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ScienceDirect
Phytochemistry 69 (2008) 1548–1554
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Muscarinic receptor binding activity of polyoxygenated flavones from *Melicope subunifoliolata*

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Received 26 October 2007; received in revised form 30 January 2008
Available online 10 March 2008

Abstract

The bark extract of *Melicope subunifoliolata* (Stapf) T.G. Hartley showed competitive muscarinic receptor binding activity. Six polymethoxyflavones [melibentin **(1)**; melisimplexin **(3)**; 3,3′,4′,5,7-pentamethoxyflavone **(4)**; meliternatin **(5)**; 3,5,8-trimethoxy-3′,4′,6,7-bismethylenedioxyflavone **(6)**; and isokanugin **(7)**] and one furanocoumarin [5-methoxy-8-geranyloxypsoralen **(2)**] were isolated from the bark extract. Compounds **2** and **6** were isolated for the first time from *M. subunifoliolata*. The methoxyflavones (compounds **1**, **3**, **4**, **5**, **6**, and **7**) show moderate inhibition in a muscarinic receptor binding assay, while the furanocoumarin (compound **2**) is inactive. The potency of the methoxyflavones to inhibit [^{3}H]NMS-muscarinic receptor binding is influenced by the position and number of methoxy substitution. The results suggest these compounds are probably muscarinic modulators, agonists or partial agonists/antagonists.

Keywords: *Melicope subunifoliolata*; Rutaceae; Flavonoid; Muscarinic receptor

1. Introduction

Compounds that interact with muscarinic receptors, such as atropine and scopolamine are among the oldest known anticholinergic drugs originally derived from the plant *Atropa belladonna* L. (Solanaceae) and *Datura* species (Solanaceae) (Brown and Taylor, 1996) respectively. The main naturally occurring alkaloid of *A. belladonna* is reported to be (−)-hyoscyamine, which forms the racemate (±)-hyoscyamine (atropine) upon heating or extraction. Both (−)-hyoscyamine and scopolamine (hyoscine) occur in *A. belladonna* and in *Datura* species, but occur at different proportions (British Pharmacopoeia Commission, 2006; Evans, 2002). However, these have limited therapeutic use due to numerous side effects such as dry mouth, impaired sweating, mydriasis, and tachycardia, as well as profound CNS dysfunction with delirium and hallucinations (Brown and Taylor, 1996). This is mainly caused by their non-selectivity towards the different muscarinic receptor subtypes designated as M1–M5. Furthermore, different muscarinic receptor subtypes have different tissue distribution (Broadley and Kelly, 2001; Ishii and Kurachi, 2006).

It is difficult to prepare ligands (agonists or antagonists) with complete selectivity to distinguish between the different muscarinic receptor subtypes. However, the approach of searching for partial agonists and antagonists, and exploring the subtype distribution in tissues has been more successful in producing useful therapeutic compounds (Broadley and Kelly, 2001; Tumiatti et al., 2007). For example, more selective agonists and antagonists for muscarinic receptor subtypes (M1–M5) with reduced side effects and improved therapeutic activities have been introduced. These include selective muscarinic receptor antagonists developed for antiulcer activity (Telenzepine; M1/M4

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0031-9422/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2008.01.024

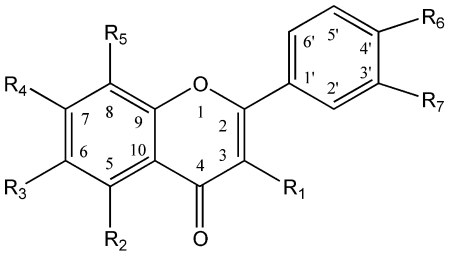
selective; Byk), bradycardia (Otenzepad; M2/M4 selective; Boehringer Ingelheim), irritable bowel syndrome (Darifenacin; M3/M1 selective; Pfizer), and antibronchospastic activity (Rispenzepine; M3/M1 selective; Dompe) (Broadley and Kelly, 2001; Eglen, 1998). Selective M1 agonists have also been shown to be potential disease-modifying agents in Alzheimer's disease (Eglen, 1998; Mutschler et al., 1995). This is mediated by the stimulation of the postsynaptic M1 receptors involved in memory and inhibition of amyloid deposition implicated in the pathogenesis of Alzheimer's disease (Broadley and Kelly, 2001). Alternatively, antagonists of central presynaptic M2 receptors improve cognition in Alzheimer's patients by increasing the central release of acetylcholine (Lai et al., 2001; Teaktong et al., 2005). Both approaches require high selectivity for one muscarinic receptor subtype for efficacy and to avoid cholinergic side effects (Clader and Wang, 2005).

