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ORIGINAL ARTICLE

# Structural elucidation of condensed tannin from the bark waste of *Acacia crassicarpa* plantation wood in Indonesia

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**Abstract** Recently, *Acacia crassicarpa* has been planted in peatland areas with acidic soil in Indonesia for use in pulp and paper materials. Its bark is not suitable to produce bleached pulp; hence, it is discarded as waste. Meanwhile, in South Africa and other countries, *Acacia mearnsii* has been planted for a long time, and its bark extracts have been used as a leather tanning agent. First, the structure of condensed tannin from the bark waste of *A. crassicarpa* is characterized. The yield of the extracts obtained from *A. crassicarpa* using a 70% acetone aqueous solution (7% based on bark weight) is less than that obtained from *A. mearnsii* (34%). A novel flavan dimer from the condensed tannin, specific to *A. crassicarpa*, is isolated from the bark extracts. To the best of our knowledge, this dimer is a new compound as evidenced from pyrolysis–gas chromatography–mass spectrometry and nuclear magnetic resonance analyses; it corresponds to a gallocatechin–catechin flavan dimer with the absence of one oxygen atom at the 3C of the pyran ring. In addition, 2,4,6-trimethoxybenzoic acid methyl ester is identified as a novel pyrolysis product obtained from the cleavage of the pyran ring.

**Keywords** Condensed tannin · *Acacia crassicarpa* · Bark waste · Pyrolysis–gas chromatography–mass spectrometry · Nuclear magnetic resonance

## Introduction

Bark, a by-product of the pulp industry, is a renewable resource at pulp mills, in addition to wood wastes and black liquor [1]. Indonesia is the third largest manufacturer of pulp and paper in Asia, with 84 pulp and paper mills [2]; therefore, the country produces a significant amount of bark by-products. Bark contains a high concentration of tannin; as a result, interest in developing methods for utilizing tannin has recently increased. Studies have reported the utilization of tannin as a tanning agent [3], a protein precipitate [4, 5], an insecticide and a herbicide [6], a wood adhesive [7], and a wood preservative [8–10]. Tannin is divided into two major classes: condensed tannin and hydrolysable tannin. Condensed tannins consist of flavan-3-ol units with 4 → 6 and 4 → 8 carbon linkages. The biological activity of tannin is closely related to structure, functional groups, and stereochemistry [11].

*Acacia crassicarpa* is a native species of Indonesia [12], which has been developed as a pulp and paper raw material owing to its better ability to grow in diverse environments as compared to *Acacia mangium* and *Acacia auriculiformis* [13]. Thus far, the increasing amount of bark waste produced by the pulp and paper industries is not offset by the utilization of its tannin content, partially because of the lack of detailed studies on the tannin from the bark of *A. crassicarpa*; only studies on the polyphenolic compounds from the knot and stem wood of *A. crassicarpa* have been reported thus far [14]. In these studies, melacacidin and isomelacacidin are identified as the main compounds, and

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low amounts of flavanone taxifolin, flavone quercetin, and flavanol catechin are also identified by gas chromatography–mass spectrometry (GC–MS).

Meanwhile, in New Zealand, South Africa, and other countries, *A. mearnsii* has long been planted, and its bark extracts have been utilized as a leather tanning agent. The structure of the condensed tannin contained in the bark of *A. mearnsii* (known as Morishima acacia in Japan) has been elucidated [11, 15] (Fig. 1).

This study aims to isolate the condensed tannins from *A. crassicarpa* and elucidate their chemical structures.

## Materials and methods

### Bark sample of *A. crassicarpa*

Bark samples were collected from 4- to 5-year-old *A. crassicarpa* trees grown in the Research and Development plantation area of PT Riau Andalan Pulp and Paper located in the Pelalawan area of Riau, Indonesia, in 2011. The samples were dried at room temperature for 2 weeks. Then, the air-dried bark was ground in a Wiley mill to obtain a powder with a mesh size of 40–80.

### Extraction and fractionation of *A. crassicarpa*

First, 40 g of bark powder (40–80 mesh size) was extracted using 300 mL of a 70% acetone aqueous solution at room temperature for 24 h. Extraction was repeated four times. The resulting solvent was evaporated to obtain the 70% acetone extract, followed by dilution using 300 mL of aqueous water and extraction three times using 300 mL of ethyl acetate, affording the ethyl acetate extract (C-EA) and the water-soluble extract (C-WS).

C-EA was purified by column chromatography (length = 70 cm and Ø = 2.6 cm) using a Sephadex LH-20 (GE Healthcare Bio-Sciences, Sweden) with ethanol as the eluent, affording four fractions (C-EA1 to C-EA4, respectively). Each fraction was further purified by two-dimensional thin-layer chromatography (2D TLC) using a mixture of ethyl acetate and *n*-hexane as the developing solution. Further separation of C-EA was performed using a 50% acetone aqueous solution to give a C-EA-AW fraction.

Using a similar procedure, C-WS was also purified by column chromatography (Sephadex LH-20) and further separated using a 50% methanol aqueous solution and 50% acetone, successively, to obtain a C-WS-AW fraction. Figure 2 shows the fractionation procedure.

