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Keizo Hosokawa, Atsuyuki Hishida, Shin Nishiumi, Eri Fukushi, Jun Kawabata and Hitoshi Ashida
Full Length Research

Isolation and identification of compounds with dioxin-induced AhR transformation inhibitory activity from the leaves of Mallotus japonicus (Thunb.) Muell. Arg.

Keizo Hosokawa1*, Atsuyuki Hishida2, Shin Nishiumi3, Eri Fukushi4, Jun Kawabata4 and Hitoshi Ashida3

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The solvent extracts of 368 plant parts from 191 different species cultivated in Tsukuba (Ibaraki Prefecture, Japan) were previously screened for aryl hydrocarbon receptor (AhR) transformation inhibitory activity. It was observed that the leaf extract of Mallotus japonicus had strong activity. To clarify the antidioxin phytochemical, a 70% acetone extraction from the leaves was fractionated with three kinds of solvent (n-hexane, ethyl acetate, 1-butanol) in order of increasing polarity. The fractions of ethyl acetate and 1-butanol with strong antidioxin activity were purified by preparative high performance liquid chromatography in combination with AhR transformation assays by a cell-free system using a rat hepatic cytosolic fraction. The structures of isolated compounds were elucidated by nuclear magnetic resonance (NMR) techniques and fast atom bombardment mass spectra. As a result, six compounds (phyllanthusiin D, quercetin, quercitrin, isoquercitrin, rutin, and kaempferol-3-O-rutinoside) as the active components were isolated. The compounds suppressed AhR transformation in a concentration-dependent manner, with 50% inhibitory concentration (IC50) values of 0.12 µM (phyllanthusiin D), 0.45 µM (quercitrin), 0.97 µM (isoquercitrin), and 16 µM (rutin) against 1 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced AhR transformation. Quercetin and kaempferol-3-rutinoside exhibited very weak activity. Thus, phyllanthusiin D, which is a hydrolysable tannin, exhibited the greatest activity. This is the first report to demonstrate the antiodioxin activity of a tannin.

Key words: Mallotus japonicus, aryl hydrocarbon receptor (AhR) transformation, flavonol, tannin.

INTRODUCTION

Dioxins are some of the most toxic substances known to humans, and cause serious health problems, such as birth defects, cancer promotion, immunosuppression, and weight loss upon ingestion. The mechanism of toxicity for dioxins involves binding to cytosolic aryl hydrocarbon receptors (AhR) and subsequent receptor transformation...
(De Vito and Birnbaum, 1994).
Moreover, the initial step of the toxic action has been shown to be AhR dependent (Craig and Gustafsson, 1997). Thus, drugs or food components that suppress AhR transformation can be used to treat or prevent dioxin toxicity. The extracts of 368 plant parts from 191 species were previously screened for activity against dioxin-induced AhR transformation (Nishiumi et al., 2006). As a result, the extract from the leaves of *Mallotus japonicus* strongly suppressed AhR transformation. *M. japonicus* is a deciduous tree that sprouts red-colored buds and is widely distributed in Japan, where the bark is used as a natural remedy. There have been several reports on the chemical constituents of the different parts of *M. japonicus*, that is, the seeds, leaves, bark, fruits, and pericarp.

Furthermore, the compounds identified were found to exhibit a range of biological activities, including cytotoxicity (Arisawa et al., 1990a), antitumoral activity (Arisawa et al., 1990a), xanthine oxidase inhibition (Arisawa et al., 1990b), and antitumor effects (Arisawa et al., 1990b). However, no other biologically active compounds from this plant have been identified. On the other hand, the extract screening results led to the isolation and structural elucidation of an active AhR transformation inhibitor from the leaves of *M. japonicus* using preparative high-performance liquid chromatography (HPLC).

The present study isolated and identified AhR transformation inhibitors from *M. japonicus* leaves and evaluated their activities. While the majority of AhR transformation inhibitors previously reported were polyphenols (Ashida et al., 2000), the present study revealed a hydrolysable tannin as one of the AhR transformation inhibitors from *M. japonicus* leaves, which exhibited high activity and has not been reported previously. Thus, it is proposed that some tannins have the ability to suppress dioxin-induced AhR activity.

