CHAPTER THREE

EXPERIMENTAL

3.1 Plant Materials

The plant materials were collected from the Sri Aman and Bintulu districts in Sarawak, East Malaysia. Identification was carried out at the Herbarium, Forest Department Headquarters, Kuching, Sarawak, where voucher specimens were deposited.

3.2 Instruments

3.2.1 Melting points

Melting points (m.p.) were taken on a hot stage Leitz Wetzler melting point apparatus and were uncorrected.

3.2.2 Optical rotations

Optical rotations were determined on a Jason DIP 370 digital polarimeter.

3.2.3 Infrared spectroscopy

Infrared spectra were recorded in $CHCl_3$ solutions on a Perkin-Elmer 1330 infrared spectrophotometer.

3.2.4 Ultraviolet spectroscopy

Ultraviolet spectra were recorded in absolute ethanol on a Shimadzu UV-3101 PC instrument.

3.2.5 NMR spectroscopy

^1H and ^{13}C NMR spectra were recorded on a JEOL JNM-GSX 270 Fourier-transform spectrometer mostly in $CDCl_3$ solution using trimethylsilane (TMS) as the internal standard at 270 MHz and 67.8 MHz, respectively. Some spectra were

recorded on a JEOL AA400 at Kyoto University, Japan and a Bruker AMX 500 at the National University of Singapore.

3.2.6 Mass spectroscopy

Mass spectra were measured on VG Prospec, VG 7070, VG ZAB-2SEQ, JEOL JMS-HX 100 mass spectrometers.

3.2.7 High performance liquid chromatography

HPLC was carried out using a WATERS 440 Liquid Chromatograph equipped with dual refractive index (RI) and ultra-violet (UV) detectors.

3.2.8 Gas chromatography

Gas chromatography analyses were carried out on a Shimadzu GC 14A Gas Chromatograph equipped with a flame ionization detector (FID) and using a 1.1 m glass column of 3 mm i.d. coated with OV-17. Analyses were also carried out on another Shimadzu GC 14A Gas Chromatograph equipped with a 25 mm x 0.53 mm i.d. aluminium clad capillary column, coated with HT 5 (siloxane-carborane phase) and a FID detector. Nitrogen was used as carrier gas at a flow rate of 2 ml/min and made-up to 17 ml/min. The operating temperature was from 80 ^{o}C to 300 ^{o}C programmed at a rate of 10 ^{o}C/min, with an initial hold of 1 min and a final hold of 10 min. Chromatograms were plotted by a Hewlett Packard 3395 integrator.

3.2.9 Gas Chromatography-Mass Spectrometry

GC-MS analyses were carried out on a HP GC 5890 Series II - MSD 5971A equipped with a MSD detector and using a Shimadzu capillary column of 25 m x 0.25 μm thickness x 0.2 mm i.d. coated with SE-30. Some GC-MS analyses were also carried out on a HPGC 5890 Series II - VG Prospec equipped with a FID detector and a capillary column of 25 m x 0.25 μm thickness x 0.25 mm i.d. coated with OV-1.

The operation conditions were the same as those for GC and samples were partially purified through column and preparative layer chromatography prior to introduction into the GC.

3.3 Chromatographic methods

Flash column-chromatography was performed using Merck silica gel 9385 (230-400 Mesh ATM). SiO_2 column chromatography was performed using Merck silica gel 7739 and 7736 (silica gel for thin layer chromatography). In this method, a filtering column (sintered glass filter of pore size 2) was packed with a slurry of the silica gel as suction was applied. With the suction off, and the eluant 2-4 cm higher than the top of the silica gel bed, a glass rod was used to stir the silica gel until a homogeneous slurry was formed. Air bubbles were removed by tapping the outside wall of the column and the excess solvent by full suction. The silica gel was then packed as tightly as possible by tapping the wall of the column and draining by partial suction the excess eluant several times until there was no more excess eluant to be drained (that is, no eluant above the top of the silica gel surface). The silica gel should be kept wet during the whole process. The sample in a minimum amount of solvent was introduced directly on the top of the silica gel bed evenly and smoothly by a Pasteur pipette. Small amounts of eluant was introduced by a Pasteur pipette along the wall gently to bring the sample down underneath the bed surface. Fractions of the SiO_2 column chromatography were collected by using Erlenmeyer flasks and monitored by thin layer chromatography.

3.3.2 Thin layer chromatography

Thin layer chromatography was routinely used to detect and separate the various components present in plant extracts and to monitor fractions collected during column chromatography. The crude plant extracts, fractions from column chromatography, fractions from chromatotron collections, HPLC fractions and the isolated pure compounds were examined by TLC using precoated 5 x 10 cm glass plates, 0.25 thickness, silica gel 60 F_{254} (Merck, Darmstadt, GFR). The TLC plates were spotted with a piece of fine glass capillary tube and then developed in saturated chromatographic tanks with various solvent systems at room temperature (28 °C). The spots were visualised by examination of the TLC plates under UV light (254 and 366 nm), followed by development in iodine vapour and also by spraying with Vanillin reagent and heating on a hot plate. For acetogenins, TLC plates were sprayed with 5% phosphomolybdic acid in methanol and heating the plates on a hot plate. The mobile phase used to develop the TLC plates were usually 5% chloroform/hexane, 5% ethyl acetate/chloroform, 1% methanol/chloroform, 5% methanol/chloroform and 10% methanol/chloroform.

