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学位論文全文に代わる要約
Extended Summary in Lieu of Dissertation

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学位論文題目：
Title of Dissertation

Biochemical Utilization of Indonesian Forest Biomass as Antioxidant, Antidiabetic, and Antihyperlipidemic Agents
（インドネシアの森林バイオマスの生化学的利用，特に抗酸化剤，抗糖尿病薬および抗高脂血症薬として）

学位論文要約：
Dissertation Summary

Introduction

Indonesia land area occupies 1.3% of earth's land area, but it has 11% of the world's vegetation species (Sukara 2014). The vegetation also highly varied and many of them are uniquely native and cannot be found elsewhere. These abundant natural recourses are very prospective for the future to provide raw materials for food, health, and energy.

Many compounds from natural products have been commercialized worldwide. Japan, United States (US), and Europe have launched 23 new natural compounds to be commercialized from 2001 to 2005 period alone (Lam 2007). The medicinal properties were ranging from antimicrobes, anticancer, Alzheimer medication, antityrosinaemia, immunosuppresion, antihyperlipidemia, antidiabetic, and analgesic action.

On the other hand, there is a large shift in the cause of human mortality in the last 15 years. World Health Organization (WHO) report showed that there was a large reduction in mortality caused by infectious diseases (WHO 2014). Meanwhile, non-communicable diseases (NCD) rise sharply to become major cause of mortality. Heart disease is the major cause of mortality by NCD followed by stroke, chronic obstructive pulmonary disease, cancer, and diabetes.

This research consisted of two stages. Objective of the first stage was to examine *in vivo* antidiabetic activity of *G. pictum* ethanolic leaves extract and discussing the role of its antioxidant in the management of diabetes. Objective of the second stage was to explore antioxidant and antihyperlipidemic activity of six Indonesian fruit species, include *Baccaurea racemosa* (Reinw. Ex Blume) Mull. Arg. fruit, *Mangifera caesia* Jack fruit, *Pouteria campechiana* (Kunth) Baehni fruit, *Phyllanthus acidus* (L.) Skeel fruit, *Sandoricum koetjape* (Burm. F.) Merr. fruit, and *Syzigium cumini* (L.) Skeel fruit as well as waste wood and bark of *Albizia falcataria*.

Antidiabetic and Antioxidant Activities of *Graptophyllum pictum*

Graptophyllum pictum is an herb distributed in the tropical region of the world. It is used as a medicinal plant to treat hepatitis, gallstone, hemorrhoid, infection, and diabetes. Olagbende-Dada *et al*. (2011) has showed that *G. pictum* leaves extract posses the antidiabetic activity *in vivo* by reducing fasting blood glucose level in alloxan induced diabetic rats. However, there has been no information about this antidiabetic mechanism.

The previous study investigated the effect of *G. pictum* ethanolic extract in inhibiting α-glucosidase activity (Nurcholis *et al.* 2012). Due to the fact that inhibiting this enzyme will delay carbohydrate digestion (Honma *et al.* 2010). Therefore, the present study examined the *in vivo* antidiabetic activity of *G. pictum* ethanolic leaves extract as well as provide a detailed mechanism on the activity in diabetic animal models.

For this experiment, fresh leaves of plant materials were washed with water, cut into small pieces, and dried. They were then ground in a grinder to a powder form and screened to give 40-80 mesh powder sizes. Thirty grams of the powder leaves were macerated using 10 x 30 mL 70% ethanol in a tightly closed round bottom flask at room temperature for a period of 24 h and filtered with Whatman filter paper (type 4). The whole process was repeated one times and the filtrate was concentrated under reduced pressure on a rotary evaporator (BUCHI, R-250, Switzerland) at 50°C temperature. The extracts were then used for the experiments.