### Pyrolysis–gas chromatography–mass spectrometry (Py-GC/MS)

The samples (150–200 μg) and the internal standard *n*-eicosane were wrapped in a 500 °C pyrofoil and pyrolyzed at 500 °C for 4 s using a JHP-5 pyrolyzer (Japan Analytical Industry, Japan), which was interfaced (interface temperature of 250 °C) with a GC–MS system (QP-5050, Shimadzu, Japan) equipped with an HP-1MS column (30 m × 0.25 mm i.d., film thickness 1.00 μm), with an electron impact of 70 eV and He as the carrier gas. The injection port (temperature 280 °C) was fit with a split liner (split ratio 50:1). The following temperature profile was used for GC analysis: hold for 1 min at 50 °C, 5 min at 50–280 °C (5 °C/min), and 13 min at 280 °C. The pyrolysis products were identified by the comparison of their retention times and mass spectra with those of the authentic samples and literature data. Catechin and epigallocatechin were purchased from Tokyo Chemical Industry (Tokyo, Japan) and used as model compounds for the tannin monomer.

Methylated Py-GC/MS was conducted by wrapping the condensed tannin samples in a 500 °C pyrofoil after the addition of 3 μL of a 25% solution of tetramethylammonium hydroxide (TMAH, Tokyo Chemical Industry Co.,

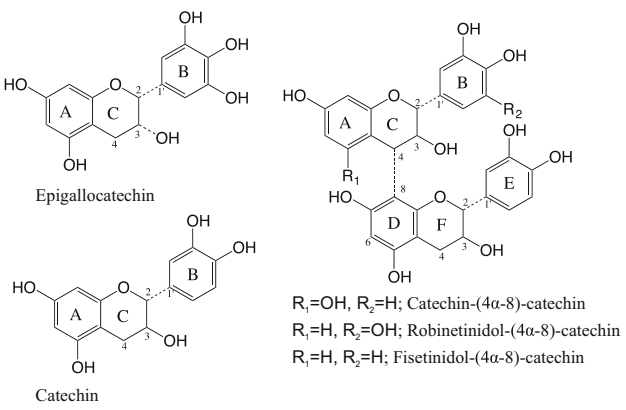



**Fig. 1** Examples of flavan-3-ol units of condensed tannin [11]

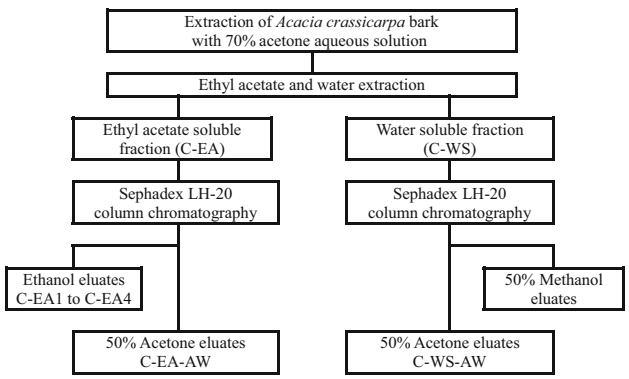



**Fig. 2** Scheme of the extraction and fractionation of *A. crassicarpa* bark

**Table 1** Yields of acacia bark extracts containing condensed tannin

| | *Acacia crassicarpa* (%) | *Acacia mearnsii* (%) |
|---|---|---|
| 70% acetone aqueous extracts | 7.0 | 34 |
| Ethyl acetate-soluble fraction | 0.8 | 12 |
| Water-soluble fraction | 6.2 | 22 |

Ltd.) in methanol. The samples were analyzed using the same procedure as described above with conventional Py-GC/MS. Gallic acid and 2,4,6-trimethoxybenzoic acid (Tokyo Chemical Industry Co., Ltd.) were used to identify the pyrolysis product of tannin, which exhibited an ion *m*/*z* 226.

### Pyrolysis–gas chromatography-milli mass spectrometry (Py–GC/Milli MS)

The identification of the pyrolysis product with an *m*/*z* value 226 was conducted using catechin, which was analyzed using a JMS-600H spectrometer (JEOL, Japan).

### Fast atomic bombardment mass spectroscopy (FAB-MS)

FAB-MS spectra of the fractions (C-EA1 to C-EA4) from the condensed tannin samples were obtained using a JMS-600H spectrometer (JEOL, Japan) with Xe as the primary beam. FAB-MS measurements were conducted with sample solutions in a glycerol matrix with Ultramax 1621 as the standard.

## $^{1}$H and $^{13}$C nuclear magnetic resonance (NMR) analyses

The isolated dimer (C-EA1) from *A. crassicarpa* was analyzed using an AVANCE-500 NMR spectrometer at the Chemical Analysis Division, Research Facility Center for Science and Technology, University of Tsukuba, Japan using ethanol-$d_6$ ($CD_3CD_2OD$) as the solvent. The $^{1}$H-NMR, $^{13}$C-NMR, and two-dimensional NMR (2D NMR), i.e., heteronuclear multiple quantum coherence (HMQC), as well as distortionless enhancement by polarization transfer (DEPT 135), spectra were recorded at 500 Hz.

Interpretation spectrum of $^{1}$H-NMR (TMS, tetramethylsilane) of C-EA1: $\delta$ 2.42 [dd (double doublet), *J* (coupling constant) = 16.1, 8.0 Hz, H-4F(a)], 2.64 [dd, $J = 16.3$, 3.6 Hz, H-3C(a)], 2.71 [dd, $J = 16.3$, 4.7 Hz, H-3C(b)], 2.74 [dd, $J = 16.1, 5.4$ Hz, H-4F(b)], 3.86 [ddd (double double doublet), $J = 8.0, 7.2, 5.4$ Hz, H-3F], 4.05 [brdd (broad double doublet), $J = 4.7, 3.6$ Hz, H-2C], 4.42 [d (doublet), $J = 7.2$ Hz, H-2F], 4.63 [s (singlet), H-8aA], 5.80 [d, $J = 2.3$ Hz, H-8A], 5.87 [s, H-6D], 5.89 [d, $J = 2.3$ Hz, H-6A], 6.33 [s, H-2′B and H-6′B), 6.42 [bs (broad singlet), H-2′E and H-5′E]. Fifteen protons were observed in the spectrum.

