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Radioligand-receptor Binding Assays in the Search for Bioactive Principles from Plants

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Pharmacological testing of plant extracts poses a number of perceived limitations to investigators. Among the difficulties which may be encountered are: the selection and acquisition of plants, choice of solvent for extraction, testing of extracts for activity, reproducibility of results from different batches of the same plant species, and the isolation of already known compounds. In recent years there has been an escalation in the number of sensitive in-vitro bioassay techniques based on specific enzymes and receptors utilizing micro-titre well assays. Such assays have been automated by industry, and the development of robotic high-throughput screening has made it possible to carry out tens of thousands of biological tests on a daily basis. Due to this technology, there has been a resurgence of interest by the pharmaceutical industry and by biotechnological companies in screening plants for a range of specific biological targets (O'Neill & Lewis 1993; Buss 1998).

Several neurotransmitters have now been characterized, including acetylcholine, glutamic acid, dopamine, 5-hydroxytryptamine (5-HT), γ-aminobutyric acid (GABA), glycine, opiate, benzodiazepine, noradrenaline and histamine (Kruk & Pycock 1990). Each neurotransmitter is associated with a set of subtypes which are responsible for a range of pharmacological actions.

Receptor-radioligand binding assays have been developed from this research and these assays are used routinely to measure and to characterize the interactions of ligands with a variety of receptors. The assays, which are rapid and sensitive, rely on radioligands, such as ^{3}H- and ^{125}I -labelled ligands, which are capable of binding to receptors specifically. The versatility of such assays has been used to examine many issues of neurobiological interest (Hulme & Birdsall 1992). The assays involve either preparations of animal tissue rich in a particular receptor, or cloned human receptors expressed on cell surfaces, being incubated with a radiolabelled ligand in the absence of, and also in the presence of, the test compound. Activity observed in such in-vitro tests needs to be confirmed by the application of functional assays.

New drugs are required for many diseases including those of the central nervous system (CNS), such as migraine, sleeping disorders, schizophrenia, Alzheimer's disease, epilepsy, stroke, Parkinson's disease and the management of pain. Plants have provided compounds with CNS activity such as the well-known alkaloids morphine, codeine, reserpine, caffeine and nicotine. There are approximately 250,000 species of plant on Earth and the vast majority of these have not been examined in any detail for their pharmacological activities. Even those plants which have been researched have mainly been examined for a single type of activity, e.g. cardiac or analgesic, and not for a range of activities. The development of receptor-radioligand binding assays offers the possibility of rapidly increasing our knowledge on pharmacologically active constituents of plants and of searching for new drug molecules.

Collaboration with two multi-national pharmaceutical companies has enabled us to apply receptor-radioligand binding assays in-vitro, utilizing animal and human cloned receptors to investigate plants for a range of CNS activities and for specific analgesic effects. The experiences gained during these investigations are briefly summarized in this paper.

Screening of 10 selected Chinese medicinal plants for CNS activity utilizing receptor-radioligand binding assays

Ten species of higher plants from six Chinese genera were selected for investigation as potential sources of CNS compounds. The plants were selected on the basis of their medicinal use or because of their close relationships with plants used in traditional medicine for the treatment of CNS

disorders. The species investigated were *Alangium platanifolium* (Sieb. et Zucc.) Harms (Alangiaceae), *Celastrus angulatus* Maxim. (Celastraceae), *C. orbiculatus* Thun. (Celastraceae), *Clerodendrum mandarinorum* Diels (Verbenaceae), *C. bungei* Steud. (Verbenaceae), *Periploca callophylla* (Wight) Falc. (Asclepiadaceae), *P. forrestii* Schlecter (Asclepiadaceae), *Schefflera bodinieri* (Levi.) Rehd. (Araliaceae), *S. delavayi* (Fr.) Harms (Araliaceae) and *Uncaria rhynchophylla* (Miq.) Jacks. (Rubiaceae). The experimental details of plant authentication, extraction and receptor-radioligand binding assays, utilizing animal tissues, have been published previously (Zhu et al 1996a). Thirteen extracts of different plant parts were prepared in 70% ethanol from the ten species, and evaporated to dryness. Each micro-titre well contained 50-μL samples of plant extract (concentration 1 mg mL^{-1}), 50 μL ^{3}H-ligand of specified concentration and 400 μL of target tissue (protein concentration of 0·5 mg mL^{-1}). After incubation under specified conditions, the radioactivity of particulate matter was measured by scintillation counting. The amount of specifically bound ligand was determined by subtracting the amount of ligand nonspecifically bound from the total amount of radioactivity bound in the absence of any extract and expressed as a percentage of the total binding. The percentage inhibition of binding for extracts is equal to 100 minus the percentage specific binding.

Choice of solvent and concentration of extracts for testing

Aqueous extracts are mainly used for traditional medicine preparations whereas laboratory investigations aimed at the isolation of constituents tend to utilize organic solvents. A series of four solvents was tested for the ten plant species and a range of concentrations prepared between 10 and 0·1 mg mL^{-1}. Extracts in 70% ethanol at a concentration of 1 mg mL^{-1} were selected for routine use on the basis of results in the receptor-radioligand binding assays.

Results of receptor-radioligand binding assays with plant extracts

Specific inhibition of 13 plant extracts against 18 receptor-radioligand binding assays has been reported previously (Zhu et al 1996a). The majority of the extracts tested showed strong inhibition (60–100%) against α_2-adrenoceptor, opiate, dopamine 1 and 2, $GABA_A$ and $GABA_B$ receptors which is indicative of the presence of CNS active compounds. Weak or zero activity was observed mainly in the assays for inhibition of binding to β-adrenoceptor, muscarinic and Na^+/K^+ ATPase recep-

Table 1. Inhibition of radioligand specific binding of four extracts of three selected species of Chinese plant against 18 receptors in-vitro.

