

Original Research Paper

Antidiabetic and Antioxidant Potential of *Vernonia amygdalina* Leaf Extract in Alloxan-Induced Sprague-Dawley Rats

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Abstract: The treatment of diabetes mellitus using drug medications have side effects such as weight gain, hypoglycemia, the risk of cardiovascular or all-cause mortality. These limitations have become one reason to use complementary therapy from plant extract, such as *Vernonia amygdalina* as an antidiabetes. This research aimed to investigate the effect of *V. amygdalina* leaf extracts as antidiabetic and antioxidant agents by measuring the fasting Blood Glucose Levels (BGLs), IC₅₀ value and the profiles of phytochemical constituents using LC-MS analysis. We used 24 Sprague-Dawley rats (200-300 g) divided into six groups: normal group, positive control group and negative control group. The other three groups were leaf extract groups in different doses: 200, 400 and 600 mg/kg BW dose of leaf extract groups, respectively. The antidiabetic effects were tested based on BGLs were observed for 16 days and examined on day 4, 8, 12 and 16. The rat renal histological changes were observed under an electron microscope using Hematoxylin Eosin (HE) dye. *V. amygdalina* leaf extract showed an effect in reducing BGL on diabetic rats with an effective dose of 200 mg/kg. Renal histopathology result showed necrosis on proximal tubules in all treatment except the normal group. In conclusion, the administration of *V. amygdalina* leaf extract can reduce BGL. However, it did not show any improvement in the histopathological structure of the kidney.

Keywords: Diabetes, Blood Glucose Levels, *Vernonia amygdalina*, Renal Histopathology

Introduction

Diabetes is a chronic disease caused by the pancreas inability to produce enough insulin hormones or ineffective use of insulin hormones in the body. Based on the data from research of basic health (Riskesdas), the proportion of people diagnosed with Diabetes Mellitus (DM) was 6.9% of the total population, which is approximately 12,191,564 people with age >15 years old were diagnosed DM in Indonesia in the year 2013. Data from International Diabetes Federation (IDF) showed that people with DM kept on rising and Indonesia was in the 7th place for the country with the most people with DM from 2013 to 2015 (Indonesia, 2015; Atlas, 2015).

The cost of treatment for DM based on IDF data for the South East Asia region was around 106 billion USD per year and 698-1246 USD per person. The Indonesian

Endocrinology Society (Perkeni) reported that DM main problem in Indonesia is not-routinely taking antidiabetes drugs or insulin injection. According to Perkeni, it is because of the limited availability of hypoglycemic drugs in the public health center. Also, problem-related to DM is the lack of access to the health centers, public health center facilities and infrastructure for people with DM in Indonesia (Soewondo *et al.*, 2013). In handling people with DM, one of the efforts of doctors in Indonesia is to utilize herbal therapy as a complementary therapy (Joeliantina *et al.*, 2016; 2019). Complications to several organs could be found if DM was not well treated. Nephropathy is one of DM complications in the kidney (Chawla *et al.*, 2016; Targher *et al.*, 2018).

DM is categorized as a degenerative disease. The main problem in degenerative disease is damaged

healthy cells in the body caused by free radicals (Ahmad *et al.*, 2017). These damaged cells make them not able to work appropriately as their function and structure. These free radicals originated from the Reactive Oxygen Species (ROS) have unpaired electron, thus making them unstable (Asmat *et al.*, 2016). Thus, antioxidant agents tend to be ROS scavengers that are needed to prevent cell damage due to oxidative stress inside our body. Some natural bioactive compounds from plants are believed to have antioxidant activities (Altemimi *et al.*, 2017).

Vernonia amygdalina is popular as an African plant in Indonesia. Its leaf is rich in antioxidant substances (Iwo *et al.*, 2017). It contains many nutrients and chemical compounds, including protein, fiber, carbohydrate fatty acids, carotenoid acid, calcium, flavonoids, alkaloids, saponins, terpenoids, tannins, glycosides, anthraquinones and luteolin (Ojimelukwe and Amaechi, 2019). This plant has been used empirically by some people for various diseases, including anticancer drugs, preventing heart disease, lowering cholesterol, preventing stroke, lowering blood sugar, antibacterial digestive disorders and as an antioxidant (Sh *et al.*, 2018; Agbogidi and Akpomorine, 2013). However, the effect of *V. amygdalina* in the ethanolic extract as an antioxidant is still unclear.

This study aimed to determine the potential of *V. amygdalina* as antidiabetic and antioxidant agents by measuring BGLs, IC₅₀ value and the profiles of phytochemical constituents of *V. amygdalina* using LC-MS analysis.

Materials and Methods

Materials

Vernonia amygdalina leaves were obtained from the Research Center for biotechnology, The Indonesian Institute of Sciences in Cibinong.

Sample Preparation

In about 500 g of clean *V. amygdalina* leaves were dried. The leaves were diluted in 70% of 1 L ethanol. The leaves were soaked for three days and filtered. The filtrate was evaporated at 55°C using a rotary evaporator. The final weight was 102.5 grams, with a yield of 20.5%.

Ethanolic Extraction of *V. amygdalin* Leaves

The dried plant sample was macerated with 96% ethanol for 24 h in a closed vessel, then filtered. This extraction was once repeated in 500 mL. The ethanolic extract was evaporated with a rotary evaporator and obtained as a concentrated extract.

