CHAPTER II

EXPERIMENTAL

1. Source of Plant Materials

The plant used in this investigation was collected in August, 1982 from Nakornpathom province, Thailand. This plant was identified to be *Cissus quadrangularis* Linn. (syn. *Vitis quadrangularis* Wall.) in family Vitidaceae, Order Rhamnales, and it was authenticated by comparison with voucher specimens at the Botany Section, Technical Division, Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand.

2. General Techniques

2.1 Thin Layer Chromatography (TLC)

Silica gel G type 60 (E. Merck) was used to prepare thin-layer plates. The plates were prepared by mixing 35 g of silica gel and 70 ml of distilled water in an erlenmeyer flask, the mixture was shaken for 2 minutes and applied to five clean glass plates (20 x 20 cm) according to the method of Stahl (40) to produce a layer equivalent to 0.25 mm thick. The plates were air-dried, activated at 110 °C for one hour and allowed to cool before storage in a desiccator. The solvent systems used for analytical thin layer chromatography were :

a. benzene : chloroform (1:1)

b. petroleum ether : benzene (1:5)

c. petroleum ether : dichloroethane (1:1)

d. chloroform : methanol (99:1)

The analytical plates were detected by Liebermann-Burchard reagent and heated at 110 °C for 10-15 minutes.

2.2 Column Chromatography (CC)

Column chromatographic techniques used in this investigation followed the method of Still (41) and were called "Short Column Chromatography". The columns used were flat bottom glass columns of the diameters 5 cm and 10 cm. Silica gel 60 (230-400 mesh, E. Merck) was used as an adsorbent. The adsorbent was packed into the column by wet packing technique according to the method of Still (41).

2.3 Melting Point (MP)

The melting points were determined by mean of a Gallenkamp Melting Point Apparatus and are uncorrected.

2.4 Infrared Absorption Spectrum (IR)

Infrared absorption spectra were determined on a Shimadzu Model IR 440 spectrophotometer and on a Perkin-Elmer 283 spectrophotometer. Absorption bands were reported in wave numbers (cm^{-1}).

2.5 Nuclear Magnetic Resonance Spectrum (NMR)

Proton NMR spectra were recorded at 90 MHz on a Varian Model FX 90 Q. instrument and on a Varian A-60D instrument. Tetramethylsilane was used as an internal standard and chemical shifts were reported on the ppm scale.

2.6 Mass Spectrum (MS)

The compounds were submitted for low resolution mass spectral study on JEOL mass spectrometer, Model DX 300 at 70 eV. The high resolution mass spectral study (at MIT) was performed on a CEC 110B Double Focusing Mattauch Herzog Spectrometer. The spectrum was recorded on Ionomet plates (deposited AgBr) (Dupont Industries, Monrovia, California.)

3. Experimental

3.1 Phytochemical Screening

Ground fresh plant material (200 g) was macerated with 95% ethanol (200 ml) for three days and filtered into an erlenmeyer flask. This extract will serve as a stock solution (350 ml) for the screening procedure.

3.1.1 Screening for Sterols and Triterpenoids

An aliquot portion (200 ml) of the stock solution was evaporated to a syrupy mass under reduced pressure in Buchi evaporator. After the contents was cooled to room temperature, it was diluted with distilled water and extracted with petroleum ether (500 ml). The petroleum ether extract was dried over anhydrous sodium sulfate and filtered. An aliquot portion of filtrate (25 ml) was used for the Liebermann-Burchard test. The sample, 3 drops of acetic anhydride, and 1 drop of conc. H_2SO_4 when mixed yielded a progression of color from blue to green indicating the presence of sterols.

3.1.2 Screening for Flavonoids

An aliquot portion of the stock solution (25 ml) was evaporated to dryness on a steam bath. After the dried residue was cooled to room temperature, it was defatted several times with petroleum ether until the last extract of petroleum ether was colorless. The defatted residue was dissolved in 80% ethanol and filtered. The filtrate was submitted to the cyanidin test. The cyanidin test consists of treating the sample with 0.5 ml of conc. HCl and magnesium ribbon ; a pink color was obtained indicating a positive result.

