Doctoral Dissertation

Evaluation of anti-diabetic effect and anti-inflammatory effect on intestinal epithelial cells of folk medicine in West Kalimantan

(西カリマンタン民間薬の抗糖尿病効果と腸上皮細胞の炎症に対する効果の評価)

by

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Kochi University

September 2017

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Summary

The West Kalimantan of Indonesia has diversified species of plants and indigenous people, especially Dayak tribes live there. They have a long tradition to use the plants as natural medicine to prevent and cure diseases. In this study, it is tried to analyze the effectiveness of several medicinal plants that are trusted by Dayak tribes to ameliorate diabetes, diarrheas, and stomachaches by examining their abilities to inhibit α-glucosidase *in vitro* (yeast maltase, rat intestinal maltase and rat intestinal sucrase), to down-regulate activity of blood glucose levels of diabetic *db/db* mice in maltose loading tests and long term administration, and to prevent the damage to human colon epithelial FPCK-1-1 cells.

First, the amount of materials extracted by methanol from wood barks of 17 plants that belong to plant families of *Anacardiaceae* (*Pentaspadon motleyi, Mangifera pajang*, *Mangifera foetida*, and *Dracontomelon dao*), *Fabaceae* (*Parkia timoriana*, *Parkia speciosa*, *Parkia intermedia*, *Parkia sp.*, and *Adenanthera sp.*), *Malvaceae* (*Durio dulcis*, and *Durio kutejensis*), *Phyllanthaceae* (*Baccaurea costulata*, and *Baccaurea angulata*), and 4 species from other plant families that are *Annonaceae* (*Goniothalamus tapis*), *Apocynaceae* (*Willughbeia angustifolia*), *Burseraceae* (*Dacryodes rostrata*), and *Clusiaceae* (*Garcinia parvifolia*) was measured. These plants were collected from Kuala Buayan Village, Sanggau Regency, and Pala Pulau Village, Putussibau Regency, West Kalimantan.

Extractive contents varied from 2.05 to 21.48% among these species. Almost all of the species belong to the high category of extractive content, especially *P. intermedia* (*Fabaceae*) had 21.48%. *D. dulcis* (*Malvaceae*) and *B. angulata* (*Phyllathaceae*), whose extractive contents were 2.05 and 2.75%, respectively, belong to a moderate category.

Second, the inhibitory effects of methanol extracts from 17 plants on the activities of yeast α-glucosidase using pNPG as a substrate (yeast maltase), rat intestinal α-glucosidase using pNPG as a substrate (rat intestinal maltase), and rat intestinal sucrase using sucrose as a substrate were analyzed.

Methanol extracts from all species of *Fabaceae* family (*P. speciosa*, *Adenanthera sp.*, *P. intermedia*, *P. timoriana*, and *Parkia sp.*) had strong inhibitory effects on yeast

α-glucosidase activity. They were able to inhibit 50% (IC_{50}) of α-glucosidase activity at lower concentrations (< 5 μg/ml). For species in *Anacardiaceae*, *Malvaceae*, *Phyllanthaceae*, and other family, IC_{50} values were diversified. In contrast, species of *D. dao*, *D. kutejensis*, and *G. parvifolia* had IC_{50} values similar to those of species of *Fabaceae* plants family against yeast α-glucosidase activity (< 5 μg/ml). *P. motleyi*, *P. speciosa*, *P. timoriana*, *D. rostrata*, *Adenanthera sp.*, and *B. costulata* showed lower inhibitory activity on rat intestinal sucrase (IC_{50} < 1000 μg/ml). All species showed low activity to inhibit rat intestinal maltase.

Third, the toxicities of *D. dulcis*, *D. kutejensis*, *P. timoriana*, *P. speciosa*, *P. intermedia*, *B. angulata*, and *D. dao* were analyzed. These species were selected based on the functional utilization as traditional medicine for diarrheas, stomachaches, and diabetes, and the ability to inhibit α-glucosidase *in vitro* with low value of IC_{50} except *B. angulata*.

Methanol extracts from wood barks of *D. dulcis*, *D. kutejensis*, *P. timoriana*, *P. speciosa*, *P. intermedia*, and *D. dao* are toxic at a concentration of 100 μg/ml for normal mouse fibroblast NIH3T3 cells. Extract of *P. speciosa* is the most toxic at a concentration of 10 μg/ml and other methanol extracts from wood barks did not show strong toxicity at concentrations of 1 μg/ml. *P. speciosa* extract is not toxic to C57BL/6J mice even after administrating 5 mg extract/mouse orally.

Fourth, anti-diabetic effects of *D. dulcis*, *D. kutejensis*, *P. timoriana*, *P. speciosa*, *B. costulata*, and *D. dao* that showed activity to inhibit α-glucosidase *in vitro* were analyzed in the maltose loading test using *db/db* mice. Maltose loading tests was conducted twice.

In the first experiment, dose of each extract or acarbose administered five minutes before the oral administration of maltose was 1 mg/0.1 ml DW/mouse and measurement of blood glucose was conducted at 70 min, 140 min, and 210 min after the maltose loading. At 70 minutes after maltose loading, the lower levels of blood glucose were found in groups administered with extracts of *P. speciosa* and *P. timoriana*. Blood glucose level of *P. speciosa* group was significantly lower than that of *B. costulata* group at 70 min, 140 min, and 210 min after maltose loading. At 140 minutes after maltose loading, only the level of blood glucose of *P. speciosa* group was significantly lower than that of the group administered with either *D. kutejensis* or *B. costulata*. At

210 min after maltose loading, levels of blood glucose of groups administered with *D. dulcis*, *P. timoriana*, and *P. speciosa* were significantly lower than those of *D. kutejensis*, and *B. costulata* groups, while that of acarbose was significantly lower than that of *D. kutejensis* group.

In the second experiment, the measurement of blood glucose was conducted at 30 min, 60 min, and 120 min after the maltose loading. The dose of each methanol extract administered to each mouse was 5 mg/0.1 ml DW/mouse and that of acarbose was 8 mg/0.1 ml DW/mouse. The levels of blood glucose of groups administered with acarbose and *P. speciosa* extract were significantly lower than that of *B. costulata* group at 30 min, 60 min, and 120 min. At 30 min after maltose loading, the level of blood glucose of acarbose group was significantly lower than that of diabetic control group. The level of blood glucose of *D. dulcis* group was significantly lower than that of *B. costulata* group at 60 min and 120 min. The level of blood glucose of *P. timoriana* group was significantly lower than that of *B. costulata* group at 120 min. In two maltose loading tests, effect of *P. speciosa* extract to down-regulate the blood glucose was confirmed at three points in each experiments compared with *B. costulata* extracts.

Fifth, it was asked whether methanol extracts of *D. dulcis*, *D. kutejensis*, *P. timoriana*, *P. speciosa*, *B. costulata*, and *D. dao* that have the activity to inhibit α-glucosidase *in vitro* (Chapter 3) and those of *D. dulcis*, *P. timoriana*, and *P. speciosa*, which had the ability to reduce the blood glucose level of *db/db* mice in two maltose loading tests (Chapter 5) have the activity to reduce the level of blood glucose of *db/db* mice after administrating them for four weeks. The levels of blood glucose before and after fasting, two, three, and four weeks after oral administration of these extracts were measured.

The level of fasting blood glucose of *P. speciosa* group was significantly lower than that of diabetic control, *D. kutejensis,* and *B. costulata* groups at three and four weeks after starting oral administration. The level of fasting blood glucose of *P. timoriana* group was significantly lower than that of diabetic control and *B. costulata* groups four weeks after administration. The levels of blood glucose before fasting of *P. timoriana* group and *D. dao* group were significantly lower than those of diabetic control, *D. kutejensis*, and *B. costulata* groups four weeks after administration.

Acarbose group had the highest food consumption among all *db/db* groups. Only *P. speciosa* and *B. costulata* groups consumed significantly lower amount of food than acarbose group four weeks after starting the oral administration. Body weight of all *db/db* mice increased continuously till four weeks after starting the administration of plant extracts except that of *B. costulata* group. No significant difference was found in body weight of mice between diabetic control group and other groups, suggesting that the methanol extracts are not toxic and safe for continuous use.

Acarbose group and groups administered with plant extracts except *B. costulata* have the tendency to have increased levels of serum cholesterol compared with diabetic control group. The levels of serum cholesterol of *P. timoriana* group and *D. dao* group were significantly higher than that of *B. costulata* group. Levels of serum triacylglycerol of *D. dulcis* group and *P. speciosa* group were significantly lower than that of diabetic control group.

Sixth, the anti-inflammatory effects of *D. dulcis*, *D. kutejensis*, *P. timoriana*, *P. speciosa*, and *D. dao* on the damage-prevention of human colon epithelial FPCK-1-1 cells were analyzed. FPCK-1-1 is a human intestinal epithelial cell line established from a tubular adenoma of male patient with familial adenomatous polyposis and it was used as a new culture model of intestinal inflammation.

D. dulcis, *D. kutejensis*, *P. timoriana*, *P. speciosa*, and *D. dao* had activities to prevent the damage of human colon epithelial FPCK-1-1 cells caused by PMA (phorbol 12-myristate 13-acetate)-stimulated THP-1 cells three days after starting the co-culture. Extracts from *P. speciosa* and *D. dao* induced FPCK-1-1 cells to produce mucopolysaccharides. It is suggested these mucopolysaccharides function as a barrier by covering the surface of FPCK-1-1 monolayer cells to prevent the damage of FPCK-1-1 monolayer cells induced by PMA-stimulated THP-1 cells.

Methanol extracts from wood barks of *D. dulcis*, *P. timoriana*, *P. speciosa*, and *D. dao* have both activities to prevent the damage of FPCK-1-1 human colon epithelial cells and down-regulate the level of blood glucose of *db/db* mice. It is suggested that methanol extracts from wood barks of *D. dulcis*, *P. timoriana*, *P. speciosa*, and *D. dao* contain non-toxic bioactive compounds which prevent the damage of human colon epithelial FPCK-1-1 cells and decrease the level of blood glucose of *db/db* mice by down-regulating the inflammation.

Contents

List of main papers

Main papers used in creating the dissertation

Peer-reviewed paper

1. Fathul Yusro, Yeni Mariani, Yuko Konishi, Takahiro Taguchi, Mari Tominaga, Satoshi Kubota and Akira Tominaga. 2016. Effects of medicinal plants in West Kalimantan Indonesia to prevent the damage of human colon epithelial FPCK-1-1 cells and regulate the levels of blood glucose and triacylglycerol of *db/db* mice. Kuroshio Science, 10: 73-88.

Additional paper

1. Fathul Yusro, Kazuhiro Ohtani and Satoshi Kubota. 2016. Inhibition of α-glucosidase by methanol extracts from wood bark of *Anacardiaceae*, *Fabaceae*, *Malvaceae* and *Phyllanthaceae* plants family in West Kalimantan, Indonesia. Kuroshio Science, 9: 108-122.

Conference presentation

1. Fathul Yusro, Yeni Mariani, Yuko Konishi, Takahiro Taguchi, Mari Tominaga, Satoshi Kubota and Akira Tominaga. Effects of medicinal plants in West Kalimantan Indonesia to prevent the damage of human colon epithelial FPCK-1-1 cells and regulate the levels of blood glucose and triacylglycerol of *db/db* mice. 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

The West Kalimantan of Indonesia has diversified species of plants and indigenous people. Especially, Dayak tribes live there. They have a long tradition to use the plants as natural medicine to prevent and cure diseases. Though modern drugs have been available in medical centers and drugstores, traditional medicine remains as an option because of less side effects, relative safety, and lower prices compared with modern medicine (Ablat *et al*. 2014). Especially, it is easier for people who live in rural areas to find traditional medicine in forests around them. Based on Balai Penelitian dan Pengembangan Kesehatan (2013), 35.2% households in Indonesia stored several kinds of medicine at home such as prescription drugs, commercial drugs, antibiotics and traditional medicine. The percentage of traditional medicine among stored medicine at home is 15.7%.

Health Ministry of Indonesia made an inventory of medicinal plants and reported that in 2012, there are 15,773 kinds of medicinal plant potions used by 209 tribes of 26 provinces (potion is a combination of several species of plants or several parts of plants that are used for the prevention or the treatment of diseases), and in 2015, there are 10,047 kinds of medicinal plant potions used by 96 tribes in 24 provinces (Balai Penelitian dan Pengembangan Kesehatan 2016). In West Kalimantan, variety of medicinal plants species has been reported (Diba *et al*. 2013, Yusro *et al*. 2013[a,b], Yusro *et al*. 2014[a], Yusro *et al*. 2015), and many species of them have functions to ameliorate diabetes, diarrheas and stomachaches. Inflammation is related to diabetes (Dandona *et al*. 2004, Esser *et al*. 2014, Wellen and Hotamisligil 2005), and chronic diarrheas, especially inflammatory bowel disease (Debnath *et al*. 2013, Zakaria *et al*. 2011).

Hyperglycemia or high blood sugar level is one of the criteria of diabetes. It is caused by the disruption of a metabolic system of carbohydrates, proteins, and fats resulting in complications of the kidneys, eyes, and cardiovascular system (Oyedemi *et al*. 2011, Patel *et al.* 2012, Zhang and Li 2015). The effects of these complications can

lead to the death of diabetic patients. Diabetes is the third cause of the death after cancer, cardiovascular and cerebrovascular diseases (Patel *et al.* 2012).

The number of diabetic patients in the world in 2014 was 386.7 million with the prevalence level of 8.3%, and this will increase up to 591.9 million in 2030 (International Diabetes Federation 2014). In 2013, the number of diabetic patients in Indonesia reached 12,191,564, with prevalence levels of diabetes in urban and rural areas are 7% and 6.8%, respectively (Balai Penelitian dan Pengembangan Kesehatan 2013, Infodatin 2014). This indicates that changes in lifestyle between urban and rural communities are not too much different, especially in terms of less exercises and high levels of food consumption with sugar and fat diets. Approximately 53.1% of Indonesia's population consume sweet drinks or food, and 40.7% of them consume high-fat diets more than once per day (Infodatin 2014), and nearly half (48%) of the total food consumed is rice, which is known to contain high levels of carbohydrates and lower levels of fibers. Lower intake of fibers into the body leads to abdominal obesity that increases risk of diabetes (Novo Nordisk 2013).

One of the ways that can be used as a therapeutic approach in the treatment of diabetes is postprandial control of blood sugar levels by delaying the glucose absorption. The α-glucosidase enzymes such as maltase and sucrase in the epithelial mucosa of small intestine have a function to break up the glycoside bonds of the complex carbohydrates to make monosaccharides and disaccharides that can be absorbed by intestinal epithelial cells. Inhibition of α-glucosidase in the digestive system will delay the digestion of carbohydrates and extend the digestion time, which decreases the rate of glucose absorption, resulting in the reduction of blood glucose level. The inhibition of α-glucosidase is therapeutic for patients with type 2 diabetes (Jaiswal *et al.* 2012). Another potential therapeutic approach is to prevent the inflammation which it known as a signal of obesity, metabolic disorder, and type 2 diabetes (Esser *et al*. 2014, Shoelson *et al*. 2007).

Currently, modern drugs had been widely available for decreasing blood sugar levels. The extensive use of these drugs, however, causes side effects such as flatulence, diarrheas, abdominal discomfort, nasopharyngitis, upper respiratory tract infections, headaches, allergy, anaphylactoid reactions, angioedema, and exfoliate dermatologic reactions (Antu *et al.* 2014, Dicker 2011, Kashtoh *et al.* 2014, Xu *et al.* 2014). Although

it has already been used intensively, sometimes these drugs are unable to control hyperglycemia and eventually the condition of diabetic patients would become worse (Ablat *et al.* 2014).

To treat diabetes with traditional herbal medicines have been accepted by the people who live in the rural area or in the urban area close to modern medical center. People know about diabetes based on some indication such as excessive urination, increase of hunger sensation, a lot of eating and drinking, reduction of body weight, listless, foot numb, and a long healing time after being injured (Soenanto 2005).

Based on the results of the previous studies, it is known that there are 22 species of plants that have been traditionally used to treat diabetes by three Dayak subethnic tribes, Iban, Kanayant and Ketungau in West Kalimantan Province, Indonesia (Yusro *et al.* 2015). Some herbs such as the followings have anti-diabetic compounds. For example, *Garcinia mangostana* has anti-diabetic compound named xanthon (Chaverri *et al.* 2008). *Tinospora crispa* was effective for patients with diabetic type 2 (Klangjareonchai and Roongpisuthipong 2012) and *Phyllanthus niruri* can decrease blood glucose levels of the model mice (Okoli *et al.* 2010). However, other medicinal plants have not yet scientifically proven to have anti-diabetic properties.

Diarrhea is a change in the frequency, consistency, weight, and volume of stool (Thomas *et al*. 2003) and chronic diarrhea, especially inflammatory bowel disease involves chronic inflammation (Debnath *et al*. 2013, Zakaria *et al*. 2011). In 2013, the prevalence level of diarrheas in Indonesia was 3.5% (Balai Penelitian dan Pengembangan Kesehatan 2013) and cases of inflammatory bowel diseases (IBD) rarely reported because of less opportunities to be examined in the tertiary health centers (Zakaria *et al*. 2011). Bowel inflammation is caused by several factors such as infection, inherited genes, immune system, and environment, and repeated inflammation often leads to colon cancer (Baumgart and Carding 2007, Kaser *et al*. 2010). Medication of chronic diarrhea is very important to recover the health and prevent cancer diseases.

In this study, it is tried to analyze the effectiveness of several medicinal plants that are trusted by indigenous people in West Kalimantan Indonesia to ameliorate diabetes, diarrhea, and stomachaches. All of plant species that were examined in this study belong to the *Anacardiaceae*, *Fabaceae*, *Malvaceae*, *Phyllanthaceae*, and other plant families (*Annonaceae, Apocynaceae, Burseraceae, Clusiaceae*) and reported activities

of these plants were summarized (**Fig. 1.1, Table 1.1**). Based on literature reviews, methanol extracts from wood barks of above plants were used as anti-diabetic reagents *in vitro* to inhibit α-glucosidase and to treat diabetes of leptin receptor deficient *db/db* mice.

Before starting experiments using these plant extracts, *in vitro* cytotoxicity assay using NIH3T3 cells and *in vivo* toxicity assay using C57BL/6J mice were performed. Furthermore, anti-inflammatory effects of the bark extracts were estimated by the prevention of the decrease in the transepithelial electrical resistance of FPCK-1-1 monolayer cells derived from a tubular adenoma of male patient with familial adenomatous polyposis.

The plant part used in this series of experiments is wood bark. The wood bark was selected because of their high content of methanol extracts with a variety of chemical compounds that may have the ability to inhibit α-glucosidase (*in vitro*) or to reduce the blood glucose level of leptin receptor deficient *db/db* mice (*in vivo*).

Saccharomyces cerevisiae and rat intestinal acetone powder are a source of α-glucosidase enzymes in the *in vitro* assay of α-glucosidase. Yeast α-glucosidase derived from *S. cerevisiae* contains maltase (Yamamoto *et al.* 2004), and crude α-glucosidase extracts from mammalian small intestine such as rat intestinal acetone powder contains maltase-glucoamylase and sucrase-isomaltase (Asano 2003, Ng *et al.* 2015). Different enzyme sources and difference of substrates in the assays will affect the inhibitory activity against α-glucosidase (Tadera *et al.* 2006, Jo *et al.* 2010).

Anti-diabetic effect of medicine in the *in vitro* assay does not necessarily suggest the anti-diabetic effect *in vivo,* because there is no metabolism in the *in vitro* assay (Soumyanath and Srijayantha 2006). Among the plant extracts that have the activity to inhibit α-glucosidase *in vitro*, some of them do not effectively inhibit α-glucosidase in a mammalian model (Shihabudeen *et al*. 2011). It is very important to prove the effectiveness of plant extracts that effectively inhibit α-glucosidase *in vitro* in the mammalian model of type 2 diabetes *in vivo*. Leptin receptor deficient *db/db* mouse is a good model of diabetes. This strain of mouse is known as obese mice that consume food more than twice as much as the wild type mice, resulting in the higher levels of blood glucose, triacylglycerol, and cholesterol and are used frequently as a model of type 2 diabetes (Dwiranti *et al*. 2012, Kobayashi *et al*. 2000).

It is necessary to decide the appropriate concentration of plant extracts before administrating to mice. The preliminary studies on the cytotoxicity of plant extracts were performed using normal mouse fibroblast NIH3T3 cells (Akter *et al*. 2014) and the most toxic extract was used to evaluate its toxicity against C57BL/6J mice. NIH3T3 cells are non-tumor cells (Orsine *et al.* 2013) established from normal mouse fibroblasts (Akter *et al*. 2014) and used as a control cell line in some cytotoxicity assays for anti-cancer drugs (Beattie *et al*. 2011, Danihelova *et al.* 2013). C57BL/6J is a strain of mice that genetically develop sugar imbalance, and is commonly used for developing obesity, hyperglycemia, hyperinsulinemia, and type 2 diabetes by administrating a high fat diet (Winters *et al.* 2003, Yamamoto *et al.* 2011).

Anti-intestinal inflammatory activity of plant extracts could be determined in an early phase damage model using FPCK-1-1 cells (Tominaga *et al*. 2012). FPCK-1-1 is an intestinal epithelial cell line established from a tubular adenoma of male patient with familial adenomatous polyposis (Kawaguchi *et al*. 1991) and it was used as a new culture model of intestinal inflammation (Tominaga *et al*. 2012, Tominaga *et al*. 2013). In this model, FPCK-1-1 cells were co-cultured with phorbol myristate acetate (PMA)-stimulated monocytic leukemia THP-1 cells resulting in the reduction of transepithelial electrical resistance (TER) of FPCK-1-1 monolayer cells (Tominaga *et al*. 2012).

In this research, it was found that methanol extracts from wood barks of *D. dulcis*, *P. timoriana*, *P. speciosa*, and *D. dao* had anti-diabetic activities to inhibit α-glucosidase *in vitro* and down-regulate the level of blood glucose in *db/db* mice. Furthermore, they prevented the damage of human colon epithelial FPCK-1-1 cells induced by PMA-stimulated THP-1 cells.

Table 1.1. Medicinal plant species and their reported activities.

No.	Local Name Scientific Name Family	Traditional Utilization*		Literature Review
		Main Use	Medication	
1	Pelanjau *Pentaspadon motleyi* *Anacardiaceae*	Fruits: consumption Wood: construction	Exudate: skin infection (tinea versicolor, ringworm, and rash)	Wood extractive of *P. motleyi* contains phenols and fatty acids (4-(1,1 dimetylpropyl)phenol, 4-(1,1,2,2 tetrametyl-butyl)phenol, nonylphenol isomer, 2-methoxy-4-(1-prophenyl)phenol, 4-nonylphenol, and 2-heptadecyl-6-hidroxy-benzoit acid) and has anti-fungal activity (Yusro *et al.* 2010). Other phytochemical compounds are flavonoids and tannins (Yusro 2011).
2	Asam kemantan *Mangifera foetida* *Anacardiaceae*	Fruits: consumption Wood: construction	Leaves: fever	Fruit of *M. foetida* have anti-oxidant compounds such as flavonoids, carotenoids, and ascorbic acid (Tyug *et al.* 2010, Mirfat *et al.* 2013).
3	Asam bawang, asam kalimantan *Mangifera pajang* *Anacardiaceae*	Fruits and skin fruits: consumption Wood: construction	Wood bark: stomachaches, edema, toothaches Fruits: increase of appetite	Phenolic compounds from fruit peel of *M. pajang* have the free radical scavenging activity (Hassan *et al.* 2011) and contain anti-oxidant carotenoids (Khoo *et al.* 2010).

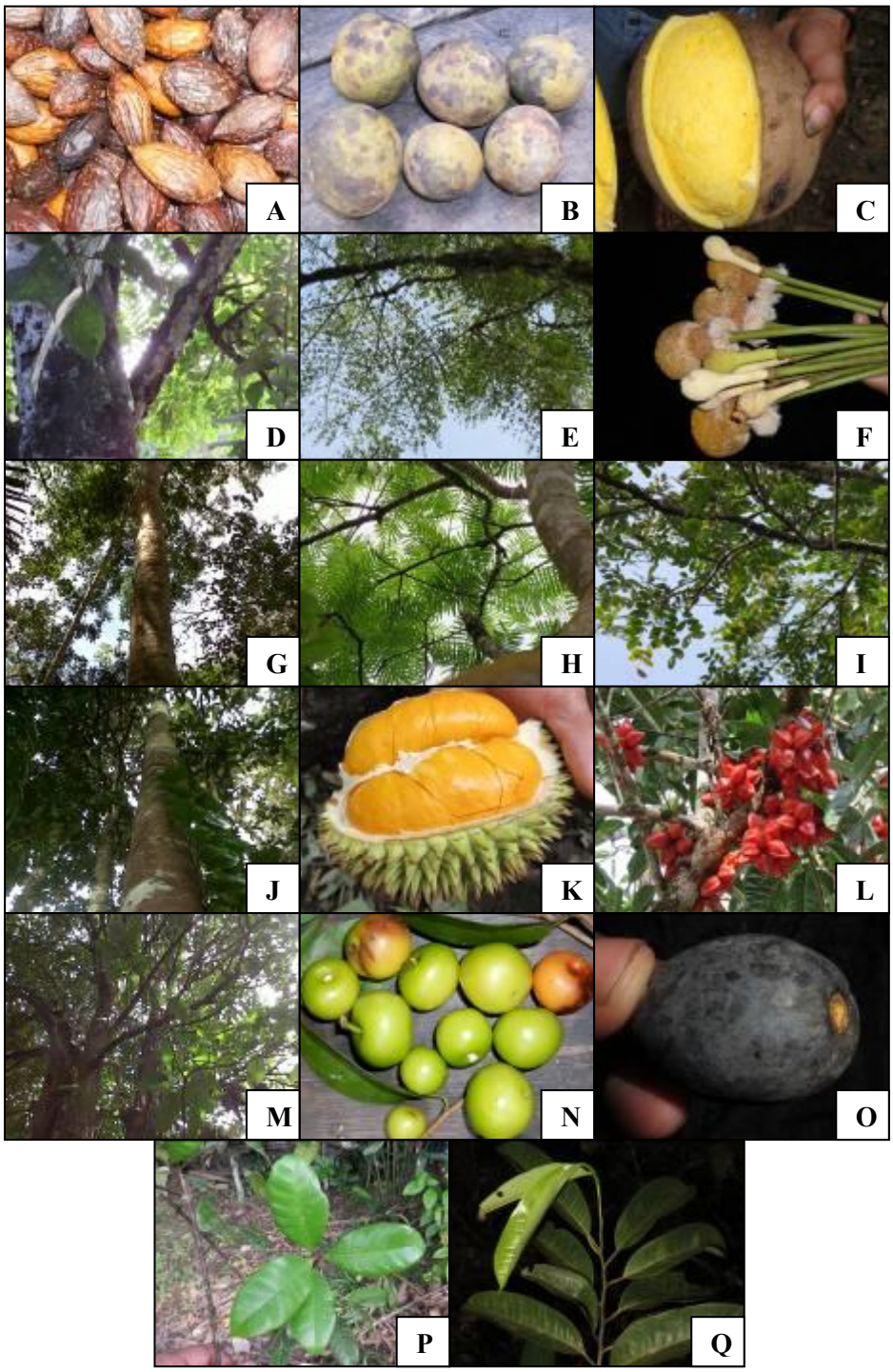
4	Sengkuang *Dracontomelon dao* *Anacardiaceae*	Fruits: consumption Wood: construction	Stem: diarrheas, stomachaches	Leaf extract of *D. dao* has anti-bacterial and anti-fungal compounds such as tannins, flavonoids, sterols, saponins, and triterpenoids (Khan and Omoloso 2002).
5	Selukai *Goniothalamus tapis* *Annonaceae*	-	Wood bark: rheumatism	Goniothalamin as bioactive compound of *G. tapis* has cytotoxic activities against cancer cell lines: P-388 (murine lymphocytic leukemia), KB (human oralnasopharyngal, also known as a subline of the HeLa), Col-2 (human colon cancer), MCF-7 (human breast cancer), Lu-l (human lung cancer), A549 (adenocarcinomic human alveolar basal epithelial cells), T24 (human urinary bladder cancer cells), and ASK (rat glioma cell) (Sangrueng *et al*. 2015). Bioactive compounds of wood bark have strong platelet-activating factor (PAF) receptor binding activity (inhibitors) (Moharam *et al.* 2012).
6	Jatak *Willughbeia angustifolia* *Apocynaceae*	Fruits: consumption	Sap: rash	-
7	Kemayau	Fruits: consumption	Fruits: recovery of	Fruits and seeds contain phenols, flavonoids,

	Dacryodes rostrata *Burseraceae*		stamina	anthocyanins, and potential anti-oxidants (Lim 2012).
8	Asam kandis *Garcinia parvifolia* *Clusiaceae*	Fruits: consumption	Sap: boil, thrush, and skin injury Flowers: treatment of mothers after delivering babies	Extract of *G. parvifolia* has anti-plasmodial, anti-oxidant, cytotoxic and anti-bacterial activities (Syamsudin *et al*. 2007). Bioactive compounds of *G. parvifolia* are porxanthone, porlanosterol, dulxanthone, parvixanthone and rubraxanthone (Kardono *et al*. 2006). Rubraxanthone of *G. parvifolia* has inhibitory effect on platelet-activating factor (PAF) binding to rabbit platelets using 3H-PAF as a ligand (Jantan *et al.* 2002). *G. parvifolia* has antiplatelet aggregation activity in human whole blood (Jantan *et al.* 2009).
9	Petai kedaung *Parkia timoriana* *Fabaceae*	Fruits: consumption	Fruits: stomachaches	All of part plants contain phytosterols (Tisnadjaja 2006), methanol extracts have hepatoprotective activity on paracetamol-induced liver damage in Wistar rats (Ajibola *et al.* 2013)
10	Petai pelepah pendek *Parkia speciosa* *Fabaceae*	Fruits: consumption	Roots: anti-diabetic, Fruits: stomachaches	Stigmast-4-en-3-one from fruit and pod of *P. speciosa* has anti-hyperglycemia activity (Jamaludin and Mohamed 1993, Jamaludin *et al.* 1995). Leaf

				extract of *P. speciosa* has anti-oxidant and anti-ulcer activities (Al Batran *et al.* 2013)
11	Petai pelepah panjang *Parkia intermedia* *Fabaceae*	Fruits: consumption	Roots: anti-diabetic	-
12	Poko utan *Parkia sp.* *Fabaceae*	Wood: construction	Roots: stomachaches	-
13	Petai betawi, sama laki *Adenanthera sp.* *Fabaceae*	Wood: construction	Leaves: constipation	-
14	Durian meranang *Durio dulcis* *Malvaceae*	Fruits: consumption Wood: construction	Inner fruits bark: stomachaches	-
15	Durian pekawai *Durio kutejensis* *Malvaceae*	Fruits: consumption Wood: construction	Inner fruits bark: stomachaches	Wood bark of *D. kutejensis* contains triterpenoids, maslinic acid, arjunolic acid, benzoquinone and fraxidin (Rudiyansyah and Garson 2006), and fruit flesh extracts have anti-oxidants and anti-melanogenesis properties with potential for hypopigmentation and skin lightening agent (Arung

				et al. 2015).
16	Enceriak *Baccaurea costulata* *Phyllanthaceae*	Fruits: consumption	Roots: anti-diabetic	-
17	Belimbing merah *Baccaurea angulata* *Phyllanthaceae*	Fruits: consumption	Roots bark: anti-diabetic	Fruit of *B. angulata* with phenolic compound has anti-oxidant activity (Ahmed *et al.* 2014, Jauhari *et al.* 2013)

* Yusro *et al*. 2013[a,b], Yusro *et al*. 2014[a], Yusro *et al.* 2015: Local people were interviewed while collecting the plant samples (Malay and Java tribes) in Kuala Buayan village, Meliau District, Sanggau Regency, West Kalimantan Province, Indonesia.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Q

Fig. 1.1. Pictures of the parts of medicinal plant species. (A): *P. motleyi* (fruits), (B): *M. foetida* (fruits), (C): *M. pajang* (fruits), (D): *D. dao* (tree), (E): *P. intermedia* (tree), (F): *P. speciosa* (flowers), (G): *P. timoriana* (tree), (H): *Parkia sp.* (tree), (I): *Adenanthera sp.* (tree), (J): *D. dulcis* (tree), (K): *D. kutejensis* (fruits), (L): *B. angulata* (fruits), (M): *B. costulata* (tree), (N): *G. parvifolia* (fruits), (O): *D. rostrata* (fruit), (P): *W. angustifolia* (leaves), and (Q): *G. tapis* (leaves).

1.2. Objectives of the research

The main objective of this research is to find scientific evidences of medicinal plants in West Kalimantan Indonesia as anti-diabetic and anti-intestinal inflammatory reagents. To achieve this goal, following sub-objectives are set:

a. Analyze the efficiency of methanol extractions from wood barks of 17 medicinal plants that belong to the *Anacardiaceae, Fabaceae, Malvaceae, Phyllanthaceae* and other plant families (*Annonaceae*, *Apocynaceae*, *Burseraceae*, *Clusiaceae*) to be used for the following assays.
b. Analyze the inhibitory effects of methanol extracts from wood barks of 17 medicinal plants that belong to the *Anacardiaceae*, *Fabaceae, Malvaceae*, *Phyllanthaceae*, and other plant families (*Annonaceae*, *Apocynaceae, Burseraceae, Clusiaceae*) on yeast α-glucosidase and rat intestinal α-glucosidase.
c. Analyze the cytotoxicity of methanol extracts from wood barks of seven medicinal plants (*D. dulcis*, *D. kutejensis*, *P. timoriana*, *P. speciosa*, *P. intermedia*, *B. angulata*, and *D. dao*) against the normal mouse fibroblast NIH3T3 cells. And analyze the toxicity of methanol extract from the most toxic plant (*P. speciosa*) *in vivo* against C57BL/6J mice.
d. Analyze the regulatory effects of methanol extracts from wood barks of six plants (*D. dulcis*, *D. kutejensis*, *P. timoriana*, *P. speciosa*, *B. costulata*, and *D. dao*) on the levels of blood glucose and triacylglycerol of *db/db* mice.
e. Analyze the activities of methanol extracts from wood barks of five plants (*D. dulcis*, *D. kutejensis*, *P. timoriana*, *P. speciosa*, and *D. dao*) to prevent the damage of human colon epithelial FPCK-1-1 cells.

