Doctoral Dissertation

Evaluation of Anti-Inflammatory Effects of Folk Medicine of West Kalimantan
（西カリマンタン民間薬の抗炎症効果の評価）

by

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Summary

The Dayak people represent an indigenous group of Kalimantan Island. They have the knowledge how to utilize medicinal plants as medicine to cure diseases. Nowadays, the knowledge of medicinal plants is fading due to the decrease of the people who use them and the deterioration of the forest environment. One of the efforts to conserve the knowledge of medicinal plants has been implemented through the continuous utilization of medicinal plants to cure diseases by Dayak Uud Danum, one Dayak sub-ethnic group who lives in the upstream areas of Ambalau and Serawai river of Sintang Regency.

In this study, the knowledge of medicinal plants of Dayak Uud Danum were summarized and the anti-inflammatory activities of most common medicinal plants used by Dayak Uud Danum to cure allergies, skin infection, fevers, edema and diarrhea were evaluated by examining their abilities to suppress the delayed-type hypersensitivity (DTH) response against 2,4,6-trinitro-1-chlorobenzene (picryl chloride) using BALB/cAJc mice and to prevent the damage of human colon epithelial FPCK-1-1 cells.

First, the knowledge of medicinal plants of Dayak Uud Danum in six villages was summarized. The medicinal plants were classified according to useful parts of plants, methods of preparation and administration of plants, and the types of diseases that can be treated with plants. There are 95 species of medicinal plants used to cure various diseases and 38 species are used to cure inflammatory diseases associated with allergic, skin infection, fever, edema, and diarrhea. Leaves are the widely used parts. The most common way to prepare the herbal medicine is decoction and the oral-topical route is the most common route of administration.

Second, the extractive content by methanol extraction from leaves of most common medicinal plants used by Dayak Uud Danum to cure allergies, skin infection, fevers, edema, and diarrhea were measured. These plants are Tekeriho (*Callicarpa longifolia* Lam.), Penahan (*Myrmeconauclea strigosa* Merr.), Tebelion (*Eusideroxylon zwageri* Teijsm & Binn.), Kerokak (*Scoparia dulcis* L.), and Bungur (*Lagerstroemia speciosa* (L.) Pers.)

The extractive contents varied from 4.33% to 8.99%. All the plant species are categorized into the group of high level of extractive content. The highest yield was obtained in the extraction from *C. longifolia*.

Third, safety dose and concentration of the methanol extracts from leaves of five plants species was determined through the *in vitro* cytotoxicity assay using mouse fibroblast NIH3T3 cells and the toxicity assay *in vivo* using BALB/cAJc mice.

Except *S. dulcis*, methanol extracts from leaves of *C. longifolia*, *M. strigosa, E. zwageri*, and *L. speciosa* were toxic at a concentration of 100 μg/ml. *L. speciosa* extract was most toxic at lower concentrations (0.1 μg/ml, 1 μg/ml, and 10 μg/ml) in the *in vitro* cytotoxicity assay using mouse fibroblast NIH3T3 cells. *L. speciosa* extract was not toxic to BALB/cAJc mice even after administrating it as much as 5 mg/0.1 DW per mouse.

Fourth, anti-inflammatory effects of *C. longifolia*, *M. strigosa, E. zwageri*, *S. dulcis*, and *L. speciosa* were analyzed on DTH in response to picryl chloride using BALB/cAJc mice by measuring the suppression of ear swelling after the challenge with the antigen used for immunization. The number of eosinophils migrated to the site inflammation were measured by using ear sections.

Dose of each extract administered to mice was 5 mg/0.1 ml DW per mouse and that of hydrocortisone was as much as 0.5 mg suspended in a volume of 0.1 ml DW. The measurement of ear thickness was conducted at 24 hours and 48 hours after challenge. At 24 hours after challenge, only one group of mice treated with *L. speciosa* did not show significant difference compared with a positive control group (immunized and challenged). In all other groups administered with plant extracts the ear thickness was significantly suppressed compared with a positive control. At 48 hours after challenge, all groups administered with plant extracts showed significant suppression compared with a positive control group and a higher level of suppression than that by hydrocortisone. The highest suppression was shown by group of mice administered with *S. dulcis* extract.

The number of eosinophils in the ear section at 48 hours after challenge was compared among all groups of experiments. The number of eosinophils in ear sections from a negative control group (immunized but not challenged) was slightly larger than

those from non-treated group. Number of eosinophils of a positive control group was more than ten-fold compared with that of a negative control group. Except *L. speciosa* group, groups administered with other plant extracts have significantly lower number of eosinophils compared with a positive control group. Furthermore, those numbers of eosinophils administered with plant extracts except *L. speciosa* were lower than that of hydrocortisone group. The lowest number of eosinophils was found in a group of mice treated with *S. dulcis* extract. It is suggested that *L. speciosa* suppresses the DTH reaction by the mechanism different from other four plants.

Fifth, anti-inflammatory activities of *C. longifolia*, *M. strigosa, E. zwageri*, *S. dulcis*, and *L. speciosa* to prevent the damage of human colon epithelial FPCK-1-1 cells derived from a patient with familial adenomatous polyposis were analyzed. Transepithelial electrical resistance (TER) was measured during the co-culture of FPCK-1-1 cells with PMA (phorbol 12-myristate 13-acetate)-stimulated THP-1 cells. The surface of monolayer cells was stained with both Alcian blue and periodic-acid-Schiff. The level of IL-22 in the supernatant of FPCK-1-1 cells in the upper chamber was measured.

The extract of *M. strigosa* most efficiently prevented the decreased of TER that would be caused by PMA-stimulated THP-1 cells on the third day of the co-culture. The high level of mucopolysaccharides was detected with Alcian blue on the surface of FPCK-1-1 monolayers cells treated with the methanol extract from leaves of *M. strigosa*. Among five plant extracts, only the extract of leaves of *C. longifolia* induced the FPCK-1-1 monolayer cells to produce the IL-22.

Methanol extracts from leaves of *C. longifolia*, *M. strigosa, E. zwageri*, *S. dulcis*, and *L. speciosa* had anti-inflammatory activities to ameliorate the DTH response and four plant extracts from *C. longifolia*, *M. strigosa, E. zwageri*, and *S. dulcis* inhibited the migration of eosinophils to the site of inflammation. Only the extract from *M. strigosa* leaves had the preventive effect on the damage of human colon epithelial cells caused by inflammation. It is suggested that methanol extract from leaves of *M. strigosa* contains non-toxic bioactive compounds that suppress the DTH response and prevent the damage of human colon epithelial FPCK-1-1 cells.

Contents

List of main papers

Main papers used in creating the dissertation

Peer-reviewed papers

1. Yeni Mariani, Fathul Yusro, Yuko Konishi, Takahiro Taguchi and Akira Tominaga. 2016. Regulatory effects of five medicinal plants used Dayak Uud Danum in West Kalimantan Indonesia on delayed-type hypersensitivity and the inflammation of human colon epithelial cells. Kuroshio Science, 10: 59-72.

Conference presentation

1. Yeni Mariani, Fathul Yusro, Yuko Konishi, Takahiro Taguchi and Akira Tominaga. 2016. Regulatory effects of five medicinal plants used Dayak Uud Danum in West Kalimantan Indonesia on delayed-type hypersensitivity and the inflammation of human colon epithelial cells. Theme: Sustainable Biodiversity for a Better life, International Conference on Biodiversity, Pontianak Indonesia. October 8-9, 2016.

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Chapter 1
Introduction

1.1. Background

Currently, 25% of modern drugs produced by the pharmaceutical company are compounds isolated from medicinal plants and their derivatives (Ahmed *et al*. 2014). In developing countries, medicinal plants are still the main resources in traditional healing systems, although the modern drugs using identified molecules are available (Mahmood *et al*. 2013). This is caused by the fact that people who live in a remote area are far from modern health care facilities and the cost of medical treatment is high (Oladele *et al*. 2011). Furthermore, that medicinal plants remedies can cure a wide variety of diseases without severe side effects (Mahmoud and Gairola 2013).

In Indonesia, the knowledge of medicinal plants is derived from indigenous people. Since they are divided into sub-ethnic groups and live throughout the nation, the knowledge of medicinal plants varies among groups. Dayak ethnic group is the indigenous people of Kalimantan Island and mainly live in a remote area. Dayak ethnic groups in West Kalimantan province are divided into 151 sub-ethnic groups and their languages are classified into 168 groups (Alloy *et al*. 2008). According to the residents who live close to the forest, Dayak people have their local wisdom especially related to the knowledge how to utilize natural resources. One of their local wisdom is the utilization of plants as medicine to cure diseases.

Nowadays, the utilization of medicinal plants is facing several threats due to the scarcity of the knowledge of medicinal plants and forest condition. This knowledge usually is owned by old generation while young generation accepts modern medicine. Thus, the knowledge of medicinal plants is fading. Generally, this knowledge is orally transferred from generation to generation (Mitra *et al*. 2007). Environmental destruction also has decreased the number of species of medicinal plants due to the destruction of their habitat. Therefore, it is necessary to collect the documentation of species of plants associated with traditional knowledge of medicinal plants to avoid the loss of this knowledge and the extinction of biological resources (Revanthi and Parimelazhagan

2010). Ethnobotanical survey is one of the efforts in the discovery of new modern drugs (Fabricant and Farnsworth 2001).

One of the efforts to conserve the knowledge of medicinal plants is through the continuous utilization of medicinal plants to treat diseases by Dayak Uud Danum, one of Dayak sub-ethnic group who lives in the upstream areas of Ambalau and Serawai river of Sintang Regency. In six villages of Dayak Uud Danum communities, there are 95 species of medicinal plants used and 38 species of them are used to cure inflammatory diseases associated with allergic, skin infection, fever, edema, and diarrhea.

Inflammation is a process of natural protection to maintain the homeostasis of a living organism against tissue injury, infection, or irritation (Mubashir *et al*. 2013). Although inflammation is necessary to protect hosts from infection and maintain homeostasis, it should be under control. There are several kinds of modern drugs to treat inflammation. Anti-inflammatory drugs are classified into non-steroidal anti-inflammatory drugs and corticosteroids. Although doctors frequently prescribe these drugs and they are available in drugstores or pharmacies to relieve inflammatory effects, sometimes it is not applicable because of their side effects. Prolonged consumption of anti-inflammatory modern drugs may lead to the disruption of gastro-duodenal mucosa (Bjarnason *et al*. 1993) and cardiovascular and renal complications (Harirforoosh *et al*. 2013). Nowadays, people restart the use of herbal medicines to treat inflammation in view of their function to ameliorate the condition through working in multiple signaling pathways (Muluye *et al*. 2014).

Nowadays people have started to use the herbal medicine already known by the indigenous people around the world to treat several diseases related to inflammation. One of West Kalimantan indigenous people, Dayak Uud Danum uses various plants to treat inflammation. Although these medicinal plants are not yet scientifically proven to have anti-inflammatory properties, they still use their medicinal plants. Therefore, it is necessary to prove the anti-inflammatory activities of these medicinal plants.

Delayed-type hypersensitivity (DTH), Type IV allergy is characterized by the activation of T cells and macrophages to elicit the tissue inflammation such as ear swelling that is caused by the challenge of the sensitized antigen (Hou *et al*. 2006, Yoshino *et al*. 2010). The activation of T lymphocytes and macrophages induced the

production of pro-inflammatory cytokines such as IFN-γ and IL-17 (Ishii *et al*. 2010, Tominaga *et al*. 2010, 2011, Yoshimoto *et al*. 2000). The symptom of DTH is not only observed in contact dermatitis or skin infection, but also in patients bearing transplanted tissues and tumors.

Diarrhea is a movement of ions and water following the osmotic gradient, resulting in the increase of stools. Inflammation can also induce diarrhea with many different mechanisms. In normal condition, the gastro-intestinal tract has huge capacity to absorb fluid and electrolytes as much as 8-9 liters and about 100-200 ml are excreted in the stool. Infections cause the disruption of secretion balance and lead to diarrhea with the increasing of fluid stool. One of the causes of the disruption of secretion balance in the intestine is the disruption of the junction of epithelial cells, leading to inflammation (Hodges and Gill 2010). Inflammation of intestinal mononuclear cells will induce the pro-inflammatory cytokines such as tumor necrosis factor (TNF-α), interferon gamma (IFN-γ), IL-4, IL-6, IL-8, IL-12, and IL-13 (Suracwiz 2010). Diarrhea is difficult to be fully recovered in a patient with an inflammatory bowel disease (Binder 2009). Prolonged inflammation of intestine may lead to the increasing risk of colon cancer (Kaser *et al*. 2010).

In this study, anti-inflammatory activities of medicinal plants used by Dayak Uud Danum to treat allergies, skin infection, fever, edema, and diarrhea were analyzed. These medicinal plants are Tekeriho (*Callicarpa longifolia* Lam.), Penahan (*Myrmeconauclea strigosa* Merr.), Tebelion (*Eusideroxylon zwageri* Teijsm & Binn.), Kerokak (*Scoparia dulcis* L.), and Bungur (*Lagerstroemia speciosa* (L.) Pers.) (**Fig. 1.1)**. Leaves were selected for these experiments, because these are the parts of plants used by Dayak Uud Danum and they allegedly have the anti-inflammatory effects.

Five medicinal plants from communities of Dayak Uud Danum were chosen. Some of their biological activities are clarified except *M. strigosa*. *C. longifolia* has anti-bacterial, anti-diabetic, analgesic, anti-pyretic, anti-fungal, and anti-inflammatory activities (Soni *et al*. 2014, Yadav *et al*. 2012a, Yadav *et al*. 2012b). *E. zwageri* has anti-melanogenesis activity (Arung *et al*. 2009). *S. dulcis* has anti-diabetic (Pari and Latha 2004, Perumal *et al*. 2014), while *L. speciosa* has anti-oxidant (Unno *et al*. 1997),

anti-gout (Unno *et al*. 2004), and anti-microbial (Nasrin *et al*. 2012) activities. Thus, except for *M. strigosa,* the activities of their plant extracts are partially reported.

Since both IFN-γ and TNF-α are involved in the inflammation of colon epithelial cells and DTH (Bruewer *et al*. 2003 and Tominaga *et al.* 2010), it is useful to examine the anti-inflammatory effects of medicinal plants on DTH and the inflammation of colon epithelial cells.

C. longifolia
(Tekeriho)

M. strigosa
(Penahan)

E. zwageri
(Tebelion)

S. dulcis
(Kerokak)

L. speciosa
(Bungur)

Fig. 1.1. Medicinal plants of Dayak Uud Danum used in this study to analyze the anti-inflammatory effects.

Anti-inflammatory activities of these five medicinal plants of Dayak Uud Danum using a model of damage of colon epithelial cells and DTH are not yet reported. Regulatory effects of medicinal plants on allergic inflammation were evaluated using a DTH response against 2,4,6-trinitro-1-chlorobenzene (picryl chloride) in BALB/cAJc mice by measuring the suppression of ear swelling after the challenge with antigen.

Anti-inflammatory effects of plant extracts on colon epithelial cells were evaluated using FPCK-1-1 colon epithelial cells established from a tubular adenoma in a male familial polyposis coli patient.

Before administering the plant extracts to the mice and epithelial cells, the safety dose and concentration were determined through cytotoxicity assay. Mouse fibroblast NIH3T3 cells were used in the *in vitro* cytotoxicity assay to determine non-toxic concentration of plant extracts for human colon epithelial cells.

In this study, the maximum safe dose was estimated by the toxicity assay *in vivo* using BALB/cAJc mice, the same strain used for DTH assay. In the toxic assay, the mice were administered with several doses of a plant extract that was most toxic in the *in vitro* cytotoxicity and the body weight of each mouse was measured.

The result showed that all of the medicinal plants have anti-inflammatory activities equivalent to hydrocortisone except *L. speciosa* in the DTH assay. Methanol extracts of all plants except *L. speciosa* suppressed the increment of ear thickness and inhibited the migration of eosinophils to the site of inflammation. Although the extract of *L. speciosa* suppressed the ear swelling in the DTH response at 48 hours after challenge, the degree of suppression was much weaker at 24 hours after challenge compared with other plant extracts. Among these plants extracts, *M. strigosa* extract showed preventive effect on the inflammation of colon epithelial cells.

1.2. Objectives of the research

The main objective of this research is to scientifically prove the biological activities of medicinal plants used by Dayak Uud Danum, one of the indigenous people of West Kalimantan, Indonesia, to ameliorate the inflammatory symptoms in allergies, skin infection, fever, edema, and diarrhea.

To achieve the goals, followings are the subjects of this research:

a. Summary the knowledge of medicinal plants of Dayak Uud Danum in six villages.
b. Classify the medicinal plants according to kind of diseases, plants part, methods of preparation, and administration.
c. Analyze the toxicity of methanol extracts from leaves of five species medicinal plants of Dayak Uud Danum using mouse fibroblast NIH3T3 cells and BALB/cAJc mice.

d. Analyze the anti-inflammatory activity of methanol extracts from leaves of five species medicinal plants of Dayak Uud Danum in DTH response against 2,4,6-trinitro-1-chlorobenzene (picryl chloride) using BALB/cAJc mice.
e. Analyze the anti-inflammatory effects of methanol extracts from leaves of five species medicinal plants of Dayak Uud Danum on the damage of human colon epithelial FPCK-1-1 cells.

Chapter 2
Medicinal plants used to cure inflammatory diseases by Dayak Uud Danum

2.1. Introduction

Dayak is a term to define people who live in the upstream of the rivers in the Kalimantan. They are indigenous people of Kalimantan (Indonesia region, Sabah and Sarawah in Malaysia). Originally, Dayak people are hunters and they harvest forest products. Therefore, natural environment, especially forest is inseparable from their lives (Joshi *et al*. 2004, Mulyoutami *et al*. 2009). Dayak Uud Danum is one of Dayak sub-ethnic group who lives in the most regions in Ambalau district of Sintang regency. Dayak Uud Danum in West Kalimantan dwells in forests and still depends on the forest to fulfill their needs, especially they use medicinal plants to cure various diseases. Dayak people in West Kalimantan use a wide variety of plants as medicine in a traditional therapeutic system and the knowledge of medicinal plants are orally transferred through the generations (Yusro *et al*. 2014, Leaman *et al*. 2003).

Nowadays, the transmission of knowledge of medicinal plants is facing several threats due to the exposure of younger generation to modern culture, resulting in the extinction of this knowledge (Kidane *et al*. 2014, Leonti *et al*. 2015). In addition, the lack of documentation in the transmission process of this knowledge accelerates the loss of the knowledge (Alves and Rosa 2007). Therefore, to prevent the loss of the knowledge of medicinal plants among Dayak Uud Danum, it is necessary to document the way to use them.

Although the documentation of the utilization of medicinal plants by several Dayak sub-ethnic groups in West Kalimantan is already reported (Diba *et al*. 2013, Yusro *et al*. 2014), the way of application of medicinal plants by Dayak Uud Danum sub-ethnic groups is not yet reported. In a Dayak people's therapeutic system, a wide variety of species of medicinal plants are used to cure various diseases. Dayak people are commonly suffered from diseases such as skin infections, gastro-intestinal inflammation, and fever caused by infections (Leaman *et al*. 2003). Furthermore,

Damayanti (1999) reported that diseases such as skin infections, diarrhea, and fever are very common among various ethnic groups in Indonesia.

