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学位論文題目 Chemical constituents with anti-allergic activity from red
peony root and a horticultural cultivar of *Paeonia lactiflora*
and monoterpenoids profiles of peony related species
(赤芍及び *Paeonia lactiflora* の園芸品種の抗アレルギー作用成
分と芍薬関連 *Paeonia* 属植物のモノテルペノイドのプロファイリング)

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論文内容の要旨

Peony root (PR), called “Shakuyaku” in Japanese, has been widely used as analgesic, antispasmodic and astringent in Kampo medicine. It is prescribed as the dried root of *Paeonia lactiflora* Pallas with no less than 2.0% paeoniflorin in Japanese Pharmacopeia (JP16). PR available in Japanese market is mainly imported from China and only a small part is produced domestically. In China, there are two kinds of PR, white peony root (WPR) and red peony root (RPR) which are used for different therapeutic purposes. WPR is prescribed as the dried root of *P. lactiflora* which has been boiled and peeled before drying, while RPR is prescribed as the dried root of *P. lactiflora* or *P. veitchii* Lynch in Chinese Pharmacopeia. However, most of RPR are derived from *P. lactiflora*, the same botanical origin as WPR. We have previously demonstrated that *P. lactiflora* derived WPR and RPR are genetically and chemically different and geographically isolated from each other. The imported PR in Japanese market and WPR in Chinese market are both derived from cultivated *P. lactiflora* produced in southern part of China, except for different processing methods. The RPR derived from wild *P. lactiflora* produced in northern part of China has been occasionally used in Japan. In the course of the studies to search new resources of PR and further to promote domestic production, several horticultural cultivars of *P. lactiflora* were selected as promising candidates which have potential as medicinal resources of PR on the basis of genetic and chemical analyses. Preliminary bioactive screening experiment shed light on a crude drug of RPR from China and a horticultural cultivar of *P. lactiflora*, “Edulis Superba” which showed relatively strong anti-allergic activity. In order to elucidate the active compounds, bioassay-guided fractionations were conducted and the anti-allergic activities of the isolated compounds were evaluated. In addition, a comparative study based on dozens of monoterpenoids, a group of representative and bioactive components of PR was further performed by liquid chromatography coupled with mass spectrometry to precisely characterize the monoterpenoids profiling of different types of PR and the related species in sect. *Paeonia*.

1. Chemical constituents with anti-allergic activity from the red peony root, the root of *P. lactiflora*¹⁾

The botanical origin of the used RPR sample (D27967) was identified as *P. lactiflora* by genetic analysis of ITS sequence. Methanolic extract of D27967 showed certain anti-allergic activity as inhibitory effect against 2, 4-dinitrophenylated bovine serum albumin (DNP-BSA) stimulated β -hexosaminidase release in immunoglobulin E (IgE)-sensitized rat basophil leukemia (RBL)-2H3 cells (IC₅₀: 0.61 mg/ml). After fractionation with Diaion HP-21 CC, 60% and 80% aqueous MeOH sub-fractions were found to exhibit significantly inhibitory effects (IC₅₀: 0.20 and 0.12 mg/ml, respectively). Therefore, these two bioactive sub-fractions were further investigated to elucidate active

compounds, which led to isolation of 29 compounds, including three new (**1-3**) and fourteen known monoterpenoids (**4-17**), as well as five flavonoids (**19-23**), six other types of compounds (**18, 24-29**) (Chart 1). Among the isolated 17 monoterpenoids, ten of them showed moderate anti-allergic activities with IC₅₀ ranging from 41.17 to 87.11 μ M. Compound **1** named as paeoniflorol exhibited the most effectiveness (IC₅₀: 41.17 μ M), followed by salicylpaeoniflorin (**8**) and galloylpaeoniflorin (**11**). The structure-activity relationships indicated that the basic skeletons of compound **1** and the paeoniflorin-type (**6-15**) were important for the activity, compared to the components with albiflorin-type skeleton (**3-5**). Of the compounds with paeoniflorin-type skeleton, hydroxyl group at C-4 was essential and *O*-hydroxybenzoyl moiety at C-8, galloyl group attaching to C-6' were beneficial to the activity.