Naturally occurring indole alkaloids such as strychnine, brucine and alcuronium bind to the five muscarinic receptor subtypes at the allosteric sites (Ellis, 1997). Other allosteric modulators include gallamine, staurosporine and indolocarbazoles (Birdsall et al., 1997; Lazareno et al., 2000). The binding of a ligand at these allosteric sites induces a conformational change in the receptor, to either increase (positive cooperativity) or decrease (negative cooperativity) the binding of other ligands at the orthosteric site (Espinoza-Fonseca and Trujillo-Ferrara, 2005). For example, the binding of brucine to the allosteric site increases the affinity of the natural ligand aceylcholine by 2-fold at the orthosteric site of M1 receptor (Dolezal and Tucek, 1998). This is potentially useful in conditions such as Alzheimer's disease where the release of acetylcholine is reduced (Wess, 2005).

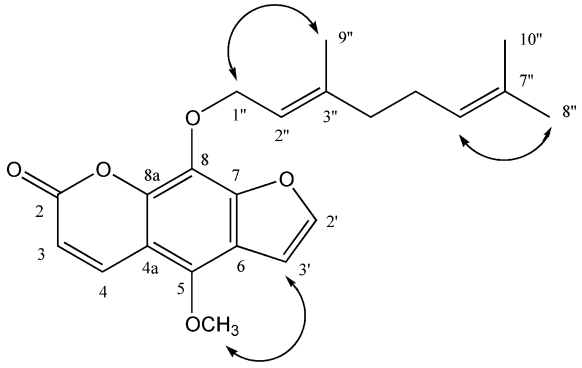
In the studies to discover plant extracts with G-protein coupled receptor activities, more than 200 Malaysian plants were screened based on availability and species related to those with ethno-botanical uses for medicine (Chung et al., 2005a, 2005b, 2006). The crude methanolic extract from the bark of *Melicope subunifoliolata* (Stapf) T.G. Hartley (Rutaceae) showed pronounced competitive muscarinic receptor binding activity. We now report the isolation and structural elucidation of six flavonoids and one furanocoumarin from this extract, and their muscarinic receptor binding activities.

2. Results and discussion

The methanolic extract of *M. subunifoliolata* showing muscarinic receptor binding activity was separated into various fractions by liquid partitioning and column chromatography. The fractions with muscarinic receptor binding activities were separated further to obtain pure compounds. The compounds characterized by NMR and MS were six polymethoxyflavones [3,5,6,7,8-pentamethoxy-3′,4′-methylenedioxyflavone (**1**) (Fauvel et al., 1981; Jong and Wu, 1989; Ritchie et al., 1965); 3,5,6,7-tetramethoxy-3′,4′-methylenedioxyflavone (**3**) (Jong and Wu, 1989; Ritchie et al., 1965); 3,3′,4′,5,7-pentamethoxyflavone (**4**) (Chen et al., 1986; de la Torre et al., 2004; Dong et al., 1999; Herunsalee et al., 1987); 3,5-dimethoxy-3′,4′,6,7-bismethylenedioxyflavone (**5**) (Fauvel et al., 1981; Higa et al., 1987; Jong and Wu, 1989; Ritchie et al., 1965); 3,5,8-trimethoxy-3′,4′,6,7-bismethylenedioxyflavone (**6**) (Fauvel et al., 1981; Higa et al., 1987; Jong and Wu, 1989); and 3,5,7-trimethoxy-3′,4′-methylenedioxyflavone (**7**) (Higa et al., 1987; Ho et al., 2003)] and one furanocoumarin [5-methoxy-8-geranyloxypsoralen (**2**) (Franke et al., 2001; Sokolova et al., 1976)]. Compounds **2** and **6** were isolated for the first time from *M. subunifoliolata*.



