NMR metabolomics for identification of adenosine A1 receptor binding compounds from *Boesenbergia rotunda* rhizomes extract

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**A B S T R A C T**

**Ethnopharmacological relevance:** *Boesenbergia rotunda* Linn. (Zingiberaceae) is traditionally used in many Asian countries as medicine for stomach pain and discomfort, viral and bacterial infection, inflammation, and as diuretic agent.

**Aim of the study:** The study aimed to identify adenosine A1 receptor binding compounds from *Boesenbergia rotunda* rhizome extract by using comprehensive extraction coupled to the NMR metabolomics method.

**Materials and methods:** Dried and powdered *Boesenbergia rotunda* rhizomes were extracted with the comprehensive extraction method to obtain several fractions with different polarity. Each fraction was divided into two: for NMR analysis and for adenosine A1 receptor binding test. Orthogonal projection to the least square analysis (OPLS) was used to study the correlation between metabolites profile and adenosine A1 receptor binding activity of the plant extracts. Based on Y-related coefficient and variable of important (VIP) value, signals in active area of OPLS loading plot were studied and the respective compounds were then elucidated.

**Results and discussions:** Based on OPLS Y-related coefficient plot and variable of importance value plot, several characteristic signals were found to positively correlate to the binding activity. By using 1D and 2D NMR spectra of one of the most active fraction, pinocembrine and hydroxy-panduratin were identified as the possible active compounds. Two signals from ring C of pinocembrine flavonone skeleton with negative coefficient correlations possibly overlapped with those of non-active methoxylated flavonones which were also present in the extract. NMR based metabolomics applied in this study was able to quickly identify bioactive compounds from plant extract without necessity to purify them. Further confirmation by isolating pinocembrine and hydroxy-panduratin and testing their adenosine A1 receptor binding activity to chemically validate the method are required.

**Conclusion:** Two flavonoid derivatives, pinocembrine and hydroxy-panduratin, have been elucidated as possible active compounds bind to adenosine A1 receptor. Flavonoid was reported to be one of natural antagonist ligand for adenosine A1 receptor while antagonistic activity to receptor is known to be associated with diuretic activity. Thus, the result of this research supports the traditional use of *Boesenbergia rotunda* rhizome extract as diuretic agent.

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1. Introduction

*Boesenbergia rotunda* (Linn.) Mansf. or *Boesenbergia pandulata* (Roxb.) Schltr. (Zingiberaceae) is widely used as a spice in some Asian countries such as Indonesia, Malaysia, and Thailand. It is also used as traditional medicine for stomach pain and discomfort, viral and bacterial infection, inflammation, and as diuretic agent.

A number of chalcones and flavonoids derivatives isolated from this plant has been reported as responsible compounds for the aforementioned medicinal uses (Abdelwahab et al., 2011; Bhamarapravati et al., 2006; Ching et al., 2007; Kiat et al., 2006; Mahmood et al., 2010; Morikawa et al., 2008; Tuchinda et al., 2002) except for diuretic activity. Antagonistic binding activity to adenosine A1 receptors have been reported to be associated with diuretics activity (Modlinger and Welch, 2003). Flavonoids form is a group of natural products which have been studied the most for its antagonistic activity to this receptor (Ji et al., 1996; Yuliana et al., 2009).

In this paper, the correlation of NMR signals of metabolites present in *Boesenbergia rotunda* extracts obtained from comprehensive extraction with its adenosine A1 receptor binding activity...
was studied. The aim was to quickly identify which compounds have strong correlation to the receptor binding activity. This combination of comprehensive extraction and NMR metabolomics approach has been successfully applied to identify seven methoxy flavonoids from _Orthosiphon stamineus_ which have binding activity to the receptor (Yuliana et al., 2011b). The method offers many benefits as compared to bioassay guided fractionation. Compounds that are important for the tested bioactivity can be easily identified to be further studied while common compounds that may cause false positive can be discarded at very early stage (Yuliana et al., 2011a).

