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Analysis of antioxidant prenylflavonoids in different parts of *Macaranga tanarius*, the plant origin of Okinawan propolis

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ABSTRACT

Objective: To analyze the antioxidant prenylflavonoids in different parts of *Macaranga tanarius* (*M. tanarius*) (Euphorbiaceae) including the leaf, petiole, stem, leaflet, flower and fruit (only in female plant), and to evaluate their antioxidant properties. **Methods:** Methanol extracts of each part of *M. tanarius* were prepared and five prenylflavonoids in them were quantitatively analyzed using HPLC. The fruits from female plant were further separated into seed, pericarp, and glandular trichome. After the quantitative analyses of prenylflavonoids in each part of *M. tanarius*, antioxidant activity of the extracts was evaluated by 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging assay. **Results:** The leaf of *M. tanarius* contained two prenylflavonoids as main components in both male and female plants. Both flowers (male and female) contained five kinds of prenylflavonoids. In the petiole, stem and leaflet of both male and female plants, the prenylflavonoids were not detected or their amounts were very low. Five kinds of prenylflavonoids were detected in the seed, pericarp and glandular trichome of female *M. tanarius*. In particular, the glandular trichome had the highest level of total prenylflavonoids (235 mg/g of fresh plant). DPPH radical scavenging activity of all parts was more than 30%. **Conclusions:** We found that different parts of *M. tanarius* contained antioxidant prenylflavonoids. In particular, not only the glandular trichome but also the leaf contained prenylflavonoids, which indicated that *M. tanarius* may be developed as a functional plant, because the leaves of this plant can be easily collected.

1. Introduction

Macaranga tanarius (*M. tanarius*, Euphorbiaceae) is a widely distributed tropical tree in Southern Asia. *M. tanarius* is a dioecious plant and is also a well-known pioneer tree and an ant-plant. Ants defend this tree against herbivores by producing food bodies that attractants[1-4]. In Thailand, the root of *M. tanarius* is used as an emetic agent, whereas the fresh leaves are used to cover wounds as an anti-inflammatory[5]. We have reported that *M. tanarius* is the plant origin of Okinawan propolis[6]. Propolis is a resinous hive product collected by honeybees. Honeybees in Okinawa, Japan use surface materials (glandular trichome) of *M. tanarius* fruits as propolis. We have found that Okinawan propolis and glandular trichome of *M. tanarius* contain several prenylflavonoids with potent antioxidant and antimicrobial activities[6,7]. Some of the prenylflavonoids identified from the glandular trichome of *M. tanarius* are present in the leaf of *M. tanarius*[8-12]. Thus, *M. tanarius* might be the plant with source of functional prenylflavonoids. However, quantitative analysis of the

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prenylflavonoids in various parts of *M. tanarius* has not been performed to date.

Thus, in this study, we collected male and female plants of *M. tanarius* and performed quantitative analysis of antioxidant prenylflavonoids present in different parts of *M. tanarius*, including the leaf, petiole, stem, leaflet, flower, and fruit (only in female plant). The fruits were further separated into seed, pericarp, and glandular trichome which is the surface material of *M. tanarius* fruits. After the quantitative analyses of prenylflavonoids in each part of *M. tanarius*, we examined its 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging activity. The qualitative characteristics of *M. tanarius* described in our study will be useful when it is used as a natural medicinal plant.

2. Materials and methods

2.1. Plant materials

M. tanarius was collected from Naha, Okinawa, Japan, in June 2008. Mature fruits of *M. tanarius* were obtained in this season. Although the age of the plant sample used in this study was unknown, the height of the tree was 2-3 m. The plant was identified by Mr. Tsuyoshi Miyagi, Okinawa Prefectural Forest Resources Research Center, Japan, where a voucher specimen was deposited.

2.2. Chemicals

Butylated hydroxytoluene was purchased from Kanto Chemicals (Tokyo, Japan). DPPH and α-tocopherol (vitamin E, VE) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Eriodictyol and naringenin were purchased from Funakoshi (Tokyo, Japan).

