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Muscarinic Receptor Activity of Some Malaysian Plant Species

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Abstract

Muscarinic receptor binding activity was tested on 224 plant extracts obtained from more than 50 plant families found in Malaysia. The plant extracts were evaluated by a 96-well microplate filtration–based radioligand competitive assay, centered on the ability of the plant extracts to competitively displace the radioligand, [^{3}H]*N*-methylscopolamine, from binding to the muscarinic membrane receptors. The screening assay was initially carried out at 50 μg/assay point, and those showing inhibition at and above 61% were retested at 10 μg/assay point. The extracts of *Ficus septica* Burm. f. (Moraceae) [65.85 ± 3.75% inhibition; mean (n = 3) ± SD], *Polyalthia microtus* Miq. (Annonaceae) (32.63 ± 1.38% inhibition), and *Popowia odoardoi* Diels (Annonaceae) (35.79 ± 7.11% inhibition) at 10 μg/assay point exhibited muscarinic properties, which are worthy of further investigation.

Keywords: *Ficus septica*, Malaysian plants, muscarinic receptor, *Polyalthia microtus*, *Popowia odoardoi*, radioligand binding assay.

Introduction

Since ancient times, human being have used natural products, including plants, as remedies to treat illnesses. Today, about one-third of the top 25 prescription drugs in the world are natural products or their derivatives (Manly et al., 2002). The sources of natural products include plants, marine and microorganisms (Zhu, 1996; Cragg et al., 1997; Shu, 1997; Manly et al., 2002), and historically, plants have been an important source of pharmacologically active compounds providing for a number of muscarinic receptor antagonists. For example, atropine from the plant *Atropa belladonna* L. and scopolamine from *Datura* sp. are the most commonly used anticholinergic drugs (Evans & Evans, 2002).

Anticholinergic drugs act on two distinct classes of receptors, muscarinic and nicotinic. The muscarinic class of acetylcholine receptors are widely distributed throughout the body and subserve numerous vital functions in both the brain and autonomic nervous system (Lefkowitz et al., 1990). Heterogeneity of muscarinic receptor was shown in the late 1980s when five subtypes (M1–M5) were identified using molecular biological techniques (Kubo et al., 1986; Bonner et al., 1987; Liao et al., 1989; Sokolovsky, 1989; Kashihara et al., 1992).

Activation of muscarinic receptors in the periphery causes a decrease in heart rate, relaxation of blood vessels, constriction in the airways of the lung, an increase in secretions, and motility of various organs of the gastrointestinal tract, increase in the secretions of lacrimal and sweat glands, constriction in the iris sphincter and ciliary muscles of the eye (Lefkowitz et al., 1990). In the brain, muscarinic receptors influence many important functions such as learning, memory, and the control of posture (Lefkowitz et al., 1990).

Classic muscarinic receptor antagonists such as atropine do not distinguish between muscarinic receptor subtypes. These nonselective compounds cover therapeutic indications such as antispasmodic, antitussive, and antibronchospastic, but their therapeutic utility is limited by the presence of side effects including mydriasis, CNS disturbances, tachycardia, and constipation. In recent studies, it has been shown that selective M1 antagonists are useful in reducing gastric acid secretion. Selective M2 antagonists may be useful in the treatment of

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Table 1. Percent inhibition of extracts (50 μg/well) on specific binding to muscarinic receptor.