3.1.3 Screening for Alkaloids

An aliquot portion of the stock solution (125 ml) was evaporated to a syrupy mass. Five milliters of 2*N* HCl was added, and the mixture was warmed on a steam bath for ten minutes with stirring. After the mixture was cooled to room temperature, it was filtered. The filtrate was used for the precipitation test with three alkaloidal reagents. The results are summerized in Table I :

Table I

Alkaloid Screening Test

Reagent	Result
Dragendorff	-
Mayer	-
Wagner	-

4. Isolation of Chemical Substances from *Cissus quadrangularis* Linn.

4.1 Extraction

Fresh plant (50 kg) was blended with 95% ethanol in a Waring electric blender. It was then macerated twice for 3 day-peroids each with 95% ethanol (70 L and 30 L).

4.2 Fractionation

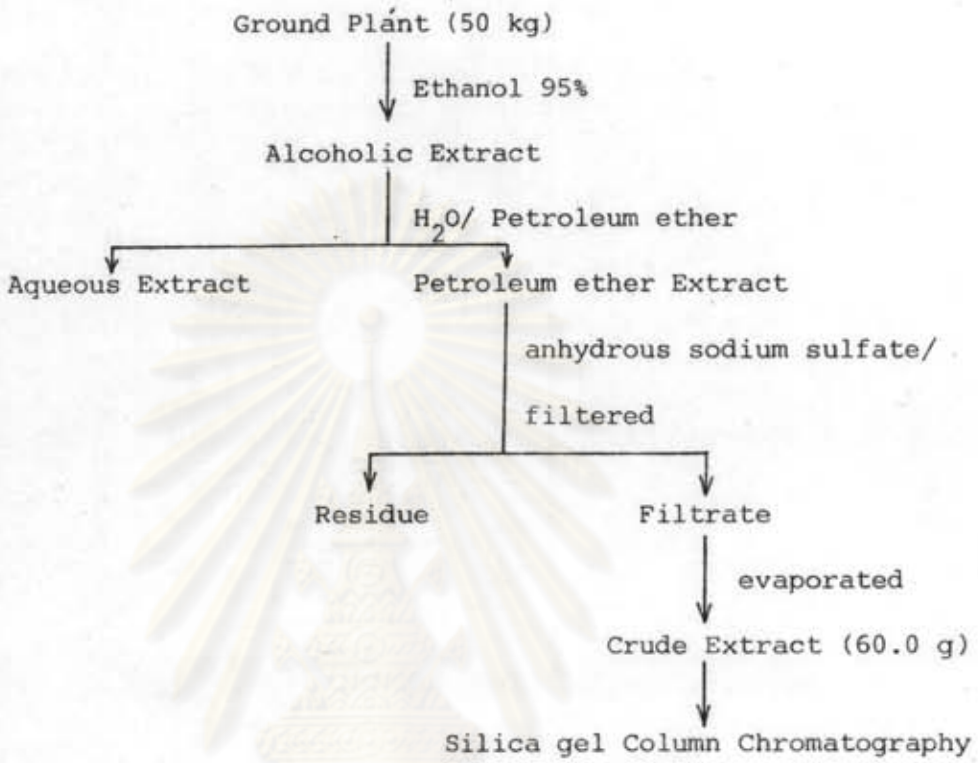
The alcoholic extracts were filtered through a Buchner funnel. The filtrates were combined and concentrated under reduced pressure in Buchi evaporator to a syrupy liquid (500 ml) and was fractionated according to the flow chart shown in Figure 1. The total crude extract was diluted with distilled water (100 ml) and partitioned several times with petroleum ether (20 L) until the last extract of petroleum ether gave negative test with Liebermann-Burchard reagent. The combined petroleum ether extract was dried over anhydrous sodium sulfate and concentrated under reduced pressure in Buchi evaporator to give a dark-green syrupy mass (60.0 g).

4.3 Silica gel Column Chromatography

The combined petroleum ether extract was examined by thin layer chromatography on silica gel plates using solvent system a as developing solvent system. The spots appeared after spraying with Liebermann-Burchard reagent.