(**1**) R_1=OCH_3, R_2=OCH_3, R_3=OCH_3, R_4=OCH_3, R_5=OCH_3, R_6, R_7=-OCH_2O-
(**3**) R_1=OCH_3, R_2=OCH_3, R_3=OCH_3, R_4=OCH_3, R_5=H, R_6, R_7=-OCH_2O-
(**4**) R_1=OCH_3, R_2=OCH_3, R_3=H, R_4=OCH_3, R_5=H, R_6=OCH_3, C_7=OCH_3
(**5**) R_1=OCH_3, R_2=OCH_3, R_3, R_4=-OCH_2O-, R_5=H, R_6, R_7=-OCH_2O-
(**6**) R_1=OCH_3, R_2=OCH_3, R_3, R_4=-OCH_2O-, R_5=OCH_3, R_6, R_7=-OCH_2O-
(**7**) R_1=OCH_3, R_2=OCH_3, R_3=H, R_4=OCH_3, R_5=H, R_6, R_7=-OCH_2O-



(**2**)

The arrows in (**2**) show the NOE correlations.

5-Methoxy-8-geranyloxypsoralen (**2**) was obtained as greenish crystals, with the melting point of 53.9 °C, which was similar to the reported value of 53–54 °C (Sokolova et al., 1976). The molecular ion *m/z* 369 ($[M+H]^+$, $C_{22}H_{24}O_5$+H) obtained by ESI-MS was similar to that reported in the literature (Franke et al., 2001), while EI-MS gave *m/z* 232 (100), 217 (83), 204 (44), 189 (54), 161 (34), 105 (12). The ^{1}H NMR and ^{13}C NMR values are shown in Table 1 and are consistent with the reported values (Franke et al., 2001; Sokolova et al., 1976).

The ^{1}H NMR, ^{13}C NMR data and the MS fragmentation pattern of **2** narrowed the possibility of compound **2**

Table 1
NMR data for 5-methoxy-8-geranyloxypsoralen (**2**) in $CDCl_3$

Position	δC (ppm)	δH (ppm) (*J* in Hz)	HMBC
2	160.84	–	H-3,H-4
3	113.04	6.27 *d* (*J* = 9.9)	–
4	139.71	8.11 *d* (*J* = 9.9)	–
4a	107.78	–	H-3,H-4
5	144.67[a]	–	5-OMe,H-4
6	114.73	–	H-3,H-2′,H3′
7	151.18	–	H-2′,H-3′
8	126.99	–	H-3,H-4,H-2′,H-3′,H-1″
8a	144.70[a]	–	H-4
2′	145.34	7.61 *d* (*J* = 2.4)	H-3′
3′	105.36	6.98 *d* (*J* = 2.4)	H-2′,5-OMe
OMe-5	61.02	4.16 *s*	–
1″	70.53	4.87 *d* (*J* = 7.2)	H-2″
2″	119.70	5.58 *t* (*J* = 7.1)	H-1″,H-4″,H-9″
3″	143.41	–	H-1″,H-4″,H-5″,H-9″
4″	39.85	1.99 *br*	H-1″,H-2″,H-6″,H-9″
5″	26.63	1.99 *br*	H-2″,H-4″,H-6″
6″	124.07	5.01 *br s*	H-4″,H-5″,H-8″,H-10″
7″	131.98	–	H-5″,H-8″,H-10″
Me-8″	25.93	1.64 *s*	H-6″,H-10″
Me-9″	16.77	1.66 *s*	H-2″,H-4″,H-5″
Me-10″	17.92	1.56 *s*	H-6″,H-8″

[a] Values with same superscript are interchangeable.

to either 5-methoxy-8-geranyloxypsoralen or 8-methoxy-5-geranyloxypsoralen. Based on NOE difference, HMBC (Table 1), and COSY long-range experiments, the structure of **2** was determined to be 5-methoxy-8-geranyloxypsoralen. Especially important was the observed HMBC correlation of OMe-protons with C-5 and NOE correlation of 5-OMe with H-3′.

The six flavones exhibit the following order of potency to inhibit [^{3}H]N-methyl scopolamine ([^{3}H]NMS)-muscarinic receptor binding assay: **5** = **6** > **3** > **7** > **4** > **1** (One-way analysis of variance; Tukey's *post hoc* comparisons, $P < 0.05$) (Table 2). Table 3 shows the comparison of the position and number of methoxy substitution on the flavones together with the K_i values obtained for each flavone in the order of decreasing activity. Generally, all the flavones that bind to the muscarinic receptor possessed 3-, 5-methoxy groups and 2,3-double bond in conjugation with 4-oxo functions. Methoxy substitution at positions C-3′, C-4′, C-6, C-7, and C-8 caused a decrease in inhibition potency (an increase of K_i value). By referring to compounds **6** and **1**, with the presence of methoxy group at C-8, substitution of the methoxy group at positions C-6 and C-7 causes an increase of 24-fold in K_i value compared to methylenedioxy substitution. When position C-8 is not substituted by the methoxy group (compounds **5** and **3**), methoxy substitution at positions C-6 and C-7 causes a 3-fold increase in K_i value compared to the methylenedioxy substitution.