No.	Voucher no.	Family	Species	Part[a]	Inhibition[b]
1	43264	Alangiaceae	*Alangium ebenaceum*	L	+
2	143521		*Alangium griffithii*	B	+
3	143521			L	±
4	43303	Anacardiaceae	*Bouea macrophylla*	L	±
5	43304		*Bouea oppositifolia*	L	3+
6	143516		*Buchanania insignis*	B	±
7	143516			L	±
8	143370		*Semicarpus leneatus*	L	+
9	143374	Annonaceae	*Artabotrys roseus*	B	±
10	143374			L	±
11	143523			B	2+
12	143523			L	±
13	143377		*Desmos chinensis*	B	±
14	143377			L	±
15	143358		*Eniscosanthum grandiflorum*	L	+
16	143362		*Goniothalamus woodii*	B	±
17	143362			L	±
18	133834		*Goniothalamus gigantifolius*	L	±
19	145372			L	+
20	145372			S	+
21	143506		*Neouvaria acuminatiisima*	B	3+
22	143506			L	+
23	143509		*Orophea corymbosa*	B	±
24	143509			L	±
25	143511		*Polyalthia insignis*	B	3+
26	143511			L	±
27	143502		*Polyalthia longipes*	B	3+
28	143502			L	+
29	145376		*Polyalthia microtus*	B	4+
30	145376			L	+
31	143524		*Polyalthia rumphii*	L	3+
32	143524			R	±
33	143395		*Popowia odoardoi*	B	4+
34	145365			S	3+
35	143368		*Uvaria rufa*	B	±
36	143368			L	±
37	143507		*Xylopia malayana*	B	+
38	143507			L	+
39	43125	Apocynaceae	*Dyera costulata*	L	±
40	145362		*Kopsia dasyrachis*	L	+
41	145362			S	2+
42	145361	Burseraceae	*Canarium denticulatum*	B	2+
43	145361			L	+
44	143522		*Canarium hirsutum*	B	+
45	143522			F	±
46	43302		*Dacryodes rugosa*	L	+
47	43306		*Santiria griffithii*	L	+
48	43127		*Santiria laevigata*	L	3+
49	43127			S	±
50	43350	Caesalpiniaceae	*Peltophorum pterocarpum*	L	2+
51	43309	Chrysobalanaceae	*Maranthes corymbosa*	S	+
52	43320	Combretaceae	*Terminalia superba*	L	±
53	143381	Cornaceae	*Chionanthus laxiflorus*	B	±
54	143381			L	±
55	43344	Cupressaceae	*Dacrydium becarii*	L	+
56	43344			S	2+
57	43345		*Dacrydium elatum*	L	+

(*Continued*)

Table 1. Continued.

No.	Voucher no.	Family	Species	Part[a]	Inhibition[b]
58	43345			S	±
59	143503	Dichapetalaceae	*Dichapetalum gelonioides*	B	±
60	143503			L	±
61	43308	Dipterocarpaceae	*Anisoptera costata*	L	+
62	43124		*Hopea dryobalanoides*	L	2+
63	43124			T	2+
64	43112		*Neobalanocarpus heimii*	L	2+
65	43134		*Shorea parvifolia*	L	+
66	43134			S	2+
67	43324		*Upuna borneensis*	L	2+
68	43324			S	2+
69	143504	Ebenaceae	*Diospyros cauliflora*	B	±
70	143504			L	±
71	143519		*Diospyros tuberculata*	B	+
72	143519			L	±
73	43273	Elaeocarpaceae	*Elaeocarpus petiolaris*	L	±
74	145393	Euphorbiaceae	*Borneodendron aenigmaticum*	B	±
75	145393			L	±
76	145400		*Mallotus griffithianus*	B	±
77	145400			L	±
78	145371		*Mallotus wrayi*	L	2+
79	43108		Phyllanthus emblica	L	+
80	43108			T	+
81	43338		*Phyllanthus pectinata*	L	+
82	43137	Fagaceae	*Castanopsis inermis*	L	+
83	43323	Flacourtiaceae	*Flacourtia rukam*	L	+
84	145388		*Homalium panayanum*	B	±
85	145388			L	±
86	43123	Gittiferae	*Mesua ferra*	L	2+
87	145398	Guttiferae	*Calophyllum blancoi*	B	±
88	145377		*Calophyllum gracilipes*	B	+
89	145377			L	±
90	145385		*Calophyllum nodosum*	B	±
91	145385			L	−
92	133842		*Garcinia brianii*	B	2+
93	133842			L	+
94	133846		*Garcinia cuspidate*	B	±
95	142698		*Garcinia parvifolia*	L	2+
96	142674		*Garcinia rostrata*	L	±
97	43279	Ixonanthaceae	*Ixonanthes reticulata*	L	2+
98	145368	Lauraceae	*Litsea garciae*	B	2+
99	145380		*Litsea sessilis*	B	2+
100	43339		*Persea Americana*	L	2+
101	143388	Leguminosae	*Dalbergia pseudo-sissoo*	B	±
102	143388			L	±
103	43129		*Millettia atropurpurea*	L	±
104	43138		*Sindora echinocalyx*	L	+
105	43138			T	+
106	143360		*Spatholobus macropterus*	B	±
107	143360			L	±
108	43144	Magnoliaceae	*Aromadendron elegans*	L	2+
109	143392	Meliaceae	*Aglaia affinis*	B	+
110	143392			L	−
111	143393		*Aglaia argentea*	B	±
112	143393			L	±
113	143366		*Aglaia shawiana*	B	±
114	143366			L	±

