氏　　名	こいるん　にさ KHOIRUN NISA
学位の種類	博士（薬科学）
学位記番号	富医薬博甲第 216 号
学位授与年月日	平成 28 年 9 月 28 日
学位授与の要件	富山大学学位規則第 3 条第 3 項該当
教育部名	富山大学大学院医学薬学教育部 博士後期課程 生命薬科学専攻
学位論文題目	Isolation and structure determination of new compounds from *Baeckea frutescens* collected in Indonesia and their antibacterial and cytotoxic activities （インドネシア産フトモモ科植物 *Baeckea frutescens* 由来の新規化合物の単離・構造決定と抗菌活性及び細胞毒性）

論文審査委員

（主査）	教　授	黒崎　文也
（副査）	教　授	笹岡　利安
（副査）	教　授	森田　洋行（指導教員）

論文内容の要旨

Introduction

Natural products have been an integral part in drug discovery. About 51% of new drugs developed between 1981 and 2013 were based on the structure of natural products. Thus, investigating of the bioactive compounds from natural resources still attracts our attention to drug discovery.

Indonesia consists of more than 18,000 islands, including Sumatra, Java, Kalimantan, and Sulawesi. Around two-thirds of the area is still covered with tropical rainforests, and over 38,000 species of tropical plants can be found in Indonesia. Among these, 7,000 species of medicinal plants have been used as Indonesian traditional medicine "Jamu". A great number of biologically active metabolites have been reported from Indonesian medicinal plants during a couple of decades. However, most of natural product resources have remained to be fully investigated. Therefore, exploratory research for novel biological active components from diverse medicinal plants in Indonesia will contribute to discover new pharmaceutical leads and candidates, as well as the development of new drugs in order to treat various diseases. This study aims to isolate new compounds, mainly by focusing on antibacterial activity since the antibiotic resistance of human pathogenic microorganisms is still a global concern.

1. Antibacterial screening of Indonesian medicinal plants

The crude MeOH and $CHCl_3$ extracts were prepared from twenty-six Indonesian medicinal plants obtained in different places. These extracts were screened for their antibacterial activities against three Gram-positive bacterial strains, *Bacillus subtilis* NBRC 3134, *Staphylococcus aureus* NBRC 15035, and *Mycobacterium smegmatis* NBRC 3082, and two Gram-negative bacterial strains, *Escherichia coli* NBRC 102203 and *Klebsiella pneumoniae* NBRC 3512. The antibacterial activity was determined using the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT) assay method. The minimum inhibitory concentration (MIC) value of the crude extracts was defined as the lowest concentration showed no visible bacterial growth after incubation at 37°C for 24 h. The antibacterial assays revealed that the $CHCl_3$ extracts of *B. frutescens* leaves and *C. mangga* rhizome showed weak activities against *S. aureus* with an MIC value of 100 µg/mL and 200 µg/mL, respectively (**Table 1**). In contrast, those of *B. frutescens* leaves and *C. mangga* rhizome exhibited antibacterial activities against *B. subtilis* with an MIC value of 200 µg/mL and 150 µg/mL, respectively.

2. Constituents of the leaves of *Baeckea frutescens* collected in Indonesia

The leaves of *B. frutescens* L. (Myrtaceae), popularly known as "Jungrahab", have been broadly utilized as a traditional folk medicine for a long time to treat fever and rheumatism in South East Asia, Australia, and Southern China. This plant is also known to possess anti-inflammatory, antipyretic, antidysentery, antibacterial, and cytotoxic activities. Previous phytochemical investigations of *B. frutescens* resulted in the isolation of phloroglucinols, sesquiterpenes, flavanones, tasmanones, chromones, and chromanones. Since the $CHCl_3$ extract of *B. frutescens* leaves exhibited the most potent antibacterial activity, the extract was selected and was subjected to further isolation of the bioactive compounds.

The leaves of *B. frutescens* were extracted with $CHCl_3$ under sonication at room temperature. The resulting $CHCl_3$ extract was subjected to silica gel Medium Pressure Liquid Chromatography (MPLC) with an *n*-hexane-EtOAc gradient solvent system to yield 10 fractions. The fractions were further separated by a series of chromatographic methods to furnish twelve new compounds (**Figure 1**) including nine phloroglucinol-type metabolites, baeckenones A−I (**1**−**6**, **10**−**12**), two cyclopentenone derivatives, frutescencenones A and B (**7** and **8**), and a furanone derivative, frutescencenone C (**9**), along with ten known compounds (**13**−**22**) (**Figure 2**). The structures of all new compounds were elucidated on the basis of physicochemical data such as HRMS and 1D and 2D NMRs. Among the known compounds, 4-hydroxy-2,2,5-trimethyl-cyclopent-4-ene-1,3-dione (**16**) has been isolated for the first time as a natural product.

