Biomedical & Pharmacology Journal Vol. **9**(2), 433-441 (2016)

The Response to oxidative stress α-Humulene Compounds Hibiscus manihot L Leaf on the Activity of 8-Hydroxy-2-Deoksiquanosin Levels Pancreatic β-Cells in diabetic Rats

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http://dx.doi.org/10.13005/bpj/956

(Received: May 19, 2016; accepted: June 20, 2016)

ABSTRACT

Increased antioxidant activity of α-humulene of Hibiscus manihot L leaf empirically may help prevent oxidative stress due to the production of compounds reactive oxygen species (ROS), thereby protecting cells from damage function. Oxidative stress can be formed through an increase in the activity of the compound 8-hydroxy-2-deoksiguanosin in the body. This study is a laboratory experimental design was randomized pre and post-test control group design. A total of 36 wistar rats were divided into 4 groups, namely P_0 (negative control), group P_1 (isolate α-humulene dose of 5 mg/kg, P_2 (isolate α-humulene dose of 10 mg/kg), and P_3 (isolate α-humlene dose of 15 mg/kg). All groups induced by alloxan dose of 140 mg/kg in order to get the condition. After the treatment for 12 weeks, serum was taken for examination of 8-OHdG levels and see a picture immunohistochemistry pancreatic tissue of wistar rats. Data were analyzed by *one-way ANOVA* and continued test *t-paired* to determine the mean difference between treatments with significance limit of 5%. the results showed that, the dose α-humulene isolate of Hibiscus manihot L resulted in a decrease in the average of 8-hydroxy-2-deoksiguanosin levels in the positive control group was (3.63 ± 0, 19) ng / mL, the treatment groups respectively P_1 by (3.94 ± 0.51) ng/ mL; P_2 was (3.49 ± 0.47) ng/mL and P_3 of (1.99 ± 0,18) ng/mL. It is also evident from the results of immunohistochemical against 8-OHdG profile repair due to damage pancreatic β-cells in the islets of Langerhans. This difference was statistically significant was shown with a value of $p<0.05$. Therefore α-humulene compounds Hibiscus manihot L leaf may help prevent oxidative stress through reduction mechanism 8-OHdG

Keywords: Oxidative Stress, α-humulene, Hibiscus manihot L, 8-OHdG, Diabetes

INTRODUCTION

Hibiscus manihot L leaf isolate is thought to prevent oxidative stress due to increased production of reactive oxygen species (ROS), which can protect cells from damage function. Oxidative stress can be formed through an increase in 8-OHdG in the body, and decrease the body's ability to neutralize free radicals. Oxidative stress is a condition associated with an increased rate of cell damage induced by oxygen and derivatives (compound reactive oxygen species/ROS). Cell damage caused by an imbalance between ROS formation and activity of antioxidant enzyme defense[4,6].

Complications of diabetes can increase the compound reactive oxygen species (ROS) either through the enzymatic process is the reaction of oxidation and phosphorylation (ox-phos) and the reaction ADPH-oxidase and through non-enzymatic by forming gluco oxidant and glycation.[3]

Diabetes is caused due to abnormalities in insulin secretion, or action of insulin disorders. The state of hyperglycemia in diabetes lead to increased formation of free radicals and anti-oxidants and a decrease in a number of events eventually called oxidative stress. Diabetes can induce an increase in the formation of compound 8-hydroxy-2-deoksiguanosin, and increased polyol pathway activity (sorbitol)[5,10].

8-OHdG compounds is one of the dominant form of free radicals caused by oxidative lesions and carcinogenesis. Studies show that 8-OHdG is a biomarker that is good for risk assessment degenerative disease or as a marker to measure oxidative damage endogenous DNA superfluous through the conducting signal will affect the cells in the body, particularly in inhibiting the tyrosine kinase activity and inhibition of the phosphorylation of a serine residue the IRS (insulin receptor substrate). The decrease of tyrosine kinase activity would result in the disruption of the insulin receptor signaling in cell membranes. The absence of downstream signaling resulted insulin can not be attached to the cell membrane and finally glucose can not get into the cells, so that there was a decrease in intracellular glucose and an increase in extracellular glucose. Such a condition called type 2 diabetes, therefore it is type 2 diabetes mellitus as insulin resistance.[8,14] Insulin resistance can occur at many levels, beginning of insulin receptors on the target organ, impaired formation of signaling transmembrane by activation of the insulin receptor kinase and occurs in a complex network of signal intracellular or defects in signal transduction which is often referred to as postreceptor step such as glucose transporter translocation and transport glucose[12].