## MATERIALS AND METHODS

### Plant material

*M. japonicus* was deposited at the Tsukuba Division, Research Center for Medicinal Plant Resources, National Institute of Biomedical Innovation, Health and Nutrition (NIBIOHN) in Japan. Its accession number is 0095-79TS. Plants were cultivated and the leaves were collected in the experimental field of the Tsukuba Division of NIBIONH. The leaves were dried at about 55 to 60°C and used for the extraction.

### Extraction and isolation

Dried leaves (440 g) were extracted using 70% aqueous acetone (1.3 L) three times. The combined extracts (3.9 L) were concentrated to 1 L under reduced pressure using a rotary evaporator. The precipitate was then filtrated, and the supernatant was extracted using n-hexane (300 mL, 3 times), ethyl acetate (300 mL, 3 times) and 1-butanol (300 mL, 3 times) in order of increasing polarity. The resultant extracts were concentrated to dryness, and 26 mg, 4.2 g, and 20.6 g were obtained, respectively. Finally, a 45.7 g extract was obtained by evaporating the water layer. Active components were isolated from the ethyl acetate and 1-butanol fractions using preparative HPLC (SHIMADZU 10A system; Shimadzu Co., Kyoto, Japan), with a Cadenza 5CD-C18 column (20 × 150 mm; Imtak Co., Kyoto, Japan). The mobile phase for the ethyl acetate fraction was 10 to 40% CH3CN in water (0 to 30 min), and 40 to 90% CH3CN in water (30 to 60 min), at a flow rate of 4.0 mL/min and detection at 300 nm. Four fractions were obtained and all fractions were re-chromatographed by preparative HPLC. The mobile phase for the fractions was 10% CH3CN in water (0 to 20 min), 10 to 40% CH3CN in water (20 to 30 min), and 40 to 90% CH3CN in water (30 to 60 min). Further, the obtained fractions were re-chromatographed by preparative HPLC. The mobile phase for the fractions was 10% CH3CN in water (0 to 20 min), 10 to 90% CH3CN in water (20 to 50 min), at a flow rate of 4.0 mL/min and detection at 300 nm. As a result, five compounds (1, 22.9 mg; 2, 4.9 mg; 3, 2.7 mg; 4, 6.2 mg; and 5, 3.1 mg) were isolated. The mobile phase for the 1-butanol fraction was 10 to 90% MeOH in water (0 to 60 min), at a flow rate of 2.0 mL/min and detection at 254 or 400 nm. Three fractions were obtained and each fraction was re-chromatographed by preparative HPLC. The mobile phase for each fraction was 10 to 70% MeOH in water (0 to 60 min), at a flow rate of 2.0 mL/min and detection at 254 or 400 nm. As a result, three compounds (4, 14.0 mg; 5, 204.8 mg; and 6, 37.6 mg) were isolated.

### Recording of NMR spectra

NMR spectra of each compound were recorded on a Bruker AMX500 instrument (1H, 500 MHz; 13C, 125 MHz). Chemical shifts were determined relative to residual signals for methanol-d4 solvent (δH 3.3 ppm, δC 49.0 ppm). Field desorption and fast atom bombardment (FAB) mass spectra were obtained using a JEOL SX102A instrument.

### Measurement of AhR transformation by SW-ELISA

The antagonistic effects of the isolated compounds on AhR transformation were assessed using the in vitro cell-free system of Nishiumi et al. (2006). Briefly, a rat hepatic cytosolic fraction containing AhR was pre-incubated with various concentrations (0.1 to 50 μM) of each compound dissolved in methanol. After 20 min, the cytosolic fraction was treated with 1 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin or dimethylsulfoxide (10 μL/mL) as a vehicle control and incubated at 20°C for 2 h in the dark. AhR transformation was measured by SW-ELISA as described previously (Fukuda et al., 2004). Briefly, the reaction mixture consisted of 10 μL of HEDG buffer (25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 1.5 mM EDTA, 1.0 mM dithiothreitol and 10% (v/v) glycerol) containing 750 μM KCl (final concentration of 150 μM) and 40 μL of the cytosolic fraction after incubation as described earlier. The reaction mixture (50 μL) containing the transformed AhR was plated on a dioxin responsive element probe-bound 96-well microtiter plate, and AhR transformation was measured.