In this study, the documentation of medicinal plants used by Dayak Uud Danum to cure inflammatory diseases was focused. Inflammation is one of process of natural protection to maintain the homeostasis of a living organism against tissue injury, infection, and irritation (Mubashir *et al*. 2013). Bacterial infection and chemical injury are one of the factors that cause inflammation, resulting in damage or death of cells. In response to infection, innate and adaptive immune system are involved in inflammation (Dinarello 2010). Several diseases manifested through inflammation such as allergy, skin infection, asthma, fever, edema, obesity, diabetes, atherosclerosis, cancer, and autoimmunity have been examined (Iwalewa *et al*. 2007). This study focuses on the documentation of knowledge of medicinal plants of Dayak Uud Danum associated with inflammatory diseases such as allergy, skin infection, fever, edema, and diarrhea. Poor sanitation is one of the major causes of those diseases caused by infection (Larson 1999, Baker *et al*. 2016). People in a remote area including Dayak Uud Danum are facing the poor sanitary conditions.

The knowledge of the utilization of medicinal plants by Dayak Uud Danum in six villages of Ambalau district to cure inflammatory diseases associated with allergy, skin infection, fever, edema, and diarrhea was summarized through an ethnobotanical survey. Several data such as species of medicinal plants, plant parts used, preparation and administration of medicinal plant remedies were collected.

In six villages of Dayak Uud Danum community, the presence of medicinal plants is still a part of their life and they are still using the medicinal plants to treat the inflammatory diseases. The knowledge of medicinal plants is owned not only by traditional healers but also by common people.

2.2. Materials and Methods

Observation Site

Ambalau district is one of the districts in Sintang Regency located in West Kalimantan Province, Indonesia. This location was chosen due to the residence of Dayak Uud Danum as a native people in Ambalau river area. The distance from Pontianak city, the capital of West Kalimantan province to Nanga Kemangai, Ambalau

district is 401.9 km (386 km by road and 15.9 km by a boat). Ambalau district has a population of 13,388. In this district, villages are isolated each other and the main transportation is by boat.

There is only one government healthcare facility, which locates in the capital of the district, and health posts in several villages in Ambalau district. Therefore, Dayak Uud Danum has a tendency to be independent from a government healthcare system.

Data Collection

The ethnobotanical survey on the traditional knowledge of medicinal plants used to cure inflammatory diseases by Dayak Uud Danum was conducted from February to March in 2015 and in March of 2016. The survey was conducted in the following six villages in Ambalau district: Nanga kemangai, Bukit tinggi, Lunjang tinggang, Sungai tambun, Riam sabon, and Buntut sabon.

Data collection was undertaken after obtaining the permission from the head of Ambalau district (Camat) and from the head of each village (Kepala Desa). Information regarding the use and practice of medicinal plants and the location of the plants were obtained through interviews and with the agreement of the respondents. The samples were randomly selected based on households and sites.

There were 96 respondents participated in this research. The questions were asked in relation to the species of medicinal plants, especially those used to cure inflammatory diseases associated with allergy, skin infection, fever, edema, and diarrhea. The plant parts used, the modes of preparation, and the routes of administration of the efficacious plants for diseases were asked to the respondents. Medicinal plants mentioned during interviews were collected to make herbarium.

Data Analysis

The medicinal plants data were classified according to efficacious diseases, plant parts used, preparation of remedies, and routes of administration. During survey, only the local names of medicinal plants were obtained. Scientific names were identified later based on IPNI (International Plant Name Index).

2.3. Results and Discussion

Among indigenous communities, the knowledge of medicinal plants has developed naturally along with other indigenous cultures. Due to the increasing demand to keep people's health, the knowledge of medicinal plants in the traditional therapeutic system has become inseparable from the maintenance of their health in modern life (Kunwar *et al*. 2015). The traditional therapeutic system is useful especially for people who live far from the public healthcare center. It has become a good choice for the people who live in a rural area, because they can decrease the medical expenses (Hembing 2000).

In this study, community members of Dayak Uud Danum in the six villages of Ambalau district were interviewed and the knowledge of their medicinal plants were collected and summarized. The medicinal plants were classified according to the efficacious diseases, parts of plants, methods of preparation, and the routes of administration.

The presence of traditional therapies and the remedies of medicinal plants including medicinal plants to cure inflammatory diseases are still an alternative medicine in Dayak Uud Danum community. The presence of traditional healers in the society, the custom of traditional therapies in the indigenous culture, and low cost of the treatment are the major reasons for the indigenous people to choose the traditional therapies (Cheikhyoussef *et al*. 2011).

Totally, there are 95 medicinal plants are used by Dayak Uud Danum community in the six villages of Ambalau district. Among these plants, 38 plants are used to cure inflammatory diseases associated with allergy, fever, skin infection, edema, and diarrhea.

In the research site, Buntut sabon village has the highest number of medicinal plants used to cure various diseases. This village also has the highest number of medicinal plants used to cure inflammatory diseases. These results suggest that Dayak Uud Danum people in this village have an extensive knowledge and use a wide variety of medicinal plants compared with other villages.

Table 2.1. The number of medicinal plants used by Dayak Uud Danum to cure inflammatory diseases associated with allergy, skin infection, fever, edema, and diarrhea in six villages of Ambalau district

No.	Village	Medicinal plants	Medicinal plants used to cure inflammatory diseases associated with allergy, skin infection, fever, edema, and diarrhea	Percentage (%)
1.	Buntut sabon	94	38	40.42
2.	Riam sabon	37	14	37.83
3.	Sungai tambun	86	35	40.70
4.	Nanga kemangai	46	22	47.83
5.	Lunjang tinggang	32	16	50
6.	Bukit tinggi	23	13	56.52

Among six villages in the research site, Bukit tinggi village has the lowest number of medicinal plants. Despite of the lowest knowledge of medicinal plants among six villages, the percentage of the medicinal plants used to cure inflammatory disease by Dayak Uud Danum in Bukit tinggi is high (56.52%) (**Table 2.1**).

Diba *et al*. (2013) reported that 68 species of medicinal plants are used by Dayak Daro and Dayak Kanaytn to cure various diseases, and 10 species are used to cure inflammatory diseases associated with allergy, skin infection, fever, edema, and diarrhea. Yusro *et al*. (2014) reported that 33 species of medicinal plants are used by the traditional healers, traditional birth attendants, shamans, and older people to reduce fever among sub-ethnic groups of Dayak (Kanayatn, Daro, Bukat, and Iban). Present study showed that 38 species of medicinal plants are used by Dayak Uud Danum to cure inflammatory diseases associated with allergy, skin infection, fever, edema, and diarrhea (**Table 2.2**). Due to the higher number of species of medicinal plants used by Dayak Uud Danum compared with those in two reports described above, it suggested the Dayak Uud Danum community has a lot of knowledge on medicinal plants. The abundant knowledge of medicinal plants of Dayak Uud Danum is allegedly due to their residence that locates in a remote area and the lack of healthcare facilities in some villages. In addition, the fact that some diseases could not be cured if treated with modern drugs may lead to the high level of usage of medicinal plants among Dayak Uud Danum community (Nunkoo and Mahomoodally 2012).

Similar situation is also reported by Kustiawan (2007) in Dayak community near the Betung Kerihun National Park in Kapuas Hulu district, West Kalimantan. Although some member of the community are willing to accept the new trend of modern drugs, elders and others that have the knowledge of medicinal are asked to use their knowledge in the practice of traditional therapies (Kustiawan 2007).

The utilization of medicinal plants in traditional therapies is still common among indigenous people, especially for those who live in a remote area, far from the city without a healthcare facility. But, in a certain area where modern drugs are available and people are provided with healthcare facilities, the role of traditional herbal medicine is slowly replaced with chemical drugs. In addition, Caniago and Siebert stated in 1988 that the availability of modern medicines and healthcare facilities has altered the presence and the knowledge of medicinal plants among indigenous people.

In this study, it was revealed that there are 38 species of medicinal plants used by Dayak Uud Danum in six villages to cure inflammatory diseases associated with allergy, skin infection, fever, edema, and diarrhea. Among of these plants, 34 species belong to 27 plant families and the scientific names of four species of medicinal plants are not yet identified (**Table 2.2**).

Table 2.2. Medicinal plants used to cure inflammatory diseases associated with allergy, skin infection, fever, edema, and diarrhea

No.	Medicinal Plants		Parts of Plants	Preparation mode and Administration routes	Village
	Scientific Name Family (Family)	Vernacular name			
1.	*Allium tuberosum* Roxb. *Allliaceae*	Kucai	Bulbs	Pounded (compress)	Buntut sabon, Sungai tambun and Nanga kemangai
2.	*Amaranthus sp* *Amaranthaceae*	Bungo dahpak	Leaves	Pounded, heated (drink, compress)	Buntut sabon, Riam sabon and Sungai tambun
3.	*Annona muricata* L. *Annonaceae*	Nangkak Belanda	Leaves	Decoction, heated, pounded (drink, compress)	Buntut sabon, Sungai tambun, Nanga kemangai and Lunjang tinggang
4.	*Artocarpus elasticus* Reinw. Ex.Bl *Moraceae*	Kaju elang	Leaves	Burn, pounded (compress)	Buntut sabon, Riam sabon and Sungai tambun

5.	*Bambusa vulgaris* Schrad. ex. J.C. Wendl *Poaceae*	Titing tingan	Roots	Decoction (drink)	Buntut sabon, Riam sabon, Sungai tambun, Nanga kemangai and Lunjang tinggang
6.	*Blumea balsamifera* (L.) DC. *Asteraceae*	Keromomum	Leaves, roots, stems	Decoction, burn, pounded (drink, take a bath, compress)	Buntut sabon, Sungai tambun, Nanga kemangai and Bukit tinggi
7.	*Callicarpa longifolia* Lam. *Lamiaceae*	Tekeriho	Leaves	Decoction (drink)	Buntut sabon, Sungai tambun, Nanga kemangai, Lunjang tinggang and Bukit tinggi
8.	*Carica papaya* L. *Caricaceae*	Tekajuk	Leaves, seeds	Decoction, pounded, heated (drink, take a bath)	Buntut sabon, Sungai tambun, Nanga kemangai, Lunjang tinggang and Bukit tinggi
9.	*Cassia alata* (L.) Roxb. *Fabaceae*	Gelinggang	Leaves	Pounded (compress)	Buntut sabon, Riam sabon and Nanga kemangai
10.	*Cordyline fruticosa* (L.) A.Chev. *Asparagaceae*	Sabang untung	Leaves	Decoction, pounded (drink, compress)	Buntut sabon, Sungai tambun, Nanga kemangai and Lunjang tinggang
11.	*Davallia sp* *Davalliaceae*	Paku pantai	Leaves, sap	Decoction, pounded (drink, eat, compress)	Buntut sabon
12.	*Dillenia sp* *Dilleniaceae*	Sekeromohing	Leaves, bark	Decoction, pounded (drink, compress)	Buntut sabon, Riam sabon, Sungai tambun and Nanga kemangai
13.	*Eusideroxylon zwageri* Teijsm & Binn. *Lauraceae*	Tebelion	Leaves, bark, wood, roots	Decoction, raw (drink, eat)	Buntut sabon, Riam sabon, Sungai tambun and Nanga kemangai
14.	*Elephantopus scaber* L. *Asteraceae*	Sawi antu	Leaves, roots	Decoction (drink)	Buntut sabon and Sungai tambun
15.	*Eurycoma longifolia* Jack *Simaroubaceae*	Pasak bumi	Leaves, stems, roots, sap	Decoction, pounded, raw (drink, compress)	Buntut sabon, Riam sabon, Sungai tambun, Nanga kemangai and Lunjang tinggang
16.	*Excoecaria cochinchinensis* Lour. *Euphorbiace*	Tapah darah	Leaves	Decoction (drink, take a bath)	Buntut sabon, Riam sabon and Lunjang tinggang
17.	*Gymnanthemum amygdalinum*	Keromomum malaisia	Leaves	Decoction (drink)	Buntut sabon, and Sungai tambun

	(Delile) Sch.Bip *Asteraceae*				
18.	*Hibiscus rosasinensis* L. *Malvaceae*	Kembang sepatu	Leaves	Pounded (compress)	Buntut sabon, Sungai tambun, Nanga kemangai, Lunjang tinggang and Bukit tinggi
19.	*Imperata cylindrica* (L.) P.Beauv. *Poaceae*	Lalang	Rhizomes	Decoction (drink)	Buntut sabon, Sungai tambun, Lunjang tinggang and Bukit tinggi
20.	*Impatiens balsamina* L. *Balsaminaceae*	Jerekak merah	Leaves	Pounded (compress)	Buntut sabon, Riam sabon and Sungai tambun
21.	*Jussiaea linifolia* Vahl. *Onagraceae*	Sabilio	Leaves	Decoction (drink)	Buntut sabon and Sungai tambun
22.	*Kalanchoe pinnata* (Lam.) Pers. *Crassulaceae*	Semomolum	Leaves	Decoction, pounded (drink, compress)	Buntut sabon, Sungai tambun, Nanga kemangai, Lunjang tinggang and Bukit tinggi
23.	*Kyllinga brevifolia* Rottb. *Cyperaceae*	Telumbang bakat	Leaves	Decoction, heated (drink, compress)	Buntut sabon, Sungai tambun and Lunjang tinggang
24.	*Lagerstroemia speciosa* (L.) Pers *Lythraceae*	Bungur	Leaves	Decoction (take a bath)	Buntut sabon, Riam sabon, Sungai tambun, Nanga kemangai, Lunjang tinggang and Bukit tinggi
25.	*Lansium domesticum* Jack *Meliaceae*	Langsat	Bark, Roots	Decoction (drink)	Buntut sabon, Sungai tambun, Nanga kemangai, Lunjang tinggang and Bukit tinggi
26.	*Myrmeconauclea strigosa* Merr. *Rubiaceae*	Penahan	Leaves, stems, roots	Decoction, pounded (drink, compress)	Buntut sabon, Riam sabon, Sungai tambun, Nanga kemangai and Lunjang tinggang
27.	*Nephrolepsis sp Nephrolepidaceae*	Paku bakai	Leaves, sap	Decoction, pounded (drink, eat, compress)	Buntut sabon and Sungai tambun
28.	*Nauclea orientalis Rubiaceae*	Kelepindih	Leaves	Decoction (drink)	Buntut sabon, Sungai tambun and Nanga kemangai
29.	*Premna cordifolia* Roxb. *Lamiaceae*	Seluoi	Leaves	Decoction (compress)	Buntut sabon, Riam sabon, Sungai tambun, Nanga kemangai and

					Lunjang tinggang
30.	*Premna sp Lamiaceae*	Pahaas	Leaves	Decoction, raw (drink, eat)	Buntut sabon, Sungai tambun and Nanga kemangai
31.	*Sauropus androgynus* Merr. *Phyllanthaceae*	Cangkok manis	Leaves	Pounded (compress)	Buntut sabon, Riam sabon, Sungai tambun, Nanga kemangai, Lunjang tinggang and Bukit tinggi
32.	*Scoparia dulcis* L. *Plantaginaceae*	Kerokak	Whole parts	Decoction (drink)	Buntut sabon, Riam sabon, Sungai tambun, Nanga kemangai, and Bukit tinggi
33.	*Urena lobata* L. *Malvaceae*	Kelenyupang	Roots, seeds	Decoction (drink)	Buntut sabon, Sungai tambun, Nanga kemangai, Lunjang tinggang and Bukit tinggi
34.	*Vitex pubescens* Vahl. *Verbenaceae*	Kolopakpak/ keleban	Leaves, bark	Decoction, chewed (drink, eat raw, take a bath)	Buntut sabon, Sungai tambun and Bukit tinggi
35.	Unidentified -	Kolotohpung	Leaves	Pounded (compress)	Buntut sabon and Sungai tambun
36.	Unidentified -	Mantan ondo	Leaves	Decoction, pounded (compress, drink)	Buntut sabon and Sungai tambun
37.	Unidentified -	Pasak bumi sokon	Roots, leaves	Decoction, pounded (drink, compress)	Buntut sabon and Sungai tambun
38.	Unidentified -	Ponjokaan	Leaves	Decoction, pounded (drink, compress)	Buntut sabon and Sungai tambun

Results revealed that *Asteraceae* and *Lamiaceae* are dominant families used to cure inflammatory diseases in traditional therapies of Dayak Uud Danum (**Table 2.2**). *Asteraceae* plant family is reported to be medicine by several indigenous people in the region such as Sarban Hills, Pakistan (Ijaz *et al*. 2016). *Lamiaceae* family is known as the sources of phenolic compounds for various biological activities including anti-inflammatory activity (Carovic-stanko *et al*. 2016).

Many indigenous people have been using medicinal plants to treat various diseases including inflammatory diseases, because many chemical compounds contained in medicinal plants are reported to be immunosuppressive (Amirghofran

2012).

It is reported that various medicinal plants used by indigenous people contain phytoestrogens, flavonoids, phytosterols, tocopherols, ascorbic acid, curcumin, genistein, quinones, sesquiterpenes, terpenoids, and others that were scientifically proved to be anti-inflammatory (Rogerio *et al*. 2010, Iwalewa *et al*. 2007). Other bioactive compounds contained in plants such as alkaloids, tannins, saponins, anthraquinenones, triterpenoids and others were also reported to have a wide range of biological activities such as anti-cancer, immunostimulatory, anti-bacterial, anti-malarial and anti-tuberculosis (Iwalewa *et al*. 2007).

Among of 38 of medicinal plants used by Dayak Uud Danum to cure inflammatory diseases associated with allergy, skin infection, fever, edema, and diarrhea, there are 5 medicinal plants that are commonly used by the people in six villages. These plants are Tekeriho (*C. longifolia*), Tebelion (*E. zwageri*), Bungur (*L. speciosa*), Penahan (*M. strigosa*) and Kerokak (*S. dulcis*) (**Table 2.2**).

In traditional therapies, many parts of plants are used to make remedies. In some cases, the whole plants were used, while in many cases different parts of the same plant such as leaves, roots, bark, sap, stems, bulbs, seeds, rhizomes are used to cure various ailments.

In Dayak Uud Danum community, leaves are most frequently used for medicinal remedies (56.14%), followed by roots (15.8%), stems (7.02%), bark (7.02%), and sap (5.26%). Besides these parts, seeds, rhizomes, bulbs, and whole plants are also used for making remedies (3.51%, 1.75%, 1.75%, and 1.75%, respectively). Due to its availability throughout the year, leaves are the part of plants that are frequently used in many plant remedies by indigenous people as reported in Rangamati Bangladesh (Kadir *et al*. 2012) and in Mauritius (Nunkoo and Mahomoodally 2012). In addition, people can use leaves in large quantities compared with other parts of plant (Tugume *et al*. 2016).

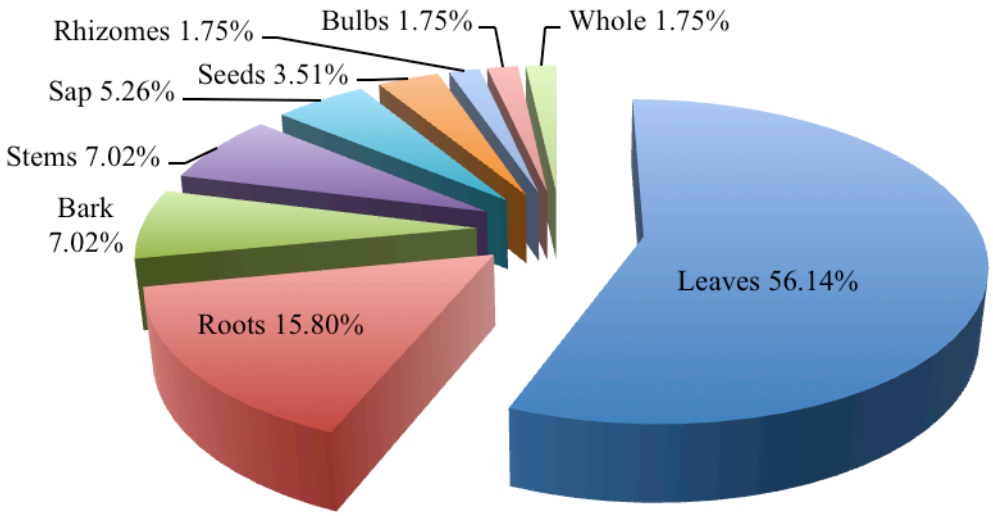


Fig. 2.1. Parts of medicinal plants frequently used for remedies

The high frequency of the usage of leaves in medicinal remedies may suggest the possibility that leaves contain higher level of bioactive compounds compared with other parts of the plants. This opinion is derived from the fact that leaves play a main role in photosynthesis and function as reservoirs that contain secondary metabolites which protect plants and are also useful as medicine (Nunkoo and Mahomodally 2012). Furthermore, the usage of leaves in medicinal remedies has lower risk to cause serious damage to the plants, suggesting the lower level of threat to the extinction of medicinal plants compared with the usage of other parts of the plants. Several threats such as the loss of the habitat, the degradation of the habitat, and overharvesting may lead to the extinction of the species of medicinal plants (Hamilton 2004).

