5. EXPERIMENTAL

5.1. Plant Material

The bark and leaves of *Alseodaphne corneri* Kosterm was identified and collected at Hutan Simpan Piah, Sg. Siput, Perak by the Phytochemistry group of the Chemistry Department University of Malaya. The herbarium series number is KL 5501.

5.2. Instrumentation

The (^{1}H, ^{13}C and 2D) NMR spectra were obtained using JEOL LA 400 FT NMR and JEOL ECA 400 FT NMR spectrometer system using deuterated chloroform as solvent. The chemical shifts were reported in ppm or δ scale and the coupling constants are given in the Hz unit.

Mass spectrum was obtained on a JEOL JMS 700 TZ spectrometer. The EIMS spectra were obtained on Shimadzu GC-MS-QP2000A Mass Spectrometer 70 eV.

UV spectra and optical rotation were obtained by using Jasco (J-815) CD spectrometer with methanol as solvent.

The infrared spectra were obtained with chloroform as solvent on a Perkin Elmer 2000 spectrometer.

5.3. Chromatography

5.3.1. Thin layer chromatography (TLC)

Aluminium supported silica gel 60 F_{254} plates were used to monitor isolate ion of compounds based on the spot of TLC. TLC spots were visualized under ultra-violet light (245-365 nm) using the model UVGL-58 after spraying with the required reagents.

5.3.2. Column Chromatography (CC)

The solvents used in this experiment were hexane, dichloromethane, chloroform, acetone and methanol. All solvents are AR grade except those that are used for bulk extractions (distilled). Silica gel 60, 70-230 mesh ASTM (Mersk 7734) was used for column chromatography. A slurry of silica gel 60 (approximately ratio of 30:1, silica gel:sample) in dichloromethane solvent was poured into a glass column of appropriate size. The crude extract was initially dissolved in minimum amount of solvent and loaded on top of the packed column. The extract was eluted with gradient solvent system at a certain flow rate.

5.3.3. Preparative Thin Layer Chromatography (PTLC)

PTLC silica gel 60 F_{254} glass plates of size 20x20 cm were used for separation of compounds that cannot be separated by conventional column. UV Light Model UVGL-58 was used to examine bands on the PTLC.

5.4. Reagents

5.4.1. Mayer's test (Potassium mercuric iodide)

1.4 g mercuric iodide in 60 ml distilled water was mixed with solution of 5.0 g potassium iodide in 10 ml distilled water. The mixture then made up to 100 ml solution.

The positive result was indicated by the formation of white precipitate when the aqueous layer (acidified) is treated with 2-3 drops of Mayer's reagent.

5.4.2. Dragendorff's Reagent (potassium bismuth iodide)

Bismuth (III) nitrates (0.85 g) are dissolved in a mixture of glacial acetic acid (10 ml) and distilled water (40 ml) for solution A. While for solution B; potassium iodides (8.0 g) are dissolved in distilled water (20 ml). To prepare the stock solution, solution A and B mixed with equal volumes. The stock solution (20 ml) then was diluted in the mixture acetic acid (20) ml and distilled water (60 ml) for spray agent. A positive result indicated by the formation of orange spots.

5.5. Extraction of *Alseodaphne corneri* Kosterm.

Plant extractions were carried out by cold extraction or exhaustive extraction using the soxhlet extractor, the general procedure is described below.

Dried, grounded barks of the plant (1.0kg) were first defatted with hexane for twice for 3-days period. The hexane extract were first concentrated by using rotary evaporator. The plant material was dried up and then soaked with 25% NH_4OH for 2 hours. They were then macerated twice for 3-days periods. The supernatant obtained was concentrated using rotary evaporator under reduced pressure to a volume of 500ml and give alkaloid in the solid form residue and the extraction was successively by checking with a Mayer's negative test after each extraction. The combined extract were then basified with concentrated ammonia solution to pH 11 followed by re extraction with dichloromethane. The dichloromethane extract was washed with distilled water

and dried over anhydrous sodium sulphate and evaporated to dryness to give crude alkaloid. Similar experimental procedures were also used for leaves of *Alseodaphne corneri* Kosterm.

5.6. Isolation of Alkaloids from *Alseodaphne corneri* Kosterm

The crude alkaloid was subjected to column chromatography over silica gel, using the following solvent system.

