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Original Article

Tea silkworm droppings as an enriched source of tea flavonoids

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ABSTRACT

Andraca droppings is the waste excreted from the tea biter *Andraca theae*. Its chemical constituents and potential medical use, unlike those of the traditional Chinese medicine silkworm droppings, have not been reported yet. To explore new nutraceuticals, the chemical constituents of this substance were investigated. Since the bioactive ingredients are generally present in the EtOAc-soluble fraction, this fraction, obtained from the ethanolic extract of the dried *Andraca* droppings by liquid–liquid partitioning, was separated by chromatographic methods, including Sephadex LH-20, centrifugal partition chromatography, and RP-18 columns, to produce 14 compounds (**1**–**14**). They were characterized as 1,7-dimethyl xanthine (**1**), three benzoic acids (**2, 3**, and **5**), and 10 flavonoids (**4, 6**–**14**). The amount of compounds **6, 7, 10, 13**, and **14** in the droppings were 1.7–15.5-fold compared to those of tea leaves. In addition, 1,7-dimethyl xanthine (**1**) was found present only in the *Andraca* droppings but absent in tea leaves. Therefore, except for compound **1**, which might be transformed from caffeine by microflora in the insect, the compounds were believed not to be absorbed by the worm gut and excreted directly. The present study suggests the *Andraca* droppings are an enriched source of the bioactive flavonoids from tea leaves and are potential as a useful nutraceutical.

1. Introduction

Silkworm feculae (also called *Can-Sha* in Chinese) is the droppings of silkworm (*Bombyx mori* L.; Bombycidae). In traditional Chinese medicine, it is used to expel wind, harmonize stomach, disperse dampness, and transform turbidity. It has been reported to promote wound healing, hematopoiesis by bone marrow, liver protection, antiulcer, antitumor, antidiabetes, and antihyperlipidemia [1]. The mulberry leaf, the fodder of silkworm, is also a traditional Chinese medicine.

Andraca theae is a common tea pest. The larvae of *A. theae* flock and bite the leaves of tea shrub (*Camellia sinensis*) and only the leaf vein remains after they grow up and disperse. The epidemic of *A. theae* is seriously harmful to the production and quality of tea leaves. Aside from its destruction, its

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droppings, which might possess similar bioactivities to *C. sinensis* leaf, are considered as a potential material for medical use.

About 160 compounds have been isolated from leaves and other parts of *C. sinensis*. The compounds are classified into triterpenoids, flavonoids, catechins, aromatic glycosides, and others [2–9]. However, no chemical studies on *Andraca* droppings have been conducted. For the reasons mentioned above, the present study aimed to isolate its chemical constituents by chromatographic methods, and deduce the relationship of the chemical constituents between *C. sinensis* leaf and *Andraca* droppings.

2. Experimental

2.1. General

Instruments to obtain the physical data for the compounds were as follows. Circular dichroism: Jasco J-710 Spectropolarimeter (Tokyo, Japan); optical rotation: Jasco DIP-370 Digital Polarimeter; nuclear magnetic resonance (NMR): a Bruker DPX-200 (200 MHz), AV-400 (400 MHz), or AVIII-600 (600 MHz) NMR (Bruker Daltonics, Billerica, MA, USA) with a dual CryoProbe, *J* in Hz, δ in ppm calibrated by δ_H 3.30/δ_C 49.0 for CD_3OD, δ_H 2.49/δ_C 39.5 for DMSO-d_6, or δ_H 7.19 for C_5D_5N, the 2D NMR spectra acquired by standard pulse sequences; ESI-MS: an Esquire 2000 Ion Trap Mass Spectrometer (Bruker Daltonics); thin-layer chromatography: Silica gel 60 F_{254} aluminum sheets (0.25 mm; Merck KGaA, Darmstadt, Germany); column chromatography: Sephadex LH-20 (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) and Lobar, Lichrospher RP-18 (size B; 40–63 μm, 310 × 25 mm; Merck); centrifugal partition chromatography (CPC): Model L.L.B-M (230 mL; Sanki Engineering Limited, Tokyo, Japan); high-performance liquid chromatography (HPLC): a Hitachi HPLC (Tokyo, Japan) equipped with a D-7000 interface, L-7100 pump, and an L-7400 UV-VIS or an L-7455 diode array detector; HPLC columns: Phenomenex Prodigy ODS (3) 100A columns (5 μm; 250 × 4.6 mm for analysis or 250 × 10 mm for semi-preparation; Torrance, CA, USA).