Antioxidant Activity Analysis Using DPPH Method

In the amount of 4 mg sample was diluted in 4 mL methanol resulting the final extract concentration of 1000 µg/mL and divided into five concentrations (20, 40, 60, 80 and 100 µg/mL) and were added with 0.5 mL DPPH solution (1 mL in methanol) and vortexed them. The mixed solutions were incubated 30 min. at room temperature. The absorbance was measured at 515 nm using spectrophotometer UV-Vis.

The calculation of DPPH scavenging inhibition was determined as follow:

$$\text{DPPH scavenging inhibition (\%)} = \left[1 - \left(\frac{A_s}{A_0} \right) \times 100\% \right]$$

where, A_s was the absorbance of the sample and A_0 was the absorbance of the blank solution. The IC₅₀ values were obtained as a percentage from the linear regression equation.

Bioactive Compound Profiling by LC-MS

Mass spectrometry was performed on an LC-MS Xevo, G2-XS QToF (waters ms technologies). The ionization type was Electrospray Ionization (ESI). The scan range was from 100 to 1200 m/z. The capillary and cone voltage was set at 0.8 and 30 kV, respectively and was used positive electron spray mode. The desolvation gas was set to 1000 L/h at a 500°C. The cone gas was set to 50 L/h and the source temperature was set to 120°C (Tang *et al.*, 2016).

The Ultra-high Performance Liquid Chromatography (UPLC) analysis was performed using a water acquity ultra-performance LC system. Chromatographic separation was carried out on an ACQUITY UPLC HSS T3 column (100×2.1 mm, 1.7 µm) at a column temperature of 40°C. The mobile phase consisted of solvent A (0.1% formic acid in the water, v/v) and solvent B (0.1% formic acid in acetonitrile), with gradient polarity from 95:0.5 (A: B) to 0.5:95 (A: B). The flow rate was set at 0.3 mL/min. The column and autosampler were maintained at 40 and 20°C, respectively. The injection volume was 1 µL. The data acquisition and processing were performed using UNIFI. The parameter used was Retention Time (RT) in the range of 1-15 min.

Preparation of In-Vitro Antidiabetic Experiment

The rat groups were designed as listed in Table 1. The rat models were given pellets and water *ad libitum*. Each group of rats was placed in a cage that was kept at room temperature.

Table 1: Rat group of *V. amygdalina* leaf extract treatments

Rat group	Number of rat/group (n)	Induced by toxic agent (3 g/kg BW)	Treatment	Treatment dose (mg/kg BW)
Normal (NG)	4	-	-	-
Positive Control (PC)	4	A single-dose alloxan	Metformin	200
Negative Control (NC)	4	A single-dose alloxan	Aquadest	-
Sample treatment 1 (VA1)	4	A single-dose alloxan	POS extract	200
Sample treatment 2 (VA2)	4	A single-dose alloxan	POS extract	400
Sample treatment 3 (VA3)	4	A single-dose alloxan	POS extract	600

Data Analysis and Management

Data collected from the normality test by using the Shapiro-Wilk test. If the data was not significant with the normal distribution analyzed by a one-way ANOVA test ($p > 0.05$), it indicated normality. For data with abnormal distribution ($p < 0.05$), it could be analyzed using the Kruskal Wallis test. If significant differences were found, additional analysis was performed using the Post Hoc test ($p < 0.05$) to see which groups had differences. Observations of rat kidney histopathologies were observed qualitatively by looking at the differences of each treatment group.

Results and Discussion

The ethanolic extraction of *V. amygdalin* leaf sample was yielded at 20.015 g (4.003%) from 500 g of dried *V. amygdalin* leaf sample.

In this study, the measurement results of the value of linear regression between concentration and percent inhibition of DPPH were obtained with a value of $y = 0.58x - 0.734$ with $R^2 = 0.996$. Meanwhile, the linear regression value between concentration and percent inhibition of quercetin was $y = 5.471x + 15.382$ with $R^2 = 0.997$. Thus, the correlation between the percent inhibition of DPPH and sample concentration has been analyzed, as shown in Table 2.

A compound expressed as it is active if the IC_{50} value < 50 ppm, strong if the IC_{50} value is between 50-100 ppm, it is moderate if the IC_{50} value is between 101-250 ppm while it is weak if IC_{50} value is 251-500 and inactive above 500 ppm (Jun *et al.*, 2003).

The results of the data percentage decrease in fasting blood glucose levels. After normality was collected and tested, the data was normally distributed (Table 3).

Alloxan Injection and Treatment

After being fasted for 12 h, rats were injected intraperitoneally with alloxan at a dose of 120 mg/kg BW. According to each treatment group, rats were treated for 16 days using a syringe inserted into the mouth of a mouse. Examination of blood glucose levels was done after giving the plant extract treatments (Abel-Salam, 2012; Handayani *et al.*, 2020).

Rat blood was collected intravenously on the tip of the rat tail. Checking blood glucose levels was done in the first 12 h every four days. The instrument used for checking blood glucose levels was an easy touch glucometer with a strip inserted with the rat blood. The percentage of blood glucose levels was calculated with the formula below:

$$PDBGL = \frac{Go - Gt}{Go} \times 100\%$$

Where:

Go = Blood glucose levels before being treated

Gt = Blood glucose levels after being treated

$PDBGL$ = Percentage decrease in blood glucose level

Preparation and Observation of Kidney Histopathology

Kidney histopathology observation was carried out after 16 days of blood glucose level observation were completed. After the rats were decapitated and dissected, the rat kidneys were taken and put into an organ tube containing 10% formalin buffer. Furthermore, the taken kidneys were observed using a microscope with Haemotoxylin and Eosin (H&E) staining to observe histological changes of rat kidneys (Handayani *et al.*, 2020).