The unique phloroglucinols have been isolated from the family Myrtaceae plants. The structures of baeckenones D, E, and G (**4**, **5**, and **10**) demonstrated unique acylphloroglucinol skeletons with *endo*-peroxide linkage, as in the case of **14**. Baeckenones D (**4**) and E (**5**) were isolated as a diastereoisomer in racemic mixtures. The relative structure of baeckenone D was determined by the X-ray crystallographic analysis and NOESY experiments. I propose that baeckenone D (**4**) and baeckenone E (**5**) would be derived from a biosynthetic precursor (**i**) via a keto-enol tautomerization of the precursor for the formation of two diene intermediates (**iia** and **iib**) and a singlet oxygen ($^1O_2$)-involving Diels-Alder type cyclization of each intermediate for the formation of baeckenone D (**4**) and baeckenone E (**5**), respectively (**Scheme 1**).

3. Biological activities of constituents isolated from *Baeckea frutescens*

The antibacterial activities of the isolated compounds (**1**−**22**) against *B. subtilis*, *S. aureus*, *M. smegmatis*, *E. coli*, and *K. pneumoniae* were assessed by using the standard microdilution methods. All compounds were inactive against the Gram-negative bacterial strains, *E. coli* and *K. pneumoniae*. In contrast, only baeckenone B (**2**) had good antibacterial activities against the Gram-positive bacterial strains, *B. subtilis* and *S. aureus* with MIC values of 40 μM and 50 μM, respectively.

The phloroglucinol derivatives reportedly exhibit cytotoxic activities against cancer cell lines such as breast and leukemia cells. Therefore, cytotoxic activities of the isolated compounds (**1**−**22**) were also assessed against human pancreatic (PSN-1), human lung (A549), and breast (MDA-MB231) cancer cell lines. The cytotoxic activities were evaluated by using the standard WST-8 assay method. 5-fluorouracil

was used as a positive control. Baeckenone F (**6**) showed moderate growth inhibitory activities, with half maximal inhibitory concentration (IC_{50}) values ranging from 33.3 μM to 39.3 μM against three tested cell lines. Baeckenone D (**4**) and the known *endo*peroxide-type phloroglucinol (**14**) exhibited selectively weak growth inhibitory activities against the A549 cancer cell line. Frutescencenone A (**7**) exhibited significant cytotoxic effects against the three cancer cell lines, human lung A549, pancreatic PSN-1, and breast MDA-MB-231, with IC_{50} values of 36.3, 29.3, and 38.2 μM, respectively. In contrast, a new furanone, frutescencenone C (**9**), showed selective cytotoxic activity against the PSN-1 cell line, with an IC_{50} value of 20.1 μM (**Table 2**).

Conclusion

In this study, $CHCl_3$ and MeOH soluble-portions prepared from twenty-six traditional medicinal plants were screened for their antibacterial activity. Among these, the $CHCl_3$ extract of the leaves of *B. frutescens* showed the highest antibacterial activity. The phytochemical investigation and bioassay-guided separation of the leaves of *B. frutescens* led to the isolation of twelve new compounds consisting nine phloroglucinols (**1**−**6, 10**−**12**), two cyclopentenones (**7, 8**), and a furanone (**9**), together with ten known compounds (**13**−**22**). Among the isolated compounds, new acylphloroglucinol, baeckenone B (**2**), showed antibacterial activity against *B. subtillis* and *S. aureus* with the MIC values of 40 μM and 50 μM, respectively. On the other hand, baeckenone F (**6**) and frutescencenones A and C (**7** and **9**) exhibited growth inhibitory activities against several cancer cell lines. A large number of phloroglucinol-producing plants have been used in traditional medicine throughout the world. Phloroglucinols and the related metabolites have been reported that these compounds possess a wide range of biological effects, such as antibacterial, antifungal, antiplasmodial, and antimalarial activities. Further elucidation of the bioactivity of the compounds obtained in this study may suggest key lead(s) in the development of new drugs.

