Adenosine A1 receptor antagonist activity of methoxy flavonoids from Orthosiphon stamineus

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Adenosine A_1 Receptor Binding Activity of Methoxy Flavonoids from *Orthosiphon stamineus*

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Key words
- *Orthosiphon stamineus*
- Lamiaceae
- adenosine A_1 receptor
- methoxy flavonoid
- diuretic
- kidney stone

Abstract

Orthosiphon stamineus Benth. (*Orthosiphon grandiflorus* Bold. or *Clerodendranthus spicatus* Thunb.) is an Indonesian medicinal herb traditionally used for diseases such as hypertension, diabetes, and kidney stones. Despite the importance of this last application, there are very few reports on it. Diuretic action is an important factor in kidney stone treatment, as an increase in the volume of fluid flowing through the kidney will help to dissolve the stones, assist their passing to avoid further retention, and flush out the deposits. Among the diverse roles of adenosine A_1 receptor antagonists in renal protection, many studies have shown that they can induce diuresis and sodium excretion. A bioassay-guided fractionation of a methanol-water extract of *Orthosiphon stamineus* leaves using the adenosine A_1 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 A_1-R. The results of this study thus provide a scientific foundation for the traditional use of *Orthosiphon stamineus* in kidney stone treatment, as the affinity of the active compounds isolated from it as adenosine A_1 receptor ligands allows them to be associated with diuretic activity, which is one possible treatment for renal lithiasis.

Abbreviations

CPA:	N^6-cyclopentyladenosine
DPCPX:	8-cyclopentyl-1,3-dipropylxanthine
GFR:	glomerular filtration rate
RBF:	renal blood flow

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Introduction

Orthosiphon stamineus Benth. (synonyms *Orthosiphon grandiflorus* Bold, or *Clerodendranthus spicatus* Thunb., Lamiaceae) is a popular medicinal herb in Indonesia that is traditionally used to treat disorders such as hypertension, diabetes, and kidney stones. Many compounds have been identified in this plant, including diterpenoids, triterpenoids, and flavonoids [1], [2], [3], [4], [5], [6], [7], [8]. In particular, flavonoids have shown *in vitro* antioxidant activity [1] and *in vitro* cytostatic activity on Ehrlich ascites tumor cells [7].

However, its diuretic activity has been scarcely investigated. One of these reports described the activity of an *Orthosiphon* crude extract in rats as being lower than that of furosemide but with a significant enhancement of ion excretion [8].

The important role of adenosine in the regulation of many cellular functions has made adenosine receptors an important target for drug development. Drugs acting as adenosine receptors have a broad therapeutic potential as, for example, sedatives, muscle relaxants, respiratory stimulants, antidepressants, cardioprotective agents, and modulators of many aspects of renal function [9]. The regulatory effect of adenosine is mediated via four adenosine receptor subtypes: A_1, A_{2A}, A_{2B}, and A_3. There are two types of compounds targeted at these receptors: agonists, which can activate a receptor in its natural state and replace the need for an endogenous ligand, and antagonists, which can prevent binding, thereby blocking the action of an endogenous ligand [10].

The distribution of adenosine receptors in the kidney has been reviewed. Some studies show that adenosine A_1 receptor antagonists can induce diuresis and sodium excretion [11]. In the kidney, adenosine A_1 receptors are expressed in

the afferent arterioles, glomerulus, proximal tubules, and collecting ducts. The mechanism of adenosine antagonists on sodium and water excretion may occur directly by inhibition of sodium reabsorption in proximal tubules or indirectly by promoting afferent arteriole dilatation [9], [11]. Diuretic action is an important factor in kidney stone treatment, as an increase in fluid volume flowing through the kidney will help to dissolve the stones, assist in their passing, thus avoiding further retention, and flush out the deposits [12]. Both A_1 receptor antagonists and A_{2A} agonists may play an important role in renal protection, but the ligand affinity for the A_{2A} receptor is lower than that for the A_1 receptor [13].