Based on the one-way ANOVA test, the value of $p < 0.001$ was obtained and showed that p was smaller than 0.05, which indicated a difference between groups. Next, a homogeneity test was performed and the results showed that the data were not homogeneous with a value of $p = 0.013$ ($p = 0.05$), as shown in Table 3.

It showed the group data has different variants. Furthermore, the Post Hoc test was utilized to compare each plant treatment group at different dosages. It found that the groups of positive control and plant extracts (200, 400 and 600 mg/kg BW) showed significantly different in the normal group ($p < 0.001$).

Table 4 showed that the normal group has a significant difference with the positive dick group and the three dosage groups of *V. amygdalina* leaf extract (200, 400 and 600 mg/kg BW). Between each group, the dosage of *V. amygdalina* leaf extract did not have a significant difference.

Kidney Histopathological Observation

Kidney organs that have been taken from Sprague-Dawley rats will then be observed using a microscope with the help of Hematoxylin and Eosin (H&E) staining.

Histological changes were depicted in Fig. 1 showed that the normal group (Fig. 1A) was no significant difference, which meant that both glomerular and proximal tubules from rat kidneys were not observed any damage to their structure. The negative control group (Fig. 1B), the 200 mg/kg BW dose group (Fig. 1D) and the 600 mg/kg BW dose group (Fig. 1F) showed the presence of

proximal tubular necrosis and infiltration of mononuclear cells (lymphocytes and macrophages). Meanwhile, hemorrhage and necrosis of the proximal tubule in the positive control group (Fig. 1C) and the 400 mg/kg BW dose group were observed.

Bioactive compound profiles in the *Vernonia amygdalina* ethanolic leaf extract were successfully investigated using LC-MS. There were eight bioactive compounds identified, as depicted in Fig. 2. The relative molecular weights of all bioactive compounds were also listed in Table 5.

Table 2: Measurement results of absorbance, percentage of inhibition, IC₅₀ value, regression equation of *Vernonia amygdalina* Del. extract and quercetin using DPPH method

Sample	Concentration (ppm)	% Inhibition	Linear regression equation	IC ₅₀ (ppm)
<i>Vernonia amygdalina</i> Del. Extract	20	11.406	$y = 0.585x - 0.734$ $R^2 = 0.996$	86.709
	40	21.094		
	60	34.625		
	80	46.560		
	100	57.187		
Quercetin	2	25.439	$y = 5.4717x + 15.382$ $R^2 = 0.9977$	6.326
	4	38.281		
	6	48.125		
	8	59.843		
	10	69.375		

Table 3: One-way ANOVA test results in decreasing of fasting Blood Glucose Levels (BGLs)

Groups	% Fasting BGLs change	<i>p</i>
Normal	0.38±3.06	<0.001
Negative control	16.89±22.22	
Positive control	75.99±9.68	
Extract 200 mg/kg BW	77.86±4.33	
Extract 400 mg/kg BW	71.34±4.52	
Extract 600 mg/kg BW	66.96±6.54	

Table 4: The result of Tamhane test in fasting BGL

No.	Group 1	Group 2	<i>p</i>
1	Normal	Negative control	0.98
		Positive control	<0.001*
		Extract 200 mg/kg BW	<0.001*
		Extract 400 mg/kg BW	<0.001*
		Extract 600 mg/kg BW	<0.001*
2	Negative control	Positive control	0.11
		Extract 200 mg/kg BW	0.15
		Extract 400 mg/kg BW	0.19
		Extract 600 mg/kg BW	0.22
3	Positive control	Extract 200 mg/kg BW	1.00
		Extract 400 mg/kg BW	1.00
		Extract 600 mg/kg BW	0.95
4	Extract 200 mg/kg BW	Extract 400 mg/kg BW	0.73
		Extract 600 mg/kg BW	0.43
5	Extract 400 mg/kg BW	Extract 600 mg/kg BW	0.99

* was significantly different ($p < 0.05$)

Table 5: The relative molecular weight of bioactive compound in *Vernonia amygdalina* leaf extracts

Compound name	Observed mass (m/z)	Neutral mass (Da)
4-O-Caffeoylquinic acid	355.1024	354.09508
5-Hydroxy-6,4'-dimethoxy-flavone-7-O-β-D-glucopyranoside	499.1242	476.13186
5-O-Methylvisamminol	291.1232	290.11542
Azedarachin C	609.2715	586.27780
Daturametelin C	469.2946	468.28757
Luteolin-7-O-glucuronide	463.0873	462.07983
Trigonelline	138.0551	137.04768
25(S)-Ruscogenin	453.2994	430.30831

