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Research Article

Nano-extract of *Acalypha hispida* Increased Cu,Zn-SOD Antioxidant in Pancreas of Diabetic Rat

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Abstract

Nanotechnology has rapidly grown in various research fields to treat oxidative stress in diabetes, including medicine and phytomedicine. Previous research showed that *Acalypha hispida* has strong antioxidant activity *in vitro* and anti-hyperglycemic activity *in vivo*. However, the size reduction in the crude extract should be applied to decrease the doses and increase the efficacy. The research objective was to evaluate the nano-extract of *A. hispida* leaves on Cu,Zn-SOD antioxidant content in the pancreas of diabetic rats. The analysis of Cu,Zn-SOD antioxidant content was carried out using immunohistochemistry. Cu,Zn-SOD content of pancreas in diabetic rats (DMC) was significantly lower than in normal rats (NLC). The antioxidant content of Cu,Zn-SOD in MET, CAH, NAH3, and NAH6 groups was significantly higher than in the DMC group of the pancreas of diabetic rats. Nano extract of *A. hispida* showed a better effect in increasing Cu,Zn-SOD antioxidant content than crude extract. The result concluded that nano-extract of *A. hispida* leaves increased Cu,Zn-SOD antioxidant content in the pancreas of diabetic rats.

Keywords: *Acalypha hispida*, Cu,Zn-SOD, Diabetes, Nanotechnology, Oxidative stress, Pancreatic rat.

Nano-ekstrak *Acalypha hispida* Meningkatkan Antioksidan Cu,Zn-SOD pada Pankreas Tikus Diabetes

Abstrak

Nanoteknologi telah berkembang pesat di berbagai bidang penelitian untuk mengatasi stress oksidatif pada diabetes termasuk kedokteran dan fitomedika. Penelitian sebelumnya menunjukkan bahwa *Acalypha hispida* memiliki aktivitas antioksidan yang kuat secara *in vitro* dan aktivitas antihiperglikemia secara *in vivo*. Penelitian ini bertujuan untuk mengetahui pengaruh pemberian nano-ekstrak daun *A. hispida* terhadap kandungan antioksidan Cu,Zn-SOD pada pankreas tikus diabetes. Analisis kandungan antioksidan Cu,Zn-SOD dilakukan secara imunohistokimia. Kandungan Cu,Zn SOD tikus diabetes (DMC) secara signifikan lebih rendah daripada tikus normal (NLC). Kandungan Cu, Zn-SOD pada kelompok MET, CAH, NAH3, dan NAH6 secara signifikan lebih tinggi daripada kelompok DMC pada pankreas tikus diabetes. Nano-ekstrak daun *A. hispida* memiliki efek yang lebih baik dalam meningkatkan kandungan Cu,Zn-SOD dibandingkan dengan ekstrak kasar. Hasil penelitian menyimpulkan bahwa nano-ekstrak daun *A. hispida* meningkatkan kandungan antioksidan Cu,Zn-SOD pada pankreas tikus diabetes.

Kata Kunci: *Acalypha hispida*, Cu,Zn-SOD, Diabetes, Nanoteknologi, Pankreas tikus, Stres oksidatif.

1. Introduction

National Nanotechnology Initiative (NNI) defined nanotechnology as the manipulation of matter to produce a device and dosage in the size of 1-100 nm.[1] In nanomedicine, a nanoparticle is accepted in the range size of a few nanometers to <1000 nm.[2] Nanotechnology has rapidly grown in various research fields, including pharmaceutic, medicine, and phytomedicine.[3] The application of nanotechnology in antioxidant-rich plants was proven to overcome the limitation of its crude extract, such as optimal less absorption of bioactive. The plant with antioxidant and anti-hyperglycemic properties is *Acalypha hispida* leaves extract.[4] The IC_{50} antioxidant of this plant is less than 10 µg/mL, showing strong antioxidant activity *in vitro*. This plant inhibits α-glucosidase and α-amylase activity, preventing the degradation and absorption of carbohydrate complex related to its bioactive compounds. The bioactive compounds of *A. hispida* leaves have been previously identified, such as flavonoid and phenolic acid.[4,5]

