A new quantification method for polyphenols in medicinal plants using quantitative NMR, and new polyphenols from pomegranate

2019

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Contents

ABBREVIATIONS

^{1}H NMR	Proton nuclear magnetic resonance
^{13}C NMR	Carbon nuclear magnetic resonance
3-DG	3-Deoxyglucosone
AGEs	Advanced Glycation End-products
AMD	Age-related macular disease
APCI-MS	Atmospheric Pressure Chemical Ionization Mass Spectrometry
Calcd	Calculated
CEL	Carboxyethyl Lysine
CD	Circular Dichroism
CML	Carboxymethyl-Lysine
COSY	^{1}H-^{1}H Correlated spectroscopy
δ	NMR chemical shift in part per million downfield from a standard
DHAP	Dihydroxyacetone phosphate
DHHDP	Dehydrohexahydroxydiphenoyl
EGCG	(-)-Epigallocatechin-3-gallate
GO	Glyoxall
GOLD	Glyoxal-lysine dimer
Glc	Glucose
HHDP	Hexahydroxydiphenoyl
HR-ESI-MS	High-resolution electrospray ionization mass spectrometry;
HSA	Human Serum Albumin
HSQC	Heteronuclear single quantum correlation
HMBC	Heteronuclear multiple-bond correlation
IC_{50}	Concentration that is inhibit in 50% of the test subjects
i.d.	Inside diameter
J	Coupling constant
MOLD	Methyl glyoxal lysine dimer
MGO	Methylglyoxal
m/z	Mass-to-charge ratio
NP-HPLC	Normal phase high-performance liquid chromatography
PTB	*N*-phenacylthiazolium Bromide
ROESY	Rotating frame nuclear Overhauser effect spectroscopy
RP-HPLC	Reverse phase high-performance liquid chromatography
t_R	Retention time
UHPLC	Ultra high-performance liquid chromatography
UPS	Ubiquitin-proteasome system

Chapter I

Introduction

Ellagitannins are polyphenolic secondary metabolites of higher plants, which belong to the class of hydrolyzable tannins. Currently, more than 500 ellagitannins have been discovered [1, 2]. They have shown potential health benefits, such as the prevention of advanced glycation end-products (AGEs) formation [3], anti-inflammatory effects [4, 5], anti-diabetic effects [6], anti-fungal effects [7], and antioxidant effects [8, 9].

The characteristic of ellagitannins is the present of hexahydroxydiphenoyl (HHDP) unit in their structure, which retrieved from the oxidative transformation of two galloyl units [10]. Chemical reaction and circular dichroism (CD) spectral data are used to determine the absolute configuration of HHDP of ellagitannin. Most of HDDP units at *O*-2, *O*-3, and *O*-4, *O*-6 positions of the D-glucopyranose have (*S*)-configuration, whereas most of the absolute configuration of the HHDP units at the *O*-3, *O*-6 position have (*R*)-configuration, such as in geraniin (**1a**) [47-48].

Geraniin is the major ellagitannin in *Geranium thunbergii*, which is one of the popular medicinal plants known as *gennoshoko* in Japan. Geraniin has been reported to have various biological activities [11]. Belonging to the dehydroellagitannin family, geraniin usually forms an equilibrium mixture of six- and five-membered in aqueous and methanol solution (Fig. 1). This condition lead to coelution problem and cause the difficulty in quantifying the analyses using HPLC due to the overlapping peaks.

NMR (Nuclear magnetic resonance) has become the famous tool for chemical structure elucidation (qualitative method). In NMR analysis, the signal intensities

of the NMR spectrum are directly proportional to the molar amount of the nucleus in the sample. Based on this principle, the NMR analysis can be used for both, quantitative and qualitative methods.

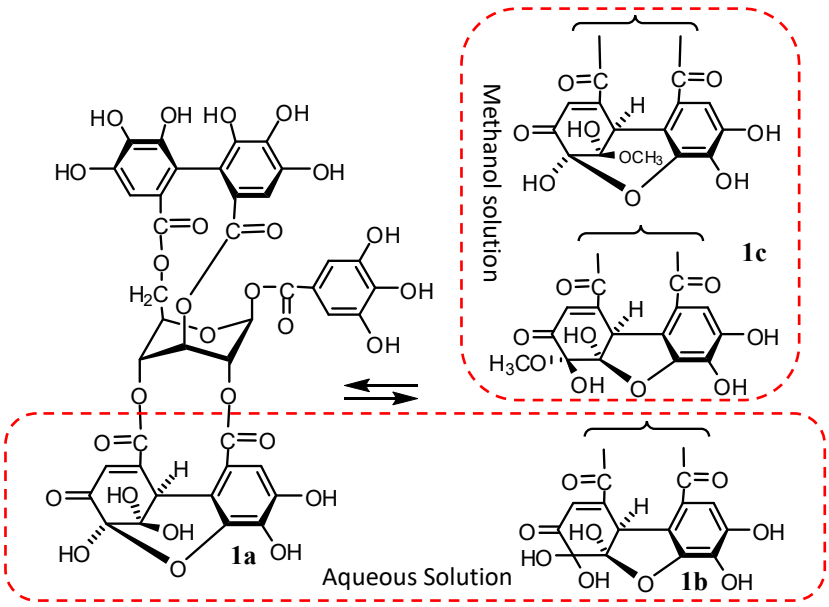


Figure 1. Chemical structure of geraniin.
Geraniin formed equilibrium mixture of six-and five-membered in aqueous (**1b**) and methanol solution (**1c**). Methanol adducts with methoxyl groups at hemiacetal in geraniin are produced in methanol solution (**1c**).

Quantifying using NMR is known as qNMR (quantitative NMR). The survey by Pauli and co-workers showed the increased of the usefulness of qNMR in numerous fields of industrial and academic research in recent years [12, 13]. The qNMR can become an alternative for quantifying the dehydroellagitannin that usually forms an equilibrium mixture of six- and five-membered in aqueous and methanol solutions. The usefulness of qNMR to quantifying the amount of polyphenols content in *Geranium thunbergii* as a traditional medicine will be discussed detail in Chapter II.

Ellagitannin also exists plentiful in pomegranate (*Punica granatum L.*). Fischer et al. (2011) has detected 48 compounds from pomegranate extracts (peels, mesocarps, and arils) which most of them are tannins [14]. Punicalin (**4**) and punicalagin (**3**) are two major ellagitannins in pomegranate. A recent research has been successfully isolated and elucidated the structure of three new natural compounds from pomegranate. Pomegraniins A (tetramer) (**7**) and pomegraniins B (pentamer) (**8**) are the new of ellagitannin oligomers from arils of pomegranate. In addition, the chemical structure of a new glucose ester of neolignan, pomegralignan (**9**) and other known as ellagitannins (oenothein B (**3**), eucalbanin B (**5**), eucarpanin T_1 (**6**), punicalagin (**3**), and punicalin (**4**)) from arils and pericarps have been isolated and elucidated their chemical structure based on spectroscopic analyses [3].

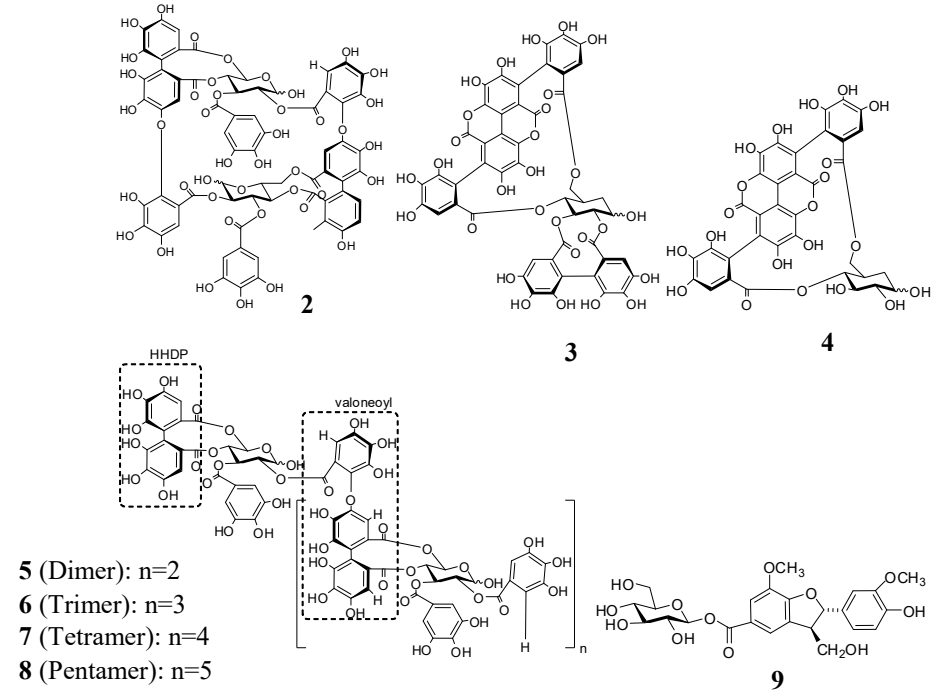


Fig 2. Chemical structure of ellagitannins from pomegranate.

Since the invention of NMR in 1946 [15], and the introduction of 2D NMR method in the mid-1980s, the isolation of natural compound and structure determination increased significantly [16]. Exploring the new natural compound is a challenge for the discovery of novel structure. We also believed that pomegranate contains various type of polyphenols which have never been reported before. For these reasons, we explored to discover new polyphenols from pomegranate. In Chapter III, the isolation and elucidation of new polyphenols from pomegranate were discussed.

Glycation is the non-enzymatic reaction between reducing sugar and free amino acid. The reaction was initiated by the formation of unstable Schiff base, then forms more stable Amadori product. Further the Amadori product are rearranged by performing oxidation, dehydration, and cyclization process to establish highly reactive intermediate dicarbonyl group that react with free amino acid lysine and arginine lead to yield the advanced glycation end-products (AGEs) [17].

A number of polyphenols have been reported for their anti-glycation activities [3, 20-23] by chelating ion activity [18, 20], trapping the carbonyl [18, 20, 22], binding the amino acid [17, 19], or cleaving the crosslink derived [21] from AGEs. Tannins were also reported for its potential to be an anti-glycation [3, 21-24]. The mechanism of anti-glycation of tannin might be similar to the common mechanism of other polyphenols as anti-glycation agents.

Ellagitannins which isolated from pomegranate arils were analyzed for their inhibitory effect on AGEs formation and AGEs crosslink cleaving activity

comparing to anti-glycation of proanthocyanidins. The Proantocyanidin was isolated and elucidated from some varieties of red-kerneleds rice from Akaonimochi, Benizomemochi, Tanegashima, Akamai, and Benimusume - Japan. The study of the anti-glycation of these samples was presented in chapter IV.

Chapter II

A new quantification method for polyphenols in medicinal plants using quantitative NMR

Geranium thunbergii contains a large amount of ellagitannin, in which dried weight of its leaves comprise 10% ellagitannins. Geraniin and corilagin are two major ellagitannins in *G. thunbergii*. [25]. There are two ways in using *G. thunbergii* to treat intestinal disorders medicinally in Japan [70, 71]. The dried aerial parts can be brewed with hot water (making a tea) or the dried plant can be boiled in water for one hour. The resulting concoctions are used for the treatment of constipation and diarrhea, respectively.

Geraniin is an ellagitannin with a dehydrohexahydroxydiphenoyl (DHHDP) group always shows multiple broad peaks on high-performance liquid chromatography (HPLC) when there is dissolved into aqueous and alcoholic solution. This anomaly occurred because of some dehydroellagitannins formed an equilibrium mixture of six- and five-membered in the aqueous or alcoholic solution (Fig. 1) [30, 31]. This phenomenon becomes a problem in quantifying ellagitannins which has a DHHDP group using HPLC. In addition, analytical methods using HPLC require pure standards for establishing the calibration curve. It is well known that obtaining commercially pure native standards of specific ellagitannins is difficult. Therefore, the large ellagitannin oligomers are more difficult to purify than the monomers [69]. For HPLC and other chromatographic studies, an initial purification of the specific ellagitannin is generally required to use in further quantification processes.

Nuclear magnetic resonance (qNMR) is one of the alternatives for quantifying the ellagitannins. The usefulness of qNMR is further accelerated by additional advantages, such as the lack of calibration curve requirements, the non-destructive

character of the NMR technique, for the lack of special sample preparation requirements, the relatively short measurement times, and the possibility of simultaneously quantifying multiple compounds in crude extracts [26-29, 72-73], therefore, qNMR is a simple and absolute quantification method, which is able to determine the purities of compounds or the absolute content of a compound in the natural source with unit traceability.

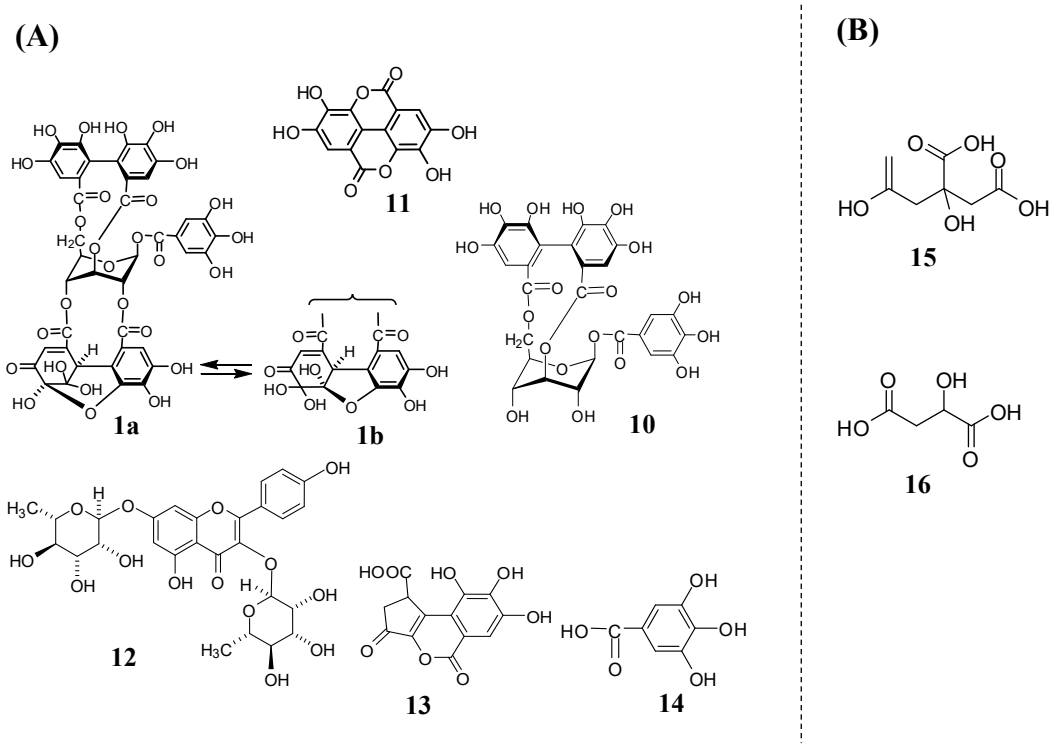


Fig 3. Structures of the main ellagitannins (A), and related compounds (B) in *G. thunbergii*.

In this study, we investigated the application of qNMR to assay the main ellagitannin and the related polyphenol contents (Fig 3) in the short-term and long-term extracts of *G. thunbergii*. To the best of our knowledge, no study has yet investigated the quantity of ellagitannin in *G. thunbergii* using qNMR. Most

researchers quantifying ellagitannin have utilized chromatographic methods, such as HPLC, UHPLC, and LC-MS/MS [44-46]. The present study also explored the main polyphenol contents in *G. thunbergii* cultivated in Japan and China. The contents of the targeted compounds were calculated from the integral of the characteristic anomeric or aromatic proton signals against that of an internal standard.

MATERIALS AND METHODS

Plant Material

Japanese pharmacopoeia standard products G. thunbergii (*gennoshoko*) cultivated in Japan and China were purchased from Japanese companies, Kokumin, Konishi, Kojuma, Daiko, Eidai, Matsuura, Japan Health, and Uchida.

HPLC Analysis

The normal phase HPLC system consisted of a pump (Jasco PU-980), UV-Vis detector (Shimadzu SPD-6A, Kyoto, Japan), and a column (YMC-Pack SIL A-003, 4.6 mm I.D × 250 mm). The mobile phase consisted of oxalic acid (450 mg/L) in *n*-hexane:methanol:tetrahydrofuran:formic acid (55:33:11:1, v/v/v/v, respectively). The extract (5 µL) was injected into the HPLC and delivered at a flow rate of 1.5 mL/min. The detection wavelength was set to 280 nm. The reversed phase HPLC system consisted of pump (Hitachi L-2130, Tokyo, Japan) equipped with a diode array detector (Hitachi L-7455). The separation employed an Intersustain C18 column (GL Science, 5µm, 4.6 mm I.D. × 150 mm) at 40^{O}C. The mobile phase consisted of eluent A (H_2O:acetonitrile:formic acid, 90:5:5 v/v/v) and eluent B (H_2O:acetonitrile:formic acid, 50:45:5, v/v/v). The eluent was programmed as follows: 0 min 0% B; 30 min. 100% B; 30.1–45 min 0% B. The flow rate of the mobile phase was set at 1.0 mL/min.