Receptor	*Schefflera bodinieri*		*Clerodendrum mandarinorum*	*Uncaria rhynchophylla*
	Leaf	Root	Root bark	Stem, hook
α_1-Adrenoceptor	+++	++	++	+
α_2-Adrenoceptor	+++	+++	+++	+++
β-Adrenoceptor	+	++	++	±
5-HT_1	++++	+++	+++	NT
5-HT_{1A}	+	+	+++	+++
5-HT_2	++++	+++	+++	++
Opiate	++++	++++	++++	+++
Adenosine 1	+++	+++	++++	+
Benzodiazepine	++	+	±	NT
Ca^{2+} channel	++++	++++	++	++
Sulphonylureas	++++	+++	+	++
Dopamine 1	++++	+++	+++	+++
Dopamine 2	++++	+++	+++	NT
Muscarinic	+	±	±	NT
Histamine 1	+++	+++	+++	+
Na^+/K^+ ATPASE	+	±	+	NT
$GABA_A$	+	++++	++++	+++
$GABA_B$	+++	++++	++++	++++

Extracts were prepared in 70% ethanol, concentrated to dryness and dissolved in 0·7% ethanol to give a concentration of 1 mg mL^{-1} for testing; 50 μL samples were tested. ++++ = inhibition of 81–100%; +++ = inhibition of 61–80%; ++ = inhibition of 41–60%; + = inhibition of 21–40%; ± = inhibition of 1–20%; NT = not tested. Inhibition values are based on the mean of three separate determinations with triplicate samples. Full experimental details have been published (Zhu et al 1996a).

tors. As an illustration of these results, the specific inhibition of binding of four extracts from three of the species against 18 receptors is given in Table 1.

Schefflera bodinieri. The genus *Schefflera* (Araliaceae) contains 200 species worldwide of which 37 are indigenous to south-west China (How 1984). The most important genera of the Araliaceae for medicinal use are *Panax*, *Acanthopanax* and *Tetrapanax* which contain mixtures of triterpenoid glycosides and oligosaccharides. Several species of *Schefflera* are used medicinally in China for the treatment of arthritis, asthma, bronchitis, lumbago, migraine, neuralgia, pain and rheumatism (Liao 1986). *S. bodinieri* has not been investigated previously and extracts from leaves and from roots proved to have strong binding to α_1- and α_2-adrenoceptors, 5-HT_1, 5-HT_2, opiate, Ca^{2+} channel, sulphonylureas, dopamine 1 and 2, histamine 1, $GABA_A$ and $GABA_B$ receptors, (Table 1). Binding to opiate, α_1- and α_2-adrenoceptors and 5-HT_1 receptors may be related to analgesic activity; binding to GABA receptors may be related to anticonvulsant and sedative effects whilst binding to histamine 1 receptors is indicative of activity for treatment of bronchitis and asthma.

Clerodendrum mandarinorum. The genus *Clerodendrum* (Verbenaceae) has 30 species indigenous to China and several of them are used medicinally. Other genera of the Verbenaceae used for medicinal purposes are *Callicarpa*, *Premna*, *Verbena* and *Vitex*. The total extract of *C. mandarinorum* root bark exhibited strong binding to α_2-adrenoceptor, 5-HT_1, 5-HT_{1A}, 5-HT_2, opiate, adenosine 1, dopamine 1 and 2, histamine 1, $GABA_A$ and $GABA_B$ (Table 1). *C. mandarinorum* has not been investigated previously for its pharmacological activities but the related species *C. trichotomum* has been shown to have antihypertensive, sedative, analgesic and anti-inflammatory effects in animals (Ding 1957; Xue 1987).

Uncaria rhynchophylla. *U. rhynchophylla* (Rubiaceae) is one of the species accepted for the Chinese Pharmacopoeia monograph 'Gouteng' which consists of the stems and hooks of several species of *Uncaria* (Anon 1992). Gouteng is used medicinally in China to arrest convulsions and as an antihypertensive (Du 1987). The extract exhibited strong binding to α_2-adrenoceptor, 5-HT_{1A}, 5-HT_2, opiate, dopamine 1, Ca^{2+}-channel, sulphonylureas and GABA receptors (Table 1). The binding of the extract to α-adrenoceptors and to Ca^{2+} channels may be correlated with the peripheral vasodilation and antihypertensive effects of Gouteng whilst binding to 5-HT receptors may be linked to its sedative activity and clinical use for the treatment of vascular headaches. Strong binding to $GABA_A$ and $GABA_B$ receptors may relate to anticonvulsant activity and use of *U. rhynchophylla* for the treatment of epilepsy.

Isolation of active compounds

Bioassay-guided fractionation of extracts utilizing receptor-radioligand binding assays and chromatography on silica gel, Sephadex and HPLC columns resulted in the isolation of a number of pure natural products.

Schefflera bodinieri. *S. bodinieri* roots yielded eight novel triterpenoid glycosides named bodinitins A, B, C, D (**1**–**4**) (Zhu et al 1996d) and related triterpenoids **5**–**8** (Zhu et al 1996e) (Figure 1). The chemical structures were determined by MS, ^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H COSY and ^{1}H-^{13}C COSY. Hydrolysis yielded sugars which were characterized by ^{1}H-NMR and TLC and aglycones which were further identified by NMR spectroscopy. The triterpenoids are of the β-amyrane-type with a double bond at either C-13 or C-14.

The extract of *S. bodinieri* leaves yielded two further novel triterpenoids (**9** and **10**, Figure 1), the closely related known compound 3α-hydroxy-20-demethylisoleuritolic-14(15)-ene-28,30-dioic acid, stigmasterol-3-*O*-β-D-glucose, D-sorbitol and two disaccharides (Zhu et al 1996c).

