400 mg/kg significantly ($p<0.05$, $p<0.01$ and $p<0.05$, respectively) decreased paw edema at 3, 4 and 5 hr compared to 2% Tween 80 and showed a maximum inhibition of paw edema of 32.14% at 3 hr after carrageenan administration. All doses of OI (25-400 mg/kg) produced less anti-inflammatory effect than IND (Table 41).

WT at the dose of 25 mg/kg significantly ($p<0.01$ and $p<0.05$, respectively) decreased paw edema at 3 and 4 hr after carrageenan administration compared to 2% Tween 80 and produced a maximum inhibition of paw edema of 36.76% at 3 hr. WT at doses of 50 and 100 mg/kg significantly ($p<0.01$) decreased paw edema at 3 and 4 hr compared to 2% Tween 80 and produced a similar maximum inhibition of paw edema of 42.65% at 3 hr. WT at the dose of 200 mg/kg significantly ($p<0.001$ and $p<0.01$, respectively) decreased paw edema at 3 and 4 hr compared to 2% Tween 80 and produced a maximum inhibition of paw edema of 47.06% at 3 hr. WT at the dose of 400 mg/kg significantly ($p<0.001$, $p<0.001$ and $p<0.05$, respectively) decreased paw edema at 3, 4 and 5 hr compared to 2% Tween 80 administration and produced a maximum inhibition of paw edema of 48.53% at 3 hr. All doses of DL (25-400 mg/kg) produced less anti-inflammatory effect than IND (Table 42).

Table 37 Change of edema volume (ml) of oral administration of 2% Tween 80, indomethacin (IND; 10 mg/kg) and the root extract of Ben-Cha-Moon-Yai remedy (BMY; 125-500 mg/kg). N=8. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ significantly different compared to 2% Tween 80.

Treatments (mg/kg)	**Paw edema (ml)±S.E.M. (% Inhibition)**					
	1 hr	**2 hr**	**3 hr**	**4 hr**	**5 hr**	**6 hr**
2% Tween 80	0.029±0.0051	0.0525±0.0031	0.0913±0.0058	0.0775±0.0082	0.0675±0.0118	0.0400±0.0091
IND 10 mg/kg	0.0250±0.0019 (-13.79%)	0.0313±0.0029* (-40.48%)	0.0400±0.0060*** (-56.19%)	0.0238±0.0037*** (-69.35%)	0.0188±0.0023** (-72.22%)	0.0338±0.0053 (-15.63%)
BMY 125 mg/kg	0.0338±0.0046 (16.38%)	0.0500±0.0063 (-4.76%)	0.0613±0.0069* (-32.91%)	0.0675±0.0053 (-12.90%)	0.0750±0.0082 (11.11%)	0.0550±0.0057 (37.50%)
BMY 250 mg/kg	0.0338±0.0053 (16.38%)	0.0575±0.0070 (9.52%)	0.0463±0.0080*** (-49.34%)	0.0375±0.0049*** (-51.61%)	0.0400±0.0073 (-40.74%)	0.0463±0.0073 (15.63%)
BMY 500 mg/kg	0.0375±0.0031 (29.31%)	0.0513±0.0040 (-2.38%)	0.0313±0.0040*** (-65.77%)	0.0338±0.0056*** (-56.45%)	0.0338±0.0068* (-50%)	0.0325±0.0041 (-18.75%)

Table 38 Change of edema volume (ml) of oral administration of 2% Tween 80, indomethacin (IND; 10 mg/kg) and *Aegle marmelos* root extract (AM; 25-400 mg/kg). N=8. $^{*}p<0.05$, $^{***}p<0.001$ significantly different compared to 2% Tween 80.

Treatments (mg/kg)	**Paw edema±S.E.M. (% Inhibition)**					
	1 hr	**2 hr**	**3 hr**	**4 hr**	**5 hr**	**6 hr**
2% Tween 80	0.0513±0.0048	0.0625±0.0041	0.0750±0.0046	0.0838±0.0037	0.0850±0.0073	0.0788±0.0058
IND 10 mg/kg	0.0400±0.0019 (-22.03%)	0.0350±0.0019*** (-44%)	0.0275±0.0025*** (-63.33%)	0.0275±0.0025*** (-67.18%)	0.0313±0.0035*** (-63.24%)	0.0375±0.0037*** (-52.41%)
AM 25 mg/kg	0.0463±0.0032 (-9.84%)	0.0625±0.0031 (0%)	0.0738±0.0026 (-1.67%)	0.0825±0.0049 (-1.55%)	0.0800±0.0066 (-5.88%)	0.0788±0.0061 (-0.06%)
AM 50 mg/kg	0.0438±0.0018 (-14.72%)	0.0600±0.0033 (-4.00%)	0.0725±0.0059 (-3.33%)	0.0800±0.0053 (-4.53%)	0.0775±0.0037 (-8.82%)	0.0763±0.0042 (-3.24%)
AM 100 mg/kg	0.0413±0.0029 (-19.59%)	0.0575±0.0041 (-8.00%)	0.0700±0.0038 (-6.67%)	0.0763±0.0050 (-9.01%)	0.0738±0.0073 (-13.24%)	0.0750±0.0089 (-4.82%)
AM 200 mg/kg	0.0413±0.0035 (-19.59%)	0.0550±0.0042 (-12.00%)	0.0650±0.0038 (-13.33%)	0.0713±0.0035 (-14.98%)	0.0700±0.0033 (-17.65%)	0.0663±0.0046 (-15.93%)
AM 400 mg/kg	0.0413±0.0023 (-19.59%)	0.0525±0.0031 (-16.00%)	0.0575±0.0016* (-23.33%)	0.0638±0.0026* (-23.93%)	0.0625±0.0059 (-26.47%)	0.0625±0.0067 (-20.69%)

Table 39 Change of edema (ml) volume of oral administration of 2% Tween 80, indomethacin (IND; 10 mg/kg) and *Dimocarpus longan* root extract (DL; 25-400 mg/kg). N=8. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ significantly different compared to 2% Tween 80.

Treatments (mg/kg)	**Paw edema±S.E.M. (% Inhibition)**					
	1 hr	**2 hr**	**3 hr**	**4 hr**	**5 hr**	**6 hr**
2% Tween 80	0.0425±0.0049	0.0725±0.0077	0.0850±0.0089	0.0825±0.0065	0.0725±0.0092	0.0613±0.0079
IND 10 mg/kg	0.0313±0.0012 (-26.47%)	0.0300±0.0038** (-58.62%)	0.0263±0.0026*** (-69.12%)	0.0238±0.0026*** (-71.21%)	0.0300±0.0042*** (-58.62%)	0.0363±0.0046 (-40.86%)
DL 25 mg/kg	0.0438±0.0026 (2.94%)	0.0725±0.0053 (0%)	0.0825±0.0065 (-2.94%)	0.0788±0.0072 (-4.55%)	0.0713±0.0051 (-1.72%)	0.0600±0.0087 (-2.12%)
DL 50 mg/kg	0.0438±0.0026 (2.94%)	0.0713±0.00581 (-1.72%)	0.0738±0.0073 (-13.24%)	0.0713±0.0064 (-13.64%)	0.0650±0.0053 (-10.34%)	0.0575±0.0075 (-6.20%)
DL 100 mg/kg	0.0413±0.0035 (-2.94%)	0.0688±0.0029 (-5.17%)	0.0700±0.0046 (-17.65%)	0.0688±0.0058 (-16.67%)	0.0575±0.0041 (-20.69%)	0.0500±0.0087 (-18.43%)
DL 200 mg/kg	0.0400±0.0042 (-5.88%)	0.0638±0.0125 (-12.07%)	0.0575±0.0073* (-32.35%)	0.0538±0.0068* (-34.85%)	0.0488±0.0040* (-32.76%)	0.0475±0.0025 (-22.51%)
DL 400 mg/kg	0.0388±0.0029 (-8.82%)	0.0625±0.0037 (-13.79%)	0.0538±0.0037* (-36.76%)	0.0500±0.0033** (-39.39%)	0.0463±0.0037* (-36.21%)	0.0425±0.0077 (-30.67%)

Table 40 Change of edema volume (ml) of oral administration of 2% Tween 80, indomethacin (IND 10 mg/kg) and *Dolichandrone serrulata* root extract (DS; 25-400 mg/kg). N=8. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ significantly different compared to 2% Tween 80.

Treatments (mg/kg)	**Paw edema±S.E.M. (% Inhibition)**					
	1 hr	**2 hr**	**3 hr**	**4 hr**	**5 hr**	**6 hr**
2% Tween 80	0.0463±0.0026	0.0575±0.0036	0.0725±0.0049	0.0800±0.0057	0.0738±0.0053	0.0688±0.0048
IND 10 mg/kg	0.0388±0.0029 (-16.13%)	0.0388±0.0035** (-32.61%)	0.0300±0.0038*** (-58.62%)	0.0288±0.0029*** (-64.06%)	0.0288±0.0029*** (-60.99%)	0.0325±0.0031** (-52.69%)
DS 25 mg/kg	0.0450±0.0019 (-2.60%)	0.0550±0.0027 (-4.35%)	0.0700±0.0046 (-3.45%)	0.0688±0.0079 (-14.06%)	0.0688±0.0079 (-6.72%)	0.0688±0.0087 (0.07%)
DS 50 mg/kg	0.0438±0.0026 (-5.30%)	0.0538±0.0026 (-6.52%)	0.0688±0.0035 (-5.17%)	0.0675±0.0036 (-15.63%)	0.0675±0.0036 (-8.41%)	0.0663±0.0050 (-3.57%)
DS 100 mg/kg	0.0438±0.0026 (-5.30%)	0.0525±0.0025 (-8.70%)	0.0650±0.0038 (-10.34%)	0.0638±0.0042 (-20.31%)	0.0638±0.0042 (-13.50%)	0.0625±0.0045 (-9.02%)
DS 200 mg/kg	0.0425±0.0016 (-8.01%)	0.0525±0.0016 (-8.70%)	0.0588±0.0029 (-18.97%)	0.0563±0.0062* (-29.69%)	0.0575±0.0065 (-21.98%)	0.0575±0.0059 (-16.30%)
DS 400 mg/kg	0.0413±0.0012 (-10.71%)	0.0513±0.0035 (-10.87%)	0.0538±0.0046* (-25.86%)	0.0525±0.0053* (-34.38%)	0.0538±0.0056 (-27.07%)	0.0538±0.0059 (-21.76%)

Table 41 Change of edema volume (ml) of oral administration of 2% Tween 80, indomethacin (IND; 10 mg/kg) and *Oroxylum indicum* root extract (OI; 25-400 mg/kg). N=8. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ significantly different compared to 2% Tween 80.

Treatments (mg/kg)	**Paw edema±S.E.M. (% Inhibition)**					
	1 hr	**2 hr**	**3 hr**	**4 hr**	**5 hr**	**6 hr**
2% Tween 80	0.0488±0.0035	0.0600±0.0033	0.0700±0.0027	0.0763±0.0026	0.0738±0.0053	0.0700±0.0065
IND 10 mg/kg	0.0375±0.0025 (-23%)	0.0275±0.0016*** (-54.17%)	0.0225±0.0016*** (-67.86%)	0.0238±0.0018*** (-68.83%)	0.0300±0.0033*** (-59.29%)	0.0338±0.0026*** (-51.79%)
OI 25 mg/kg	0.0488±0.0035 (0.10%)	0.0613±0.0044 (2.08%)	0.0700±0.0042 (0%)	0.0738±0.0026 (-3.22%)	0.0738±0.0042 (0.07%)	0.0725±0.0059 (3.57%)
OI 50 mg/kg	0.0475±0.0053 (-2.46%)	0.0600±0.0063 (0%)	0.0675±0.0062 (-3.57%)	0.0725±0.0070 (-4.86%)	0.0713±0.0058 (-3.32%)	0.0700±0.0053 (0%)
OI 100 mg/kg	0.0475±0.0041 (-2.46%)	0.0575±0.0025 (-4.17%)	0.0650±0.0053 (-7.14%)	0.0713±0.0044 (-6.50%)	0.0688±0.0061 (-6.72%)	0.0688±0.0051 (-1.79%)
OI 200 mg/kg	0.0450±0.0033 (-7.60%)	0.0500±0.0042 (-16.67%)	0.0525±0.0045* (-25%)	0.0563±0.0037* (-26.18%)	0.0588±0.0055 (-20.28%)	0.0613±0.0061 (-12.50%)
OI 400 mg/kg	0.0425±0.0025 (-12.73%)	0.0463±0.0032 (-22.92%)	0.0475±0.0041* (-32.14%)	0.0525±0.0056** (-31.10%)	0.0538±0.0050* (-27.07%)	0.0575±0.0056 (-17.86%)

Table 42 Change of edema volume (ml) of oral administration of 2%Tween 80 (10 ml/kg), indomethacin (IND; 10 mg/kg) and *Walsura trichostemon* root extract (WT; 25-400 mg/kg). N=8. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ significantly different compared to 2%Tween 80.

Treatments	**Paw edema±S.E.M. (% Inhibition)**					
(mg/kg)	**1 hr**	**2 hr**	**3 hr**	**4 hr**	**5 hr**	**6 hr**
2%Tween 80	0.055±0.0042	0.0675±0.0036	0.085±0.0060	0.0863±0.0050	0.0763±0.0042	0.0675±0.0036
IND 10 mg/kg	0.0438±0.0032 (-20.45%)	0.0325±0.0025*** (-51.85%)	0.0225±0.0016*** (-73.53%)	0.0238±0.0018*** (-72.45%)	0.0288±0.0029*** (-62.27%)	0.0325±0.0041** (-51.85%)
WT 25 mg/kg	0.0500±0.0075 (-9.09%)	0.0525±0.0075 (-22.22%)	0.0538±0.0086** (-36.76%)	0.0588±0.0089* (-31.84%)	0.0688±0.0074 (-9.78%)	0.0700±0.0084 (-3.70%)
WT 50 mg/kg	0.0513±0.0055 (-6.82%)	0.0525±0.0049 (-22.22%)	0.0488±0.0051** (-42.65%)	0.0563±0.0046** (-34.74%)	0.0663±0.0046 (-13.06%)	0.0688±0.0048 (1.85%)
WT 100 mg/kg	0.0488±0.0040 (-11.36%)	0.0500±0.0038 (-25.93%)	0.0488±0.0074** (-42.65%)	0.0563±0.0075** (-34.74%)	0.0638±0.0080 (-16.34%)	0.0675±0.0084 (0%)
WT 200 mg/kg	0.0475±0.0041 (-13.64%)	0.0500±0.0033 (-25.93%)	0.0450±0.0033*** (-47.06%)	0.0500±0.0042** (-42%)	0.0575±0.0041 (-24.54%)	0.0613±0.0051 (-9.26%)
WT 400 mg/kg	0.0475±0.0031 (-13.64%)	0.0475±0.0049 (-29.63%)	0.0438±0.0046*** (-48.53%)	0.0463±0.0042*** (-46.35%)	0.0525±0.0025* (-31.10%)	0.0563±0.0032 (-16.67%)

Anti-nociceptive activity

Mouse hot-plate test

To demonstrate the validity of the hot-plate analgesic testing following drug administration, mice received morphine sulphate (MO; 10 mg/kg) intraperitoneally (i.p.) and were tested during the subsequent 240 min period. As expected MO significantly (p<0.001) increased the hot-plate latency producing an area of analgesia of 12,835.65±1,909.33 %MPE-min compared with that of normal saline solution (NSS) (-10,873.10±4,166.42 %MPE-min; Figure 68).

Initial studies utilizing the hot-plate test in mice to examine the efficacy of Ben-Cha-Moon-Yai remedy and five root species extracts in producing analgesia. Mice were administered orally 2% Tween 80 or various doses of Ben-Cha-Moon-Yai remedy (BMY; 125, 250 and 500 mg/kg) and five root species extracts (25, 50, 100, 200 and 400 mg/kg).

All doses of BMY (125, 250 and 500 mg/kg) significantly (p<0.05, p<0.001 and p<0.01, respectively) increased the hot-plate latency when compared to the vehicle group. BMY 250 mg/kg significantly (p<0.05) increased the hot-plate latency when compared to BMY 125 mg/kg (Figure 69). The analgesic peak effects of BMY 125, 250 and 500 mg/kg were reached within 90, 240, 120 min after oral administration, respectively.

AM 400 mg/kg significantly (p<0.05) increased the hot-plate latency when compared to the vehicle group (Figure 70). The analgesic peak effect of AM 400 mg/kg was reached within 120 min after oral administration.

DS at the doses of 200 and 400 mg/kg significantly (p<0.01, p<0.05, respectively) increased the hot-plate latency when compared to the vehicle group (Figure 70). The analgesic peak effects of DS 200 and 400 mg/kg were reached within 120 and 90 min after oral administration, respectively.

All doses of DL and OI (25-400 mg/kg) did not produce analgesic response when compared to the vehicle group (Figure 71 and 73).

WT at the doses of 100, 200 and 400 mg/kg significantly ($p<0.05$, $p<0.01$ and $p<0.01$, respectively) increased the hot-plate latency when compared to the vehicle group (Figure 74). The analgesic peak effects of WT 100, 200 and 400 mg/kg were reached within 120, 30 and 90 min after oral administration, respectively.

In order to investigate any role of the opioid receptor in AM, DS and WT actions, mice were then administered NSS (10 ml/kg, i.p.), naloxone (NAL; 5 mg/kg, i.p.), a short-acting opioid receptor antagonist, 2% Tween 80 (10 ml/kg, p.o.), AM (400 mg/kg, p.o.), DS (200 mg/kg, p.o.), WT (400 mg/kg, p.o.) or the combination of NAL and AM (5/400 mg/kg), the combination of NAL and DS (5/200 mg/kg) and the combination of NAL and WT (5/400 mg/kg). NAL alone failed to produce significant response when compared to vehicle group. AM, DS, WT at the dose tested produced significant ($p<0.001$) response when compared to vehicle group. The inclusion of naloxone with AM, DS and WT significantly ($p<0.001$) attenuated the analgesic response due to AM, DS and WT indicating that opioid receptors are involved in the analgesic response produced by AM, DS and WT (Figure 75, 76, 77, respectively).

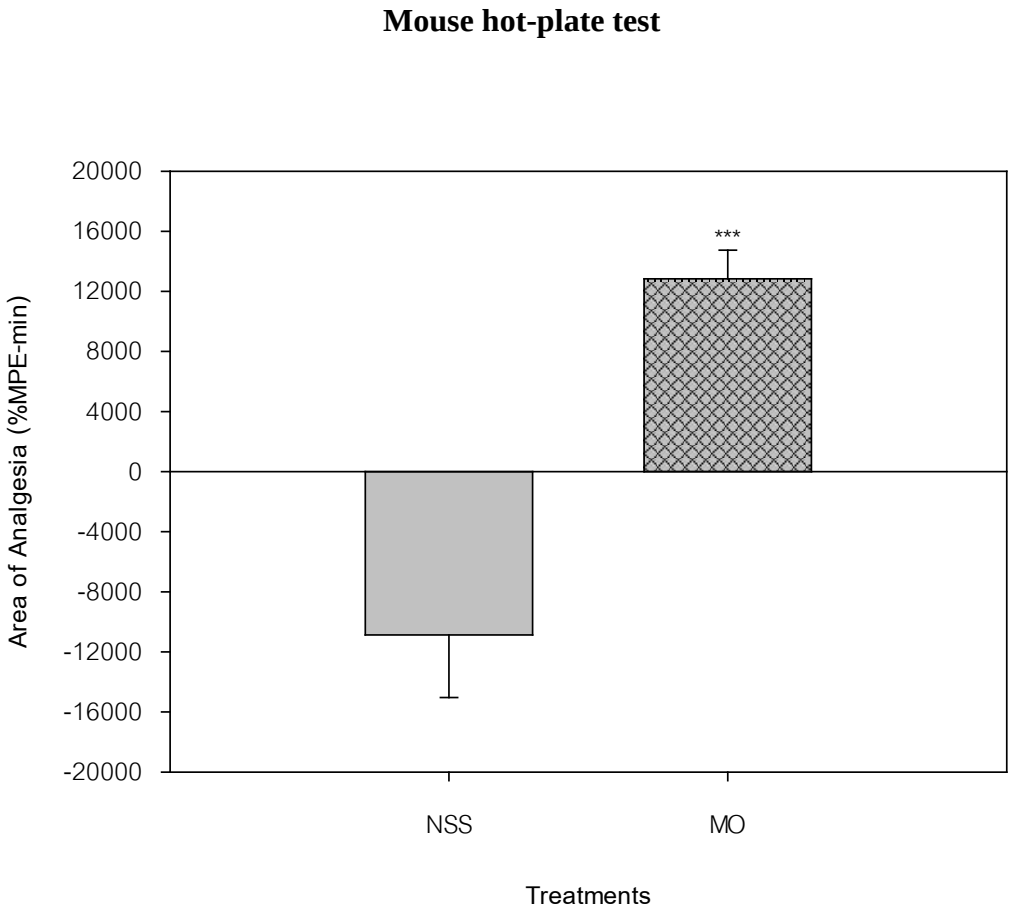


Figure 68 Area of analgesia (%MPE-min) from 0-240 minutes after intraperitoneal administration of normal saline solution (NSS; 10 ml/kg) and morphine sulphate (MO; 10 mg/kg). N=10 for all groups. $^{***}p<0.001$ significantly different compared to NSS.

Mouse hot-plate test

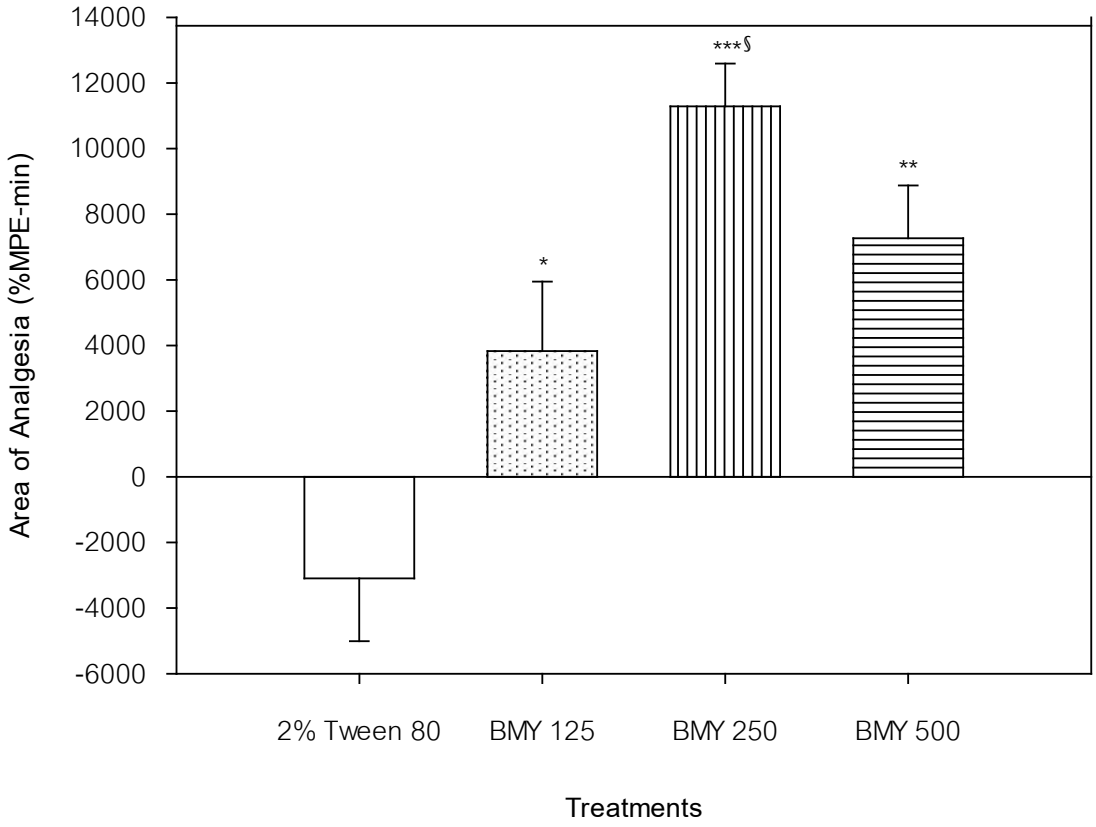


Figure 69 Area of analgesia (%MPE-min) from 0-240 minutes after oral administration of 2% Tween 80 (10 ml/kg) and various doses of the root extract of Ben-Cha-Moon-Yai remedy (BMY; 125-500 mg/kg). N=10 for all groups.

$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ significantly different compared to 2% Tween 80.

$^{\S}p<0.05$ significantly different compared to BMY 125 mg/kg.

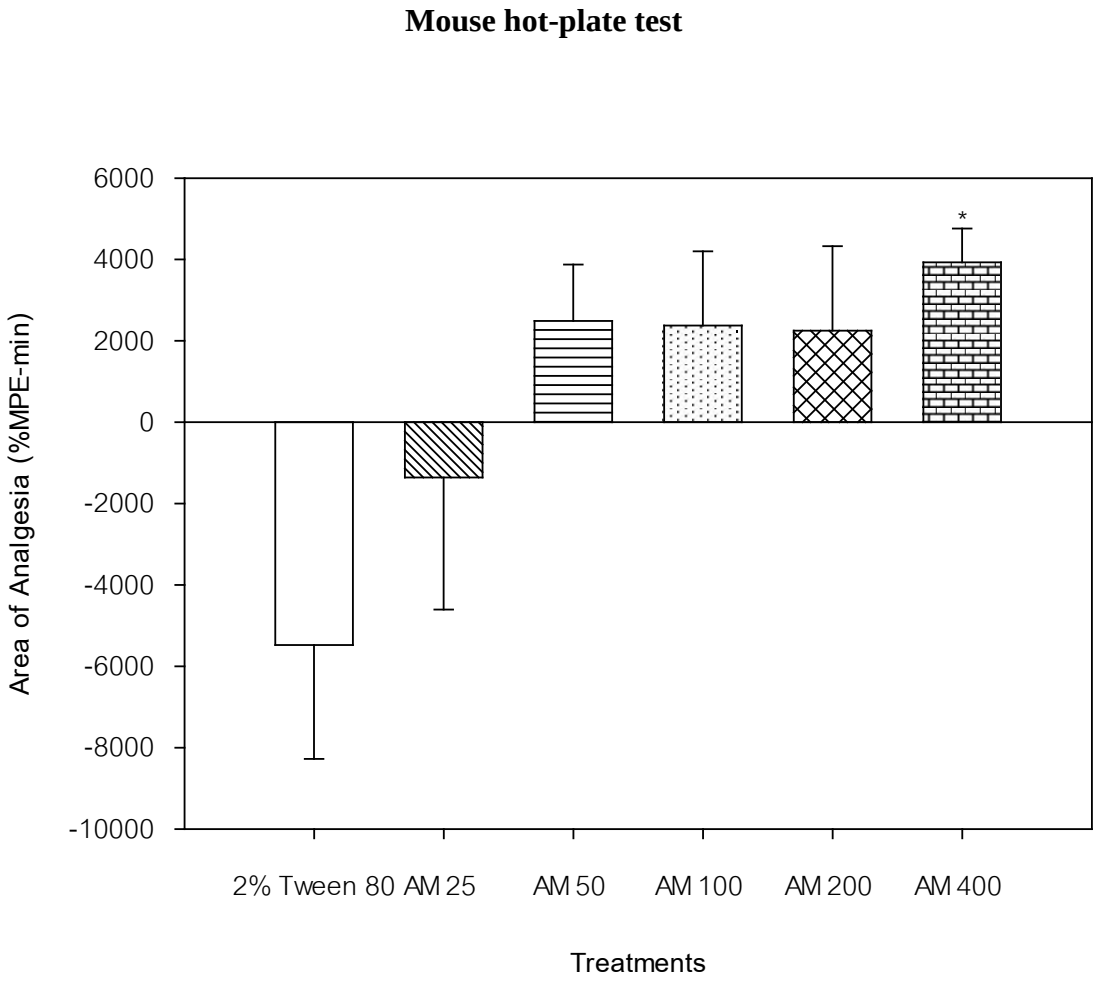


Figure 70 Area of analgesia (%MPE-min) from 0-240 minutes after oral administration of 2% Tween 80 and various doses of *Aegle marmelos* root extract (AM; 25-400 mg/kg). N=10 for all groups. $^{*}p<0.05$ significantly different compared to 2% Tween 80.

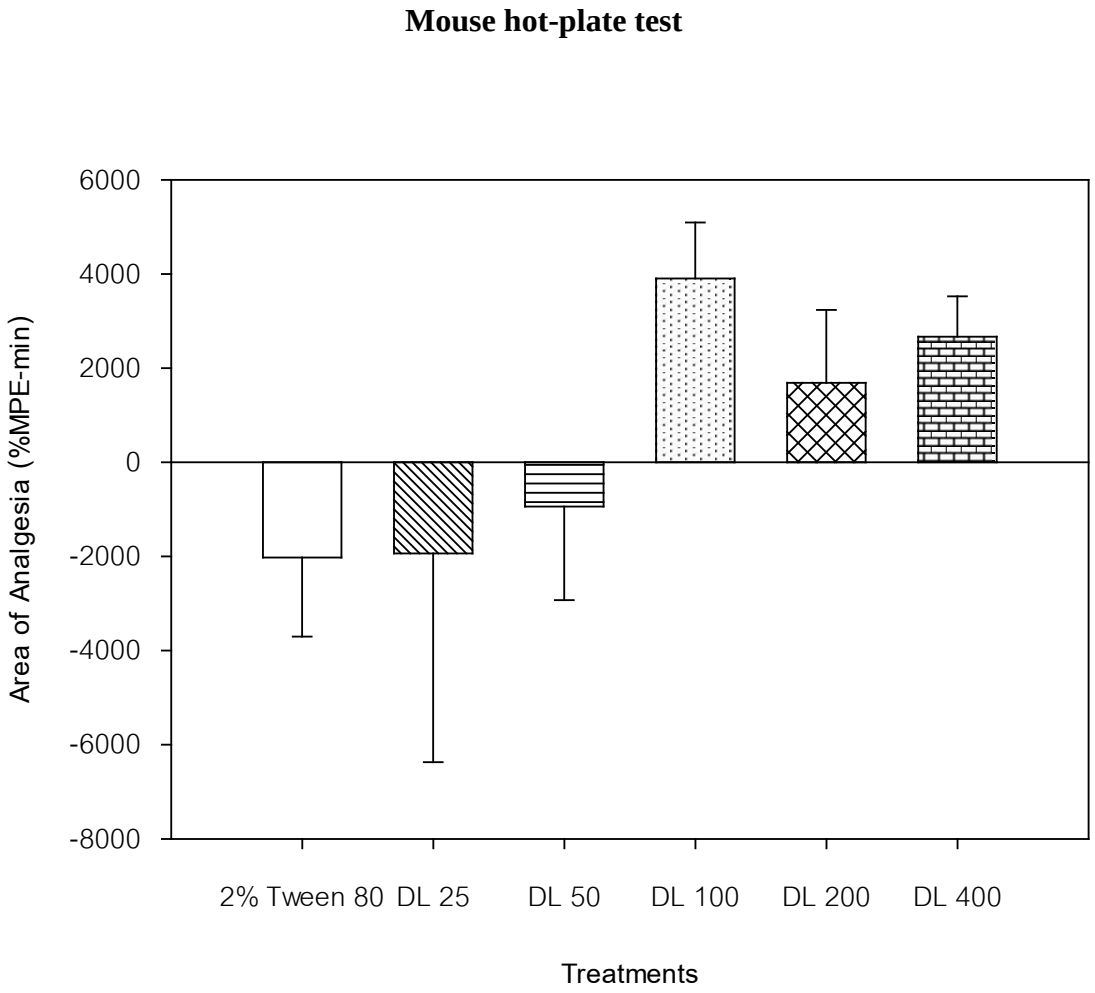


Figure 71 Area of analgesia (%MPE-min) from 0-240 minutes after oral administration of 2% Tween 80 and various doses of *Dimocarpus longan* root extract (DL; 25-400 mg/kg). N=10 for all groups.

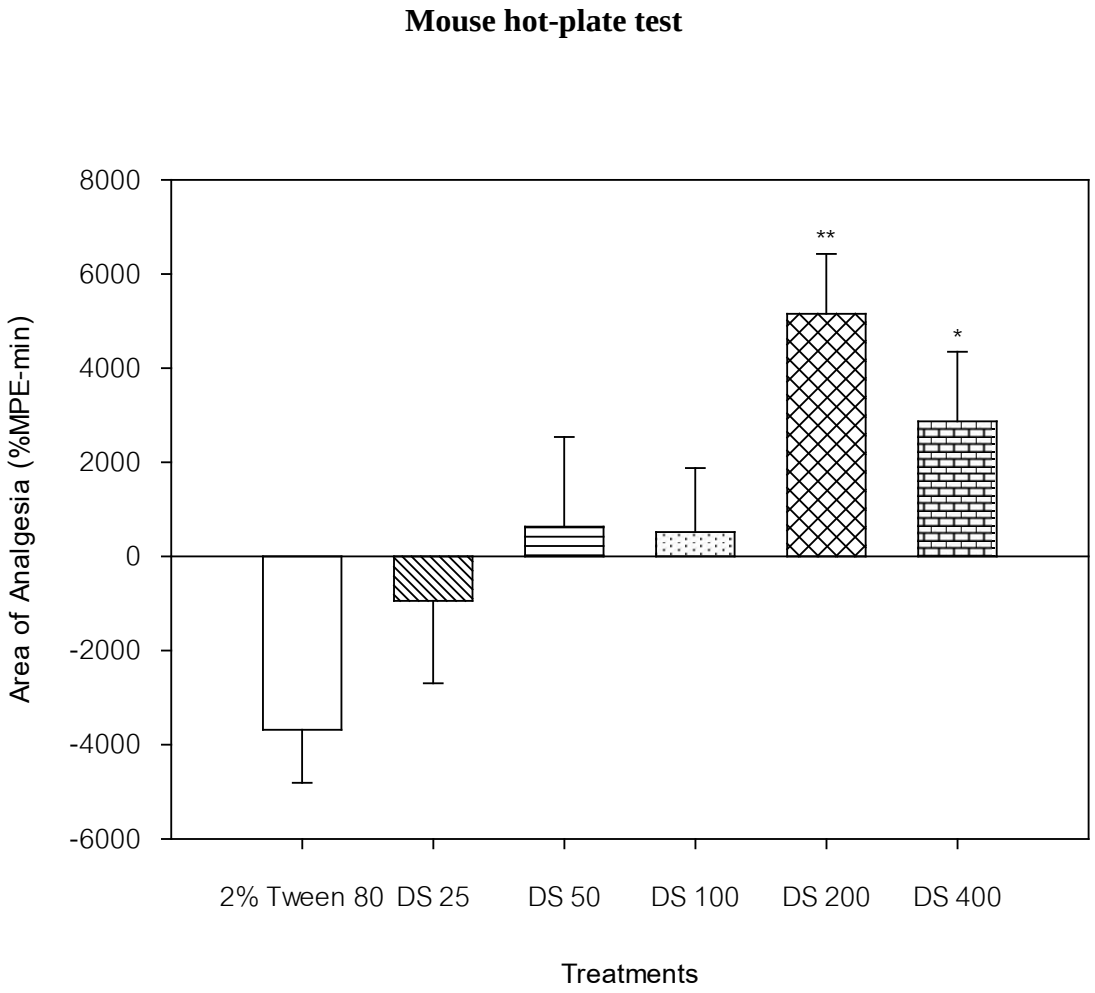


Figure 72 Area of analgesia (%MPE-min) from 0-240 minutes after oral administration of 2% Tween 80 and various doses of *Dolichandrone serrulata* root extract (DS; 25-400 mg/kg). N=10 for all groups. $^{*}p<0.05$, $^{**}p<0.01$ significantly different compared to 2% Tween 80.