NMR Spectroscopy

All NMR analyses were carried out on a Varian NMR System 600 MHz (Palo Alto, CA, USA) operating at 600 MHz for ^{1}H-NMR. The measurement parameters were as follows: pulse flip angle, 90^{O}; number of acquisitions, 8; acquisition time, 4 s; number of points, 48.077; delay time, 60 s; spin, off; temperature, room temperature.

qNMR Analysis

70% aqueous acetone extracts of the short-term and long-term decoction (20 mg) of *G. thunbergii* were dissolved in acetone-d_6:D_2O:CF_3COOD (70:25:5, v/v/v) (1 mL) + 1,4-BTMSB-d_4 (0.1 mg) (Fujifilm, Wako Pure Chemical Corporation, Tokyo, Japan). 1,4-BTMSB-d_4 (99.9% purity) was used as an internal standard. Sample quantification was performed according to Equation (1):

$$P_{\text{sample}} = \frac{I_{sample} / H_{sample}}{I_{1,4-BTMSB-d4} / H_{1,4-BTMSB-d4}} \text{ x } \frac{M_{sample} / W_{sample}}{M_{1,4-BTMSB-d4} / W_{1,4-BTMSB-d4}} \text{ x} P_{1,4-BTMSB-d4} \qquad (1)$$

Where: P_{sample} = purity of desired compound; $P_{1,4\text{-BTMSB-}d4}$ = 99.9%; I_{sample} = area measured by integration of the desired compound on ^{1}H NMR spectum; $I_{1,4\text{-BTMSB-}d4}$ = 100; H_{sample} = number of hydrogen(s) of the desired signal of compound; $H_{1,4\text{-BTMSB-}d4}$ = 18; M_{sample} = molar mass of desired compound; $M_{1,4\text{-BTMSB-}d4}$ = 226.5; W_{sample} = weight of sample (20mg); $W_{1,4\text{-BTMSB-}d4}$ = 0.1mg. To determine the weight of compound in the sample, an absolute equation was used (Equation 2).

$$m_{compound} = \frac{P_{sample}}{P_{1,4-BTMSB-d4}} \text{x} W_{sample} \quad (2)$$

Short and long-term decoction

The short-term decoction was performed by brewing 10 g of dried *G. thunbergii* in 600 mL boiling water for 1min, then filtered and dried by evaporator. The dried filtrate obtained 2.2 g of extract. The long-term decoction was performed by boiling 10 g of dried *G. thunbergii* in 600 mL water until the water reduced to 300 mL. After filtration, the filtrate was dried by evaporator and obtains 0.34 g of extract. These methods imitated the traditional method to treat gastrointestinal disease by using *G. thunbergii* in Japan.

The long-term decoction fractionation was carried out by extracting 2.2 g of dry samples sequentially using diethyl ether, ethyl acetate, n-butanol, and water solvents (250 mL each solvent). The results of the extracts obtained from each solvent are diethyl ether 44.6 mg; ethyl acetate 173.1 mg; *n*-butanol 269.1 mg; and water 1.72 g.

RESULTS

HPLC Analysis of Geraniin and the Related Polyphenols in G. thunbergii

In this study, problems of coelution on HPLC during the analysis of geraniin were found. The HPLC chromatograph of *G. thunbergii* extracts after brewing in hot water showed the geraniin (**1**) peak. After boiling for 1 h, geraniin was completely hydrolyzed to corilagin (**10**), brevifolincarboxylic acid (**13**), ellagic acid (**11**), and gallic acid (**14**). However, after boiling for 1 h, selected signals at the same retention time to geraniin remained on the chromatograph in normal phase HPLC implied that other compounds overlapped with the retention time signal corresponding to geraniin (Fig 4). Furthermore, ellagic acid, gallic acid, and brevifolincarboxylic acid elute closely on normal phase HPLC. The reversed phase HPLC profile also revealed that brevifolincarboxylic acid, geraniin, and corilagin eluted close together. This HPLC coelution phenomenon is one of the reasons why we began to explore the other methods to quantify ellagitannins and the related polyphenols.

^{1}H-NMR Fingerprint

There are two critical issues for determining the type of solvent to employ, i.e. the ability to dissolve the compounds of interest and the signal separation. Dimethyl sulfoxide (DMSO)-d_6 was a very good choice for solvating ellagitannins, but it did not provide sufficient ^{1}H-NMR signal separation.

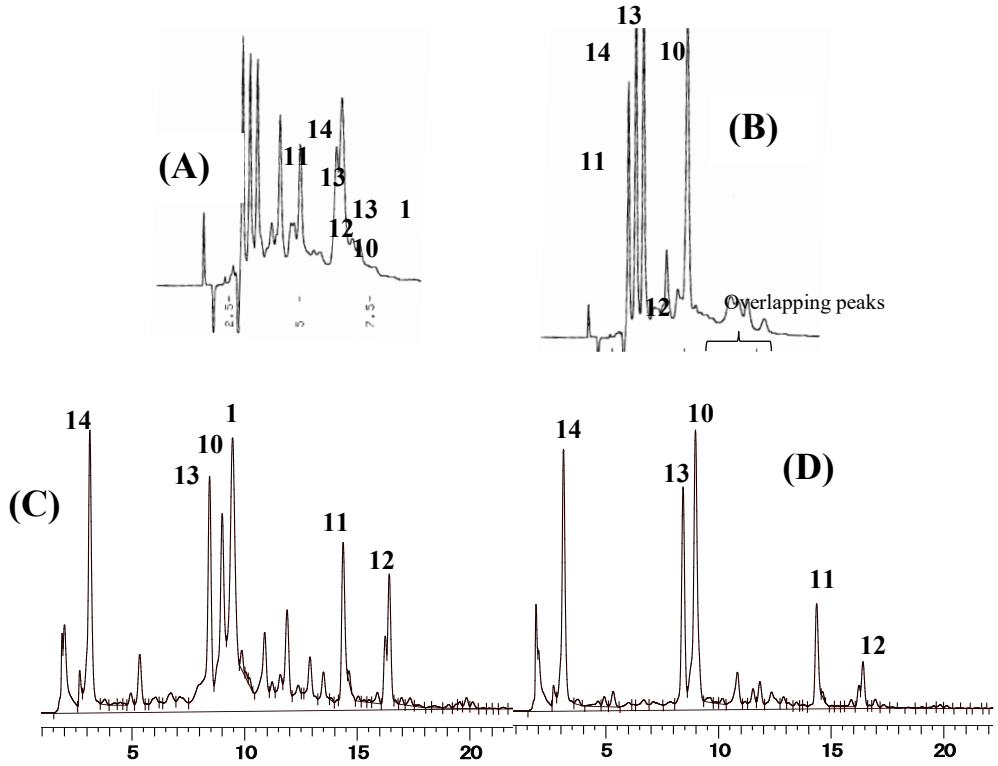


Fig 4. HPLC profiles for the extracts of *G. thunbergii*.
(A): short-term decoction on normal phase HPLC; (B): long-term decoction on normal phase HPLC; (C): short-term decoction on reversed phase HPLC; (D): long-term decoction on reversed phase HPLC. (Reprint: Bastian, et al., 2018 [61])

Despite the addition of CF_3COOD-D_2O and employing variable temperature techniques, sufficient peak separation could not be obtained. Alternatively, acetone-d_6 could provide adequate ^{1}H-NMR signal separation, but it was inadequate at dissolving some of the target compounds. We could not obtain valuable data in the solvent with good solubility such as methanol-d_4 since necessary signals corresponding to some of the candidate compounds were overlapped with a signal of HDO in the solvent. On the other hand, acetone-d_6 has less overlapping area with HDO. Furthermore, a small amount of D_2O was added to raise the solubility and CF_3COOD was added to provide sharp signals due to

polyphenols. With peak separation being the most important factor in the qNMR analysis, acetone-d_6 was selected and D_2O and CF_3COOD were added to obtain a final ratio of 70:25:5, respectively. This solvent ratio resulted in good ^{1}H-NMR peak separation.

In order to develop the ^{1}H-NMR fingerprints indicative of geraniin and its metabolites, sugar, and organic acids, ^{1}H-NMR spectrum analyses of the individual compounds were carried out. The indicative signals of the candidate compounds for qNMR are presented in Table 1 and Fig 5. Based on the integrations of these signals, the contents of ellagitannin and the related compounds, in various extracts of *G. thunbergii* were quantified by using the method described in the section of materials and methods.

Table 1 Specific ^{1}H NMR data of main ellagitannins and the other compound of *G. thunbergii* for qNMR analysis.

Compounds	MW	^{1}H NMR data
Geraniin (**1**)	952	δ 6.54 (**1a** form glucose H-1; 1H, br s) δ 6.51 (**1b** form glucose H-1; 1H, br s)
Corilagin (**10**)	634	δ 6.30 (glucose H-1; 1H, br s)
Brevifolincarboxylic acid (**13**)	292	δ 7.36 (aromatic proton; 1H, s)
Kaempferitrin (**12**)	578	δ 7.78 (B-ring H-2',6'; 2H, d, *J* =9.0 Hz)
Ellagic acid (**11**)	302	δ 7.55 (2H, s)
Gallic acid (**14**)	170	δ 7.04 (2H, s)
Glucose	180	δ 4.52 (*β*-form H-1; 1H, d, *J*=8.4 Hz) δ 5.11 (*α*-form H-1; 1H, d, *J*=3.6 Hz)
Malic acid (**16**)	134	δ 4.44 (1H, dd, *J*=4.2, 7.2 Hz)
Citric acid (**15**)	210	δ 2.85 (1H, d, *J*=15.6 Hz)

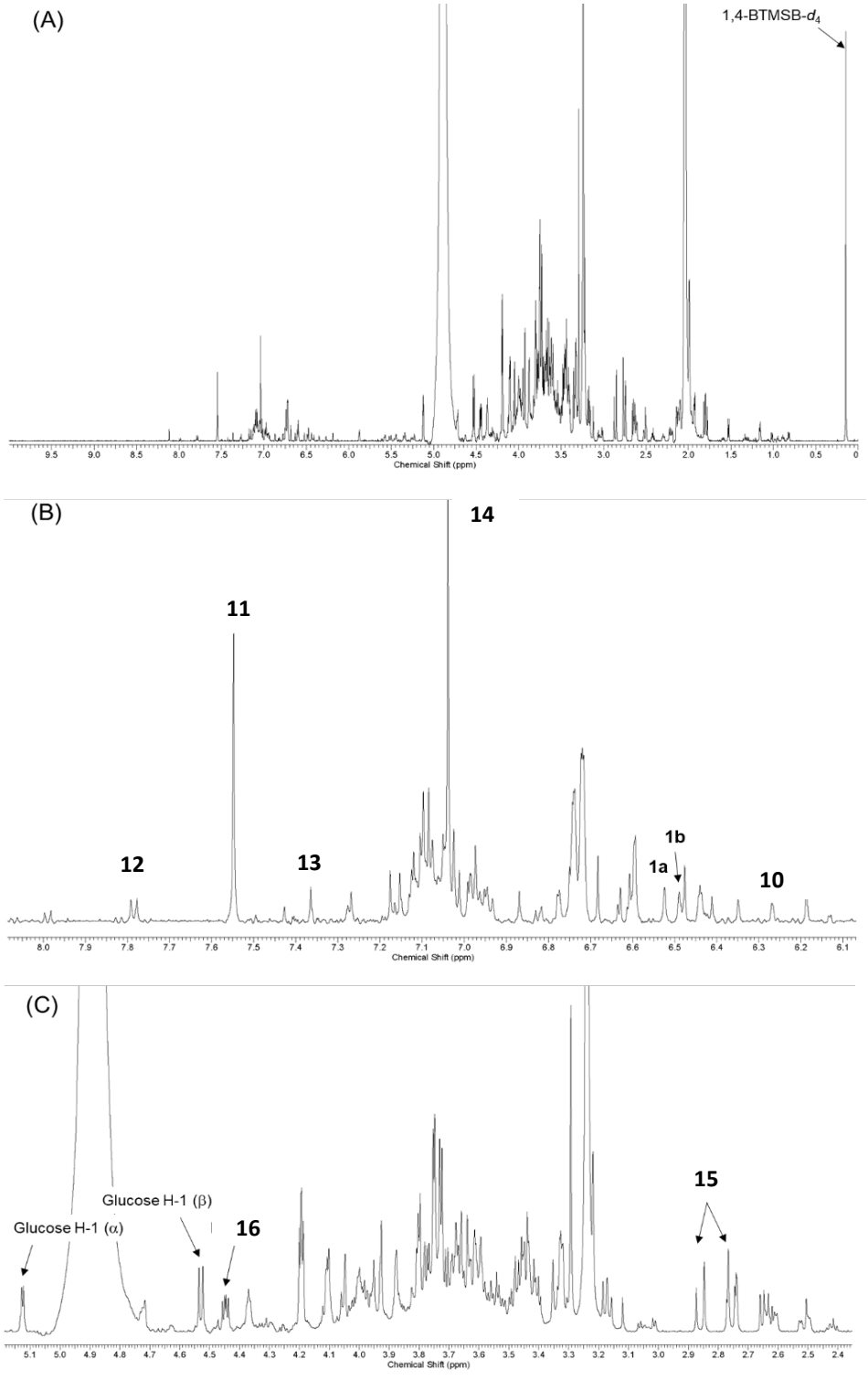


Fig 5. ^{1}H NMR spectra of the extract from short-term decoction of *Geranium thunbergii* in 600 MHz.
(A) Full-scale, (B) Expansion of the low-field region, (C) Expansion of the high-field region. (Reprint: Bastian, et al., 2018 [61])

Short-Term and Long-Term Decoction

Based on the selected peaks for qNMR in Table 1, we evaluated the composition differences of the target polyphenols in *G. thunbergii* obtained from extraction in both short-term and long-term decoctions. Extraction by short-term decoction showed geraniin as the predominant ellagitannin and the related polyphenols (62.0% of the total polyphenols). In contrast, corilagin (corresponding to the hydrolysate of geraniin) was a major extraction compound from long-term decoction. During the short-term decoction, the amount of corilagin was only 13.2%. After the long-term decoction, the amount of corilagin increased to 49.0% (Table 2). Additionally, the percentages of gallic acid, ellagic acid, and brevifolincarboxylic acid also increased. However, geraniin was not detected and no significant difference was found between the amount of kaempferitrin before and after long-term decoction. These results indicate that geraniin was hydrolyzed to corilagin, gallic acid, ellagic acid, and brevifolincarboxylic acid after 1h of decoction.

Table 2. Relative contents (%) to the amount of the main polyphenols in the short- and long-term extracts of *G. thunbergii*.

Main polyphenols	Contents (%)	
	Short-term decoction	Long-term decoction
Geraniin (**1**)	62.0	0.0
Corilagin (**2**)	13.2	49.0
Brevifolincarboxylic acid (**3**)	5.1	13.1
Kaempferitrin (**4**)	6.0	5.4
Ellagic acid (**5**)	6.5	16.1
Gallic acid (**6**)	7.2	16.4

Fig 6 presents a graph of the change in main polyphenol content during 50 min of decoction. Geraniin increased until 10 min of decoction; thereafter, it decreased sharply. In contrast, the amount of corilagin increased steadily over the entire 50 min decoction. The amounts of brevifolincarboxylic acid, gallic acid, and ellagic acid also increased steadily.

