CHAPTER III

EXPERIMENTAL

1. Source of Plant Material

The fruits of *Sapindus rarak* DC. were collected from Prae province, Thailand in December 2002.

The plant material was authenticated by comparison with the voucher specimen No. BKF 120357, deposited in the herbarium of the Royal Forest Department, Bangken, Bangkok, Thailand.

2. General Techniques

2.1 Analytical Thin Layer Chromatography (TLC)

Technique	:	One dimension, ascending
Adsorbent	:	Silica gel 60 F_{254} precoated plate (E. Merck)
Layer thickness	:	0.2 mm.
Developing distance	:	6.0 cm.
Temperature	:	Laboratory room temperature (30-35 °C)
Detection	:	1. Ultraviolet light at wavelength of 254 nm.
	:	2. *p*-anisaldehyde reagent in concentrated sulphuric acid

2.2 Column Chromatography (CC)

2.2.1 Quick Column Chromatography

Adsorbent : Silica gel 60 (No.7734) (E. Merck) Particle size 0.063-0.200 nm. (70-230 mesh ASTM)

Packing method : Dry packing method

Sample loading : The sample was dissolved in a small amount of organic solvent, mixed with a small quantity of adsorbent, triturated, dried and then placed gently on top of the column.

Detection : Fractions were examined using TLC technique. In order to detect the compounds, the TLC plate was observed under UV light at wavelength of 254 nm and then exposed to *p*-anisaldehyde reagent in concentrated sulphuric acid.

2.2.2 Conventional Column Chromatography

Adsorbent : 1. Silica gel 60 (No.7734) (E. Merck) Particle size 0.063-0.200 nm. (70-230 mesh ASTM)

: 2. Silica gel 60 (No.9385) (E. Merck) Particle size 0.063-0.200 nm. (70-230 mesh ASTM)

Packing method : Wet packing method

Sample loading : The sample was dissolved in a small amount of eluent, then applied gently on top of the column.

Detection : Fractions were examined using TLC technique. In order to detect the compounds, the TLC plate was observed under UV light at wavelength of 254 nm and then exposed to *p*-anisaldehyde reagent in concentrated sulphuric acid.

2.2.3 Column Chromatrography on Diaion HP-20

Adsorbent : Diaion HP-20 porous polymer resin

(Mitsubishi Chemical Co, Tokyo, Japan)

Packing method : Wet packing method

Sample loading : The sample was dissolved in a small amount of eluent, then applied gently on top of the column.

Detection : Fractions were examined using TLC technique. In order to detect the compounds, the TLC plate was observed under UV light at wavelength of 254 nm and then exposed to *p*-anisaldehyde reagent in concentrated sulphuric acid.

2.3 Spectroscopic Techniques

2.3.1 Infrared (IR) Absorption Spectra

IR spectra were recorded on a Perkin-Elmer FT-IR 1760X spectrometer at the Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

2.3.2 Mass Spectra (MS)

Electron Impact Mass Spectra (EIMS) of isolated compounds were obtained on a Micromass Platform II mass spectrometer at 70 eV. at the National Science and Technology Development Agency (NSTDA).

2.3.3 Nuclear Magnetic Resonance Spectrometer (NMR)

The ^{1}H NMR spectra, ^{13}C NMR spectra and 2D-NMR spectra were measured with a Bruker AVANCE 400 NMR spectrometer at the National Science and Technology Development Agency (NSTDA).

Deuterated pyridine (pyridine-d_5) was used as the NMR solvent throughout this study. Spectral data were reported in ppm scale using the solvent chemical shift as the reference frequency.

2.4 Physical Property Measurement Apparatus

2.4.1 Melting Points

Melting points were determined on a Gallenkamp Melting Point Apparatus at the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

2.4.2 Optical Rotations

Optical rotations were measured on a Perkin-Elmer Polarimeter model 341 at the Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

2.5 Solvents

Organic solvents used in extraction were of commercial grade. For column chromatography, solvents were redistilled prior to use.

2.6 Chemical test for detection.

p-anisaldehyde reagent

The reagent consists of 15 ml MeOH, 2 ml acetic acid, 10 ml sulfuric acid and 0.2 ml *p*-anisaldehyde. Reagent was sprayed to the spots on TLC plates for detection of the spots of steroid or triterpenoid compounds. If the spots change to dark blue or greenish blue, the sample may contain steroid or triterpenoid compounds.