2.3. Preparation of extracts

Male and female of *M. tanarius* plants were separated into leaf, petiole, stem, leaflet, flower, and fruit. The fruits were further separated into seed, pericarp, and glandular trichome. Each part (1 g) was air-dried for 3 days and ground using a grinder. Then, each sample was extracted with 10 mL of methanol at room temperature for 3 days. The methanol extracts were filtered, and the filtrates were concentrated using a rotary evaporator.

2.4. Analysis of prenylflavonoids

Prenylflavonoids in the methanol extracts of each part of *M. tanarius* were analyzed using a Jasco Gulliver HPLC system (Tokyo, Japan) with a photo-diode array detector. The dried methanol extracts of the samples were dissolved in methanol again, and the extracts were then filtered with a 0.45-μm membrane filter. For the qualitative and quantitative analyses of the samples, we used a Capcell Pak UG120 C18 column (250.0 mm × 4.6 mm i.d.; Shiseido, Tokyo, Japan). The mobile phase consisted of 0.1% (v/v) trifluoroacetic acid in water (A phase) and 0.1% (v/v) trifluoroacetic acid in acetonitrile (B phase). The gradient was 20%-80% B phase (0-60 min), 80%-100% B phase (60-80 min), and 100% B phase (80-90 min), and the flow rate was 1.0 mL/min. Qualitative analysis of the prenylflavonoids was performed by monitoring the UV spectra in the 195-650 nm range at a rate of 0.8 spectra/s and a resolution of 4.0 nm. On the other hand, the peak areas by the absorption of UV at 280 nm were used for quantitative analysis. Prenylflavonoids isolated from Okinawan propolis were used as authentic compounds to identify each peak[13,14].

Another set of the extracts dissolved in methanol was analyzed by LC/MS to obtain more information for identifying each peak. A portion of the filtrate (5 μL) was subjected to SI-1 HPLC system (Shiseido, Tokyo, Japan) on a Capcell Pak UG120 C18 column (250.0 mm × 2.0 mm i.d., 5 μm). The mobile phase consisted of 0.1% (v/v) formic acid in water (C phase) and 0.1% (v/v) formic acid in acetonitrile (D phase). The gradient was 20%-80% D phase (0-60 min), 80%-100% D phase (60-80 min), and 100% D phase (80-90 min), and the flow rate was 0.2 mL/min. The elution of extracts was monitored at 270 nm and then introduced into the API-2000 mass spectrometer (AB Sciex, Framingham, MA, USA) equipped with an electrospray ion source. The operating parameters are as follows: source voltages, 5 kV; electrospray capillary voltage, -10 V; and capillary temperature, 250 ℃. All MS data were acquired in negative ionization mode. The antioxidant prenylflavonoids 1-5 from each part of *M. tanarius* were quantitatively analyzed. The calibration curve for each compound was obtained from replicate injections (n=3) of known amounts of the corresponding standards with the same flavonoid skeleton (eriodictyol for prenylflavonoids 1, 2, 3 and 5, and naringenin for prenylflavonoid 4). The limit of detection and quantification were determined at a signal-to-noise (S/N) ratio of 3 and 10, respectively. The limit of detection and quantification were determined using a serially diluted reference solution of each compound.

The reproducibility of the determinations was confirmed by analyzing five technical replicates of the standard solution. The precision was expressed using relative standard deviation. The reproducibility was evaluated on the basis of the result obtained at different times by different analysts

in the same laboratory. A recovery test was performed using the method of standard addition.

2.5. Free radical scavenging activity on DPPH

This assay was performed according to the method of Chen and Ho with some modifications[15]. The reaction mixture contained 2 mL of ethanol, 125 μmol/L DPPH, and 0.5 mg/mL of test samples. After incubation for 1 h at room temperature, the absorbance was recorded at 517 nm. The control solution contained only ethanol and DPPH. Results are expressed as percentage decrease with respect to control values. Butylated hydroxytoluene and VE at the same concentration were used as the positive controls.

3. Results

The contents of water and extract of each part of *M. tanarius* are shown in Table 1. Water content in each part was determined by subtracting dry weight from fresh weight. The weights shown in Table 1 were each weight (mg) per gram of the plant. Extraction efficiency (%) was determined from the yields of the methanol extracts.