2.2. Material

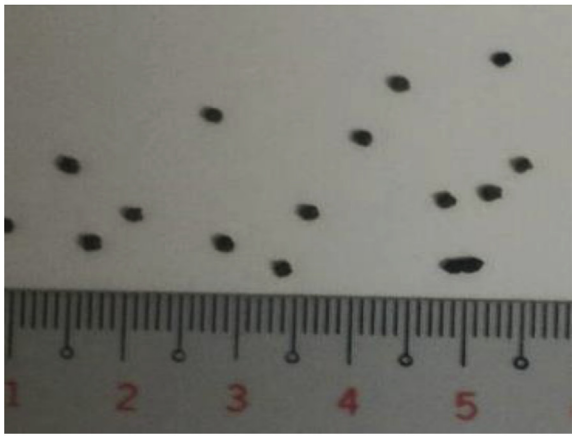
Dried *Andraca* droppings (Figure 1) (380 g) were provided by Tea Research and Extension Station, Council of Agriculture, Executive Yuan, Taoyuan, Taiwan, R.O.C., on November 10, 2014.

2.3. Extraction and isolation

The *Andraca* droppings (380 g) were extracted by 95% EtOH (3 × 2 L) and concentrated under reduced pressure at 45°C to give the EtOH extract (40 g). The suspension of the EtOH extract (25 g) in H_2O (200 mL) was partitioned in sequence against $CHCl_3$, EtOAc, and *n*-BuOH, each 3 × 200 mL, to give three corresponding fractions (1.9 g, 2.4 g, and 2.4 g, respectively), and H_2O soluble (17.9 g) and insoluble (358.2 mg) fractions (see Figure 1).

Most of the EtOAc-soluble fraction (2.1 g) were fractionated by a Sephadex LH-20 column (4 cm outer diameter × 8 cm) into

A



B

Figure 1 – *Andraca* droppings. (A) General appearance in piles; (B) individual appearance.

16 fractions (fraction E1–16). Fraction E4 (149.9 mg) was separated by a Lobar RP-18 column (size B; MeOH–H_2O 3:7; 2 mL/min) to give compound **1** (6.2 mg). Fraction E5 (127.7 mg) was separated by the same column (MeOH–H_2O 9:11; 2.0 mL/min) to give nine fractions, of which fraction 6 (5.8 mg) was compound **4**, fractions 2 (25.2 mg) and 3 (25.0 mg) yielded compounds **2** (5.0 mg; t_R 9.5 minutes) and **3** (3.4 mg; t_R 10.2 minutes), respectively, upon separation on the semi-preparative HPLC column, eluted individually by MeOH–H_2O (9:11) and (11:9) with a flow rate of 2.0 mL/min and detection at UV 254 nm. Recrystallization of an aliquot of fraction E6 (35 mg out of 266.7 mg) from MeOH yielded compound 5 (7.8 mg). Separation of fraction E8 (116.8 mg) on a CPC, using the lower and upper layers of $CHCl_3$–MeOH–H_2O–*n*-BuOH (10:10:6:1) as mobile and stationary phases, respectively, with a flow rate of 1.2 mL/min and rotation speed 800 rpm, to give three fractions (fraction E8-1–3), then the delivery system was reversed to give fraction E8-4. Separation of fraction E8-2 (82.0 mg) by the semipreparative HPLC column (MeOH–H_2O 28:72; 2.0 mL/min; UV 254 nm) to give compounds **6** (13.1 mg; t_R 8.5 min) and **7** (26.1 mg; t_R 14.2 min). Separation of fraction E9 (127.7 mg) on the Lobar RP-18 column (MeOH–H_2O 35:65; 2 mL/min) to give