References

1. Nisa, K.; Ito, T.; Subehan.; Matsui, T.; Kodama, T, Morita, H. New acylphloroglucinol derivatives from the leaves of *Baeckea frutescens*. *Phytochem. Lett.* **2016**, *15*, 42−45.
2. Nisa, K.; Ito, T.; Kodama, T.; Tanaka, M.; Okamoto, Y.; Asakawa, Y.; Imagawa, H.; Morita, H. New cytotoxic phloroglucinols, baeckenones D–F, from the leaves of Indonesian *Baeckea frutescens. Fitoterapia*. **2016**, *109*, 236−240.
3. Ito, T.; Nisa, K.; Kodama, T.; Tanaka, M.; Okamoto, Y.; Ismail.; Morita, H. Two new cyclopentenones and a new furanone from *Baeckea frutescens* and their cytotoxicities. *Fitoterapia*. **2016**, *112*, 132−135.

Table 1. Antibacterial activities of $CHCl_3$ extracts from *B. frutescens* and *C. mangga*

Plant extracts	MIC (μg/mL)				
	S. aureus	*M. smegmatis*	*B. subtilis*	*K. pneumoniae*	*E. coli*
Baeckea frutescens	100	> 200	200	> 200	> 200
Curcuma mangga	200	> 200	150	> 200	> 200
Ampicillin [a]	0.1	0.1	0.1	0.1	0.1

[a] Positive control

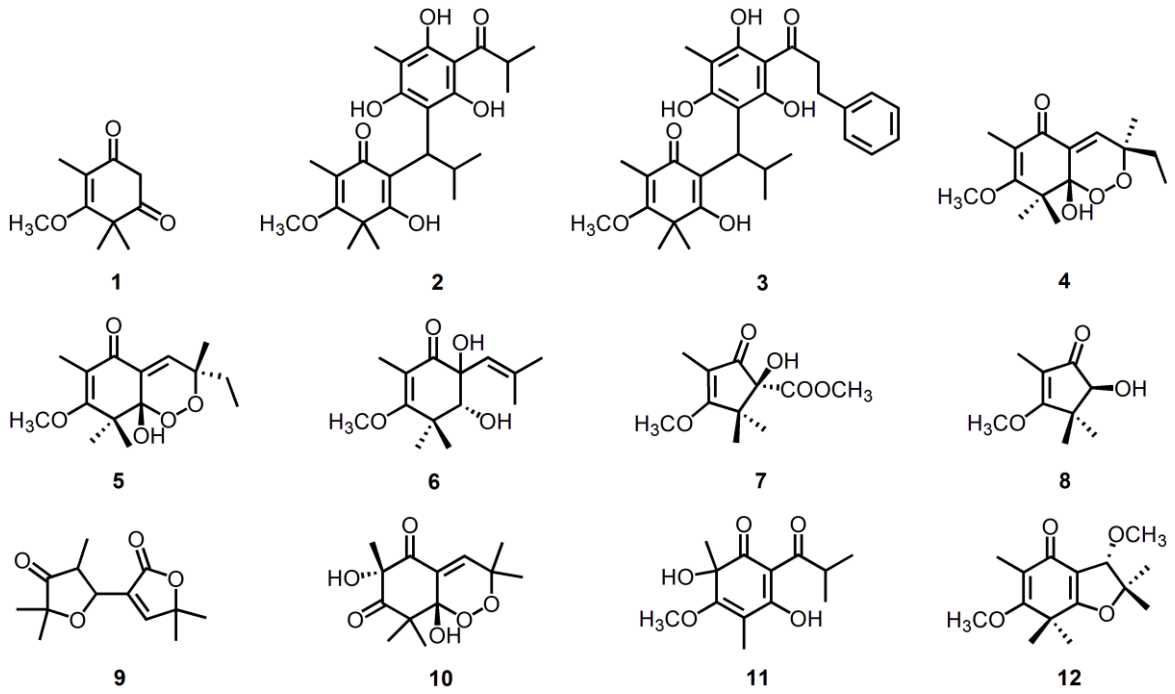


Figure 1. Structure of new compounds isolated from the $CHCl_3$ extract of *B. frutescens* leaves