3. Molluscicidal activity testing

3.1 Materials

3.1.1 Tested animals

The golden apple snails *Pomacea canaliculata* with 2.0-2.5 cm. shell length and average weigh of about 7-9 grams were used for molluscicidal test. The snails were collected from Amphoe WangNoi, Ayuthaya Province, Thailand, in 2005.

3.1.2 Chemicals

- Niclosamide 70 % WP (wettable powder) (Bayerthai, Co. Ltd.)
- Metaldehyde 80 % WP (Sotus International, Co. Ltd.)
- CU-1 (tea seed granule with 12 % saponins)
  (Siriwat Product, Co. Ltd.)

3.2 Methods

3.2.1 Preparation of tested snails

The snails used for this study were the golden apple snails *Pomacea canaliculata* Lamarck. For preparation of dechlorinated water for golden apple snails, tap water was left at room temperature at least 24 h. before used. The snails were collected from Amphor Wang Noi, Ayuthaya Province and were acclimated to laboratory conditions before testing (at least 3 days) by feeding them with water mimosa and banana leafs. Before molluscicidal testing, the snails were selected by size of shell length at about 2.0-2.5 cm.

3.2.2 Molluscicidal test [Huang et al., 2003]

The experiment was carried out in two steps that were preliminary molluscicidal test and definitive molluscicidal test. For preliminary molluscicidal test, the range finding test was guided to find out the range which was defined as the interval between the lowest concentration that killed all snails (LC_{100}) and the highest concentration that killed none of the snails (LC_00). The experiment in preliminary molluscicidal range finding test consisted of six concentrations of crude extract (from *Sapindus rarak* DC. fruits) and extracting solvent. The amount of mortal snails of range finding test was recorded and these data were then used to establish a more narrow concentration range for the definitive molluscicidal tests.

The experiment in definitive molluscicidal tests consisted of five different concentrations for each compound with three replicates. The control was done in parallel except that the snails were submerged in the dechlorinated water without any chemicals addition.

For comparison, Niclosamide [70 %WP], Metaldehyde [80 %WP] and CU1 (tea seed cake containing 12 % saponins) were used as positive controls. Five different concentrations for each compound were prepared and three replicates (20 snails for each) were kept in a 1L plastic jar containing 300 ml of solution. Snails were submerged in the respective test compound solution in a jar covered with net to prevent them from escaping. The snails were fed for 24 h. and checked for dead snails by probing with a needle to detect their response and the number of dead snails was calculated after 24 hours.

3.2.3. Statistical Procedure

Percentage mortality was corrected by Abbott's formula [Abbott 1925] If the control replication had snail death between 5-20 %, the mortality percentage was used the Abbott's formula as follows:

$$\% \text{ Mortality} = \frac{\% \text{ test mortality} - \% \text{ control mortality}}{100 - \% \text{ control mortality}} \times 100$$

% test mortality is percentage of total death snail in extract and chemicals solution at 24 hours.

% control mortality is percentage of total death snail in dechlorinated water at 24 hours.

If the control replication had snail death more than 20 %, The experiment was canceled and a new experiment was initiated.

Data on accumulated mortality of snails after 24 h. were analyzed by Probit Analysis [Meeposom, 2004] that was done by SPSS computer software (Version 15). The 95% confidence intervals of the LC_{50} were

determined as a measure of the toxicity. The details of Probit Analysis were calculated in appendix B.

4. Extraction and Isolation

4.1 Extraction

The dried powdered fruits from *Sapindus rarak* DC. (500 g) were macerated with methanol for three days. The obtained extract was evaporated under reduced pressure at a temperature of approximately 40 °C to give 25.4 g (5.1 % w/w) of methanol extract (Scheme 1).

The methanol extract were subjected to molluscicidal activity evaluation against golden apple snails (*Pumacea canaliculata*). The snail deaths were observed, at 24 hours intervals, and used for calculation of mortality (Appendix B). The results of molluscicidal testing are shown in Table 6.

Table 6 The results of molluscicidal activity evaluation against *P. canaliculata* at 24 hours intervals of methanol extract and chemical controls.