Diabetes mellitus (DM) is a metabolic illness marked by high blood sugar levels. The prevalence of DM was predicted to increase by 783 million people with diabetes in 2045 worldwide.[6] Prolonged hyperglycemia in diabetes stimulates oxidative stress by producing reactive oxygen species (ROS) excess in various tissues, including pancreatic cells. Pancreatic β-cells express the high-Km glucose transporter-2 (GLUT2) as glucose influx in the cells, which means it does not have a high affinity for glucose and only begins to uptake glucose at high blood glucose levels.[7] Whenever exposed to a high glucose level, GLUT2 has a high absorption efficiency.

When exposed to oxidative stress, pancreatic β cells may be highly susceptible to ROS assault. Pancreatic β cells have a relatively low expression of antioxidant enzymes such as copper,zinc-superoxide dismutase (Cu,Zn-SOD), glutathione peroxidase (GPx), and catalase (Cat) than other tissues.[7] Cu,Zn-SOD enzyme is the first primary antioxidant defence in the cells against oxygen radicals: in this way, this enzyme is essential for aerobic organisms, but not for anaerobes. This enzyme catalyzes superoxide anion (O_2•-) to hydrogen peroxide (H_2O_2), and then H_2O_2 is converted to a water molecule (H_2O) and oxygen (O_2) by GPx and Cat.[8] Cu,Zn-SOD are secreted either naturally or as a result of depolarization caused on by high extracellular K^+ concentration. This enzyme physiologically exhibits an unedited effect in addition to oxygen radical dismutation.[9] In physiological conditions, the balance between the oxidant and antioxidant systems is maintained by Cu,Zn-SOD in combination with non-enzymatic ROS scavengers such as vitamin E, A, and C.[10] There are three isoforms of SOD, namely Cu,Zn-SOD, Manganese-SOD (Mn-SOD), and extracellular SOD (Ec-SOD). However, Cu,Zn-SOD is abundant in the nucleus and cytoplasm.[11,12] Previous research showed that diabetic rats exhibited decreasing Cu,Zn-SOD antioxidant content.[12] Furthermore, the objective of the research was to evaluate the effect of nano-extract of *A. hispida* leaves on Cu,Zn-SOD antioxidant content in the pancreas of diabetic rats.

2. Methods

2.1. Instrument

The equipment in this study was a spray dryer, planetary ball milling (FRITSCH, Pulverisette 7, Germany), rotary microtome (Yamato RV-240, Japan), micropipette, tissue embedding console (Tissue-Teck Sakura, Japan) and light microscope with a camera (Olympus CX31).

2.2. Materials

The materials were *A. hispida* leaves (TropBRC, IPB University), adult male rats (Sprague-Dawley; 260-290 grams), streptozotocin (S0130, Sigma), Cu,Zn-SOD primary antibody (S2147, Sigma), and starr trekTM universal link (STU700H, Biocare).

2.3. Procedure

2.3.1. Nano-extract preparation

The extraction and nano-extract preparation were referred to Alfarisi et al.[13] The leaves were dried and ground, then extracted using maceration methods. After