$^{13}$C-NMR (TMS) of C-EA1: $\delta$ 28.0 (C-4F), 28.8 (C-3C), 67.2 (C-2C), 68.5 (C-3F), 79.5 (C-8aA), 82.5 (C-2F), 95.3–96.3 (C-6A, C-8A, C-6D), 99.6–100.4 (C-1′B, C-4C, C-4aD, C-6′E), 106.7 (C-2′E, C-5′E), 107.0 (C-2′B and C-6′B), 130.9–133.5 (C-4aA, C-4′B, C-8D, C-1′E), 146.1 (C-3′E, C-4′E), 146.3 (C-3′B and C-5′B), 156.3–157.4 (C-5A, C-7A, C-5D, C-7D, C-8aD).

The purified condensed tannin polymers from *A. crassicarpa* and *A. mearnsii* were analyzed using the AVANCE-500 NMR spectrometer (solvent: $CD_3CD_2OD$)

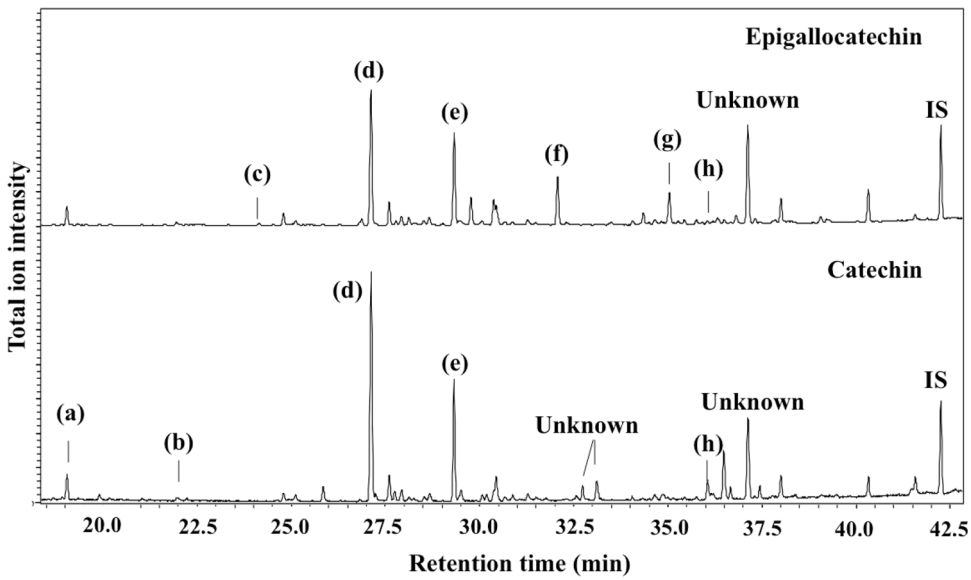



**Fig. 3** Total ion chromatogram from methylated Py-GCMS of catechin and epigallocatechin **a** 1,2-Dimethoxybenzene; **b** 3,4-dimethoxytoluene; **c** 1,2,3-trimethoxybenzene; **d** 1,3,5-trimethoxybenzene; **e** 2,4,6-trimethoxytoluene; **f** 3,4,5-trimethoxybenzaldehyde; **g** 3,4,5-trimethoxybenzoic acid methyl ester; **h** 2,4,6-trimethoxy benzoic acid methyl ester; and IS (internal standard): *n*-eicosane

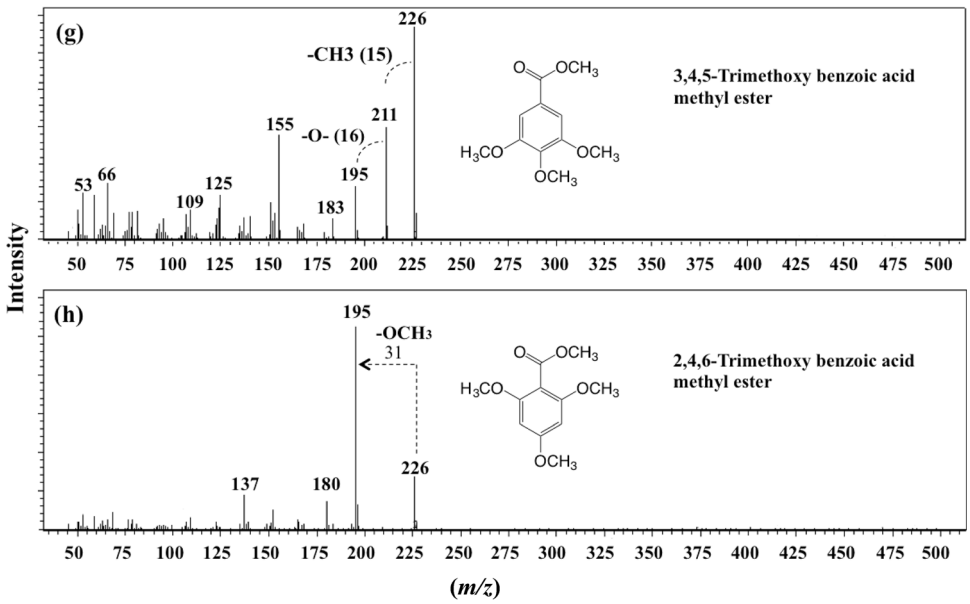



**Fig. 4** Mass spectra of 2,4,6-trimethoxy benzoic acid methyl ester and 3,4,5-trimethoxy benzoic acid methyl ester obtained by methylated Py-GC/MS of 2,4,6-trimethoxy benzoic acid and gallic acid