In the preparation of remedies of medicinal plants, some remedies are derived from a single part of plant, more than two parts of plant, or a mixture of several parts from several plants. Thus, in the preparation of remedies from medicinal plants, there are several methods that are usually used by many indigenous people such as decoction, pound, squeeze, or direct use (raw) (Xavier *et al*. 2014, Ong and Kim 2014).

In Dayak Uud Danum people, decoction is the most popular method to prepare the remedies of medicinal plants to cure inflammatory diseases followed by pounded, raw, and squeezed (58%, 32%, 8%, and 2%, respectively). Decoction or boiling in hot water

is the most popular method in the preparation of the remedies of medicinal plants in several communities of indigenous people (Mahmood *et al*. 2013, Nunkoo and Mahomodally 2012).

Decoction was prepared by boiling the parts of medicinal plants in water until the volume of water reduced to two-thirds or the required level. Pounded remedies were prepared by crushing the parts of medicinal plants to make the paste. Juice is obtained by squeezing the parts of fresh plants or by cutting the parts of the plants to obtain the liquid. Moreover, some of the remedies of medicinal plants can be used without any treatment (raw).

In Dayak Uud Danum community, water is the main solvent usually used for the preparation of remedies, especially for the decoction method. Nunkoo and Mahomodally (2012) stated the following reasons for the indigenous people to choose water to prepare remedies from medicinal plants: water is an inert excipient, universally available, and water is suitable to avoid the break down of the active compounds in medicinal plants.

Generally, the fresh materials are preferred to use in remedies, especially for the raw method. It may be due to the belief among the indigenous people that the bioactive compounds in medicinal plants might be vaporized and or destroyed on drying or heating (Mengesha 2016). Although fresh materials are the most preferred, some dried materials are also used in remedies of medicinal plants. The usage of dried plants is advantageous when medicinal plants are collected far from the village or the materials are available only in a certain season.

Wahyono *et al*. (2015) classified the route of administration of remedies of medicinal plants in Indonesia into three groups (oral, topical, inhale). In some cases, the external application of remedies of medicinal plants (topical) and both internal and external (oral-topical) routes of administration were used. Based on that classification, the remedies of medicinal plants of Dayak Uud Danum can be categorized into two groups (oral, topical) and a mixed way of administration (oral-topical). The most common route of administration of remedies from medicinal plants to cure inflammatory diseases in Dayak Uud Danum community is oral-topical (44.74%) followed by oral (31.58%), and topical (23.68%). The topical route is considered to be

safer than oral route, because the effects of the remedies are restricted in the area where the remedies were applied (Abe and Ohtani 2013).

As well as other indigenous people, Dayak Uud Danum people use the local unit in the preparation of remedies from medicinal plants such as finger length (for bark, roots, and stems), pinch (for powder), and numbers (for leaves, seeds and fruits). In many indigenous tribes, the lack of precision and standardization of the dosage of remedies of medicinal plants prescribed by the traditional healer is the weakness of a traditional therapeutic system. In addition, the same dose of medicinal remedies is prescribed regardless of the patient's age, body weight, and sex (Yirga 2010).

The scientific research to prove the efficacy of medicinal plants derived from indigenous people's knowledge is necessary to define the appropriate dose of their medicinal plants. The scientific evidence may encourage the indigenous people and outside the community to use the medicinal plants.

This precious knowledge accumulated over centuries among indigenous people may disappear within a couple of generation if the knowledge transmission is not supported by documentation. The documentation of plant species associated with traditional knowledge of medicinal plants prevents the loss of this knowledge and the extinction of biological resources (Revanthi and Parimelazhagan 2010). In addition, the lack of transmission process of medicinal plants and the formal education about the knowledge of medicinal plants is the major cause of the decrease of the knowledge of medicinal plants (Lense 2012).

2.4. Conclusion

The knowledge of the species of medicinal plants of Dayak Uud Danum in six villages of Ambalau district was summarized. In the research site, the knowledge of medicinal plants of 96 respondents was documented.

As a result, 95 species of medicinal plants used by Dayak Uud Danum were documented. The medicinal plants are used to cure various diseases. Among of them, 38 species are used to cure inflammatory diseases associated with allergy, skin infection, fever, edema, and diarrhea. The most common medicinal plants used by Dayak Uud Danum in six villages to cure the inflammatory disease are Tekeriho (*C. longifolia*),

Tebelion (*E. zwageri*), Bungur (*L. speciosa*), Penahan (*M. strigosa*), and Kerokak (*S. dulcis*).

The main part of medicinal plants used by Dayak Uud Danum people is leaf. Most of the remedies of medicinal plants are prepared by decoction or boiling in hot water. To administer the remedies of medicinal plants, the oral-topical route of administration is the most common way.

Chapter 3
Methanol extraction from five species of medicinal plants used by Dayak Uud Danum

3.1. Introduction

It is well known that many indigenous people around the world use plants not only to treat human diseases but also to keep livestock healthy. In developing countries, many people have to rely on a traditional health care system due to the inaccessibility to the government health care center, unaffordable medical costs, and cultural factors (Yirga 2010).

Due to the globalization, there are several problems that occur in maintaining our health, economy, and environment. Medicinal plants and the utilization of the knowledge on them can extenuate the problem on health. Medicinal plants are able to support the primary healthcare of people who live in a remote area and play a key role in providing an important way for a sustainable development (Toda *et al*. 2016).

Dayak Uud Danum as, a member of Dayak people in the indigenous community of West Kalimantan is accustomed to use medicinal plants as a source in their traditional health care system. Due to their residential areas that are far from a government health care center, they have to rely on their natural resources to treat their diseases. Based on the results of field research, in six villages of Dayak Uud Danum community, there are 95 species of medicinal plants used by Dayak Uud Danum. Among those plants, 38 species are used to cure inflammatory diseases associated with allergy, skin infection, fever, edema, and diarrhea. Leaves, roots, stems, bark, sap, seeds, rhizomes, bulbs, and whole parts are used to make various remedies. According to Hosseinzadeh *et al*. (2015), medicinal plant remedies consist of variety compounds of secondary metabolites that have therapeutic properties. Many natural products derived from medicinal plants are proven to have biological active compounds, leading to the new innovative drugs by pharmaceuticals companies (Palombo 2009).

Extraction is an initial stage of medicinal plant analysis. Extraction with selective solvent can be used in order to obtain chemical compounds from medicinal plants.

Through the extraction process, bioactive chemical compounds of medicinal plants can be obtained for the further separation and characterization (Sasidharan *et al*. 2011). In the extraction process, ethanol, methanol, acetone, and ethyl acetate are frequently used as polar solvents. Extraction with methanol is quite effective, and chemical compounds such as oils, fats, waxes, alkaloids, flavones, polyphenols, tannins, saponins, glycosides, and aglycones are generally obtained (Filho 2006, Houghton and Raman, 1998).

In Dayak Uud Danum community, there are leaves of five species of medicinal plants that are commonly used to cure inflammatory diseases associated with allergy, skin infection, fever, edema, and diarrhea. Leaves of these plant species may have chemical compounds that can be used as anti-inflammatory and anti-intestinal inflammatory reagents. Through the extraction process with methanol, we measured the percentage of extractive contents from leaves of five species of medicinal plants. Cytotoxicity of the methanol extracts was examined against mouse fibroblast NIH3T3 cells *in vitro*, and against BALB/cAJ mice *in vivo*. Furthermore, anti-inflammatory effects of these methanol extracts on delayed-type hypersensitivity by using BALB/cAJc mice and the damage prevention of human colon epithelial FPCK-1-1 cells were evaluated.

3.2. Materials and Methods

Sample collection

All samples described below are the species of medicinal plants used by Dayak Uud Danum and collected from Ambalau District of Sintang Regency, West Kalimantan Province, Indonesia: Tekeriho (*C. longifolia*), Penahan (*M. strigosa*), Tebelion (*E. zwageri*), Kerokak (*S. dulcis*), and Bungur (*L. speciosa*) (**Fig. 1.1**). After being dried in the air for 2 weeks, 40 grams of dried leaves were milled with an electric grinder (Oster, Sunbeam Products, Inc.) to obtain fine powder. Voucher specimens of all plants samples were deposited in the Laboratory of Wood Technology, Tanjungpura University Pontianak to identify the scientific name of each plant.

Extraction

Thirty grams of powdered leaves were extracted with 100 ml of methanol (99.7%) by using a soxhlet extractor (Yamato Water Bath BS660, Yamato Scientific Co. Ltd)

for 1 hour at 70°C. The extraction was repeated three times followed by the filtration with Whatman filter paper No. 2. The filtrated samples were evaporated with a vacuum rotary evaporator (Eyela No-1000, Tokyo, Japan) with a speed of 5 rpm at 40°C. The evaporated samples were dried for one day in a wind dryer (Pierce, Reacti-Therm and Reacti-Vap, Thermo Fisher Scientific Inc., Waltham, MA). The drying process was continued with a vacuum dryer (Ettas, AVO-250NB, Active Co. Saitama, Japan) for one day and the final residue was obtained. The percentage of the extractive content was determined at this point.

$$\text{Extractive content (\%)} = \left(\frac{\text{weight of extract (g)}}{\text{weight of powdered leaves (g)}} \right) \text{x100\%}$$

3.3 Results and discussion

In this study, the level of extractive yield of leaves of five species of medicinal plants used by Dayak Uud Danum to treat allergy, skin infection, fever, edema, and diarrhea were measured. From present results, all the plant species are categorized into higher level of extractive content after the methanol extraction with a yield ranging from 4.33% to 8.99% (**Fig. 3.1**). The highest yield was the extraction from *C. longifolia* followed by *M. strigosa*, *E. zwageri, L. speciosa*, and *S. dulcis*.

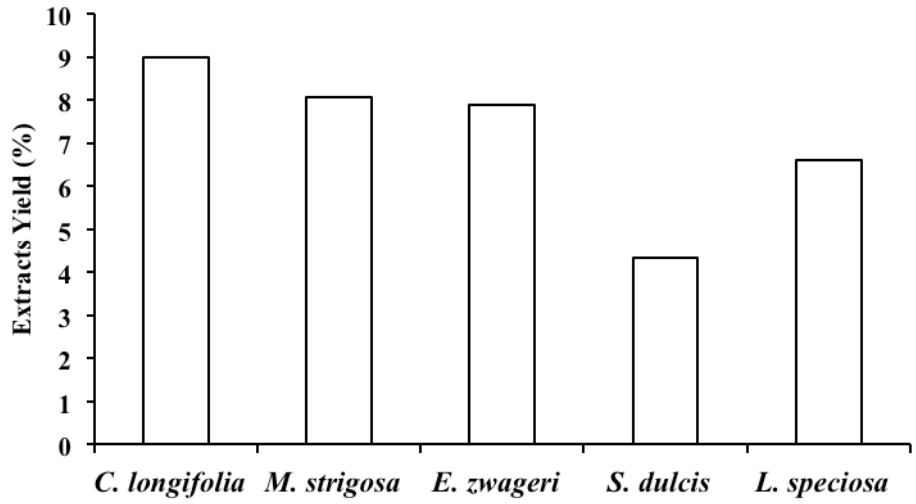


Fig. 3.1. The percentage yield of methanol extracts from plant leaves.

Extractives or secondary metabolites are low molecular weight compounds present in plants and they have a wide spectrum of function. The levels of extraction

yield of contents vary among the plant species. In general, extractive contents of plants range from less than 1% to more than 10%, while it is about 20% in a tropical wood (Tsoumis 1991). The different part of plants like woods, leaves, bark, roots, and branches give differences in yields and types of chemical compounds. Generally, extraction from bark will give higher extraction yield followed by that from leaves, roots, and stems (Sjostrom 1981). All extracts used in this study are derived from leaves. It is assumed that higher levels of extraction yields of all plants are related to the presence of chlorophyll. In the extraction process, the usage of polar solvent will dissolve chlorophyll, resulting in the higher yield (Harborne 1996).

As polar solvents such as methanol, ethanol, and water are very effective to isolate bioactive compounds (Filho 2006). Methanol is generally used to acquire the intracellular components not only because of its high efficiency in extraction, but also because of its expeditious penetration into cell membranes (Silva *et al*. 1998).

Except *M. strigosa* leaves extract, other methanol extracts contain already identified bioactive compounds. *C. longifolia* leaves are reported to have alkaloids, phenolic compounds, flavonoids, saponins, and steroid compounds (Erwin *et al*. 2015). Tannins, steroids (tritepenoids), alkaloids, flavonoids, and scoparinol (a diterpene) were found in the extract from leaves of *S. dulcis* (Abere *et al*. 2015, Wankhar *et al*. 2015, Murti *et al*. 2012).

Triterpenes, steroids (triterpenoids), tannins, glycosides, flavones, and ellagic acid were found in the extract of leaves of *L. speciosa* (Chan *et al*. 2014, Nasrin *et al*. 2012, Priya *et al*. 2008). Wood part of *E. zwageri* was reported to have tetratetracontane, eicosane, iso-elemicins, methyl elaidate, and heneicosane (Ping 2012). Yoosu *et al.* (2009) also reported eusiderin A and eusiderin I in the wood part of *E. zwageri*.

Although the bioactive compound was not yet identified in the extract from leaves of *M. strigosa*, some chemical compounds such as oils, fats, waxes, alkaloids, flavones, polyphenols, tannins, saponins, and glycoside aglycones may be obtained from the methanol extract (Filho 2006, Houghton and Raman 1998). Alkaloids, flavonoids, condensed tannins, gallotannins, flavones, flavonols, flavanones, anthocyanidins, isoflavonoids, coumarins, isoquinolines, indoles, diterpenes, saponins, sterols, terpenoids, and essential oils are chemical compounds from secondary metabolites of

plants that are known for their anti-inflammatory activities (Mohammed *et al*. 2014, Pengelly 2004).

3.4. Conclusion

The levels of extraction yield of methanol extracts from leaves of five species of medicinal plants used by Dayak Uud Danum were measured. The percentages of extraction yield varied from 4.33% to 8.99%. The highest yield was from *C. longifolia* followed by *M. strigosa*, *E. zwageri, L. speciosa*, and *S. dulcis*. Methanol extracts from leaves of medicinal plants used by Dayak Uud Danum may have potential bioactive compounds that are able to suppress the allergic inflammation of delayed-type hypersensitivity and prevent the damage of human colon epithelial cells.

Chapter 4

Toxic activities of methanol extracts from leaves of medicinal plants were determined using NIH3T3 cells and BALB/cAJc mice

4.1. Introduction

Many indigenous people around the world use medicinal plants in their traditional therapies. Nowadays the usages of medicinal plants remedies are becoming the alternative options, not only in the rural areas but also in the urban areas. The usages of medicinal plants remedies have increased recently, because the prices are low and they are accessible or readily available and acceptable in community culture. They have a wide spectrum of activities and less side effects compared with modern synthetic medicines (Chikezie *et al*. 2015a). Furthermore, the usages of medicinal plants are believed to be safe based on the experiences in a long period of consumption (Otang *et al*. 2014).

Despite the consumption of medicinal plants remedies are considered to be safe, recent scientific reports showed that many of medicinal plants are potentially toxic, mutagenic, and carcinogenic (Otang *et al*. 2014). Medicinal plants contain bioactive compounds with a wide variety of biological functions such as anti-microbial, anti-cancer, anti-diabetic, and anti-inflammatory activities. Even medicinal plants that contain beneficial bioactive compounds may also contain toxic or poisonous chemical compounds. It was reported that several poisonous compounds such as myristicin, phytohemagglutinins, or lectins are derived from plants (Chikezie *et al*. 2015b). Although these compounds are poisonous, these compounds can be used for medicines at a proper dose or an appropriate concentration. Myristicin is found in nutmeg and used as an anticholinergic drug (Demetriades *et al*. 2005), while phytohaemagglutinins or lections are found in certain legumes and used as anti-tumor, anti-insects, and anti-viral drugs (Lam and Bun Ng 2010).

Generally, in traditional therapies, the administration and preparation of remedies from medicinal plants are conducted without precise doses or concentrations. In

addition, the same dose of medicinal remedies is prescribed regardless of the patient's age, body weight, and sex (Yirga 2010).

In the indigenous traditional therapies, Dayak Uud Danum people drink the decoction of medicinal plants. In general, to prepare medicinal plant remedies, they just use a handful of parts of medicinal plants. For example, leaves of medicinal plants (3, 7, or 15 sheets) were boiled in hot water to prepare a decoction. To avoid the severe impact of harmful or toxic compounds included in the remedies of medicinal plants, it is necessary to scientifically prove the proper activity of remedies of medicinal plants. Through cytotoxicity assays, the effective and safe dose for the oral administration of herbal remedies will be determined. Toxic effects of medicinal plants extracts were conducted using *in vitro* and *in vivo* models.

In this study, the anti-inflammatory activities of methanol extracts from leaves of medicinal plants traditionally used by Dayak Uud Danum to treat several diseases related to inflammation were analyzed (**Fig. 1.1**). The anti-inflammatory activities of these plants will be examined through an *in vivo* model on the Delayed-type hypersensitivity (DTH) using BALB/cAJc mice and an *in vitro* damage-prevention model of human colon epithelial cells. Before conducting these the anti-inflammatory assays, it is necessary to determine the safety doses or concentrations of plant extracts to avoid the toxicity of these extracts to mice and cells.

Normal murine fibroblast NIH3T3 cells (Theiszova *et al*. 2005) were used in the *in vitro* cytotoxicity assay to determine the non-toxic concentration of methanol extracts from herbal plants for human colon epithelial cells. Based on this *in vitro* cytotoxicity assay, the extract of *L. speciosa* was chosen as a most toxic one and used for the *in vivo* toxicity assay. The *in vivo* toxicity assay was performed using the same strain of mice used for DTH assay. During the toxicity assay, the body weight of each mouse was measured. The result showed that all methanol extracts from medicinal plants of Dayak Uud Danum are toxic at higher concentrations, and *L. speciosa* extract was most toxic in terms of the numbers of viable cells in an *in vitro* assay. The safe concentration for the *in vitro* assay using FPCK-1-1 cells was estimated to be one μg/ml. The safe concentration of methanol extracts from medicinal plants for mice was estimated to be less than 5 mg/0.1 ml DW/mouse.