3.3.3 Preparative layer chromatography (PLC)

Preparative layer chromatography (PLC) was carried out on silica gel 60 F_{254} (Merck 7730) on 20 x 20 cm glass plates of layer thickness 0.75 mm (15 g silica gel in 27 ml distilled water per plate). The plates were air dried for one hour and then activated overnight in an oven at 110 °C before use. Preparative layer chromatography was used routinely for the isolation as well as purification of the compounds. The weight of the sample applied to each plate was approximately 25 mg. Samples were introduced onto the plates as a continuous streak 1 cm from the

base of the plate using fine capillary tubes and then developed in saturated chromatographic tanks at room temperature (28 °C).

The solvent systems used were various percentages of ethyl acetate in chloroform and methanol in chloroform.

The developed plates were visualized under UV light and sometimes by spraying on one side of the plate in a narrow streak with the rest of the plate covered by a piece of paper. Required bands were scrapped off using a micro spatula and extracted exhaustively via a small vertical column with a cotton plug at the bottom. The extracting solvent used was 5% methanol in chloroform.

3.3.4 Centrifugal thin layer chromatography (Chromatotron)

Chromatography was performed in a thin layer of silica gel PF_{254} with $CaSO_4.½H_2O$ type 60 TLC, Merck 7749 on a motor driven-rotor. Fifty grams of the silica gel was mixed with 100 ml of distilled water of temperature 0-5 °C. The mixture was poured on to the rotor which was manually rotated by turning the central rod. This would give a layer thickness of 1 mm. For a 2 mm thickness, 65 g of the same silica gel mixed with 130 ml of distilled water was used. The silica gel layer was allowed to set at room temperature for 1 hour and then dried and activated in an oven at 70 °C overnight. The cold rotor was then scrapped to obtain a smooth surface of silica gel. A solution of the compound to be separated was applied to the silica gel absorbent via the inlet and wick. Elution by solvent formed concentric bands of separated substances which were collected when they leave the edge of the rotor together with solvents.

3.3.5 Spray reagents

Vanillin solution: 1 g of vanillin crystals and 1 ml of concentrated sulphuric acid were dissolved in 98 ml of absolute ethanol.

Dragendorf's reagent: Solution A : 0.85 g of bismuth nitrate was dissolved in a mixture of 10 ml glacial acetic acid and 40 ml of distilled water. Solution B: 8 g of potassium iodide was dissolved in 20 ml of distilled water.

A stock solution was prepared by mixing equal volumes of solution A and B. Dragendorf's reagent was made by mixing 1 ml of stock solution with 2 ml of glacial acetic acid and 10 ml of distilled water. Orange spots on the developed TLC plates indicated the presence of alkaloids.

Phosphomolybdic acid solution: 5 g of phosphomolybdic acid was dissolved in 95 ml of methanol and heated up to dissolve the acid. Greyish-blue spots on the developed TLC plates (heated to 100 ^{o}C) indicated the presence of acetogenins.

3.4 Bioassay procedures

3.4.1 Culturing of Aedes aegypti mosquitoes

Aedes aegypti mosquitoes were cultured to ensure a continuous supply of mosquito eggs. The following procedure was carried out to culture the mosquitoes from the egg to the adult stage.

1. A piece of filter paper which had fresh mosquito eggs on it was submerged in de-chlorinated tap water. A little larval food was added. Mosquito larvae hatched after 24 hours. The larvae were allowed to grow into pupa (usually after 5-6 days upon hatching).

2. The pupa were then transferred into a porcelain bowl and the bowl placed in a mosquito cage. The mosquito cage was of dimension 30 cm x 30 cm x 30 cm, had aluminium frames with stainless steel wire mesh and a full size door complete with cotton trunk of approximately 15 cm in length. Food was supplied in the form of a sugar solution containing vitamin B complex. This solution was absorbed onto a muslin cloth supported by a stick in the sugar solution container.

3. After one or two days, adult mosquitoes emerged from the pupa stage. Normally, the male would emerge before the female. The mosquitoes were then blood-fed on a guinea pig for one night. The guinea pig was introduced in an animal restrainer into the mosquito cage and left there for one night. Twenty-four hours later, the female mosquitoes started laying eggs. The eggs were laid onto a partially-folded piece of wet filter paper. This wet filter paper provided the female a suitable breeding ground. The filter paper covered with eggs were then collected, air-dried and kept for future use.

4. To hatch larvae for bioassay testings, a small piece was cut from the egg-containing filter paper, submerged in de-chlorinated water and the larvae allowed to hatch.

3.4.2 Procedure for carrying out the susceptibility or resistance of mosquito larvae to insecticides test

1. One hundred milligram of the sample was dissolved in 20 ml of absolute ethanol to prepare a standard stock solution of 5000 µg/ml concentration.