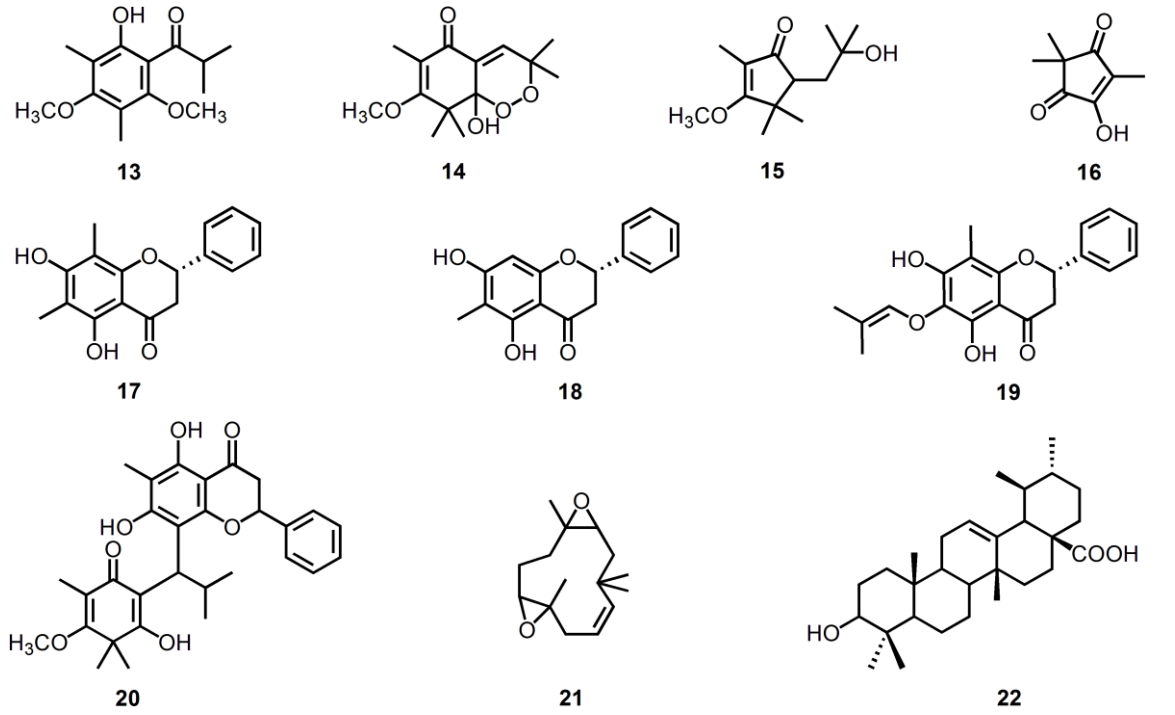
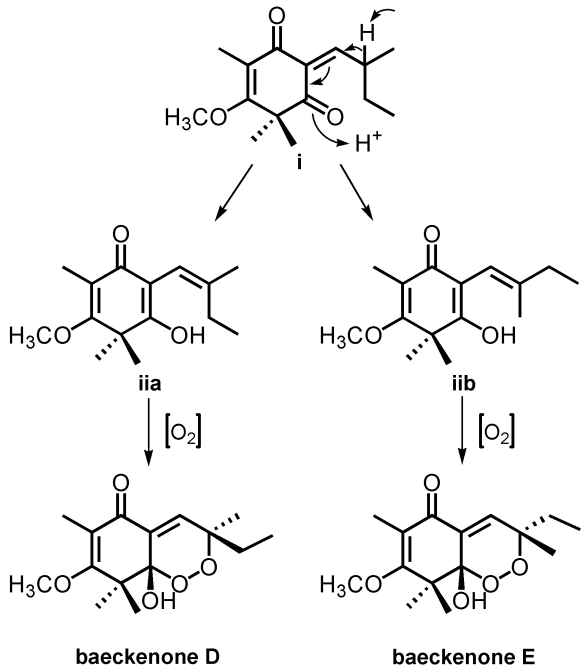


Figure 2. Structure of known compounds isolated from the $CHCl_3$ extract of *B. frutescens* leaves



Scheme 1. Proposed racemic formation of baeckenone D and baeckenone E

Table 2. Cytotoxic activities of some compounds isolated from *B. frutescens* leaves

Compound	IC_{50} (μM)		
	A549	PSN-1	MDA-MB-231
Baeckenone D	60.0	> 100	> 100
Baeckenone F	34.0	33.3	39.3
Frutescencenone A	36.3	29.3	38.2
Frutescencenone C	88.0	20.1	> 100
5-FU [a]	1.8	2.4	4.0

[a] 5-Florouracil (5-FU): Positive control