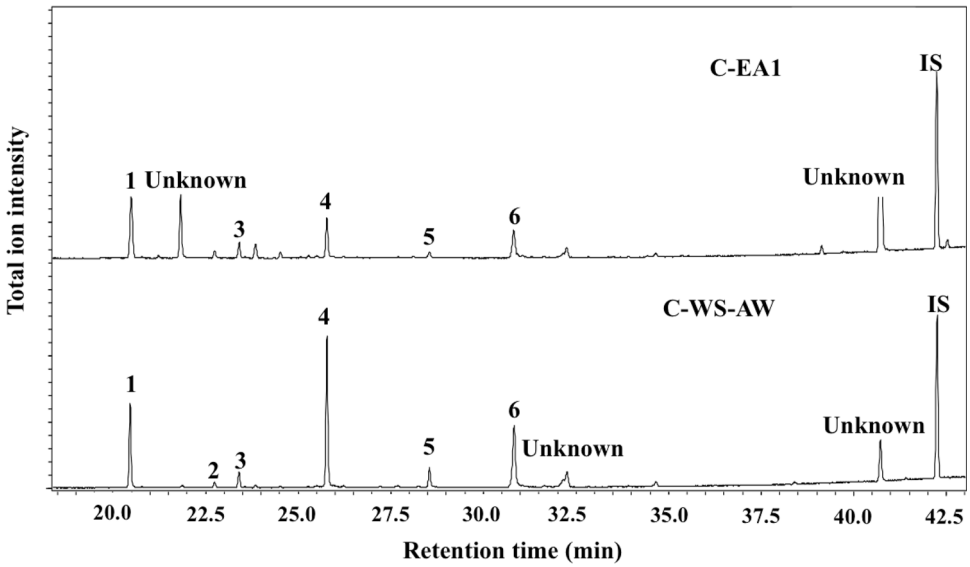



**Fig. 5** Pyrogram of conventional Py-GCMS of C-EA1 and C-WS-AW from *A crassicarpa*. *1* catechol; *2* resorcinol; *3* 4-methylcatechol; *4* pyrogallol; *5* 5-methyl pyrogallol; *6* phloroglucinol; and IS (internal standard): *n*-eicosane

and a JEOL JNM-LA400 spectrometer (solvent: $CD_3$-$COCD_3$), respectively.

## Results and discussion

### Extraction of condensed tannin

The yield of the extract obtained from *A. crassicarpa* (7.0%) using a 70% acetone aqueous solution was one-fifth of that obtained from *A. mearnsii* (Table 1). This result is consistent with a previous study, which confirms that *A. mearnsii* is a good source of tannin [15]. In addition, the yield of the extract obtained from the bark of *A. crassicarpa* was less than that of other species, including *A. mangium, A. auriculiformis, Rhizophora apiculata,* and *Larix leptolepis*, with yields of 37.9, 28.6, 20.2, and 11.0%, respectively [16]. The age of the tree and the location of sampling are known to affect the tannin yields; as compared to the outer or whole bark, the inner part of the bark has a higher amount of tannin because of the higher metabolite concentration [14].

### Structural elucidation of condensed tannin by Py-GC/MS

#### *Py-GC/MS of catechin and epigallocatechin*

Catechin and epigallocatechin were used as monomer standards for flavanol. The main products from pyrolysis were catechol and 4-methylcatechol from the B-ring of

