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Comprehensive extraction and NMR-based Metabolomics : novel approaches to natural products lead finding in drug discovery

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Citation

Yuliana, N. D. (2011, June 9). *Comprehensive extraction and NMR-based Metabolomics : novel approaches to natural products lead finding in drug discovery*. Retrieved from <https://hdl.handle.net/1887/17704>

Version: Not Applicable (or Unknown)

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Note: To cite this publication please use the final published version (if applicable).

Chapter 3

Adenosine A1 receptor binding activity of methoxy flavonoids from *Orthosiphon stamineus* Benth.

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Abstract

Orthosiphon stamineus Benth. (*Orthosiphon grandiflora* Bold, or *Chlerodendratius spicatus* Thumb.) is an Indonesian medicinal herb traditionally used for various diseases such as hypertension, diabetes, and kidney stones. It is also used as one of ingredients of commercial slimming preparation. Despite the importance of these activities, there are very few studies reported. One of the targets for developing anti-obesity drugs is the adenosine A1 receptor. Antagonists of this receptor have been reported to associate with lipolytic activity, diuretic activity, and glucose tolerance improvement. A bioassay guided fractionation of a methanol-water extract of *Orthosiphon stamineus* leaves using the adenosine A1 receptor binding assay resulted in the isolation of seven methoxy flavonoids as active ligands with K_i values in the micromolar range. The Hill slope values are not significantly different from unity (within 0.9 – 1.4), which indicates the antagonist effect to A1-R. The results of this study thus provide evidence for the traditional use of *Orthosiphon stamineus* as diuretic or lipolytic agent as it may affect body weight, and the diuretic effect might be connected with the use to treat kidney stones.

Key words: *Orthosiphon stamineus*, adenosine A₁ receptor, methoxy-flavonoid, lipolysis

Yuliana *et al.*, Adenosine A1 receptor binding activity of methoxy flavonoids from *Orthosiphon stamineus* Benth. *Planta Med* 2009; 75: 132–136.

Introduction

Orthosiphon stamineus Benth. (synonyms *Orthosiphon grandiflora* Bold, or *Chlerodendratius spicatus* Thumb., Lamiaceae) is a popular medicinal herb in Indonesia, traditionally used to treat various disorders such as hypertension, diabetes, and kidney stones. Many compounds have been identified in this plant including diterpenoids, triterpenoids, and flavonoids (297, 298, 313-315). Regardless its commercial use as one of the ingredients of diuretics and slimming herbal preparations, scientific support for this traditional use is scarce. One report described the diuretic activity of an *Orthosiphon* crude extract in rats as being lower to that of furosemide but with a significant enhancement of ion excretion (316). Administration of 0.5g/kg body weight of *Orthosiphon* extract for 14 days significantly reduced plasma glucose, triglyceride, and HDL cholesterol concentration in diabetic rats as compared to control (317).

The important role of adenosine in the regulation of many cellular functions has made adenosine receptors an important target for drug development. Drugs acting on adenosine receptors have a broad therapeutic potential as, for example, sedatives, muscle relaxants, respiratory stimulants, antidepressants, cardioprotective agents, and modulator in many aspects of renal functions (318). The regulatory effect of adenosine is mediated via four adenosine receptors subtype A1, A2A, A2B, and A3. There are two type of compounds targeted at those receptors: agonists, which can activate a receptor in its natural state and replace the need of an endogenous ligand, or antagonists which can prevent binding of the natural ligand but not activating the receptor, therefore blocking the action of the endogenous ligand (290).

The distribution of the adenosine receptors in the kidney has been reviewed. Some studies showed that adenosine A1 receptor antagonists can induce diuresis and sodium excretion (319). In the kidney, adenosine A1 receptors are expressed in the afferent arterioles, glomerulus, proximal tubules and collecting ducts. The mechanism of adenosine antagonists on sodium and water excretion may be based on direct inhibition of sodium re-absorption in proximal tubules, or indirectly by promoting afferent arteriole dilatation (318, 319). Both A1 receptor antagonists and A2A agonists may play an important role in renal protection but the ligand affinity of A2A receptor is lower than of the A1 receptor (320). Adenosine A1 receptor is also highly expressed in

adipocytes, and affects fat cell metabolism. Incubation of adipocytes of *fa/fa* rats with the adenosine A1 antagonist DPCPX significantly increased glycerol release, indicating lipolysis stimulation (321). Similarly, the antagonist 8-phenyltheophylline stimulates cAMP and lipolysis of obese adipocytes while having little effect on the lean adipocytes (322). The blockage of the adenosine A1 receptor increases the cAMP level which further activates pKinase A and HSL, two important lipolytic enzymes (192). The administration of the adenosine A1 antagonist 1,3-dipropyl-8-(p-acrylic)-phenylxanthine (BW-1433) improves glucose tolerance in obese Zucker rats (323). The mechanism is not known yet, although the authors suggested that this effect is not exerted by inhibiting hepatic gluconeogenesis since adenosine A1 receptors are only found in small amount in liver.