4.2. Materials and Methods

Plants materials

Methanol extracts from leaves of five medicinal plants of Dayak Uud Danum were prepared as described in the previous chapter (**Chapter 3**). For *in vitro* cytotoxicity assay, 10 mg of methanol extracts from plant leaves (dry weight) was dissolved in 1 ml of DMSO (Dimethyl sulfoxide, 10 mg/ml) to make a stock solution. In this test, four concentrations of plant extracts were tested (0.1 μg/ml, 1 μg/ml, 10 μg/ml and 100 μg/ml). As a control, diluted DMSO was used. The effects of DMSO were negligible.

In the *in vivo* toxicity assay, as much as 500 mg of methanol extracts of each plant (dry weight) were grinded in a mortar and suspended in 10 ml of distilled water (DW) (Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan) to make a concentration of 50 mg/ml. Then, these samples were homogenized with a Polytron (Kinematica, Luzern, Switzerland). The homogenized extracts were heated at 75°C for 30 minutes and left at room temperature overnight and this step was repeated twice. Samples were stored at 4°C before use. Three concentrations were employed in this cytotoxicity assay (0.5 mg/0.1 ml DW/mouse, 2.5 mg/0.1 ml DW/mouse and 5 mg/0.1 ml DW/mouse).

In vitro cytotoxicity assay

Cell lines

NIH3T3 cells are non-tumor cells and derived from healthy mouse fibroblast (Danihelova *et al*. 2013). The cells were maintained in Dulbecco's-modified Eagle Medium (DMEM-high glucose, Sigma-Aldrich, Louis, MO, USA) supplemented with 8% FCS, 20 U/ml penicillin, 50 μg/ml kanamycin in 5% CO_2 at 37°C for several days before starting the experiment.

Inhibitory rate viability of NIH3T3 cells

The determination of viability of NIH3T3 cells was conducted according to the manufacturer's protocol using Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Inc. Rochville, USA). Briefly, a volume of 99 μl of NIH3T3 cells at a density 5000 cells/well was added to each well of a 96 well plate, then the plate was pre-incubated for 24 hours in a humidified incubator (37^0C, 5% CO_2). A volume of one

μl of methanol extracts at four levels of concentration in DMSO (10 μg/ml, 100 μg/ml, 1 mg/ml and 10 mg/ml) was added into the plate and incubated for 48 hours. As a control, one μl of DMSO was added in a separate well with the same number of NIH3T3 cells. After incubated, 10 μl of CCK-8 solution were added into each well and incubated again for 4 hours. The absorbance was measured at 450 nm using a microplate reader (THERMO max, Molecular Devices, LLC., Sunnyvale, CA, USA).

The percent inhibition of viable cells was determined with equation:

$$\text{Inhibitory rate (\%)} = \left(1 - \frac{\text{Absorbance of treated well}}{\text{Absorbance of control well}}\right) \text{x } 100\ \%$$

***In vivo* toxicity assay**

Animals

Six weeks old BALB/cAJc female mice were purchased from CLEA Japan, Inc. (Osaka, Japan) and used at the age of 7 weeks, the mice were maintained for one week before starting experiments. This experiment was obtained approval by the Animal Care and Use Committee for Kochi University.

Toxicity assay

In this assay, the mice were separated into 4 groups. The first group consist of three mice and was administered with 0.1 ml distilled water (DW)/mouse, the second group consist of four mice and was administered with 5 mg plant extract/0.1 ml DW/mouse (250 mg/kg body weight), the third group consist of three mice and was administered with 2.5 mg plant extract/0.1 ml DW/mouse (125 mg/kg body weight), and the fourth group consist of three mice and was administered with 0.5 mg plant extract/0.1 ml DW/mouse (25 mg/ kg body weight). Based on *in vitro* toxicity assay, the methanol extract from leaves of *L. speciosa* had the highest of level of toxicity. Therefore, methanol extract of *L. speciosa* was orally administered to the mice using a polyethylene capillary sonde. The extract was administered every other day for one week (three times) and body weight of each mouse was measured on indicated days. In this assay, the average body weight of mice was 20 g and it was used to determine the doses for the administration of the plant extract.

Statistics

The SPSS 16 was used for statistical analysis of data. Data were analyzed using one-way ANOVA (Tukey-HSD post hoc test), where differences were considered significant at P value < 0.05.

4.3. Results and Discussion

Generally, the medicinal plants employed in traditional therapies are derived from the traditional knowledge that has accumulated for centuries and it may be accidentally discovered (Hanisa *et al*. 2014). Thus, precise biological activities of many medicinal plants are not yet scientifically proven. Active compounds in medicinal plants are derived from secondary metabolites. Extractive contents of plants function as a protector from a foreign organism such as pest insects. Therefore, many of plants extractives are toxic. Although many of extractives are toxic, there are dosages or concentrations that can be useful as a medicine. In fact, many medicines are derived from poisonous compounds of plants. Pharmaceutical companies have developed therapeutic drugs by synthesizing beneficial derivatives from poisonous compounds.

Remedies of medicinal plants contain a variety of compounds from secondary metabolites that have therapeutic properties (Hosseinzadeh *et al*. 2015). Many natural products derived from medicinal plants are proven to have biologically active compounds, leading to the new chemical innovation of pharmaceutical companies (Palombo 2009, Donno *et al* 2013). Currently, 25% of modern drugs produced by the pharmaceutical companies are derivatives of isolated compounds from medicinal plants (Ahmed *et al*. 2014).

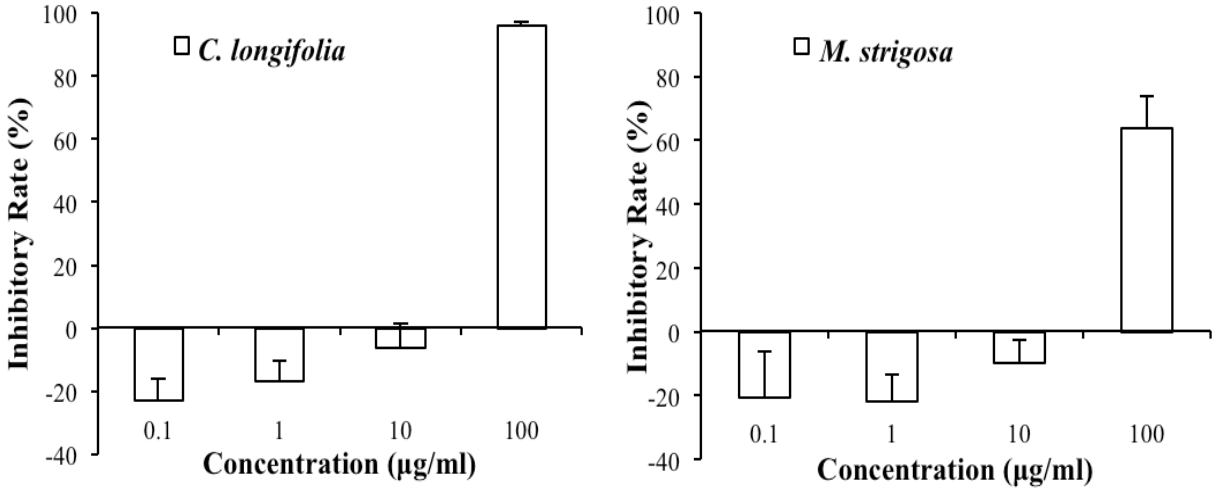
Nowadays, people have started to use remedies of medicinal plants already known by the indigenous people around the world to treat several diseases. Remedies of medicinal plants are believed to have lower side effects, comparatively safer in a long period of consumption. Therefore, it is necessary to scientifically estimate the safe dosage of remedies of medicinal plants through toxicity assays. Toxicity assay is an essential initial stage in examining the toxicity of both natural and synthetic drugs before starting more complex and advanced analyses (Lopez-Garcia *et al*. 2013). Toxicity assays were conducted in the following two ways: *in vitro* cytotoxicity assay

using a murine fibroblast cell line NIH3T3 and *in vivo* toxicity test using BALB/cAJc mice.

***In vitro* cytotoxicity of methanol extracts from leaves of medicinal plants on NIH3T3 cells**

In this study, the cytotoxicity of methanol extracts from leaves of five species of medicinal plants was investigated. These medicinal plants are *C. longifolia, M. strigosa, E. zwageri, S. dulcis*, and *L. speciosa* used by Dayak Uud Danum to cure several diseases in which inflammation is involved such as allergic, skin infection, fever, edema, and diarrhea. The leaves were selected for the analysis of anti-inflammatory activities, because Dayak Uud Danum used them to cure inflammatory diseases.

In drug discovery, *in vitro* cytotoxicity assay has become indispensable, because it is convenient and comprehensive to characterize the beneficial function of new chemical entities (Niles *et al*. 2009). NIH3T3 cells are derived from a normal murine fibroblast and commonly used to evaluate the toxicity of new biological materials by performing the viability test and apoptotic or necrotic assay (Tomankova *et al*. 2011).



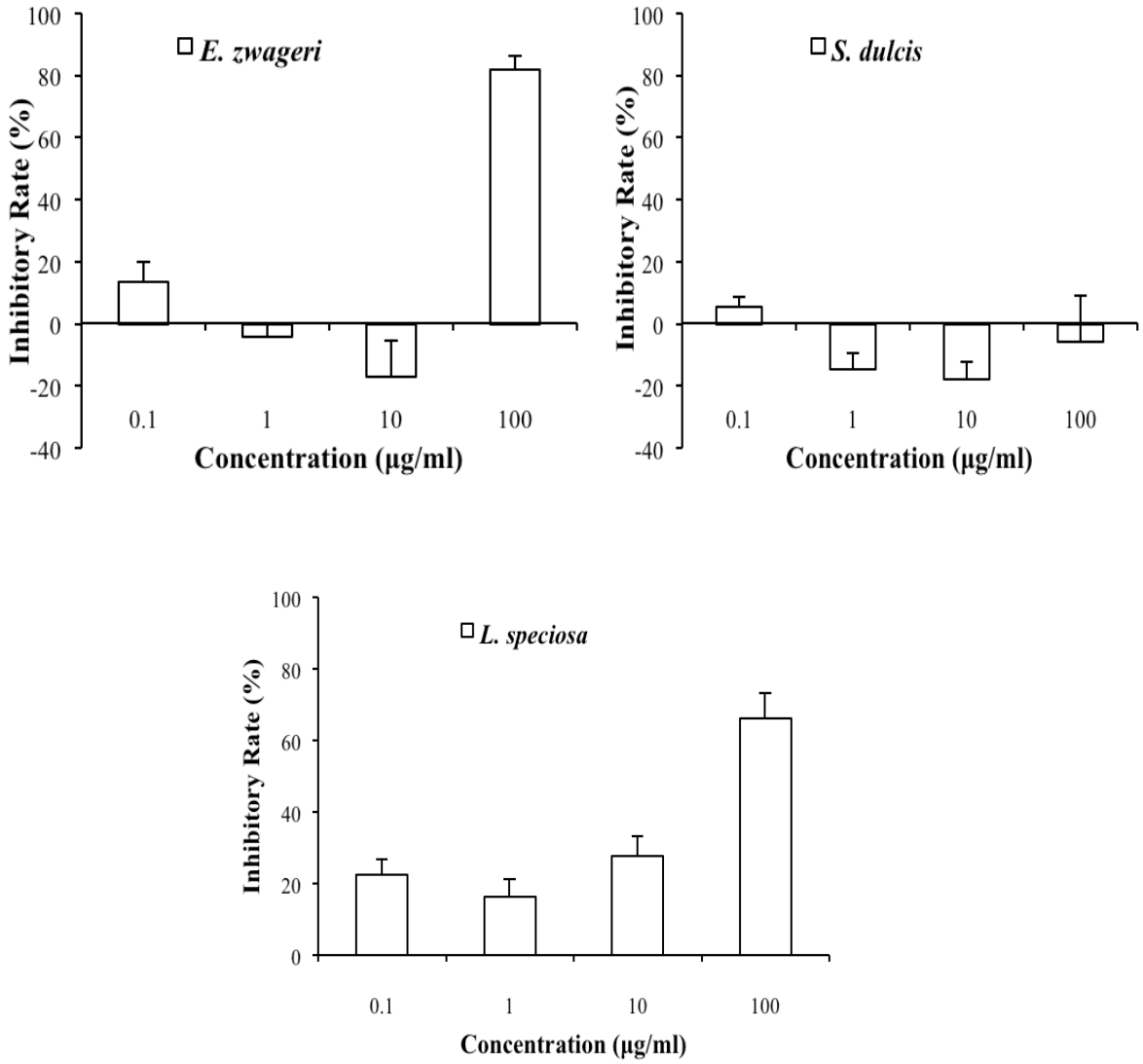


Fig. 4.1. Inhibitory rate of methanol extracts from five medicinal plants against NIH3T3. (A). *C. longifolia*, (B). *M. strigosa*, (C). *E. zwageri*, (D). *S. dulcis*, (E). *L. speciosa*. Results are shown as the mean ± SE (standard error) (n=4).

Result of *in vitro* cytotoxicity assay showed that all of the methanol extracts from leaves were toxic at a concentration of 100 μg/ml (**Fig. 4.1**). The highest inhibitory rate at this concentration was shown by *C. longifolia* (95.97%, **Fig. 4.1.A**). The most toxic extract was *L. speciosa* (**Fig. 4.1.E**). *L. speciosa* extract had higher inhibitory rates than other methanol extracts: the inhibitory rate of *L. speciosa* extract at a concentration of 0.1 μg/ml, 1 μg/ml, and 10 μg/ml were 22.5%, 16.43%, and 27.6, respectively. When

NIH3T3 cells were treated with methanol extracts from plant leaves at a concentration below 10 μg/ml, the inhibitory rates were negligible except *L. speciosa* extract.

Based on the inhibitory rate, cytotoxicity of a material can be classified into 4 levels: severe cytotoxicity (more than 60%), moderate (40-60%), mild (20-40%), and absence of cytotoxicity (less than 20%) (Kucekova *et al*. 2014). At a concentration 100 μg/ml, almost of all plants extracts were classified into the level of severe cytotoxicity except *S. dulcis* extract that was classified as absence of cytotoxicity. Due to the inhibitory rate at lower concentration (0.1 μg/ml, 1 μg/ml and 10 μg/ml), almost all plants extracts were classified as absence of cytotoxicity except *L. speciosa* extract.

Except *M. strigosa* leaves extract, other methanol extracts contain already identified bioactive compounds. Several chemical compounds such as alkaloids, phenolic compounds, flavonoids, saponins, steroids, sterols, condensed tannins, gallotannins, flavones, flavonols, flavanones, anthocyanidins, isoflavonoids, coumarins, isoquinolines, indoles, diterpenes, terpenoids, triterpenes, ellagic acid, and essential oils are found in *C. longifolia*, *E. zwageri*, *S. dulcis* and *L. speciosa*. Although the bioactive compound was not yet identified in the extract from leaves of *M. strigosa*, some chemical compounds such as oils, fats, waxes, alkaloids, flavones, polyphenols, tannins, saponins, glycosides aglycones are obtained from the methanol extract (Filho 2006, Houghton and Raman 1998). The methanol extract of *Clerodendrum inerme* contains cardiac glycosides, anthraquinones, phenolics, flavonoids, saponins, tannins, diterpenes, triterpenes, sterols, and steroids. It is reported that the methanol extract of *Clerodendrum inerme* did not show cytotoxic effect on NIH3T3 cells in terms of the inhibition of cell growth (IC_{50}) > 2.5 mg/ml (Al-Snafi 2016, Uddin *et al*. 2011).

Based on this result, a concentration of 1 μg/ml of methanol extracts from five medicinal plants of Dayak Uud Danum was used in the damage-prevention model of human colon epithelial cells. Since methanol extract *of L. speciosa* showed the highest inhibitory rate on NIH3T3 cells at a lower concentration, this extract was chosen to administer BALB/cAJc mice in the *in vivo* toxicity assay at 5 mg/0.1 DW/mouse or lower concentrations.

***In vivo* toxicity of methanol extract from leaves of *L. speciosa* in BALB/cAJc mice**

In order to examine the toxic effects of a material against a complex organism, an animal model is usually used. In this assay, several concentrations of plant extracts were used to monitor the body weight and the behavior of animals (Murbach *et al*. 2014). Based on the *in vivo* cytotoxicity assay, the safe dosage of the methanol extracts from medicinal plants will be obtained to administer to mice.

Since the methanol extract of *L. speciosa* showed the highest toxicity at lower concentrations in the *in vitro* cytotoxicity test (**Fig. 4.1**), this extract was chosen to administer to mice to examine the cytotoxicity of the plant extract. Therefore, in this present study, following are three doses employed: 5 mg/0.1 ml DW/mouse, 2.5 mg/0.1 DW/mouse, and 0.5 mg/0.1 ml DW/mouse. The methanol extract from leaves of *L. speciosa* was administered to mice on day 0, 2, and 4. BALB/cAJc was used as a mouse strain, because this strain will be used in the measurement of the DTH response. BALB/cAJc is a mouse strain that is commonly used in the immuno-regulatory assay (Ishii *et al*. 2015, Reis *et al*. 2013, Wang *et al*. 2016)

There was no significant difference in body weight among groups administered with different doses of the methanol extract from leaves of *L. speciosa* (**Fig. 4.2**).

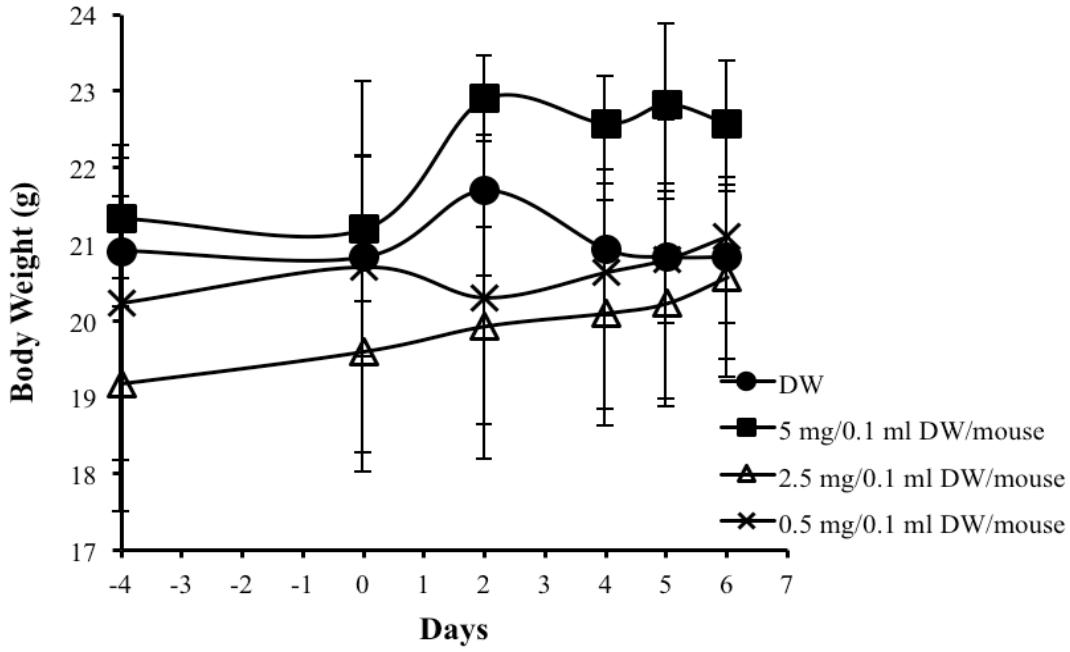


Fig. 4.2. Effects of the administration of *L. speciosa* methanol extract on the body weight of BALB/cAJc mice. *L. speciosa* methanol extract was administered on day 0, 2, and 4. The results are shown as the mean ± SE (n=3). No significant differences were found among groups administered with the extract at any concentration (Tukey-HSD post hoc test, one-way ANOVA, $P < 0.05$).