Table 5.1: Solvent System CH_2Cl_2 : MeOH used in Column Chromatography

Dichloromethane	**:**	**Methanol**
100	:	0
99	:	1
98	:	2
96	:	4
95	:	5
94	:	6
92	:	8
90	:	10
80	:	20
0	:	100

The fractions collected were grouped into a series of fractions, monitored with TLC and the fractions with similar compounds were then combined. Each series were then treated separately to isolate and purify its alkaloid content by PTLC and small column chromatography. The alkaloids that showed potent activity toward antiplasmodial and

antihypertensive activity based on the literature reviews, were tested for their bioactivities.

Structural identification of the isolated compounds were carried out by using spectroscopic methods; ^{1}H NMR, ^{13}C NMR, COSY, HSQC, HMBC, DEPT, IR, UV, optical rotation and mass spectroscopy. Compounds isolated from the leaves and barks of *Alseodaphne corneri* are tabulated in table 5.2 and table 5.3. The isolation of alkaloids from *Alseodaphne corneri* was summarized in the flow diagram shown in scheme 5.1 (leaves) and scheme 5.2 (bark).

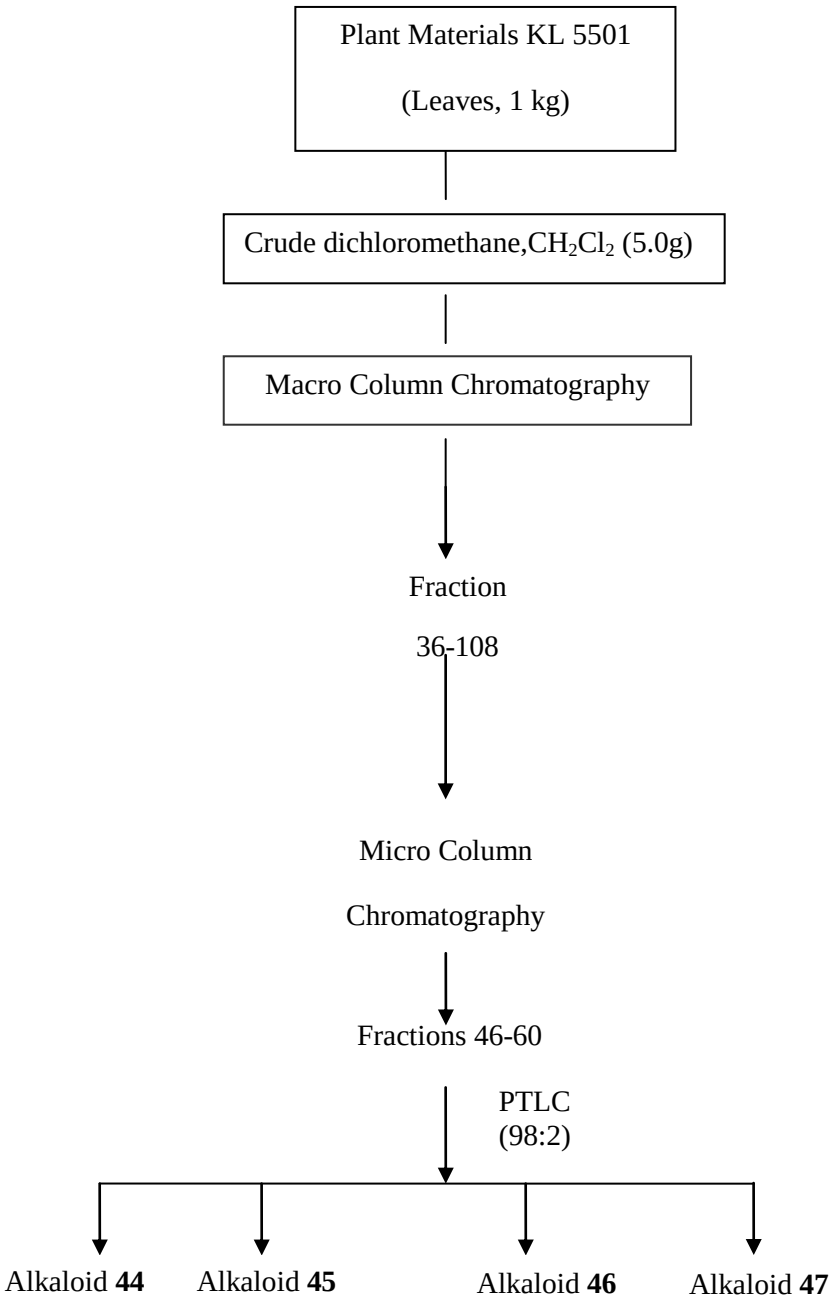
Table 5.1: Solvent System used to isolate Alkaloids **44-47** from the Leaves of *A.corneri*