According Todorwal *et al*, [13] reported that one of the traditional medicinal plants that have the potential to be developed as an antidiabetic drug is *Hibiscus manihot* L, since traditionally the leaves of *Hibiscus manihot* L has been used to treat various diseases, such as diabetes, ulcers, diseases heart disease, high blood pressure, osteoporosis, renal impairment, seizures, and depression. The results showed that of the *Hibiscus manihot* L leaf isolate contains several chemical compounds, including; flavonoids, terpenoids, alkaloids, tannins, polyphenols, saponins and serotonin. While the á-humulene compounds that are derived sesquiterpene that is believed to have antioxidant activity of exogenous and works to increase the number of pancreatic β-cells through a decrease in blood glucose levels and cholesterol in diabetic wistar rats[1]. According Prangdimurti, *et al.,*[9] also states that terpenoid intake suji leaf extract is rich in antioxidants can increase the activity of superoxidase dismutase and catalase in diabetic mice liver, so it can protect cells against oxidative stress.

α-humulene is one of the hormones produced in the body, with molecular formula $C_{15}H_{24}$, and has a molecular weight of 204 356 g/mol. Another name of α-humulene are: 3,7,10-humulatriene, 2,6,6,9-tetramethyl cycloundeca 1,4,8-triene. α-humulene works by lowering the levels of glucose in the blood or as a diabetes drug. α-humulene is a long-acting form of insulin that is slightly different from other forms of insulin that are not man-made[2].

α-humulene also known as α-caryophyllene is a natural monocyclic sesquiterpene derived from farnesyl diphosphate (FPP). Diphosphate biosynthesis begins with the loss of sesquiterpene synthesis enzymes, generating cation alilik highly susceptible to intramolecular. This biosynthesis can be done in the laboratory by preparing stanane alilik of farnesol, called the synthesis of Corey. There are various ways to synthesize α-humulene in the laboratory, involving the closure of which is different from the C-C bonds. McMurry synthesis using a titanium catalyst coupling reactions of carbonyl; Takahashi synthesis using an intramolecular alkylation of allyl halides by anion cyanohydrin as pelidungi. α-humulene can also be synthesized using a combination of the four components of the assembly and the palladium-mediated cyclization, synthesis is essential to the simplicity of the C-C bond and cyclization steps, which is believed will prove in the synthesis polyterpenoid. The mechanism of formation of α-humulene compound through farnesyl diphosphate[7,15] as shown in Figure 1

MATRERIALS AND METHODS

Preparation of plant extract

Hibiscus manihot L Leaf is cleaned and dried in a way placed an open place with open air circulation and is not exposed to direct sunlight. Furthermore, blended to a powder. *Hibiscus manihot* L leaf powder which has been dried and weighed as much as 700 g in maceration using methanol for 24 hours. The extract obtained is filtered and evaporated using a rotary vacuum evaporator until a viscous extract methanol. Then hydrolysed with 2N HCl for 2-3 hours. Results hydrolysis re-extracted with n-hexane is then evaporated to obtain a thick n-hexane extract as much as 12.64 g. Extract thick n-hexane followed phytochemical screening test to determine the class of sesquiterpene and the separation and purification by thin layer chromatography and column chromatography

Mass Spectroscopy Analysis

Mass spectroscopy method is one of the analytical techniques to determine the structure of a compound based on the separation of beam ions according to mass ratio with the load and measuring the intensity of a beam of ions tertsebut. Isolates of *Hibiscus manihot* L leaf relatively pristine identified using mass spectroscopy. The mass spectrum obtained compared to the spectrum WILEY229.LIB Library.

