

## The Effect of Brotowali Stem Extract (*Tinospora Crispa*) Towards Increasing Number of Lymphocytes in the Healing Process of Traumatic Ulcer on Diabetic Wistar Rat

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### Abstract

One of the common complication of diabetes delayed wound healing in oral traumatic ulcer. Lymphocyte cell plays an important role in late inflammatory phase during the process of wound healing. Brotowali (*Tinospora crispa*) contains flavonoid and terpenoid which can help to control blood glucose levels and accelerate wound healing. *Tinospora crispa* extract was made with oven-dried method at 50°C. Dry specimens are then made into a powder and macerated with ethanol 80% (1:10 w/v). The experimental animals were divided into 3 groups, control group 1: Normal Wistar Rat with traumatic ulcer, control group 2: Diabetic Wistar rat with traumatic ulcer, and treatment group: Diabetic Wistar rat with traumatic ulcer treated with 250 mg/kg *Tinospora crispa* extract once a day. All samples were euthanized on day 3, 5, and 7 after traumatic ulcer was made. Then histopathology preparation was made to count the number of lymphocytes. Blood glucose levels measurement was conducted on day 3, 5, 7, and 14 after traumatic ulcer was made. Kruskal-Wallis test showed a significant difference of blood glucose levels between the control group 1, control group 2 and treatment group. One Way Anova test showed a significant difference of number of lymphocytes between control group 1, control group 2 and treatment group. Brotowali stem extract (*Tinospora crispa*) is proven to affect blood glucose levels on day 3, 5, 7, and 14. It is also proven that Brotowali stem extract affects the number of lymphocytes in healing of traumatic ulcer among diabetic Wistar rats on day 3, 5, and 7.

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### Introduction

Diabetes is a type of disease where imbalance of blood glucose levels in a patient's body take place.<sup>1</sup> Type 2 diabetes is the most common diabetes among the patients. Type 2 Diabetes is a metabolic disorder characterized by a rise in blood glucose due to decrease in insulin secretion by pancreatic beta cell function and insulin resistance.<sup>2</sup>

International Diabetes Federation (IDF) reported that the prevalence of diabetes in Indonesia is about 4.8% and more than half of

the cases of diabetes (58.8%) had not been diagnosed. IDF also states that approximately 382 million people worldwide suffer from diabetes in 2013 with undiagnosed diabetes category about 46%. It is estimated that prevalence will continue to rise and reach 592 million by 2035.<sup>3</sup>

Oral health complications such as inflammation, traumatic ulcers and pathology of oral mucosa is often associated with diabetes.<sup>4</sup> Oral mucosal lesions such as traumatic ulcers often occur in patients with diabetes. Several studies have shown a prevalence of 80% of diabetic patients had oral mucosal lesions.<sup>5</sup> The prevalence and levels of development of oral mucosal lesions appear to be higher in patients with diabetes. Diabetes can cause vascularization disorder, decreased blood flow, decreased innate immunity, decreased production of growth factors and psychological stress involved in oral mucosal healing among diabetic patients.<sup>6</sup> Prevalence of traumatic ulcer

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in oral mucosal is quite high and it is about 83.6%.<sup>7</sup> The treatment of traumatic ulcer is basically by eliminating traumatic effect.<sup>8</sup>

Brotowali (*Tinospora crispa*) from Menispermaceae family are reported to have biological activities such as anti-inflammatory and immunomodulatory.<sup>9</sup> Brotowali contains various chemical compound such as flavonoids, alkaloids, soft resin, starch, glycosides, picroretoside, harsha, bitter substances picroretine, tinocrisposide, berberine, palmatine, columbine, and caoculine.<sup>10</sup> Flavonoids in the Brotowali (*Tinospora crispa*) stem extract have anti-oxidant properties as well as effective cytotoxic properties.<sup>11</sup>

Lymphocyte plays an important role in wound healing by releasing lymphokines which affect the number of another inflammatory cell. Lymphokines induce the proliferation of fibroblasts with the help of fibroblast activating factor (FAF), which plays a role in wound healing.<sup>12</sup> Lymphokines such as interleukin-2 (IL-2) and transforming growth factor beta (TGF- $\beta$ ) bind to flavonoids which assist the process of lymphocyte proliferation.<sup>13</sup> Based on these thinking, author is encouraged to conduct research on the influence of Brotowali (*Tinospora crispa*) stem extract at a dose of 250 mg/kg<sup>23</sup> towards decreasing of blood glucose levels on day 3, 5, 7, and 14, as well as increasing number of lymphocytes in the healing process of traumatic ulcer on diabetic Wistar rats on day 3, 5, and 7.

