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学位の種類	博士（薬科学）
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教育部名	富山大学大学院医学薬学教育部　博士後期課程 薬科学専攻
学位論文題目	Studies on chemical constituents of *Kaempferia marginata* and *Crinum asiaticum* collected from Vietnam and their NO production inhibitory activities （ベトナム産 *Kaempferia marginata* 及び *Crinum asiaticum* の化学成分と NO 生産抑制活性に関する研究）
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様式 3

論　文　要　旨

論文題目 Studies on chemical constituents of *Kaempferia marginata* and *Crinum asiaticum* collected from Vietnam and their NO production inhibitory activities

課程･専攻名　博士後期課程･薬科学専攻

氏名：KIEP MINH DO

Inflammation is an immune response to harmful stimuli, such as pathogens, damage cells, toxic components, and irradiation that helps in the restoration of the damaged tissues and the maintenance of host homeostasis. However, excessive or uncontrolled inflammation could be detrimental, contributing to the pathogenesis of numerous diseases, including rheumatoid arthritis, asthma, cancer, diabetes, and inflammatory-associated diseases. In inflammatory response, endothelial cells are activated, and leukocytes are emigrated through the vessel wall. The principal leukocytes in acute inflammation are neutrophils followed by macrophages. Activated macrophages produce a variety of inflammatory mediators, such a nitric oxide (NO), prostaglandins, and pro-inflammatory cytokines. The balance of proinflammatory and anti-inflammatory functions in macrophages is one of the important regulatory factors for maintaining cell and tissue homeostasis. In the macrophages, the inducible nitric oxide synthase plays a central role for producing a significant amount of NO. However, excessive NO production can be detrimental to homeostasis. Therefore, compounds with the macrophage NO production inhibitory activity could be important for the development of anti-inflammatory drugs.

In this study, six species of plants, including *Boesenbergia pandurata* (Zingiberaceae), *Crinum asiaticum*. L. var. *anomalum* Baker (Amaryllidaceae), *Curcuma sahuynhensis* (Zingiberaceae), *Globba pendula Roxb* (Zingiberaceae), *Kaempferia champasakensis* (Zingiberaceae), and *Kaempferia marginata* Carey ex Roscoe (Zingiberaceae) were collected from Vietnam, and their methanol, ethyl acetate, chloroform, and *n*-hexane extracts with the five different concentrations were tested for the NO production inhibitory activity against lipopolysaccharides (LPS)-stimulated RAW264.7 cells. The assay revealed the NO production inhibitory activities of the chloroform and methanol extracts of *C. asiaticum* whole plants, the *n*-hexane and ethyl acetate extracts of *C. sahuynhensis* rhizomes, the chloroform extract of *G. pendula* roots, and the *n*-hexane extract of *K. marginata* rhizomes. Among them, the chloroform extract of the *C. asiaticum* whole plants and the *n*-hexane extract of the *K. marginata* rhizomes showed the most and second most potent NO production inhibitory activity (IC_{50} = 30.89 ± 0.62 μg/mL and 54.18 ± 0.61 μg/mL, respectively). Thus, this study aims to discover new natural NO production inhibitors from the chloroform extract of the whole *C. asiaticum* and the *n*-hexane extract of the *K. marginata* rhizomes.

1. Constituents of *Kaempferia marginata* collected from Vietnam and their NO inhibitory activities[1,2)]

Kaempferia marginata Carey ex Roscoe is a small perennial herb that belongs to the Zingiberaceae family. It is mostly distributed in India, China, Myanmar, Thailand, and Vietnam. The rhizomes have been used as an herb in food as well as a traditional medicine in Asian countries. In Thailand, the roots and leaves are used by local people for curries as a flavoring, while the rhizomes are used as traditional medicine for the treatment of allergy symptoms, fevers, and swollen legs. In China, the rhizomes are used as a food spice and also prescribed in traditional medicine to treat abdominal pains, toothaches, coughs, and inflammatory tumors. In Vietnam, its tubers are consumed as a fresh vegetable as well as a crude drug for treating nausea, while the rhizome alcohol infusion is topically applied for the relief of muscle aches and backaches. Chemical constituents of *K. marginata* are reported to be diterpenes, monoterpenes, cinnamate derivatives, and steroids. Among them, pimarane diterpenes reportedly shows various biological activities, including antiplasmodial, anti-tuberculosis, antifungal, and anti-inflammatory.