Experimental Design

This study is a laboratory experimental design with *The Randomized Pre and posttest control group design* to prove the oxidative stress response α-humulene compound leaf isolate of *Hibiscus manihot* L on the activity levels of 8-OHdG in wistar rats with diabetes. The next step immunohistochemical examination to see the profile of wistar rat pancreatic â-cells in the islets of Langerhans fifths of visual field

Experimental Animals

A total of 36 wistar rats aged 3 months and weighing 200-250 g given standard formula diet enriched vitamin B_{12} for 1 month. All rats adapted for 1 week. Once the animal is in uniform conditions, the rats were made diabetic for 3 days in a way induced alloxan dose of 140 mg/kg, then made four experimental groups, namely one kelopok control and 3 treatment groups were each given α-humulene isolate of leaves of *Hibiscus manihot* L at a dose of 5 mg/kg, 10 mg/kg and 15 mg/kg for 7 weeks. Giving α-humulene compound isolate of *Hibiscus manihot* L leaf made by dissolving into 5 ml of distilled water, then homogenized and presented orally to rats in accordance treatments by disonde. After a seven-week treatment period was terminated wistar rats (euthanasi) with CO_2 quickly and sterile. Pancreatic tissue is taken and washed with phosphate buffer saline, drained and weighed. Furthermore, immunohistochemical examination to see improvements profile wistar rat pancreatic β-cells

Measurement of the Activity of 8-OHdG Levels in Rats

Laboratory tests performed at the time of treatment until there is diabetes, with testing procedures as follows:

a. Determination of the levels of 8-OHdG is done using KIT and done in accordance with the instructions on the KIT. KIT purchased from STA-325: OxiSelect ™ Oxidative RNA Damage ELISA Kit (8-OHdG) for the analysis of 8-OHdG.
b. Examination Imunohistochemical structure of pancreatic tissue wistar rats performed using binocular microscope with Gomori-Nuclear fast red staining, magnification 400x

Data Analysis

The data collected from this research was statistically analyzed by the following procedures. Statistical analysis was conducted using SPSS 13.0 application program for windows (Triton, 2006) for.

1. Normal distribution using Shapiro-Wilk within α = 0,05;
2. Homogeneity of variance were analyzed using Levene's test to determine whether variations in respective homogeneous group.
3. Analisis of differentiation on mean levels increase of 8OHdG from each group were analyzed using one-way ANOVA. Further analysis is one way anova Post Hoc Test; assuming homogeneous variance is then selected Post Hoc Test was LSD at significance level α = 0.05.

4. Further t-paired test to see if each group before and after treatment gave significantly different results with a significance limit of $p<0.05$

RESULTS AND DISCUSION

Characteristics Compound α-Humulene *Hibiscus manihot* L Leaf

Extract thick n-hexane leaves of *Hibiscus manihot* L extraction provides as much as 47.68% yield reddish yellow. While the test results of antioxidant activity against DPPH (1,1-diphenyl-2-pikril hydrazine) isolates compound α-humulene leaves *Hibiscus manihot* L of the separation and purification, has the ability as inhibiting the activity of DPPH (1,1-diphenyl-2-pikril hydrazine) amounted to 79.65% within 60 minutes. This means that the compound á-humulene isolate leaf *Hibiscus manihot* L is a strong oxidizing agent linked to the ability as an anti-free radicals (free radical scavenger). This test has a positive correlation with the increase in antioxidant capacity as a result of oxidative stress, or reactive oxygen species. Further test results phytochemical with Liebermann-Burchard reagent, isolate of *Hibiscus manihot* L leaf tested positive for class sesquiterpene compounds that give color purple color with strong intensity.

Mass Spectroscopy Analysis

The results of the mass spectroscopy measurements provide clues to the fragments occur in the molecular ion can be determined so possible structure and relative molecular mass of α-humulene compound leaves of *Hibiscus manihot* L. Results fragmentation mass spectrum with a retention time of 16.492 minutes (17.40%) gave molecular ion at m/z 204 [M^+] with a base peak at m/z 93. the other fragment ions can be interpreted in Figures 2 and 3

The figures show that the α-humulene compound have molecular ions parent with mass number m/z 204 [M^+], then experience the release of molecules [M^+-CH_3] giving m/z 189, followed by ion fragmentation of m / z 161 [M^+-C_2H_4], fragment ion m/z 147 [M^+-C_3H_6], fragment ion m/z 121 [M^+-

Table 1: Results of the purity test

Mobile phase	The number of stains	Rf Large	UV	Color sightings Liebermann-Burchad
Benzene-Chloroform (10:1)	1	0,423	Brown	Purple+++ (sesquiterpen)
n-Hexane-benzena (3:1)	1	0,517	Brown	Purple+ (sesquiterpen)
n-Hexane-ethylacetate (8:2)	1	0,658	Brown	Purple+ (sesquiterpen)
Benzene-methanol(8:2)	1	0,715	Brown	Purple+ (sesquiterpen)