2. Into three 250 ml drinking glasses containing 25 ml of chlorine-free water were introduced 0.5 ml, 1.0 ml and 1.5 ml of the stock solution. When made-up to 50

ml, these solutions would have concentrations of 50, 100 and 150 µg/ml. A control was prepared by introducing 1.5 ml of absolute alcohol into another drinking glass containing 25 ml of chlorine-free water.

3. Ten late 3rd-instar mosquitoes were introduced into each glass by means of a dropper. The volume was then made up to 50 ml with chlorine-free water.

4. A little larval food (ground roasted cow liver) was added.

5. After twenty-four hours, mortality counts were made.

6. From this range-finding experiment, further experiments were carried out to narrow down the range. The experiments were carried out in a series of at least five concentrations in duplicates or triplicates.

7. The mortality data were analyzed with a personal computer programmed with probit analysis in order to obtain the LC_{50} and LC_{90} values.

8. Used larvae were discarded by pouring them onto dry grass and left to die under the hot sun.

3.4.3 Procedure for carrying out an *in vitro* anti-cancer bioassay

Cell survival was measured by using the MTT-microculture tetrazolium assay described by Mosmann (1983) and Scudiero *et al.*, (1988). Briefly, cells at the exponential growth phase were harvested and centrifuged at 200 g for 5 min, resuspended in complete medium to 0.5×10^5 or 1.0×10^5 cells per ml and 180 µl of the cells wee added to each well of a flat bottom 96-well plate with a multichannel pipette. After 24 hour incubation in a 5% CO_2 humidified incubator at 37 °C, 20 µl of

the test agent was added in 6 replicates to give a final concentration of 100 µg/ml, 10 µg/ml and 1 µg/ml medium. The concentration of ethanol or DMSO used to dissolve the extract was adjusted to 0.5% and this concentration of solvent was used in control wells. After 48 hours (for MOLT 4 and P388) or 72 hours (for KB and SW 620) incubation at 37 °C, MTT (stock solution 5 mg/ml PBS, diluted 5 times with RPMI medium without FBS prior to use, 50 µl per well) was added and the plates were incubated again for 4 hours, after which the medium was removed by aspiration. 150 µl of DMSO were added to each well and the formazan dissolved by trituration. The plates were read immediately in a microplate reader (Ceres UV900c, Bio-Tek Instruments, USA) at 570 nm. Wells with complete medium, test agent and MTT but without cells were used as blanks.

3.5 Extraction of compounds

The stem bark was dried and ground before extraction. The ground bark was then extracted with n-hexane for forty-eight hours. The hexane extract was filtered and the residue was then reextracted with a fresh portion of n-hexane and the two extracts combined. The combined hexane extract was concentrated down under reduced pressure in a rotary evaporator.

The bark was then extracted with ethyl acetate for forty-eight hours, twice. The ethyl acetate extract was filtered and evaporated to dryness under reduced pressure.

The bark was finally extracted with 95% ethanol for forty-eight hours, twice. Similarly, the filtered extracts were combined and evaporated to dryness in a rotary evaporator.

The ethanol extract was partitioned between chloroform and distilled water. The chloroform solubles was concentrated down under reduced pressure and further partitioned between n-hexane and 90% aqueous methanol. The methanol soluble fraction was collected and evaporated to dryness under reduced pressure.

3.6 Isolation of bioactive principles

3.6.1 Isolation of bioactive principles from *Goniothalamus andersonii*

The chopped bark (1 kg) of *Goniothalamus andersonii* was air-dried and ground. The sample was soaked in n-hexane for 48 hours twice and the filtrate collected and evaporated to dryness. About 15 g of crude extract was obtained. The extract was redissolved in a minimum amount of 95% ethanol and left to stand to recrystallise goniothalamin (GA-1). Crystals of goniothalamin (6.5 g) was obtained. The remaining hexane extract was subjected to SiO_2 column chromatography. The solvent system used was hexane, hexane-chloroform and chloroform. This gave a complex inseparable mixture of essential oils of very high R_f (0.78, 5% ethyl acetate/hexane) value. The mixture after partial separation by gas chromatography (OV-17 packed in a 1.1 m glass column of 3 mm I.D., FID detector) was identified by GC-MS (OV-1). This indicated the presence of sesquiterpene mixtures.

The bark was then extracted with ethyl acetate twice for forty-eight hours and the filtrate collected and evaporated to dryness. The dried extract (39 g) was dissolved in ethanol and had a little hexane added to it to induce crystallisation of

goniothalamin. Goniothalamin crystals (20 g) were filtered out and the mother liquor rotary evaporated to dryness. The crude ethyl acetate extract (73 g) was purified by SiO_2 column chromatography using hexane, hexane-chloroform and chloroform as the eluting solvent. This gave goniothalamin (GA-1) (14 g), goniothalamin epoxide (GA-2) (0.2 g), goniodiol (GA-4) (1.0 g) and (-)-iso-5-deoxygoniopypyrone (EELA 25) (1.2 g). Figure 3.1. shows the flow chart for the scheme of extraction of the crude extracts of *Goniothalamus andersonii*.