2. Chemical constituents with anti-allergic activity from the root of Edulis Superba, a horticultural cultivar of *P. lactiflora*²⁾

Edulis Superba is a horticultural cultivar of *P. lactiflora*, which is identified as RPR-type of *P. lactiflora* by genetic analysis of ITS sequence. The EtOAc and n-BuOH soluble fractions partitioned from methanolic extract of its root, were found to possess the promising anti-allergic activity (IC₅₀: 0.32 and 0.58 mg/ml, respectively). Subsequent phytochemical investigation led to isolate one new norneolignan, named paeonibenzofuran (**30**), and 25 known compounds including five monoterpene glycosides (**4, 6, 8, 13, 31**), ten flavonoids (**19-21, 32-38**), and ten other types of compounds (**18, 24-25, 28-29, 39-43**) (Chart 2). Bioactive assay indicated that mudanpioside E (**31**) with paeoniflorin-type skeleton and quercetin (**37**) showed potent inhibitory activity against β -hexosaminidase release with IC₅₀ values of 40.34 and 25.05 μ M, respectively.

To date, several components such as 1,2,3,4,6-penta-*O*-galloyl- β -D-glucose (**18**), paeoniflorin (**6**), paeonol (**24**) from PR have been revealed to exhibit potent anti-allergic activity both *in vitro* and *in vivo*. In our above studies, several other compounds, especially the characteristic monoterpenoids, were unambiguously documented to make contribution to the anti-allergic activity as inhibitory effect against IgE-mediated degranulation.

3. Characterization and quantification of monoterpenoids in different types of peony root and the related species in sect. *Paeonia* by LC-ESI-IT-TOF-MS³⁾

A comparative study on monoterpenoids profiles using a liquid chromatography-electrospray ionization-ion trap-time of flight mass spectrometry (LC-ESI-IT-TOF-MS) was conducted to elucidate the characteristic monoterpenoids composition in different types of PR and the related species including *P. lactiflora*, *P. veitchii*, *P. anomala* Linn. and *P. japonica* Miyabe et Takeda as well as eleven horticultural cultivars of *P. lactiflora*. MS/MS fragmentation pathways of monoterpenoids with paeoniflorin-, albiflorin- and sulfonated paeoniflorin-types of skeletons were reconstructed by using the authenticated compounds obtained in the above study, which provides basic clues for subsequent elucidation of monoterpenoids profiles. Thirty-one monoterpenoids were unambiguously assigned or tentatively identified (Fig.1A). Six monoterpenoids (**4, 6, 9, 11, 13, 17**) were found to be predominate compounds in all the samples. 4-*O*-methyl-paeoniflorin (**7**) was only detected in *P. veitchii* and *P.*

anomala, and mudanpioside C (**10**) was only detected in *P. lactiflora*. Paeoniflorin-type monoterpenoid sulfonates (**PS1-PS6**) were easily detected in more than half of commercial WPR samples collected from Chinese market, indicating considerable part of WPR was processed by sulfur fumigation and such processing resulted in sulfonation of paeoniflorin-type monoterpenoids. Quantification of fifteen monoterpenoids revealed that the total contents of fifteen monoterpenoids were obviously higher in *P. lactiflora* (26.34-65.03 mg/g) and *P. veitchii* (33.74-74.49 mg/g) than in *P. japonica* (30.36 mg/g) and *P. anomala* (11.20-17.71 mg/g). Of the commercial samples derived from *P. lactiflora*, RPR showed obviously high contents of six paeoniflorin-type monoterpenoids (**6, 8, 10, 12, 13, 14**), but low contents of albiflorin-type monoterpenoids (**3, 4, 5**). *P. veitchii* showed the highest content of salicylpaeoniflorin (**8**) and galloylpaeoniflorin (**11**), differed clearly from the samples derived from other three *Paeonia* species. In addition, monoterpenoids composition between RPR-type and WPR-type of *P. lactiflora* cultivars showed high similarities, which differed from the solution between the commercial WPR and RPR. Principle component analysis (PCA) based on the contents of 21 monoterpenoids revealed five separated groups in the scores plot (Fig. 1B). Besides the respective groups of *P. veitchii* and *P. anomala*, samples derived *P. lactiflora* were clearly classified into three groups that were RPR group, WPR/ PR group and sulfonated WPR group.