(*Continued*)

Table 1. Continued.

No.	Voucher no.	Family	Species	Part[a]	Inhibition[b]
115	43105		*Aglaia korthalsii*	L	±
116	143361		*Aglaia rivularis*	B	±
117	143361			L	±
118	143376		*Chisocheton polyandra*	B	±
119	143376			L	±
120	143512		*Chisocheton erythrocarpus*	B	±
121	143512			F	±
122	143512			L	+
123	145367		*Chisocheton macranthus*	B	±
124	145367			F	+
125	145367			L	2+
126	143513		*Chisocheton pentandrus*	B	2+
127	143513			L	+
128	142663		*Chisocheton polyandrous*	L	±
129	143355		*Dysoxylum ramiflorum*	B	±
130	143355			L	+
131	143364		*Dysoxylum rugulosum*	B	+
132	143364			L	±
133	43104		*Sandoricum koetjape*	L	2+
134	43104			T	3+
135	43143		*Walsura chrysogene*	L	2+
136	145378	Menispermaceae	*Fibraurea chloroleuca*	F	±
137	145378			L	+
138	143518	Moraceae	*Ficus septica*	L	3+
139	145397	Myrtaceae	*Decaspermum fruticosum*	B	2+
140	145397			L	2+
141	145392	Ochnaceae	*Gomphia serrata*	B	+
142	145392			L	±
143	43111	Olaceae	*Ochanostachys amentacea*	L	±
144	145396	Oleaceae	*Chionanthus crispus*	B	±
145	145396			L	±
146	143359	Piperaceae	*Piper officinarum*	L	±
147	143359			S	±
148	43307	Podocarpaceae	*Xanthophyllum stipitatum*	L	+
149	43329	Rhamnaceae	*Maesopsis eminii*	L	±
150	145373		*Vantilago dichotoma*	S	+
151	143525	Rhizophoraceae	*Carallia borneensis*	L	±
152	43348		*Carallia suffruticosa*	L	2+
153	145363	Rubiaceae	*Gardenia tubifera*	L	+
154	145395		*Morinda rigida*	B	±
155	145395			L	±
156	145395			S	±
157	145364		*Praravinia suberosa*	B	±
158	145364			L	±
159	145382		*Psychotria sarmentosa*	L	±
160	145384		*Timonius flavescens*	B	±
161	145384			L	±
162	145366	Rutaceae	*Clausena excavata*	B	±
163	145366			L	±
164	143391		*Melicope accedens*	B	±
165	143391			L	−
166	143375		*Melicope incana*	B	±
167	143375			L	−
168	143367		*Melicope luna-akenda*	L	±
169	143369		*Melicope subunifoliata*	B	3+
170	143369			L	2+
171	143389		*Micromelum minutum*	B	±

(*Continued*)

Table 1. Continued.