Table 2: 8-OHdG Levels Pre and Posttest Treatment

Treatment	Observation of 8-OHdG Levels (ng/mL)		
	Pretest The mean ± SD	Posttest The mean ± SD	Difference of8-OHdG Levels (ng/mL)
Negative (K-) and Positive (K+)	6,75 ± 0,46	3,63 ± 0,19	3,119 ^{bc}
α-humulene isolate a dose of 5 mg	6,97 ± 0,55	3,94 ± 0,51	3,029 ^{c}
α-humulene isolate a dose of 10 mg	6,92 ± 0,49	3,49 ± 0,47	3,433 ^{b}
α-humulene isolate a dose of 15 mg	6,95 ± 0,58	1,99 ± 0,18	4,967 ^{a}

Note: Difference in average values followed by different letters in the same column, shows the test results were significantly different ($p<0.05$) LSD test for posstest

K^- (Negative Control)

K^+ (Positive Control/Glibenclamide)

C_2H_4], fragment ion m/z 107 [M^+-CH_2], fragment ion m/z 93 [M^+-C_2H_3], fragment ions m/z 80 [M^+-CH], fragment ion m/z 67 [M^+-CH], fragment ion m/z 53 [M^+-CH_2], fragment ion m/z 41 [M^+-C_2H_2], and fragment ion m/z 29 [M^+-C].

Examination of the activity of 8-OHdG Levels in Diabetic Rats

Test results mean 8-OHdG levels in diabetic wistar rats pre- and posttest are presented in Table 2. While the of 8-OHdG levels profiles of the various doses of α-humulene compound isolat of *Hibiscus manihot* L leaf can be seen in Figure 4

Test results with the *Shapiro-Wilks* normality and homogeneity test with *Levene's test* shows that the data mean levels of 8-OHdG Wistar rats pre and post administration of various *Hibiscus manihot* L leaf isolate showed dose throughout the data were normally distributed and homogeneous variants (p>0,05).

Results of analysis and one way *ANOVA* followed by *LSD test* showed that there were significant differences between the levels of 8-OHdG Wistar rat control group (K^+) treatment group after the administration isolat of *Hibiscus manihot*