Compounds	LC_{50} (ppm) (95 % confidence Limits of LC_{50})	LC_{90} (ppm) (95 % confidence Limits of LC_{90})
Niclosamide	0.27	1.12
Metaldehyde	34.98	89.57
CU1	60.32	99.32
Methanol extract	11.19	19.82

From molluscicidal activity test, the methanol extract showed the highest activity level with LC_{50} value of 11.19 ppm. The results of LC_{50} molluscicidal activity of methanol extract as compound with chemical controlled substances showed high molluscicidal activity. These promising results prompted us to further purify bioactive constituents from this plant.

4.2 Isolation

The methanol extract, which was found to be active against the golden apple snails. The methanol extract from *Sapindus rarak* fruits (20g) was suspended in 100 ml water and partitioned with chloroform (3x50ml) at room temperature. Each layer was evaporated under reduced pressure to give 8.5 g of chloroform layer (42.5 % w/w) and 10.5 g of aqueous layer (52.5 % w/w).

Table 7 The partition of methanol extract

Solvent	Appearance	Weight (g)	% w/w of powder (20g)
$CHCl_3$	Brown powder	8.5	42.5
H_2O	Brown powder	10.5	52.5

The chloroform layer and aqueous layer were subjected to molluscicidal activity evaluation against golden apple snails (*Pumacea canaliculata*). The snails death were observed, at 24 hours intervals,

and used for calculation of mortality (Appendix B). The results of molluscicidal testing are shown in Table 8.

Table 8 The results of molluscicidal activity testing of chloroform layer and aqueous layer against *P. canaliculata* at 24 hours intervals.

Fraction	LC_{50} (ppm) (95% confidence Limits of LC_{50})	LC_{90} (ppm) (95% confidence Limits of LC_{90})
$CHCl_3$	10.84	19.85
H_20	9.62	18.80

From molluscicidal activity test, fraction aqueous Layer displayed the highest molluscicidal activity level with LC_{50} value of 9.62 ppm. These promising results prompted us to further purify bioactive constituents from this fraction.

The aqueous layer (10g) was fractionated by quick column chromatography using on a silica gel column. A mixture solvent of ethyl acetate : methanol : H_2O (8 : 2 : 1) is the mobile phase. Fractions (50ml) were collected and combined according to their TLC patterns to yield of 4 fractions (A-1-A-4) as shown in Table 9.

Table 9 Combination of fractions from aqueous layer

Fraction code	Number of fraction	Weight(g)	% w/w of powder
A-1	1- 20	5.63	56.3
A-2	20 - 27	2.26	22.6
A-3	28 - 35	1.03	10.3
A-4	36-45	0.72	7.2

Each fraction was subjected to molluscicidal activity evaluation against golden apple snails (*Pumacea canaliculata*). The snails death were observed, at 24 hours intervals, and used for calculation of mortality (Appendix B). The results of molluscicidal testing are shown in Table 10.

Table 10 The results of molluscicidal activity testing of fraction A-1-A-4 against *P. canaliculata* at 24 hours intervals.

Fraction code	LC_{50} (ppm) (95 % confidence Limits of LC_{50})	LC_{90} (ppm) (95 % confidence Limits of LC_{90})
A-1	8.47	16.86
A-2	8.99	17.69
A-3	9.61	17.94
A-4	9.28	17.34

From molluscicidal activity test, fraction A-1 displayed the highest molluscicidal activity level with LC_{50} value of 8.47 ppm. These promising results prompted us to further purify bioactive constituents from this fraction.

Fraction A-1 (5g) was further chromatographed on a Diaion HP-20 porous polymer resin column and eluted with H_2O, 20, 40, 60, 80, 100% aq. in MeOH, successively. Fractions (20ml) were collected and combined according to their TLC patterns to yield 6 fractions (B-1-B-6) as shown in Table 11.

Table 11 Combination of fractions from A-1

Eluants	Fraction code	Number of fraction	Weight(g)	% w/w of powder
H_2O	B-1	1-25	0.8	16
20%MeOH	B-2	26-38	0.85	17
40%MeOH	B-3	39-62	0.95	19
60%MeOH	B-4	63-80	1	20
80%MeOH	B-5	81-98	1.15	23
100%MeOH	B-6	99-120	1.3	26

Each fraction was subjected to molluscicidal activity evaluation against golden apple snails (*Pumacea canaliculata*). The snails death were observed, at 24 hours intervals, and used for calculation of mortality (Appendix B). The results of molluscicidal testing are shown in Table 12.