### Materials and methods

This type of research is a laboratory experiment. Variable measurement is performed after treatment and sampling is done randomly and control groups are used. Thus, the study design is Randomized Post Test Only Control Group Design. The samples to be used are samples of Wistar rats (*Rattus norvegicus*). Determination of the minimum sample size is done with Lemeshow formula.

Fresh specimens of Brotowali stem are washed and cut into small pieces and then dried in an oven with a temperature of 50°C. Dry specimens are then made into a powder and macerated with 80% ethanol solution (1:10 w / v) for 24 hours. Then the extract was filtered and separated between the pulp and the filtrate. The pulp is macerated with ethanol. The procedure is

repeated until the test results of TLC (Thin Layer Chromatography) and H<sub>2</sub>SO<sub>4</sub> do not show anycolour of pink. The pulp and filtrate are collected and evaporated from the first until the last day with a rotary evaporator to remove any excessive solvent. The extract is then stored at -20°C.<sup>11</sup>

Male Wistar rats weighing 150-200g were kept in the same cage with a room temperature of 25  $\pm$  2°C, food pellets are given and distilled water ad libitum for 7 days before the trial begins.<sup>11</sup> Animals were divided into 3 groups which are control group 1, control group 2, and treatment group. Each group contains 8 experimental animals.

Wistar rats in control group 2 and treatment group are diabetes induced through intraperitoneal injection of alloxan (120 mg / kg) which dissolved in 0.05 M citrate buffer of pH 3. Control group 1 was given only sterile saline solution. 3 days after the injection, blood was taken from lateral vein of rats to determine blood glucose levels. Animals declared diabetes when blood glucose levels  $\geq$ 200 mg/dL after 72 hours' post alloxan induction.<sup>14</sup>

Wistar rats in all treatment group were anesthetized prior to the injection of ketamine (60mg/kg) and xylazine (60mg/kg) intraperitoneal.<sup>15</sup> After anesthesia, each animal is placed on a surgical table in dorsal decubitus position. Then the buccal mucosa is sterilized with a swab moistened with 0.12% chlorhexidine digluconate. After sterilization, the ulceration of buccal mucosa is created by wounding the buccal mucosa by using a scalpel blade no. 15 with a cross-section diameter wound of 8 mm.<sup>16</sup>

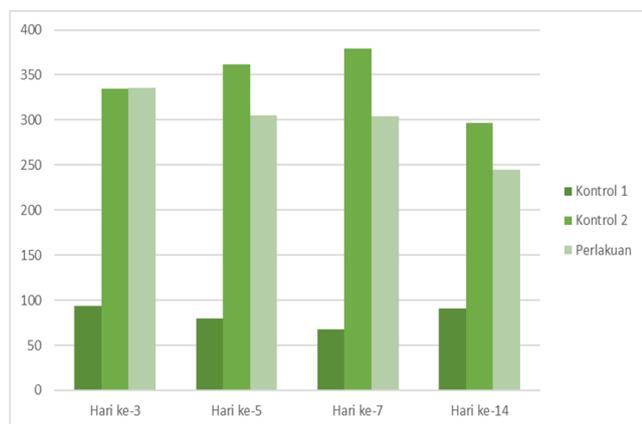
Brotowali stem extract at a dose of 250 mg/kg was administered once per day in treatment group with an oral sonde when traumatic ulcer is formed. Extract of brotowali stem is given on day 3, 5, 7, and 14 after the formation of traumatic ulcer.