### RESULTS AND DISCUSSION

As reported previously in Nishiumi et al. (2006), the leaves of *M. japonicus* strongly suppressed AhR transformation. The dried leaves of *M. japonicus* were...
extracted with 70% aqueous acetone, and the crude extract was fractionated using different solvents and then subjected to preparative HPLC to yield six compounds (1-6, Figure 1) that exhibited antidioxin activity. Compounds 1-5 and 4-6 were obtained from the ethyl acetate and 1-butanol extracts, respectively. Thus, compounds 4 and 5 were isolated from both fractions.

The FAB mass spectrum of compound 1 showed [M – H]⁻ and [M + Na]⁺ ion peaks at m/z 991 and 1015, respectively, which is in good agreement with the mass calculated for C₄₄H₃₂O₂₆ (Table 1). Analysis of the ¹H and ¹³C NMR spectra indicated the presence of glucose, and four kinds of gallic acid derivatives (ring-A to D, Table 2) were observed. These data were almost identical to those of Yoshida et al. (1992). Accordingly, compound 1 was determined to be phyllanthusiin D. Yoshida et al. (1992) raised the possibility that phyllanthusiin D (1) found in *Phyllanthus flexuosus* leaves could be an artifact. As the phyllanthusiin D (1) isolated in the present experiment was not detected in the initial stage of the extraction, it may also be an artifact.

The FAB-mass spectrum of compounds 2 to 6 showed [M – H]⁻ ion peaks at m/z 301, 449, 463, 609 and 593, which agrees well with the masses calculated for C₁₅H₁₀O₇, C₂₁H₂₀O₁₁, C₂₁H₂₀O₁₂, C₂₇H₃₀O₁₆ and C₂₇H₃₀O₁₅, respectively (Table 1). Each ¹H NMR and ¹³C NMR spectra of the aglycones in compounds 2 to 5 were highly similar to each other. On the other hand, both spectra of the positions 2' (δH 8.06, δC 132.4), 3' (δH 6.89, δC 116.2) and 6' (δH 8.06, δC 132.4) in compound 6 were different from those of compounds 2 to 5. It was revealed that the aglycone of compounds 2 to 5 was quercetin and that of compound 6 was kaempferol. The sugar moiety in compounds 3 and 4 was a monoglycoside and that in compounds 5 and 6 was a diglycoside. From the spectral analysis of compounds 3 to 6, the sugar moieties were

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**Table 1. FAB-MS data for compounds 1 to 6.**

<table>
<thead>
<tr>
<th>Mass ion (m/z)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
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<tbody>
<tr>
<td>[M – H]⁻</td>
<td>991</td>
<td>301</td>
<td>449</td>
<td>463</td>
<td>609</td>
<td>593</td>
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<tr>
<td>[M + H]⁺</td>
<td>-</td>
<td>303</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>[M + Na]⁺</td>
<td>1015</td>
<td>-</td>
<td>471</td>
<td>487</td>
<td>-</td>
<td>617</td>
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</table>
identified as rhamnose, glucose, rutinose and rutinose, respectively. Ultimately, the isolated compounds were identified by comparison of their analytical data (Tables 1 and 3) with those in the literature (2, quercetin (Awaad et al., 2006); 3, quercitrin (Mendez et al., 1995); 4, isoquercitrin (Mendez et al., 1995); 5, rutin (Slimestad et al., 2008); and 6, kaempferol-3-rutinoside (Kazuma et al., 2003).

