

## Phytochemical Content and Protective Effect of *Kleinhovia hospital* Leaves Extract on Pancreatic Cytotoxicity in Hyperglycemic Rats

(KANDUNGAN FITOKIMIA DAN EFEK PROTEKTIF  
EKSTRAK DAUN PALIASA (*KLEINHOVIA HOSPITA* Linn)  
PADA SITOTOKSISITAS PANKREAS TIKUS HIPERGLIKEMIA)

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### ABSTRACT

Diabetes mellitus is a wide problem nowadays. Many traditional plants are used to overcome the complication of this disease. One of them is paliasa (*Kleinhovia hospital* Linn) leaves. This study aims to investigate phytochemical contents and the protective effects of paliasa leaves extract on pancreatic cytotoxicity based on microscopic lesion such as hemorrhagic score and necrotic appearances, in alloxan-induced hyperglycemic rats. Male Wistar rats (three months old) were divided into four groups consisted of seven rats each. Group I was diabetic control, Group II was diabetic groups, paliasa extract (300mg/kgBW) were given by sonde for a period of 14 days prior to alloxan injection (150 mg/kg intraperitoneal). Group III was diabetic rats given 600 mg/kg BW paliasa extract and group IV was diabetic rats given 900 mg for 14 days. At the end of the study, rats were sacrificed. Tissue sample of pancreas was then processed for slide preparation and was stain with hematoxylin eosin. Pancreas hemorrhagic score was divided into four criteria, focal (score 1), multifocal (score 2), extensive (score 3), and diffuse (most severe, score 4). Microscopic examination was done using binocular microscope, at Laboratory of Veterinary Pathology, Disease Investigation Centre Denpasar, Bali. Data were then analyzed by using analysis of variances. Study showed that paliasa extract could lower hemorrhagic score on pancreas of diabetic rats, even though it was not significant compared to control group (p 0,205). Phytochemical analysis showed that paliasa leaves extract contained alkaloid, terpenoid, and flavonoid. Necrotic appearances were varied from pycnosis, karyorheksis, karyolysis, and vacuolization. In conclusion, paliasa leave extract may have protective effect on pancreas cytotoxicity.

Key words: alloxan; paliasa extract; diabetes; phytochemical; rats

### ABSTRAK

Diabetes mellitus merupakan masalah yang sering dijumpai di dunia. Banyak tanaman tradisional digunakan untuk mengatasi komplikasi penyakit ini. Salah satu di antaranya adalah daun paliasa (*Kleinhovia hospital*). Penelitian ini bertujuan untuk mengetahui kandungan fitokimia dan efek proteksi ekstrak daun paliasa terhadap sitotoksitas pancreas (tingkat perdarahan dan gambaran nekrosis) pada tikus Wistar hiperglikemia yang diinduksi aloksan. Penelitian menggunakan rancangan *Control Group Design*, menggunakan 28 ekor tikus berumur tiga bulan. Tikus-tikus percobaan dibagi secara acak menjadi empat kelompok, yaitu kelompok kontrol, paliasa dosis 300mg/kg berat badan (BB), 600 mg/kg BB, dan 900 mg/kg BB. Setelah diberikan perlakuan selama 14 hari, tikus dikorbankan nyawanya dan dilakukan pemeriksaan pankreas dengan pengecatan hematoxylin eosin. Tingkat perdarahan pankreas dibagi menjadi empat skor lokal (skor 1), multifokal (skor 2), ekstensif (skor 3), dan difus (paling berat, diberi skor 4). Pemeriksaan mikroskopis memakai mikroskop cahaya binokuler. Pemeriksaan dilakukan di Laboratorium Patologi Veteriner, Balai Besar Veteriner (BB Vet) Denpasar, di Pegok, Denpasar, Bali. Analisis data dilakukan dengan sidik ragam. Penelitian menunjukkan bahwa ekstrak paliasa dapat menurunkan tingkat perdarahan pancreas tikus hiperglikemik, namun tidak signifikan (p 0,205). Uji fitokimia dilakukan di Jurusan Farmasi Fakultas MIPA Universitas Udayana. Hasil penelitian menunjukkan uji fitokimia ekstrak tanaman paliasa mengandung alkaloid, terpenoid, dan flavonoid. Gambaran nekrosis bervariasi dari piknosis, karioreksis, kariolisis, dan vakuolisasi. Ekstrak tanaman paliasa memiliki efek protektif pada sitotoksitas pancreas.