The rats used were divided into 7 groups (n=5). Diabetes condition in alloxan-induced diabetic rats were prepared in the 6 groups by overnight-fasted rats using a single intraperitoneal injection of freshly prepared alloxan 150 mg/kg body weight (bw) (Midha *et al.* 2012). After 72 hours of induction, the rats were treated by overnight fast and the blood glucose concentration was monitored in the venous blood drawn from the tail vein using a glucometer GlucoDr AGM-3000. Blood glucose level of more than 200 mg glucose per 100 mL blood was considered as diabetic and was enrolled in the study. Positive control group was given oral administration of glibenclamide 3 mg/kg bw, while negative control group was untreated (Sy *et al.* 2005). The other groups were given oral administration of *G. pictum* 70% ethanolic extract (GPE) with the dose of 25, 50, 100, 200 mg/kg bw respectively. Normal group was injected with sterile isotonic solution and was given oral administration of water only. Glibenclamide and extract were dissolved in 0.9% NaCl. Treatment was given orally for 10 days after the rats were considered diabetic. Body weight and food consumption were measured daily (Figure 1). At the end of treatment, rat's liver and pancreas were decapitated. Lipid peroxide concentration was measured from the liver, moreover histological and immunohistochemical examination of pancreas were also carried out.

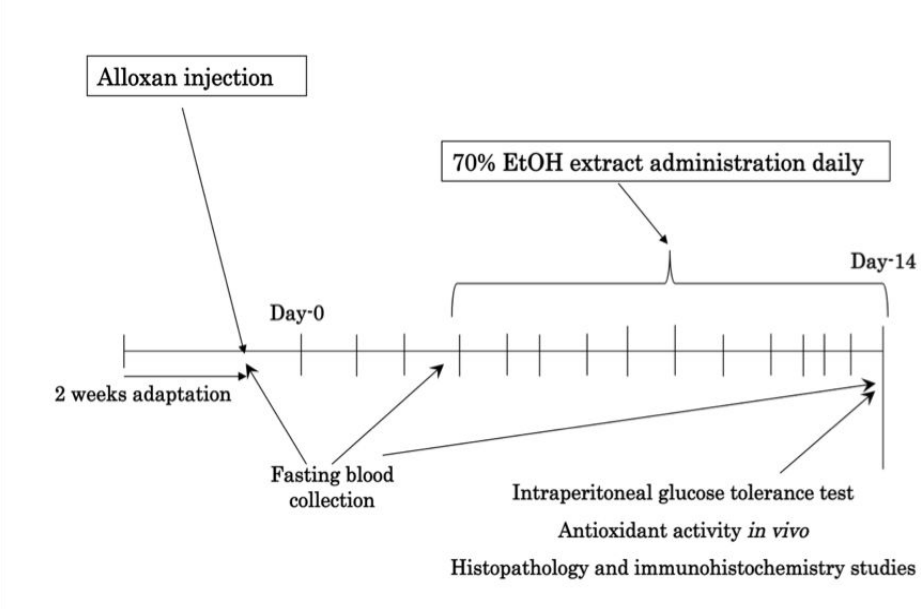

Figure 1 Experimental design of *Graptophyllum pictum* (L.) Griff *in vivo* antidiabetic activity

Figure 2 shows the oral glucose tolerance test for GPE. After glucose injection, the administration of 50 mg/kg bw of GPE decrease the elevation of blood glucose level significantly at 150 minutes. In the present study, we examined the effects of GPE administration on sensitivity to glucose absorption. We observed that blood glucose concentration returned to its baseline concentration in 120 minutes after glucose injection in glibenclamide-administered group. Glibenclamide directly stimulates insulin secretion from pancreas and sustains concentration of circulating insulin that lowers glucose concentration in response to hyperglycemic state (Boyla *et al*. 2014). The effective dose is found to be at 50 mg/kg bw GPE administration. Increasing the dose produces lower ability to absorb glucose due to the receptor sites are almost fully occupied.

Figure 3 shows concentration of rat liver lipid peroxides, after 14 days treatment. In the alloxan-induced diabetic rats, the liver lipid peroxide was increased compared to the normal group. Administration of 50 mg/kg body weight (bw) to 200 mg/kg bw of *G. pictum* 70% ethanolic extract (GPE) were able to reduce liver lipid peroxide concentration in the rats compared to negative control, with the best result was given by the group that administered with 100 mg/kg bw GPE daily.

Lipid peroxides are formed by oxidative degradation of lipids. This process is initiated by the presence of free radicals such as reactive oxygen species, which turn the unsaturated structures (unsaturated fatty acids) in the lipids into lipid radicals. These reactions stop when the radicals react with antioxidants. In these reactions, one of the final degradation products is malondialdehyde (Diedrich *et al*. 2001).

