CHAPTER 4

BIOACTIVITY

CHAPTER 4

4.1 Radioligand receptor binding assays

4.1.1 Introduction

Plants have provided a number of active compounds with action on the Central Nervous System (CNS) including the well-known alkaloids such as morphine, codeine, reserphine, caffeine and nicotine[213]. The developments of receptor radioligand binding assays offer the possibility of rapidly increasing the knowledge on the pharmacologically active constituents of plants and searching for novel drug molecules is still continuing. For that purposes the author studied eleven alkaloids isolated from the genus *Phoebe* and *Dehaasia* in order to examine their CNS activity using radioligand receptor binding assays.

4.1.2 Receptor binding methodology

4.1.2.1 Preparation of alkaloids

Pure alkaloids were obtained from the selected plants as shown in the Experimental section (Chapter 6).

4.1.2.2 Radio ligand and receptor binding assay for screening of alkaloids

The receptors and ligands utilized are tabulated in Table 4.1. The general procedure is as follows.

Table 4.1: Radioligand receptor binding methods

Receptor	Radiactive Ligand (nM)	Displacing Ligand (uM)	Buffer	Tissue
Muscarinic	^{3}H-scopolamine (5)	Atropine (1)	Tris-HCl pH 7.4	Rat whole brain
Dihyropyridine	^{3}H-(+)-[methyl-^{3}H] PN 200-110	Nifedipine (10)	Tris-HCl pH 7.4	Rat whole brain

Alkaloids were initially dissolved in 10% etanol or 50% DMSO to give a final concentration of 10^{-4}M. Each alkaloid (50 µl, pH 7.5 at 25°C) and required ^{3}H-ligand (50 µl at specified concentration given in Table 4.1) were mixed with target tissue (400 µl, final protein concentration of 0.5 mg/ml). The mixture was incubated for a specified time at a specified temperature (90/0; min/°C), and then filtered on GF/B filters by using a pressure reduced Brandel Cell Harvester (Brandel, Gaithors-berg, MD) and washed with ice-cold buffer. The filters covered with samples were punched into vials and soaked in scintillation solution for at least 1h before counting. The scintillation counters (LS-600 TA, Beckman Ltd) gave sample counts in CPM.

4.1.3 Membrane preparation

Removed rat brain (Dissect other structures leaving just the cortex)

Weight brain

↓

Suspend the brain in 10 x vol. (X mls) of iced cold Tris-HCl, remove other structure leaving only the cortex and finely chopped the brain with a scissor

↓

Utra-Turax for 2 x of a 10 s

↓

Homogenised at 800 rpm for 20 X (up & down = 2 x) With pestle and glass tube

↓

Transfer to a 20 ml tube and centrifuge at 40,000 g (18,000 rpm) using a type 28 Beckman at 4°C for 15 min

↓

Retain pellet and resuspend in 5 mls of Tris-HCL using homogenization for 20 sec

↓

Repeat centrifugation and resuspension twice

↓

Final pellet is resuspended in 5 ml of Tris-HCl

Notes

1. All procedure needs to be done at 4° C. Cool all glasswares and solution first beforehand.
2. Protein determination done using Lowry test on final pellet.
3. Preparation of solutions:
   a. Tris-HCl solution (1 M, pH 7.4)

      Dissolve 1.21 g of Tris base and add 100 ml of d-water. The adjust pH to 7.4 with conc. HCl at 4° C.

4.1.4 Results and Discussion

The general assay procedure involves preparation of animal tissue rich in particular recerptor being incubated with a radiolabelled ligand in the absence of and also in the presence of, a test compound.

A radioactive drug, which has a high affinity and high degree of selectivity, is used in the experiment. In this study the radioactive drugs (^{3}H – scopolamine and (+) – [methyl – ^{3}H] {PN200 – 110} were used to label two receptors (muscarinic) and Dihydropriridine respectively as shown in Table 4.1. These two radioligands are capable of binding to membrane receptors specifically.

