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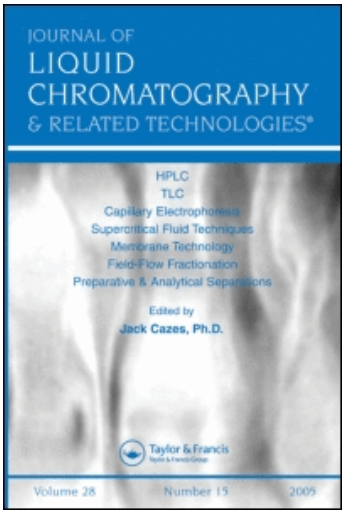

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Identification of Possible Compounds Possessing Adenosine A1 Receptor Binding Activity in the Leaves of *Orthosiphon stamineus* Using TLC and Multivariate Data Analysis

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Abstract: A novel approach to identify compounds possessing adenosine A1 receptor binding activity in the leaves of *O. stamineus* was developed. *O. stamineus* extract is one of the components of a functional beverage used in Indonesia for the treatment of kidney stones. In this study, adenosine A1 receptor binding, which is related to the diuretic action in the treatment of kidney stones was tested. A combination of thin layer chromatography of different extracts prepared by extraction with diverse solvents (*n*-hexane, chloroform, *n*-butanol and water), and multivariate data analysis based on orthogonal partial least squares proved to be a promising approach to determine these active compounds. Several methoxyflavonoids, fatty acids or terpenoids were estimated to be related

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to this activity. The results of this study support the traditional use in Indonesia of *O. stamineus* as a functional drink to treat kidney stones.

Keywords: Adenosine, Kidney stone, Multivariate data analysis, Orthogonal partial least square, *Orthosiphon staminues*, TLC

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INTRODUCTION

Orthosiphon stamineus Benth is one of the ingredients of *jamu*, an Indonesian traditional functional beverage. *Jamu* is consumed to assist in the treatment of several disorders such as hypertension, diabetes, and kidney stones.[1] Though a great amount of biologically active compounds have been isolated from *O. stamineus* very few scientific reports have been published on the use of this plant to reduce kidney stones.[2–5]

Diuretic action is an important factor in kidney stone treatment since an increase in volume of fluid flowing through the kidney helps to dissolve the stones, assist their passing to avoid further retention, and flush out the deposits. This activity involves adenosine A1 receptor antagonists, which are able to induce diuresis and sodium excretion.[6] Moreover, adenosine A_1 antagonists have been proven to have fewer side effects as diuretics.[7]

Our group reported the isolation of seven methoxyflavonoids possessing adenosine A1 receptor binding activity from this plant.[8] However, clearly, there are other active compounds which have yet to be identified, but this task is time consuming and might not necessarily be successful since the activity detected in isolated compounds often differs from that exhibited in the presence of other compounds, due to synergism and/or antagonism with these. Therefore, the development of a holistic approach to identify the possible compounds responsible for the activity of a crude extract can be more fructiferous.

Many scientists have proposed the possibility of the application of systems biology for this purpose.[9–11] In this new approach, the traditional research methodology which focuses on single compounds and single targets is replaced by a holistic approach, which allows the analysis of the whole body of compounds in a sample. Similarly to traditional medicinal systems such as the *ayurvedic* herbal medicines and Traditional Chinese Medicine, which are based on the activity of crude extracts of herbs containing a mixture of compounds, this approach centers attention on mixtures of compounds rather than on single pure compounds as in the classic ''modern'' approach. Thus, effects such as the synergism or antagonism between compounds in plants which were ignored in the classical phytochemical research, can be detected and studied from the systems biology approach.

In order to develop this system, it is necessary to find holistic analytical techniques to identify as many plant metabolites as possible.