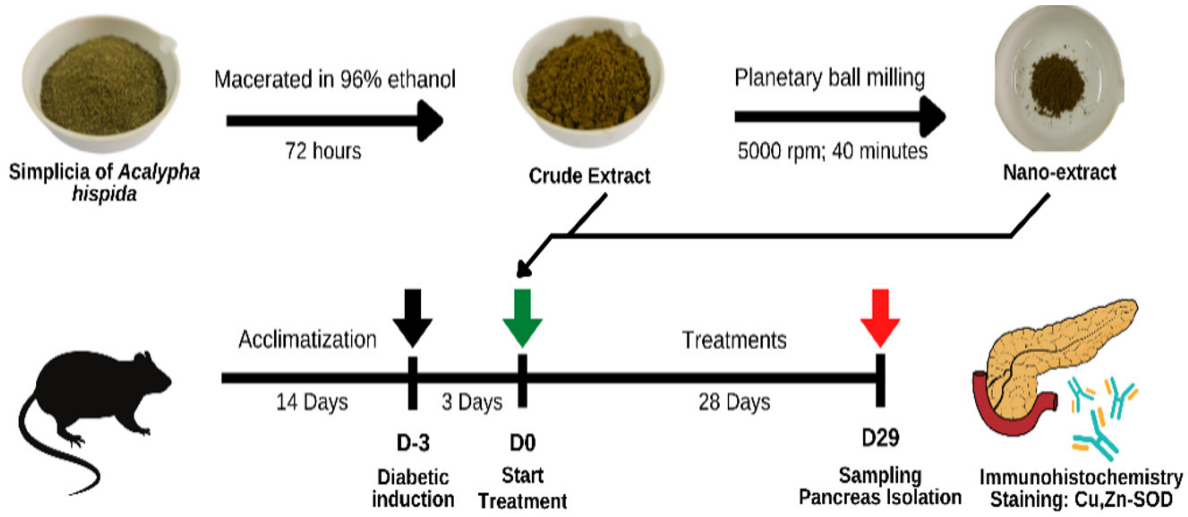

Figure 1 The experimental route of nano-extract of *Acalypha hispida* treatment in diabetic rats.

that, the filtrate was run in a spray dryer. The dry extract was milled in the planetary ball milling at 5000 rpm for 40 minutes to produce a nano-extract.[13] The characterization of nano-extract was evaluated using particle size analyzer (PSA) and scanning electron microscope (SEM).[13]

2.3.2. Animal Experiments

Adult male rats (260–290 g; 8–12 weeks) were purchased from the National Drug and Food Control (BPOM), Republic of Indonesia. The acclimatization was conducted for two weeks in the following conditions: 25-27°C; humidity 60-70%; 12-hour light/dark cycle. Rats were given antibiotics, anthelmintics, and vitamins before grouping. The water and feed were given ad libitum. Animal Ethics Committee, School of Veterinary Medicine and Biomedicine-IPB University approved the experimental procedures (number: 143/KEH/SKE/VI/2019). Twenty fours rats randomly were divided into six groups (n=4): (1) normal rat control (NLC), (2) diabetic rat treated with 1% carboxymethyl cellulose (CMC) as diabetes mellitus control (DMC), (3) diabetes mellitus+metformin at 88 mg/kg body weight (BW) (MET), (4) DM+crude extract at 300 mg/kg BW (CAH), (5) DM+nano-extract at 30 mg/kg BW (NAH3), and (6) DM+nano-extract at 60 mg/kg BW (NAH6).

Diabetic rats were induced with a single dose of streptozotocin (STZ) at 55 mg/kg BW (intraperitoneal injection). All rats were kept for three days to ensure stable hyperglycemia. Rats with glucose levels at 300 mg/dL were considered diabetic rats. The treatments were administered for a period of 28 days. The rats were sacrificed on the 29th day, then pancreatic tissue was collected and fixed in 4% paraformaldehyd. The experimental route of nano-extract treatment in diabetic rats was illustrated in Figure 1.

2.3.3. Immunohistochemical staining

The pancreatic rats were processed with routine histology. It was trimmed, dehydrated in alcohol, cleared in xylene, and embedded in paraffin. The block was sectioned at five µm with a rotary microtome. Immunohistochemical staining of Cu,Zn-SOD antioxidant referred to the method previously described by Wresdiyati et al.[14] The pancreatic sections were incubated in Cu,Zn-SOD primary antibody (1:200) for two overnight. The 3,3'-diaminobenzidine (DAB) chromogen was used for visualization. The quantitative analysis of the Langerhans islet and the acinar cell was observed in five fields of view (40X) under a light microscope (Olympus CX31 with camera). The immunoreaction Cu,Zn-SOD product was examined based on the colour intensity of the nucleus; strong positive (+++) indicated by dark brown colour throughout the cell nucleus, moderate positive (++) indicated by dark brown colour in some parts of the nucleus, and weak positive (+) indicated by a light brown colour in the nucleus, and negative reactions (-) indicated by blue colour