Kata-kata kunci: aloksan; ekstrak paliasa; diabetes; fitokimia; tikus

## INTRODUCTION

Diabetes mellitus is a worldwide disease and is depicted in blood glucose increasing above normal. World Health Organization predicted that more than 220 million people in the world will suffer from diabetes and this number will be doubled in 2030 (Hamid and Moustafa, 2013). In Indonesia, it is estimated that 50% of diabetic patients had not been diagnosed (Muhtadi *et al.*, 2015).

Oral anti-diabetic and insulin sensitizer can repair insulin insensitivity, increase insulin production, and lower blood glucose. However, those medicines have some side effects, such as hypoglycemia at higher dosage, low oral bioavailability, and low permeability when entering intestine epithelium. This will urge increasing demand of anti diabetic herbal products with relatively fewer side effects (Malekpour *et al.*, 2012). One of traditional herbal plants that used to treat diabetes is paliasa. Paliasa (*Kleinhovia hospital* Linn) is found at South Sulawesi. Paliasa leaves can be used as vegetables, supportive medicine for liver disease, and combat hair flea (Dini, 2009).

Diabetes mellitus induced by alloxan is type-1 diabetes mellitus, insulin dependent diabetes mellitus (Viana *et al.*, 2004). It happened due to autoimun disorder and beta cell pancreas damage in Langerhans islets. First, beta cell pancreas decrease in number and volume, then insulin deficiency will happen permanently (Waer and Helmy, 2012). Diabetes mellitus treatment without any side effects is a big challenge for future. WHO declared that anti-diabetic herbal medicine need special concerns (Patil *et al.*, 2011).

Hiperglycemia stimulates *reactive oxygen species* from many ways such as oxidative fosforilation, glucose autooxidation, lipooxygenase, and nitrit oxide sintase. Natural anti oxidant in our body is not sufficient to neutralize free radicals that can cause tissue damage. Thus, herbal anti oxidant is needed as an alternative for the treatment (Hamzah *et al.*, 2012).

In diabetic rats, pancreas cells showed granular degeneration, pycnosis, karyorrhesis, and karyolysis (Liu *et al.*, 2014). In histopathology of alloxan induced diabetic rats, Mir *et al.* (2013) found changes in exocrine and pancreatic beta cells. On the islets of Langerhans cell degranulation occurs, accompanied by the formation of ghost islets with loss of whole cells.

Bleeding is also found in all parts of the gland. This study aims were to investigate phytochemical contents and the protective effects of paliasa leaves extract on pancreatic cytotoxicity (hemorrhagic score and necrotic appearances) in alloxan-induced hyperglycemic rats.

## RESEARCH METHODS

### Plant Material Collection and Preparation

Paliasa leaves were harvested from Makassar, a city of South Sulawesi Province. The process of extraction was done as following: 1 kg of dry paliasa leaves, were poured with 15 litres methanol in room temperature (26°C) for 14 hours, then they were filtered with Whatman paper. Filtrate was dried by vacuum through rotary evaporation (Arung *et al.*, 2009).

### Animals

Three month-old male Wistar rats (180-200 g body weight) bred in Pharmacology Department, Faculty of Medicine, University of Udayana, animal house were used. The rats were housed in cages with room temperature (26°C). This experimental protocol was approved by Ethics Committee of Animal Usage for Research and Education from Faculty of Veterinary Medicine, Udayana University (Letter Number: 191/KE-PH-Lit-2/V/2015 on 12 May 2015). Alloxan was purchased from Pharmacological Laboratory of Widya Mandala University, Surabaya, Indonesia.

### Experimental Design

A total of 28 rats were randomly divided into four groups of seven each and induced with alloxan 120 mg/kg BW intraperitoneal in saline. All rats were found to be diabetic after 72 h. Rats with a blood glucose level above 150 mg/dL were considered to be diabetic (Agunbiade *et al.*, 2015). Group I served as the diabetic control; Group II were given paliasa leaves extract by using sonde orally with dose 300 mg/kg BW; Group III were given paliasa leaves extract by using sonde orally with dose 600 mg/kg BW; Group IV were given paliasa leaves extract by using sonde orally with dose 900 mg/kg BW. The curative effect of paliasa extract was then evaluated for a period of 14 days after alloxan administration and animals were sacrificed by using chloroform. Pancreases were collected immediately for histopathological examination.