Although it is very difficult to develop this kind of technique, nuclear magnetic resonance (NMR) spectroscopy has been considered to be one of the analytical methods, which allows the detection of a variety of compounds in a single run with appropriate robustness.[12–13] However, NMR alone is unable to detect all metabolites due to its low sensitivity and the presence of overlapping peaks. Thus, according to the case, the use of more sensitive instruments, e.g., GC-MS and LC-MS is often required. Another possibility to be explored is the use of thin layer chromatography (TLC). TLC is known to be a fast tool for the detection of compounds. Additionally, due to the diverse developing reagents that can be applied and the fact that compounds can be visualized even when retained, it often allows the detection of more compounds than HPLC and GC, albeit with poorer resolution.

Data obtained from TLC chromatograms can be recorded with the support of several software programs such as SIMCA codec. The link between the obtained data and activity can then be determined statistically by means of multivariate data analysis in order to identify the spots related to the activity. There is no report available on the use of TLC for such systems biology work.

The purpose of this study was to identify compounds possessing adenosine A1 receptor binding activity in the leaves of *Orthosiphon stamineus* using a systems biology approach by combining thin layer chromatography, activity data from the sample, and multivariate data analysis.

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EXPERIMENTAL

Plant Material

Orthosiphon stamineus dried plant material (leaf and adjacent stem) was obtained from van der Pigge Drugstore, Haarlem, The Netherlands.

Chemicals and Reagents

Methanol, *n*-hexane, chloroform, ethyl acetate, ethanol, HCl, NaOH, and DMSO were purchased from Biosolve BV (Valkenswaard, The Netherlands), Tris buffer from Gibco BRL (New York, USA); *n*-butanol from JT Baker BV (Deventer, The Netherlands), and DMSO-d_6 NMR solvents from Euriso-top (Yvette, France). All solvents and reagents were analytical grade. TLC plates (10 cm × 20 cm) were purchased from Merck, Darmstadt, Germany.

Sample Preparation

Samples of 1 g each of powdered *O. stamineus* were extracted with 300 mL *n*-hexane, 300 mL chloroform, 300 mL *n*-butanol, or 300 mL water by sonication for 30 min. After paper filtration, the supernatant was evaporated using a vacuum rotary evaporator at 45°C to obtain the dry extracts.

Thin Layer Chromatography

Aliquots of 2 mg of the dry extracts were redissolved in 500 μL *n*-hexane, 500 μL chloroform, 500 μL *n*-butanol, and 500 μL water. A volume of 10 μL of each sample was spotted on the TLC plate (10 cm × 20 cm) using an automatic TLC spotter. The TLC plates were then placed in saturated TLC chambers containing 10 mL of mobile phases of mixtures of chloroform and ethyl acetate, chloroform and methanol, chloroform and ethanol, and *n*-hexane and ethyl acetate. The TLC plates were developed until 2 cm below the upper edge of the plate and dried using an air dryer (80°C). The spots on the TLC plates were visualized with an UV lamp ($\lambda = 254$ nm) and the images were recorded with an image scanner (Fuji Life Science, FL, USA).

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NMR Measurements

A sample of 20 mg of dried extract was dissolved in 900 μL DMSO-d_6, and 800 μL of this solution was transferred to an NMR tube. ^{1}H NMR was recorded at 25°C on a 400 MHz Bruker AV-400 spectrometer, operating at a proton NMR frequency of 400.13 MHz. Each ^{1}H NMR spectrum consisted of 128 scans requiring 10 min acquisition time with the following parameters: 0.25 Hz/point, pulse width (PW) = 30° (4.0 μsec), and relaxation delay (RD) = 5.0 sec. The resulting spectra were manually phased, baseline corrected, and calibrated to TSP at δ 0.0, all using XWIN NMR (version 3.5, Bruker).

