CHAPTER III

INVESTIGATION OF PHARMACOLOGICAL ACTIVITIES OF ISOLATED COMPOUNDS

3.1 Experimental Section

3.1.1 Antimalarial Assay

In vitro antimalarial activity was determined by using the [^{3}H]hypoxanthine incorporation method. Briefly, 25 µl aliquots of drugs solutions of different concentrations were placed in 96-well plate together with 200 µl of a 1.5% cell suspension of parasitized erythrocytes containing 1-2% parasitemia at the early ring stage. The mixture were incubated in a candle jar at 37°C. After 24 hr of incubation, 25 µl (0.25 µCi) of [^{3}H]hypoxanthin was added to each well. The mixture were incubated further under the same condition for 18-24 hr. DNA parasites was harvested onto glass filter paper (Unifilter®, Packard). The filter were dried, and liquid scintillation fluid was added for radioactivity measurement in a 6-probe liquid scintillation counter (Packard). An IC_{50} value was determined from the sigmoid curve of percent [^{3}H]hypoxanthin incorporation against drug concentration.[45]

3.1.2 Cytotoxicity Assay

In vitro cytotoxicity was determined by using MTT assay. Briefly, cells were diluted to 10^5 cells/ml. Test compounds were dissolved with few drops of DMSO and diluted with water to make the concentration of DMSO less than 0.1%, added into 96-well plates and incubated at 37 ◦C for 5 days. MTT solution (2 mg/ml) was added into each well and then incubated at 37 ◦C for 4 h. The MTT formazan crystals were dissolved in 200 µl for 100% DMSO and 25 µl of Sorensen's glycine buffer. After 20 min, the optical density (OD) was measured with microtiter plate reader at wavelength of 510 nm. Doxorubincine hydrochloride was used as a positive control and DMSO was used as a negative control.[46]

3.2 Results and Discussion

3.2.1 Antimalarial assay of isolated compounds from *A. paniculata.*

Pure isolated compounds isolated from *A. paniculata* were evaluated for their activity to inhibit in vitro the growth of chloroquine resistant strain K1 of *Plasmodium falciparum*. Dihydroartimisinin was used as a positive control. The results are shown in **Table 3.1**. Andrographolide (**1**) and 5,2'-Dihydroxy-7,8-dimethoxyflavone (**5**) displayed significant antimalarial activity with IC_{50} values of 17 μM and 6.46 μM, respectively. However, these compounds had to be considered as moderately active, when compared to Dihydoartimisinin.

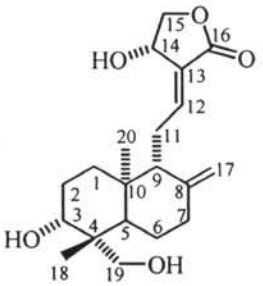
Table 3.1 In vitro antimalarial activity of isolated compounds from *A. paniculata.*

Compounds	IC_{50} (μM)
Andrographolide (**1**)	17.0
14-Deoxy-11,12-didehydroandrographolide (**2**)	Inactive
Neoandrographolide (**3**)	Inactive
Andrographiside (**4**)	Inactive
5,2'-Dihydroxy-7,8-dimethoxyflavone (**5**)	6.46
8-*O*-Acetylharpagide (**6**)	Inactive
Antirrinoside (**7**)	ND
Dihydroartimisinin	0.004

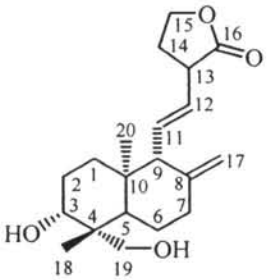
ND = not determined

Dihydroartimisinin = Positive control

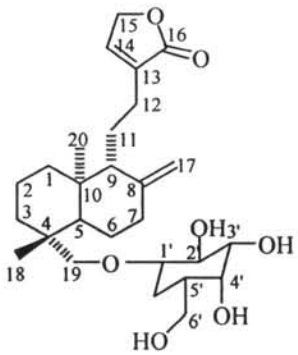
For the Andrographolide series, the C-14 hydroxyl group is probably a major influence on the activity. It was found that the absence of the C-14 hydroxyl group in 14-Deoxy-11,12-didehydroandrographolide (**2**), Neoandrographolide (**3**) and the presence of a glucose ring in Andrographiside (**4**) led to the activity diminished considerably.