Although the methanol extract of *L. speciosa* was the most toxic one in the *in vitro* cytotoxicity assay, oral administration of this extract to BALB/cAJc mice did not significantly reduce the mice body weight. During six days observation, the mice group administered with the methanol extract of *L. speciosa* at the dose of 2.5 mg/0.1 ml DW/mouse and 0.5 mg/0.1 ml DW/mouse showed a tendency of the increase in body weight. The body weight of mice in a group administered with the highest dose (5 mg/0.1 ml DW/mouse) and those administered with distilled water (DW) were almost constant during the last three days.

Mergia *et al*. (2014) reported that methanol extracts from leaves of *Clutia abyssnica* contain alkaloids, anthraquinones, flavonoids, glycosides, steroids, phenolics, tannins, and terpenes. It is reported that the lethal dosage (LD_{50}) of this extract was

above 2000 mg/kg body weight and no evidence of acute toxicity was shown in healthy Swiss albino mice.

In the *in vivo* toxicity assay, the measurement of the body weight of the animals may partially indicate whether a test sample contains harmful chemical compounds. If the body weight of animals decreases rapidly, it indicates that the extract may contain harmful chemical compounds. The harmful chemical compounds damage tissues and this may lead to the impairment of the organ to decrease the body weight (Manaharan *et al*. 2014). Beside the body weight, the observation of general aspects of animal behavior such as appetite and vitality may indicate the presence of toxic compounds. During this experiment, all the mice were healthy without showing any abnormal behaviors. No adverse effects including vomiting and diarrhea were observed during the experiment. Based on the result of this present study, it is suggested that the methanol extract of *L. speciosa* is not harmful to mice at least by administrating this dose.

It is reported that the methanol extract of *L. speciosa* contain triterpenes, steroids (triterpenoids), tannins, glycosides, flavones, and ellagic acid (Chan *et al.* 2014, Nasrin *et al*. 2014, Priya *et al*. 2008). Regarding the result of this study, it is suggested that the presence of these chemical compounds does not show acute toxicity in mice at a dose of 5 mg/0.1 ml DW/mouse. Therefore in the DTH assay, BALB/cAJc mice will be administered with the methanol extract of *L. speciosa* at a dose of 5 mg/0.1 ml DW/mouse.

4.4. Conclusion

The cytotoxicity of methanol extracts from the leaves of five medicinal plants used by Dayak Uud Danum was investigated in *in vitro* assay against normal mouse fibroblast cells (NIH3T3). As result, the methanol extracts from all the plants except *S. dulcis* were toxic at a concentration of 100 μg/ml, while at lower concentrations (0.1 μg/ml, 1 μg/ml, 10 μg/ml) only *L. speciosa* extract was toxic. Therefore, a concentration of 1 μg/ml will be used as a final concentration in the damage-prevention model of human colon epithelial cells.

In the *in vivo* toxicity assay, the administration of methanol extract of *L. speciosa* did not show any significant difference in the body weight of mice at the following

doses: 5 mg/0.1 ml DW/mouse, 2.5/0.1 ml DW/mouse, and 0.5 mg/0.1 ml DW/mouse. For the anti-inflammatory effects of methanol extracts of medicinal plants on DTH response, a dose of 5 mg/0.1 ml DW/mouse will be used as a safe starting dose.

Recapitulation showing the example of data calculation of cytotoxicity assay of methanol extracts from leaves of five medicinal plants using NIH3T3

Table 4.1. Average of absorbance and cytotoxicity inhibitory rate effects of plant extract on NIH3T3 cells (100 µg/ml)

Sample	Average of absorbance ± SE (n=4)	Inhibition ± SE (%) (n=4)
Control	1.0130±0.1091	
C. longifolia	0.0407±0.0109	95.9773 ± 1.0850
M. strigosa	0.3670±0.0998	63.7216 ± 9.8626
Control	1.2550±0.0199	
E. zwageri	0.0220±0.0497	82.044 ± 4.0220
S. dulcis	1.3068±0.1811	5.8097 ±14.6616
Control	1.1500±0.1497	
L. speciosa	0.3875±0.0811	66.3043 ± 7.0555

Example: The calculation of cytotoxicity inhibitory rate (100 µg/ml)

The inhibitory activity of methanol extract from wood bark of *L. speciosa*

$$\text{Inhibitory rate (\%)} = \left(1 - \frac{\text{Absorbance of treated well}}{\text{Absorbance of control well}}\right) \text{x } 100\ \%$$

$$\text{Inhibitory rate (\%)} = \left(1 - \frac{0.3875}{1.1500}\right) \text{x } 100\ \%$$

$$\text{Inhibitory rate (\%)} = \left(1 - 0.3369\right) \text{x } 100\ \%$$

$$\text{Inhibitory rate (\%)} = 66.3\%$$

Chapter 5

Anti-inflammatory effects of five medicinal plants used by Dayak Uud Danum on the delayed-type hypersensitivity

5.1. Introduction

Inflammation is one of process of natural protection to maintain the homeostasis of a living organism against tissue injury, infection, and irritation (Mubashir *et al*. 2013). At initial level of inflammation, plasma proteins, interstitial fluid, and leukocytes migrate into perturbed tissues when tissues are damaged. The migration of these blood-derived products to the site of inflammation induces the vasodilation, resulting in the increases of vascular permeability and the blood flow (Ashley *et al*. 2012). Inflammation occurs in response to infection, antigen challenge, or tissue injury. Although inflammation is a natural host defense mechanism, prolonged and exaggerated inflammation may lead to the dysfunction of organs. Therefore, inflammation must be regulated properly.

Delayed-type hypersensitivity (DTH) called Type IV allergy is one of the diseases related to inflammation and usually the symptoms of DTH relate to contact dermatitis. The skin inflammation in DTH caused by continuous skin exposure to allergen such as haptens is mediated by T-cells and macrophages. Haptens are low molecular weight chemicals that bind to body epidermal proteins eliciting immune responses. In animal models, the DTH reaction can be measured through ear swelling (Erkes and Selvan 2009). In DTH reactions, besides T cells and macrophages, several pro-inflammatory cytokines are involved (Sampson 2000).

Cytokines are the proteins released by cells at the site of inflammation to regulate the inflammatory reactions (Zhang and An 2007). Some cytokines that have a role to up-regulate the inflammation are called pro-inflammatory cytokines, while others that have a role to down-regulate the inflammation are called anti-inflammatory cytokines (Dinarello 2000). So, the regulation of the pro-inflammatory cytokines is one way to control an inflammation.

Eosinophils are leukocytes that appear in various inflammatory diseases and have important roles in the regulation of inflammation. Increasing number of eosinophils in the blood or in the tissues characterizes the symptoms of allergic or parasitic disorder (Rogerio *et al*. 2010, Texeira 2001). Eosinophils release cytotoxic granular proteins, resulting in the tissue damages (Rankin *et al*. 2000). In DTH, the challenge to paint haptens on ears elicits the increment of ear thickness, resulting in the proliferation and migration of eosinophils to the site of inflammation.

It is well known that many indigenous people around the world use medicinal plants as anti-inflammatory medicine to treat several diseases. In Dayak Uud Danum community, there are several plants that have been used to treat inflammation diseases (**Fig. 1.1**). These five medicinal plants assumed to have bioactive compounds that can suppress the inflammation.

In this study, anti-inflammatory activities of these plants were analyzed through DTH in response to 2,4,6-trinitro-1-chlorobenzene using BALB/cAJc mice by measuring the suppression of ear swelling after the challenge with the antigen used for immunization. Anti-inflammatory activities of plant extracts were evaluated by measuring the ear thickness and numbers of eosinophils migrated to the site of inflammation using ear sections. Through this assay, the local knowledge of Dayak Uud Danum in treating inflammatory diseases will be scientifically proved. The result showed that all plant extracts of Dayak Uud Danum can suppress the DTH responses and extracts of all plants except that of Bungur (*L. speciosa*) were able to inhibit the migration of eosinophils to the site of inflammation.

5.2. Materials and Methods

Animals

Six weeks old BALB/cAJc female mice were purchased from CLEA Japan, Inc. (Osaka, Japan) and used at the age of 8 weeks. All experiments were performed after being approved by the Animal Care and Use Committee for Kochi University.

Chemicals

Picryl chloride (PCl; 2,4,6-trinitro-1-chlorobenzene) was purchased from Nacalai Tesque (Kyoto, Japan) and used as a hapten. Cyclophosphamide (CY) was purchased

from Shionogi Pharmaceutical Co. (Osaka, Japan). Hydrocortisone was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of plant extracts

Methanol extracts from leaves of 5 medicinal plants were prepared as described in a previous Chapter (**Chapter 3**). As much as 500 mg of methanol extracts of each plant (dry weight) grinded in a mortar and suspended in 10 ml of distilled water (Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan) at a concentration 50 mg/ml. Then, these samples were homogenized with a Polytron (Kinematica, Luzern, Switzerland). The homogenized extracts were heated at 75°C for 30 minutes and left at room temperature overnight and this step was repeated twice. Samples were stored at 4°C before use. The dose of each sample (5 mg/0.1 ml distilled water (DW)/mouse or 250 mg/kg body weight) was determined by *in vivo* model of toxicity assay of the plant extract as described in a previous chapter (**Chapter 4**).

Immunization and Challenge

Two days before being immunized with PCl, mice were injected with cyclophosphamide subcutaneously (150 mg/kg in distilled water) at the nape of the neck. This injection was to remove proliferating immunosuppressive cells (Satoh *et al*. 1997). Abdominal coat hairs were removed and mice were immunized by painting abdominal skin with 0.05 ml of 7% PCl in ethanol: acetone (3:1). This process of immunization was repeated on the next day. Five mg of each plant extract suspended in a volume of 0.1 ml DW was orally administered to each mouse by using a polyethylene capillary sonde every other day for two weeks after immunization. In one group, each mouse was orally administered with 0.5 mg hydrocortisone suspended in a volume of 0.1 ml DW every other day for two weeks after immunization. Mice of a positive control group (immunized and challenged) were administered with the same volume of DW. Two weeks after administering plant extracts, mice were challenged by painting 0.02 ml of 1% of PCl in acetone: olive oil (1:4) on each ear lobe. Ear thickness of each mouse was measured by using a dial thickness gauge (Peacock G-1M, Ozaki Mfg. Co.

Ltd., Tokyo) before and after challenge. The suppression percentage of DTH reaction was determined with equation:

$$\%\ \text{Supression} = 1 - \left(\frac{\text{the increment of ear thickness of each group}}{\text{the increment of ear thickness of a positive control group}} \right) \text{x}100$$

Tissue Eosinophil Counts

Mice were sacrificed 48 hours after the challenge. The ear lobe of each mouse was removed and fixed with 8% paraformaldehyde in a 0.2 M Phosphate buffer with pH 7.2, and embedded in paraffin. Next, the sections of ears were stained with a hematoxylin/eosin solution. At the level of magnification of 400x the number of eosinophils was counted and expressed as number of cells in the observed area. The number of eosinophils was counted in 10 squares of 100 μm x 50 μm of ear sections from each group.

Statistics

The SPSS 16 was used for statistical analysis of data. One-way ANOVA (Tukey-HSD post hoc test) was used to evaluate the statistical significance. A P value < 0.05 was considered statistically significant.

5.3. Results and Discussion

In this study, anti-inflammatory activities of five species of medicinal plants used by Dayak Uud Danum to treat several diseases in which inflammation is involved were analyzed (**Fig 1.1)**. The examination of anti-inflammatory effects was conducted, especially on DTH reaction. Plant extracts were orally administered every other day for two weeks after immunization till one day before challenge and the ear thickness was measured at 4, 24, and 48 hours after challenge.

In this assay, PCl, that is a hapten usually used in *in vivo* model of T cell-mediated immunity assays, was used to induce DTH. In order to enhance the elicitation response to the challenge with PCl, cyclophosphamide was administered to mice before immunization (Satoh *et al*. 1997). By painting the PCl solution on the abdomen, the hapten molecules immediately bind to keratinocytes, Langerhans cells, and dermal

dendritic cells and induce these cells to release several pro-inflammatory cytokines such as IL-1β, IL-18, IL-6, IL-12, TNF-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF), resulting in the presentation of the PCl-derived antigen to naive T cells (Erkes and Selvan 2009, Grabbe and Schwarz 1998). Furthermore, another cytokines such as IFN-γ and IL-17 are also released from activated T-lymphocytes (Ishii *et al*. 2010, Tominaga *et al*. 2010, 2011, Yoshimoto *et al*. 2000).

Epidermal immunization with a hapten like PCl causes the activation of epidermal Langerhans cells and dendritic cells, leading to the migration of these cells to regional lymph nodes (Ruckert *et al*. 2002). After mice were immunized with a hapten, the swelling of ears is induced when ears are challenged with same antigen (Erkes and selvan 2009).

5.3.1. DTH response

All mice except a negative control and normal mice exhibited inflammatory symptoms after being challenged with PCl that were recognized as ear thickness and active behaviors of mice such as scratching ears. At 24 hours (h) after challenge, the increment of ear thickness of mice in a positive control was 148 μm in average. Mice administered with *C. longifolia* and *M. strigosa* extracts showed lower increment of ear thickness (78 μm and 86 μm, respectively) than those with hydrocortisone (96 μm), followed by *S. dulcis*, *E. zwageri,* and *L. speciosa* (97 μm, 98 μm, and 122 μm, respectively).

At 48 h after challenge, the increment of ear thickness of mice in a positive control was 129.5 μm in average. Interestingly, *S. dulcis* extract strongly suppressed the increment of ear thickness (44.5 μm) followed by extracts from *M. strigosa*, *E. zwageri*, *C. longifolia* and *L. speciosa* (50 μm, 56.5 μm, 57.5 μm, and 65 μm, respectively). The increment of ear thickness of mice administered with hydrocortisone was 66 μm. The ear thickness of mice at 0 h, 4 h, 24 h, and 48 h after challenge are shown in **Fig 5.1. A**.

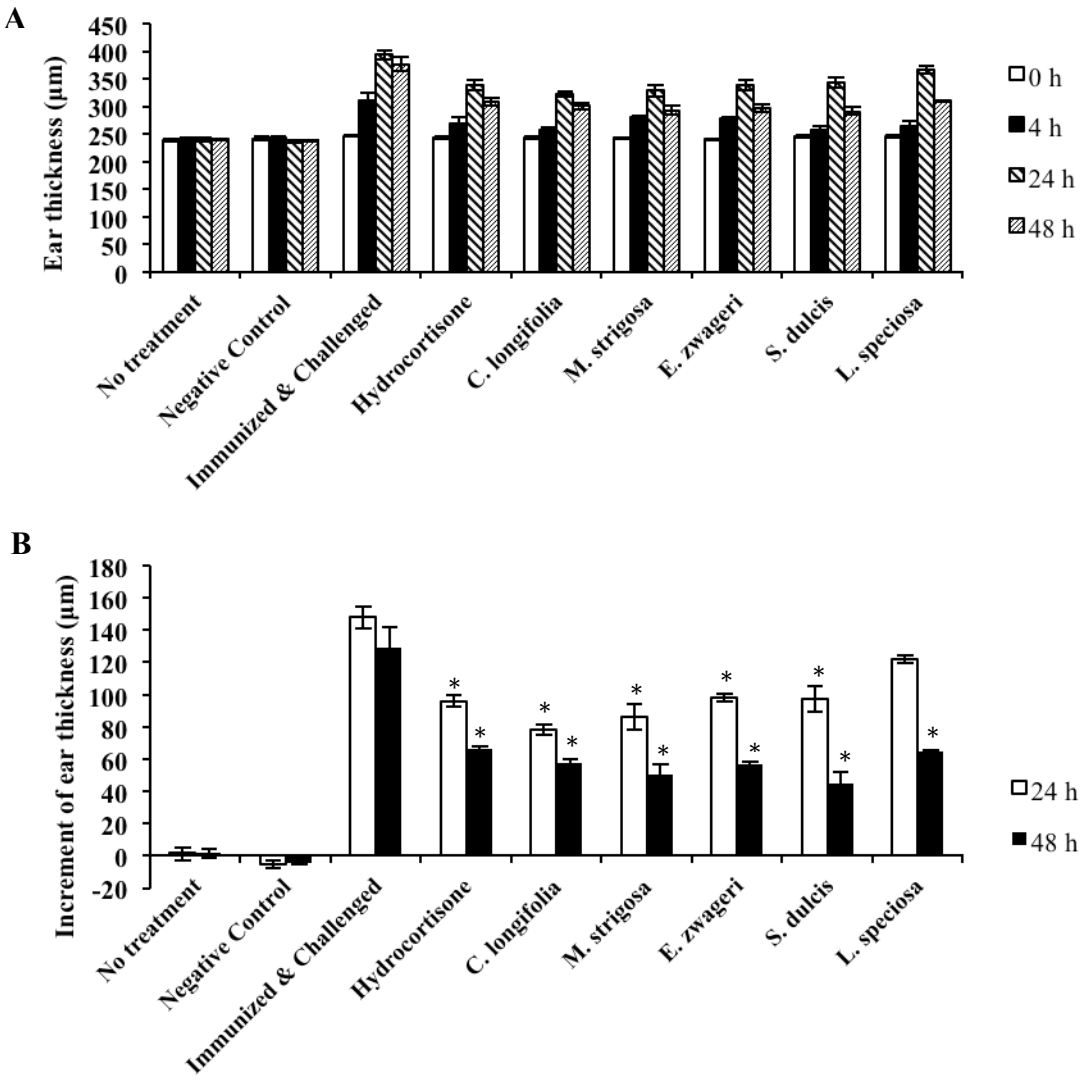


Fig. 5.1. Effects of methanol extracts from five medicinal plants of Uud Danum on DTH response. DTH response was elicited against PCl as described in Materials and Methods. Hydrocortisone was administered orally at a dose of 0.5 mg/0.1ml DW/mouse. Plants extracts were administered orally at a dose of 5 mg/0.1ml DW/mouse. All reagents were administered every other day for two weeks after immunization. Increment of ear thickness was measured before (0 h) and after challenge (4, 24 and 48 h). Results are shown as the mean ± SE (n=5). **Panel A**: Ear thickness of each group after the challenge with PCl. **Panel B**: Increment of ear thickness was shown by subtracting the ear thickness before challenge from the ear thickness of each mouse. *Asterisks indicate that there are significant differences between positive

control group and other groups (Tukey-HSD post hoc test, one-way ANOVA, $P < 0.01$).

As shown in **Fig. 5.1.A**, normal mice (no treatment) and negative control mice (immunized but not challenged) did not show any increment of ear thickness, but in the group of positive control mice (immunized and challenged), the ear thickness gradually increased after challenge. The increment of ear thickness was highest at 24 h after challenge and the ear thickness gradually decreased.

At 24 h after challenge, only one group of mice treated with *L. speciosa* did not show significant difference compared with a positive control group. But at 48 h after challenge, all groups of plant extracts showed significant suppression compared with a positive control group.

As shown in **Fig. 5.1.B**, all methanol extracts had the anti-inflammatory activities to suppress the DTH response efficiently at 48 h after challenge. At 24 h after challenge, *C. longifolia* extract showed the highest suppression the DTH response (47.3%) followed by extracts from *M. strigosa, E. zwageri*, and *S. dulcis* with suppression of 41.9%, 33.8% and 34.5%, respectively. At 48 h after challenge, *S. dulcis* extract showed the highest suppression (65.6%) followed by extracts from *M. strigosa*, *E. zwageri* and *C. longifolia* with suppression of 61.4%, 56.4%, and 55.6%, respectively. *L. speciosa* showed the lowest suppression on 24 h after challenge (17.6%), but at 48 h after challenge it finally suppressed about 50% of the increment of ear thickness (49.8%) of a positive control. At 24 h after challenge only one group of mice treated with *L. speciosa* did not show significant difference with a positive control group. But at 48 h after challenge, all groups of plant extracts showed significant difference with a positive control group and higher level of suppression than that of hydrocortisone. The levels of suppression of ear swelling at 24 and 48 hours after challenge in the group administered with hydrocortisone were 35.1% and 49.0%, respectively (**Fig. 5.1.B**).