Table 12 The results of molluscicidal activity testing of fraction B-1-B-6 against *P. canaliculata* at 24 hours intervals.

Fraction code	LC_{50} (ppm) (95 % confidence Limits of LC_{50})	LC_{90} (ppm) (95 % confidence Limits of LC_{90})
B-1	8.14	14.87
B-2	7.61	13.84
B-3	7.18	13.05
B-4	6.79	12.56
B-5	7.24	13.33
B-6	6.21	10.59

From molluscicidal activity test, fraction B-6 displayed the highest molluscicidal activity level with LC_{50} value of 6.21 ppm. These promising results prompted us to further purify bioactive constituents from this fraction.

Fraction B-6 (100 % MeOH) (1g) was further chromatographed on a Diaion HP-20 column and eluted with 40, 60, 80 and 100% acetone in water, successively. Fractions (20ml) were collected and combined according to their TLC patterns to yield of 4 fractions (C-1-C-4) as shown in Table 13.

Table 13 Combination of fractions from B-6

Eluants	Fraction code	Number of fraction	Weight(g)	% w/w of powder
40%Acetone	C-1	1-30	0.32	32
60%Acetone	C-2	31-45	0.27	27
80%Acetone	C-3	46-58	0.22	22
100%Acetone	C-4	59-74	0.17	17

Each fraction was subjected to molluscicidal activity evaluation against golden apple snails (*Pumacea canaliculata*). The snails death were observed, at 24 hours intervals, and used for calculation of % mortality (Appendix B). The results of molluscicidal testing are shown in Table 14.

Table 14 The results of molluscicidal activity testing of fraction C-1-C-4 against *P. canaliculata* at 24 hours intervals.

Fraction code	LC_{50} (ppm) (95 % confidence Limits of LC_{50})	LC_{90} (ppm) (95 % confidence Limits of LC_{90})
C-1	5.54	9.56
C-2	5.82	11.84
C-3	5.71	11.06
C-4	5.80	11.31

From molluscicidal activity test, fraction C-1 displayed the highest molluscicidal activity level with LC_{50} value of 5.54 ppm. These promising results prompted us to further purify bioactive constituents from this fraction.

Fraction C-1 (40 % Acetone) (0.3g) was further chromatographed on a silica gel column and eluted with $CHCl_3$/MeOH, 21 : 1 → 2 : 1. Fractions (20ml) were collected and combined according to their TLC patterns to yield 4 fractions (D-1-D-4) as shown in Table 15.

Table 15 Combination of fractions from C-1

Fraction code	Number of fraction	Weight(mg)	% w/w of powder
D-1	1-23	24	8
D-2	24-35	75	25
D-3	36-49	51	17
D-4	50-67	147	49

Each fraction was subjected to molluscicidal activity evaluation against golden apple snails (*Pumacea canaliculata*). The snails death were observed, at 24 hours intervals, and used for calculation of mortality (Appendix B). The results of molluscicidal testing are shown in Table 16.

Table 16 The results of molluscicidal activity testing of fraction D-1-D-4 against *P. canaliculata* at 24 hours intervals.

Fraction code	LC_{50} (ppm) (95 % confidence Limits of LC_{50})	LC_{90} (ppm) (95 % confidence Limits of LC_{90})
D-1	5.06	8.25
D-2	4.84	7.91
D-3	4.68	7.57
D-4	4.44	7.24

From molluscicidal activity test, fraction D-4 displayed the highest molluscicidal activity level with LC_{50} value of 4.44 ppm. These promising results prompted us to further purify bioactive constituents from this fraction.

Fraction D-4 (0.1g) was further chromatographed on a silica gel column and eluted with CH_2Cl_2:MeOH (6 : 1). Fractions (10ml) were collected and combined according to their TLC patterns to yield 2 fractions (E-1 and E-2). as shown in table 12. Compound Sp1 (55.32mg) (55.32 % w/w) was obtained as a white powder from fraction E-1 and compound Sp2 (44.68mg) (44.68%w/w) was obtained as a white powder from fraction E-2.