The Adenosine A1 Receptor Binding Assay

The adenosine A1 receptor binding assay was carried out on rat cortical brain membranes, which were prepared according to the method, except that the membranes were incubated with 2 IU/mL adenosine deaminase at 37°C before storage.[14–15] The protein concentration of this membrane was measured with the bicinchonic acid method.[16]

A sample of 4 mg were dissolved in 1 mL of DMSO and diluted 10-fold with Tris/HCl 50 mM pH 7.4 buffer to a final concentration of 0.4 mg/mL. An aliquot of 100 μL of this solution was mixed with 100 μL of [^{3}H] DPCPX 0.4 nM in Tris/HCl 50 mM pH 7.4 buffer as a radioligand, 100 μL of buffer tris/HCL 50 mM pH 7.4, and 100 μL of rat cortical brain membranes (protein concentration = 30 μg/100 μL in buffer Tris/HCl 50 mM pH 7.4). After incubation of the mixture at 25°C during 60 minutes it was filtered under pressure over a glass fiber filter (GF/B Whatman) using the harvester. The filtrate was washed 3 times with 2 mL of ice cold 50 mM buffer tris/HCl pH 7.4 and then 3.5 mL of scintillation liquid was added and stirred. The radioactivity of the filtrate was counted for 4 minutes with a liquid scintillation counter.

Non specific binding was determined using 100 μL of 10^{-5} M cyclopentiladenosine instead of the sample, and total binding was measured in the absence of sample, which was replaced by 50 mM buffer Tris/HCl pH 7.4. The adenosine A1 receptor binding activity (% specific binding) of the sample was determined applying the equation below:

$$SB = (SpB - NSB)/(TB - NSB) \times 100\% \quad (1)$$

$$\% \text{ replacement} = 100\% - SB \quad (2)$$

SB = % specific binding
SpB = sample binding
NSB = non specific binding
TB. = Total binding

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Multivariate Data Analysis

Data recorded from TLC chromatograms and the activity data of each extract were transferred to SIMCA codec software (v. 10.0, Umetrics, Umeå, Sweden). Statistical analysis was performed using this software based on orthogonal partial least squares.

RESULTS AND DISCUSSION

We chose to use TLC analysis of the extracts due to its sensitivity and its capacity for detecting almost all kind of compounds in the extracts. Its reproducibility, which is increased by the use of automatic spotting, was assessed using several standard compounds (data not shown) and proved to be adequate.

This experiment focused on flavonoids because many of these compounds have been proven to bind the adenosine A1 receptor.[8,17–18]

The TLC solvent system for flavonoids, which proved to be the best for flavonoids in the different *O. stamineus* extracts prepared chloroform: ethylacetate (5:5). Other solvent systems consisting of mixtures of chloroform and methanol, chloroform and ethanol, *n*-hexane and ethylacetate allowed for the detection of less spots compared to that of the chosen mixture.

Table 1 shows the adenosine A1 replacement activity of the tested *O. stamineus* as extracts. As can be observed, only the *n*-hexane and chloroform extract were active. In order to detect the compounds which could contribute to this activity, the extracts were analyzed by TLC and the data obtained in the corresponding chromatograms was transferred to SIMCA codec software to correlate their activity and calculate their statistic importance by means of multivariate data analysis (orthogonal-partial least square-OPLS).

Figure 1 shows the score scatter plot of OPLS based on the TLC chromatogram. The similarity of the samples with respect to the intensity of spots and activity correlates with their grouping pattern. The active samples (A–F) were separated from the non active samples (G–L).

The loading plot of OPLS (Figure 2) showed the correlation of the spot to the activity. The color intensity in the loading plot determine the degree of correlation of these spots to the activity, the more active being those which are whiter. As can be observed, spots 1–6 and 8 were thus considered to be active while spot 7 was a non active spot as it was black. However, some spots such as 3 and 5 consist of white spots surrounded by black spots and can thus also be considered as active spots, since the black spots surrounding both spots correspond to non active compounds

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Table 1. Results of adenosine A1 receptor-binding assay with extracts prepared from *O. stamineus*. Concentration of assayed samples was 0.25 mg/ml in DMSO

