Determination of gallic acid and rutin in extracts
*Cassia alata* and *Andrographis paniculata*

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**ABSTRACT**: This study determines gallic acid and rutin in ethanol, methanol, and acetic acid extracts of *Cassia alata* and *Andrographis paniculata*. A preliminary study using thin layer chromatography and 0.2% DPPH (2,2-diphenyl-1-picrylhydrazyl) spraying found that gallic acid was present in all three solvent extracts. Antioxidant components of all extractions were then analysed using high performance liquid chromatography with a C\(^18\) reverse-phase column at a flow rate of 0.50 ml/min and 25 °C. The results showed high components of gallic acid and rutin in all extracts of *C. alata*. The amounts of gallic acid found in *C. alata* extracted with ethanol, methanol, and acetic acid were 366, 410, and 258 mg/g crude extract, respectively. Amounts of gallic acid found in *A. paniculata* extracted with ethanol, methanol, and acetic acid were 309.4, 351.2, and 309.1 mg/g crude extract, respectively. Rutin concentrations found in *C. alata* extracted with ethanol, methanol and acetic acid were 194.4, 193.6, and 120.0 mg/g crude extract, respectively. The highest gallic acid level was found in *C. alata* extracted with methanol, while the highest rutin levels were found in *C. alata* extracted with ethanol and methanol.

**KEYWORDS**: antioxidant, DPPH, TLC, HPLC

**INTRODUCTION**

Reactive oxygen species (ROS), including superoxide radical anion (O\(^2^-\)), hydrogen peroxide (H\(_2\)O\(_2\)), hydroxyl radical (OH\(^-\)), singlet oxygen (O\(^*\)), are generated during normal physiological activities of the human body. ROS have been linked with ageing and several diseases such as cancer, inflammation, immune system decline, cardiovascular disease, neurological disease, and atherosclerosis. Since synthetic antioxidants have potential health hazards, natural free-radical scavengers have become more interesting to scientists. Antioxidants occurring in fruits and vegetables are believed to prevent and even repair oxidative stress which is thought to damage body cells and to be linked to diseases such as cancer, heart disease, Alzheimer’s disease, and Parkinson’s disease. Vitamin C and vitamin E, carotenoids, and selenium are the principal antioxidants available in the body. Clinical trials using supplements of vitamin C, vitamin E, and carotenoids have shown inconsistent results and have led to the belief that whole fruits and vegetables rather than the individual compounds they contain are likely to give more positive results in disease prevention\(^1\).

Rutin or quercetin-3-rhamnosyl-glucoside, is a natural flavone derivative with significant scavenging properties on oxidizing species such as OH\(^-\), O\(^2^-\), and the peroxyl radical. Due to its biological effects, it has been widely used in treating disease. Rutin pharmacological properties include: antiallergic, anti-inflammatory and vasoactive, antitumour, antibacterial, antiviral, and antiprotozoal, in addition to hypolipidaemic, cytoprotective, antispasmodic, and anticarcinogenic properties\(^2\).

*Andrographis paniculata* (Burm.f.) Nees., known in Thai as ‘fa-thalai-chon’ (Fig. 1), is an important medicinal plant in India, China, Thailand, and Scandinavia. The aerial parts of the plant (leaves and stems) are normally used for the extraction of the
active phytochemicals. Extracts of the plant and their constituents have been reported to exhibit a wide spectrum of biological activities of therapeutic importance including antibacterial, antiviral, anti-inflammatory, antimalarial, immunostimulant, hepatoprotective, antithrombotic, anticancer, hypoglycaemic, and hypotensive properties\(^5\).

Where *Cassia alata* L., known in Thai as ‘chumhet-thet’ (Fig. 2), is recommended for primary health care in Thailand to treat ringworm, constipation, and skin disease. Several reports have described antimicrobial substances from *C. alata*\(^6,7\).

The objectives of this study were to determine whether the antioxidant compounds, gallic acid and rutin, are present in *C. alata* and *A. paniculata* extracts using HPLC.

**MATERIALS AND METHODS**

**Materials**

The samples of *A. paniculata* were collected from Chiang Mai Province and the samples of *C. alata* were collected from Phitsanulok Province, between May and November 2011.

The HPLC grade gallic acid, catechin, tannic acid, and rutin were from Sigma (St. Louis, MO, USA). Acetonitrile, distilled water, 99% acetic acid, 99% methanol, 99% ethanol, chloroform, and silica gel 60 F\(_{254}\) TLC plate were purchased from Merck (Darmstadt, Germany). DPPH (2,2-diphenyl-1-picrylhydrazyl) was from Sigma (St. Louis, MO, USA).

