氏　　名	フェブルアディ　バスティアン
授与した学位	博　士
専攻分野の名称	栄養学
学位授与番号	博甲第１２９号
学位授与の日付	平成３１年３月２２日
学位論文の題目	A new quontification method for polyphenols in medicinal plants using quontitative NMR, and new polyphenols from pomegranate
学位審査委員会	主査　伊東　秀之　副査　山下　広美　副査　新田　陽子 副査　荻野　哲也　副査　中村　光

学位論文内容の要旨

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, and antioxidant effects.

Geraniin is the major ellagitannin in *Geranium thunbergii*, which is one of popular medicinal plant known as *gennoshoko* in Japan. There are two ways to use *G. thunbergii* medicinally to treat intestinal disorders in Japan. The dried aerial parts can be brewed with hot water (making a tea) for treat the constipation or boiling the dried plant in water for one hour for treat the diarrhea. Geraniin belonging to the dehydroellagitannins contain dehydrohexahydroxydiphenoyl (DHHDP) group usually forms an equilibrium mixture of six- and five-membered in aqueous and methanol solution. This condition affected the coelution problem and causing difficulty in quantifying analyses using HPLC because the peaks overlapped each other. In addition, analytical methods using HPLC require pure standards for establishing the calibration curve. Furthermore, it is difficult to purchase commercially standards of specific ellagitannins. Quantifying using nuclear magnetic resonance (qNMR) is one of the alternatives for quantifying the ellagitannins which contain DHHDP group. There are some additional advantages of qNMR such as the lack of calibration curve requirements, the non-destructive character of the NMR technique for the lack of special sample preparation requirements, short measurement times, and the possibility of simultaneously quantifying multiple compounds in crude extracts, so that we can determine the purities of compounds or the absolute content of a compound in the natural source with unit traceability by qNMR method.

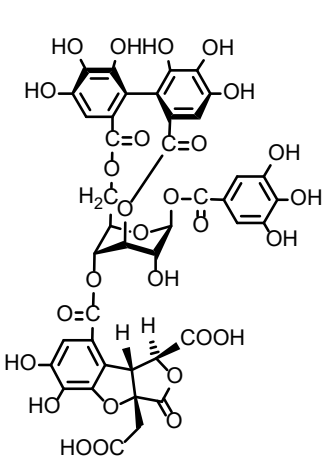
In this study, we developed ^{1}H-NMR fingerprints for quantifying ellagitannins, especially in *G. thunbergii* extract. Several specific proton signals from these ellagitannins were identified for geraniin, corilagin, corilagin, ellagic acid, brevifolincarboxylic acid, flavonoid glycoside,

kaempferitrin, and organic acids such as malic acid and citric acid. We also evaluated the quantification of polyphenols using the qNMR method in short-term and long-term decoctions of *G. thunbergii,* and comparing the polyphenols content of *G. thunbergii* plants from six cultivated in Japan and three cultivated in China. The results showed that geraniin was the major compound (62.0%) in the short-term decoction extract and corilagin was the major compound (49.0%) from the long-term decoction extracts. The amount of geraniin reached a maximum after 10 min of extraction. Geraniin was not observed by 40 min thereafter due to hydrolysis. Instead, corilagin became the dominant compound. These findings suggested that geraniin shows constipation effect while corilagin plays a key role in diarrhea treatment.

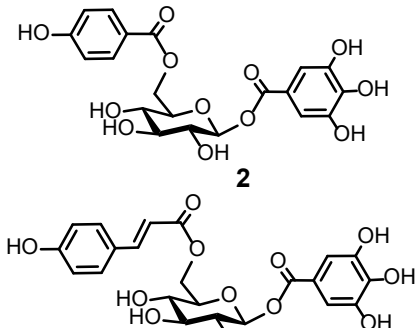
G. thunbergii cultivates from Japan contained higher levels of geraniin and corilagin than those from Zhejiang. Overall, the polyphenol content of cultivates from Japan was higher than those from Zhejiang. Furthermore, the six cultivates of *G. thunbergii* from Japan contained kaempferitrin, whereas the three cultivates from Zhejiang did not contain kaempferitrin.