IFN-γ is engaged not only in the DTH but also in the host defense by killing tumors and intracellular microorganisms. IFN-γ works not only as a potent macrophage activator, but also as an immuno-regulatory cytokine. IL-17 is also engaged in the DTH, the host defense against bacterial infection, and the angiogenesis. In the DTH against

PCl, both anti-IFN-γ and anti-IL-17 antibodies partially inhibit the ear swelling (Tominaga *et al*. 2010). Not only IFN-γ, but also IL-12 is a major pro-inflammatory cytokine in DTH response (Grabbe and Schwarz 1998).

T-helper 1 cells (TH1) are the major inducer of DTH and secrete cytokines such as IFN-γ and IL-12, resulting in the activation of macrophage followed by the activation of natural killer (NK) and cytotoxic T-cells. This is the host defense mechanism to protect against intracellular pathogens (Farhadi *et al*. 2014). Furthermore, it is reported that DTH reaction is suppressed by injecting anti-TNF-α antibodies (Higashi *et al*. 1994).

So, it is suggested that the down-regulation of the level of these pro-inflammatory cytokines (IFN-γ, IL-12, IL-17, and TNF-α) can be a target of these plant extracts to suppress the DTH response. These results suggest that medicinal plants used by Dayak Uud Danum can be applied to reduce the inflammatory response in which T-lymphocytes and macrophages are involved.

Currently, it is well known that secondary metabolites from natural products such as medicinal plants have a broad spectrum of biological activities, and the anti-inflammatory activity is one of them. Many reports have been published to prove that chemical compounds from medicinal plants have immunosuppressive effects (Amirghofran 2012) and medicinal plants have been used to treat diseases caused by the malfunction of the immune system. Flavonoid group, one of the secondary metabolites, has anti-inflammatory activity and is used to develop a new type anti-inflammatory agent (Kim *et al*. 1993).

During inflammation, there are several potent mediators such as histamine, tryptase, nitric oxide, and cytokines. One way to regulate inflammation is to control the production of inflammatory mediators.

Among these medicinal plants used in this study, there are various bioactive compounds that might contribute to the suppression of ear thickness in this model of DTH response. It is also conceivable that secondary metabolites of medicinal plants may have anti-inflammatory activities and affect to several pathways of various phases of inflammation (Dawid 2013). Presence of potent bioactive compounds such as

phenolic and flavonoid compounds may work as anti-inflammatory reagents (Oskoueian *et al*. 2012).

It is reported that anti-inflammatory activity of plant phenolic compounds may depend on oxygen or nitrogen bond (Othman *et al*. 2015). Most of modern medicines contain polyphenol compounds derived from plants and are used as antioxidant, anti-inflammatory, sedating, wound-healing, antimicrobial and antiviral drugs (Wink 2015).

Medicinal plant remedies have a different mode of action from modern drugs in treating diseases. Bioactive compounds of medicinal plants have a wide spectrum of pharmacologic activities compared with many synthetic drugs. Another beneficial effects of medicinal plants are that they have less side effects and higher level of tolerance (Chikezie *et al*. 2015b). Modern drugs contain a single active compound that works on a specific pathway, while medicinal plant remedies have several molecules working together on several pathways (Kumar *et al*. 2013). Therefore, it is suggested that medicinal plant remedies have less and weaker side effects than modern drugs. In the Dayak Uud Danum community, although the commercial drugs are available, they still use their traditional medicinal plants to treat some diseases related to inflammation.

5.3.2. Eosinophils

Eosinophils are multifunctional granulocytic leukocytes that can be identified with acidic eosin dye. The increasing number of eosinophils in the tissues is a sign of various diseases such as helminth infection, allergy, asthma, and eosinophilic gastrointestinal disorders (Stone *et al*. 2010, Texeira 2001). During inflammation, pro-inflammatory mediators released by eosinophils induce tissue damage and physiologic abnormalities (Sa-Nunes *et al*. 2006). Therefore, the down-regulation of eosinophils is one of the important criteria to find new therapeutic drugs for inflammatory disease.

At 48 h after challenge, a number of eosinophils were measured in the ear section and compared among groups of experiments, including a non-treated group, a negative control group, a positive control group, the group administered with hydrocortisone, and groups administered with extracts of five medicinal plants used by Dayak people. In 10 fields (100 μm x 50 μm) of ear sections from each group, a number of easionophils were measured and the average number of eosinophils was determined (**Fig. 5.2.B, Fig.**

5.3.). The number of eosinophils in ear sections from a negative control group was slightly larger than those from non-treated group. The increase of eosinophils in a negative control group is probably caused by the immunization with PCl.

Number of eosinophils of a positive control group (6.0) was more than ten-fold compared with that of a negative control group. The lowest number of eosinophils was found in a group treated with *S. dulcis* extract (1.8) followed by groups treated with *M. strigosa*, *E. zwageri,* and *C. longifolia* extract (2.2, 2.9, and 3, respectively). Interestingly, the group of mice administered with hydrocortisone had slightly higher level of eosinophils (3.6) than those treated with plant extracts from *S. dulcis, M. strigosa*, *E. zwageri,* and *C. longifolia* except the mice treated with *L. speciosa* (5.6) (**Fig. 5.3**).

In groups with higher level of increment of ear thickness, more eosinophils were found, suggesting the importance of the migration of eosinophils to the site of inflammation in this DTH response (Tominaga *et al*. 2011).

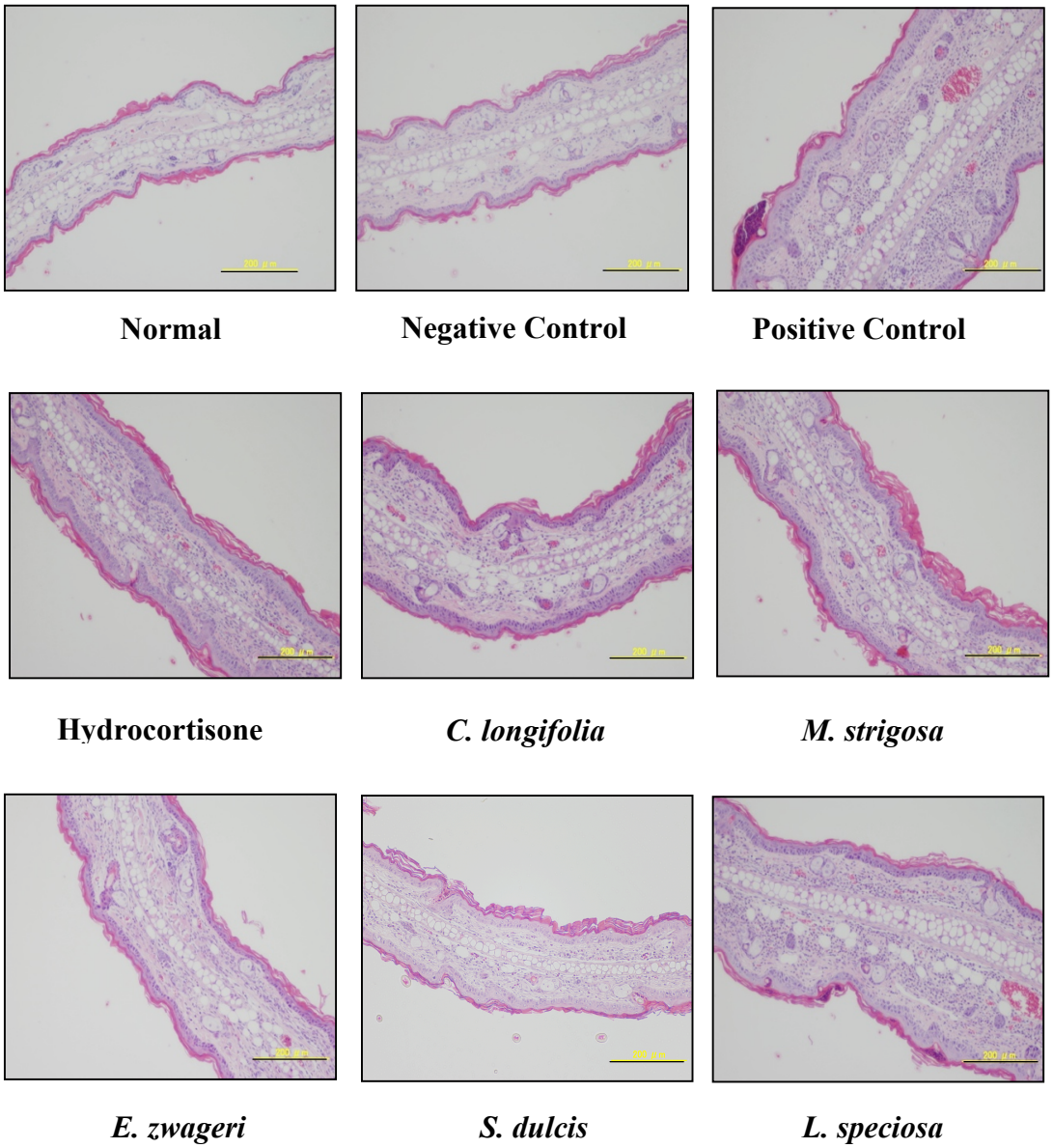
In normal condition, only small numbers of eosinophils are found in blood stream or in tissue. Eosinophils contribute to the innate immune system and are able to recognize and process antigens. Eosinophils are also reported to have features of NK cells and macrophages (Hamaguchi-Tsuru *et al*. 2004).

Eosinophils can be activated due to the response of a various stimuli such as the infection with pathogens and become a marker of several diseases and inflammation (Metcalfe *et al*. 2016). The activated eosinophils induce the secretion of granules from eosinophils. Due to the presence of toxic arginine-rich proteins in the granules, the eosinophils are able to kill microbes, parasites, and tumor cells and cause damages to tissues. These cytotoxic granular proteins of eosinophils are the major basic protein (MBP), eosinophil peroxidase (EPO), the eosinophil derived neurotoxin (EDN), and eosinophil cationic protein (ECP) (Bystrom *et al*. 2011, Long *et al*. 2016).

Eosinophil cationic protein (ECP) is a potent cytotoxic protein with anti-bacterial and anti-viral properties. Higher level of ECP in host tissues characterizes the eosinophilic inflammation diseases such asthma and allergic reaction (Bystrom *et al*. 2011, Munthe-Kaas *et al*. 2007). Regarding the function of eosinophils in host protection, in addition to cytotoxic granular proteins, other inflammatory mediators

such as cytokines, chemokines, lipid mediators, and superoxide derived from eosinophils must also be considered (Long *et al*. 2016).

A



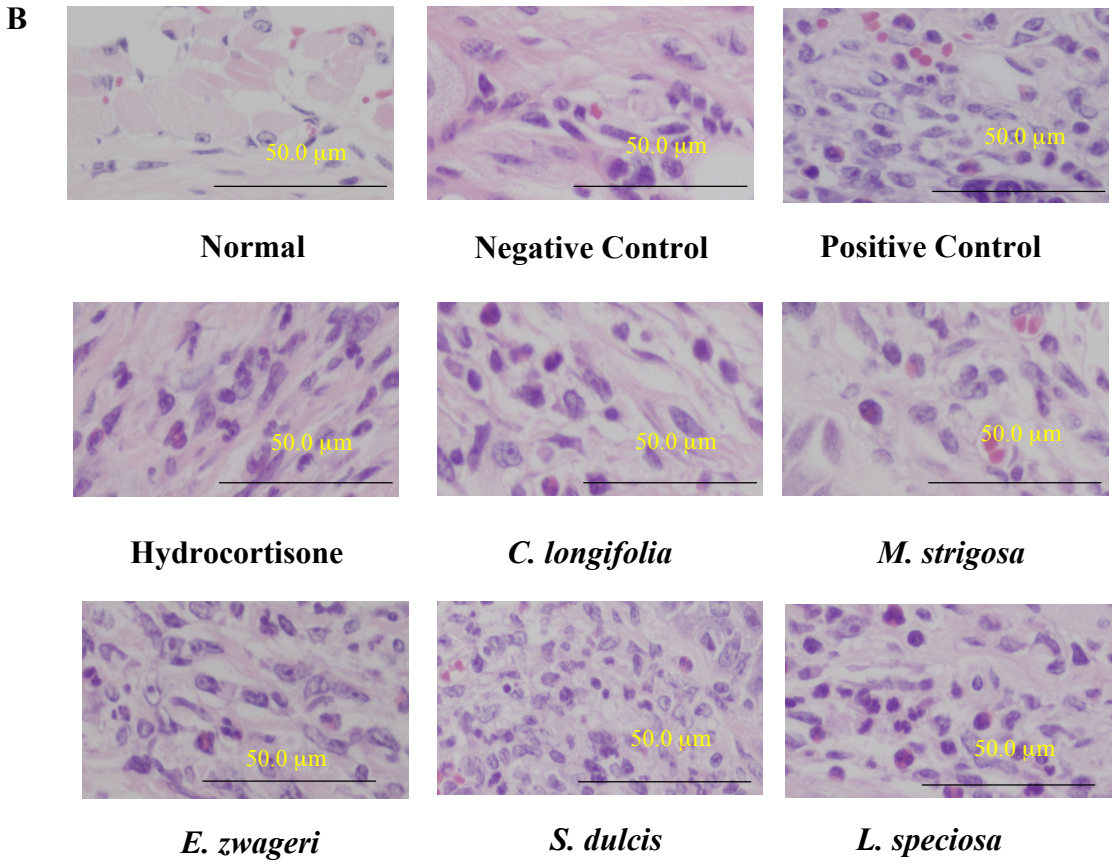


Fig. 5.2. Effects of plant extracts on the DTH response were shown as the ear swelling and the suppression of eosinophils migrated to the site of inflammation. DTH response was elicited against PCl as described in Materials and Methods. Forty-eight hours after challenge, ears were removed and fixed with 8% paraformaldehyde in a 0.2 M Phosphate buffer with pH 7.2, and embedded in paraffin, then stained with hematoxylin and eosin. **Panel A**: Mice ear sections with 100 x magnification. Bars, 200 µm. **Panel B**: Mice ear sections with 400 x magnification. Bars, 50 µm. Leukocytes with red cytoplasm are eosinophils.

Eosinophils are derived from $CD34^+$ hematopoietic progenitor cells. While eosinophils migrate from bone marrow to the peripheral blood, differentiation and proliferation of eosinophil precursors are regulated by cytokines such as IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-5. Among these

cytokines, IL-5 is a most potent and selective proliferation factor of eosinophils (Satoh *et al*.1997, Rogerio *et al*. 2010, Rosenberg *et al*. 2013).

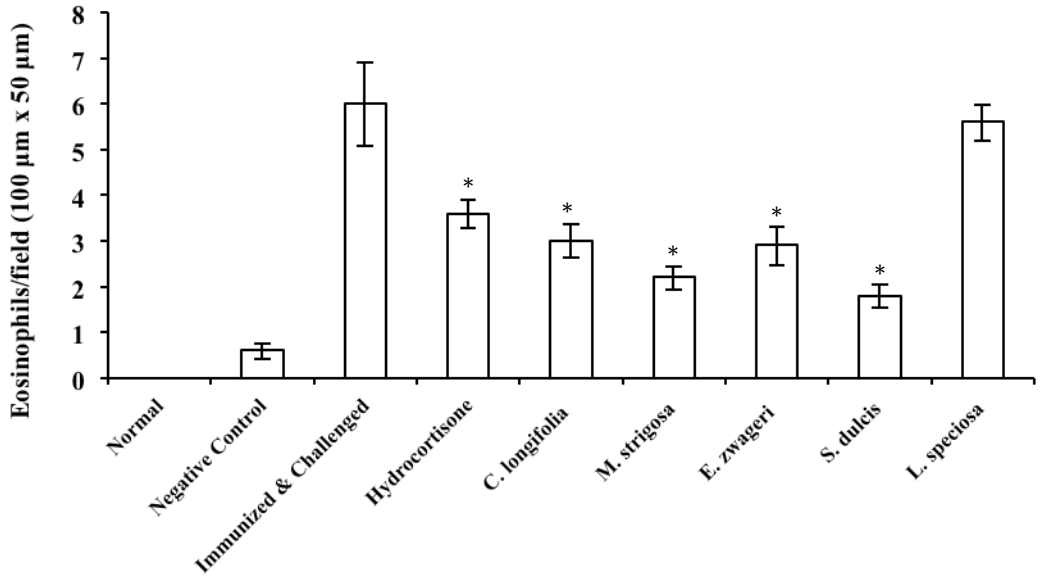


Fig. 5.3. Number of eosinophils migrated to the site of inflammation. Eosinophils of each group were counted at the magnification level of 400 x. Results are shown as the average number of eosinophils in 10 squares of 100 µm x 50 µm of ear sections ± SE (n=10). *Asterisks indicate that there are significant differences between positive control and other groups (Hydrocortisone, *C. longifolia*, *M. strigosa*, *E. zwageri,* and *S. dulcis*) (Tukey HSD, $P< 0.05$).

In an allergic inflammatory response, several eotaxins such as CCL11 (eotaxin), CCL24 (eotaxin-2), and CCL26 (eotaxin-3) are also involved in the migration of eosinophils. The decreased level of IL-4, IL-5, CCL11, and leukotriene B_4 (LTB_4) may lead to the reduction of eosinophils at the site of inflammation (Fortunato *et al*. 2012, Menzies-Gow *et al*. 2002).

Interrupting cell adherence or chemotaxis of eosinophils by the administration of hydrocortisone is an alternative way to down-regulate the proliferation and the migration of eosinophils (Altman *et al*. 1981). Teixeira *et al.* (2001) reported that the expression of adhesion molecules (CAMs) on endothelial cells and leucocytes also have a role in eosinophil migration, while the activation of eosinophils are induced by the

presence of chemokines and LTB_4. In DTH, the challenge by painting PCl on ears elicits the increment of ear thickness, resulting in the proliferation and migration of eosinophils to the site of inflammation.

Most of the groups administered with extracts from medicinal plants had lower number of eosinophils at the site of inflammation except *L. speciosa* group that had the number of eosinophils equivalent to that of a positive control. At 48 h after challenge, *L. speciosa,* however, still suppressed ear thickness. It is clear that extracts from *S. dulcis*, *M. strigosa*, *E. zwageri*, and *C. longifolia* have anti-eosinophilia activity in their methanol extracts of leaves and may have the ability to down-regulate the inflammatory cytokines such as IL-5 or eotaxin, resulting in the inhibition of the proliferation of eosinophils in bone marrow and spleen and the inhibition of the migration of eosinophils to the site of inflammation. Or, these plant extracts may contain molecules that suppress the migration of eosinophils.

Although the extract from *L. speciosa* suppressed the ear thickness moderately, it did not inhibit the migration of eosinophils, suggesting that *L. speciosa* extract has an anti-inflammatory compounds which does not inhibit the migration of eosinophils to the site of inflammation. In other words, *L. speciosa* plant extract may have suppressed the inflammation by inhibiting the process other than the one involved in eosinophilia. It may be a good idea to administer the extract of *L. speciosa* together with that of *M. strigosa* or *S. dulcis* that has a potent activity to suppress the migration of eosinophils to the site of inflammation.