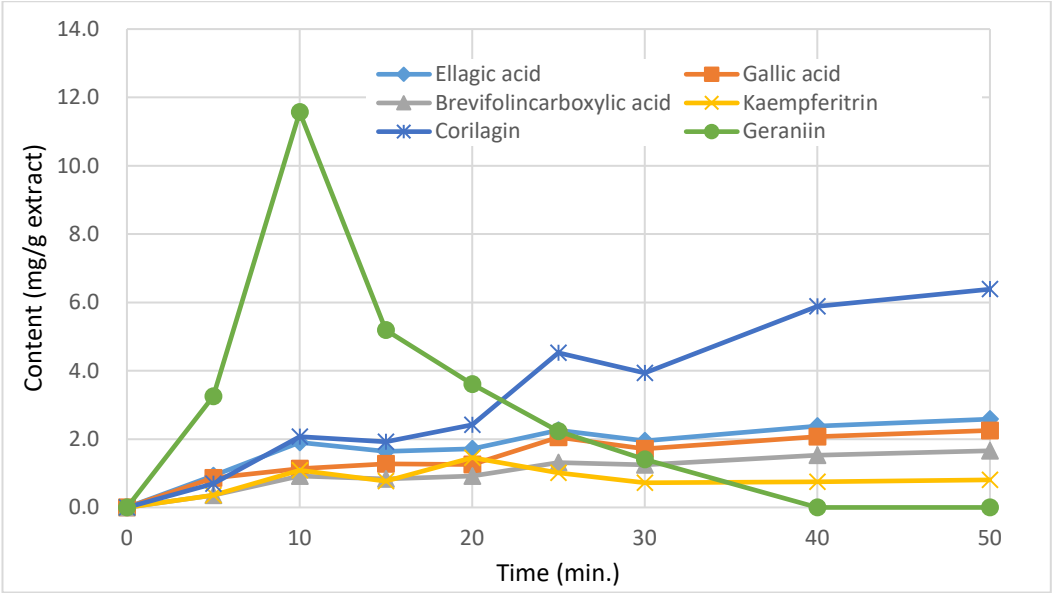


Fig 6. Time course of change in contents of the main polyphenols during decoction of *G. thunbergii*. (Reprint: Bastian, et al., 2018 [61])

The contents of main polyphenols in each extract of G. thunbergii

The extract obtained from the long-term decoction of *G. thunbergii* was successively extracted with diethyl ether, ethyl acetate, and water-saturated n-butanol. The ether, ethyl acetate, n-butanol, and water-soluble extracts were measured for main polyphenols and organic acid content using the qNMR method. The ether extract contained gallic acid exclusively. Gallic acid was also found in the ethyl acetate extract and n-butanol extract. Corilagin accounted for half of the

extracts in ethyl acetate and n-butanol. (Figure 5). These data indicate that ellagitannins, such as corilagin, could be effectively extracted by ethyl acetate and *n*-butanol

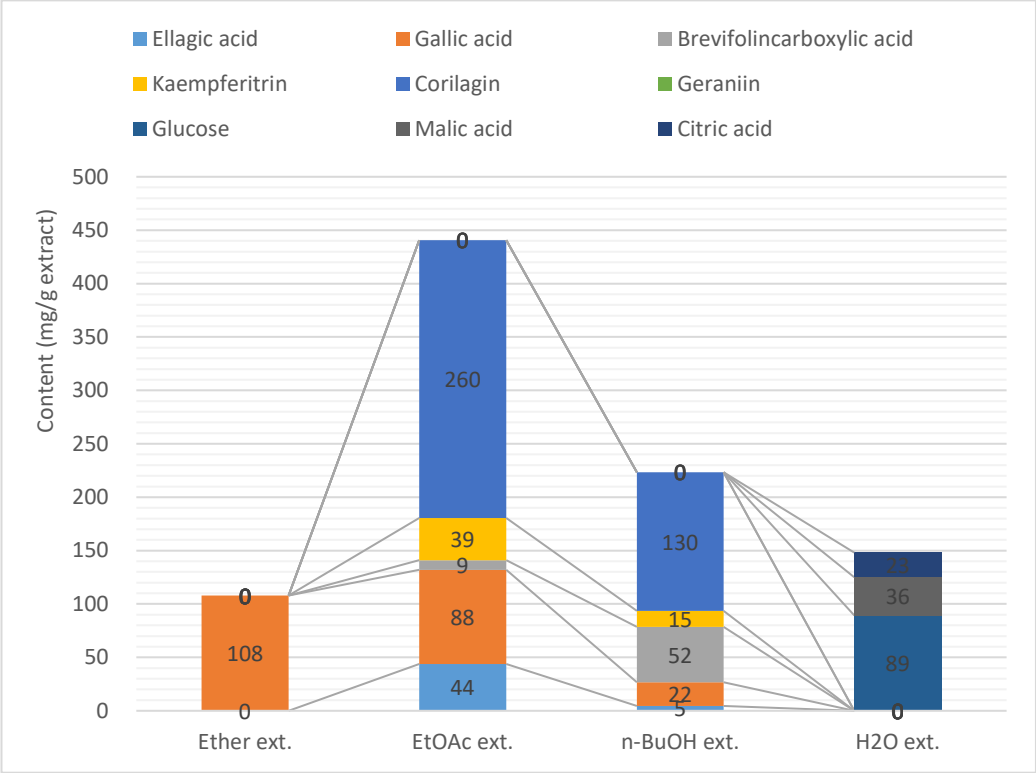


Fig 6. The content of the main polyphenols and organic acids in each extract in the extract obtained by long-term decoction of *G. thunbergii*. (Reprint: Bastian, et al., 2018 [61])

Amounts of main polyphenols of G. thunbergii cultivated in Japan and China

We estimated the amount of main polyphenols in *G. thunbergii* from nine cultivates in Japan and China using our qNMR method. All samples were extracted with 70% aqueous acetone. As shown in Table 3, *G. thunbergii* obtained from Japan contained geraniin in the range of 3.49 to 7.41 mg/g (average: 5.21 mg/g) whereas geraniin content in *G. thunbergii* from Zhejiang province in China was in the range of 0.44 to 2.82 mg/g (average: 1.61 mg/g). The amounts of

corilagin, gallic acid, ellagic acid, and brevifolincarboxylic acid were very similar between the Japanese and Zhejiang cultivates. However, kaempferitrin was found in Japan cultivates in the range of 0.13 to 0.56 mg/g (average: 0.2 mg/g) while the three cultivates from Zhejiang did not contain any kaempferitrin.

Table 3. The amounts of main polyphenols of *G. thunbergii* cultivated in Japan and China (mg/g dried weight).

Polyphenols	Japan						China		
	Fukushima	Nagano 1	Nagano 2	Nagano 3	Hyogo	Miyazaki	Zhejiang 1	Zhejiang 2	Zhejiang 3
Geraniin (**1**)	3.49	4.15	4.23	3.57	8.65	7.14	2.82	1.57	0.44
Corilagin (**2**)	0.65	0.43	0.61	0.23	0.53	0.43	0.49	0.23	0.17
Brevifolincarboxylic acid (**3**)	0.17	0.14	0.25	0.07	0.22	0.09	0.12	0.06	0.05
Kaempferitrin (**4**)	0.13	0.25	0.56	0.12	0.36	0.35	0.00	0.00	0.00
Ellagic acid (**5**)	0.33	0.37	0.41	0.23	0.47	0.30	0.28	0.13	0.12
Gallic acid (**6**)	0.21	0.23	0.31	0.21	0.23	0.03	0.14	0.08	0.09

DISCUSSION

We developed ^{1}H-NMR fingerprints for quantifying ellagitannins, especially in *G. thunbergii* extract. Several specific proton signals from these ellagitannins were identified for geraniin, corilagin, corilagin, ellagic acid, brevifolincarboxylic acid, and organic acids, such as malic acid and citric acid. Table 1 and Figure 3 show the chemical shift used for further quantifying the *G. thunbergii* extracts in this study.

Furthermore, we evaluated the quantification of polyphenols using our qNMR assay in short-term and long-term decoctions of *G. thunbergii.* Geraniin was the major compound (62.0%) in the short-term decoction extract and corilagin was the major compound (49.0%) for the long-term decoction extract (Table 2). The amount of geraniin reached a maximum after 10 min of extraction. Geraniin was not observed in the next 40 min thereafter due to hydrolysis, instead, corilagin became the dominant compound.

In Japan, there are two methods of using *G. thunbergii* as a folk medicine. The short and long decoctions are used for treating constipation and diarrhea, respectively. Our findings suggested that both geraniin and corilagin possess the ability to treat gastrointestinal diseases, but that they may show distinct functions. It might be speculated that geraniin has the ability to treat constipation while corilagin plays a key role in diarrhea treatment.

Fractionation of the long-term decoction showed that corilagin was obtained in the ethyl acetate and *n*-butanol fractions. Based on our qNMR method,

corilagin was the major compound in both fractions, obtained at 59% and 58% in ethyl acetate and n-butanol, respectively. Among the solvents tested (diethyl ether, ethyl acetate, *n*-butanol, and water), ethyl acetate and *n*-butanol were found suitable for extraction of the main ellagitannin. In addition, our data indicated that only gallic acid could be extracted by diethyl ether. Malic acid, citric acid, and glucose were all obtained in the water-soluble portion (Fig 6).

The main ellagitannin contents of *G. thunbergii* extracts from plants cultivated in Japan and Zhejiang province in China are presented in Table 3. We found that the *G. thunbergii* cultivates from Japan contained higher levels of geraniin and corilagin than those from Zhejiang. Overall, the polyphenol content of cultivates from Japan was higher than those from Zhejiang. Furthermore, the six cultivates of *G. thunbergii* from Japan contained kaempferitrin, whereas the three cultivates from Zhejiang did not contain kaempferitrin. Differences in the content of these polyphenols in *G. thunbergii* may be affected by soil and weather conditions.

All samples were directly quantified using qNMR after the extracting solvents were removed. The advantages of qNMR include accuracy, simplicity, and speediness. It occurred due to the initial separation of target analyses into pure compounds is unnecessary. Therefore, further research should be undertaken to investigate other specific chemical shifts from other ellagitannins to enrich the number of unique qNMR ellagitannin identifier peaks.

Chapter III

New polyphenols from pomegranate

Ellagitannins are a member of hydrolyzable tannin, a type of polyphenol. Beside contain galloyl groups, ellagitannins also contain various type of hexahydroxydiphenol (HHDP) units which bonded to sugar moiety. At the present, approximately 500 type of ellagitannins have been isolated from various plants [43].

Punica granatum L (Punicaceae), known as pomegranate, is edible fruit as intact arils or juice. Pomegranate was reported to contain ellagitannins, which mainly located in the fruit peel, arils, and mesocarp of pomegranate. Punicalagin and punicalin are two major monomeric of ellagitannins in pomegranate. In addition, other oligomeric of ellagitannin such as oenothein B, eucalbanin B, eucarpanin T_1, pomegraniin A, and pomegraniin B also have been reported [3]. Ellagitannins of pomegranate have demonstrated various biological activities including anti-inflammatory [32], anticancer [33, 34], anti-diabetic [35], anti-diarrheal [36], anti-carcinogenic [37], antifungal [38], antimicrobial [39], and antioxidant [40-42] activities.

Exploring the new natural compounds and their structure elucidation have increased significantly since the invention of NMR. In this study, NMR spectroscopy including 2D-NMR analyses played an important role to determine the structure of compounds. Combining with circular dichroism (CD) analysis and chemical reaction, the relative and absolute configurations of the new compound can be elucidated.

We believe that pomegranate contains various other types of polyphenols which have never been reported and might have biological activities. It was very interesting to us to explore the new compound from pomegranate. Therefore, the aim of this study is to explore the new natural compounds from pomegranate.

MATERIALS AND METHODS

General

NMR. The ^{1}H and ^{13}C NMR Spectra were analyzed in acetone-d_6-D_2O (9:1) or only acetone-d_6 at 24 ± 1 ^{O}C was acquired on a Varian NMR System (600 MHz for ^{1}H NMR and 151 MHz for ^{13}C NMR; Palo Alto, CA, USA). The standard pulse sequences programmed for the instrument were used for the different 2D NMR analyses (i.e., ^{1}H-^{1}H COSY, ROESY, HSQC, and HMBC).

CD Spectral. Optical rotations were acquired using Jasco DIP-1000 polarimeter (Jasco, Tokyo, Japan). CD Spectral was acquired on a Jasco J-720 W Spectrometers.

ESI-MS. The mass spectra were obtained on a Bruker MicrOTOF II Spectrometer (Bruker, Billerica, MA) using a HR-ESI-MS source in negative-ion mode and APCI-MS source in a positive-ion mode.

NP-HPLC. The Normal Phase HPLC system (Shimadzu) consisted of a liquid chromatograph (model LC-10ATvp), a UV-vis detector (model SPD-10A), and a Column (YMC-Pack SIL A-003, 46 mm i.d x 250mm). The mobile phase to separate the injected extracts consisted of *n*-hexane: methanol: tetrahydrofuran: formic acid (47:39:13:1, v/v, respectively) contain oxalic acid (450 mg/L). The wavelength on UV-vis detector was set at 280 nm.

RP-HPLC. Reversed Phase HPLC system consisted of a pump (Hitachi ELITE la Chrom) and equipped with diode array detector (Hitachi Diode Array Detector L-7455). The separation using Inert sustain C18 column (GL Science, 5

μm 4.6 i.d x 150 mm) at 40 ^{O}C. The mobile phase was applied by two solutions, i.e. eluent A (90% water: 5% acetonitrile: 5% formic acid) and eluent B (50% water: 45% acetonitrile: 5% formic acid). The eluent was programmed as follow: 0 min 0% B; 30 minute 100% B; and 30.1-45 minute 0% B. The flow rate of mobile phase was set at 1mL/min.

Column chromatography. Fractionation and purification by column chromatography were conducted on Diaion HP-20 (Mitsubishi Kasei Co., Tokyo, Japan), Toyopearl HW-40 (coarse grade) (Tosoh Co., Japan), MCI EL CHP-20P (75-15 μm) (Mitsubishi Kasei Co., Tokyo, Japan), and YMC-gel ODS-AQ 120-50S (YMC Co., L.td). Mega Bond Elut C-18 6mL Agilent Technologies also used for purification.

Extraction and Isolation

The extract of arils juice (0.5 kg) of pomegranate was subjected to a Diaion HP-20 column chromatography (10 cm i.d. x 70 cm) and extracted sequentially with H_2O, 10%-100% aqueous MeOH, MeOH, and 70% aqueous acetone. A portion (5.0 g) of the 30% MeOH fraction (35.64 g) was further chromatographed over Toyopearl HW-40 (coarse grade) (35 cm x 2.2 cm i.d., 350 drops/fr.) with 50% aqueous MeOH, to obtain corilagin (88.2 mg) (fr. 106-117) and crude of **1** (74.9 mg) (fr. 150-168). The crude pomegraniic acid was purified by Mega Bond Elut C-18 with H_2O and aqueous MeOH (10-50%) to obtain a new compound pomegraniic acid (**17**) (19.7mg) (0.0034%) from the 30% aqueous MeOH fraction.

The extract of arils juice of pomegranate (2137 g) subjected to Diaion HP-20 (10 cm i.d x 70 cm) column chromatography with 10, 30, and 50% aqueous MeOH. Fraction 50% aqueous MeOH (1.84 g) was separated by Toyopearl HW-40C (2.2 cm i.d. x 30 cm, 400 drops/fr) with 50-70% aqueous MeOH, MeOH, MeOH:H_2O:acetone (7:2:1), MeOH: H_2O: acetone (7:1:2), and 70% aqueous acetone, respectively. From fraction 50% aqueous MeOH (Fractions 46-49), a new compound 1-*O*-Galloyl-6-*O*-(4-hydroxybenzoyl)-β-D-glucose(**23**) (12.8 mg) was obtained.

The fresh leaves of pomegranate (590 g) were extracted with 70% aqueous acetone to obtain acetone extract (150 g). Acetone extracts (20 g) was further extracted using ether, ethyl acetate (EtOAc), *n*-butanol, and H_2O. Fraction EtOAc extract (5.2 g) was separated by Toyopearl HW-40C (36 cm x 2.2 cm i.d., 300 drops/fr) column chromatography (1st CC) with 50%-70% aqueous MeOH, and 70% aqueous acetone. This separation yielded granatin A (377 mg) and granatin B (1.1 g). Fractions 46-67 from 1st CC was separated by Sephadex LH-20 (1.1 cm i.d. x 37 cm, 300 drops/fr) column chromatography (2nd CC) using EtOH and MeOH. Fraction EtOAc extract (8.5 g) from leaves pomegranate also was separated by Toyopearl HW-40C (36cm x 2.2cm i.d., 300 drops/fr) column chromatography (3rd CC) with 50-70% aqueous MeOH, MeOH, and 70% aqueous acetone to obtain granatin A (251.6 mg). Fractions 50-87 from 3rd CC (965.5 mg) was subjected to Sephadex LH-20 (1.1 cm i.d x 37 cm, 200 drops/fr) column chromatography (4th CC) with EtOH, EtOH:MeOH (1:1), and then MeOH, respectively. Fractions 86-97 (63.3 mg) from 4th CC was purified on YMC Gel

ODS-AQ-HG (1.1 cm i.d. x 20 cm, 300 drops/fr) column chromatography (6^{th} CC) with H_2O, 10-40% aqueous MeOH to yield a new compound 6-*O*-*p*-coumaroyl-1-*O*-galloyl-β-D-glucose(**24**) (3.1 mg). Fractions 17-19 from 2^{nd} CC, 98-100 from 4^{th} CC, and 1-80, 93-95 from 5^{th} CC were combined and separated by MCI gel CHP 20P (1.1 cm i.d. x 35 cm, 250 drops/fr) with 20-70% aqueous MeOH, MeOH, and 70% aqueous acetone, respectively to yield another new compound 4-*O*-(6'-*O*-Galloyl-β-D-glucosyl)–coumaric acid(**25**).