No.	Voucher no.	Family	Species	Part[a]	Inhibition[b]
172	143389			L	±
173	43325	Sapindaceae	*Amesiodendron chinense*	L	+
174	43325			S	2+
175	43337		*Dimocarpus longan*	L	2+
176	43337			S	2+
177	43327		*Lepisanthes alata*	F	±
178	43327			S	2+
179	43106		*Nephelium lappaceum*	T	±
180	43310		*Nephelium maingayi*	L	2+
181	43341		*Nephelium rambutanake*	L	+
182	43341			S	2+
183	43110		*Pometia pinnata*	L	2+
184	43110			T	2+
185	143514		*Walsura pinnata*	B	+
186	143514			L	±
187	43334	Sapotaceae	*Mimusops elengi*	L	2+
188	43334			S	±
189	43347		*Palaquim maingayi*	L	+
190	43347			S	+
191	143520	Scyphostegiaceae	*Scyphostegia borneensis*	B	±
192	143520			L	±
193	43145	Simaroubaceae	*Irvingia malayana*	L	2+
194	133822		*Quassia indica*	B	±
195	43311	Sterculiaceae	*Heritiera simplicifolia*	S	2+
196	145381		*Sterculia stipulata*	B	±
197	145381			L	±
198	143385	Theaceae	*Schima wallichi*	B	+
199	143385			L	−
200	145383		*Schima wallichii*	B	±
201	145383			L	±
202	43275	Tiliaceae	*Pentace triptera*	L	±
203	145391	Ulmaceae	*Gironniera subaequaelis*	L	±
204	145375	Urticaceae	*Dendrocnide elliptica*	B	±
205	145375			L	±
206	145387		*Leucosyke winklerii*	B	+
207	145387			L	±
208	143378	Verbenaceae	*Callicarpa havillandii*	B	−
209	143378			L	±
210	106702		*Callicarpa erioclona*	B	+
211	133850		*Callicarpa farinose*	B	±
212	133849		*Callicarpa fulvohirsuta*	B	+
213	145389		*Callicarpa havilandii*	B	+
214	145389			L	±
215	145374		*Callicarpa longifolia*	L	+
216	106701		*Callicarpa stapfii*	B	2+
217	106701			L	2+
218	143372		*Sphenodesme triflora*	B	−
219	143372			L	±
220	145386		*Stachytarpeta jamaicencis*	S	−
221	43322		*Vitex pubescens*	L	±
222	145370	Vitaceae	*Leea indica*	B	2+
223	145370			L	±
224	145379	Zingerberaceae	*Alpinia fraseriana*	S	±

Extracts were dissolved in 50% DMSO to give a concentration of 2 mg/ml for testing; 25-µl aliquots tested.
[a]B, bark; F, fruit; L, leaf; R, root; S, stem; T, twigs.
[b]4+, inhibition of 81–100%; 3+, inhibition of 61–80%; 2+, inhibition of 41–60%; +, inhibition of 21–40%; ±, inhibition of 1–20%; −, no inhibition.

bradycardia and Alzheimer disease, whereas selective M3 antagonists affect smooth muscles (chronic obstructive airway disease, irritable bowel syndrome, and incontinence) (Eglen, 1998). To reduce side effects and to improve therapeutic activities, selective muscarinic receptor antagonists have been developed for antiulcer activity (telenzepine; M1/M4 selective; Byk), bradycardia (otenzepad; M2/M4 selective; Boehringer Ingelheim), irritable bowel syndrome (darifenacin; M3/M1 selective; Pfizer), and antibronchospastic activity (rispenzepine; M3/M1 selective; Dompe) (Eglen, 1998).

As part of an ongoing screening program for potential receptor agonists and antagonists Malaysian plants that showed significant muscarinic receptor competitive binding activity were identified. The active crude extracts are subjected to further bioassay-guided isolation of active constituents. Upon successful isolation, the active constituents will be evaluated for their muscarinic receptor subtype selectivity.

Materials and Methods

Chemicals and reagents

[^{3}H]*N*-Methylscopolamine was supplied by Amersham Pharmacia Biotech UK Ltd. All other reagents were of analytical grade and obtained from standard commercial sources (Little Chalfont, Buckinghamshire, UK).

Plant materials

Plant samples were collected from the Forest Research Institute Malaysia, Kuala Lumpur, Malaysia (voucher no.: 5-digits series), and the state of Sabah, Malaysia (voucher no.: 6-digits series). The voucher specimens were kept at the herbaria of Forest Research Institute Malaysia (5-digits series) and Forest Research Center, Sepilok, Sandakan, Malaysia (6-digits series). Different parts of the plants were dried separately at 40°C. The dried materials (100–200 g) were powdered and soaked with sufficient methanol in conical flasks for 7 days with sonication (5 × sufficient volume of methanol to cover the plant materials). Methanol extracts were collected and filtered at 48, 96, and 168 h, and the conical flasks with plant materials were replaced with fresh methanol to continue extraction. The pooled extracts were evaporated at 50°C *in vacuo* and the residues freeze-dried and kept in sample bottles at −20°C until use.