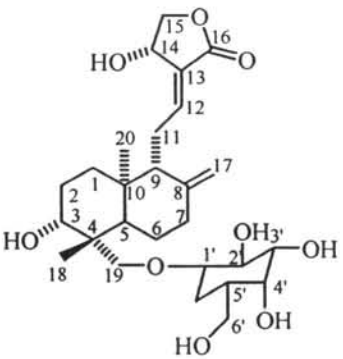
Andrographolide (**1**)



14-Deoxy-11,12-didehydroandrographolide (**2**)



Neoandrographolide (**3**)



Andrographiside (**4**)

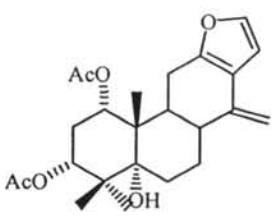
3.2.2 Antimalarial assay of isolated compounds from *C. bonduc*.

Nine isolated cassane diterpenoids (**8-16**) have been reported to have in vitro antimalarial activity against chloroquine-sensitive FCR-3/A2 strain of *Plasmodium falciparum*,[38] the results are shown in **Table 3.2**. Thus, we did not test for their antimalarial activity by ourselves. Only Caesalpinin Q was evaluated for its antimalarial activity against chloroquine-resistant K1 strain of *P. falciparum* and it displayed to be active at the primary screening (10 μg/ml, > 50% inhibition).

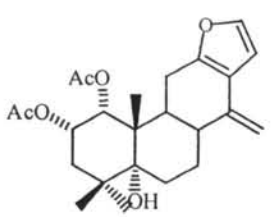
Table 3.2 In vitro antimalarial activity of isolated compounds from *C. bonduc*

Compounds	IC_{50} (μM)
Caesalpinin C (**8**)	0.79
Caesalpinin P (**9**)	1.7
14(17)-Dehydrocaesalpin F (**10**)	0.20
ε-Caesalpin (**11**)	>10
Bonducellpins C (**12**)	0.12
Caesalpinin K (**13**)	0.40
Caesalmin B (**14**)	0.80
Caesalpinin I (**15**)	>10
2-Acetoxycaesaldekarin e (**16**)	6.5
Chloroquine	0.29

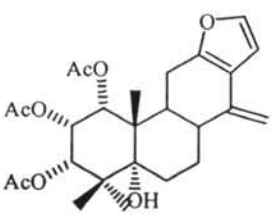
Chloroquine = Positive control



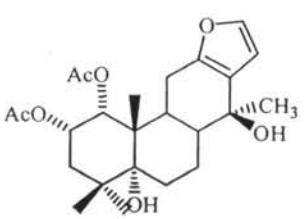
Caesalpinin C (**8**)



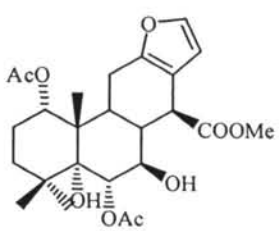
Caesalpinin P (**9**)



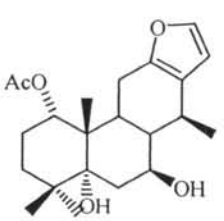
14(17)-Dehydrocaesalpin F (**10**)



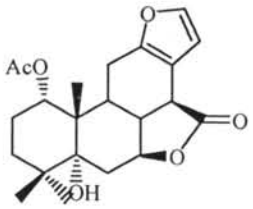
ε-Caesalpin (**11**)



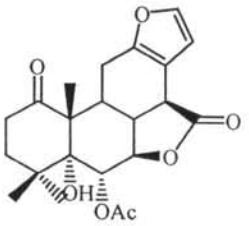
Bonducellpins C (**12**)



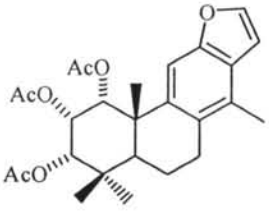
Caesalpinin K (**13**)



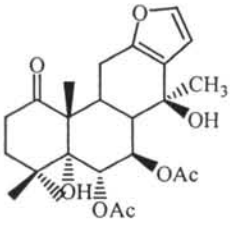
Caesalmin B (**14**)



Caesalpinins I (**15**)



2-Acetoxycaesaldekarin e (**16**)



Caesalpinin Q (**17**)

