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

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Chapter 5

Identification of 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. 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 methoxy flavonoids, fatty acids and terpenoids were estimated to be related to this activity. This report offers a simple and quick method to identify active compounds as compared to bioassay guided fractionation method.

Keywords: TLC, MVDA, adenosine receptor, *Orthosiphon stamineus*

Khatib and Yuliana *et al*., Identification of possible compounds possessing adenosine A1 receptor binding activity in the leaves of *Orthosiphon stamineus* using TLC and multivariate data analysis. Journal of Liquid Chromatography & Related Technologies 2009; 32 (19): 2906-2916.

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 (419). Though a great amount of biologically active compounds have been isolated from *Orthosiphon* stamineus (297, 298, 313-315), very few scientific reports have been published to support these reported activities.

Our group reported the isolation of seven methoxy flavonoids possessing adenosine A1 receptor binding activity from this plant by conventional bioassay guided fractionation (420). This approach sometimes can be useful but it is an elaborative task 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 is the objective of this study.

Many scientists have proposed the possibility of the application of systems biology for this purpose (4, 338, 341). In this new approach, the traditional research methodology which focuses on single compounds and single targets is replaced by a holistic approach which aims at the analysis of all 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 the 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 with 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 (13). Another possibility to be explored is the use of thin layer

chromatography (TLC). Thin layer chromatography is known to be a simple and 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 *O. stamineus* using a systems biology approach by combining TLC, activity data from the sample, and multivariate data analysis.

Material and methods

Plant material

Orthosiphon stamineus dried plant material (leaves) 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, NY, 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. Thin layer chromatography plates (10 cm x 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^{o}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 x 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^{o}C). The spots on the TLC plate were visualized with an UV lamp (λ= 254 nm) and the images were recorded with Fuji image scanner (Fujifilm Life Science, Stamford, CT, USA).

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. The ^{1}H NMR was recorded at 25^{o}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^{o} (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 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 adenosine A1 receptor bioassay can be found in Chapter 2.

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.

Result 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 proved to bind to the adenosine A1 receptor (300, 326, 420). The TLC solvent system which proved to be the best for flavonoids in the different *O. stamineus* extracts was chloroform: ethyl acetate (1:1). Other solvent systems consisting of mixtures of chloroform and methanol, chloroform and ethanol, *n*-hexane and ethyl acetate detected less spots compared to that of the selected eluent.

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 were transferred to SIMCA codec software to correlate their activity and calculate their statistic importance by means of supervised multivariate data analysis (orthogonal partial least square-OPLS).

Figure 1A 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 (412).

The loading plot of OPLS, as can be seen in Figure 1B(ii), showed the correlation of the spot to the activity. The color intensity in the loading plot determines

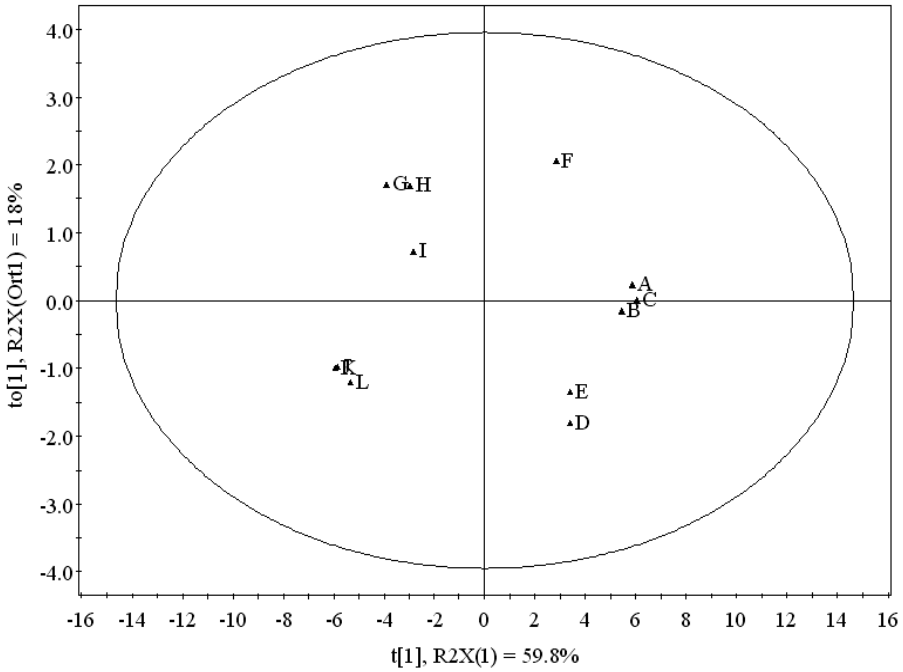
the degree of correlation of these spots to the activity, the more active being those which are whiter. As can be observed, spots 1-5 and 7 were considered thus to be active while spot 6 was non active spot as it was black. However, some spots such as 3 and 5 consist of white spots surrounded by black spots and can thus also be considered as active spots since the black spots surrounding both spots correspond to non active compounds overlapping with the active one. Both spots 3 and 5 are therefore a mixture of active and non active compounds.