All the compounds were tested with competitive muscarinic receptor binding assay and the methoxyflavones showed competitive muscarinic receptor binding at high concentrations with [^{3}H]NMS as the radioligand, except compound **2** (5-methoxy-8-geranyloxypsoralen) was inactive (Table 2). Using CS Chem3D (Version 6.0, CambridgeSoft Corporation, Cambridge, MA, USA) to model low energy conformations with MM2, the ring systems of polymethoxyflavones (**1**, **3**, **4**, **5**, **6** and **7**) assumed similar near-planar structures and were superimposable; however, 5-methoxy-8-geranyloxypsoralen (**2**) had a branched structure, with the side chain perpendicular to the planar ring system (not shown). This difference probably did not allow 5-methoxy-8-geranyloxypsoralen (**2**) to interact with the receptor site occupied by [^{3}H]NMS, and hence, accounts for its lack of muscarinic receptor binding activity.

For comparison, IC_{50} and K_i values are shown for other muscarinic antagonists such as atropine, scopolamine and dicyclomine, and other standard compounds (Table 4). The K_i values of the polymethoxyflavones that show muscarinic receptor binding are at about 10^{-6} M, which are higher than that of atropine, scopolamine or dicyclomine (Student's *t* test; $P < 0.05$). This suggests that these polymethoxyflavones bind to the muscarinic receptor at high

Table 2
IC_{50} and K_i values for compounds isolated from *Melicope subunifoliolata* on muscarinic receptor binding activity

Compound	IC_{50} (M)	K_i (μM)
3,5,6,7,8-Pentamethoxy-3′,4′-methylenedioxyflavone (**1**)	$7.24 \pm 0.15 \times 10^{-4}$	76.5
5-Methoxy-8-geranyloxypsoralen (**2**)	$1.86 \pm 0.07 \times 10^{-2}$	NE
3,5,6,7-Tetramethoxy-3′,4′-methylenedioxyflavone (**3**)	$7.41 \pm 0.07 \times 10^{-5}$	7.82
3,3′,4′,5,7-Pentamethoxyflavone (**4**)	$4.79 \pm 0.13 \times 10^{-4}$	50.5
3,5-Dimethoxy-3′,4′,6,7-bismethylenedioxyflavone (**5**)	$2.29 \pm 0.20 \times 10^{-5}$	2.42
3,5,8-Trimethoxy-3′,4′,6,7-bismethylenedioxyflavone (**6**)	$3.02 \pm 0.14 \times 10^{-5}$	3.19
3,5,7-Trimethoxy-3′,4′-methylenedioxyflavone (**7**)	$1.62 \pm 0.06 \times 10^{-4}$	17.0

Inhibition experiments were performed in triplicates by incubating 200 μl of rats brain membrane homogenate (36 μg protein/well) with 25 μl of [^{3}H]NMS (0.5 nM) in the presence of each compound (11 concentrations for each); the values shown for IC_{50} were the mean ($n = 3$) ± SEM; K_i values were calculated using Cheng–Prusoff equation; NE = could not be estimated.

Table 3
Comparison of methoxy and methylenedioxy substitutions for flavones isolated from *Melicope subunifoliolata* on K_i in muscarinic receptor binding activity

Compound	Methoxy substitution	Methylenedioxy substitution	K_i (μM)
5	C3, C5	C3′,4′; C6,7	2.42
6	C3, C5, C8	C3′,4′; C6,7	3.19
3	C3, C5, C6, C7	C3′,4′	7.82
7	C3, C5, C7	C3′,4′	17.0
4	C3, C3′, C4′, C5, C7	–	50.5
1	C3, C5, C6, C7, C8	C3′,4′	76.5

1 = 3,5,6,7,8-pentamethoxy-3′,4′-methylenedioxyflavone; **3** = 3,5,6,7-tetramethoxy-3′,4′-methylenedioxyflavone; **4** = 3,3′,4′,5,7-pentamethoxyflavone; **5** = 3,5-dimethoxy-3′,4′,6,7-bismethylenedioxyflavone; **6** = 3,5,8-trimethoxy-3′,4′,6,7-bismethylenedioxyflavone; **7** = 3,5,7-trimethoxy-3′,4′-methylenedioxyflavone.