**Sample preparation**

Plants were cleaned and cut into small pieces before being dried at room temperature and subjected to extraction with three solvents; 99% acetic acid, 99% methanol, and 99% ethanol. Each extraction step was completed within 24 h. The extracts were filtered (Whatman No. 1) and concentrated in a rotavapor apparatus at approximately 40°C. Finally, the concentrated extracts were dried using a freeze-vacuum dryer (FTS System, Flexi-Dry) and stored at −40°C for the measurement of antioxidant compounds.

**Thin layer chromatography analysis**

Crude extracts were spotted on aluminium plates of silica gel 60 F\(_{254}\) thin layer chromatography (TLC) plates (Merck, Darmstadt, Germany) and were developed in a mobile phase consisting of chloroform, acetic acid, and methanol solution (80:16:4 v/v/v).
After development, TLC plates were dried at room temperature. The resulting bands were sprayed with 0.2% (w/v) DPPH solution in methanol. Gallic acid, catechin, tannic acid, and rutin were used as reference compounds (standards). Free radical scavenging zones and $R_f$ values were identified immediately as yellow areas against a purple background. High-performance liquid chromatography (HPLC) was then used in the next step to quantify antioxidant compounds.

**High-performance liquid chromatography analysis**

HPLC analysis was performed using a Varian Series PS 240, PS 325, PS 400 system equipped with an automatic injector and UV detector. The column used was a 5 µm Varian C18 column (250 mm × 4.6 mm). The temperature was maintained at 25 °C, with an injection volume of 20 µl, a flow rate of 0.5 ml/min, and a run time of 7 min. The sample and standards (gallic acid, catechin, and rutin) were eluted using a reverse-phase Varian C18 column with distilled water-acetonitrile (50:50 v/v) mobile phase and detected at 210 nm. The calibration graph was obtained by plotting the peak area versus concentration and the calibration curve was linear from 10–50 µg/ml.

**Statistical analysis**

Three replicate values for the antioxidant compounds from each sample were used for the statistical analysis. The ANOVA and means were carried out using a computer-based statistical program. Data were subjected to ANOVA and means ± SD compared by Duncan methods, which differences at $p \leq 0.05$ considered to be significant.

**RESULTS**

**Dry weight extracts**

Dry weight of *C. alata* and *A. paniculata* were obtained from three solvent extraction methods, i.e. ethanol, methanol, and acetic acid (Table 1). The results showed that acetic acid was the best solvent for *C. alata* (37.2 mg/g fresh) where methanol was the best solvent extraction for *A. paniculata* (59.6 mg/g fresh).

**TLC analysis**

Thin layer chromatography screening using chloroform, acetic acid and methanol (80:16:4 v/v/v) separated many compounds (Fig. 3). The compounds were identified by comparison with the standard used in the study. There were about two to four compounds presented in the *C. alata* and *A. paniculata* extracts.

### Table 1 Dry weight of *C. alata* and *A. paniculata* extracts.

<table>
<thead>
<tr>
<th>Solvents</th>
<th>C. alata (mg/g fresh)</th>
<th>A. paniculata (mg/g fresh)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ethanol</td>
<td>31.2</td>
<td>30.5</td>
</tr>
<tr>
<td>methanol</td>
<td>37.2</td>
<td>34.6</td>
</tr>
<tr>
<td>acetic acid</td>
<td>26.0</td>
<td>59.6</td>
</tr>
</tbody>
</table>

The $R_f$ values (ratio of the distance travelled by the solute to the distance travelled by mobile phase) of antioxidant compounds in the sample extracts and the standard are listed in Table 2.

Qualitative detection of antioxidant compounds in *C. alata* extract revealed compounds with $R_f$ values of 0.25, 0.27, and 0.25 extracted with ethanol, methanol, and acetic acid, respectively. (Fig. 3a), the first of which represented gallic acid ($R_f = 0.24$) where the other compound found at $R_f$ 0.41, 0.45, and 0.56.
Table 2  
$R_f$ values of *C. alata* and *A. paniculata* extracts and standards.

<table>
<thead>
<tr>
<th>Solvents</th>
<th><em>C. alata</em> $R_f$</th>
<th><em>A. paniculata</em> $R_f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ethanol</td>
<td>0.25, 0.41</td>
<td>0.23, 0.41, 0.47, 0.92</td>
</tr>
<tr>
<td>methanol</td>
<td>0.27, 0.45</td>
<td>0.22, 0.35, 0.41, 0.92</td>
</tr>
<tr>
<td>acetic acid</td>
<td>0.25, 0.56</td>
<td>0.23, 0.40, 0.43</td>
</tr>
<tr>
<td>catechin</td>
<td>0.11</td>
<td>0.11</td>
</tr>
<tr>
<td>gallic acid</td>
<td>0.24</td>
<td>0.24</td>
</tr>
<tr>
<td>Rutin</td>
<td>0.083</td>
<td>0.083</td>
</tr>
<tr>
<td>Tannic acid</td>
<td>0.15</td>
<td>0.15</td>
</tr>
</tbody>
</table>