Various secondary plant metabolites extracted from barks, stems, fruits, leaves, and roots such as flavonoids, alkaloids, polyphenols, quionones, sesquiterpenes, terpenoids, and diterpenoids are scientifically proved to reduce the eosinophil migration to the site of inflammation (Rogerio *et al*. 2010). Quercetin, one of flavonoid member is a potent bioactive compound extracted from plants that is able to reduce eosinophilia in asthma (Fortunato *et al*. 2012). Furthermore, Rogerio *et al*. (2003) reported that the ethanol extract of *Lafoensia pacari* is able to reduce the production of IL-5 to inhibit the migration of eosinophil to the site of inflammation in *Toxocara canis* infection. *L. pacari* is a medicinal plant of Mato Grosso (Brazil) and traditionally used to treat gastric ulcers and inflammation.

Among all five plants, *M. strigosa* is the indigenous plant of Kalimantan (Maschwitz *et al*. 1989). Until now, there is no report about its biological activity. Therefore, this is the first report of the methanol extract from *M. strigosa* leaves on the anti-inflammatory activity of DTH reaction.

5.4. Conclusion

The anti-inflammatory effects of methanol extracts from leaves of five medicinal plants of Dayak Uud Danum on the delayed-type hypersensitivity were investigated. As a result, the methanol extracts from all species of medicinal plants used in this study had anti-inflammatory activity to suppress the ear swelling of mice. Among all plant extracts, kerokak (*S. dulcis*), penahan (*M. strigosa*), tebelion (*E. zwageri*), and tekeriho (*C. longifolia*) could inhibit the migration of eosinophils to the site of inflammation. Although bungur (*L. speciosa*) suppress the DTH reaction by suppressing the ear swelling of mice, it did not inhibit the migration of eosinophils to the site of inflammation.

Chapter 6

Anti-inflammatory effects of five medicinal plants used by Dayak Uud Danum on the prevention of damaged human colon epithelial cells

6.1. Introduction

In a developing country like Indonesia, diarrhea is still a public health problem due to a high number of its morbidity and mortality. World Health Organization reported that approximately 2.2 million or 4% of all the deaths were caused by diarrhea in the world (Surawicz 2010). In Indonesia, prevalence of diarrhea caused by infection is 3.5%, and the occurrence of diarrhea is higher in rural area than in the urban area (Balai Penelitian dan Pengembangan Kesehatan 2013, Pusat Data dan Informasi 2011).

Diarrhea is an abnormal movement of ions and water across an epithelium to disrupt the osmotic balance between bowel contents and the blood, resulting in the increase of stools (Binder 2009). Gastrointestinal infections cause the intestinal disruption of secretion balance and leads to diarrhea with the increasing of fluid stool. The disruption of the junction of epithelial cells or the increasing of mucosal permeability leads to an inflammatory diarrhea (Hodges and Gill 2010, Surawicz 2010). Diarrhea is difficult to be fully recovered in a patient with an inflammatory bowel disease (Binder 2009). Diarrhea accompanied by inflammation leads to the damage of epithelial mucosal surface (Guttman and Finlay 2008). Chronic inflammation of intestine may lead to the increasing risk of colon cancer (Kaser *et al*. 2010).

Among the structural components between epithelial cells, tight junctions or zonula occludens are located in the most apical of intercellular junction and seal the space between adjacent cells (Balda *et al*. 1996). A major role of tight junctions is to function as a physical barrier to control physiological flux and several growth factors such as hormones, cytokines, and drugs regulate the tight junctions and barrier function (Harhaj and Antonetti 2004). By measuring the transepithelial electrical resistance (TER), the condition of barrier function of epithelial cells or the permeability of the

tight junction can be examined (Chen *et al*. 2015). The increase of the epithelial paracellular permeability will lead to the damage of the junction in intestinal mucosa.

The upper side of epithelial cells covered by mucus layer acts as a front line of host defense. The functional integrity of this layer is controlled by the coordinated regulation of the mucus layer, the intercellular tight junction between epithelial cells, and host immune response (Kim and Ho 2010). There are several cytokines engaged in the inflammation of intestinal epithelial cells such as IFN-γ, TNF-α, IL-1, IL-2, IL-4, IL-6, IL-8, IL-10, and IL-13. For example, TNF-α causes the damage of tight junctions of epithelial cells by inducing cell apoptosis, leading to the increase of tissue permeability (Capaldo and Nusrat 2009, Tominaga *et al*. 2013). Therefore, it is possible to prevent the damage of epithelial cells by regulating the production of pro-inflammatory cytokines, resulting in the reduction of the symptoms of diarrhea.

Dayak Uud Danum live in a remote area and their villages are isolated from others. Dayak Uud Danum is familiar to the of use medicinal plants to treat diarrhea. Among 95 species of medicinal plants used by Dayak Uud Danum, there are several plants used to treat inflammation diseases such as diarrhea (**Fig. 1.1**). These five medicinal plants are expected to have bioactive compounds that would ameliorate the inflammation in human colon epithelial cells, leading to the reduction of the severity of symptoms of the diarrhea.

In this study, anti-inflammatory activities of Dayak Uud Danum medicinal plants were analyzed to prevent the damage of human colon epithelial FPCK-1-1 cells derived from a patient with familial adenomatous polyposis. In this *in vitro* assay, PMA (Phorbol 12-myristate 12-acetate) was used to stimulate macrophage-like THP-1 cells to induce the inflammatory response in FPCK-1-1 cells. During the co-culture of FPCK-1-1 cells with PMA-stimulated THP-1 cells, the TER of FPCK-1-1 monolayer cells was measured and the surface of monolayer cells was stained with both Alcian blue and periodic acid-Schiff (PAS). Furthermore, the level of IL-22 in the supernatant of FPCK-1-1 cells in the upper chamber were measured.

The results showed that among five medicinal plants of Dayak Uud Danum, only the extract from Penahan (*M. strigosa*) could prevent the damage of human colon epithelial cells caused by the inflammation.

6.2. Materials and Method

Plants material

Methanol extracts from leaves of 5 medicinal plants were prepared as described in a previous stage (**Chapter 3**). Ten mg of methanol extracts of each plant (dry weight) was dissolved in one ml of DMSO (1 mg/ml) to make a stock solution.

Cell lines

FPCK-1-1 cells are precancerous colon epithelial cells derived from a male patient with familial adenomatous polyposis (Kawaguchi *et al*. 1991). THP-1 cells (Human monocytic leukemia) were purchased from Health Science Research Resources Bank, Japan Health Science Foundation, Osaka, Japan (Tsuchiya *et al*. 1982). Both FPCK-1-1 and THP-1 cells were suspended in Dulbecco's-modified Eagle Medium (DMEM-high glucose) supplemented with 8% FCS, 20 U/ml penicillin, 50 μg/ml kanamycin and cultured in 5% CO_2 at 37°C for six days. FPCK-1-1 cells were sub-cultured on a Transwell permeable membrane (insert) with pores of 0.4 μm and area 1.1 cm^2 pre-coated with an equimolar mixture of collagen types I and III (3493, Corning, Ithaca, USA).

Co-culture system to treat colon epithelial cells

FPCK-1-1 monolayer cells cultured at a density of 2 x 10^5 cells/insert in 12 wells plate (Corning 3513). THP-1 cells were cultured at density of 1 x 10^5/well of 12 wells plate in the presence of 20 nM phorbol 12-myristate 13-acetate (PMA) for one day. After six days, the inserts with FPCK-1-1 cells were transferred into the wells where the THP-1 cells were cultured with PMA (lower chamber). The co-culture was maintained for three days to monitor the transepithelial electrical resistance of the FPCK-1-1 monolayer cells. In other co-culture, the methanol extracts of *C. longifolia*, *M. strigosa*, *E. zwageri*, S. *dulcis*, and L. *speciosa* were added to the upper chamber (insert) of the co-culture system at the time when co-culture started. The final concentration of each methanol extract in the upper chamber was 1 μg/ml. The scheme of early phase damage model and co-culture system is presented in **Fig. 6.1. and 6.2.**

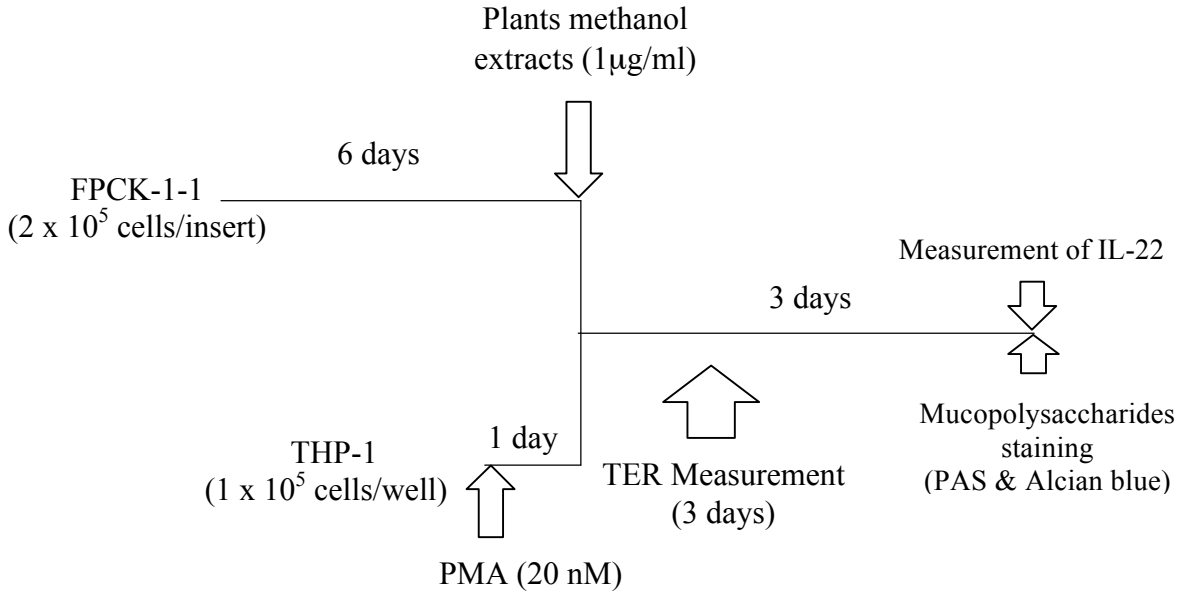


Fig. 6.1. Scheme of early phase damage model on FPCK-1-1 monolayer cells

Measurement of transepithelial electrical resistance (TER)

The TER of FPCK-1-1 monolayer cells was determined by measuring the electrical resistance between the lower chamber (well) and the upper chamber (insert) using a Millicell-ERS voltmeter with a MERSSTX01 electrode (Millipore, Bedford, MA, USA). Two hours before measurement, the medium in the transwell was changed, and the electrode was soaked in 70% ethanol and rinsed with sterile DMEM whose temperature was maintained close to 37°C. The measurement of TER was repeated four times and the mean value was calculated. The TER value of FPCK-1-1 monolayer cells at the time of starting a co-culture was expressed as 100 % (TER value for FPCK-1-1 monolayer cells was 222 – 267 Ω x cm^2 in this experiment).

Staining of mucopolysaccharides that cover the FPCK-1-1 cell monolayer cells on the filter membrane

FPCK-1-1 monolayers cells were stained with Alcian blue and periodic-acid-Schiff (PAS) (Muto Pure Chemicals Co., Ltd, Tokyo, Japan) according to manufacturer's protocol.

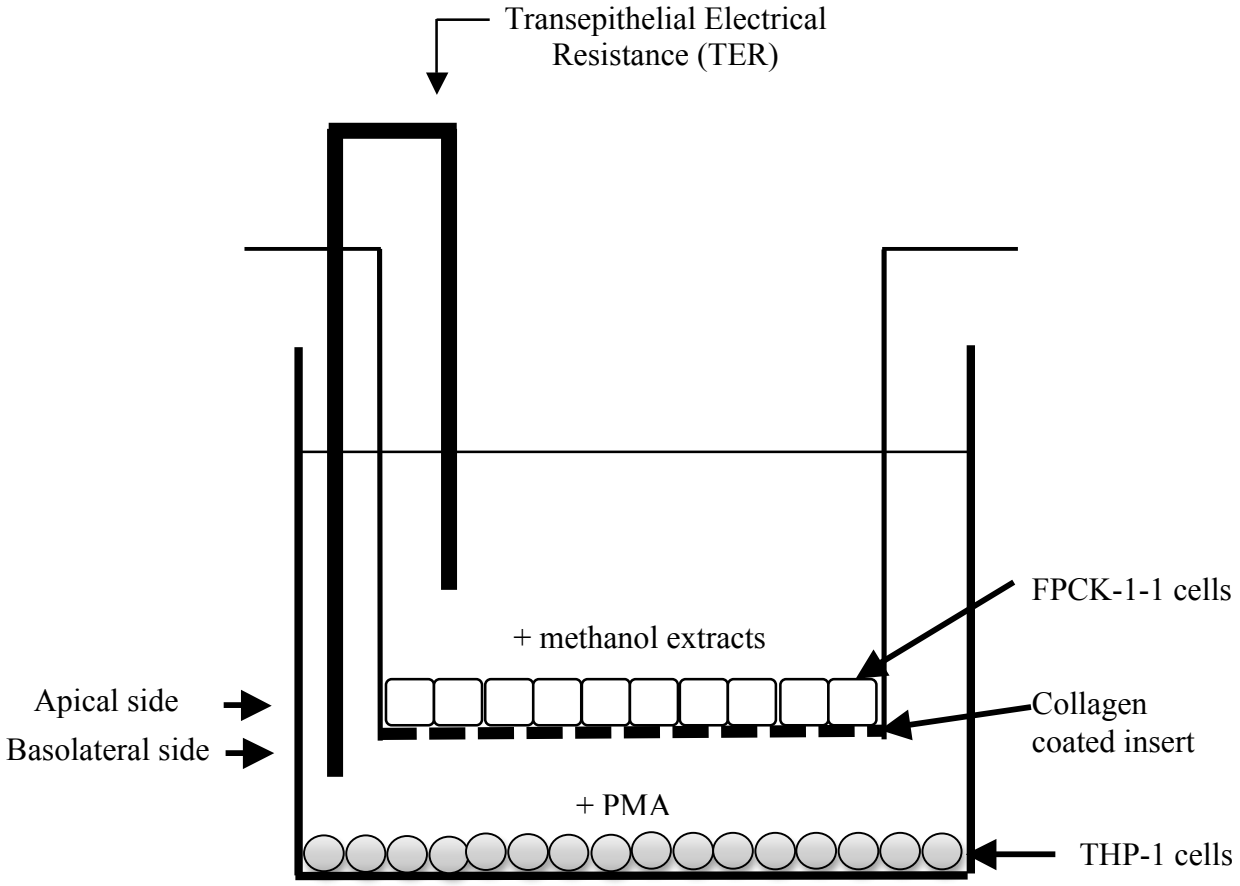


Fig. 6.2. Co-culture system of FPCK-1-1 monolayer cells with PMA-stimulated THP-1 cells.

IL-22 measurement in the supernatant

IL-22 released from FPCK-1-1 cells in the upper chamber was measured according to the manufacturer's protocol using a Human IL-22 Quantikine ELISA kit (R&D System, Inc. Minneapolis, MN, USA).

Statistics

The SPSS 16 was used for statistical analysis of data. One-way ANOVA (Tukey-HSD post hoc test) was used to evaluate the statistical significance. A P value < 0.05 was considered statistically significant.

6.3. Results and Discussion

In order to analyze the anti-inflammatory activity of methanol extracts of five species of medicinal plants used by Dayak Uud Danum to treat several diseases that are related to inflammation (**Fig. 1.1)**, an *in vitro* damage-prevention model of colon epithelial cells was employed as described (Tominaga *et al*. 2013). This assay examines the preventive effects of extracts from medicinal plants on the damage of epithelial cells.

In this assays, PMA was used as a stimulant for macrophage-like THP-1 cells. THP-1 cell line is commonly used as human macrophage. PMA is an activator of protein kinase C (PKC), and the activation of PKC induces THP-1 cells to secrete pro-inflammatory cytokines (Maeß *et al*. 2014). Methanol extracts from leaves were added to the apical side of the co-culture (upper chamber containing FPCK-1-1 cells). During the co-culture with PMA-stimulated THP-1 cells, TER of FPCK-1-1 monolayer cells was measured.

On the third day of the co-culture, there was 13.4% decrease in TER when FPCK-1-1 monolayer cells were co-cultured with PMA-stimulated THP-1 cells (**Fig. 6.3**). *M. strigosa* extract showed highest TER restoration and there is no significant difference between FPCK-1-1/non-stimulated THP-1 group (FPCK-1-1/THP-1) and *M. strigosa* group on the third day of the co-culture. Other plant extracts did not show any significant activity in this damage-prevention model of human colon epithelial cells.

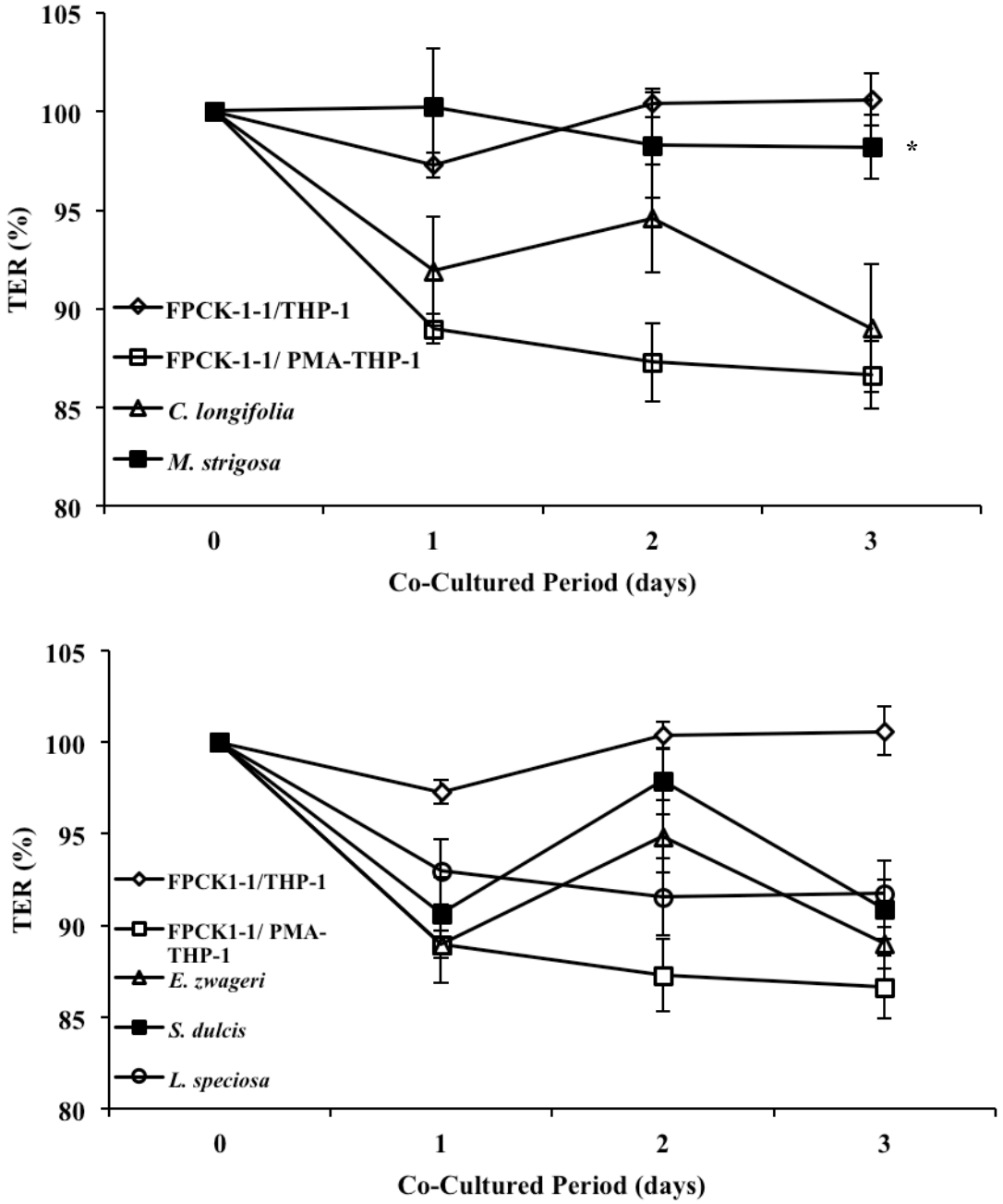


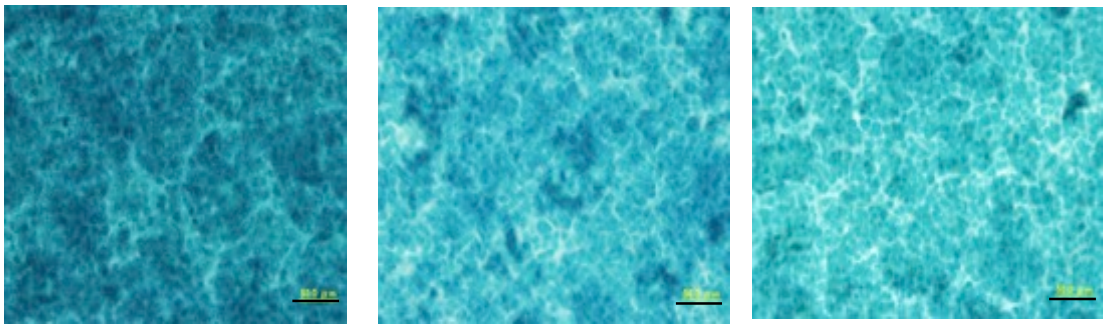
Fig. 6.3. Effects of plant extracts on the damage of FPCK-1-1 monolayer cells induced by PMA-stimulated THP-1 cells. TER was measured during the co-culture of FPCK-1-1 cells with PMA-stimulated THP-1 cells. Results are shown as the average ± SE (n=4). *An asterisk indicates that there is a significant difference between FPCK-1-1/PMA-THP-1 group and *M. strigosa* group. There is no significant difference between control FPCK-1-1/THP-1 group and *M. strigosa* group ($P < 0.01$, Tukey-HSD post hoc test).