Table 17 Combination of fractions from D-4

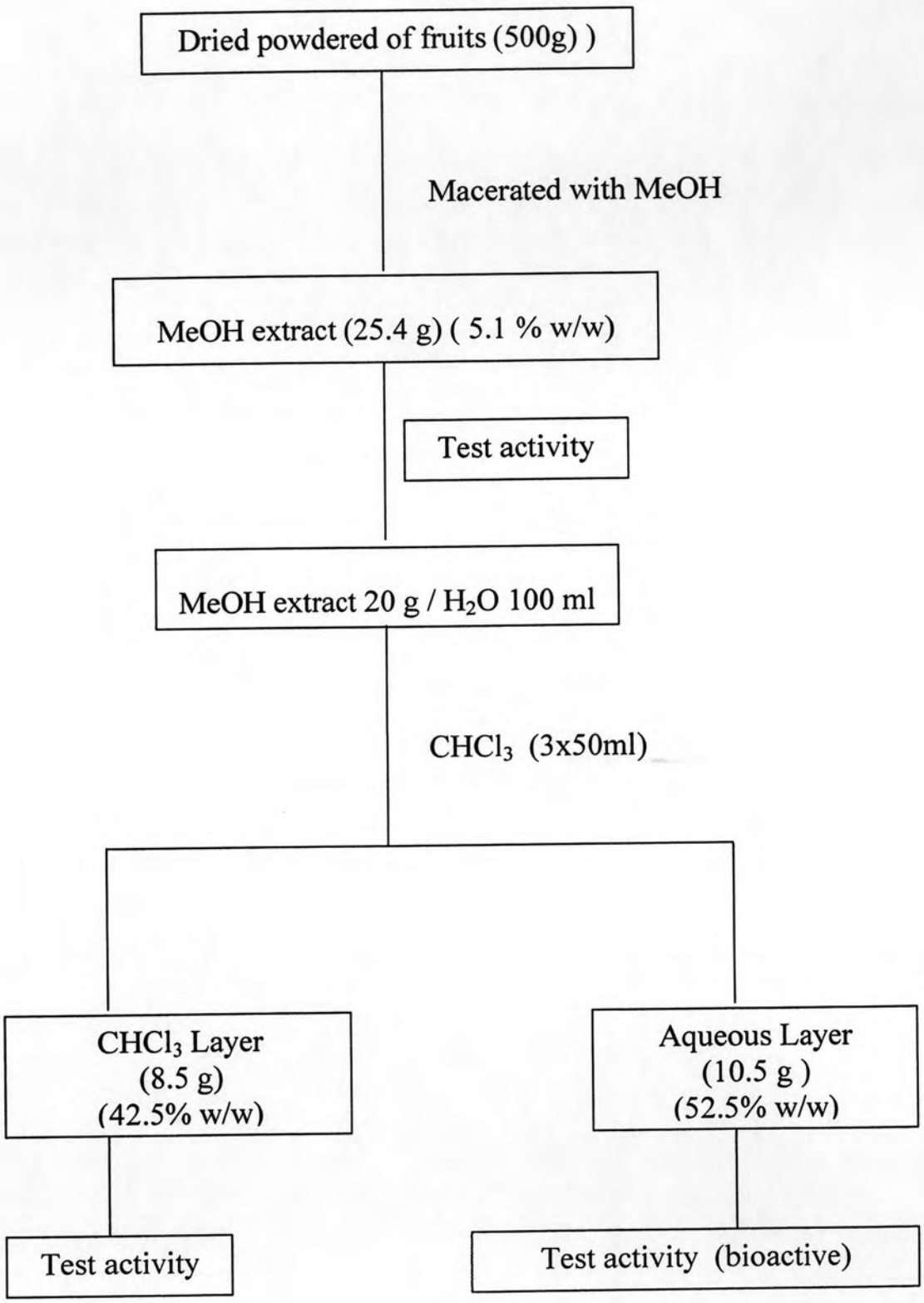
Fraction code	Compounds	Number of fraction	Weight(mg)	% w/w of powder
E-1	Sp1	1-25	55.32	55.32
E-2	Sp2	26-40	44.68	44.68

Each compound was subjected to molluscicidal activity evaluation against golden apple snails (*Pomacea canaliculata*). The snails death were observed, at 24 hoursr intervals and used for calculation of % mortality (Appendix B). The results of molluscicidal esting are shown in Table 18.