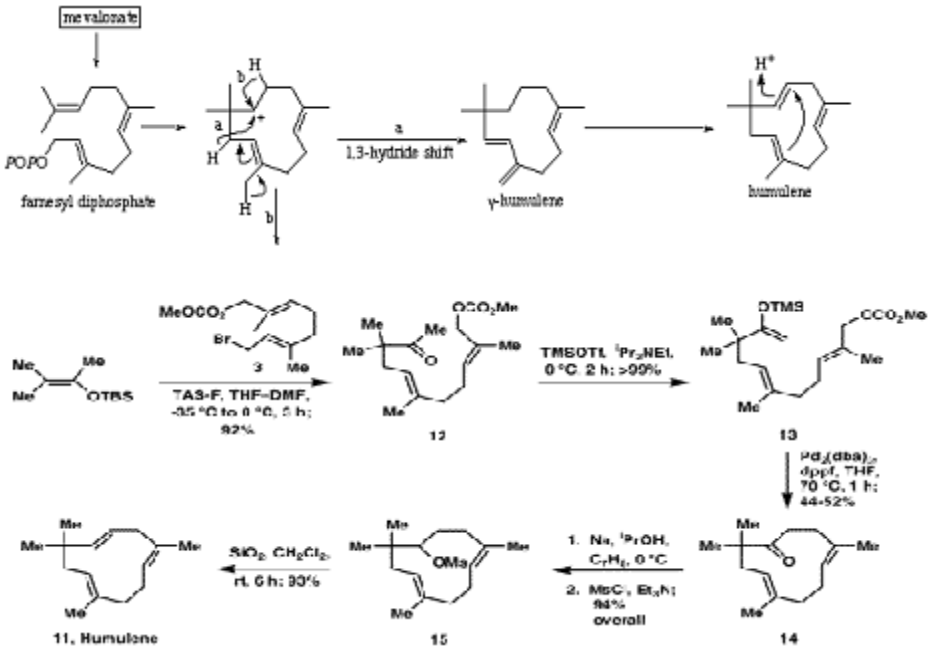


Fig. 1: The Mechanism α-humulene Compound Through Farnesil Diphosphate

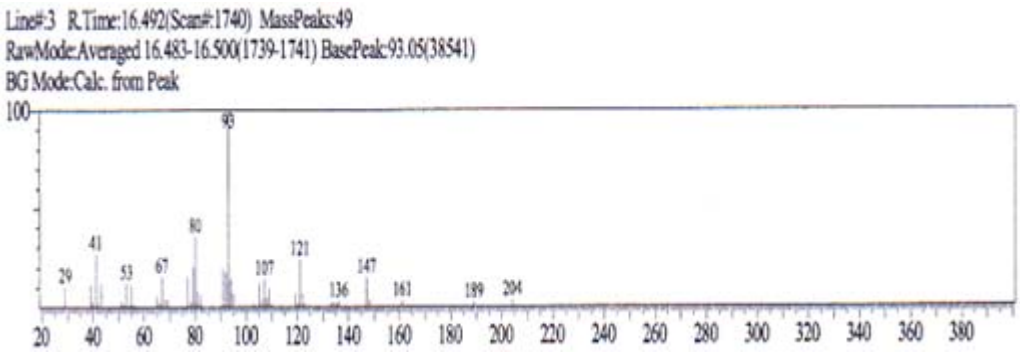


Fig. 2: The Massa Spectrum α-Humulene Compound of *Hibiscus manihot* Leaf

L leaf a dose of 5 mg/kg bw, a dose of 10 mg/kg bw and a dose of 15 mg/kg bw with a value of $p<0.05$. The results also showed that the control group (K^+) gives the equivalent effect of the treatment group a dose of 10 mg/kg bw.

Furthermore, the limit of significance with *paired t-test* showed a significant difference in the mean decrease in 8-OHdG levels between the control group (K^-) with control group (K^+) with $p<0.05$. In contrast, the treatment group a dose of 5 mg/kg bw, a dose of 10 mg/kg bw and treatment group a dose of 15 mg/kg bw significant differences with $p<0.05$.

Related to the reference, the study found that the average reduction in the levels of 8-OHdG significantly different ($p<0.05$) between the control group (dose 0 mg/kg bw/day) with treatment group P_1 is awarding *Hibiscus manihot* L leaf isolate with a dose of 5 mg/kg bw/day and the control group with the treatment group 2 P_2 seed extract pranajiwa a dose of 10 mg/kg bw/day as well as the control treatment group kolompok P_3 pranajiwa seed extract a dose of 15 mg/kg bw/day. This situation indicated that the decrease in the average levels of 8-OHdG (posttest) in the control group by 3.63 ± 0.19 ng/ml, the P_1 of 3.95 ± 0.51 ng/ml; groups P2 by 3.49 ± 0 , 47 ng/ml and the P_3 of 1.99 ± 0.18 ng/ml.

In general it can be explained that the *Hibiscus manihot* L leaf isolate in rats wistar diabetes will affect the levels of 8-OHdG. This situation can be explained that *Hibiscus manihot* L leaf isolate contains antioxidant compounds such as phenolic compounds and α-humulene a role in increasing insulin secretion by β-cells through a mechanism of pancreatic beta cells in maintaining a functioning and the α-humulene compound which is a derivative sesquiterpen compounds have reactive groups on the molecular structure so that the ability to capture free radicals is also getting stronger and the impact would be a decline in the levels of 8-OHdG as a sign of DNA damage caused to oxidative stress.

Examination Results Immunohistochemistry Pancreatic β-cells Wistar Rats

Gomori-Nuclear fast red staining done to see qualitative changes in the structure of rat pancreatic tissue treatment. Staining is composed