All isolated compounds were only active in the μM range as illustrated by compound **5** which had an IC50 value of 1·83 μM against dopamine 2 receptor (butaclamol IC50 2·08 nM) and by compounds **9** and **10** with IC50 values of 0·91 and 3·57 μM, respectively, against the muscarinic receptor (atropine IC50 0·16 nM) (Zhu 1994).

Clerodendrum mandarinorum. The roots of *C. mandarinorum* yielded 14 known compounds which were identified as the triterpenoids friedelanone (**11**), lupeol (**12**) betulinic acid (**13**), the steroids 24S-stigmasta-5,25-dien-3β-ol (**14**), 24E,24S-stigmasta-5,22,25-trien-3β-ol (**15**), the flavonoids cirsimaritin (**16**), cirsimaritin-4′-glucoside (**17**), quercetin-3 methylether (**18**), tetrahydro-α-pyrone, sucrose, α-D and β-D-glucopyranose, ethyl α-D-glucopyranoside and 2-ethyl-β-D-fructofuranoside (Figure 2) (Zhu et al 1996b). The triterpenoids were identified on the basis of their MS, ^{1}H-NMR, ^{13}C-NMR, $[\alpha]_D$ and melting points, the flavonoids by UV, ^{1}H-NMR and co-TLC with authentic compounds. The remaining compounds

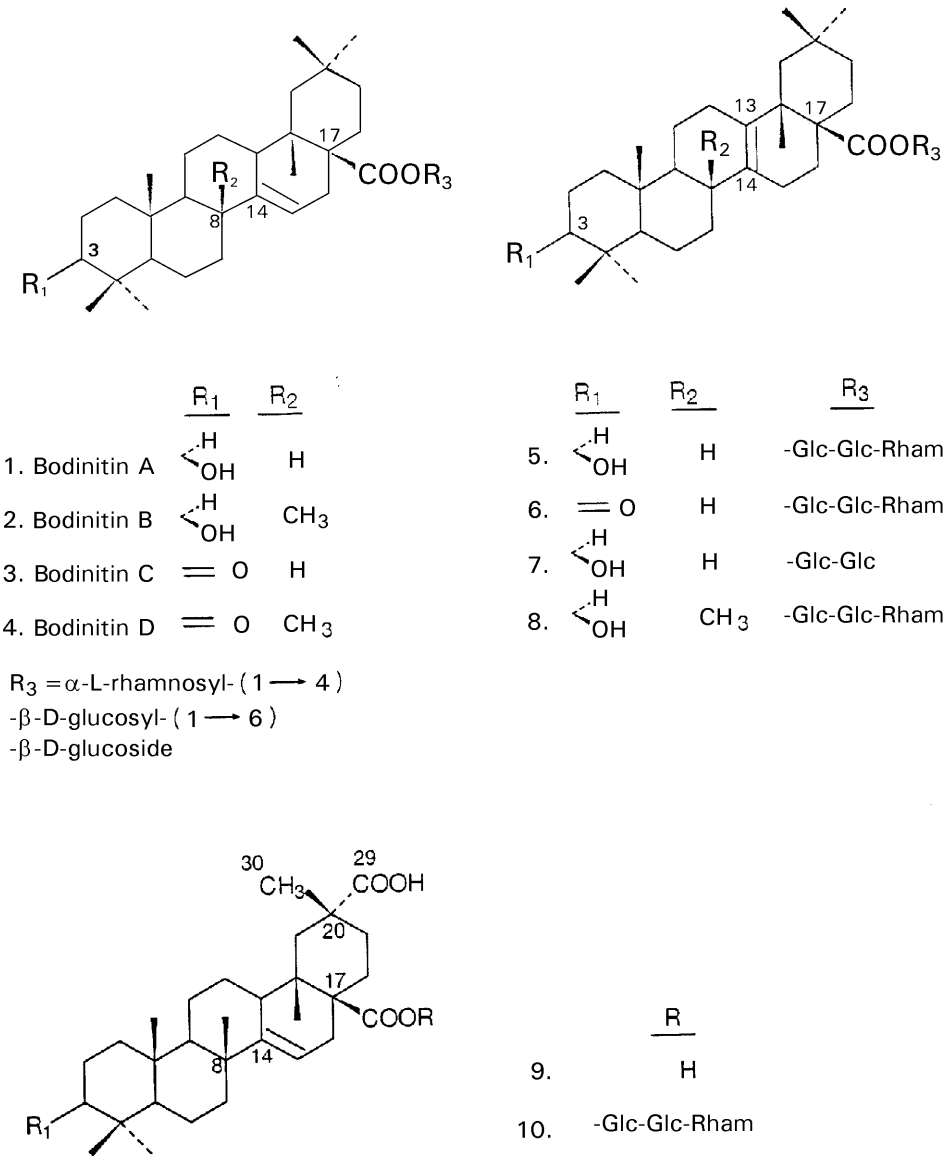
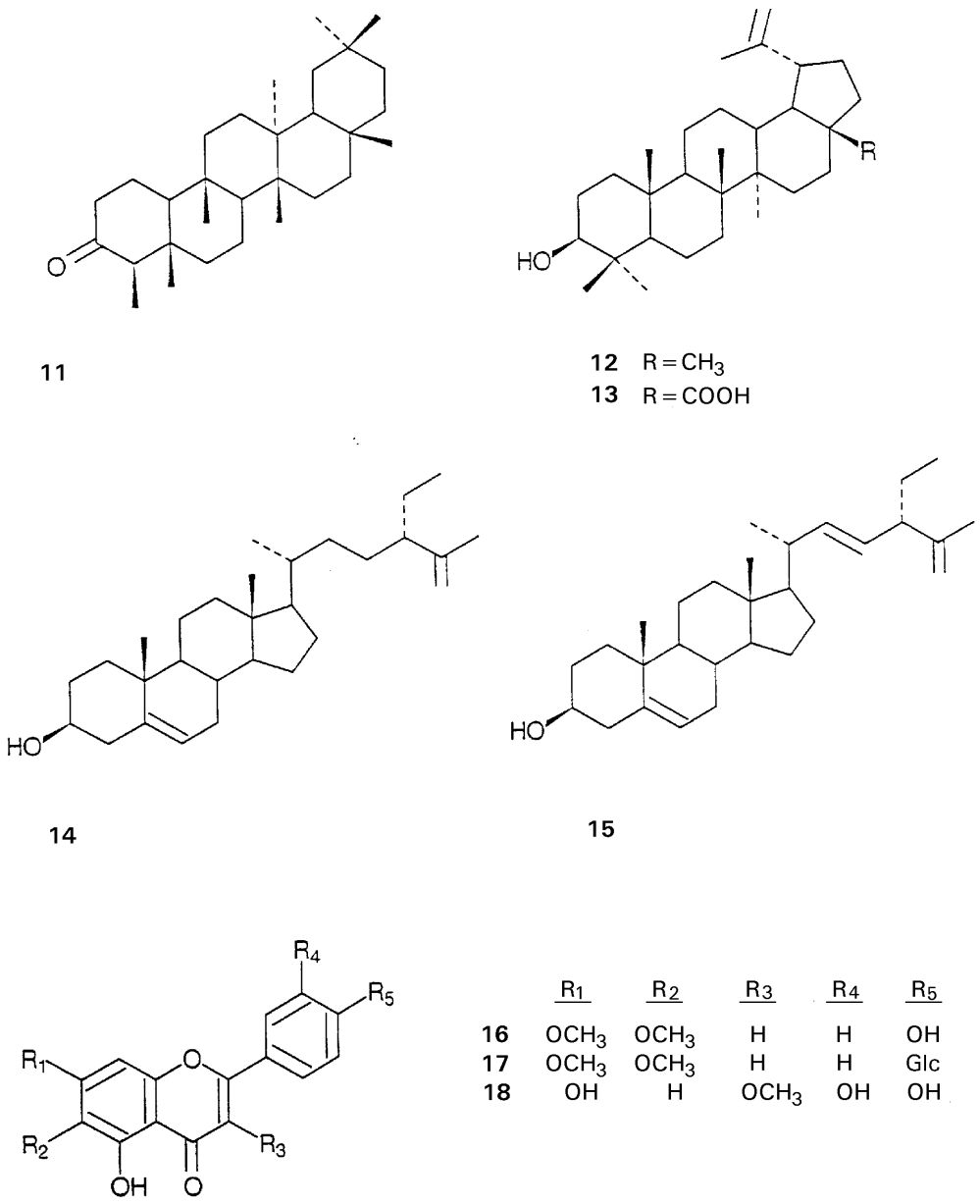