Chapter 2
Efficiency of methanol extractions from wood barks of 17 medicinal plants

2.1. Introduction

Medicinal plants in West Kalimantan are a part of life for people in rural and remote areas, especially for Dayak tribes. The main medical center is far from their villages and is not equipped well with clinical instruments and drugs. Sometimes, nurses or doctors are not always on standby even in the tertiary medical center. These situations make them to think of the treatment of diseases with medicinal plants from the surrounding nature. Variety of plants used for the treatment of diseases such as skin infection, fever, malaria, diarrhea, stomachaches, broken bone, cancer, diabetes, and etc. Based on the previous research, West Kalimantan has 208 medicinal plant species (Yusro *et al.* 2013[a], Yusro *et al.* 2014[a]). Among them, 68 plant species were used by Dayak tribes in three Regency (Diba *et al.* 2013), 29 species were used by Dayak Kanayant in Kubu Raya Regency (Yusro *et al.* 2013[b]), 33 species were commonly used to reduce the fever by four Dayak subethnic (Yusro *et al.* 2014[b]), 22 species were used by Dayak tribes to treat diabetics, and 13 species were used to treat cancer patients (Yusro *et al.* 2015). Shrubs, herbs, climbers, and trees are the most common life forms of plants that are used to make variety of potions, and plant parts used are leaves, flowers, fruits, roots, and wood bark.

Wood bark is outer part of plants that covers stems, branches, and roots and consists of 10-15% weight of trees (Sjostrom 1981). Major chemical compounds in the wood barks are prepared from extractives. The main function of the extractives from the wood bark of trees is to protect themselves from invading and destroying organisms, by healing wounds of woods. Some of the extracted compounds from barks are the precursors of other chemical compounds in the wood (Rowell *et al*. 2006). Chemical compounds in wood bark have a function as toxin to protect plants and they have a value to human as medicine.

Many compounds can be extracted using polar and non-polar solvents (Hillis 1987). Polar solvents such as methanol, ethanol, and water are frequently used to extract materials from plants. The proportion of extractive contents generally varies depending on the species, part of plants, and solvent used (Sjostrom 1981). Generally, extractive contents from bark are higher than those from woods (Fengel and Wegener 1995).

Extracts from wood barks of all plant species used by Dayak people to treat diabetes, diarrheas, and stomachaches (**Table 1.1**) can be used as anti-diabetic and anti-intestinal inflammatory reagents by showing scientific evidences for the traditional knowledge of Dayak people in West Kalimantan. Before conducting the following assays to obtain scientific evidences for the effectiveness of medicinal plants, the percentage of extractive contents with methanol from wood barks of seventeen medicinal plants were measured: **1.** Cytotoxicity against mouse fibroblast NIH3T3 cells *in vitro*. **2.** Toxicity assay against C57BL/6J mice *in vivo*, **3.** Inhibitory effects on α-glucosidase *in vitro*, anti-diabetic effects in leptin receptor deficient *db/db* mice, **4.** Anti-inflammatory effects to prevent the damage of FPCK-1-1 human colon epithelial cells.

2.2. Materials and Methods

Sample collection

Medicinal plants have been grown in Kuala Buayan Village, Meliau District, Sanggau Regency, and Pala Pulau Village, Putussibau Utara District, Putussibau Regency, West Kalimantan Province, Indonesia. Sixteen plants species (*P. motleyi*, *M. foetida*, *M. pajang*, *D. dao*, *P. timoriana*, *P. speciosa*, *P. intermedia*, *Parkia sp.*, *Adenanthera sp.*, *D. dulcis*, *D. kutejensis*, *B. costulata*, *B. angulata, W. angustifolia*, *D. rostrata*, and *G. parvifolia*) were collected from Kuala Buayan Village and one species (*G. tapis*) was collected from Pala Pulau Village (**Fig. 1.1**).

The plant part collected is wood bark taken from the trunk of the tree one meter high from the ground. The wood barks cleaned from the outer skin to avoid contamination such as a dirt or moss, and small square-shaped pieces like a chip were made and then dried in the air for 4 weeks. Fifty grams of dried wood barks were grounded to get fine powder using an electric grinder (Oster, Sunbeam Products, Inc). They were kept in plastic bags until further use. The voucher specimens of the plants

were made for identifying the scientific name and deposited in the Laboratory of Wood Technology, Tanjungpura University Pontianak, Indonesia.

Extraction

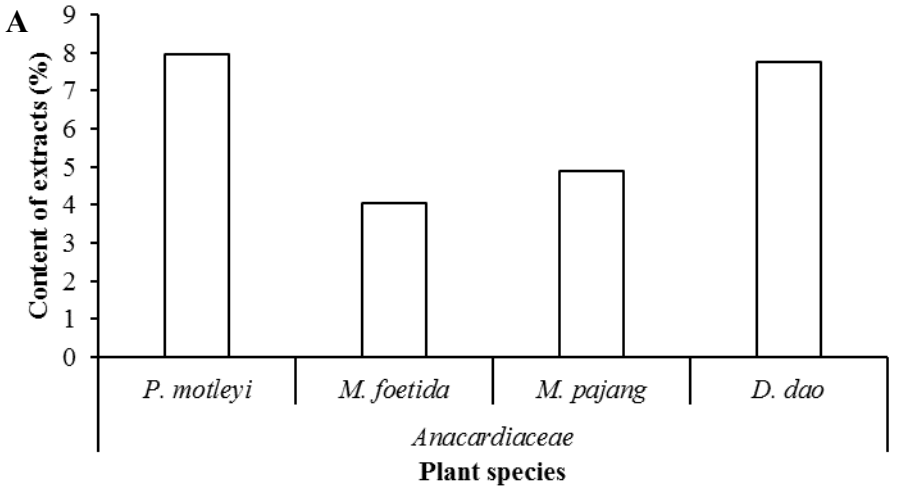
Thirty grams of bark powder were extracted three times with 100 ml of methanol (99.7%) using a soxhlet extractor (Yamato Water Bath BS660, Yamato Scientific Co.Ltd) for 1 hour at temperature 70°C. This extract was filtered through a Whatman filter paper (No. 2) and the filtrate was collected, evaporated in a vacuum rotary evaporator (Eyela N-1000, Tokyo, Japan) at 40°C with a rotary speed at 5 rpm, dried for one day in a wind dryer (Pierce, Reacti-Therm and Reacti-Vap, Thermo Fisher Scientific Inc., Waltham, MA), and dried for one day in a vacuum dryer (Ettas, AVO-250NB, Active Co., Saitama, Japan) to obtain final residues (dry weight). The percentage of the methanol extract contents was calculated by the following equation:

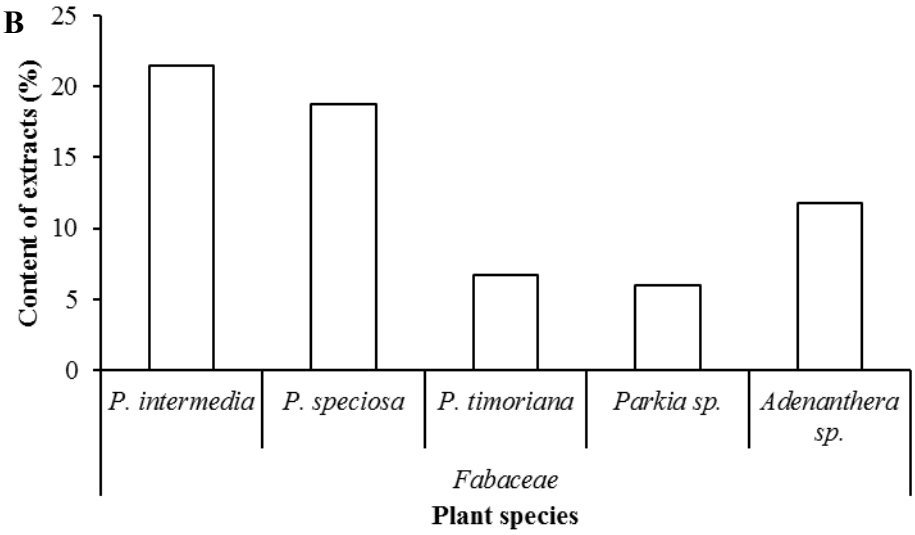
$$\text{Extractive content}\,(\%) = \left(\frac{\text{Weight of extracts}\,(\text{g})}{\text{Weight of bark powder}\,(\text{g})} \right) \text{x}\,100\%$$

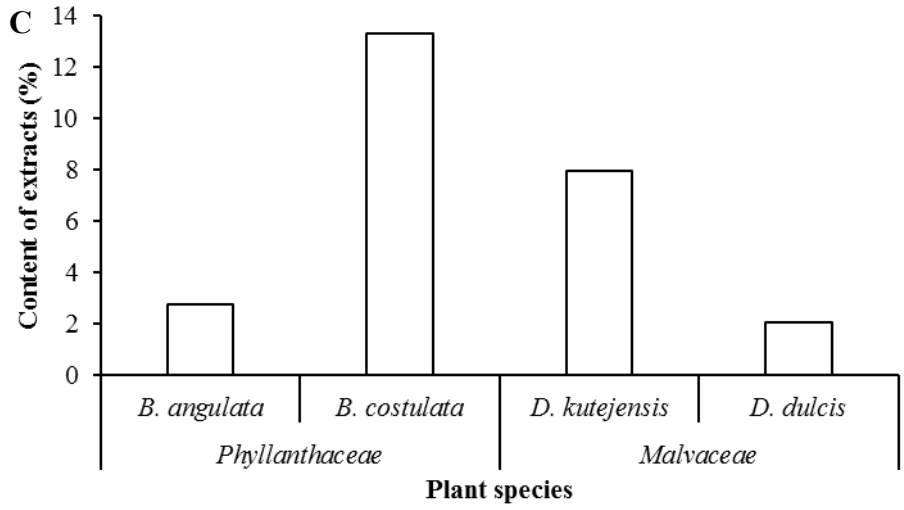
2.3. Results and Discussion

Woody plants composed of cellulose, hemicellulose, lignin, and small amounts of extractives (Rowell *et al*. 2006). Materials in the wood or bark could be extracted using polar and non-polar solvents (Hillis 1987). The proportion of extractive contents generally varies depending on the species, part of plants, and solvent used (Sjostrom 1981). Extractive contents from the bark are higher compared with those from the wood stems (Fengel and Wegener 1995).

In this study, the amount of materials extracted with methanol from wood barks of 17 plants that belong to plant families of *Anacardiaceae* (*P. motleyi*, *M. pajang*, *M. foetida*, and *D. dao*), *Fabaceae* (*P. timoriana*, *P. speciosa*, *P. intermedia*, *Parkia sp*., and *Adenanthera sp*.), *Malvaceae* (*D. dulcis,* and *D. kutejensis*), *Phyllanthaceae* (*B. costulata*, and *B. angulata*), and 4 species that belong to other plant families that are *Annonaceae* (*G. tapis*), *Apocynaceae* (*W. angustifolia*), *Burseraceae* (*D. rostrata*), and *Clusiaceae* (*G. parvifolia*) were measured. Results of the experiments showed that extractive contents vary among species as shown in **Fig. 2.1**.


A
Content of extracts (%)
0
1
2
3
4
5
6
7
8
9
P. motleyi
M. foetida
M. pajang
D. dao
Anacardiaceae
Plant species


B
Content of extracts (%)
0
5
10
15
20
25
P. intermedia
P. speciosa
P. timoriana
Parkia sp.
Adenanthera sp.
Fabaceae
Plant species


C
Content of extracts (%)
0
2
4
6
8
10
12
14
B. angulata
B. costulata
D. kutejensis
D. dulcis
Phyllanthaceae
Malvaceae
Plant species

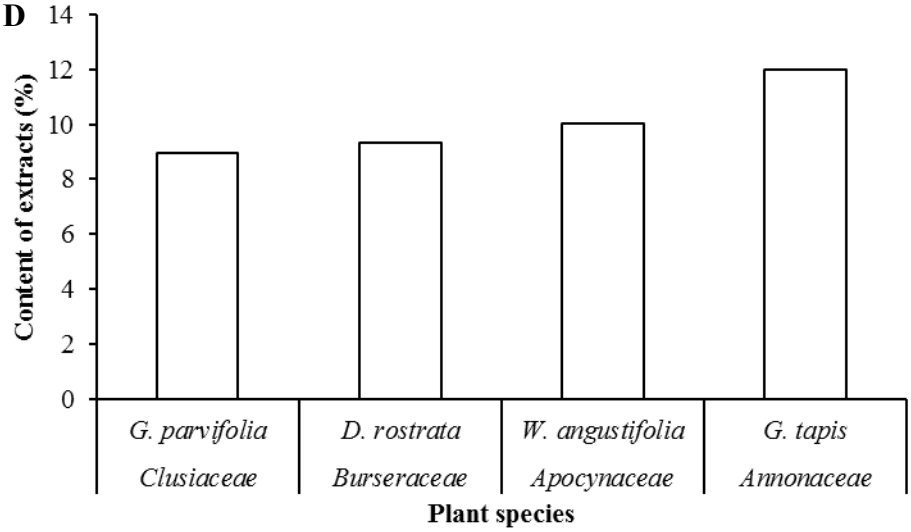


Fig. 2.1. The contents (%) of wood barks methanol extracts. (A) Plant species of *Anacardiaceae*. (B) Plant species of *Fabaceae*. (C) Plant species of *Phyllanthaceae* and *Malvaceae*. (D) Plant species of other plant families (*Annonaceae, Apocynaceae, Burseraceae, Clusiaceae*).

The extractive contents varied from 2.05 to 21.48% among these species. In *Anacardiaceae* plants family, extractive contents varied from 4.05 to 7.96%. The extractive content of *P. motleyi* is the highest and that of *M. foetida* was the lowest in this family. (**Fig. 2.1.A**). In *Fabaceae* family, the extractive contents varied from 5.93 to 21.48% and the plant that had highest extractive content was *P. intermedia,* while that had the lowest extractive content was *Parkia sp*. (**Fig. 2.1.B**). In *Malvaceae* family, the extractive content of *D. kutejensis* was 7.93% and that of *D. dulcis* was 2.05%. In *Phyllanthaceae* family, the extractive content of *B. costulata* was 13.31% and that of *B. angulata* was 2.75% (**Fig. 2.1.C**).

In other plant families, extractive contents varied from 8.94 to 11.97%, in which the highest was *G. tapis* (*Annonaceae*) and the lowest was *G. parvifolia* (*Clusiaceae*) (**Fig. 2.1.D**). Based on the classification of Indonesia classes of wood chemical components (extracted contents: < 2%; lower, 2-4%; moderate, and > 4%; higher) (Departemen Pertanian 1976). Almost all of the species have extractive contents more than 4%. Especially, *P. intermedia* (*Fabaceae*) had 21.48%. In contrast, *D. dulcis* (*Malvaceae*) and *B. angulata* (*Phyllathaceae*) had 2.05 and 2.75% content, respectively.

The proportion of extractive contents generally varies depending on the species. Extractive contents range from less than 1% to more than 10% and those of tropical wood species were around 20% (Tsoumis 1991). Different parts of the same tree such as branches, stems, roots, bark, and leaves usually have differences at the levels and in the composition of the amounts of extractive contents (Sjostrom 1981).

The technique most frequently used to recover the plant extracts is solvent extraction (Anwar and Przybylski 2012). Methanol was used as the solvent to extract materials from wood barks because of its high efficiency to extract materials. Methanol efficiently penetrates in the cell membrane. So, it is the ideal solvent to obtain endocellular components (Silva *et al.* 1998). Extraction process using polar solvent such as methanol, ethanol, and water are very effective to isolate bioactive compounds (Filho 2006). Methanol is an efficient solvent to extract lower molecular weight polyphenols (Anwar and Przybylski 2012, Do *et al.* 2014) and anti-oxidant compounds (Boeing *et al*. 2014).

Many of the bioactive compounds from species, especially those from wood barks examined in this study are not yet identified. In case of methanol extracts, however, some of compounds are identified such as oils, fats, waxes, alkaloids, flavones, polyphenols, tannins, saponins, glycosides, and aglycones (Houghton and Raman 1998, Filho 2006). Based on the **Table 1.1**, compounds extracted from another parts (fruits, leaves, wood) of plants are known to have bioactive compounds as medicine. So, extracted materials from wood barks are expected to have bioactive compounds and allegedly have ability to inhibit α-glucosidase *in vitro* and regulate the levels of blood glucose and triacylglycerol of *db/db* mice. These materials may prevent the damage human colon epithelial FPCK-1-1 cells.

Many kinds of bioactive compounds isolated from secondary metabolites of medicinal plants have high inhibitory effects on α-glucosidase activity such as alkaloids, phenolics, flavonoids, isoflavones, flavonolignans, flavanones, flavonols, anthocyanins, anthraquinones, anthrones, xanthones, glycosides, feruloylglucosides, acetophenone glucosides, stilbenoids, terpenoids, triterpenoids, curcuminoids, lignan, acids, phytosterols, and myoinositol (Benalla *et al.* 2010, Kumar *et al*. 2011).

Methanol extracts from wood barks have a large amount of phenolic constituents such as flavanoid compounds including a group of condensed tannins (phenolic acids)

and monomers of flavonoids such as quercetins and dihydroquercetins (taxifolins) (Sjostrom 1981). Tadera *et al.* (2006) reported that six groups of flavonoid compounds especially flavonol, flavanone, isoflavone and anthocyanidin effectively inhibit yeast α-glucosidase. Jo *et al.* (2010) reported quercetin compounds have high inhibitory activity against rat intestinal maltose digesting enzymes.

Phenols and flavanoids have ability to control the expression level of proinflammatory cytokines such as IL-1, IL-6, IL-10, and TNF-α (Debnath *et al*. 2013) and quercetins have the ability to regulate the level of blood glucose after starvation through increasing the insulin sensitivity by inhibiting α-glucosidase and enhancing the insulin signaling in *db/db* mice (Kim *et al*. 2011).

2.4. Conclusions

The efficiency of methanol extraction of wood barks from 17 plant species that belong to *Anacardiaceae*, *Fabaceae*, *Malvaceae*, *Phyllanthaceae*, and other plant families were measured. Percentages of methanol extractives from wood barks of 17 plant species are varied from 2.05 to 21.48% among these species. Almost all of the species belong to the high category of extractive contents. Especially, *P. intermedia* (*Fabaceae*) had the extractive content of 21.48%. The extractive contents of *D. dulcis* (*Malvaceae*) and *B. angulata* (*Phyllathaceae*) were 2.05 and 2.75%, respectively, belong to a moderate category. It is highly possible that methanol extracts from wood barks may have non-toxic bioactive compounds in terms of inhibiting α-glucosidase *in vitro*, regulating the levels of blood glucose and triacylglycerol of *db/db* mice, and preventing the damage of human colon epithelial FPCK-1-1 cells.

Chapter 3

In vitro α-glucosidase inhibition by methanol extracts from wood barks of 17 medicinal plants

3.1. Introduction

High blood sugar level is one indicator of disruption of metabolic systems that refers to diabetes and induce others complication diseases such as neuropathy, retinopathy, stroke, and ulcers (Balai Penelitian dan Pengembangan Kesehatan 2013, Kaskoos 2013, Novo Nordisk 2013). The control of postprandial blood sugar levels by delaying the glucose absorption is one of the ways that can be used as a therapeutic approach to treat diabetes.

Alpha-glucosidase is a key enzyme that plays an important role in the digestion of carbohydrates (Wu *et al.* 2012). In the epithelial mucosa of the small intestine, α-glucosidase enzymes such as maltase and sucrase break the glycoside bonds of the complex carbohydrates to make mono- and disaccharides that can be absorbed by intestinal epithelial cells. Inhibition of α-glucosidase in the digestive system will delay the digestion of carbohydrates and decrease the rate of glucose absorption, resulting in the reduction of blood glucose level (Jaiswal *et al.* 2012).

An *in vitro* model of α-glucosidase inhibition is frequently used for the preliminary screening of the anti-diabetic effects of secondary metabolites from medicinal plants. The *in vitro* assay in ethno-pharmacological research is beneficial because of lower cost, less required materials, the reduction of animal use, rapid measurement, and lower level of variability compared with animal experiments (Soumyanath and Srijayantha *et al*. 2006).

Yeast and intestinal acetone powder from rat are a source of α-glucosidase enzymes that are used in the *in vitro* assay of α-glucosidase inhibition. These enzymes are different in their substrate specificity (Tadera *et al.* 2006). Yeast α-glucosidase derived from *Saccharomyces cerevisiae* (*S. cerevisiae*) contain maltase enzyme (Yamamoto *et al.* 2004), and mammalian small intestine α-glucosidases in rat intestinal acetone powder contain maltase-glucoamylase (MG) and sucrase-isomaltase (SI)

enzymes (Asano 2003, Ng *et al*. 2015). Para-nitrophenyl-α-D-glucopyranoside (pNPG) is a substrate that was used to analyze the maltase activity (Chapdelaine *et al.* 1978) in yeast and intestinal acetone powder from rat. The other substrate that was used in most studies to examine the inhibitory activity of the rat intestinal α-glucosidase is sucrose (Tadera *et al.* 2006).

In this study, the effectiveness of methanol extracts of 17 plants on the inhibition of yeast α-glucosidase using pNPG as a substrate (yeast maltase), rat intestinal α-glucosidase (rat intestinal maltase) using pNPG as a substrate, and rat intestinal sucrase using sucrose as a substrate were analyzed. All plants that were analyzed belong to the families of *Anacardiaceae* (*P. motleyi*, *M. pajang*, *M. foetida*, and *D. dao*), *Fabaceae* (*P. timoriana*, *P. speciosa*, *P. intermedia*, *Parkia sp*., and *Adenanthera sp*.), *Malvaceae* (*D. dulcis*, and *D. kutejensis*), *Phyllanthaceae* (*B. costulata*, and *B. angulata*), and 4 species from other plant families that are *Annonaceae* (*G. tapis*), *Apocynaceae* (*W. angustifolia*), *Burseraceae* (*D. rostrata*), and *Clusiaceae* (*G. parvifolia*). Differences in enzymes and substrate sources affect the inhibitory effects on α-glucosidase activities (Tadera *et al.* 2006, Jo *et al.* 2010). In this study, it was found that several of these plants have inhibitory effects on yeast α-glucosidase (maltase) and rat intestinal sucrase.

3.2. Materials and Methods

Plants material

Methanol extracts from wood barks of 17 medicinal plants were prepared as described in a previous chapter (**Chapter 2**). One mg of methanol extract (dry weight) was dissolved in 1 ml of methanol (stock solution), and diluted with phosphate buffer (PB) 0.1 mol/l (pH 6.8) to make a series of different concentrations.

Chemicals and reagents

Para-nitrophenyl-α-D-glucopyranoside (pNPG) was purchased from Sigma-Aldrich (St Louis, MO, USA). Sucrose was purchased from Junsei Chemical Co., Ltd (Tokyo, Japan). Alpha-glucosidase from yeast (*Saccharomyces cerevisiae*) EC.3.2.1.20 was purchased from Wako Chemicals (Osaka, Japan). Rat intestinal acetone powder

was purchased from Sigma-Aldrich (St Louis, MO, USA). Glucose C-II Test kit was purchased from Wako Chemicals (Osaka, Japan).

Alpha-glucosidase assays

a. Yeast (*S. cerevisiae*) α-glucosidase activity with pNPG as a substrate

The yeast α-glucosidase activity measured according to Bothon *et al.* (2013) with slight modification. The assay was performed by adding 10 µl of phosphate buffer 0.1 mol/l (pH 6.8), 150 µl of *p*-nitrophenyl-α-D-glucopyranoside (pNPG) (5 mmol/ml), 20 µl of methanol extracts from each plant with varying concentrations (200, 20, 15, 10, 5, 2, 1 µg/ml), and 20 µl of α-glucosidase enzyme solution (5 µg/ml) in each well of 96 well plates. The change in absorbance (Multiskan JX, Thermo Electron Co., Waltham, MA) at 405 nm was recorded at an interval of one minute for 10 minutes. All measurements were made in triplicate. The α-glucosidase inhibitory activity was calculated with the following equation:

$$\text{Inhibition}\,(\%) = \left(1 - \frac{\text{Slope of absorbance treatment}}{\text{Slope of absorbance control}}\right) \text{x}\,100\%$$

The half maximal inhibitory concentration (IC_{50}) value was calculated using a linear regression analysis. IC_{50} is the concentration of the plant extract required to inhibit 50% of α-glucosidase activity under the assay condition. At the end of this Chapter, the method of calculation of inhibition (%) and IC_{50} were described (Motulsky and Christopoulus 2004, Zhang *et al*. 2015).

b. Rat intestinal maltase activity with pNPG as a substrate (rat intestinal maltase)

Five hundred mg of rat intestinal acetone powder were dissolved in 5 ml of 0.1 M phosphate buffer at pH 6.8 and then centrifuged at 2500 rpm for 5 minutes. The supernatant was used as a maltase (α-glucosidase) solution. The maltase inhibition assay was performed using a 96 well plate. The assay was performed by adding 10 µl of 0.1 mol/l phosphate buffer (pH 6.8), 150 µl of *p*-nitrophenyl-α-D-glucopyranoside (pNPG) (5 mmol/ml), 20 µl of methanol extracts from each plant (500 µg/ml), and 20 µl of α-glucosidase enzyme solution (5 µg/ml) in each well of 96 well plates. The change in absorbance (Multiskan JX, Thermo Electron Co., Waltham, MA) at 405 nm was recorded at an interval of one minute for 10 minutes. All measurements were made in triplicate. The maltase inhibitory activity was calculated with the following equation:

$$\text{Inhibition}(\%) = \left(1 - \frac{\text{Slope of absorbance treatment}}{\text{Slope of absorbance control}}\right) \text{x } 100\%$$

At the end of this Chapter, the method of calculation of inhibition (%) were described (Zhang *et al*. 2015).

c. Rat intestinal sucrase activity with sucrose as a substrate (rat intestinal sucrase)

The rat intestinal sucrase activity was determined according to Ikarashi *et al.* (2011) with slight modifications. Five hundred mg of rat intestinal acetone powder were dissolved in 5 ml of 0.1 M phosphate buffer (pH 6.8) and then centrifuged at 2500 rpm for 5 minutes. The supernatant was used as a sucrase (one of the α-glucosidase) solution. The sucrase inhibition assay was performed using a 96 well plate. Each well of the 96 well plate contains 10 µl of 0.1 M phosphate buffer (pH 6.8), 150 µl sucrose (5 mg/ml), 20 µl of methanol extract from each plant with two concentrations (500 and 1000 µg/ml), and 20 µl of sucrase enzyme solution (100 mg/ml). Reaction mixture was incubated in an incubator (Low Temperature O_2/CO_2 Incubator, Wakenyaku, Japan) at temperature 37°C for 30 minutes and heated up in the Cool-Hotter Dry Bath Incubator (MS, Major Science) at temperature 70°C for 3 minutes. Twenty µl of each reaction mixture was transferred to another well of a 96 well plate. To detect glucose, 150 µl of glucose C-II test solution was added to each well and the plate was incubated at room temperature for 15 minute. The change in absorbance (Multiskan JX, Thermo Electron Co., Waltham, MA) at 492 nm was recorded one minute after starting the incubation. All measurements were performed in triplicate. The sucrase inhibitory activity was calculated with the following equation:

$$\text{Inhibition}(\%) = \left(1 - \frac{\text{Absorbance treatment} - \text{Absorbance blank}}{\text{Absorbance control} - \text{Absorbance blank}}\right) \text{x } 100\%$$

IC_{50} value was calculated using a linear regression analysis. At the end of this Chapter, the method of calculation of inhibition (%) and IC_{50} were described (Motulsky and Christopoulus 2004, Zhang *et al*. 2015).

3.3. Results and Discussion

In this study, as described in **Table 1.1,** the effects of methanol extracts from wood barks of 17 plant species from *Anacardiaceae*, *Fabaceae*, *Malvaceae*, *Phyllanthaceae*, and other plant families (*Annonaceae*, *Apocynaceae*, *Burseraceae*, *Clusiaceae*) were examined on the inhibition of the α-glucosidase which is the target of the therapy for type 2 diabetes. High blood glucose levels in diabetic patients are induced by the rapid absorption of glucose derived from maltose and sucrose digested by α-glucosidase in the small intestine, and the inhibition of these enzymes will decrease the blood sugar level (Jo *et al.* 2010). In this report, three kinds of enzymes of α-glucosidase were examined. In case of yeast maltase, pNPG was used as a substrate. The activity of rat maltase in the intestinal acetone powder was measured by using pNPG as a substrate. Rat sucrase in the intestinal acetone powder was examined by using sucrose as a substrate.

3.3.1. Inhibition of α-glucosidase by the methanol extracts from *Anacardiaceae* plants family

Results show that different sources of enzymes and methods affect the inhibition of α-glucosidase by the extracts from *Anacardiaceae* plants family. The higher inhibition levels were shown against yeast α-glucosidase compared with rat intestinal maltase and rat intestinal sucrase. The complete results are described in **Fig. 3.1.**

As shown in **Fig. 3.1.A**, all methanol extracts from *Anacardiaceae* family have strong activity to inhibit yeast α-glucosidase. Methanol extract from *D. dao* inhibited 66.14% and 90.29% of the enzyme activity at the concentration of 5 μg/ml and 10 μg/ml, respectively. This activity is higher than those from *P. motleyi* (10 μg/ml: 62.01%), *M. pajang* (10 μg/ml: 43.09%) or *M. foetida* (10 μg/ml: 23.90%). In **Fig. 3.1.B**, all extracts have lower inhibitory activities against rat intestinal maltase compared with those on yeast α-glucosidase, varying from 15.43 to 18.2%. As presented in **Fig. 3.1.C**, methanol extract only from *P. motleyi* inhibited 52.82% of the rat intestinal sucrase activity at the concentration of 1000 μg/ml, whereas methanol extracts from other species inhibited less than 50% of the enzyme activity.

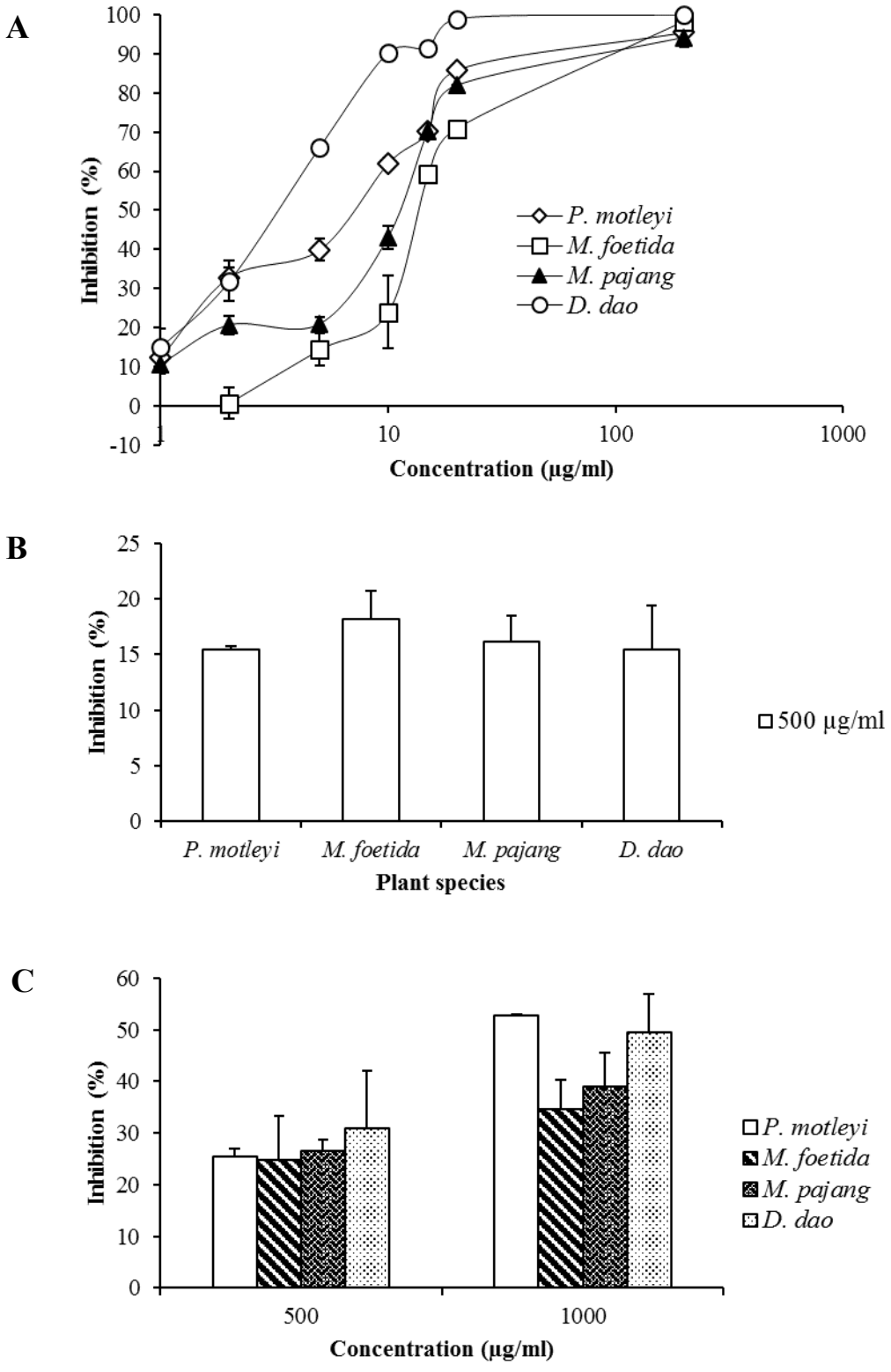
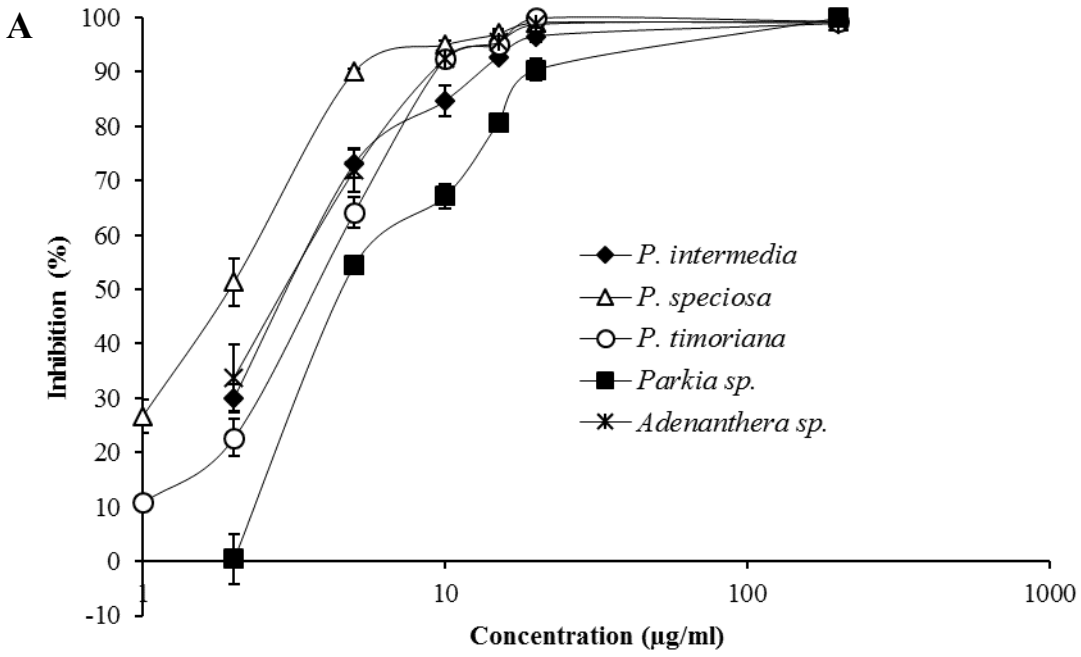


Fig. 3.1. Inhibition of α-glucosidase by methanol extracts from the wood barks of *Anacardiaceae* plants family. (A) Inhibition against yeast α-glucosidase

(maltase). (B) Inhibition against rat intestinal maltase. (C) Inhibition against rat intestinal sucrase. The data are shown as mean ± Standard Error (SE) (n=3).