Blood glucose levels in experimental animals of all treatment groups are checked with a glucometer. Then euthanasia of experimental animals are proceeded with injection of ketamine (180mg/kg) and xylazine (180 mg/kg). Buccal mucosa tissues from all treatment groups are obtained and it is put into formalin solution prior to the processing of preparations. Wistar rats were then buried after obtaining of buccal mucosa tissues.

The number of lymphocytes are

determined through the observation of histopathology preparations stained with H&E under a microscope with a magnification of 400x. The the number of lymphocytes in male Wistar rats are calculated with a graticulae ocular tool which mounted on the lens of a microscope with per field technique conducted in three-way field.<sup>17</sup>

### Results

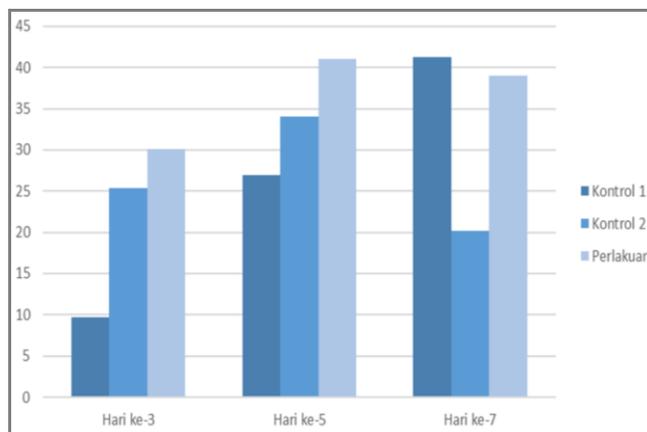


**Figure 1.** Average blood glucose levels on day 3, 5, 7, and 14.

According to the data, the results of blood glucose levels can be concluded that the average blood glucose levels of Wistar rats on day 3 in control group 1 was 94 mg/dL, control group 2 was 334 mg/dL and treatment group was 335 mg/dL. The average blood glucose levels of Wistar rats on day 5 in control group 1 was 79 mg/dL, control group 2 was 361 mg/dL and treatment group, 304 mg / dL. The average blood glucose levels of Wistarrats on day 7 in control group 1 was 67 mg/dL, control group 2 was 378 mg/dL and treatment group was 304 mg/dL. Lastly, the average blood glucose levels of Wistar rats on day 14 in control group 1 was 90 mg/dL, control group 2 was 296 mg/dL and treatment group only consists 244 mg/dL. From these data, it can be concluded that the lowest average blood glucose levels was control group 1 on day 3, 5, 7, and 14.

Based on the graph, the average blood glucose levels on day 3 had significant differences ( $p < 0.05$ ) between blood glucose levels of control group 1, control group 2 and treatment group. On day 5, blood glucose levels of control group 1 and treatment group decreased whereas control group 2 increased. On day 7, blood glucose levelsof control group 1 decreased andcontrol

group 2 increased whereas treatment group remained unchange. On the day 14, blood glucose levels of control group 1 increased whereas control group 2 and treatment group decreased.



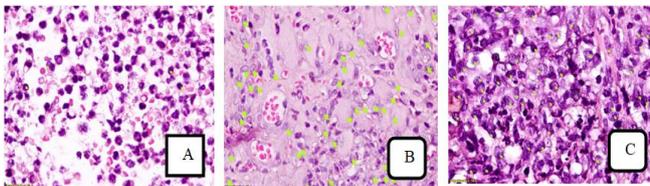
**Figure 2.** Average number of lymphocytes on day 3, 5, and 7.

From the results of data, the highest average number of lymphocytes in day 3 was the treatment group where Wistarrats were given ulcer wound, induced diabetes and Brotowali stem extract has an average number of 30.10. In control group 2 where Wistar rats were given only ulcer wound and induced diabetes without being given Brotowali stem extract has an average number of 25.34. While in control group 1 where Wistar rats were given only ulcer wound without induced diabetes and Brotowali stem extract has an average number of lymphocytes as much as 9.67.