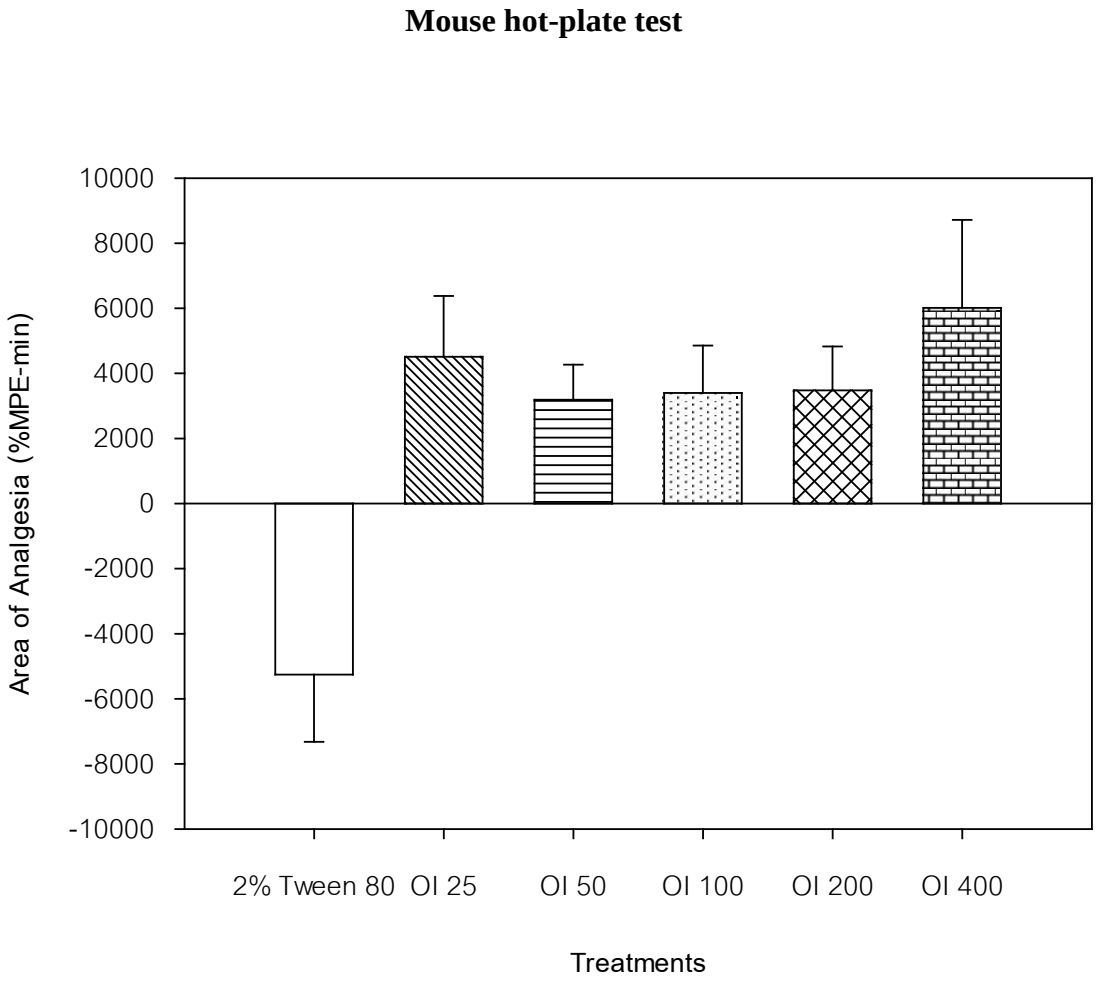


Figure 73 Area of analgesia (%MPE-min) from 0-240 minutes after oral administration of 2% Tween 80 and various doses of *Oroxylum indicum* root extract (OI; 25-400 mg/kg). N=10 for all groups.

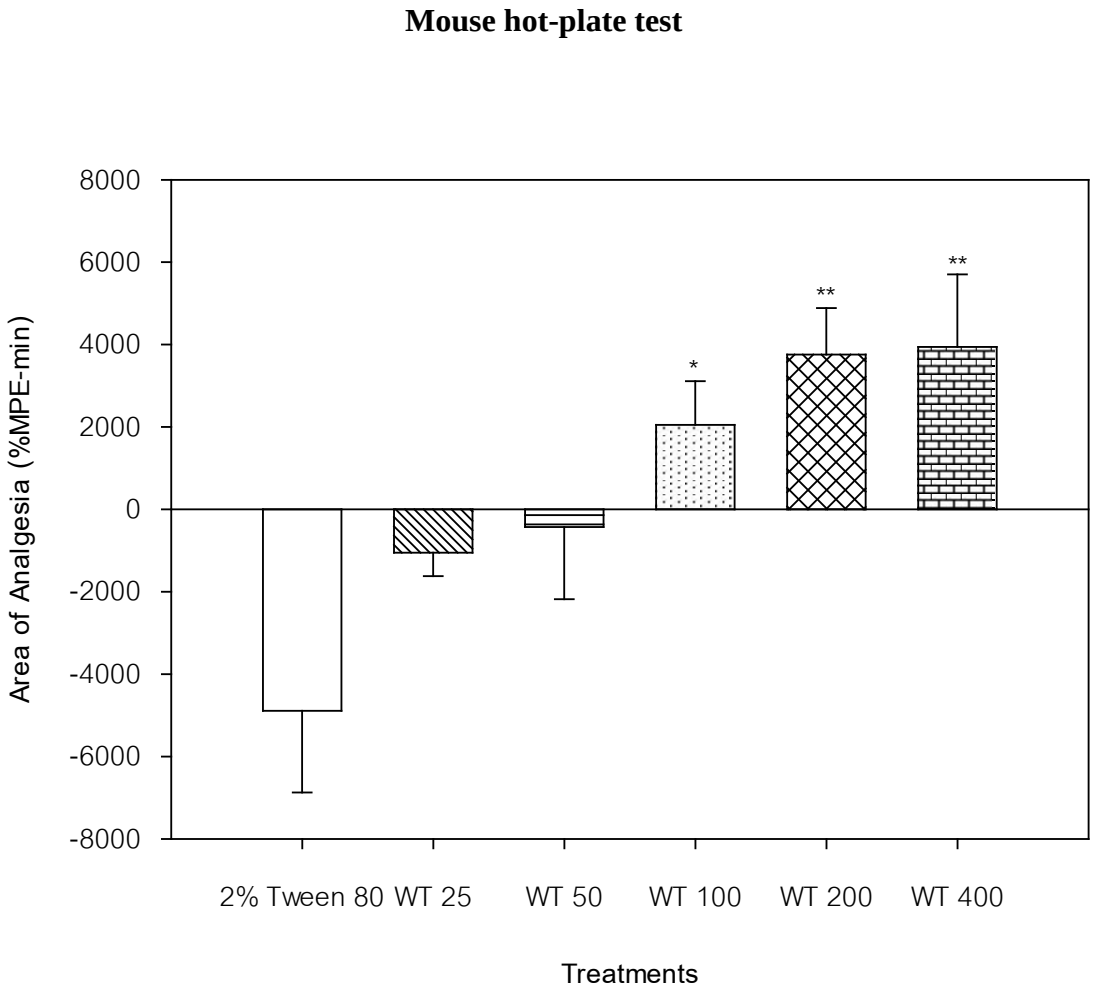


Figure 74 Area of analgesia (%MPE-min) from 0-240 minutes after oral administration of 2% Tween 80 and various doses of *Walsura trichostemon* root extract (WT; 25-400 mg/kg). N=10 for all groups. $^{*}p<0.05$, $^{**}p<0.01$ significantly different compared to 2% Tween 80.

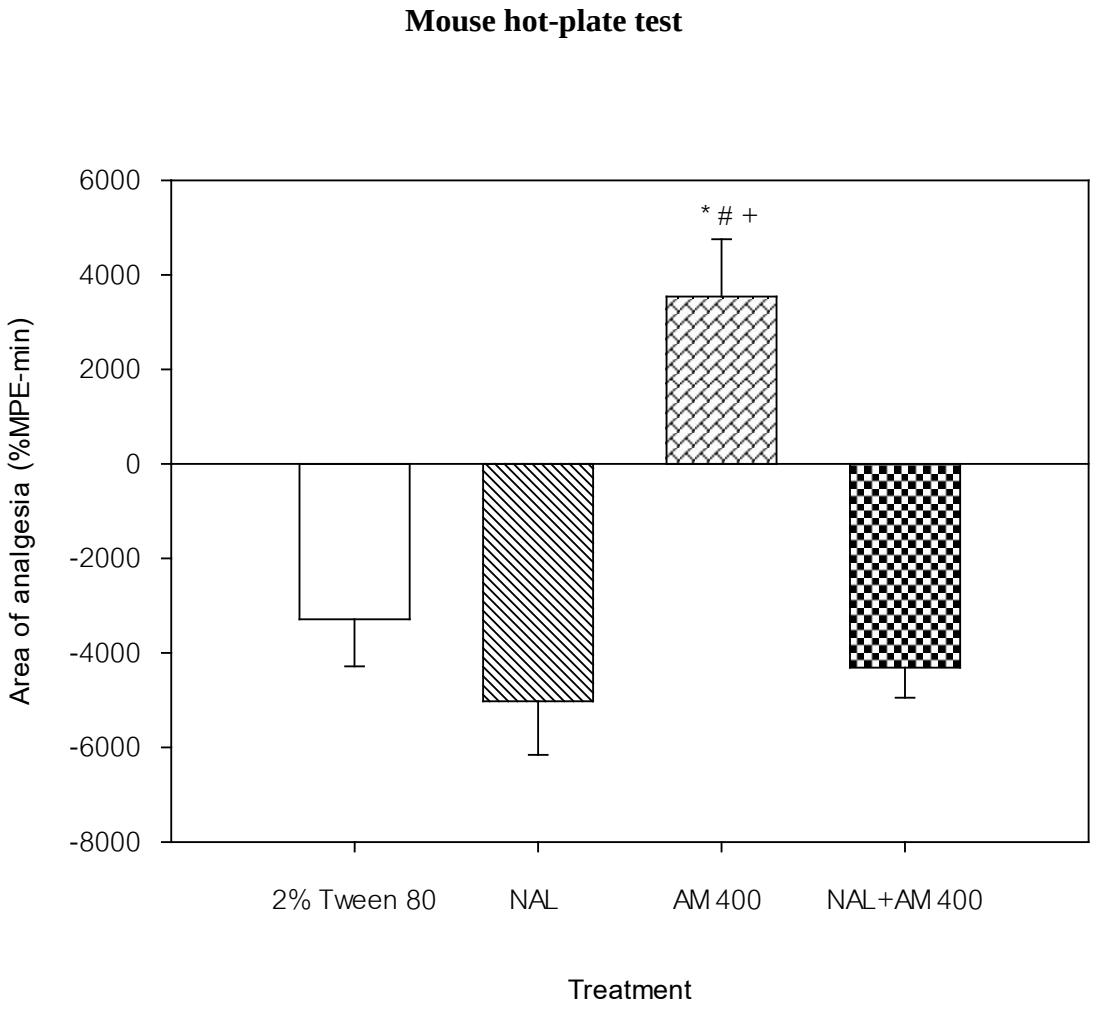


Figure 75 Area of analgesia (%MPE-min) from 0-240 minutes after administration of 2% Tween 80 (10 ml/kg, p.o.), naloxone (NAL; 5 mg/kg, i.p.), *Aegle marmelos* root extract (AM; 400 mg/kg, p.o.) and the combination of naloxone and AM (5/400 mg/kg). N=10 for all groups.

$^{*}p<0.001$ significantly different compared to 2% Tween 80.

$^{\#}p<0.001$ significantly different compared to NAL.

$^{+}p<0.001$ significantly different compared to NAL+AM 400.

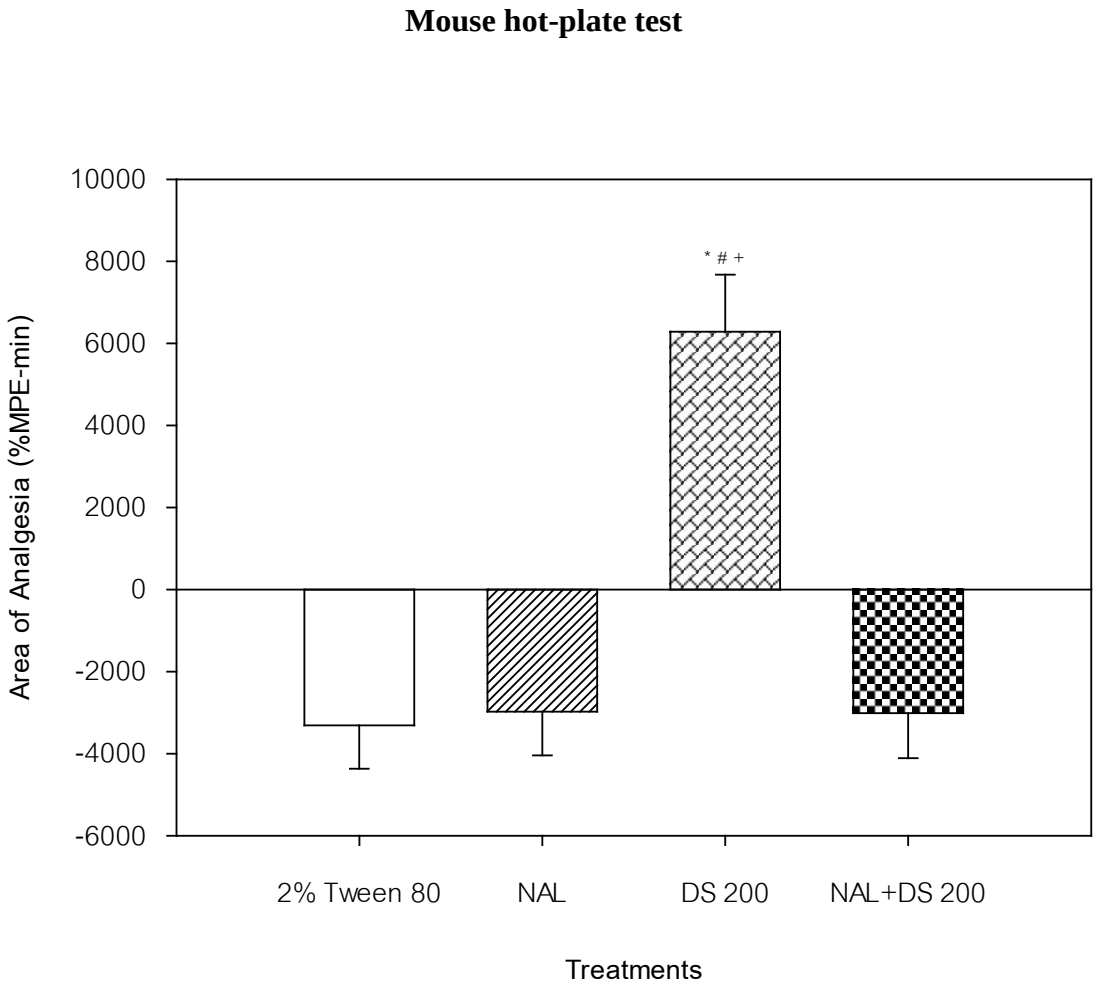


Figure 76 Area of analgesia (%MPE-min) from 0-240 minutes after administration of 2% Tween 80 (10 ml/kg, p.o.), naloxone (NAL; 5 mg/kg, i.p.), *Dolichandrone serrulata* root extract (DS; 200 mg/kg, p.o.) and the combination of naloxone and DS (5/200 mg/kg). N=10 for all groups.

$^{*}p<0.001$ significantly different compared to 2% Tween 80.

$^{\#}p<0.001$ significantly different compared to NAL.

$^{+}p<0.001$ significantly different compared to NAL+DS 200.

Mouse hot-plate test

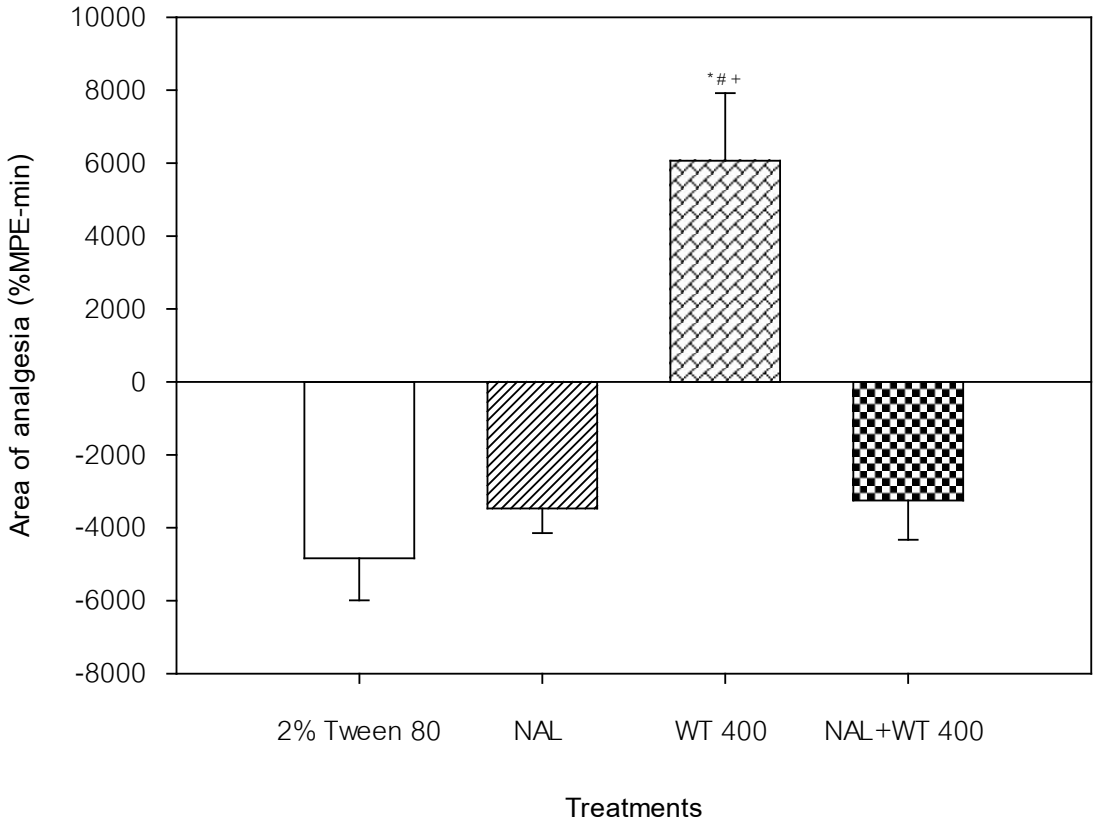


Figure 77 Area of analgesia (%MPE-min) from 0-240 minutes after administration of 2% Tween 80 (10 ml/kg, p.o.), naloxone (NAL; 5 mg/kg, i.p.), *Walsura trichostemon* root extract (WT; 400 mg/kg, p.o.) and the combination of naloxone and WT (5/400 mg/kg). N=10 for all groups.

$^{*}p<0.001$ significantly different compared to 2% Tween 80.

$^{\#}p<0.001$ significantly different compared to NAL.

$^{+}p<0.001$ significantly different compared to NAL+WT 400.

Formalin test in mice

To demonstrate the validity of formalin test following drug administration, mice received morphine sulfate (MO; 10 mg/kg) intraperitoneally or indomethacin (IND; 10 mg/kg) and were observed for paw licking at early phase (0-5 min) and late phase (15-30 min). As expected MO significantly (p<0.001 and p<0.001, respectively) decreased the licking time of both early and late phases by 90.87% and 97.69%, respectively producing mean time spent on paw licking of 7.48±1.84 and 3.67±1.08 sec compared with that of NSS (81.92±8.60 and 158.80±6.08 sec, respectively; Figure 78, Table 43).

Study then utilized the formalin test in mice to examine the efficacy of BMY and five herbal root extracts (AM, OI, DL, DS and WT) in producing analgesia. Mice were administered orally 2% Tween 80, IND (10 mg/kg), various doses of BMY (125, 250 and 500 mg/kg) or AM, OI, DL, DS and WT (25, 50, 100, 200 and 400 mg/kg).

Only BMY 250 mg/kg significantly (p<0.05) decreased the licking time by 20.03% producing mean time spent on paw licking of 90.82±6.93 sec compared with that of vehicle group (113.56±4.83 sec) in the early phase. For the late phase, All doses of BMY (125, 250 and 500 mg/kg) significantly (p<0.01) decreased the licking time by 48.96%, 56.85% and 51.30%, respectively producing mean time spent on paw licking of 80.97±18.20, 68.47±14.99 and 77.27±18.43 sec, respectively when compared with that of vehicle group (158.66±13.07 sec). The reference drug, IND (10 mg/kg) also caused significant (p<0.01) inhibition of the late phase of formalin-induced nociception, producing 56.58% inhibition when compared to the vehicle group (Figure 79, Table 44).

AM at dose of 400 mg/kg significantly (p<0.05) decreased the licking time in the early phase by 34.55% producing mean time spent on paw licking of 64.19±4.08 sec compared with that of vehicle group (98.06±5.45 sec). Furthermore, AM 400 mg/kg also significantly (p<0.01 and p<0.05, respectively) decreased the licking time in the early phase compared with that of AM 25 and 100 mg/kg (103.42±6.64 and 98.60±4.03 sec, respectively). IND (10 mg/kg), the reference drug, caused significant

(p<0.001) inhibition of the late phase of formalin-induced nociception, producing 61.71% inhibition when compared to the vehicle group. AM 400 mg/kg significantly (p<0.001) decreased the licking time by and 59.85% producing the mean time spent on paw licking of 58.44±5.50 sec compared with that of vehicle group (145.57±17.16 sec). The antinociceptive efficacy of AM 400 mg/kg is comparable to IND. Additionally, AM 400 mg/kg significantly (p<0.001, p<0.001, p<0.001 and p<0.01, respectively) decreased the licking time in the late phase compared with that of AM 25, 50, 100 and 200 mg/kg (Figure 80, Table 45).

All doses of OI failed to decrease the licking time in the early phase induced by formalin. However, OI at the doses of 100, 200 and 400 mg/kg significantly (p<0.01, p<0.001, p<0.001, respectively) decreased the licking time by 49.40%, 61.78% and 68.28%, respectively producing mean time spent on paw licking of 73.07±9.98, 55.18±9.03 and 45.80±17.08 sec, respectively compared with that of vehicle group (144.41±10.68 sec) during the late phase. IND (10 mg/kg) caused significant (p<0.01) inhibition of the late phase of formalin-induced nociception, producing 48.96% inhibition when compared to the vehicle group. In addition, OI 100 mg/kg significantly (p<0.05) decreased the licking time in the late phase compared with that of OI 25 mg/kg. OI 200 mg/kg significantly (p<0.001 and p<0.01, respectively) decreased the licking time in the late phase compared with that of OI 25 and 50 mg/kg. OI 400 mg/kg significantly (p<0.001) decreased the licking time in the late phase compared with that of OI 25 and 50 mg/kg. The antinociceptive efficacy during the late phase of OI 50-400 mg/kg is comparable to IND (Figure 81, Table 46).

All doses of DL failed to decrease the licking time in the early phase induced by formalin. However, DL 200 and 400 mg/kg significantly (p<0.01 and p<0.001, respectively) decreased the licking time during the late phase by 53.57% and 88.14%, respectively producing mean time spent on paw licking of 56.90±12.12 and 14.52±6.54 sec, respectively compared with that of vehicle group (122.54±15.26 sec). IND (10 mg/kg) caused significant (p<0.01) inhibition only in the late phase of formalin-induced nociception, producing 56.39% inhibition when compared to the vehicle group. Furthermore, in the late phase, DL 200 mg/kg significantly (p<0.01 and p<0.05, respectively) decreased the licking time compared with that of DL 25 and

50 mg/kg. DL 400 mg/kg also significantly (p<0.001) decreased the licking time in the late phase compared with that of DL 25-100 mg/kg. The antinociceptive efficacy during the late phase of DL 200 and 400 mg/kg are comparable to IND (Figure 82, Table 47).

DS 400 mg/kg significantly (p<0.001) decreased the licking time in the early phase by 38.17% producing mean time spent on paw licking of 64.53±3.99 sec compared with that of vehicle group (104.38±5.50 sec). DS 400 mg also significantly (p<0.01, p<0.05, p<0.01 and p<0.05, respectively) decreased mean time spent on paw licking in the early phase compared with that of IND and DS at the doses of 25, 50 and 100 mg/kg, respectively. For the late phase, DS 200 and 400 mg/kg significantly (p<0.01, p<0.001, respectively) decreased the licking time in the late phase by 45.22% and 55.31%, respectively producing mean time spent on paw licking of 73.99±7.81 and 60.36±5.32 sec, respectively compared with that of vehicle group (135.07±10.01 sec). DS 200 mg/kg significantly (p<0.01) decreased the licking time in the late phase compared with that of DS 25 mg/kg. DS 400 mg/kg significantly (p<0.001) decreased the licking time compared with that of DS 25 mg/kg. IND (10 mg/kg) caused significant (p<0.001) inhibition of the late phase of formalin-induced nociception, producing 61.15% inhibition when compared to the vehicle group. The antinociceptive efficacy during the late phase of DS 200 and 400 mg/kg are comparable to IND (Figure 83, Table 48).

In the early phase, WT 400 mg kg significantly (p<0.05) decreased the licking time by 31.66% producing mean time spent on paw licking of 72.32±4.15 sec compared with that of vehicle group (105.03±6.11 sec). WT 400 mg/kg significantly (p<0.01 and p<0.05, respectively) decreased the licking time compared with that of WT 50 and 100 mg/kg. For the late phase, WT 200 and 400 mg/kg significantly (p<0.01 and p<0.001, respectively) decreased the licking time by 44.49% and 53% producing mean time spent on paw licking of 78.59±7.71 and 66.55±5.82 sec, respectively compared with that of vehicle group (141.59±13.19 sec). WT 400 mg/kg also significantly (p<0.01) decreased the licking time compared with that of WT 25 mg/kg. IND (10 mg/kg) caused significant (p<0.001) inhibition of the late phase of formalin-induced nociception, producing 61.74% inhibition when compared to the

vehicle group. The antinociceptive efficacy during the late phase of WT 200 and 400 mg/kg are comparable to IND (Figure 84, Table 49).

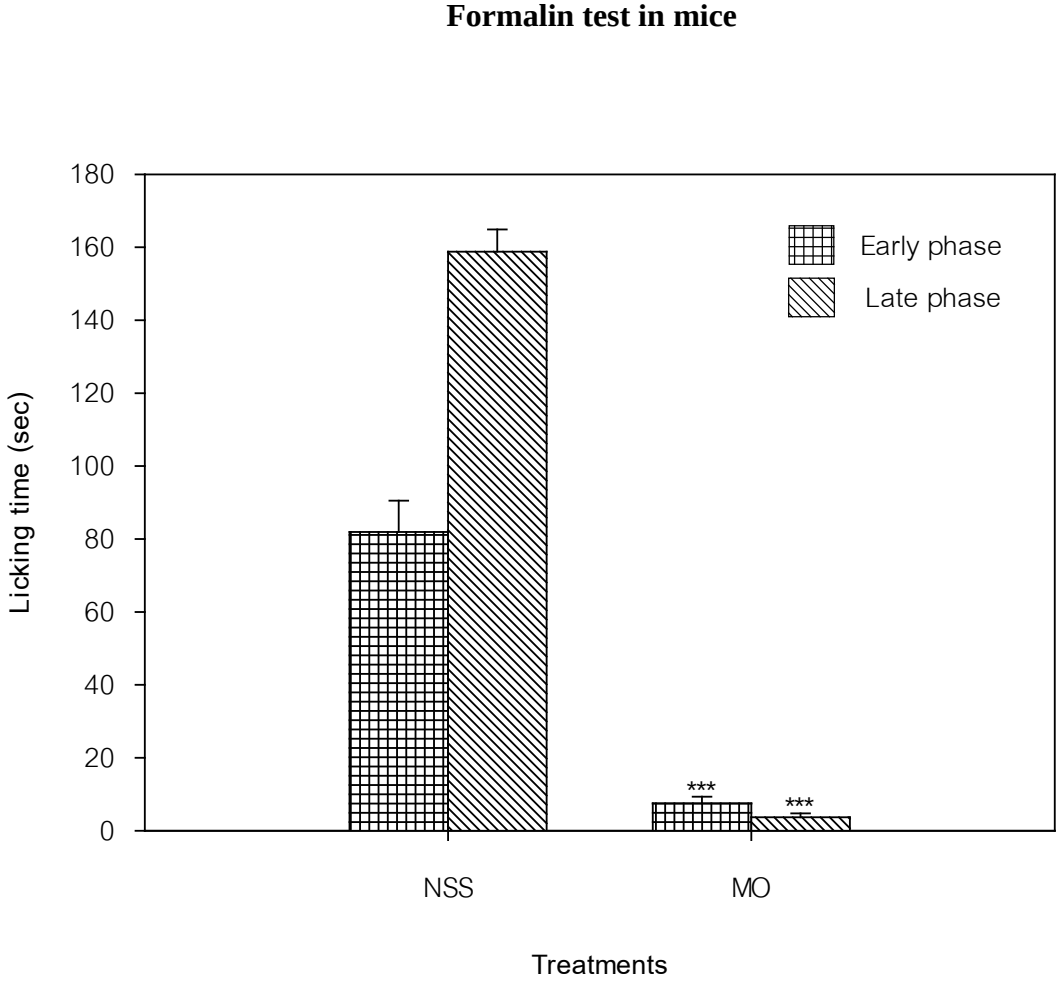


Figure 78 Time spent on paw licking after intraperitoneal administration of 0.9% normal saline solution (NSS; 10 ml/kg) and morphine sulphate (MO; 10 mg/kg). N=8 for all groups. $^{***}p<0.001$ significantly different compared to NSS.

Formalin test in mice

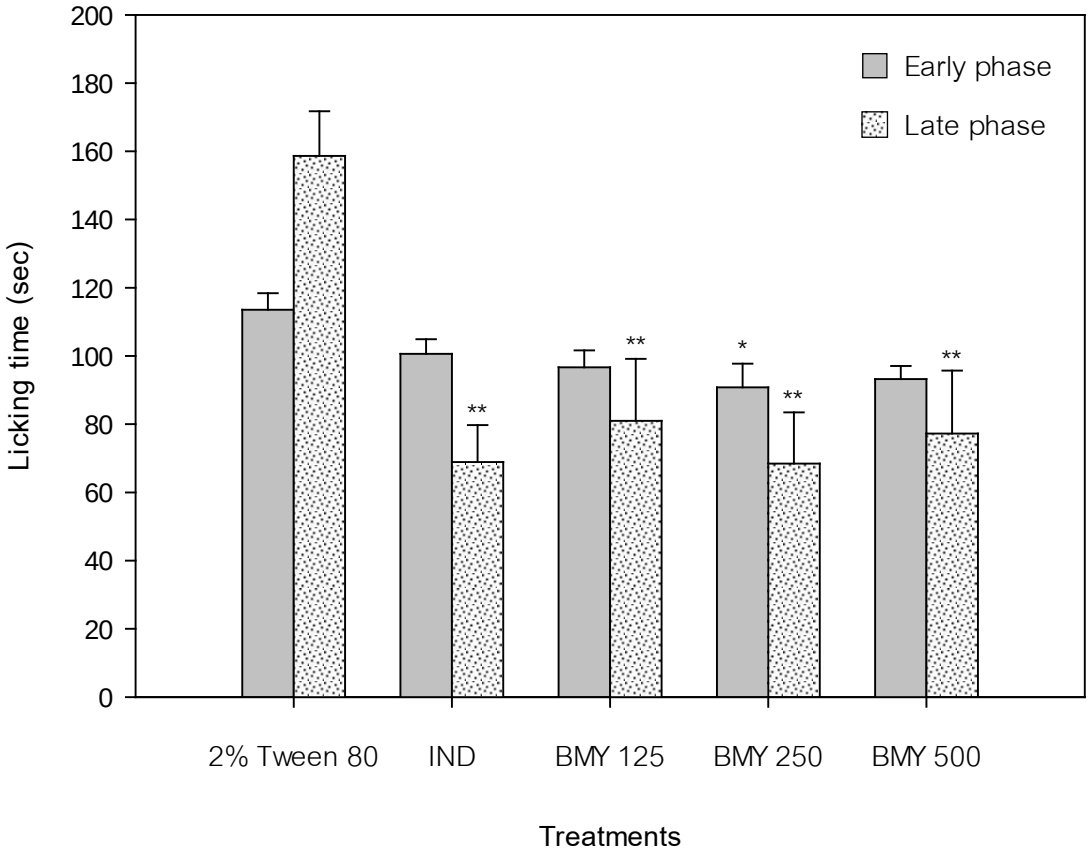


Figure 79 Time spent on paw licking after oral administration of 2% Tween 80 (10 ml/kg), indomethacin (IND; 10 mg/kg) and various doses of the root extract of Ben-Cha-Moon-Yai remedy (BMY; 125-500 mg/kg). N=8 for all groups. $^{*}p<0.05$, $^{**}p<0.01$ significantly different compared to 2% Tween 80.

Formalin test in mice

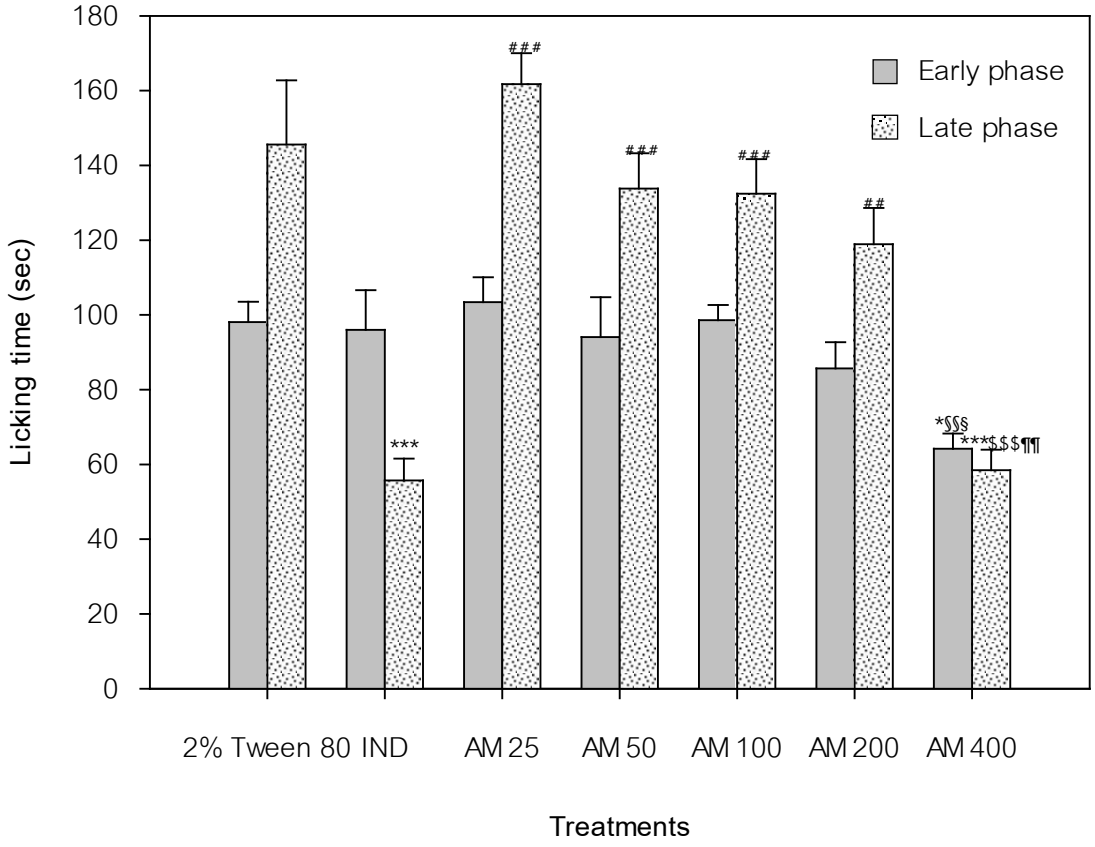


Figure 80 Time spent on paw licking after oral administration of 2% Tween 80 (10 ml/kg), indomethacin (IND; 10 mg/kg) and various doses of *Aegle marmelos* root extract (AM; 25-400 mg/kg). N=8 for all groups.

$^{*}p<0.05$, $^{***}p<0.001$ significantly different compared to 2% Tween 80.

$^{\S}p<0.05$ significantly different compared to AM 100 mg/kg.

$^{¶¶}p<0.01$ significantly different compared to AM 200 mg/kg.

$^{\S\S}p<0.01$ significantly different compared to AM 25 mg/kg.

$^{\$\$\$}p<0.001$ significantly different compared to AM 25-100 mg/kg.

$^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ significantly different compared to IND 10 mg/kg.