3.3.2. Inhibition of α-glucosidase by the methanol extracts from *Fabaceae* plants family

Five species of *Fabaceae* plants family were investigated in terms of α-glucosidase inhibiting activity. Results show that the higher levels of inhibition were observed against yeast α-glucosidase compared with rat intestinal maltase and rat intestinal sucrase. The results are shown in **Fig. 3.2**.



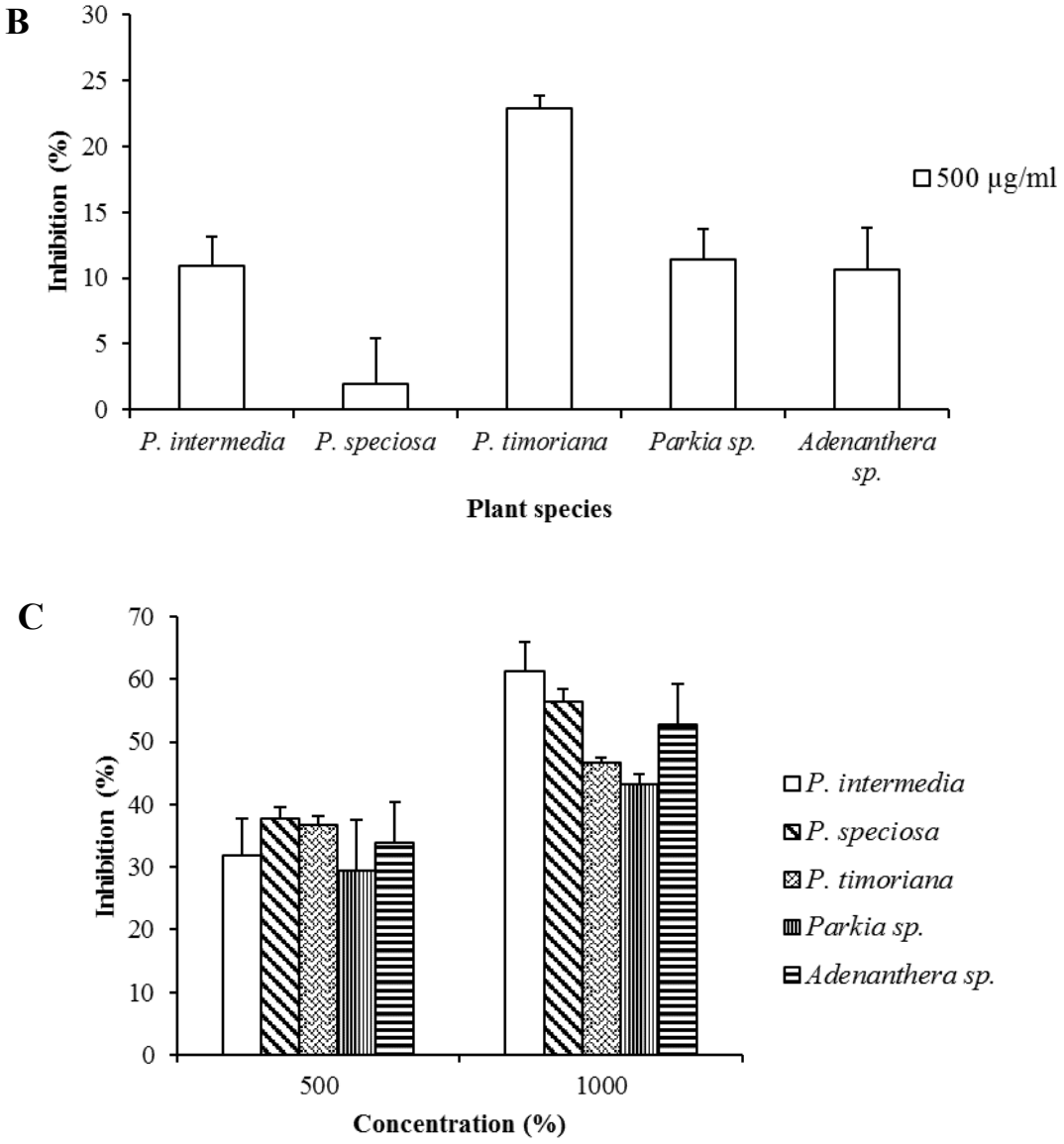


Fig. 3.2. Inhibition of α-glucosidase by methanol extracts from the wood barks of *Fabaceae* plants family. (A) Inhibition against yeast α-glucosidase. (B) Inhibition against rat intestinal maltase. (C) Inhibition against rat intestinal sucrase. The data are shown as mean ± SE (n=3).

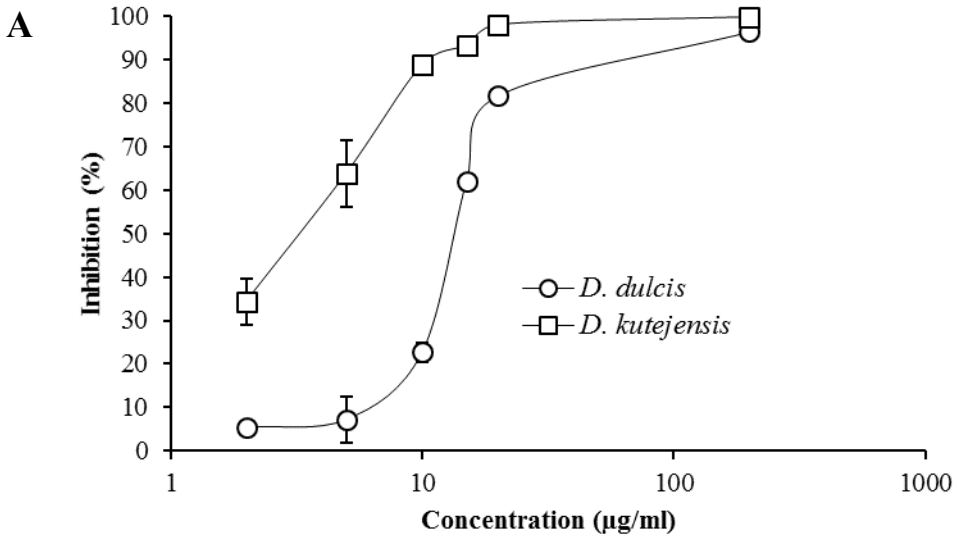
In **Fig. 3.2.A**, methanol extracts from the wood barks of *Fabaceae* plants family showed a strong activity to inhibit yeast α-glucosidase. *P. speciosa* extracts inhibited 51.36% of the enzyme activity at a concentration of 2 µg/ml. This inhibitory activity is higher than that of *P. intermedia* (5 µg/ml: 73.08%), *Adenanthera sp.* (5 µg/ml:

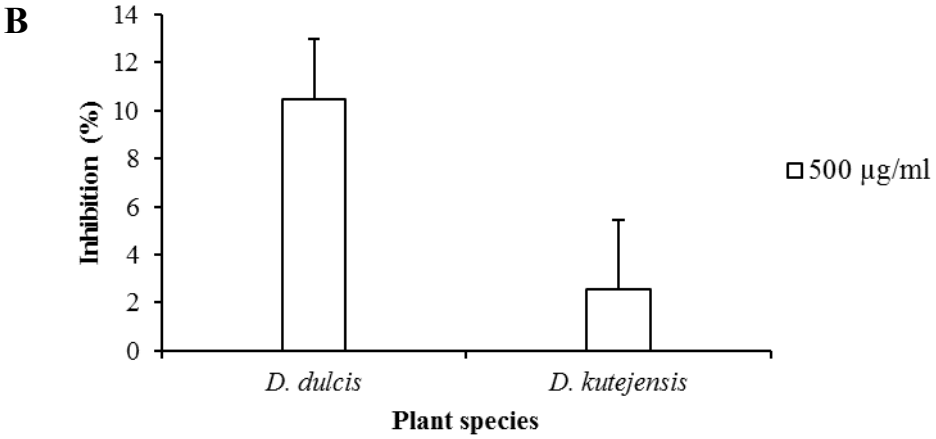
71.89%), *P. timoriana* (5 µg/ml: 64.11%), and *Parkia sp.* (5 µg/ml: 54.48%). However, in **Fig. 3.2.B**, methanol extracts of all species have low inhibitory effects on rat intestinal maltase with the level of inhibition ranging from 1.99 to 22.86%. The methanol extract of *P. timoriana* showed highest inhibition against rat intestinal maltase. As shown in **Fig. 3.2.C**, at a concentration of 1000 µg/ml, methanol extracts from *P. intermedia*, *P. speciosa*, and *Adenanthera sp*. inhibited 61.29%, 56.32%, and 52.82% of the rat intestinal sucrase activity, respectively. The methanol extract from *Parkia sp*. showed lowest inhibitory effect on rat intestinal sucrase.

3.3.3. Inhibition of α-glucosidase by the methanol extracts from *Malvaceae* plants family

Two species of *Malvaceae* plants family from genus of *Durio* were examined. Results show that the higher inhibition levels were shown against yeast α-glucosidase compared with rat intestinal maltase and rat intestinal sucrase. The results were presented in **Fig. 3.3**.

As shown in **Fig. 3.3.A**, methanol extract from the wood bark of *D. kutejensis* inhibited 63.82% of the yeast α-glucosidase activity at a concentration of 5 µg/ml and that from *D. dulcis* inhibited 61.90% of the enzyme activity at a concentration of 15 µg/ml. As shown in **Fig. 3.3.B**, methanol extracts from *D. dulcis* and *D. kutejensis* inhibited 10.46% and 2.56% of rat intestinal maltase at a concentration of 500 µg/ml. As shown in **Fig. 3.3.C**, methanol extracts from *D. kutejensis* and *D. dulcis* inhibited less than 50% of rat intestinal sucrase at a concentration of 1000 µg/ml.


A
100
90
80
70
60
50
40
30
20
10
0
Inhibition (%)
D. dulcis
D. kutejensis
1
10
100
1000
Concentration (µg/ml)


B
14
12
10
8
6
4
2
0
Inhibition (%)
500 µg/ml
D. dulcis
D. kutejensis
Plant species

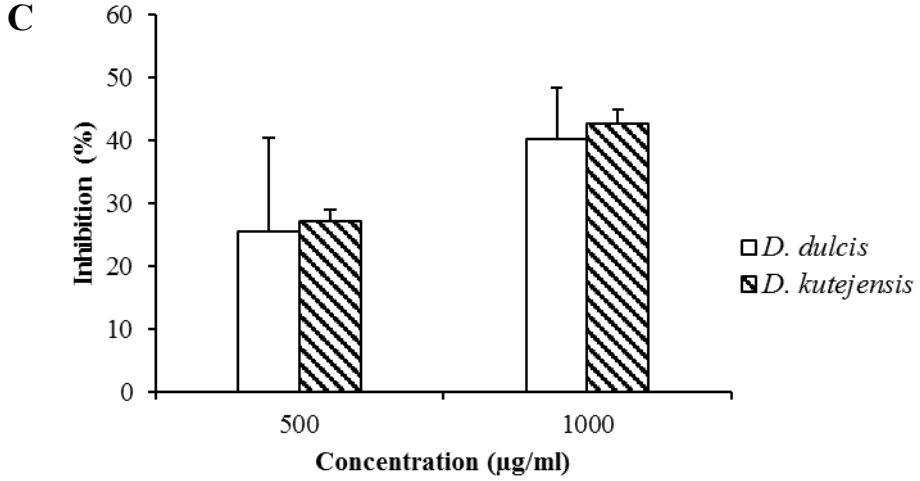
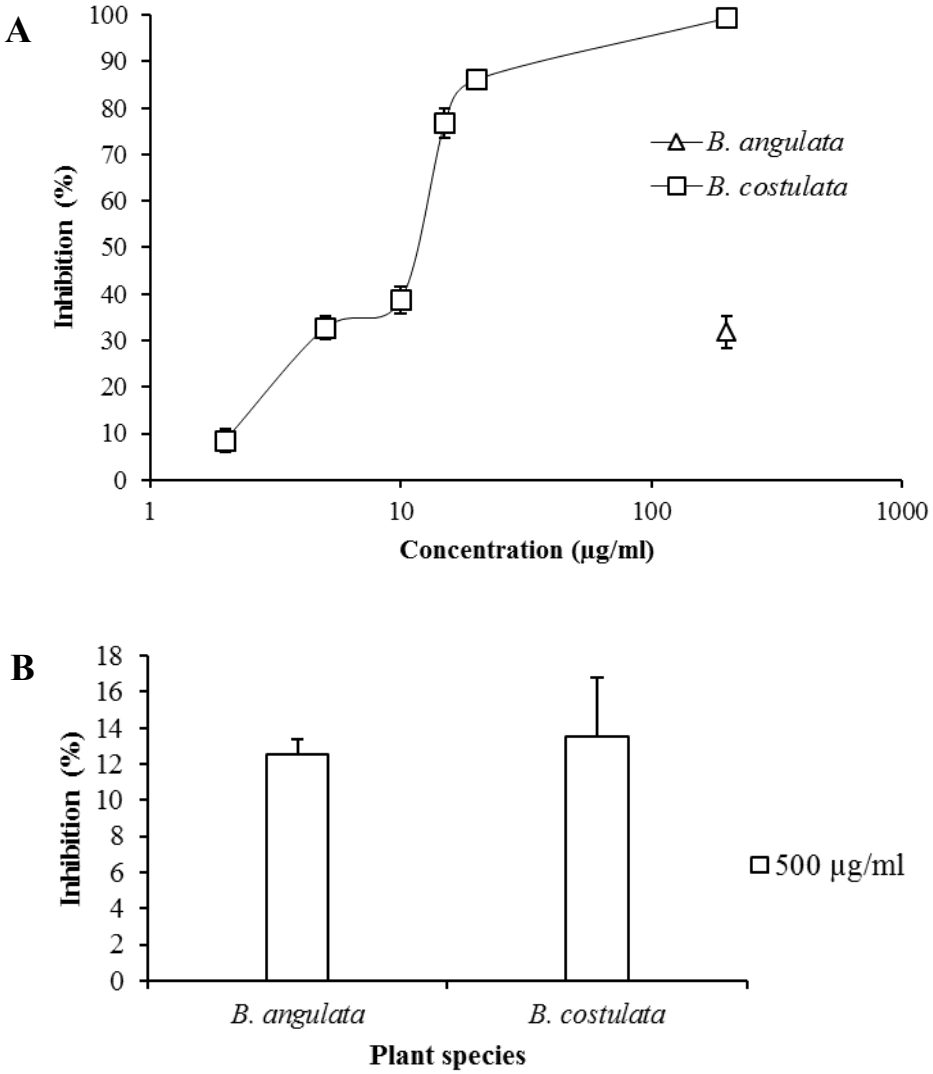

C
60
50
40
30
20
10
0
Inhibition (%)
D. dulcis
D. kutejensis
500
1000
Concentration (µg/ml)

Fig. 3.3. Inhibition of α-glucosidase by methanol extracts from the wood barks of *Malvaceae* plants family. (A) Inhibition against yeast enzyme. (B) Inhibition against rat intestinal maltase. (C) Inhibition against rat intestinal sucrase. The data are shown as mean ± SE (n=3).

3.3.4. Inhibition of α-glucosidase by the methanol extracts from *Phyllanthaceae* plants family

Two species of *Phyllanthaceae* plants family from genus of *Baccaurea* were examined. Only *B. costulata* showed higher level of inhibition against yeast α-glucosidase and rat intestinal sucrase. The results are presented in **Fig. 3.4**.



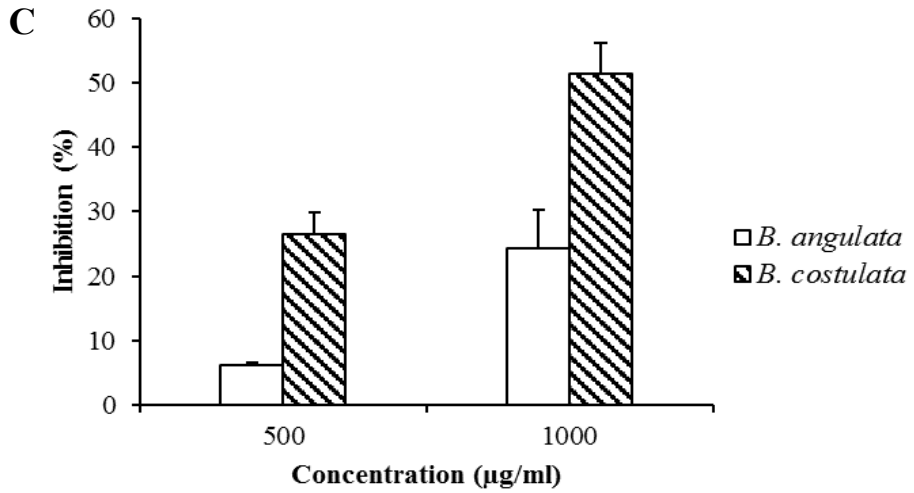


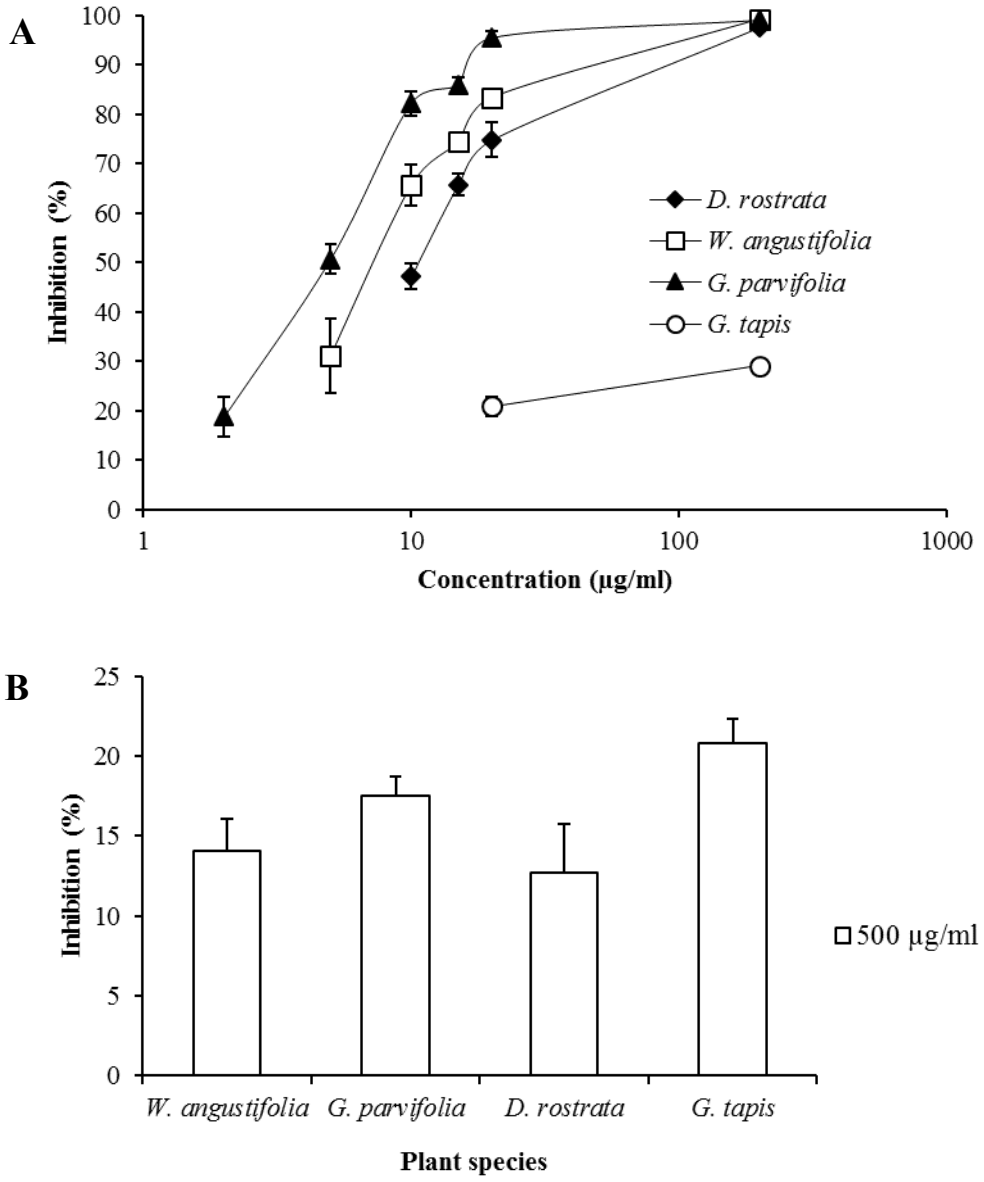
Fig. 3.4. Inhibition of α-glucosidase activity by methanol extracts from the wood barks of *Phyllanthaceae* plants family. (A) Inhibition against yeast enzyme. (B) Inhibition against rat intestinal maltase. (C) Inhibition against rat intestinal sucrase. The data are shown as mean ± SE (n=3).

As shown in **Fig. 3.4.A**, methanol extract from the wood bark of *B. costulata* inhibited the 76.81% of the yeast α-glucosidase activity at a concentration of 15 µg/ml. In contrast, that from *B. angulata* inhibited 31.81% of the enzyme activity at a concentration of 200 µg/ml. As shown in **Fig. 3.4.B**, methanol extracts from *B. costulata* and *B. angulata* inhibited 13.5% and 12.56% of the rat intestinal maltase, respectively at a concentration of 500 µg/ml. As shown in **Fig. 3.4.C**, methanol extracts from *B. costulata* and *B. angulata* inhibited 51.36% and 24.21% of rat intestinal sucrase, respectively at a concentration of 1000 µg/ml.

3.3.5. Inhibition of α-glucosidase by the methanol extracts from other plant families

Four species, *G. tapis* (*Annonaceae*), *W. angustifolia* (*Apocynaceae*), *D. rostrata* (*Burseraceae*) and *G. parvifolia* (*Clusiaceae*) were examined for their inhibitory activities of α-glucosidase. Results show that the higher levels of inhibition were

observed against yeast α-glucosidase compared with rat intestinal maltase and rat intestinal sucrase. The results are presented in **Fig. 3.5**.



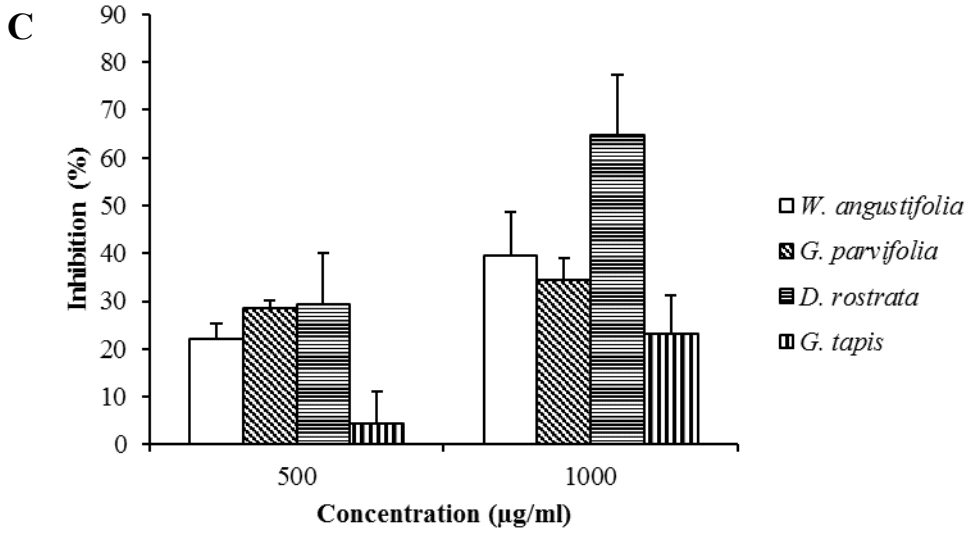


Fig. 3.5. Inhibition of α-glucosidase activity by methanol extracts from the wood barks of other plant families. (A) Inhibition against yeast enzyme. (B) Inhibition against rat intestinal maltase. (C) Inhibition against rat intestinal sucrase. The data are shown as mean ± SE (n=3).

As presented in **Fig. 3.5.A**, there are three plant species that have strong activity to inhibit yeast α-glucosidase. Methanol extract from *G. parvifolia* inhibited 82.25% of the enzyme at a concentration of 10 µg/ml. This activity is higher than those from *W. angustifolia* (10 µg/ml: 65.66%) and *D. rostrata* (10 µg/ml: 47.22%). The methanol extract of *G. tapis* inhibited 29.09% of yeast α-glucosidase activity at a concentration of 200 µg/ml. As shown in **Fig. 3.5.B**, all species showed low levels of inhibition against rat intestinal maltase ranging from 12.74 to 20.81%. As shown in **Fig. 3.5.C**, the methanol extract only from *D. rostrata* inhibited 64.65% of the rat intestinal sucrase activity at a concentration of 1000 µg/ml, whereas other species inhibited less than 50% of the enzyme activity.

Differences of extractive substances in each extract from individual plant species affect the level of inhibition of α-glucosidase. It is suggested that higher the level concentration of bioactive compound results in higher level of inhibition of α-glucosidase activity. According to Kardono (2003), various level of α-glucosidase inhibition in medicinal plant is due to differences in bioactive compounds contained in a plant.

Yeast α-glucosidase enzyme was more susceptible than rat intestinal maltase and rat intestinal sucrase to methanol extracts from wood barks of *Anacardiaceae*, *Fabaceae*, *Malvaceae*, *Phyllanthaceae*, and other plant families.

A commercial anti-diabetic drug, acarbose has high inhibitory activity against mammalian α-glucosidase and low inhibitory activity against yeast α-glucosidase, and quercetin has a significant ability to inhibit yeast α-glucosidase (Eyla *et al.* 2012). The IC_{50} of acarbose against yeast α-glucosidase and rat intestinal maltase using pNPG substrate are 133 μg/ml and 10.7 μg/ml, respectively (Sancheti *et al*. 2011). The IC_{50} of acarbose against rat intestinal sucrase is 0.49 μg/ml (Ikarashi *et al*. 2011). Results of this research showed that methanol extracts from almost all the species have high inhibitory effects on yeast α-glucosidase activity except that from *B. angulata* (*Phyllanthaceae*) and *G. tapis* (*Annonaceae*). In contrast, methanol extracts from all plant species showed low levels of inhibition against rat intestinal maltase, and those from some species showed higher level of inhibition against rat intestinal sucrase compared with rat intestinal maltase. *Fabaceae, Phyllanthaceae, Anacardiaceae* and *Burseraceae* showed inhibitory activities above 50% against rat intestinal sucrase at a concentration of 1000 μg/ml.

Yeast α-glucosidase enzyme is a pure enzyme that is available for screening anti-diabetic inhibitors (Hogan *et al*. 2010). Yeast (*S. cerevisiae*) contains two α-glucosidases, α-1,4-glucosidase (E.C. 3.2.1.20, maltase) and oligo-1,6-glucosidase (E.C. 3.2.1.10, isomaltase), and these enzyme will react with the substrates of maltose, isomaltose and α-D-glucopyranoside (Yamamoto *et al*. 2004). In this study, E.C. 3.2.1.20 that contain only maltase was used. Mammalian α-glucosidase source such as rat intestinal acetone powder has more complex enzyme including that are maltase-glucoamylase (MG) and sucrase-isomaltase (SI) (Ng *et al.* 2015). In these cases, pure yeast α-glucosidase enzyme seems to be more sensitive than complex enzymes to methanol extracts from plants. This insensitivity of complex enzymes to methanol extracts from plants may be caused by other enzymes in rat intestine besides maltase and sucrase to hydrolyze the substrates such as pNPG or sucrose, developing color as an indicator of the enzyme activity (Hogan *et al*. 2010). This may result in the lower level inhibition of rat intestinal α-glucosidase.

Based on terminal domains, small intestinal α-glucosidase is divided in four types that are N-terminal membrane-proximal domain maltase-glucoamylase (ntMGAM; maltase), C-terminal luminal domain maltase-glucoamylase (ctMGAM; glucoamylase), N-terminal membrane-proximal domain sucrase-isomaltase (ntSI; isomaltase), and C-terminal luminal domain sucrase-isomaltase (ctSI; sucrase) (Lee *et al*. 2016, Sim *et al*. 2010). Substrate specificities of these four types of small intestinal α-glucosidases for pNPG, maltose, and sucrose are different, and are summarized in **Table 3.1**. The differentiation of substrate specificities of enzymes is expressed as the catalytic efficiency (K_{cat}/K_m) (Berg 2007). For example, the K_{cat}/K_m of ntMGAM for maltose is about 20 times higher compared with that for pNPG (Sim *et al*. 2010). This is probably the reason why rat intestinal maltase activity in our research shows lower level of inhibition value for maltose than for pNPG even at a higher concentration (500 μg/ml) of plant extracts.

Table 3.1. Substrate specificities of intestinal α-glucosidases.

No.	Substrates	Specificities
1	pNPG	In human intestinal α-glucosidases, catalytic efficiency (K_{cat}/K_m) of ntMGAM and ntSI are 1.4 ± 0.1 and 13 ± 1, respectively (Sim *et al*. 2010).
2	Maltose	In rat small intestinal α-glucosidases, catalytic efficiency (K_{cat}/K_m) of ctMGAM, ntMGAM, ctSI and ntSI are 51.0 ± 7.0, 12.7 ± 3.0, 2.7 ± 0.5 and 1.7 ± 0.4, respectively (Lee *et al*. 2016). In human intestinal α-glucosidases, catalytic efficiency (K_{cat}/K_m) of ntMGAM and ntSI are 26 ± 8 and 19 ± 4, respectively (Sim *et al*. 2010).
3	Sucrose	In rat small intestinal α-glucosidases, percent hydrolysis of ctMGAM, ntMGAM, ctSI and ntSI are 14.8 ± 0.6, 0.6 ± 0.1, 73.1 ± 0.1 and 0.9 ± 0.0, respectively (Lee *et al*. 2016).

Currently, it is already known that some of the medicinal plants such as *Anacardiaceae*, *Fabaceae*, *Malvaceae*, *Apocynaceae*, *Annonaceae*, *Burseraceae*, and

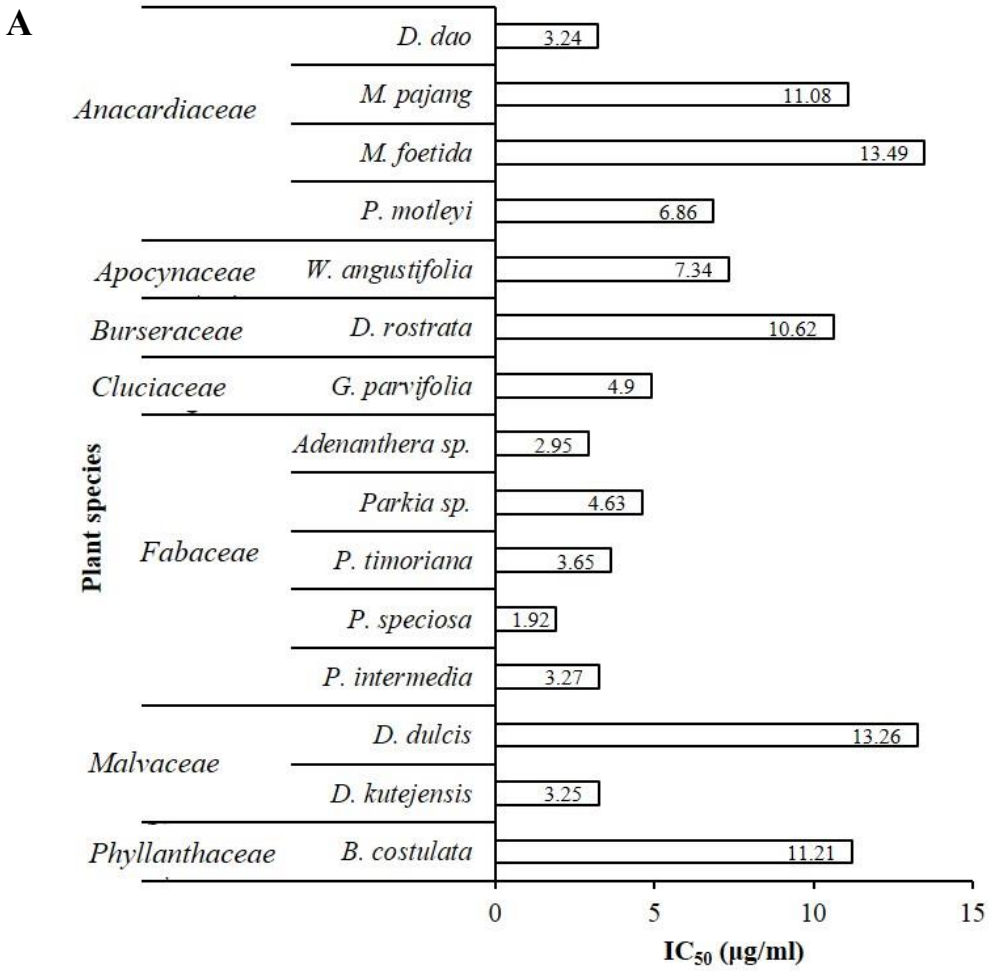
Clusiaceae plant families have anti-diabetic activity. Khera and Bhatia (2014) reported that 17 species of *Fabaceae*, 2 species of *Malvaceae*, 3 species of *Anacardiaceae*, 2 species of *Annonaceae* and 1 species of *Apocynaceae* have compounds that function as anti-diabetic reagents. Eyla *et al*. (2012) reported that 14 species of *Apocynaceae* and 7 species of *Clusiaceae* have inhibitory activities on yeast α-glucosidase. Simmonds and Howes (2006) reported that 10 species of *Anacardiaceae*, 78 species of *Fabaceae*, 11 species of *Malvaceae*, 17 species of *Apocynaceae*, and 1 species of *Burseraceae* have compounds that function as anti-diabetic reagents, using with leaves, barks, seeds, stems, all part, nuts, roots, flowers, fruits, woods, and bulbs. From these all species, only fruits of *P. speciosa* species are already reported to have a function as anti-diabetes agent.