The investigation of NO production inhibitors from the active *n*-hexane extract of the *K. marginata* rhizomes led to the isolation of twelve new diterpenoids, marginols A–K (**1**–**11**), 14-*epi*-boesenberol F (**12**), and nineteen known diterpenoids, boesenberol F (**13**), boesenberol J (**14**), kaemgalangol A (**15**), kaemgalangol C (**16**), kaempulchraols B–D (**17**–**19**), kaempulchraols E, K, L, and W (**20**–**23**), (5β,9β,10α,13α)-pimara-6,8(14)15-trien-18-oic acid (**24**), 6-actetoxysandaracopimaradien-9-ol-1-one (**25**), 6-acetoxysandaracopimaradien-1,9-diol (**26**), sandaracopimaradien-1,9-diol (**27**), sandaracopimaradien-6β,9β-diol-1-one (**28**), sandaracopimaradien-1,6,9-triol (**29**), virescenol B (**30**), and virescenol C (**31**) (Figure 1). Notably, **9** and **10** contained a naturally very rare 6-oxabicyclo[3.2.1]octane-5-ol ring, while **11** had a naturally rare oxepan-2-one ring in its structure. The structures of all isolated compounds were determined by analyses of their spectroscopic data (1D and 2D NMR spectra, MS, and CD spectrum) and by comparing these data with those reported in the literature.

The isolated compounds **2**, **4**–**7**, **9**–**12**, **15**, **16**, **20**, **21**, **25**, **27**, and **28** without showing cytotoxicity against the RAW264.7 cells were further investigated for the NO production inhibitory activities against LPS-stimulated RAW274.7 cells. L-*N*MMA was used as a positive control (IC_{50} = 40.73 ± 0.60 μM). Compounds **2**, **4**–**7**, **9**, **11**, **12**, **15**, **25**, and **28** exhibited potent inhibitory activities for the NO production in LPS-stimulated RAW264.7 cells, with the IC_{50} values ranging from 65.04 to 96.10 μM. In contrast, **10**, **16**, **20**, **21**, and **27** did not show any NO production inhibitory activity (Table 1). These findings provide insight into the chemodiversity of the Vietnamese *K. marginata* rhizomes.

2. Constituents of *Crinum asiaticum* collected from Vietnam and their NO inhibitory activities[3)]

Crinum asiaticum L. var. *anomalum* Baker is a perennial plant that belongs to the Amaryllidaceae family. It is locally called “Ngai to” or “Nang” in Vietnam, where extracts of its leaves have been used as folk remedies for treating coughs, fevers, and inflammatory diseases, without scientific evidence for a long time. The genus *Crinum* has attracted much attention due to its alkaloidal constituents with various biological activities, such as antiplasmodial, antibacterial, antiviral, anti-malarial, and cytotoxic activities. However, to the best of our knowledge, no investigations of the chemical constituents and biological activities of any parts of *C. asiaticum* var. *anomalum* have been ever reported.

Investigation of the NO production inhibitors from the active chloroform extract of the *C. asiaticum* whole plants led to isolation of three new flavanols, (2*R*,3*S*)-7-methoxy-flavan-3-ol (**32**), (2*R*,3*S*)-7-hydroxy-flavan-3-ol (**33**), and (2*R*,3*S*)-2′-hydroxy-7-methoxy-flavan-3-ol (**34**) and two