In order to investigate whether the bioactivities exhibited by *Orthosiphon stamineus* could be justified by the presence of compounds with this type of activity, a bioassay guided fractionation using the adenosine A1 receptor binding assay (A1-R) of a water:methanol extract of its leaves was carried out, leading to the isolation of seven active methoxy flavonoids.

Material and Methods

Plant material, chemicals, and reagents

Orthosiphon stamineus material was obtained from van der Pigge Drugstore (Haarlem, The Netherlands). The plant materials were identified by one of the authors (Nancy Dewi Yuliana) and the voucher specimen (ORST-Fcog-NL-230506) was deposited in Division of Pharmacognosy, Institute of Biology, Leiden University. Methanol, *n*-hexane, chloroform, ethyl acetate, HCl, NaOH, glacial acetic acid, H₂SO₄, and DMSO were purchased from Biosolve BV (Valkenswaard, The Netherlands). Tris buffer was purchased from Gibco BRL (Grand Island, NY, USA). Anisaldehyde was obtained from Acros Organic (Geel, Belgium) and *n*-butanol was obtained from JT Baker BV (Deventer, The Netherlands), [³H]DPCPX (8-cyclopentyl-1,3-dipropylxanthine) was from DuPont NEN ('s Hertogenbosch, The Netherlands), CPA (N⁶-cyclopentyladenosine) was from RBI Inc. (Zwijndrecht, The Netherlands). Chloroform and DMSO-*d*₆ were purchased from Euriso-top (Gif-sur-Yvette, France).

Reference compounds luteolin and quercetin (98% purity) were purchased from Sigma Aldrich Chemie BV (Zwijndrecht, The Netherlands).

Column and thin layer chromatography

Column chromatography was performed with silica gel 60 (230-400 mesh) from Merck (Darmstadt, Germany) in a 5 x 58 cm column and Sephadex LH-20 gel from Sigma Aldrich Chemie BV (Zwijndrecht, The Netherlands) in a 2.5 x 40 cm column. Preparative and analytical TLC were performed using silica gel 60 F 254 TLC plates (20 x 20 cm) from Merck (Darmstadt, Germany).

Plant material extraction

The powdered dried plant materials (500 g) were extracted 3 times with 2 L, 1 L, and 0.5 L of methanol 80% by ultrasonication at room temperature for 1 h. Extracts were pooled and taken to dryness and the resulting residue (21.24 g) was suspended in 300 mL of deionized water. This was partitioned successively with *n*-hexane, chloroform and *n*-butanol (500 mL x 3) and the resulting fractions were taken to dryness yielding residues of 0.78 g (*n*-hexane), 3.54 g (chloroform), 3.26 g (*n*-butanol), and 13.18 g (water). The activity of the crude extract and those four fractions was tested with the A₁-R assay. The methanol extract showed 3% displacement of [³H] DPCPX radioactive ligand to the adenosine A₁ receptor, *n*-hexane fraction 1%, and chloroform fraction 19%, while *n*-butanol and water fractions did not show any activity.

Compound Isolation

The chloroform fraction (3 g) was placed on a silica gel column and eluted with a stepwise gradient (chloroform-ethyl acetate 1:0 to 0:1, methanol, each 900 mL, 2 mL/minute) to give nine fractions which were further tested for their activities. All fractions exhibited activity in the range of 21% - 91% displacement of [³H] DPCPX radioactive ligand to the adenosine A₁ receptor. Based on their activity, the yield, and TLC patterns, three fractions: B (0.41 g), C (0.22 g), and F (0.16 g) were chosen for further separation with a Sephadex LH-20 column, each were eluted with 200 mL of methanol and flow rate of 2 mL/minute. Five fractions were obtained from fr. B (B1-5). Fraction B3 (15.60 mg) was further separated with a Sephadex LH-20 column (200 mL methanol, 2 mL/minute) to give 5 fractions (B3a-B3e). Fraction B3e was identified as