Physicochemical data

Pomegraniic acid (**17**). Pale brown amorphous powder; t_R on normal-phase HPLC: 6.68 min; ^{1}H-NMR (600 MHz, acetone-d_6:D_2O (9:1), δ): 6.24 (d , *J*=4.2Hz, 1H, Glc-H1), 4.30 (d, *J*=4.8Hz, 1H, Glc-H2), 5.06 (d, *J*=3.6 Hz, 1H, Glc-H3), 5.69 (d, *J*=3.6Hz, 1H, Glc-H4), 4.63 (t, *J*=4.62Hz, 1H, Glc-H5), 4.54 (br.dd, Glc-H6), δ 4.39 (br.dd, Glc-H6), 6.74 (s, 1H, HHDP-Ring A-H3), 6.87 (s, 1H, HHDP-Ring B-H3), 7.13 (s, 2H, galloyl-H2,6), 7.41 (s, 1H, Ring D-H3), 5.04 (d, *J*=1.8 Hz, 1H, Ring E-H1), 5.11 (d, *J*=1.8 Hz, 1H, Ring E-H4), 3.24, 3.14 (dd, *J*=16.8 Hz, 2H, Ring E-H5). ^{13}C-NMR (151 MHz, acetone-d_6:D_2O (9:1), δ): 94.0 (Glc-C1), 69.7 (Glc-C2), 71.3 (Glc-C3), 64.6 (Glc-C4), 74.0 (Glc-C5), 63.9 (Glc-C6), 114.7 (HHDP-Ring A-C1), 124.5 (HHDP-Ring A-C2), 107.6 (HHDP-Ring A-C3), 144.4 (HHDP-Ring A-C4), 135.6 (HHDP-Ring A-C5), 144.1 (HHDP-Ring A-C6), 168.0 (HHDP-Ring A-C7), 115.7 (HHDP-Ring B-C1), 124.3 (HHDP-Ring B-C2), 109.0 (HHDP-Ring B-C3), 144.0 (HHDP-Ring B-C4), 136.3 (HHDP-Ring B-C5), 144.1 (HHDP-Ring B-C6), 166.3 (HHDP-Ring B-C7),

119.4 (galloyl-C1), 109.5 (galloyl-C2,6), 145.2 (galloyl-C3), 138.9 (galloyl-C4), 145.2 (galloyl-C5), 165.1 (galloyl-C7), 121.4 (Ring D-C1), 146.7 (Ring D-C2), 112.9 (Ring D-C3), 164.0 (Ring D-C4), 134.9 (Ring D-C5), 146.5 (Ring D-C6), 164.0 (Ring D-C7), δ 50.5 (Ring E-C1), 87.9 (Ring E-C2), 174.0 (Ring E-C3), 81.0 (Ring E-C4), 37.6 (Ring E-C5), 169.6 (Ring E-C6), δ 170.5 (Ring E-C7). HR-ESI-MS 969.0853 *m/z* $[M-H]^-$ (calcd for $C_{41}H_{29}O_{28}$ -H, *m/z* 969.0851).

1-*O*-Galloyl-6-*O*-(4-hydroxybenzoyl)-β-D-glucose (**23**). Pale brown amorphous powder. ^{1}H-NMR (600 MHz, acetone-d_6-D_2O (9:1), δ): 7.83 (d, 2H, *J*=8.4 Hz, H2",6") , 7.11 (s, 1H, galloyl-H), 6.85 (d, 2H, *J*=8.4 Hz, H-3",5"), 5.66 (d, 1H, *J*=8.4 Hz, Glc-H1), 4.56 (dd, 1H, *J*=1.8 Hz, 12 Hz, Glc-H6α), 4.33 (dd, 1H, *J*=5.4 Hz, 12 Hz, Glc-H6β), 3.75-3.84 (overlapped with solvent peak, Glc-H5), 3.60 (dd, 1H, *J*=8.4 Hz, 9 Hz, Glc-H2), 3.55 (t, 1H, *J*=9 Hz, Glc-H3), 3.53 (t, 1H, *J*=9 Hz, Glc-H4). ^{13}C-NMR (151 MHz, acetone-d_6:D_2O (9:1), δ): 166.2 (C7"), 165.1 (C7'), 162.1 (C4"), 145.2 (C3', 5'), 138.8 (C4'), 131.7 (C2", 6"), 120.8 (C1"), 119.5 (C1'), δ 115.1 (C3", 5"), 109.3 (C2', 6'), 94.7 (Glc-C1), 76.5 (Glc-C5), 74.8 (Glc-C2), 72.6 (Glc-C3), 70.0 (Glc-C4), 63.5 (Glc-C6). HR-ESI-MS: *m/z* 451.0859 $[M-H]^-$ (calcd.for $C_{20}H_{20}O_{12}$ -H, *m/z* 451.0882).

6-*O*-*p*-coumaroyl-1-*O*-galloyl-β-D-glucose (**24**). Pale brown amorphous powder. ^{1}H-NMR (600 MHz, acetone –d_6:D_2O (9:1) δ): 7.59 (d, *J*=16.2 Hz, 1H, Hβ), 7.49 (d, *J*=8.4 Hz, 2H, H-2", 6"), 7.15 (s, 2H, H2', 6'), 6.86 (d, *J*=8.4 Hz, 2H, H3", 5"), 6.33 (d, *J*=16.2 Hz, 1H, Hα), 5.67 (d, *J*=7.2 Hz, 1H, Glc-H1), 4.49 (dd, *J*= 1.2, 12.0 Hz, 1H, Glc-H6), 4.28 (dd, *J*=6.0, 12.0 Hz, 1H, Glc-H6), 3.72 (ddd, *J*=1.2, 6.0 9.6 Hz, 1H, Glc-H5), 3.58 (t, *J*=9.6 Hz, 1H, Glc-H3), 3.54 (dd,

J=7.2, 9.6 Hz, 1H, Glc H2), 3.48-3.44 (overlapped with solvent peak, Glc H4). ^{13}C-NMR (151 MHz, acetone–d_6:D2O (9:1), δ): 170.0 (C7”), 165.0 (C7’), 160.0 (C4”), 145.2 (C3’, 5’), 145.2 (Cβ), 138.8 (C4’), 130.2 (C2”, 6”), 125.7 (C1”), 119.5 (C1’), 115.8 (C3”, 5”), 114.0 (Cα), 109.3 (C2’, 6’), 94.6 (Glc C1), 76.6 (Glc C3), 74.9 (Glc C5), 72.7 (Glc C2), 69.9 (Glc C4), 63.3 (Glc C6). HR-ESI-MS: *m/z* 477.1037 [M-H]$^-$ (calcd.for $C_{22}H_{21}O_{12}$ -H, *m/z* 477.1038).

4-*O*-(6’-*O*-Galloyl-β-D-glucosyl)–coumaric acid (**25**). Pale brown amorphous powder. ^{1}H-NMR (600 MHz, acetone–d_6:D_2O (9:1) δ): 7.58 (d, *J*=9.0 Hz, 2H, H2’, 6’), 7.54 (d, *J*=16.2 Hz, 1H, Hβ), 7.17 (s, 2H, H-2”, 6”), 7.13 (d, *J*=9.0 Hz, 2H, H3’, 5’), 6.26 (d, *J*=16.2 Hz, 1H, Hα), 5.05 (d, *J*=7.8 Hz, 1H, Glc-H1), 4.66 (dd, *J*=1.8, 11.4 Hz, 1H, Glc-H6), 4.26 (dd, *J*=8.4, 12.0 Hz, 1H, Glc-H6), 3.91 (t, *J*=8.4 Hz, 1H, Glc-H5), 3.59 (t, *J*=9.0 Hz, 1H, Glc-H3), 3.54 (dd, *J*=7.8, 9.0 Hz, 1H, Glc-H2), 3.49-3.43 (overlapped with solvent peak, Glc-H4). ^{13}C-NMR (151 MHz, acetone–d_6:D_2O (9:1), δ): 168.7 (C7’), 166.0 (C7”), 159.2 (C4’), 145.4 (C3”, 5”), 143.6 (Cβ), 138.2 (C4”), 129.6 (C2’, 6’), 128.7 (C1’), 120.5 (C1”), 117.4 (Cα), 116.7 (C3’, 5’), 109.0 (C2”, 6”), 100.5 (Glc-C1), 76.7 (Glc C3), 74.2 (Glc C5), 73.5 (Glc C2), 70.4 (Glc C4), 63.9 (Glc C6). HR-ESI-MS: *m/z* 477.1050 [M-H]$^-$ (calcd. for $C_{22}H_{21}O_{12}$ -H, *m/z* 477.1038).

Methylation, Methanolysis, and hydrolysis of pomegraniic acid.

Methylation. A mixture of pomegraniic acid crude (10 mg), acetone super dehydrated (5 ml), potassium carbonate anhydrous (100 mg), dimethyl sulfat (0.1 ml) were stirred overnight at room temperature and then refluxed for 30 minutes.

After removal the solvent, the sample was purified by preparation TLC (toluena:acetone, 5:1 (v/v)) to obtain the pomegraniic acid methylated.

Methanolysis. A mixture of pomegraniic acid methylated (PA-Me) and 1% sodium methanolate methanol (NaOMe) in MeOH (1 ml) were left overnight at room temperature. The reaction was stopped by adding 3 drops acetic acid (AcOH) then added trimethyl silyldiazomethane ($C_4H_{10}N_2Si$) (0.5 ml) for an hour reaction. After removal of the solvent by evaporator, residues were purified by TLC preparation using *n*-hexane:acetone, 3:1 (v/v) as mobile phase solvent.

Partial Hydrolysis of pomegraniic acid. Hydrolysis of **17** (10 mg) in 1 ml of HCl 1 M yielded **5** (0.7 mg). Hydrolysis was conducted for two hours in boiled water (95 ^{O}C). After removal of the acid by H_2O using Mega bond elut C18, residues were purified by semi-preparative HPLC by eluting with H_2O:CH_3CN:CH_2O_2 (90:5:5, v/v) using Inert sustain C18 column (GL Science, 5 µm 4.6 i.d x 150 mm) at 40 ^{O}C.

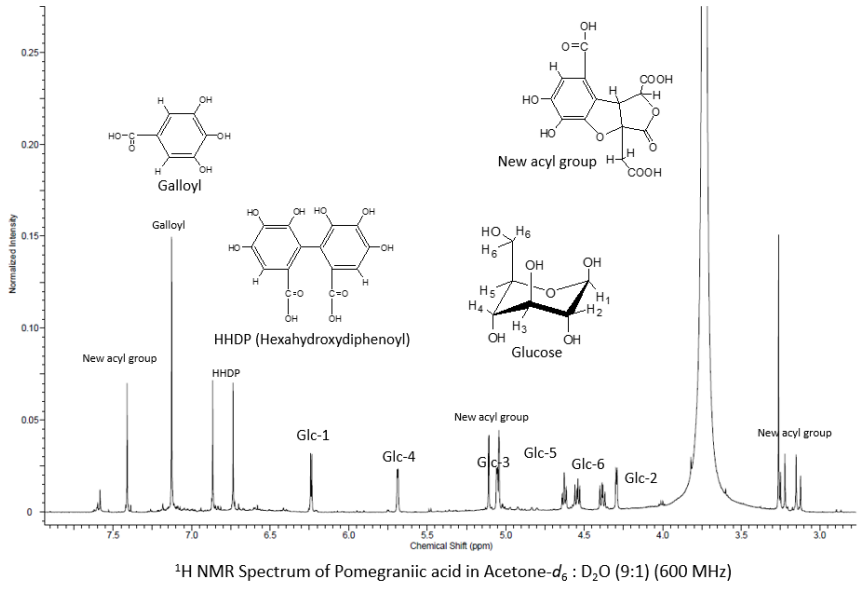
R E S U L T S

1. New monomeric ellagitannin

A new monomeric ellagitannin named pomegraniic acid (**17**) was obtained as a pale brown amorphous powder and exhibited a molecular ion peak at *m/z* 969.0853 $[M–H]^-$ (calcd for $C_{41}H_{29}O_{28}$, *m/z* 969.0851) by HR-ESI-MS. The molecular formula was determined to be $C_{41}H_{30}O_{28}$.

NMR Analyses

The ^{1}H-NMR spectrum of **17** exhibited one 2H singlet (δ_H 7.13) and three 1H singlets (δ_H 6.74, 6.87, and 7.41) on the aromatic region. The 2H singlet (δ_H 7.13) indicated a galloyl group, while two of 1H singlet peaks at δ_H 6.74 and 6.87 were assignable to HDDP. The other 1H singlet peak at δ_H 7.410 and was indicated an aromatic group on the new 4-acyl group (Fig 7).



^{1}H NMR Spectrum of Pomegraniic acid in Acetone-d_6 : D_2O (9:1) (600 MHz)

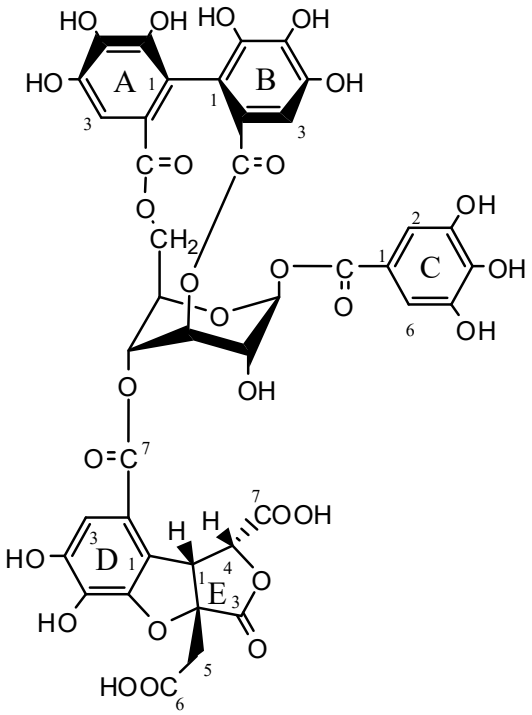
Fig 7. ^{1}H-NMR spectrum of pomegraniic acid in acetone-d_6 : D_2O (9:1) (600 MHz)

Table 4. ^{1}H and ^{13}C NMR spectroscopic data of pomegraniic acid (17)

	Position	δ_H	δ_C
Ring A (HDDP)	1		114.7
	2		124.5
	3	6.74 (s)	107.6
	4		144.4
	5		135.6
	6		144.1
	7		168.0
Ring B (HDDP)	1		115.7
	2		124.3
	3	6.87 (s)	109.0
	4		144.0
	5		136.3
	6		144.1
	7		166.3
Glucose	1	6.24 (d, *J*=4.2 Hz)	94.0
	2	4.30 (d, *J*=4.8 Hz)	69.7
	3	5.06 (d, *J*=3.6 Hz)	71.3
	4	5.69 (d, *J*=3.6 Hz)	64.6
	5	4.63 (t, *J*=7.8 Hz)	74.0
	6	4.54 (dd, *J*=7.2, 11.4 Hz) 4.39 (dd, *J*=7.8, 11.4 Hz)	63.9
Ring C (Galloyl)	1		119.4
	2,6	7.13 (s)	109.5
	3		145.2
	4		138.9
	5		145.2
	7		165.1
Ring D	1		121.4
	2		146.7
	3	7.41 (s)	112.9
	4		164.0
	5		134.9
	6		146.5
	7		164.0
Ring E	1’	5.04 (d, *J*=1.8 Hz)	50.5
	2’		87.9
	3’		174.0
	4’	5.11 (d, *J*=1.8 Hz)	81.0
	5’	3.15 (dd, *J*=16.8; 59.4 Hz)	37.6
	6’		169.6
	7’		170.5

The NMR spectrum also showed signals attributable to the sugar protons (δ_H 4.30 – 6.24). Comparing with other known ellagitannins, the ^{1}H NMR spectrum of

the sugar is characterized by $^{1}C_4$ glucopyranose [50-53]. It was supported by the small coupling constant of the anomeric proton, Glc-2, Glc-3, and Glc-4 (3.6 - 4.2 Hz) (Table 4).



17

Fig 8. Structure of pomegraniic acid

The 2D-NMR Hetero-nuclear Multiple-bond Connectivity (HMBC) spectra indicated that the galloyl, HHDP, and the new 4-acyl group attached to *O*-1, *O*-3~*O*-6 and *O*-4 position at glucose, respectively. This is also supported by the appearance of signals attributable to four ester carbonyl carbon that observed in the range of δ_C 164-168 on the ^{13}C-NMR spectrum. Each ester carbonyl carbon signals of HHDP at δ_C 168.0 and 166.3 showed HMBC correlation peaks with proton signal of Glc-6 and Glc-3, respectively. The ester carbonyl carbon of the

galloyl at δ_C 165.1 has HMBC correlation peak with two proton signals of methine on the benzene ring of galloyl and proton signal of the glucose anomeric proton signal. Meanwhile ester carbonyl carbon at δ_C 164.032 has HMBC correlation peak with the proton signal of Glc-4 and an aromatic proton on the aromatic ring of new 4-acyl group (Fig 9).

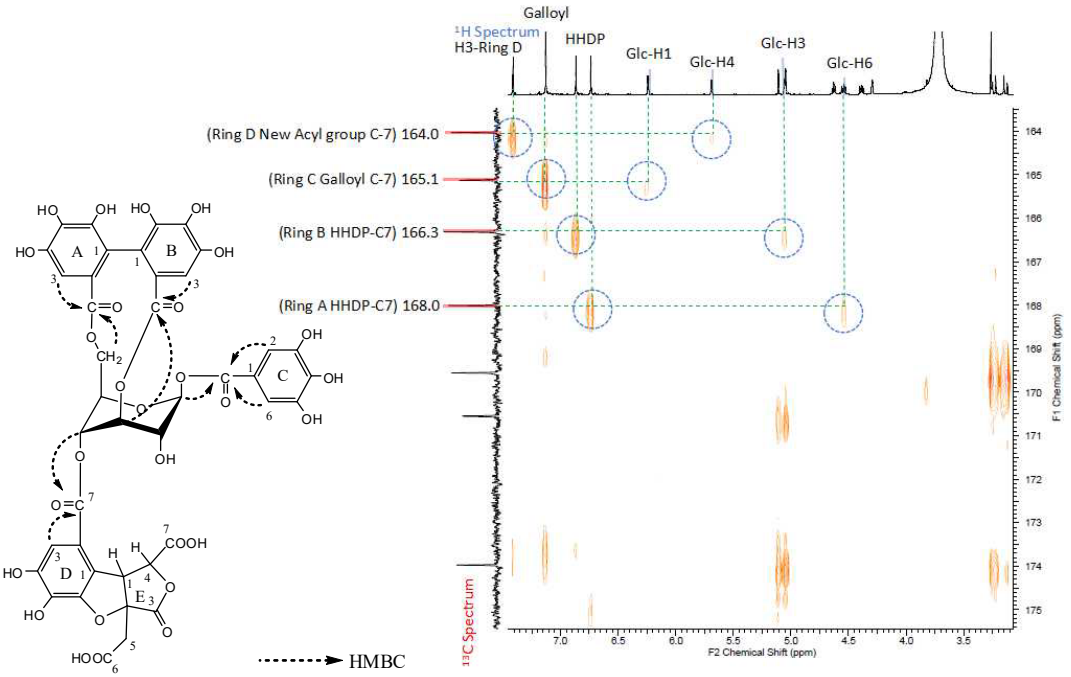


Fig 9. Key ^{1}H-^{13}C Hetero-nuclear Multiple-bond Connectivity (HMBC) correlations of pomegraniic acid

^{1}H-^{1}H ROESY spectrum showed that methine proton of galloyl at δ_H 7.13 has a correlation to methylene proton of Glc-6 at δ_H 4.54 and δ_H 4.39. Beside that anomeric proton of glucose at δ_H 6.24 has a correlation to δ_H 7.41 of the new 4-acyl group (Fig 10). Base on this finding, the anomeric position of glucose was confirmed as β-configuration.