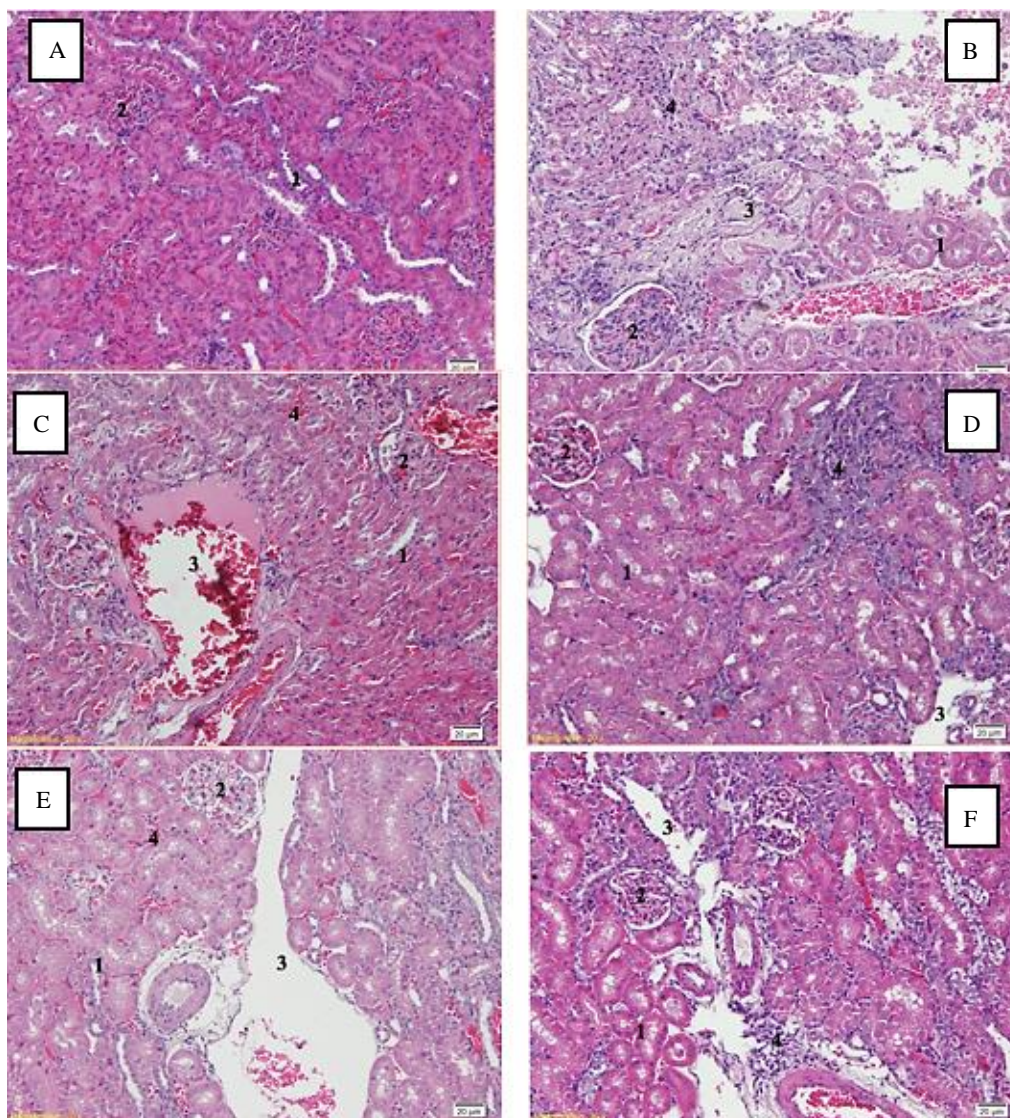
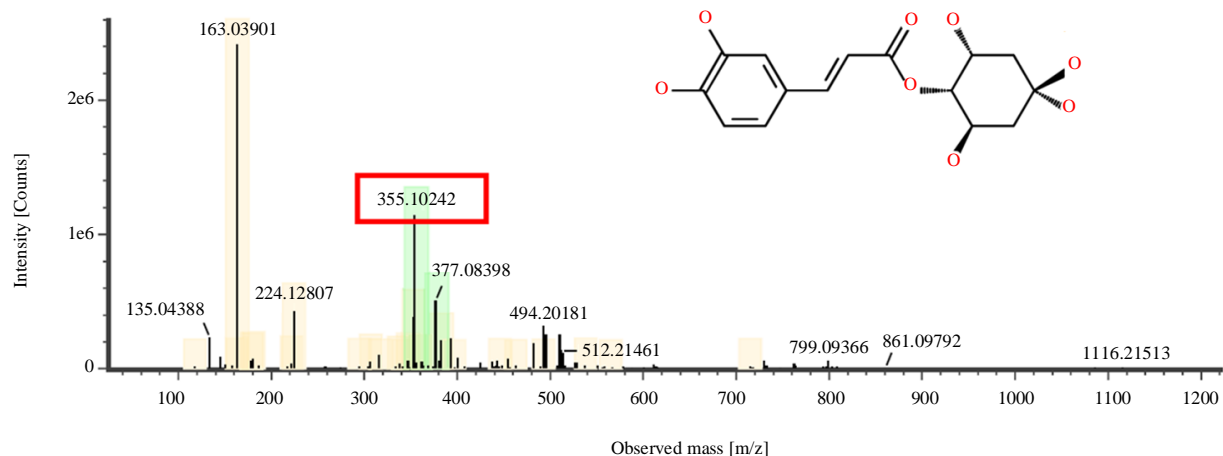
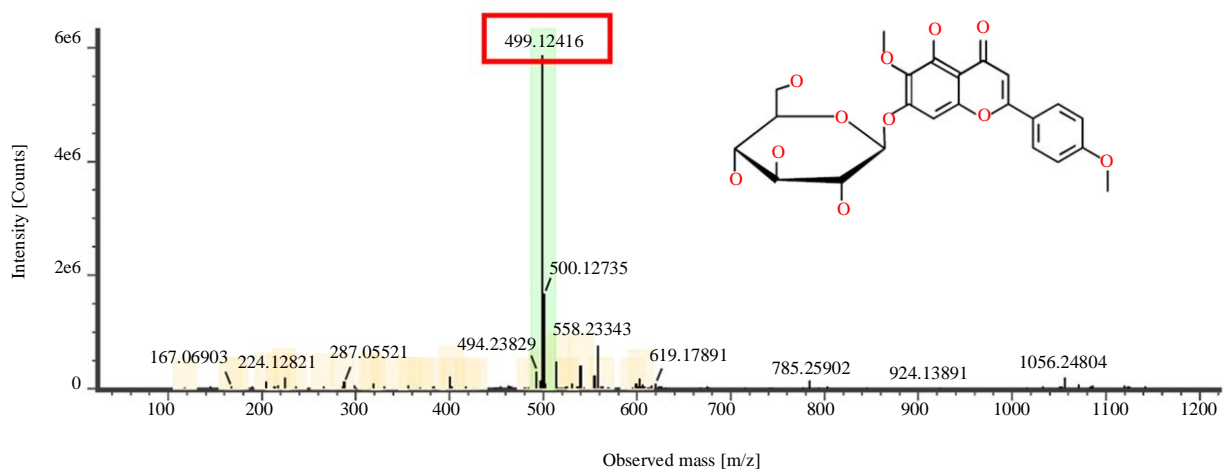