Crude extract dilution

DMSO (1 ml) was added to 4 mg of crude plant extracts and vortexed vigorously giving an initial concentration

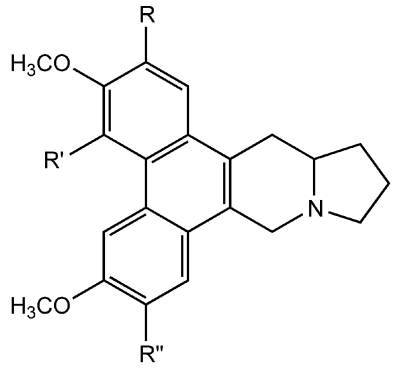
Table 2. Percent inhibition of extracts (10 μg/well) on specific binding to muscarinic receptor.

No.	Voucher no.	Family	Species	Part[a]	Inhibition[b]
1	43304	Anacardiaceae	*Bouea oppositifolia*	L	±
2	143506	Annonaceae	*Neouvaria acuminatiisima*	B	+
3	143511		*Polyalthia insignis*	B	±
4	143502		*Polyalthia longipes*	B	±
5	145376		*Polyalthia microtus*	B	+
6	143524		*Polyalthia rumphii*	L	+
7	145365		*Popowia odoardoi*	S	+
8	43125	Apocynaceae	*Dyera costulata*	L	+
9	43123		*Mesua ferra*	L	±
10	43127	Burseraceae	*Santiria laevigata*	L	±
11	43124	Dipterocarpaceae	*Hopea dryobalanoides*	L	+
12	43112		*Neobalanocarpus heimii*	L	+
13	145368	Lauraceae	*Litsea garciae*	B	±
14	43104	Meliaceae	*Sandoricum koetjape*	T	±
15	43104			L	±
16	143518	Moraceae	*Ficus septica*	L	3+
17	43111	Olaceae	*Ochanostachys amentacea*	L	+
18	43325	Sapindaceae	*Amesiodendron chinense*	S	±
19	43110		*Pometia pinnata*	L	+
20	43110			T	±
21	106701	Verbenaceae	*Callicarpa stapfii*	B	+

Extracts were dissolved in 50% DMSO to give a concentration of 0.4 mg/ml for testing; 25-μl aliquots tested.

[a]B, bark; F, fruit; L, leaf; R, root; S, stem; T, twigs.

[b]4+, inhibition of 81–100%; 3+, inhibition of 61–80%; 2+, inhibition of 41–60%; +, inhibition of 21–40%; ±, inhibition of 1–20%; −, no inhibition.