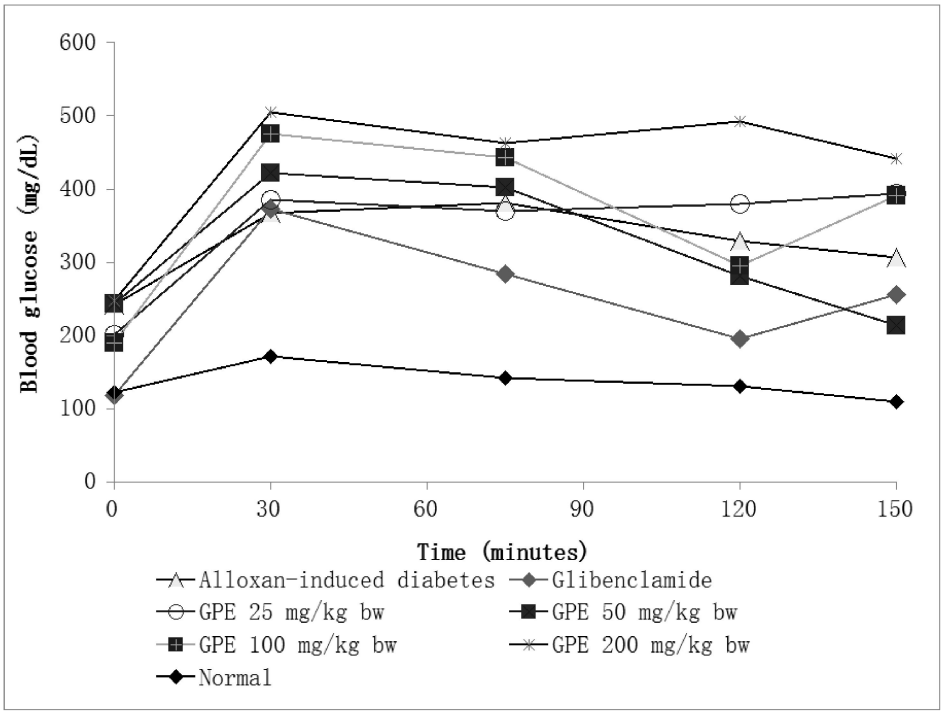

Figure 2 Antihyperglycaemic effect of alloxan, glibenclamide, and *Graptophyllum pictum* leaves extract on intraperitoneal glucose tolerance test. Error bars indicate standard deviation with 5 replications.

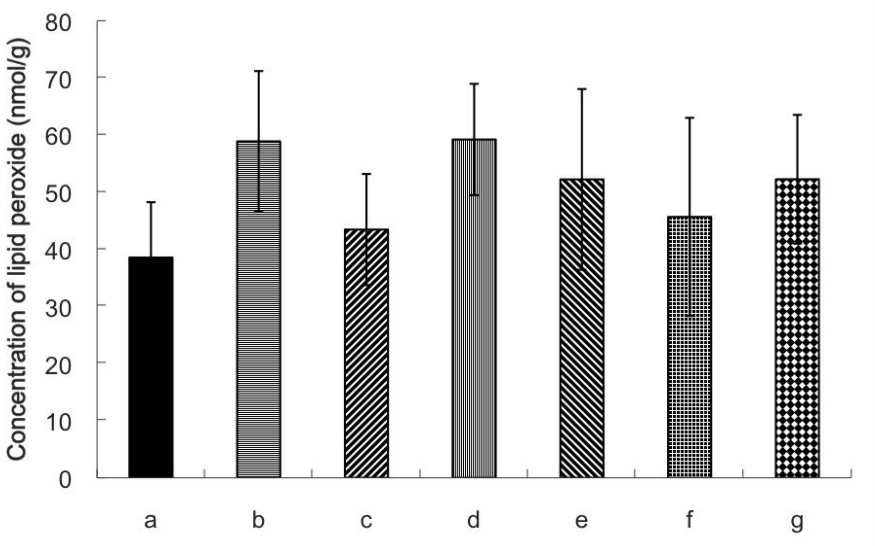

Figure 3-1 Concentration of rat liver lipid peroxides after 14 days treatment. a: Normal group. b: Alloxan-induced diabetic group (negative control). c: Alloxan-induced diabetic group with glibenclamide administered (positive control). d: Alloxan-induced diabetic group with 25 mg/kg bw of GPE administered. e: Alloxan-induced diabetic group with 50 mg/kg bw of GPE administered. f: Alloxan-induced diabetic group with 100 mg/kg bw of GPE administered. g: Alloxan-induced diabetic group with 200 mg/kg bw of GPE administered (n=5) ($P > 0.05$)