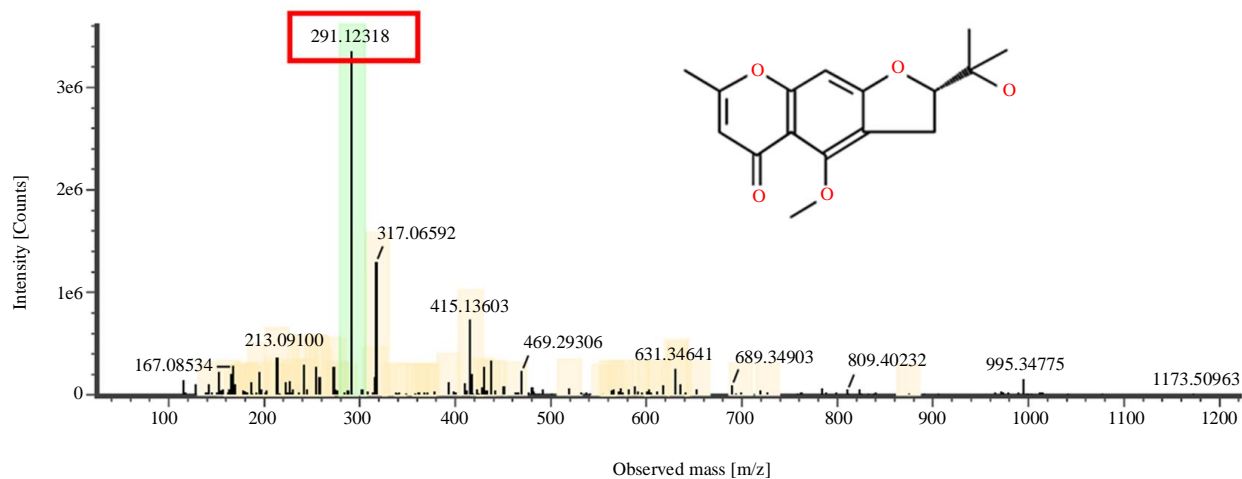
Fig. 1: Histological changes in liver cells using H&E staining: (A) normal group: There were no specific abnormalities; (B) negative control group: There were proximal tubular necrosis, infiltration of mononuclear cells (lymphocytes and macrophages); (C) positive control groups: Hemorrhage and tubular necrosis proximalis; (D) group of 200 mg/kg BW dosage: Proximal tubular necrosis and infiltration of mononuclear cells (lymphocytes and macrophages); (E) group of 400 mg/kg BW dosage: Proximal tubular necrosis and inflammation of mononuclear cells (lymphocytes and macrophages); (F) group of 600 mg/kg BW dosage. proximal tubular necrosis and infiltration of mononuclear cells (lymphocytes and macrophages). 1 = proximal tubule, 2 = glomerulus. 3 = proximal tubular necrosis, 4 = infiltration of mononuclear cells (lymphocytes and macrophages)



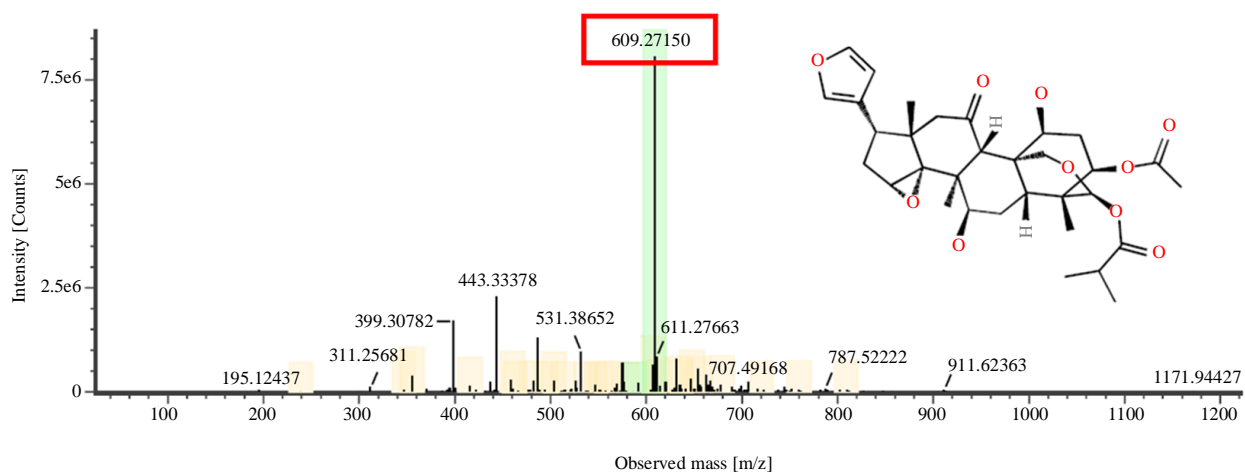
(a)