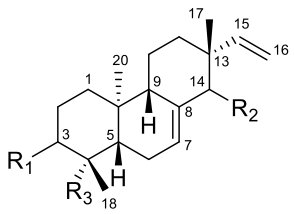
known flavans, (2*S*)-4′-hydroxy-7-methoxyflavan (**35**) and (2*S*)-7,4′-dihydroxy-7-methoxyflavan (**36)** (Figure 2). The isolated compounds, except **33** due to insufficient amount, were evaluated for the NO production inhibitory activity toward LPS-stimulated RAW264.7 cells. Compounds **32** and **34**–**36** showed significant inhibitory effects on the NO production, with IC_{50} values of 11.60, 12.33, 12.43, and 11.15 μM, respectively, which were stronger than that of the positive control L-*N*MMA (IC_{50}: 39.30 μM) (Table 2). Compound **35** did not exhibit any cytotoxicity against the RAW264.7 cells at 3.13 μM. Hence, effects of the active compounds on the production of specific cytokine, IL-6, were investigated, by using immunosorbent assay. The immunosorbent assay indicated that **32** and **34**–**36** inhibited the IL-6 production at 10 μM (Figure 3). Furthermore, **32** and **34**–**36** inhibited the LPS-induced phosphorylation of the p65 subunit of NF-κB in RAW264.7 cells at 10 μM, without significantly affecting the phosphorylation status of p65 under the unstimulated conditions (Figures 4a and 4b). These results suggested that **32** and **34**–**36** could exert potent anti-inflammatory effects by inhibiting the activation of the NF-κB signaling pathway in specific pathogenic conditions, such as LPS-stimulation.

Conclusion

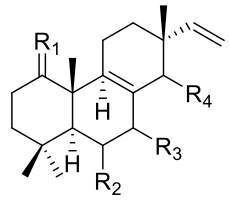
In this study, the chemical investigation of the active *n*-hexane and chloroform extracts of the *K. marginata* rhizomes and the *C. asiaticum* whole plants led to the isolation of 36 compounds including 15 unreported ones, namely marginols A–K (**1**–**11**), 14-*epi*-boesenberol (**12**), (2*R*,3*S*)-7-methoxy-flavan-3-ol (**32**), (2*R*,3*S*)-7-hydroxy-flavan-3-ol (**33**), and (2*R*,3*S*)-2′-hydroxy-7-methoxy-flavan-3-ol (**34**), and 21 known compounds **13**–**31**, **35** and **36**. The biological screening showed that the isopimarane diterpenoids **2**, **4**–**7**, **9**, **11**, **12**, **15**, **25**, and **28** and flavonoids **32** and **34**–**36** showed potent NO production inhibitory activities against LPS-stimulated RAW264.7 macrophage cells. In particular, flavonoids **32** and **34**–**36** were the strong inhibitors for the NO production. Further assay indicated that these compounds showed the activities by inhibiting the NF-κB signaling pathway through the inhibition of the IL-6 production. Compounds **32** and **34**–**36** were also found to inhibit the LPS-induced phosphorylation of the p65 subunit of NF-κB in RAW264.7 cells, without significantly affecting the phosphorylation status of p65 under the unstimulated conditions. These findings thus provided insights into not only the diversity of anti-inflammatory natural products, but also the traditional usage of *K. marginata* rhizomes and the *C. asiaticum* whole plants the for the treatment of inflammatory-associated diseases, from the viewpoint of the chemical constituents.

References

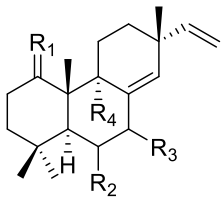
1. Do KM, Kodama T, Shin MK, Ton Nu LH, Nguyen HM, Dang SV, Shiokawa K, Hayakawa Y, Morita H (2022) Marginols A–H, unprecedented pimarane diterpenoids from *Kaempferia marginata* and their NO inhibitory activities. *Phytochemistry* 196: 113109.
2. Do KM, Kodama T, Nguyen HM, Ikumi N, Soeda C, Shiokawa K, Morita H (2023) Seco- and isopimarane diterpenoids from *Kaempferia marginata* rhizomes and their NO inhibition activities. *Phytochemistry* 205: 113510.
3. Do KM, Shin MK, Kodama T, Win NN, Prema, Nguyen HM, Hayakawa Y, Morita H (2022) Flavanols and flavanes from *Crinum asiaticum* and their effects on LPS signaling pathway through the inhibition of NF-κB activation. *Planta Medica* 88: 913–920.