Conclusion

In the present study, 43 compounds, including three new monoterpenoids and one new norneolignan were isolated from the roots of RPR-type of *P. lactiflora*. They are mainly monoterpenoids, as well as flavonoids, lignan and other types of compounds. Monoterpenoids with paeoniflorin-type skeleton exhibited relatively strong inhibitory effect against IgE-mediated degranulation. Monoterpenoids profiling clarified the obviously different chemical characteristics of the four species, and the marker constituents for their discrimination. Quantification of fifteen monoterpenoids further revealed considerable variation in the contents of the respective monoterpenoids, and the total contents in *P. lactiflora* and *P. veitchii* were obviously higher than in *P. anomala* and *P. japonica*. The four species as well as the different types of PR showed their feature composition of the paeoniflorin-type monoterpenoids. Moreover, the horticultural cultivar of *P. lactiflora*, Edulis Superba was selected as a candidate of new resource of RPR with anti-allergic activity, and its active constituents and monoterpenoids profiling were evaluated.

Reference

- 1) YH Shi, S Zhu, YW Ge, YM He, K Kazuma, ZT Wang, K Yoshimatsu, K Komatsu. Monoterpene derivatives with anti-allergic activity from red peony root, the root of *Paeonia lactiflora*. Fitoterapia 2016, 108: 55-61.
- 2) YH Shi, S Zhu, T Tamura, M Kadowaki, ZT Wang, K Yoshimatsu, K Komatsu. Chemical constituents with anti-allergic activity from the root of Edulis Superba, a horticultural cultivar of *Paeonia lactiflora*. J Nat Med, 2016, 70: 234-240.
- 3) YH Shi, S Zhu, K Toume, ZT Wang, J Batkhuu, K Komatsu. Characterization and quantification of Monoterpenoids in different types of peony root and related *Paeonia* species by liquid chromatography coupled with ion trap and time-of-flight mass spectrometry. (Submitted)