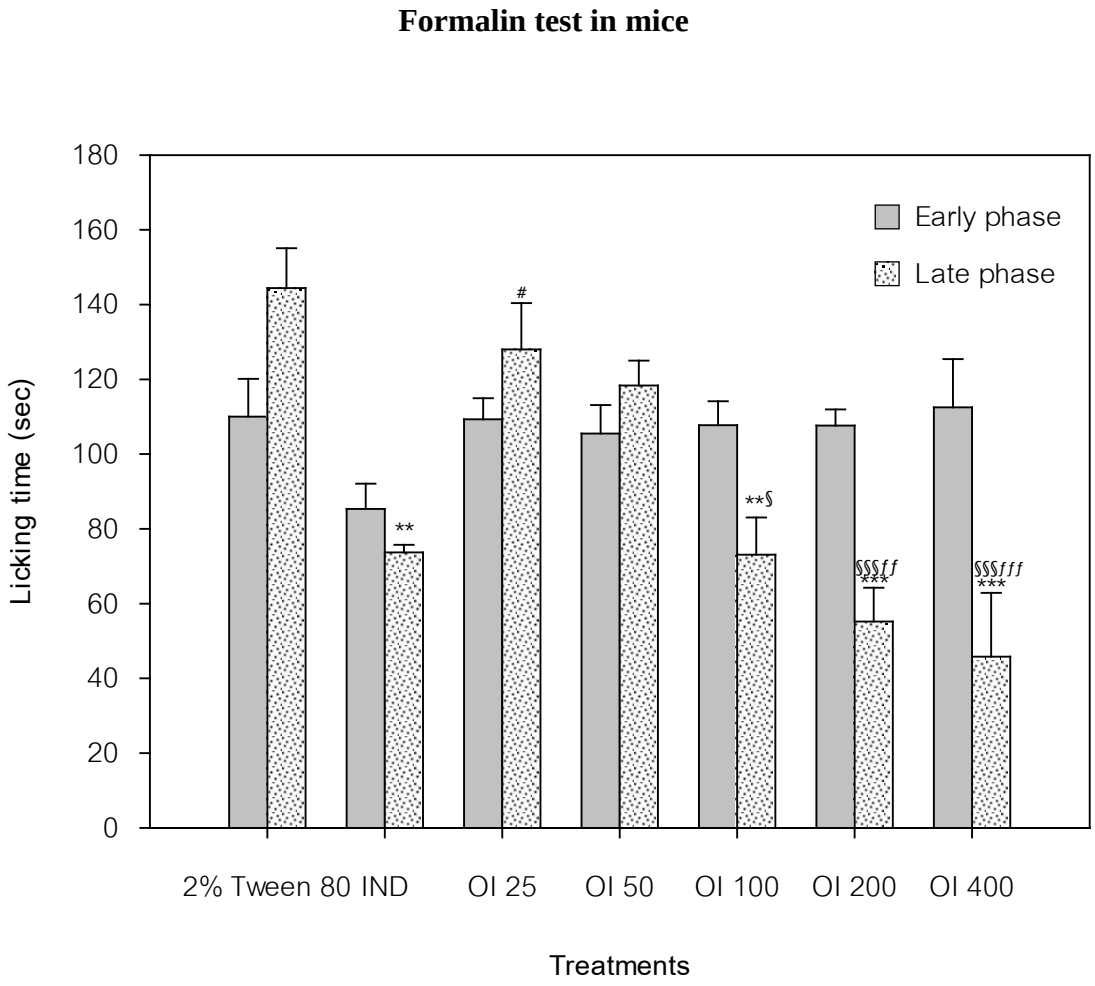


Figure 81 Time spent on paw licking after oral administration of 2% Tween 80 (10 ml/kg), indomethacin (IND; 10 mg/kg) and various doses of *Oroxylum indicum* root extract (OI; 25-400 mg/kg). N=8 for all groups.

$^{**}p<0.01$, $^{***}p<0.001$ significantly different compared to 2% Tween 80.

$^{\S}p<0.05$, $^{\S\S\S}p<0.001$ significantly different compared to OI 25 mg/kg.

$^{ff}p<0.01$, $^{fff}p<0.001$ significantly different compared to OI 50 mg/kg.

$^{\#}p<0.05$ significantly different compared to IND 10 mg/kg.

Formalin test in mice

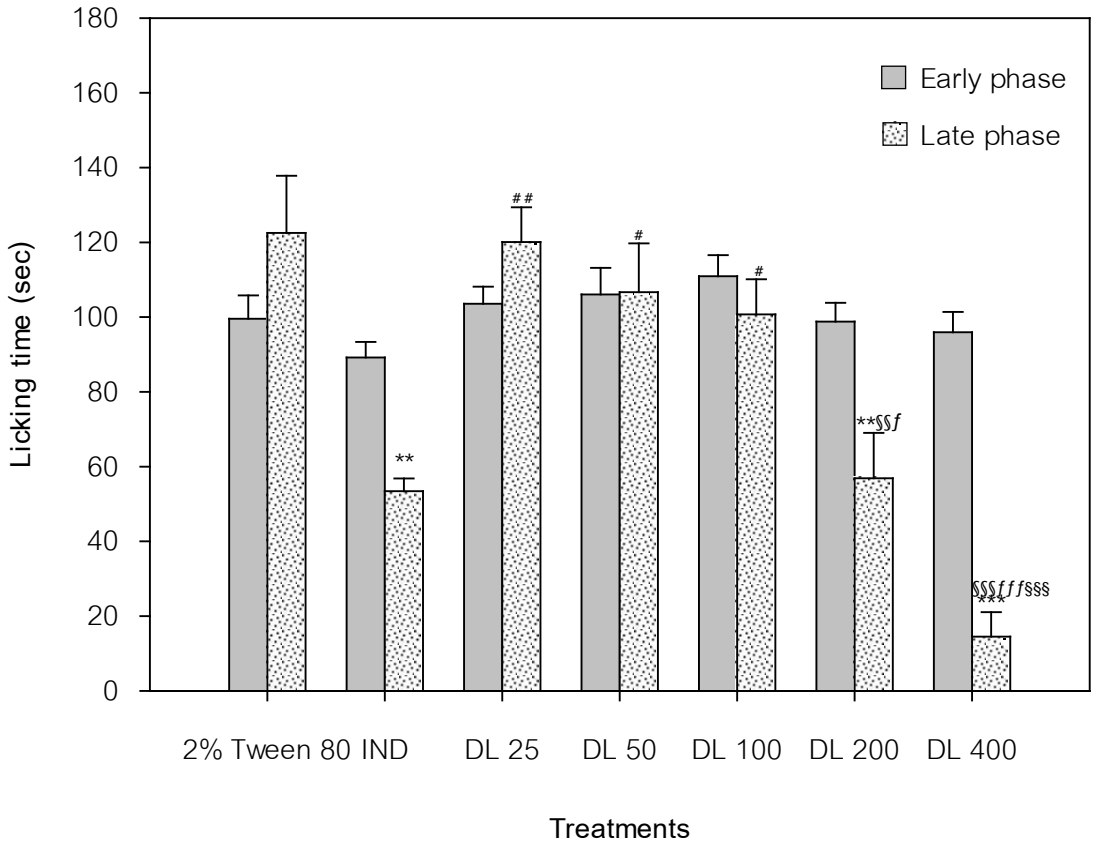


Figure 82 Time spent on paw licking after oral administration of 2% Tween 80 (10 ml/kg), indomethacin (IND; 10 mg/kg) and various doses of *Dimocarpus longan* root extract (DL; 25-400 mg/kg). N=8 for all groups.

$^{**}p<0.01$, $^{***}p<0.001$ significantly different compared to 2% Tween 80.

$^{f}p<0.05$, $^{fff}p<0.001$ significantly different compared to DL 50 mg/kg.

$^{§§}p<0.01$, $^{§§§}p<0.001$ significantly different compared to DL 25 mg/kg.

$^{§§§}p<0.001$ significantly different compared to DL 100 mg/kg.

$^{\#}p<0.05$, $^{\#\#}p<0.01$ significantly different compared to IND 10 mg/kg.

Formalin test in mice

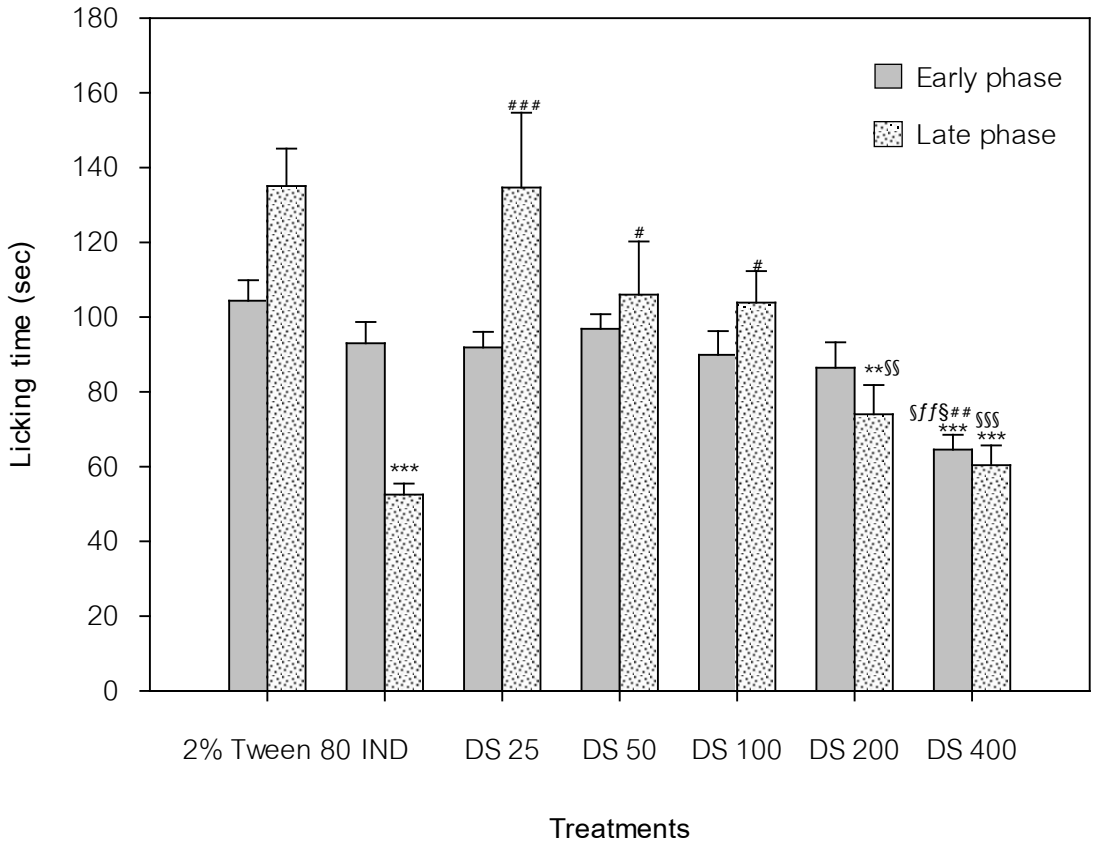


Figure 83 Time spent on hind paw licking after oral administration of 2% Tween 80 (10 ml/kg), indomethacin (IND; 10 mg/kg) and various doses of *Dolichandrone serrulata* root extract (DS; 25-400 mg/kg). N=8 for all groups.

$^{**}p<0.01$, $^{***}p<0.001$ significantly different compared to 2% Tween 80.

$^{\S}p<0.05$ significantly different compared to DS 100 mg/kg.

$^{\S}p<0.05$, $^{\S\S}p<0.01$, $^{\S\S\S}p<0.001$ significantly different compared to DS 25 mg/kg.

$^{ff}p<0.01$ significantly different compared to DS 50 mg/kg.

$^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ significantly different compared to IND 10 mg/kg.

Formalin test in mice

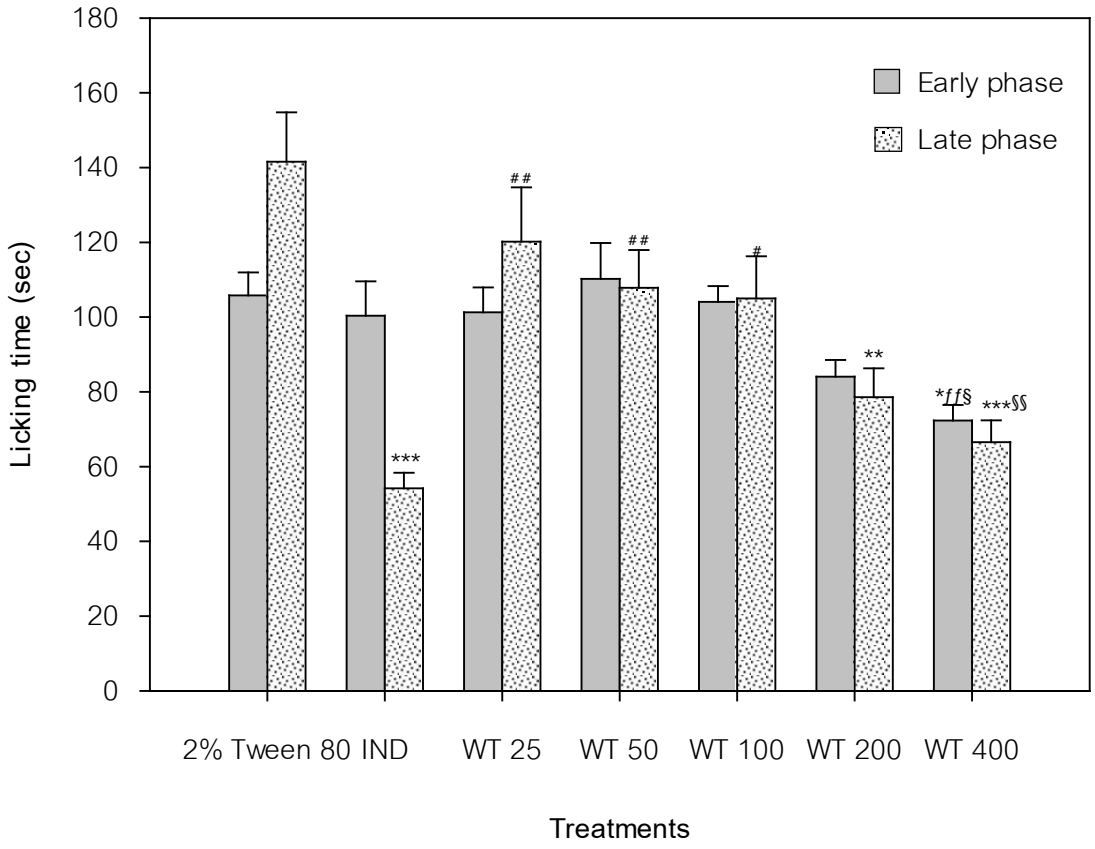


Figure 84 Time spent on paw licking after oral administration of 2% Tween 80 (10 ml/kg), indomethacin (IND; 10 mg/kg) and various doses of *Walsura trichostemon* root extract (WT; 25-400 mg/kg). N=8 for all groups.

$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ significantly different compared to 2% Tween 80.

$^{\S}p<0.05$ significantly different compared to WT 100 mg/kg.

$^{\S\S}p<0.01$ significantly different compared to WT 25 mg/kg.

$^{ff}p<0.01$ significantly different compared to WT 50 mg/kg.

$^{\#}p<0.05$, $^{\#\#}p<0.01$ significantly different compared to IND 10 mg/kg.

Table 43 Time spent on paw licking after intraperitoneal administration of 0.9% normal saline solution (NSS; 10 ml/kg) and morphine sulfate (MO; 10 mg/kg). Each value represents mean±S.E.M. N=8 for all groups. Inhibition is reported as percentage compared to vehicle control.

Treatment	**Dose (mg/kg)**	**Licking time (sec) (% inhibition)**	
		Early phase	**Late phase**
NSS		81.92±8.60	158.80±6.08
MO	10	7.48±1.84*** (90.87%)	3.67±1.08*** (97.69%)

***p<0.001 significantly different compared to NSS.

Table 44 Time spent on paw licking after oral administration of 2% Tween 80 (10 ml/kg), indomethacin (IND; 10 mg/kg) and various doses of Ben-Cha-Moon-Yai remedy (BMY; 125-500 mg/kg). Each value represents mean±S.E.M. N=8 for all groups. Inhibition is reported as percentage compared to vehicle control.

Treatments	**Dose (mg/kg)**	**Licking time (sec) (% inhibition)**	
		Early phase	**Late phase**
2% Tween 80		113.56±4.83	158.66±13.07
IND	10	100.61±4.33 (-11.40%)	68.89±10.85** (-56.58%)
BMY	125	96.67±4.97 (-14.87%)	80.97±18.20** (-48.96%)
	250	90.82±6.93* (-20.03%)	68.47±14.99** (-56.85%)
	500	93.20±3.89 (-17.93%)	77.27±18.43** (-51.30%)

*p<0.05, **p<0.01 significantly different compared to 2% Tween 80.

Table 45 Time spent on paw licking after oral administration of 2% Tween 80 (10 ml/kg), indomethacin (IND; 10 mg/kg) and various doses of *Aegle marmelos* root extract (AM; 25-400 mg/kg). Each value represents mean±S.E.M. N=8 for all groups. Inhibition is reported as percentage compared to vehicle control.

Treatments	**Dose (mg/kg)**	**Licking time (sec) (% inhibition)**	
		Early phase	**Late phase**
2% Tween 80		98.06±5.45	145.57±17.16
IND	10	95.98±10.65 (-2.12%)	55.73±5.83*** (-61.71%)
AM	25	103.42±6.64 (5.45%)	161.76±8.25$^{\#\#\#}$ (11.12%)
	50	94.09±10.62 (-4.05%)	133.80±9.44$^{\#\#\#}$ (-8.08%)
	100	98.60±4.03 (0.55%)	132.43±9.26$^{\#\#\#}$ (-9.02%)
	200	85.68±7.03 (-12.62%)	118.91±9.70$^{\#\#}$ (-18.31%)
	400	64.19±4.08$^{*\,\S\S}$ (-34.55%)	58.44±5.50$^{***\,\$\$\$\,\P}$ (-59.85%)

$^{*}p<0.05$, $^{**}p<0.01$ significantly different compared to 2% Tween 80.

$^{\S}p<0.05$ significantly different compared to AM 100 mg/kg.

$^{\S\S}p<0.01$ significantly different compared to AM 25 mg/kg.

$^{\P\P}p<0.01$ significantly different compared to AM 200 mg/kg.

$^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ significantly different compared to IND 10 mg/kg.

$^{\$\$\$}p<0.001$ significantly different compared to AM 25-100 mg/kg.

Table 46 Time spent on paw licking after oral administration of 2% Tween 80 (10 ml/kg), indomethacin (IND; 10 mg/kg) and various doses of *Oroxylum indicum* root extract (OI; 25-400 mg/kg). Each value represents mean±S.E.M. N=8 for all groups. Inhibition is reported as percentage compared to vehicle control.

Treatments	**Dose (mg/kg)**	**Licking time (sec) (% inhibition)**	
		Early phase	**Late phase**
2% Tween 80		110.03±10.08	144.41±10.68
IND	10	85.31±6.77 (-22.47%)	73.70±2.00** (-48.96%)
OI	25	109.31±5.63 (-0.65%)	128.02±12.38$^{\#}$ (-11.35%)
	50	105.48±7.62 (-4.13%)	118.32±6.67 (-18.06%)
	100	107.74±6.40 (-2.08%)	73.07± 9.98**§ (-49.40%)
	200	107.65±4.31 (-2.16%)	55.18±9.03$^{***\S\S\S\,ff}$ (-61.78%)
	400	112.52±12.87 (2.26%)	45.80±17.08$^{***\S\S\S\,fff}$ (-68.28%)

$^{**}p<0.01$, $^{***}p<0.001$ significantly different compared to 2% Tween 80.

$^{\S}p<0.05$, $^{\S\S\S}p<0.001$ significantly different compared to OI 25 mg/kg.

$^{ff}p<0.01$, $^{fff}p<0.001$ significantly different compared to OI 50 mg/kg.

$^{\#}p<0.05$ significantly different compared to IND 10 mg/kg.

Table 47 Time spent on paw licking after oral administration of 2% Tween 80 (10 ml/kg), indomethacin (IND; 10 mg/kg) and various doses of *Dimocarpus longan* root extract (DL; 25-400 mg/kg). Each value represents mean±S.E.M., N=8 for all groups. Inhibition is reported as percentage compared to vehicle control.

Treatments	**Dose (mg/kg)**	**Licking time (sec) (% inhibition)**	
		Early phase	**Late phase**
2% Tween 80		99.55±6.25	122.54±15.26
IND	10	89.21±4.14 (-10.39%)	53.44±3.40** (-56.39%)
DL	25	103.56±4.56 (4.02%)	120.08±9.32$^{\#\#}$ (-2.01%)
	50	106.04±7.11 (6.52%)	106.66±13.05$^{\#}$ (-12.96%)
	100	110.94±5.62 (11.44%)	100.72±9.42$^{\#}$ (-17.81%)
	200	98.77±5.04 (-0.78%)	56.90±12.12$^{**\S\S f}$ (-53.57%)
	400	95.92±5.47 (-3.64%)	14.52±6.54$^{***\S\S\S fff}$ (-88.14%)

$^{**}p<0.01$, $^{***}p<0.001$ significantly different compared to 2% Tween 80.

$^{\S\S}p<0.01$, $^{\S\S\S}p<0.001$ significantly different compared to DL 25 mg/kg.

$^{f}p<0.05$, $^{fff}p<0.001$ significantly different compared to DL 50 mg/kg.

$^{\S\S\S}p<0.001$ significantly different compared to DL 100 mg/kg.

$^{\#}p<0.05$, $^{\#\#}p<0.01$ significantly different compared to IND 10 mg/kg.

Table 48 Time spent on paw licking after oral administration of 2% Tween 80 (10 ml/kg), indomethacin (IND; 10 mg/kg) and various doses of *Dolichandrone serrulata* root extract (DS; 25-400 mg/kg). Each value represents mean±S.E.M. N=8 for all groups. Inhibition is reported as percentage compared to vehicle control.

Treatments	**Dose (mg/kg)**	**Licking time (sec) (% inhibition)**	
		Early phase	**Late phase**
2% Tween 80		104.38±5.50	135.07±10.01
IND	10	93.00±5.66 (-10.89%)	52.48±2.98*** (-61.15%)
DS	25	91.88±4.18 (-11.97%)	134.68±19.98$^{\#\#\#}$ (-0.29%)
	50	96.84±3.94 (-7.22%)	105.98±14.25$^{\#}$ (-21.53%)
	100	89.92±6.32 (-13.84%)	103.88±8.44$^{\#}$ (-23.10%)
	200	86.42±6.84 (-17.20%)	73.99±7.81**§§ (-45.22%)
	400	64.53±3.99$^{***ff\S\S\#\#}$ (-38.17%)	60.36±5.32***§§§ (-55.31%)

$^{**}p<0.01$, $^{***}p<0.001$ significantly different compared to 2% Tween 80.

$^{\S}p<0.05$ significantly different compared to DL 100 mg/kg.

$^{\S}p<0.05$, $^{\S\S}p<0.01$, $^{\S\S\S}p<0.001$ significantly different compared to DL 25 mg/kg.

$^{ff}p<0.01$ significantly different compared to DL 50 mg/kg.

$^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ significantly different compared to IND 10 mg/kg.

Table 49 Time spent on paw licking after oral administration of 2% Tween 80 (10 ml/kg), indomethacin (IND; 10 mg/kg) and various doses of *Walsura trichostemon* root extract (WT; 25-400 mg/kg). Each value represents mean±S.E.M. N=8 for all groups. Inhibition is reported as percentage compared to vehicle control.

Treatments	**Dose (mg/kg)**	**Licking time (sec) (% inhibition)**	
		Early phase	**Late phase**
2% Tween 80		105.03±6.11	141.59±13.19
IND	10	100.38±9.15 (-5.13%)	54.16±4.17*** (-61.74%)
WT	25	101.28±6.64 (-4.28%)	120.20±14.52$^{\#\#}$ (-15.10%)
	50	110.20±9.60 (4.14%)	107.85±10.11$^{\#\#}$ (-23.82%)
	100	104.08±4.19 (-1.64%)	104.98±11.27$^{\#}$ (-25.85%)
	200	84.05±4.46 (-20.57%)	78.59±7.71** (-44.49%)
	400	72.32±4.15$^{*\,ff\S}$ (-31.66%)	66.55±5.82***§§ (-53.00%)

$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ significantly different compared to 2% Tween 80.

$^{\S}p<0.05$ significantly different compared to DL 100 mg/kg.

$^{\S\S}p<0.01$ significantly different compared to DL 25 mg/kg.

$^{ff}p<0.01$ significantly different compared to DL 50 mg/kg.

$^{\#}p<0.05$, $^{\#\#}p<0.01$ significantly different compared to IND 10 mg/kg.

Acetic-acid induced writhes in mice

The acetic acid-induced writhing method to examine the analgesic efficacy of BMY and five herbal root extracts (AM, OI, DL, DS and WT). Each mouse was administered orally 2% Tween 80, indomethacin (IND; 10 mg/kg), various doses of BMY (125, 250, 500 mg/kg) or AM, OI, DL, DS and WT (25, 50, 100, 200, 400 mg/kg).

To demonstrate the validity of acetic acid-induced writhing method, IND 10 mg/kg was used as a positive control. As expected IND significantly (p<0.001) decreased writhing response by 85.48% compared with 2%Tween 80. All doses of BMY (125, 250 and 500 mg/kg) significantly (p<0.05, p<0.01, p<0.001, respectively) decreased the number of writhes induced by acetic acid by 32.78%, 43.57% and 59.34%, respectively when compared to vehicle control. The antinociceptive efficacy of BMY 500 mg/kg is comparable to IND (Figure 85).

AM at doses of 200, 400 mg/kg significantly (p<0.001) decreased the number of writhes induced by acetic acid by 69.36% and 87.54%, respectively when compared to vehicle control. AM at doses of 200, 400 mg/kg significantly (p<0.001) decreased the number of writhes when compared to AM (25, 50 and 100 mg/kg). IND significantly (p<0.001) decreased writhing response by 83.16% compared with 2% Tween 80. The antinociceptive efficacy of AM 200 and 400 mg/kg are comparable to IND (Figure 86).

OI at doses of 100, 200, 400 mg/kg significantly (p<0.05, p<0.05 and p<0.001, respectively) decreased the number of writhes induced by acetic acid by 33.84%, 37.16% and 58.31%, respectively when compared to vehicle control. IND significantly (p<0.001) decreased the number of writhes by 78.55% when compared to vehicle control. The antinociceptive efficacy of OI 400 mg/kg is comparable to IND (Figure 87).

DL at doses of 100, 200, 400 mg/kg significantly (p<0.05, p<0.01, p<0.001, respectively) decreased the number of writhes by 33.55%, 38.98% and 67.73%, respectively when compared to vehicle control. DL 400 mg/kg significantly (p<0.01,

$p<0.01$ and $p<0.05$, respectively) decreased the number of writhes when compared to DL at doses of 25, 50 and 100 mg/kg. IND significantly decreased the number of writhes by 75.40% when compared to vehicle control. The antinociceptive efficacy of DL 400 mg/kg is comparable to IND (Figure 88).

DS at doses of 200, 400 mg/kg significantly ($p<0.05$ and $p<0.01$, respectively) decreased the number of writhes by 37.18% and 45.49%, respectively when compared to vehicle control. DS 400 significantly ($p<0.05$) decreased the number of writhes when compared to DS 25 mg/kg. IND significantly ($p<0.001$) decreased the number of writhes by 85.92% when compared to vehicle control. IND has higher antinociceptive efficacy than DS 25-400 mg/kg (Figure 89).

WT at doses of 200, 400 mg/kg significantly ($p<0.001$) decreased the number of writhes by 49.98 % and 61.50%, respectively when compared to vehicle control. WT 200 mg/kg significantly decreased the number of writhes ($p<0.001$, $p<0.01$, respectively) when compared to WT 25 and 50 mg/kg. WT 400 mg/kg significantly ($p<0.001$ and $p<0.01$, respectively) decreased the number of writhes when compared to WT 50 and 100 mg/kg. IND significantly ($p<0.001$) decreased the number of writhes by 93.52% when compared to vehicle control. IND has higher antinociceptive efficacy than WT 25-400 mg/kg (Figure 90).

Acetic acid-induced writhing in mice

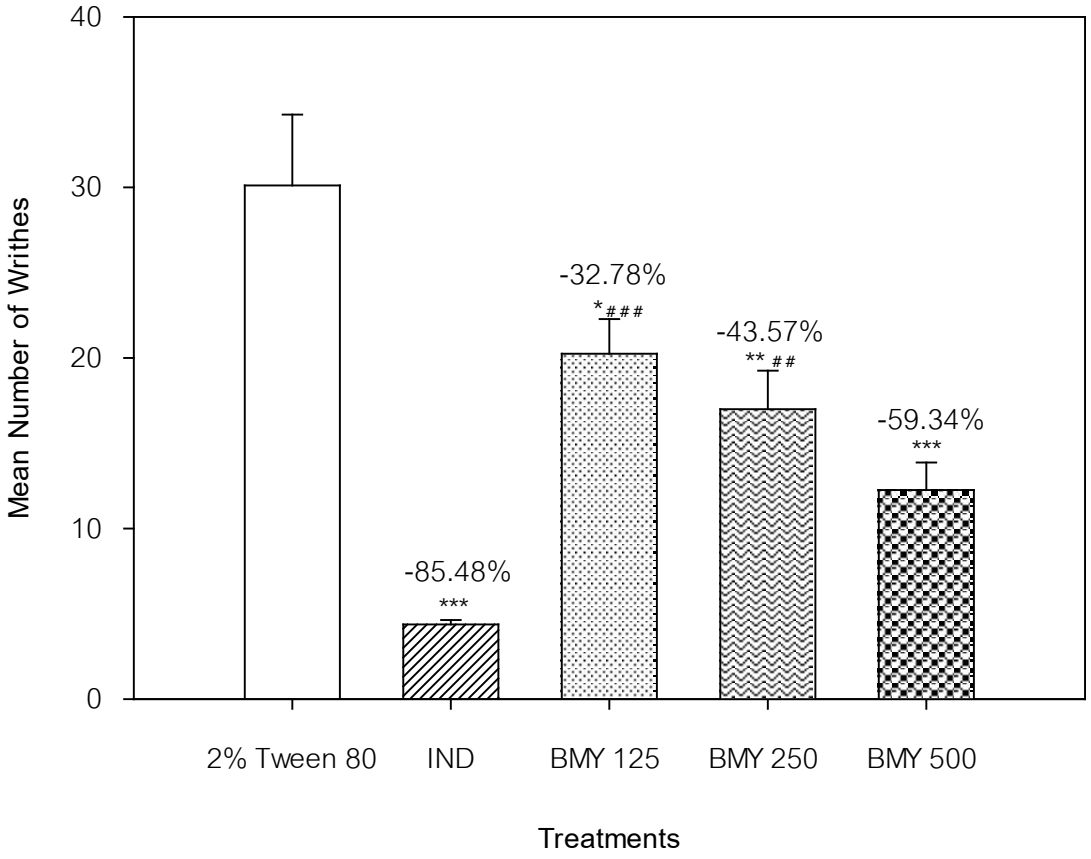


Figure 85 Mean number of writhes after oral administration of 2% Tween 80 (10 ml/kg), indomethacin (IND; 10 mg/kg) and various doses of the root extract of Ben-Cha-Moon-Yai remedy (BMY; 125-500 mg/kg). N=8 for all groups.

$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ significantly different compared to 2% Tween 80.
$^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ significantly different compared to IND 10 mg/kg.

Acetic acid-induced writhing in mice

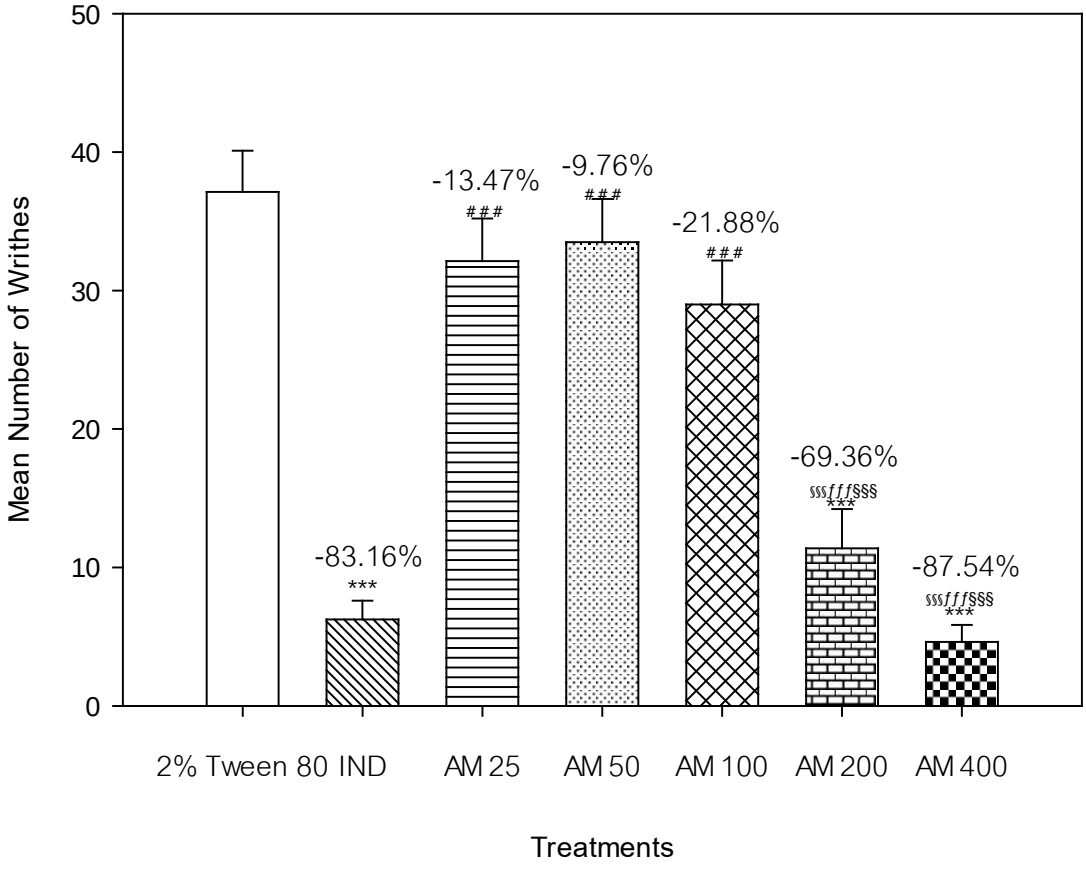


Figure 86 Mean number of writhes after oral administration of 2% Tween 80 (10 ml/kg), indomethacin (IND; 10 mg/kg) and various doses of *Aegle marmelos* root extract (AM; 25-400 mg/kg). N=8 for all groups.

$^{***}p<0.001$ significantly different compared to 2% Tween 80.

$^{§§§}p<0.001$ significantly different compared to AM 25 mg/kg.

$^{fff}p<0.001$ significantly different compared to AM 50 mg/kg.

$^{§§§}p<0.001$ significantly different compared to AM 100 mg/kg.

$^{###}p<0.001$ significantly different compared to IND 10 mg/kg.

Acetic acid-induced writhing in mice

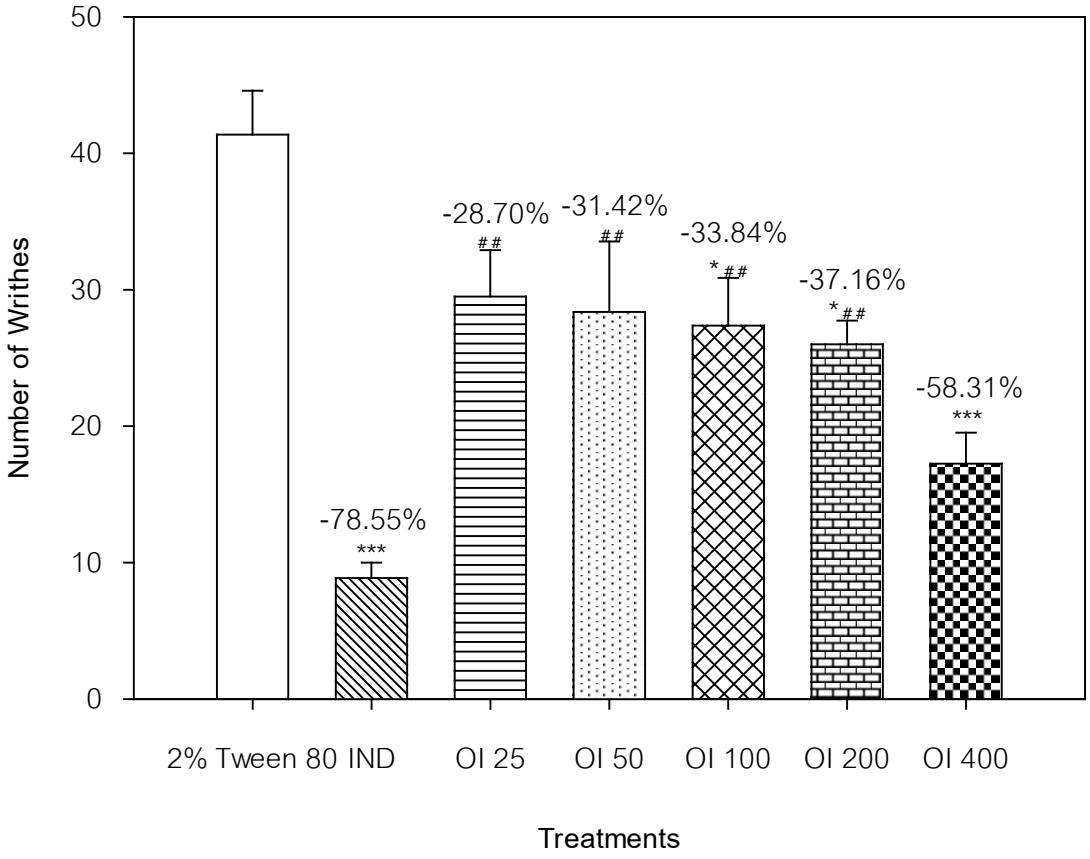


Figure 87 Mean number of writhes after oral administration of 2% Tween 80 (10 ml/kg), indomethacin (IND; 10 mg/kg) and various doses of *Oroxylum indicum* root extract (OI; 25-400 mg/kg). N=8 for all groups.