Extract	Replication	Sample code	Adenosine A_1 replacement (%)
n-Hexane	1	A	76.02
	2	B	85.97
	3	C	69.86
$CHCl_3$	1	D	61.87
	2	E	68.82
	3	F	71.06
n-BuOH	1	G	21.76
	2	H	35.91
	3	I	28.46
Water	1	J	24.10
	2	K	19.62
	3	L	20.80

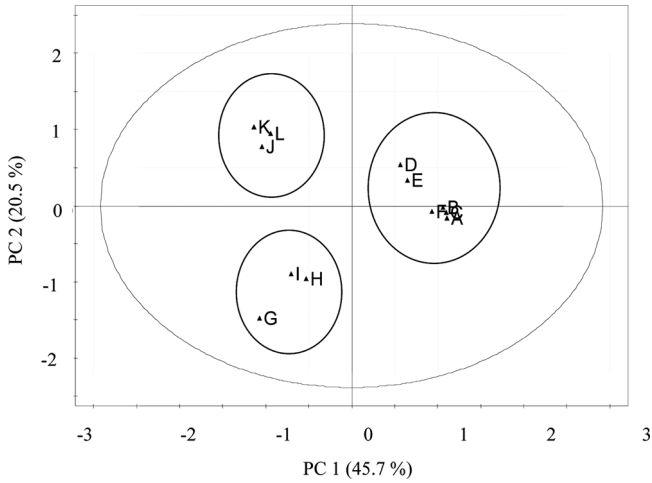

Figure 1. Score scatter plot of orthogonal partial least squares of *O. stamineus* leaves extract using principal component 1 (PC1) and principal component 2 (PC2) based on TLC chromatogram. PC 1 and PC 2 could explain the 45.7% and 20.5% variation, respectively. A–L are the sample codes related to different extraction solvents: A–C = *n*-hexane extract, D–F = chloroform extract, G–I = *n*-butanol extract, and J–L = water extract.

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overlapping with the active one. Both spots 3 and 5 are, therefore, a mixture of active and non active compounds.

In our previous work on this plant, 7 methoxy flavonoids which bind adenosine A1 receptor had been isolated.[8] Those flavonoids were analyzed by TLC using the same solvent system as that of the loading scatter plot (Figure 2). The retention time and color of these methoxyflavonoids coincided with those of spots 2, 3, 4, and 5. Spots 2, 3, and 4 were found to actually be produced by the overlapping of two methoxy flavnonoids each. In the case of spot 2, pillion and eupatorin were identified while spot 3 consisted in a mixture of 3′-hydroxy-4′,5,6,7- tetramethoxyflavone and sinensetin and spot 4 had tetramethylscutellarein and eupatoretin. Spot 5 was identified as 5,6-dihydroxy-7,4′-dimethoxyflavone and spots 1, 6, 7, and 8 corresponded to unknown compounds.

In order to confirm the chemical structures of the compounds of the TLC chromatogram, all extracts were analyzed by ^{1}H NMR spectroscopy. The data derived from ^{1}H NMR spectroscopy and the activity data of the extracts were analysed statistically by OPLS. The resulting scatter plot coincided with the pattern observed when using TLC. However, the loading scatter plot did not show methoxy flavonoids as the compounds responsible for the activity (Figure 3), since none of the chemical shifts close to activity (δ 1.34, 2.18, 5.74, and 6.14) could be attributed