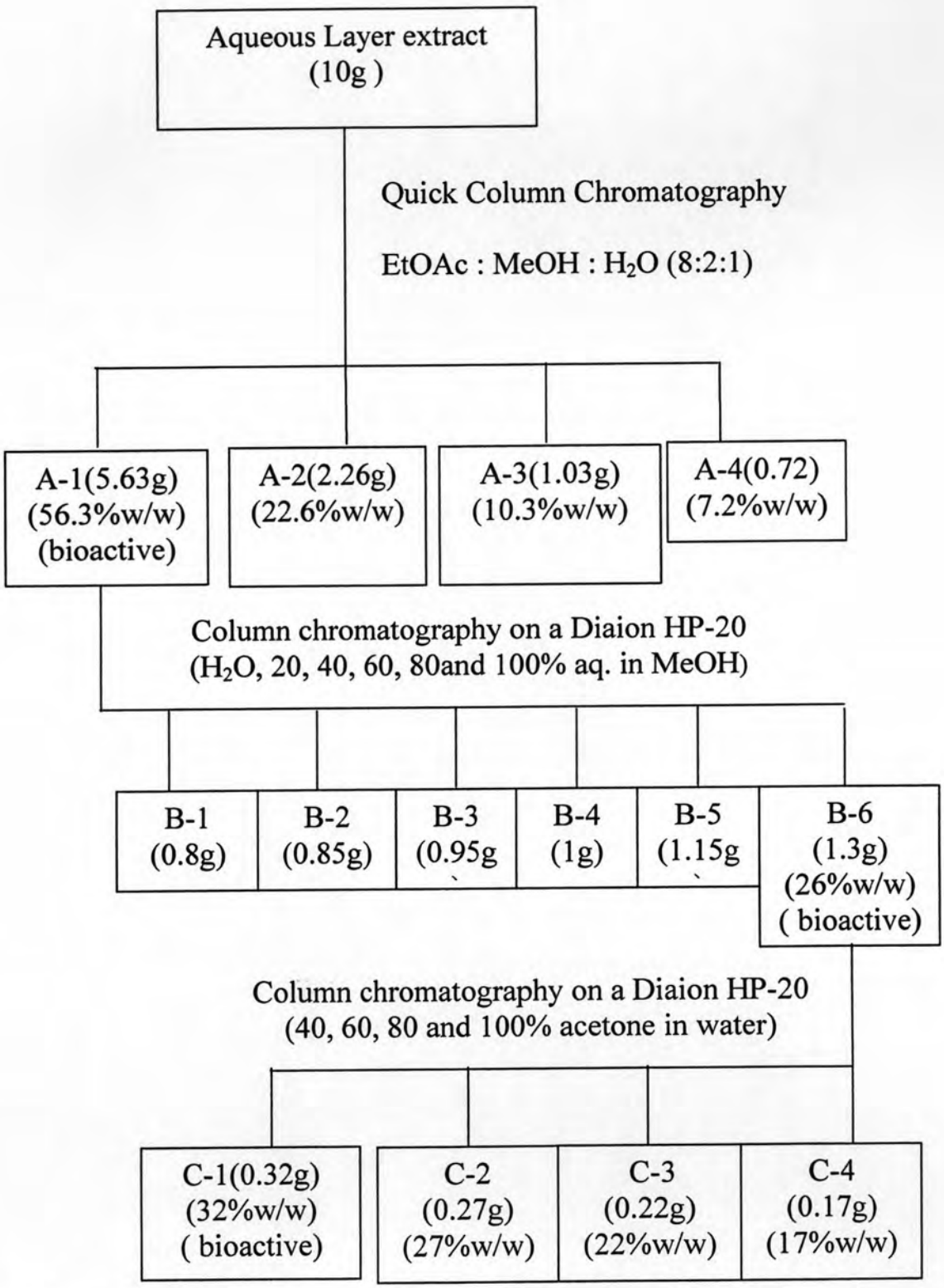
Table 18 The results of Molluscicidal activity testing of compound Sp1 and compound Sp2 against *P. canaliculata* at 24 hours intervals.

Compounds	LC_{50} (ppm) (95 % confidence Limits of LC_{50})	LC_{90} (ppm) (95 % confidence Limits of LC_{50})
Sp1	4.34	7.21
Sp2	4.28	6.84

From molluscicidal activity test, compound Sp1 and compounds Sp2 showed the molluscicidal activity level with LC_{50} values at 4.34 and 4.28 ppm, respectively after treating 24 h.