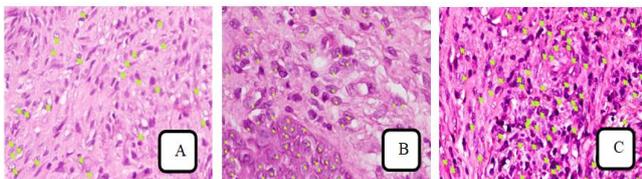
The average number of lymphocytes on day 5 increased in each group and the highest number of lymphocytes is the treatment group. The treatment group where Wistar rats were given ulcer wound, induced diabetes and Brotowali stem extract has an average number of lymphocytes as much as 41.05. In control group 2 where Wistar rats were given only ulcer wound and induced diabetes without being given Brotowali stem extract has an average number of lymphocytes as much as 34.05. While in control group 1 where Wistar rats were given only ulcer wound without induced diabetes and Brotowali stem extract has an average number of lymphocytes as much as 26.92.

The average number of lymphocytes on day 7 increased and decreased simultaneously. Control group 1 and treatment group increased

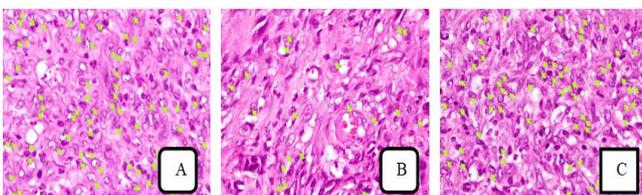
whereas control group 2 decreased in number of lymphocytes. Control group 1 has the highest average number of lymphocytes. Control group 1 was given ulcer wound only has an average number of lymphocytes as much as 41.29. In treatment group was given ulcer wound, induced diabetes and Brotowali stem extract has an average number of lymphocytes as much as 39.00. While in control group 2 where Wistar rats were given only ulcer wound and induced diabetes without Brotowali stem extract has an average number of lymphocytes as much as 20.1.



**Figure 3.** Microscopic appearance of lymphocytes (green arrows) on day 3. (A) Control group 1, (B) Control group 2 and (C) Treatment group on day 3 with HE staining and 400x magnification.



**Figure 4.** Microscopic appearance of lymphocytes (green arrows) on day 5. (A) Control group 1, (B) Control group 2 and (C) Treatment group on day 5 with HE staining and 400x magnification.



**Figure 5.** Microscopic appearance of lymphocytes (green arrows) on day 7. (A) Control group 1, (B) Control group 2 and (C) Treatment group on day 7 with HE staining and 400x magnification.

## Discussion

Based on the data, the results of the average blood glucose levels on day 3 showed no difference between control group 2 and

treatment group because blood glucose levels of diabetes Wistar rats was still high. On day 5, blood glucose levels in treatment group decreased whereas control group 2 increased slightly. On day 7 and 14, blood glucose levels of control group 2 and treatment group decreased. The average blood glucose levels in treatment group always decreased on day 5, 7, and 14, and always lower than the levels of blood glucose of control group 2. This is because control group 2 was not given brotowali stem extract whereas treatment group was given brotowali stem extract. Based on research, it is shown that the bioactivities of Brotowali stem extract (*Tinospora crispa*) can lower blood glucose levels by 10% on the day 14 after the administration of Brotowali stem extract at a dose of 250 mg/kg.<sup>11</sup> The average blood glucose levels in control group 1 remained unchanged. This happened because Wistar rats are experiencing stress and this may lead to changes in blood glucose levels.

Based on the results, some blood glucose levels which were not significant because the duration of administration of Brotowali stem extract was not long enough. Brotowali can lower blood glucose levels because of the content, terpenoids which contain borapetoside A which increases phosphorylation of insulin receptor and protein kinase B as well as increasing the expression of GLUT2 in the liver. This leads to increase glucose utilization in peripheral tissues and reduced gluconeogenesis in the liver and thus it lowered blood glucose levels.<sup>18</sup> According to Sharma<sup>19</sup> studies, significant results required a duration of 60 days. In this study, the time between manufacture of extracts and treatment time is about one month. Thus, this may lead to the instability of Brotowali stem extract.