The bark of the *G. andersonii* was then soaked in ethanol and the filtrate collected and evaporated to dryness. GC indicated the presence of goniothalenol (GMg). The ethanol extract (56 g) was partitioned between chloroform and water. The chloroform-soluble fraction was then evaporated to dryness and further partitioned between 90% methanol and hexane. SiO_2 column chromatography (33 g) gave goniothalenol (GMg) (0.05 g). No acetogenins were detected.

The hexane, ethanol and methanol soluble fractions were all bioassayed for larvicidal. *In vitro* cytotoxicity screening on P388 cell lines were also carried out. Pooled fractions collected from the SiO_2 column chromatography of the methanol-soluble fraction were bioassayed against the larvae of the *Aedes aegypti*.

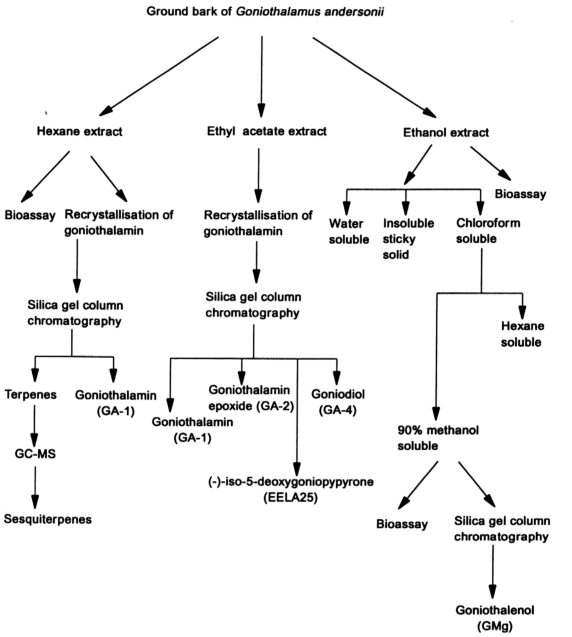

Figure 3. 1: Scheme of extraction of the bark of *Goniothalamus andersonii*

3.6.2 Isolation of bioactive principles from *Goniothalamus dolichocarpus*

The dry ground bark of *Goniothalamus dolichocarpus* (1 kg) was soaked in hexane twice for forty-eight hours. The hexane extract was filtered and vacuum-evaporated. The extract (20 g) was redissolved in ethanol and a small amount of

hexane was added to induce recrystallisation of goniothalamin (GA-1) (4 g). The mother liquor was subjected to a SiO_2 column chromatography separation. This gave a complex mixture of essential oils of very high R_f value (0.79, 5% ethyl acetate/hexane). The mixture was subjected to a partial separation by gas chromatography (OV-17 placed in a 1.1 m glass column of 3 mm i.d., FID detector) and then separation by GC-MS (OV-17). GC-MS indicated the presence of sesquiterpene mixtures.

The ground bark was then soaked in ethyl acetate for more than forty-eight hours twice. Goniothalamin (GA-1) (4 g) was recrystallised from the residue of the crude extract (44 g). The mother liquor was then separated by SiO_2 column chromatography. The sample was packed via dry packing and eluted using hexane and various percentages of hexane-chloroform, chloroform and methanol-chloroform. This gave (-)-iso-5-deoxygoniopypyrone (EELA 25) (250 mg), (+)-goniothalamin epoxide (GA-2) (200 mg), (+)-goniodiol (GA-4) (10 mg), (-)-pinocembrin (LB5) (16 g), (-)-naringenin (LB6) (7 g) and (+)-5β-hydroxygoniothalamin (LB 21C1) (140 mg). These compounds were separately purified by SiO_2 column chromatography using the same solvent system as for the first column, preparative layer chromatography using ethyl acetate/chloroform or methanol/chloroform or recrystallisation process. Pinocembrin (LB5) and naringenin (LB6) recyrstallised from acetone and goniothalamin epoxide (GA-2) from 10% dichloromethane in cyclohexane.

Another sample of the ground bark (1.16 kg) was extracted with ethanol to give 49 g of the crude extract. Recrystallisation of goniothalamin (GA-1) was carried out. About 7 g of goniothalamin (GA-1) was obtained. The mother liquor was then

evaporated to dryness and partitioned between chloroform and water. The chloroform soluble fraction was then partitioned between hexane and 90% methanol/water to give 29 g of the methanol-soluble fraction. This fraction was separated by SiO_2 column chromatography using chloroform and various percentages of methanol-chloroform as the solvent. This gave pinocembrin (LB5) (10 g), naringenin (LB6) (4.6 g), a minor amount of goniothalamin (GA-1) (2.5 g) and the mono-THF annonaceous acetogenin, annonacin (LBMF8b) (80 mg). Annonacin was purified by SiO_2 column chromatography, preparative layer chromatography and centrifugal thin layer chromatography (the chromatotron). Annonacin was silylated with BSTFA at room temperature and vacuum-pumped dry under high vacuum to get rid of unreacted BSTFA.