$^{*}p<0.05$, $^{***}p<0.001$ significantly different compared to 2% Tween 80.

$^{\#\#}p<0.01$ significantly different compared to IND 10 mg/kg.

Acetic acid-induced writhing in mice

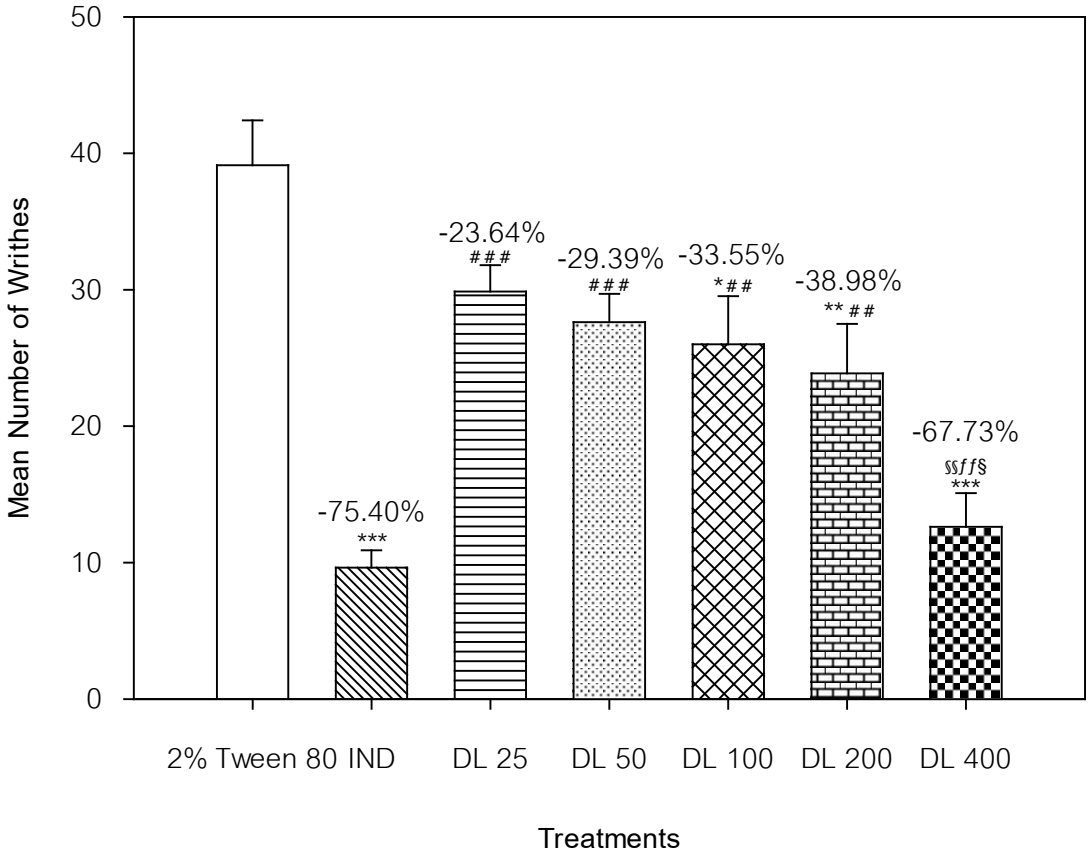


Figure 88 Mean number of writhes after oral administration of 2% Tween 80 (10 ml/kg), indomethacin (IND; 10 mg/kg) and various doses of *Dimocarpus longan* root extract (DL; 25-400 mg/kg). N=8 for all groups.

$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ significantly different compared to 2% Tween 80.

$^{\S}p<0.05$ significantly different compared to DL 100 mg/kg.

$^{\S\S}p<0.01$ significantly different compared to DL 25 mg/kg.

$^{ff}p<0.01$ significantly different compared to DL 50 mg/kg.

$^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ significantly different compared to IND 10 mg/kg.

Acetic acid-induced writhing in mice

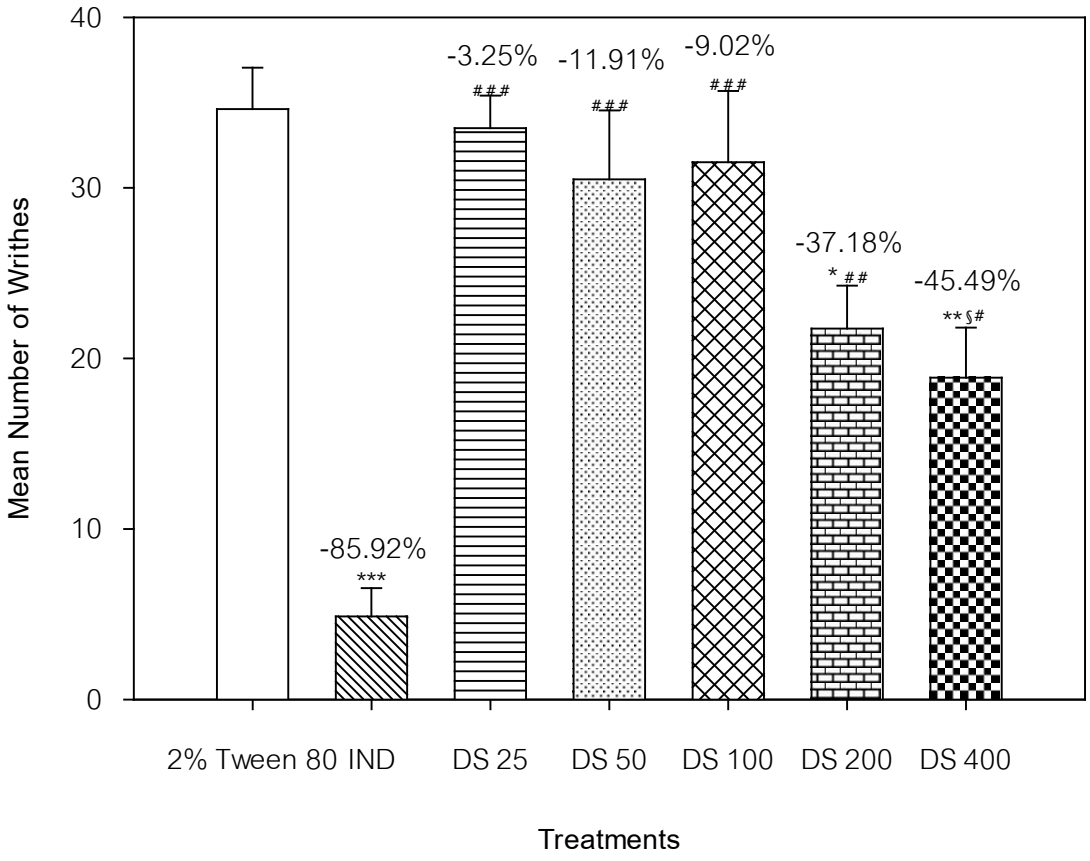


Figure 89 Mean number of writhes after oral administration of 2% Tween 80 (10 ml/kg), indomethacin (IND; 10 mg/kg) and various doses of *Dolichandrone serrulata* root extract (DS; 25-400 mg/kg). N=8 for all groups.

$^{*}p<0.05$, $^{**}p<0.01$ significantly different compared to 2% Tween 80.

$^{\S}p<0.05$ significantly different compared to DS 25 mg/kg.

$^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ significantly different compared to IND 10 mg/kg.

Acetic acid-induced writhing in mice

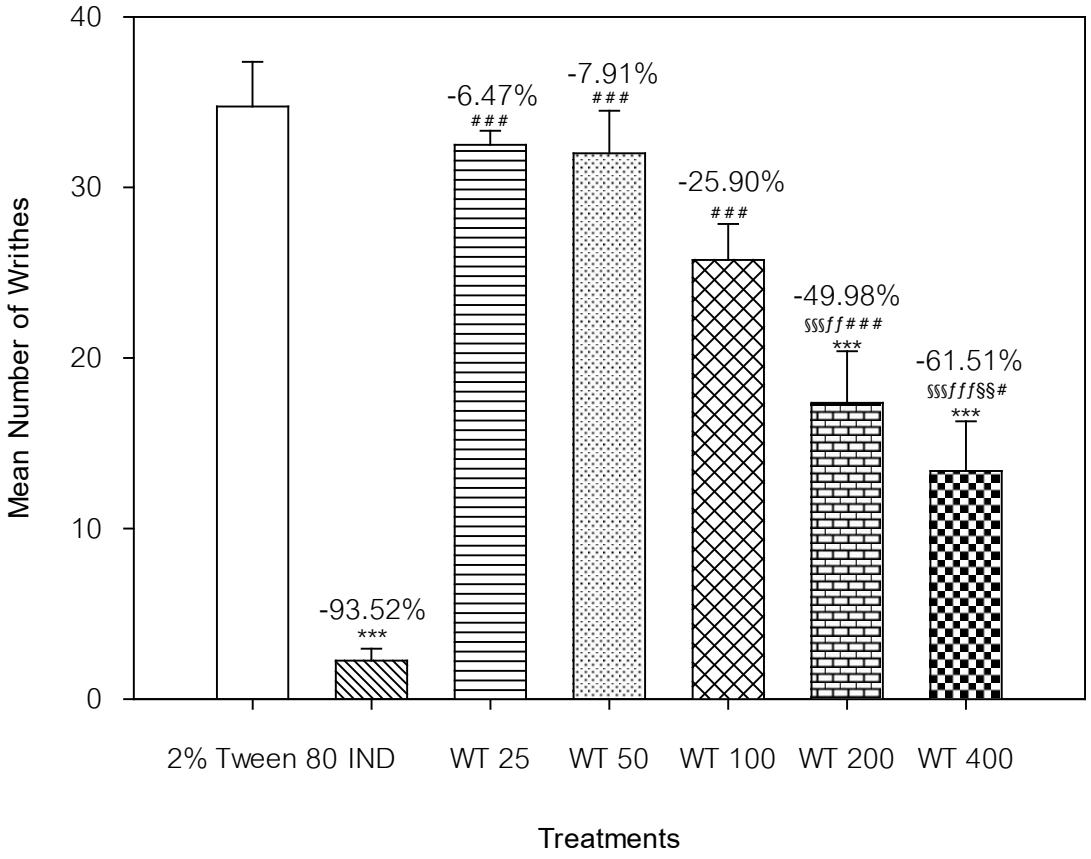


Figure 90 Mean number of writhes after oral administration of 2% Tween 80 (10 ml/kg), indomethacin (IND; 10 mg/kg) and various doses of *Walsura trichostemon* root extract (WT; 25-400 mg/kg). N=8 for all groups.

$^{***}p<0.001$ significantly different compared to 2% Tween 80.

$^{§§}p<0.01$ significantly different compared to WT 100 mg/kg.

$^{ff}p<0.01$, $^{fff}p<0.001$ significantly different compared to WT 50 mg/kg.

$^{§§§}p<0.001$ significantly different compared to WT 25 mg/kg.

$^{\#}p<0.05$, $^{\#\#\#}p<0.001$ significantly different compared to IND 10 mg/kg.

Rota-rod performance test in mice

In order to determine the effect of BMY and five herbal root extracts (AM, OI, DL, DS and WT) on motor response, mice were administered 2% Tween 80, BMY (500 mg/kg), AM, OI, DL, DS or WT (400 mg/kg) orally and tested on the rota-rod apparatus for 5 subsequent trials at 30, 60, 90, 120 and 240 min after drug administration. The results showed that BMY, AM, OI, DL, DS and WT at doses tested did not affect the motor response of the animals (Figure 91).

Rota-rod performance test in mice

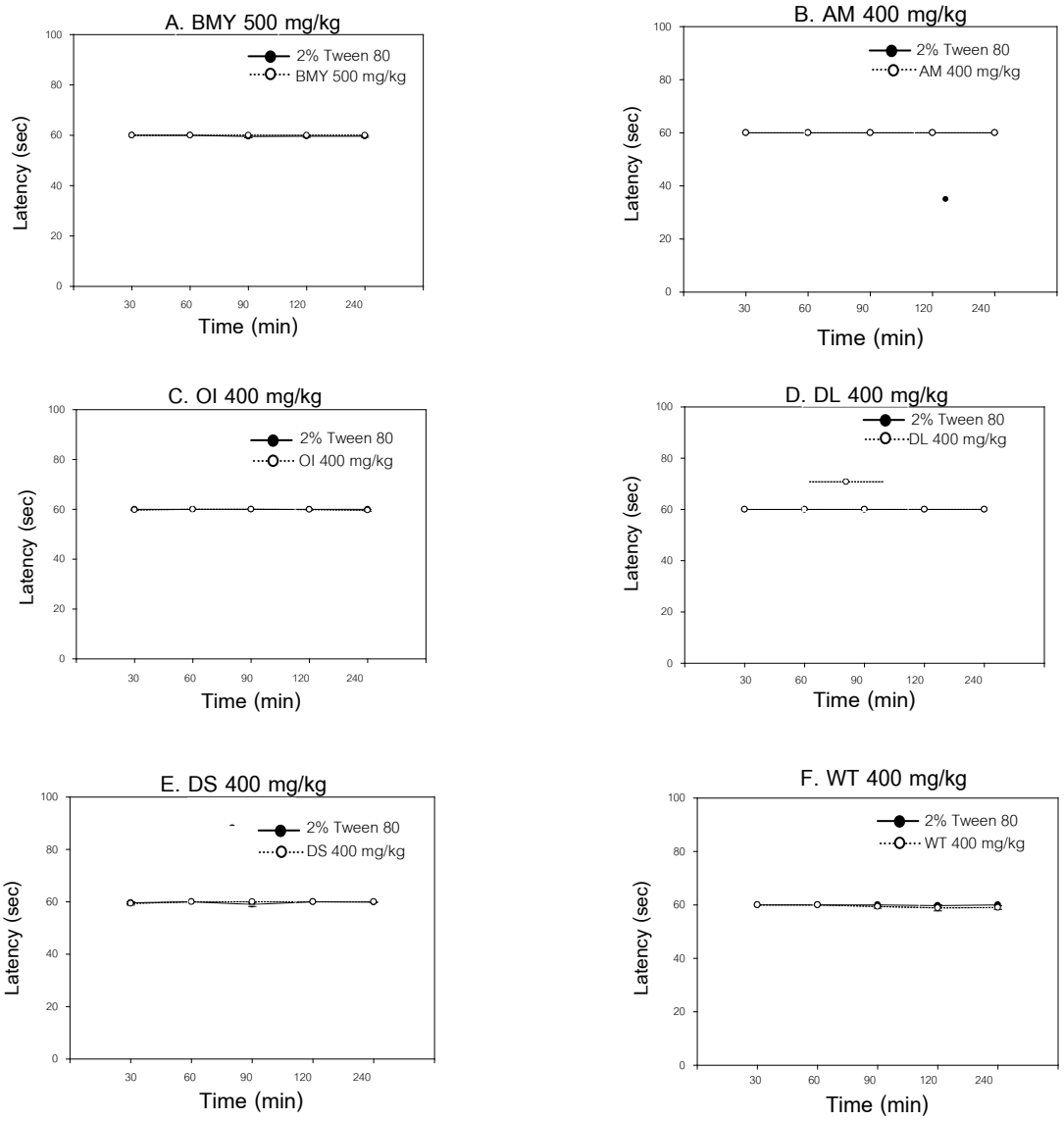


Figure 91 Rota-rod latency of each extract after oral administration compared to 2% Tween 80 (10 ml/kg). N=8 for all groups.

A. Ben-Cha-Moon-Yai remedy extract (BMY; 500 mg/kg). B. *Aegle marmelos* root extract (AM; 400 mg/kg). C.*Oroxylum indicum* root extract (OI; 400 mg/kg). D. *Dimocarpus longan* root extract (DL; 400 mg/kg). E. *Dolichandrone serrulata* root extract (DS; 400 mg/kg). F. *Walsura trichostemon* root extract (WT; 400 mg/kg).

CHAPTER V

DISCUSSION

Pharmacognostic specification

The quality control methods play an important role in traditional medicine which conserve as a tool for identification, authentication and quality control of herbal drug [14]. WHO has published the “Quality control methods for medicinal plant materials” which describes a recommended test procedure to evaluate the identity, purity and quality of the plant materials. These standardization parameters are essential to publish in the pharmacopoeia. The majority of the information can be obtained from its macroscopy, microscopy, physio-chemical parameters and chemical fingerprint of medicinal plant materials.

Macroscopic and microscopic methods are the simplest and cheapest methods to establish the correct identity of the plant materials [18]. The macroscopic of root indicated that its shape, size, colour, surface characteristics, texture, fracture and appearance of the cut surface. Transverse section were prepared with free-hand section of each root and stained with safranine to confirm its lignifications. Microscopy of the powder was also carried out and the specific diagnostic characteristics were recorded. This examination gives a clear idea about the specific histological characteristics of crude drugs, besides the macro-morphological and cyto-morphological characters. While these diagnostic features enable the analyst to know the nature and characteristics of the crude drugs, further evaluation of numerical parameters indicate their acceptability by criteria other than the morphological characteristics [5].

The physico-chemical parameters are mainly used in judging the purity and quality of the drug. The procedures normally adopted to get the qualitative information about the purity and standard of a crude drug including the determination of various parameters [190]. A high ash value is indicative of contamination, substitution, adulteration or carelessness in preparing the crude drug for marketing. The residue remaining after incineration of plant material indicates presence of

various impurities includes both "physiologic ash" which is derived from the plant tissue itself and "non-physiologic ash" which is the residue of the extraneous matter adhering to the plant surface [191]. Acid insoluble ash is frequently necessary to evaluate the crude drugs, which indicates the residue obtained after treating the total ash with about 2 N HCL, then weighing the residue. This ash value indicates contamination with siliceous material or acid insoluble matter e.g. earth and sand. These ash values are important quantitative standards which constitutes the inorganic matter after incineration of that particular herbal ingredient, specifications have been set up to limit them.

Moisture is also an inevitable component of crude drugs which should be eliminated as far as practicable. Excess moisture can result in the breakdown of important constituents by enzymatic activity and may encourage the growth of yeast and fungi during storage. Direct measurement of water content and total measurement of water as well as volatile matters in term of loss on drying needed to be tested. Extractive values give an idea about the chemical constituents of crude drug soluble in a particular solvent which yields a solution containing different phyto-constituents [192]. This study proposed the upper limits for unwanted properties of Ben-Cha-Moon-Yai remedy crude drugs, such as loss on drying, total ash, acid-insoluble ash and water contents, together with the lower limits for extractable matters such as the ethanol and water extractive values as shown in Table 7, 9, 11, 13, and 15 [193].

The fingerprinting analyses is nowadays getting momentum for the quality control of multi-component herbal medicines and has been widely accepted as a useful tool to determine authenticity and reliability of chemical constituents of herbal drug and formulations [26]. A combination of high performance liquid chromatography and online UV spectrum detection *via* diode array configuration also adds a value to conventional botanical methods used in the quality assurance of crude drugs and compound preparations [120]. The selection of the HPLC conditions was guided by the requirement for obtaining chromatograms with better resolution of adjacent peaks within short time, especially when large amount of samples were analyzed [194]. In this study, TLC, 3D-HPLC and LC-MS profiles of five root species

and Ben-Cha-Moon-Yai remedy were established as their characteristic fingerprint and employed to assess their consistency and difference.

However, different herbal materials are traditionally used as the same herbal medicine. The quality is different not only between materials of different species used as the same herbal medicine, but also between materials of the same species growing from different areas. The chemical composition of the same herbal material collected at different times or with different processing methods is different, so the production should be strictly specified in order to control the product quality and minimize variations between different product batches [190]. The results obtained from macroscopic and microscopic inspections, physico-chemical parameters, and development of TLC, 3D-HPLC and LC-MS fingerprint can be used to standardize all five root species in Ben-Cha-Moon-Yai remedy.

Genotoxic and anti-mutagenic activity

The Ames Salmonella assay is a short-term *in vitro* testing which has gained popularity from the large number of chemical compounds to investigate their genotoxicity and modulation effect on the mutagenic response [128] toward *Salmonella typhimurium* tester strains due to it was a quick and relatively inexpensive assay [129].

The *Salmonella typhimurium* tester strains TA98 and TA100 with histidine-requiring (*his*$^-$) auxotrophs were used for detecting and classifying mutagens. Each strain was deficient in excision repair of DNA damage (*uvr*B), ampicillin-resistant R-factors and presence of *pKM101* [129]. They can be reverted back to the wild type by particular mutagens [131]. In this study, the mutagenic and antimutagenic acitivity of root extracts and BMY remedy were study in the absence of enzyme activating system using the pre-incubation method of Maron and Ames in 1983 [130] to observe the response of the extracts in an acidic condition. Most of the extracts exhibited non-mutagenicity without nitrite treatment in the Ames test toward both strains of *Salmonella tyhimurium* under acidic condition without metabolic activation. However, the water extract of *A. marmelos* revealed the mutagenicity on both strains in the present study, whilst the study of Kruawan and Kangsadalampai in 2006 [195]

demonstrated that the fruit extract from this plant were not mutagenicity toward *Salmonella typhimurium* TA100 in the Ames test.

Most of the extract, except the ethanol extracts of *D. serrulata*, *D. longan* and *W. trichostemon* and the water extracts of *D. serrulata*, were mutagenicity on *Salmonella typhimurium* strains TA98 and TA100 after being treated with sodium nitrite, similar observation was reported by Higashimoto et al., in 1993 [196]. It was found that three species and Thai medicinal plant extracts were not mutagenic for both strains of *Salmonella typhimurium*, but when the extracts were treated with sodium nitrite, the mutagenicity were observed toward strain TA100. The result was in accordance with the previous study that the nitrosated fraction from the bark of *O. indicum* had been found the mutagenicity on *Salmonella typhimurium* TA98 and TA100 [98]. Besides the roots extract and BMY remedy, many medicinal plants, foods and chemical compounds, show direct-acting mutagenicity after nitrite treatment without metabolic activation. Wakabayashi et al., in 1985 [197] and Kato *et al*., in 1991 [198] also demonstrated that the reaction mixtures showed the mutagenicity to *Salmonella typhimurium* TA98 and TA100 strains after 1-aminopyrene treated with amount of nitrite at pH3 at 37 °C for 4 hr without metabolic activation.

In the recent years, many researches has been employed the *in vitro* assay to determine the genotoxic carcinogen by treatment with the nitrosation reaction mixture or directly with *N*-nitroso compounds; similar to the *in vivo* assays of its biological activity [199]. Previous studied found that people who exposed to the high levels of nitrate have the raise incidences of gastric and liver cancer. Therefore, it has been denoted that the *N*-nitroso compounds is an etiology of human cancer [200].

Nitrite occurs in nitrite-preserved meat of fish, spoiled foods, even nitrate mostly found in foods and vegetables which can be reduced to nitrite by the microbial enzymes. It is the most important precursor to generate the nitrosating agents [201] [31]. It has been denoted that *N*-nitroso compounds are formed by the interaction of nitrogenous compounds with nitrosating agents, the most important of which is acid nitrite [202].

Therefore, when the extracts containing the nitrogen trixodie (N_2O_3) or dihitrogen tetroxide (N_2O_4) with primary, secondary, or tertiary amines, or with secondary amide [203] reacts with the sodium nitrite (nitrosating agent precursor), it can be generate the carcinogenic *N*-nitroso compounds under the acidic condition with gastric pH [204]. This finding may be common since most natural compounds generally reacted with nitrite and expresses their products that can induce mutations.

In conclusion, this study confirmed that nitrite is a direct-mutagen in certain *Salmonella typhimurium* histidine dependent strains sensitive to frame-shift (TA98) and base-pair substitutions (TA100) mutations. These results can be suggested that some mutagens and carcinogens may be produced in the human stomach.

The screening for antimutagenicity of plant extract is important in the discovery of new effective anticarcinogenic therapeutic drug [4]. The rationale was due to plant extracts exhibiting antimutagenicity is indication of a possible anticarcinogen [205]. A great number of naturally and synthetic compounds has been known to inhibit the nitrosation reaction. 1-Aminopyrene is a derivative of 1-nitropyrene found in human gastrointestinal tract. Anaerobic bacteria metabolize 1-nitropyrene to 1-aminopyrene. Previous studied showed that 1-aminopyrene is an important contributor to the direct-acting mutagenicity, as measured by the Ames assay of the diesel particulate extracts [206].

The antimutagenic effect of the roots extract and BMY remedy against the mutagenic reaction product produced from the reaction of 1-aminopyrene treated with nitrite under acidic condition pH 3-3.5 were exhibited in the Ames test. It revealed that most of the extracts exhibited antimutagenic potential ranged from negligible (0-20%) to strong (> 60%) effects toward both strains of *Salmonella typhimurium*. These extracts demonstrated a dose-dependent by inhibitory effect towards *Salmonella typhimurium* TA98 and TA100. This data agree with the previous studied that the methanol extract from the fruit of *O. indicum* also exhibited the strong antimutagenic effect against *Trp*-P-1 in an Ames test [86]. The same results were demonstrated in the determination of mutagenic and antimutagenic effects of Ya-rid-si-duang-mahakal which is the one of Thai traditional medicine to treat hemorrhoid and some flower

grown in Thailand. It has been denote that there were mutagenic after nitrite treatment and provided the antimutagenic effects against the same condition of this study [207-208]. The antimutagenic potential was also demonstrated in the ethanol extract of *Mucuna collettii* in the Ames test against AF-2 and B(a)P mutagens toward strains TA98 and TA100 of *Salmonella typhimurium* and *rec* assays [209]. Moreover, it has been reported that the fifteen kinds of Thai vegetables exhibited the antimutagenic effects against direct and indirect activating mutagens by using the Ames test with *Salmonella typhimurium* strain TA100 [210].

The present study can be implied that most of the extracts contained certain precursors that could react with nitrite under acidic condition to produce direct mutagenic product causing frame-shift (TA98) and base-pair substitution (TA100) mutation. It provided the evidence to support the safe consumption of Ben-Cha-Moon-Yai remedy and its ingredients at low dose. However, during the use of these remedy or its ingredients, consumer should avoid nitrite containing food items. In addition, the result indicated that the direct acting mutagens formed from interaction between nitrite treated 1-aminopyrene in acid solution could be suppressed by some component, in the extracts.

The preliminary toxicity investigation is brine shrimp lethality testing described by Meyer *et al*., 1982 and used as a “Benchtop bioassay” for natural medicine discovery [126]. The results indicated the present of potent cytotoxic component of Ben-Cha-Moon-Yai remedy extract and the water extract from *A. marmelos* based on the studied of Meyer et al., 1982 which classified the cytotoxicity of crude extracts into toxic (LC_{50} value < 1000 µg/ml) and non-toxic (LC_{50} value > 1000 µg/ml). On the contrary, the fruit [211] and leaves extracts [212] from *A. marmelos* demonstrated non acute-toxicity belong to 6 g/kg in mice and non short-term toxicity for 14 consecutive days in rats, respectively.

The comet assay has been used as a standard test to assess genotoxicity of novel pharmaceutical or other chemical *in vitro* and is also becoming an important tool for evaluating the genotoxic potential of compound *in vivo* and used successfully to monitor DNA damage in human populations. The *in vitro* comet assay has several

advantages over cytogenetic test such as the micronucleus test that is commonly used for genotoxicity screening [213-214].

Advantages of the comet assay for assessing DNA damage in mammalian cell includes (1) damage to the DNA in individual cell is measured, (2) only small number of cells are needed to carry out the assay (<10,100), (3) the assay can be performed on virtually any eukaryotic cell type, including cells obtained from exposed human populations for environmental monitoring, (4) and it is a very sensitive method for detecting DNA damage [135].

Positive results from *in vitro* comet assay indicate that the test extract induces DNA damage in cultured mammalian cells. These include a dose-response relationship and a pairwise comparison of each dose group against the control group to identify significant effects at individual doses. Negative results indicate that under the test conditions, the test extracts does not induce DNA damage in cultured mammalian cells [137].

The result of this study indicate that both ethanolic and water extracts from the root of *A. marmelos* and *D. longan* were considered as the positive results. A concentration related increase or decrease in DNA migration was observed after these extract treatment. Compared with the untreated control, these extract induced DNA damage even at a low concentration (25 µg/ml), as indicated by the presence of the DNA tail whereas the increase of the DNA tail of the remaining extract was not significantly different from the negative control.

However, plant extracts exhibiting a mutagenic or antimutagenic and also cytotoxic and genotoxic effects need to be extensively investigated to determine their possible genotoxicity and cytotoxicity to humans as their safe use in traditional medicine.

Antimicrobial activity

The use of antimicrobial and other therapeutic drugs derived from medicinal plant materials become increasingly interested from conventional medicine [215]. The ideal of antimicrobial activity testing should be simple, rapid, reproducible, inexpensive and maximized sample throughput in order to screen a various number of plant extracts [216].

The agar diffusion method is suitable for preliminary testing which allow a rapid selection of the active extracts and allows to simultaneously testing a large number of antimicrobials in a relatively easy and inexpensive manner. The results of diffusion method are considered as qualitative because it can only reveal the susceptibility of antimicrobials against the bacteria tested with diameter of inhibition zone. The major disadvantage of the method is unable to generate the MIC value [217]. The agar-well technique was performed in this study its advantages to improve the complete diffusion of plant extracts into the Mueller-Hinton agar [218].

Broth microdilution is another quantitative method routinely used as a fast screening method for MIC determination [216]. MICs are considered the ‘gold standard’ for determining the susceptibility of organisms to antimicrobials and are therefore used to judge the performance of all other methods of susceptibility testing [219]. The advantages of the method include considerable saving in media usage, requirement of a small quantity of sample and test the susceptibility of multiple antimicrobials at the same time. Moreover, it decreased the intensive labor and time cost compared to with agar-based method. The MIC value was observed as the lowest concentration where no viability was observed in the wells of 96-microwell plate after incubation [217].

Among five root species and Ben-Cha-Moon-Yai remedy extract, the ethanolic extract from *O. indicum* root was the most active extract that can inhibited a maximum of 9 microorganisms in agar-well diffusion assay including; *S. aureus, S. epidermidis, B. cereus, M. luteus, P. aeruginosa, S. typhi, Shigella sp., C. albican, S. cerevisiae*. The lowest MIC value of *O. inducum* was found in *B. cereus, B. subtilis, M. luteus* and *S. cerevisiae* which ranged from 500-2000 µg/ml.

From the result of agar-well diffusion assay, it demonstrated that the ethanolic extract from *O. indicum* root inhibited the growth of a maximum of 9 microorganisms (69.23%) followed by the ethanolic and water extracts from *D. longan* root that prevented the growth on 7 microorganisms (53.85%) of the 13 tested microorganisms. Other plant extracts showed selective activity against 11 tested microorganisms and there were no crude extracts active against *E. aerogenes* and *E. coli*. According to the antimicrobial activity evaluation suggested by Alves et al., 2000, [147] the activity was classified into 4 classes by the zone of inhibition (mm) as inhibition zones < 9 mm classified as inactive, inhibition zones between 9-12 mm classified as less active, inhibition zones between 13-18 mm classified as active and inhibition zones > 18 m classified as very active. The ethanolic extract of *O. indium* root classified as an active extract against *S. aureus*, *M. luteus* and *C. albican* and as a less active extract against *B. cereus*, *B. subtilis*, *P. aeruginosa*, *S. typhi*, *Shigell sp*., and *S. cerevisiae*. The ethanolic extract from *D. longan* root was classified as an active extract against *B. subtilis* and *M. luteus* while the water extract of this plant was classified as an active extract against *S. aureus* and as a less active extract against *B. subtilis* and *M. luteus*. Ben-Cha-Moon-Yai remedy was classified as an active extract against *M. luteus* and as a less active extract against *S. aureus*, *B. cereus*, *S. typhimurium* and *Shigella sp*.

The inhibition of a maximum of nine bacterial and fungal strains by ethanolic extract of *O. indicum* may be attributed to the presence of soluble phenolic and flavonoid compounds in the extracts. The result are in accordance with a recent study of Moirangthem et al., in 2013 [101] which it was shown that the methanol extract of *O. indicum* stem bark contained the highest amount of total phenolic and flavonoid content and inhibited the growth of both bacterial and fungal test organisms. In addition, the previous study has been reported that the methanolic extract from root bark of this plant also exhibited the antimicrobial activity against all tested microorganisms [110]. Therefore, the result of this study could be suggested that the antimicrobial activity of the extract may be related to some phenolic and flavonoid component in the extracts. The antibacterial effects of the extracts could be explained by disturbance of the permeability barrier of the bacterial membrane structure [220, 221]

As commonly known that *D. longan* is a rich sources of phenolic compound, the result in this study also demonstrated that both ethanol and water extract of this plant demonstrated the good antimicrobial activity against 7 pathogenic tester strains. Phenolic toxicity to microorganisms is due to the site and number of hydroxyl groups present in the phenolic compound. Plants have limitless ability to synthesize aromatic secondary metabolites, most of which are polyphenol or their oxygen-substituted derivative. Important subclasses in this group of compounds include phenols, phenolic acids, quinines, flavones, flavonoids, flavonols, tannins and courmarins. These group compounds show antimicrobial effect and serves as plant defense mechanisms against pathogenic microorganisms [215].

Most of the active extract showed antimicrobial against gram positive bacteria. The main reason for the differences in bacterial susceptibility could be attributed to the difference in morphological constitutions between these microorganisms. The outer membrane surrounding the cell wall in gram-negative bacteria is restricts diffusion of compounds through its lipopolysaccharide covering, on the other hand, Gram-positive bacteria only have an outer peptioglycan layer which is not as an effective permeability barrier as the former [216, 222].

Antioxidant activity

Assessments of antioxidant properties of natural compounds from medicinal plant materials are very important because of their uses in medicine, food and cosmetics. The natural antioxidants are known to minimize the adverse effects of free radicals in living system. Many of these naturally occurring antioxidants are now isolated, fully characterized, and available for various applications [223]. Antioxidant activity can be evaluated both *in vitro* and *in vivo*. There are potential models for evaluation of the antioxidant activity. In vitro methods consist of chemical methods in which free radicals can be generated using chemical reaction [10]. The antioxidant activities of the five root species and Ben-Cha-Moon-Yai remedy extracts were assessed on the basis of radical scavenging activity against the DPPH radical and nitric oxide radical and lipid peroxidation inhibition using β-carotene bleaching assay.

DPPH assay

DPPH$^{\cdot}$ is a stable free radical which has been used for estimation of free radical scavenging potential of an antioxidant molecule [224]. The assay is considered as one of the standard and easy colorimetric method for the evaluation of antioxidant activities of plant extracts [223]. DPPH$^{\cdot}$ is a stable nitrogen-centered free radical which appear purple colour absorbing of 515-520 nm in methanolic solution [225]. The vital role of antioxidants is their interaction with oxidative free radicals. The assumption of DPPH method is that the antioxidants react with the stable free radical [226]. This assay is based on the principle that DPPH on accepting a hydrogen (H) atom from the scavenger molecule resulting into reduction of α, α-diphenyl-β-picrylhydrazyl (deep violet colour) and convert to α, α-diphenyl-β-picrylhydrazine (yellow colour), the purple colour changes to yellow with concomitant decrease in absorbance at 517 nm. The discolouration degree indicates the scagening potentials of the plant extracts. The colour change is monitored by spectrophotometrically and utilized for the determination of parameters for antioxidant properties.

The addition of the extracts to the DPPH solution caused a rapid decrease n the optical dentity at 517 nm. In this study, the ethanolic extract of *D. longan* and the water extract of *W. trichostemon* were able to decolorized DPPH free radical. The discoloration degree indicates the scavenging potentials of the extracts and IC_{50} values were calculated. The results indicate that the DPPH radical scavenging activity of the extract the ethanolic extract from *D. longan* was higher compared to quercetin which the extract exhibited IC_{50} of 9.3 µg/ml compared to quercetin with IC_{50} of 9.8 µg/ml. The DPPH radical scavenging activity of the water extract from *W. trichostemon* was also higher compared to BHT which the extract exhibited IC_{50} of 16.1 µg/ml while BHT exhibited IC_{50} of 22.3 µg/ml.

Lipid peroxidation testing using β-carotene bleaching assay

The antioxidant activity is measured by the ability of a compound to inhibit the coupled oxidation of linoleic acid and β-carotene in an emulsified aqueous system. As β-carotene loses their double bonds by oxidation, the compound loses its chromophore and characteristic orange colour, so colorimetric can be used to investigate the decline in colour in the initial absorbance at 450 nm and is slowed down in the presence of an antioxidant [227-228].