### 2. Materials and methods

#### 2.1. Plant material extraction

Dried _Boesenbergia rotunda_ rhizomes were purchased from a traditional market in Bandung, Indonesia and were identified by one of the author (N.D. Yuliana). The voucher specimen was stored at Natural Products Laboratory, Leiden University. The rhizomes were powdered and subjected to comprehensive extraction with protocol as follow: 0.70 g of _Boesenbergia rotunda_ powder was mixed with 0.05 g Kieselguhr, packed into stainless steel extraction column (L=4.00 cm, d=1.80 cm). The column was closed at both ends with fat free cotton and connected to a Waters 600E pump (Waters, Milford, MA). Organic solvents and filtered milli-pore water (500 mL each) were ultrasonicated and degassed before use. The combination of solvents used was n-hexane (A), acetone (B), and acetone–water 1:1 (C). The solvent was continuously delivered into the column in gradient (see Table 1). The fractions were collected in 10 mL tubes every 2 min with an automatic fraction collector and every 2 samples were combined to obtain 17 fractions at the end of extraction. The extraction was performed in 3 replicates. From each extraction 4 ml was sampled for bioassay, other 12 ml for NMR. All were dried under N2 and put overnight in freeze drier before analysis. Concentration of the extracts for the bioassay and NMR were adjusted to 1.4 mg/ml DMSO and 5–10 mg/ml MeOD, respectively.

#### 2.2. NMR measurement and data analysis

NMR measurements were performed according to Kim et al. (2010). The solvent used was MeOD. The 1H NMR spectra were automatically reduced to ASCII files. Bucketing was performed by AMIX software (Bruker, Karlsruhe, Germany). Spectral intensities were scaled to total intensity and reduced to integrated regions of equal width (0.04) corresponding to the region of δ 0.3–10.0. The regions of δ 4.75–4.90 and δ 3.28–3.34 were excluded from the analysis because of the residual signal of D2O and MeOD, respectively. Orthogonal projection to the latent structure (OPLS) analysis were performed with the SIMCA-P software (v. 12.0, Umetrics, Umeå, Sweden) with scaling based on the Pareto method.

#### 2.3. Adenosine A1 receptor bioassay

The assay was performed as previously described (Chang et al., 2004) 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 [3H] DPCPX (8-cyclopentyl-1,3-dipropylxanthine) (Kd=1.6 nM). Membranes were prepared from Chinese hamster ovary (CHO) cells stably expressing human adenosine receptors by a method previously described (Dalpiaz et al., 1998). Non-specific binding was determined by using 10 μM CPA (N6-cyclopentyladenosine). The mixture consisting of 50 μL [3H] 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.

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**Table 1** Comprehensive extraction scheme.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Gradient</th>
<th>Flow rate (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–12</td>
<td>A 100%</td>
<td>4</td>
</tr>
<tr>
<td>12–32</td>
<td>A 100%–B 100%</td>
<td>4</td>
</tr>
<tr>
<td>32–44</td>
<td>B 100%</td>
<td>4</td>
</tr>
<tr>
<td>44–64</td>
<td>B 100%–C 100%</td>
<td>4</td>
</tr>
<tr>
<td>64–80</td>
<td>C 100%</td>
<td>4</td>
</tr>
</tbody>
</table>

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**Fig. 1.** Adenosine A1 receptor binding activity profile of _Boesenbergia rotunda_ fractions obtained from comprehensive extraction.
3. Results and discussion

3.1. Adenosine A1 receptor binding activity profile

Adenosine A1 receptor binding activity profile of Boesenbergia rotunda fractions can be seen in Fig. 1. Apparently this plant is a potential source for adenosine A1 receptor ligands. The highest binding activity (> 60%) was found in fraction 1–4 and 7–12. Boesenbergia rotunda has been previously reported as a rich source of flavonoid and chalcone derivative compounds (Abdelwahab et al., 2011; Bhamarapravati et al., 2006; Ching et al., 2007; Kiat et al., 2006; Mahmood et al., 2010; Morikawa et al., 2008; Tuchinda et al., 2002). To study if indeed these compounds contribute to Boesenbergia rotunda’s excellent bioactivity profile, we further observe metabolite – bioactivity correlation pattern by using multivariate data analysis (OPLS).