Figure 1

(Fractionation of the Ethanol Extract)



4.4 Isolation of Chemical Substances

The combined petroleum ether extract (60.0 g) was divided into six portions, each portion was subjected to silica gel column chromatography in the same manner. Each portion (approx. 10.0 g) was dissolved in 20 ml of eluting solvent, [benzene : chloroform (3:1)] and placed on top of a 10 cm diameter column of silica gel (300 g). Fractions of 25 ml each were collected and examined by thin layer chromatography (TLC). Those fractions of similar patterns were combined as shown in Table II.

Table II

Elution Pattern of the Silica gel Column (10.0 mg)

Fraction	Eluent Component	Weight (mg)
1-12	Benzene : Chloroform (3:1)	580
13-19	"	176
20-24	"	47
25-43	"	185
44-46	"	65
47-77	"	571
78-90	"	407
91-115	"	2931
116	Methanol	1630

The first combination (fractions 1-12, 580 mg) gave negative test with Liebermann-Burchard reagent.

The second combination (fractions 13-19, 176 mg) was shown by TLC to contain one spot with hRf value of 47 (see Figure 5 page 53). It was crystallized in n-Hexane to give white crystalline needles (28 mg) and designated as CQ-1. Additional amounts of CQ-1 (5.3 mg) was obtained from the crystallization of the third combination (fractions 20-24, 47 mg) in n-Hexane. The total weight of CQ-1 obtained from six columns was 112 mg.

The fourth combination (fractions 25-43, 185 mg) was shown by TLC to contain one spot with hRf value of 31 (see Figure 5 page 53). It was crystallized in petroleum ether to give white crystalline solid (23 mg) and designated as CQ-2. Additional amounts of CQ-2

(2.5 mg) was obtained from rechromatographing of the fifth combination (fractions 44-46, 65 mg) on 5 cm silica gel column chromatography, using benzene : chloroform (3:1) as eluent. The total weight of CQ-2 obtained from six columns was 120 mg.

The sixth combination (fractions 47-77, 571 mg) was shown by TLC to contain one spot with hRf value of 23 (see Figure 5 page 53). It was crystallized in n-Hexane to give white crystalline solid (30 mg) and designated as CQ-3. Additional amounts of CQ-3 (43 mg) was obtained from rechromatographing of the seventh combination (fractions 78-90, 407 mg) on 5 cm silica gel column chromatography, using benzene : chloroform (3:1) as eluent. The total weight of CQ-3 obtained from six columns was 150 mg.

The eighth combination (fractions 91-115, 2931 mg) was shown by TLC to contain one spot with hRf value of 15 (see Figure 5 page 53). It was crystallized in absolute alcohol to give white crystalline needles (1.358 mg) and designated as CQ-4. Additional amounts of CQ-4 (15 mg) was obtained from rechromatographing of the seventh combination (fractions 78-90, 407 mg) on 5 cm silica gel column chromatography, using benzene : chloroform (3:1) as eluent. The total weight of CQ-4 obtained from six columns was 3.729 g.

5. Characterization of Isolated Compounds

The compounds were characterized by studies on melting points and recording infrared, nuclear magnetic resonance, and mass spectra.

CQ-1 was obtained as white crystalline needles. It is soluble in petroleum ether, ether, chloroform, and acetone.

hRf values

a. 47 (see Figure 5 page 53)

b. 33 (see Figure 6 page 54)

c. 47 (see Figure 7 page 55)

d. 71 (see Figure 8 page 56)

Melting point

165-166 °C

Infrared absorption spectrum (KBr disc) (see Figure 9 page 57)

ν_{max} (cm^{-1}): 3090, 2950, 2875, 1720, 1655, 1460, 1380, 890, 875.

Molecular weight

424 (mass spectrometry)

Proton nuclear magnetic resonance spectrum (see Figure 10 page 58)

^1H nmr (90 MHz, $CDCl_3$) δ : 0.80 (3H, s), 0.94 (3H, s), 0.96 (3H, s), 1.03 (3H, s), 1.07 (6H, s), 1.68 (3H, s), 4.64 (2H, d).

Mass spectrum

m/e (% rel. int.) (see Figure 11 page 59)

424 (M^+, 33%), 409 (11%), 381 (9%), 314 (13%), 245 (16%), 218 (25%), 205 (100%), 203 (29%), 189 (36%), 109 (88%), 99 (100%).