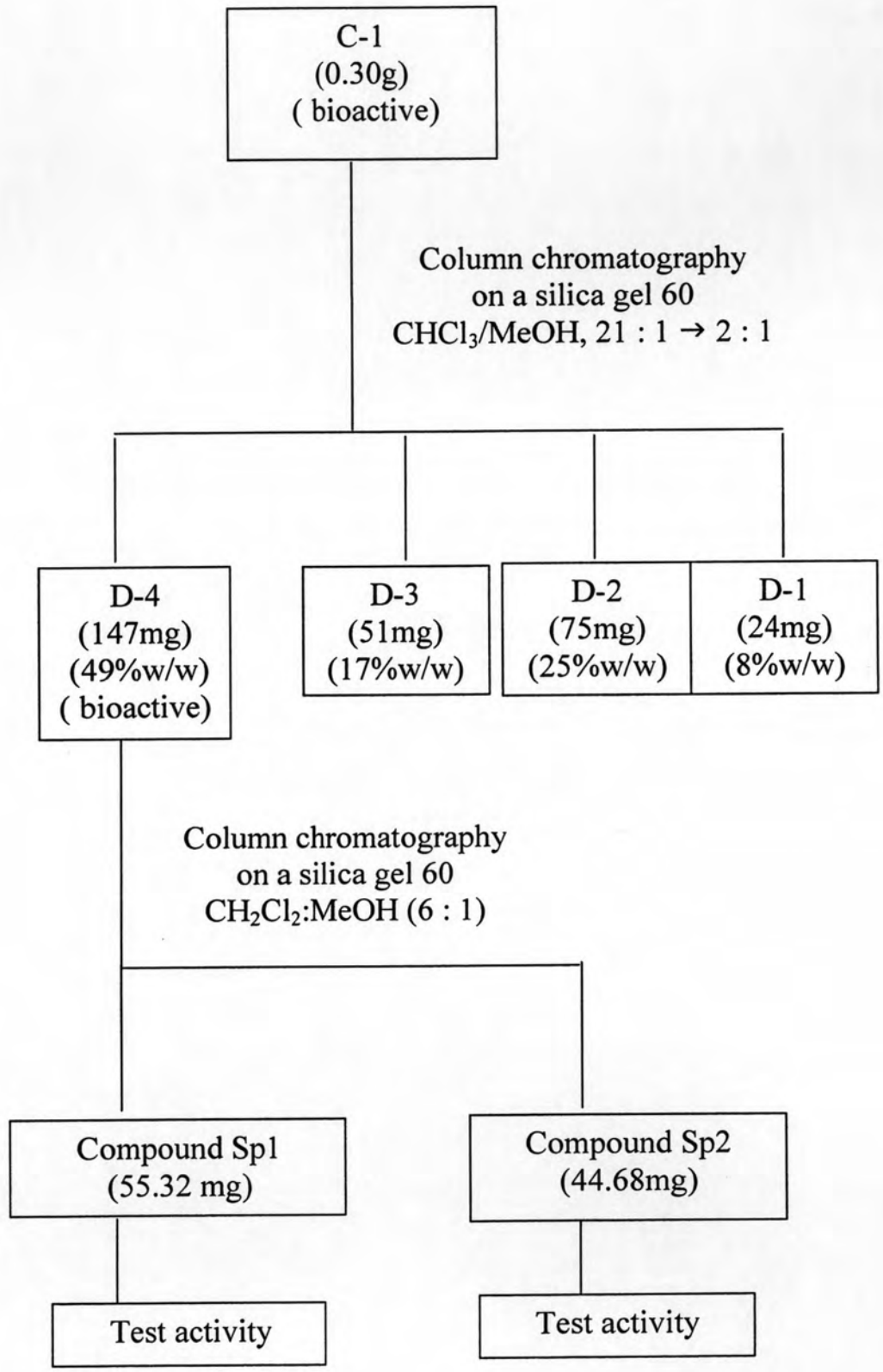


Scheme 1 Extraction and isolation of the fruits from *Sapindus rarak*



Scheme 2 Extraction and isolation of the fruits from *Sapindus rarak*

(cont.)



Scheme 3 Extraction and isolation of the fruits from *Sapindus rarak* (Cont.)