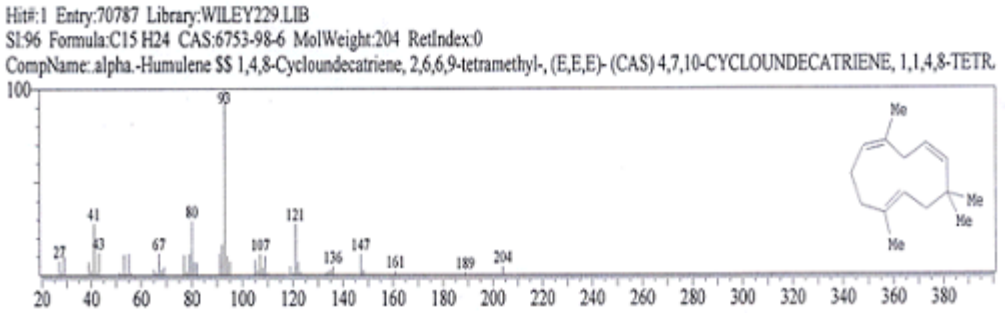


Fig. 3: The Mass Spectrum α-Humulene Compound Standar Library

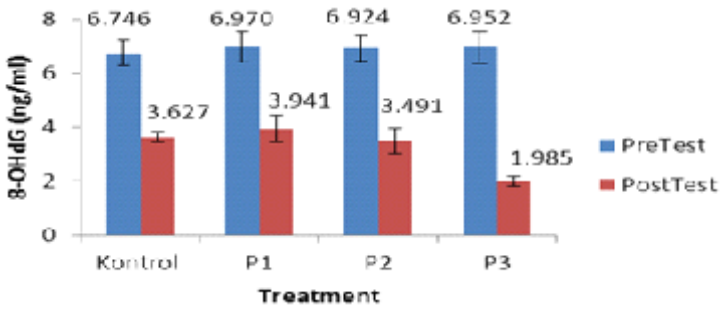
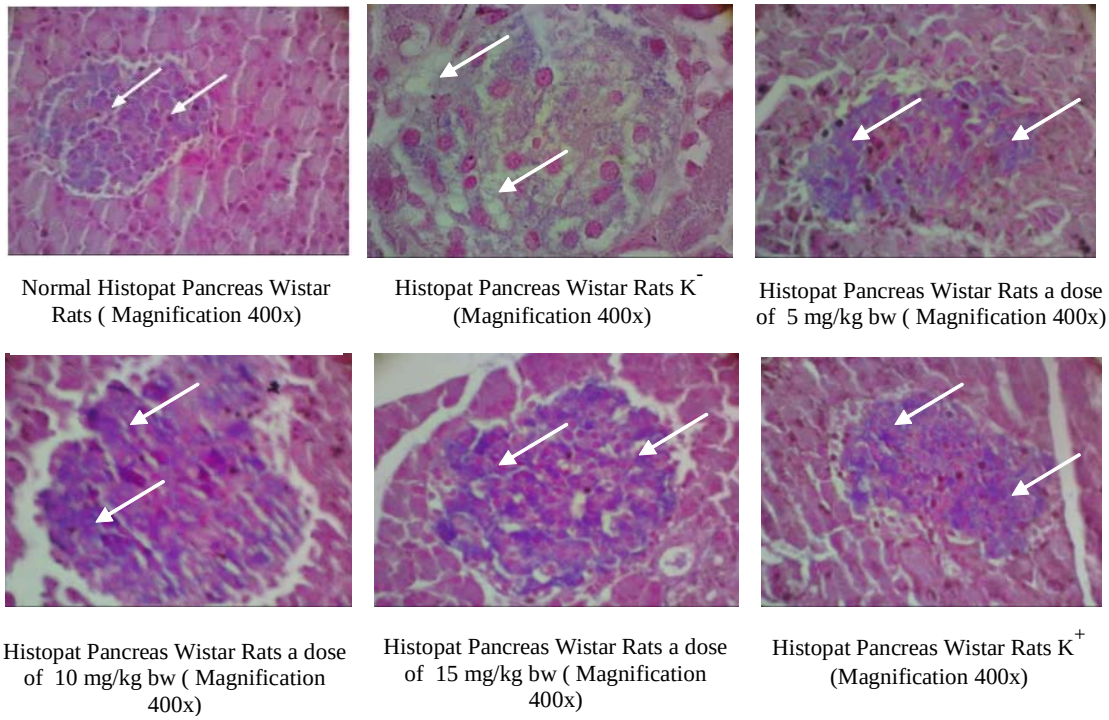


Fig. 4: Profile of 8-OHdG Levels Pre and Posttest Treatment

of two color components, and the Gomori-Nuclear fast red. Gomori an alkaline dye in order to color the cell nucleus that are acidic while Nuclear fast red is an acidic dye that can stain the cytoplasm is alkaline.Histopathological changes in pancreatic tissue morphology Wistar rats with 400 times magnification and staining Gomori-Nuclear fast red from the normal state to occur alloxan-induced diabetes caused a dose of 140 mg/kg bw can be seen in Figure 5.