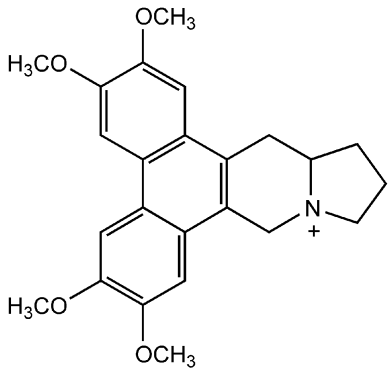


R=H; R'=H; R"=OCH_3 (+)-Isotylocrebrine

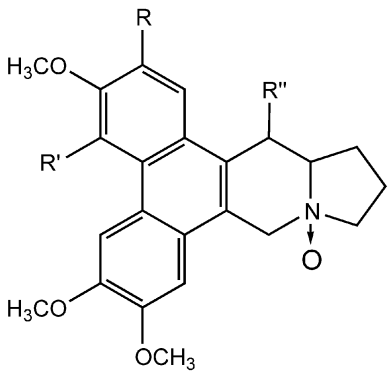
R=OCH_3; R'=H; R"=H (+)-Antofine

R=OCH_3; R'=OCH_3; R"=H (+)-Tylocrebrine

R=OCH_3; R'=H; R"=OCH_3 (+)-Tylophorine



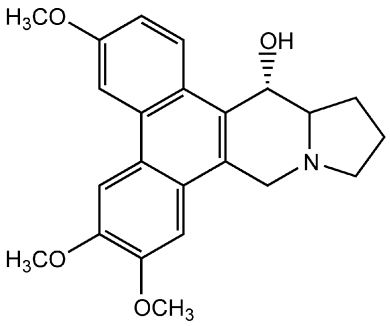
Dehydrotyloporine



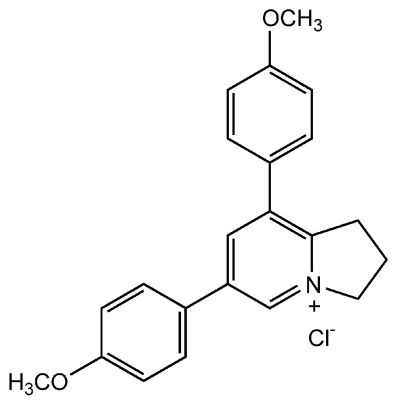
R=OCH_3; R'=H; R"=H (+)-Tylophorine N-oxide

R=OCH_3; R'=OCH_3; R"=OH Ficuseptine-A

R=H; R'=OCH_3; R"=OH 14α-Hydroxyisotylocrebrine



O-Methyltylophorinidine



Ficuseptine

Figure 1. Some alkaloids isolated from *Ficus* species (Baumgartner et al., 1990; Buckingham, 1994; Peraza-Sanchez et al., 2002; Wu et al., 2002).

of 4 mg/ml. The extracts were tested at 50 and 10 μg/assay points.

Membrane preparation

Total rat brain (minus cerebellum) membrane was prepared according to the protocol described by Gattu et al. (1995) with minor modifications. Male Sprague-Dawley rats (250–300 g) were decapitated and the brains removed. The cerebellum was dissected out and the rest of the brain finely chopped with scissors, homogenized in 10 volumes of ice-cold 50 mM Tris-HCl, pH 7.4, buffer using Ultra-Turax (2×10 s) and followed by glass-Teflon pestle homogenization at 800 rpm for 20 strokes.

The homogenate was centrifuged at $40{,}000 \times g$ using Beckman type 28 rotor at 4°C for 15 min. The pellet was retained and washed twice by resuspending in ice-cold 50 mM Tris-HCl, pH 7.4, buffer (centrifuged at $40{,}000 \times g$ for 15 min). The final pellet was suspended in 5 ml ice-cold 50 mM Tris-HCl, pH 7.4, buffer, aliquoted and kept at −80°C until use. Protein was determined using the Sigma Total Protein Reagent using