Figure 3.2 shows the flow chart for the scheme of extraction of the crude extracts of *Goniothalamus dolichocarpus*.

The hexane, ethanol and methanol soluble extracts were all bioassayed for larvicidal and *in vitro* cytotoxicity activities on P388 cell lines.

Fractions collected from the SiO_2 column chromatography of the methanol-soluble fraction which contain similar components were pooled together and bioassayed against *Aedes aegypti* larvae.

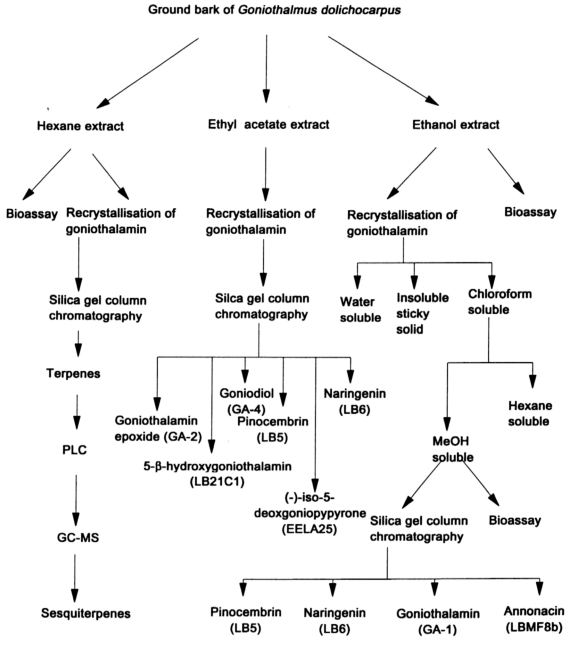

Figure 3. 2: Scheme of extraction of the bark of *Goniothalamus dolichocarpus*

3.6.3 Isolation of bioactive principles from *Goniothalamus malayanus*

The dry ground bark of *Goniothalamus malayanus* (800 g) was soaked in n-hexane once for forty-eight hours. TLC of the extract (12 g) indicated the presence of goniothalamin (GA-1) only. The crude extract was partially purified by SiO_2 column

chromatography using hexane and ethyl acetate/hexane (various percentages) as eluting solvent. This gave goniothalamin (GA-1) (20 mg).

The ground bark was then soaked in ethanol twice for forty-eight hours to give 60 g of crude dry extract. The extract was then partitioned between chloroform and water. The chloroform soluble fraction was then partitioned between 90% methanol/water and hexane. The methanol-soluble fraction was evaporated to dryness to give 25 g of the crude dry extract. The extract was then purified by SiO_2 column chromatography using chloroform and various percentages of methanol-chloroform as the eluting solvent. This gave (+)-annonacin (GMC) (1.3 g), (+)-goniothalamicin (minor amount, not separated from annonacin), (+)-goniothalenol (GMg) (0.6 g), ouregidione (GMf) (90 mg) and aristolactam BII (GM5-7) (0.5 g). These compounds were further purified by preparative layer chromatography using various percentages of methanol-chloroform, by centrifugal thin layer chromatography and recrystallisation.

The hexane, ethanol and methanol-soluble fraction extracts were tested for larvicidal activity using the resistance of mosquito larvae to insecticides test. *In vitro* cytotoxicity screening on P388 cell lines were also carried out. Fractions containing-similar components collected from the SiO_2 column chromatography of the methanol soluble fraction were pooled together and bioassayed against *Aedes aegypti* to identify bioactive fractions.

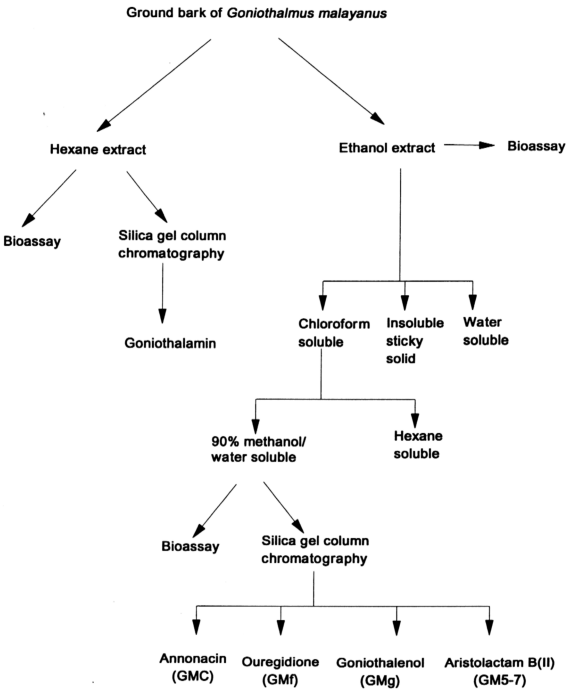

Figure 3. 3: Scheme of extraction for the bark of *Goniothalamus malayanus*

3.6.4 Isolation of bioactive principles from *Goniothalamus velutinus*

The dry ground bark of *Goniothalamus velutinus* (1 kg) was soaked in n-hexane once for 48 hours. This gave 22 g of the crude extract. Preparative layer chromatography of the hexane extract gave goniothalamin (GA-1) (0.1 g) and a mixture of terpenes. GC-MS indicated a mixture of sesquiterpenes.