Eluent	**Fraction**	**Alkaloid isolated**
CH_2Cl_2 : MeOH		

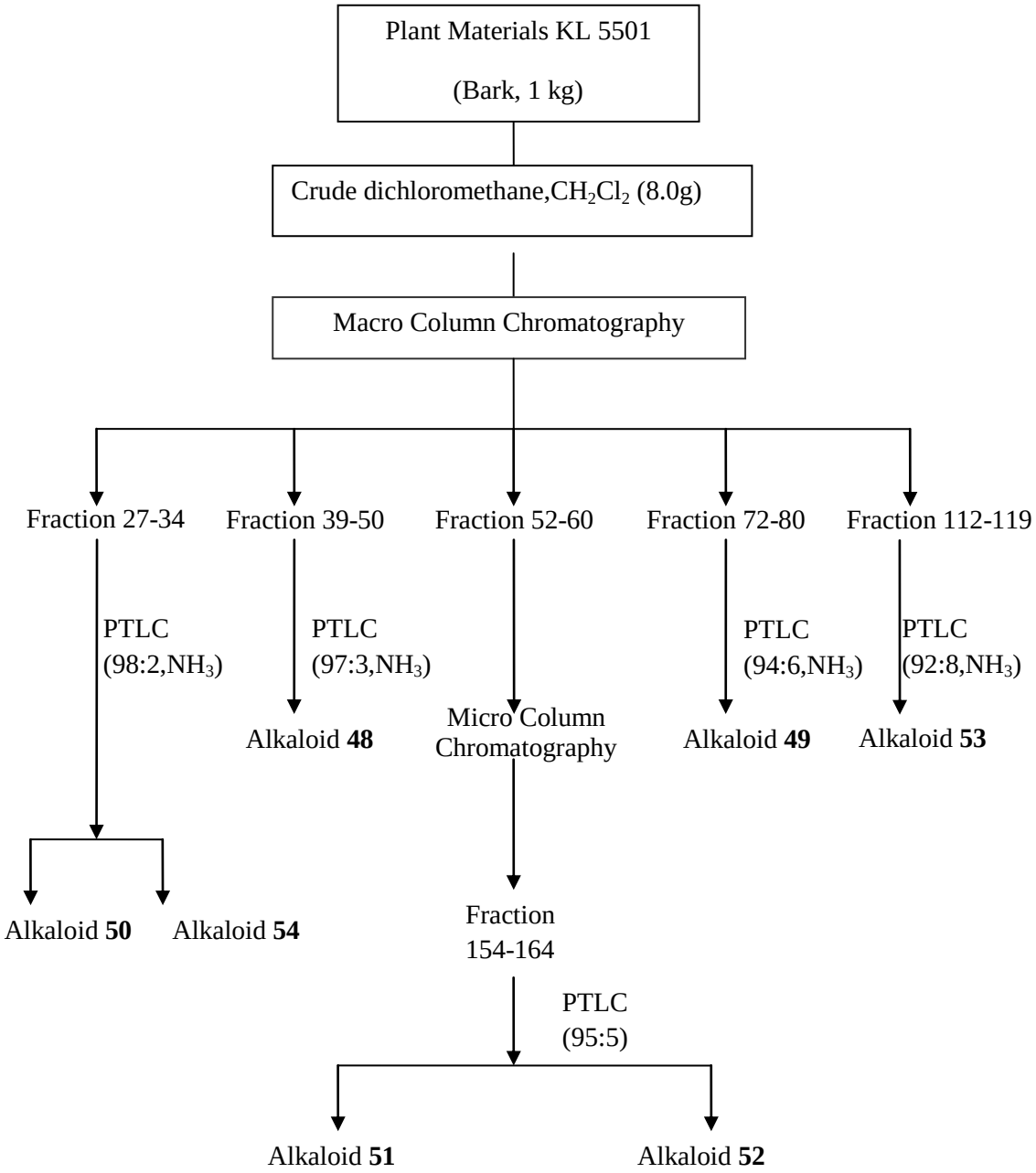
98 : 2	46-60	Alkaloid **44**; Isocorydine
98 : 2	46-60	Alkaloid **45**; Norisocorydine
98 : 2	46-60	Alkaloid **46**; *N*-Methyl Laurotetanine
98 : 2	46-60	Alkaloid **47**; *N*-methyl Lindcarpine

Table 5.2: Solvent System used to isolate Alkaloids **48-54** from the Barks of *A.corneri*

Eluent CH_2Cl_2 **: MeOH**	**Fraction**	**Alkaloid isolated**
98 : 2	27-34	Alkaloid **50**; Gyrolidine
98 : 2	27-34	Alkaloid **54**; Stephasubimine
97 : 3	39-40	Alkaloid **48**; Laurotetanine
95 : 5	52-60	Alkaloid **51**; Norstephasubine
95 : 5	52-60	Alkaloid **52**; 3', 4'-dihydronorstephasubine
94 : 6	72-80	Alkaloid **49**; Norboldine
92 : 8	112-119	Alkaloid **53**; 7'-O-demethylstebisimine



Scheme 5.1: Isolation of Alkaloids from the Leaves of *Alseodaphne corneri* Kosterm.