3.2. Multivariate data analysis (OPLS)

The OPLS score plot of Boesenbergia rotunda fractions (Fig. 2) showed that fractions which have higher binding activity (coloured in yellow to brown) are grouped separately from the less active ones (coloured in green to blue). The cumulative $R^2_Y$ and $Q^2$ value were 0.858 and 0.828, respectively. Cross validation with ANOVA gave the $p$ value of 7.316 × 10⁻¹⁰. These showed that the OPLS is statistically valid. To further investigate which compounds are responsible for the grouping, the OPLS loading Bi-plot (Fig. 3) was studied.

In the loading bi-plot, the right part of the plot is the active area. The study was then focused on fractions and NMR chemical shifts located in this area. It is shown that the active NMR signals can be divided into 4 groups, those are several signals located between 0.60 and 2.88 ppm (1), 4.60 and 5.50 ppm (2), 5.50 and 8.00 ppm (3), and 9.00 and 10.00 ppm (4). Area 1 represents typical signals for unsaturated fatty acids. Unsaturated fatty acids such as linoleic acid, has been reported to bind unspecifically to the adenosine A1 receptor (Ingkaninan et al., 1999). Signals in this area can also be attributed to the methyl or methylenes group of prenylated flavonoids (rotundaflavone) or prenylated chalcones (krachaizin) since Boesenbergia rotunda was also reported to contain several compounds belong to this group (Morikawa et al., 2008). Area 2 represents signals from methine and olefinic...
protons which may belong also to prenyl units of chalcones and flavonoids. Signals in area 3 are characteristic signals for protons in aromatic ring, while area 4 can be attributed to hydroxyl protons of these chalcones and flavonoids. (Fig. 4)

Apart from the active area, it can be noticed that signals between 3.00 and 4.60 ppm are located in less active area. Among compounds isolated from *Boesenbergia rotunda*, some of them have methoxyl groups attach to the aromatic rings, such as krachaizin A, krachaizin B, rotundaflavone 1a, panduratin A, isopanduratin A, pinostrobin, alpinetin, and cardamonine. In the proton NMR spectra of *Boesenbergia rotunda* fractions, methoxyl signals appears as tall singlets at 3.75–3.95 ppm. These signals are more abundant in less active fractions (the last four fractions of all replications) as mentioned in its Xvar plots. Then it can be predicted that the responsible compounds for adenosine A1 receptor binding activity of *Boesenbergia rotunda* are not compounds with methoxyl substituents. The two possibilities could be hydroxy-panduratin, a cyclohexenyl chalcone derivative, and pinocembrin (5,7-dihydroxyflavanone), a flavanone derivative (Fig. 5).

Fraction 1a as one of the most active fractions was taken for 2D NMR analysis and the presence of hydroxy-panduratin and pinocembrin was then elucidated based on the previously reported NMR data (Ching et al., 2007; Tuchinda et al., 2002). Typical signals of these two compounds were found to be present in the fraction. There are slightly differences in chemical shifts since the compounds were present in the mixture, not as the pure one as those in the previous reports.

The assignment of NMR signals of pinocembrin and hydroxy-panduratin are described below while the summary of Y-related coefficient and variable of important (VIP) value are presented in Table 2.

### 3.2.1. Pinocembrin

Multiplet at 7.45 ppm attributed to H-2', H-3', H-4', H-5' and H-6' protons confirmed with J-resolved. In HMBC the protons are correlated to carbon at position 2' and 6' (125.93 ppm), and to carbon at position 3', 4', 5', and 6' (128.30 ppm). Double doublets at 5.52 ppm (J = 3, J = 3) attributed to proton at position 2 as confirmed with J-resolved. In COSY spectra the proton is correlated to 2 protons at position 3 (2.84 and 2.75 ppm).