As diuretics, adenosine A_1 antagonists are considered to have fewer side effects than furosemide. Furosemide, the most widely used diuretic agent, may cause in many cases a decrease in the glomerular filtration rate (GFR) as a side effect [11], [14]. GFR indicates the volume of fluid filtered by glomerular capillaries into the Bowman's capsule and must be maintained within a certain narrow range. A change in GFR will change renal blood flow (RBF), and while an excessive rate of RBF will damage the glomerulus, a low RBF will deprive the kidneys of oxygen. Adenosine antagonists are able to enhance sodium and water excretion while preserving the GFR and RBF and thus inhibiting the deterioration of renal function [11], [14]. In a more recent report, KW-3902 (rolofylline) was able to reduce the amount of furosemide used in acute decompensated heart failure patients. The use of this A_1-R antagonist together with furosemide was shown to induce diuresis while preventing renal deterioration as compared with a placebo (using only furosemide) [15].

In order to investigate whether the diuretic activity exhibited by *Orthosiphon stamineus* could be justified by the presence of compounds with this type of activity, a bioassay-guided fractionation using the adenosine A_1 receptor binding assay (A_1-R) of a water-methanol extract of its leaves was carried out, leading to the isolation of seven active methoxy flavonoids.

Materials and Methods

Plant material, chemicals, and reagents

Orthosiphon stamineus was obtained from van der Pigge Drugstore. The plant materials were identified by one of the authors (N.D. Yuliana), and a voucher specimen (ORST-Fcog-NL-230506) was deposited in the Division of Pharmacognosy, Institute of Biology, Leiden University. Methanol, *n*-hexane, chloroform, ethyl acetate, HCl, NaOH, glacial AcOH, H_2SO_4, and DMSO were purchased from Biosolve BV. Tris buffer was purchased from Gibco BRL. Anisaldehyde was obtained from Acros Organic, and *n*-BuOH was obtained from JT Baker BV. [^{3}H]DPCPX (8-cyclopentyl-1,3-dipropylxanthine) was from DuPont NEN, and CPA (N^6-cyclopentyladenosine) was from RBI Inc. $CDCl_3$ and DMSO-d_6 were purchased from Euriso-top. Reference compounds luteolin and quercetin (98% purity) were purchased from Sigma Aldrich Chemie BV.

Column and thin-layer chromatography

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

Plant material extraction

The powdered, dried plant materials (500 g) were extracted 3 times with 2 L, 1 L, and 0.5 L of MeOH 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*-BuOH (500 mL×3), and the resulting fractions were evaporated to dryness, yielding residues of 0.78 g (*n*-hexane), 3.54 g (chloroform), 3.26 g (*n*-BuOH), and 13.18 g (water). The activity of the crude extract and these four fractions was tested with the A_1-R assay. The MeOH extract showed 3% displacement of [^{3}H] DPCPX radioactive ligand to the adenosine A_1 receptor, the *n*-hexane fraction showed 1% displacement, the chloroform fraction showed 19% displacement, and the *n*-BuOH and water fractions showed no activity.

Compound isolation

The chloroform fraction (3 g) was placed on a silica gel column and eluted with a stepwise gradient ($CHCl_3$/EtOAc 1:0 to 0:1, MeOH, each 900 mL, 2 mL/min) to give nine fractions that were further tested for their activities. All fractions exhibited activity in the range of 21–91% displacement of [^{3}H] DPCPX radioactive ligand to the adenosine A_1 receptor. Based on their activity, 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 was eluted with 200 mL of MeOH at a flow rate of 2 mL/min.

Five fractions were obtained from fraction B (B1-B5). Fraction B3 (15.60 mg) was further separated with a Sephadex LH-20 column (200 mL MeOH, 2 mL/min) to give five fractions (B3a-B3e). Fraction B3e was identified as compound **4** (0.65 mg). Further purification of fraction B3c with a preparative TLC using a $CHCl_3$/EtOAc (7:3) solvent system resulted in the isolation of compound **2** (3.95 mg).

Eight fractions were obtained from fraction C (C1-C8). Fraction C2 (33 mg) was separated further with a Sephadex LH-20 column (200 mL MeOH, 2 mL/min) to give two fractions (C2a and C2b). Preparative TLC was applied to fractions C2a and C2b using $CHCl_3$/EtOAc/CH_3COOH (3:7:0.1) as solvent system, yielding compounds **1** (1.4 mg) and **5** (3.03 mg).