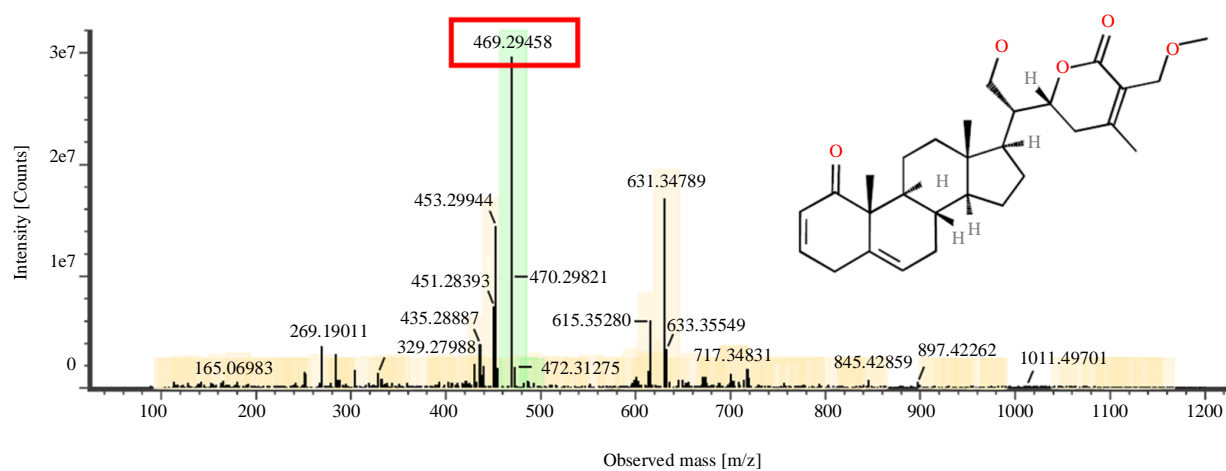
(b)



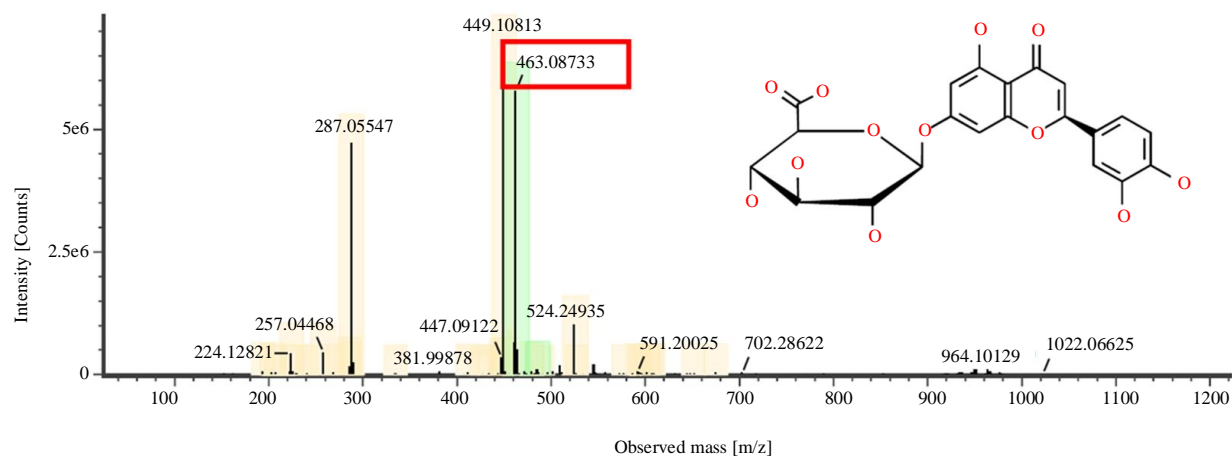
(c)



(d)



(e)



(f)

