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., Thailand)
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., Thailand)
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.

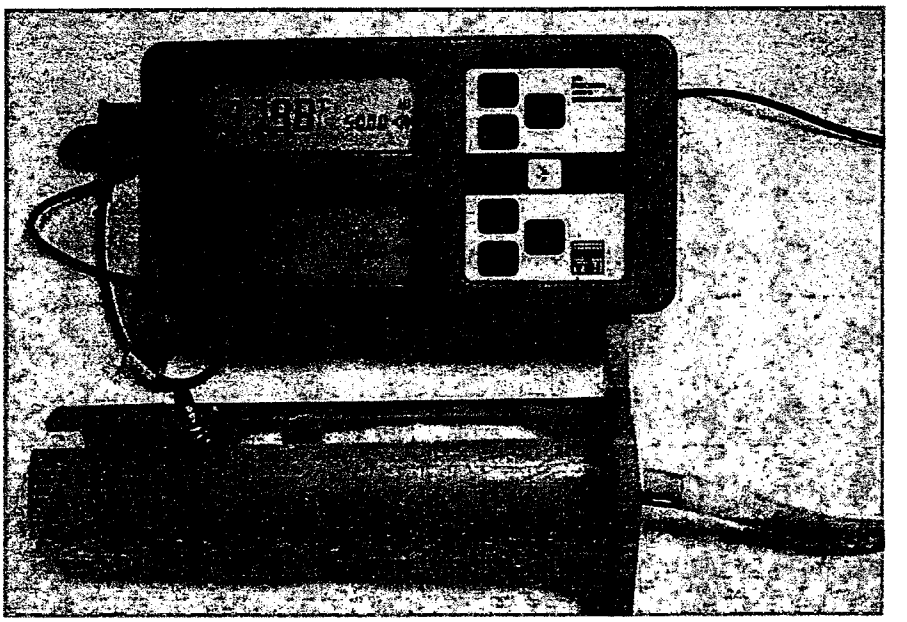

Figure 8 Digital Thermometers (YSI PrecisionTM 4000A)

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.

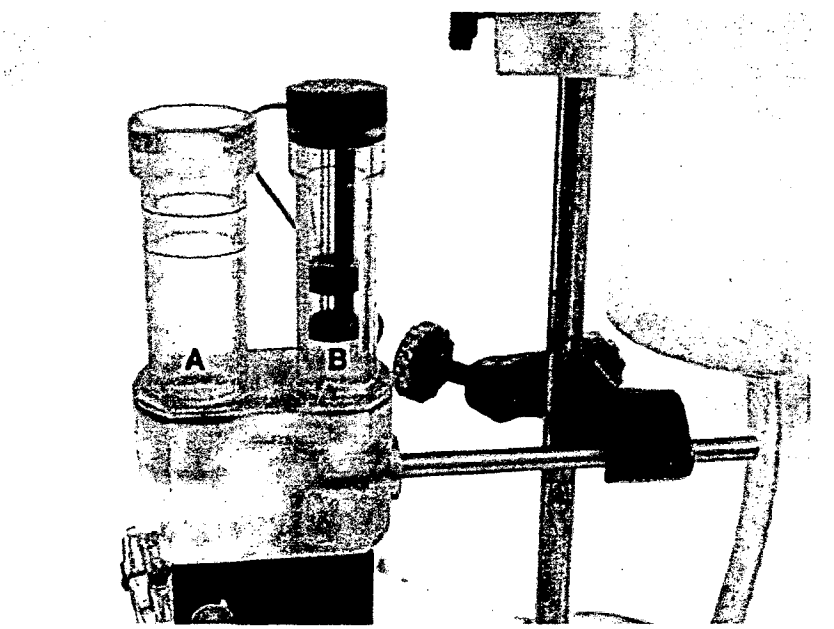

Figure 9 Plethysmometer. The larger one (A) is used to measure fluid displaced by the paw, a volume change that is precisely mirrored in the smaller tube (B) containing a transducer which is linked to a decoder capable of digitally displaying volumes.

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:

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

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).