One of the criteria to prevent the damage of FPCK-1-1 colon epithelial cells is the mucus production of epithelial monolayer cells. Three days after starting the co-culture, the surface of FPCK-1-1 monolayer cells in the upper chamber was stained either with Alcian blue or PAS to analyze the level of mucopolysaccharides.

In terms of the staining levels of mucopolysaccharides, the FPCK-1-1 monolayer cells after being treated with each methanol extract of leaves were compared with those co-cultured with PMA-stimulated THP-1 cells in the absence of plant extracts.

In this study, two kinds of staining technique were used, Alcian blue and PAS. The presence of mucopolysaccharides was detected with both of Alcian blue (**Fig. 6.4**) and PAS staining (**Fig. 6.5**).

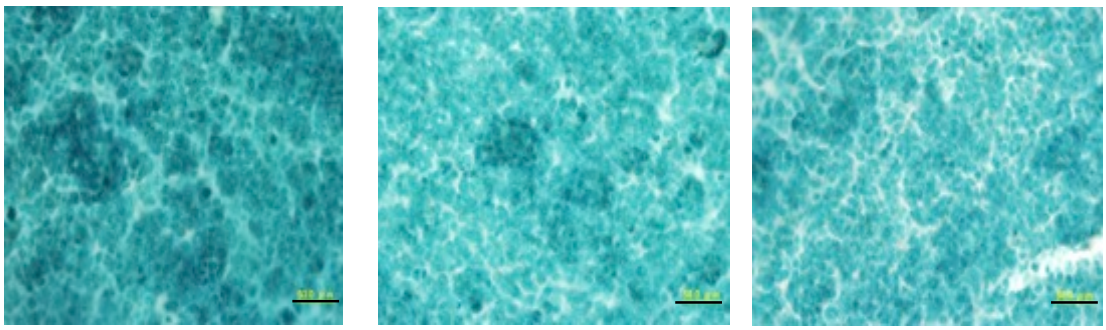
As shown in **Fig. 6.4** and **Fig. 6.5**, the level of mucopolysaccharides on FPCK-1-1 monolayer cells was decreased when the cells were co-cultured with PMA-THP-1 cells. When the FPCK-1-1 monolayer cells treated with methanol extract from leaves of *M. strigosa*, however, the level of mucopolysaccharides on the surface of FPCK-1-1 cells was recovered.



Control (FPCK-1-1/THP-1)

FPCK-1-1/PMA-THP-1

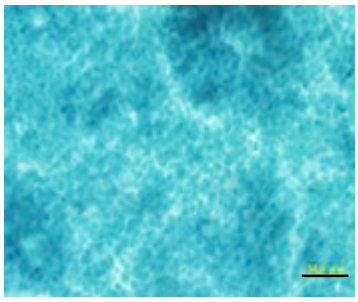
FPCK-1-1/PMA-THP-1 + *C. longifolia*



FPCK-1-1/PMA-THP-1 + *M. strigosa*

FPCK-1-1/PMA-THP-1 + *E. zwageri*

FPCK-1-1/PMA-THP-1 + *S. dulcis*



FPCK-1-/PMA-THP-1 + *L. speciosa*

Fig. 6.4. Alcian-blue staining of FPCK-1-1 monolayer cells treated with plant extracts. FPCK-1-1 monolayer cells were stained with Alcian-blue as described in Materials and Methods. Three days after starting the co-culture with PMA-stimulated THP-1 cells plant extracts were added at the beginning of the co-culture. Bar: 50 μm.

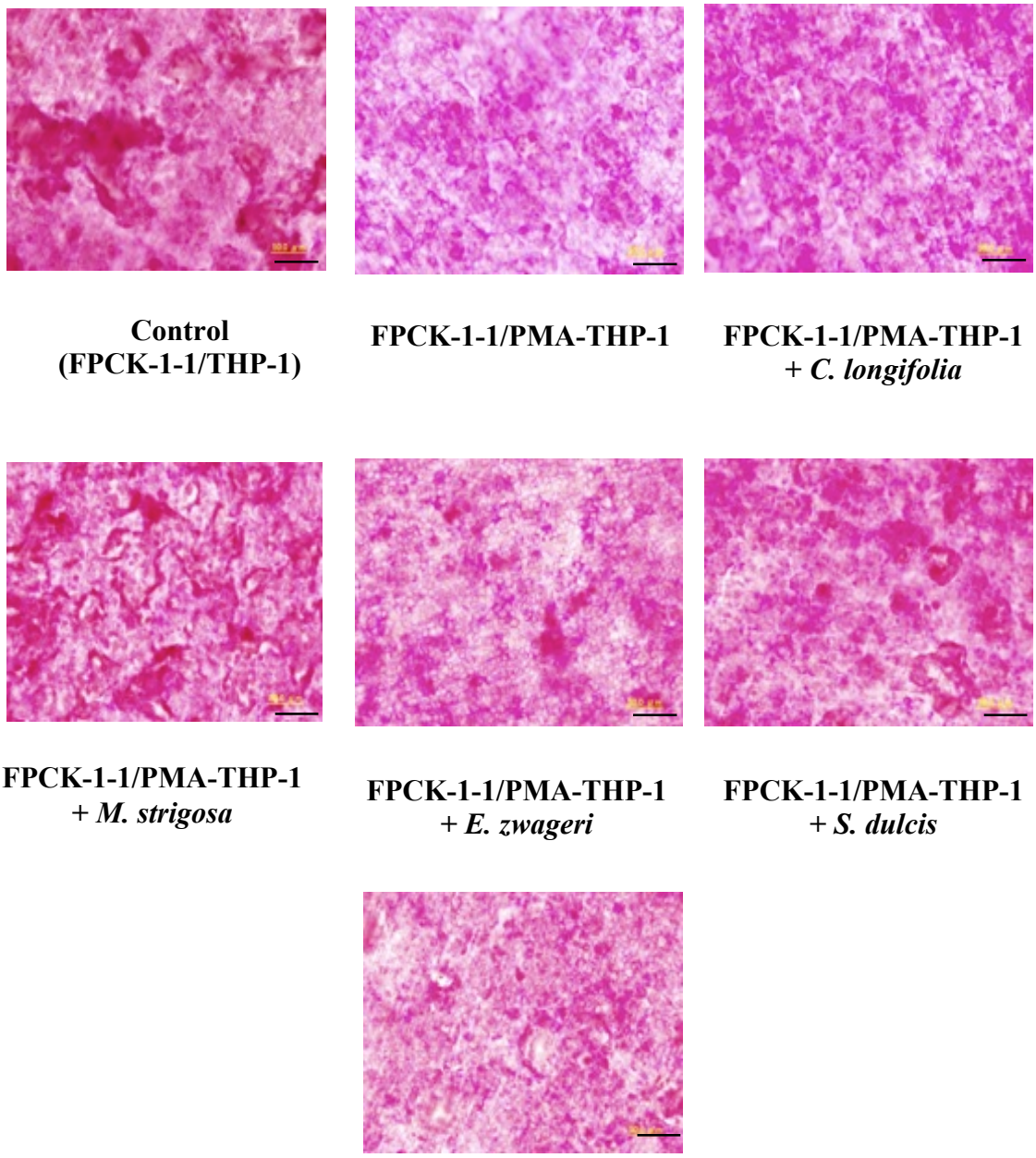


Fig. 6.5. PAS staining of FPCK-1-1 monolayer cells treated with plant extracts. FPCK-1-1 monolayer cells were stained with PAS as described in Materials and Methods. Three days after starting the co-culture with PMA-stimulated THP-1 cells Plant extracts were added at the beginning of the co-culture. Bar: 50 μm.

The presence of mucopolysaccharides layer on the surface of FPCK-1-1 cells was detected with Alcian blue and PAS staining. Alcian blue is a cationic dye that is widely used for staining macromolecules like mucopolysaccharides and acidic mucins in tissues and cells (Dong *et al*. 2012), and PAS is commonly used to stain acid mucopolysaccharides (Fujii *et al*. 1965).

FPCK-1-1 monolayer cells treated with methanol extract from leaves of *M. strigosa* showed higher level of staining with Alcian blue and PAS compared with other groups, suggesting that mucopolysaccharides are produced by the monolayer cells in response to *M. strigosa* extract. It is relevant to the higher level of TER value suggesting that *M. strigosa* extract has the strongest protection against the damage signal from PMA-stimulated THP-1 cells. It is suggested that the presence of mucopolysaccharides that covers the FPCK-1-1 epithelial monolayer cells increases the TER value. The mucopolysaccharides that cover the surface of FPCK-1-1 epithelial monolayer cells may function as a shield to inhibit the movement of ions (Tominaga *et al*. 2013). Through TER measurement, the paracellular barrier function and tight junction integrity can be determined (Ren *et al*. 2012). The decreased of TER value represents, in part, the increased level of the permeability of the tight junction (Balda *et al*. 1992). The results suggest that *M. strigosa* extract has the ameliorating effects on the barrier function of intestinal epithelial cells by reducing the paracellular permeability.

The increased paracellular permeability caused by the breakdown of tight junction drives materials to penetrate from lumen to the basolateral side of the epithelium, resulting in the inflammation that finally causes diarrhea (Guttman *et al*. 2006, Ren *et al*. 2012). Epithelial barrier disruption may cause the secretion of fluid and electrolytes from epithelial cells, inducing the loss of mucosal proteins and mal-absorption (Strauman *et al*. 2010). To preserve the function of intestinal barrier by preventing paracellular permeation induced by harmful agents to lumina, it is important to keep the epithelial tight junction intact (Al-Sadi and Ma 2007).

Next, IL-22 released in the apical side of FPCK-1-1 monolayer cells treated with various plants extracts were measured (**Table 6.1**). The measurements were conducted using the supernatants from the upper chamber where FPCK-1-1 monolayer cells were cultured, while PMA-stimulated THP-1 cells were cultured in the lower chamber.

FPCK-1-1 monolayer cells were cultured in the upper chamber in the presence of plant extracts for three days.

Most plant extracts did not induce FPCK-1-1 colon epithelial cells to produce IL-22 except the extract from *C. longifolia*. *M. strigosa* extract was expected to induce FPCK-1-1 cells to produce IL-22, because this extract prevented the decrease of TER by inducing FPCK-1-1 cells to produce mucopolysaccharides. However, it only induced the marginal level of IL-22 **(Table 6.1**).

Table 6.1. Effects of plant extracts on the production of IL-22 by FPCK-1-1 monolayer cells.

Co-culture & addition of plant extracts	IL-22 (pg/ml) (mean ± SE)
FPCK-1-1/THP-1	4.6 ± 1.6
FPCK-1-1/PMA-THP-1	3.0 ± 3.1
+ *C. longifolia*	23.8 ± 2.2
+ *M. strigosa*	3.8 ± 2.9
+ *E. zwageri*	0
+ *S. dulcis*	3.0 ± 2.6
+ *L. speciosa*	0

C. longifolia group is significantly different from FPCK-1-1/THP-1 group (Tukey-HSD post hoc test, one-way ANOVA, $P<0.01$).

It is reported that IL-22 produced by FPCK-1-1 cells has a role to recover the intestinal mucosal layer (Tominaga *et al*. 2012, 2013). IL-22 is one of a member of IL-10 cytokine family secreted mainly from CD4 positive T cells, NK cells, and dendritic cells. In intestinal inflammation, main role of IL-22 is to induce the epithelial cells to produce proteins and strengthen the mucus barrier by promoting mucin secretion and increase the epithelial cells through the restitution of goblet cells, resulting in the intestinal recovery (Mizoguchi 2012). IL-22 regulates the tight junction between intestinal epithelial cells through promoting the cell proliferation and the wound healing (Dudakov *et al*. 2015). In addition, due to the activation of Jak-STAT-3 (Janus kinase/signal transducer and activator of transcription-3) IL-22 promotes the production

of anti-microbial peptides to prevent tissue destruction and tissue restoration (Karin and Clevers 2016, Zundler and Neurath 2016, Li *et al*. 2014).

TNF-α is a pro-inflammatory cytokine primarily produced by activated macrophages and T-lymphocytes. TNF-α has role a role to protect hosts against infections by bacteria, parasites, and viruses (Bradley 2008). In the inflammation of intestinal epithelial cells, however, TNF-α causes the intestinal damages. It induces the apoptosis of cells and the disruption of tight junctions through disturbing the structure and function of the tight junction, resulting in the increase of paracellular permeability (Bruewer *et al*. 2003, Li *et al*. 2010). It is reported that the TER value of epithelial cells was recovered by adding the antibodies against TNF-α (Biasi *et al*. 2013, Tominaga *et al*. 2013).

Furthermore, Vezza *et al* (2016) reported that following flavonoid family compounds from several plants have potent anti-inflammatory activities of intestine: glycosides, aglycones, quercetins, flavonols, flavonones, flavones, catechins, isoflavons, anthocyanidins, and chalones. These plants secondary metabolites are scientifically proved to have curative effects on colonic damage. These results suggest that methanol extract from *M. strigosa* leaves may have secondary metabolites that are able to block the signaling pathway of TNF-α, in the absence of IL-22.

6.4. Conclusion

Anti-inflammatory activities of methanol extracts from leaves of five medicinal plants of Dayak Uud Danum were investigated by using the *in vitro* damage-prevention model of human colon epithelial cells. As result, among five medicinal plants species, only penahan (*M. strigosa*) showed the highest TER restoration and induced colon epithelial cells to produce the highest level of mucopolysaccharides. Only the extract from *C. longifolia* induced FPCK-1-1 colon epithelial cells to produce IL-22.

Chapter 7
Conclusions

This is the study to summarize the knowledge of medicinal plants by Dayak Uud Danum in six villages of Ambalau district. Among Dayak Uud Danum communities in six villages, there are 95 species of medicinal plants used to cure various diseases and 38 species of medicinal plants are used to cure inflammatory diseases associated with allergic, skin infection, fever, edema, and diarrhea. Leaves are the main parts used to make various remedies. Most of the remedies are prepared by decoction or boiling leaves in hot water, and the oral-topical route is the most common way to administer the herbal medicine.

Among the biological activities of medicinal plants used by Dayak Uud Danum, most commonly used anti-inflammatory activities of these plants were examined. The examinations were focused on five species of medicinal plants that were used by Dayak Uud Danum to cure allergies, skin infection, fever, edema, and diarrheas in which inflammation is involved. These medicinal plants are Tekeriho (*Callicarpa longifolia* Lam.), Penahan (*Myrmeconauclea strigosa* Merr.), Tebelion (*Eusideroxylon zwageri* Teijsm & Binn.), Kerokak (*Scoparia dulcis* L.), and Bungur (*Lagerstroemia speciosa* (L.) Pers.).

At first, toxicity of plant extracts was determined *in vitro* and *in vivo* by using murine fibroblast NIH3T3 cells and BALB/cAJc mice, respectively. Anti-inflammatory activities were analyzed at a safe dose in a delayed-type hypersensitivity (DTH) response against 2,4,6-trinitro-1-chlorobenzene (picryl chloride) using BALB/cAJc mice by measuring the suppression of the ear swelling after the challenge with the antigen. The anti-inflammatory effects of medicinal plants were also examined through *in vitro* assays by using human colon epithelial FPCK-1-1 monolayer cells.

The percentages of extraction yield from leaves of five plant species varied from 4.33% to 8.99%. All the plant species are categorized into the group of high level of extractive content. The highest yield was obtained in the extraction from *C. longifolia*.

Methanol extracts from leaves of all plant species were toxic to normal mouse

fibroblast NIH3T3 cells at a concentration of 100 μg/ml. *L. speciosa* extract was most toxic at lower concentrations (0.1 μg/ml, 1 μg/ml, and 10 μg/ml). *L. speciosa* extract was not toxic to BALB/cAJc mice even after administrating as much as 5 mg/0.1 DW per mouse.

All methanol extracts from leaves of five species of medicinal plants had the anti-inflammatory activities to suppress the DTH response (ear swelling of mice) efficiently at 48 hours after challenge. Among all plants extracts, extracts from *S. dulcis*, *M. strigosa*, *E. zwageri*, and *C. longifolia* inhibited the migration of eosinophils to the site of inflammation. Although *L. speciosa* extract suppressed the DTH response by suppressing the ear swelling of mice, it did not inhibit the migration of eosinophils to the site of inflammation.

Methanol extract of *M. strigosa* showed the highest activity to prevent the damage of human colon epithelial FPCK-1-1 cells that is caused by the co-culture with PMA-stimulated THP-1 cells. It also induced FPCK-1-1 cells to produce the highest level of mucopolysaccharides revealed by the staining with Alcian Blue. Among extracts from five plant species, only the methanol extract from *C. longifolia* induced FPCK-1-1 colon epithelial cells to produce IL-22.

The results showed that methanol extract from leaves of *M. strigosa* has both DTH-suppressing activity in mice and damage-preventing activity of human colon epithelial FPCK-1-1 cells against inflammation. To clarify these anti-inflammatory activities of this plant extract, it is necessary to purify and identify the bioactive compounds from this plant. This will provide us the way to regulate the inflammation more appropriately.

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