The bark was then soaked in ethanol twice for forty-eight hours to give 55 g of crude extract. The crude extract was then partitioned between chloroform and water. The chloroform soluble fraction was then partitioned between hexane and 9:1 methanol:water. The methanol-soluble fraction was vacuum-evaporated. The dry extract (48 g) was purified by SiO_2 column chromatography. This gave ouregidione (GV26SF2) (2.0 g), goniothalenol (GV9b) (0.5 g), aristolactam BII (GV3b-2) (0.2 g) and annonacin (GV26OR) (3.0 g).

Annonacin (GV26OR) was purified by preparative layer chromatography and then HPLC using WATERS μ Bondapak C18 3.9 x 300 mm column and 88% methanol/water as the mobile phase. A Phenomenex Bond clone 10, C18 300 x 7.8 mm column but with 90% methanol/water as the mobile phase was also used for 1 mg injections of the crude compound.

Ouregidione (GV26SF2) was purified by multiple preparative layer chromatography and recrystallisation in chloroform.

Goniothalenol (GV9b) and aristolactam BII (GV3b-2) were purified by preparative layer chromatography and recrystallisation.

The hexane, ethanol and methanol soluble extracts were bioassayed for larvicidal and *in vitro* cytotoxicity activities on P388 cell lines. Fractions containing similar compounds collected from the SiO_2 column chromatography of the methanol-soluble fraction were pooled together and bioassayed to detect bioactive fractions.

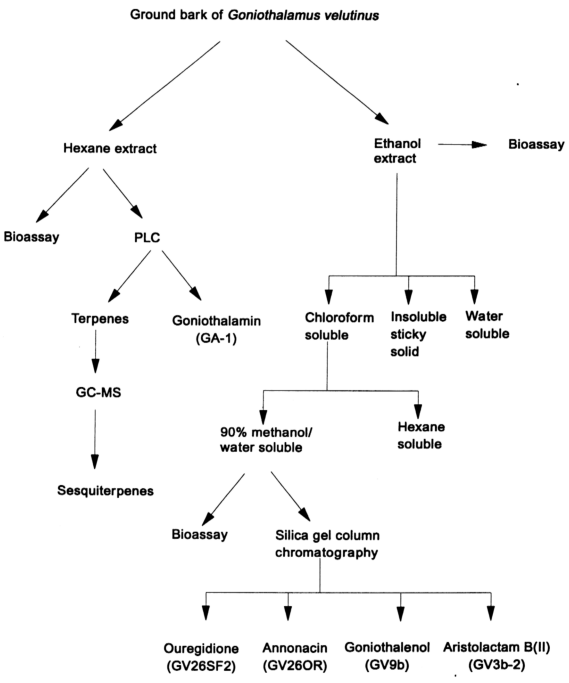

Figure 3. 4: Scheme of extraction for the bark of *Goniothalamus velutinus*

3.6.5 Isolation of bioactive principles from *Disepalum anomalum*

The dry ground bark of *Disepalum anomalum* (2 kg) was extracted with n-hexane twice for 48 hours. The amount of extract obtained was 80 g. This extract was not studied in detail since TLC did not show anything interesting. Moreover, bioassay against the mosquito of the *Aedes aegypti,* did not indicate good bioactivity.

The bark was then soaked in ethanol twice for more than forty-eight hours. This gave 86 g of crude ethanol extract. The dry ethanol extract was partitioned between chloroform and water. The chloroform solubles was then partitioned between hexane and 9:1 methanol:water. This gave about 30 g of the methanol soluble fraction. The methanol-soluble fraction was purified by SiO_2 column chromatography. The solvent system used was chloroform and various percentages of methanol-chloroform. Fractions containing similar components were pooled together and bioassayed for larvicidal activity. Two compounds were obtained from the SiO_2 column chromatography. Liriodenine (LS 13-4y-13) present in fractions 13-17 was purified by multiple preparative layer chromatography, centrifugal thin layer chromatography and recrystallisation from chloroform. After purification 10 mg of liriodenine (LS 13-4y-13) was obtained. Fractions 18-22 contained a new mono-THF acetogenin disepalin (F7) with an acetate group at C-20. Disepalin was partially purified by PLC using 2% methanol/chloroform as the mobile phase. The semi-pure disepalin was then purified by HPLC using a Machery Nagel (MN) ET 4.0 x 250 mm nucleosil normal phase column and 1% methanol/chloroform as the mobile phase. A waters μ Porasil 125 Å, 10 μm, 3.9 x 30 mm normal phase HPLC column was also used to isolate the pure disepalin. 1 mg of sample was injected each time. The

fractions were collected at a flow rate of 1 ml/min by viewing the peaks that appear at the RI detector. About 300 mg of pure disepalin was obtained.