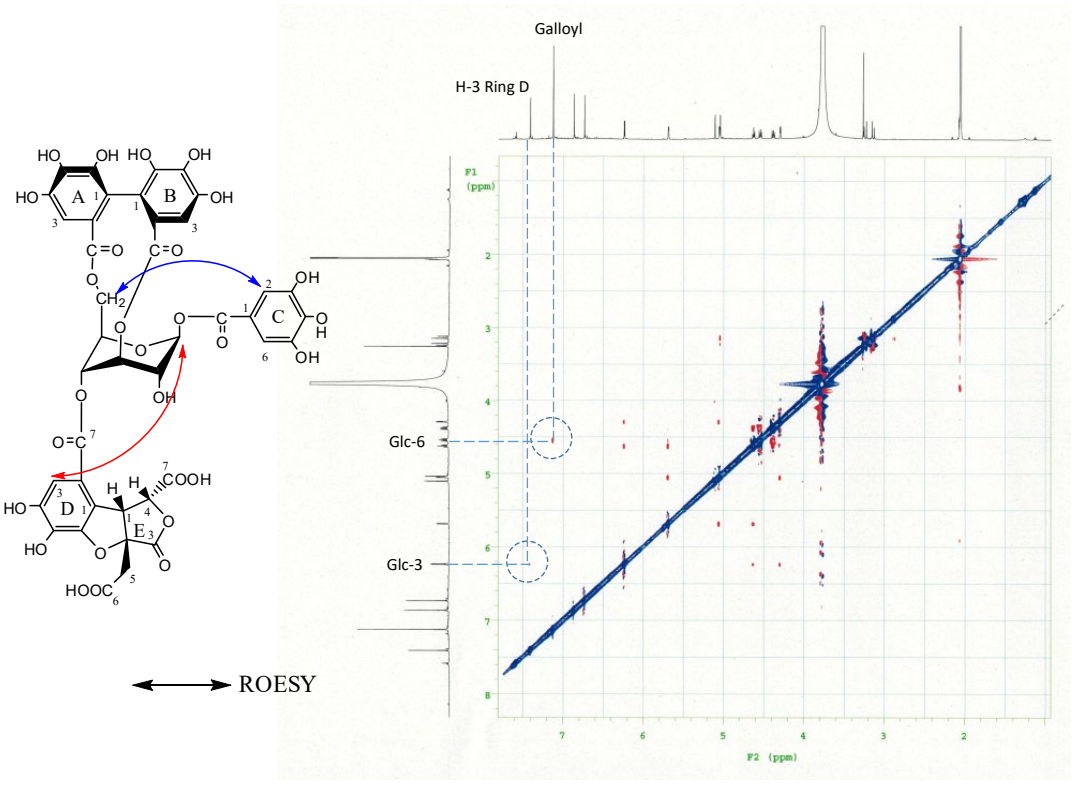


Fig 10. ^{1}H-^{1}H ROESY correlations 2D NMR of pomegraniic acid to confirm glucose configuration

Determining the new 4-acyl group

The structure of the new 4-acyl group was elucidated based on NMR spectrum and further detail spectroscopic data including HMBC, HSQC, ROESY, CD Spectrum, and also chemical reaction evidence was detailed.

On the basis of ^{13}C NMR spectrum, 4-acyl group consists of six aromatic carbons (δ_C 121.4, 146.7, 112.9, 164.0, 134.9, 146.5), a tertiary carbon (δ_C 87.9), four ester carbonyl carbons (δ_C 174.0, 169.6, 170.5, 164.0), two methine carbons (δ_C 50.5, 81.0), and a methylene carbon (δ_C 37.6) (Table 4).

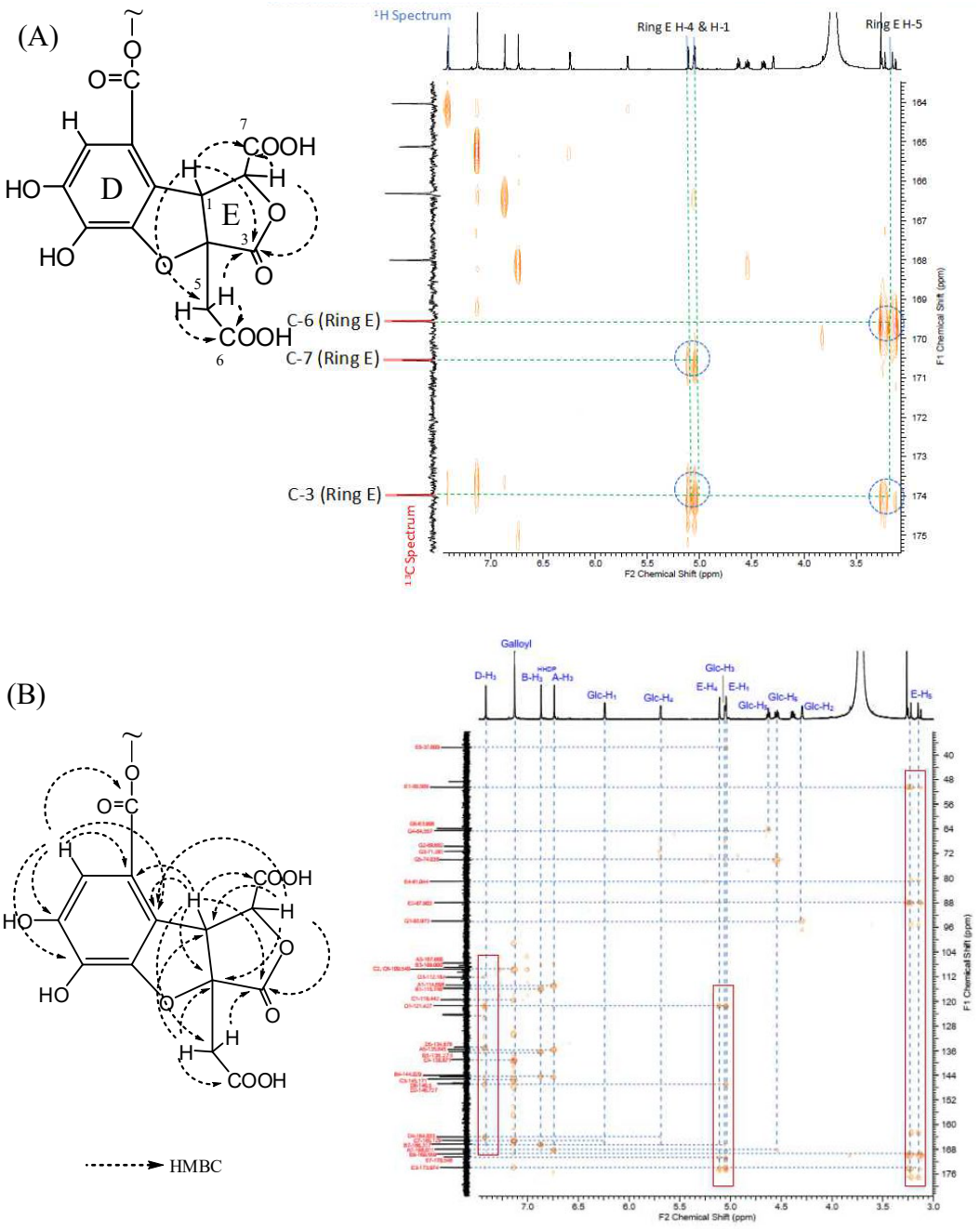


Fig 11. The key (A) and the comprehensive (B) ^{1}H-^{13}C HMBC correlations of 4-acyl group in pomegraniic acid

The proton at δ_H 7.41 has HMBC correlation peaks to others carbon at benzene ring (δ_C 121.4, 146.7, 164.0, and 134.9) and also to carboxylic proton at δ_C 165.1. The proton at δ_H 5.04 which attached to benzylic carbon showed HMBC

correlation to others carbon, i.e. carbons-3, 2 at benzene ring-D (δ_C 112.9, 146.7), the carbon-7 at carboxylic acid (δ_C 164.0), carbon-3 at ester group (δ_C 174.0), the tertiary carbon-2 (δ_C 87.9), and the methylene carbon-5 (δ_C 37.6). Furthermore, the HMBC spectrum showed a methine proton at δ_H 5.11 has a correlation with carboxylic acid carbon-7, benzylic carbon-1, tertiary carbon-2, and ester carbon-3 (Fig 11). On the basis of the HSQC spectrum, the methylene proton attached at carbon-5 (δ_C 37.6).

The relative configuration of the 4-acyl group was also confirmed by ^{1}H-^{1}H ROESY spectrum (Fig 12). The ROESY spectrum showed that methine proton at carbon-1 has correlations to proton at methylene carbon-5and methine carbon-4. This fact gives information that these protons have the same directions, and both of carboxyl groups on ring E (carbon-5 and 7) have the opposite directions each other.

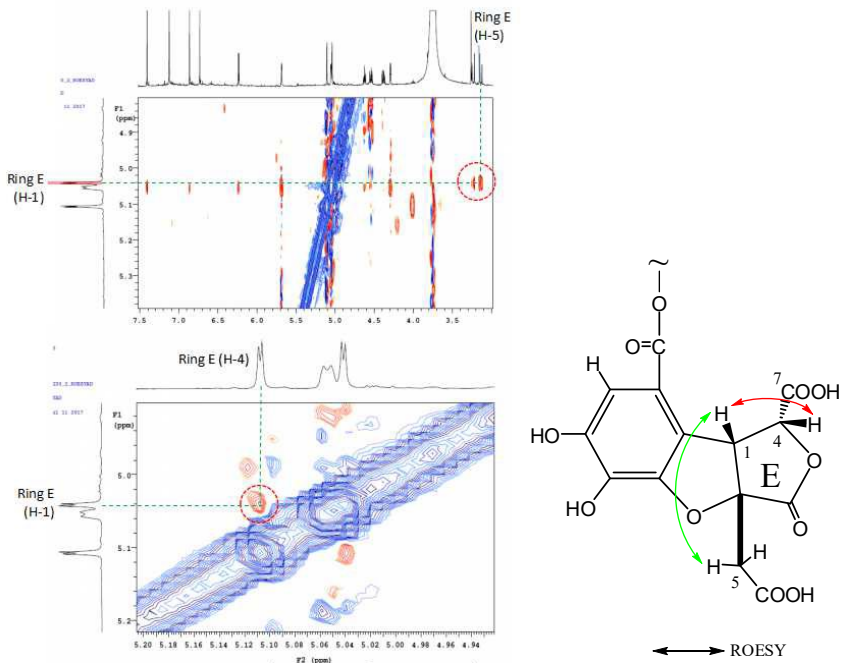


Fig 12. The ROESY correlation of 4-acyl group of pomegraniic acid

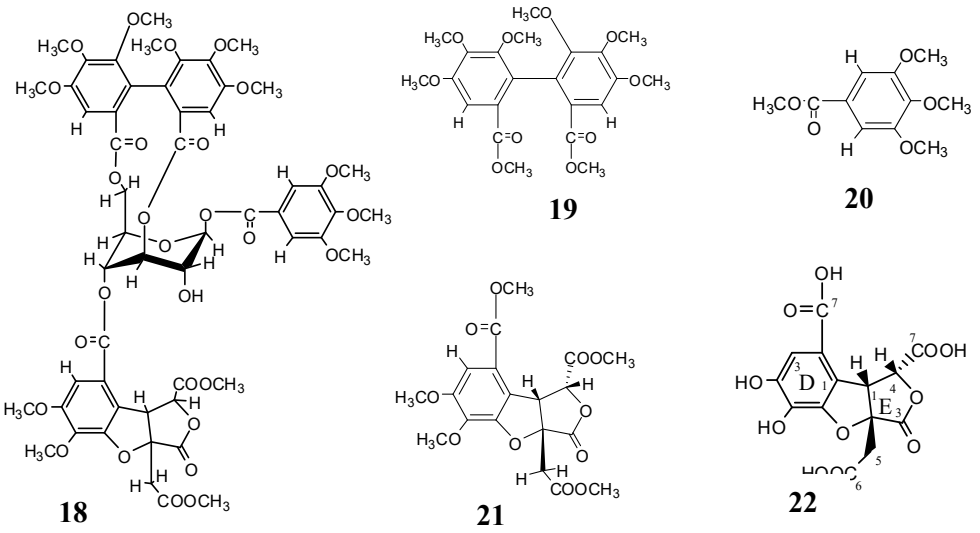


Fig 13. Trideca-*O*-methyl Pomegraniic acid methylated (18), Acyl group derivatives methylated (19, 20, 21), and 4-acyl group hydrolysate (22).

Methylation, Methanolysis, and hydrolysis of pomegraniic acid.

Methylation and methanolysis processed to obtain clear evidence of the pomegraniic acid structure. Methylation process was conducted to confirm 13 -OH which attached to benzene ring and the carboxylic group, while methanolysis process was conducted to confirm the acyl group of pomegraniic acid. The ^{1}H NMR spectrum of trideca-*O*-methyl pomegraniic acid (**18**) showed 39 H of 13 methylene groups (δH 3.60 – 4.00) after methylation of **17** (Fig 14) indicating that 13 hydroxyl groups of HDDP, galloyl, and 4-acyl group of **17** were converted to methylene groups and they were detected on ^{1}H NMR spectrum. The molecular mass of **18** was confirmed by ESI-MS: *m/z* 1152.30 $[M-H]^-$ (calcd. for $C_{54}H_{55}O_{28}$, *m/z* 1153.30).

Acyl groups were also confirmed by chemical degradation using methanolysis. Compound **18** was degraded by sodium methanolate methanol (NaOMe) and successfully yielded **19**, **20**, and **21**. The molecular mass of **19**, **20**,

and **21** were further confirmed by analysis APCI-MS (*m/z* 451.16 $[M+H]^+$, *m/z* 227.09 $[M+H]^+$, *m/z* 425.10 $[M+H]^+$, respectively).

Compound 4-acyl group (**22**) were yielded from hydrolysis reaction by HCl 1 M during for 2 hours, then isolated by Mega Bond Elut C18. The hydrolysis of pomegraniic acid was conducted to complete the absolute configuration of the **22** by analyses of CD spectrum.