Administration of Brotowali stem extract method plays an important role in blood glucose levels of *Wistar* rats. Previous research stated that Brotowali stem extract may be administrate through two types of method, namely orally<sup>11</sup> or intraperitoneal.<sup>20</sup> Both methods have advantages and disadvantages. Oral method is easier to administrate than intraperitoneal method but the use of oral sonde might cause residue extract on oral sonde. Intraperitoneal method is more accurate than oral method however its drawback is hard to administrate on animals and it will die if syringe needle enters peritoneal cavity or chest.<sup>21</sup>

Observation of lymphocytes cell count were also done related to healing process of

traumatic ulcer. Healing process of traumatic ulcer may be interrupted on diabetic patient because of the dysfunction in inflammatory response, angiogenesis disorders, and increasing lymphocytes apoptosis.<sup>6</sup> Wound healing process can be observed histologically by counting the number of lymphocytes to determine cell proliferation. Based on research data, the average number of lymphocytes on day 3, 5, and 7 showed treatment group and followed by control group 2 are higher than control group 1. This is because Wistar rats in treatment group were given Brotowali stem extract whereas control group 1 and control group 2 were not given any Brotowali stem extract. Brotowali stem extract contains flavonoids which act as antioxidants and play a role in declining of Reactive Oxygen Species (ROS) where cellular response against bacteria and macromolecular damage that occurs from traumatic ulcer wound.<sup>22</sup> Thus, the average number of lymphocytes in treatment group is higher than control group 1 and control group 2. The average number of lymphocytes began to increase on day 3 because day 3 is the phase of inflammation and lymphocytes will initiate to maintain immunology on wound sites.<sup>23</sup> Lymphocytes play an important role in immune system and the number of lymphocytes will increase in inflammatory phase until it peaks on day 3. On day 7, the number of lymphocytes start to decrease.<sup>24</sup> However, the results showed that the average number of lymphocytes peak on day 5 and began to decline on the day 7. This happened because acute inflammatory process is slow and thus on day 5, lymphocytes which are late inflammatory cells start to infiltrate the wound sites. The average number of lymphocytes on day 7 decreased and it indicates that antigen is no longer present, the inflammatory phase is over and the wound began to enter proliferation phase.<sup>25</sup> Control group 1 on day 7 showed the average number of lymphocytes was higher than control group 2 and treatment group altogether. This is because Wistar rats might be stressed and thus period of wound healing is longer. The results showed that treatment group is the most effective group in increasing number of lymphocytes in the process of wound healing (traumatic ulcer).

Brotowali lowers blood glucose levels because of the content of terpenoids in plants containing borapetoside A. Borapetoside A

increases phosphorylation of insulin receptor and protein kinase B as well as increasing the expression of GLUT2 in the liver. It also leads to increase of glucose utilization in peripheral tissues and reduced gluconeogenesis in the liver so that it can lower blood glucose levels.<sup>26</sup> Other contents in Brotowali stem extract are luteolin and quercetin. Luteolin and quercetin are able to trap superoxide radicals in human body. Luteolin and quercetin also have antioxidant effects that can cause declining in Reactive Oxygen Species (ROS) which led to apoptosis of lymphocytes decrease.<sup>27</sup>

Flavonoids of Brotowali act as an antioxidant and it can stimulate the production of nitric oxide which led to dilation of blood vessels and potentially as a free radical scavenger. Quercetin is a flavonoid compound that is potential as an antioxidant that trap free radicals so that cells which have been damaged by free radicals get a chance to regenerate.<sup>1</sup>

## Conclusions

Based on the results, it can be concluded that Brotowali stem extract (*Tinospora crispa*) at a dose of 250 mg/kg for 14 days can lower blood glucose levels in diabetic Wistar rats. It can also increase the number of lymphocytes in day 3, 5, and 7 in the healing process of traumatic ulcer on oral mucosal of diabetic Wistar rats.

## Declaration of Interest

The authors report no conflict of interest and the article is not funded or supported by any research grant.

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