seven fractions, of which fractions 3 and 4 were compounds **6** (2.3 mg) and **7** (17.7 mg), respectively, and fraction 6 (15.2 mg) gave compounds **8** (2.8 mg; t_R 8.7 min) and **9** (3.2 mg; t_R 10.4 min) upon separation over the semipreparative HPLC column (MeOH–H_2O 11:9; 2.0 mL/min; UV 254 nm). Separation of fraction E10 (175.8 mg) by the Lobar column (MeOH–H_2O 2:8, 2 mL/min) to give compounds **10** (22.0 mg) and **11** (17.6 mg), respectively. Separation of fraction E13 (176.5 mg) by the Lobar column (MeOH–H_2O 33:67, 2 mL/min) gave compound **12** (17.1 mg). Fractionation of fraction E14 (548.1 mg) by a Sephadex LH-20 column (2.0 outer diameter × 29 cm; MeOH–H_2O 6:4), followed by the Lobar column (23%–28% MeOH/H_2O, 2 mL/min) to give five fractions, of which fraction 4 was compound **13** (46.6 mg) and fraction 5 gave compound **14** (11.8 mg; t_R 9.7 min) upon separation by the semipreparative HPLC column (MeOH–H_2O 35:65, 2.0 mL/min; UV 254 nm).

3. Results

A total of 14 compounds (**1**–**14**) (Figure 2) were isolated from the EtOAc-soluble fractions of the dried *Andraca* droppings by chromatographic methods, including Sephadex LH-20, CPC, and RP-18 column chromatography. Ten of them were flavonoids, including seven flavanol derivatives (compounds **6**, **7**, **10**–**14**) and three flavonol 3-*O*-glycosides (compounds **4**, **8**, **9**).

The ^{1}H NMR spectrum of compound **1** showed three singlets at δ 7.79 (H-8), 3.95 (7-Me), and 3.31 (1-Me) and thus compound **1** was identified as 1,7-dimethyl xanthine (paraxanthine) [10]. This suggestion was confirmed by further comparison with the ^{13}C NMR data [11].

Compounds **6** and **7** were identified as catechin and *l*-epicatechin [12–14], respectively. Their ^{1}H NMR spectra showed the distinct signals of H-2 at δ 4.55 (d, J = 7.5 Hz) and 4.80 (br. s), respectively. The CD spectra of both compounds are similar in shape and showed respective a positive Cotton effect around 240 nm and a negative CE around 280 nm, supporting 2R– configuration [14,15]. However, the optical property of compound **6** with $[\alpha]_D$ value close to 0 was not consistent with that of *d*-catechin present in tea leaf. This optical property indicates compound **6** to be a mixture of *d*- and *l*- catechins, with *d*-from enantiomeric excess as verified from CD data.

Similarly, compounds **10** and **11** were identified as gallocatechin and *l*-epigallocatechin, respectively. Their ^{1}H NMR spectra are similar to those of compounds **6** and **7** except that the ABX system in the B ring of compounds **6** and **7** (6: $\delta_{H\text{-}2'}$ 6.82, d, J = 1.8 Hz; $\delta_{H\text{-}5'}$ 6.75, d, J = 8.0 Hz; $\delta_{H\text{-}6'}$ 6.69, dd, J = 8.0, 1.8 Hz) was replaced by a two-proton singlet (δ ~6.40). As with compound **6**, the optical property of compound 10 with $[\alpha]26^D$ +0.5 (c 1.0, MeOH) was not consistent with that of *d*-gallocatechin present in tea leaf, indicating compound 10 to be a mixture with *d*-from enantiomeric excess.

Compounds **12** and **13** were identified as *l*-3″-*O*-methylepigallocatechin gallate and *l*-epigallocatechin gallate [16–18], respectively. The ^{1}H NMR spectrum of compound **13** was similar to that of compound 11 except for showing the much more downfield shifted H-3 (δ 5.52 vs. 4.16) and an additional two-proton singlet ($\delta_{H\text{-}2''/6''}$ 6.94). The ^{1}H NMR spectrum of compound **12** was similar to that of compound **13** except for showing an additional *O*-methyl singlet (δ 3.80) and replacement of a two-proton singlet for H-2″/6″ by an AB system (δ 7.01 and 7.05, J = 1.9 Hz). Compound **14** was identified as *l*-epicatechin gallate [19]. Its ^{1}H NMR spectrum was similar to that of compound **7** except for showing the much more downfield shifted H-3 (δ 5.52 vs. 4.15) and an additional two-proton singlet ($\delta_{H\text{-}2''/6''}$ 6.94) (see Figure 2).