compound **4** (0.65 mg). Further purification of fr. B3c with preparative TLC using chloroform/ethyl acetate (7:3) solvent system resulted in the isolation of compound **2** (3.95 mg). Eight fractions were obtained from fr. C (C1-C8). Fraction C2 (33 mg) was separated further with Sephadex LH-20 column (200 mL methanol, 2 mL/minute) to give 2 fractions (C2a-C2b). The preparative TLC was applied to fraction C2a and C2b using chloroform/ethyl acetate/ acetic acid (3:7:0.1) as solvent system, yielding compound **1** (1.4 mg) and **5** (3.03 mg). Seven fractions were obtained from fr. F (F1-F7). Fraction F3 was identified as compound **6** (1.3 mg), fr. F4 as compound **3** (4 mg), and fr. F5 as compound **7** (0.6 mg). Fractionation and isolation were monitored by TLC using a chloroform/ethyl acetate solvent system with visualization under UV (254 and 365 nm) and anisaldehyde-H₂SO₄ spray reagent followed by heating. The chemical structures of the isolated compounds were elucidated on the basis of ¹H, ¹³C, J resolved, HMQC, HMBC, COSY NMR, and APCI-MS spectra. The obtained data were compared with previous reports. The purity of the isolated flavonoids was evaluated by ¹H-NMR spectrum. They were more than 90%.

NMR measurement

Each pure compound was dissolved in CDCl₃ or in DMSO-*d*₆. The NMR spectra were recorded on 500 MHz Bruker DMX 500 Spectrometer. All NMR experiments were performed at 25°C. Chemical shifts (δ) are given in ppm and coupling constants (*J*) are reported in Hz.

APCI Mass Spectrometry

Spectra were recorded on Agilent 1100 MSD single quadrupole mass spectrometer using probe positive-ion and Phenomenex RP 18 (4.6 x 150 mm, 5 micron) column. The mass scan range was 100 – 800 *m/z*. The solvent system was methanol/water/formic acid (90:10:0.1) with flow rate of 1 mL/min. and injection volume of 5 µL/min.

Adenosine A1 Receptor Assay

The assay was performed as previously described by Chang *et al.* (290) except that the volume of total mixture in the assay was 200 µL. The membranes were prepared

from Chinese Hamster Ovary (CHO) cells stably expressing human adenosine receptors by method previously described by Dalpiaz *et al.*(291). More detail on the bioassay can be found in Chapter 2.

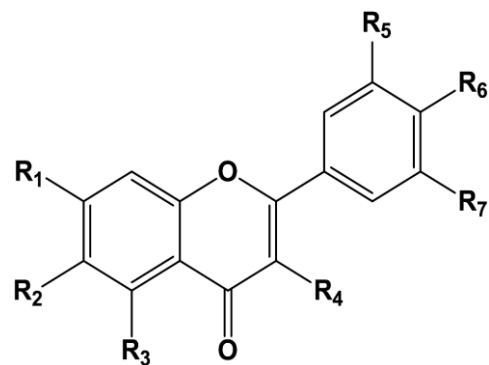
Statistical analysis

The counting results were analyzed using the software package Graphpad Prism (Graphpad Software Inc., San Diego, CA, USA). The K_i values were calculated from IC_{50} values as described previously (324). The pK_i values of each compound were determined using several different concentrations. Data are means \pm standard deviation of three individual determinations, each performed in duplicate.

Result and discussion

Most compounds that have been synthesized and tested as antagonists of adenosine A1 receptor (A1-R) have a xanthine-based structure which, however, is usually associated to several problems, i.e., poor selectivity over the other adenosine receptors, poor solubility and bioavailability. Therefore, the search for a non-xanthine like compound is of high interest (318). Among non-xanthine like compounds from natural sources, flavonoids are well-known to be A1-R antagonists (325). A broad screening for A1-R antagonists focused on the flavones as members of the larger class of flavonoids has resulted in a series of active flavones with K_i value in the micromolar range (326).

Bioassay guided fractionation of 80% methanol extracts of *Orthosiphon stamineus* using A1-R assay resulted in the isolation of seven compounds (compounds **1** – **7**) as A₁-R active ligands with pK_i values in the micromolar range (Fig. 1). The ¹H-NMR spectra profile allowed these compounds to be identified as methoxy-flavonoids. The characteristic ¹H -resonances of flavones are the signal in the range of δ 6.0 -7.0 attributed to the protons adjacent to the hydroxyl or methoxy in A and/or B ring, or the proton signal of H-3 in C ring; and the signals above δ 7.0 which are attributed to proton with *meta* position to hydroxyl or methoxy in B ring; while the upfield signals between δ 3.7 – 4.0 are assigned as methoxy groups.