Figure 1. Novel triterpenoids of *Scheffiera bodinieri*.

were identified by ^{1}H- and ^{13}C-NMR, and by ^{1}H-^{1}H COSY.

Compounds were only active in the μM range as exemplified by betulinic acid with an IC50 value of 7·5 μM against sulphonylurea receptor (glibencamide IC50 0·79 nM), cirsimaritin-4′-glucoside with an IC50 value of 3·0 μM against adenosine 1 receptor (cyclohexyladenosine IC50 14·1 nM) and ethyl-α-D-glucopyranoside with an IC50 value of 5·5 μM against muscarinic receptor (atropine IC50 0·16 nM).

Uncaria rhynchophylla. Bioassay-guided fractionation of an extract of *U. rhynchophylla* stems and hooks is summarized in Figure 3. Isolated compounds were identified on the basis of their MS, ^{1}H-NMR and ^{13}C-NMR spectra and by co-chromatography with authentic compounds. A chloroform fraction was separated into further fractions on a silica gel column and fractions C1–C28 (Figure 3) combined on the basis of their TLC profiles and activity in the receptor-radioligand binding assays. These fractions showed more than 50% binding to the opiate receptor and only low activity in the other 11 binding assays (Zhu et al 1997b). The major compound isolated from the combined fractions was the known triterpenoid ursolic acid. Fractions C29–C54 from the same column were combined on the basis of TLC profile and activity in the binding assays. Each fraction inhibited binding of radioligands by more than 80% to α_2-adrenoceptor, 5-HT$_{1A}$, 5-HT$_2$ and opiate receptors



	R1	R2	R3	R4	R5
16	OCH_3	OCH_3	H	H	OH
17	OCH_3	OCH_3	H	H	Glc
18	OH	H	OCH_3	OH	OH

Figure 2. Compounds isolated from *Clerodendrum mandarinorum*.

(Figure 3) and contained alkaloids. It is known that *U. rhynchophylla* and related species which comprise Gouteng are rich sources of oxindole-type (**19**, **20**) and indole-type (**21**, **22**) alkaloids (Figure 4) (Phillipson et al 1978). The indole alkaloids hirsutine (**21**, R = ethyl, pseudo) and epiallocorynantheine (**21**, R = vinyl, epiallo) (Figure 4) were isolated from these combined fractions. Combined fractions El–E30 were obtained similarly from separation of an ethylacetate fraction on a Sephadex column. The fractions exhibited more than 80% inhibition of binding of radioligands to 5-HT_2 and opiate receptors. Compounds isolated from these combined fractions were identified as (+)-catechin (**23**), (−)-catechin (**24**), (+)-gallocatechin (**25**) and (−)-gallocatechin (**26**) (Figure 5). The compounds isolated had weak binding properties with IC50 values in the nM range. The IC50 values of some of the isolated compounds as inhibitors to binding of radioligands to receptors are given in Table 2. Ursolic acid had IC50 values of 6·63 and 2·83 μM against muscarinic and sulphonylurea receptors, respectively, whereas hirsutine had IC50 values of 0·15, 4·53, 3·47, 1·60, 0·11 and 0·14 μM against α_2- and β-adrenoceptors, 5-HT_{1A}, 5-HT_2, opiate and sulphonylurea receptors, respectively. Epiallocorynantheine had IC50 values of 6·73, 3·48, 3·54 and 0·65 μM against β-adrenoceptor, 5-HT_{1A}, 5-HT_2 and opiate receptors, respectively. The activity of the isolated compounds was not