Compounds **2**, **3**, and **5** are simple benzoic acid derivatives and were identified as 3-*O*-methylgallic acid [20], 4-hydroxybenzoic acid [21], and gallic acid [22], respectively, by comparison of their ^{1}H and ^{13}C NMR data with those reported.

Compounds **4**, **8**, and **9** were 3-*O*-glycosyl flavonols as exemplified by their ^{1}H NMR spectra, showing an AX system

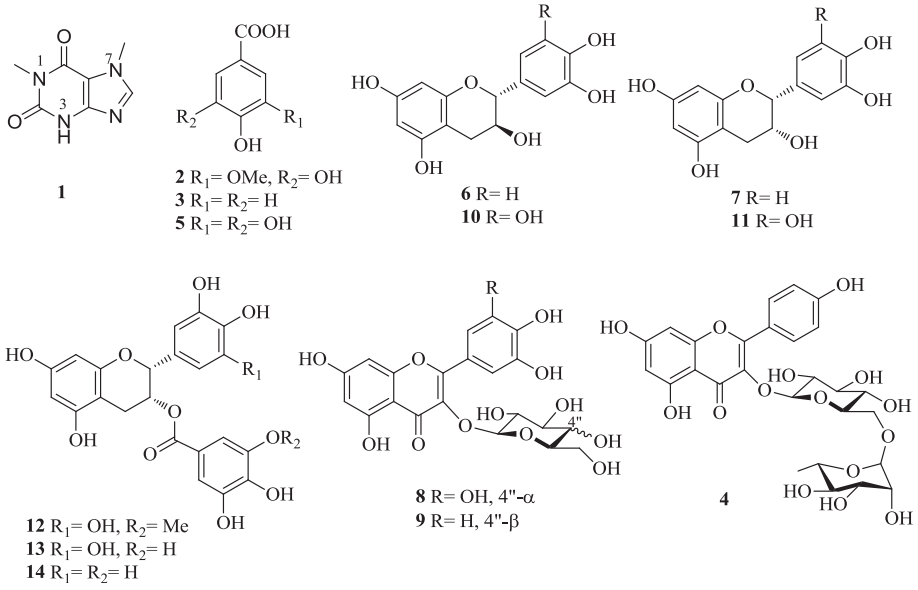


Figure 2 – Structures of compounds (1–14) from the *Andraca* droppings.

for H-6 (δ ~6.20) and H-8 (δ ~6.40), an AA′XX′ (4) or A_2 (8) or AMX (9) system for protons in ring B, and characteristic signals for the anomeric protons, e.g. δ 5.12 (d, $J = 7.4$ Hz) for Glc H-1 and δ 4.51 (d, $J = 1.1$ Hz) for Rha H-1 in compound **4**. They were identified as kaempferol 3-O-rutinoside (compound **4**) [23], isomyricitrin (compound **8**) [23], and quercetin 3-O-β-D-galactopyranoside (compound **9**) [13] by comparison of their physical data (^{1}H and ^{13}C NMR, $[\alpha]_D$) data with those reported.

Among these isolated compounds, compound **12** is the most abundant (2.22%, w/w) while the content of the unusual 3-desmethylated caffeine (compound **1**), having not been reported from tea leaf (see Table 1), is about 0.30%.

Comparison of the content of the corresponding compounds isolated from *Andraca* droppings in the present study to our unpublished data and the data from those reported in the literature [4–6, 24–26] indicated that most of them are obviously more abundant, e.g. up to 15.5 folds for catechin (compound **6**), except the content of one compound (**11**, 1.0-fold) was identical (Table 1). From the aforementioned data we speculated that the *Andraca* cannot digest these flavonoids efficiently, leading to the concentration of these compounds in the digestion processes.