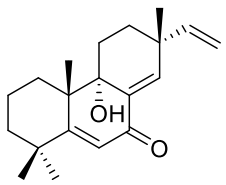
	R_1	R_2	R_3
1	α-OH	H	CHO
2	H	α-OH	COOH
3	H	α-OAc	COOH



	R_1	R_2	R_3	R_4	R_5
4	O	β-OH	H	β-OH	H
5	H	H	β-OH	α-OH	H
12	H	β-OH	α-OH	α-OMe	H
13	H	β-OH	α-OH	β-OMe	H
16	α-OH	β-OH	H	α-OH	H
17	H	β-OH	H	β-OMe	H
18	H	β-OH	H	α-OH	H
19	H	β-OH	H	β-OH	H



	R_1	R_2	R_3	R_4
6	O	β-OH	α-OH	OH
7	O	H	α-OAc	OH
14	H	β-OH	H	OH
20	α-OH	β-OH	H	H
21	H	β-OAc	H	OH
22	H	β-OH	H	OMe
23	H	β-OH	β-OH	OH
25	O	β-OAc	H	OH
26	α-OH	β-OAc	H	OH
27	α-OH	H	H	OH
28	O	β-OH	H	OH
29	α-OH	β-OH	H	OH



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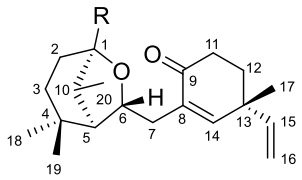
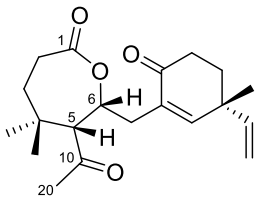


	R
9	OH
10	OMe



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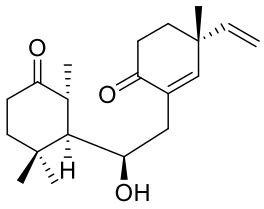

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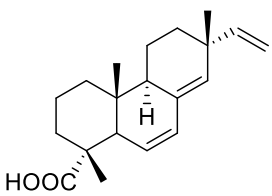

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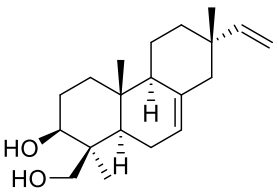

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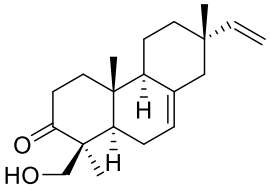

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Figure 1. Structures of compounds (**1**–**31**) isolated from the *n*-hexane extract of *K. marginata*.

Table 1. NO production inhibitory activities of **1**–**31** from the *K. marginata* rhizomes

Compounds	IC_{50}[a] (μM)	Compounds	IC_{50}[a] (μM)
1	NT[b]	**17**	NT[b]
2	86.59 ± 1.93[c]	**18**	NT[b]
3	NT	**19**	NT[b]
4	89.02 ± 2.13[c]	**20**	> 100
5	96.10 ± 4.64[c]	**21**	> 100
6	68.51 ± 1.86[c]	**22**	NT[b]
7	74.97 ± 0.73[c]	**23**	NT[b]
8	NT[b]	**24**	NT[b]
9	81.93 ± 0.58[c]	**25**	78.03 ± 1.13[c]
10	> 100	**26**	NT[b]
11	87.70 ± 3.38[c]	**27**	> 100
12	65.04 ± 0.76[c]	**28**	86.63 ± 2.03[c]
13	NT[b]	**29**	NT[b]
14	NT[b]	**30**	NT[b]
15	84.97 ± 1.74[c]	**31**	NT[b]
16	> 100	L-*N*MMA[d]	40.73 ± 0.60[c]

[a] IC_{50}, half-maximal inhibitory concentration

[b] NT, not tested due to the cytotoxicity

[c] Data are presented as mean ± SD of three independent experiments performed in duplicated