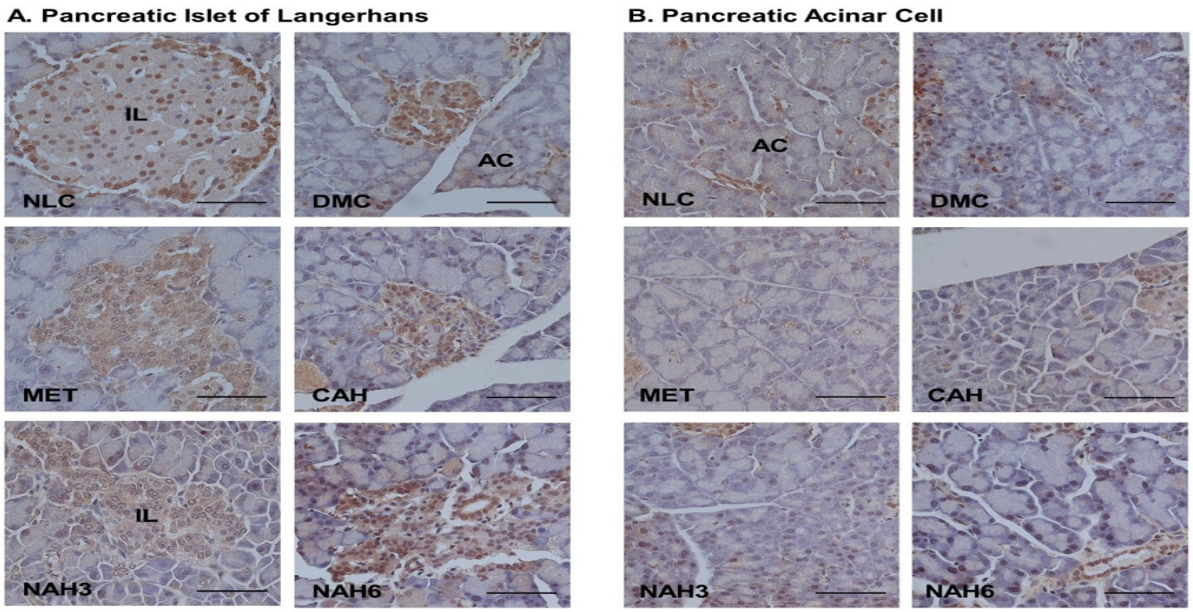

Figure 2 The photomicrograph of Cu,Zn-SOD localization in the pancreatic islet of Langerhans (A) and acinar cells (B). NAH6, NAH3, CAH, and MET groups showed the increased Cu,Zn-SOD antioxidant content compared to the DMC group in the pancreatic islet of Langerhans and acinar cells. NLC, normal control; DMC, diabetes mellitus (DM) control; MET, DM+metformin 88 mg/kg BW; CAH, DM+crude extract 300 mg/kg BW; NAH3, DM+nano-extract at 30 mg/kg BW; NAH6, DM+nano-extract at 60 mg/kg BW. Bar=50 µm. IL: islet of Langerhans; AC: acinar cells.

in the cell nucleus.

2.3.4. Data analysis

The image analysis was conducted using ImageJ 1.50 (FIJI). A one-way analysis of variance (ANOVA) was used to compare the data with a significance of $p<0.05$. The post hoc analysis used Duncan's test if there were significant effects.

3. Result

Immunohistochemical staining of pancreatic rats on Cu,Zn-SOD antioxidant enzyme was shown in Figure 2. The brown colour showed the reaction product of the enzyme in the cell nucleus of the pancreatic islet of Langerhans and acinar cells. Cu,Zn-SOD antioxidant content in the islet of Langerhans and acinar cells of pancreatic diabetic rats (DMC) showed a significant decrease compared to normal rats (NLC) (Figure 3). In contrast, Cu,Zn-SOD antioxidant content in MET, CAH, NAH3, and NAH6 groups were significantly higher than DMC group, as shown by the percentage of cells in the islet of Langerhans and acinar cells with a negative reaction of these groups lower than the DMC group.