Structure-activity relation of cassane diterpenoids isolated from *C. bonduc*

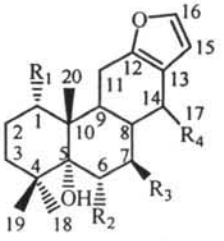
Based on reported data and our results, the structure-activity relationship of cassane- type diterpenes on antimalarial activity as follows;

R_1 : OAc >> OH >> H

R_2 : H > OAc > OH

R_3 : OH >> OAc >>H

R_4 : =O > COOMe > CH_3



Majority of diterpenes isolated from seed kernels of *C. bonduc* possessed the acetoxyl group at either of C-1, C-2, C-3, C-6 or C-7. In general, the simple cassane-type diterpenes having an acetoxyl substituent at C-1 showed stronger activity than without substituent at C-1 (**14** > **15**). Among the diacetoxyl-substituted diterpenes, those having acetoxyl substituents at C-1 and C-3 were more potent than those at C-1 and C-2 (**8** > **9**). And the presence of a hydroxyl group at C-7 plays crucial role for strong antimalarial activity. The diterpenes having a hydroxyl group at C-7 showed stronger activity than those with an acetoxyl subsituent at C-7 or without substituent at C-7. The strong activity of Bonducellpins C (**12**, IC_{50}, 0.12 μM) might be attributable to the presence of hydroxyl group at C-7.

3.3.3 Cytotoxicity of isolated compounds from *A. paniculata* and *C. bonduc*.

Pure isolated compounds from *A. paniculata* and *C. bonduc* were evaluated for cytotoxic effects against five tumor cell lines: BT474, Chago, Hep-G2, KATO-3, and SW-620 by MTT colorimetric method. Doxorubicine was used as a positive control. Results are presented in **Table 3.3**. 14-Deoxy-11,12-didehydroandrographolide (**2**) and flavone **5** showed strong cytotoxic activity against the KATO-3 cell line with IC_{50} values of 5.79 and 8.41 μg/ml, and showed moderate cytotoxicity against the Hep-G_2 and SW620 cell. Andrographolide (**1**) showed only weak cytotoxicity against the Hep-G_2 cell line with IC_{50} = 9.07 μg/ml. Iridoid glucoside **7** and most of cassane diterpenes were inactive (IC_{50} > 10 μg/ml), except for Caesalpinin I (**15**) showed weak cytotoxic against the KATO-3, SW620 cell lines.

Table 3.3 In vitro cytotoxicity of isolated compounds from *A. paniculata* and *C. bonduc*

Compounds	IC_{50} (μg/ml)				
	BT 474	**Chago**	**Hep-G_2**	**KATO-3**	**SW620**
Andrographolide (**1**)	> 10	> 10	9.07	> 10	> 10
14-Deoxy-11,12-didehydroandrographolide (**2**)	> 10	> 10	6.07	5.79	6.03
Neoandographolide (**3**)	> 10	> 10	> 10	> 10	> 10
Andrographiside (**4**)	ND	ND	ND	ND	ND
5,2'-Dihydroxy-7,8-dimethoxyflavone (**5**)	> 10	> 10	7.11	8.41	7.48
8-*O*-Acetylharpagide (**6**)	ND	ND	ND	ND	ND
Antirrinoside (**7**)	> 10	> 10	> 10	> 10	> 10
Caesalpinin C (**8**)	ND	ND	ND	ND	ND
Caesalpinin P (**9**)	ND	ND	ND	ND	ND
14(17)-Dehydrocaesalpin F (**10**)	> 10	> 10	> 10	> 10	> 10
ε-Caesalpin (**11**)	ND	ND	ND	ND	ND
Bonducellpins C (**12**)	> 10	> 10	> 10	> 10	> 10
Caesalpinin K (**13**)	ND	ND	ND	ND	ND
Caesalmin B (**14**)	ND	ND	ND	ND	ND
Caesalpinin I (**15**)	> 10	> 10	> 10	10	10
2-Acetoxycaesaldekarin e (**16**)	> 10	> 10	> 10	> 10	> 10
Caesalpinin Q (**17**)	> 10	> 10	> 10	> 10	> 10
Doxorubicine	0.01	0.9	0.61	> 10	0.73

ND = not determined

Doxorubicin = Positive control

- BT 474 = Breast ductol carcinoma
- Chago = Human undifferentiated lung carcinoma
- Hep-G_2 = Liver hepatoblastoma
- KATO-3 = Human gastric carcinoma
- SW620 = Colon adenocarcinoma