In this study, methanol extracts from wood barks of *Anacardiaceae*, *Fabaceae*, *Malvaceae*, *Phyllanthaceae*, and other plant families (*Apocynaceae, Burseraceae* and *Clusiaceae*) were reported to have anti-diabetic activity. According to Patel *et al.* (2012), several plants in different families have been used as anti-diabetic medicinal plants, and one of the most potential plants to decrease blood glucose belongs to *Fabaceae* family. These reports were supported by our results, where all species in *Fabaceae* plants family tested have high inhibitory activity against yeast α-glucosidase.

In order to find bioactive compounds from plants, functional materials can be traced by three approaches: random, ethno-pharmacological and taxonomical ones (Filho 2006). In this study, to identify the plants as anti-diabetic medicine, it was used two approaches based on ethno-pharmacology and taxonomy. In ethno-pharmacology, among 17 plant species studied, traditionally used as an anti-diabetic medicine are only 4 species (*Fabaceae* and *Phyllanthaceae*). In the taxonomic approach, plants species from *Malvaceae*, *Anacardiaceae*, *Apocynaceae*, *Annonaceae*, *Burseraceae*, and *Clusiaceae* are useful as anti-diabetic, and it was tried to examine 13 other species as anti-diabetic drug candidates. Results of this study showed that almost all species have inhibitory activities on yeast α-glucosidase, except *B. angulata* and *G. tapis* that have lower activities in all tests using yeast α-glucosidase, rat intestinal maltase, and rat intestinal sucrase.

3.3.6. IC_{50} of α-glucosidase inhibition

Based on the α-glucosidase inhibition assays, IC_{50} of each species extract was calculated. IC_{50} is the concentration of the extract required to inhibit 50% of α-glucosidase activity under the assay condition. In this study, some plant extracts inhibited less than 50% of α-glucosidase activity at the maximum concentration. In these cases, the IC_{50} values of these plant extracts were not calculated.



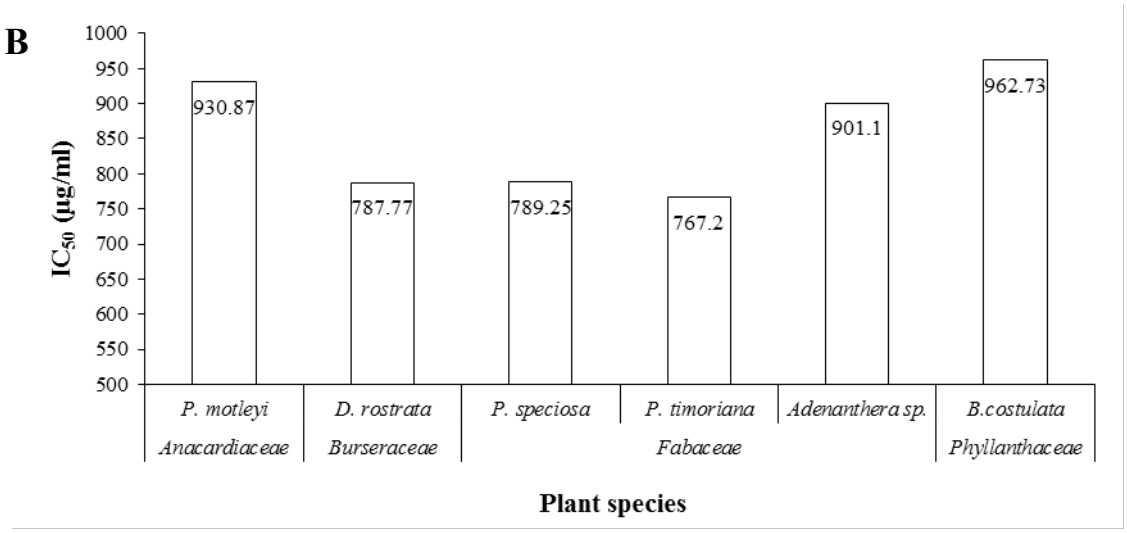


Fig. 3.6. The α-glucosidase IC_{50} for the extracts of Dayak medicinal plants. (A) The IC_{50} of yeast α-glucosidase, and (B) The IC_{50} of rat intestinal sucrase.

As shown in **Fig. 3.6.A**, methanol extracts from all species of *Fabaceae* family (*P. speciosa*, *Adenanthera sp.*, *P. intermedia*, *P. timoriana*, and *Parkia sp.*) had strong inhibitory effects on yeast α-glucosidase activity, because they are able to inhibit 50% of α-glucosidase activity at lower concentrations (< 5 µg/ml). For species in *Anacardiaceae*, *Malvaceae*, *Phyllanthaceae*, and other family, IC_{50} values were diversified. In contrast, species of *D. dao*, *D. kutejensis*, and *G. parvifolia* had IC_{50} values similar to species of *Fabaceae* plant family against yeast α-glucosidase activity (< 5 µg/ml). It is reported that ethanol extracts from *Symplocos cochinchinensis* has IC_{50} value of 82.07 ± 2.10 µg/ml (Antu *et al.* 2014). All species in this research had lower IC_{50} values than that of *Symplocos cochinchinensis* in terms of inhibition on yeast α-glucosidase activity. In the assay with rat intestinal sucrase (**Fig. 3.6.B**), *P. timoriana* has the lowest IC_{50} values among the methanol extracts from six species.

The following three assays of α-glucosidase were used: yeast α-glucosidase derived from *S. cerevisiae* with pNPG as a substrate, rat intestinal maltase in the acetone powder with pNPG as a substrate, rat intestinal sucrase in the acetone powder with sucrose as a substrate. Methanol extracts from wood barks of all the Dayak plants showed differences in the value of inhibitory activity. In these experiments, IC_{50} values against yeast α-glucosidase were much lower than those against rat intestinal sucrase. These results may be relevant to studies conducted by Tadera *et al.* (2006) that

inhibitory activities of six groups of flavonoids compounds especially flavonol, flavanone, isoflavone, and anthocyanidin against yeast α-glucosidase were higher than those against the rat small intestinal α-glucosidase.

Inhibitory activities of plant extracts against rat intestinal α-glucosidase using pNPG as a substrate (rat intestinal maltase) are very low. The IC_{50} values are not shown, because inhibitory activities were examined only at a concentration of 500 μg/ml of each plant extract. Ng *et al.* (2015) reported that some of flavonoids compound such as flavonols, flavonones, flavanols, and isoflavons have low activities to inhibit the rat intestinal α-glucosidase with pNPG as a substrate. Hogan *et al*. (2010) reported that grape pomace extracts are more effective to inhibit yeast α-glucosidase rather than rat intestinal α-glucosidase with pNPG as a substrate.

The methanol extracts of wood barks contain large amounts of phenolic constituents, such as flavonoid compounds included in the group of condensed tannins (phenolic acids) and monomer of flavonoids such as quercetins and dihydroquercetin (taxifolin) (Sjostrom 1981). Methanol extracts of wood barks that were used in this research allegedly contains flavonoids and quercetins as bioactive compounds. In this study, the strong inhibitory activities of methanol extracts from medicinal plants against yeast α-glucosidase suggest that they are more efficient to inhibit yeast maltase rather than inhibiting complex enzymes in rat intestine.

Plant extract of *Morinda lucida* that contains flavanoids, tannins and terpenoids has activity to inhibit yeast α-glucosidase. It is speculated that there are competitive and noncompetitive inhibitors in the crude extracts. Non-competitive inhibitors in the extracts do not compete with the substrates to bind to the active site of enzyme. Instead, these inhibitors bind to the other side of enzyme that changed the conformation of the enzyme, resulting in the delay of the breakdown of disaccharides to monosaccharides (Kazeem *et al*. 2013). In non-competitive inhibition, the structure of inhibitors and the substrates do not have similarity, but inhibitors could bind to both of free enzymes and enzyme-substrate complexes (Sharma 2012). Damsud *et al*. (2013) reported that phenylpropanoyl amides isolated from *Piper sarmentosum* function as non-competitive inhibitors against yeast α-glucosidase by forming enzyme-inhibitor and substrate-enzyme-inhibitor complexes. In this study, it is speculated that methanol extracts from wood barks inhibit yeast α-glucosidase in a mode of non-competitive inhibition.

The main dietary carbohydrate digested to produce an absorbable glucose in the small intestine is maltose (Tadera *et al.* 2006), and the delay of digestion of maltose in small intestinal will decrease the rate of glucose absorption, resulting in the reduction of blood glucose level of diabetic patients.

Local people in West Kalimantan Indonesia traditionally use roots as a part of medicinal plants to treat diabetic patients. They used to make extracts from a handful of roots using boiled water and drink it once or three times a day. In this research, methanol was used to extract materials from wood barks and results show that methanol extracts have the ability to inhibit yeast α-glucosidase *in vitro* at a low concentration and almost all of these plants have a potential to be used as anti-diabetic medicine.

Due to the potentiality of these plants as anti-diabetic medicine, it is necessary to examine the effects of these plant extracts *in vivo* so that it can be estimated the effectiveness of these medicinal plants to inhibit intestinal α-glucosidase before applying to humans, especially to patients with type 2 diabetes mellitus.

3.4. Conclusions

The inhibitory effects of methanol extracts from wood barks of 17 plant species of *Anacardiaceae*, *Fabaceae*, *Malvaceae*, *Phyllanthaceae*, and other plant families (*Annonaceae, Apocynaceae, Burseraceae* and *Clusiaceae*) were examined on α-glucosidase activities using three kinds of α-glucosidase assays: yeast α-glucosidase with pNPG as a substrate, rat intestinal acetone powder with pNPG as a substrate (rat intestinal maltase), and rat intestinal acetone powder with sucrose as a substrate (rat intestinal sucrase). As a result, it was found that almost all the species have high inhibitory activities against yeast α-glucosidase, except *B. angulata* (*Phyllanthaceae*) and *G. tapis* (*Annonaceae*) with lower levels of inhibitory activities. Inhibitory effects of these plant extracts on rat intestinal maltase and sucrase activities are much lower compared with those on yeast α-glucosidase. Only methanol extracts from barks of the families of *Fabaceae* (*P. intermedia*, *P. speciosa*, and *Adenanthera sp*.), *Phyllanthaceae* (*B. costulata*), *Anacardiaceae* (*P. motleyi*), and *Burseraceae* (*D. rostrata*) inhibited more than 50% of rat intestinal sucrase activity at a concentration of 1000 μg/ml. Further study is required to clarify the mechanism of the inhibition of α-glucosidase by

using animal model of type 2 diabetes mellitus before applying to human type 2 diabetes patients.

Example of raw data and calculations of α-glucosidase inhibition by methanol extracts.

Table 3.2.A. Estimation of the inhibitory activities against yeast α-glucosidase by methanol extracts (10 μg/ml) from wood barks of 7 medicinal plants.

Sample	Average of absorbance (n=3)*										Average of slope absorbance ± SE (n=3)	Inhibition ± SE (%) (n=3)
	1 min	2 min	3 min	4 min	5 min	6 min	7 min	8 min	9 min	10 min		
Control	0.1403	0.1613	0.1893	0.2027	0.2137	0.2247	0.2380	0.2503	0.2633	0.2783	0.01441212±0.0007	
Pelanjau (*P. motleyi*)	0.0333	0.0440	0.0497	0.0553	0.0593	0.0640	0.0693	0.0750	0.0810	0.0857	0.00547475±0.0002	62.01±1.35
Asam kemantan (*M. foetida*)	0.0467	0.0587	0.0690	0.0780	0.0877	0.0990	0.1130	0.1250	0.1350	0.1443	0.01096768±0.0013	23.90±9.34
Asam bawang (*M. pajang*)	0.0333	0.0407	0.0487	0.0550	0.0620	0.0690	0.0780	0.0873	0.0987	0.1087	0.00820202±0.0004	43.09±2.98
Sengkuang (*D.dao*)	0.0167	0.0183	0.0203	0.0223	0.0233	0.0243	0.0253	0.0263	0.0293	0.0293	0.00140000±0.0001	90.29±0.62
Durian meranang (*D. dulcis*)	0.0497	0.0667	0.0783	0.0850	0.0947	0.1077	0.1210	0.1330	0.1430	0.1507	0.01113737±0.0003	22.72±2.25
Kedaung (*P. timoriana*)	0.0143	0.0170	0.0183	0.0190	0.0193	0.0213	0.0223	0.0237	0.0240	0.0247	0.00109495±0.0002	92.40±1.25
Enceriak (*B. costulata*)	0.0393	0.0530	0.0603	0.0670	0.0757	0.0853	0.0953	0.1057	0.1123	0.1193	0.00882828±0.0004	38.74±2.82

*: The number is truncated to four decimal places. In the following calculation, the number up to ten decimal places is used.

Table 3.2.B. Estimation of the inhibitory activities against yeast α-glucosidase by methanol extracts (10 μg/ml) from wood barks of 8 medicinal plants.

Sample	Average of absorbance (n=3)*										Average of slope absorbance ± SE (n=3)	Inhibition ± SE (%) (n=3)
	1 min	2 min	3 min	4 min	5 min	6 min	7 min	8 min	9 min	10 min		
Control	0.1707	0.1957	0.2143	0.2377	0.2550	0.2787	0.2960	0.3117	0.3297	0.3483	0.01952929±0.0006	
Jatak (*W. angustifolia*)	0.0760	0.0837	0.0897	0.0960	0.1020	0.1083	0.1147	0.1230	0.1293	0.1380	0.00670707±0.0008	65.66±4.13
Asam kandis (*G. parvifolia*)	0.0240	0.0323	0.0347	0.0390	0.0407	0.0447	0.0470	0.0503	0.0560	0.0573	0.00346667±0.0005	82.25±2.46
Kemayau (*D. rostrata*)	0.0857	0.0963	0.1097	0.1203	0.1313	0.1413	0.1500	0.1597	0.1690	0.1793	0.01030707±0.0005	47.22±2.58
Petai betawi (*Adenanthera sp.*)	0.0227	0.0250	0.0257	0.0280	0.0290	0.0313	0.0317	0.0337	0.0350	0.0360	0.00147475±0.0002	92.45±1.16
Petai panjang (*P. intermedia*)	0.0377	0.0410	0.0463	0.0493	0.0513	0.0537	0.0563	0.0600	0.0627	0.0657	0.00300202±0.0005	84.63±2.76
Durian pekawai (*D. kutejensis*)	0.0293	0.0353	0.0393	0.0407	0.0423	0.0457	0.0467	0.0483	0.0497	0.0510	0.00219192±0.0002	88.78±1.22
Petai pendek (*P. speciosa*)	0.0087	0.0077	0.0083	0.0087	0.0087	0.0087	0.0090	0.0337	0.0100	0.0103	0.00096364±0.0001	95.07±0.61
Poko utan (*Parkia sp.*)	0.0563	0.0660	0.0737	0.0790	0.0847	0.0913	0.0977	0.1037	0.1103	0.1160	0.00642424±0.0005	67.10±2.31

*: The number is truncated to four decimal places. In the following calculation, the number up to ten decimal places is used.

Table 3.2.C. Estimation of the inhibitory activity against yeast α-glucosidase by methanol extract (200 µg/ml) from wood bark of *B. angulata.*

Sample	Average of absorbance (n=3)*										Average of slope absorbance ± SE (n=3)	Inhibition ± SE (%) (n=3))
	1 min	2 min	3 min	4 min	5 min	6 min	7 min	8 min	9 min	10 min		
Control	0.1080	0.1243	0.1347	0.1497	0.1673	0.1827	0.1940	0.2033	0.2127	0.2207	0.01287273±0.0005	
Bel. merah (*B. angulata*)	0.0613	0.0713	0.0807	0.0900	0.0983	0.1077	0.1157	0.1237	0.1323	0.1413	0.00877778±0.0004	31.81±3.49

*: The number is truncated to four decimal places. In the following calculation, the number up to ten decimal places is used.

Table 3.2.D. Estimation of the inhibitory activity against yeast α-glucosidase by methanol extract (200 µg/ml) from wood bark of *G. tapis.*

Sample	Average of absorbance (n=3)*										Average of slope absorbance ± SE (n=3)	Inhibition ± SE (%) (n=3)
	1 min	2 min	3 min	4 min	5 min	6 min	7 min	8 min	9 min	10 min		
Control	0.1690	0.1983	0.2280	0.2577	0.2870	0.3170	0.3473	0.3763	0.4060	0.4353	0.02964444±0.0009	
Selukai (*G. tapis*)	0.0417	0.0663	0.0910	0.1117	0.1330	0.1537	0.1743	0.1940	0.2133	0.2323	0.02102222±0.0003	29.09±1.12

*: The number is truncated to four decimal places. In the following calculation, the number up to ten decimal places is used.

Example: calculation of the inhibitory activity against yeast α-glucosidase by methanol extract (10 µg/ml) from wood bark of pelanjau (*P. motleyi*).

$$\text{Slope of absorbance(m)} = \frac{\overline{XY} - \overline{X}\,\overline{Y}}{\overline{X^2} - \overline{X}^2}$$

	Time (X)	Average of absorbance (Y)*	X^2	XY	Y^2
Control	1	0.1403333333	1	0.1403333333	0.0196934444
	2	0.1613333333	4	0.3226666667	0.0260284444
	3	0.1893333333	9	0.5680000000	0.0358471111
	4	0.2026666667	16	0.8106666667	0.0410737778
	5	0.2136666667	25	1.0683333333	0.0456534444
	6	0.2246666667	36	1.3480000000	0.0504751111
	7	0.2380000000	49	1.6660000000	0.0566440000
	8	0.2503333333	64	2.0026666667	0.0626667778
	9	0.2633333333	81	2.3700000000	0.0693444444
	10	0.2783333333	100	2.7833333333	0.0774694444
Total	55	2.1620000000	385	13.0800000000	0.4848960000
Average	5.5	0.2162000000	38.5	1.3080000000	0.0484896000

*: This values based on the Table 3.2.A.

$$\text{Slope of absorbance(m)} = \frac{\overline{XY} - \overline{X}\,\overline{Y}}{\overline{X^2} - \overline{X}^2}$$

$$\text{Slope of absorbance control (m)} = \frac{1.308 - 5.5\text{x}0.2162}{38.5 - 5.5^2}$$

$$\text{Slope of absorbance control (m)} = \frac{1.308 - 1.1891}{38.5 - 30.25}$$

$$\text{Slope of absorbance control (m)} = \frac{0.1189}{8.25}$$

$$\text{Slope of absorbance control(m)} = 0.01441212$$

	Time (X)	Average of absorbance (Y)*	X^2	XY	Y^2
P. motleyi	1	0.0333333333	1	0.0333333333	0.0011111111
	2	0.0440000000	4	0.0880000000	0.0019360000
	3	0.0496666667	9	0.1490000000	0.0024667778
	4	0.0553333333	16	0.2213333333	0.0030617778
	5	0.0593333333	25	0.2966666667	0.0035204444
	6	0.0640000000	36	0.3840000000	0.0040960000
	7	0.0693333333	49	0.4853333333	0.0048071111
	8	0.0750000000	64	0.6000000000	0.0056250000
	9	0.0810000000	81	0.7290000000	0.0065610000
	10	0.0856666667	100	0.8566666667	0.0073387778
Total	55	0.6166666667	385	3.8433333333	0.0405240000
Average	5.5	0.0616666667	38.5	0.3843333333	0.0040524000

*: This values based on the Table 3.2.A.

$$\text{Slope of absorbance} P.\,motleyi\,(\text{m}) = \frac{\overline{\text{XY}} - \overline{\text{X}}\,\overline{\text{Y}}}{\overline{\text{X}^2} - \overline{\text{X}}^2}$$

$$\text{Slope of absorbance } P.\,motleyi\ (\text{m}) = \frac{0.3843333333 - 5.5\text{x}0.0616666667}{38.5 - 5.5^2}$$

$$\text{Slope of absorbance } P.\,motleyi\ (\text{m}) = \frac{0.3843333333 - 0.3391666667}{38.5 - 30.25}$$

$$\text{Slope of absorbance } P.\,motleyi\ (\text{m}) = \frac{0.0451666667}{8.25}$$

$$\text{Slope of absorbance } P.\,motleyi\ (\text{m}) = 0.00547475$$

$$\text{Inhibition}\,(\%) = \left(1 - \frac{\text{Slope of absorbance treatment}}{\text{Slope of absorbance control}}\right) \text{x}\,100\%$$

$$\text{Inhibition of } P.\,motleyi(\%) = \left(1 - \frac{\text{Slope of absorbance } P.\,motleyi}{\text{Slope of absorbance control}}\right) \text{x } 100\%$$

$$\text{Inhibition of } P.\,motleyi(\%) = \left(1 - \frac{0.00547475}{0.01441212}\right) \text{x } 100\%$$

$$\text{Inhibition of } P.\,motleyi(\%) = 62.01\%$$

Example: calculation of the yeast α-glucosidase IC_{50} by methanol extract from wood bark of pelanjau (*P. motleyi*).

No	X= Concentration (µg/ml)	Y= Inhibition (%)
1	5	39.87
2	10	62.01

$$IC_{50} \text{ of pelanjau } (P.\,motleyi) = 10^{\frac{\text{Log}\left(\frac{X_2}{X_1}\right).(50-Y_1)}{(Y_2-Y_1)} + \text{Log } X_1}$$

$$IC_{50} \text{ of pelanjau } (P.\,motleyi) = 10^{\frac{\text{Log}\left(\frac{10}{5}\right).(50-39.87)}{(62.01-39.87)} + \text{Log } 5}$$

$$IC_{50} \text{ of pelanjau } (P.\,motleyi) = 10^{\frac{\text{Log } (2).(10.13)}{(22.14)} + 0.699}$$

$$IC_{50} \text{ of pelanjau } (P.\,motleyi) = 10^{\frac{(0.301).(10.13)}{(22.14)} + 0.699}$$

$$IC_{50} \text{ of pelanjau } (P.\,motleyi) = 10^{\frac{(3.049)}{(22.14)} + 0.699}$$

$$IC_{50} \text{ of pelanjau } (P.\,motleyi) = 10^{0.138 + 0.699}$$

$$IC_{50} \text{ of pelanjau } (P.\,motleyi) = 10^{0.837}$$

$$IC_{50} \text{ of pelanjau } (P.\,motleyi) = 6.86 \text{ µg/ml}$$

Table 3.3.A. Estimation of the inhibitory activities against rat intestinal maltase by methanol extracts (500 μg/ml) from wood barks of 9 medicinal plants.

Sample	Average of absorbance (n=3)*										Average of slope absorbance ± SE (n=3)	Inhibition ± SE (%) (n=3)
	1 min	2 min	3 min	4 min	5 min	6 min	7 min	8 min	9 min	10 min		
Control	0.3123	0.3483	0.3770	0.3950	0.4163	0.4380	0.4597	0.4823	0.5050	0.5280	0.02290909±0.0012	
Enceriak (*B. costulata*)	0.3030	0.3283	0.3493	0.3703	0.3900	0.4090	0.4277	0.4457	0.4653	0.4850	0.01981616±0.0007	13.50±3.25
Pelanjau (*P. motleyi*)	0.2963	0.3203	0.3400	0.3597	0.3783	0.3973	0.4157	0.4347	0.4540	0.4740	0.01936364±0.0001	15.48±0.29
Asam kemantan (*M. foetida*)	0.2987	0.3190	0.3380	0.3570	0.3753	0.3940	0.4127	0.4237	0.4510	0.4713	0.01873939±0.0005	18.20±2.52
Asam bawang (*M. pajang*)	0.2883	0.3100	0.3297	0.3493	0.3677	0.3860	0.4053	0.4237	0.4433	0.4637	0.01919798±0.0005	16.19±2.26
Sengkuang (*D. dao*)	0.2853	0.3063	0.3260	0.3457	0.3657	0.3847	0.4033	0.4223	0.4413	0.4607	0.01937374±0.0009	15.43±3.93
Belimbing merah (*B. angulata*)	0.2623	0.2840	0.3050	0.3253	0.3443	0.3640	0.3833	0.4037	0.4240	0.4443	0.02003030±0.0001	12.56±0.77
Jatak (*W. angustifolia*)	0.2663	0.2860	0.3067	0.3267	0.3453	0.3643	0.3843	0.4043	0.4240	0.4443	0.01968687±0.0004	14.06±2.04
Durian meranang (*D. dulcis*)	0.3093	0.3337	0.3543	0.3743	0.3947	0.4150	0.4343	0.4547	0.4760	0.4967	0.02051111±0.0005	10.46±2.49
Selukai (*G. tapis*)	0.2537	0.2700	0.2867	0.3043	0.3220	0.3407	0.3593	0.3780	0.3970	0.4163	0.01814141±0.0003	20.81±1.57

*: The number is truncated to four decimal places. In the following calculation, the number up to ten decimal places is used.

Table 3.3.B. Estimation of the inhibitory activities against rat intestinal maltase by methanol extracts (500 μg/ml) from wood barks of 7 medicinal plants.

Sample	Average of absorbance (n=3)*										Average of slope absorbance ± SE (n=3)	Inhibition ±SE (%) (n=3)
	1 min	2 min	3 min	4 min	5 min	6 min	7 min	8 min	9 min	10 min		
Control	0.2867	0.2990	0.3100	0.3210	0.3327	0.3443	0.3560	0.3680	0.3800	0.3923	0.01166465±0.0006	
Asam kandis (*G. parvifolia*)	0.2187	0.2273	0.2360	0.2463	0.2557	0.2643	0.2750	0.2850	0.2953	0.3043	0.00961616±0.0001	17.56±1.19
Kemayau (*D. rostrata*)	0.2660	0.2770	0.2867	0.2963	0.3057	0.3160	0.3263	0.3373	0.3477	0.3583	0.01017778±0.0003	12.74±3.05
Petai betawi (*Adenanthera sp.*)	0.2383	0.2507	0.2617	0.2730	0.2830	0.2917	0.3010	0.3120	0.3230	0.3350	0.01042828±0.0003	10.59±3.26
Petai panjang (*P. intermedia*)	0.2157	0.2277	0.2387	0.2493	0.2597	0.2703	0.2793	0.2890	0.3000	0.3107	0.01038586±0.0002	10.96±2.14
Poko utan (*Parkia sp.*)	0.2200	0.2313	0.2407	0.2507	0.2610	0.2703	0.2807	0.2923	0.3030	0.3140	0.01033535±0.0002	11.39±2.34
Petai pendek (*P. speciosa*)	0.2327	0.2440	0.2560	0.2680	0.2780	0.2893	0.3007	0.3120	0.3250	0.3360	0.01143232±0.0004	1.99±3.45
Petai kedaung (*P. timoriana*)	0.2290	0.2393	0.2483	0.2577	0.2653	0.2737	0.2833	0.2917	0.3020	0.3117	0.00899798±0.0001	22.86±0.96

*: The number is truncated to four decimal places. In the following calculation, the number up to ten decimal places is used.

Table 3.3.C. Estimation of the inhibitory activity against rat intestinal maltase by methanol extract (500 μg/ml) from wood bark of durian pekawai (*D. kutejensis*)

Sample	Average of absorbance (n=3)*										Average of slope absorbance ± SE (n=3)	Inhibition ± SE (%) (n=3)
	1 min	2 min	3 min	4 min	5 min	6 min	7 min	8 min	9 min	10 min		
Control	0.2360	0.2517	0.2610	0.2697	0.2790	0.2883	0.2973	0.3070	0.3170	0.3270	0.00968889±0.0069	
Durian pekawai (*D. kutejensis*)	0.2837	0.3010	0.3117	0.3187	0.3267	0.3360	0.3447	0.3543	0.3640	0.3743	0.00944040±0.0003	2.56±2.89

*: The number is truncated to four decimal places. In the following calculation, the number up to ten decimal places is used.

Example: calculation of the inhibitory activity against rat intestinal maltase by methanol extract (500 μg/ml) from wood bark of durian pekawai (*D. kutejensis*).

$$\text{Inhibition}\,(\%) = \left(1 - \frac{\text{Slope of absorbance treatment}}{\text{Slope of absorbance control}}\right) \text{x}\, 100\%$$

$$\text{Inhibition of durian pekawai}\,(D.\,kutejensis)\,(\%) = \left(1 - \frac{0.00944040}{0.00968889}\right) \text{x}\, 100\%$$

$$\text{Inhibition of durian pekawai}\ (D.\,kutejensis)\,(\%) = 2.56\%$$

Table 3.4. Estimation of the inhibitory activities against rat intestinal sucrase by methanol extracts (1000 µg/ml) from wood barks of 17 medicinal plants.

Sample	Average of Absorbance ±SE (n=3)	Inhibition ±SE (%) (n=3)
Blank	0.0441±0.0023	
Control	0.2281±0.0088	
Pelanjau (*P. motleyi*)	0.1309±0.0003	52.82±0.14
Asam kemantan (*M. foetida*)	0.1643±0.0125	34.67±5.48
Asam bawang (*M. pajang*)	0.1565±0.0153	38.91±6.70
Sengkuang (*D.dao*)	0.1371±0.0171	49.46±7.47
Petai kedaung (*P. timoriana*)	0.1422±0.0016	46.68±0.72
Petai pendek (*P. speciosa*)	0.1244±0.0047	56.32±2.05
Petai betawi (*Adenanthera sp.*)	0.1309±0.0148	52.82±6.49
Petai panjang (*P. intermedia*)	0.1153±0.0103	61.29±4.53
Poko utan (*Parkia sp.*)	0.1485±0.0036	43.26±1.61
Durian meranang (*D. dulcis*)	0.1543±0.0186	40.10±8.17
Durian pekawai (*D. kutejensis*)	0.1497±0.0052	42.60±2.31
Enceriak (*B. costulata*)	0.1336±0.0109	51.36±4.80
Belimbing merah (*B. angulata*)	0.1836±0.0140	24.21±6.13
Jatak (*W. angustifolia*)	0.1554±0.0209	39.51±9.19
Asam kandis (*G. parvifolia*)	0.1648±0.0105	34.40±4.63
Kemayau (*D. rostrata*)	0.1091±0.0291	64.65±12.77
Selukai (*G. tapis*)	0.1855±0.0182	23.15±8.00

Example: calculation of the inhibitory activity against rat intestinal sucrase by methanol extract (1000 µg/ml) from wood bark of pelanjau (*P. motleyi*).

$$\text{Inhibition}(\%) = \left(1 - \frac{\text{Absorbance treatment} - \text{Absorbance blank}}{\text{Absorbance control} - \text{Absorbance blank}}\right) \text{x } 100\%$$

$$\text{Inhibition of pelanjau } (P.\,motleyi)\,(\%) = \left(1 - \frac{0.1309 - 0.0441}{0.2281 - 0.0441}\right) \text{x } 100\%$$

$$\text{Inhibition of pelanjau } (P.\,motleyi)\,(\%) = \left(1 - \frac{0.0868}{0.184}\right) \text{x } 100\%$$

$$\text{Inhibition of pelanjau } (P.\,motleyi)\,(\%) = (1 - 0.47173) \text{x } 100\%$$

$$\text{Inhibition of pelanjau } (P.\,motleyi)\,(\%) = 52.82\%$$

Example: calculation of the rat intestinal sucrase IC_{50} by methanol extract from wood bark of pelanjau (*P. motleyi*).

No	X= Concentration (µg/ml)	Y= Inhibition (%)
1	500	25.53
2	1000	52.82

$$IC_{50} \text{ of pelanjau } (P.\,motleyi) = 10^{\frac{\text{Log}\left(\frac{X_2}{X_1}\right).(50 - Y_1)}{(Y_2 - Y_1)} + \text{Log } X_1}$$

$$IC_{50} \text{ of pelanjau } (P.\,motleyi) = 10^{\frac{\text{Log}\left(\frac{1000}{500}\right).(50 - 25.53)}{(52.82 - 25.53)} + \text{Log } 500}$$

$$IC_{50} \text{ of pelanjau } (P.\,motleyi) = 10^{\frac{\text{Log } (2).(24.47)}{(27.29)} + 2.699}$$

$$IC_{50} \text{ of pelanjau } (P.\,motleyi) = 10^{\frac{(0.301).(24.47)}{(27.29)} + 2.699}$$

$$IC_{50} \text{ of pelanjau } (P.\,motleyi) = 10^{\frac{(7.365)}{(27.29)} + 2.699}$$

$$IC_{50} \text{ of pelanjau } (P.\,motleyi) = 10^{0.27 + 2.699}$$

$$IC_{50} \text{ of pelanjau } (P.\,motleyi) = 10^{2.969}$$

$$IC_{50} \text{ of pelanjau } (P.\,motleyi) = 930.87 \text{ µg/ml}$$

Chapter 4
Toxic effects of methanol extracts from wood barks on NIH3T3 cells and C57BL/6J mice

4.1. Introduction

Medicinal plants in West Kalimantan, Indonesia traditionally are used without any specific doses or concentrations. Usually, they use a handful of medicinal plants and extract them using hot water, and drink the hot water extracts. Extractives contain toxic chemical compounds from plants that function as a chemical barrier against insects (Rowell *et al.* 2006) such as toxic phenols in *Anacardiaceae* plants family that are defensive against pest insects and fungi (Aguilar-Ortigoza and Sosa 2004). Toxic compounds in the plants can be used as medicine for human, but the concentration of extracts should be clarified, because some of plants such as *Anacardiaceae* plants family cause edema, burning, stinging sensation, exudation, crusting, and death with anaphylactic shock at higher concentrations (Wiart 2006).