MET, CAH, NAH3, and NAH6 groups exhibited the same ability to maintain the antioxidant content of Cu,Zn-SOD in the pancreatic islet of Langerhans. On the other hand, the MET group did not show Cu,Zn-SOD content as good as CAH, NAH3, and NAH6 groups in the pancreas of acinar cells. Based on these results, the administration of crude extract and nano-extract of *A. hispida* increased the antioxidant content of Cu,Zn-SOD in the pancreatic islet of Langerhans and acinar cells of diabetic rats.

4. Discussion

The study exhibited that streptozotocin-induced diabetic rats showed a significant decrease in Cu,Zn-SOD content (Figure 3). This condition indicates oxidative stress in the pancreas of diabetic rats. It is caused by STZ that selectively enters the pancreatic β cells via GLUT2 and impairs pancreatic β cells through triggering DNA fragmentation and acting as nitrite oxide (NO) donor.[15] STZ destructs β cells population and induces insulin insufficiency and chronic hyperglycemia.[16] In the same way, hyperglycemia also contributes

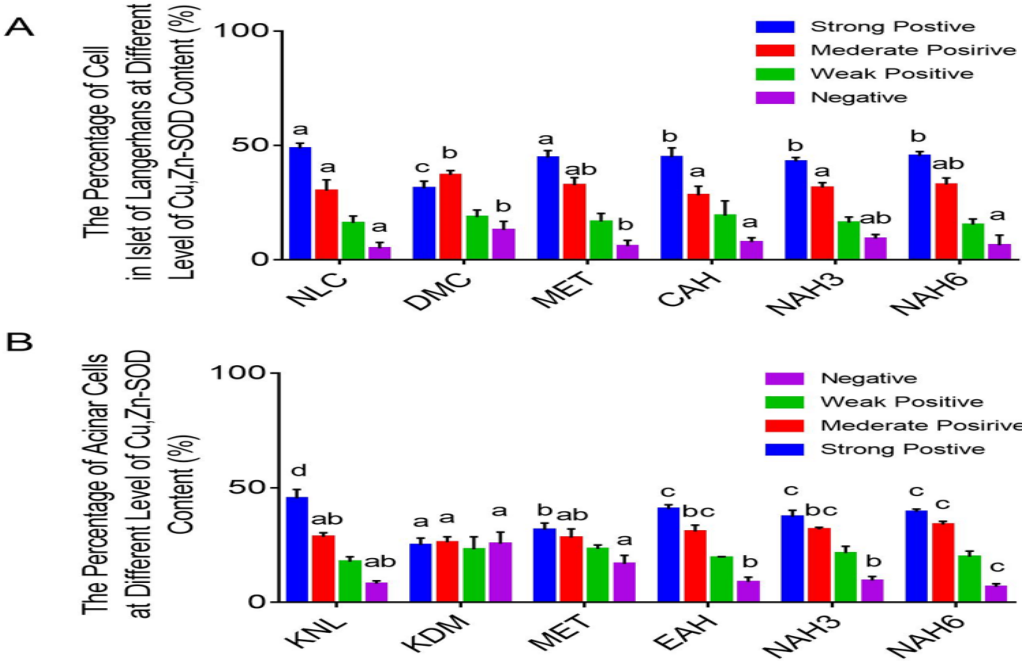

Figure 3 The percentage number of the antioxidant content of Cu,Zn-SOD in the pancreatic islet of Langerhans (A) and acinar cells (B). Nano-extract of A. hispida (NAH6) increased antioxidant content of Cu,Zn-SOD in pancreatic and acinar cells of diabetic rats (DMC). NLC, normal control; DMC, diabetes mellitus (DM) control; MET, DM+metformin 88 mg/kg BW; CAH, DM+crude extract 300 mg/kg BW; NAH3, DM+nano-extract at 30 mg/kg BW; NAH6, DM+nano-extract at 60 mg/kg BW. Data were expressed as mean±SD. The same letters (a-d) are not significantly different from Duncan's test results ($\alpha = 0.05$).