Disepalin was acetylated using acetic anhydride and pyridine. The acetylated compound was purified by HPLC using the Waters μ Porasil 3.9 x 300 μm normal phase column and chloroform as the mobile phase.

Disepalin was also silylated by reacting a small amount with BSTFA. The product was vacuum-pumped to get rid of excess BSTFA.

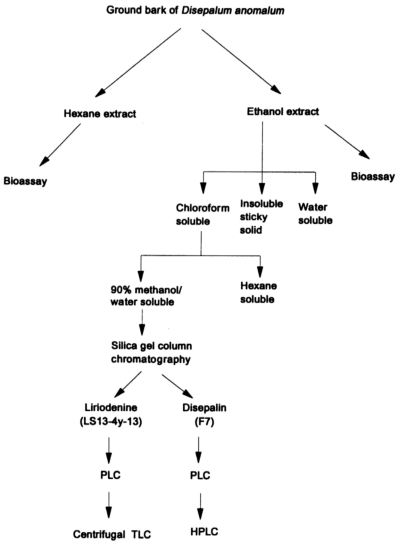

Figure 3. 5: Scheme of extraction for the bark of *Disepalum anomalum*

3.6.6 Isolation of bioactive principles from *Mezzetia umbellata*

The ground bark of *Mezzetia umbellata* (1 kg) was extracted twice with hexane for more than forty eight hours. The hexane extract was bioassayed for larvicidal activity. TLC indicated very little spots to be present in this extract.

The bark was then soaked in ethanol twice to give 70 g of the crude extract. Again TLC indicated very few spots. The extract was bioassayed for larvicidal activity and partitioned in the usual manner. The ethanol extract was partitioned between chloroform and water. The chloroform-soluble fraction was then partitioned between hexane and 90% methanol/water. Twenty grams of the methanol-soluble extract was obtained. This methanol-soluble fraction was bioassayed for larvicidal activity. Pooled fractions were bioassayed. SiO_2 column chromatography gave two compounds aristolactam BII (GM5-7) (20 mg) and annonacin (KHP13b) (100 mg). Both compounds were further purified by PLC. Annonacin (KHP13b) was aectylated using acetic anhydride and pyridine. It was also silylated with BSTFA.

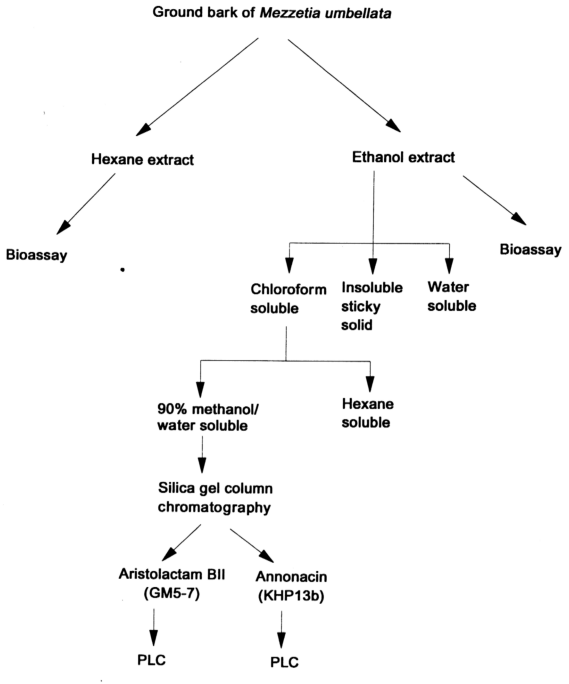

Figure 3. 6: Scheme of extraction for the bark of *Mezzetia umbellata*

3.6.7 Gas chromatography screening of the stem bark of the various *Goniothalamus* species

Fifty grams of *Goniothalamus ridleyi*, *G. macrophyllus*, *G. uvarioides*, *G. macranii*, *G. sinclairinius*, *G. gigantifolius*, *G. umbrosus* and *G. montanus* were

extracted twice with n-hexane followed by ethanol. The ethanol extract was partitioned in the usual manner. The hexane, ethanol and methanol soluble extracts were bioassayed for larvicidal activity. Some extracts were sent for preliminary *in vitro* cytotoxicity testings on P388 cell lines. All extracts were compared with other compounds previously isolated by thin layer chromatography and gas chromatography.

Hexane extracts were partially purified by preparative layer chromatography before injecting into GC.

3.7 Syntheses

3.7.1 Synthesis of goniothalamin epoxide and isogoniothalamin epoxide

Goniothalamin (1 g, 5 mmol) in 20 ml dichloromethane was epoxidized with *m*-chloroperoxybenzoic acid (1.8 g, 10 mmol) over twenty-four hours at room temperature. To the suspension was added 120 ml of 10% dichloromethane in cyclohexane. 20 ml of 10% aqueous $NaHCO_3$ was added to the milky mixture to neutralize the excess acid. The organic layer was collected and dried with K_2CO_3. The solution was filtered under vacuum, washed with 10% CH_2Cl_2 in cyclohexane and the filtrate rotary evaporated until concentrated. Goniothalamin epoxide and isogoniothalamin epoxide were fractionally crystallized as plates and needles respectively in the ratio 1.8:1.0 (0.36 g and 0.2 g).