The presence of different extracts can hinder the extent of β-carotene bleaching by neutralizing the linoleate-free radical and other free radical formed in the system. As the results shown in figure 1 and 2, most of the extracts showed higher ability to inhibit the bleaching of β-carotene by scavenging linoleate-derived free radicals than negative control, except the water extract of *D. serrulata* and *A. marmelos*. The extracts with showed the lowest of β-carotene discolorations exhibited the highest antioxidant activity. The orders of antioxidant efficiency from the ethanolic extracts of five root species and Ben-Cha-Moon-Yai remedy were decreased in the following order: *D. longan* > *A. marmelos* > Ben-Cha-Moon-Yai remedy > *W. trichostemon* > *D. serrulata* > *O. indicum*. While, the orders of antioxidant efficiency from the water extract of five root species were decreased in the following order: *D. longan* > *W. trichostemon* > *O. indicum* > *D. serrulata* > *A. marmelos*. Both synthetic antioxidant such as BHT and quercetin showed the highest ability to prevent the bleaching of β-carotene than the extracts.

Nitric oxide scavenging assay (Griess reagent assay)

In the present study, the crude extract from five root species and Ben-Cha-Moon-Yai remedy were evaluated for their scavenging activity on nitric oxide production from Sodium nitroprusside in aqueous solution at physiological pH. The extract inhibits nitrite formation by directly competing with oxygen in the reaction with nitric oxide [229]. The present result showed that the ethanolic extract from *D. longan* root was the highest potential scavenger of NO with IC_{50} of 23 µg/ml, followed by the ethanolic extract of *W. trichostemon* with exhibited IC_{50} of 25 µg/ml. The scavenging effects of all extracts were expresses as dose-dependent manner.

However, the activity of quercetin was very more pronounced than all of the extracts which showed the lowest IC_{50} of 9.17 µg/ml.

Total phenolic contents

The result indicated that the water extract of *D. longan* and *W. trichostemon* showed the highest total phenolic content obtained from Folin-ciocalteu reagent. These *in vitro* assays indicate that the extracts from *D. longan* and *W. trichostemon* are a significant source of natural antioxidant. The result could be indicated that phenolic compounds were the main antioxidant components and its total content was directly proportional to the antioxidant activity [230]. Therefore, further investigation is needed to isolate and identify the antioxidant compounds present in the active extracts and the *in vivo* antioxidant of this extract need to be assessed prior to clinical used

Anti-pyretic activity

Pyrexia is a secondary effect of infection, tissue damage, inflammation, malignancy, and other inflammatory disease conditions to the body [231]. The processes that underlie fever initiation and maintenance are multiple, different and complex. In the classic model, the fever condition entails enhanced formation of cytokines such as IL-1α, IFN and TNF- α, and the cytokines increase the synthesis of PGE2. Aspirin suppresses this response by inhibiting the synthesis of PGE_2 [232]. In this alternative model, it is generally believed that the release of LPS from Gram-negative bacteria cell wall during most infections can cause a fever by stimulating peripheral macrophages to synthesize and release pyrogenic cytokines such as interleukin 1 (IL-1), interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) and subsequent induction of prostaglandin (PG) synthesis in the central nervous system (CNS) and fever. The released PGE_2 then stimulates hepatic vagal afferents that convey the pyrogenic message to the POAH, provoking the release of NE, thereby causing successive hyperthermic actions [157, 233, 234].

In the present study, the rat hyperthermia induced by LPS was employed to investigate the antipyretic activity of the five root species and Ben-Cha-Moon-Yai

remedy extracts. This study employed ASA as a reference drug. Orally administered ASA, the positive control, significantly attenuated fever in LPS-treated rats at all times tested. This could be due to inhibition of cyclooxygenase (COX) and therefore interference with the cascade of the synthesis of prostaglandins (PGs) which induces fever. BMY (125-500 mg/kg), AM, OI, DS, DL and WT (25-400 mg/kg) suspending in 2% Tween 80 solution were administered orally. The oral administration was chosen in order to imitate the normal consumption of Ben-Cha- Moon-Yai remedy, the Thai traditional antipyretic herbal medicine.

The whole extract of Ben-Cha-Moon-Yai remedy at all doses tested demonstrated anti-pyretic efficacy. The highest dose of BMY had the fastest onset of antipyretic action and seemed to have the highest antipyretic efficacy. BMY displayed antipyretic activity in the LPS-induced fever model of rats over 1-7 hr after LPS injection, supporting the view that BMY may be involved in the inhibition of some processes or some substances involving fever. Only AM at the dose of 400 mg/kg showed antipyretic activity starting from 2 hr and the effect was sustained for up to 7 hr after LPS injection. This result is consistent with the previous study of Arul *et al*., in 2005 [48] that showed antipyretic activity of all serial extracts of *Aegle marmelos* leaves (50 mg/kg) in mice made hyperthermic by dried yeast injection. OI at the doses of 25, 100 and 400 mg/kg showed antipyretic activity starting from 2 hr and the effect was sustained for up to 7 hr after LPS injection. OI 400 mg/kg seemed to have the highest antipyretic efficacy. DS did not showed antipyretic activity in the LPS-induced fever model in treatment groups. DL showed antipyretic effect only at 2-3 hr after LPS injection suggesting that DL could display a very short duration of antipyretic action when compared with other herbal roots. All doses of WT except the highest dose showed antipyretic activity starting from 2 or 4 hr after LPS injection and the effect was sustained for up to 7 hr. WT 100 mg/kg seemed to have the highest antipyretic efficacy. The highest dose of WT had no antipyretic effect which may be due to high toxicity or minimal absorption of the extract.

When comparing between the most effective doses of BMY and individual components. BMY (500 mg/kg), AM (400 mg/kg), OI (400 mg/kg) and WT (100 mg/kg) had comparable antipyretic efficacy. According to the lowest dose used, WT

seemed to be the most potent antipyretic agent. This can be concluded that the antipyretic efficacy of BMY is due to the combinations of AM, OI, and WT.

In conclusion, BMY 500 mg/kg seemed to be the most potent antipyretic agent and more potent than individual components due to additive and/or synergistic effects of some herbal roots in the remedy. This might be a reason why Thai traditional doctors use Ben-Cha-Moon-Yai remedy as an antipyretic agent instead of using individual roots. Although DS and DL showed negligible antipyretic efficacy, they might be included in the remedy in order to reduce toxicity of other roots (if any) or contribute other pharmacological effects that help relieve all symptoms accompanied fever. However, they might be useful for other indications since Ben-Cha-Moon-Yai remedy has also been used for treating other symptoms including anti-inflammation and antiflatulence by Thai traditional doctors.

This is the first study that helps clarifying the pharmacological action of this herbal remedy and provides additional scientific support for this Thai traditional medicine. Additional studies are required to better understand their potential antipyretic mechanism of action. The other studies may provide important clues to help understand the mechanism underlying the antipyretic of each herbal root extracts of Ben-Cha-Moon-Yai remedy and the extract of Ben-Cha-Moon-Yai remedy and further support the use of this Thai traditional medicine in a clinical setting.

Anti-inflammatory testing by carrageenan-induce paw edema in mice

Anti-inflammatory activity was assessed utilizing carrageenan-induced mouse paw edema, an acute inflammation model. The carrageenan-induced paw edema test in mice is a suitable method for evaluation of anti-inflammatory on natural products which has been modified by Levy in 1969. This method causes a reproducible inflammatory reaction and remains the standard irritant for examining acute inflammation and anti-inflammatory drug [235].

Inflammation induced by carrageenan is the acute, nonimmune, well-researched and highly reproducible. Cardinal signs of inflammation (edema, hyperalgesia and erythema) develop immediately following subcutaneous carrageenan

injection, resulting from actions of proinflammatory agents. The oedema at 3 hr after the application of carrageenan was considered to reach the highest response [236-237]. The inflammatory response resulted from carrageenan can be modulated by inhibitors of specific molecules within the inflammatory cascade. Carrageenan-induced paw edema test, a standard experimental model of acute inflammation is characterized by a biphasic response. The development of edema induced by carragenan is a biphasic phases: early phase (1-2 h after injection carrageenan) is due to the release of serotonin, bradykinin and histamine liberation, while late phase (over 2) is associated with the release of prostaglandins especially those of the E series [238-240]. Continuity between two phases is believed to be mediated by kinins [231, 241].

The results demonstrated that IND significantly reduced paw edema at 2 hr or more after carrageenan administration (during second phase). The effect of IND in decreasing paw edema only at the second phase could be explained by the fact that IND is a cyclooxygenase inhibitor and contributes to the reduction of prostaglandins synthesis. These results are consistent with the previous study which showed that IND caused strong inhibition of the second phase without affecting the development of the first phase [241].

All doses of BMY showed significant reduction of paw edema at 3 hr or more, suggesting that BMY produces an anti-edematous effect at the second phase. The highest dose of AM (400 mg/kg), OI (200-400 mg/kg), DL (200-400 mg/kg), DS (200-400 mg/kg) and all doses of WT (25-400 mg/kg) significantly reduced paw edema at 3 hr or more, suggesting that these five root extracts produce anti-inflammatory effect during the second phase which involves prostaglandin synthesis. This effect may be due to the interference by BMY and all five root species extracts on the liberation of prostaglandins, or the blockade of the prostaglandin receptors. Results of AM and OI were consistent with previous studies that showed anti-edematogenic effect of the extract of *Aegle marmelos* fruits and leaves and the extract of *Oroxylum indicum* root bark at the second phase of carrageenan-induced paw edema in rats [48, 242, 243]. This is the first study that demonstrated the anti-inflammatory properties of BMY, DS and WT. These studies also provide additional

scientific support to the use of *Aegle marmelos*, *Dolichandrone serrulata*, *Oroxylum indicum* and *Walsura trichostemon* roots as anti-inflammatory drugs in Thai traditional medicine.

Anti-nociceptive activity

Antinociceptive property was assessed utilizing thermally-induced (hot-plate) and chemically-induced (formalin and writhing tests) pain models in mice. The involvement of opioid receptors in the analgesic effects of each herbal root extracts of Ben-Cha-Moon-Yai remedy was also investigated. The hot plate test is useful for evaluation of centrally mediated anti-nociceptive activity which is known to elevate the pain threshold of mice towards thermal stimulus [244-245]. Pain reflexes in response to a thermal stimulus measured using a hot-plate analgesia meter from Ugo Basile Instruments. The latency of nociceptive response (reaction times) of each mouse that was identified by the time for licking or jumping of a hind limb was recored [178]. Both behaviors are considered to be supraspinally integrated responses [246].

Firstly, analgesic effect of BMY and all five herbal root extracts (AM, OI, DL, DS, WT) was evaluated utilizing the standard mouse hot-plate test [174], a central analgesic activity testing model. This model usually employs morphine (MO) as a reference drug. In this study, MO showed potent analgesic effect on the response indicating the sensitivity of this test. BMY and AM, OI, DL, DS, WT were administered orally to the animals by suspending in 2% Tween 80. The oral administration was chosen in order to imitate the normal consumption of Ben-Cha-Moon-Yai remedy, the Thai traditional antipyretic and anti-inflammatory medicine.

Results from the present study indicated that all doses of BMY (125-250 mg/kg), *Aegle marmelos* root extract (AM; 400 mg/kg), *Dolichandrone serrulata* root extract (DS; 200 and 400 mg/kg) and *Walsura trichostemon* root extract (WT; 100-400 mg/kg) have significant analgesic action in the hot-plate test. The antinociceptive peak response of BMY (125-500 mg/kg) and AM (400 mg/kg), DS (200 and 400 mg/kg) and WT (100, 200 and 400 mg/kg) was observed at different time points

starting from 90-240 min after orally administration. This may partly due to variable absorption of the herbal root extracts from the gastrointestinal tract of rodents causing a delay effect. The results supported that BMY, AM, DS and WT at specified doses have central analgesic effect. The rest of the extracts including *Oroxylum indicum* root extract (OI) and *Dimocarpus longan* root extract (DL) showed negligible analgesic action in the hot-plate model. These results are consistent with the previous studies. Shankarananth *et al*., in 2007 [47] demonstrated the analgesic activity of *Aegle marmelos* leaves extract in a thermal-induced nociception model, tail-flick test. Zaveri and Jain in 2009 reported the analgesic activity of *Oroxylum indicum* root bark extract in the same model [243].

Naloxone, a short acting opioid antagonist, was utilized to investigate the involvement of opioid receptors in the analgesic effects of the effective root extracts including AM (400 mg/kg), DS (200 mg/kg) and WT (400 mg/kg) utilizing hot-plate test. The results demonstrated the attenuation of the analgesic response of AM, DS and WT by naloxone suggesting the involvement of opioid receptors in analgesia produced by these three herbal root extracts.

In order to measure the analgesic effect of BMY, AM, OI, DL, DS and WT against chemical stimuli, formalin test was chosen. The formalin test is a valid and reliable model of nociception and inflammatory pain [245]. In This test, animals displayed nociceptive behaviors consisting of shaking, licking and biting the affected paw in two distinct phases. The early phase (acute pain) initiates immediately after formalin injection and lasts for five minutes as a result of chemical stimulation of primary afferent nociceptors. The late phase (inflammatory pain) initiates 20 min after formalin injection and lasts for 10 min arising from peripheral inflammation and functional changes in the dorsal horn of the spinal cord. Previous studies reported that formalin test involves neurogenic response with release of substance P and bradykinin participated in the early phase, whereas histamine, serotonin, prostaglandin, nitric oxide and bradykinin are involved in late phase [176, 177, 247]. Each phase of formalin test reflects different mechanisms; drugs that act predominantly on central nervous system inhibit both phases equally while peripherally acting drugs inhibit

only the late phase. In addition, the late phase is selectively attenuated by cyclooxygenase inhibitors.

In this study, we employed MO and IND as reference drugs. MO, a central analgesic drug, demonstrated potent analgesic effects in both phases while IND, a peripheral acting drug and demonstrated analgesic response only in the late phase. BMY at doses of 125 and 500 mg/kg produced significant analgesic action only in the late phase, while BMY at the dose of 250 mg/kg produced significant analgesic action in both phases. BMY at the dose of 250 mg/kg is likely to be the most effective dose in this model. The highest dose of AM (400 mg/kg) demonstrated significant analgesic responses in both phases of formalin-induced nociception test indicated that AM possess analgesic property in both acute and inflammatory pain. OI at doses of 100-400 mg/kg and DL at doses of 200 and 400 mg/kg produced significant analgesic responses only during the late phase suggesting antinociceptive activity of OI and DL in inflammatory pain. DS at the dose of 200 mg/kg produced significant analgesic action only in the late phase, while the highest dose of DS (400 mg/kg) produced significant analgesic action in both phases suggesting analgesic property in both acute and inflammatory pain. WT at the dose of 200 mg/kg produced significant analgesic action only in the late phase, while the highest dose of WT (400 mg/kg) produced significant analgesic action in both phases. The results suggested the analgesic property of WT in both acute and inflammatory pain. All the results from the formalin test indicated that BMY, AM, DS and WT displayed central analgesic action, while OI and DL exhibited peripheral analgesic action.

Studies were then undertaken to investigate the peripheral analgesic effect of BMY, AM, OI, DL, DS and WT utilizing the acetic acid-induced writhing test. The writhing model is a sensitive method for screening peripheral analgesic efficacy agents and it is more sensitive to non-steroidal analgesics. The writhing test is based on the postulation that acetic acid acts by releasing endogenous mediators that stimulate the nociceptive neurons as a result of prostanoids mediators and is used as a screening tool for the assessment of analgesic properties of a test compound.

The writhing response is presumed to be induced by local peritoneal receptor activation [248]. The nociceptive properties of acetic acid may be due to the release of cytokines, including TNF-α, interleukin-1β, and interleukin-8 by resident peritoneal macrophages and mast cells (Ribeiro et al., 2000). In mice acetic acid was reported to cause an increase in the peritoneal fluid levels of PGE_2 and PGF_2, as well as lipooxygenase products [249], and the release of sympathetic nervous system mediators [250]. This response can be prevented by various inhibitors of prostaglandin biosynthesis including nonsteroidal anti-inflammatory agents, non-narcotic analgesics, some monoamine oxidase inhibitors and antioxidants prevented prostaglandin release [249].

In the present study, indomethacin (IND), a nonsteroidal anti-inflammatory drug, produced significant analgesic response in the acetic acid-induced writhing test. All dose of BMY, AM (200 and 400 mg/kg), OI (100-400 mg/kg), DL (100-400 mg/kg), DS (200 and 400 mg/kg) and WT (200 and 400 mg/kg) showed significant analgesic responses in this model indicating the peripheral antinociceptive property of these extracts. These results were consistent with previous studies that reported analgesic activity of *Aegle marmelos* leaves extract [47-48], *Oroxylum indicum* root bark extract [243] and the active ingredient of *Dimocarpus longan* extract [251] in the acetic acid-induced writhing method. The proposed mechanism of BMY and all five root extracts may be due to the reduction on the liberation of those inflammatory mediators or by direct blockade of receptors resulting in a peripheral analgesic action.

Results from hot-plate, formalin and writhing tests indicated both central and peripheral antinociceptive properties of BMY. AM, DS and WT also displayed both central and peripheral analgesic activities, while OI and DL exhibited only peripheral analgesic effects. This is the first study that demonstrated the analgesic properties of BMY, DS and WT. These studies also provide additional scientific support to the use of *Aegle marmelos* (Ma-tum) and *Walsura trichostemon* (Kad-lin) roots as analgesic drugs in Thai traditional medicine.

Furthermore, to exclude the possible cause of non-specific disturbances of motor coordination caused by BMY, AM, OI, DL, DS and WT, the rota-rod test was performed. This test has been used to determine a compound's ability to produce skeletal muscle relaxation, convulsions and depression of the CNS. Results from this study indicated neither detectable relaxant nor sedative effects of the highest doses of BMY, AM, OI, DL, DS and WT. Therefore, the behavioral responses observed in the hot-plate, writhing and formalin tests were likely not the motor dysfunction but rather than a true antinociceptive effect.

In order to investigate the root extract that contribute to the analgesic or anti-inflammatory effects of BMY, each root extract at the same dose was compared to BMY. The analgesic and anti-inflammatory effects of BMY at all doses tested (125-500 mg/kg) were more potent than each individual root extract at doses of 25-100 mg/kg. The analgesic property of all doses of BMY was most likely resulted from all five root extracts. The anti-inflammatory property of the lowest dose of BMY (125 mg/kg) was most likely resulted from all five root extracts, while for the higher doses of BMY (250 and 500 mg/kg) was mainly due to AM and WT. This could be due to additive and/or synergistic effects of some herbal roots in the remedy. It is believed that some herbal roots in the remedy may reduce the toxicity of other roots and exert other pharmacological effects that are beneficial. This might be a reason why Thai traditional doctors prefer to use Ben-Cha-Moon-Yai remedy instead of single root as an anti-inflammatory or antipyretic agent.

The present study demonstrated that BMY, AM, DS and WT possess both central and peripheral antinociceptive properties, while OI and DL possess only peripheral analgesic property. The analgesic mechanisms of AM, DS and WT are most likely involved with the opioid pathway. Additionally, BMY and all five herbal root extracts also demonstrated anti-inflammatory property. The anti-inflammatory mechanism of all five herbal root extracts may involve the interference on the liberation of prostaglandins or inhibition of prostaglandin E_2 effects. The mechanism of action of BMY and all five herbal root extracts requires further investigation. These findings may eventually lead to the development of novel therapies with minor adverse effects in treating pain and inflammatory conditions.

Table 50 Summary of safety and efficacy of five root species and Ben-Cha-Moon-Yai remedy

Experiment	*A. marmelos*		*D. longan*		*D. serrulata*		*O. indicum*		*W. trichostemon*		BMY
	E	W	E	W	E	W	E	W	E	W	
Brine shrimp lethality testing	+++										++
Mutagenicity assay (without nitrite)		+++									
Mutagenicity assay (with nitrite)	++	+++		+++			+++	++			+++
Comet assay	+++	+++	++	+++							
Anti-mutagenicity assay	+++		+++	+++	+++		+++	+++	+++	+++	+++
Antimicrobial activity			+++	+++			+++				++
Total phenolic content			+++						+++		
DPPH assay	++		+++	++	++		++		+++	+++	++
β-carotene bleaching assay	++		+++	++					++		++
Nitric oxide scavenging assay			+++						+++		
Anti-pyretic activity	+++						+++		+++		+++
Anti-inflammatory activity	+++		+++		+++		+++		+++		+++
Anti-nociceptive activity	+++		++		+++		++		+++		+++

+ Positive effect (+ mild effect, ++ moderate effect, +++ high effect); E-ethanolic extract, W-water extract

In conclusion, this study provided the pharmacognostic specifications of the five root species in Ben-Cha-Moon-Yai remedy which could be used as the standardization data to authenticate and evaluate the quality of plant materials before used as therapeutic drugs. Furthermore, provides preclinical evidences in biological activities and possible toxicities with postulated mechanisms of Ben-Cha-Moon-Yai remedy together with all five root species extracts. This research protocol can be applied to other traditional medicine formulars.

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APPENDICES

APPENDIX A

Data of pharmacognostic specifications of five root species
in Ben-Cha-Moon-Yai remedy

Table 51 Pharmacognostic characters (% by weight) of *Aegle marmelos* root

	Sources	No	Loss on drying	Total ash content	Acid-insoluble ash content	Water content	Ethanol extractive value	Water extractive value
1	Tak (Meung)	1	7.08	3.47	0.56	10.43	5.01	4.46
		2	7.23	3.46	0.54	10.34	4.96	4.41
		3	7.71	3.46	0.50	10.42	5.18	4.13
2	Lop Buri	1	6.95	3.34	0.50	8.43	4.61	4.26
		2	6.90	3.39	0.45	9.90	4.29	4.23
		3	6.96	3.24	0.62	9.40	4.11	4.40
3	Buri Ram	1	7.08	4.56	0.80	10.42	6.58	6.05
		2	6.96	4.73	0.91	10.32	6.17	6.78
		3	6.89	4.55	0.90	10.34	7.01	5.96
4	Phetchaboon	1	5.41	5.59	0.87	8.44	7.19	6.19
		2	5.51	5.46	0.78	8.44	7.50	6.22
		3	5.40	5.43	0.82	6.95	7.31	6.31

	Sources	No	Loss on	Total ash	Acid-insoluble	Water	Ethanol	Water

			drying	content	ash content	content	extractive value	extractive value
5	Tak (Maesot)	1	6.17	3.40	0.65	7.94	4.37	4.33
		2	6.21	3.33	0.48	9.04	4.34	4.62
		3	6.25	3.35	0.54	8.74	4.43	4.80
6	Ubon Ratchathani	1	7.14	4.09	1.26	9.42	6.88	6.59
		2	7.29	4.12	1.16	8.94	6.30	6.75
		3	7.39	4.08	1.13	5.95	6.81	6.93
7	Uthai Thani	1	6.78	2.50	0.29	9.71	4.80	6.64
		2	6.41	2.58	0.29	9.93	4.62	6.69
		3	6.47	2.59	0.38	9.54	4.71	6.58
8	Kalasin	1	6.52	4.33	1.00	8.92	7.75	8.10
		2	6.45	4.29	0.98	8.82	7.76	6.77
		3	6.50	4.24	0.92	8.72	6.99	7.20

	Sources	No	Loss on drying	Total ash content	Acid-insoluble ash content	Water content	Ethanol extractive	Water extractive

							value	value
9	Uttaradit	1	7.79	5.51	0.57	10.68	1.58	13.59
		2	7.76	5.45	0.56	10.79	1.60	13.61
		3	7.80	5.43	0.51	9.69	1.74	13.49
10	Nong Khai	1	7.73	3.83	0.85	12.80	4.51	7.01
		2	7.65	5.45	0.56	11.02	4.50	7.03
		3	7.80	5.43	0.51	10.91	4.39	7.09
11	Mukdahan(Meung)	1	7.94	3.73	0.76	10.40	5.14	4.78
		2	7.87	3.89	0.76	10.71	5.23	4.70
		3	7.86	3.84	0.88	10.54	5.37	5.02
12	Mukdahan(Nongsung)	1	7.11	2.90	0.35	9.80	4.57	5.56
		2	7.34	2.91	0.36	9.83	4.40	5.79
		3	7.34	2.87	0.43	10.00	4.39	6.13

Table 52 Pharmacognostic characters (% by weight) of *Dimocarpus longan* root

	Sources	No	Loss on drying	Total ash content	Acid-insoluble ash content	Water content	Ethanol extractive value	Water extractive value
1	Uthai Thani	1	6.71	2.49	0.28	9.09	5.51	4.26
		2	6.72	2.51	0.27	8.91	5.65	3.79
		3	6.48	2.72	0.26	8.47	5.70	4.63
2	Lop Buri	1	6.92	2.70	0.39	10.94	8.67	5.55
		2	6.96	2.79	0.37	10.32	8.47	6.04
		3	6.90	2.83	0.35	10.34	8.69	5.73
3	Buri Ram	1	7.75	2.66	0.52	11.18	10.61	6.49
		2	7.71	2.91	0.56	11.63	10.62	5.61
		3	7.67	2.87	0.56	12.28	11.76	7.32
4	Phetchabun	1	8.96	4.03	0.64	10.26	6.95	4.49
		2	8.96	4.22	0.58	9.92	7.37	4.89
		3	8.96	4.26	0.65	10.15	6.90	Broke
	Sources	No	Loss on drying	Total ash content	Acid-insoluble ash content	Water content	Ethanol extractive value	Water extractive value
5	Tak (Meung)	1	7.23	5.54	1.33	12.46	6.10	3.60

		2	7.25	5.43	1.27	12.71	6.57	3.80
		3	7.38	5.24	1.23	11.46	7.61	3.92
6	Tak (Maesot)	1	8.76	3.38		9.81	5.36	5.77
		2	8.85	3.68	0.48	8.91	5.38	5.28
		3	8.77	3.70	0.79	9.13	4.90	5.48
7	Ubon Ratchathani	1	8.11	3.20	1.10	10.11	9.20	5.08
		2	7.88	5.85	3.79	10.88	9.40	5.53
		3	7.28	3.37		10.59	8.91	4.67
8	Chiang Rai	1	7.43	3.64	0.64	10.44	4.00	3.67
		2	7.76	3.27	0.54	10.38	5.46	3.05
		3	7.75	4.10	0.99	9.63	4.12	3.69

	Sources	No	Loss on drying	Total ash content	Acid-insoluble ash content	Water content	Ethanol extractive value	Water extractive value
9	Kalasin	1	8.37	3.84	0.60	9.91	7.69	4.86
		2	8.39	3.43	0.59	10.82	7.99	4.82

		3	8.52	3.44	0.44	10.65	8.25	4.72
10	Uttaradit	1	10.24	2.49	0.35	11.91	10.42	5.96
		2	10.22			13.18	9.26	5.61
		3	10.22	2.25	0.31	11.93	9.90	5.42
11	Nong Khai	1	7.65	2.03	0.37	9.42	10.29	6.63
		2	8.00	2.03	0.38	9.63	9.73	7.27
		3	7.74	2.01	0.35	10.05	10.21	5.89
12	Mukdahan	1	7.40	7.58	5.69	10.04	6.96	6.08
		2	7.41	7.11	5.75	9.95	6.16	5.80
		3	7.37	6.76	5.21	10.31	6.19	5.84
13	Nan	1	8.18	2.59	0.42	11.54	7.09	4.16
		2	8.19	2.62	0.41	11.46	7.20	4.07
		3	8.14	2.61	0.39	11.44	7.41	3.82

Table 53 Pharmacognostic characters (% by weight) of *D. serrulata* root

	Sources	No	Loss on drying	Total ash content	Acid-insoluble ash content	Water content	Ethanol extractive value	Water extractive value
1	Buri Ram (Prakonchai)	1	7.89	3.97	1.09	14.37	4.06	7.56
		2	7.95	3.96	0.95	13.69	4.13	7.37

		3	7.95	3.98	0.81	13.38	4.07	7.04
2	Tak (Maesot)	1	7.35	4.11	0.80	10.98	4.39	5.59
		2	7.29	4.35	0.82	9.79	4.40	5.15
		3	7.31	4.19	0.79	9.79	4.41	4.66
3	Uttaradit	1	9.10	2.58	0.74	14.17	3.03	11.36
		2	9.08	2.62	0.44	13.59	3.00	12.16
		3	9.05	2.64	0.79	12.59	2.84	11.90
4	Nakhon Ratchasima	1	8.63	2.83	0.38	14.19	1.58	3.72
		2	8.69	2.85	0.48	15.18	0.16	3.65
		3	8.73	2.84	0.37	15.59	1.61	3.56
5	Nan	1	7.75	4.36	0.58	12.59	4.14	16.41
		2	7.70	4.30	0.61	12.19	4.29	15.13
		3	7.69	4.13	0.63	13.79	4.49	15.38

	Sources	No	Loss on drying	Total ash content	Acid-insoluble ash content	Water content	Ethanol extractive value	Water extractive value
6	Nong Khai	1	7.45	1.14	0.38	10.88	4.28	10.38
		2	7.63	1.11	0.43	12.08	4.52	10.67
		3	7.51	1.11	0.31	11.27	4.14	9.77

7	Tak	1	6.67	4.20	0.62	10.67	2.48	8.62
		2	6.72	4.13	0.58	10.97	2.25	8.79
		3	6.66	4.15	0.53	10.98	2.22	7.82
8	Lop Buri	1	7.21	4.12	0.60	13.57	6.11	12.92
		2	7.19	4.14	0.60	11.60	6.25	12.77
		3	7.22	4.08	0.59	11.59	6.26	12.12
9	Chaiyaphum	1	8.40	4.10	3.10	12.79	5.55	5.81
		2	8.51	4.05	4.13	12.00	5.80	5.60
		3	8.47	4.05		12.60	5.62	5.42
10	Ubon Ratchathani	1	7.11	2.63	0.50	10.56	7.26	13.17
		2	7.17	2.61	0.35	10.98	7.63	13.11
		3	7.14	2.60	0.45	11.65	7.59	14.24
Sources		No	Loss on drying	Total ash content	Acid-insoluble ash content	Water content	Ethanol extractive value	Water extractive value
11	Uthai Thani	1	7.39	5.34	0.65	10.19	7.08	20.44
		2	7.50	5.37	0.80	11.19	7.10	19.80
		3	7.37	5.17		10.79	7.01	18.77

12	Buri Ram (Meung)	1	9.32	3.45	0.75	15.77	4.20	7.41
		2	9.25	3.43	0.67	13.99	4.29	7.95
		3	9.34	3.43	0.72	14.09	4.11	7.43
13	Phetchabun	1	7.78	4.96	0.78	13.19	4.59	11.60
		2	7.48	5.01	0.73	13.99	4.62	11.76
		3	7.70	4.78	0.57	9.19	4.53	11.31
14	Mukdahan	1	7.83	3.25	0.67	14.78	4.54	10.10
		2	7.65	3.20	0.51	13.19	4.15	9.80
		3	7.57	3.19	0.63	12.98	3.77	8.79

Table 54 Pharmacognostic characters (% by weight) of *O. indicum* root

	Sources	No	Loss on drying	Total ash content	Acid-insoluble ash content	Water content	Ethanol extractive value	Water extractive value
1	Lop Buri	1	8.12	5.93	0.90	10.38	12.62	24.49
		2	8.03	6.15	1.05	10.48	13.23	26.13
		3	8.14	6.12	1.03	12.46	13.28	26.67

2	Kalasin	1	6.61	8.49	2.62	8.98	7.14	13.24
		2	6.62	8.22	2.18	9.18	7.64	14.09
		3	6.60	8.40	2.50	8.99	7.84	15.09
3	Chiang Rai	1	9.89	6.69	1.07	9.47	5.02	10.14
		2	7.27	6.95	1.25	9.17	4.66	9.96
		3	7.25	6.91	1.20	8.47	4.06	9.72
4	Ubon Ratchathani	1	6.07	7.27	2.68	8.98	9.93	23.01
		2	6.10	7.13	2.59	8.18	9.95	25.03
		3	6.10	7.08	2.52	8.48	9.41	25.48
5	Mukdahan	1	8.08	3.93	0.67	10.57	9.90	22.50
		2	6.82	3.92	0.69	10.58	10.02	22.77
		3	7.50			11.56	10.51	22.68
	Sources	No	Loss on drying	Total ash content	Acid-insoluble ash content	Water content	Ethanol extractive value	Water extractive value
6	Uthai Thani	1	6.74	4.15	0.60	9.48	10.95	26.96
		2	9.06	4.12	0.52	9.78	10.37	30.64
		3	6.77	4.32	0.63	9.18	8.86	29.93

7	Phetchabun	1	7.38	5.16	0.95	11.67	7.79	15.90
		2	7.40	5.15	0.82	11.38	8.04	52.47
		3	7.32	5.20	0.93	12.36	7.85	16.25
8	Chaiyaphum	1	6.87	3.97	0.76	10.79	13.46	24.84
		2	6.94	3.96	0.74	13.19	13.56	26.12
		3	6.93	3.96	0.74	11.99	14.63	26.52
9	Tak (Meung)	1	6.99	8.46	2.44	10.49	3.54	8.08
		2	7.01	8.51	2.61	10.89	3.39	7.89
		3	6.97	8.38	2.31	10.09	3.49	7.92
10	Nan	1	7.30	3.60	0.72	11.89	6.59	25.48
		2	7.19	3.59	0.63	10.19	5.95	25.30
		3	7.25	3.60	0.68	11.78	4.46	16.88
	Sources	No	Loss on drying	Total ash content	Acid-insoluble ash content	Water content	Ethanol extractive value	Water extractive value
11	Tak (Maesot)	1	6.06	6.65	1.21	11.98	7.10	17.86
		2	5.83	6.59	1.36	11.57	7.41	16.88
		3	5.86	6.61	1.19	11.68	6.85	17.94

12	Nong Khai	1	6.55	5.59	1.58	12.77	6.22	10.62
		2	6.55	5.67	1.76	11.17	6.11	10.40
		3	6.53	5.69	1.56	10.59	5.97	28.56
13	Buri Ram	1	5.51	2.05	0.35	11.19	4.14	6.87
		2	5.49	2.11	0.42	10.78	3.70	7.36
		3	5.49	2.11	0.38	10.98	3.53	6.80

Table 55 Pharmacognostic characters (% by weight) of *Walsura trichostemon* root

	Sources	No	Loss on drying	Total ash content	Acid-insoluble ash content	Water content	Ethanol extractive value	Water extractive value
1	Lop Buri	1	5.87	1.85	0.31	11.57	5.04	3.84
		2	5.81	1.85	0.30	12.17	4.78	3.58
		3	5.70	1.90	0.35	11.48	4.73	3.72
2	Nan (Nanoi)	1	7.15	2.44	0.55	13.97	10.70	7.41

		2	7.15	2.41	0.53	13.17	9.98	7.25
		3	7.13	2.35	0.50	11.57	10.50	6.76
3	Mukdahan (Meung)	1	6.46	2.02	0.83	11.57	3.09	3.21
		2	6.62	2.14		12.57	3.16	3.79
		3	6.54	2.11	0.87	12.47	2.91	3.71
4	Phetchabun	1	7.98	3.92	1.00	15.38	7.14	6.19
		2	7.98	3.93	0.89	15.77	6.92	6.02
		3	8.23	3.81	0.91	15.18	7.00	6.43
5	Mukdahan (Nongsung)	1	7.01	2.58	0.45	11.19	6.20	3.94
		2	7.01	2.51	0.37	11.98	6.21	4.09
		3	6.91	2.58	0.40	12.47	5.89	4.14
	Sources	No	Loss on drying	Total ash content	Acid-insoluble ash content	Water content	Ethanol extractive value	Water extractive value
6	Chaiyaphum	1	8.28	1.60	0.32	15.49	3.19	3.81
		2	8.15	1.61	0.33	16.17	3.46	3.75
		3	8.24	1.59	0.29	16.36	3.39	3.47
7	Buri Ram (Meung)	1	6.80	2.27	0.38	13.56	6.22	10.42