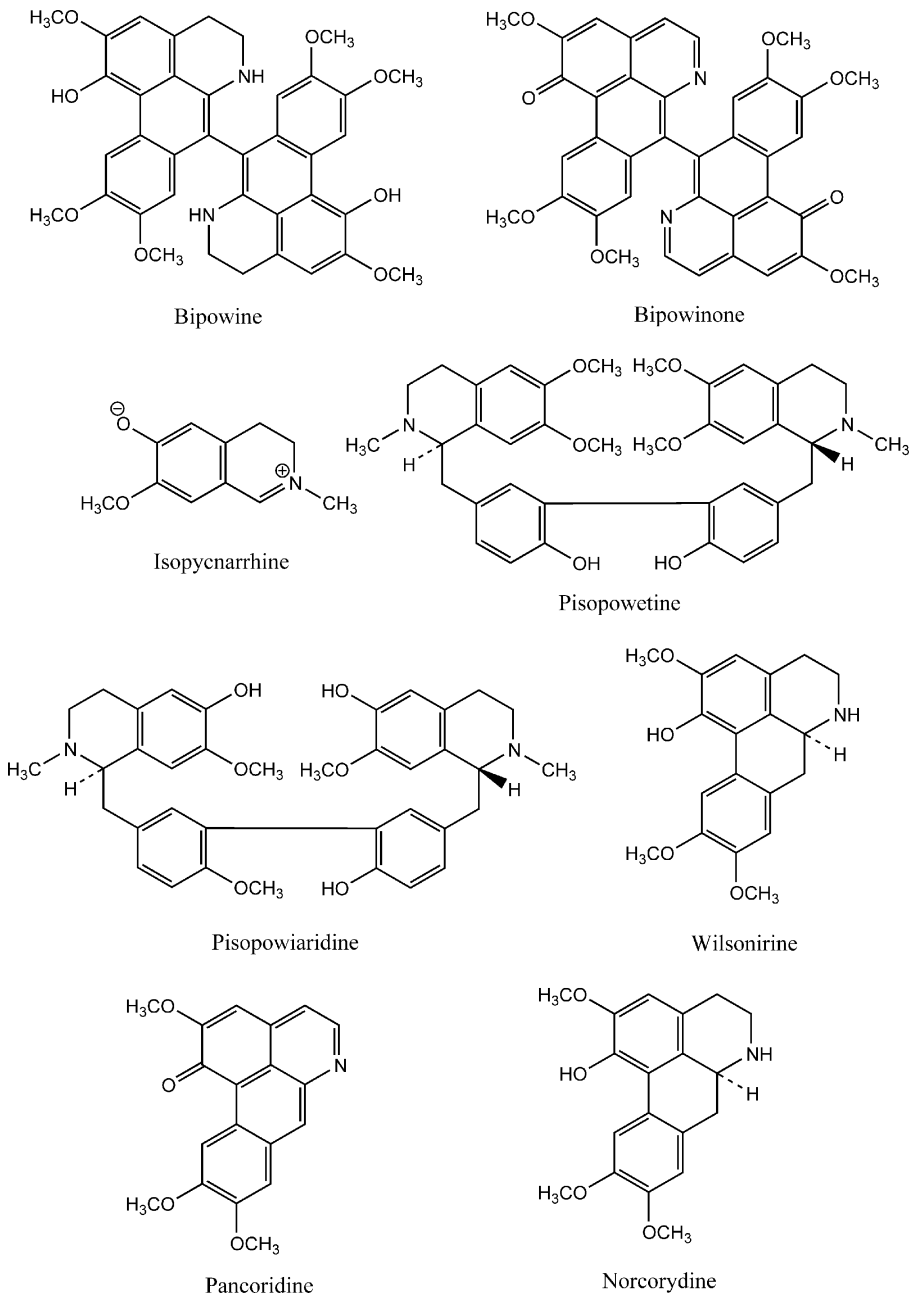


Figure 2. Some alkaloids isolated from *Popowia* species (Jossang et al., 1986).

bovine serum albumin as the standard, and the protein content corresponded to about 20 mg protein/ml.

Muscarinic receptor binding assay

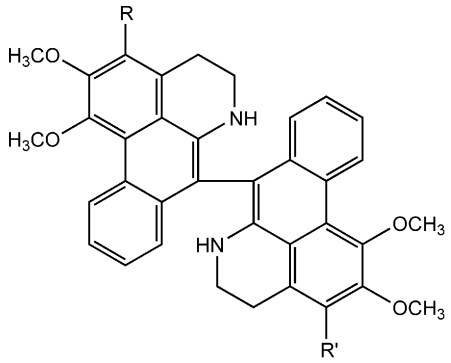
The muscarinic receptor binding was assayed by a modification of the method of Bockman et al. (2001). Briefly, the membranes were thawed on ice and diluted to 0.75 mg/ml protein using 50 mM Tris-HCl, pH 7.4, buffer. The reference ligand (atropine) and radioligand ([^{3}H]*N*-methylscopolamine) were diluted to 100 µM and 5 nM final concentration using 50% DMSO in deionized water and binding buffer, respectively.

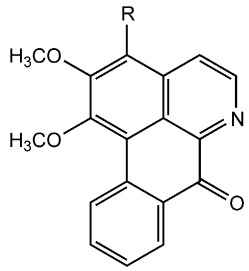
With its cover removed, the Multiscreen plate (GF-B, Millipore Billerica, MA, USA) was placed on vacuum manifold and the filter of each well prewetted with 200 µl of 50 mM Tris-HCl, pH 7.4, buffer. Radioligand (25 µl) was added to each well of the Multiscreen plate, followed by addition of 25 µl of 50% DMSO (total binding, 3 wells), reference compound (nonspecific binding, 3 wells), or crude extracts to the corresponding well in the plate. The reaction was initiated by adding 200 µl of diluted membranes to each well. The plate was then covered, vortexed gently, and incubated at 22°C for 90 min.