In the Figure above there have been improvements morphology of pancreatic tissue histopathology at the Wistar rat islets of Langerhans from application isolate *Hibiscus manihot* L leaf a dose of 5 mg/kg bw compared to the negative control group, although the number of β-cells of the pancreas degenerated until necrosis somewhat reduced. This means giving isolate *Hibiscus manihot* L leaf a dose of 5 mg/kg bw can help tissue repair and pancreas were damaged by alloxan induced. Conversely at a dose of 10 mg/kg bw to changes in the morphological structure of the pancreatic tissue is stimulated cell proliferation. There is an increasing amount of β-cells of the pancreas means in accordance with the theory that when the cells undergo stimulation of the injury when something is potentially reversible changes that are irreversible. Repair mechanisms pancreas from application isolates of *Hibiscus manihot* L leaf a dose of 10 mg/kg bw is the possibility of isolated of *Hibiscus manihot* L leaf a dose of 10 mg/kg bw led to a higher production of insulin is greater in comparison treatment group a dose of 5 mg/kg bw so that the destruction of β-cells of the pancreas can be corrected quickly and almost close to normal. The number of pancreatic β-cells in the provision of isolat *Hibiscus manihot* L leaf a dose of 10 mg/kg bw more than the number of pancreatic â-cells a dose of 5 mg/kg bw.

At the a dose of 15 mg/kg bw did not appear to have cell degeneration to necrosis in Wistar rat pancreatic tissue around the islands of Langerhans that show clear boundaries between β-cells with α-cells. Likewise, the number of cytoplasmic granules in the β-cell nucleus has increased to nearly normal conditions so that the pancreatic tissue repair process can take place quickly. Furthermore, the administration of antidiabetic drug (*Glibenclamide*) as positive control (K^+) structure changes Wistar rat pancreatic tissue



Normal Histopat Pancreas Wistar Rats (Magnification 400x)

Histopat Pancreas Wistar Rats K^- (Magnification 400x)

Histopat Pancreas Wistar Rats a dose of 5 mg/kg bw (Magnification 400x)

Histopat Pancreas Wistar Rats a dose of 10 mg/kg bw (Magnification 400x)

Histopat Pancreas Wistar Rats a dose of 15 mg/kg bw (Magnification 400x)

Histopat Pancreas Wistar Rats K^+ (Magnification 400x)

Fig. 5: Histopat Pancreatic β-Cells Wistar Rats

morphology in the islands of Langerhans. Still visible presence of cytoplasmic granules and cell boundaries clear between β-cells with α-cells. Quantitatively number of pancreatic β-cells, Wistar rats was lower than the number of pancreatic β-cells due to administration of a dose of 15 mg/kg bw, so that the process of improvement of pancreatic β-cells do not appear clearly.

Observance of the β-cells of the pancreas performed quantitatively by calculating the amount of β-cells of the pancreas tissue Wistar rats in each group and the control group both treatment groups. Â-cells were detected by staining Gomori-Nuclear fast red and 400 times magnification is shown in Figure bluish purple cells in the islets of Langerhans cell while the other is red. It can be concluded that the isolated *Hibiscus manihot* L leaf containing α-humulen compound that play a role in increasing insulin secretion by pancreas β-cells through a mechanism in maintaining β-cell functioning and also α-humulen compound which are derivatives sesquiterpene have reactive groups on the molecular structure so that the ability to capture free radicals are also getting stronger and the impact will be decreased levels of 8-OHdG as a sign of DNA damage caused by free radicals.

CONCLUSION

This study proves that the isolated of *Hibiscus manihot* L leaf contain á-humulen compounds sesquiterpene derivatives which have activity as an antidiabetic towards decreased of 8-OHdG levels in Wistar rats and prevent oxidative stress due to the formation of free radicals. It also ensures further research in the form of clinical trials on human subjects with diabetes, whether the compound α-humulene isolate leaf *Hibiscus manihot* L have the same effect than the diabetes drug glibenclamide, so as to explain the mode of action.

ACKNOWLEDGMENTS

In this moment I would like to thank the University of Udayana financial support through grants main universities and all parties directly involved in the study.

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