Table 4
K_i and IC_{50} values obtained from the competition experiments with standard unlabelled ligands on muscarinic receptor binding activity

Unlabelled ligand	Pharmacological action	IC_{50} (M)	K_i (M)
Atropine	Muscarinic receptor antagonist	$2.83 \pm 0.19 \times 10^{-9}$	2.99×10^{-10}
Scopolamine	Muscarinic receptor antagonist	$4.88 \pm 0.22 \times 10^{-10}$	5.15×10^{-11}
Dicyclomine	Muscarinic receptor antagonist	$1.50 \pm 0.17 \times 10^{-8}$	1.59×10^{-9}
(±)-8-OH-DPAT	5-HT_{1A} receptor agonist	$3.91 \pm 0.46 \times 10^{-5}$	4.12×10^{-6}
Serotonin	5-HT receptor agonist	NE	NE
Propanolol	β-adrenergic receptor antagonist	NE	NE

The values shown for IC_{50} were the mean ($n = 3$) ± SEM; NE = could not be estimated (10^{-4} or higher); (±)-8-OH-DPAT = (±)-8-hydroxy-2-(di-*n*-propylamino)-tetralin.

concentrations, and they are only moderately active compared to muscarinic receptor antagonists such as atropine (K_i, 2.99×10^{10} M), scopolamine (K_i, 5.15×10^{11} M) and dicyclomine (K_i, 1.59×10^{-9} M). However, the K_i values are similar to muscarinic agonists such as milameline and carbachol as reported by Schwarz et al. (1999) and Barrett et al. (1995). Furthermore, their values are also similar to that of (±)-8-hydroxy-2-(di-*n*-propylamino)-tetralin ((±)-8-OH-DPAT) (K_i, 4.12×10^{-6} M) (Table 4), a 5-HT_{1A} receptor agonist, which has been reported to act as an antagonist at muscarinic receptors and influences muscarinic receptor function in the rabbit iris-ciliary body (Chidlow and Osborne, 1997).

Muscarinic ligands that bind to the primary or allosteric sites are usually assumed to contain basic nitrogen groups, and surrogates for the acetate group of acetylcholine and/or aromatic groups. However, the structures of these ligands are highly diverse, and they have been reviewed else where (Broadley and Kelly, 2001; Clader and Wang, 2005; Ishii and Kurachi, 2006; Widzowski et al., 1997). The structures of the methoxyflavones tested here differ from these known ligands for muscarinic receptors, for example, they clearly lack the nitrogen moiety. The methylenedioxyphenyl groups of the methoxyflavones, however, resemble part of the structures of darifenacin (M3 antagonist) and zamifenacin (M3 antagonist) (Broadley and Kelly, 2001). Although methoxyflavones lack nitrogen, a few uncharged competitive antagonists, namely, "carbo" analogues, have been described previously (Barlow and Tubby, 1974; Waelbroeck et al., 1996). Furthermore, it has been reported recently that the presence of nitrogen is not essential for the allosteric actions of the steroidal analogues of WIN 51,708 and WIN 62,577 (Lazareno et al., 2002). The allosteric modulator, KT5720 has been proposed to interact with M1 receptors through van der Waals, hydrophobic and π-interactions using computation modeling (Espinoza-Fonseca and Trujillo-Ferrara, 2006). Therefore, it is conceivable that the non-nitrogen containing polymethoxyflavones described here may act as allosteric modulators, agonists or partial agonists/antagonists with potential therapeutic uses.

3. Experimental

3.1. General experimental procedures

^1H and ^{13}C NMR spectra were obtained on a 500 MHz Varian INOVA spectrometer (Palo Alto, CA, USA). COSY, HMBC, HMQC, and NOESY NMR experiments were also conducted. $CDCl_3$ was used as the solvent and TMS as internal reference. MS data were recorded on a Finnigan MAT LCQ mass spectrometer (San Jose, CA, USA). Melting points were determined on a Stuart Scientific SMP3 apparatus (Bridgeville, PA, USA).