In HMBC the proton is correlated to its direct carbon at position 2 (98.45 ppm). Two double doublets at 2.75 (J = 17, J = 3) and 3.01 (J = 17, J = 12) ppm are attributed to H-3 as confirmed with J-resolved spectra. In HMBC, the protons are correlated to direct carbon at 42.62 ppm. Next, doublet at 5.80 ppm is attributed to two protons at position 6 and 8 (J = 2) as confirmed in J-resolved and in HMBC it is correlated to its direct carbon at 92.39 ppm.

### 3.2.2. Hydroxy-panduratin

Multiplets at δ 7.17 and δ 7.41 are attributed to 5 protons of monosubstitute benzene (H-2', H-3', H-4', H-5', H-6'), in HMBC they correlate to δ 124.96 (C-4''), δ 125, 89 (C-2' and C-6'') and δ 127.81 (C-3' and C-5''). A broad singlet at δ 5.89 attributed to H-3 and H-5 and correlate to δ 94.74 (C-3 and C-5), γ,γ-dimethyl allyl protons appeared as broad singlet at δ 1.53 (H-4'') which correlate to its direct carbon at δ 24.34 (C-4''). Multiplet at δ 5.49 is assigned as H-4' which correlate to its direct carbon C-4' (125.90).

All hydroxy-panduratin characteristic signals were found to have positive Y-related coefficients. However, two signals of H-3 for pinocembrin have negative Y-related coefficient value, while others are positive and having high coefficient value (> 0.50). It is possible that the signals overlaps with those of methoxylated flavonones found in this plant, such as methoxylated pinocembrin,
pinostrobilin, sakuranetin, panduratin, or boesenbergin. The last is predicted as not active in OPLS loading bi-plot.

The NMR based metabolomics strategy to quickly identify bioactive compounds presented in this study is a promising tool to solve complications related to the natural occurrence of natural products as a complex mixture. However, there are several limitations that should be taken into consideration to avoid a false conclusion when one wants to use this approach. In this study, the NMR data was normalised by scaling to total intensity. With such a data-processing approach, the normalised NMR signals of compounds found in different fractions with the same concentration may significantly differ if the concentration and/or concentrations of other compounds in these fractions are different. This may lead to a non-linear correlation of the activity value of the samples and the normalised intensity levels of its NMR signal. In another case, when the samples contain very highly active compounds, or when it contain several compounds whose activity levels are greatly different, the weak NMR signals of highly active compounds may merge into the noise or be overlapped/covered by the signals of other active compounds.

Looking to the previously mentioned limitations of the presented method, further works to chemically validating bioactivity prediction of pinostrobilin and hydroxy-panduratin need to be conducted. The two compounds have to be isolated and tested to the respective receptor.

4. Conclusion

Comprehensive extraction coupled to NMR metabolomics was established here to study the correlation between adenosine A1 receptor binding activity and metabolite profile of the resulted *Boesenbergia rotunda* fractions. Two compounds previously reported to be present in this plant, pinostrobilin and hydroxy-panduratin, were predicted to be compounds responsible for the adenosine A1 binding activity of *Boesenbergia rotunda* fractions. The prediction based on their positive Y-related coefficient and high VIP value (> 0.50). Two signals of pinostrobilin have negative Y-related coefficient value. The possibility is that the signals overlap with those of other flavanone derivatives which are not active to the receptor. Further confirmation by isolating and testing these two compounds to the reported activity is required. Identification of active compounds from plant extracts by using the combination of comprehensive extraction and NMR metabolomics was found to be more efficient than bioassay guided fractionation since one can focus to the real actives and ignore others which are reported as false positive in the respective bioassay test.

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References