Drug (D) + Receptor (R) → Drug-Receptor complex (RD)

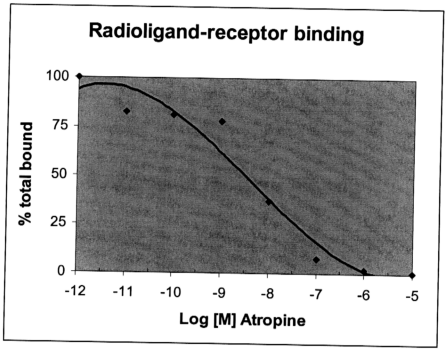
Drug-receptor complex yielded from the reaction can be measured since it is radioactive and is known as Bound (Drug that is bound to the receptor). The amount of bound or drug, which is bound to the receptor, can be subtracted from the

concentration of drug. This will leave behind the Free drug. The Free drug is referring to the amount of radioactive drug, which is not binding to the tissue. The method such as filtration was used to separate Bound from Free.

Non-specific binding was estimated in the presence of a high concentration of a receptor specific non-radioactive compound. The amount of ligand specifically bound was determined by subtracting the amount of ligand non-specifically bound from the total amount of radioactivity bound in the absence of any compound and expressed as a percentage of the total binding. The percentage inhibition of binding for plant extracts is equal to 100% - % specific binding as given below.

$$\% \text{ Inhibition} = [(\text{bound total} - \text{bound by alkaloid}) / \text{bound total}] \times 100\%$$

The figures (Figure 4.1 and 4.2) show the competitive binding curves of 3H-Scorpolamine and ^{3}H-PN200-110 binding to rat brain homogenates. Based on the comparison of these two figures, % inhibitions of eleven alkaloids have been compared at concentration 10^{-4} M. The alkaloids, which have % inhibition more than 50 % are considered an active compound[213]. The activity of eleven alkaloids is shown in Table 4.2.



Brain tissue homogenate 0.6 mg/ml (final conc.)
^{3}H-Scopolamine 10^{-5} M (final conc.)
Incubation for 90 min at 21 degrees centigrade

Figure 4.1: Competitive binding assay of ^{3}H-scopolamine in the presence of atropine ($10^{-12} - 10^{-5}$ M).

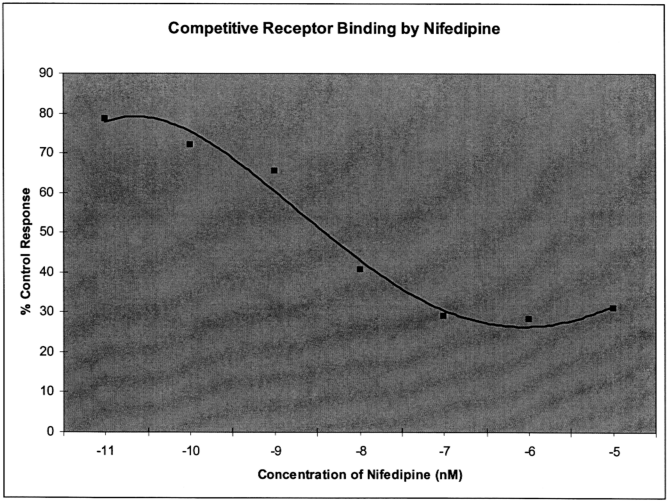


Figure 4.2: Competitive receptor binding of ^{3}H-PN200-110 in the presence of nifedipine (10^{-11} – 10^{-6} M).

Table 4.2: Inhibition of radioligand specific binding of 11 alkaloids of three selected species of higher plant.

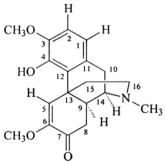
Drug/ Alkaloids	Receptor	
	Muscarinic	Dihydropyridine
	Activity[a]	Activity
Milonine **190**	+/-	NT[b]
Pallidine **189**	+/-	NT
Sinoacutine **81**	+/-	NT
Grandine C **183**	+/-	+/-
Norhexamecambrine A **187**	3+	+/-
Pallidinine **188**	+/-	NT
Norboldine **98**	+/-	+/-
Grandine B **180**	+/-	+/-
Phoebescortechiniine A **175**	+/-	+/-
Phoebegrandine B **29**	+/-	+/-
Tetrahydropronuciferine **177**	+/-	NT

Alkaloids were prepared in10% etanol or 50% DMSO to give a final concentration of 10^{-4} M for testing.