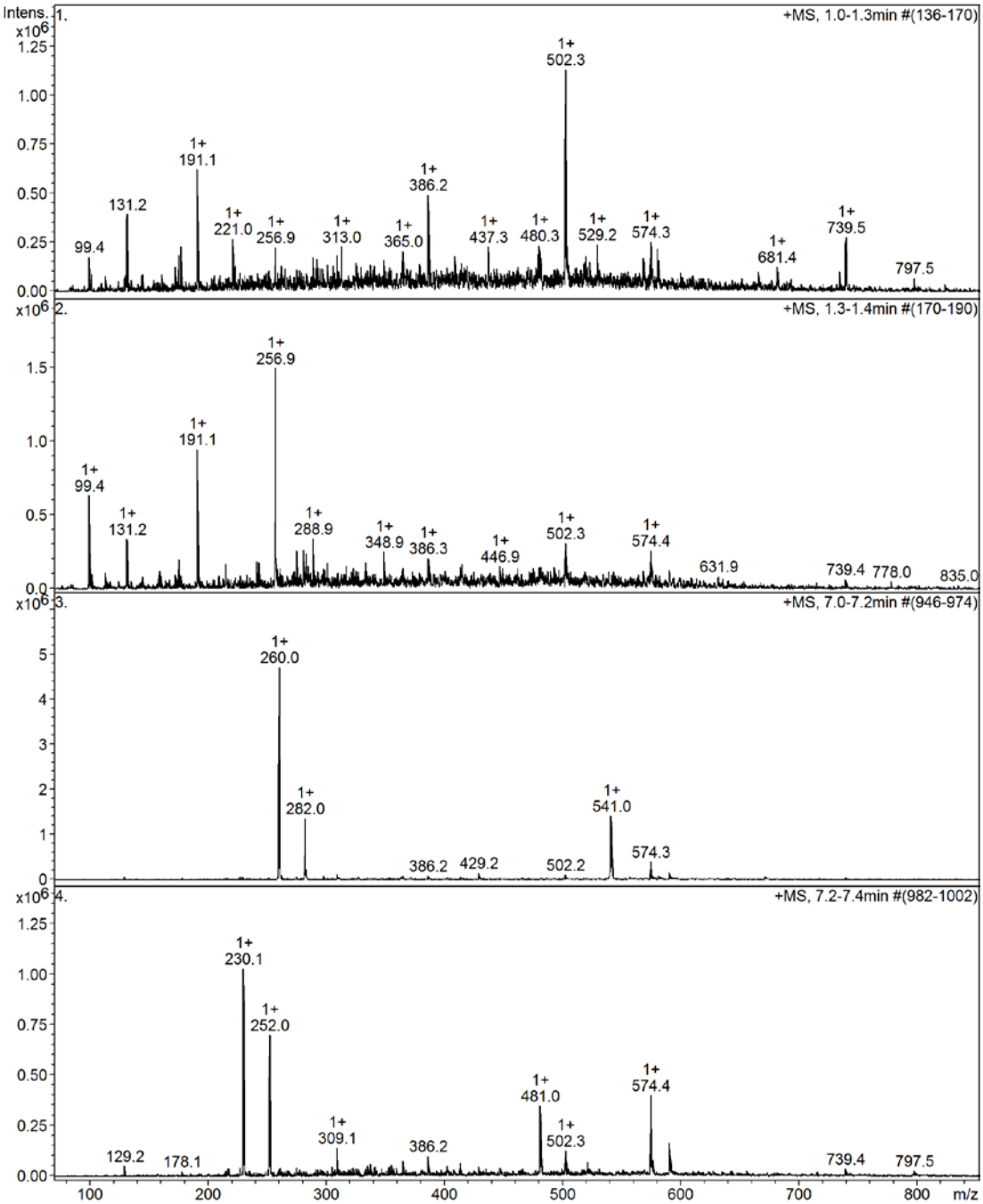
		2	6.71	2.33	0.39	13.96	6.43	9.37
		3	6.72	2.28	0.38	14.35	6.53	8.61
8	Uthai Thani	1	6.38	4.26	0.88	10.98	5.59	6.16
		2	6.44	4.34	0.76	12.17	5.74	6.48
		3	6.32	4.24	0.89	12.76	5.62	6.12
9	Nong Khai	1	6.43	2.13		13.40	4.17	4.45
		2	6.55	2.18	0.48	13.58	4.16	4.12
		3	6.54	2.17	0.46	12.77	4.26	4.59
10	Buri Ram (Prakonchai)	1	6.39	2.47	0.48	11.77	6.62	10.12
		2	6.46	2.47	0.47	11.25	6.94	10.09
		3	6.33	2.46	0.45	12.98	6.70	9.44
	Sources	No	Loss on drying	Total ash content	Acid-insoluble ash content	Water content	Ethanol extractive value	Water extractive value
11	Tak	1	7.86	2.84	0.48	16.19	8.82	4.93
		2	7.75	2.88	0.46	15.77	8.51	4.94
		3	7.87	2.58	0.38	13.18	8.94	4.94
12	Surin	1	7.47	2.51	0.38	12.99	5.75	5.63

	2	9.53	2.52	0.37	12.79	5.78	5.53
	3	7.50	2.56	0.36	12.19	5.81	5.88
13 Nan (Weing Sa)	1	7.34	5.95	1.00	12.69	10.01	9.41
	2	7.36	5.71	0.94	12.57	10.58	8.48
	3	7.49	5.73	0.94	12.98	10.89	8.50

APPENDIX B

Liquid Chromatography and Mass Spectrometry

Figure 92 A representative LC/MS spectrums from *Aegle marmelos* root of compound 1-8



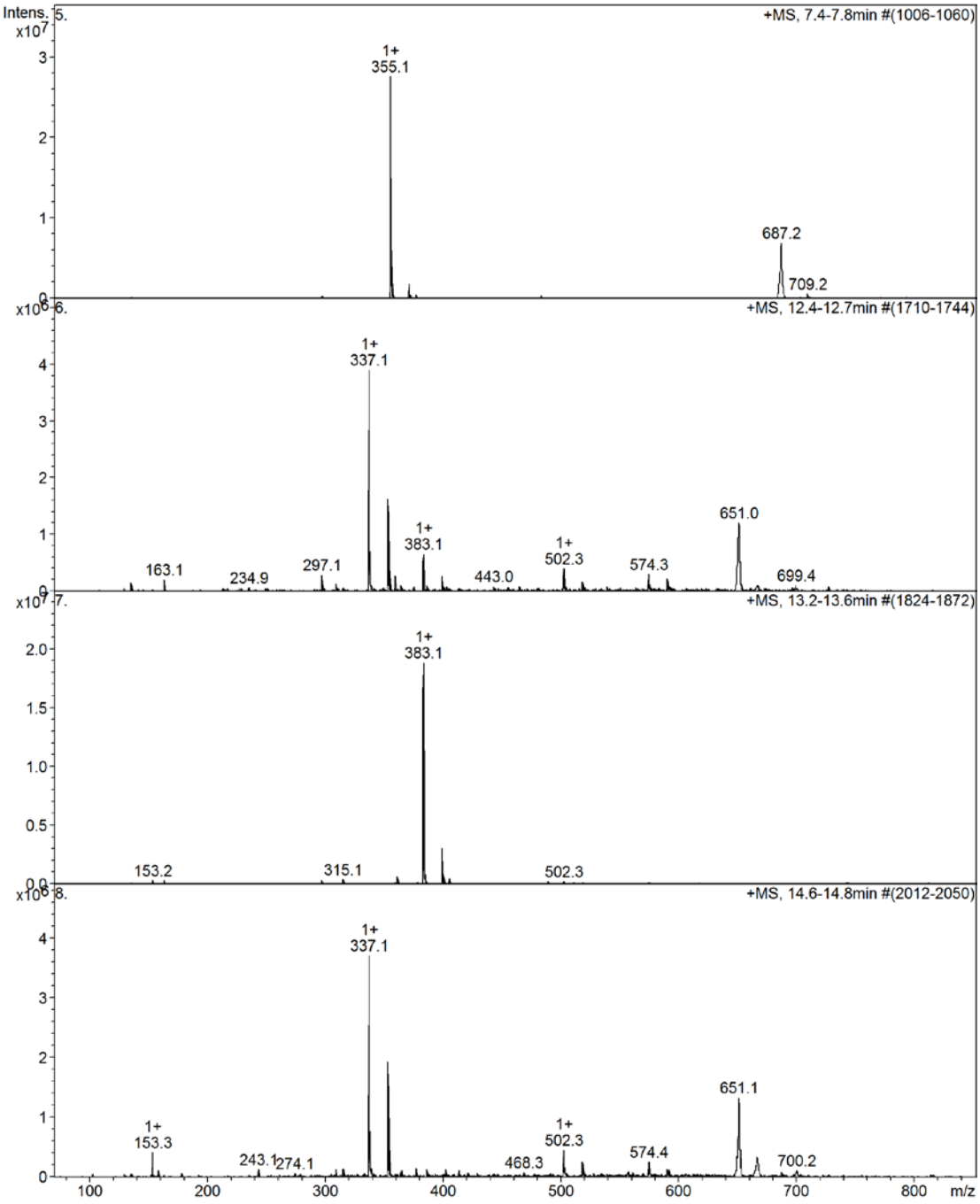
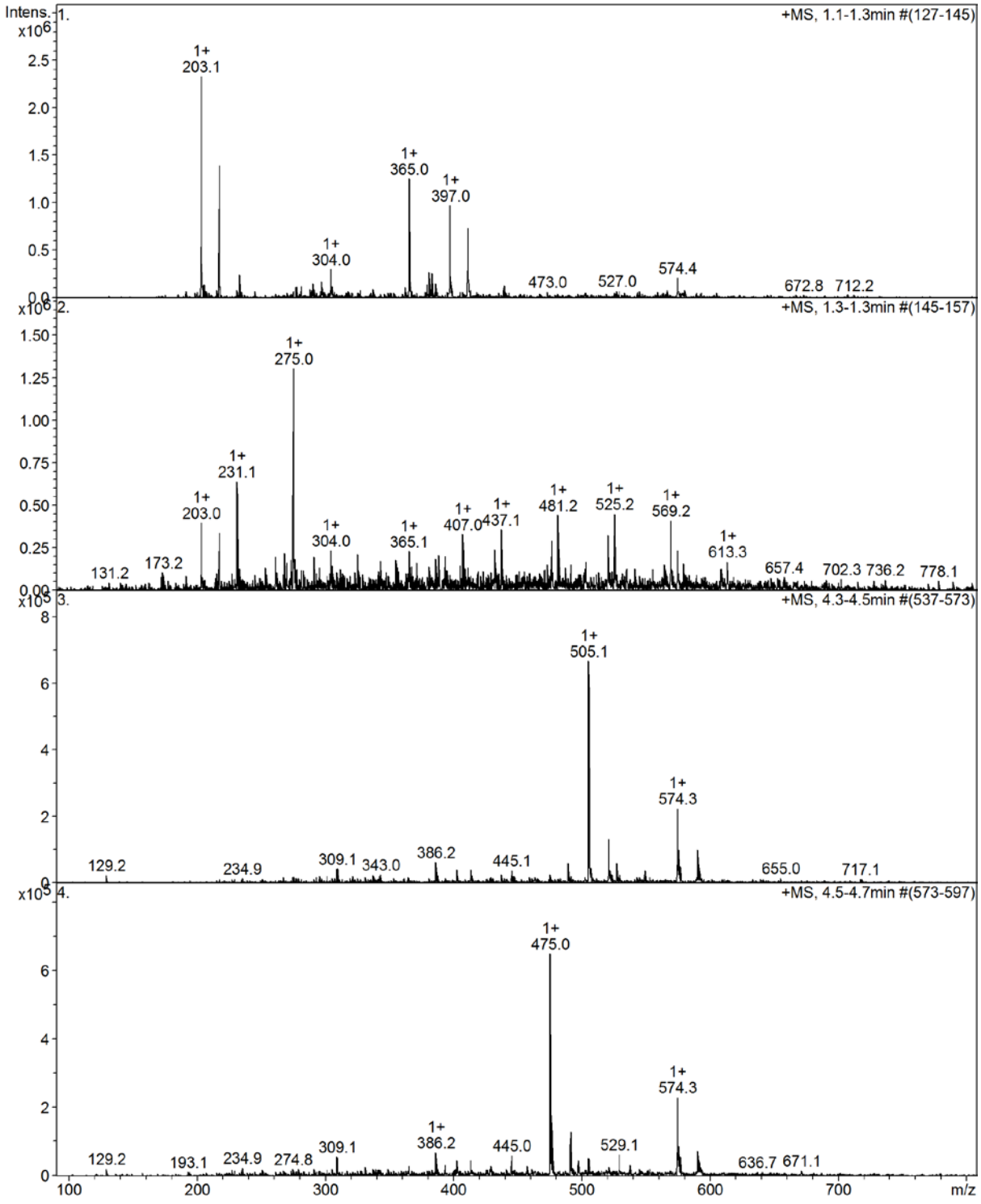

Intens.
x10^7
5.
+MS, 7.4-7.8min #(1006-1060)
1+
355.1
687.2
709.2
x10^6
6.
+MS, 12.4-12.7min #(1710-1744)
1+
337.1
1+
383.1
1+
502.3
651.0
163.1
234.9
297.1
443.0
574.3
699.4
x10^7
7.
+MS, 13.2-13.6min #(1824-1872)
1+
383.1
153.2
315.1
502.3
x10^6
8.
+MS, 14.6-14.8min #(2012-2050)
1+
337.1
651.1
1+
153.3
1+
502.3
243.1
274.1
468.3
574.4
700.2
100
200
300
400
500
600
700
800
m/z

Figure 93 A representative LC/MS spectrums from *Dimocarpus longan* root of compound 1-9



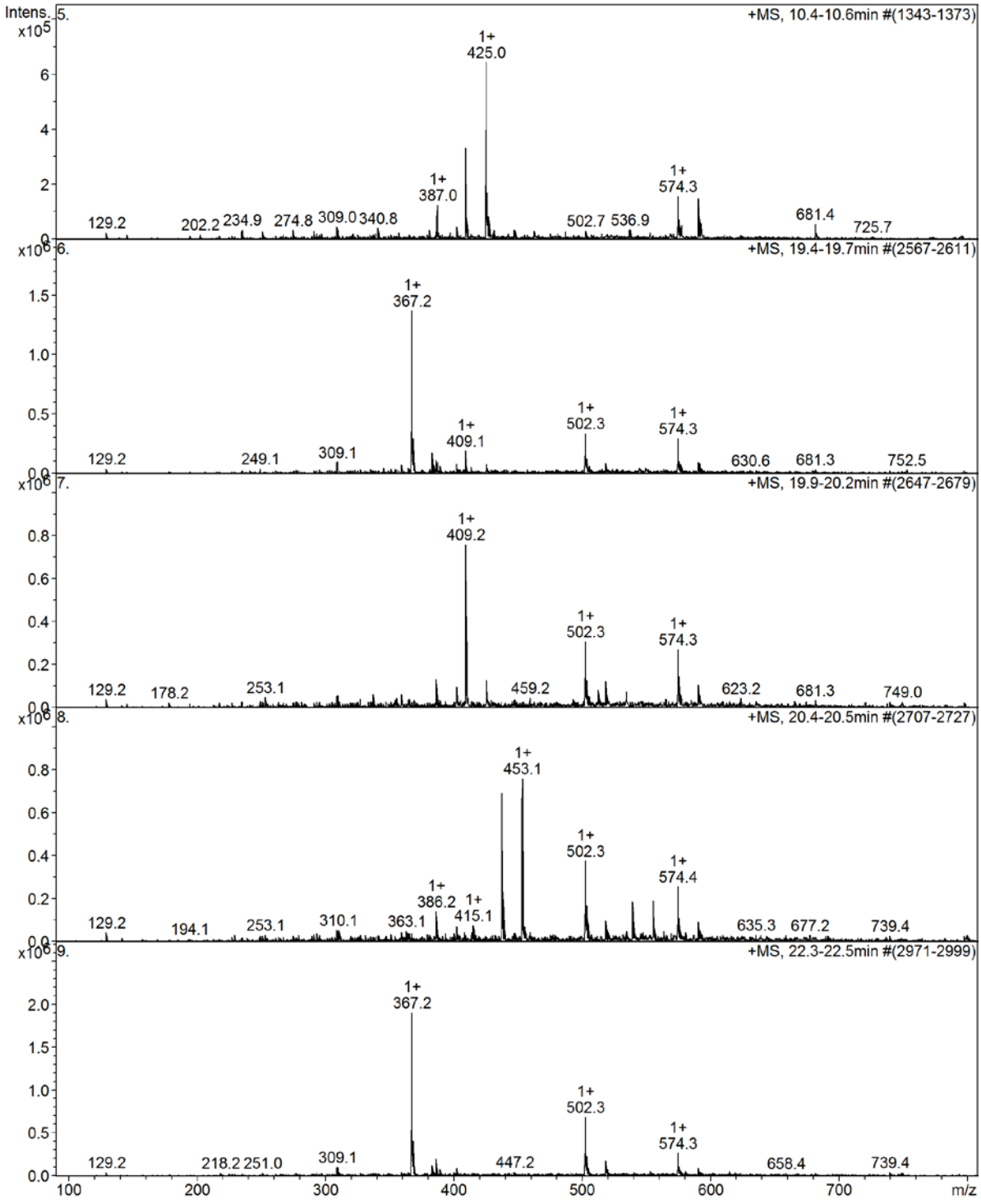
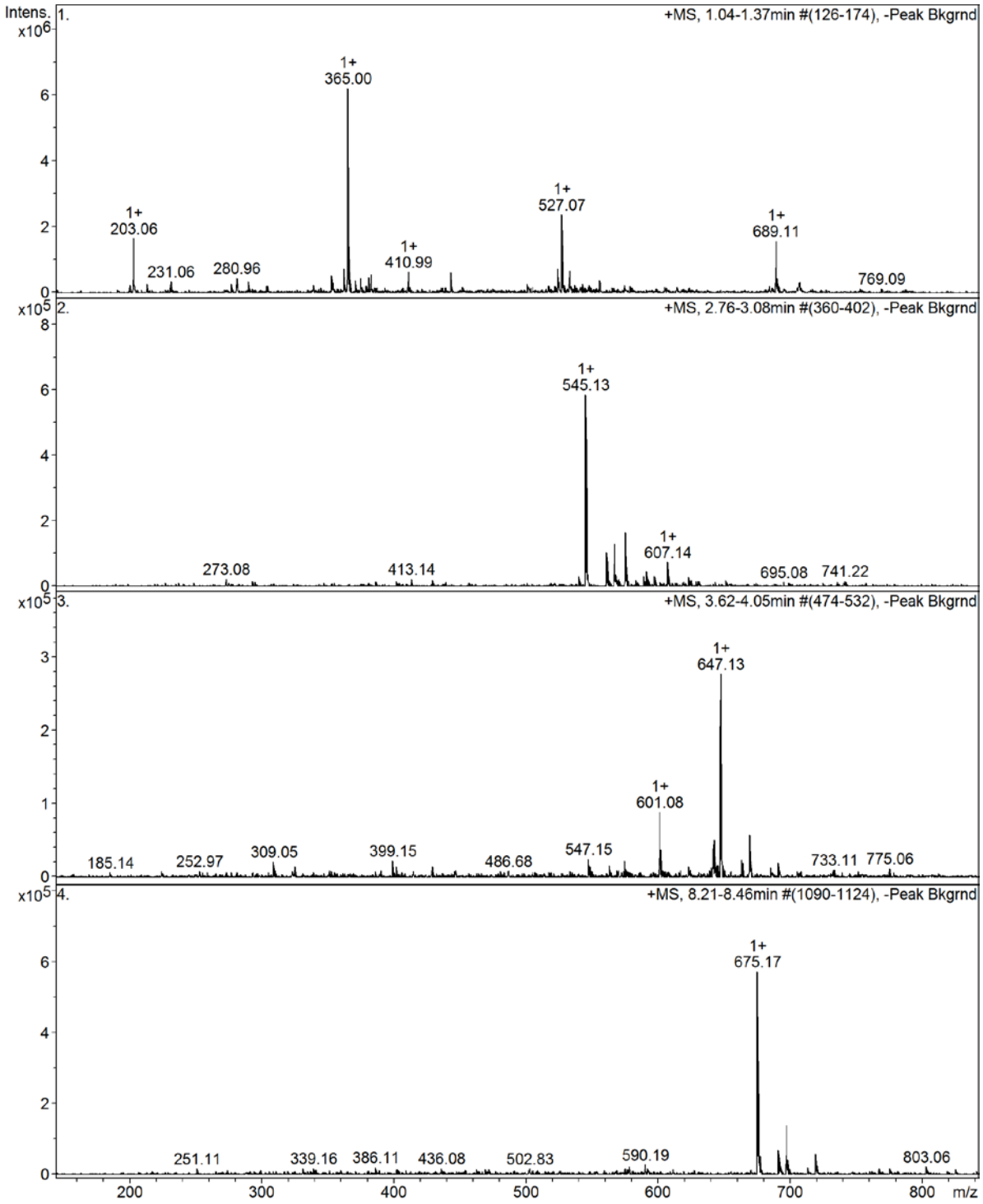

Intens.
x10^5
5.
+MS, 10.4-10.6min #(1343-1373)
1+ 425.0
1+ 387.0
1+ 574.3
129.2 202.2 234.9 274.8 309.0 340.8 502.7 536.9 681.4 725.7
x10^6
6.
+MS, 19.4-19.7min #(2567-2611)
1+ 367.2
1+ 409.1
1+ 502.3
1+ 574.3
129.2 249.1 309.1 630.6 681.3 752.5
x10^6
7.
+MS, 19.9-20.2min #(2647-2679)
1+ 409.2
1+ 502.3
1+ 574.3
129.2 178.2 253.1 459.2 623.2 681.3 749.0
x10^6
8.
+MS, 20.4-20.5min #(2707-2727)
1+ 453.1
1+ 502.3
1+ 574.4
1+ 386.2
1+ 415.1
129.2 194.1 253.1 310.1 363.1 635.3 677.2 739.4
x10^6
9.
+MS, 22.3-22.5min #(2971-2999)
1+ 367.2
1+ 502.3
1+ 574.3
129.2 218.2 251.0 309.1 447.2 658.4 739.4
100 200 300 400 500 600 700 m/z

Figure 94 A representative LC/MS spectrums from *Dolichandrone serrulata* root of compound 1-11



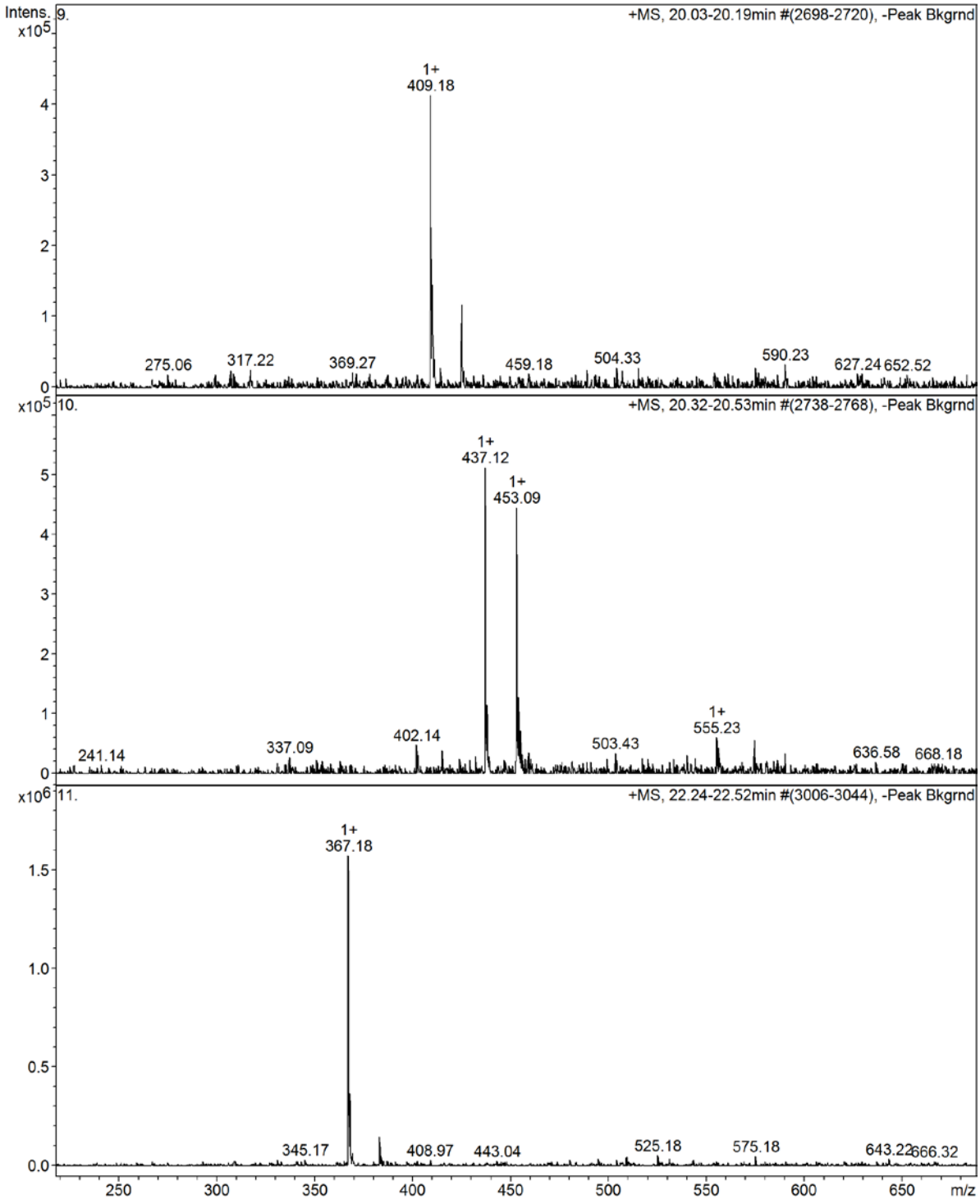
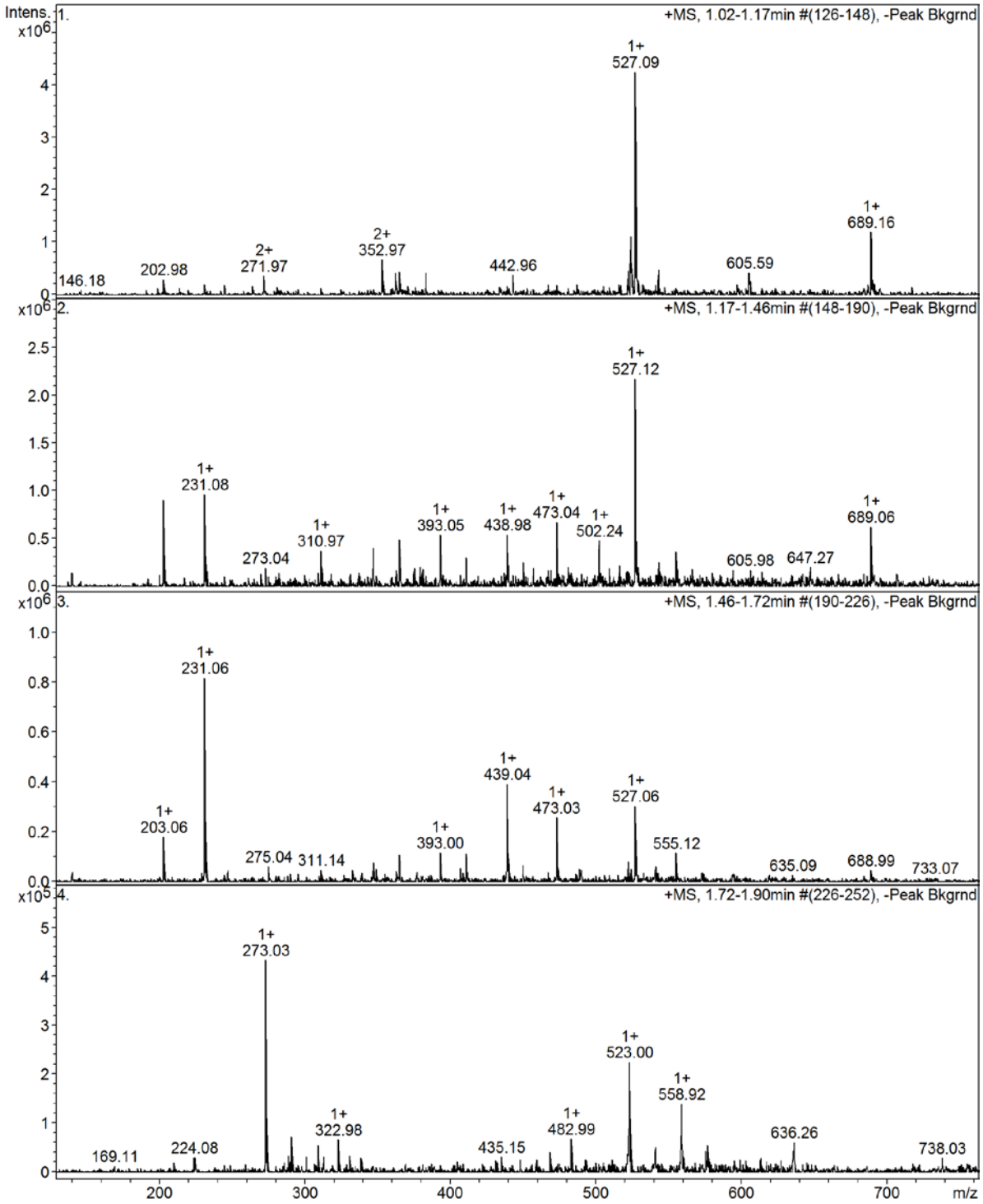
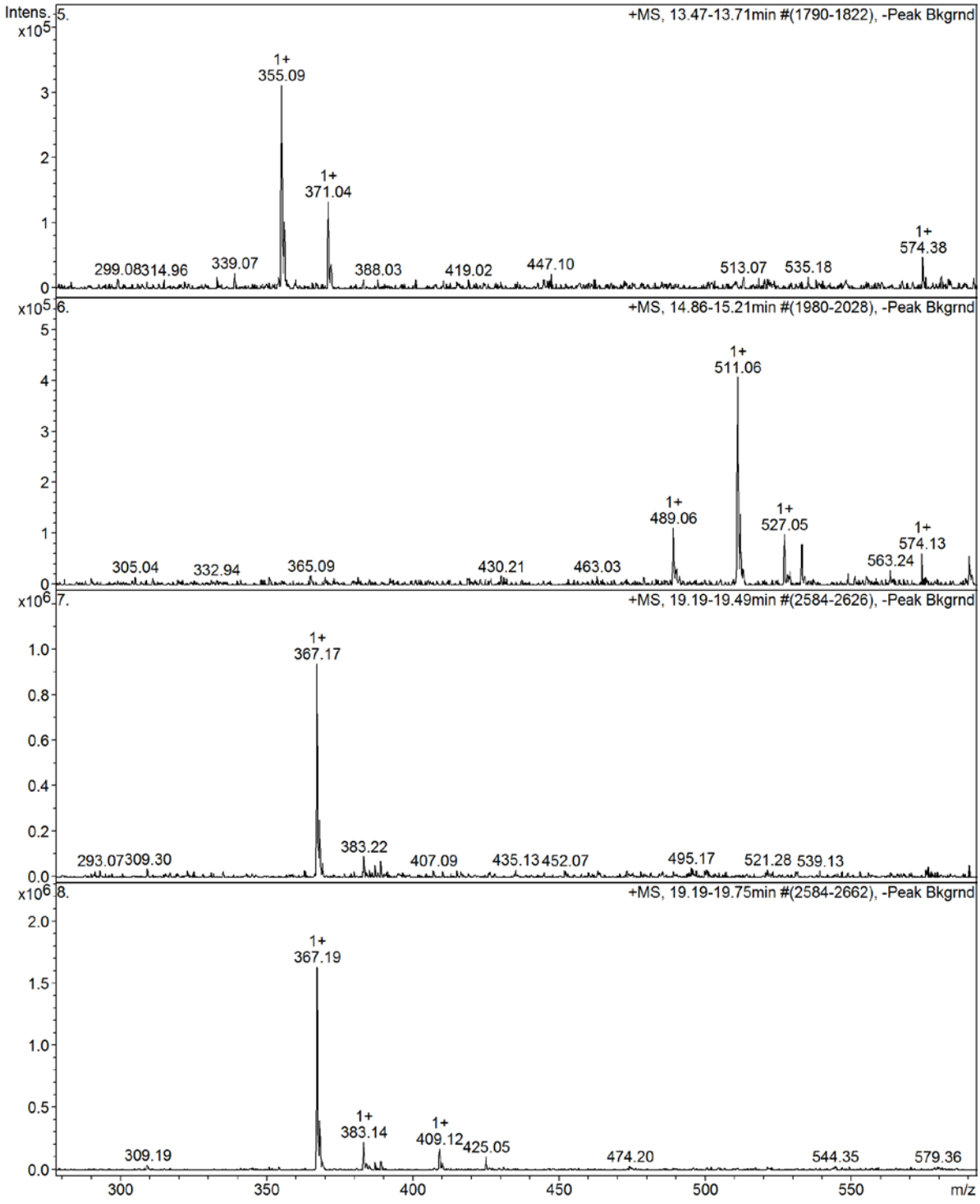
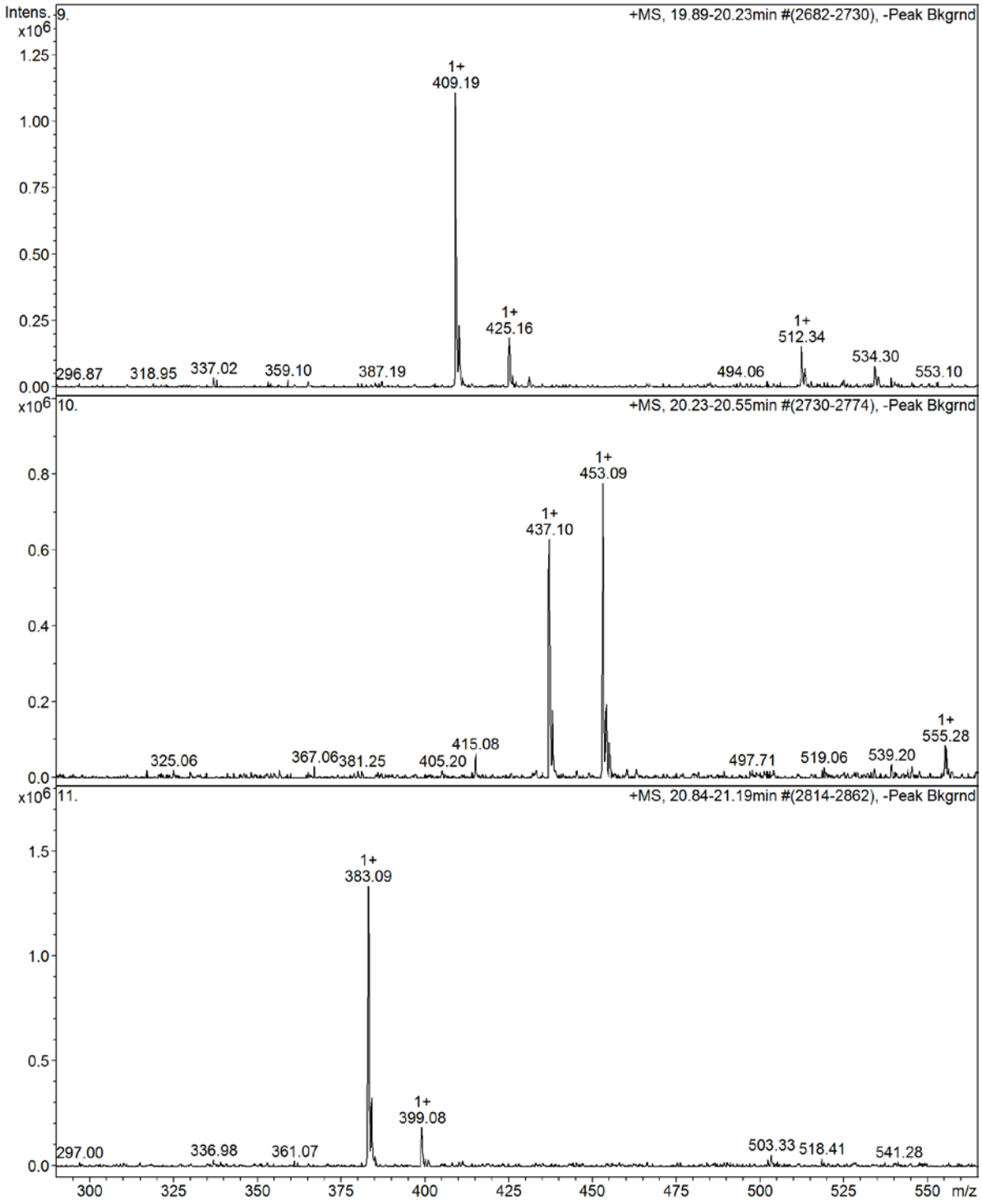

Intens.
x10^5
9.
+MS, 20.03-20.19min #(2698-2720), -Peak Bkgrnd
1+
409.18
275.06
317.22
369.27
459.18
504.33
590.23
627.24
652.52
x10^5
10.
+MS, 20.32-20.53min #(2738-2768), -Peak Bkgrnd
1+
437.12
1+
453.09
1+
555.23
241.14
337.09
402.14
503.43
636.58
668.18
x10^6
11.
+MS, 22.24-22.52min #(3006-3044), -Peak Bkgrnd
1+
367.18
345.17
408.97
443.04
525.18
575.18
643.22
666.32
m/z

Figure 95 A representative LC/MS spectrums from *Oroxylum indicum* root of compound 1-11