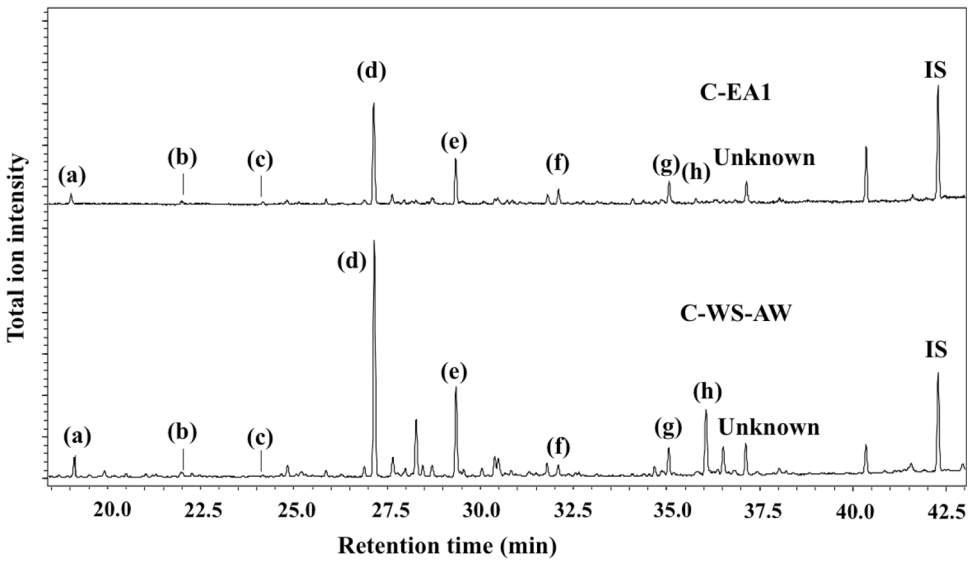



**Fig. 6** Pyrogram of methylated Py-GC/MS of C-EA1 and C-WA-AW from *A. crassicarpa*. **a** 1,2-dimethoxybenzene; **b** 3,4-dimethoxytoluene; **c** 1,2,3-trimethoxybenzene; **d** 1,3,5-trimethoxybenzene; **e** 2,4,6-trimethoxytoluene; **f** 3,4,5-trimethoxybenzaldehyde; **g** 3,4,5-trimethoxybenzoic acid methyl ester; **h** 2,4,6-trimethoxy benzoic acid methyl ester; and IS (internal standard): *n*-eicosane

catechin and epicatechin (Fig. 1), while pyrogallol was the main product from epigallocatechin or gallocatechin. In this study, the intensity of the catechol peak was greater than that of 4-methylcatechol. This result confirmed that it is easier to cleave the C1′–C2 bond as compared to that of a pyran ring, which has been previously reported [11]. The presence of catechol or pyrogallol in the pyrogram is a very useful marker for the B-ring product, which can be used to identify the tannin structure. The yields of catechol and pyrogallol from catechin and gallocatechin have been reported to be 10.8 and 13.0 mol%, respectively, by conventional Py-GC [11].

In addition, a low-intensity peak corresponding to phloroglucinol was detected as a marker for the A-ring product. By conventional Py-GC/MS analysis, it is difficult to detect the A-ring products from pyrolysis as compared to the B-ring products because of their high polarity and instability under the conditions of the technique.

To overcome the polarity problem, methylated Py-GC/MS can be used as an alternative method for analyzing the A-ring pyrolysis products from condensed tannin. In this method, the A-ring products are more easily detected than B-ring products. Figure 3 shows the methylated Py-GC/MS pyrogram of catechin and epigallocatechin obtained using TMAH as the methylating agent. Both the monomer standards, catechin and epigallocatechin, afforded the A-ring pyrolysis products, 1,3,5-trimethoxybenzene (d), and 2,4,6-trimethoxytoluene (e). Low amounts of the B-ring products, 1,2-dimethoxybenzene (a) and 3,4-dimethoxytoluene (b), were also obtained from catechin, while 1,2,3-trimethoxybenzene (c), 3,4,5-trimethoxybenzaldehyde (f), which have been reported previously [17], and 3,4,5-trimethoxybenzoic acid methyl ester (g) were obtained from the B-ring of epigallocatechin.

In the methylated Py-GC/MS pyrogram of catechin and epigallocatechin, an unidentified mass ion (*m*/*z*) peak of 226 (h) was observed at a retention time of 36 min. Based on the Py-GC/Milli MS data, this pyrolysis product (h) had a chemical formula of $C_{11}H_{14}O_5$. However, the fragmentation trace and retention time of this peak did not match those of 3,4,5-trimethoxybenzoic acid methyl ester (g), which have been previously reported as a methylated Py-GC/MS product of wine tannin [18–20], and the ion fragmentation of 2,4,6-trimethoxybenzoic acid methyl ester (h) has not been reported. Hence, pure compounds 2,4,6-trimethoxybenzoic acid and gallic acid are subjected to methylated Py-GC/MS to identify the unknown fragmentation ion, and the results confirmed that the pyrolysis product (h) from catechin and epigallocatechin is 2,4,6-trimethoxybenzoic acid methyl ester (Fig. 4). It is formed from the cleavage of the pyran ring (C-ring) by the methylation of the tannin monomers, indicating that chain cleavage occurs at different locations in condensed tannins. 2,4,6-trimethoxybenzoic acid methyl ester was identified as a novel pyrolysis product. This property can be useful for determining the structures of unidentified condensed tannins.

### *Py-GC/MS of C-EA1 and the tannin polymer fraction from A. crassicarpa*

Figure 5 shows the pyrogram of C-EA1 obtained by conventional Py-GC/MS. Catechol (1) and pyrogallol (4) were detected as the B-ring pyrolysis products, while phloroglucinol (6) was obtained as the A-ring product, and resorcinol was not observed. Figure 6 shows the methylated Py-GC/MS pyrogram of C-EA1 from *A. crassicarpa*. 1,3,5-Trimethoxybenzene (d), 2,4,6-trimethoxytoluene (e), and 2,4,6-trimethoxybenzoic acid methyl ester (h) were

