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· 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· 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. 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* and *Walsura trichostemon* 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.