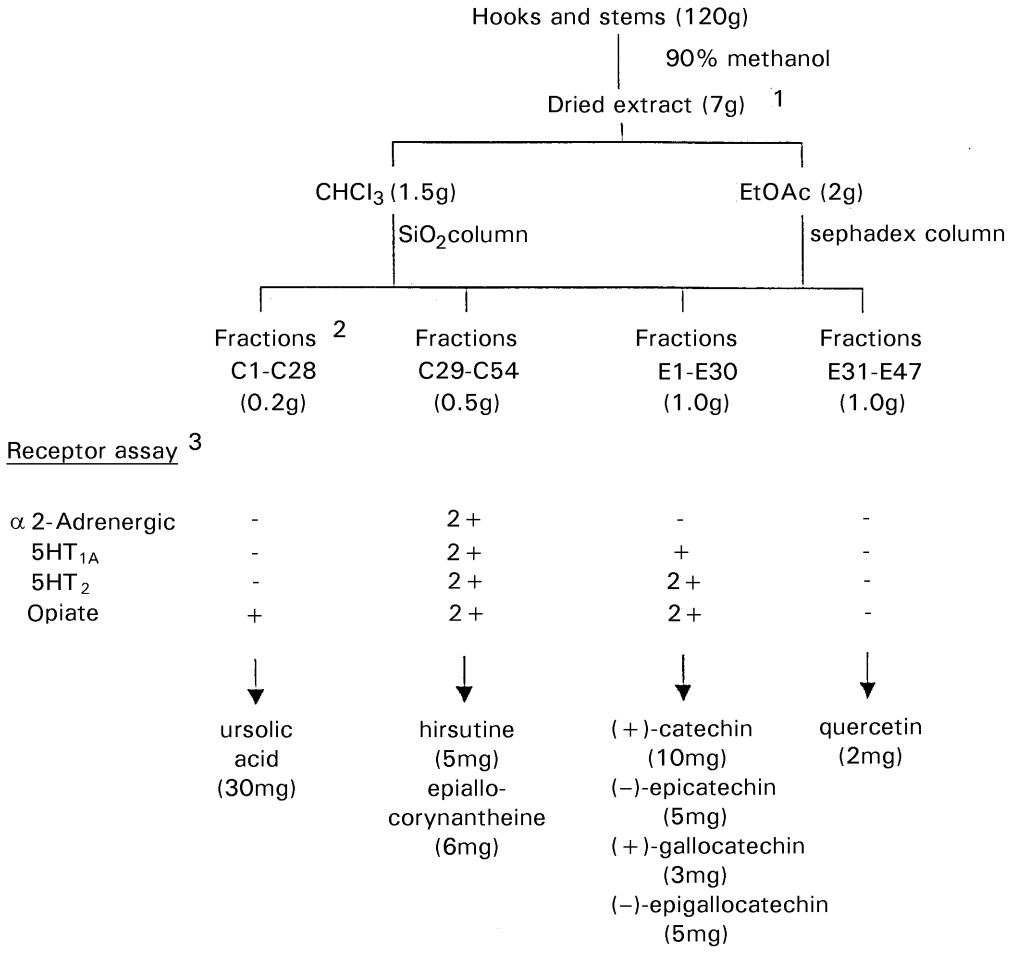
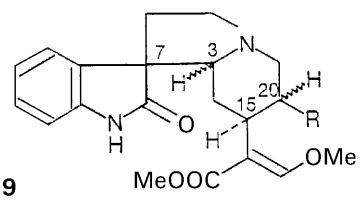


Figure 3. Bioassay-guided fractionation of *Uncaria rhychophylla*. [1]Extracts showed >60% inhibition of specific radioligand to dopamine 1, α2-adrenoceptor, 5-HT_{1A} and opiate receptors. [2]Fractions were collected every 100 mL eluting solvent and combined on the basis of TLC profiles. [3]Inhibition of radioligand binding to receptor, + 50–79% at 1 mg mL^{-1}, 2+ 80–100% at 1 mg mL^{-1}.

always comparable directly with the activity of the fractions from which they were obtained, and this may be due to a number of reasons, including the possibility of combination effects of several compounds acting at the same time, or possibly due to incomplete isolation of all of the compounds present in a particular fraction.

The results of our investigation differ from some of those reported by other workers. Bioassay-guided fractionation of extracts of the related species *U. sinensis*, using 5-HT receptor-binding assays led to the isolation of corynantheine (**21**, R = vinyl, normal) and dihydrocorynantheine (**21**, R = ethyl, normal) as the active principles (Kanatani et al 1985). The corresponding alkaloids with C-3H β configurations, hirsutine (**21**, R = ethyl, pseudo) and hirsuteine (**21**, R = vinyl, pseudo) were reported to have only low binding activity. The major pharmacologically active compound isolated from *U. rhynchophylla* has been reported to be the oxindole alkaloid rhynchophylline (**19**, R = ethyl, normal, B) (Du 1987). All these alkaloids are known to us through phytochemical investigation over many years (Phillipson et al 1978) and they were not detected as the active principles during our bioassay-guided fractionation utilizing 11 receptor-radioligand binding assays (Zhu et al 1997b). To confuse matters further, it has been reported that the glycosidic alkaloids 3α-dihydrocadambine and 3β-isodihydrocadambine, minor constituents isolated from an *Uncaria* species, (possibly *U. sinensis*), are the active antihypertensive principles (Endo et al 1983).