[d] L-*N*MMA was used as a positive control

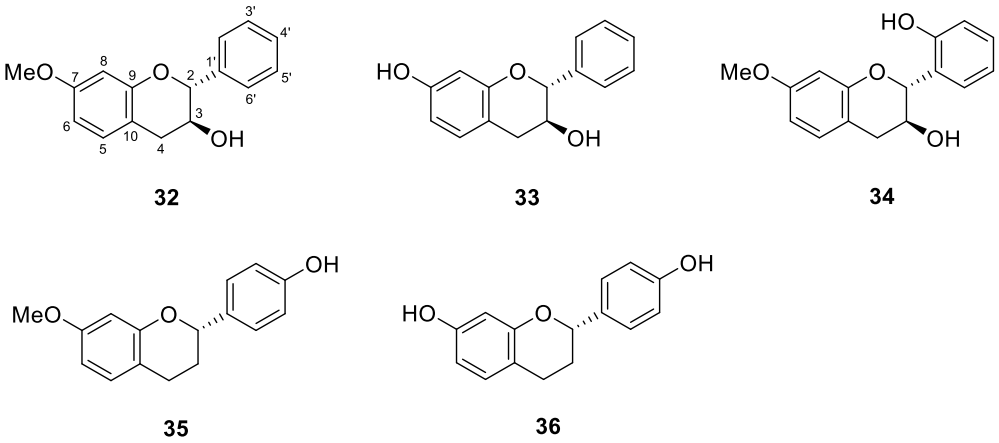

Figure 2. Structures of compounds (**32**–**36**) isolated from the chloroform extract of the *C. asiaticum* whole plants

Table 2. NO production inhibitory activities of **32**–**36** from the *C. asiaticum* whole plants

Compounds	IC_{50}[a] (μM)
32	11.60 ± 0.27[b]
34	12.33 ± 0.40[b]
35	12.43 ± 0.16[b]
36	11.15 ± 0.69[b]
L-*N*MMA[c]	39.30 ± 2.23[b]

[a] IC_{50}, half-maximal inhibitory concentration

[b] Data are presented as mean ± SD of three independent experiments performed in duplicated

[c] L-*N*MMA was used as a positive control

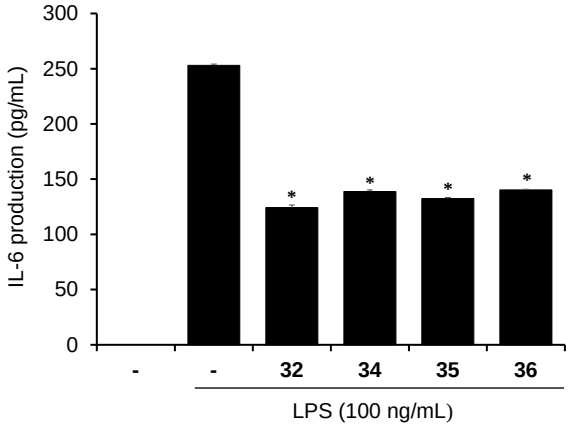

Figure 3. Inhibitory effects of **32** and **34**–**36** on IL-6 production. RAW264.7 cells were treated with 10 μM of **32** and **34**–**36** for 1 h, and then stimulated with LPS (100 ng/mL) for 24 h. The supernatant was collected, and pro-inflammatory cytokine (IL-6) levels was measured by ELISA as described in materials and methods. Data are expressed as the mean ± SD of three independent experiment. *p <0.05 as compared with LPS alone.