Seven fractions were obtained from fraction F (F1-F7). Fraction F3 was identified as compound **6** (1.3 mg), fraction F4 as compound **3** (4 mg), and fraction F5 as compound **7** (0.6 mg).

Fractionation and isolation were monitored by TLC using a $CHCl_3$-EtOAc solvent system with visualization under UV (254 and 365 nm) and anisaldehyde-H_2SO_4 spray reagent followed by heating. The chemical structures of the isolated compounds were elucidated on the basis of ^{1}H-NMR, ^{13}C-NMR, J-resolved NMR, HMQC, HMBC, COSY NMR, and APCI-MS. The obtained data were compared with previous reports.

The purity of the isolated flavonoids was evaluated by a ^{1}H-NMR spectrum. In all cases, it was greater than 90%.

NMR measurement

Each pure compound was dissolved in $CDCl_3$ or DMSO-d_6. NMR spectra were recorded on a 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 an Agilent 1100 MSD single quadrupole mass spectrometer using a probe positive-ion and Phenomenex RP 18 (4.6 × 150 mm, 5 micron) column. The mass scan range was 100 – 800 *m/z*. The solvent system was MeOH/H_2O/formic acid (90 : 10 : 0.1) with a flow rate of 1 mL/min and an injection volume of 5 μL/min.

Adenosine A_1 receptor assay

The assay was performed as previously described by Chang et al. [16], except that the volume of the total mixture in the assay was 200 μL. The radioactive ligand used for the assay was 0.4 nM [^{3}H] DCPCX (8-cyclopentyl-1,3-dipropylxanthine) (K_d = 1.6 nM). Membranes were prepared from Chinese hamster ovary (CHO) cells stably expressing human adenosine receptors by a method previously described by Dalpiaz et al. [17], and 10 μM CPA (N^6-cyclopentyladenosine) was used to determine non-specific binding. The mixture consisting of 50 μL [^{3}H] DPCPX, 50 μL CPA/50 mM Tris-HCl buffer/test compounds in different concentrations, 50 μL 50 mM Tris-HCl buffer pH 7.4, and 50 μL of membrane was incubated at 25 °C for 60 min and then filtered over a GF/B Whatman filter under reduced pressure. The filters were washed three times with 2 mL ice-cold 50 mM Tris/HCl buffer, pH 7.4, and 3.5 mL scintillation liquid was added to each filter. The radioactivity of the washed filters was counted by a Hewlett-Packard Tri-Carb 1500 liquid scintillation detector. Non-specific binding was determined in the presence of 10^{-5} M CPA. The K_i values were calculated from IC_{50} values as described previously [18].

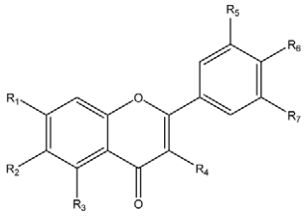
Statistical analysis

The counting results were analyzed using the software package Prism (Graph Pad, Inc.). The pK_i values of each compound were determined using several different concentrations. Data are means ± SD of three individual determinations, each performed in duplicate.

Results and Discussion

Most synthetic compounds that have been synthesized and tested as antagonists of the adenosine A_1 receptor (A_1-R) have a xanthine-based structure, which is usually associated with problems such as poor selectivity over the other adenosine receptors and poor solubility and bioavailability. Therefore, the search for a non-xanthine-like compound is highly desirable [9]. Among non-xanthine-like compounds from natural sources, flavonoids have been known to be rigid A_1-R antagonists [19]. A broad screening of A_1-R antagonist ligands focused on the flavones as members of the larger class of flavonoids has resulted in a series of active flavones with K_i values in the micromolar range [20].

Bioassay-guided fractionation of the 80% MeOH extracts of *Orthosiphon stamineus* using the A_1-R assay resulted in the isolation of seven compounds (compounds **1 – 7**) as A_1-R-active ligands with pK_i values in the micromolar range (**Fig. 1**). The ^{1}H-NMR spectra profile allowed identification of these compounds as methoxy flavonoids. The characteristic ^{1}H resonances of flavones are the signals in the range of δ = 6.0 – 7.0 attributed to the protons adjacent to the hydroxy or methoxy in the A and/or B ring or the proton signal of H-3 in the C ring. The signals above δ = 7.0 are attributed to protons with a *meta* position to the hydroxy or methoxy in the B ring, while the upfield signals between δ = 3.7 and δ = 4.0 are assigned as methoxy groups. The ^{13}C-NMR spectra of compounds **2**, **3**, and **5** were compared with previously reported data [21], [22]. The spectra of compounds **1**, **4**, **6**, and **7** were referred to these three compounds, with the differences being limited to the number and position of methoxy and/or hydroxy groups. The molecular weight of these seven compounds was confirmed by LC/MS measurement.