Methanol extracts from wood barks of several plants have potential value as anti-diabetic reagents with high levels of inhibition of α-glucosidase *in vitro* especially against yeast α-glucosidase (**Chapter 3**). The process of α-glucosidase inhibition *in vitro* does not include any metabolic pathways (Soumyanath and Srijayantha 2006). Many plant extracts are reported to have the activity to inhibit α-glucosidase *in vitro*, but some of them do not effectively inhibit α-glucosidase in mammals (Shihabudeen *et al*. 2011). It is very important to prove that plant extracts effectively inhibit α-glucosidase in an *in vivo* model of type 2 diabetes. Other functions of these plants are anti-diarrheas and anti-stomachaches (**Table 1.1**) that are related to inflammation. Thus, it is very important to prove that plant extracts have anti-inflammatory activities using human intestinal cells *in vitro*.

Before administrating plant extracts to leptin receptor deficient *db/db* mice, model mice of type 2 diabetes and applying to FPCK-1-1 cells, human colon epithelial cells used as a model of anti-intestinal inflammation, the safe range of doses and concentrations of plant extracts must be clarified. The evaluation of cytotoxicity of plant

extracts was performed by using normal mouse fibroblast NIH3T3 cells (Akter *et al*. 2014). C57BL/6J mice were also used to find a suitable dose to avoid the toxic effect of plant extracts.

NIH3T3 cells are non-tumor cells (Orsine *et al.* 2013) derived from a normal mouse fibroblast (Akter *et al*., 2014) that frequently used as a control cell line in some cytotoxicity assays for anti-cancer drugs (Beattie *et al*. 2011, Danihelova *et al.* 2013). C57BL/6J is a strain of mice that genetically develop sugar imbalance, and are commonly used for developing obesity, hyperglycemia and hyperinsulinemia by administrating a high fat diet, and has been used as a model of type 2 diabetes (Winters *et al.* 2003, Yamamoto *et al.* 2011). In many experiments, C57BL/6J mice were used as an animal model of toxicity assay (Da Silva *et al.* 2015, Xing *et al*. 2016). Leptin receptor deficient *db/db* mice have the background of C57BLKS/J inbred strain.

In this experiment, the following seven plants were selected for measuring the cytotoxicity: *D. dulcis*, *D. kutejensis*, *P. timoriana*, *P. speciosa*, *P. intermedia*, *B. angulata*, and *D. dao.* These species were selected based on the functional utilization as traditional medicine for diarrheas, stomachaches, anti-diabetes, and the ability to inhibit α-glucosidase *in vitro* with low value of IC_{50} except *B. angulata*. It was found that methanol extracts of *D. dulcis*, *D. kutejensis*, *P. timoriana*, *P. speciosa*, *P. intermedia*, and *D. dao* are toxic at higher concentrations, and *P. speciosa* is the most toxic one in the cytotoxicity assay using NIH3T3 cells *in vitro*. Concentration of ≤ 5 mg/0.1ml DW/mouse and 1 μg/ml are safe dose and concentration to C57BL/6J mice and FPCK-1-1 cells, respectively.

4.2. Materials and Methods

Plants material

Methanol extracts from wood barks of *D. dulcis*, *D. kutejensis*, *P. timoriana*, *P. speciosa*, *P. intermedia*, *B. angulata*, and *D. dao* were prepared as described in **Chapter 2**. For the cell cytotoxicity test *in vitro*, 10 mg of methanol extracts from wood barks (dry weight) were dissolved in 1 ml DMSO (10 mg/ml) as a stock solution, and diluted to various concentrations. For animal experiments (*in vivo*), 500 mg (dry weight) of methanol extracts were suspended in 10 ml of distilled water (50 mg/ml) (Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan), grinded in a mortar, and homogenized

using a Polytron homogenizer (Kinematica, Luzern, Switzerland). Homogenate of extracts was heated in a water bath Incubator BT-22 (Yamato Scientific Co., Ltd., Tokyo, Japan) at 75°C for 30 minutes, while mixing every five minutes (Vortex Genie 2, Scientific Industries, Inc., New York) and stored at room temperature for overnight. The heating procedure was repeated twice and extracts were stored at 4°C before use.

***In vitro* cytotoxicity test**

Cell lines

NIH3T3 cells are non-tumor cells (Orsine *et al.,* 2013) derived from normal mouse fibroblast (Akter *et al*., 2014). NIH3T3 cells were maintained at 37°C in 5% CO_2 in a high glucose Dulbecco's-modified Eagle Medium (DMEM) supplemented with 8% fetal calf serum (FCS), 20 U/ml penicillin, and 50 µg/ml kanamycin.

Inhibitory rate of NIH3T3 cells

Cytotoxicity tests were conducted based on manufacturer's protocols using Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc. Rochville, MD, USA). Briefly, 100 µl of NIH3T3 cells at a density 5000 cells/well were plated in a 96-well plate. Pre-incubate the plate for 24 hours in a humidified incubator (37°C, 5% CO_2). One µl of each wood barks methanol extract were added to the wells to give final four levels concentration (100, 10, 1 and 0.1 µg/ml) and incubated for 48 hours in the incubator. As a control, one µl of DMSO was added to each well. Ten µl of CCK-8 solution was added to the each well and the plates were incubated for four hours in the incubator. The absorbance was measured at 450 nm using a microplate reader (THERMO max, Molecular Devices, LLC., Sunnyvale CA, USA). The inhibitory rate was calculated with the following equation:

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

***In vivo* toxicity test**

Animals

Female C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan) at six weeks of age. All mice were maintained for one week before starting experiments in Animal Facility of Kochi University Medical School. All experiments were approved by

the Animal Care and Use Committee for Kochi University and conducted under Specific Pathogen Free (SPF) condition.

Toxicity of *P. speciosa* extract to C57BL/6J mice

In this experiment, mice were divided into 4 groups and methanol extract of *P. speciosa* was used as the most toxic one in the *in vitro* cytotoxicity test among samples prepared. Each group consists of 3 mice except Group 2. Group 1 (three mice) received 0.1 ml/mouse of distillated water (DW), Group 2 (four mice) received 0.5 mg/0.1ml DW/mouse of extract (29.4 mg/kg bodyweight), Group 3 (three mice) received 2.5 mg/0.1ml DW/mouse of extract (147 mg/kg bodyweight) and Group 4 (three mice) received 5 mg/0.1ml DW/mouse of extract (294 mg/kg bodyweight). The plant extracts were administered to mice orally using a polyethylene capillary sonde every other day for one week (three times of oral administration). Bodyweight was measured every other day. In this experiment, average bodyweight of C57BL/6J mice was 17 g.

Statistics

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

4.3. Results and Discussion

In vitro cytotoxic activities of methanol extracts from wood barks against NIH3T3 cells

Crude extracts from varieties of plant contain many unknown phytocompounds that may have beneficial effects for the treatment of diseases, but they may contain toxic molecules for human (Kandhare *et al*. 2015). *In vitro* cytotoxicity test is one of the first steps that is recommended for the assessment of hazardous unwanted effects of plant extracts (Theiszovaa *et al*. 2005). A cytotoxicity test is useful for preliminary screening of crude extracts, because it can be used to separate toxic compounds from nontoxic ones and can provide evidence of safety of plant extracts. A cytotoxicity test is a rapid, standardized, sensitive, and inexpensive to examine whether a plant extract contains noxious compounds (Merit *et al*. 2006). Many types of cells have been used for cytotoxicity tests, and one of them is NIH3T3 cell line (Ekwall *et al*. 1990).

In this study, cytotoxicity of methanol extracts from wood barks of *D. dulcis*, *D. kutejensis*, *P. timoriana*, *P. speciosa*, *P. intermedia*, *B. angulata*, and *D. dao* that have the activities to inhibit α-glucosidase *in vitro* and are traditionally used as herbal medicine to treat diarrheas, stomachaches, and diabetes on the normal mouse fibroblast NIH3T3 cells were evaluated. NIH3T3 cells are non-tumor cells (Orsine *et al.* 2013) that frequently used in biological research for study of apoptosis and cell cycle regulation. This cell line has an advantage of not carrying mutations in oncogenes and preserving an intact cell cycle during examination (Seluanov *et al*. 2010).

Results show that, almost all methanol extracts from wood barks are toxic at a concentration of 100 µg/ml except *B. angulata* (**Fig. 4.1**). The most toxic extract is from *P. timoriana* (inhibitory rate 92.67%; **Fig. 4.1.C**), followed by that from *D. dao* (91.73%; **Fig. 4.1.G**). Diversity of toxicity occur at lower concentrations (≤10 µg/ml). At a concentration of 10 µg/ml, the percentages of inhibition are low and almost all plant extracts have inhibition under 50% except those from *P. speciosa* (72.34%; **Fig. 4.1.D**) and *P. timoriana* (56.47%; **Fig. 4.1.C**). All samples show lower toxicity effect at a concentrations of 1 µg/ml.

Samples with percentages of inhibition higher than 60% are classified into a group of high level of cytotoxicity (severe cytotoxicity), those with 40-60% inhibition are classified into a group of moderate level of cytotoxicity, those with 20-40% inhibition are classified into a group of a mild level of cytotoxicity, and those under 20% inhibition are classified into a group of a low level of cytotoxicity (Kucekova *et al*. 2014). Almost all the methanol extracts from wood barks at a concentration of 100 µg/ml were classified to have severe cytotoxicity, except *B. angulata*. Extracts from plant species belonging to the genus of *Parkia* were more toxic than those of other species at a concentration of 10 µg/ml, especially *P. speciosa* belongs to a group with severe cytotoxicity and *P. timoriana* belongs to a group with moderate cytotoxicity. At lower concentrations (1 and 0.1 µg/ml), all methanol extracts from wood barks belong to the groups with mild or low level of cytotoxicity.

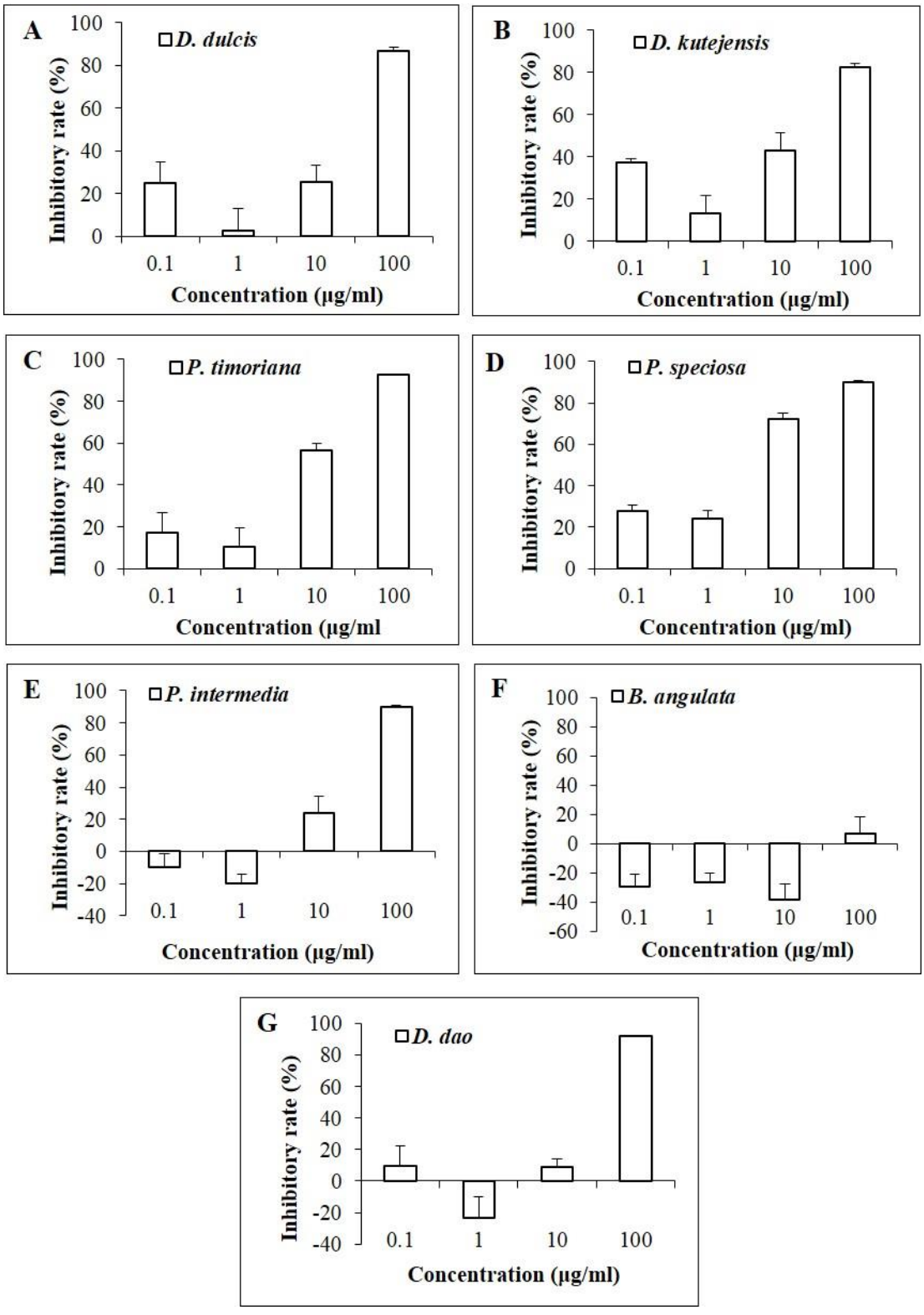


Fig 4.1. Cytotoxicity of methanol extracts from wood barks against NIH3T3 cells.

(A): *D. dulcis,* (B): *D. kutejensis,* (C): *P. timoriana,* (D): *P. speciosa,* (E): *P.*

intermedia, (F): *B. angulata,* and (G): *D. dao.* The values are shown as mean ± SE (n=4).

Many of the bioactive compounds from wood barks examined in this study are not yet identified. In case of methanol extracts from wood barks, however, some of compounds are identified such as oils, fats, waxes, alkaloids, flavones, polyphenols, tannins, saponins, glycosides and aglycones (Houghton and Raman 1998, Filho 2006). The use of polar solvents such as methanol in extracting the wood bark will give us phenolic constituents in large amounts, such as flavanoid compounds included in the group of condensed tannins (phenolic acids) and monomer of flavonoids such as quercetin and dihydroquercetin (taxifolin) (Sjostrom 1981).

Bittencourt *et al*. (2013) reported that guaraná extracts (*Paullinia cupana*) that contain condensed tannins, catechins, caffeins, and theobromines show no cytotoxicity against fibroblast NIH3T3 cells at 20 mg/ml. Serrano-Diaz *et al*. (2014) reported that the extracts of saffron floral bio-residues (*Crocus sativus*) which contain polyphenols, flavonols, quercetin and anthocyanins show no cytotoxic effect on 3T3 fibroblast cells at 900 μg/ml. Tajudin *et al*. (2012) reported that methanol extracts of *Cynometra cauliflora* which contain high level of total polyphenols show no cytotoxicity at 15 μg/ml.

Since crude extracts have complex compounds and many of them are unidentified, probably other compounds besides tannins, polyphenols, flavonols, and quercetin have toxic effects on NIH3T3 cells at a high concentration (100 μg/ml), but at lower concentrations almost all compounds in the methanol extracts from wood barks are not harmful. Based on this result, it is concluded that the final concentration of one μg/ml is suitable for the analysis of anti-intestinal inflammation using FPCK-1-1 cells. For toxicity assay using C57BL/6J mice, it is decided to use *P. speciosa* extracts because of its highest cytotoxicity among plant extracts at lower concentrations.

Toxicity of the methanol extract from wood bark of *P. speciosa* in C57BL/6J mice

Based on the results *in vitro* cytotoxicity test, all samples had low level of cytotoxicity below a concentration of 10 μg/ml. Since *P. speciosa* was the most toxic one against NIH3T3 cells at a concentration of 10 μg/ml, it was selected as a sample to estimate the toxicity of methanol extracts from wood barks *in vivo* using C57BL/6J

mice. *In vivo* toxicity test is very important to identify the range or levels of doses that could be used subsequently, and to know the possible toxic signs that occur during examination (Mopuri and Meriga 2014).

One of the important parameters to measure toxic effects of plant extracts in mice is body weight (Gonzalez *et al*. 2012, Hor *et al*. 2012, Teo *et al*. 2002). Organic and functional changes that occur in the metabolic system cause the difference in the metabolism of digested food, resulting in the diversity in body weight (Gonzalez *et al*. 2012). To administer methanol extracts from wood barks to mice, 0.5 mg/0.1ml DW/mouse, 2.5 mg/0.1ml DW/mouse, and 5 mg/0.1ml DW/mouse were chosen. Methanol extract from wood barks of *P. speciosa* was administered to mice on day 0, 2 and 4.

Results revealed no significant difference among all groups administered with methanol extract of *P. speciosa* in terms of body weight of C57BL/6J mice (**Fig. 4.2)**.

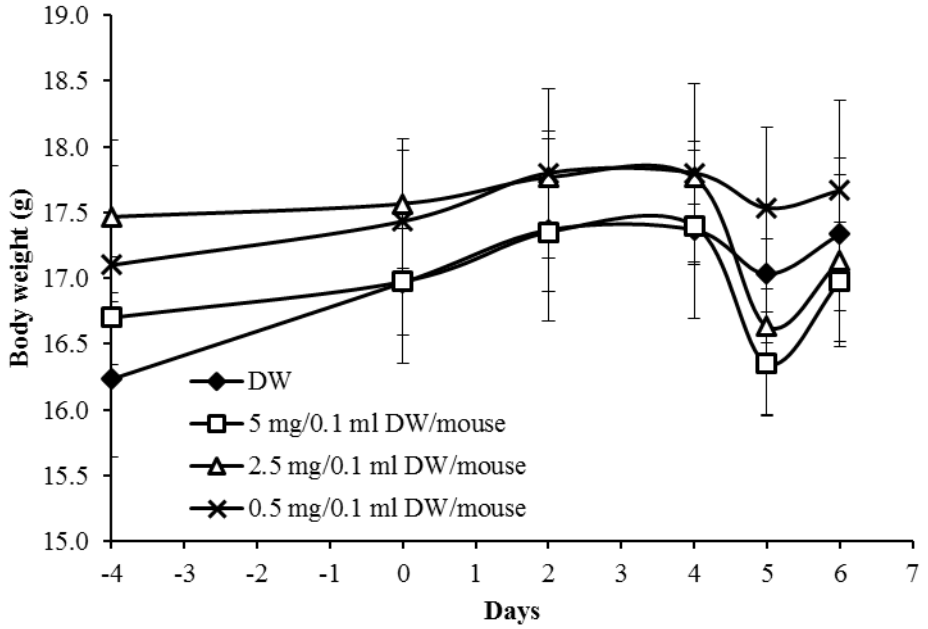


Fig. 4.2. Toxicity of methanol extract from wood bark of *P. speciosa* in C57BL/6J mice. Methanol extract from wood bark of *P. speciosa* was administered on day 0, 2, and 4. The values are shown as mean ± SE (n=3 for Groups of DW, 2.5 mg/mouse, and 0.5 mg/mouse. n=4 for Group of 5 mg/mouse). No significant differences are found in body weight among groups in any

combination of dosage groups (P<0.05, LSD post hoc test, one-way ANOVA)

As presented in **Fig. 4.2**, after the first and second oral administration, the increase of average body weight of mice in all groups was observed. One day after the third oral administration (day 5), the body weight of all group of mice decreased. Two day after the third administration (day 6), the body weight of all groups recovered to the previous levels. These results indicate that after oral administration, mice might have stress and lost body weight by loosing appetite. It seems that the recovery process takes two days after oral administration. After three times administration, there was no significant differences between DW group and other groups.

Signs of toxicity of plant extracts against mice are the symptoms such as reducing of body weight, but not the mortality (Hor *et al.* 2012). At any dose of methanol extract from wood bark of *P. speciosa*, the extracts did not show any sign of toxic symptoms such as reduced body weight (**Fig. 4.2**)

Substances with LD_{50} between 5000 and 15000 mg/kg body weight are classified as non-toxic (Hor *et al.* 2012). LD_{50} is the dose that causes the death of half of the tested population in the *in vivo* model (Zhang *et al*. 2007). Even though the LD_{50} were not measured because of no mortality in the toxicity test, it is concluded that *P. speciosa* extracts is not toxic and safe under the given condition. Because after administrating three times of *P. speciosa* extract at a dose of 5 mg/0.1ml/mouse (294 mg/kg body weight), the average body weight of mice was not significantly different from that of mice administered with DW.

Hor *et al.* (2012) reported that the methanol extract from red dragon fruit (*Hylocereus polyrhizus*) containing phenols and 2-furancarboxaldehyde is not toxic at a dose of 5000 mg/kg body weight to male and female Sprague-Dawley (SD) rats. Mopuri and Meriga (2014) reported that ethanol extract from bark of *Terminalia paniculata* containing polyphenols, tannins, alkaloids, and triterphenoids is not toxic at a dose of 1000 mg/kg body weight in Sprague Dawley rats. Possible bioactive compounds such as tannins, triterpenoids, and phenols in the methanol extracts of *P. speciosa* are expected to be non-toxic at a low dose of 5 mg/0.1 ml DW/mouse (294 mg/kg body weight).

Based on the results described above, it is conclude that 5 mg/0.1 ml DW/mouse of methanol extracts of *P. speciosa* is safe for the continuous administration to leptin receptor deficient *db/db* mice.

4.4. Conclusions

The cytotoxic effects of methanol extracts from wood barks of *D. dulcis*, *D. kutejensis*, *P. timoriana*, *P. speciosa*, *P. intermedia*, *B. angulata*, and *D. dao* on normal mouse fibroblast NIH3T3 cells were evaluated. Almost all methanol extracts from wood barks are toxic at a concentration of 100 μg/ml, except *B. angulata*. Methanol extract from *P. speciosa* is the most toxic at a concentration of 10 μg/ml and all methanol extracts from wood barks are low toxic at concentrations of 1 μg/ml.

P. speciosa extract is not toxic in C57BL/6J mice even after administrating 5 mg extract/mouse. There was no significant difference in body weight between the mice administered with 5 mg *P. speciosa* extract/mouse and those administered with DW. It was determined that the final concentration of *P. speciosa* extract to be added to the anti-intestinal inflammation assay using FPCK-1-1 cells is 1 μg/ml and the safe dose for the administration to leptin receptor-deficient *db/db* type 2 diabetes mellitus mice is 5 mg/0.1ml DW/mouse.

Example of raw data and calculations of cytotoxicity on NIH3T3 cells by methanol extracts.

Table 4.1. Estimation of the cytotoxicity inhibitory rate by methanol extracts (100 µg/ml) from wood barks of 7 medicinal plants.

Sample	Average of absorbance ± SE (n=4)	Inhibitory rate ± SE (%) (n=4)
Control	1.0466±0.1313	
Durian meranang (*D. dulcis*)	0.1370±0.0171	86.91±1.64
Durian pekawai (*D. kutejensis*)	0.1810±0.0161	82.71±1.55
Control	1.4430±0.1077	
Petai kedaung (*P. timoriana*)	0.1057±0.0081	92.67±0.56
Petai pendek (*P. speciosa*)	0.1445±0.0117	89.98±0.81
Control	1.0310±0.1063	
Petai panjang (*P. intermedia*)	0.1075±0.0107	89.57±1.04
Enceriak (*B. costulata*)	0.9630±0.1211	6.59±11.75
Control	1.0946±0.0511	
Sengkuang (*D.dao*)	0.0905±0.0045	91.73±0.41

Example: calculation of cytotoxicity inhibitory rate by methanol extract (100 µg/ml) from wood bark of durian meranang (*D. dulcis*).

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

$$\text{Inhibitory rate of durian meranang}\,(D.\,dulcis)\,(\%) = \left(1 - \frac{0.1370}{1.0466}\right) \text{x}\, 100\%$$

$$\text{Inhibitory rate of durian meranang}\,(D.\,dulcis)\,(\%) = (1 - 0.1309)\, \text{x}\, 100\%$$

$$\text{Inhibitory rate of durian meranang}\,(D.\,dulcis)\,(\%) = 86.91\%$$

Chapter 5
Effects of six medicinal plants on maltose loading tests in *db/db* mice

5.1. Introduction

Controlling of postprandial blood glucose level is the early treatment to minimize the risk of chronic vascular complications of patients with type 2 diabetes (Shihabudeen *et al*. 2011). Carbohydrate is a main source of human diet that is composed of long-chain of glycoside bonds, and is digested by α-glucosidase to produce disaccharides such as maltose and sucrose (Jaiswal *et al.* 2012). Maltose is abundant digested products of carbohydrates in small intestine. Maltase, one of α-glucosidase enzymes in the mucosal brush border of the small intestine catalyzes the maltose digestion to produce two glucoses (Shihabudeen *et al*. 2011). Inhibition of α-glucosidase in the digestive system will delay the digestion of maltose and extend the digestion time to produce two glucoses, which decreases the rate of glucose absorption, resulting in the reduction of blood glucose level.

Maltose loading test is a rapid test to evaluate the effects of plant extracts to reduce blood glucose level after oral administration of maltose. Ability of plant extracts to improve maltose tolerance indicates the potential of plant extracts as new agents for the treatment of type 2 diabetes.

Previous research showed that methanol extracts of *D. dulcis*, *D. kutejensis*, *P. timoriana*, *P. speciosa*, *B. costulata*, and *D. dao* have activity to inhibit α-glucosidase *in vitro* (**Chapter 3**). Anti-diabetic effect of natural medicine in the *in vitro* assay does not necessarily suggest the anti-diabetic effect *in vivo,* because there is no metabolism in the *in vitro* assay (Soumyanath and Srijayantha 2006). Among the plant extracts that have activity to inhibit α-glucosidase *in vitro*, some of them do not effectively inhibit α-glucosidase in a mammalian model (Shihabudeen *et al*. 2011). It is very important to prove the effectiveness of plant extracts that inhibit α-glucosidase *in vitro* in the model of type 2 diabetes *in vivo*.

Leptin is a peptide hormone that mainly produced by white adipose tissue and is involved in the regulation of appetite. This hormone decreases the food consumption, elevates energy expenditures, and down-regulates metabolic efficiency (Lago *et al*. 2008, Otero *et al*. 2005). The disruption of leptin hormone increases the appetite, resulting in the increase of body weight. Obesity induced adipose tissue inflammation and insulin resistance that cause type 2 diabetes (Hsieh 2011). Leptin receptor is encoded by diabetes (*db*) gene (Lago *et al*. 2008, Otero *et al*. 2005).

Leptin receptor-deficient *db/db* mouse is a good model of type 2 diabetes. This strain is well known as a mouse model of obesity that consume food more than twice as much as the wild type mice, resulting in the higher levels of blood glucose, triacylglycerol, and cholesterol (Dwiranti *et al*. 2012, Kobayashi *et al*. 2000). It was found that *D. dulcis*, *P. timoriana*, and *P. speciosa* have activity to down-regulate the blood glucose level of *db/db* mice in maltose loading tests.

5.2. Materials and Methods

Plant extracts

Methanol extracts from wood barks of *D. dulcis*, *D. kutejensis*, *P. timoriana, P. speciosa*, *B. costulata*, and *D. dao* were prepared as described in the previous Chapter (**Chapter 2**). Five hundred mg (dry weight) of the methanol extracts were suspended in 10 ml of distilled water, DW (50 mg/ml) (Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan), grinded in a mortar, and homogenized using a Polytron homogenizer (Kinematica, Luzern, Switzerland). Homogenate of extracts was heated in a water bath Incubator BT-22 (Yamato Scientific Co., Ltd., Tokyo Japan) at 75°C for 30 minutes, while mixing every five minutes (Vortex Genie 2, Scientific Industries Inc., New York) and stored at room temperature for overnight. The heating procedure was repeated twice and extracts were stored at 4°C before use.

Anti-diabetes assay

Animals

Female mice of leptin receptor-deficient strain, BKS.Cg-+*Lepr*^{db}/+*Lepr*^{db}/Jcl (*db/db*) and female mice of the parental strain, BKS.Cg-m^{+}/m^{+}/Jcl (+/+) were purchased from CLEA Japan (Tokyo, Japan) at six weeks of age. All mice were maintained for one

week before the start of experiments in the Animal Facility of Kochi University Medical School. The first maltose-loading test was conducted at 7 weeks old. The second maltose-loading test was performed at 8 weeks old. All experiments are approved by the Animal Care and Use Committee for Kochi University and conducted under Specific Pathogen Free (SPF) conditions.

Maltose loading tests

In this experiment, mice were divided into 9 groups. Group 1 (non-diabetic control) consists of eight female mice of BKS.Cg-m^{+}/m^{+}/Jcl (+/+). BKS.Cg-+$Lepr^{db}$/+$Lepr^{db}$/Jcl (*db/db*) mice were divided into 8 groups, Groups 2-9 and each group consists of six female mice. Maltose loading tests were conducted twice and grouping, concentration of reagents, and time of measurement of blood glucose are described in **Table 5.1**. Acarbose and plant extracts were administered orally using a polyethylene capillary sonde to 14 hours-fasted mice five minutes before the oral administration of maltose. One drop of blood was taken from a lateral tail vein of each mouse and levels of blood glucose were measured with Accu-Chek (Roche Diagnostics K.K., Tokyo Japan) at indicated times.

Statistics

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

Table 5.1. Grouping of mice in maltose loading tests.

Exp. No.	Group (G)		Concentration*		Age of mice	Time of measurement blood glucose
			Maltose	Extracts		
I	G1	Wild type	27 mg/0.1ml/mouse	-	7 weeks	0 minutes
	G2	Diabetic control	54 mg/0.2ml/mouse	-		70 minutes
	G3	+ Acarbose	54 mg/0.2ml/mouse	1 mg/0.1ml/mouse		140 minutes
	G4	+ *D. dulcis*	54 mg/0.2ml/mouse	1 mg/0.1ml/mouse		210 minutes
	G5	+ *D. kutejensis*	54 mg/0.2ml/mouse	1 mg/0.1ml/mouse		
	G6	+ *P. timoriana*	54 mg/0.2ml/mouse	1 mg/0.1ml/mouse		
	G7	+ *P. speciosa*	54 mg/0.2ml/mouse	1 mg/0.1ml/mouse		
	G8	+ *B. costulata*	54 mg/0.2ml/mouse	1 mg/0.1ml/mouse		
	G9	+ *D. dao*	54 mg/0.2ml/mouse	1 mg/0.1ml/mouse		
II	G1	Wild type	27 mg/0.1ml/mouse	-	8 weeks	0 minutes
	G2	Diabetic control	54 mg/0.2ml/mouse	-		30 minutes
	G3	+ Acarbose	54 mg/0.2ml/mouse	8 mg/0.1ml/mouse		60 minutes
	G4	+ *D. dulcis*	54 mg/0.2ml/mouse	5 mg/0.1ml/mouse		120 minutes
	G5	+ *D. kutejensis*	54 mg/0.2ml/mouse	5 mg/0.1ml/mouse		
	G6	+ *P. timoriana*	54 mg/0.2ml/mouse	5 mg/0.1ml/mouse		
	G7	+ *P. speciosa*	54 mg/0.2ml/mouse	5 mg/0.1ml/mouse		
	G8	+ *B. costulata*	54 mg/0.2ml/mouse	5 mg/0.1ml/mouse		
	G9	+ *D. dao*	54 mg/0.2ml/mouse	5 mg/0.1ml/mouse		

*Following body weight of mice was used to decide a dose of reagents. All extracts were suspended in DW as described in Materials and Methods. Experiment I: Non-diabetic parental strain of mice (wild type); 16 g. *db/db* mice; 36 g. Experiment II: Non-diabetic parental strain of mice; 17 g, *db/db* mice; 40 g.

5.3. Results and Discussion

Down-regulation of the level of blood glucose to the normal range is very important for patients with type 2 diabetes to prevent another complication caused by the hyperglycemia. Inhibition of α-glucosidase is one of the ways to reduce blood glucose by delaying of glucose absorption in small intestine (Jaiswal *et al*. 2012). It is very important to find scientific evidences *in vivo* that traditionally used plants to treat diabetic patients have a function to reduce the level of blood glucose by inhibiting α-glucosidase.

The previous research showed that *D. dulcis*, *D. kutejensis*, *P. timoriana*, *P. speciosa*, *B. costulata*, and *D. dao* have activity to inhibit α-glucosidase *in vitro*. To find out if these plants have activities to reduce blood glucose *in vivo*, maltose loading tests were conducted using the leptin receptor-deficient *db/db* mice. Maltose is a major digestion product of carbohydrates and abundant in small intestine. The *db/db* mice are known as obese mice that eat diet more than twice as much as wild type mice, and this strain of mouse is a good model of type 2 diabetes (Dwiranti *et al*. 2012, Kobayashi *et al*. 2000).

Maltose loading tests were conducted twice. In the first experiment, concentration of extract and acarbose is equal, 30 mg/kg body weight (1 mg/0.1 ml/mouse). Results showed that only *D. dulcis*, *P. timoriana*, and *P. speciosa* reduced the level of blood glucose after maltose loading compared with *D. kutejensis* and *B. costulata* as shown in **Fig. 5.1**.