Water contents in the parts other than the glandular trichome were 600-800 mg/g (Table 1). In the present study, each part of the plant was dried at room temperature for 3 days. The glandular trichome did not show a significant change in weight. Water content of the glandular trichome was very low (10 mg/g). Among all parts of the plant, the glandular trichome showed the highest extraction efficiency using methanol (48.5%). This indicates that the glandular trichome of *M. tanarius* consists mostly of hydrophobic constituents. On the other hand, the extraction efficiency of the leaf was more than 20.0%, which indicates that the leaf of *M. tanarius* also contains hydrophobic compounds.

We identified prenylflavonoids 1-5 in the methanol extracts of different parts of *M. tanarius* by HPLC with photo-diode array and MS detection. The chemical structures of the compounds identified are shown in Figure 1. We used the prenylflavonoids isolated from the Okinawan propolis as authentic compounds to identify each peak. First, the peaks were assigned by comparing the retention times and UV spectra of authentic standards by photo-diode array detection. Further, LC/MS analysis was performed to confirm the assignments of each peak to obtain the profiles of compounds present in the samples. Negative electrospray ion-MS of each HPLC peak corresponded to the molecular ions.

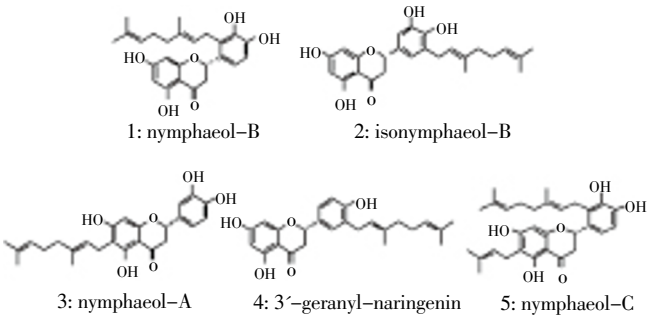

Figure 1. Structures of prenylflavonoids identified from *M. tanarius*.

The results of the quantitative analysis of the extracts from each part of *M. tanarius* are shown in Table 2. Values are expressed as means of triplicate analyses for each sample. A calibration curve for each prenylflavonoid was constructed and tested for linearity. Good linearity was observed for the compounds between peak areas and concentrations over the range test (r>0.998). The limit of detection and quantification values for each compound were 2.5 and 8.0 μg/mL, respectively. The results for precision and reproducibility were good. The percentage recovery value was found to be approximately 95% by the recovery test.

The leaf of *M. tanarius* contained the prenylflavonoids 1 and 5 as main components in both male and female plants

Table 1
Content of water and extract of each part of *M. tanarius*.

Plant part	Dry weight (mg/g)		Water content (mg/g)		Extract (mg/g)		Extraction efficiency (%)	
	Male	Female	Male	Female	Male	Female	Male	Female
Leaf	400	366	601	634	94.3	90.8	23.6	24.8
Petiole	205	264	795	736	16.3	12.5	8.0	4.7
Stem	138	455	862	545	11.4	9.3	8.3	2.0
Leaflet	295	275	705	726	35.8	27.1	12.1	9.9
Flower	276	238	724	762	71.1	47.0	25.8	19.7
Seed		338		662		41.6		12.3
Pericarp		236		764		22.4		9.5
Glandular trichome		990		10		480.0		48.5

All data are per gram of plant.

Table 2
Contents of prenylflavonoids in fresh samples of *M. tanarius* (mg/g).

Plant part	Prenylflavonoid 1		Prenylflavonoid 2		Prenylflavonoid 3		Prenylflavonoid 4		Prenylflavonoid 5		Total	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Leaf	1.10±0.03	1.07±0.02	0.12±0.01	ND	0.24±0.02	0.15±0.00	ND	ND	3.08±0.05	2.98±0.05	4.54	4.20
Petiole	ND	0.02±0.00	ND	0.01±0.00	ND	ND	ND	0.01±0.00	ND	0.03±0.00	ND	0.07
Stem	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.01±0.00	ND	0.01
Leaflet	ND	0.30±0.00	ND	ND	ND	ND	ND	ND	0.02±0.00	0.03±0.00	0.02	0.33
Flower	2.78±0.01	0.81±0.00	0.14±0.00	0.31±0.01	0.10±0.01	1.22±0.01	0.23±0.00	0.90±0.01	0.63±0.01	0.79±0.01	3.88	4.03
Seed		0.19±0.01		0.11±0.00		0.18±0.01		0.19±0.01		0.14±0.01		0.81
Pericarp		0.52±0.01		0.30±0.00		0.52±0.01		0.49±0.01		0.31±0.00		2.14
Glandular trichome		60.14±0.25		35.22±0.29		55.40±0.53		48.50±0.25		35.92±0.37		235.18