Since the invention of NMR in 1946, and 2D-NMR method was first introduced in the mid-1980s, the isolation of natural compound and structure determination improved greatly. Exploring new natural compound is a challenge to the discovery of novel structure. We also believed that in pomegranate contain various polyphenols which have never been reported before. For these reasons, we explored to discover new polyphenols from pomegranate. New compounds were separated and isolated using series chromatography method. NMR spectroscopy including 2D-NMR analyses plays an important role to determine the structure. Combining with circular dichroism (CD) analyses and chemical reaction, the relative and absolute configuration of the new compound have been elucidated. A new monomeric ellagitannin named pomegraniic acid (**1**) were successfully isolated from pomegranate arils and elucidated their structure. Pomegraniic acid 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 $C_{41}H_{30}O_{28}$. The pomegraniic acid consists of a galloyl group, an hexahydroxydiphenoyl (HHDP) group, and a new acyl group unit. The connectivities of each moiety were determined by HMBC. The galloyl, HHDP, and new acyl group attached to *O*-1, *O*-3~*O*-6 and *O*-4 position at glucose, respectively. The HHDP moiety was established an (*R*)-configuration by the negative cotton effect at 229 nm of CD spectral data. Small coupling constants of the anomeric proton, Glc-2, Glc-3, and Glc-4 (3.6 - 4.2 Hz) indicating that pomegraniic acid indicate $^1C_4$ conformation of D-glucose. ROESY data indicated that the galloyl group attached at β-position of the anomeric position of glucose. The detail structure of the new acyl group of pomegraniic acid has been determined based on 2D NMR, CD data, and chemical reactions. The relative configuration of the new acyl group was determined by ROESY correlations. The absolute configuration of three asymmetric carbons in the new acyl group was determined by aromatic quadrant rule of CD method. Based on these data, the

structure of pomegraniic acid including the absolute configuration was concluded to be represented by the formula **1**. Three new galloyl glucose derivatives also were isolated from pomegranate arils and leaves and elucidated their structures based on NMR techniques. New compound **2** was isolated as a pale brown amorphous powder from arils of pomegranate. 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). This compound consists of a galloyl group, and a 4-hydroxybenzoyl group which attached at *O*-1 and *O*-6 positions of glucose by 2D-NMR data. Consequently, the structure of compound **2** was determined as 1-*O*-galloyl-6-*O*-(4-hydroxybenzoyl)-β-D-glucose. New compounds **3** and **4** are isomer which consist of a galloyl and coumaroyl groups. Compound **3** was obtained from leaves of pomegranate as a pale brown amorphous powder. 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 its consistent with the molecular formula $C_{22}H_{23}O_{12}$. Compound **4** was isolated as a pale brown amorphous powder. On the basis of the 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). The 2D-NMR data indicated that the coumaroyl and galloyl groups of compound **3** attached through ester bond at *O*-6 and *O*-1 of glucose, respectively. The galloyl group of compound **4** attached through ester bond at 6 position of glucose, and anomeric position connected to coumaroyl unit by glycoside bond based on 2D-NMR data. The structures of compounds **3** and **4** were elucidated as 6-*O*-*trans*-4-coumaroyl-1-*O*-galloyl-β-D-glucose and 4-*O*-(6'-*O*-galloyl-β-D-glucosyl)–coumaric acid, respectively.



Pomegraniic acid (**1**)



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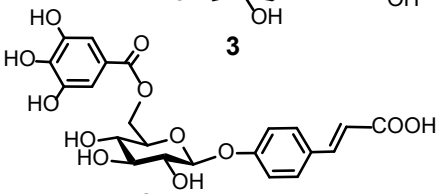