3.2. Plant material

The barks of *M. subunifoliolata* were collected in 2001, Sabah, Malaysia. Voucher specimens (SAN143369) were deposited at the herbarium of the Forest Research Centre, Sepilok, Sandakan, Sabah, Malaysia.

3.3. Extraction, fractionation and purification

The air-dried barks (200 g) were powdered and extracted exhaustively with MeOH and after the removal of the solvent *in vacuo* yielded 33 g of crude extract. A portion of the MeOH extract (15 g) was dissolved in MeOH/water (1:9) and partitioned with hexane and then EtOAc. The fractions were then dried *in vacuo* to give hexane (3.0 g), ethyl acetate (8.0 g), and MeOH/water (1.9 g) extracts. A brown precipitate was formed during the partitioning of MeOH/water fraction with EtOAc. After the removal of the EtOAc layer, the brown precipitate was separated from the MeOH/water fraction by filtration and dried to give a brown residue (1.2 g).

The oily hexane extract showed inhibition on [^{3}H]N-methyl scopolamine ([^{3}H]NMS)-muscarinic receptor binding (50 μg/well: 67.30 ± 0.71% inhibition). Trituration of the oil with methanol yielded crystals of compound **1** (17 mg).

The EtOAc extract showed inhibition on [^{3}H]NMS-muscarinic receptor binding (50 μg/well: 92.70 ± 2.19% inhibition; 10 μg/well: 80.47 ± 2.35% inhibition). The EtOAc extract (0.67 g) was fractionated on a silica gel chromatography column (silica gel 60, mesh 0.040–0.063 mm, Merck, Darmstadt, Germany) using a CH_2Cl_2/MeOH gradient system (100% CH_2Cl_2 to 9:1), and 11 fractions (F-1 to F-11) were collected and combined according to TLC monitoring. Fraction F-4 (65 mg) showed 41.99 ± 1.34% inhibition (50 μg/well) on [^{3}H]NMS-muscarinic receptor binding. A quantity of 20 mg of fraction F-4 was further separated by prep. TLC on silica gel 60 F^{254} (0.5 mm thickness, glass plate, Merck, Darmstadt, Germany) using

CH_2Cl_2/MeOH (99:1; 2 × development) to give compound **2** (R_f 0.67; 14 mg) as the major compound. Fraction F-6 (348 mg) showed the highest inhibition on [^{3}H]NMS muscarinic receptor binding (50 μg/well: 87.01 ± 0.66% inhibition). A quantity of 100 mg of fraction F-6, by column chromatography (silica gel 60, mesh 0.040–0.063 mm, Merck), using CH_2Cl_2/MeOH gradient system (100% CH_2Cl_2 to 96:4), gave eight fractions (F-12 to F-19). Fraction F-16 (25.9 mg) was separated by prep. TLC on silica gel 60 F^{254} (0.5 mm thickness, glass plate, Merck) using CH_2Cl_2/MeOH (98:2; 3 × development), to give four fractions (A–D). Compound **1** (R_f 0.72; 8 mg) was isolated from fraction A. Recrystallization of fraction B (R_f 0.68; 18 mg) with EtOH/Me_2CO yielded compound **3** (11.7 mg). Fraction F-7 (111 mg) showed 78.11 ± 0.66% inhibition (50 μg/well) on [^{3}H]NMS-muscarinic receptor binding, and on recrystallization from MeOH gave compound **4** (30.7 mg).

The brown residue obtained from the precipitate formed during the partitioning of the MeOH/water fraction of the crude extract with EtOAc showed 77.49 ± 1.14% inhibition (50 μg/well) on [^{3}H]NMS muscarinic receptor binding. Recrystallization of this brown residue (105 mg) using MeOH/$CHCl_3$ yielded 35 mg of compound **5**. The brown residue (0.6 g) was also subjected to column chromatography on silica gel (silica gel 60, mesh 0.040–0.063 mm, Merck) using a CH_2Cl_2/MeOH gradient (99:1–96:4) to give 10 fractions (F-20 to F-29). Fraction F-25 (151 mg) was recrystallized with hexane/CH_2Cl_2 to give compound **5** (51 mg). The residue after recrystallization showed 83.47 ± 0.76% inhibition (50 μg/well) and 83.15 ± 0.96% inhibition (10 μg/well) on [^{3}H]NMS muscarinic receptor binding. The residue (72 mg) was further separated by prep. TLC on silica gel 60 F^{254} (0.5 mm thickness, glass plate, Merck) using CH_2Cl_2/MeOH (98:2; 2 × development), which resulted in the isolation of **4** (R_f 0.18; 3.1 mg), **5** (R_f 0.33; 54.9 mg), **6** (R_f 0.38; 2.8 mg), and **7** (R_f 0.24; 2.0 mg), respectively.