### Histopathological Studies

Pancreatic tissues from all groups were subjected to histopathological studies and collected in jar contained with approximately 10% buffered formalin solution. Sections of approximately 5  $\mu$ m thickness of the pancreas were made and stained with hematoxylin and eosin (HE tissue staining) and they were examined for pancreatic hemorrhagic score. Stained sections were qualitatively evaluated using an electric binocular microscope (Olympus Japan), with magnification 400 times.

Pancreas hemorrhagic score was divided into four groups, i.e. local (score 1), multifocal (score 2), extensive (score 3), and diffuse (score 4).

### Phytochemical Analysis

Phytochemical analysis was done at Pharmacy Department, of the Faculty of Mathematics and Natural Science, Udayana University, in order to investigate the main content of paliasa extract that has effect in protecting pancreas.

### Atsiri Oil

Extract was added with ethanol. Paliasa leaves extract has no aromatic smell although it was evaporated until dry.

**Alkaloid.** Two mililiters extract was steamed until it get residu. Residu was added with 5 mL HCl 2N, then it was divided to five reaction tube. First tube was added with weak acid that acted as blanko. Second tube was added with three drops Dragendorff, third tube was added with three drops Mayer reaction, fourth tube was added wagner reaction. The paliasa extract showed orange sediment in second tube and white yellowish sediment in third tube. It showed there was alkaloid.

**Sterol and terpenoid.** One mililiter extract was steamed, and then it was added by 0.5 mL chloroform, and added by 0.5 mL anhidrat acetic acid. Then it was dropped by 12 mL sulphate acid. The extract showed brownish or violet ring in the boundary, it revealed there was terpenoid.

**Saponin.** Three mililiters paliasa extract was shaken vertically for 10 seconds, then it waited for 10 seconds. The extract had no stable foam, it meant there was no saponin.

**Polyphenol.** One mililiter extract was added with iron/ferri (III) chloride 10%, if it turned to dark blue, blue black or black green, then it meant there was polyphenol there. The extract showed negative for polyphenol.

**Glycoside.** It is done by Liebermann-Burchard reaction. One mililiter extract was evaporated; the rest was soluted in 5 mL anhidrate acetat acid, and then added with 10 drop sulfate acid. The extract has black brown colour, it meant negative for glycoside.

**Flavonoid.** One mililiter extract solution was evaporated, the rest was wetted by aseton, enhanced by a little of smooth borac acid powder and oxalic acid, then it was heated carefully. After that, the rest was mixed with 10 mL eter. By using UV<sub>366</sub>; the extract showed intensive yellowish fluorescence, and it meant there was flavonoid. Phytochemical test showed that paliasa contains alkaloid, terpenoid, and flavonoid

### Statistical Analysis.

All the grouped data were statistically evaluated with one way analysis of variance (Anova),  $P < 0.05$  was considered statistically significant.

## RESULTS AND DISCUSSION

Result of treatment effect was shown on Table 1 (electric microscope, 400x)

The Table 1 showed the mean pancreatic hemorrhagic score between groups after being given treatment did not differ significantly ( $p > 0.05$ ). Necrosis appearances were varies, from pycnosis, karyolysis, and karyorhexis as shown in Figure 1.

Alloxan is beta cytotoxin. Alloxan chemically induces diabetes by destroying beta cells that produce insulin. A decrease in the release of insulin lowers insulin usage to neutralize blood glucose in the body. Increased oxygen free radicals occur due to high blood glucose levels.

Table 1. Mean pancreatic hemorrhagic score of diabetic rats after treatment with paliasa extract (n=7).

Treatment Group	Mean pancreatic hemorrhagic score $\pm$ SD
Control	3,14 $\pm$ 0,690
Paliasa 300	2,71 $\pm$ 0,756
Paliasa 600	2,57 $\pm$ 0,535
Paliasa 900	2,43 $\pm$ 0,545