The reaction mixture was filtered on the vacuum manifold and washed four-times with 200 µl of ice-cold 50 mM Tris-HCl, pH 7.4. The plate was cleaned with tissues to remove excess buffer and air-dried. The filters were punched out and transferred into 5-ml scintillation vials and 4 ml of scintillation cocktail added. The vials were capped, the content shaken for a few minutes, and radioactivity counted for 3 min per vial, using a scintillation counter (Packard, Meriden, CT, USA).

Data analysis

The percentage inhibitory specific binding in the presence of the test compounds was calculated using a standard





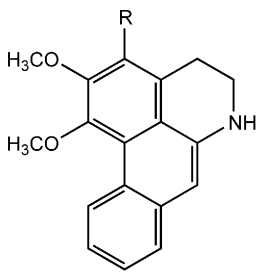
R=H Lysicamine

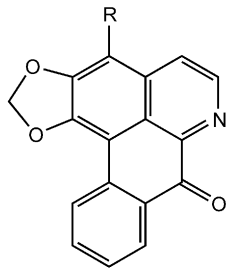
R=OCH$_3$ O-Methylmoschatoline

R=OCH$_3$; R'=OCH$_3$ 7,7'-bisdehydro-O-methylisopiline

R=H; R'=OCH$_3$ 7-dehydronornuciferinyl-7'-dehydro-O-methylisopiline

R=H; R'=H Urabaine





R=OCH$_3$ Dehydro-O-methylisopiline

R=H Dehydronornuciferine

R=H Iiriodenine

R=OCH$_3$ Atherospermidine

Figure 3. Some alkaloids isolated from *Polyalthia* species (Jossang et al., 1977; Marsaioli et al., 1977; Connolly et al., 1996; Said et al., 2003).

data reduction algorithm, and it is as follows:

$$\frac{[T - NSP] - [B - NSP]}{[T - NSP]} \times 100$$

where B = binding in the presence of test extract, NSP = nonspecific binding in the presence of excess inhibitor (reference ligand), and T = total binding.

Results and Discussion

The crude extracts were initially screened at 100 μg/assay point and above, and under this condition, some of the extracts were not soluble in the assay buffers, which prevented proper filtration of the plates. Consequently, the extracts were screened at 50 μg/assay point (Table 1), and extracts that showed inhibition at and above 61% were retested at 10 μg/assay point. Under this condition, the samples remained soluble, and proper filtration was achieved. The extracts were screened in triplicate and the percentage inhibition averaged (Table 2).

Of these 224 extracts tested at 50 μg/assay point, 42 (19%) exhibited 41–60% inhibition and 12 (5%) exhibited 61% or higher inhibition. To reduce false-positive results, extracts exhibiting above 61% inhibition and comparators were then tested at 10 μg/well. Of these, only *Ficus septica* gave more than 60% inhibition ($65.85 \pm 3.75\%$). Other extracts such as *Polyalthia microtus* (Annonaceae) and *Popowia odoardoi* (Annonaceae) showed $32.63 \pm 1.38\%$ and $35.79 \pm 7.11\%$ inhibition, respectively.

Our preliminary chemical studies and the literature indicate these plants, *Ficus septica* Burm. f., *Polyalthia microtus* Miq., and *Popowia odoardoi* Diels, and related species contain alkaloidal compounds, and some of the reported structures are as shown in Figures 1, 2, and 3. It is conceivable that the active constituents, which inhibited binding of the radioligand, [^{3}H]*N*-methylscopolamine to muscarinic receptors, are probably alkaloids, as known muscarinic receptor agonists and antagonists possess nitrogen moieties. We have, therefore, selected *Ficus septica* Burm. f., *Polyalthia microtus* Miq., and *Popowia odoardoi* Diels for bioassay-guided fractionation to identify the muscarinic active constituents and to ascertain their activities.

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