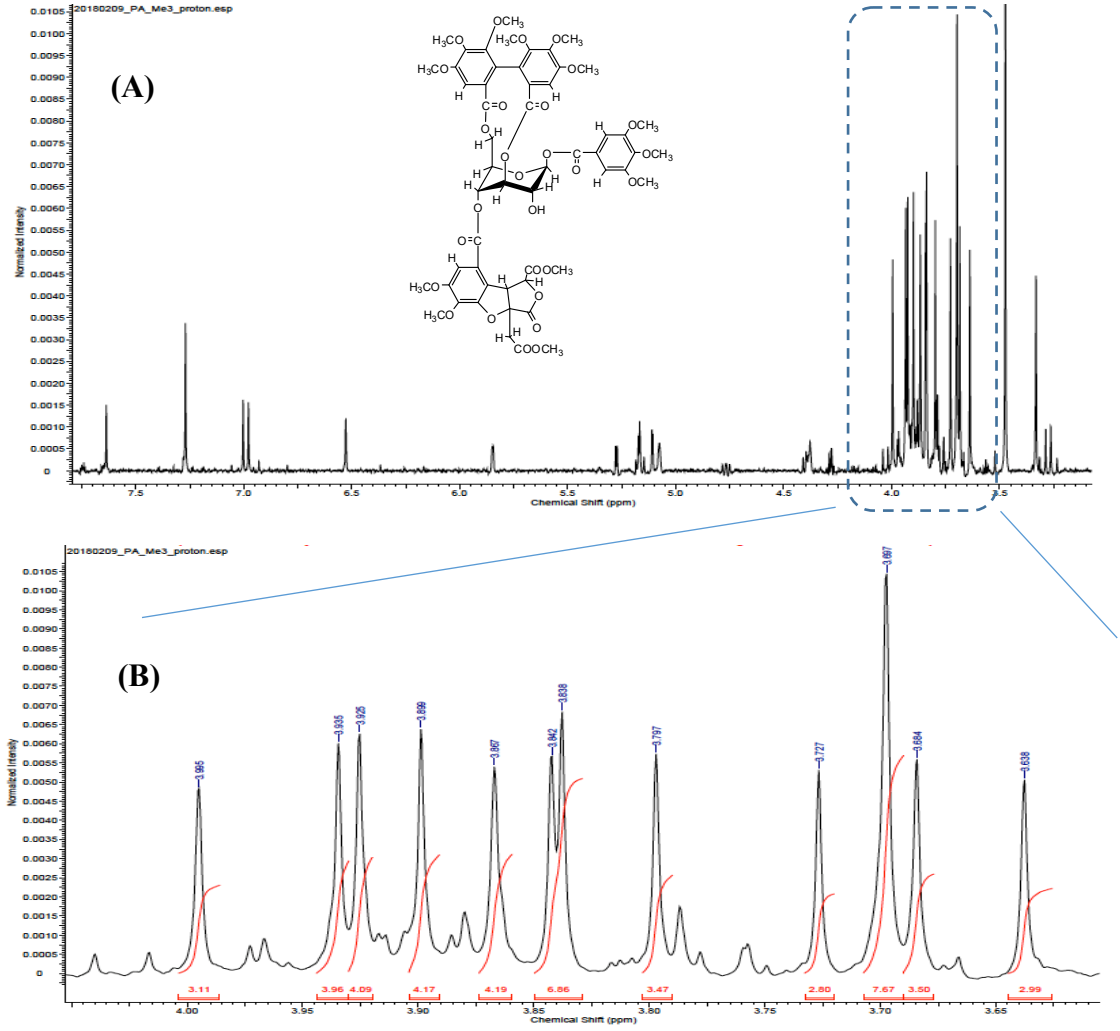


Fig 14. ^{1}H-NMR spectra of trideca-*O*-methyl pomegraniic acid methylated (A) and the expansion of methoxyl group of the ^{1}H-NMR spectrum of trideca-*O*-methyl pomegraniic acid methylated (B)

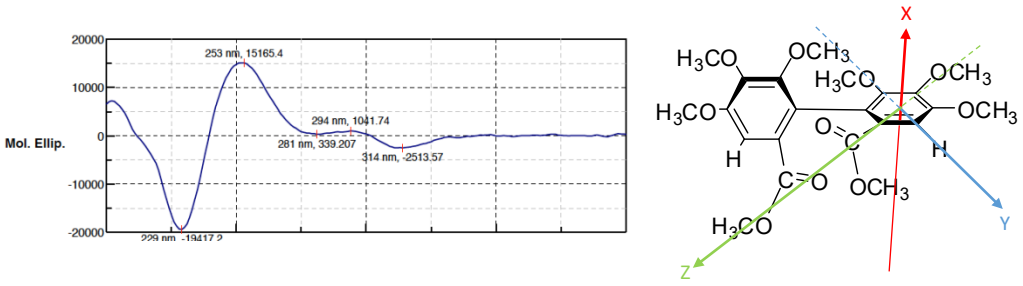


Fig 15. CD Spectrum of HHDP-Me showed (*R*)-configuration

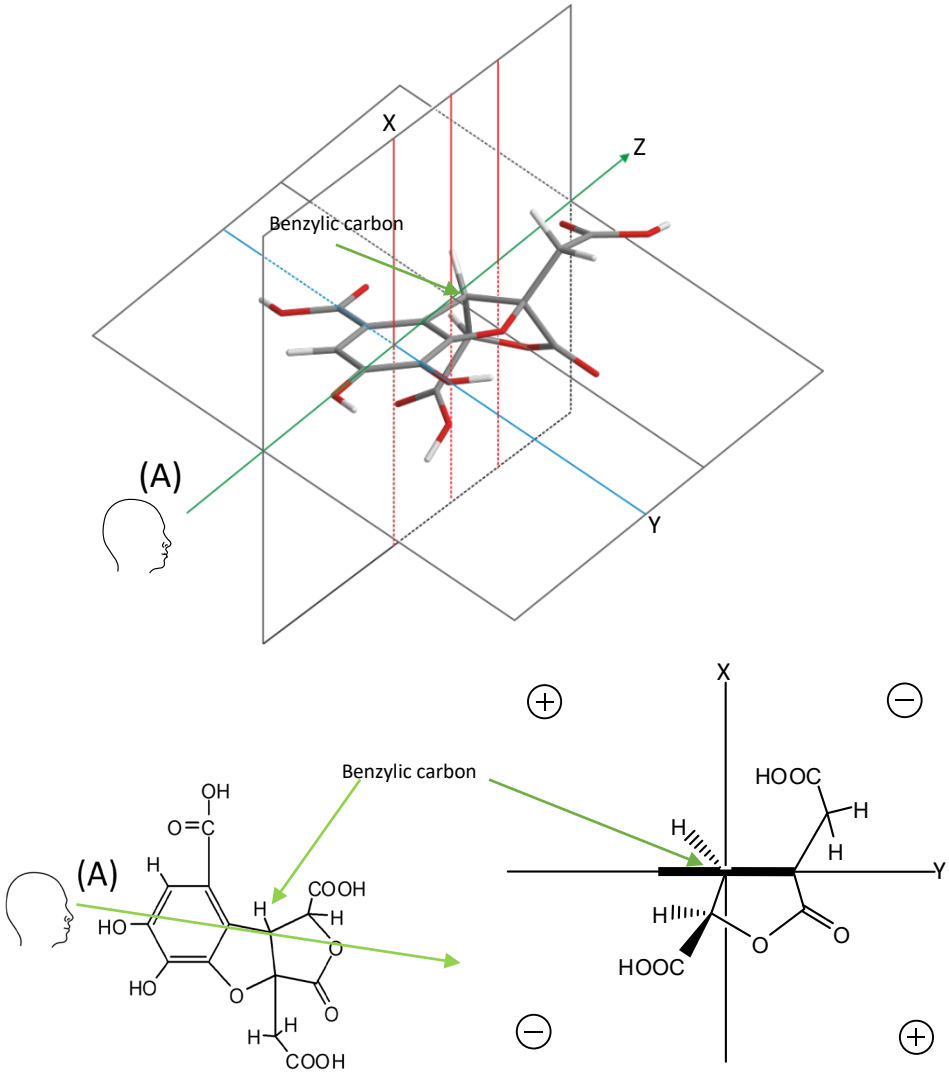


Fig 16. Quadrant projection of 4-acyl group of pomegraniic acid. (A) Projection in the direction of arrow.

CD Spectral analysis of HHDP and 4-acyl group

The CD spectrum of compound **19** showed a negative Cotton effect at 229 nm and a positive cotton effect at 253 nm. These results indicate that HHDP-Me has the (*R*)-configuration (Fig 15).

The direction of benzylic carbon of 4-acyl group (**22**) was determined based on the aromatic quadrant rules [54, 55]. The CD spectra of the 4-acyl group showed the negative cotton effect at 237 nm, indicating that 4-acyl group has an *R*-position of the benzylic carbon as shown in the quadrant projection on Fig 16.

2. New galloyl glucose derivatives

1-O-galloyl-6-O-(4-hydroxybenzoyl)-β-D-glucose

Compound **23** was isolated as a pale brown amorphous powder. The molecular formula of $C_{20}H_{21}O_{12}$ was established based on the $[M-H]^-$ ion the HR-ESI-MS at *m/z* 451.0859 (calcd. for $C_{20}H_{20}O_{12}$, *m/z* 451.0882).

The ^{1}H-NMR spectrum of **23** showed the compound consists of a glucose, a galloyl group, and a hydroxybenzoyl group. On the aliphatic proton region of ^{1}H-NMR, the presence of large proton coupling constants ($J_{1,2}$=8.4 Hz, $J_{2,3}$=9 Hz, $J_{3,4}$=9 Hz) among proton signals indicated the glucose has $^4C_1$ conformation and the glucose anomeric was β-configuration [56]. The occurrence of a galloyl moiety was indicated by the presence of a proton singlet 2H at δ_H 7.11. Two doublets at δ_H 7.83 (2”, 6”) and 6.85 (3”, 5”) have coupling constants *J*=8.4 Hz indicated the presence of a hydroxybenzoic group. The galloyl moiety was located at *O*-1 of glucose. It was determined by the HMBC correlation of the glucose anomeric methine proton (δ_H 5.66) with the ester carbon (δ_C 168.1) of the galloyl

group. Methylene proton (δ_H 4.56 and 4.33) of the Glc-6 possessed a correlation with the ester carbon (δ_C 166.2) at the hydroxybenzoyl group. Finally, the structure of compound **23** was decided as 1-*O*-Galloyl-6-*O*-(4-hydroxybenzoyl)-β-D-glucose.

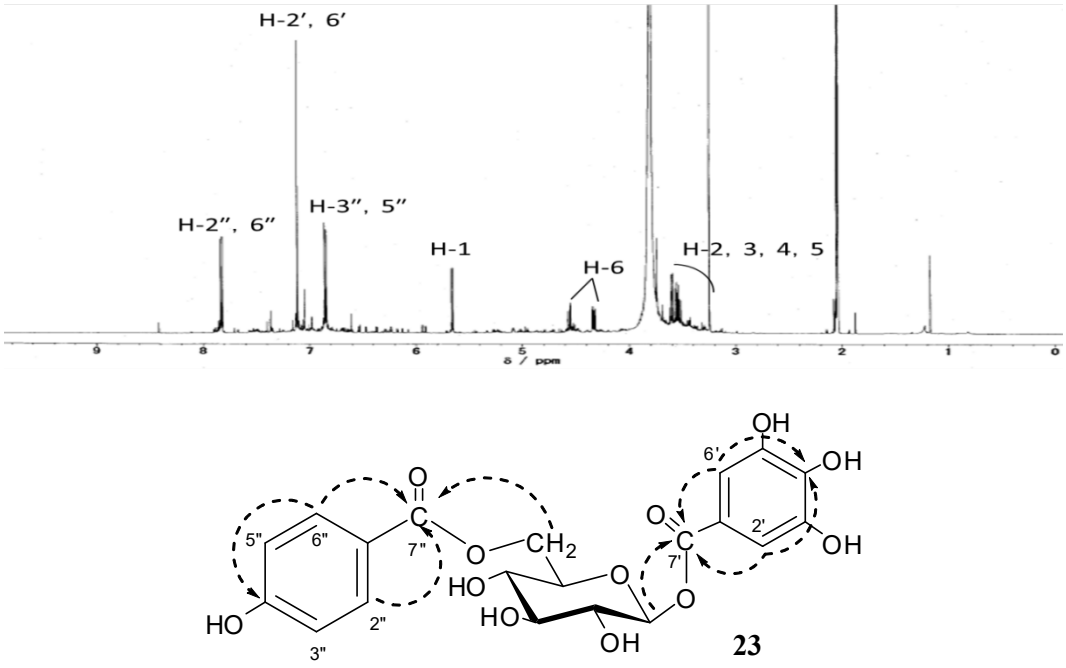
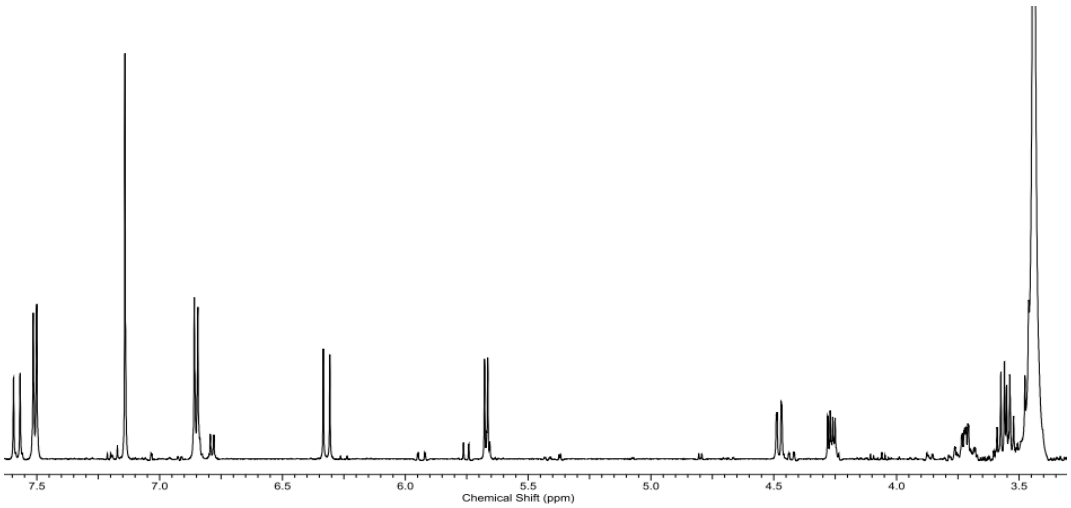
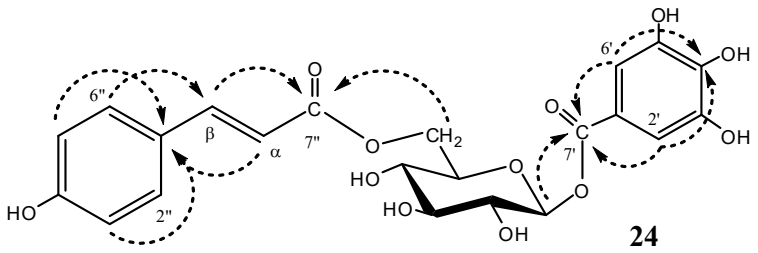


Fig 17. ^{1}H-NMR spectrum, and Chemical structure with key HMBC correlation of 1-*O*-galloyl-6-*O*-(4-hydroxybenzoyl)-β-D-glucose

6-O-trans-p-coumaroyl-1-O-galloyl-β-D-glucose

Compound (**24**) was obtained from leaves of pomegranate as a pale brown amorphous powder. This compound consists of a glucose, a galloyl, and a coumaroyl group. The HR-ESI-MS showed a molecular peak at *m/z* 477.1049 $[M-H]^-$ (calcd. for $C_{22}H_{22}O_{12}$, *m/z* 477.1038) and it accorded with the molecular formula of $C_{22}H_{23}O_{12}$.





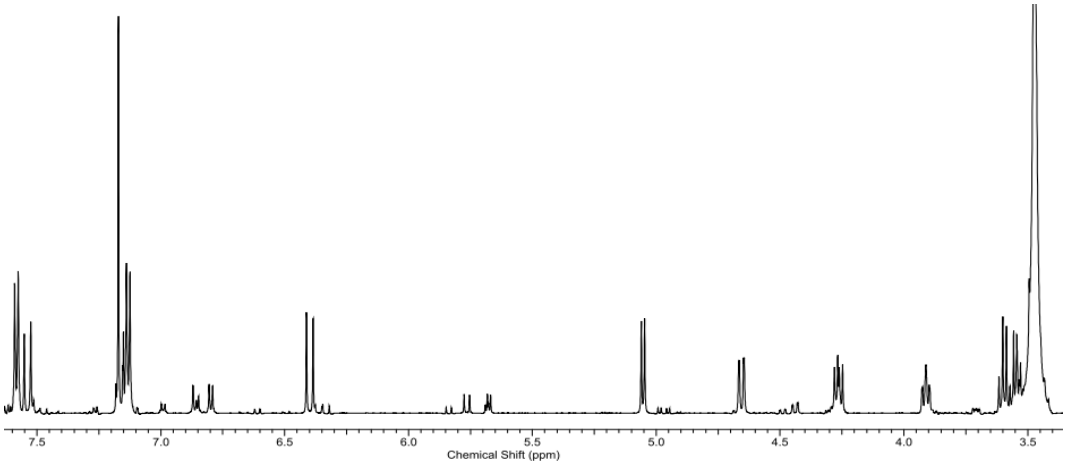
24

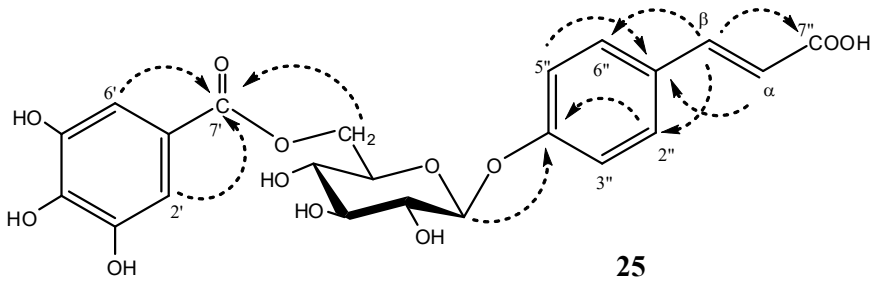
Fig 18. ^{1}H-NMR spectrum and Chemical structure with key HMBC correlation of 6-*O*-*trans*-*p*-coumaroyl-1-*O*-galloyl-β-D-glucose

The aliphatic proton region of ^{1}H NMR spectrum showed seven sets of well-resolved signals of glucose (δ_H 3.54-6.67). The presence of the large coupling constant of glucose protons of Glc-1, 2, 4, 5 (Table 5) indicated the presence of β-glucopyranose core in the $^{4}C_1$ conformation of glucose. A two-proton singlet (δ_H 7.15) indicates the manifestation of a galloyl. The presence of four protons on two doublet signals (δ_H 7.39 (2H) and δ_H 6.86 (2H)) and two doublet signals at δ_H 6.33 (1H, d, H-α) and δ_H 7.59 (1H, d, H-β) indicated that compound **3** has a *p*-coumaric acid moiety. The coupling constant of 16.2 Hz between H-α and H-β confirmed the *trans* geometry of coumaric acid group [57-58].