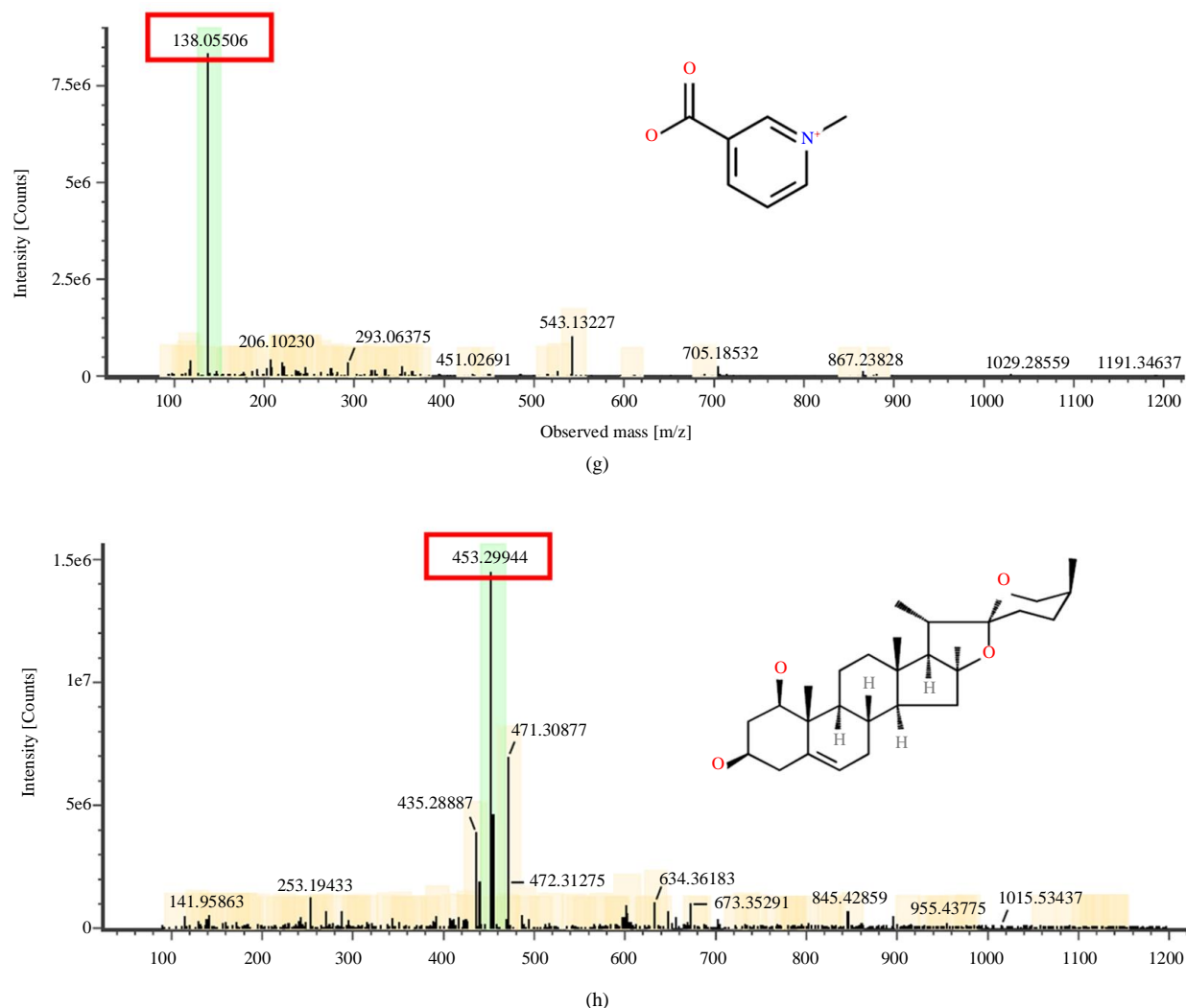


Fig. 2: LC-MS profile of bioactive compounds in *Vernonia amygdalina* ethanolic leaf extract. (a) 4-O-caffeoylquinic acid; (b) 5-hydroxy-6,4'-dimethoxy-flavone-7-O- β -D-glucopyranoside; (c) 5-O-methylvisamminol; (d) azedarachin C; (e) daturametelin C; (f) luteolin-7-O-glucuronide; (g) trigonelline; (h) 25(S)-ruscogenin. The red boxes are the observed mass (m/z) of the bioactive compounds

Effect of V. amygdalina Leaf Extract on Fasting Blood Glucose Levels

In this study, an antidiabetic activity was observed by observing a decrease in fasting blood glucose levels in Sprague-Dawley rats. Based on the results of previous studies, extracts from *V. amygdalina* leaves have several ingredients, such as luteolin, saponins and tannins (Asante *et al.*, 2016). The antidiabetic effect of *V. amygdalina* leaf extract was an increase in GLUT-4 (Glucose Transporter-4) translocation and glycogenesis and played a role in inhibiting gluconeogenic enzymes such as Glucose-6-Phosphatase (G6Pase) (Ong *et al.*, 2011). Luteolin is known to have the ability to inhibit the enzymes α -glucosidase and α -amylase (function to catalyze

the hydrolysis of internal α -1,4-glycosidic linkage contained in polysaccharides), so that it functions to reduce Post-Prandial Hyperglycemia (PPHG) (Asante *et al.*, 2016; Johnson *et al.*, 2014; Ong *et al.*, 2011).

Tannin is a polyphenol compound with a strong binding ability and can precipitate proteins, amino acids and alkaloids. Tannins are classified into two types, namely condensed tannins and hydroxylated tannins (Dai *et al.*, 2020). It can also inhibit the enzyme activities of α -amylase and α -glucosidase (Li *et al.*, 2018). Tannins have benefit in slowing down glucose absorption in the small intestine and producing insulin-like effects in insulin-sensitive tissues. It reduces blood glucose levels and regulates the antioxidant environment in cells (Asante *et al.*, 2016; Konyanga *et al.*, 2011; Laddha and Kulkarni, 2019).