The Chinese Pharmacopoeia monograph for Gouteng permits the use of a number of Uncaria species including *U. rhynchophylla* Miq., *U. macrophylla* Wall., *U. hirsuta* Havil., *U. sessilifructus* Roxb. and *U. sinensis* (Oliv.) Havil. (Anon 1992).



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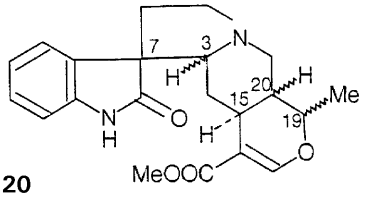
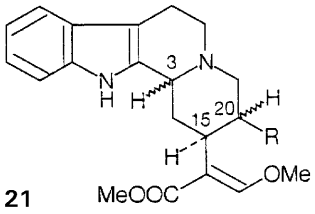
	R	Configuration
Isorhynchophylline	ethyl	A, normal
Rhynochophylline	ethyl	B, normal
Isocorynoxeine	vinyl	A, normal
Corynoxine	vinyl	B, normal
Corynoxine A	ethyl	A, allo
Corynoxine B	ethyl	B, allo



20

	R	Configuration	C-19Me
Isomitraphylline		A, normal	α
Mitraphylline		B, normal	α
Uncarine A		A, normal	β
Uncarine B		B, normal	β
Isopteropodine		A, allo	α
Pteropodine		B, allo	α
Speciophylline		A, epiallo	α
Uncarine F		B, epiallo	α



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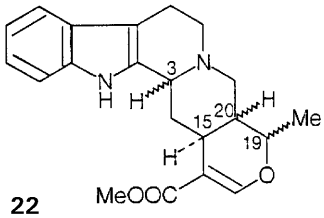
	R	Configuration
Dihydrocorynantheine	ethyl	normal
Corynantheine	vinyl	normal
Hirsutine	ethyl	pseudo
Hirsuteine	vinyl	pseudo
Epiallo-corynantheine	vinyl	epiallo



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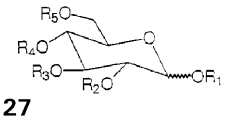
	Configuration	C-19Me
Ajmalicine	normal	α
3-Isoajmalicine	pseudo	α
Akuammigine	epiallol	α

Figure 4. Oxindole (**19**, **20**) and heteroyohimbine (**21**, **22**) alkaloids of *Uncaria rhynchophylla* and related species which constitute the Chinese medicinal drug, Gouteng. Four possible diastereo isomers exist for alkaloidal types **19**–**22**, defined as allo (C-3Hα, C-2OHα), epiallo (C-3Hβ, C-20 Hα), normal (C-3Hα C-20Hβ), pseudo (C-3Hα, C-20Hβ). Oxindole alkaloidal types **19**,**20** can exist as A or B isomers depending on the configuration of C-7 spiro carbon; the lactam carbonyl may lie above (A) or below (B) the plane of the C-D rings.



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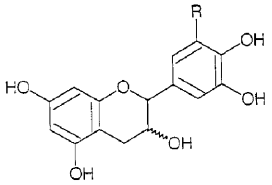
R1 to R5 = H, galloyl or hexhydrodiphenoyl



	R	C-3 OH
23 (+)-catechin	H	β
24 (–)-epicatechin	H	α
25 (+)-gallocatechin	OH	β
26 (–)-epigallocatechin	OH	α

Figure 5. Polyphenolic constituents of tannins, proanthocyanidin-type (**23**–**26**) and galloyl or hexahydrodiphenoyl esters of glucose (**27**).

Table 2. IC50 values of compounds isolated from *Uncaria rhynchophylla* in inhibition of radioligand-receptor binding assays in-vitro.

Compound	Receptor	IC50 (μM)
Ursolic acid	Muscarinic	6·63
	Sulphonylurea	2·83
Hirsutine	α_2-Adrenoceptor	0·15
	β-Adrenoceptor	4·53
	5-HT_{1A}	3·47
	5-HT_2	1·60
	Opiate	0·11
	Sulphonylurea	0·14
Epiallo-corynantheine	β-Adrenoceptor	6·73
	5-HT_{1A}	3·48
	5-HT_2	3·54
	Opiate	0·65

The IC50 values of control compounds used were: atropine 0·16 nM (muscarinic receptor); glibenclamide 0·79 nM (sulphonylurea receptor); phentolamine 5·64 nM (α_2-adrenoceptor); propranolol 1·41 nM (β-adrenoceptor); 8-OH DPAT 1·00 nM (5-HT_{1A} receptor); spiperone 5·62 nM (5-HT_2 receptor); naloxone 1·02 nM (opiate receptor). Full experimental details have been published (Zhu et al 1997b).

Furthermore, it is reported that Gouteng may also be obtained from *U. lancifolia* Hutch. and *U. scandens* (Smith) Hutch. (Du 1987). All of these species contain similar heteroyohimbine and oxindole alkaloids (**19**–**22**, Figure 4) but they are not necessarily identical in each of the species. In addition, it is known that some of these species exhibit infraspecific variation in their contained alkaloids (Phillipson et al 1978). Due to the conflicting reports as to the active constituents of Gouteng, it has to be concluded that there is still considerable scope for further investigation.

Choice of plants for biological testing – selection or random collections?