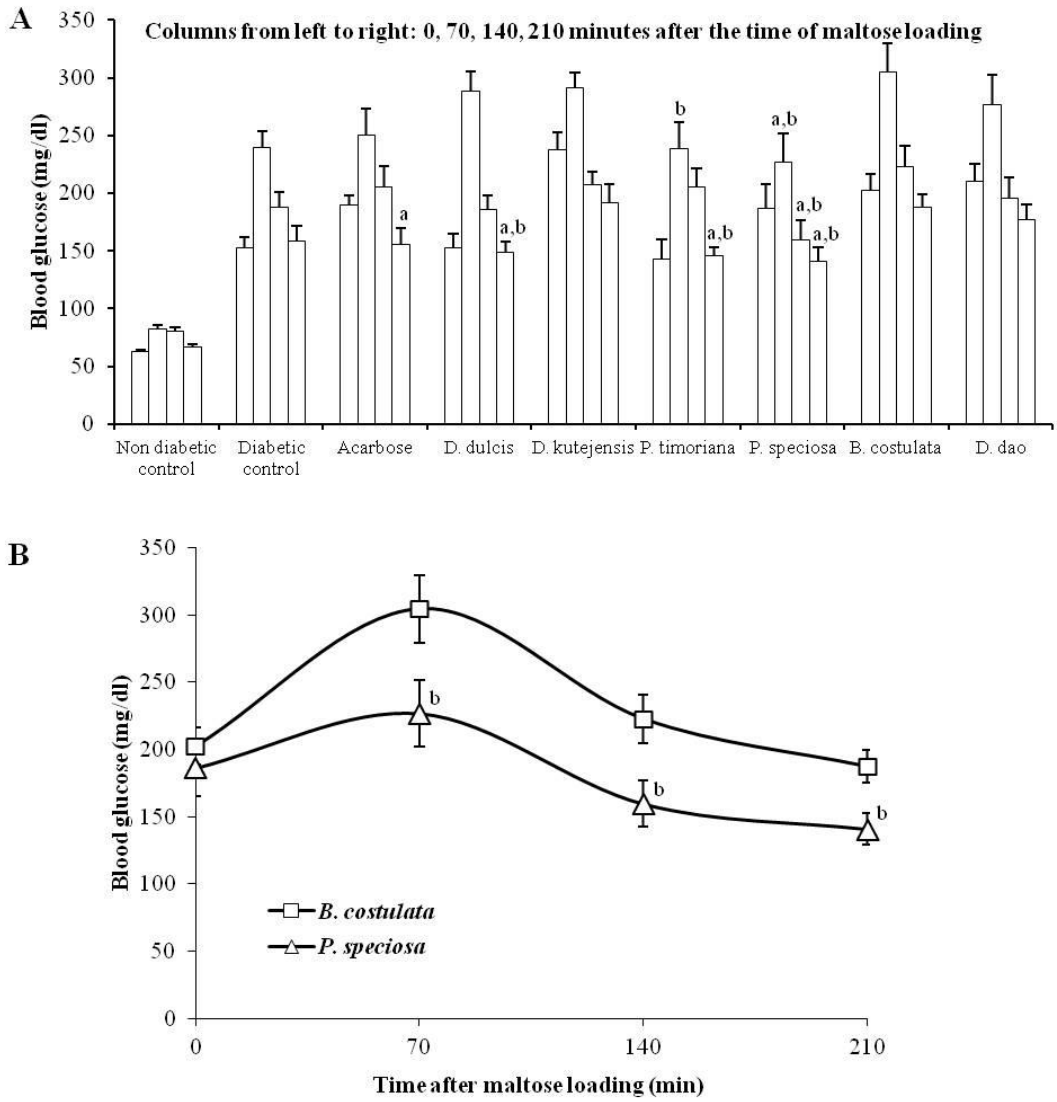


Fig. 5.1. Effects of methanol extracts from wood barks on the blood glucose levels of *db/db* mice in a maltose loading test I. Blood glucose level was measured at 0, 70, 140 and 210 minutes after maltose loading. Acarbose (30 mg/kg body weight) and plants extracts (30 mg/kg body weight) were administrated orally using a polyethylene capillary sonde to fasted mice five minutes before oral administration of maltose (1.5 g/kg body weight). Levels of blood glucose were measured as described in Materials and Methods. **Panel A**: All data of blood glucose levels of parental line (non diabetic control), and *db/db* mice. **Panel B**: There are significant differences between *P. speciosa* group vs. *B. costulata* group at 70 min, 140 min, and 210 min ($P < 0.05$). Results are shown

as the average ± SE (n=8 for parental line and n=6 for *db/db* mice). Significant differences between *D. kutejensis* vs. other groups and *B. costulata* vs. other groups are indicated by letters a and b, respectively ($P < 0.05$, LSD post hoc test, one-way ANOVA).

As presented in **Fig. 5.1**, almost all groups of *db/db* mice have variety of fasting blood glucose (0 minutes) even though the average level of casual blood glucose of each group was adjusted before experiment from 390 to 405 mg/dl. At 70 minutes after maltose loading, all groups had absorbed maltose to increase the levels of blood glucose. The lower levels of blood glucose were found in groups administered with *P. speciosa* and *P. timoriana* extracts. There was a significant difference between *P. speciosa* group and *B. costulata* group, at 70 min, 140 min, and 210 min after maltose loading (**Fig. 5.1.B**). At 140 minutes after maltose loading, only the level of blood glucose of *P. speciosa* group was significantly lower than that of the group administered with either *D. kutejensis* or *B. costulata*. At 210 min after maltose loading, levels of blood glucose of groups administered with acarbose, *D. dulcis*, *P. timoriana*, and *P. speciosa* were significantly lower than those of *D. kutejensis* group and *B. costulata* group.

In the second experiment (**Fig. 5.2**), the measurement of blood glucose was conducted at 30 min, 60 min, and 120 min after the maltose loading. The dose of methanol extract administered to mice was 125 mg/kg body weight of mouse (5 mg/0.1 ml/mouse) and that of acarbose was 200 mg/kg body weight (8 mg/0.1 ml/mouse). Results showed that levels of blood glucose of groups administered with acarbose and *P. speciosa* extracts were significantly lower than that of *B. costulata* group at 30 min, 60 min, and 120 min. At 30 min after maltose loading, the level of blood glucose of acarbose group was significantly lower than that of diabetic control group. The level of blood glucose of *D. dulcis* group was significantly lower than that of *B. costulata* group at 60 min and 120 min. The level of blood glucose of *P. timoriana* group was significantly lower than that of *B. costulata* group at 120 min.

In two maltose loading tests, effect of *P. speciosa* extract to down-regulate the blood glucose was confirmed at three points in each experiments compared with *B. costulata* extract.

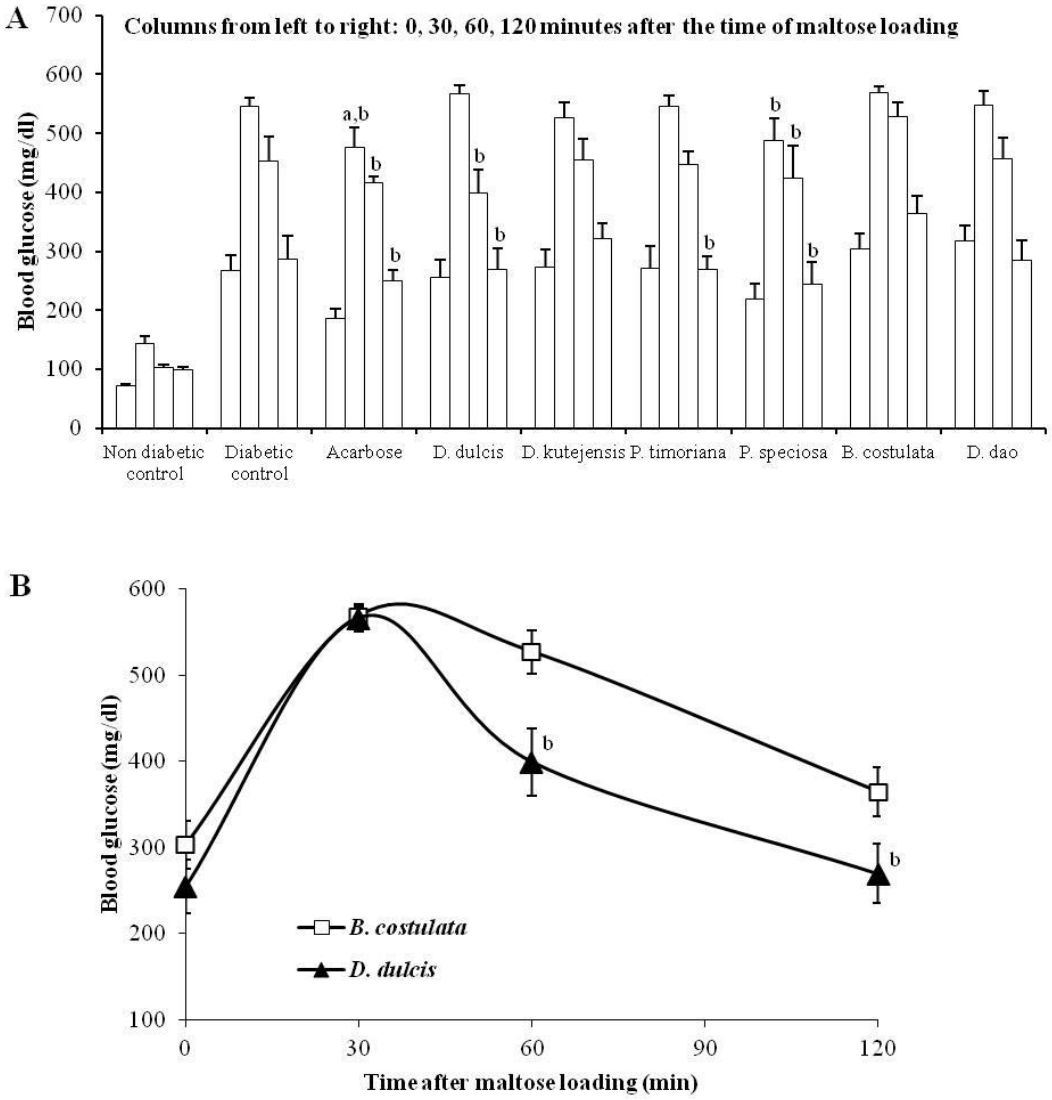


Fig. 5.2. Effects of methanol extracts from wood barks on the blood glucose levels of *db/db* mice in a maltose loading test II. Blood glucose level was measured at 0, 30, 60 and 120 minutes after the maltose loading. Acarbose (200 mg/kg body weight) and plants extracts (125 mg/kg body weight) were administered orally using a polyethylene capillary sonde to fasted mice five minutes before oral administration of maltose (1.35 g/kg body weight). The blood glucose levels were measured as described in Materials and Methods. **Panel A**: All data of blood glucose levels of parental line (non diabetic control), and *db/db* mice. **Panel B**: There are significant differences between *D. dulcis* group vs. *B. costulata* group at 60 min and 120 min. Results are shown as the average ± SE

(n=8 for parental line and n=6 for *db/db* mice). Significant differences between diabetic control group vs. acarbose group and *B. costulata* vs. other group are shown by letters a and b, respectively ($P < 0.05$, LSD post hoc test, one-way ANOVA).

Regulation of postprandial blood glucose is needed to minimize some cardiovascular complication of diabetic patients (Kim *et al*. 2011). Some methanol extract of medicinal plants such as *Salacia reticulate*, *S. oblonga* (Matsuda *et al*. 2002), *Cinnamomum zeylanicum* (Shihabudeen *et al*. 2011) and *Acorus calamus* (Prisilla *et al*. 2012) are already reported to have the ability to regulate the level of blood glucose after loading of maltose and sucrose in rat. Another report shows that extract of *Zingiber mioga* reduced the blood glucose level of *db/db* mice in the sucrose loading test (Jo *et al*. 2016). It is also reported that extracts from *Vigna nakashimae* (Yeo *et al*. 2011) and *Angelica dahurica* (Park *et al*. 2016) regulated the level of the blood glucose in the glucose tolerance test.

In this experiment, six methanol extracts from wood barks of medicinal plants in West Kalimantan, Indonesia that inhibited yeast α-glucosidase *in vitro* were administered to *db/db* mice in maltose loading tests. Although many of the plant extracts have the ability to inhibit yeast α-glucosidase, some of them do not effectively inhibit α-glucosidase in a mammalian model (Shihabudeen *et al*. 2011). So, it is essential to prove the effectiveness of plant extracts to ameliorate the diabetes *in vivo* model of type 2 diabetes.

Mice were fasted for 14 hours before oral administration of methanol extracts from wood barks followed by the administration of maltose. Fasting is very important to observe maltose utilization in the intestine of mice, because digestion of maltose and the transfer of digested glucose to blood must be conducted without the influence of glycogen stores (Ayala *et al*. 2010, Dwiranti *et al*. 2012). Increased level of blood glucose after the maltose loading indicates that maltose is digested by α-glucosidase to be absorbed by small intestine and the decreased level of blood glucose of each group of mice administered with each plant extract suggests that the methanol extract of the plant has the inhibitory effect on the intestinal α-glucosidase. The major source of absorbable glucose as digestive product of carbohydrates in the small intestine is maltose (Tadera *et*

al. 2006). The delay of maltose digestion in small intestine will decrease the rate of glucose absorption, resulting in the reduction of the blood glucose level in diabetic mice. In addition, the inhibition of glucose transport from small intestine to blood stream, or the stimulation of transfer of glucose from blood stream into cells is necessary to suppress the blood glucose level after maltose loading (Nerio *et al*. 2012).

Although extracts from *D. dulcis*, *P. timoriana*, and *P. speciosa* effectively decreased the level of blood glucose of *db/db* mice, they did not recover the level of blood glucose to the normal range. These results may be relevant to the level of inhibition of α-glucosidase *in vitro* by these extracts, because extracts from *P. speciosa* and *P. timoriana* have low IC_{50} values (IC_{50} is a concentration of the extract required to inhibit 50% of α-glucosidase activity under the assay condition). In contrast, *D. kutejensis* and *D. dao* that have also low IC_{50} value did not significantly reduce the level of blood glucose of *db/db* mice in two maltose loading tests.

In mammal intestine, there are α-glucosidase such as sucrase-isomaltase and maltase-glucoamylase. They have different substrate specificities and are involved in the digestion of sugars and starches (Asano 2003). This is the reason why the maltose loading tests *in vivo* were performed. To clarify the discrepancy described above, it is necessary to examine the effectiveness of plant extracts by administrating them for a long term.

Methanol extracts from wood barks have a large amount of phenolic constituents such as flavanoid compounds including a group of condensed tannins (phenolic acids) and monomers of flavonoids such as quercetins and dihydroquercetins (taxifolins) (Sjostrom 1981). Tadera *et al.* (2006) reported that six groups of flavonoid compounds especially flavonol, flavanone, isoflavone, and anthocyanidin effectively inhibit α-glucosidase. Jo *et al.* (2010) reported that quercetin compounds have high levels of inhibition against maltose-digesting enzymes in rat intestine. Kim *et al*. (2011) reported that quercetin had reduced blood glucose level of STZ-induced diabetic rats after starch loading. Methanol extracts from wood barks of *D. dulcis*, *P. timoriana*, and *P. speciosa* allegedly contain flavonoids and quercetins as bioactive compounds that may be responsible to reduce the blood glucose levels of *db/db* mice after maltose loading.

In two maltose loading tests, methanol extract from wood bark of *P. speciosa* showed significant effects to reduce blood glucose levels. It is also reported that extracts

of seeds and empty pods of *P. speciosa* reduce the blood glucose level in the glucose tolerant test using Alloxan-Sprague Drawley rats (Jamaludin and Mohamed 1993, Jamaludin *et al.* 1995). In this experiment, a potential bioactive compound is stigmast-4-en-3-one (Jamaludin and Mohamed 1993, Jamaludin *et al.* 1995). Phytochemical substances of *P. speciosa* are alkaloids, phenols, terpenoids, tannins, and saponins (Kamisah *et al*. 2013). Bioactive compounds from seeds and pods such as thiazolidine-4-carboxylic acid, thioproline, tetrathiane, tetrathiepane, trithiolane, and pentathiocane are reported to be potential reagents for anti-cancer, anti-oxidant, and anti-microbial activities (Kamisah *et al*. 2013).

It is reported that *P. timoriana* have hepatoprotective on paracetamol-induced liver damage in Wistar rats (Ajibola *et al.* 2013) and phytochemical substances of this plants are saponins, tannins, terpenes, phenols, sterols, and flavonoids (Ajibola *et al.* 2013, Tisnadjaja 2006). *D. dulcis* is native plant from Kalimantan and Sumatra (Lim 2012), and until now no activities of this plant is reported. Probably this is the first report of biological activities of *D. dulcis*, especially as anti-diabetes reagent.

In **Chapter 3**, extracts of medicinal plants of West Kalimantan that have a strong inhibitory activity against yeast α-glucosidase *in vitro* were reported. In this report, it was found that extracts from *D. dulcis*, *P. timoriana*, and *P. speciosa* inhibited the increase of blood glucose in two maltose loading tests.

5.4. Conclusions

Anti-diabetic effects of methanol extracts from wood barks of *D. dulcis*, *D. kutejensis*, *P. timoriana*, *P. speciosa*, *D. dao*, and *B. costulata* that are traditionally used to treat diarrheas, stomachaches, and diabetes in West Kalimantan, Indonesia were analyzed. Results showed that *D. dulcis*, *P. timoriana*, and *P. speciosa* have activity to down-regulate the blood glucose levels of *db/db* mice in maltose loading tests. The activity of *P. speciosa* extract to down-regulate the blood glucose was confirmed at three points in two maltose loading tests compared with *B. costulata* extract. Further study is required to examine the effectiveness of plant extracts by administrating them for a long term.

Chapter 6

Anti-diabetic effects of long-term administration of methanol extracts from wood barks of six medicinal plants in *db/db* mice

6.1. Introduction

The prevalence levels of type 2 diabetes and obesity has rapidly increased in the world (Yeo *et al*. 2011) and 90% cases of type 2 diabetes are caused by excess of body weight (Song *et al*. 2013). The changes in lifestyle, represented by high calorie foods and less exercises are main factors of this incident. Obesity, insulin resistance, and type 2 diabetes are linked with inflammation and an activation of immune system (Dandona *et al*. 2004, Esser *et al*. 2014, Wellen and Hotamisligil 2005). Inflammation is a biological response employed by both innate and adaptive immune systems that is triggered by harmful stimuli, such as the malfunction and infection of tissue (Ashley *et al*. 2012, Medzhitov 2008). Markers of inflammation in type 2 diabetes are tumor necrosis factor (TNF-α), interleukin-1β (IL-1β), and IL-6 (Dandona *et al*. 2004, Esser *et al*. 2014, Wellen and Hotamisligil 2005). Sites of inflammation in obesity are liver, muscle, pancreas, and adipose tissue (Esser *et al*. 2014). Leptin that is mainly produced by white adipose tissue (Otero *et al*. 2005) have a function to control of appetite (Fantuzzi 2005). The disruption of leptin hormone increases the appetite resulting in the increase of body weight (obesity), insulin resistance, and type 2 diabetes (Otero *et al*. 2005).

Leptin receptor-deficient *db/db* mice have been used as animal model of type 2 diabetes (Chen *et al*. 1996, Hummel *et al*. 1966, Lee *et al*. 1996). This strain of mouse has the higher levels of body weight (obese), food consumption, blood glucose, cholesterol, and triacylglycerol compared wild type mice (Dwiranti *et al*. 2012, Kobayashi *et al*. 2000). Many of medicinal plants have activities to reduce the blood glucose level of *db/db* mice after long-term administration (De La Garza *et al*. 2014, Kang *et al*. 2010, Kim *et al*. 2011, Yeo *et al.* 2011), suggesting that they have the potential as new natural medicine for type 2 diabetes.

In the previous Chapters, methanol extracts of *D. dulcis, D. kutejensis, P. timoriana, P. speciosa, B. costulata* and *D. dao* are reported to have the activity to inhibit α-glucosidase *in vitro* (**Chapter 3**), and three of them, *D. dulcis, P. timoriana*, and *P. speciosa* have the ability to decrease the blood glucose levels of *db/db* mice after maltose loading (**Chapter 5**). Mechanism of methanol extracts to reduce the blood glucose level of *db/db* mice in maltose loading tests is probably through the inhibition of α-glucosidase in the brush border of intestinal epithelial cells.

Since maltose loading tests show us the effect of a single oral administration of each plant extract, the long-term effects of each plant extract in *db/db* mice cannot be evaluated. To find evidence about other effects of methanol extracts from wood barks in *db/db* mice, a long-term oral administration of plant extracts to *db/db* mice were carried out and their effects on the levels of blood glucose before and after fasting, food consumption, body weight, adipose tissue, cholesterol, and triacylglycerol were evaluated. It was found that methanol extracts are not toxic and safe for continues use in *db/db* mice at a concentration of 5 mg/mouse. Some species such as *P. timoriana, P. speciosa*, and *D. dao* reduced the blood glucose levels of *db/db* mice after administering them for four weeks, and *D. dulcis* and *P. speciosa* reduced the blood levels of triacylglycerol in *db/db* mice.

6.2. Materials and Methods

Plant extracts

Methanol extracts from wood barks of *D. dulcis, D. kutejensis, P. timoriana, P. speciosa, B. costulata*, and *D. dao* were prepared as described in the previous chapter (**Chapter 2**). Five hundred mg (dry weight) of the methanol extracts were suspended in 10 ml of distilled water (50 mg/ml) (Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan), grinded in a mortar, and homogenized using a Polytron homogenizer (Kinematica, Luzern, Switzerland). Homogenates of plants extracts were heated in a water bath Incubator BT-22 (Yamato Scientific Co., Ltd., Tokyo Japan) at 75°C for 30 minutes, while mixing every five minutes (Vortex Genie 2, Scientific Industries Inc., New York) and stored at room temperature for overnight. The heating procedure was repeated twice and extracts were stored at 4°C before use.

Anti-diabetes assay

Animals

Female leptin receptor-deficient BKS.Cg-+*Lepr*^{db}/+*Lepr*^{db}/Jcl (*db/db*) mice and female mice of the parental strain, BKS.Cg-*m*$^{+}$/*m*$^{+}$/Jcl (+/+) were purchased from CLEA Japan (Tokyo, Japan) at six weeks of age. All mice were maintained for one week before the start of experiments in the Animal Facility of Kochi University Medical School. After finishing two maltose loading tests, long-term administration was started at the age of 10 weeks old. All experiments are approved by the Animal Care and Use Committee for Kochi University and conducted under Specific Pathogen Free (SPF) conditions.

Oral administration of methanol extracts from wood barks in a long-term administration

Eight female mice of BKS.Cg-*m*$^{+}$/*m*$^{+}$ (+/+) in Group 1 (non-diabetic control) are not treated. BKS.Cg-+*Lepr*^{db}/+*Lepr*^{db}/Jcl (*db/db*) mice were divided into eight groups as described above (**Chapter 5, Table 5.1**). Group 2 received 0.1 ml of distillated water (DW), Group 3 received acarbose (200 mg/kg body weight), Group 4 received *D. dulcis* (125 mg/kg body weight), Group 5 received *D. kutejensis* (125 mg/kg body weight), Group 6 received *P. timoriana* (125 mg/kg body weight), Group 7 received *P. speciosa* (125 mg/kg body weight), Group 8 received *B. costulata* (125 mg/kg body weight), and Group 9 received *D. dao* (125 mg/kg body weight). The extracts administrated orally using a polyethylene capillary sonde every other day for four weeks. After two, three, and four weeks of oral administration, one drop of blood was taken from a lateral tail vein of each mouse and levels of blood glucose were measured with Accu-Chek (Roche Diagnostics K.K., Tokyo Japan) before and after fasting (14 hours-fasted mice). Following body weights of mice were used to decide a dose of reagents: Non-diabetic control mice (parental strain); 17 g, *db/db* mice; 40 g.

Measurement of serum levels of total cholesterol and triacylglycerol

Blood was collected from orbital sinus from each of mouse using a glass capillary sonde under anesthetization and serums were prepared. Serum levels of total cholesterol and triacylglycerol were measured using Hitachi Clinical Analyzer S40 (Hitachi, Ltd., Tokyo, Japan) with S-Test Cartridges for cholesterol and triacylglycerol (Enzymatic method: GPO-POD method without Free Glycerol).

Statistics

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

6.3. Results and Discussion

The high levels of blood glucose, cholesterol, and triacylglycerol are some indicators of type 2 diabetes. Many kinds of modern medicines were used to reduce the levels of blood glucose, cholesterol, and triacylglycerol of diabetes patients. However, these medicines have adverse side effects and the effectiveness decreases in time (drug-resistance). Furthermore, modern medicines are not effective in some patients with long-term diabetic complication and medical expenses are high (Hsu *et al*. 2009). Currently, the use of medicinal plants is an option for the treatment of diabetes due to less side effects, relative safety, and lower prices compared with modern medicine (Ablat *et al*. 2014).

In this study, medicinal plants from West Kalimantan were examined whether they have the activity to reduce the levels of blood glucose after administrating them for a month. The levels of blood glucose before and after fasting, two, three, and four weeks after oral administration were measured (**Figs. 6.1 and 6.2**).

As shown in **Fig. 6.1**, before starting the oral administration of plant extracts, the level of average casual blood glucose of all groups of *db/db* mice were adjusted from 390 to 405 mg/dl without fasting. The levels of blood glucose of all groups continued to increase with age, except *D. dao* group whose level of blood glucose increased till two weeks and declined at four weeks after starting oral administration. Two and four weeks after oral administration of methanol extracts from wood barks, levels of blood glucose were differentiated. The levels of blood glucose of *P. timoriana* group and *D. dao* group were lower than those of diabetic control, *D. kutejensis*, and *B. costulata* groups four weeks after administration.

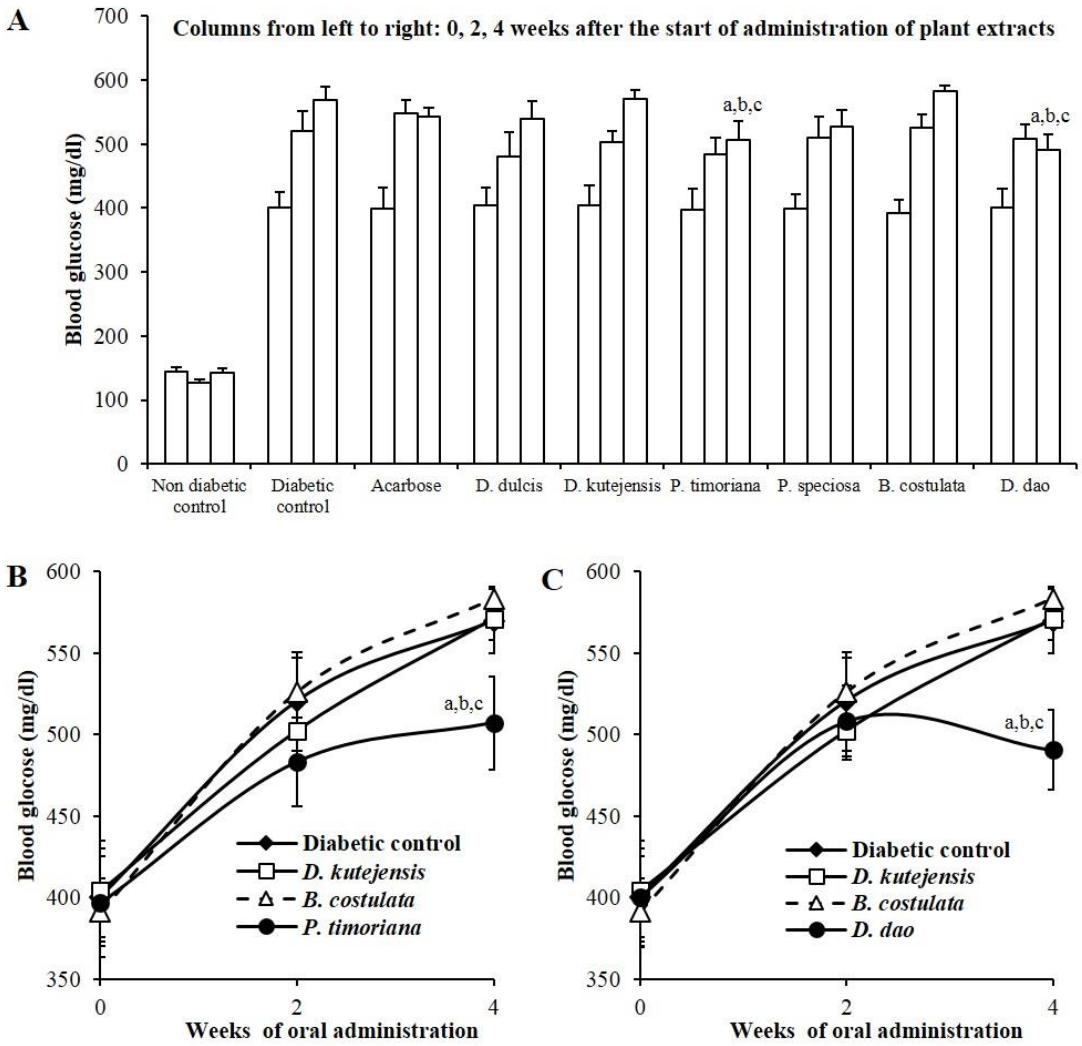


Fig. 6.1. The levels of blood glucose of *db/db* mice before fasting, two and four weeks after oral administration of methanol extracts from wood barks of medicinal plants. Acarbose (200 mg/kg body weight) and plant extracts (125 mg/kg body weight) were administrated orally using a polyethylene capillary sonde every other day for four weeks. One drop of blood was taken from a tail vein of each mouse and the blood glucose were measured as described in Materials and Methods. **Panel A**: All data of blood glucose levels of all groups including a parental strain (non diabetic control) and *db/db* mice. **Panel B**: There is a significant difference between the following groups: diabetic control vs. *P. timoriana* (a); *D. kutejensis* vs. *P. timoriana* (b); *B. costulata* vs. *P. timoriana* (c) four weeks after oral administration. **Panel C**: There is a significant difference between the following groups: diabetic

control vs. *D. dao* (a); *D. kutejensis* vs. *D. dao* (b); *B. costulata* vs. *D. dao* (c) four weeks after oral administration. The values are shown as mean ± SE (n=8 for parental line and n=6 for *db/db* mice). ($P < 0.05$, LSD post hoc test, one-way ANOVA).

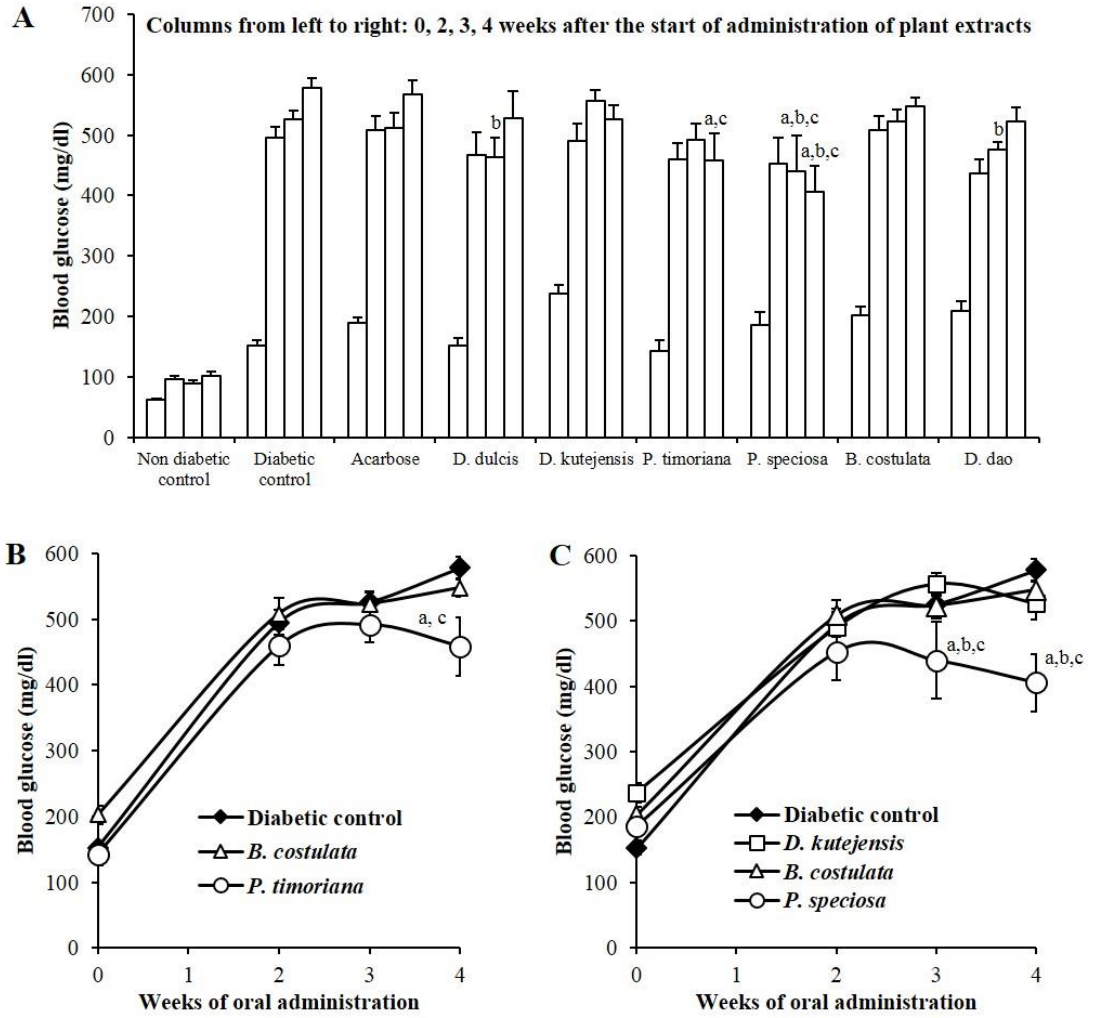


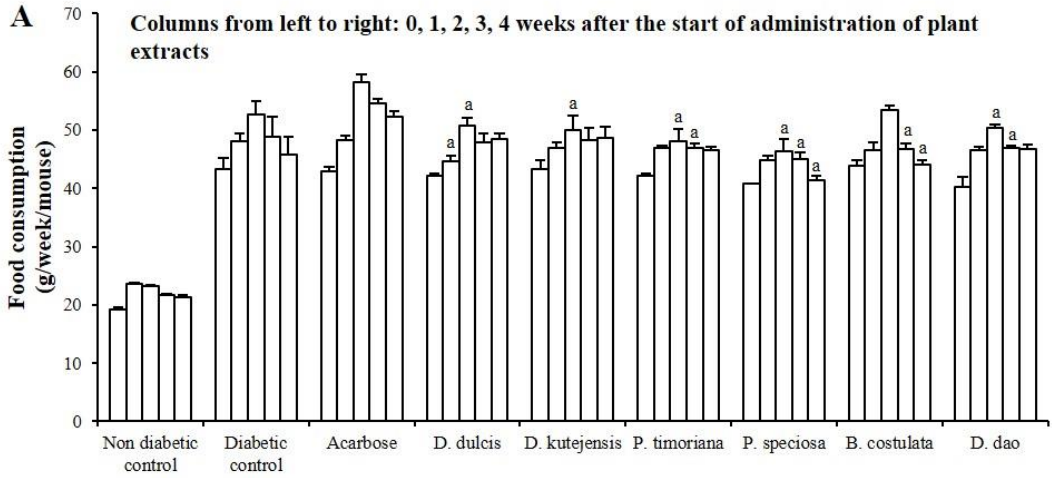
Fig. 6.2. The levels of fasting blood glucose of *db/db* mice, two, three and four weeks after oral administration of methanol extracts from wood barks of medicinal plants. Acarbose (200 mg/kg body weight) and plant extracts (125 mg/kg body weight) were administrated orally using a polyethylene capillary sonde every other day for four weeks. One drop of blood was taken from a tail vein of each mouse and the blood glucose was measured as described in Materials and Methods. **Panel A**: All data of blood glucose levels of all groups including a parental strain (non diabetic control) and *db/db* mice.

