CHAPTER III

METERIALS AND METHODS

ANIMALS

Male ICR mice, weighing 18 - 25 g and male Wistar rats weighing 130-180 g (National Laboratory Animal Center, Salaya, Mahidol University, Thailand) served as experimental subjects. Animals were housed in the animal facility, Faculty of Pharmaceutical Sciences, Chulalongkorn University under standard conditions of temperature (25 °C), 12h /12h light / dark cycles and had accessed to standard pellet diet (C.P. Company, Thailand) and tap water *ad libitum*. Animals were allowed to acclimate to the facility for at least a week before starting the experiments. At the end of each experiment, animals were sacrificed with diethylether. The number of animals used in each experiment was typically eight to ten. The study protocols had been approved by the Ethical Committee of Faculty of Pharmaceutical Sciences, Chulalongkorn University.

PREPARATION OF THE *Cissus quadrangularis* DRIED STEM EXTRACTS

1. Fresh dried stems of *Cissus quadrangularis* were collected from Amphur Kamphaengsaen, Nakhon Pathom, Thailand, All stems used in this study were collected during February - March, 2003.

2. Two kilograms of air dried stems were dried with oven and crushed to coarsely powder of 2000 g.

3. The dried stem powder was successively extracted with 95% ethanol for 72 h at room temperature. The whole extract (dark-green solution) was filtered and transferred into 3 L conical flask. The filtrate was evaporated to dryness using hot water bath.

4. The paste of *Cissus quadrangularis* was kept in tight container at 2-8.°C until the time of treatment.

A result from 2000 grams of dried stems of *Cissus quadrangularis*, crushed and

extracted with 95% ethanol was filtered. The filtrate was evaporated resulting in the paste of 8.4 g. Therefore, *Cissus quadrangularis* dried stems contained approximately 0.004% *w/w* yield of evaporated *Cissus quadrangularis* paste. The paste appeared dark-green color.

The dark-green paste of the extract was dissolved in 0.9% normal saline and the fine suspension of the extract was used for the pharmacological study. The doses employed are expressed in terms of paste (mg/kg body weight).

Chemical Identification of the dried-stem paste of C. *quadrangularis*

Thin-layer chromatography (TLC)-densitometer was used to identify the chromatogram of the tested paste compared to the reference chromatogram. The method was modified from the method of a previous study (ธัญวรรัตน์ จันทรชนะ และ พงศธร หลิมศิริวงษ์, 2543).

Reagents

1. Chloroform
2. Benzene
3. 2M HCL
4. Anisaldehyde
5. Sulfuric acid
6. Ethanol

Procedures

1. The constituents in 500 microlitre of the *Cissus quadrangularis* dried stem crude extract were dissolved by ethanol and separated by TLC, which was conducted on a TLC plate coated with silica gel F-254. TLC plate was of 0.25 nm thickness (Merck) and separation was achieved in a developing solvent system of benzene: chloroform: 2M HCL, 20:20:1 v/v.
2. The TLC plate was then analyzed using densitometer at a wavelength of 200 nm.

3. The condition of densitometer was as following :

Lamp source	:	Deuterium lamp
Determination mode	:	Reflection absorption photometry
Slit width	:	1 nm
Slit height	:	5 nm
Wavelength detector	:	200 nm

4. After the densitometric determination, the TLC plate was sprayed with the mixture solution of anisaldehyde: sulfuric acid: ethanol: water (2: 3: 90: 1). The plate was then placed in hot air oven at 105°C for 5 min.