Each value represents the mean±SD (n=3). ND: not detected. The prenylflavonoids 1–5 are nymphaeol–B, isonymphaeol–B, nymphaeol–A, 3′–geranyl–naringenin, and nymphaeol–C, respectively.

(Table 2). Further, both flowers (male and female) contained all five kinds (1–5) of prenylflavonoids. However, in the petiole, stem, and leaflet of both male and female plants, these prenylflavonoids were not detected or their amounts were very low. All five kinds of prenylflavonoids were detected in the seed, pericarp, and glandular trichome of female *M. tanarius*. In particular, the glandular trichome had the highest level of prenylflavonoids (235 mg/g of fresh plant).

Next, we evaluated the DPPH radical scavenging activity of each part of *M. tanarius* (Table 3). We reported that prenylflavonoids in the glandular trichome of *M. tanarius* have strong DPPH radical scavenging activity[6]. In the present study, DPPH radical scavenging activity of all parts was more than 30.0%. Particularly, the leaf, in which the prenylflavonoid contents were lower than those in glandular trichome, also had DPPH radical scavenging activity. This result suggests that compounds other than prenylflavonoids in each part also contribute to the DPPH radical scavenging activity.

Table 3
DPPH radical scavenging activity of each part of *M. tanarius* (%).

Plant part	Male	Female
Leaf	85.22±2.44	74.65±0.30
Petiole	32.87±1.90	39.40±0.86
Stem	37.85±1.58	39.05±0.65
Leaflet	42.58±3.24	48.92±1.76
Flower	65.28±2.82	63.57±3.30
Seed		48.20±5.32
Pericarp		80.45±1.70
Glandular trichome		79.65±0.90
BHT		14.82±1.42
VE		75.66±1.53

Each value represents the mean±SD (n=3). Butylated hydroxytoluene and α–tocopherol (vitamin E, VE) at the concentration of 0.5 mg/mL were used as positive control.

4. Discussion

Recently, few studies have reported the biological activities of the leaf extracts of *M. tanarius*. The aqueous methanol extracts of *M. tanarius* leaves inhibit α–glucosidase, and ellagitannins are the active compounds present in them[16]. On the other hand, the methanolic leaf extracts of *M. tanarius* also have antibacterial activity[17]. Therefore, *M. tanarius* is receiving increasing attention as a natural medicinal plant. We have also reported that the ethanol extracts of Okinawan propolis from glandular trichome of *M. tanarius* have antioxidant and antimicrobial activities and that the prenylflavonoids in them contribute the activities[6,7]. Further, it has been developed and patented an antimicrobial agent, containing *M. tanarius* extracts with prenylflavonoids, and as an active ingredient that is useful in oral products for preventing and treating dental caries, gingivitis and gum inflammation[18]. However, quantitative analysis of functional prenylflavonoids in the leaf and other parts of *M. tanarius* has not been performed as far as we know.

In the present study, we quantitatively analyzed five kinds of prenylflavonoids in different parts of *M. tanarius*. All these prenylflavonoids have antioxidant and antimicrobial activities[6,7]. Although the prenylflavonoids analyzed in this study are known, the report of the quantitative analysis of the prenylflavonoids in various parts of *M. tanarius* is for the first time. In particular, not only the glandular trichome but also the leaf contained prenylflavonoids, which indicates that *M. tanarius* may be developed as a functional plant, because the leaves of this plant can be easily collected. These qualitative characteristics of *M. tanarius* reported in our study will be important when *M. tanarius* is used as a natural medicinal plant.

Conflict of interest statement

We declare that we have no conflict of interest.

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