Table 1. Results of adenosine A1 receptor-binding assay with extracts prepared from *O. stamineus*. Concentration of assayed samples was 35 µg/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
Chloroform	1	D	61.87
	2	E	68.82
	3	F	71.06
n-Butanol	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

In our previous work on this plant, seven methoxy flavonoids which bind to adenosine A1 receptor were isolated (420). Those flavonoids were analyzed by TLC using the same solvent system as that of the loading scatter plot. The retention time and color of these methoxy flavonoids coincided with those of spots 2, 3, 4 and 5. Spots 2, 3 and 4 were each found to actually be produced by two overlapping methoxy flavonoids. In the case of spot 2 pillion and eupatorin were identified while spot 3 consisted of a mixture of 3'-hydroxy-4',5,6,7-tetramethoxyflavone and sinensetin and spot 4 of tetramethylscutellarein and eupatoretin. Spot 5 was identified as 5,6-dihydroxy-7,4'-dimethoxyflavone and spots 1, 6 and 7 corresponded to unknown compounds.

A.


4.0
3.0
2.0
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to[1], R2X(Ort1) = 18%
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t[1], R2X(1) = 59.8%
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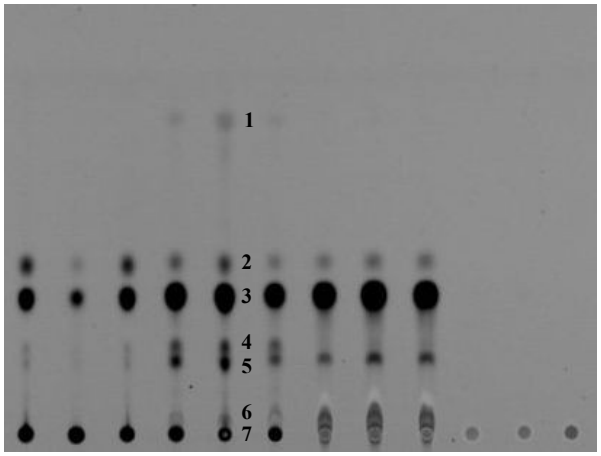
1
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A B C D E F G H I J K L

i


1
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ii

Figure 1. A. Score scatter plot of orthogonal partial least squares of *O. stamineus* leaves extract based on TLC chromatogram. 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. **B.** (i). TLC chromatograms of different extracts of *O. stamineus* leaves under UV detection (λ= 254 nm): The TLC solvent system, chloroform:EtOAc (5:5), was able to detect 7 spots. (ii). The loading plot of orthogonal partial least squares based on the TLC chromatograms. Whiter spots exhibit most activity.

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 analyzed statistically by OPLS. The resulting scatter plot coincided with the pattern observed when using TLC. In loading bi-plot, δ 3.74 can be attributed to methoxy flavonoids. Although it does not appear as the only group of compounds responsible for the activity (Fig. 2) since there are other chemical shifts closer to activity (δ 1.34, 2.18, 5.74 and 6.14). The loading bi-plot of NMR analysis revealed the possibility of the contribution of some unsaturated 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.

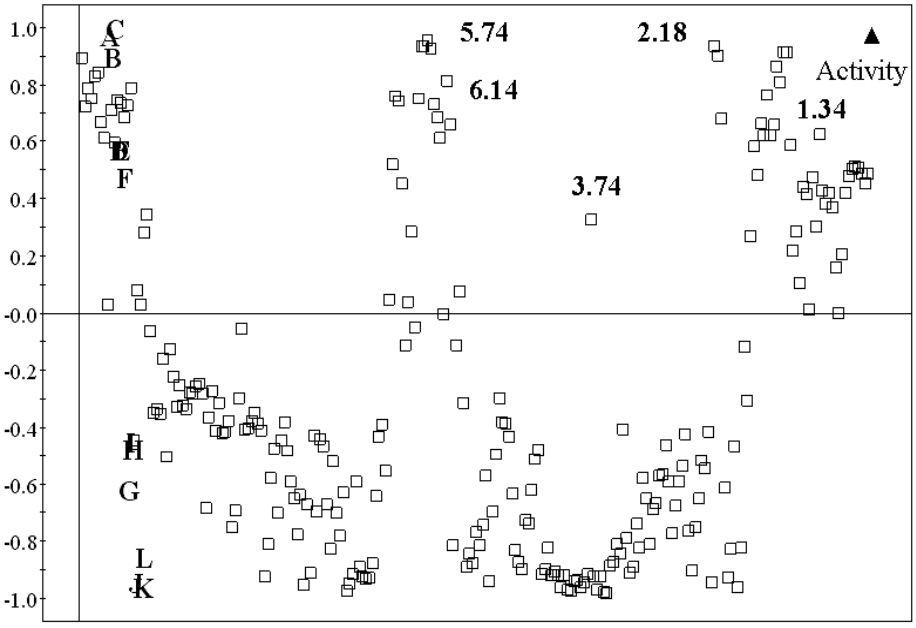

Figure 2. Loading bi-plot of orthogonal-partial least squares analysis of *O. stamineus* leaves extracts based on ^{1}H NMR. Adenosine A1 receptor binding activity was related to δ 1.34, 2.18, 5.74 and 6.14.

This is the first report on the application of this combination of TLC of different polarity extracts, bioassay, and multivariate data analysis to identify the active compounds from plant extracts. This approach was effective to correlate some methoxy flavonoids to the activity, which is in agreement with our previous isolation work (420).

Conclusions

System 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 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*.