DNP Derivative of CQ-1

Two milligrams of CQ-1 was dissolved in cold methanol (5 ml). The slightly acidic methanolic solution (5 ml of dil. HCl + 10 ml of methanol) of dinitrophenyllhydrazine was added. The solution mixture was concentrated on a steam bath to yield yellow solid of the DNP derivative. It was crystallized from chloroform-methanol mixture to

give crystalline needles, mp. 205-207°C.

CQ-2 was obtained as white crystalline solid. It is soluble in ether, chloroform, acetone, and ethanol.

hRf values

a. 31 (see Figure 5 page 53)

b. 18 (see Figure 6 page 54)

c. 33 (see Figure 7 page 55)

d. 64 (see Figure 8 page 56)

Melting point

272-275°C

Infrared absorption spectrum (KBr disc) (see Figure 12 page 60)

$\nu_{max}(cm^{-1})$: 3620, 3480, 2920, 2870, 1450, 1380, 995, 975, 915.

Molecular weight

428 (mass spectrometry)

Proton nuclear magnetic resonance spectrum (see Figure 13 page 61)

^1H nmr (90 MHz, $CDCl_3$) δ : 0.69 (3H, s), 0.86 (3H, s), 1.00 (3H, s), 1.14 (3H, s), 1.35 (3H, s), 1.42 (3H, s), 1.50 (3H, s), 1.52 (3H, s), 3.72 (1H, m).

Mass spectrum

m/e (% rel. int.) (see Figure 14 page 62)

429 (33%), 414 (34%), 411 (100%), 396 (63%), 219 (39%), 192 (29%), 150 (25%), 124 (48%), 109 (71%), 107 (47%), 95 (100%).

CQ-3 was obtained as white crystalline solid. It is soluble

in benzene, ether, and chloroform.

hRf values

a. 27 (see Figure 5 page 53)

b. 12 (see Figure 6 page 54)

c. 18 (see Figure 7 page 55)

d. 54 (see Figure 8 page 56)

Melting point

275-278 °C

Infrared absorption spectrum (KBr disc) (see Figure 16 page 64)

ν_{max} (cm^{-1}) : 3475, 2950, 2875, 1630, 1475, 1450, 1385, 1375, 1365, 1060, 1030, 980.

Molecular weight

426 (mass spectrometry)

Proton nuclear magnetic resonance spectrum (see Figure 17 page 65)

^{1}H nmr (90 MHz, $CDCl_3$) δ : 0.60 (3H, s), 0.68 (3H, s), 0.74 (3H, s), 0.80 (3H, s), 0.96 (3H, s), 0.99 (3H, s), 1.10 (6H, s), 1.27-1.87 (2H, m), 3.20 (1H, m), 5.21 (1H, m).

Mass spectrum

m/e (% rel. int.) (see Figure 18 page 66)

426 (M^+, 75%), 411 (100%), 408 (31%), 394 (27%), 393 (81%), 259 (79%), 241 (44%), 137 (47%), 133 (39%), 123 (40%), 121 (48%), 119 (52%), 109 (65%), 107 (52%), 95 (95%).

Acetate Derivative of CQ-3

Five milligrams of CQ-3 was heated on a steam bath for 2 hours

with 5 ml of acetic anhydride and 5 ml of pyridine. The solution was concentrated under reduced pressure. The residue was crystallized several times from n-Hexane to give crystalline needles, mp. 285-288 °C.

CQ-4 was obtained as white crystalline needles. It is soluble in chloroform, dichloroethane, ethyl acetate, and acetone.

hRf values

a. 14 (see Figure 5 page 53)

b. 8 (see Figure 6 page 54)

c. 11 (see Figure 7 page 55)

d. 40 (see Figure 8 page 56)

Melting point

139 °C

Infrared absorption spectrum (KBr disc) (see Figure 19 page 67)

$\nu_{max}(cm^{-1})$: 3450, 2920, 2860, 1649, 1500. 1370, 1004.

Proton nuclear magnetic resonance spectrum (see Figure 20 page 68)

^{1}H nmr (90 MHz, $CDCl_3$) δ : 0.68 (3H, s), 1.01 (3H, s), 3.49 (1H, broad s), 5.51 (1H, d).