of ethanol, methanol, and acetic acid, respectively, have not been identified. In *A. paniculata* extracts, compounds appeared with $R_f$ values of 0.23, 0.22, and 0.23 when extracted with ethanol, methanol, and acetic acid, respectively, (Fig. 3b) which represented gallic acid (standard $R_f = 0.24$). Rutin was not found in *C. alata* or *A. paniculata* (Fig. 3c). The identification and quantification of some compounds in the sample extracts were also confirmed by HPLC analysis.

![HPLC chromatograms](https://www.scienceasia.org)

**Fig. 4** HPLC chromatogram of *C. alata* extracted with (a) acetic acid, (b) methanol, and (c) ethanol and *A. paniculata* extracted with (d) acetic acid, (e) methanol, and (f) ethanol.

Table 3  Total gallic acid and rutin contents of *C. alata* and *A. paniculata* extracted with ethanol, methanol, and acetic acid (mg/g crude extracts).

<table>
<thead>
<tr>
<th>Solvent</th>
<th><em>C. alata</em></th>
<th><em>A. paniculata</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ethanol</td>
<td>366.3 ± 6.1$^b$</td>
<td>194 ± 29$^a$</td>
</tr>
<tr>
<td>methanol</td>
<td>410.0 ± 7.8$^a$</td>
<td>193.6 ± 9.5$^a$</td>
</tr>
<tr>
<td>acetic acid</td>
<td>257.6 ± 3.1$^c$</td>
<td>120.0 ± 3.0$^b$</td>
</tr>
<tr>
<td>Rutin</td>
<td>194 ± 29$^a$</td>
<td>351.2 ± 5.6$^a$</td>
</tr>
<tr>
<td></td>
<td>N/A</td>
<td>309.1 ± 4.7$^b$</td>
</tr>
</tbody>
</table>

N/A = not detected. Superscript letters a, b, and c indicate significant differences at $p < 0.05$.

High performance liquid chromatography analysis

The qualitative and quantitative analyses of *C. alata* and *A. paniculata* extracts were carried out using HPLC with a UV detector (Table 3 and Fig. 4). The components catechin, gallic acid, and rutin were identified by comparing retention times, with the retention times of catechin, gallic acid, and rutin standards being 4.659, 3.307, and 3.795, respectively (Fig. 5). Quantitative data for the sample extracts were calcu-
Fig. 5 HPLC chromatogram of standard solutions: (a) catechin, (b) gallic acid, and (c) rutin. Eluent was distilled water:acetonitrile (50:50 v/v); detection was done at 210 nm.

lated from their calibration curves for gallic acid and rutin. Values (mg/g) are expressed as mean ± standard error. Gallic acid was found to be the main identified component in the ethanol, methanol, and acetic acid in the C. alata and A. paniculata extracts. Gallic acid in the ethanol, methanol and acetic acid extracts of C. alata was found to be 366.3 ± 6.1, 410.0 ± 7.8, and 257.6 ± 3.1 mg/g crude extract, respectively, while gallic acid in the ethanol, methanol and acetic acid extracts of A. paniculata was found to be 309 ± 13, 351.2 ± 5.6, and 309.1 ± 4.7 mg/g crude extract, respectively. The rutin in the ethanol, methanol and acetic acid extracts of the C. alata was found to be 194 ± 29, 193.6 ± 9.5, and 120.0 ± 3.0 mg/g crude extract, respectively. However, rutin was not found in the three extracts of A. paniculata. Thus it was observed that the levels of gallic acid in the methanol extract of C. alata and A. paniculata were higher than that of the ethanol and acetic acid extracts, while the rutin content in the ethanol and methanol extracts of C. alata was comparable with others but was higher than that of the acetic acid extract.

DISCUSSION

This study found that methanol was the best solvent for the extraction of C. alata and A. paniculata since it produced the highest gallic acid levels. A similar observation has been reported by Phongpaichit et al. who found antifungal activity in leaf extracts of C. alata, C. fistula, and C. tora. In addition, Panichayupakaranant and Kaewsuwan, who used DPPH radical scavenging assay to investigate the antioxidant activity of crude methanol extracts from leaves, flowers, and pods of C. alata found that the leaf extract exhibited a stronger antioxidant activity than the flowers and pods. The highest gallic acid content was found in A. paniculata extracted with methanol, while rutin was not found in any of the three extracts. This finding agrees with the previous report of Akowuah et al. on the free radical scavenging activity of methanol extracts compared to water extract of A. paniculata.

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