3.7.2 Synthesis of goniodiol

Isogoniothalamin epoxide (82.5 mg, 0.35 mmol) in 8 ml of ether was stirred with 1 M perchloric acid (2 ml) at room temperature for one hour. After drying with K_2CO_3 and removal of ether, 79 mg (85% yield) of (+)-goniodiol was obtained.

Similarly goniothalamin epoxide was reacted with 1M perchloric acid to give the (6R,7S,8S)-goniodiol.

3.7.3 Synthesis of *threo*-goniodiol

Goniothalamin (0.72 g, 3.62 mmol) in 5 ml ether was reacted with Osmium tetroxide (0.85 g, 3.35 mmol) in 10 ml t-butanol for twenty-four hours. The crude osmium adduct was stirred with 15 ml of 10% mannitol in water. The mixture was extracted with ether and the ether layer dried with Na_2SO_4. This gave 0.42 g (68% yield) of a diastereomeric diols. The diols were separated by PLC.

3.7.4 Synthesis of goniodiol diacetate

(+)-Goniodiol (78 mg, 0.33 mmol) was reacted with acetic anhydride (0.1 ml, 1.1 mmol) in pyridine (0.1 ml) at room temperature for 24 hours. After 24 hours, the mixture was rotary evaporated to remove excess acetic anhydride and pyridine. Excess n-hexane was added and the mixture rotary evaporated. 101 mg (94% yield) of goniodiol diacetate was obtained.

3.7.5 Synthesis of (-)-iso-5-deoxygoniopypyrone

Goniodiol (8 mg, 3.4×10^{-5} mol) was reacted with a catalytic amount of DBU in dry THF (25 µl in 2 ml) under nitrogen atmosphere for 48 hours at room temperature. GC was able to detect the presence of a very minute amount of (-)-iso-5-deoxygoniopypyrone.

3.7.6 Synthesis of 5β-hydroxygoniothalamin and 5α-hydroxygoniothalamin

Goniothalamin (1 g, 5 mmol) was reacted with selenium dioxide (600 mg, 5.5 mmol) in 16 ml of dioxane. The mixture was refluxed for three hours. A few drops of distilled water was added and the mixture boiled for half an hour. The cold

solution was then evaporated to dryness in a rotary evaporator. Excess n-hexane was added to help the evaporation of dioxane. The crude product (0.2 g) consisted of two diastereomers which was separated by preparative layer chromatography. (+)-5β-hydroxygoniothalamin (10 mg, 0.6%) and its diastereomer (+)-5α-hydroxygoniothalamin (116 mg, 6.8%) were obtained. 5α-hydroxygoniothalamin recrystallised from dichloromethane-cyclohexane as plates. 5β-hydroxygoniothalamin was an oil.

3.7.7 Acetylation of 5α-hydroxygoniothalamin and 5β-hydroxygoniothalamin

5α-hydroxygoniothalamin (0.73 g, 3.39 mmol) was reacted with acetic anhydride (0.9 ml, excess) in 1 ml pyridine at room temperature for 24 hours.

Similarly 5 β-hydroxygoniothalamin (8.4 mg, 3.85 x 10^{-5} mol) was reacted with 0.05 ml of acetic anhdyride (excess) in 0.5 ml pyridine at room temperature for 48 hours.

Both work-ups were the same. The reacted mixtures were rotary evaporated to dryness with addition of excess hexane. 5.8 mg (69% yield) of 5β-acetoxy-goniothalamin was obtained. Similarly, 0.5 g (70% yield) of 5α-acetoxygoniothalamin was obtained. Both acetates were recrystallised in chloroform to give needles.

3.7.8 Silylation of disepalin

The pure acetogenin (3.5 mg) was reacted with 250 ml of BSTFA in a sealed ampoule at 70 °C for 24 hours. The reaction mixture was vacuum-pumped dry to remove unreacted excess BSTFA.

3.7.9 Acetylation of disepalin

The crude acetogenin (100 mg) was reacted with dry, excess acetic anhydride (10 ml) in 1.5 ml of pyridine at room temperature for 24 hours. This gave 100 mg of crude product which was purified by HPLC using a normal phase WATERS μ porasil 3.9 x 300 mm column and $CHCl_3$ as the mobile phase.

3.7.10 Acetylation of (-)-iso-5-deoxygoniopypyrone

600 mg (2.55 mmol) of (-)-iso-5-deoxygoniopypyrone was reacted with 0.12 ml (1.2 mmol) of acetic anhydride in 1.2 ml pyridine at room temperature for 24 hours. Excess reagent and catalyst were removed by rotary evaporation. The crude acetate (0.95 g) was purified by SiO_2 column chromatography and recrystallised from dichloromethane/ethanol. Needle-shaped crystals were obtained.