DRUGS

The *Cissus quadrangularis* dried stems extract and reference drugs were dissolved or suspended in 0.9% sodium chloride solution (The Government Pharmaceutical Organization, Thailand) and given intraperitoneally to the animals. The control animals were given an equivalent volume of vehicle in the same route. Morphine sulphate (10 mg/kg, Thai FDA), acetylsalicylic acid (150 mg/kg, Sigma Chemical Co., USA), and indomethacin (5 mg/kg, Sigma Chemical Co., USA) were used as standard analgesic drugs. Naloxone (1 mg/kg, Sigma Chemical Co., USA), Naltrexone (5 mg/kg, Sigma Chemical Co., USA) and NMDA (0.38 mg/kg, Sigma Chemical Co., USA), carrageenan (1 % in 0.9 % sodium chloride solution 50 (μl/rat, Sigma Chemical Co., USA).

EXPERIMENTAL METHODS

Hot-plate Analgesic Testing

The male ICR mice weighing 18-25 g were used. Analgesic testing was determined using the hot-plate method. The surface of the hot plate (measuring 28 x 28 cm was set at 50 °C ± 0.5 °C and was 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. On the day of testing, animals were randomly assigned to one of eight treatment 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 utilized in these studies. Mice were then administered various doses of treatments and retested. Each mouse was placed on the hot-plate from an elevation of 5 cm and the latency to the licking of a rear paw or a 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 for its paw-lick latency and returned to its cage (the maximum time allowed for an animal to remain on the surface of the plate during testing was 45 sec). The average of the last two trials served as the baseline pre-drug paw-lick latency.

Immediately, after the third baseline trial on the hot-plate, the drug administration took place with intraperitoneal (i.p.) vehicle (10 ml/kg) or test drugs, morphine sulphate (10 mg/kg), acetylsalicylic acid (150 mg/kg) or various doses of *Cissus quadrangularis* dried stems extract (CQ; 43.5, 87.5, 135, 350, and 700 mg/kg). All animals were placed on the hot-plate for 7 subsequent trials at 15, 30, 45, 60, 90, 120, and 240 min after injection. Thus, ED_{50} were computed and dose- time response curve were generated.

Mouse Tail-flick Analgesic Testing

These studies employed the tail-flick assay described by D'Amour and Smith (1941), with minor modifications. Male ICR mice weighing 18-25 g were used (n = 10 per group). Mice were placed in individual Plexiglas restrainers with an opening to allow the tail to protrude. Each tail rested in a shallow groove housing a light sensitive sensor. A beam of radiant heat (24-V, high amperage 150- watt light bulb situated 8 cm above the tail) was aimed at the middle of the marked dorsal portion of the distal part of each subject's tail that has been blackened length 1 cm with a black ink maker pen in order to absorb the maximum amount of heat and for uniform heat absorption (about 4 cm from the tip). The device (Harvard Tail-flick Analgesia meter) automatically recorded (in 0.1 sec) the latency between the onset of the light beam stimulus and the response to heat, at which point the light beam was terminated. The maximum duration of each test was set at 4.0 sec to minimize the potential for thermal injury. The stimulus intensity was set so that the baseline tail-flick latencies were approximately 1.0 -1.5 sec (intensity 3.7 A). The intensity was not changed for any animals within any given experiments. Animals failing to respond within 1.5 sec were excluded from testing. On the day of testing, all animals were test 3 predrug tail-flick baseline trials conducted at 10-15 min intervals. The score from the third trial served as the baseline measure for each subjects.

Immediately, after the third baseline trial on the tail-flick test, the drug administration took place with either intraperitoneal (i.p.) vehicle or test drugs, morphine sulphate (10 mg/kg), acetylsalicylic acid (150 mg/kg) and CQ (43.5, 87.5, 135, 350, and 700 mg/kg). Tail-flick latencies were recorded at 15, 30, 45, 60, 90, 120, and 240 min after injection. Thus, dose-and time response curve were generated. Analgesics will prolong the reaction time significantly and the doses required to induce this effect in 50% of the animals (Effective Dose-50, ED_{50}) can be computed.