Panel B: There is a significant difference between the following groups: diabetic control vs. *P. timoriana* (a); *B. costulata* vs. *P. timoriana* (c) four weeks after oral administration. **Panel C**: There is a significant difference between the following groups: diabetic control vs. *P. speciosa* (a); *D. kutejensis* vs. *P. speciosa* (b); *B. costulata* vs. *P. speciosa* (c) three and four weeks after oral administration. The values are shown as mean ± SE (n=8 for parental line and n=6 for *db/db* mice). ($P < 0.05$, LSD post hoc test, one-way ANOVA).

As presented in **Fig. 6.2**, the levels of fasting blood glucose of *db/db* mice were varied from 153 to 238 mg/dl. According to the increase of age, levels of fasting blood glucose increased up to more than 500 mg/dl four weeks after starting oral administration, except *P. timoriana* and *P. speciosa* groups. Significant differences were found between the following groups three and four weeks after starting oral administration: diabetic control vs. *P. speciosa*; *D. kutejensis* vs. *P. speciosa,* and *B. costulata* vs. *P. speciosa* (**Fig. 6.2, Panel C**). Significant differences were also found between the following groups four weeks after starting oral administration: diabetic control vs. *P. timoriana*; *B. costulata* vs. *P. timoriana* (**Fig. 6.2, Panel B**).

Methanol extracts from wood barks have a large amount of phenolic constituents such as flavanoid compounds including a group of condensed tannins (phenolic acids) and monomers of flavonoids such as quercetins and dihydroquercetins (taxifolins) (Sjostrom 1981). Methanol extracts from wood barks of *D. dulcis, P. timoriana, P. speciosa,* and *D. dao* allegedly contain flavonoids and quercetins as bioactive compounds. In **Chapter 3**, it was reported that extracts of medicinal plants of West Kalimantan have a strong inhibitory activity against yeast α-glucosidase *in vitro*. In **Chapter 4**, it was reported that extracts from *D. dulcis, P. timoriana,* and *P. speciosa* inhibited the increase of blood glucose in the two maltose loading test. In this report, it was found that extracts from *P. timoriana, P. speciosa,* and *D. dao* down-regulated the levels of blood of *db/db* mice four weeks after starting the oral administration.

Tadera *et al.* (2006) reported that six groups of flavonoid compounds especially flavonol, flavanone, isoflavone, and anthocyanidin effectively inhibit α-glucosidase. Jo *et al.* (2010) reported that quercetin compounds have high levels of inhibition against maltose-digesting enzymes in rat intestine. Kim *et al*. (2011) reported that quercetin has the ability to reform the level of fasting blood glucose through the elevation of insulin sensitivity by inhibiting α-glucosidase and enhancing the insulin signaling in *db/db* mice. Kang *et al.* (2010) reported that Welsh onion (*Allium fistulosum*) extract could reduce the glucose toxicity by decreasing the fasting blood glucose and increasing insulin sensitivity of *db/db* mice. These results suggest that methanol extracts from *D. dulcis, P. timoriana,* and *P. speciosa* have the abilities to delay the maltose digestion in small intestine (**Figs. 5.1 and 5.2**) and those from *P. timoriana, P. speciosa*, and *D. dao* may enhance insulin sensitivity resulting in the decrease of blood glucose in *db/db* mice (**Figs. 6.1 and 6.2**). Although acarbose reduced the blood glucose at a concentration of 8 mg/mouse in the maltose loading test (**Fig. 5.2**), no significant effect was observed by a long-term oral administration. Yeo *et al*. (2011) reported that acarbose effectively inhibited sucrose absorption in sucrose loading test of normal mice, however, acarbose did not show significant effect to reduce fasting blood glucose after 15 days of oral administration in long-term administration of *db/db* mice.



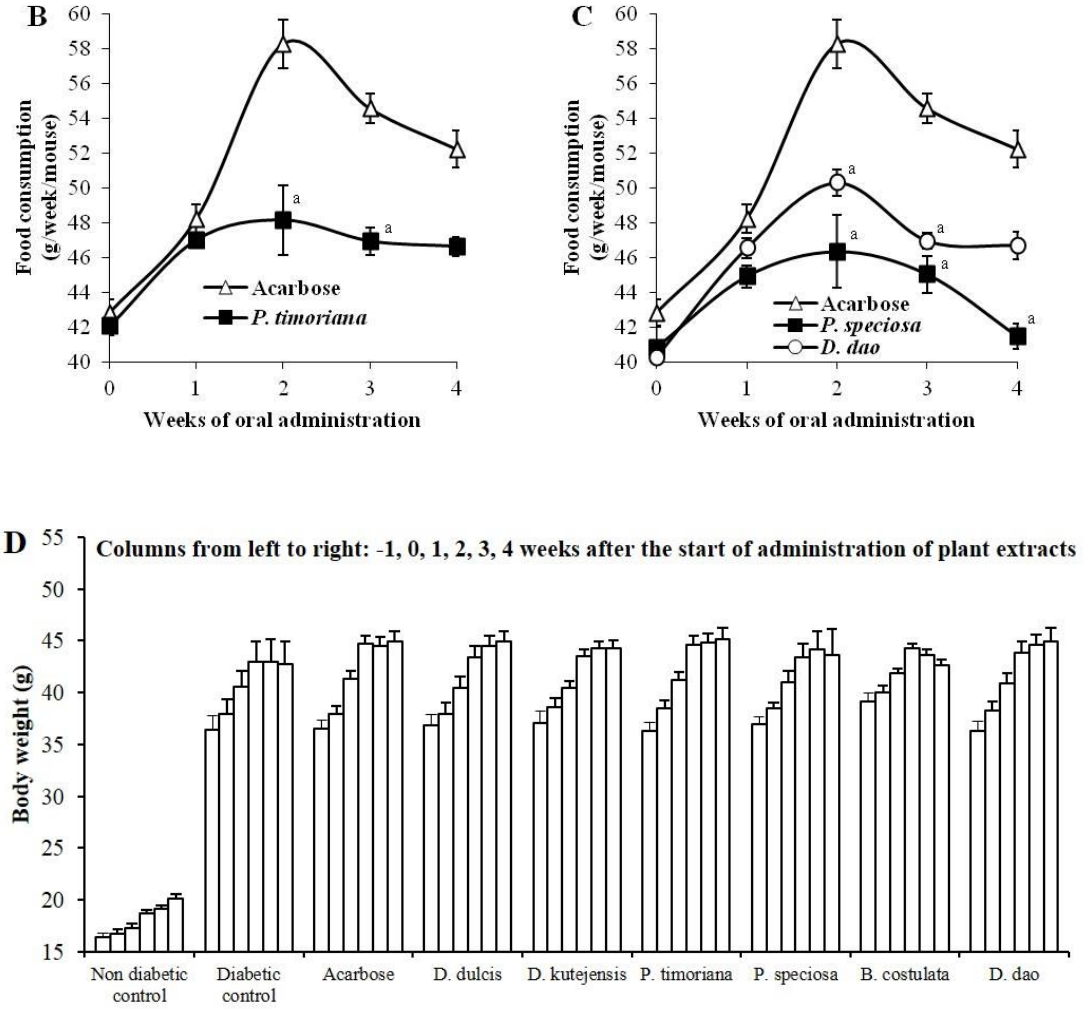


Fig. 6.3. Effect of methanol extracts from wood barks on food consumption and the body weight in *db/db* mice. Food consumption and body weight of each mouse were measured every week. **Panel A**: Data of all groups of food consumption in parental line (non diabetic control) and *db/db* mice. **Panel B**: There is a significant difference in food consumption between *P. timoriana* group and acarbose group. **Panel C**: There are significant differences between the following groups: *P. speciosa* group vs. acarbose group; *D. dao* group vs. acarbose group. **Panel D**: No significant differences are found in body weight among *db/db* mice groups in any combination. Results are shown as the average ± SE (n=8 for parental line and n=6 for *db/db* mice). Significant differences between acarbose vs. other group are shown by letter a ($P < 0.05$, LSD post hoc test, one-way ANOVA).

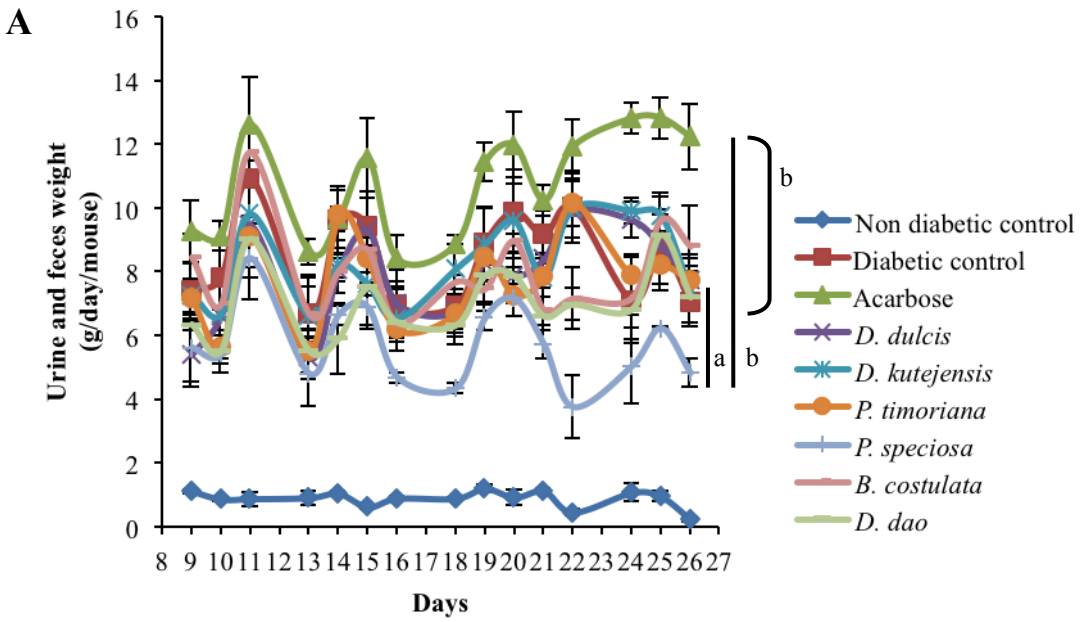
As shown in **Fig. 6.3.A**, most *db/db* mice consumed food more than twice as much as parental mice. Food consumption of mice continuously increased till two weeks after starting oral administration of plant extracts, and then the food consumption declined in groups administered with plant extracts. At the end of the administration of acarbose and plant extracts, *db/db* mice consumed from 41.48 to 52.22 g/week/mouse. Acarbose group had highest food consumption (52.22 g/week/mouse) among all *db/db* groups. Only *P. speciosa* and *B. costulata* groups consumed significantly lower amount of food than acarbose group four weeks after starting the oral administration. Kim *et al.* (2014) reported that food consumption of male *db/db* mice in long-term supplementation of low molecular weight chitosan oligosaccharide was less than that of acarbose.

There was no significant difference between any plant extract group and diabetic control group in food consumption (**Fig. 6.3.A**). However, there were significant differences between the following groups at indicated weeks after starting administration: acarbose group vs. *D. dulcis* at one and two weeks; acarbose group vs. *D. kutejensis* group at two weeks; acarbose group vs. *P. timoriana* at two and three weeks; acarbose group vs. *P. speciosa* at two, three, and four weeks; acarbose group vs. *B. costulata* at three and four weeks; acarbose vs. *D. dao* at two and three weeks (**Fig. 6.3.A, B and C**). It was suggested that methanol extracts from wood barks weakly suppress the appetite of *db/db* mice. Kim *et al*. (2011) reported that quercetin effectively reduce blood glucose level of *db/db* mice without any effect on food consumption and body weight. Yeo *et al.* (2011) reported that extract of *Vigna nakashimae* did not show significant effect on body weight and food consumption of *db/db* mice. Kang *et al.* (2010) reported that Welsh onion (*Allium fistulosum*) has an activity to control blood glucose level of *db/db* mice without showing significant effects on body weight and food consumption.

Body weight of all *db/db* mice increased continuously till four weeks after starting the administration of plant extracts except that of *B. costulata* group. No significant difference was found in body weight of mice between diabetic control group and other groups, suggesting that the methanol extracts are not toxic and safe for continuous use (**Fig. 6.3.D**). Reducing body weight is one of the indicators of toxicity of plants (Gonzalez *et al*. 2012, Hor *et al*. 2012, Teo *et al*. 2002). It is reported that the loss of body weight is due to the alteration of metabolic pathways in the body (Gonzalez *et al*.

2012). These results suggest that all the methanol extracts from wood barks of West Kalimantan plants are not toxic when administered to *db/db* mice at a dose of 5 mg/mouse every other day for four weeks.

As presented in **Fig. 6.4**, *db/db* mice groups had higher levels of urine and feces compared with wild type mice, and acarbose group is the highest among all *db/db* mice. At the end of treatment, almost all of *db/db* mice groups show significant effects to reduce the weight of urine and feces compared with acarbose group, except *B. costulata* (**Fig. 6.4.A**). Especially, *P. speciosa* showed significant differences between the following combinations on the 26th days after starting the administration: *P. speciosa* vs. diabetic control group; *P. speciosa* vs. acarbose group (**Fig. 6.4.B**).



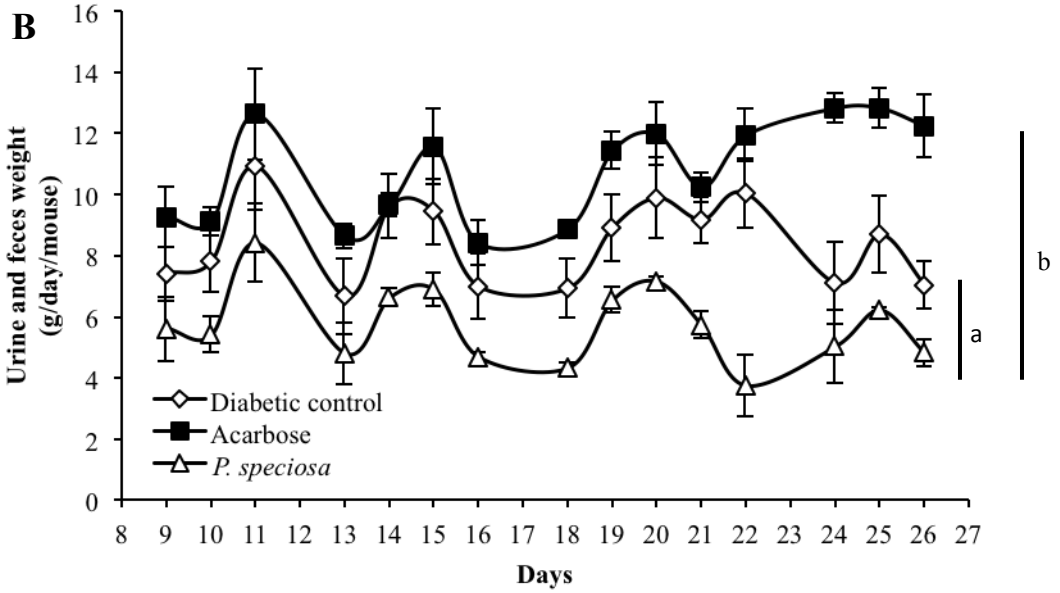


Fig. 6.4. Effect of methanol extracts from wood barks on the weight urine and feces in *db/db* mice. Weight of urine and feces in each cage were measured every day after one weak of oral administration. Each cage contains two mice. **Panel A**: Data of all groups in weight of urine and feces including parental line (non diabetic control) and eight groups of *db/db* mice. **Panel B**: There are significant differences in the weight of urine and feces between the following groups on the 26th days after starting the administration: *P. speciosa* group vs Diabetic control group; *P. speciosa* group vs Acarbose group. Results are shown as the average ± SE (n=8 for parental line and n=6 for *db/db* mice). Significant differences between Diabetic control vs. other groups and Acarbose vs. other groups are indicated by letters a, and b, respectively ($P < 0.05$, LSD post hoc test, one-way ANOVA).

One of signs of diabetes is polyuria, or high in urine quantities (Soenanto 2005). Polyuria is triggered by excess of glucose in plasma that affect to osmosis and urination (Antai *et al*. 2010). In this experiment, urine and feces were measured. Although the weight of feces is not the sign of diabetes, feces were measured because it is difficult to separate urine and feces in one cage of mice. One weak after oral administration, it was noticed that *P. speciosa* group showed lower level in the weight of urine and faces compared other groups.

Antai *et al*. (2010) reported that *Rothmannia hispida* extract reduced the urine output of alloxan-male albino Wistar rats after 14 days of oral administration with improved insulin sensitivity. Arakawa *et al*. (2001) reported that T-1095 (3-(benzo[b]furan-5-yl)-2',6'-dihydroxy-4'methylpropiophenone2'-O-(6-Omethoxycarbonyl-b-D-glycopyranoside) had reduced urine volume and water intake of *db/db* mice by down-regulating urinary glucose excretion. These results suggest that methanol extracts of *P. speciosa* may improve insulin sensitivity and urinary glucose excretion of *db/db* mice compared with acarbose.

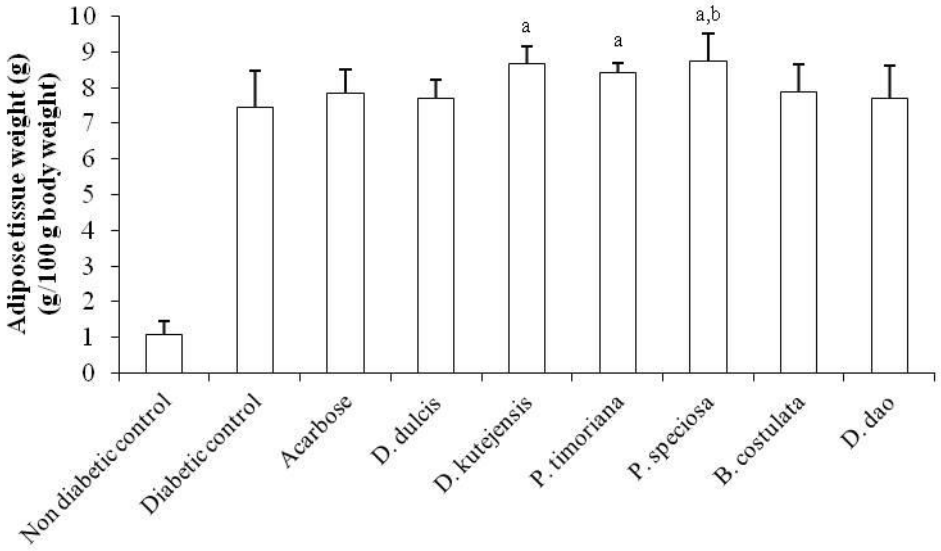


Fig. 6.5. Effect of methanol extracts from wood barks on the weight of adipose tissue of *db/db* mice. At the end of treatment (four weeks of oral administration every other day), the weight of adipose tissue around the uterus was measured. The weight of fat tissues was expressed as g/100 g body weight. Results are shown as the average ± SE (n=8 for parental strain and n=6 for *db/db* mice). Significant differences were found between the following groups: Diabetic control group vs. *D. kutejensis* group; Diabetic control group vs. *P. timoriana*; Diabetic control group vs. *P. speciosa* (indicated by letter a, $P < 0.05$, LSD post hoc test, one way ANOVA). There is a significant difference between *B. costulata* group and *P. speciosa* group indicated by letter b ($P < 0.05$, LSD post hoc test, one-way ANOVA).

As shown in **Fig. 6.5**, the average weight of adipose tissue around uterus in *db/db* mice was seven times heavier than that in parental mice. *D. kutejensis*, *P. speciosa*, and *P. timoriana* groups had significantly heavier adipose tissue than that of diabetic control group. The weight of adipose tissue of *P. speciosa* group was significantly higher than that of *B. costulata* group.

Adipose tissue has important roles to regulate the appetite, energy consumption, insulin sensitivity, and immune response against inflammation (Fantuzzi *et al*. 2005). White adipose tissue that constitutes a major part of adipose tissue has a function to store energy (Fantuzzi *et al*. 2005). De La Garza *et al*. (2014) reported that *Helichrysum italicum* and *Citrus x paradise* extracts significantly reduced the level of blood glucose of *db/db* mice, while percentage of total adipose tissue was slightly increased although no significant difference was shown statistically. Since it is expected that *P. speciosa* and *P. timoriana* groups use blood glucose more efficiently than other groups, it is suggested that these two groups may store glucose as fat more efficiently compared with other groups (**Figs. 6.2 and 6.5**). In other words, blood glucose in *P. speciosa* and *P. timoriana* groups may be converted to fatty acids to be stored in adipose tissue, resulting in having higher level of white adipose tissue compared with other groups.

As shown in **Fig. 6.6.A**, acarbose group and groups administered with plant extracts except *B. costulata* extract have the tendency to have increased levels of cholesterol compared with diabetic control group. There were significant differences at the level of serum cholesterol between *P. timoriana* group vs. *B. costulata* group and between *D. dao* group vs. *B. costulata* group. As shown in **Fig. 6.6.B**, levels of serum triacylglycerol in *D. dulcis* group and *P. speciosa* group were significantly lower than that of diabetic control group.

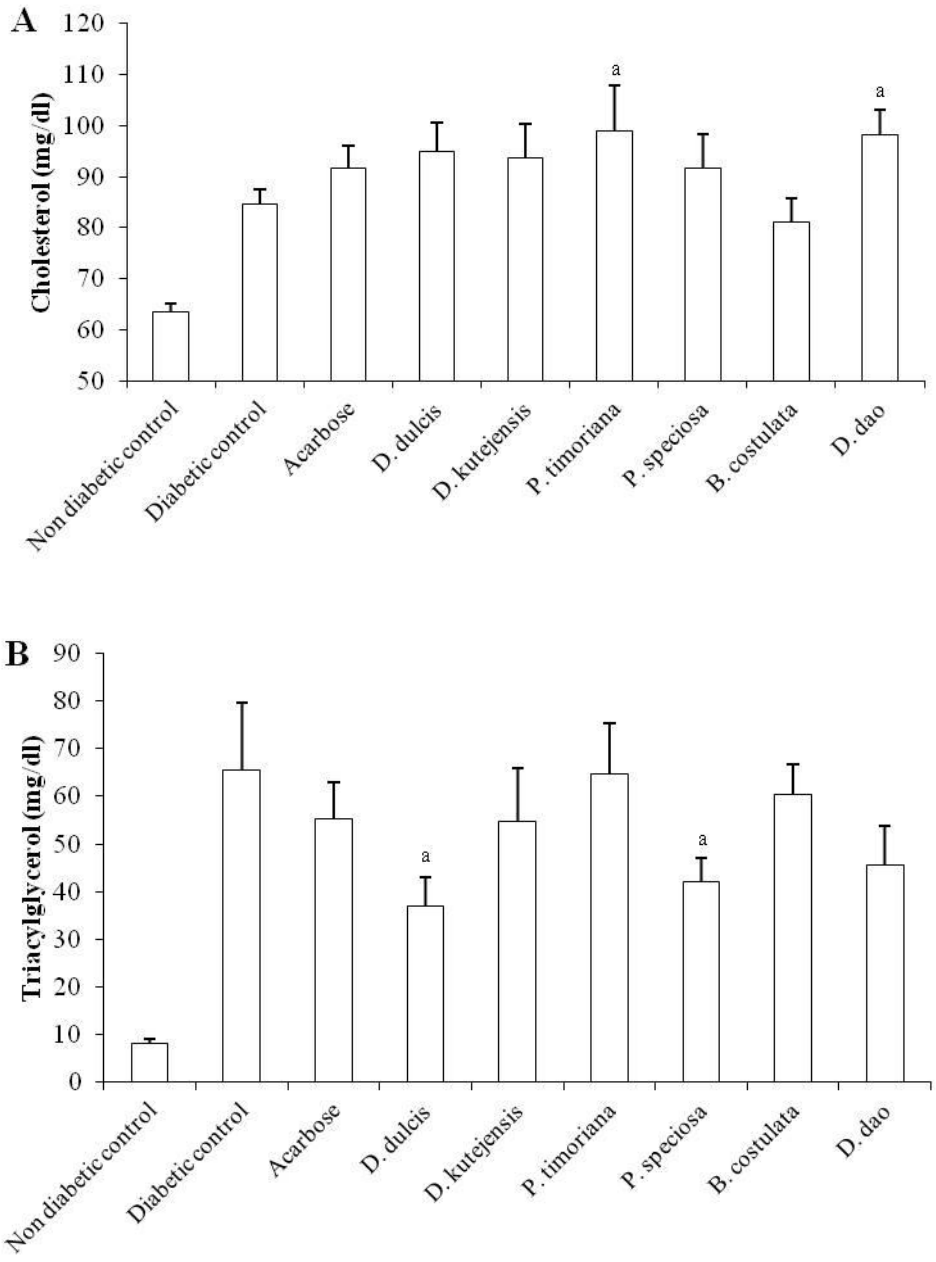


Fig. 6.6. Effects of methanol extracts from wood barks on the serum levels of cholesterol and triacylglycerol in *db/db* mice. At the end of treatment (four weeks of oral administration), serum of each mouse was prepared as described in Materials and Methods. **Panel A**: all data of cholesterol of parental mice and *db/db* mice. There were significant differences between the following groups: *P. timoriana* group vs. *B. costulata* group; *D. dao* group vs. *B. costulata* group. Significant difference between *B. costulata* vs. other

group was indicated by letter a ($P < 0.05$, LSD post hoc test, one-way ANOVA). Results are shown as the means ± SE (n=8 for parental line and n=6 for *db/db* mice). **Panel B**: all data of triacylglycerol of parental mice and *db/db* mice. There were significant differences between the following groups: *D. dulcis* group vs. Diabetic control group; *P. speciosa* group vs. Diabetic control group. Significant difference between diabetic control vs. other group was indicated by letter a ($P < 0.05$, LSD post hoc test, one-way ANOVA). The values of triacylglycerol are without free glycerol. Results are shown as the means ± SE (n=8 for parental line and n=6 for *db/db* mice. n=5 for diabetic control, *P. timoriana*, and *B. costulata*).

Dwiranti *et al.* (2012) reported that homogenate of *Ecklonia kurome* gametophytes reduced the levels of blood glucose and level of serum triacylglycerol in *db/db* mice. The metabolism of glucose and triacylglycerol are regulated, in part, by leptin and IFN-γ (Dwiranti *et al*. 2012). Resistance of insulin induced high level of triacylglycerol. *Scutellaria baicalensis* extract reduced the level of triacylglycerol of *db/db* mice by ameliorating insulin sensitivity (Song *et al*. 2013). Methanol extracts from wood barks of *D. dulcis*, *P. timoriana, P. speciosa*, and *D. dao* may influence the signaling systems of leptin, IFN-γ, and insulin to regulate the levels of glucose, cholesterol, and triacylglycerol in the blood of *db/db* mice.

Inflammation is reported to be involved in diabetes (Dandona *et al*. 2004, Esser *et al*. 2014, Wellen and Hotamisligil 2005). Increment of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 causes the insulin resistance that is associated with obesity and type 2 diabetes (Esser *et al*. 2014). In general, modern medicine, especially in the treatment of diabetes, works in one pathway (Ishak *et al*. 2013). Combination of medicine is used to obtain maximum result to reduce the level of blood glucose and minimize side effects (Ishak *et al.* 2013, Kim *et al*. 2011). Synthetic medicine of α-glucosidase inhibitors has side effects such as flatulence, stomachaches, and diarrheas (Hollander 2007, Kim *et al*. 2011, Yeo *et al*. 2011). Because type 2 diabetes is correlated with the inflammatory disease, another alternative therapies such as administering anti-inflammatory reagents would be very useful to ameliorate the disease

(Esser *et al*. 2014, Shoelson *et al*. 2007) and reduce the side effect on gastrointestinal tract.

Bioactive compounds such as phenols, flavanoids, quercetins and dihydroquercetins were extracted from wood barks by methanol (Sjostrom 1981). Phenols and flavanoids have the ability to control the expression level of proinflammatory cytokines such as TNF-α, IL-1, and IL-6 (Debnath *et al.*, 2013) and quercetins have the ability to regulate the level of fasting blood glucose through increasing insulin sensitivity by inhibiting α-glucosidase and enhancing the insulin signaling in *db/db* mice (Kim *et al*. 2011). De La Garza *et al*. (2014) reported that extracts from *Helichrysum italicum* and *Citrus x paradise* regulate hyperglycemia and TNFα-mediated inflammation of *db/db* mice. In this study, it was found that blood glucose levels of *db/db* mice were reduced by methanol extracts from wood barks of *P. timoriana, P. speciosa,* and *D. dao*, and level of triacylglycerol were reduced by *D. dulcis* and *P. speciosa* after four weeks of administration. These extracts are not toxic and safe for continues use in a dose of 5 mg/mouse.

Methanol extracts from wood barks of medicinal plants in West Kalimantan Indonesia have abilities to down-regulate the levels of blood glucose and triacylglycerol of *db/db* mice probably by regulating the inflammation. To prove the anti-inflammatory effects of plant extracts in type 2 diabetes, it is useful to examine the anti-inflammatory activity of plant extracts using epithelial FPCK-1-1 cells of human intestine.

6.4. Conclusions

Anti-diabetic effects of long-term administration of methanol extracts from wood barks of *D. dulcis, D. kutejensis, P. timoriana, P. speciosa, D. dao*, and *B. costulata* in *db/db* mice were analyzed. Results showed that after four weeks of oral administration, blood glucose levels of *db/db* mice were down-regulated by *P. timoriana, P. speciosa,* and *D. dao,* and triacylglycerol were reduced by *P. speciosa* and *D. dulcis*. Activities of methanol extracts to down-regulate the levels of blood glucose and triacylglycerol of *db/db* mice are probably related to the down-regulation of the inflammation. It is very useful to use the inflammation model of human colon epithelial FPCK-1-1 cells to prove the effectiveness of plant extracts as anti-inflammatory reagents for type 2 diabetes.

Chapter 7

Effects of methanol extracts from wood barks of five medicinal plants to prevent the damage of human colon epithelial FPCK-1-1 cells

7.1. Introduction

Synthetic α-glucosidase inhibitors have been used to treat type 2 diabetes. This type of medicine has adverse side effects such as flatulence, stomachaches, and diarrheas (Hollander 2007, Kim *et al*. 2011, Yeo *et al*. 2011). Inflammation is reported to be involved in diabetes (Dandona *et al*. 2004, Esser *et al*. 2014, Wellen and Hotamisligil 2005), diarrheas, and stomachaches (Debnath *et al.* 2013, Zakaria *et al*. 2011). Increment of pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-1β and IL-6 causes inflammation of intestine (Debnath *et al*. 2013) and the insulin resistance is associated with obesity and type 2 diabetes (Esser *et al*. 2014). Since type 2 diabetes is correlated with the inflammatory disease, another alternative therapies such as administering anti-inflammatory reagents would be very useful to ameliorate the disease (Esser *et al*. 2014, Shoelson *et al*, 2007) and reduce the side effects on gastrointestinal tract.

Medicinal plants in West Kalimantan have the potential to cure diabetes that is caused by inflammation and intestinal inflammation. In the previous chapters, it was reported that *D. dulcis, D. kutejensis, P. timoriana, P. speciosa*, and *D. dao* have activities to inhibit α-glucosidase *in vitro* (**Chapter 3**). *D. dulcis, P. timoriana*, and *P. speciosa* had reduced the level of blood glucose of *db/db* mice in two maltose loading tests (**Chapter 5**). *P. timoriana, P. speciosa*, and *D. dao* had down-regulated blood glucose level of *db/db* mice in long term administration (**Chapter 6**). *D. dulcis* and *P. speciosa* reduced the level of triacylglycerol of *db/db* mice (**Chapter 6**). Traditionally, all of these plants have been used as traditional medicine to ameliorate diarrheas and stomachaches (**Table 1.1, Chapter 1**). Diarrhea is a change in frequency, consistency, weight and volume of stool (Thomas *et al*. 2003), and chronic diarrhea, especially inflammatory bowel disease (IBD) is caused by continuous inflammation (Debnath *et al*. 2013, Zakaria *et al*. 2011). Bowel inflammation is caused by several factors such as

infection, inherited genes, immune system, and environment, and repeated inflammation often leads to colon cancer (Baumgart and Carding 2007, Kaser *et al*. 2010). To find the evidences of anti-inflammatory activities of plant extracts that have curative properties for type 2 diabetes and chronic diarrhea, it is necessary to examine the anti-inflammatory effects of plant extracts against the intestinal inflammation.