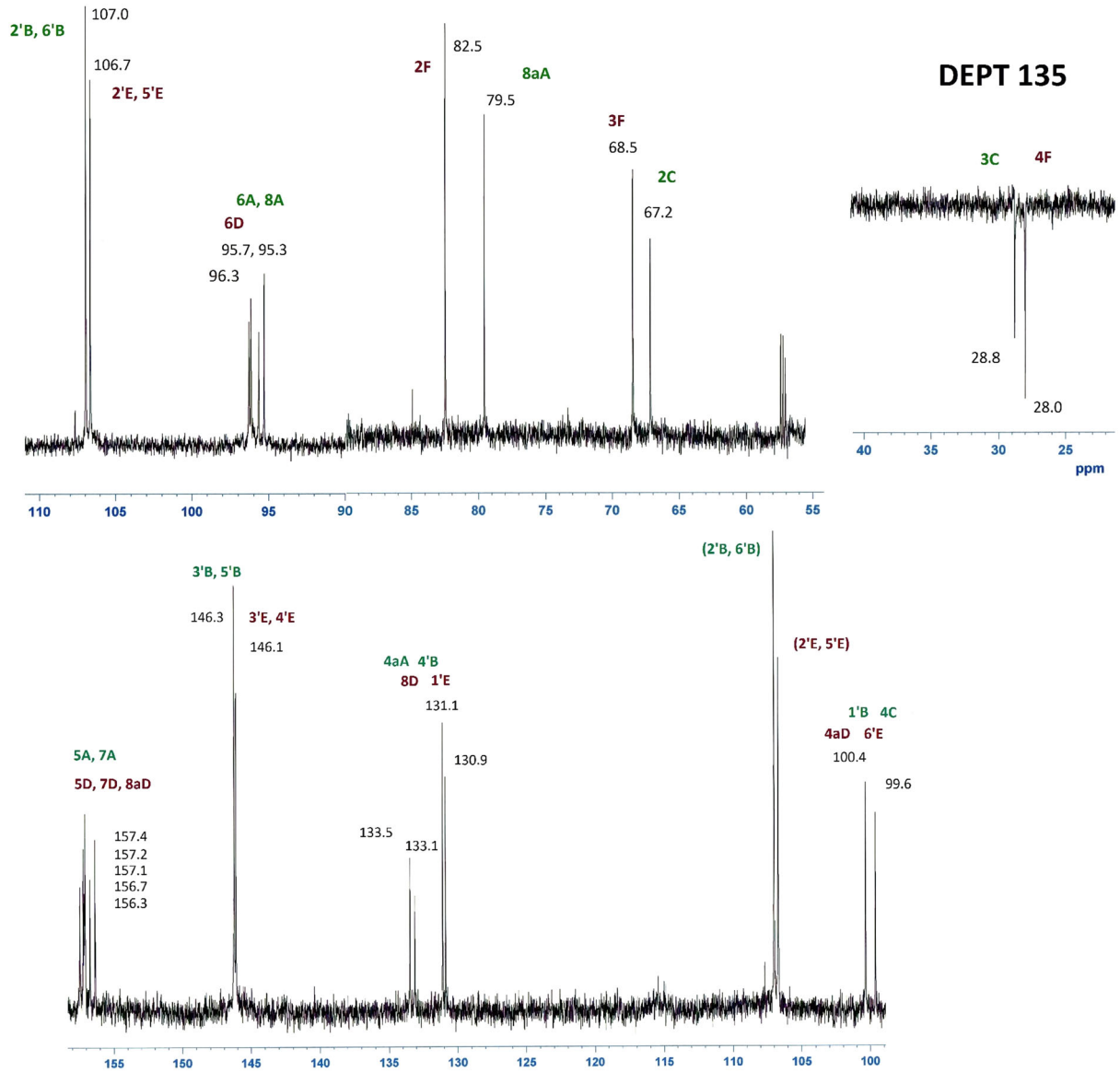



**Fig. 7** $^{13}$C-NMR and DEPT 135 spectrum of the compound purified from C-EA1

detected as TMAH markers for the A-ring products. Meanwhile, the B-ring pyrolysis products—1,2,3-trimethoxybenzene (c), 3,4,5-trimethoxybenzoic acid methyl ester (g), 1,2-dimethoxybenzene (a), and 3,4-dimethoxytoluene (b)—were observed, with low intensities for the latter two compounds. Interestingly, an intense peak for 3,4,5-trimethoxybenzaldehyde (f) was detected as a result of the cleavage of $C_2$–$C_3$ bond on the C-ring. Based on these results, C-EA1 is a procyanidin or prodelphinidin, but it cannot be fisetinidol-(4-8)-catechin or robinetinidol-(4-8)-catechin.

The purified tannin polymer fraction C-WS-AW contained catechol (1), 4-methylcatechol (3), pyrogallol (4), and 5-methylpyrogallol (6) as the B-ring pyrolysis products in the Py-GC/MS pyrogram. In addition, low amounts of phloroglucinol (6) and resorcinol (2) were observed from the A-ring (Fig. 5). In the methylated Py-GC/MS pyrogram, C-WS-AW contained 1,3,5-trimethoxybenzene (d), 2,4,6-trimethoxytoluene (e), and 2,4,6-trimethoxybenzoic acid methyl ester (h) as the A-ring products (Fig. 6). Together, these results clearly indicated that the condensed tannins of *A. crassicarpa* are predominantly composed of procyanidin and prodelphinidin.

## Characterization of dimers isolated from *A. crassicarpa* by FAB-MS and $^1$H and $^{13}$C-NMR analyses

C-EA1, C-EA2, C-EA3, and C-EA4 were collected from 1 to 64 fractions of the ethyl acetate-soluble fraction of