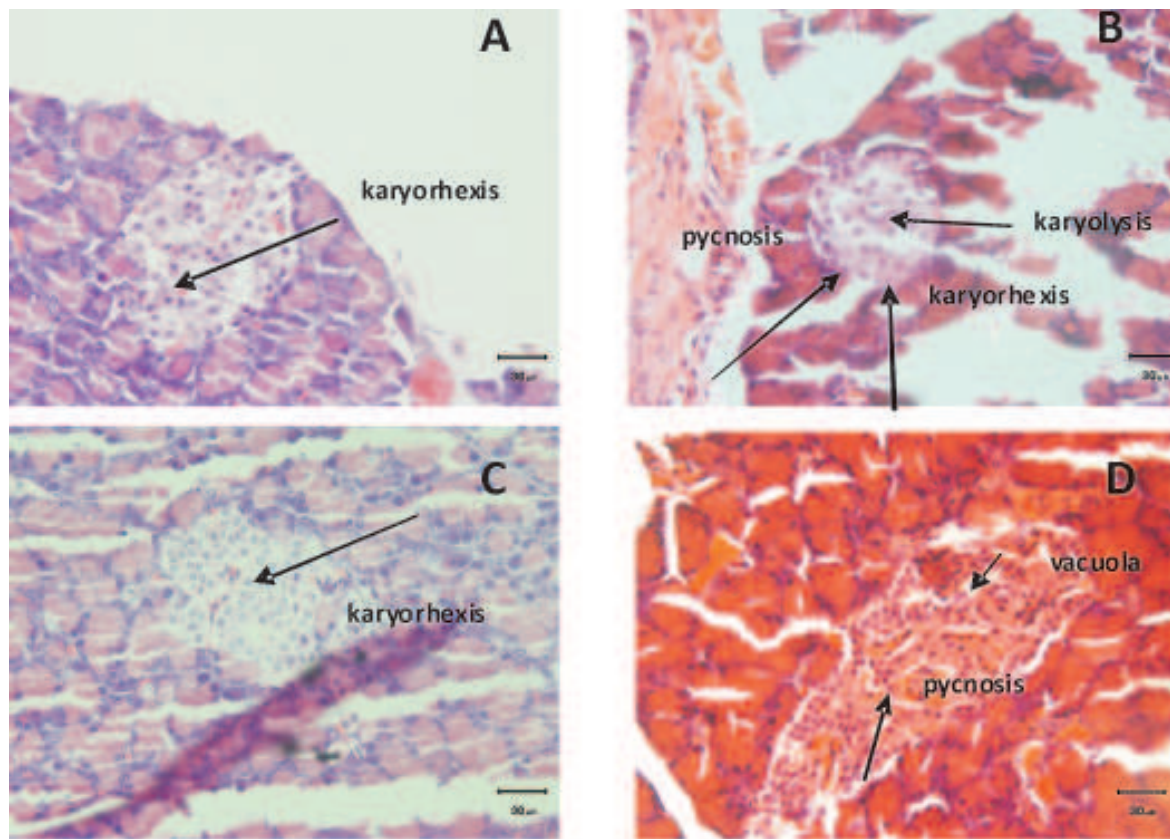


Figure 1. Necrosis appearance in pancreas of four study group (HE, 400x). Figure A is pancreas of hyperglycemic (arrow showed karyorhexis). Figure B is pancreas of hyperglycemic rats with 300 mg/kg BW paliasa extract (there is pycnosis, karyorhexis, and karyolysis). Figure C is pancreas of hyperglycemic rats with 600 mg/kg BW paliasa extract (there is karyorhexis). Figure D is pancreas of hyperglycemic rats with 900 mg/kg BW paliasa extract (there is pycnosis and vacuola).

Auto oxidation produces free radicals and this is exacerbated by the effects of diabetogenic alloxan (Hamzah *et al.*, 2012).

Alloxan and its reduction product, dialuric acid, cause a redox cycle through the formation of superoxide radicals. Superoxide radicals experience dismutase releases hydrogen peroxide. Highly reactive hydroxyl radicals are formed by Fenton reaction (Rohilla and Adi, 2012).

The effects of reactive oxygen species (ROS) along with an increase in cytosolic calcium concentration would lead to massive destruction of beta cells rapidly (Hamid and Moustafa, 2013). The severity of tissue damage and the degree of oxidative stress may depend on an imbalance between excessive ROS production and antioxidant defenses in the cells of the pancreas (Kikumoto *et al.*, 2010). Reactive oxygen species are involved in various signaling pathways of angiotensin II. Increased activity of the renin-

angiotensin system locally on the pancreas will increase pancreatic damage induced by ROS (Leung, 2007).

Beta cells of diabetic rats showed vacuolization and a decrease in secretory granules, fusion of some granules and pycnotic nuclei (Hamid and Moustafa, 2013). Giving alloxan causes morphological changes in diabetic rats in the form of severe damage to the beta cells of the pancreas, which is characterized by a decrease in the number of cells, cell damage, and even cell death (Dahecha *et al.*, 2011).