Intens.
x10^5
5.
+MS, 13.47-13.71min #(1790-1822), -Peak Bkgrnd
1+
355.09
1+
371.04
1+
574.38
299.08
314.96
339.07
388.03
419.02
447.10
513.07
535.18
x10^5
6.
+MS, 14.86-15.21min #(1980-2028), -Peak Bkgrnd
1+
511.06
1+
489.06
1+
527.05
1+
574.13
305.04
332.94
365.09
430.21
463.03
563.24
x10^6
7.
+MS, 19.19-19.49min #(2584-2626), -Peak Bkgrnd
1+
367.17
293.07
309.30
383.22
407.09
435.13
452.07
495.17
521.28
539.13
x10^6
8.
+MS, 19.19-19.75min #(2584-2662), -Peak Bkgrnd
1+
367.19
1+
383.14
1+
409.12
309.19
425.05
474.20
544.35
579.36
300
350
400
450
500
550
m/z


Intens.
9.
+MS, 19.89-20.23min #(2682-2730), -Peak Bkgrnd
x10^6
1.25
1.00
0.75
0.50
0.25
0.00
1+
409.19
1+
425.16
1+
512.34
534.30
553.10
296.87
318.95
337.02
359.10
387.19
494.06
10.
+MS, 20.23-20.55min #(2730-2774), -Peak Bkgrnd
x10^6
0.8
0.6
0.4
0.2
0.0
1+
453.09
1+
437.10
1+
555.28
325.06
367.06
381.25
405.20
415.08
497.71
519.06
539.20
11.
+MS, 20.84-21.19min #(2814-2862), -Peak Bkgrnd
x10^6
1.5
1.0
0.5
0.0
1+
383.09
1+
399.08
297.00
336.98
361.07
503.33
518.41
541.28
300
325
350
375
400
425
450
475
500
525
550
m/z

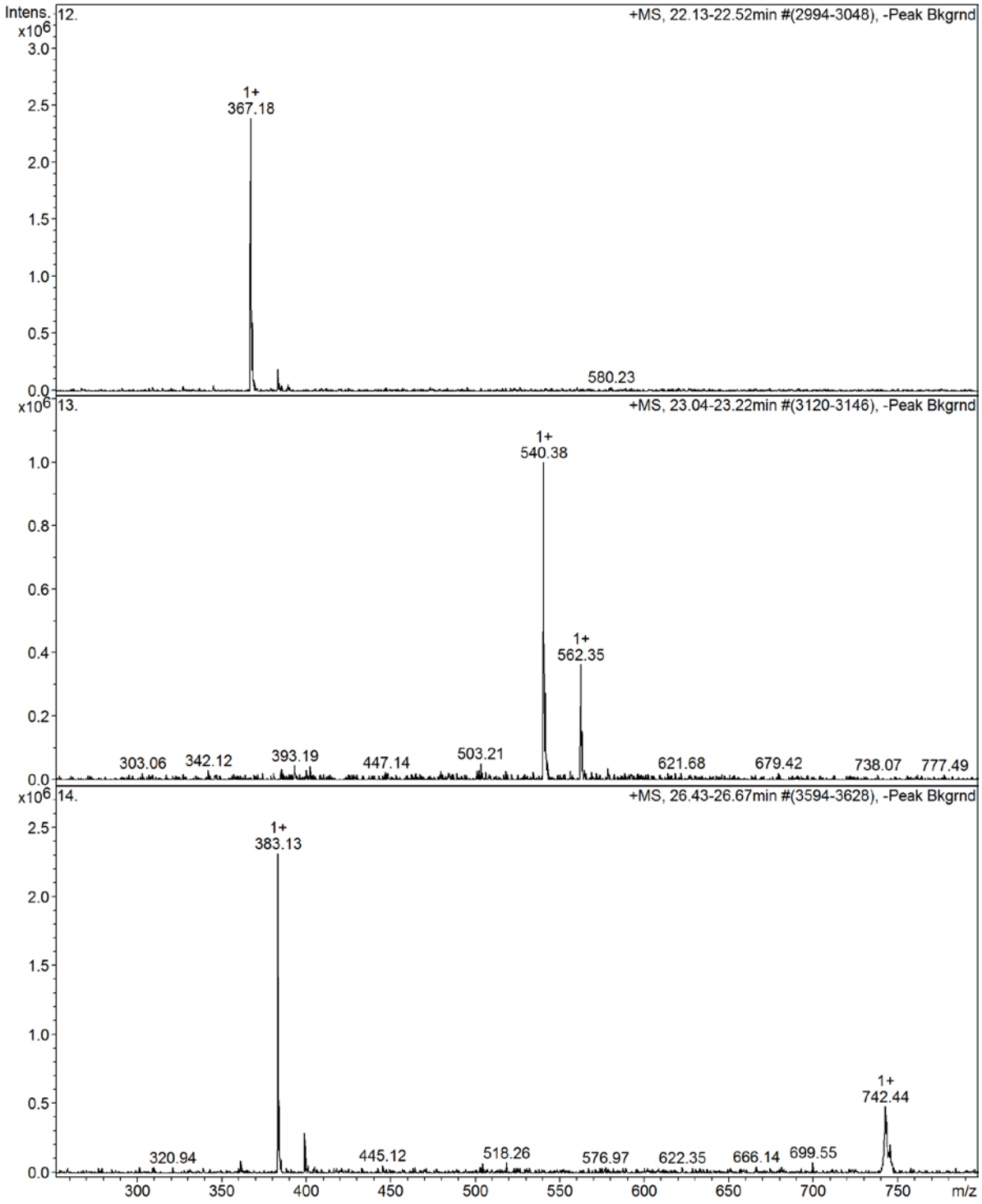
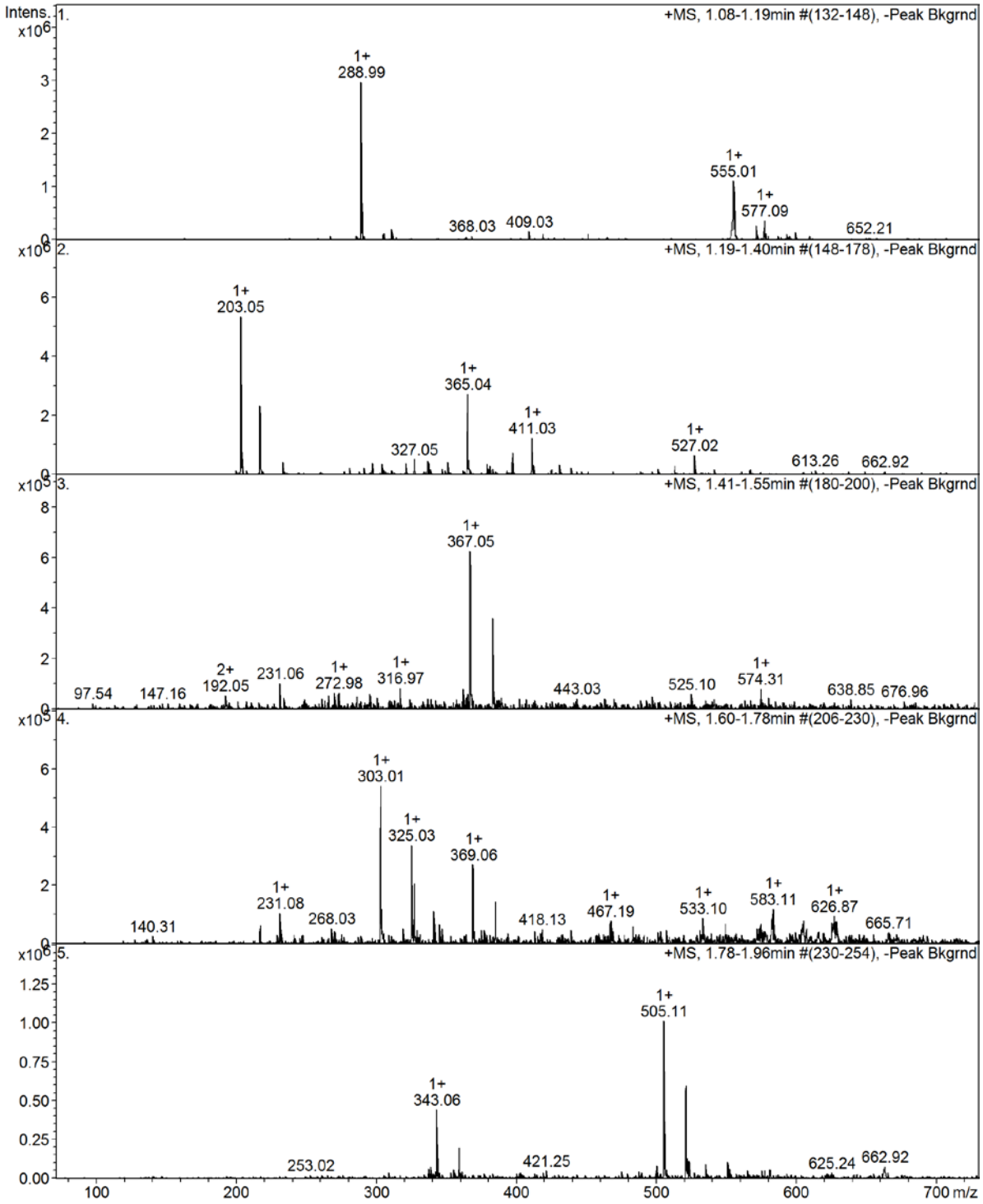
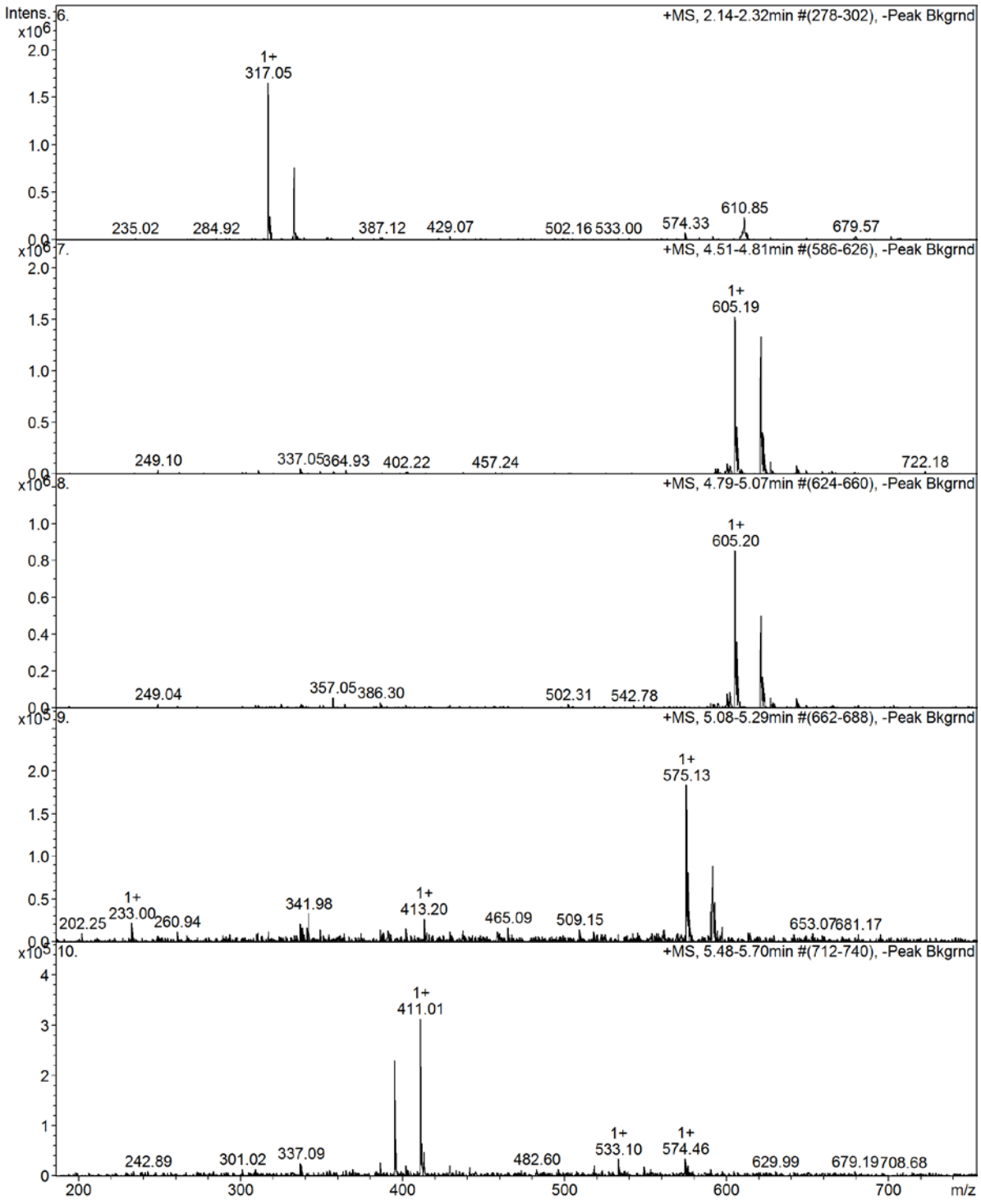
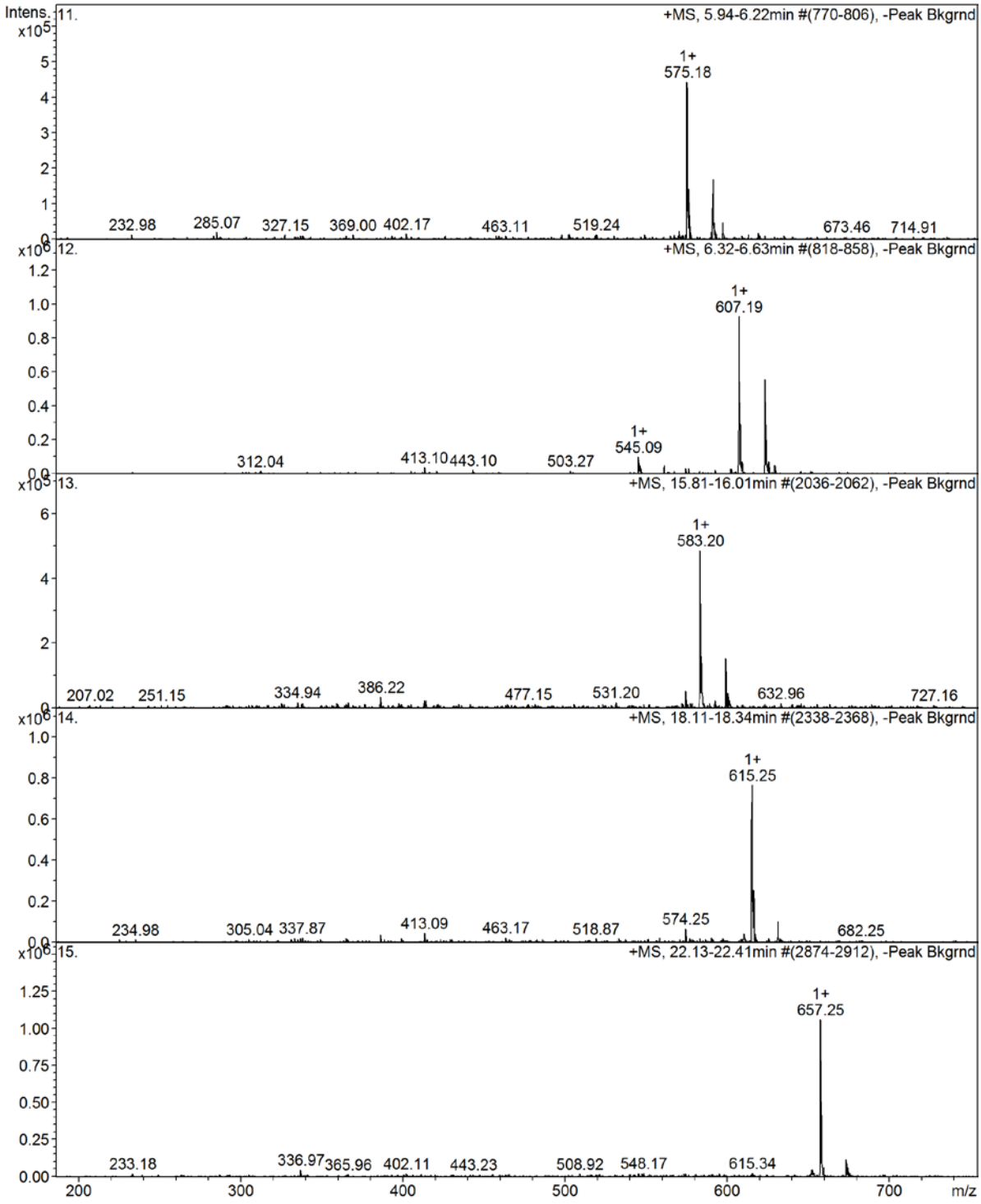
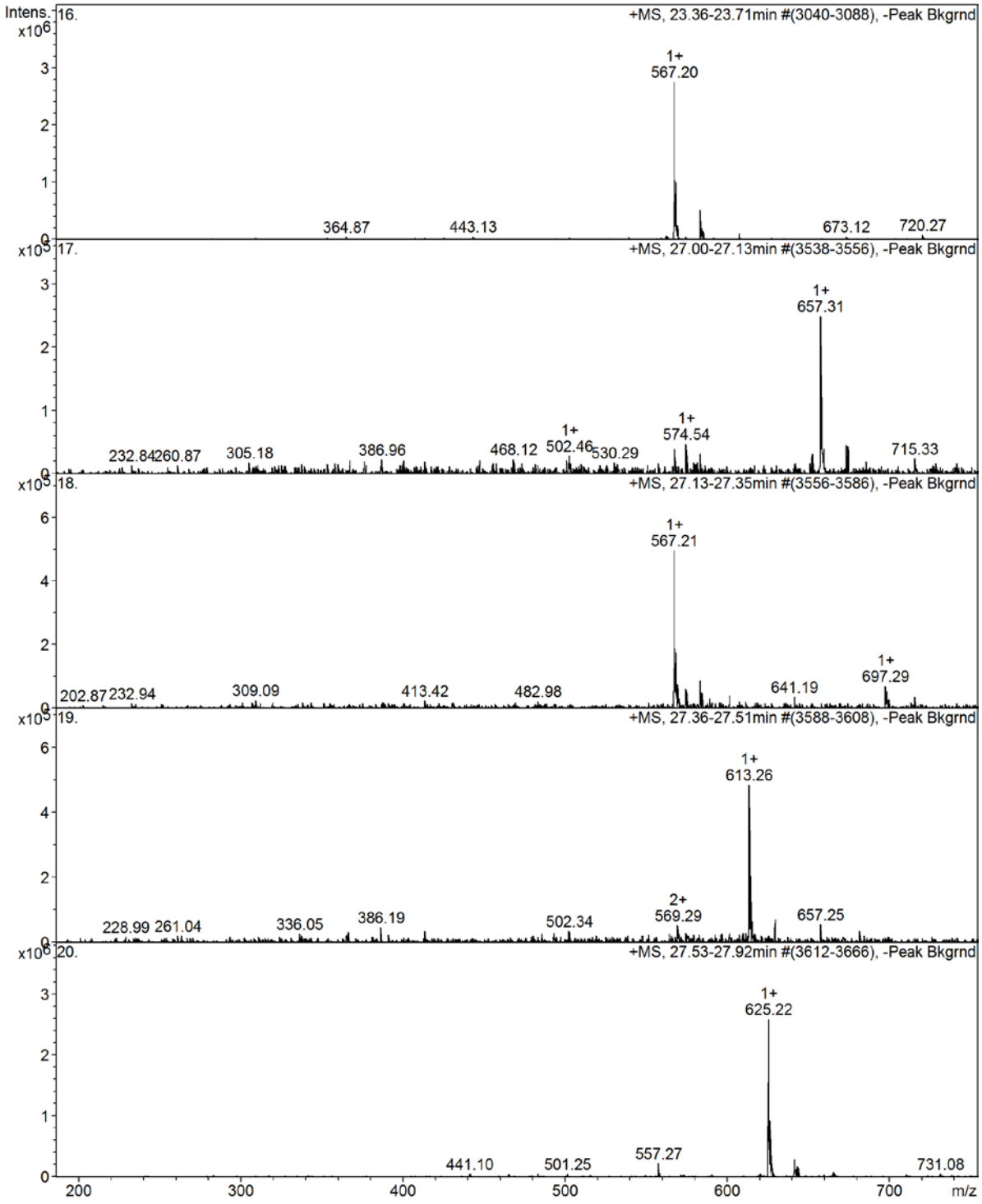

Intens.
x10^6
12.
+MS, 22.13-22.52min #(2994-3048), -Peak Bkgrnd
1+
367.18
580.23
x10^6
13.
+MS, 23.04-23.22min #(3120-3146), -Peak Bkgrnd
1+
540.38
1+
562.35
303.06
342.12
393.19
447.14
503.21
621.68
679.42
738.07
777.49
x10^6
14.
+MS, 26.43-26.67min #(3594-3628), -Peak Bkgrnd
1+
383.13
1+
742.44
320.94
445.12
518.26
576.97
622.35
666.14
699.55
m/z

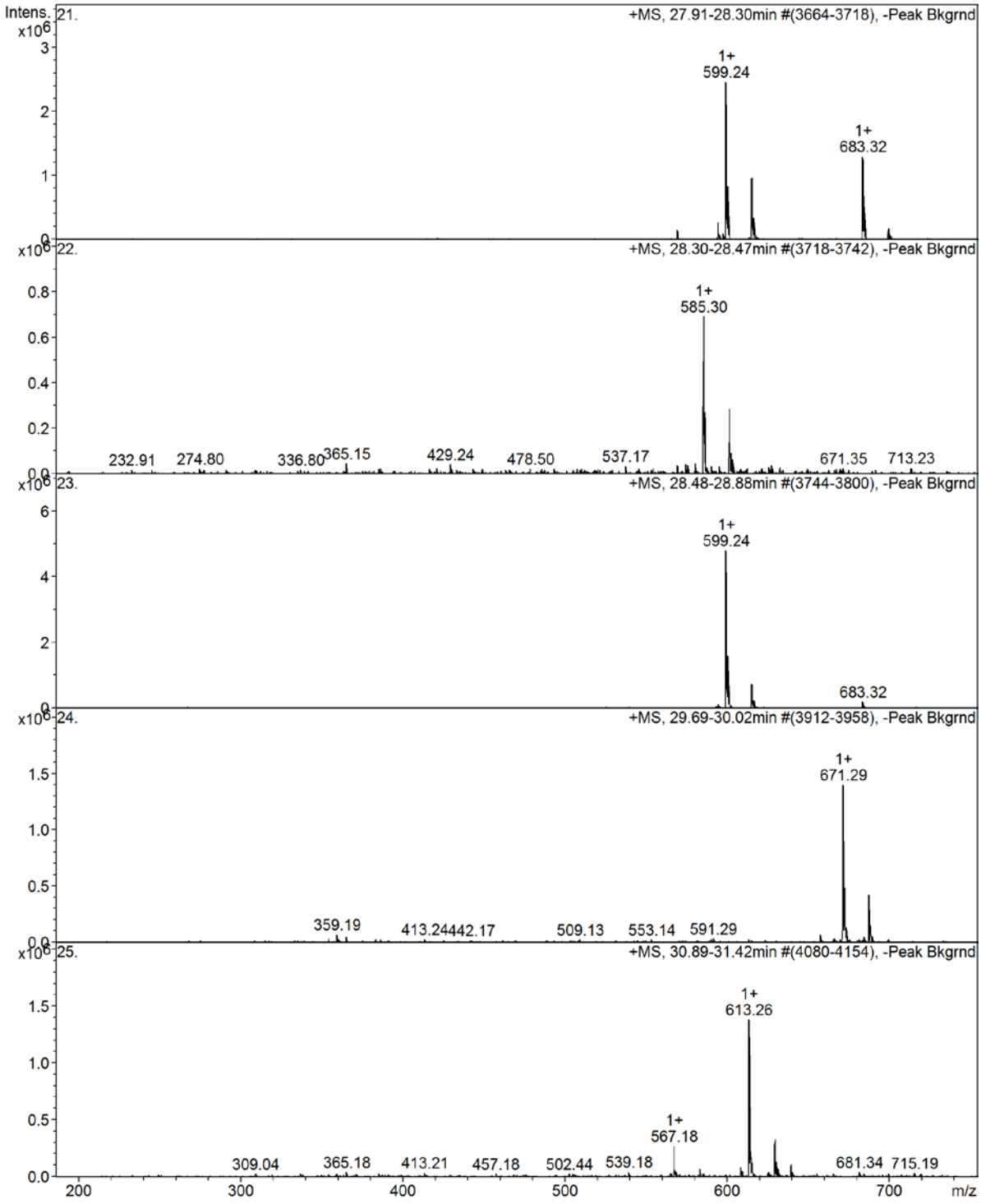
Figure 96 A representative LC/MS spectrums from *Walsura trichostemon* root of compound 1-56

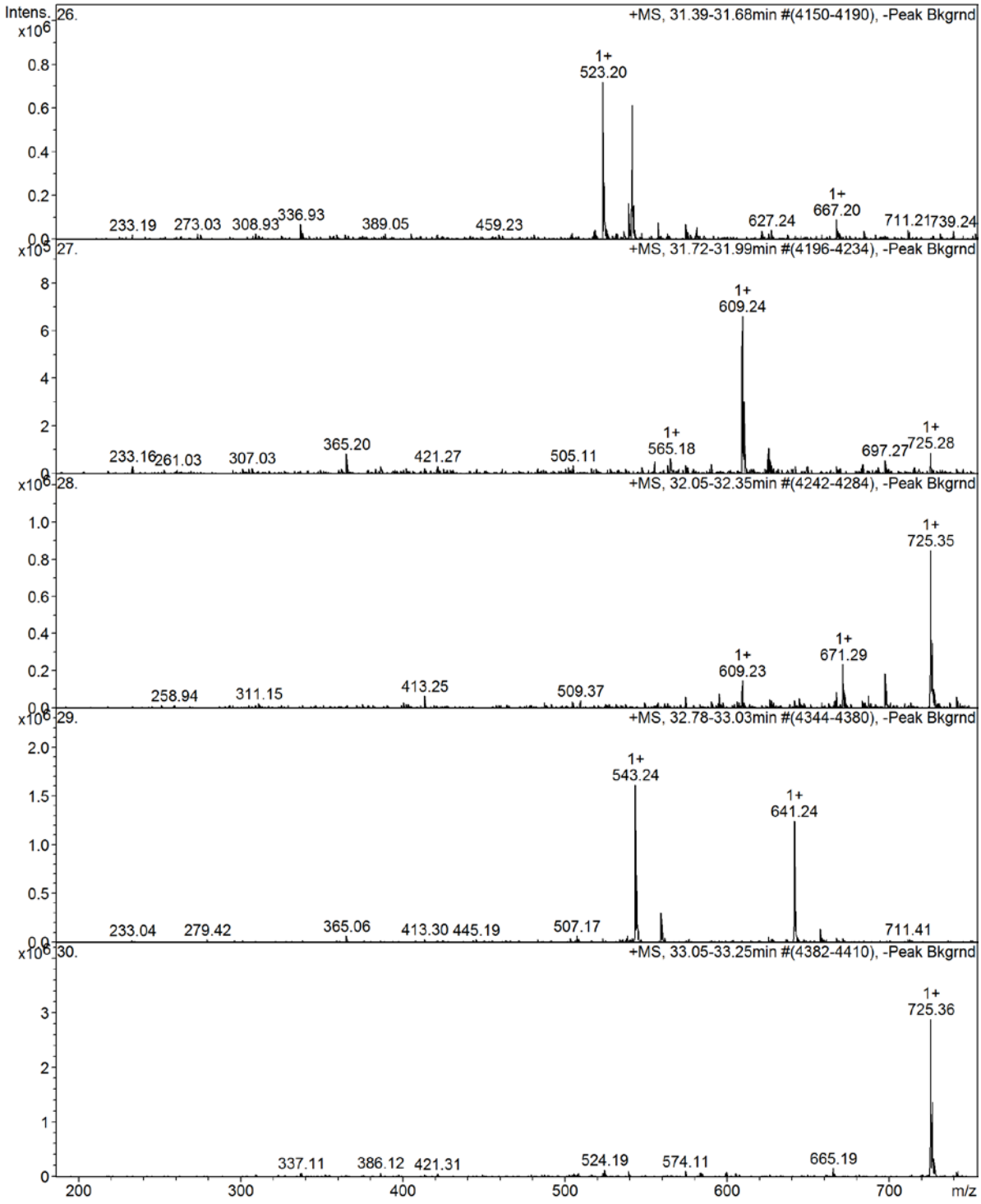


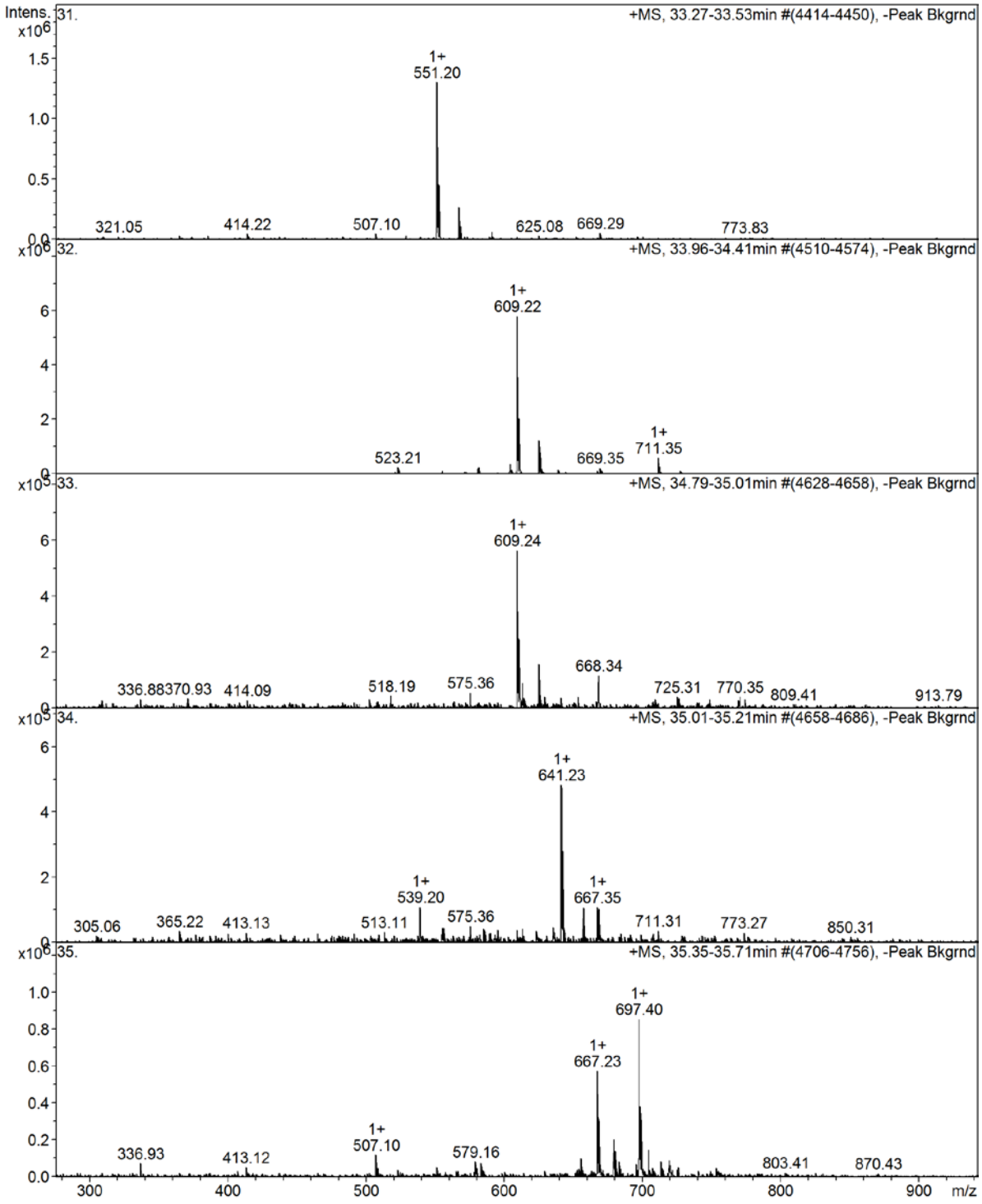

Intens.
6.
x10^6
+MS, 2.14-2.32min #(278-302), -Peak Bkgrnd
1+
317.05
235.02
284.92
387.12
429.07
502.16
533.00
574.33
610.85
679.57
7.
x10^6
+MS, 4.51-4.81min #(586-626), -Peak Bkgrnd
1+
605.19
249.10
337.05
364.93
402.22
457.24
722.18
8.
x10^6
+MS, 4.79-5.07min #(624-660), -Peak Bkgrnd
1+
605.20
249.04
357.05
386.30
502.31
542.78
9.
x10^5
+MS, 5.08-5.29min #(662-688), -Peak Bkgrnd
1+
575.13
1+
233.00
1+
413.20
202.25
260.94
341.98
465.09
509.15
653.07
681.17
10.
x10^5
+MS, 5.48-5.70min #(712-740), -Peak Bkgrnd
1+
411.01
1+
533.10
1+
574.46
242.89
301.02
337.09
482.60
629.99
679.19
708.68
200
300
400
500
600
700
m/z

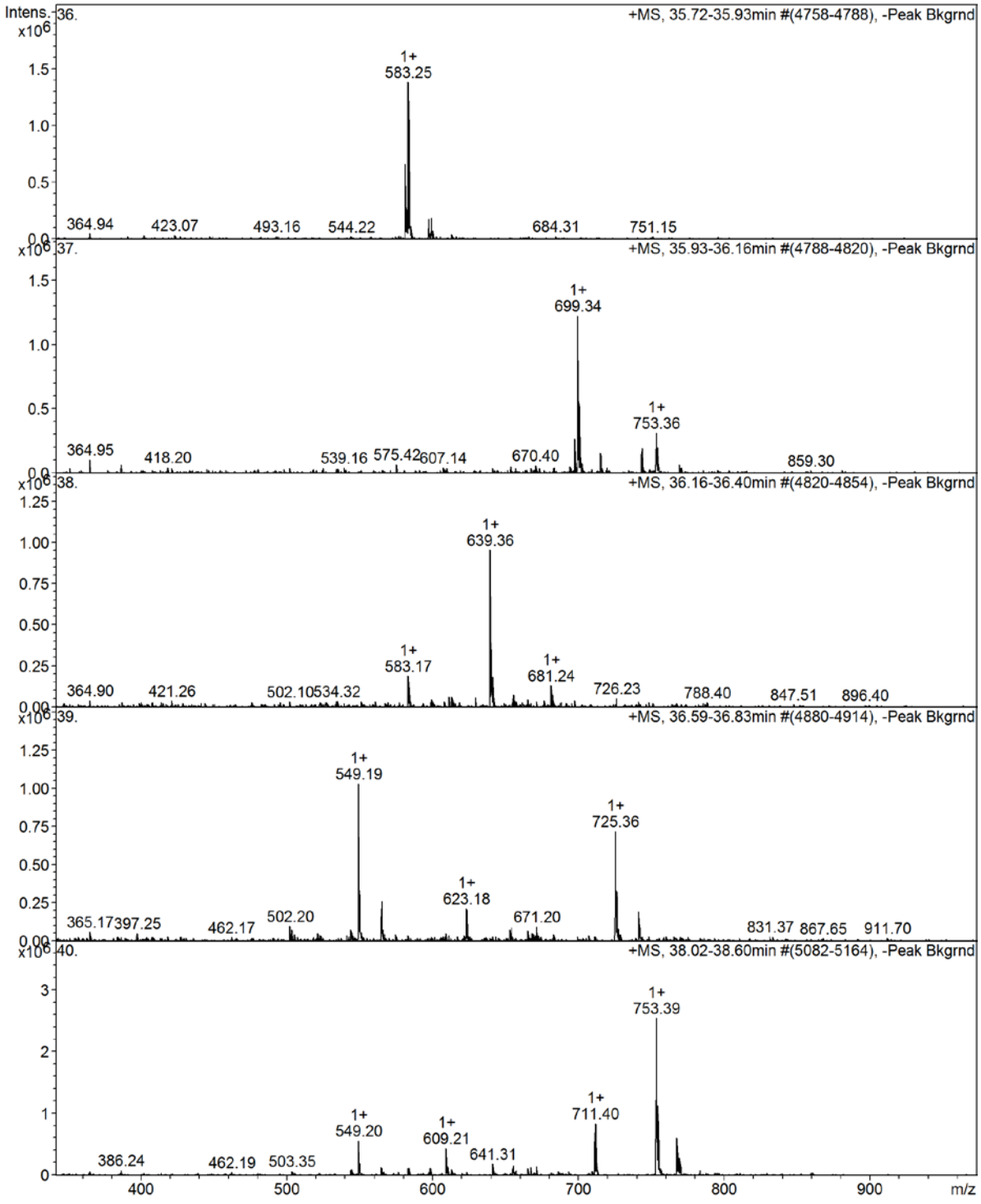

Intens.
11.
+MS, 5.94-6.22min #(770-806), -Peak Bkgrnd
1+
575.18
232.98
285.07
327.15
369.00
402.17
463.11
519.24
673.46
714.91
12.
+MS, 6.32-6.63min #(818-858), -Peak Bkgrnd
1+
607.19
1+
545.09
312.04
413.10
443.10
503.27
13.
+MS, 15.81-16.01min #(2036-2062), -Peak Bkgrnd
1+
583.20
207.02
251.15
334.94
386.22
477.15
531.20
632.96
727.16
14.
+MS, 18.11-18.34min #(2338-2368), -Peak Bkgrnd
1+
615.25
234.98
305.04
337.87
413.09
463.17
518.87
574.25
682.25
15.
+MS, 22.13-22.41min #(2874-2912), -Peak Bkgrnd
1+
657.25
233.18
336.97
365.96
402.11
443.23
508.92
548.17
615.34
200
300
400
500
600
700
m/z