The saponins content is known to reduce blood glucose levels by reducing oxidative stress and has an antidiabetic activity to stimulate insulin secretion and action and pancreatic beta-cell regeneration (El Barky *et al.*, 2016). In addition, saponins can also reduce gluconeogenesis by inhibiting the enzymes Glucose-6-phosphatase (G6Pase) and fructose-1,6-bisphosphate (Laddha and Kulkarni, 2019; El Barky *et al.*, 2016).

The Effective dose of V. amygdalina Leaf Extract

The result of fasting BGL decrease showed the highest effectiveness was 200 mg/kg BW, followed by the groups of positive control, the treatment of 400 and 600 mg/kg BW, respectively. One-way ANOVA showed a *p*-value <0.001. Post hoc tests showed the comparison between the decrease of fasting BGL possessed significant difference (*p*<0.05), between normal group and positive control, treatment with 200, 400 and 600 mg/kg groups.

Kidney Histopathology

The condition of hyperglycemia affects kidney tissue, characterized by an increase in Reactive Oxygen Species (ROS), Reactive Nitrogen Species (RNS), lipid peroxidation and protein carbonylation. This state of oxidative stress can induce NF-KB transcription factors, which control the regulation of the activation of various genes that play a role in inflammation, such as cytokines and chemokines (Patel and Santani, 2009). Inflammation that is continuously triggered can cause bleeding (hemorrhagic) in the tissues as in the positive control group and the group with an extract dose of 400 mg/kg BW (Lenzen, 2008).

In hyperglycemia, ROS and the final glycation product, which increases in diabetes mellitus, is a potent stimulator in chemokines production (Interleukin-8, monocyte-chemoattractant protein 1 and macrophage inflammatory protein-1). The resulting chemokines cause infiltration of macrophages and lymphocytes in the kidneys. This situation triggers an inflammatory response in tissue and cell damage that can develop into fibrosis. It explains the macrophage and lymphocyte infiltration that can be seen in the negative control group histopathological observations, the extract dose group 200 mg/kg BW and the extract dose 600 mg/kg BW (Lim and Tesch, 2012).

A previous study reported that rats with diabetes were observed to have high blood plasma viscosity. This situation causes a decrease in blood flow and perfusion of oxygen to the tissues, resulting in necrosis in various organs, including the kidneys. In the kidney, tubular epithelial tissue undergoes cell degeneration and turns into connective tissue, resulting in a state of necrosis (Andreucci *et al.*, 2014). It explained the results of histopathological observations in the form of necrosis in the proximal tubules found in all groups except the normal group.

The Relationship of V. amygdalina Leaves Extract to Renal Histopathology

The content of luteolin can increase the activity of Superoxide Dismutase (SOD), Glutathione Peroxide (GPx), Catalase (CAT) and Glutathione Reductase (GRed). SOD as the first line in fighting free radicals and oxidative stress with GSH together with glutathione peroxidase serves to reduce the level of oxidative stress that arises due to hyperglycemia by reducing (Hydrogen peroxide) (H₂O₂) to (water) (H₂O) and (oxygen) (O₂). A decrease in oxidative stress can also reduce the risk of inflammation in cells (Adeoye *et al.*, 2018). In addition, luteolin affects increasing the expression of Heme Oxygenase-1 (HO-1). HO-1 has functioned as an antioxidant and cytoprotectant (Sangeetha, 2019). The administration of saponins showed improvement in kidney creatinine levels, which could prevent the incidence of nephropathy due to diabetes (Wang *et al.*, 2019). Therefore, luteolin and saponin function to prevent inflammation, kidney tissue destruction and nephropathy (Ojimelukwe and Amaechi, 2019; Sangeetha, 2019; Wang *et al.*, 2019).

Although extracts from the leaves of *V. amygdalina* contain compounds with nephron-protective effects, the present study showed no improvement in the kidney histological structures. Several factors can be caused, including air, food, drinking water, laboratory conditions, diseases, stress levels and immunity (Ibegbu *et al.*, 2018; Ibrahim *et al.*, 2020).

Conclusion

The effect of ethanol extract of *Vernonia amygdalina* Del. leaf in compensating the oxidative stress was evident in vitro, shown by the IC₅₀ value = 86.709 ppm, which was categorized as vigorous antioxidant agent and it was found eight components that contained in *V. amygdalina* Del. based on the LC-MS analysis that contributed as antioxidant agents. This plant leaf extract could reduce blood glucose levels in group of hyperglycemic rats. An effective dose in reducing blood glucose levels of 200 mg/kg BW. Based on histopathological results, the extract administration did not show improvement by showing the proximal tubule necrosis in all treated groups other than the normal group. Further investigation is needed to find the optimal dose for histological improvement.

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Author's Contributions

Fatmawaty Yazid, Nina Bonita Hasanah, Rosmalena, Muhammad Hanafi and Vivitri Dewi Prasasty: Performed the experiments, analyzed the data, preparation of the manuscript for publication.

Fatmawaty Yazid: Designed the experiments, principal investigator for the implementation of research work.

Ethics

This manuscript has not been published, presented elsewhere and is not under consideration by another journal. All the authors have approved the manuscript and agree in submitting to OnLine Journal of Biological Sciences. There is no conflict of interest in this matter.

The animal study has been approved by The Research Ethical Committee of the Faculty of Medicine, Universitas Indonesia (Ethical Letter No: 096/UN2.F1/etik/PPM.00.02/2019).

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