Scheme 5.2: Isolation of Alkaloids from the Barks of *Alseodaphne corneri* Kosterm.

5.7. Antiplasmodial Assay

Extracts were tested against the chloroquine-resistant FcB1/ Colombia strain of *P. falciparum* (Frappier et al., 1996). *P. falciparum* was maintained continuously *in vitro* in human erythrocytes according to Trager and Jensen (2005). The antiplasmodial activity was determined according to Desjardins et al. (1979). The extracts were dissolved in dimethylsulfoxide (DMSO) and tested at a concentration of 10 µg/ml. Compounds showing significant inhibition rates were submitted to serial dilutions with culture medium before being added to asynchronous parasite cultures (1% parasitemia and 1% final hematocrite) in 96-well microplates for 24 h at 37 ºC. A concentration of 0.5 µCi of [3H] hypoxanthine was then added to each well, and parasites were maintained for an additional 24 h. The growth inhibition for each compound concentration was determined by comparing the radioactivity incorporated in the treated culture with that in the control culture maintained on the same plate. The concentrations causing 50% inhibition of parasite growth (IC_{50}) were calculated from the drug concentration-response curves. The human diploid embryonic lung cells MRC-5 were seeded into 96-well microplates at 2000 cells per well. The cytotoxicity assays were performed according to a published procedure (Tempete et al., 1995). Taxotere was used as a control compound.

5.8. Vasorelaxant Assay

Vasodilation Assay of alkaloid in bark of *Alseodpahne corneri* is to examine the action of compounds on the smooth muscle of the rat aorta and to elucidate the mechanism of the relaxant effect.[92]A male Wistar rat weighting 260 g was sacrificed by bleeding from carotid arteries under an anesthetization. A section of the thoracic aorta between the aortic arch and the diaphragm was removed and placed in oxygenated, modified Krebs-Henseleit solution (KHS: 118.0 mM NaCl, 4.7 mM KCl, 25.0 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM $MgSO_4$, and 11.0 mM glucose).

The aorta was cleaned of loosely adhering fat and connective tissue and cut into ring preparations 3 mm in length. The tissue was placed in a well-oxygenated (95% O_2, 5% CO_2) bath of 5 mL KHS solution at 37 °C with one end connected to a tissue holder and the other to a force-displacement transducer (Nihon Kohden, TB-611T). The tissue was equilibrated for 60 min under a resting tension of 1.0 g. During this time the KHS in the tissue bath was replaced every 20 min.

After equilibration, each aortic ring was contracted by treatment with 3×10^{-7} M Phenylepherine. Acetycholine was used as standard to make the aortic ring in relaxation mode. The presence of functional endothelial cells was confirmed by demonstrating relaxation to 10^{-5} M acetylcholine (ACh), and aortic ring in which 80% relaxation occurred, were regarded as tissues with endothelium. When the PE-induced contraction reached a plateau, each sample (**50-52**, 3×10^{-5}) was added.

These animal experimental studies were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University and under the supervision of the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Science, Sports Culture, and Technology of Japan.

5.9. Physical and Spectral Data of the Isolated Compounds

Alkaloid 44: Isocorydine

Isocorydine	: $C_{20}H_{24}NO_4$
UV λ_{max} nm	: 270 and 310
IR v_{max} cm^{-1}	: 3150
$[\alpha]_D^{25}$	: +196.0º(c=0.10, MeOH)
Mass spectrum m/z	: 342.1720
^1H NMR ($CDCl_3$) δ	: see table 3.1
^{13}C NMR ($CDCl_3$) δ	: see table 3.1

Alkaloid 45: Norisocorydine

Norisocorydine	: $C_{19}H_{21}NO_4$
UV λ_{max} nm	: 270 and 310
IR v_{max} cm^{-1}	: 3500, 2936
$[\alpha]_D^{25}$	: +158.5º(c=0.10, MeOH)
Mass spectrum m/z	: 328.1538
^1H NMR ($CDCl_3$) δ	: see table 3.2
^{13}C NMR ($CDCl_3$) δ	: see table 3.2