4.3 Physical and spectral data of the isolated compounds.

4.3.1 Compound Sp1

Compound Sp1 was obtained as white powder (55.32mg)

Melting point : 238 - 240 °C

$[\alpha]_D^{29}$: + 14.0 ° (CH_3OH, *c* 0.10)

FT-IR : V_{max} cm^{-1}, KBr disc; (Figure 11)

3319, 2942, 1695, 1453, 1386, 1256, 1136, 1054

EIMS : $[M-H]^-$ at m/z 881.3 m/z ; Fragment ion peaks at m/z 749$[(M-H)-132]^-$, 603$[(M-H)-(132+146)]^-$,and 471 $[(M-H)-(132+146+132)]^-$ (Figure 12)

^{1}H-NMR : $\delta_H$ppm, 400 MHz, in pyridine-d_5; (Figure 13-16)

^{13}C-NMR : δ_C ppm, 100 MHz, in pyridine-d_5; (Figure 17)

4.3.2 Compound Sp2

Compound Sp2 was obtained as white powder (44.68mg)

Melting point : 192-195 °C

$[\alpha]_D^{29}$: + 23.2 ° (CH_3OH, *c* 0.10)

FT-IR : V_{max} cm^{-1}, KBr disc ; (Figure 22)

3321, 2932, 1727, 1455, 1375, 1250, 1136, 1050

EIMS : $[M-H]^-$ peak appeared at m/z 923.5; Fragment ion peaks at m/z 881$[(M-H)-42]^-$, 749$[(M-H)-132]^-$, 603$[(M-H)-(132+146)]^-$,and 471$[(M-H)-(132+146+132)]^-$ (Figure 23)

^{1}H-NMR : δ_H ppm, 400 MHz, in pyridine-d_5; (Figure 24-28)

^{13}C-NMR : δ_C ppm, 100 MHz, in pyridine-d_5; (Figure 29)

5. Biological activity test

5.1 Cytotoxicity test

Cytotoxicity test was carried out at the Institute of Biotechnology and Genetic Engineering. Bioassay of cytotoxic activity against human tumor cell culture in vitro was performed by the MT (3-(4,5-dimethylthaizol-2-yl)-2,5-diphenyltetrazoliumbromide) colorimetric method [Carmichael *et al.*, 1987; Twentyman and Luscombe, 1967]. In principle, the viable cell number / well is directly proportional to the production of formazan which, following solubilization, can be measured spectrophotometrically.

The human tumor cell line was harvested from exponential-phase maintenance culture (T-75 cm^2 flask), counted by trypan blue exclusion, and dispensed within replicate 96-well culture plates in 100 μl volumes using a repeating pipette. Following a 24-h incubation at 37 °C, with 5% CO_2, 100% relative humidity and 100 μl of culture medium. Culture medium containng sample was dispensed within appropriate wells (Control group, N=3). Peripheral wells of each plate (lacking cells) were

utilized for sample blank (N=2) and medium / tetrazolium reagent blank (N=6)" back ground" determinations. Culture plates were then incubated for 4 days prior to the addition of tetrazolium reageagent. MTT stock solution was prepared as follows : 5 mg MTT / ml PBS was steriled and filtered with O.45 μm filtered units. MTT working solution was prepared just prior to culture application by diluting MTT stock solution 1:5 (v/v) in prewarmed standard culture medium. MTT working solution (50 μl) was added to each culture well resulting in 50 μg MTT / 250 μl total medium volume and cultures were incubated at 37 °C for 4 to 24 h. depending upon individual cell line requirements. Following incubation, cell monolayers and formazan were inspected microscopically: culture plates containing suspension lines or any detached cells were centrifuged at low speed for 5 min. All 10-20 μl of culture medium supernatant was removed from wells by slow aspiration through a blunt 18-guage needle and replaced with 150 μl of DMSO using a pipette. Following through formazan solubilization, the absorbance of each well was measured using a microculture plate reader at 540 nm (single wavelength, calibration factor = 1.00)

Cell line growth and growth inhibition were expressed in terms of mean (1 SD) absorbance units and / or percentage of control absorbance (1 SD%) following subtraction of mean absorbance.

Samples were also tested for cytotoxic activity towards 6 cancer cell lines, including HS27 (fibroblast), HEP-G2 (hepatoma), SW 620 (colon), Chago (lung), Kato-3 (gastric) and BT474 (breast), following the experimental method for bioassay of cytotoxic activity.