to β-cell damage through excess reactive oxygen species (ROS) production, resulting in oxidative stress.[17] This finding confirmed previous research that STZ- and alloxan-induced diabetic rats exhibited a reduced Cu,Zn-SOD content in the pancreas.[12,18] This was the first report that metformin-treated diabetic rats showed an increase in Cu,Zn-SOD content in the pancreas (Figure 3). The previous result exhibited that diabetic rats treated with metformin increased total SOD, GPx, and catalase.[19,20]

The study demonstrated that crude extract and nano-extract of *A. hispida* leaves increased the content of Cu,Zn-SOD antioxidant in the pancreatic islet of Langerhans and acinar cells of diabetic rats (Figure 3). Cu,Zn-SOD is one of three isoforms of SOD in mammals, which is present in the cell nucleus, cytoplasm, and space between the mitochondrial membrane.[21] In addition, Cu,Zn-SOD plays a crucial role as a primary enzymatic antioxidant, and its distribution in the various aerobic organism have significant implications: first, superoxide radicals are continuously produced because it related to molecular metabolism in mitochondria and cellular membrane; and second, the high levels of reactive oxygen species (ROS), hydrogen peroxide (H_2O_2), and hydroxyl radical (•OH) cause oxidative stress and cell toxicity.[9,22] This enzyme catalyzes the conversion of the toxic superoxide anion (O_2•-) to the less toxic substance (H_2O_2). H_2O_2 then converts into water molecule (H_2O) and oxygen (O_2) by GPx and Cat. However, Cu,Zn-SOD expression in pancreatic β cells was 1.4 times lower than in non-β cells. Likewise, The expression of GPx and Cat in pancreatic cells was 15-fold and 3-fold lower, respectively.[23] In a previous study, similar research demonstrated that hydrolyzed protein of sea cucumber (HDL) and Swietenia mahagoni enhanced the Cu,Zn-SOD antioxidant content of pancreatic diabetic rats.[12,24] In addition to the pancreas, Cu,Zn-SOD expression also increased in the liver and kidney of diabetic rats after nano-extract of A. hispida and Rosmarinus officinalis essential oils administration.[25,26] On the contrary, the grape seed procyanidin extract did not change the Cu, Zn-SOD expression profile in the liver of STZ-induced

diabetic rats.[27]

Interestingly, the nano-extract of *A. hispida* leaves extract was lower in 10-fold and 5-fold doses (30 mg/kg BW and 60 mg/kg BW, respectively) than the crude extract dose (300 mg/kg BW); however, the nano-extract showed the same ability in increasing Cu,Zn-SOD antioxidant content (Figure 3). It was related to smaller particle size, leading to enter easily into the cells; although, gallic acid and catechin content of nano-extract reduced by 5% and 10% than crude extract, respectively.[13] Previous research found that nano-extract of *A. hispida* in the size of 512 nm might enter the cells through phagocytosis and pinocytosis.[28] It may be related to the characteristic of nanoparticle, which has strong diffusion power due to a large high area against its volume ratio.[29]

The results show that the therapeutic effect of *A. hispida* leaves extract may improve β cells function by increasing Cu,Zn-SOD expression (antioxidant pathway). Our previous research found that nano-extract of *A. hispida* leaf contained gallic acid and catechin.[13] Gallic acid simulates the Nrf2/Keap1 signalling pathway to translocate Nrf2 from the cytoplasm to the nucleus, which then binds to an antioxidant response element (ARE) in the nucleus, leading to the expression of Cu,Zn-SOD.[30,31] Pharmacological intervention by increasing antioxidant capability such as Cu,Zn-SOD plays a significant role in protecting pancreatic β cells in diabetic conditions, leading to help in antidiabetic therapy.[12,14,17] Another therapeutic way to reduce oxidative stress in pancreatic β cells is the protection of β cells through decreasing mitogen-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) inhibition.[17] Furthermore, the nano-extract of *A. hispida* has the potential to be tested in clinical study and developed into complementary antidiabetic drugs or antidiabetic drugs.

5. Conclusion

The nano-extract of *A. hispida* leaves increased Cu,Zn-SOD antioxidant content in the pancreas of diabetic rats. Nano-extract of *A. hispida* showed a better effect in increasing Cu,Zn-SOD antioxidant content than crude extract despite the doses being lower, 10-fold and 5-fold.

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