In a parallel investigation to the one described above on ten selected Chinese medicinal plants, receptor-radioligand binding assays were used to ascertain whether or not ethnomedical data conferred any advantages for selection of plants for novel leads to analgesic agents (Sampson et al 1994). Several species of higher plant have been used traditionally as analgesics and some have yielded compounds which can be used to relieve pain, such as *Papaver somniferum*. There is still a current need for novel analgesics, particularly for analgesics without adverse reactions. More than 20 endogenous neuropeptides including bradykinin, calcitonin gene-related peptide (CGRP), cholecystokinin, neurokinin, endorphins and enkephalins are implicated in the mediation of pain. At the start of our investigation, three receptors were of particular interest to our industrial collaborator, namely, bradykinin II, CGRP and neurokinin 1. Bradykinin II is a nine-amino acid neuropeptide which acts on sensory fibres and neurones (Steranka et al 1988) and is implicated in the pathophysiological processes that accompany tissue damage (inflammation and hyperalgesia). Neurokinin 1 is an eleven-amino acid peptide implicated in the mediation of acute pain (Pernow 1983) and CGRP is a 37-amino acid peptide implicated in the mediation of migraine and other vascular headaches (Moskowitz 1990).

Three hundred plants were selected from the scientific and medicinal literature of China, South America, West Indies and West Africa on the basis of their traditional use for the relief of pain. Methanol extracts (10 μL 1% w/v), were screened in a bradykinin II receptor-radioligand assay using human cloned receptors expressed in CHO cells (Sampson 1996). The results were compared with those obtained from 300 similar extracts prepared from plants which had not been selected because of any ethnomedical criteria. Of these 300 non-selected plants, 22 (7·3%) proved to be positive in the bradykinin II assay contrasting with 65 (22%) from the 300 selected plants. The methanol extracts were then treated with polyvinylpyrrolidone (PVP) to remove phenolic compounds such as tannins (O'Neill & Lewis 1993) and there were only two positive results (0·6%) from the nonselected group and 20 (6%) from the ethnomedically selected group. Although these results indicated that ethnomedical selection significantly improved the possibility of obtaining active compounds from plants, they are of no real significance industrially because high-throughput screens are capable of dealing with large numbers of test extracts.

Each positive extract was subsequently examined for selectivity of action by assessing its ability to bind to neurokinin 1 and CGRP receptors. Ten plants with high activity in either the bradykinin II or neurokinin 1 or CGRP screens were selected for further study, namely, *Croton tiglium* (Euphorbiaceae), *Panax ginseng* (Araliaceae) and *Sinomenium acutum* (Menispermaceae) from China, *Ageratum conyzoides* (Compositae) and *Solidago vigaurea* (Compositae) from America, *Symplocos leptophylla* (Symplococaceae) from Asia, *Barringtonia edulis* (Barringtonaceae) and *Physostigma venenosum* (Legumosae) from Africa, *Typhonium giganteum* (Araceae) from the West Indies and *Iopoema pes-caprae*. The results of binding against bradykinin II, neurokinin 1 and CGRP receptors of methanol extracts, treated with PVP to remove tannins, of these ten species are given in Table 3 (Sampson 1996). *Symplocos leptophylla* and *Barringtonia edulis* extracts showed marked binding to the bradykinin II receptor (100% and 81%, respectively) and not to the other two receptors. *Physostigma venenosum* and *Typhonium giganteum* extracts were selective in the CGRP assay (55% and 69%, respectively) whereas *Ipomoea pes-caprae* and *Panax ginseng* extracts showed marked activity against bradykinin II and neurokinin 1 receptors but little activity against the CGRP receptor (Table 3). The results obtained from these three receptor-binding assays showed that four of the ten plant extracts tested were selective for one of the receptors but not to the other two pain receptors and that two of the extracts had marked activity against two of the receptors but not to the third receptor. Further investigation, particularly in functional assays and for the identification of the active principles, is warranted.

Should polyphenols be removed from plant extracts prior to testing for receptor binding activity?

The results of testing for analgesic activity in 600 species of higher plant, as described above, clearly

Table 3. Inhibition of radioligand binding to bradykinin II, neurokinin 1 and CGRP receptors by methanol extracts (PVP-treated) of ten species of higher plant.

Plant	Part used	Inhibition of binding to receptor (%)		
		Bradykinin II	Neurokinin 1	CGRP
Ageratum conyzoides	Whole plant	4	17	5
Barringtonia edulis	Leaf	81	1	10
Croton tiglium	Seed	60	17	30
Ipomoea pes-caprae	Seed	66	100	14
Panax ginseng	Root	100	100	0
Physostigma venenosum	Seed	27	12	55
Sinomenium acutum	Seed	48	21	20
Solidago virgaurea	Seed	40	35	23
Symplocos leptophylla	Stem and bark	100	19	11
Typhonium giganteum	Rhizome	39	13	69

IC50 values of control drugs: Hoe-140 2·07 nM (bradykinin-II receptor); CGRP 0·67 nM (CGRP receptor); Substance P 0·75 nM (neurokinin 1 receptor); full experimental details have been published (Sampson 1996).

show that treatment of extracts with PVP to remove phenolic compounds such as tannins results in significant loss of binding to receptors for many of the extracts. Tannins and other phenolic constituents are reportedly the active ingredients of many medicinal herbs. In a survey of 141 medicinal herbs which are ingredients of over-the-counter herbal medicines sold in UK pharmacies it has been reported that 68 of the herbs are rich in tannins (Newall et al 1996). Individual polyphenols from plants have a wide range of biological activity including antibacterial, antiviral, anti-inflammatory, antihepatotoxic, antioxidant, anthelmintic, cytotoxic and inhibitory activity against many enzymes including glucosyltranferases, monoamine oxidase, ornithine decarboxylase and xanthine oxidase (Haslam 1989, 1996). Epigallocatechin-3-gallate, a component of green tea, inhibits urokinase and it has been proposed that it may be responsible for reducing the incidence of cancer in man (Jankun et al 1997). Dragon's blood, the red sap of the South American species of *Croton* comprises a complex mixture of polyphenols (Cai et al 1991). A proanthocyanin polymeric mixture with an average of seven monomeric units containing a high proportion of (+)-gallocatechin (**25**) and (−)-epigallocatechin (**26**) is the antiviral principle isolated from the sap showing significant activity against respiratory syncytial virus 1 and 2, influenza and herpes (US Patent 1993; Ubillas et al 1994).