In this study, the anti-inflammatory effects of *D. dulcis, D. kutejensis, P. timoriana, P. speciosa*, and *D. dao* were analyzed using human colon epithelial FPCK-1-1 cells. FPCK-1-1 is an intestinal epithelial cell line established from a tubular adenoma of a male patient with familial adenomatous polyposis (Kawaguchi *et al*. 1991) and it has been used as a new culture model of intestinal inflammation (Tominaga *et al*. 2012, Tominaga *et al*. 2013). In this model, FPCK-1-1 cells were co-cultured with PMA-stimulated monocytic leukemia THP-1 cells, resulting in the reduction of transepithelial electrical resistance (TER) of FPCK-1-1 monolayer cells (Tominaga *et al*. 2012). It was found that methanol extracts from wood barks of *D. dulcis, D. kutejensis, P. timoriana, P. speciosa*, and *D. dao* have activities to prevent the damage of human colon epithelial FPCK-1-1 cells induced by PMA-stimulated THP-1 cells.

7.2. Materials and Methods

Plant extracts

Methanol extracts from wood barks of *D. dulcis, D. kutejensis, P. timoriana, P. speciosa*, and *D. dao* were prepared as described in the previous chapter (**Chapter 2**). For a damage-prevention assay *in vitro* using human colon epithelial FPCK-1-1 cells, one mg of methanol extracts from wood barks (dry weight) was dissolved in 1 ml DMSO (1 mg/ml) and used at a final concentration of 1 μg/ml in a culture medium.

FPCK-1-1 cells assay

Cell lines

FPCK-1-1 are precancerous cells originated from a colonic polyp in a 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 (JCRB0112, Tsuchiya *et al.* 1980). FPCK-1-1 cells and THP-1 cells were maintained at 37°C in 5% CO_2 in the high glucose

Dulbecco's-modified Eagle Medium (DMEM) supplemented with 8% fetal calf serum (FCS), 20 U/ml penicillin, and 50 µg/ml kanamycin. FPCK-1-1 cells were sub-cultured on 1.1 cm^2, Transwell permeable inserts with 0.4 µm pore (Transwell, used as upper chambers) pre-coated with equimolar mixture of types I and III collagen (3493, Corning, Ithaca, NY).

Co-culture system and the treatment of intestinal epithelial FPCK-1-1 cells

Anti-inflammatory activities of methanol extracts from barks of five medicinal plants were evaluated in an early phase damage model as described (Tominaga *et al*. 2012). Briefly, FPCK-1-1 cells were cultured to form a monolayer on insert membranes of Transwell set in 12 well cell culture plates (Corning 3513) at a density of 2 x 10^5 cells/insert for five day. THP-1 cells were cultured for one day in 12 well culture plates at a density of 1 x 10^5/well in the presence of phrobol 12-myristate 13-acetate (PMA 20 nM). The inserts containing FPCK-1-1 cells were transferred into the wells where THP-1 cells are cultured. Methanol extracts from wood barks were added to the apical side (upper chamber containing FPCK-1-1 cells) of the co-culture (final concentration: 1 µg/ml) (**Figs 7.1 and 7.2**).

Early phase damage model

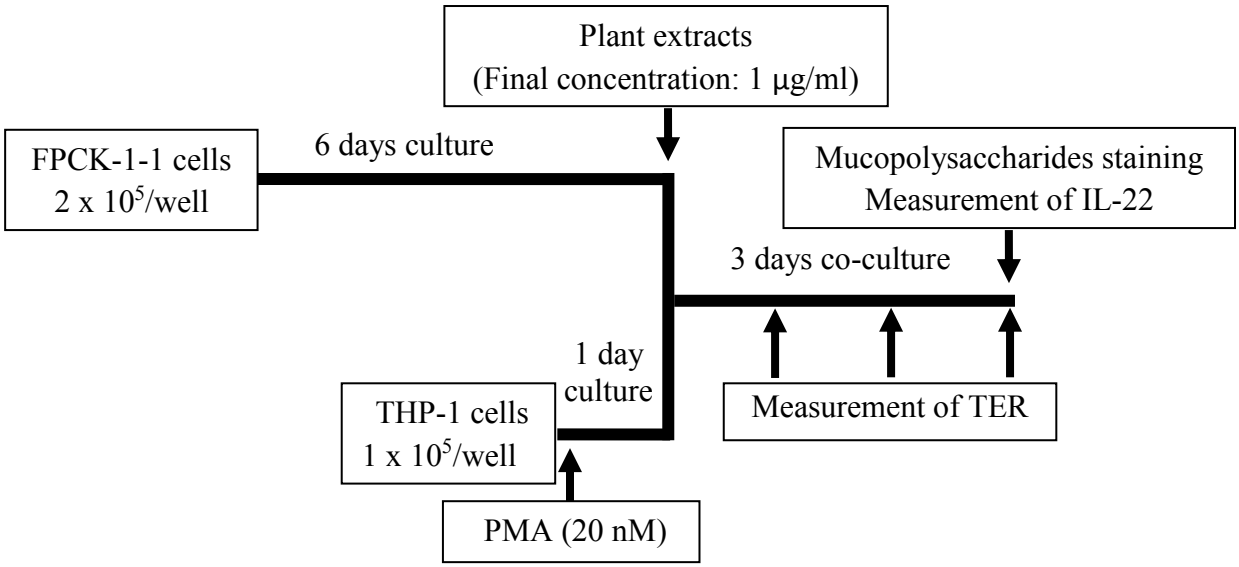


Fig. 7.1. Flowcharts of the early phase damage model of intestinal epithelial cells.

Measurement of transepithelial electrical resistance (TER)

Transepithelial electrical resistance (TER) was measured two hours after changing the medium in the Transwell. Sterile DMEM was used to rinse the electrode after soaking in 70% ethanol. Measurement of electrical resistance between the lower chamber (well) and the upper chamber (filter insert) was conducted using a voltmeter Millicell-ERS and an electrode MERSSTX01 (Millipore, Bedford, MA). To prevent changes in resistance due to temperature alteration, the temperature was maintained close to 37°C. The values of TER of FPCK-1-1 monolayer cells at the start of co-culture were expressed as 100%. Real values of TER were 50 - 80 Ω・cm^2 for medium alone and 222 - 274 Ω・cm^2 for FPCK-1-1 monolayer cells. TER was measured four times and the mean was calculated.

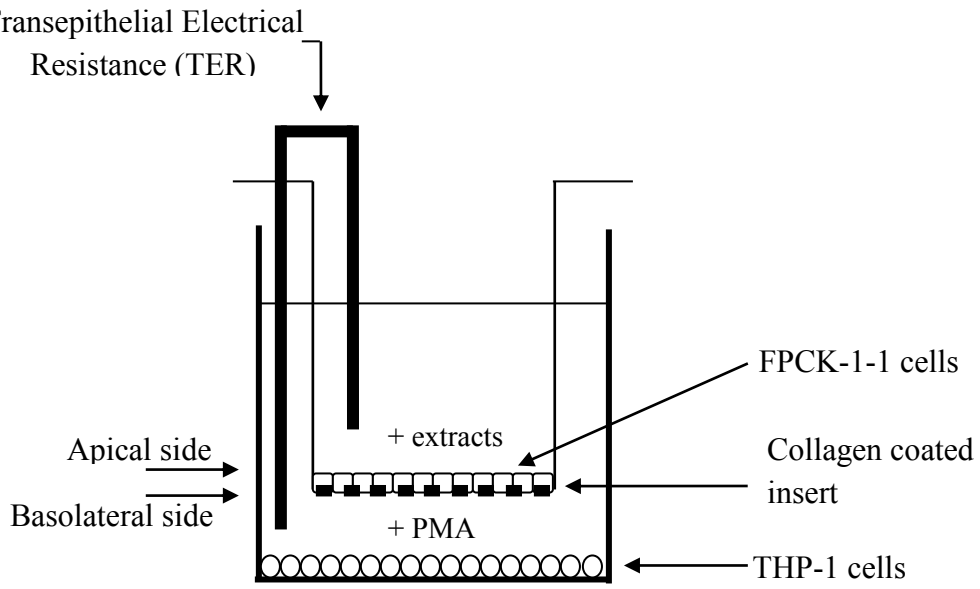


Fig. 7.2. Co-culture system of FPCK-1-1 cells with PMA-stimulated THP-1 cells.

Histochemical staining of polysaccharides produced by FPCK-1-1 cells

Staining of FPCK-1-1 monolayer cells were conducted using an Alcian blue and PAS solution (Muto Pure Chemicals Co., Ltd, Tokyo, Japan) for the staining of acidic carbohydrates according to a manufacturer's protocol.

Interleukin 22 (IL-22) measurement in the supernatant

IL-22 (PeproTech Inc., Rocky Hill, NJ) released in the supernatant of FPCK-1-1 cells in the upper chamber was measured according to the manufacturer's protocol using an ELISA kit (R&D System, Inc. Minneapolis, MN).

Statistics

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

7.3. Results and Discussion

Recently, it is reported that diabetes is linked to inflammation (Wellen and Hotamisligil 2005). To explore this possibility, the effects of plant extracts to prevent the damage of FPCK-1-1 human colon epithelial cells caused by inflammation were examined. Unique feature of this model is that precancerous FPCK-1-1 cells are derived from a tubular adenoma in a male patient of familial polyposis coli (Kawaguchi *et al*. 1991). In this model, FPCK-1-1 cells were co-cultured with PMA-stimulated monocytic leukemia THP-1 cells, resulting in the reduction of TER of FPCK-1-1 monolayer cells (Tominaga *et al*. 2012). The measurement of TER is frequently used to analyze the characteristics of monolayer integrity and quantify the permeability changes of the epithelial monolayer cells (Azzini *et al*. 2016)

As shown in **Fig. 7.3**, all the samples had inhibitory effects on the decrease of TER of FPCK-1-1 monolayer cells in response to PMA-stimulated THP-1 cells at various degrees. The values of TER of treated with *D. dao* and *P. speciosa* extracts had no significant difference with control (FPCK-1-1 cells co-cultured with non-stimulated THP-1 cells) on day 3 after starting co-culture. These results suggest that methanol extracts from *D. dao* and *P. speciosa* completely prevented the decrease of TER of FPCK-1-1 monolayer cells caused by PMA-stimulated THP-1 cells.

The values of TER of FPCK-1-1 monolayer cells treated with *P. timoriana, D. dulcis*, and *D. kutejensis* extracts were significantly lower than that of control, but higher than that of co-cultured only with PMA-stimulated THP-1 cells on day 3 after starting

the co-culture. These results suggest that the methanol extracts from barks of these three plants have some preventive effects on the damage of FPCK-1-1 monolayer cells caused by PMA-stimulated THP-1 cells.

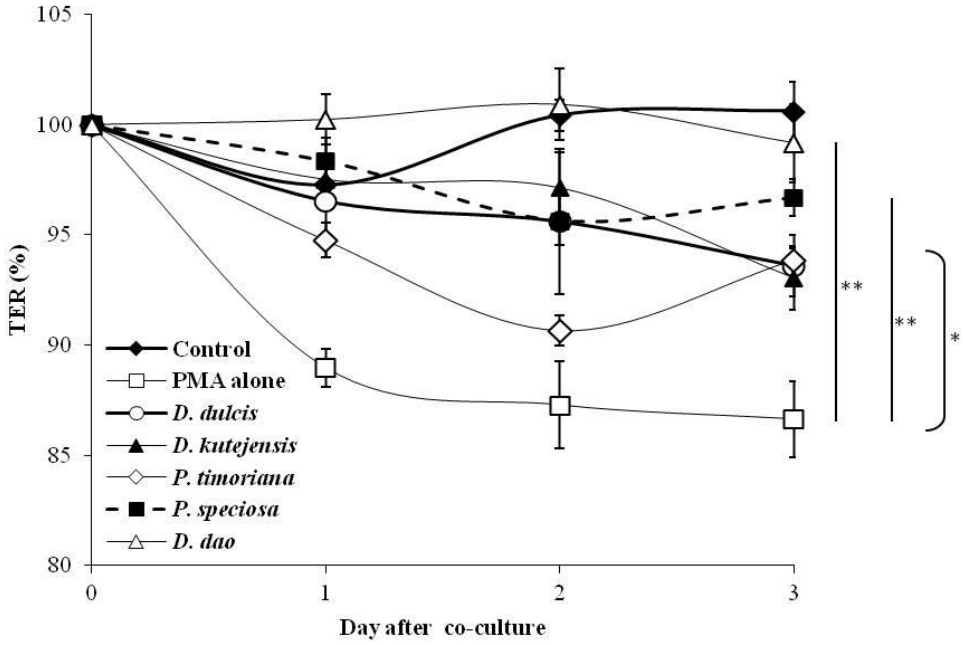


Fig. 7.3. Effect of methanol extracts from wood barks on the TER of human colon epithelial FPCK-1-1 cells co-cultured with PMA-stimulated THP-1 cells. PMA was added one day before the start of co-culture to THP-1 cells in the lower chamber. Methanol extracts from wood barks (final concentration: 1 μg/ml) were added to the upper chamber where FPCK-1-1 cells were cultured. Results are shown as the average ± SE (n=4). Asterisks show significant differences between methanol extract groups and PMA alone group on day 3 (*: $P < 0.05$, **: $P < 0.01$. LSD post hoc test, one-way ANOVA).

The increment of intestinal permeability at paracellular and transcellular pathways causes the production of proinflammatory cytokines as a sign of intestinal bowel disease (Menard *et al*. 2010). Several proinflammatory cytokines such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-13 (IL-13), IL-17 (Menard *et al*. 2010), and IL-1β (Al-Sadi and Ma 2007) could decrease the transepithelial electrical resistance (TER) and the combination of IFN-γ and TNF-α induces the damage of epithelial barriers and changes permeability of tight junctions (Bruewer *et al*. 2003, Menard *et al*.

2010). Tominaga *et al*. (2013) reported that TNF-α is responsible for the injury of human colon epithelial FPCK-1-1 monolayer cells. They showed that anti-TNF-α antibodies recovered the decreased level of TER of FPCK-1-1 cells damaged by PMA-stimulated THP-1 cells.

Nitric oxide (NO) is reportedly involved in the protection of the barrier function of intestinal epithelial cells during the acute inflammation by inhibiting the toxic oxidant formation or scavenging lipid radicals (Katsube *et al*. 2007). On the other hand, there is a report that carcinogenesis of FPCK-1-1 cells is caused by chronic inflammation-derived NO (Tazawa *et al.* 2013). Although the level of NO in this assay were not measured, methanol extracts of wood barks may act as scavengers for NO and protect the barrier function of intestinal epithelial cells.

To explore the mechanism involved in the recovery of TER values by methanol extracts, the observation were focused on the production of mucopolysaccharides as one of the barrier function of intestinal epithelial cells. Alcian blue staining were performed to visualize the mucopolysaccharides on the surface of FPCK-1-1 cells after being treated with plant extracts. Alcian blue is a cationic dye that is frequently used to stain and visualize the mucopolysaccharides and acidic mucins through electrostatic force (Dong *et al*. 2012).

As presented in **Fig. 7.4**, Alcian blue showed the higher level of staining on the surface of FPCK-1-1 monolayer cells in the presence of methanol extracts from *D. dao* and *P. speciosa*. Higher levels of mucopolysaccharides stained by Alcian blue are related to the higher levels of TER of FPCK-1-1 cells. It was suggested that methanol extracts from *D. dao* and *P. speciosa* induced FPCK-1-1 cells to produce mucopolysaccharides that cover the surface of FPCK-1-1 monolayer cells and these mucopolysaccharides function as a barrier to prevent the damage of FPCK-1-1 monolayer cells induced by PMA-stimulated THP-1 cells.

Another staining technique to examine the presence of mucopolysaccharides in the surface of FPCK-1-1 cells is the PAS (periodic acid-schiff) staining. As shown in **Fig. 7.5**, the surface of control FPCK-1-1 cells is covered by mucopolysaccharides. After the addition of PMA to THP-1 cells, the level of mucopolysaccharides on the surface of FPCK-1-1 cells decreased, suggesting that PMA-stimulated THP-1 cells caused the damage of barrier functions of intestinal epithelial FPCK-1-1 cells. Methanol extracts

from all species did not show the activity to induce FPCK-1-1 cells to produce mucopolysaccharides after 3 days of co-culture. These results are different from those obtained with Alcian blue staining that show higher levels of mucopolysaccharides in the presence of methanol extracts from *D. dao* and *P. speciosa*.

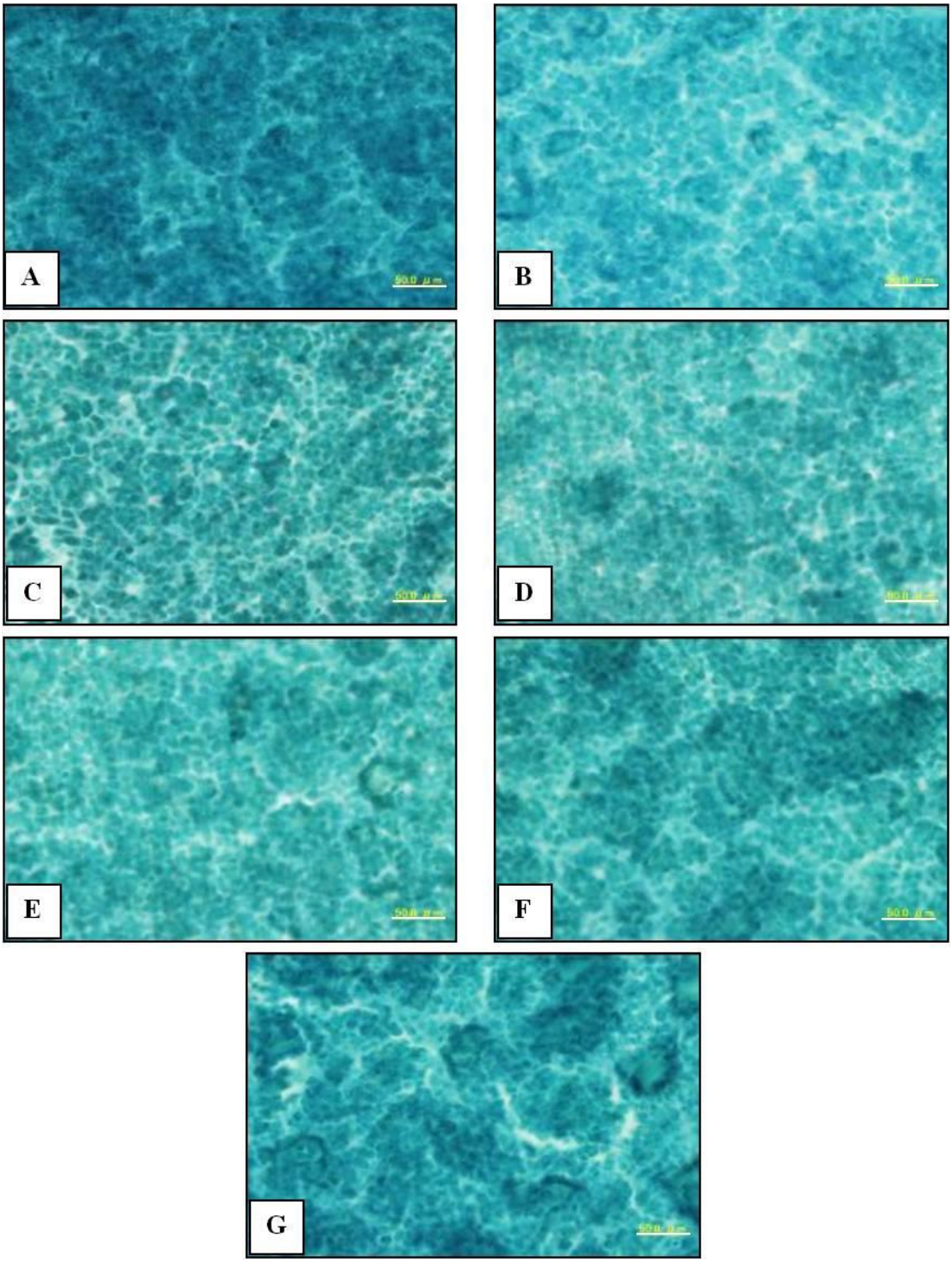


Fig. 7.4. Staining of FPCK-1-1 monolayer cells using Alcian blue in response to wood bark extracts. FPCK-1-1 monolayer cells were stained with Alcian-blue as described in Materials and Methods three days after starting the co-culture with PMA-stimulated THP-1 cells. Plant extracts were added at the beginning of the co-culture. (A): Control, (B): PMA alone, (C): *D. dulcis,* (D): *D. kutejensis,* (E): *P. timoriana,* (F): *P. speciosa,* (G): *D. dao.* Bars, 50 µm

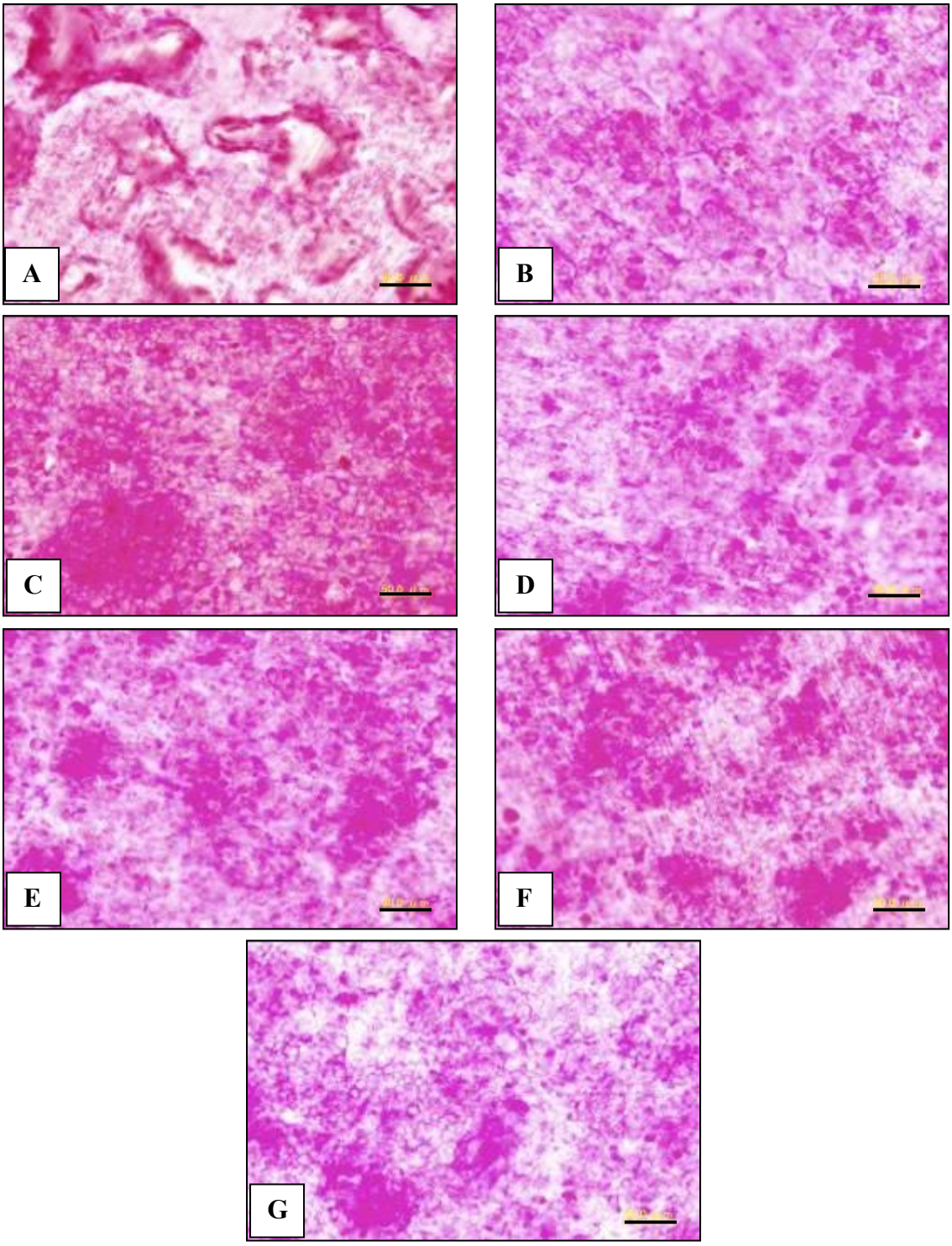


Fig. 7.5. Staining of FPCK-1-1 monolayer cells using PAS in response to wood bark extracts. FPCK-1-1 monolayer cells were stained with PAS as described in Materials and Methods three days after starting the co-culture with PMA-stimulated THP-1 cells. Plant extracts were added at the beginning of the co-culture. (A): Control, (B): PMA alone, (C): *D. dulcis,* (D): *D. kutejensis,* (E): *P. timoriana,* (F): *P. speciosa,* (G): *D. dao*. Bars, 50 μm

The discrepancy between the results of Alcian blue staining and those of PAS staining is probably due to the periodic oxidation decomposes mucopolysaccharides on the surface of FPCK-1-1 cells that are produced in the presence of methanol extracts. According to Dong *et al*. (2012), visualization of mucopolysaccharides, glycoproteins, and mucins could be analyzed by PAS staining. However, since periodic oxidation decomposes glycan, PAS staining is not suitable to detect glycan.

Surface of FPCK-1-1 cells are covered by mucopolysaccharides (Tominaga *et al*. 2013) that function as one of the barriers. It is suggested that methanol extracts from wood barks of *D. dao* and *P. speciosa* induced FPCK-1-1 cells to produce and/or maintain sulfated and carboxylated mucopolyasaccharides or glycoproteins, because the Alcian blue revealed the higher level of staining on the surface of FPCK-1-1 cells in the presence of the extracts from *D. dao* or *P. speciosa* (**Fig.7.4**). These results suggest that plant extracts used in this assay may have the ability to inhibit the expression of proinflammatory cytokines such as IL-1β, IL-6, IFN-γ, and TNF-α. Thus, these plant extracts may prevent the decrease of TER of FPCK-1-1 monolayer cells in response to PMA-stimulated THP-1 cells.

Another factor that is responsible for the recovery of the TER value of FPCK-1-1 monolayer cells is IL-22 (Tominaga *et al*. 2012, Tominaga *et al*. 2013). IL-22 belongs to IL-10 cytokine family that is mainly produced by both adaptive and innate immune cells, especially by T helper-17 (Th-17) cells, γδT cells, natural killer T (NKT), and innate lymphoid cell (ILC) (Dudakov *et al*. 2015, Munoz *et al*. 2015). In intestinal inflammation, this cytokine elevates the intestinal integrity and the innate immunity of epithelial cells (Li *et al*. 2014).

To confirm whether methanol extracts could release IL-22 that is involved in the recovery the TER value of FPCK-1-1 monolayer cells, the levels of IL-22 in the apical

side of FPCK-1-1 monolayer cells were measured. Results show that IL-22 was not detected in the supernatants of FPCK-1-1 cells in response to the plant extracts in this assay (**Table 7.1**). These results suggest that IL-22 is not involved in the recovery of TER value and the prevention of the damage of human colon epithelial FPCK-1-1 cells caused by PMA-stimulated THP-1 cells in the presences of methanol extracts from wood barks of *D. dulcis, D. kutejensis, P. timoriana, P. speciosa*, and *D. dao.*

Table 7.1. Effect of methanol extracts on production of interleukin 22 (IL-22) by FPCK-1-1 monolayer cells.

No.	Co-culture and plant extracts	Interleukin 22 (IL-22) (pg/ml)
1	Control	4.56 ± 3.15
2	PMA alone	3.00 ± 6.21
3	*D. dulcis*	-3.26 ± 3.52
4	*D. kutejensis*	-2.28 ± 2.82
5	*P. timoriana*	-1.5 ± 2.86
6	*P. speciosa*	-1.5 ± 4.26
7	*D. dao*	-5.02 ± 2.26

In the previous chapters, it was reported that *D. dulcis*, *D. kutejensis*, *P. timoriana*, *P. speciosa*, and *D. dao* have activities to inhibit α-glucosidase *in vitro* (**Chapter 3**). *D. dulcis*, *P. timoriana*, and *P. speciosa* had reduced the level of blood glucose of *db/db* mice in two maltose loading tests (**Chapter 5**). *P. timoriana*, *P. speciosa*, and *D. dao* down-regulated blood glucose level of *db/db* mice after four weeks of oral administration (**Chapter 6**). *D. dulcis* and *P. speciosa* had reduced the blood level of triacylglycerol of *db/db* mice (**Chapter 6**). In this chapter, it was reported that *D. dulcis*, *D. kutejensis*, *P. timoriana*, *P. speciosa*, and *D. dao* have effects to prevent the damage of human colon epithelial FPCK-1-1 cells caused by PMA-stimulated THP-1 cells. Extracts from *P. speciosa* and *D. dao* were very effective to prevent the decrease of TER values of colon epithelial monolayer cells by inducing FPCK-1-1 cells to produce mucopolysaccharides.

D. dulcis, *P. timoriana*, *P. speciosa*, and *D. dao* have both effects to prevent the damage of human colon epithelial FPCK-1-1 cells and regulate the blood glucose level

of *db/db* mice. These results suggest that methanol extracts from wood barks of *D. dulcis*, *P. timoriana*, *P. speciosa*, and *D. dao* contain non-toxic bioactive compounds which prevent the damage of human colon epithelial FPCK-1-1 cells and down-regulate the level of blood glucose of *db/db* mice by regulating the inflammation.

7.4. Conclusions

The anti-intestinal inflammatory effects of methanol extracts from wood barks of *D. dulcis*, *D. kutejensis*, *P. timoriana*, *P. speciosa*, and *D. dao* that are traditionally used to treat diarrheas, stomachaches, and diabetes in West Kalimantan, Indonesia were analyzed. Results showed that *D. dulcis*, *D. kutejensis*, *P. timoriana*, *P. speciosa*, and *D. dao* have effects to prevent the damage of human colon epithelial FPCK-1-1 cells caused by PMA-stimulated THP-1 cells. Extracts from *P. speciosa* and *D. dao* were very effective to prevent the decrease of TER values of colon epithelial monolayer cells by inducing them to produce mucopolysaccharides. Purification and identification of bioactive compounds from these plants are advantageous to develop an efficient way of administration to use them as anti-inflammatory medicine.

Chapter 8
Conclusions

This is the study to evaluate plants that are trusted by Dayak people in West Kalimantan Indonesia to ameliorate diabetes, diarrheas, and stomachaches by examining their ability to inhibit α-glucosidase *in vitro* (yeast maltase, rat intestinal maltase and rat intestinal sucrase), the cytotoxicity against NIH3T3 cells and the toxicity to C57BL/6J mice, the down-regulating activities of blood glucose levels of *db/db* mice in maltose loading tests and long term administration, and the prevention from the damage of human colon epithelial FPCK-1-1 cells. The plants species examined are *Dracontomelon dao*, *Mangifera foetida*, *Mangifera pajang*, *Pentaspadon motleyi* (*Anacardiaceae*), *Parkia timoriana, Parkia speciosa*, *Parkia intermedia, Parkia sp.*, *Adenanthera sp.* (*Fabaceae*), *Durio dulcis*, *Durio kutejensis* (*Malvaceae*), *Baccaurea angulata*, *Baccaurea costulata* (*Phyllanthaceae*), *Goniothalamus tapis* (*Annonaceae*), *Willughbeia angustifolia* (*Apocynaceae*), *Dacryodes rostrata* (*Burseraceae*), and *Garcinia parvifolia* (*Clusiaceae*).

The percentage of methanol extract contents of wood barks varied from 2.05 to 21.48%, and almost all species belong to high category in extractive contents, except *B. angulata* and *D. dulcis* (moderate category). *P. speciosa*, *Adenanthera sp.*, *D. dao*, *D. kutejensis*, *P. intermedia*, and *P. timoriana* had strong inhibitory activity on yeast maltase with the IC_{50} values of 1.92, 2.95, 3.24, 3.25, 3.27, and 3.65 μg/ml, respectively. In contrast, *P. motleyi*, *P. speciosa*, *P. timoriana*, *D. rostrata*, *Adenanthera sp.*, and *B. costulata* showed lower inhibitory activity on rat intestinal sucrase with the IC_{50} values of 930.87, 789.25, 767.20, 787.77, 901.1, and 962.73 μg/ml, respectively. All species showed low activities to inhibit rat intestinal maltase.

Methanol extracts from wood barks of *D. dulcis*, *D. kutejensis*, *P. timoriana*, *P. speciosa*, *P. intermedia*, and *D. dao* are toxic at a concentration of 100 μg/ml against normal mouse fibroblast NIH3T3 cells. *P. speciosa* is the most toxic at a concentration of 10 μg/ml and all methanol extracts from wood barks of these plants are low toxic at concentrations of 1 μg/ml. *P. speciosa* extract is not toxic to C57BL/6J mice even after administrating 5 mg extract/mouse.

D. dulcis, *P. timoriana*, and *P. speciosa* effectively decreased the level of blood glucose of *db/db* mice in two maltose loading tests. After four weeks of oral administration, *P. timoriana*, *P. speciosa*, and *D. dao* significantly decreased the level of blood glucose. Although mice administered with extracts from *P. timoriana* or *P. speciosa* consumed less food than those administered with acarbose, there was no significant difference in body weight among groups four weeks after starting administration. *D. dulcis* and *P. speciosa* significantly reduced the level of blood triacylglycerol.

D. dulcis, *D. kutejensis*, *P. timoriana*, *P. speciosa*, and *D. dao* have activities to prevent the damage of human colon epithelial FPCK-1-1 cells caused by PMA-stimulated THP-1 cells. Extracts from *P. speciosa* and *D. dao* effectively prevented the decrease of transepithelial electrical resistance (TER) of human colon epithelial FPCK-1-1 cells caused by the co-culture with PMA-stimulated THP-1 cells three days after starting the co-culture. Both of these extracts induced FPCK-1-1 cells to produce mucopolysaccharides.

Methanol extracts from wood barks of *D. dulcis*, *P. timoriana*, *P. speciosa*, and *D. dao* have both activities to prevent the damage of FPCK-1-1 human colon epithelial cells and down-regulate the level of blood glucose of *db/db* mice. These results suggest that methanol extracts from wood barks of *D. dulcis*, *P. timoriana*, *P. speciosa*, and *D. dao* contain non-toxic bioactive compounds which prevent the damage of human colon epithelial FPCK-1-1 cells and down-regulate the level of blood glucose of *db/db* mice by regulating the inflammation. Purification and identification of bioactive compounds from these plants are advantageous to develop an efficient way of administration to use them as anti-inflammatory and anti-diabetic medicine.

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