Compound	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇	pK _i	Hill slope
3'-hydroxy-4',5,6,7- tetramethoxyflavone (1)	OMe	OMe	OMe	H	OH	OMe	H	5.4 ± 0.2	1.1 ± 0.1
3',5-dihydroxy-4',6,7 trimethoxy-flavone (eupatorin) (2)	OMe	OMe	OH	H	OH	OMe	H	5.5 ± 0.1	1.0 ± 0.2
4',5,6,7-tetramethoxyflavone (tetramethylscutellarein) (3)	OMe	OMe	OMe	H	H	OMe	H	5.4 ± 0.1	1.0 ± 0.1
3'5-dihydroxy-4',7 dimethoxyflavone (piloin) (4)	OMe	H	OH	H	OH	OMe	H	4.3± 0.1	0.9 ± 0.1
3',4',5,6,7- pentamethoxyflavone (sinensetin) (5)	OMe	OMe	OMe	H	OMe	OMe	H	5.5 ± 0.1	0.9 ± 0.1
5,6-dihydroxy-7,4'-dimethoxyflavone (6).	OMe	OH	OH	H	H	OMe	H	5.1 ± 0.1	1.4 ± 0.3
3,3'-dihydroxy-5,6,7,4'- tetramethoxyflavone (eupatoretin) (7)	OMe	OMe	OMe	OH	OH	OMe	H	5.4 ± 0.1	1.0 ± 0.2
Luteolin (reference compound)	OH	H	OH	H	OH	OH	H	5.4 ± 0.2	1.3 ± 0.4
Quercetin (reference compound)	OH	H	OH	OH	OH	OH	H	5.8± 0.1	1.1 ± 0.2

Figure 1. Structures of compound 1 – 7, their pK_i and Hill slope values.

The ^{13}C NMR spectra of compound **2**, **3**, and **5** were compared to previously reported data (327, 328). The spectra of compound **1**, **4**, **6**, and **7** were referred to those three compounds, the differences being limited to the number and positions of methoxy and/or hydroxyl groups. The molecular weight of those 7 compounds was confirmed by LC/MS measurement.

All flavonoids isolated in this experiment exhibited one site competition curve with Hill slope value not significantly different from unity (within 0.9 – 1.4, see Fig. 2), which indicates an antagonist effect on A1-R. Of the isolated methoxy flavonoids, compound **7** (eupatoretin) was isolated for the first time from *Orthosiphon stamineus*. The name of this compound is associated to *Eupatorium semiserratum* of which it was first isolated (329).

The K_i values of some A1-R antagonist compounds have been reported. Theophylline and caffeine were the first compounds reported as A1-R antagonists with a K_i value of 8.5 and 29 μM , respectively (330). The A1-R affinity for compound **3** and both reference compounds (luteolin and quercetin) used in this study have also been previously reported (300, 330).

The hydroxyl group of naturally occurring flavones is not essential for the adenosine receptor affinity activity (300), as can be noticed in compound **3** and **5** which have no hydroxyl group but exhibit higher affinity compared to **4** which has three hydroxyls and no significant affinity compared to **1**, which has one hydroxyl at position 3'. There is no clear structure activity relationship that can be concluded from the presence of methoxy groups in various numbers and positions in compound **1-7**. The positive correlation between A1-R affinity and hydrophobicity which has been previously suggested (300) could not be observed in this experiment since compound **5** with five methoxy substitutes is not the most active one.