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 (pilloin) (**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

Fig. 1 Structures of compounds **1 – 7** and their pK_i and Hill slope values.

All flavonoids isolated in this experiment exhibited a one-site competition curve with a Hill slope value not significantly different from unity (within 0.9 – 1.4, see **Figs. 1 – 3**), which indicates the antagonist effect to A_1-R. Of the isolated methoxy flavonoids, compound **7** (eupatoretin) was isolated from *Orthosiphon stamineus* for the first time. The name of this compound is associated with *Eupatorium semiserratum*, from which it was first isolated [23].

The K_i values of some A_1-R antagonist compounds have been reported. Theophylline and caffeine were the first compounds reported as A_1-R antagonists, with a K_i value of 8.5 μM and 29 μM, respectively [24]. The A_1-R affinity for compound **3** and both reference compounds (luteolin and quercetin) used in this study has also been reported previously [24], [25].

The hydroxy group of naturally occurring flavones is not essential for adenosine receptor affinity activity [25], as can be seen in compounds **3** and **5**, which have no hydroxy group but exhibit higher affinity than does **4**, which has three hydroxys and no significant affinity compared with **1**, which has one hydroxy 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 compounds **1 – 7**. The positive correlation between A_1-R affinity and hydrophobicity that has been previously suggested [25] could not be observed in this experiment, as compound **5** with five methoxy substitutes is not the most active one.

Currently, modern therapies such as extracorporeal shock wave lithotripsy (ESWL), ureteroscopy, and some oral drugs are avail-