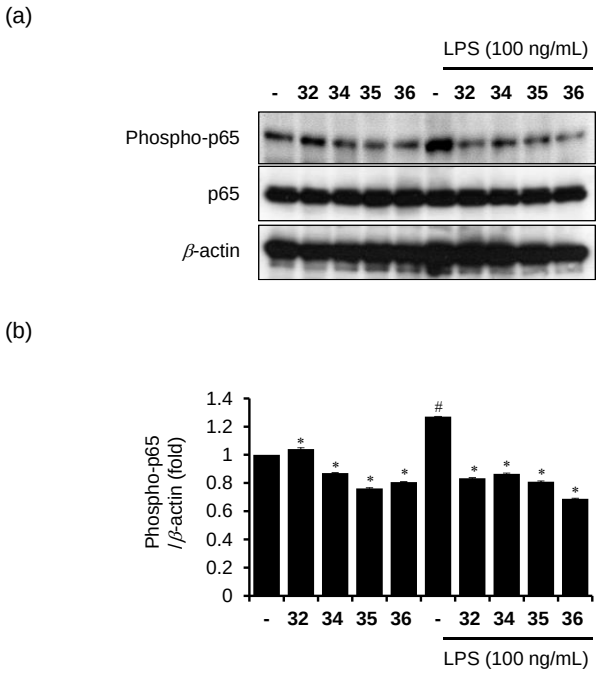

Figure 4. Inhibitory effects of **32** and **34**–**36** on NF-κB pathway. RAW264.7 cells were pre-treated with 10 μM of **32** and **34**–**36** for 1 h and stimulated with or without LPS (100 ng/mL) for 15 min. Western blot analysis was performed using antibodies against phospho-NF-κB p65 (A). The relative expression of phospho-NF-κB p65 (B). The data are expressed as the means ± SD of three independent experiments. #p < 0.05 as compared with untreated group, *p <0.05 as compared with LPS alone.

様式７

学位論文審査の要旨

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（論文題目）（英語の場合は和訳，日本語の場合は英訳を付記すること。英語（訳）は，最初の文字のみを大文字で表記し，他は小文字で表記すること（ただし，学名等を除く）。）

Studies on chemical constituents of *Kaempferia marginata* and *Crinum asiaticum* collected from Vietnam and their NO production inhibitory activities

（ベトナム産 *Kaempferia marginata* 及び *Crinum asiaticum* の化学成分と NO 生産抑制活性に関する研究）

（判定）

合格

（論文審査の要旨）（２頁以内）

炎症は，病原体，損傷細胞，毒性成分，放射線などの有害な刺激に対する免疫反応であり，宿主が損傷組織の修復や恒常性を維持する上で必要不可欠な生命現象である。一方で，過剰な炎症は，関節リウマチ，喘息，癌，糖尿病，炎症性疾患などの多くの疾患の誘発に関与することが知られている。これらの炎症反応においては，様々な因子が関与することが知られているが，それらの中で，マクロファージが産生する一酸化窒素（NO）の過剰産生は，敗血症やエンドトキシンショック，サイトカイン誘導に伴う血圧低下，関節炎を含む炎症性組織障害などに関与していることが明らかになっている。また，近年では，NO過剰産生が癌の血管新生，増殖速度や無酸素血症の悪化に深く関与していることも報告されている。そこで，申請者は，新たな抗炎症薬の開発を指向し，リポポリサッカライド（LPS）で刺激したマクロファージ由来RAW264.7細胞のNO産生に対して抑制活性を示したベトナム産ショウガ科植物*Kaempferia marginata*根茎の*n*-ヘキサン抽出物とベトナム産ヒガンバナ科植物*Crinum asiaticum*全草のクロロホルム抽出物から化学成分の単離･構造決定を実施し，計36化合物を単離した。さらに，単離した化合物の中で，4種の化合物がRAW264.7細胞のNO産生を強く抑制することを新たに見い出すに至った。本研究に関する内容の骨子と審査結果は，下記に示すとおりである。

1．ベトナム産*K. marginata*根茎の化学成分と単離した化合物のNO産生抑制活性

K. marginata 根茎は，ベトナムでは，そのエタノール抽出溶液が，解熱･鎮痛などの目的で伝統的に用いられている。しかし，これまでは，その成分に関しては桂皮酸メチル誘導体のみが報告されているのみである。申請者は，ベトナム産 *K. marginata* 根茎の *n*-ヘキサン抽出物について，各種クロマトグラフィーを用いて分画･精製した結果，12 種の新規ピマラン型ジテルペン**(1**–**12)** を 19 種の既知ピマラン型ジテルペン**(13**–**31)**とともに単離することに成功した。得られた化合物の化学構造は，各種スペクトルデータを詳細に解析して決定した。これにより，ベトナム産 *K. marginata* 根茎には，タイや中国の同植物には含まれていないタイプのピマラン型ジテルペンが含まれていること，さらには，天然においても希なセコ型であり，かつ，オキサビシクロオクタン環を有した新規骨格のピマラン型ジテルペンが含まれていることを