[a] Activity: 4+, inhibition of 81-100%; 3+, inhibition of 61-80%; 2+, inhibition of 41-60%; +/-, inhibition of 1-20%; inhibition values based on mean of three separate determinations with triplicate samples.
[b] NT: not tested

Eleven pure alkaloids were tested on two different receptors as shown in Table 4.2. Norhexamecambrine A **187** showed significant activity (3+) to inhibit binding of ^{3}H-scopolamine, which is indicative of the presence of muscarinic CNS active

component and further investigation need to be undertaken. The rest of the alkaloids only showed weak activity (+/-). Grandine C **183**, norhexamecambrine A **187**, norboldine **98**, grandine B **180**, phoebescortechiniine A **175** and phoebegrandine B **29** showed weak activity (< 50%) to inhibit binding upon ^{3}H-PN200-110, which labels the Ca^{2+} dihydropyridine receptor. The results showed that these ligand-binding assays are useful for understanding the mode of action of those alkaloids and may be used as a bioassay to guide the purification and isolation of the active ingredients from plant.



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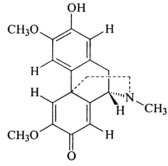

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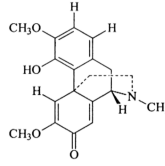

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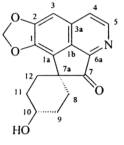

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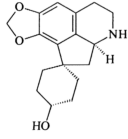

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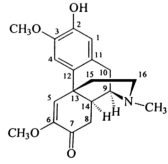

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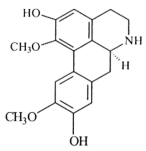

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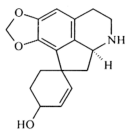

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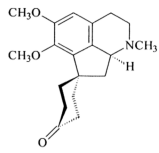

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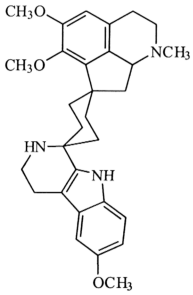

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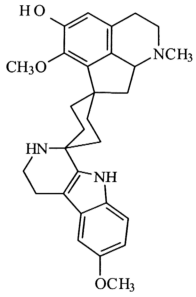

29

4.2 Parasite lactate dehydrogenase test (pLDH) for anti malaria in-vitro drug screening

Phoebe grandis, Phoebe scortechinii and *Dehaasia longipedicellata* CH_2Cl_2 crude extracts have the IC_{50} values below 8 μg/ml (upper unit for the parasite lactate dehydrogenase test, pLDH is 8 μg/ml). These alkaloidal extracts demonstrate good properties as anti-plasmodial activities against sensitive and resistant strain of *P. falciferum in viro* as shown in Table 4.3 and 4.4 below.

Table 4.3: pPLDH result for *P. falcifarum*, D10 strain (sensitive strain)

Code No.	PLDH IC_{50} (μg/ml)	Comment
KL4224 *Phoebe grandis*	0.0854	Potential for further study
KL4886 *Phoebe scortechinii*	0.1527	Potential for further study
KL4719 *Dehaasia longipedicellata*	0.7742	Potential for further study

Table 4.4: pPLDH result for *P. falcifarum*, Gombak A isolate (resistant strain)

Code No.	pLDH IC_{50} (μg/ml)	Comment
KL4224 *Phoebe grandis*	0.0894	Potential for further study
KL4886 *Phoebe scortechinii*	0.1042	Potential for further study
KL4719 *Dehaasia longipedicellata*	0.7637	Potential for further study

This selection of alkaloidal activities, which is far from complete, clearly showed that many alkaloids inhibit or overstimulate central proceses at the cellular and organ level. Only a limited number of structures have been studied. In many instances, plants contain mixtures of related alkaloids, which only differ for particular substitution patterns but these mixture also shows good bioactivity such as anti plasmodial activity.