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Glycation is the non-enzymatic reaction in which a carbonyl group of reducing sugar is covalently coupled to free amino acid from protein, lipids, and nucleic acids forming the advanced glycation end-products (AGEs). AGEs not only forming in intracellular but also up taken from the exogenous sources such as tobacco smoke and dietary food. Sugar was converted into highly reactive dicarbonyl during glycolytic or Maillard reaction processes. Maillard reaction forms the

reactive dicarbonyl intermediates product (glyoxal, methylglyoxal, and 3-deoxyglucosone) that react with arginine and lysine residues to irreversibly generate crosslinked AGEs. In this study, inhibitory effect of ellagitannins isolated from pomegranate in comparison with proanthocyanidins from red-kernels rice on AGEs formation and AGEs crosslink cleaving activities. The tested ellagitannins and proanthocyanidins showed more significant inhibitory effects against the AGEs formation than aminoguanidine as a positive control. However, the tested ellagitannins proanthocyanidins showed weaker activities on AGEs crosslink cleaving than *N*-phenacylthiazolium bromide (PTB) as a positive control. C-6 and C-8 position of flavonoid structure in catechin and proanthocyanidin are the active position to trap the dicarbonyl unit.

In this study, the qNMR method can be used to perform accurate, simple, and rapid analysis of target analyte content without the need for intricate separation steps or authentic materials for calibration. A new monomeric ellagitannin and three new galloyl glucose derivatives were isolated and elucidated their structures from pomegranate. Ellagitannins from pomegranate and proanthocyanidin from red-kerneled rice showed potent inhibitory effects on the AGEs formation. These findings suggested that pomegranate or red-kerneled rice might be beneficial in preventing the progress of age-related diseases.

主業績

No.	論文題目	著者名	発表誌名
1	Simultaneous quantification of ellagitannins and related polyphenols in *Geranium thunbergii* using quantitative NMR	Februadi Bastian Yurie Ito Erika Ogahara Natsuki Ganeko Tsutomu Hatano Hideyuki Ito	Molecules, Volume 23, Issue 6, Page 1346 (11 pages), 4 June 2018

副業績

No.	論文題目	著者名	発表誌名
2	Proanthocyanidin and anthocyanins from the hulls and beards of red-kerneled rice and their antiglycation properties	Natsuki Ganeko Nana Kato Saki Watanabe Februadi Bastian Masateru Miyake Hideyuki Ito	Bioscience, Biotechnology, and Biochemistry, Published online: 05 Dec 2018.

その他の業績

なし

論文審査結果の要旨

多くの薬用植物や食品中に含まれるエラジタンニンは，抗酸化作用に基づく多様な機能性を有することが報告され，特定保健用食品や機能性表示食品の関与成分としても注目されている。素材中のエラジタンニン含量は，汎用の高速液体クロマトグラフ（HPLC）分析により求められているが，アノマー混合物や互変異性体の平衡混合物にピークの複雑さや分析対象化合物のほとんどは市販されていないため検量線が作成できず，HPLCによる定量は問題点が多く残されているのが現状である。本論文では，定量核磁気共鳴（qNMR）を用いてゲンノショウコ中のエラジタンニンおよび関連化合物の一斉定量法を確立し，各種抽出法や素材の産地別のエラジタンニン含量の算出を可能とした。本法はその他の各種薬用植物や食品に含まれるエラジタンニンの定量にも有用であることが示唆された。

機能性エラジタンニンの探索の一環として，ザクロに含まれるエラジタンニンの探索を行い，４種の新規成分を単離し，それらの化学構造を明らかにした。そのうち，Pomegraniic acidと命名したエラジタンニン単量体は，分子内に３つの不斉炭素原子を有する新規アシル基を含むが，各種化学反応およびスペクトルデータの解析結果に基づいて全ての絶対配置を明らかにし，その絶対立体構造を解明した。また，単離したエラジタンニン類および赤米から分離したプロアントシアニジンについて，終末糖化産物（AGEs）の産生抑制作用およびAGEs中間体の分解活性を評価し，エラジタンニンオリゴマーに強いAGEs産生抑制作用を有することが認められ，ザクロが抗糖化素材として有用であることを示唆する科学的基礎データを提供した。なお，論文発表会における質疑応答では，いずれの質問に対しても的確に真摯な態度で応答していた。

以上の結果より，学術上，実際上寄与するところが少なくない。よって，本論文は博士（栄養学）の学位論文として価値あるものと認める。