明らかにし，*K. marginata* 根茎の産地の違いによる化学成分の多様性とピマラン型ジテルペンの骨格多様性について，新たな知見を与えた。さらに，単離した **1**–**31** の RAW264.7 細胞における NO 産生阻害活性を精査することで，単離した化合物の中の 10 種類が弱いながらも抗炎症活性を有することを明らかにした。*K. marginata* 根茎は，ベトナムでは炎症を抑えることができる薬用植物として用いられているが，その化学成分とその成分に抗炎症活性のあることを示したのは，本研究が最初である。

2. ベトナム産 *C. asiaticum* 全草の化学成分と単離した化合物の NO 産生抑制活性

*C. asiaticum*の葉は，ベトナムでは，咳，発熱，炎症性疾患の治療に伝統的に用いられている。しかし，いずれの部位についても，その化学成分に関する報告がこれまでにない。申請者は，*C. asiaticum*全草のクロロホルム抽出物について各種クロマトグラフィーを用いて分画･精製した結果，3種の新規フラバノン(**32**–**34**)を2種の既知フラバノン(**35**, **36**)とともに単離することに成功した。さらに，単離した**32**及び**34**–**36**のRAW264.7細胞におけるNO産生阻害活性を精査した結果，これら4種のフラバノンは，コントロールとしたN^{G}-メチル-L-アルギニン酢酸塩（L-*N*MMA）よりも強いNO産生抑制活性を示すことを明らかにした。いずれにおいても，細胞毒性は確認されなかった。そこで，これらのフラバノンが炎症性サイトカインであるIL-6の産生に及ぼす影響を，免疫吸着法を用いて検討した。その結果，これらのフラバノンが，10 μMの濃度でIL-6の産生を阻害することが明らかになった。さらに，これらのフラバノンは，10 μMの濃度でLPSで刺激したRAW264.7細胞のNF-kBのp65サブユニットのリン酸化を阻害するが，非刺激条件下では，p65サブユニットのリン酸化に有意な影響を及ぼさないことを明らかにした。これらの結果から，これらの4種のフラバノンが炎症状態においてNF-kBシグナル伝達経路を阻害することで，その抗炎症作用を示すことを明らかにし，本植物の伝統的な抗炎症剤としての使用に化学的観点から新たな知見を与えた。

以上のように，Kiep Minh Doは，ベトナム産植物2種から15種の新規化合物を含む36化合物を単離・同定し，これらの植物にはNO産生抑制活性を有する多くの化合物が含まれていることを初めて明らかにし，これらの薬用植物の抗炎症剤としての伝統的使用に化学的観点から新たな知見を与えた。さらに，単離した4種のフラバノンが，NF-kBシグナル伝達経路の阻害を介してRAW264.7細胞のNO産生を強く抑制することを新たに明らかにした。強い活性を示したこれらの4種のフラバノンについては，NO産生を抑制する新たな抗炎症薬の開発シードとなることが期待される。また，*K. marginata*根茎には，他地域とは異なるピマラン型ジテルペンが含まれていること，及び*C. asiaticum*全草の化学成分を初めて報告したことは，植物の化学成分の多様性について新たな科学的知見を与えたと言える。

主査及び副査は，論文内容と面接試験を通して，申請者 Kiep Minh Doに，博士（薬科学）の学位を授与するに十分に値すると判定した。

（学位論文のもとになる論文　著者名, 論文題目, 掲載誌名, 巻, 最初の頁と最後の頁, 年を記載）

1. Do KM, Kodama T, Shin MK, Ton Nu LH, Nguyen HM, Dang SV, Shiokawa K, Hayakawa Y, Morita H (2022) Marginols A–H, unprecedented pimarane diterpenoids from *Kaempferia marginata* and their NO inhibitory activities. *Phytochemistry* 196: 113109.
2. Do KM, Kodama T, Nguyen HM, Ikumi N, Soeda C, Shiokawa K, Morita H (2023) Seco- and isopimarane diterpenoids from *Kaempferia marginata* rhizomes and their NO inhibition activities. *Phytochemistry* 205: 113510.
3. Do KM, Shin MK, Kodama T, Win NN, Prema, Nguyen HM, Hayakawa Y, Morita H (2022) Flavanols and flavanes from *Crinum asiaticum* and their effects on LPS signaling pathway through the inhibition of NF-κB activation. *Planta Medica* 88: 913–920.