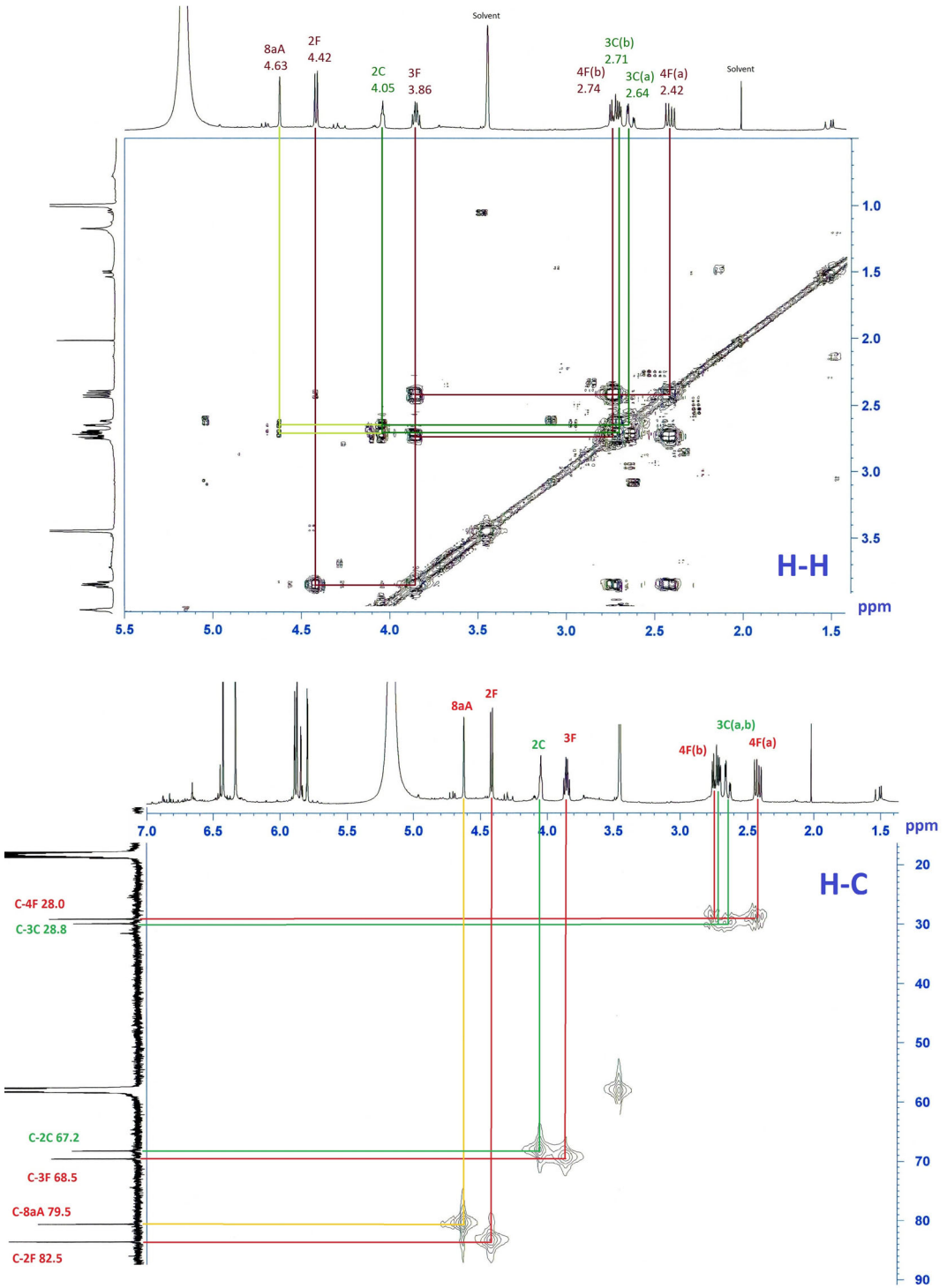



**Fig. 8** $^{1}$H and $^{13}$C-NMR HMQC spectrum of the compound purified from C-EA1

*A. crassicarpa* (C-EA), and a reddish brown amorphous powder (92.7 mg) was obtained at Rf values: 0.77(*n*-hexane:ethyl acetate, 1:1) and 0.40 (6% acetic acid) of 2D-TLC as C-EA1 (1–16 fractions). The FAB-MS spectra of the compound C-EA1 $[M + H]^{+}$ is 579.

The $^{1}$H-NMR (TMS) and $^{13}$C-NMR (TMS) spectra of C-EA1 are described in the experimental section: 15 protons were observed.

The $^{13}$C-NMR of DEPT 135 spectrum of C-EA1 exhibited two methylene (–$CH_2$–) carbons with negative signals at 28.0 and 28.8 ppm, in addition to 11 methine (–

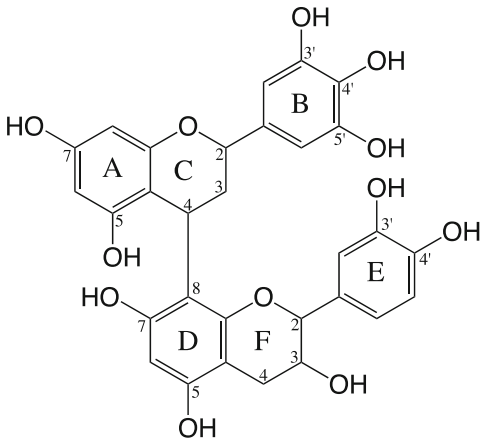
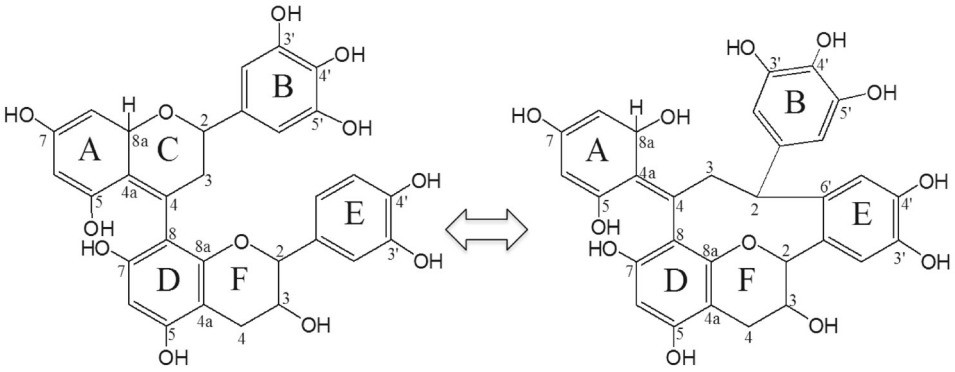