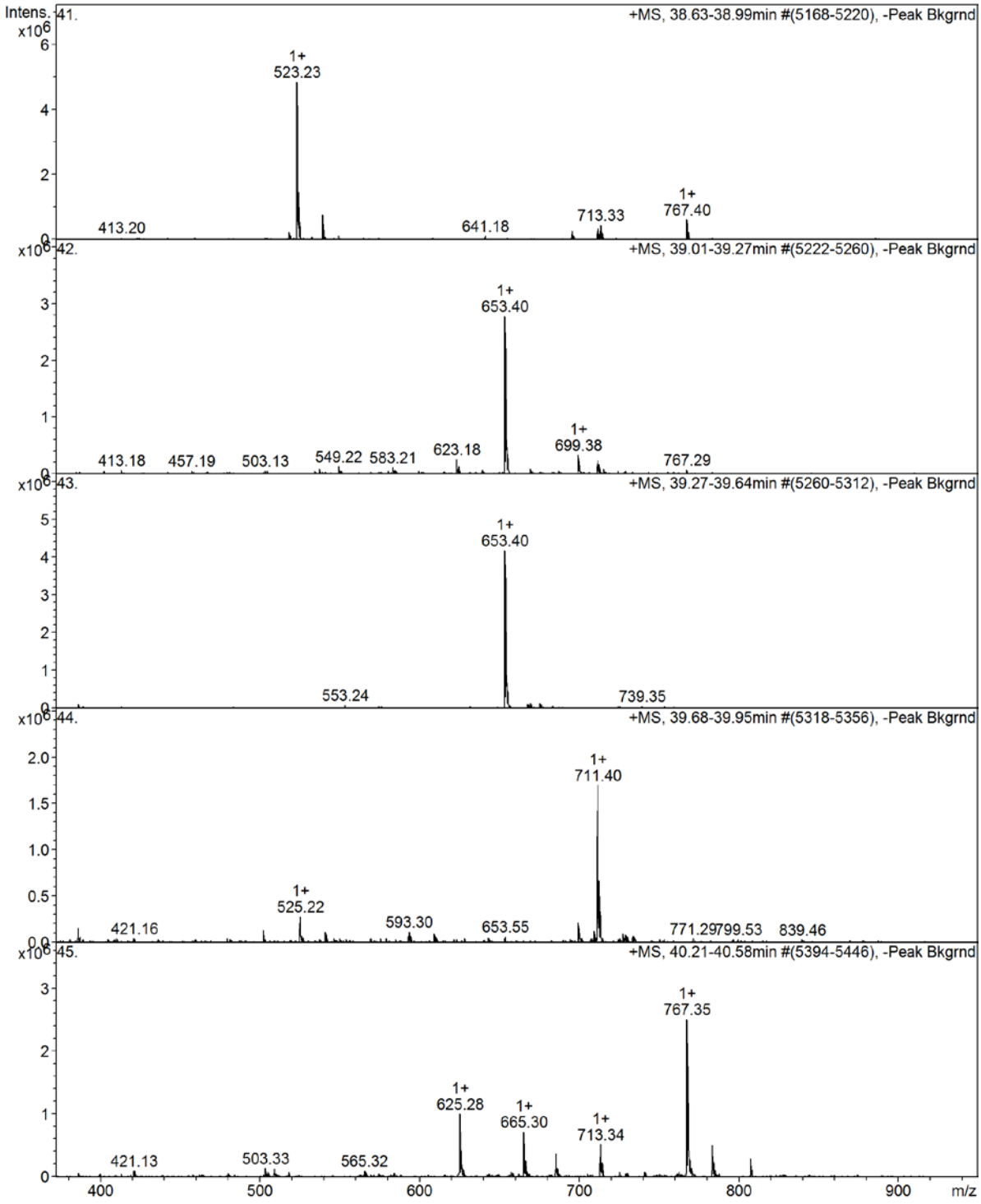

Intens.
16.
+MS, 23.36-23.71min #(3040-3088), -Peak Bkgrnd
1+
567.20
364.87
443.13
673.12
720.27
17.
+MS, 27.00-27.13min #(3538-3556), -Peak Bkgrnd
1+
657.31
1+
574.54
1+
502.46
232.84
260.87
305.18
386.96
468.12
530.29
715.33
18.
+MS, 27.13-27.35min #(3556-3586), -Peak Bkgrnd
1+
567.21
1+
697.29
202.87
232.94
309.09
413.42
482.98
641.19
19.
+MS, 27.36-27.51min #(3588-3608), -Peak Bkgrnd
1+
613.26
2+
569.29
228.99
261.04
336.05
386.19
502.34
657.25
20.
+MS, 27.53-27.92min #(3612-3666), -Peak Bkgrnd
1+
625.22
441.10
501.25
557.27
731.08
200
300
400
500
600
700
m/z

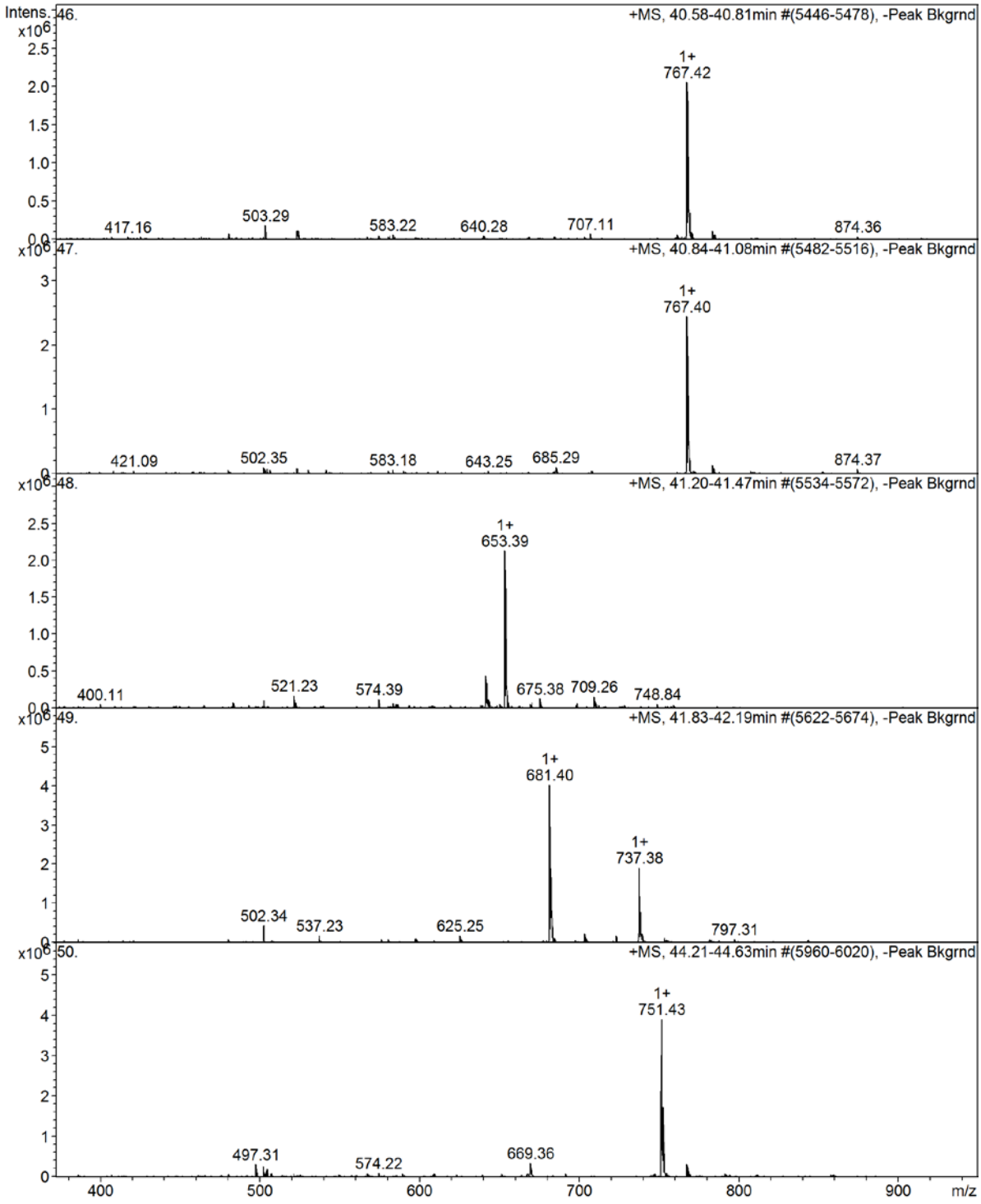

Intens.
x10^6
21.
+MS, 27.91-28.30min #(3664-3718), -Peak Bkgrnd
1+
599.24
1+
683.32
x10^6
22.
+MS, 28.30-28.47min #(3718-3742), -Peak Bkgrnd
1+
585.30
232.91
274.80
336.80
365.15
429.24
478.50
537.17
671.35
713.23
x10^6
23.
+MS, 28.48-28.88min #(3744-3800), -Peak Bkgrnd
1+
599.24
683.32
x10^6
24.
+MS, 29.69-30.02min #(3912-3958), -Peak Bkgrnd
1+
671.29
359.19
413.24442.17
509.13
553.14
591.29
x10^6
25.
+MS, 30.89-31.42min #(4080-4154), -Peak Bkgrnd
1+
613.26
1+
567.18
309.04
365.18
413.21
457.18
502.44
539.18
681.34
715.19
200
300
400
500
600
700
m/z


Intens.
26.
+MS, 31.39-31.68min #(4150-4190), -Peak Bkgrnd
1+
523.20
1+
667.20
233.19
273.03
308.93
336.93
389.05
459.23
627.24
711.21
739.24
27.
+MS, 31.72-31.99min #(4196-4234), -Peak Bkgrnd
1+
609.24
1+
565.18
1+
725.28
233.16
261.03
307.03
365.20
421.27
505.11
697.27
28.
+MS, 32.05-32.35min #(4242-4284), -Peak Bkgrnd
1+
725.35
1+
671.29
1+
609.23
258.94
311.15
413.25
509.37
29.
+MS, 32.78-33.03min #(4344-4380), -Peak Bkgrnd
1+
543.24
1+
641.24
233.04
279.42
365.06
413.30
445.19
507.17
711.41
30.
+MS, 33.05-33.25min #(4382-4410), -Peak Bkgrnd
1+
725.36
337.11
386.12
421.31
524.19
574.11
665.19
m/z


Intens.
31.
+MS, 33.27-33.53min #(4414-4450), -Peak Bkgrnd
1+
551.20
321.05
414.22
507.10
625.08
669.29
773.83
32.
+MS, 33.96-34.41min #(4510-4574), -Peak Bkgrnd
1+
609.22
523.21
669.35
1+
711.35
33.
+MS, 34.79-35.01min #(4628-4658), -Peak Bkgrnd
1+
609.24
668.34
336.88370.93
414.09
518.19
575.36
725.31
770.35
809.41
913.79
34.
+MS, 35.01-35.21min #(4658-4686), -Peak Bkgrnd
1+
641.23
1+
539.20
1+
667.35
305.06
365.22
413.13
513.11
575.36
711.31
773.27
850.31
35.
+MS, 35.35-35.71min #(4706-4756), -Peak Bkgrnd
1+
697.40
1+
667.23
1+
507.10
336.93
413.12
579.16
803.41
870.43
300
400
500
600
700
800
900
m/z


Intens.
36.
+MS, 35.72-35.93min #(4758-4788), -Peak Bkgrnd
1+
583.25
364.94
423.07
493.16
544.22
684.31
751.15
37.
+MS, 35.93-36.16min #(4788-4820), -Peak Bkgrnd
1+
699.34
1+
753.36
364.95
418.20
539.16
575.42
607.14
670.40
859.30
38.
+MS, 36.16-36.40min #(4820-4854), -Peak Bkgrnd
1+
639.36
1+
583.17
1+
681.24
364.90
421.26
502.10
534.32
726.23
788.40
847.51
896.40
39.
+MS, 36.59-36.83min #(4880-4914), -Peak Bkgrnd
1+
549.19
1+
725.36
1+
623.18
365.17
397.25
462.17
502.20
671.20
831.37
867.65
911.70
40.
+MS, 38.02-38.60min #(5082-5164), -Peak Bkgrnd
1+
753.39
1+
711.40
1+
549.20
1+
609.21
386.24
462.19
503.35
641.31
400
500
600
700
800
900
m/z


Intens.
x10^6
41.
+MS, 38.63-38.99min #(5168-5220), -Peak Bkgrnd
1+
523.23
413.20
641.18
713.33
1+
767.40
x10^6
42.
+MS, 39.01-39.27min #(5222-5260), -Peak Bkgrnd
1+
653.40
413.18
457.19
503.13
549.22
583.21
623.18
1+
699.38
767.29
x10^6
43.
+MS, 39.27-39.64min #(5260-5312), -Peak Bkgrnd
1+
653.40
553.24
739.35
x10^6
44.
+MS, 39.68-39.95min #(5318-5356), -Peak Bkgrnd
1+
711.40
1+
525.22
421.16
593.30
653.55
771.29
799.53
839.46
x10^6
45.
+MS, 40.21-40.58min #(5394-5446), -Peak Bkgrnd
1+
767.35
1+
625.28
1+
665.30
1+
713.34
421.13
503.33
565.32
400
500
600
700
800
900
m/z


Intens.
46.
+MS, 40.58-40.81min #(5446-5478), -Peak Bkgrnd
1+
767.42
417.16
503.29
583.22
640.28
707.11
874.36
47.
+MS, 40.84-41.08min #(5482-5516), -Peak Bkgrnd
1+
767.40
421.09
502.35
583.18
643.25
685.29
874.37
48.
+MS, 41.20-41.47min #(5534-5572), -Peak Bkgrnd
1+
653.39
400.11
521.23
574.39
675.38
709.26
748.84
49.
+MS, 41.83-42.19min #(5622-5674), -Peak Bkgrnd
1+
681.40
1+
737.38
502.34
537.23
625.25
797.31
50.
+MS, 44.21-44.63min #(5960-6020), -Peak Bkgrnd
1+
751.43
497.31
574.22
669.36
400
500
600
700
800
900
m/z

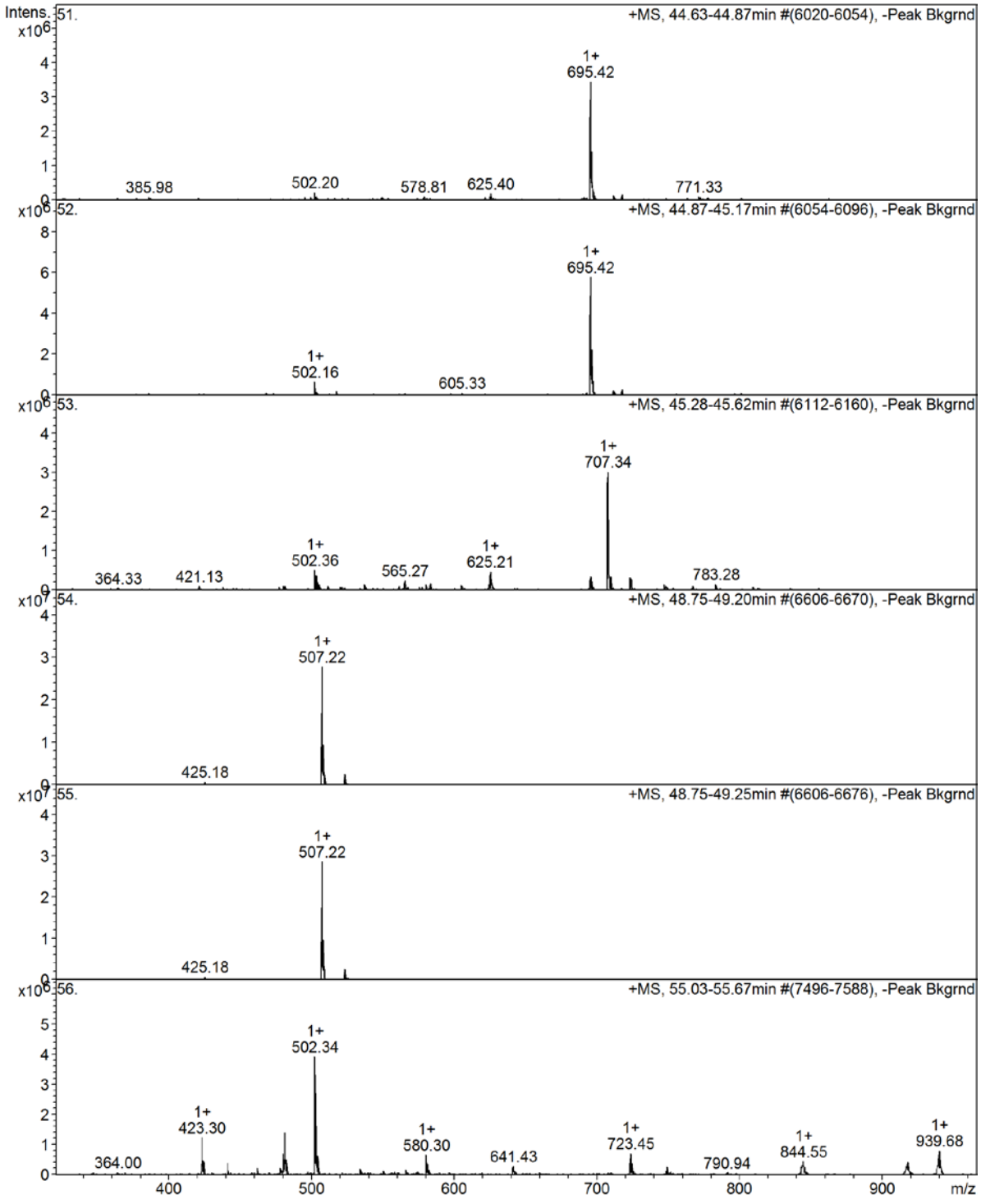
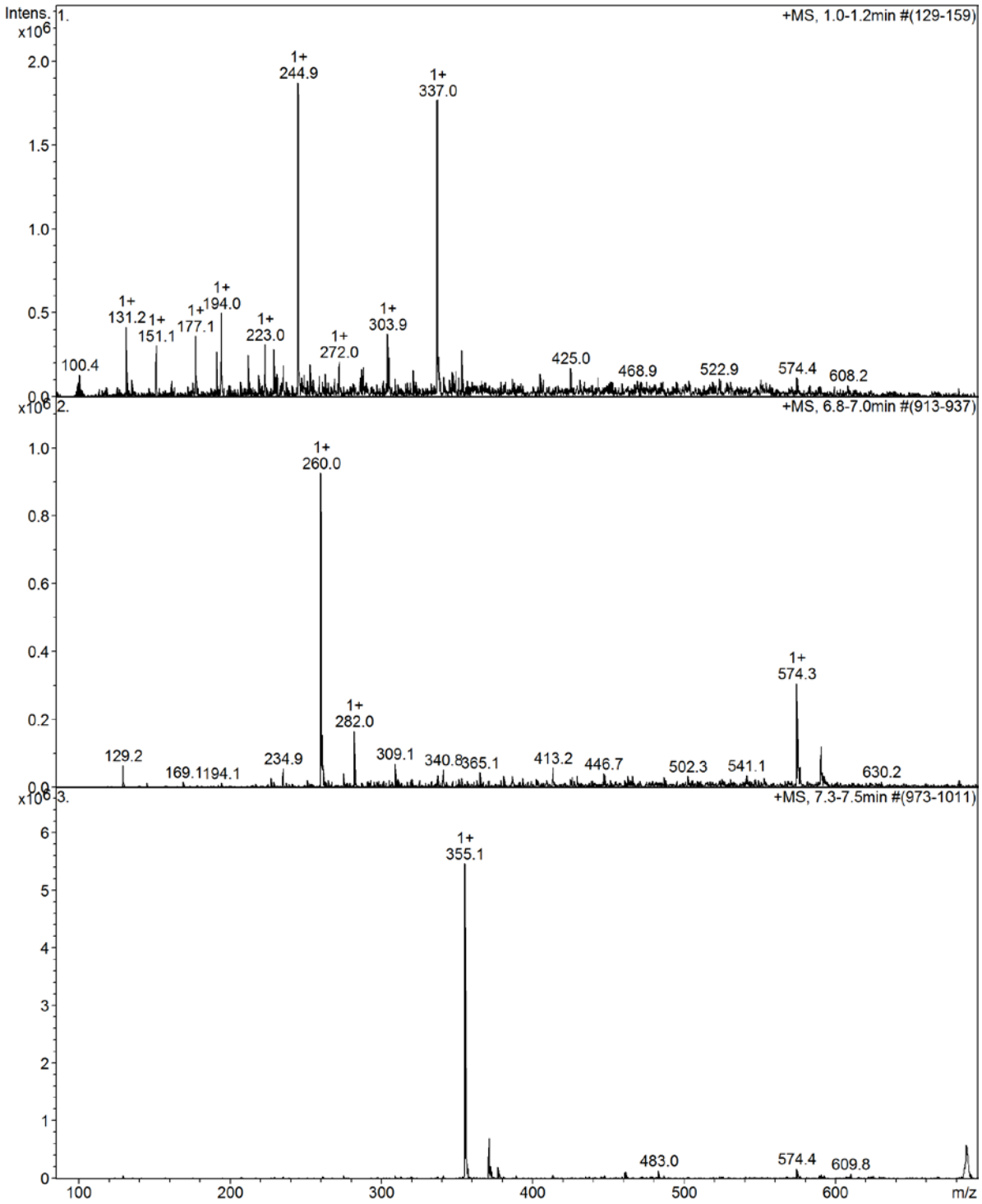
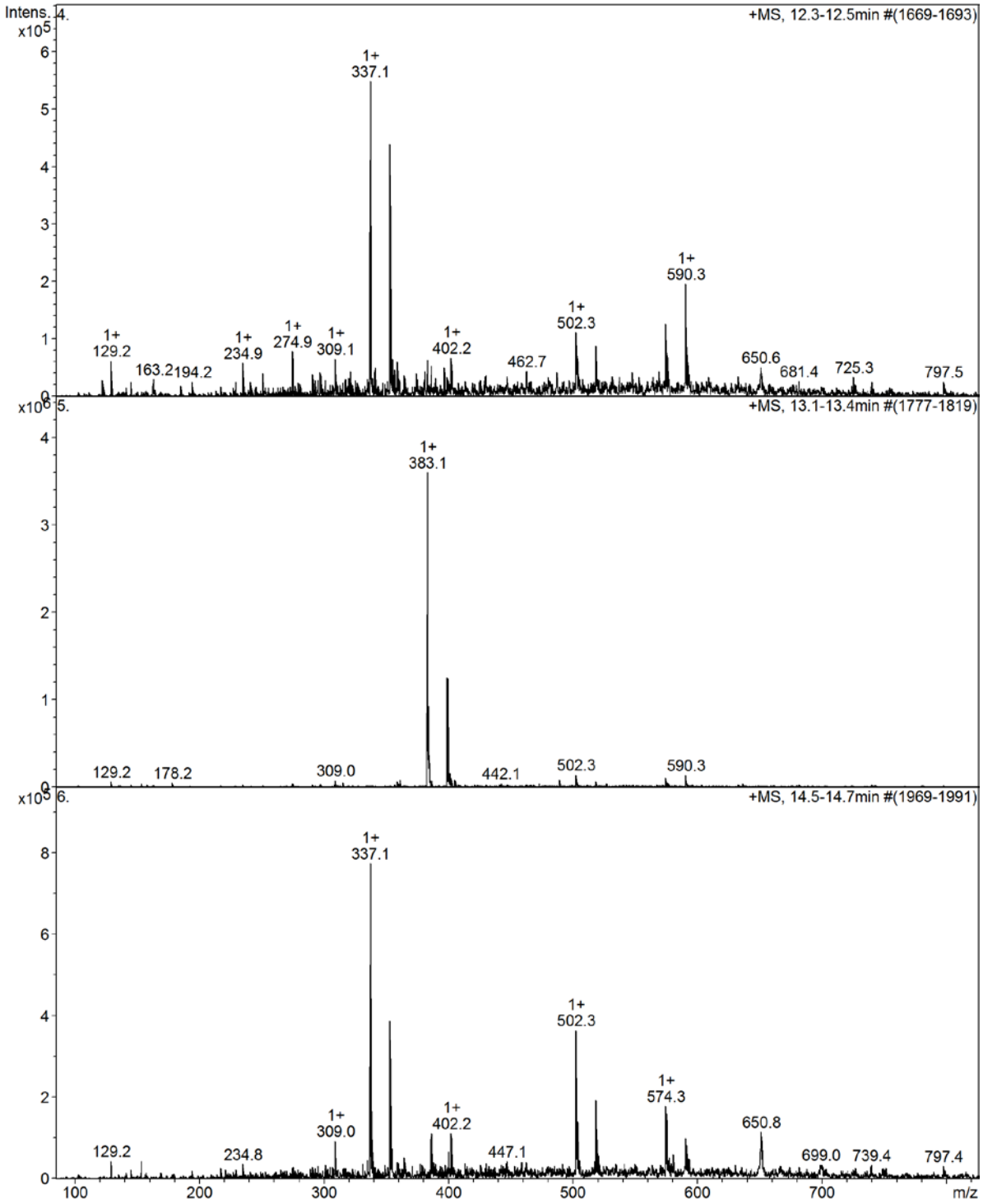

Intens.
51.
+MS, 44.63-44.87min #(6020-6054), -Peak Bkgrnd
1+
695.42
385.98
502.20
578.81
625.40
771.33
52.
+MS, 44.87-45.17min #(6054-6096), -Peak Bkgrnd
1+
695.42
1+
502.16
605.33
53.
+MS, 45.28-45.62min #(6112-6160), -Peak Bkgrnd
1+
707.34
1+
502.36
1+
625.21
364.33
421.13
565.27
783.28
54.
+MS, 48.75-49.20min #(6606-6670), -Peak Bkgrnd
1+
507.22
425.18
55.
+MS, 48.75-49.25min #(6606-6676), -Peak Bkgrnd
1+
507.22
425.18
56.
+MS, 55.03-55.67min #(7496-7588), -Peak Bkgrnd
1+
502.34
1+
423.30
1+
580.30
1+
723.45
1+
844.55
1+
939.68
364.00
641.43
790.94
400
500
600
700
800
900
m/z

Figure 97 A representative LC/MS spectrums from Ben-Cha-Moon-Yai remedy extract of compound 1-6




Intens.
x10^5
4.
+MS, 12.3-12.5min #(1669-1693)
1+ 337.1
1+ 590.3
1+ 502.3
1+ 129.2
1+ 234.9
1+ 274.9
1+ 309.1
1+ 402.2
163.2
194.2
462.7
650.6
681.4
725.3
797.5
x10^5
5.
+MS, 13.1-13.4min #(1777-1819)
1+ 383.1
129.2
178.2
309.0
442.1
502.3
590.3
x10^5
6.
+MS, 14.5-14.7min #(1969-1991)
1+ 337.1
1+ 502.3
1+ 574.3
1+ 309.0
1+ 402.2
129.2
234.8
447.1
650.8
699.0
739.4
797.4
100
200
300
400
500
600
700
m/z

APPENDIX C

Mutagenic activity

Table 56 Mutagenicity of Ben-Cha-Moon-Yai Remedy and its ingredients (200 mg/ml) in acid solution pH3.0-3.5 on *Salmonella typhimurium* **TA98** (frameshift mutation) without metabolic activation

Sample	Amount of extract (mg/plate)	Number of revertants/plate[a]			
		Ethanolic extract		Water extract	
		w/o nitrite	With nitrite	w/o nitrite	With nitrite
Ben-Cha-Moon-Yai Remedy	Positive[b]		1562.33± 205.06		
	Spontaneous[c]	19.33 ± 4.04	17.5 ± 0.71		
	0.4	29 ± 2.65	102 ± 17.09		
	0.8	27.67 ± 7.09	191 ± 75.43		
	1.6	18.33 ± 4.93	251.67 ± 63.57		
	3.2	18 ± 2.65	347 ± 18.36		
Aegle marmelos	Positive[b]				3800 ± 746.70
	Spontaneous[c]	17.5 ± 6.36	44.67 ± 16.26	34.67 ± 8.33	15.67 ± 5.69
	0.4	23.5 ± 13.44	83.67 ± 13.65	37 ± 4.24	35.67 ± 8.02
	0.8	14.67 ± 5.51	136.33 ± 41.43	47 ± 21	50 ± 7
	1.6	14.67 ± 0.58	151.33 ± 7.37	102.33±39.11	131 ± 42.43
	3.2	17 ± 4	221 ± 7	787.6 ± 26.84	183.5 ± 16.26
Oroxylum indicum	Positive[b]		1562.33 ±205.06		
	Spontaneous[c]	17.5 ± 6.36	17.5 ± 0.71	34.67 ± 8.33	17.5 ± 0.71
	0.4	23.33 ± 4.04	217.67 ± 39.26	23.33 ± 0.58	20 ± 10.54
	0.8	23.33 ± 4.16	210.33 ± 25.50	25 ± 8.49	51 ± 10.82
	1.6	13.67 ± 6.43	253.67 ± 35.23	28.67 ± 8.39	90.67 ± 19.86
	3.2	16 ± 3.46	155.67 ± 19.22	21.67 ± 7.23	123.67 ± 32.87
Dimocarpus longan	Positive[b]		1562.33± 205.06		3800 ± 746.70
	Spontaneous[c]	19.33 ± 4.04	17.5 ± 0.71	34.67 ± 8.33	15.67 ± 5.69
	0.4	22.33 ± 3.79	217.67 ± 39.26	27.67 ± 6.66	45 ± 15.56
	0.8	24 ± 3.61	210.33 ± 25.50	27 ± 1.41	208.5 ± 4.95
	1.6	22.67 ± 3.79	253.67 ± 35.23	25.67 ± 1.53	73 ± 16.97
	3.2	13 ± 1.41	155.67 ± 19.22	18.33 ± 6.81	125.55 ± 90.79

Walsura trichostemon	Positive[b]				1872.33± 48.18
	Spontaneous[c]	17.5 ± 6.36	44.67 ± 16.26	34.67 ± 8.33	36.33 ± 5.13
	0.4	27 ± 4.36	27.33 ± 2.08	19 ± 3	58 ± 8.19
	0.8	27.33 ± 3.51	29.67 ± 7.37	20 ± 0	80.33 ± 19.86
	1.6	28 ± 17.06	57.33 ± 14.29	29.33 ± 3.21	102.33 ± 6.66
	3.2	24 ± 5.66	159.67 ± 75.53	28.33 ± 4.93	68.67 ± 5.51
Dolichandrone serrulata	Positive[b]		1872.33 ± 48.18		
	Spontaneous[c]	19.33 ± 4.04	36.33 ± 5.13	34.67 ± 8.33	17.5 ± 0.71
	0.4	51 ± 18.38	32 ± 2.65	28.33 ± 10.07	16.67 ± 2.52
	0.8	58.67 ± 22.28	29.67 ± 5.69	20.67 ± 7.02	21.33 ± 5.51
	1.6	72 ± 1.41	23.33 ± 5.13	32.67 ± 11.06	23.33 ± 2.08
	3.2	57.33 ± 3.21	21.33 ± 2.08	40.33 ± 12.22	26 ± 5

Table 57 Mutagenicity of Ben-Cha-Moon-Yai Remedy and its ingredients (200 mg/ml) in acid solution pH3.0-3.5 on *Salmonella typhimurium* **TA100** (basesubstitution mutation) without metabolic activation

Sample	Amount of extract (mg/plate)	Number of revertants/plate[a]			
		Ethanolic extract		Water extract	
		w/o nitrite	With nitrite	w/o nitrite	With nitrite
Ben-Cha-Moon-Yai Remedy	Positive[b]		481 ± 51.16		
	Spontaneous[c]	80 ± 1.53	49.67 ± 3.06		
	0.4	78.67 ± 11.85	215 ± 35.64		
	0.8	94 ± 25.12	194.67 ± 24.99		
	1.6	79.33 ± 18.82	276 ± 19.52		
	3.2	79.33 ± 18.82	392.33 ± 62.61		
Aegle marmelos	Positive[b]				864.33 ±151.66
	Spontaneous[c]	127 ± 9.54	134.67 ± 43.14	127 ± 9.54	126.67 ± 14.01
	0.4	104.67 ± 16.20	195.67 ± 8.74	112±12.73	285.67 ± 40.53
	0.8	119.33 ± 0.94	205.33 ± 50.05	135.67±12.01	371.67 ± 20.43
	1.6	146 ± 1.15	296 ± 2.83	169±44.24	425.67 ± 7.37
	3.2	134.33 ± 22.23	141.67 ± 38.55	819.5±6.36	513.33 ± 37.00
Oroxylum indicum	Positive[b]		481 ± 51.16		
	Spontaneous[c]	80 ± 1.53	49.67 ± 3.06	127 ± 9.54	49.67 ± 3.06
	0.4	82 ± 24.25	244.33 ± 86.38	85.33±30.27	175.67 ± 51.78
	0.8	86 ± 22.65	343.33 ± 88.08	116.33±39.55	206.67 ± 37.31
	1.6	87 ± 11.53	366.5 ± 143.39	128.33±11.06	313.33 ± 27.30
	3.2	98.67 ± 34.53	428 ± 78.46	139.5±7.78	370.33 ± 25.54
Dimocarpus longan	Positive[b]		481 ± 51.16		864.33 ±151.66
	Spontaneous[c]	80 ± 1.53	49.67 ± 3.06	127 ± 9.54	126.67 ± 14.01
	0.4	71.33 ± 8.02	131.67 ± 24.34	97±25.12	338.67± 103.36
	0.8	62.33 ± 2.08	140.33 ± 26.58	106±20.30	293.33 ± 84.11
	1.6	61 ± 4	198.67 ± 39.00	81.33±32.01	350.67 ± 94.16
	3.2	62 ± 6.93	187.33 ± 36.12	86±20.07	406 ± 52.94

Walsura trichostemon	Positive[b]				864.33 ±151.66
	Spontaneous[c]	127 ± 9.54	134.67 ± 43.14	127 ± 9.54	126.67 ± 14.01
	0.4	108±0.85	116.5 ± 26.16	71.67±17.16	305.33 ± 72.98
	0.8	108.33±42.90	160.67 ± 22.59	93.33±16.07	396 ± 83.44
	1.6	88±28.83	222 ± 44.24	122.33±15.14	346 ± 33.87
	3.2	96.97±30.27	206 ± 33.51	90.33±19.86	432 ± 66.47
Dolichandrone serrulata	Positive[b]				
	Spontaneous[c]	80 ± 1.53	134.67 ± 43.14	127 ± 9.54	49.67 ± 3.06
	0.4	61.67 ± 10.26	118 ± 28.48	111.33±11.50	98.33 ± 18.45
	0.8	63.67 ± 5.69	98.67 ± 12.10	112±21.66	116.33 ± 23.97
	1.6	99.67 ± 7.02	71 ± 23.64	112±18.52	152.33 ± 23.63
	3.2	76 ± 15.52	70.33 ± 21.96	113±20.07	162.33 ± 29.14

*a is a number of revertant colonies per plate

b is a number of revertant colonies after nitrite treated 1-aminopyrine

c is a number of spontaneous revertant colonies

VITA

Name: Miss Rawiwan Manohan **Born:** November 17, 1985, Chiang Rai, Thailand.
Education: Bachelor of Applied Thai Traditional Medicine (2nd class honor), School of Health Sciences, Mae Fah Luang University, Thailand in 2008.

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Poster Presentation:

1. Manohan, R., Palanuvej, C., Ruangrungsi, N. "Cytotoxic and Free radical scavenging activities of Ben-Cha-Moon-Yai Remedy" The 9th Joint Seminar of NRCT-JSPS Core University Program on Natural Medicine in Pharmaceutical Sciences, December 8-9, 2010, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.
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3. Manohan, R., Palanuvej, C., Ruangrungsi, N. "Mutagenicity of Ben-Cha-Moon-Yai Remedy using Ames test" The Proceeding of 34th Pharmacological and Therapeutic Society of Thailand Meeding, March 22-24, 2011, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand.
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1. Manohan, R., Palanuvej, C., Ruangrungsi, N. "Mutagenicity and Cytotoxicity of Ben-Cha-Moon-Yai Remedy" The 1st International Postgraduate Conference on Pharmaceutical Sciences, June 29-30, 2012.
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