Alkaloid 46: *N*-Methyllaurotetanine

N-Methyllaurotetanine	: $C_{20}H_{22}NO_4$

UV λ_{max} nm	: 215, 283, 305
IR v_{max} cm^{-1}	: 3500, 2936
$[\alpha]_D^{25}$	: +111.0(c=0.86, MeOH)
Mass spectrum m/z	: 342.1705
^1H NMR ($CDCl_3$) δ	: see table 3.3
^{13}C NMR ($CDCl_3$) δ	: see table 3.3

Alkaloid 47: *N*-Methyllindcarpine

N-Methyllindcarpine	: $C_{19}H_{21}NO_4$
UV λ_{max} nm	: 270, 303
IR v_{max} cm^{-1}	: 3336
$[\alpha]_D^{25}$	: +160.0 (c=0.21, MeOH)
Mass spectrum m/z	: 327
^1H NMR ($CDCl_3$) δ	: see table 3.4
^{13}C NMR ($CDCl_3$) δ	: see table 3.4

Alkaloid 48: Laurotetanine

Laurotetanine	: $C_{20}H_{22}NO_4$

UV λ_{max} nm	: 220, 281, 302, 312
IR v_{max} cm^{-1}	: 3429
$[\alpha]_D^{25}$	: +125.0 (c=2.28, MeOH)
Mass spectrum m/z	: 328.1566
^1H NMR ($CDCl_3$) δ	: see table 3.5
^{13}C NMR ($CDCl_3$) δ	: see table 3.5

Alkaloid 49: Norboldine

Norboldine	: $C_{18}H_{19}NO_4$
UV λ_{max} nm	: 283, 304
IR v_{max} cm^{-1}	: 3584, 3162
$[\alpha]_D^{25}$	: +102.5 (c= 0.01, MeOH)
Mass spectrum m/z	: 313
^1H NMR ($CDCl_3$) δ	: see table 3.6
^{13}C NMR ($CDCl_3$) δ	: see table 3.6

Alkaloid 50: Gyrolidine

Gyrolidine	: $C_{38}H_{42}N_2O_6$

UV λ_{max} nm	: 244, 286
IR v_{max} cm^{-1}	: 1070, 1126, 1231, 1268, 1511, 1606
$[\alpha]_D^{25}$	: -115.0 (c=1.1, MeOH)
Mass spectrum m/z	: 622
^1H NMR ($CDCl_3$) δ	: see table 3.7
^{13}C NMR ($CDCl_3$) δ	: see table 3.7

Alkaloid 51: Norstephasubine

Norstephasubine	: $C_{35}H_{32}N_2O_6$
UV λ_{max} nm	: 219, 290, 327
IR v_{max} cm^{-1}	: 1606, 2929
$[\alpha]_D^{25}$	: +309.0 (c=1.0, MeOH)
Mass spectrum m/z	: 576
^1H NMR ($CDCl_3$) δ	: see table 3.8
^{13}C NMR ($CDCl_3$) δ	: see table 3.8

Alkaloid 52: 3', 4'-dihydronorstephasubine

3', 4'-dihydronorstephasubine	: $C_{35}H_{34}N_2O_6$

UV λ_{max} nm	: 203, 286
IR v_{max} cm^{-1}	: 1604, 2934
$[\alpha]_D^{25}$	: +22 (c 0.5,MeOH)
Mass spectrum m/z	: 579.2535
^1H NMR ($CDCl_3$) δ	: see table 3.9
^{13}C NMR ($CDCl_3$) δ	: see table 3.9

Alkaloid 53: 7'-O-demethylstebisimine

7'-*O*-demethylstebisimine	: $C_{35}H_{34}N_2O_6$
UV λ_{max} nm	: -
IR v_{max} cm^{-1}	: 2200, 3583
$[\alpha]_D^{25}$	: -
Mass spectrum m/z	: 577.20
^1H NMR ($CDCl_3$) δ	: see table 3.10
^{13}C NMR ($CDCl_3$) δ	: see table 3.10

Alkaloid 54: Stephasubimine

Stephasubimine	: $C_{35}H_{31}N_2O_6$

UV λ_{max} nm	: 248, 281, 323
IR v_{max} cm^{-1}	: 1602, 3392
$[\alpha]_D^{25}$	: -
Mass spectrum m/z	: 575.2164
^1H NMR ($CDCl_3$) δ	: see table 3.11
^{13}C NMR ($CDCl_3$) δ	: see table 3.11

References

1. Keng, H.,(1978). *Order and families of Malayan seed plants*. 2 ed.; Singapore University Press.