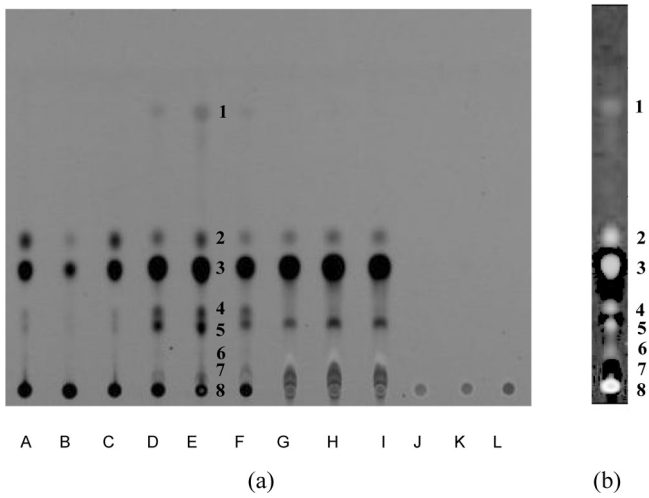

Figure 2. TLC chromatograms of different extracts of *O. stamineus* leaves under UV detection ($\lambda = 254$ nm): A–C = *n*-hexane extract, D–F = chloroform extract, G–I = *n*-butanol extract, and J–L = water extract. The TLC solvent system, $CHCl_3$:EtOAc (5:5), was able to detect 8 spots (a). The loading plot of orthogonal partial least squares based on the TLC chromatograms. Whiter spots exhibit most activity (b).

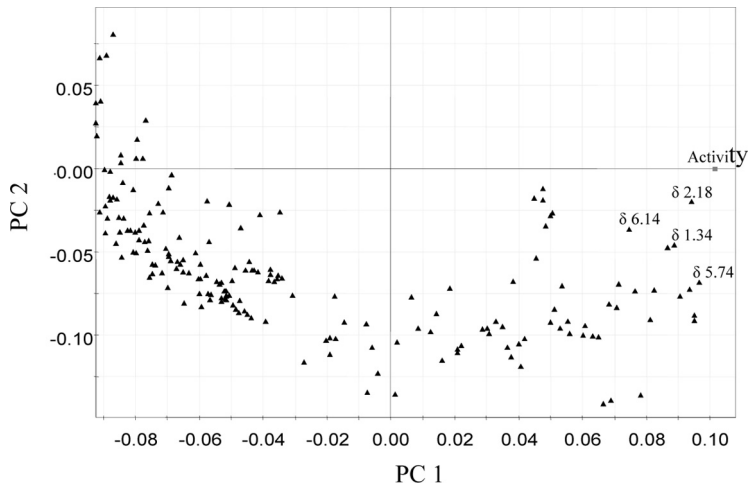

Figure 3. Loading scatter plot of orthogonal partial least squares analysis of *O. stamineus* leaves extracts using principal component 1 (PC1) and principal component 2 (PC2) based on ^{1}H NMR. Adenosine A1 receptor binding activity was related to δ 1.34, 2.18, 5.74, and 6.14.

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to methoxy flavonoids. This could be due to the absence of a significant contribution of these compounds due to the low intensity of their signals as compared to that of major compounds. In this case, the sensitivity of NMR spectroscopy proved to be insufficient to detect the minor amount of methoxy flavonoids present in the extracts.

The loading scatter plot of NMR analysis revealed the possibility of the contribution of some fatty acids or terpenoids to the activity since δ 1.34, 2.18, 5.74, and 6.14 correspond to the chemical shifts of these compounds. This was in agreement with the result from TLC analysis, since spot 1 was suspected to correspond to a non-polar compound.

This is the first report on the application of the combination of TLC of different polarity extraction, bioassay, and multivariate data analysis to identify the active compounds from plant extracts. This approach was effective to estimate some methoxy flavanoids to be responsible for the activity, which is in agreement with our previous isolation work.[8]

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CONCLUSION

Systems biology is a good approach to identify possible active compounds possessing adenosine A1 receptor binding activity in *O. stamineus*. The combination of TLC, activity data from the sample, and multivariate data analysis based on orthogonal partial least squares, is useful for this approach. Methoxy flavonoids, fatty acids, or terpenoids are the compounds which could be responsible for the tested activity. The results obtained in this study are thus in agreement with our previous work on the isolation of methoxy flavonoids possessing this activity from leaves of *O. stamineus*.

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