The final structure of **24** was confirmed by ^{1}H-^{13}C HMBC analysis and lead to obtain further evidence. The correlation between proton δ_H 5.67 (anomeric carbon) and ester carbon (δ_C 119.5) of galloyl identified the location of the galloyl moiety. Subsequently, placement of the *trans-p*-coumaric acid was determined by the correlation of ester carbon (δ_C 166.2) with double-doublet methylene proton at δH 4.28. Thus, the structure of **24** was determined as 6-*O*-*trans-p*-coumaroyl-1-*O*-galloyl-β-D-glucose.

4-O-(6'-O-galloyl-β-D-glucosyl)–coumaric acid





25

Fig 18. ^{1}H-NMR spectrum and Chemical structure with key HMBC correlation of 4-*O*-(6'-*O*-Galloyl-β-D-glucosyl)–coumaric acid

Together with compound **24**, compound **25** was isolated from pomegranate leaves. This compound was also isolated as a pale brown amorphous powder and has similarity moieties with the compound **24**. In addition, the basis of HR-ESI-MS showed the similarity of the molecular mass peak at *m/z* 477.1050 (calcd for $C_{22}H_{22}O_{12}$, *m/z* 477.1038).

On the basis of ^{1}H NMR spectrum, exhibiting of a β-glucopyranose core with $^{4}C_{1}$ was confirmated by presences six protons at δ_H 3.54-5.05 with large coupling constants ($J_{1,2}$=7.8, $J_{2,3}$=9.0 $J_{3,4}$=9.0, $J_{4,5}$=8.4 Hz). A singlet proton at δ_H 7.17 (2H) is the characteristics of the galloyl. This proton and methylene at Glc-6 (δ_H 4.26) have a HMBC correlation with the acyl carbon (δ_C 166.0) of galloyl. This indicates that the galloyl was attached to *O*-6 on glucose.

Coumaroyl moiety was identified by signal protons at δ_H 7.58 and δ_H 7.13 (both 2H, *J*=9.0 Hz, H6”/H2”, H3”/H5”) and two doublet signal at δ_H 6.26 (H-α) and δ_H 7.54 (H-β). HMBC correlation between the methine proton (δ_H 5.67) at anomeric glucose and the with *para*-carbon (δ_C 159.2) at the benzene ring assigned the position of coumaroyl moiety. Thus, the position of coumaric acid can be decided at *O*-1 glucose and the full structure of **25** was confirmed as 4-*O*-(6’-*O*-galloyl-β-D-glucosyl)-coumaric acid.

Table 5. ^{1}H and ^{13}C NMR spectroscopic data of new galloyl glucose derivatives 23, 24, and 25

Unit	Position	Compound **23**		Compound **24**		Compound 4	
		δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
Galloyl	1’		119.5		119.5		120.5
	2’	7.11 (s)	109.3	7.15 (s)	109.3	7.17 (s)	109.0
	3’		145.2		145.2		145.4
	4’		138.8		138.8		138.2
	5’		145.2		145.2		145.4
	6’	7.11 (s)	109.3	7.15 (s)	109.3	7.17 (s)	109.0
	7’		165.1		165		166.0
Glucose	1	5.66 (d, *J*=8.4 Hz)	94.7	5.67 (d, *J*=7.2Hz)	94.6	5.05 (d, *J*=7.8 Hz)	100.5
	2	3.60 (dd, *J*=8.4, 9 Hz)	74.8	3.54 (dd, *J*=7.2, 9.6 Hz)	72.7	3.54 (dd, *J*=7.8, 9.0 Hz)	73.5
	3	3.55 (t, *J*=9 Hz)	72.6	3.58 (dd, *J*=9.6 Hz)	76.6	3.59 (t, *J*=9.0 Hz)	76.7
	4	3.53 (t, *J*=9 Hz)	70.0	3.48-3.44 (overlapping)	69.9	3.49-3.43 (overlapping)	70.4
	5	3.75-3.84 (overlapping)	76.5	3.72 (ddd, *J*=1.2, 6.0, 9.6 Hz)	74.9	3.91 (t, *J*=8.4 Hz)	74.2
	6	4.56 (dd, *J*=1.8, 12.0 Hz, α); 4.56 (dd, *J*=5.4, 12.0 Hz, β)	63.5	4.49 (dd, *J*=1.2, 12.0 Hz) 4.28 (dd, *J*=6, 12.0 Hz)	63.3	4.26 (dd, *J*=8.4. 12 Hz)	63.9
p-Hydroxy-Benzoyl	1”		120.8				
	2”	7.83 (d, *J*=8.4 Hz)	131.7				
	3”	6.85 (d, *J*=8.4 Hz)	115.1				
	4”		162.1				
	5”	6.85 (d, *J*=8.4 Hz)	115.1				
	6”	7.83 (d, *J*=8.4 Hz)	131.7				
	7”		166.2				
P-Coumaric acid	1”				125.7		128.7
	2”			7.49 (d, *J*=8.4 Hz)	130.2	7.58 (d, *J*=9 Hz)	129.6
	3”			6.86 (d, *J*=8.4 Hz)	115.8	7.13 (d, *J*=9 Hz)	116.7
	4”				160.0		159.2
	5”			6.86 (d, *J*=8.4 Hz)	115.8	7.13 (d, *J*=9 Hz)	116.7
	6”			7.39 (d, *J*=8.4 Hz)	130.2	7.58 (d, *J*=9 Hz)	129.6
	7”				166.2		168.7
	α			6.33 (d, *J*=16.2 Hz)	114.0	6.26 (d, *J*=16.2 Hz)	117.4
	β			7.49 (d, *J*=16.2 Hz)	145.2	7.54 (d, *J*=16.2 Hz)	143.6

DISCUSSION

Pomegraniic acid (**17**) was isolated from arils of pomegranate. This compound has four moieties i.e. HHDP, galloyl, glucose, and new 4-acyl group. The present of HHDP and galloyl groups indicated that compound **17** is an ellagitannin. The connectivity of each moiety has proved by HMBC data. The galloyl, HHDP, and new 4-acyl group attached to *O*-1, *O*-3~*O*-6 and *O*-4 position at glucose, respectively.

The HHDP moiety of **17** was established an (*R*)-configuration by the negative cotton effect at 229 nm of CD spectral analysis. Remarkably, (*R*)-configuration was indicated by a negative Cotton effect around 230 nm in its respective CD spectra. This fact also supported by the HMBC correlation, which showed that HHDP linked to the D-glucosyl *via**O*-3, *O*-6 position. Most ellagitannin with HHDP unit attached *via* the *O*-2, *O*-4 and *O*-3, *O*-6 position seems to lead to the (*R*)-configuration of HHDP unit [47-48].

Coupling positions of HHDP moiety to the glucopyranose could determine the D-glucose conformation. $^{4}C_{1}$ conformation could be determined in the case *O*-2, *O*-3 or *O*-4, *O*-6 HHDP coupling, while an *O*-1, *O*-6, or *O*-2, *O*-4, or *O*-3, *O*-6 HHDP coupling always favors the thermodynamically less stable $^{1}C_{4}$ conformation of D-glucose [49].

In addition, small coupling constant of the anomeric proton of Glc-2, Glc-3, and Glc-4 (3.6 - 4.2 Hz) indicated that pomegraniic acid has a $^{1}C_{4}$ conformation of

D-glucose. In addition, ROESY data provides information that the anomeric position of glucose was confirmed as β-configuration (Fig 10).

New 4-acyl group of pomegraniic acid was determined by 2D NMR, CD spectra, and chemical reaction analyses. The relative configuration of the new 4-acyl group was determined by ROESY correlations and showed that two methines and methylene in the ring E have the same direction.

The absolute configuration of the new 4-acyl group could not be resolved directly by using **17**. After hydrolysis, the new 4-acyl group was retrieved by separation process using TLC. Aromatic quadrant rule was used to establish the position of benzylic carbon of new 4-acyl group. Using CD analysis, the absolute configuration of the new 4-acyl group can be determined, whether the compound new 4-acyl group has (*S*)- or (*R*)-configuration. If the Cotton effect is positive at 220-240 nm, this means that the bulky group position of the compound new 4-acyl group position is in positive quadrant axis. Thus, the benzylic acid position has a (*S*)-configuration. Meanwhile, if the Cotton effect showed negative at 220-240 nm, the bulky group position is in opposite and has a (*R*)-configuration. CD spectrum exhibited a negative Cotton effect around 230 nm which indicated of a new 4-acyl group with an (*R*)-configuration.

Based on these findings, the absolute configuration of the structure of the new 4-acyl group unit structure was determined and represented by the formula **22.** The full structure of pomegraniic acid was represented by formula **17**.

The presence of a galloyl group and the absence of HHDP group have characterized the compound **17** as a gallotannin and this compound was isolated for the first time from the pomegranate arils.

Three others new polyphenols were also isolated from pomegranate. Compound **23** was isolated from arils of pomegranate from fraction 50% of Methanol, whereas compound **24** and **25** were isolated from fraction ethyl acetate of pomegranate leaves. All of these new compounds have a galloyl group attached on glucose core and the glucose have $^4C_1$ conformation.

Compound **23** has a galloyl and a hydroxybenzoyl groups attached on *O*-1, and *O*-6 position of glucose, respectively. Compound **24** and **25** are isomer which consists of a galloyl and coumaroyl groups. The coumaroyl group of **24** was attached at *O*-6 of glucose, while the galloyl at *O*-1 of glucose. Meanwhile, the coumaroyl and galloyl unit of compound **25** has opposite positions of those of compound **24,** the coumaroyl of compound **25** wasattached at *O*-1 and the galloyl was attached at *O*-6 position of glucose.

In summary, this study describes the discovery of four new polyphenols. Pomegranic acid, a new monomeric ellagitannin, and three new polyphenols were successfully isolated from arils and leaves of pomegranate and elucidated their structure. Further experiments were needed to know the advantages and benefits of these compound for human health.

Chapter IV

Anti-glycation effects of polyphenols from pomegranate and proanthocyanidin from red-kernel rice

Glycation is the non-enzymatic reaction in which a carbonyl group of reducing sugar is covalently coupled to free amino acid from protein, lipids, and nucleic acids to form the advanced glycation end-products (AGEs). AGEs are occurred both endogenously (in the human body) and exogenously (in the food). First, sugar was converted into highly reactive dicarbonyl during glycolytic or Maillard reaction process. Briefly, in Maillard reaction is in intracellular, reducing sugar (e.g fructose, galactose, glucose, ribose) and free amino groups forming the reversible unstable Schiff bases that evolve into stable Amadori products, although the reaction is still reversible. Finally, the Amadori products tend to undergo re-arrangement, dehydration, or cyclization to form the reactive dicarbonyl intermediates product (glyoxal, methylglyoxal, and 3-deoxyglucosone) that react with arginine and lysine residues to irreversibly generate crosslinked AGEs [59] (Fig 19).

AGEs are not only forming in intracellular, but also can be taken from the exogenous sources such as tobacco smoke and dietary food. The contribution of exogenous AGEs is approximately at 30% of the total AGEs accumulate in the human body. [59]. Mechanism of AGEs formation in food is based on Maillard reaction. Naturally, AGEs are available in uncooked animal-derived foods. The cooking process results in the formation of new AGEs within these cooked foods. Notably, grilling, roasting, and frying increase and accelerate the AGE formation and consequently increase the AGEs accumulation in the body. [60].

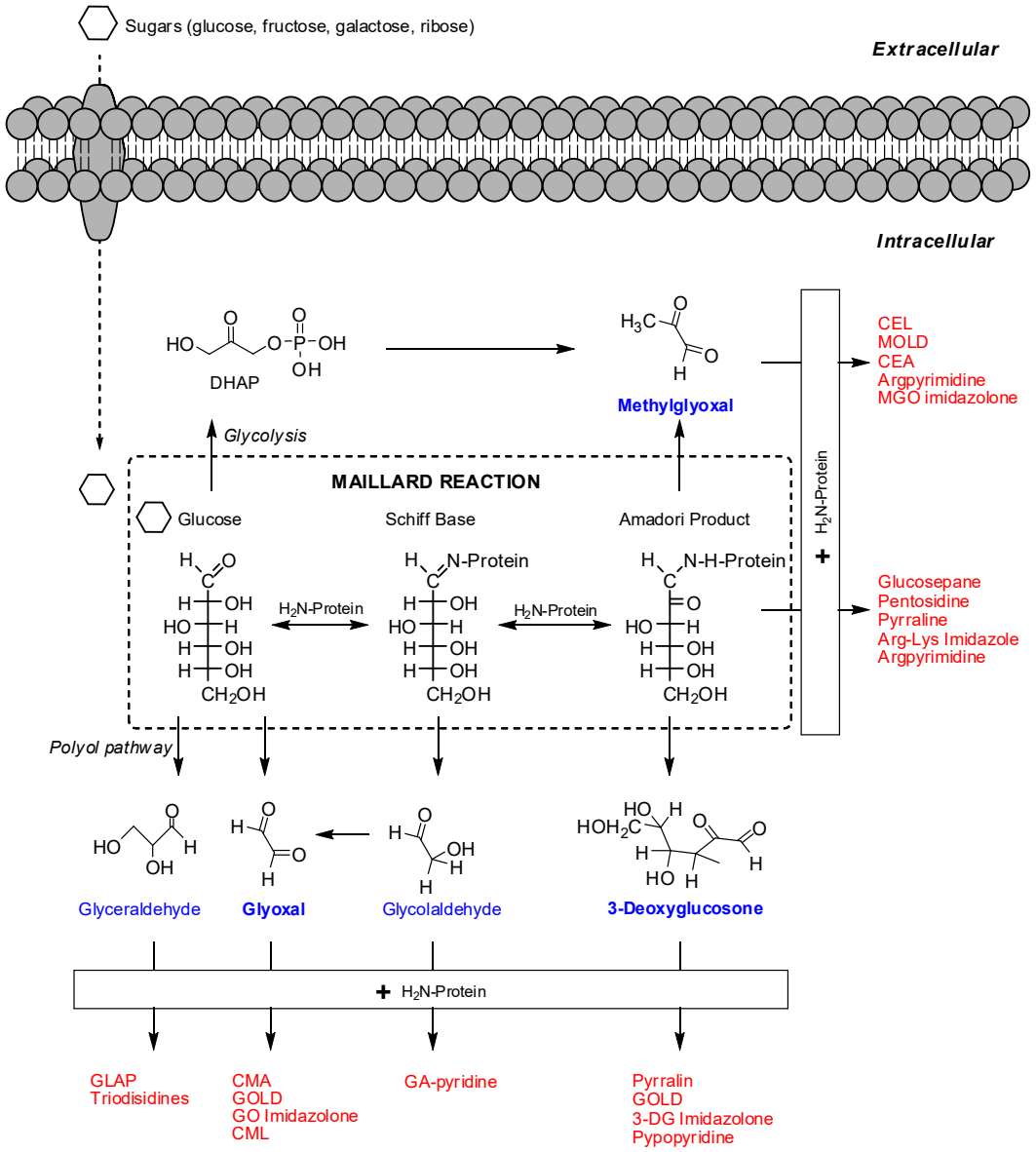


Fig 19. Formation of advanced glycation end-products from dietary sugar. AGEs are derived by some pathways i.e. Maillard reaction, polyol pathway, and glycolysis pathway, which mainly the formation of AGEs are through the Maillard reaction. AGEs precursors are highlighted in blue, and AGEs are highlighted in red.

AGEs also known as glycotoxins, which are related to their ability to promote oxidative stress and inflammation by forming cross-linking to the protein in the surface cell then altering their structures and functions. Intracellular AGEs accumulation was associated with age-related macular degeneration (AMD),

Alzheimer's and Parkinsonian disease, whereas glycation of extracellular is a common feature of aging, and diabetic complication [61].

Glyceraldehyde, glyoxal, glycoaldehyde, 3-deoxyglucosone, and methylglyoxal are glycating moieties of carbohydrate metabolism which they are derived from Maillard reaction, and glycolysis and polyol pathways. They are the precursor to forming AGEs. Among of precursors, glyoxal (GO), methylglyoxal (MGO), and 3-deoxyglucosone (3-DG) are major glycating moieties, which have α-dicarbonyl in their structure, and the formation of AGEs commonly occurs through the reaction of these dicarbonyl. To keep the low AGEs concentration low in the body, MGO and GO can be detoxified by enzymatic glyoxalase system [62, 63], and 3-DG can be detoxified by 3-DG reductase enzyme. These enzymes affect the AGEs precursors not to reactive in forming AGEs [64, 65]. This mechanism prevents the AGEs formation by re-arrangement AGEs precursors. Besides that, there are other mechanisms degradation to break down AGEs in the human body. The Ubiquitin-proteasome system (UPS) and autophagy in intracellular have been reported to removal AGEs. Unfortunately, the mechanisms of detoxification in the body decrease during the high glycemia condition and aging processes [66-67].

A number of polyphenols have been reported for their anti-glycation activities [3, 20-23] through chelating ion activity [18, 20] trapping the carbonyl [18, 20, 22], binding the amino acid [17, 19], or cleaving the crosslink derived [21] from AGEs. Tannins were also reported have potential to be an anti-glycation [3,

21-24]. The mechanisms of anti-glycation of tannin might be similar to the common mechanism of other polyphenols as anti-glycation agents.