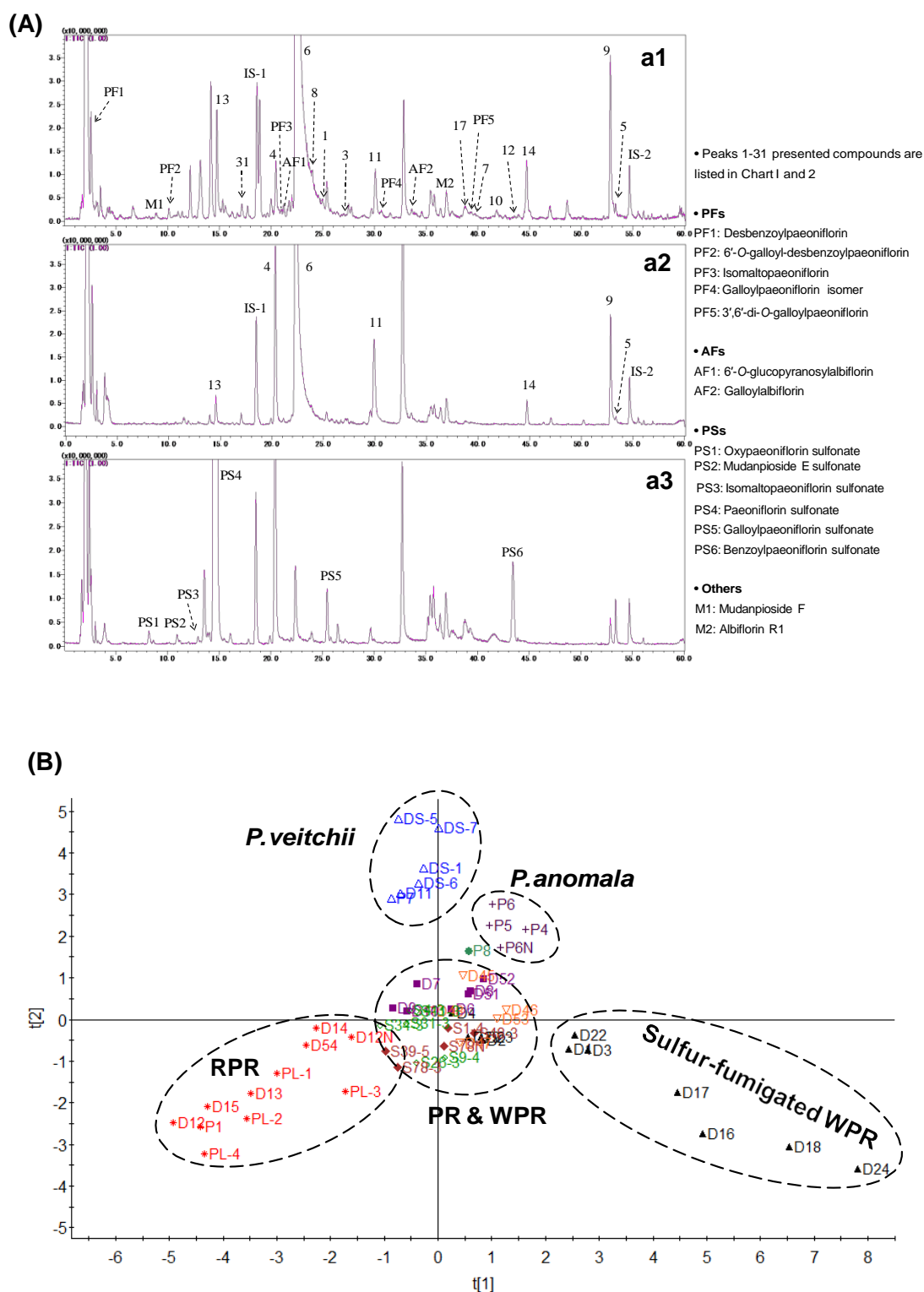


Fig. 1 Representative total ion chromatograms of peony root by LC-ESI-IT-TOF-MS (A), and PCA scores plot of 21 monoterpeneoids in fifty-six *Paeonia* samples (B). **a1**: a plant specimen of *P. lactiflora* (P1, RPR-type), **a2**: a commercial drug of *P. lactiflora* (D52, WPR), **a3**: WPR processed by sulfur fumigation (D24). Scores plot, Triangle: WPR processed by sulfur fumigation in Chinese market; Open triangle: RPR derived from *P. veitchii*; Star: RPR derived from *P. lactiflora* and produced in Inner Mongolia, China; Cross: *P. anomala*; Dot: *P. japonica*; Box: PR produced in Japan; Open inverted triangle: PR produced in China; Circle: Cultivars in Japan, WPR-type of *P. lactiflora* cultivar; Diamond: Cultivars in Japan, RPR-type of *P. lactiflora* cultivar.

学 位 論 文 審 査 の 要 旨

芍薬は *Paeonia lactiflora* の根を基原とし、漢方方剤の約 1/3 に配合される。年間使用量の 95%を中国からの輸入品に依存する。中国の芍薬には白芍と赤芍があり、白芍は日本と同様に鎮痙、鎮痛薬として使用され、赤芍は駆瘀血薬として使用される。赤芍の基原種として *P. veichii* も使用されるが、大多数は *P. lactiflora* であり、同種に由来する白芍との区別法について定説がなかった。当研究室ではこれまで各地の *P. lactiflora* と 2 生薬及び日本産芍薬について遺伝子多型の解析並びに主要 8 成分の定量分析を行い、中国産白芍と日本産芍薬は同系統で成分組成も類似しているが、赤芍とは異なることを明らかにした。さらに共同研究により、赤芍に抗アレルギー作用（粘膜型マスト細胞における抗原刺激脱顆粒抑制作用）を見出した。一方、国産芍薬の栽培拡充を目的として富山ブランド芍薬の開発に着手し、富山県薬用植物指導センターで栽培されている約 70 品種の根について同様の検討を行い、赤芍と類似する成分組成を示す品種を選抜した。

申請者は赤芍の抗アレルギー作用に着目し、その活性成分を明らかにすること、また本作用を有する栽培品種を選抜し活性成分を明らかにしてブランド芍薬の開発に寄与することを目的にして探索研究を行った。さらに、活性成分を含むモノテルペノイドに関する網羅的解析を上記の芍薬類及び *Paeonia* 属植物の根について行った。それにより、下記に示す知見を得た。