3.4. 96-well microplate muscarinic receptor binding assay

Crude extracts and partitioned fractions were tested at 50 μg/well and 10 μg/well. Combined fractions collected from column chromatography were further tested at 1 μg/well. Seven compounds isolated from *M. subunifoliolata* were tested at 10 concentrations (10^{-3}–10^{-12} M) in order to obtain K_i values. All assays were performed in triplicate and inhibition results shown are the mean ($n = 3$) ± SEM.

Rat brain membrane (without cerebellum) was prepared according to the method described by Gattu et al. (1995) with minor modifications (Chung et al., 2005b). Membrane protein concentration was determined by a modified Lowry et al. method (1951) using Sigma Total Protein Reagent (Product No: 541-2) with BSA as standard.

The 96-well microplate muscarinic receptor assay was optimized and performed as described by Yap (2005). Around 200 μL/well membrane suspensions (36 μg/well of protein) were added to [^{3}H]N-methyl scopolamine ([^{3}H]NMS) (0.5 nM, final concentration) (Amersham Pharmacia Biotech., Little Chalfont, Buckinghamshire, UK) to give total binding (TB). Non-specific binding (NSB) was determined by adding 25 μL of atropine (10^{-5} M, final concentration) to the reaction mixture. TB was subtracted by NSB to give specific binding (SB). The reaction mixture was incubated at 21 °C and pH 7.4 for 90 min. UniFilter 96-well GF/C filter plate was loaded onto the UniFilter cell harvester (Perkin–Elmer, Boston, MA, USA) and prewetted with ice-cold Tris–HCl buffer. After incubation, the reaction mixture was filtered with GF/C filter plate and washed with 200 μL/well of Tris–HCl buffer (four times). The seal was then applied at the bottom of the plate and 25 μL aliquots of [^{3}H]NMS was spotted directly onto the GF/C filter plate in triplicate to determine the total radioactivity per well. The plate was air-dried and followed by addition of 25 μL/well of scintillation cocktail (MicroScint-O) (Perkin–Elmer). The top side of the plate was then sealed with TopSeal A, and agitated at 400 rpm for 5 min before counting for 1 min/well with TopCount NXT microplate scintillation counter (Perkin–Elmer).

Data analysis was performed with PRISM® Software, version 3.03 (GraphPad Software, Inc., San Diego, USA). Saturation analysis was made by nonlinear regression to avoid distortion of the experimental error through data transformation (Scatchard transformation) and thus violation of the assumption in linear regression (Motulsky, 1994). The formula for one-site binding nonlinear regression with ligand depletion was fitted to the total binding data to determine B_{max} and K_D (Swillens, 1995). Rosenthal plot and Hill plot were also used to ascertain the occurrence of one site binding at the radioligand concentrations that were used to calculate B_{max} and K_D (Hill, 1910; McKinney, 1998; Rosenthal, 1967; Scatchard, 1949). K_i values were calculated according to the equation of Cheng and Prusoff (1973):

$$K_i = IC_{50}/(1 + [L]/[K_D]),$$

where K_i, inhibition constant for ligand; IC_{50}, 50% inhibition concentration of ligand; L, radioligand concentration; and K_D, dissociation constant of the radioligand. One-way analysis of variance (ANOVA) was used to assess the statistical significance of the difference between the means of the IC_{50} (K_i) of the methoxyflavones. Student's t test was used to compare the means of the IC_{50} (K_i) of the individual methoxyflavones and the standard muscarinic receptor antagonists (atropine, scopolamine and dicyclomine).

Acknowledgements

This work was supported by a TopDown Grant (IRPA 26-02-03-0127) from the Ministry of Science and Innovation, Government of Malaysia. K.F. Yap was a recipient

of the National Science Fellowship, Ministry of Science and Innovation, Government of Malaysia. The authors would like to thank L. Madani for assisting in the identification of the plant material.

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