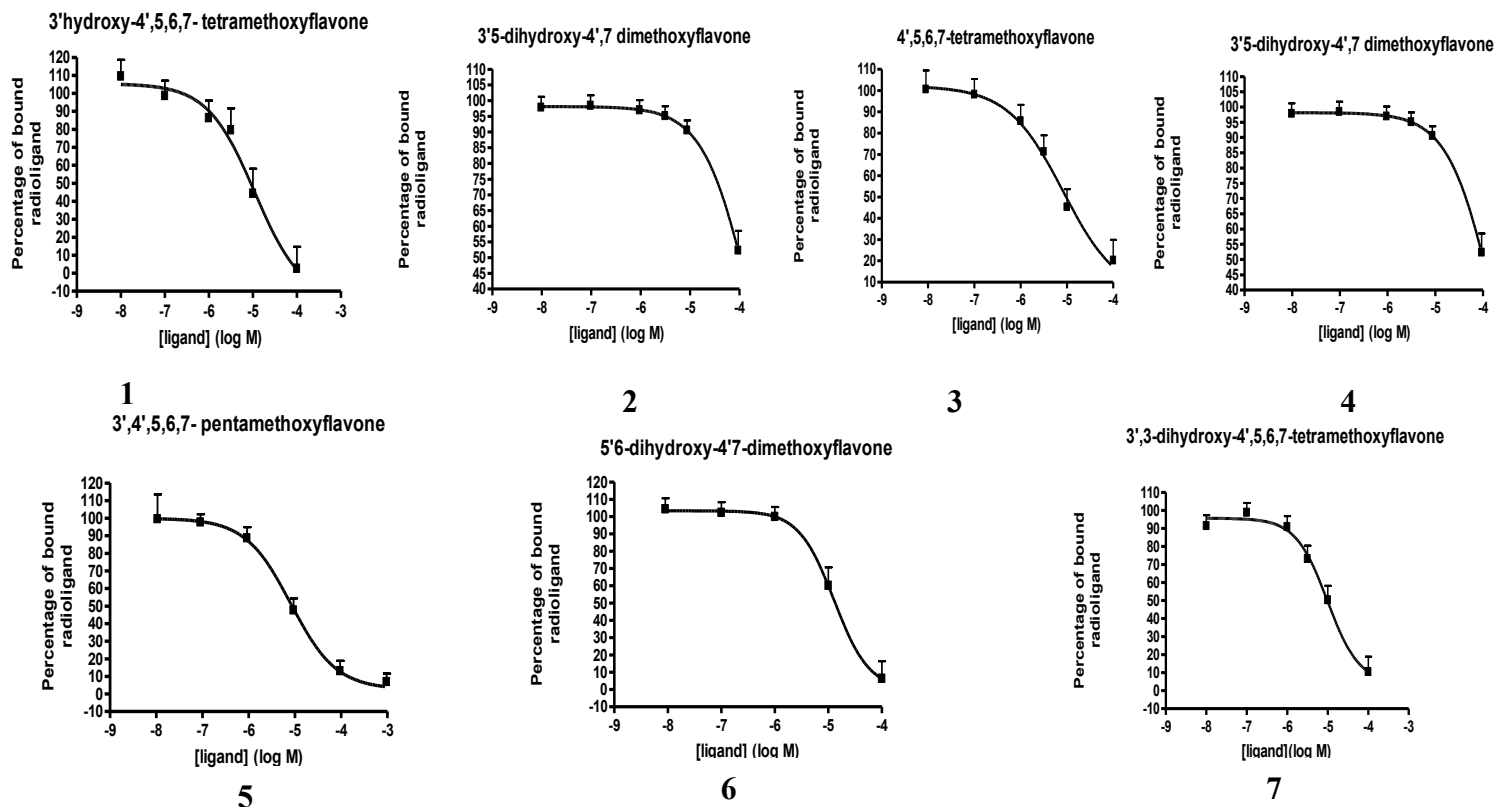


Figure 2. The radioligand-binding curves of compound **1** – **7** were determined by incubation of CHO cells expressing adenosine A₁ human receptors for 60 minutes with 0.4 nM [³H]-DPCPX and an increasing concentration of the compounds at 25°C in 50 mM Tris-HCl buffer (pH 7.4); nonspecific binding was obtained in the presence of 10 μM CPA; the values are mean ± SD from 3 replicates; K_i value was calculated (324).

Methoxylated flavones such as 7-methoxyflavone, 7,4'-dimethoxyflavone, 5,7-dimethoxyflavone, and 5,7,4'-trimethoxyflavone have been reported to exhibit higher metabolic stability and intestinal absorption, thus better bioavailability than the unmethylated ones like 7-hydroxyflavone, 7,4'-dihydroxyflavone, chrysin (5,7-dihydroxyflavone), and apigenin (5,7,4'-trihydroxyflavone) (331). This study shows that methoxy flavonoids are promising A₁-R antagonist ligands from natural sources.

Conclusions

The result of this experiment justifies the traditional use of *Orthosiphon stamineus* as diuretic, anti-diabetic, and slimming agent since the affinity found for the isolated compounds to adenosine A₁ receptor and their antagonistic effect, are associated with these bioactivities.

Further research regarding the above mentioned activities by an *in-vivo* method, as well as toxicity and bioavailability of the methoxy-flavonoids if compared to synthetic ligands is necessary in order to assess the potential of the methoxy-flavonoids as A₁-R antagonists.

Acknowledgments

Financial support from The Dutch Government via Netherlands Education Support Office (NESO) and The Embassy of Dutch Government in Indonesia for this project is gratefully acknowledged.