Negative control group, 25 mg/kg bw of GPE administered group, and 200 mg/kg bw of GPE administered group as the groups with high blood glucose concentration (Figure 2) shows high liver lipid peroxide concentration (Figure 3). Oppositely, normal group, positive control group, and 100 mg/kg bw of GPE administered group as the groups with low blood glucose concentration show relatively low liver lipid peroxide concentration as well. It was shown that there was formation of reactive oxygen species in the animal and it seemed to correlate with fasting blood glucose level.

Histological evaluation of the pancreas from the normal rats shows normal architecture of Langerhans islets. The pancreas of the alloxan induced-rats (negative control) show hyperplasia condition. The animals treated with glibenclamide and 50, 100, and 200 mg/kg bw of GPE show normal architecture of Langerhans islets compared to the normal group. Hence, there is mild inflammation in the group receiving 25 mg/kg bw of GPE.

Immunohistochemical examination showed that insulin-producing cells in normal group are uniformly distributed in the islet of Langerhans marked with no light nodules in the dark spot. Intense dark color points that insulin concentration in the cells is high. On the other hand, pancreas of diabetic group shows large bright color hole in the center of islet of Langerhans. It indicates the destruction of pancreatic β-cells. The color contrast between the islet of Langerhans and the background is low. Thus indicates that insulin production decrease compared to normal group.

There are only minor light spots in the group that received 50 mg/kg GPE, it means that this group have less damage compared to other treatment group. Moreover, dark color intensity is high enough to justifiy that those cells produce insulin near normal concentration.

Other groups show variation in the number of healthy cells and insulin production. Orders of insulin production from high to low are as follow; glibenclamide administered group, 25 mg/kg bw of GPE administered group, 100 mg/kg bw of GPE administered group, and 200 mg/kg bw of GPE administered group.

It was found that the concentration of the lipid peroxides in extract-treated groups was lower than that of the negative control group. The immunohistochemical examinations show that treating the rats with the extract of *G. pictum* leaves protected pancreas β-cell function in secreting insulin. In conclusion, we found that antioxidant activity of 70% ethanolic extract of *G. pictum* leaves has positive correlation to its in vivo antidiabetic activity by protecting the cells from free radical oxidation.

Screening of Antioxidant and Antihyperlipidemic Potencies of Indonesian Underutilized Fruits

Non-timber forest products such as fruits contain various bioactive compounds. Many fruits are also utilized as traditional medication against various diseases. It is well known that several tropical fruits have activities as antihyperlipidemia, antiinflammation, and antioxidant, which have relation to atherosclerosis; those were *Artocarpus heterophyllus* (Bumrungpert *et al.* 2009), *Garcinia mangostana* (Udani *et al.* 2009), *Punica malus* (Mirmiran *et al.* 2010), and *Diospyros kaki* (Dewanjee *et al.* 2009). Fruits therapy is common to be done in order to treat hypercholesterolemia and to maintain body health condition. Therefore, it is crucial to evaluate the potential of various underutilized fruits in order to find bioactive compounds for the development of new medicine.

Therefore, this research has been conducted to explore the bioactivity six Indonesian fruit species, include *Baccaurea racemosa* (Reinw. Ex Blume) Mull. Arg. fruit, *Mangifera caesia* Jack fruit, *Pouteria campechiana* (Kunth) Baehni fruit, *Phyllanthus acidus* (L.) Skeel fruit, *Sandoricum koetjape* (Burm. F.) Merr. fruit, and *Syzigium cumini* (L.) Skeel fruit.