Because plant polyphenols have such a range of interesting biological activities and their study may well lead to the development of new drug entities, the question has to be asked as to why they are removed in some industrial screening programmes. Among the reasons given are the following. Polyphenols bind non-selectively to protein and this results in ''false leads'' when receptor binding and enzyme assays are used. They occur as complex mixtures of unstable compounds which are liable to oxidize thus changing their chemical composition. Many polyphenols are known molecular structures and will not provide useful new leads to novel drugs. Their absorption, distribution, metabolism and excretion characteristics are not fully understood. In an attempt to discover the effects of polyphenols on receptors, we assessed 20 polyphenols for their ability to inhibit binding of radioligands to 16 receptors in-vitro (Zhu et al 1997a). The polyphenols tested had molecular weights ranging from 290 to 1874 Da and were representative of the two major groups of tannins. One group consisted of either monomeric or dimeric proanthocyanidins mainly comprising (+)-catechin (**23**), (−)-epicatechin (**24**), (+)-gallocatechin (**25**) or (−)-epigallocatechin (**26**) (Figure 5). The other group comprised galloyl or hexahydroxy-diphenoyl acid esters of glucose (**27**) (Figure 5). It was anticipated that all of these polyphenols would bind non-specifically to the proteins of the receptors present in the assay preparations. Surprisingly, all 20 polyphenols tested at 10^{-5} M concentrations failed to inhibit radioligand binding to ten of the 16 receptors under the assay conditions. These ten receptors were α_1-adrenoceptor, 5-HT_{1A}, 5-HT_2, adenosine 1, benzodiazepine, Ca^{2+} channel, sulphonylureas, muscarinic, histamine 1 and ATPase. The most susceptible receptors to competitive binding by the 20 polyphenols were the β-adrenoceptor (5 compounds), 5-HT_1 (four compounds) and opiate (five compounds) (Zhu et al 1997a). Some 16 of the 20 polyphenols were active in the radioligand-receptor binding assays against a single receptor, under the assay conditions used and four polyphenols were active against two of the receptors (Zhu et al 1997a). No correlations were

observed between the molecular weights of the polyphenols tested and their ability to inhibit binding of radioligands to receptors, or between mono-, di- or tri-hydroxy substituents in the B ring of monomeric flavan-3-ols. These results indicate that further investigations are required to learn more about the specificity of polyphenol/tannin-receptor interactions and that polyphenol research should feature in drug discovery programmes (Zhu et al 1997a; Phillipson et al 1998). Tannins may well be regarded as unsuitable for drug development but they exhibit a wide range of activity and they are believed to be the active ingredients of many medicinally used herbs. Modern techniques of isolation coupled with in-vitro tests, such as receptor-radioligand binding assays should help in the isolation of more single-entity, well-defined polyphenolics with specific biological activities.

Conclusions

Sensitive in-vitro assays based on enzymes, or receptor-radioligand binding, require only minute amounts of test material and provide fast and specific methods for testing a range of biological activities in-vitro. Industry has established high-throughput robotic screens which enable tens of thousands of compounds to be assessed for activity on a daily basis. Such assays can be used for bioassay-guided fractionation of plant extracts as illustrated by the research described in this paper. Sensitive in-vitro screens coupled with chromatographic techniques for separation and isolation of single compounds, coupled with X-ray crystallography and spectroscopic methods, particularly MS and NMR, facilitate the identification of biologically active substances. In theory, the technology is now available for a thorough investigation of the biological activities of the estimated 250,000 species of higher plants. In practice, high-throughput screening programmes generate masses of data and much of this information will remain buried in company files.

From the industrial standpoint, the objective is to identify compounds with specific activity for a particular receptor, or subset of receptors, with minimal activity against related receptors. Pinpointing such compounds facilitates the development of novel drug entities whether they be natural products as isolated or synthetic analogues of active natural products. The retention of large numbers of plant extracts means that they can be tested repeatedly as new biological screens come into use. Industry is currently focussing its attention on combinatorial synthesis as a logical way of increasing the numbers of compounds available for biological screening and there is less emphasis on natural products. It should not be forgotten that natural products have made an undoubted contribution to the development of new clinical drugs. Six of the top twenty pharmaceutical drugs sold in 1996 were natural products and 50% of the top twenty are directly linked to natural product research (Buss 1998).

Academic research units cannot match the capability of industry in terms of screening such large numbers of compounds or extracts. Some biological screens may only last for a few months before being replaced by newer ones which offer further advantages of specificity. Hence, it is necessary for academics to select research targets (Phillipson 1995). The industrial approach to investigation of plants does not necessarily equate with that of scientists studying the biological effects of plants used in traditional medicine where the objectives may well include rationalization of medicinal use, irrespective of specificity of action of isolated compounds or novelty of chemical structures. One possible role for academic researchers is to follow ethnopharmacological leads, collaborating with physicians in the investigation of the efficacy of traditional medicines, identifying active compounds and establishing quality assurance techniques for standardization of such medicines. Another possible role for academics would be to collaborate with industry, either in a particular area of therapy, or within the separation and identification of natural products. It is essential, for example, that commonly occurring natural products be recognized by analytical techniques at an early stage of an investigation. Phenolics may not be regarded as ideal drug targets by some companies whereas saponins may disrupt membranes of cellular targets and the isolation of well known compounds, such as fatty acids, does not justify the expense of industrial screening (O'Neill & Lewis 1993). Partially purified extracts of plants in which common metabolites are not present may prove to be an attractive source of screening material for industrial projects.

Whichever line of research is to be followed, there is no doubt that receptor-radioligand binding assays are important tools for identifying biologically active substances in plant extracts as illustrated by the search for CNS and analgesic compounds discussed in this article.

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