These flavonoids possess various bioactivities, such as antioxidative, anti-inflammatory and anticancer activities [2–9]. The most abundant compound, **13** (EGCG; 2.22%), is reported to be the most effective cancer chemopreventive polyphenol in green tea [27]. The second and third abundant epicatechin (compound **7**; 1.24%) and gallocatechin (compound **10**; 1.05%) also possess anticancer activities [28]. The methylated EGCG (compound **12**) exhibits antiallergic functions by inhibition of mast cell activation and suppression of leukotriene and interleukin-2 secretion, and suppression of TNF-α and MIP1-α production, and its potency is higher than EGCG (**13**) [29]. In addition, 1,7-dimethyl xanthine (paraxanthine; **1**), the only compound not found in the fodder, *C. sinensis* leaf, is a nonselective antagonist for phosphodiesterase [30] and adenosine receptor [31]. Paraxanthine, the 3-*N*-demethylated metabolite of caffeine in humans via cytochrome P-450 oxidation [32], might be produced by microbial transformation in the gut of *Andraca* worm.

This study indicates that *Andraca* droppings are a much better source of the bioactive ingredients than tea leaf. The findings also indicated that AD should possess at least similar bioactivity to tea leaf and has great potential to be developed as a useful nutraceutical.

4. Discussion

Table 1 lists the percentage and the relative content of the isolated compounds in the fodder, *C. sinensis* leaf, and *Andraca* droppings. This comparison provided important hint about how these compounds were digested by the gut microflora. All the flavanol derivatives (compounds **6**, **7**, **10**–**14**) were enriched in *Andraca* droppings, indicating them not readily metabolized by insect gut and microflora, where 3-*N*-demethylation on caffeine was undertaken efficiently to give compound **1**. The optical properties of catechin (compound **6**) and gallocatechin (compound **10**) revealed that some biotransformations occurred during the digestion process, including oxidation at C-3 hydroxy group, followed by C-2 epimerization and reduction at the formed C-3 ketone. Through these, the *l*-form product will be produced from the natural *d*-form. From the relative content of flavonol glycosides, β-glucosidase seems abundant but β-galactosidase absent in the gut system since the content of compound **8** is much lesser but that of compound **9** much more (42-fold) in *Andraca* droppings than in tea leaf. In addition, mono-O-

Table 1 – Estimated amount (g) of compounds 1–14 in 100 g of the corresponding extracts[a] from *Camellia sinensis* leaf and *Andraca theae*.

Compound	Droppings (A)	*C. sinensis* (B)[b]	Relative content (A/B)
Flavanol derivatives			
Catechin (**6**)	0.62	0.04	15.5
Epicatechin (**7**)	1.24	0.12	10.3
Gallocatechin (**10**)	1.05	0.12	8.8
Epigallocatechin (**11**)	0.84	0.82	1.0
Epigallocatechin 3″-O-methyl-gallate (**12**)	0.81	NE	—
Epigallocatechin gallate (**13**)	2.22	1.30	1.7
Epicatechin gallate (**14**)	0.56	0.24	2.3
Flavonol glycosides			
Kaempferol 3-O-rutinoside (**4**)	0.28	NE	—
Isomyricitrin (**8**)	0.11	NE	—
Quercetin 3-O-β-D-galactopyranoside (**9**)	0.84	NE	—
Xanthine			
1,7-dimethyl xanthine (**1**)	0.30	—	
Benzoic acids			
gallic acid (**5**)	0.37	NE	—
3-O-methylgallic acid (**2**)	0.24	NE	—
4-hydroxybenzoic acid (**3**)	0.16	NE	—

NE = not estimated.

[a] Extracts were soluble in ethanol and the concentrate of the ethyl acetate-soluble part from *C. sinensis* leaf and *A. theae* droppings, respectively.

[b] The data for compounds 6–7 and 10–14 were provided by Tea Research and Extension Station.

methylation on the phenolic –OH in the galloyl moiety seems to be a general metabolic pathway by the gut system as verified in compounds **2** and **12**.

This study demonstrates that *Andraca* droppings might become a new nutraceutical similar to the droppings of silkworm. A lot of substandard tea, so-called vice tea, is discarded during tea preparation but is good fodder for the insect *Andraca theae*. Accompanied with the present finding of the potential use of *Andraca* droppings, sufficient utilization of vice tea to nurture this distinct insect might create a new agriculture-industry joined business and should be beneficial to human welfare and economic development.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jfda.2016.11.011.

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