The plants were collected in West Java Province, Indonesia. Plants were identified by Research Center for Biology, Indonesian Institute of Sciences, Bogor, Indonesia. Six plants were collected for antioxidant and antihyperlipidemic potencies screening. Our samples were separated between fruit peel or rind, fruit pulp, and seed. These samples are underutilized biomass in Indonesia. Some of the fruits are believed to be able to heal cardiovascular related diseases. Antioxidant activity was measured based on DPPH radical scavenging activity (Blois 1958). Antihyperlipidemic activity was determined based on extract inhibition against HMG CoA reductase activity (Gholamhoseinan *et al.* 2010).

Among our samples, *S. cumini* (raw seed acetone extract) and *M. caesia* (raw seed acetone and methanol extract) exhibited remarkable antioxidant activity with IC_{50} less than trolox (IC_{50} 5.88 μg/mL) with *S. cumini* raw seed acetone showed the highest activity (IC_{50} 4.49 μg/mL). Three extracts have partially good potent activity with IC_{50} less than positive control of α-tocopherol (IC_{50} 10.02 μg/mL). Twelve samples have moderate antioxidant activity (IC_{50} varying from 10.96 μg/mL to 121.20 μg/mL) whereas the other 3 samples have comparatively low activity (IC_{50} more than 150 μg/mL). We separated active fractions in *S. cumini* raw seed for the next step.

Raw seed of *S. cumini* (2000 g) was extracted three times with acetone and methanol similarly to give an acetone and a methanol extract. Both the acetone and methanol extract were successively fractionated with dichloromethane, ethyl acetate, and water, to give dichloromethane fraction, ethyl acetate fraction, and water

fraction. The ethyl acetate fraction from the acetone extract gave strongest activity (IC_{50} 3.63 μg/ml).

Ethyl acetate soluble fraction from *S. cumini* acetone extract was separated on preparative TLC plates with ethyl acetate as mobile phase to give 5 bands. Bands 3 and 5 show strong antioxidant activity. Band 4 shows medium antioxidant activity. While bands 1 and 2 do not show any antioxidant activity.

Band 5 was purified by preparative HPLC with methanol: water (45:55) as eluent. This separation resulted 6 major fractions (Figure 6-3). Fraction 3 shows the highest antioxidant activity. Fraction 2 and 6 indicates medium antioxidant activity and the other fractions did not show any antioxidant activity.

Baliga *et al*. (2011) described that the seed of *S. cumini* from India contains jambosine, gallic acid, ellagic acid, corilagin, 3,6-hexahydroxy diphenoylglucose, 4,6-hexahydroxy diphenoylglucose, 1-galloylglucose, 3-galloylglucose, quercetin, and β-sitosterol.

Among 52 samples, *P. acidus* (fruit acetone) and *B. racemosa* (ripe fruit peel and raw fruit peel acetone extract) showed 80.00%, 71.65%, and 70.37% inhibitory effect on HMG CoA reductase activity, respectively. Five extracts showed strong inhibitory effect more than 50% enzyme activity and 8 extracts showed moderate inhibition activity (more than 25% inhibition). The rest of samples showed weak or no inhibition of HMG CoA reductase activity in this study.

Five hundred grams of *P. acidus* fruit powder was extracted to give 66 g acetone extract and 138 g methanol extract. The extract was further successively fractionated to give 9 g dichloromethane fraction, 10 g ethyl acetate fraction, and 52 g water fraction. Each of the fractions was tested for HMG CoA reductase inhibition assay and the ethyl acetate fraction from the acetone extract gave the strongest activity (inhibition 87.30%).

Cytotoxic, antibacterial, and antioxidant activity of *P. acidus* fruit petroleum ether extract was reported by Habib *et al*. (2011) and *P. acidus* leaf activity as antiinflamatory, antinociceptive, and antioxidant have been reported by Chakraboty *et al*. (2012). However, the use of *P. acidus* fruit extract for antihyperlipidemic agent has not yet been reported before.

Chemical constituents from *P. acidus* fruit have not been reported. Leeya *et al*. (2010) isolated adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic acid, and kaempferol from *P. acidus* leaves found in Thailand. Among adenosine, 4-hydroxybenzoic acid, caffeic acid, hypogallic acid, and kaempferol from *P. acidus* leaves found in Thailand, previous findings showed that only caffeic acid possessed strong antihyperlipidemic activity. However, caffeic acid also had strong DPPH radical scavenging activity with IC_{50} 10 μg/mL (Choi *et al*. 2014). We suggest that *P. acidus* fruit extract contained new antihyperlipidemic compounds that have not been reported.