The antioxidin activity of the isolated compounds (1-6) is shown in Table 4. Phyllanthusiin D (1) showed the highest activity (IC₅₀ 0.12 μM), quercitrin (3) and isoquercitrin (4) exhibited moderate activities (IC₅₀ 0.45 and 0.97 μM, respectively), and rutin (5) exhibited weak activity (IC₅₀ 16 μM). The two other compounds (quercetin (2) and kaempferol-3-rutinoside (6)) showed low activities. The types of compounds previously reported to exhibit antioxidin activity were mainly flavonoids and catechins (Ashida et al., 2000; Xue et al., 2017). It is known that the antagonistic effect of flavonoids is attributable to its action as a ligand of AhR and the important moiety of structure are the non-polar or less polar molecules (Ashida et al., 2000), although the important structures of flavonoids for AhR activation are number of hydroxy groups (Jin et al., 2018).

On the other hand, catechins act as antagonists by binding to the AhR chaperone protein, hsp90 (Palermo et al., 2005; Yin et al., 2009). The important structure of catechins as antagonist is phenolic groups of the A-ring (Khandelwal et al., 2013). Although phyllanthusiin D (1) has many hydroxy groups and does not have the aromatic ring like A-ring of catechins, the strong activity was exhibited. The antagonistic effect of phyllanthusiin D (1) may be expressed by binding to both AhR and hsp90, since it is known that tannins have several biological and pharmacological activities (Okuda, 2005). A main property of tannins is protein precipitation, which is termed

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Table 2. ¹H and ¹³C NMR data for 1 (methanol-d₄).

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<tr>
<th>Positions</th>
<th>δ_H (mult., J in Hz)</th>
<th>Positions</th>
<th>δ_C</th>
<th>Positions</th>
<th>δ_C</th>
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<tr>
<td>Glucose</td>
<td></td>
<td>Glucose</td>
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<td>Ring-D</td>
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</tr>
<tr>
<td>H-1</td>
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<td>C-1</td>
<td>91.6</td>
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<td>H-2</td>
<td>5.45 s</td>
<td>C-2</td>
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<td>C-5</td>
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<td>63.5</td>
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<td></td>
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<td></td>
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<td></td>
<td>Ring-A</td>
<td></td>
<td>Ring-E</td>
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<td>C-2</td>
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<td>Ring-B/C</td>
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<td>C-3</td>
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Table 3. $^1$H and $^{13}$C NMR data for 2-6 (methanol-$d_4$).

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<td>$\delta_H$ (multu., J in Hz)</td>
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<td>$\delta_H$ (multu., J in Hz)</td>
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Further, tannins contain a polyphenolic moiety. It is speculated that tannins might be potential candidates as an antidioxin substance.

Conclusion

Six compounds (phyllanthusiin D, quercetin, quercitrin, isorhamnetin, rutin and kaempferol-3-rutinoside) were isolated and identified from the leaves of *M. japonicus* as antagonists of dioxin. Phyllanthusiin D exhibited the highest activity. The mechanism of action of the antagonists should be
further investigated. This work shows that the tannin phyllanthusiin D has antiodin activity and could be applied to the development of functional foods.

CONFLICT OF INTERESTS
The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS
The authors thank Ms. Mika Umino and Mr. Taishi Harada of the Department of Nutritional Management, Faculty of Health Science, Hyogo University for their assistance with this work.

REFERENCES

Table 4. ICso values against TCDD-induced AhR transformation of six compounds isolated from M. japonicas leaf extract.

<table>
<thead>
<tr>
<th>Compound</th>
<th>ICso (μM) against TCDD-induced AhR transformation</th>
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<tbody>
<tr>
<td>Phyllanthusiin D (1)</td>
<td>0.12 ± 0.02</td>
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<td>Quercetin (2)</td>
<td>.a</td>
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<tr>
<td>Quercitrin (3)</td>
<td>0.45 ± 0.04</td>
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<td>Isoquercitrin (4)</td>
<td>0.97 ± 0.11</td>
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<td>Rutin (5)</td>
<td>16 ± 1.0</td>
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<tr>
<td>Kaempferol-3-rutinoside (6)</td>
<td>.a</td>
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</table>

*aActivity that was measured between 0 and 100 μM was very low, so ICso value was not indicated.*
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