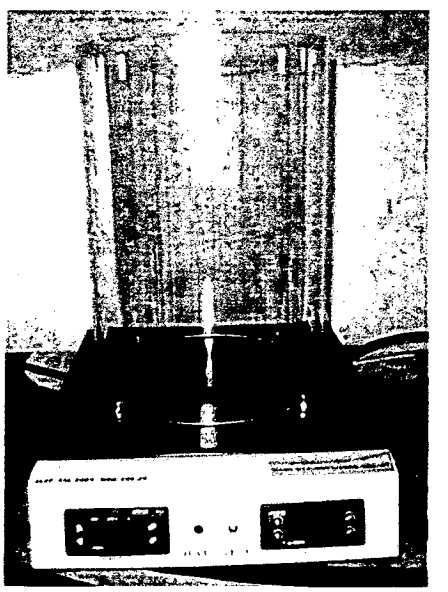

Figure 10 Hot-Plate Analgesiometer

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:

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

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

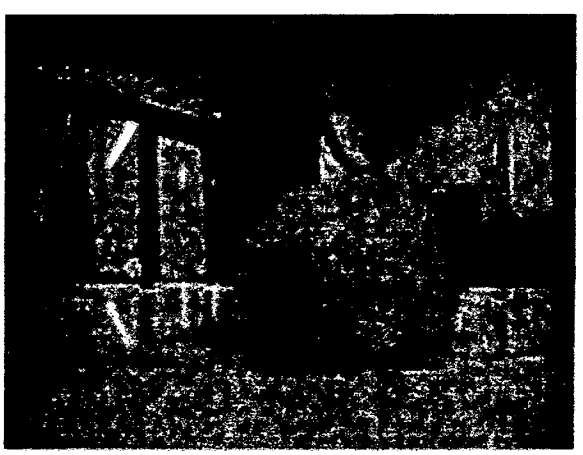

Figure 11 Formalin-induced paws licking in mouse

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.

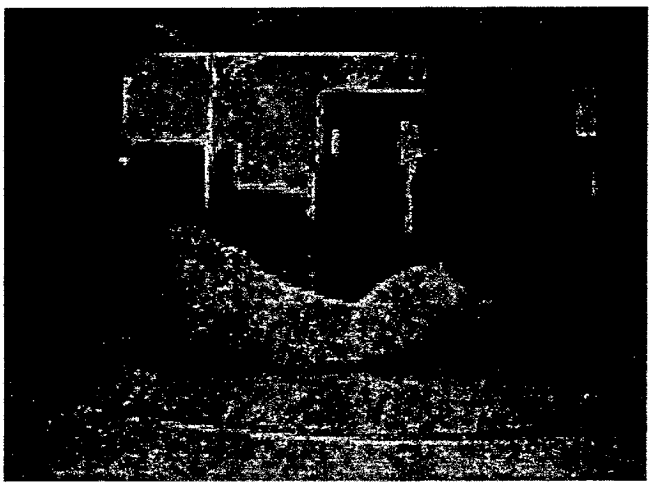

Figure 12 Writhing response in mouse

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].

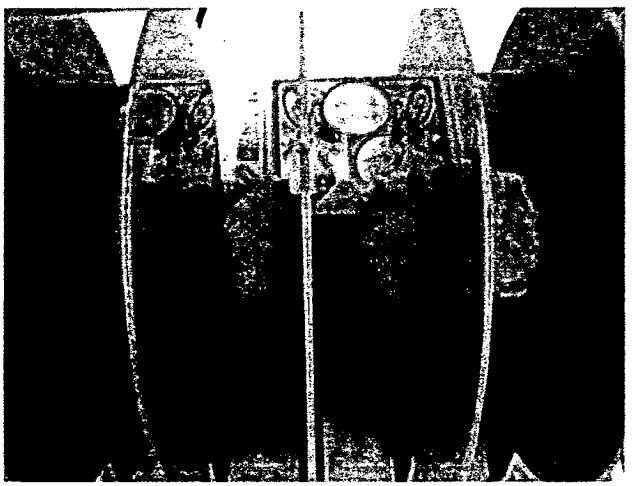

Figure 13 Rota-rod test in mice

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 $p<0.05$.