**Fig. 9** A possible structure of condensed tannin dimer from *A. crassicarpa* (MW: 578, $C_{30}H_{26}O_{12}$)



Isomerization Condensation

CH–) carbons and 17 quaternary carbons (–C–), and 30 $^{13}$C peaks were observed in the spectrum (Fig. 7). The $^{13}$C-NMR results indicated a clear difference between the chemical shift of C-3C ($\delta$ 28.8) and C-3F ($\delta$ 68.5). These assignments are supported by DEPT 135 data, which showed a negative signal for C-3C, as methylene (–$CH_2$–). Furthermore, the analysis of the $^{1}$H and $^{13}$C-NMR HMQC spectra was utilized to define the interaction between the protons and carbons in the compound (Fig. 8). The signal of $^{13}$C-NMR (HMQC) ($\delta$ 28.8) exhibited cross-peaks with H-3C (a) and (b) ($\delta$ 2.64 and 2.71), corresponding to a methylene carbon with no oxygen. This result confirmed the assignments of the F-ring (pyran ring) with the presence of hydroxyl group (–OH).

Based on the interpretation by $^{1}$H-NMR and $^{13}$C-NMR, isomerization and condensation were observed during the preparation of the C-EA1 sample. The dimer should change its structure according to the isomerization of a proton at C-4C to C-8aA and condensation of the benzyl cation (at C-2C) of the B-ring to E-ring (at C-6′E).

Based on the above discussion, the dimer from the condensed tannin isolated from the bark of *A. crassicarpa* (C-EA1) is characterized as 2′-(3,4-dihydroxyphenyl)-2-(3,4,5-trihydroxyphenyl)-3,3,3′,4,4′,4′-hexahydro-2*H*,2′*H*-4,8-′bichromene-3′,5,5′,7,7′-pentol (Fig. 9), or 5,7,3′,4′,5′-pentahydroxy-flavan-(4-8)-catechin. This is a novel dimer of condensed tannin, which contains a catechin and a flavan structure without an oxygen atom at the 3C of the pyran ring. The previously reported dimers fisetinidol-(4-8)-catechin and robinetinidol-(4-8)-catechin [21] were isolated from *A. mearnsii* extracts.

Unfortunately, 2D-NMR HMBC and NOESY data were not available because the compound was used for termite preservation tests, leaving insufficient amount of the compound for the analysis. Results from the analysis and termite tests will be separately reported in due course.

## Characterization of condensed tannin polymer by NMR analysis

Polymer tannins are predominantly composed of repeating units of flavan-3-ols, which are linked via $C_4 \rightarrow C_6$ or $C_4 \rightarrow C_8$ bonds [22]. It is complicated to elucidate the structure of condensed tannin polymers because of their large molecular weights ranging from 2000 to 5000. This study approached the structural elucidation via the analysis

of the flavanol units in polymer tannins, which consisted of pyrogallol- and catechol-type structures. In the Py-GC/MS pyrogram of C-WS-AW, the intensity of the pyrogallol peak was greater than that of catechol, indicating that the content of pyrogallol in polymer tannin is greater than that of catechol.

The C-WS-AW fraction was purified according to a previously reported method [11, 15, 21]. It may still contain impurities. For further studies, this fraction needs to be purified to a greater extent so as to obtain a clear $^{13}$C-NMR result.

The $^{13}$C-NMR spectrum was investigated for the condensed tannin polymers isolated from both *A. mearnsii* and *A. crassicarpa*. (Figs. ESM Appendix 1 and Appendix 2, respectively) The Py-GC/MS result, showing the presence of pyrogallol- and catechol-type structures, was supported by the presence of high-intensity chemical shifts in the $^{13}$C-NMR spectrum of WS-AW from *A. mearnsii* corresponding to C2′, C6′ (B-ring pyrogallol-type) and C2′, C5′ (B-ring catechol-type) at 108 ppm and 116 ppm, respectively [23, 24]. In the case of C-WS-AW from *A. crassicarpa*, however, those peaks were slightly different, and the peaks were detected at 107.8 ppm for the pyrogallol-type and 106.8 ppm for the possible other type structures. The peak at 106 ppm was possibly related to the condensed catechol type according to the results of the isolated dimer C-EA1.

## Conclusions

The structure of condensed tannins from the bark wastes of *A. crassicarpa* were characterized for the first time. A novel dimer of flavan units specific to *A. crassicarpa* was isolated from the bark extracts. The new compound is a gallocatechin–catechin flavan dimer with no oxygen at the 3C of the pyran ring, which was characterized as 2′-(3,4-dihydroxyphenyl)-2-(3,4,5-trihydroxyphenyl)-3,3,3′,4,4′,4′-hexahydro-2H,2′H4,8-′bichromene-3′,5,5′,7,7′-pentol, or 5,7,3′,4′,5′-pentahydroxy-flavan-(4-8)-catechin.

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