Paw Pressure Test (Randall Selitto Test)

Male Wistar rats weighing 130-180 g were used in this test with an Analgesymeter (Ugo Basile, Milan). A force increasing at a constant rate was applied to the rat's hind paw by placing the animal's paw on a small plinth under a cone-shaped pusher with a round tip. The applied force was continuously monitored by an indicator moving along a linear scale calibrated in grams. The Analgesymeter was fitted with a single weight such that the maximum pressure exerted on the paw was 500 g. The pressure on the paw was increased at a constant rate of 32 g/sec. The application of force was stopped when the rat started to struggle (vigorous attempt to withdraw the paw) to a noticeable degree (whether or not accompanied by vocalization), the paw pressure threshold was determined at the withdrawal response. The time at which the rat removed the paw, or struggled to do so, was recorded as the end point. Animals failing to react to a 500 g force were given scores corresponding to the full scale (500 g). The animal was held during this test but not restrained. On the day of testing, all animals underwent 3 pre-drug paw pressure baseline trials conducted at 10-15 min intervals. The score from the last two trials served as the baseline measure for each animal.

Immediately, after the third baseline trial, the drug administration took place with either intraperitoneal (i.p.) vehicle or test drugs morphine sulphate (10 mg/kg), acetylsalicylic acid (150 mg/kg) and CQ (43.5, 87.5 135, 350, and 700 mg/kg). All animals were test for the paw pressure threshold test for 6 subsequent trials at 15, 30, 60, 90, 120, and 240 min after injection. Thus, dose-and time response curve were generated.

Carrageenan-Induced Inflammatory Hyperalgesia Testing

Male Wistar rat weighing 130-180 g (n = 10 per group) were used in this experiment using the model described by Hargreaves et al. (1998). On the day of testing, all animals were pretreat with either i.p. vehicle or test drugs (Santos et al., 1999; Mendess et al., 2000; Johanek et al., 2001; Otuki et al., 2001) and 30 min later the plantar surface of the right hind paw in each rat was injected with 50 µl of 1 % carrageenan and returned to its cage for testing. Two hours after carrageenan injection (Hargreaves, K., 1988; Megaraughty et al., 2001), animals were tested for mechanical hyperalgesia with an Ugo Basile Analgesymeter. Using a wedge-shaped probe, an increasing weight was applied to the paw (750 g maximum) and the withdrawal threshold determined as the first sign of a pain response. Each animal's withdrawal threshold was an average of two trials, which were separated by at least 10 min. The left hind paw was not injected with carrageenan but was similarly allowing direct comparisons between inflamed and non-inflamed paws for each animal.

Analysis of the analgesic mechanism of action of the crude extract of *Cissus quadrangularis* dried stem

The possible participation of the opioid system in the antinociceptive effect of CQ was investigated. To analyze this mechanism we also used the model of mice hot-plate test. Animals were pretreated with naloxone 1 mg/kg i.p. (Pieretti et al., 1999; Perrot et al., 2001), naltrexone 5 mg/kg i.p. (Lattanzi, 2002), or NMDA 0.38 mg/kg i.p. (Davis and Inturrisi, 1999) 30 min before CQ (135, 350 and 700 mg/kg i.p.) administered.

DATA TREATMENT AND STATISTICAL ANALYSE

Statistical analyses were performed on the dose-response curves by analysis of variance (ANOVA) and, where appropriate, were followed by Turkey' *post hoc* testing (SPSS version 13.0 for windows). The time-course of hot-plate latency, tail-flick latency and paw-pressure threshold are expressed as the mean percent maximum possible effect (%MPE) according to the following formula:

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

Note: cut-off time for hot-plate test = 45 seconds
cut-off time for tail-flick test = 4 seconds
cut-off time for paw pressure test = 250 g

Dose-effect curves for the hot-plate, tail-flick and paw pressure assays were derived by computing the area under the corresponding 0-240 min time-course-%MPE curves; areas were calculated using the trapezoidal rule (Tallarida and Murray, 1987). The minimum level of statistical significance was set at $p < 0.05$.