Antioxidant and Antihyperlipidemic Activities of *Albizia falcataria* Waste Biomass

The waste wood of *Albizia falcataria* were given by Nankai Plywood Co., Ltd. Japan and originated from Indonesia. The bark samples were taken from Bogor Agricultural University, Indonesia. Waste wood and bark samples were cut into small chip size. Both were then ground using wood mill to make powder.

Waste wood powder was extracted three times with acetone (1 g powder: 10 mL solvent) at room temperature in Erlenmeyer flask at room temperature for a period of 48 h. Bark powder was extracted with acetone (1 g powder: 5 mL solvent) with the same manner. Mixture was filtered using Whatman filter paper (No. 2), the solution was collected to become acetone extract and methanol was added to the residue with the same ratio. The methanol mixture was filtered and the solution was collected to become methanol extract. Both acetone and methanol extract were concentrated under reduced pressure on rotary evaporator. The concentrated extracts were then used for the experiments.

Both of the extracts were successively fractionated with dichloromethane, ethyl acetate, and water to give acetone-dichloromethane soluble fraction, acetone-ethyl acetate soluble fraction, acetone-water soluble fraction, methanol-dichloromethane soluble fraction, methanol-ethyl acetate soluble fraction, and methanol-water soluble fraction, respectively. Further separation was carried out using preparative thin layer chromatography (TLC) with stationary phase silica gel 60 F_{254} (0.50 mm thickness) for the most active fraction.

In the present study, waste wood and bark of *Albizia falcataria* were evaluated for their *in vitro* antihyperlipidemic and antioxidant activities. Two kinds of extract with acetone and methanol were prepared and their inhibitory effects on HMG CoA reductase activity and DPPH scavenging activity were examined. The highest antihyperlipidemic activity was given by methanol extract of *A. falcataria* bark (50.5% inhibition). Both acetone and methanol extract of waste wood of *A. falcataria* gave medium antihyperlipidemic activity, while acetone extract of *A. falcataria* bark did not express antihyperlipidemic activity.

All bark extract exhibited strong DPPH radical scavenging activity with the strongest is in *A. falcataria* bark acetone extract (IC_{50} 4.49 μg/mL). Successive fractionation was carried out on the samples of waste wood and bark of *Albizia falcataria*. Among all of the samples, high antioxidant activities were shown by ethyl acetate soluble fraction (IC_{50} 2.48 μg/mL) and water soluble fraction (IC_{50} 4.06 μg/mL) from acetone extract of the bark and ethyl acetate soluble fraction from methanol extract of bark (IC_{50} 4.73 μg/mL), respectively.

We selected ethyl acetate fraction of methanol extract from the bark of *A. falcataria* to be further separated because it gave the highest antihyperlipidemic activity as well as strong DPPH radical scavenging activity. Ethyl acetate soluble fraction from *A. falcataria* methanol extract was separated on preparative TLC plates with ethyl

acetate as mobile phase to give 4 bands. Band 4 showed strong antioxidant activity, band 3 indicated medium activity, and band 1 and 2 did not exhibit antioxidant activity.

Affandi *et al*. (1998) identified 3 chemical constituents from ether soluble fraction of methanolic extract of *A. falcataria* bark, those were lupeol, stigmasta-4,22-dien-3-ol, and lupenone. Syringaresinol also had been reported to be isolated from the bark of *A. falcataria* (Kokila *et al*. 2013).

The observed HMG CoA reductase inhibition and antioxidant activities of the ethyl acetate soluble fraction of methanol extract of *A. falcataria* indicated that this plant possess potential medicinal value. Isolation and identification of chemical constituents should be carried out to find the responsible compounds of the antihyperlipidemic and antioxidant activities.

Finally, we found several candidiates for the development of antioxidant, antidiabetic, and antihyperlipidemic drugs. In order to reveal more characteristics and study the mechanism of action, future reseach is needed. More information is required in term of the chemical constituents responsible for the action of those activities.

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