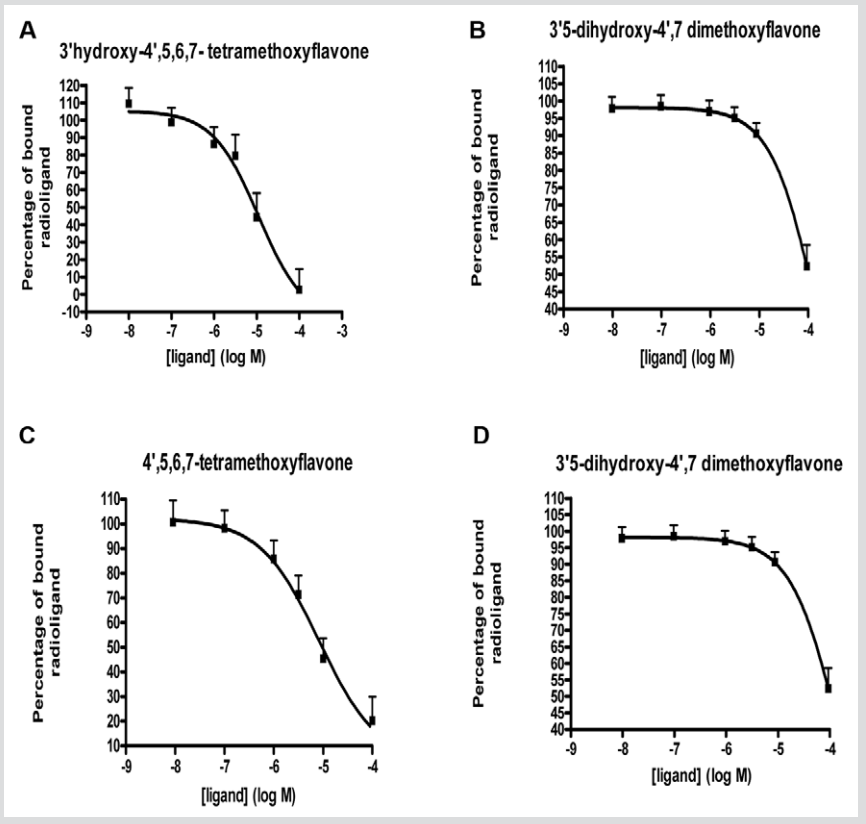


Fig. 2 Radioligand-binding curves of compounds **1 – 4** were determined by incubation of CHO cells expressing adenosine A_1 human receptors for 60 min with 0.4 nM [^{3}H]-DPCPX and an increasing concentration of the compounds at 25 °C in 50 mM Tris-HCl buffer (pH 7.4). Non-specific binding was obtained in the presence of 10 μM CPA, and K_i value was calculated [18]. All values (mean ± SD) are based on triplicates.

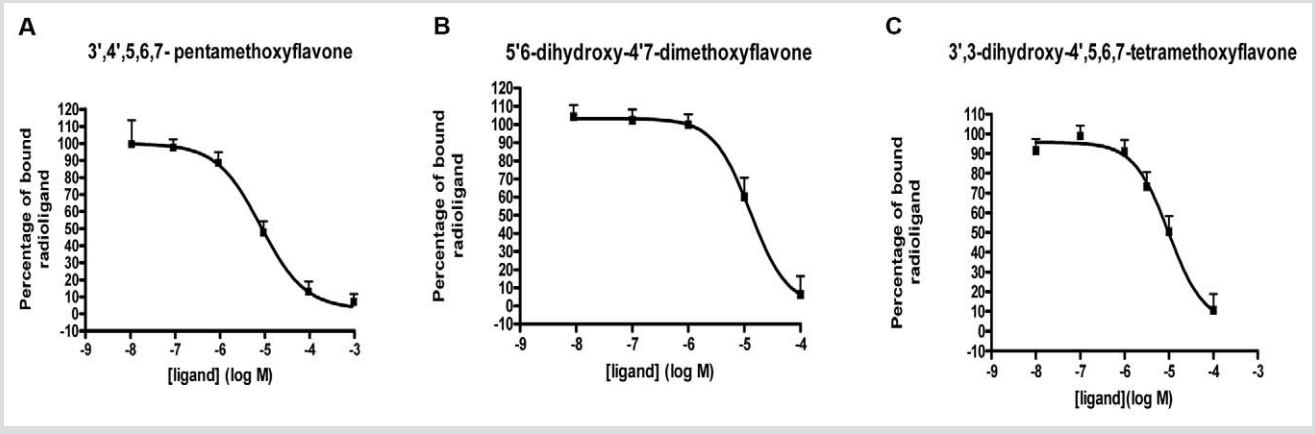


Fig. 3 Radioligand-binding curves of compounds **5 – 7** were determined by incubation of CHO cells expressing adenosine A_1 human receptors for 60 min with 0.4 nM [^{3}H]-DPCPX and an increasing concentration of the compounds at 25 °C in 50 mM Tris-HCl buffer (pH 7.4). Non-specific binding was obtained in the presence of 10 μM CPA, and K_i value was calculated [18]. All values (mean ± SD) are based on triplicates.

able. Despite the lack of scientific evidence, the use of herbal therapy to treat kidney stones has become an interesting choice because of the low cost and because modern treatments such as ESWL are unavailable for many people. A review of literature published on the use of phytotherapeutic agents to treat kidney stones revealed that diuretic activity is one of the mechanisms behind the anti-lithogenic effect exhibited by some herbal remedies [26]. The result of this experiment justifies the traditional use of *Orthosiphon stamineus* in urolithiasis treatment due to its diuretic activity, proved by the affinity of the isolated compounds to the adenosine A_1 receptor antagonist, which as explained above, is associated with diuretic activity.

Further research regarding diuretic activity evaluation by an *in vivo* method, toxicity, and bioavailability of these methoxy flavonoids related to synthetic ligands is necessary in order to observe the potential of these methoxy flavonoids as A_1-R antagonists.

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 and thus better bioavailability than unmethylated flavones such as 7-hydroxyflavone, 7,4′-dihydroxyflavone, chrysin (5,7-dihydroxyflavone), and apigenin (5,7,4′-trihydroxyflavone) [27]. These reports allow methoxy flavonoids

to be proposed as promising A_1-R antagonist ligands from natural sources.

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