In this study, the anti-glycation effects (inhibitory of AGEs formation and AGEs crosslink cleaving) of ellagitannins and proanthocyanidin were analyzed. Ellagitannins were isolated from pomegranate arils and proanthocyanidins were isolated from some varieties of red-kernel rice in Japan.

MATERIALS AND METHODS

Materials

Ellagitannins (i.e pomegraniic acid (**17**), punicalagin (**3**), punicalin (**4**), eucarpanin T_1 (**6**), and corilagin (**1**)) were isolated from arils of pomegranate. Proantocyanidins were isolated from some varieties of red-kerneled rice from Akaonimoch, Benizomemochi, Tanegashima, Akamai, and Benimusume - Japan.

Inhibitory effect on AGEs formation

The inhibitory activities to prevent the formation of AGEs was evaluated using the method described by Kato et al. [23]. Glucose (2.0 M), 8.0 mg/mL human serum albumin (HSA), 83.3 mM phosphate buffer (pH 7.2) and sample solution were mixed at a 6:1:1:1:1 volume ratio. As a control, the vehicle was added instead of the sample solution. For each blank, glucose was replaced with distilled water. The total volume was 1 mL. After incubation of the mixture at 60 ^{O}C for 40h, the solution was diluted 8-fold and dispensed into a black microplate in 200 µl portion then following the measurement of the fluorescence of plasma with wavelengths 465 nm and emission 370 nm using a Power Scan HT (DS Pharma Biomedical Co. Ltd.,). The inhibitory rate was calculated as follows:

$$\textit{Inhibitory rate (\%)} = 100 - [(S\text{-}SB)/(C\text{-}CB)] \times 100 \quad (3)$$

Where S and C represent the relative intensities of the sample and control solutions, respectively, and SB and CB represent the glucose-omitted blank

solutions. The IC_{50} values were calculated by probit analysis, and performed in triplicate.

AGE crosslink-cleaving activity

AGE crosslink-cleaving activity was evaluated according to the modified method reported by Kato et al [23]. A solution of 1-phenyl-1,2-propanedione (PPD; Sigma-Aldrich Japan K.K., Tokyo, Japan), in 50% aqueous acetonitrile was used as a reactive substrate in the AGE-crosslink model. For the measurement of AGE-derived crosslink-cleaving activity, the samples (100 μL) were mixed with 1.13 mmol/L PPD 900 μL, and incubated at 37 °C for 4 h. The reaction was stopped by adding 2 mol/L HCl (200 μL), followed by centrifugation at 10,000 rpm for 2 min. The amount of benzoic acid in the supernatant was measured by reversed-phase HPLC [column: InertSustain C18, 75 × 4.6 mm i.d. (GL Sciences Inc., Tokyo, Japan); A gradient system was established with the mobile phase consisting of A, containing 50 mM phosphate buffer (pH 2.2) and acetonitrile with ratio 80:20, respectively, and B, containing 50 mM phosphate buffer (pH 2.2) and acetonitrile with ratio 50:50. The assay was started with 0% B in 0-15 min, 0→100% B in 20 min, 100% B in 20-30 min, 100→0% B 30.1 min, 0% B in 40 min; flow rate: 1.0 mL/min; column temperature, 40 ^{O}C; detection: UV at 230 nm. All sample were performed in triplicate. The relative ratio of samples was compared to *N*-phenacylthiazolium Bromide (PTB) as the positive control and assumed to be 100.

RESULTS

Together with pomegraniic acid, others ellagitannin i.e. punicalagin, punicalin, eucarpanin T_1, and corilagin were also isolated from pomegranate arils. Proanthocyanidins were isolated from some varieties of red-kernels rice from Akaonimoch, Benizomemochi, Tanegashima, Akamai, and Benimusume – Japan. Then Proantocyanidins inhibitory effect on AGEs formation and AGEs crosslink cleaving activities were analyzed.

Table 6 summarized anti-glycation activities (inhibitory effect and crosslink cleaving activity of AGEs) of ellagitannins and proanthocyanidins from pomegranate and red-kernel rice. In inhibitory effects on AGEs analysis, all tested samples contain the formation of AGEs by glycation reaction of HSA with glucose. Among all samples, Eucarpanin T_1 exhibited the highest level of inhibition with an IC_{50} value of 0.10 μM, and corilagin showed the highest capability to cleaving the AGEs crosslink (184%).

All ellagitannins from pomegranate exhibited the potential of cleaving the AGEs crosslinks than the positive control (117-184%). Comparing to ellagitannins, tested proanthocyanidins showed weaker activities on AGE crosslink cleaving than PTB as a positive control. However, the beards extract of red-kernel rice exhibited a cleaving activity stronger than the positive control.

Table 6. Inhibitory effects on AGE formation in HSA/glucose, and AGE-derived crosslink-cleaving activities of ellagitannin from pomegranate, and proanthocyanidin oligomers from hulls, and extract of beards of red-kerneled rice.[a)]

SAmple	IC_{50} Inhibitory effects on AGEs formation		AGEs Crosslink-cleaving activities[b)]
	μg/mL	μM	Relative ratio (%)
Ellagitannins from pomegranate			
Pomegraniic acid	2.50 ± 0.60	2.58 ± 0.62	149
Punicalagin	0.86 ± 0.61	0.79 ± 0.56	165
Punicalin	0.89 ± 0.07	1.14 ± 0.09	126
Eucarpanin T_1	0.23 ± 0.01	0.10 ± 0.01	117
Corilagin	2.67 ± 1.05	4.19 ± 1.65	184
Proantocyanidins and beard extracts from red-kerneled rice			
Akaonimochi (2012)	1.00 ± 0.10	0.40 ± 0.00	69.0 ± 2.9
Akaonimochi (2013)	1.40 ± 0.10	0.50 ± 0.00	66.4 ± 5.0
Benizomemochi	1.20 ± 0.10	0.40 ± 0.00	59.1 ± 3.9
Tanegashima (ng)	1.70 ± 0.10	0.80 ± 0.00	42.3 ± 0.1
Tanegashima (g)	1.9 ± 0.0	0.8 ± 0.00	63.3 ± 5.3
Akamai (ng)	1.00 ± 0.10	0.40 ± 0.00	75.5 ± 4.5
Benimusume	1.3 ± 0.10	0.5 ± 0.00	69.1 ± 7.1
Beard extracts	2.80 ± 0.10	-	124.0 ± 5.0
Positive control			
Aminoguanidine	48.8 ± 3.5	565.32 ± 22.63	NT[c)]
PTB	NT	NT	100

[a)] Data are expressed as means ± SE (n = 3), [b)] Concentrations of tested samples are 100 g/mL, [c)]N.T.: Not tested.

DISCUSSION

Overall, all ellagitannins and proanthocyanidins have the higher capabilities to inhibite the AGEs formation than positive control aminoguanidine. However, comparing with ellagitannins from pomegranate and positive control, tested proanthocyanidins showed weaker activities on AGE crosslink cleaving than PTB as a positive control.

Numerous studies have shown the capability of polyphenols to trap both carbonyl and dicarbonyl. Sang et al. [22] and Bhuiyan et al. [20] have explained the mechanism of dicarbonyl trapped by (-)-epigallocatechin-3-gallate (EGCG) and quercetin, respectively. Both EGCG and quercetin are flavonoid, which has three rings (C_6-C_3-C_6) basic flavonoid structure (Fig 20). The flavonoids with an OH at C-3' are stronger AGEs inhibitors than those with OH at C-3. In addition, the hydroxyl groups on position 3',4',5', and 7 of flavonoid increase the AGEs inhibitory abilities[68].

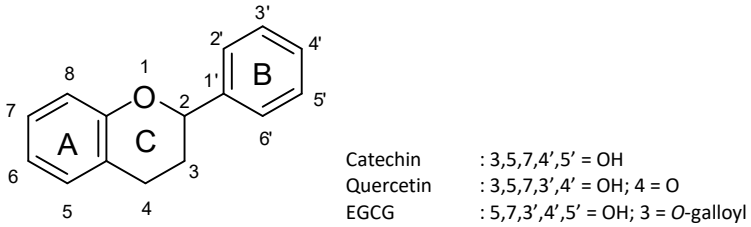


Fig 20. Basic structure of flavonoid, hydroxyl group position and *O*-galloyl position of catechin, quercetin, and (-)-epigallocatechin-3-gallate

Proanthocyanidin was arranged by monomeric of catechin, which the catechin has similarity structure with quercetin. The differences between catechin

and quercetin are on its hydroxyl position on C-ring, and the present of double bond oxygen on the C-4 position of the quercetin. EGCG and quercetin trapped the MGO (dicarbonyl of AGEs precursor) on C-6 and C-8 position of the ring A forming 6,8-di-MGOEGCG (**26**) and 6,8-di-MGO-quercetin (**27**), respectively. This mechanism has been proven by chemical reaction and NMR analyses by Sang et al. [22] and Bhuiyan et al. [20],which could explain the ability of catechin and proanthocyanidin in inhibiting the AGEs formation.

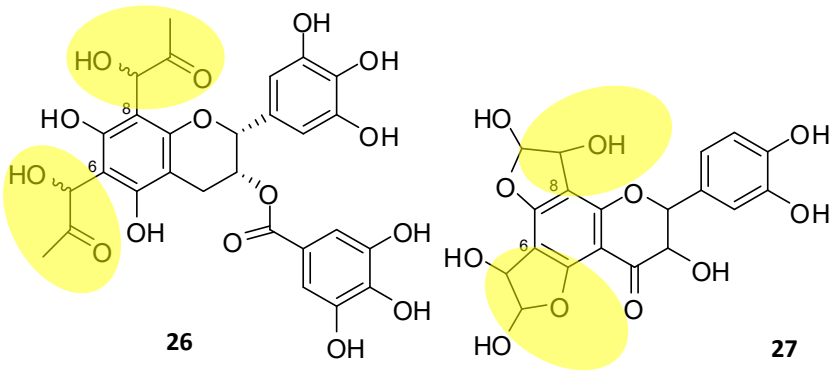


Fig 21. EGCG and quercetin binding the Methylglyoxal (MGO) on 6,8 position of Ring A [20, 22]

In addition, by using molecular docking modeling study, Qais et al. [19] and Alam et al. [17] have explained the mechanism of cinnamic acid and quercetin to binding the amino acid into the benzylic ring to reduce the glycation site in HSA. HSA protein at the hydrophobic core was bounded by conventional hydrogen bond, pi-sigma bond, or pi-alkyl bond by quercetin [19].

Although quercetin or cinnamic acid are not belonging to the ellagitannin group, their anti-glycation mechanism may play the same role as the ellagitannnin

groups to prevent and breakdown the AGEs formation. Ellagitannin also has the variety of hydroxybenzene structure, especially tryhydroxybenzene group that might be involved in the mechanism of chelation metal ion catalysts to blocking the oxidation of the Amadori intermediate to trap the reactive carbonyl and dicarbonyl of AGEs precursor compounds, to bind the amino acid to reduce the glycation site in HSA, or to cleave the AGEs formations. The AGEs crosslink cleaving activity of hydroxybenzene compounds have been analyzed by Yagi et al [21]. The benzene ring with three hydroxyl group on 1, 2, and 4 position (hydroxyquinol) and 1, 2, and 3 position (pyrogallol) have higher activities than other hydroxybenzene. Ellagitannins have abundant HHDP and galloyl group, and its hydroxyl position is similar to pyrogallol. This fact might explain the AGEs crosslink activity of ellagitannin.

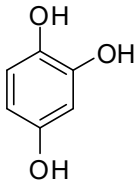
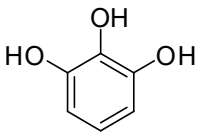
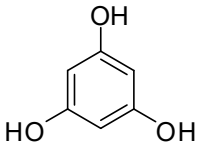
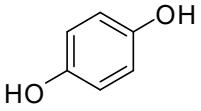
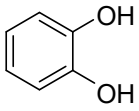
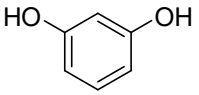
Tryhydroxybenzene			
Chemical Structure			
Name	Hydroxyquinol (1,2,4-trihydroxybenzene)	Pyrogallol (1,2,3-trihydroxybenzene)	Phloroglucinol (1,3,5-trihydroxybenzene)
AGEs crosslink cleaving activity	51.62 %	42.13 %	6.06 %
Dihydroxybenzene			
Chemical Structure			
Name	Hydroquinone (1,4-dihydroxybenzene)	Pyrocatechol (1,4-dihydroxybenzene)	Resolcinol (1,3-dihydroxybenzene)
AGEs crosslink cleaving activity	15.73 %	14.7 %	6.88 %

Fig 22. Chemical structure and AGEs crosslink cleaving activity of hydroxybenzene compounds (adapted from Yagi et al. [21]**)**

Several studies have reported that ellagitannins have the potential to be an anti-glycation [3, 21, 23, 24]. However, most of the investigations are limited *in vitro* experimental study and do not explain how ellagitannin work against the glycation. Therefore, the further investigation about ellagitannin mechanism as anti-glycation should examine the *in vivo* and explore the mechanism of ellagitannin as an anti-glycation agent.

Chapter V

Conclusion Remark

Ellagitannins are polyphenolic secondary metabolic of higher plants, which belong to the class of hydrolyzable tannins. With more than 500 ellagitannins have been discovered. They have shown potential health benefits, such as the prevention of advanced glycation end-products (AGEs) formation, anti-inflammatory effects, anti-diabetic effects, anti-fungal effects, antioxidant effects, and other benefits.

Geraniin is an ellagitannin, which exists abundant in *Geranium thunbergii* and it has been used as traditional medicines. Geraniin has a dehydrohexahydroxydiphenoyl (DHHDP) group which always shows multiple broad peaks on high-performance liquid chromatography (HPLC) when dissolved into aqueous and alcoholic solution and became a problem to quantify the amount. Therefore, qNMR was employed to quantify the amount of main ellagitannins and other relative compounds in short-term and long-term decoction. By using qNMR, we found that geraniin is the main compound in short-term decoction, while corilagin was the main compound in long-term decoction. In Japan, *G. thunbergii* has been using as a folk medicine. The short and long decoctions are used to treat constipation and diarrhea, respectively. Our findings might be speculated that geraniin has the ability to treat constipation while corilagin plays a key role in diarrhea treatment. The contents of main polyphenols in *G. thunbergii* from six cultivates in Japan and three cultivates in China using our qNMR also were estimated by qNMR. In general, *G. thunbergii* from Japan has the higher amount of polyphenols than *G. thunbergii* from China.

In this study, we also successfully isolated and elucidated a new monomeric ellagitannin namely pomegraniic acid, and three new galloyl glucose derivatives (1-*O*-galloyl-6-*O*-(hydroxybenzoyl)-β-D-glucose, 6-*O*-*trans*-*p*-coumaroyl-1-O-galloyl-β-D-glucose, and 4-O-(6‘-*O*-galloyl-β-D-glucosyl)-coumaric acid) from arils and leaves of pomegranate. Pomegraniic acid consists of a galloyl, an HHDP (*R*-configuration), and a new 4-acyl group (*R*-configuration), which attached to *O*-1, *O*-3~*O*-6 and *O*-4 position at D-glucose, respectively. Their relative and absolute configuration has been determined by spectroscopic (NMR and CD spectrum) and chemical reaction (methylation, methanolysis, and hydrolysis) analyses.

Glycation is the non-enzymatic reaction initiated by the reaction of the free reducing sugar with free amino acid, protein, or lipid. The anti-glycation activities of five ellagitannins from pomegranate and proanthocyanidins from seven varieties of red-kernel rice were analysed in this study. Comparing to aminoguanidine as the positive control, both ellagitannin and proanthocyanidin have higher capacity to prevent the AGEs formation. Only beard extracts and ellagitannin have higher AGEs crosslink-cleaving activities than positive control. The anti-glycation activities of ellagitannins and proanthocyanidins are related to the capability of their structure to binding the dicarbonyl, trapping the amino acid, or cleave the AGEs crosslink.

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Acknowledgement

It is a pleasure to thank the many people who made this dissertation possible.

I would like to express my gratitude to Prof. Hideyuki Ito as my supervisor for the continuous support of my Ph.D. study and research. With his motivation, patience, enthusiasm, and immense knowledge, I successfully overcame difficulties and learned a lot.

I also appreciate the feedback offered by Prof. Hiromi Yamashita, Assoc. Prof. Yoko Nitta, Prof. Tetsuya Ogino, and Prof. Hikaru Nakamura.

I owe my deepest gratitude to Natsuki Ganeko, Ph.D., Yuji Iwaoka, Ph.D. and all Ito Lab member for all kind assistance and support during I pursue my doctoral course. I am not just get friends, but also I got family here.

I would like to offer my special thanks to Prof. Yoshiaki Amakura and Assoc. Prof Morio Yoshimura from Matsuyama University for the CD analysis, also I would like thank Prof. Tsutomu Hatano from Okayama University for the discussion. And I would like to sincerely thank Erika Orahara, Masateru Miyake, Ph.D., Yurie Ito, Nana Kato, and Saki Watanabe as co-authors and collaborators in my research.

I am indebted to all teachers in the Department of Nutritional Science, Faculty of Health and Welfare Science, Okayama Prefectural University for the help, support and kind advices.

Lastly, I wish to special thank my parents, my wife, and my sons for their patience, support, and their love.