Necrosis is usually found in the middle of Langerhans islet because this part is initially influenced by alloxan (Singh and Gupta, 2007). Karyolysis, the loss of the cell nucleus, as well as the presence of remnants (residue) cells that have vacuole shaped found in research Jelodar *et al.* (2005). At the core of beta cells that undergo karyolysis, also showed debris in the form of the



fragment mass surrounding the cell nucleus (Boudreau *et al.*, 2006).

Pancreas is more susceptible to oxidative stress compared to other organs as the cells in the pancreas have lower anti-oxidant enzymes (Robertson, 2006). Oxidative stress that induced by alloxan will cause breakdown of DNA and activate polysintetase resulting in impaired synthesis of insulin in the pancreas (Takemoto *et al.*, 2014). Pancreas functions as a glucose sensor and insulin-requiring oxygen-rich environment as well as glucose, so that it can generate sufficient signal for insulin secretion and providing adequate insulin to the target tissues. These special circumstances make pancreatic beta cells are highly susceptible to oxidative stress (Lenzen, 2008). Hyperglycemic conditions lead to an increase in the concentration of  $H_2O_2$  and induce catalase activity as a defense mechanism against free radicals. However catalase levels may not be sufficient to prevent the onset of diabetes (Kikumoto *et al.*, 2010).

Vacuolization is one indication of structural disruption of membrane permeability, cause increase in fluid and electrolyte transport into the cell. Permeability disorders occur due to reactive oxygen species (Hamid and Moustafa, 2013). Variations of histopathological pancreas may occur because of differences in the status of anti-oxidants, as well as the size and maturity of cells (Mir *et al.*, 2013).

Anti-oxidants contained in the plants may act to neutralize free radicals. Free radicals are associated with the development of degenerative disorders such as diabetes mellitus and cardiovascular related diseases (Renjith *et al.*, 2013). A phytochemical test result of paliasa plant contains alkaloids, terpenoids and flavonoids. Alkaloids may act to stimulate the multiplication of existing cells in Langerhans islets and differentiation into new cells. Alkaloids maybe also important for the recovery of partial beta cell (Singh and Gupta, 2007). Alkaloid has anti-hyperglycemic, anti-inflammatory, and anti-oxidants effects (Adeneye and Crook, 2015). Alkaloids lower insulin-mediated glucose disposal and reduce activities of two enzymes that are essential for the production of glucose, namely glycogen phosphorylase and glucose-6-phosphatase (Ezuruike and Prieto, 2014).

Terpenoids and flavonoids have anti-diabetic properties. Flavonoids are able to regenerate the damaged beta cells in alloxan-induced diabetic rats and act as insulin secretagogous

(Balamurugan *et al.*, 2014). Liu *et al.* (2007) found that flavonoids can enhance insulin sensitivity. Terpenoids and flavonoids may be able to improve the integrity of the B-endocrinocyte through increasing the release of insulin or insulin activity improvement (Snigur *et al.*, 2008).

Flavonoids can modulate the activity of glycolytic and gluconeogenic enzymes (Renjith and Rajamohan, 2012). Flavonoids inhibit the glucose transporter epithelial cells in the small intestine and regenerate the damaged beta cells in the alloxan induced of pancreatic beta cells (Sharma *et al.*, 2013). The formation of advanced glycated end products (AGEs) can be prevented, and there is no atherosclerosis, nephropathy, and retinopathy (Rahimi *et al.*, 2005).

An efficacy hypoglycemic herb are obtained through a process of increasing insulin secretion, inhibit glucose absorption from the intestine, inhibits glucose production from hepatocytes, and increase glucose uptake by muscle and fat (Hui *et al.*, 2009). Giving phytonutrients may be an effective strategy to overcome the complications of diabetes by regulating the enzymes that responsible for metabolizing glucose (Renjith and Rajamohan, 2012). The active substance in traditional crops can activate the regeneration of pancreatic beta cells (Jelodar *et al.*, 2005). Pancreatic function recovery occurs through the facilitation of metabolites in insulin-dependent process (Balamurugan, 2014).

## CONCLUSION

The conclusion obtained from the study is: paliasa plant extracts contain alkaloids, terpenoids and flavonoids, and paliasa plant extract given to hyperglycemic rats may not significantly reduce the level of hemorrhagic pancreas.

## SUGGESTION

Further studies are needed to investigate each phytochemical content of paliasa extract towards pancreas chemical markers.

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