1. *Paeonia lactiflora* の根に由来する赤芍に含有される抗アレルギー作用成分

抗アレルギー作用は、Rat basophil leukemia (RBL)-2H3 細胞における抗原刺激脱顆粒抑制作用で評価した。本作用は赤芍の熱水抽出エキスよりメタノールエキスを強かったことから、このエキスをカラムに付し水・メタノール系で溶出し活性を調べたところ、60%及び 80%メタノール溶出画分で活性が認められた。これらの画分についてカラムクロマトグラフィー及び分取 HPLC を行い、新規 3 化合物を含むモノテルペノイド 17 化合物、フラボノイド 5 化合物及びその他 7 化合物を単離し、HR-ESI-MS、 ^1H -及び ^{13}C -NMR、2D NMR などにより構造決定した。それらの抗原刺激脱顆粒抑制作用を検討した結果、多くのモノテルペノイド化合物、1,2,3,4,6-penta-*O*-galloyl- β -D-glucose (PGG)及び methyl gallate が中程度の抑制活性を示した。新規化合物の paeoniflorol (1)はポジティブコントロールの baicalein と同程度の活性を示し (IC₅₀ 41.17 μM)、salicylpaeoniflorin (8) と galloylpaeoniflorin (11)がそれに続いた。Paeoniflorin タイプの骨格を持つ化合物に活性があり、活性発現には pinane 骨格の 4 位のヒドロキシ基が重要であり、8 位のオルトヒドロ

キシベンゾイル基及び糖部の 6'位のガロイル基の存在により活性が増強されることが示唆された。

2. *Paeonia lactiflora* の園芸品種エジュリスパーバの根に含有される抗アレルギー作用成分

ブランド芍薬候補として選抜した 6 品種の根の熱水抽出エキスについて RBL-2H3 細胞における抗原刺激脱顆粒抑制作用を調べた結果、2 品種で活性が認められた。その内エジュリスパーバのメタノールエキスが強い活性を示したことから、本エキスを酢酸エチル、*n*-ブタノールで順次分配した。活性が認められた酢酸エチル及び *n*-ブタノール画分をさらに分画し、分取 HPLC を行い、新規ノルネオリグナンの paeonibenzofuran を単離し、構造決定した。その他、モノテルペノイド、フラボノイドなど 25 化合物を単離同定し、活性を調べた結果、mudanpioside E (**31**) と quercetin が **1** と同等の脱顆粒抑制作用を示した (IC₅₀ はそれぞれ 40.34 μ M、25.05 μ M)。

3. 芍薬類及び *Paeonia* 属 4 種の LC-MS によるモノテルペノイドのプロファイル解析

抗アレルギー作用成分の含有が赤芍の特徴であること及び選抜した品種エジュリスパーバの有用性を明らかにすることなどを目的にして、中国及び日本市場に流通する芍薬類、*P. lactiflora* の中国野生品と日本の栽培品種、近縁種の *P. veichii*、*P. anomala* 及び *P. japonica* の根について、LC-ESI-IT-TOF-MS によるモノテルペノイドのプロファイル解析を行った。Paeoniflorin タイプ、albiflorin タイプ及び paeoniflorin のスルホン酸エステルタイプの骨格を持つ化合物の MS フラグメントパターンの特徴を明らかにした後、LC クロマトグラム上の 31 ピークを同定した。15 成分について定量した結果、paeoniflorol (**1**) 及び paeoniflorin タイプのモノテルペノイド (**8**, **11**, **31** など) の含量が赤芍で高いことが判明した。エジュリスパーバもこれとほぼ同様の成分組成を示し、主成分分析では赤芍のグループに分類された。*P. veichii* では特に **8** と **11** の含量が高く、抗アレルギー活性が高い可能性が示唆された。

以上、赤芍及びエジュリスパーバから新規 4 化合物を含む 43 化合物を単離、同定した。その内新規化合物の paeoniflorol 及び paeoniflorin タイプのモノテルペノイド数化合物に中程度の抗アレルギー作用を見出し、赤芍の新たな効果の一根拠が得られた。同様な特徴はエジュリスパーバにも認められ、本品種を基原とする芍薬の付加価値を証明することができた。さらに *Paeonia* 属植物のケモタキシノミーにおける新知見を得ることもできた。これらの研究成果は学位論文として十分に評価し得るものである。

主査及び副査は、申請者 石 燕紅 の論文内容について審査を行うとともに面接試験を行い、博士（薬科学）を授けるに値するものと判定した。

- 1) Shi Y. H., Zhu S., Ge Y. W., He Y. M., Kazuma K., Wang Z. T., Yoshimatsu K., Komatsu K.: Monoterpene derivatives with anti-allergic activity from red peony root, the root of *Paeonia lactiflora*. *Fitoterapia*, 108: 55-61, 2016.
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