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The effects of *Aquilaria malaccensis* leaf extracts on blood glucose level, kidney profile and liver function in streptozotocin induced diabetic rats

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Abstract

Diabetes mellitus is clinically defined as hyperglycemia or an abnormality caused due to increased glucose intake and a disordered metabolism. *Aquilaria malaccensis* has been shown its beneficial effects in reduction of postprandial glucose intake. Overall, the aim of this study was to evaluate the antidiabetic effects of *A. malaccensis* methanolic and aqueous leaf extracts on Streptozotocin-induced (STZ-induced) diabetic rats.The STZ-induced diabetic rat model was used to compare the effect of 500 mg/kg *A. malaccensis* methanolic and aqueous leaf extracts on blood glucose levels as compared to the standard drug 500 mg/kg metformin. Treatment with *A. malaccensis* methanolic and aqueous leaves extracts of 500 mg/kg body weight for 5 days resulted in a significant (p <0.05) decrease in blood glucose level, with percentage of glucose lowering effects of 57.08% and 55.48%, respectively, compared to metformin, which showed a percentage of glucose lowering effects of 68.79%. Furthermore, the biochemical parameters assessment on diabetic rats treated with both extracts showed that the extract did not enhance damage in serum protein. This could suggest that the extracts did not cause any significant damage in the internal organs such as liver and kidneys. These findings indicated that *A. malaccensis* methanolic and aqueous extracts have the potential to lower blood glucose levels without causing harm to the animals and could be used as a supplementary food in the management of diabetes mellitus.

Keywords: *antidiabetic, Aquilaria malaccensis, streptozotocin, Sprague-Dawley, hyperglycemia*

Introduction

Diabetes mellitus is a type of metabolic disorder characterised by hyperglycemia resulting from defects in insulin secretion and action. Diabetes mellitus is classified into two major categories: type 1 diabetes (formerly known as insulin dependent diabetes mellitus, or T1DM), and type 2 diabetes (formerly known as non insulin dependent diabetes mellitus, or T2DM). Type 1 diabetes is caused by the body's inability to produce insulin, which is the hormone that liberate the cells, allowing glucose to enter and fuel them. Since glucose cannot enter the cells, it builds up in the blood and the body's cells become starve to death.

Meanwhile, type 2 diabetes is caused by the interaction between a genetic vulnerability, behavioural and environmental risk factors (Mustaffa et al. 2011). Type 2 diabetes results from the body's inability to produce enough or use insulin properly. Often type 2 diabetes can be controlled through diet and exercise, but sometimes these are insufficient and either oral medications or insulin must be applied. Based on epidemiological statistics by International Diabetes Federation (IDF) diabetes affects approximately 3% of the population worldwide, where 90% of which suffer from type 2 diabetes. According to *Malaysian Diabetes Association*, people with type 2 diabetes frequently develop the disease after the age of 30, but are unaware of their condition until they are treated for one of its serious complications.

To date, at least 400 plants worldwide have been documented as beneficial in the treatment of diabetes (Bailey et al. 2006). *A. malaccensis* is one of them but there is no report and detailed systemic documentation on antidiabetic potential. Agarwood leaf extract was

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found to possess antipyretic, laxative, and antimicrobial activities (Zhou et al. 2008) and some studies revealed that agarwood has remarkable anticancer activity (Hashim et al. 2014).

Currently, most of scientific investigations on potential antidiabetic activity of *A. malaccensis* leaves extract have not yet progressed to clinical stage. However, there are promising results generated from few pre-clinical studies to evaluate the effects of leaves extract from several species of Aquilaria including *A. malaccensis* on blood glucose level in animal models (Pranakhon et al. 2011; Manoka et al. 2016). These studies have reported on the lowering of blood glucose level in streptozotocin (STZ)-induced rats following oral treatment using leaves extract of Aquilaria. The significance of antihyperglycaemic effects produced by the extracts was proved statistically against the activities of widely used commercial antidiabetic drug, acarbose. These findings may provide the basis to the traditional uses of Aquilaria leaves in alternative treatment of diabetes.

On the other hand, an observational study on antidiabetic potential of Aquilaria leaves was reported by Pranakhon et al. (2011). The study reported on a singular case of antihyperglycaemic effects of Aquilaria leaves in a diabetic patient, who consumed the leaves infused water for a period of six months. However, the report may be considered as statistically insignificant as there was no further systematic investigation has been carried out to prove the observation. While oral consumption of Aquilaria leaves extract may be proved safe according to investigation at pre-clinical level, further toxicology tests via systematic clinical trials should be carried out to prove its safety for human consumption. Despite promising findings, there are still limited scientific and clinical evidence available to prove the antidiabetic potential of Aquilaria leaves extract.

Diabetes is a chronic health problem with devastating, yet preventable consequences. It is evident that diabetes mellitus is increasing in the community in both developed and developing country. Therefore, the objective of this study is to investigate the effects of methanolic and aqueous extracts of *A. malaccensis* in STZ-induced diabetic rats. The new finding on the component of *A. malaccensis* leaf extracts which suggest promising potential antidiabetic agents that are safe for human consumption which will also be analysed and evaluated. The overall findings from current study unravel how feasible *A. malaccensis* leaf extracts with antihyperglycemic potential in human diabetes treatment.

Methods

Plant material

Leaves of *A. malaccensis* were collected during December 2011 from Malaysia's tropical forest and plantations assisted by of Malaysian Timber Industry Board (MTIB) and Universiti Putra Malaysia (UPM) for identification (voucher no. SK 2422/14).

Preparation of test materials

The fresh leaves of *A. malaccensis* were dried at room temperature for 30 days. The dried leaves were ground and the powder materials were extracted using methanol solvent extraction and hot aqueous extraction methods. For methanolic extract method, the dried leaves powder of *A. malaccensis* were extensively extracted with methanol solvent in a ratio of 1:20 at room temperature for 48 hours and filtered through a Whatman No. 1 filter paper. The filtrate was then concentrated at 60 °C using rotary vacuum evaporator to give a residue. For aqueous extract, the extract was prepared by immersion of the powdered material in boiled distilled water, and allowed to infuse for 15 minutes. The suspension was filtered through a Whatman No. 1 filter paper and subjected to freeze drying to obtain a dry powder. The crude extract was kept in universal bottle and stored at 4 °C prior to use.

Antidiabetic activity of the extracts in STZ-induced diabetic rats

A total of 36 Male Sprague-Dawley rats (200 – 280 g) were purchased and maintained in an airconditioned room (25 ± 1 °C), with 12 hours light – 12 hours dark cycle and fed with standard diet and water *ad libitium*. Rats were acclimatised for 7 days before starting the experiment. Rats were diabetes induced by a single subcutaneous injection (55 mg/kg body weight) of streptozotocin (STZ) dissolved in commercial sterile NaCl solution (normal saline). After induction, rats were normally fed with standard diet and allowed to drink 10% glucose solution. After seven days of STZ injection, blood was collected from rat tail to determine fasting blood glucose level using glucometer. Only the rats with fasting blood glucose over 7.0 mmol/L, were considered diabetic and used for the experiments (*Image 1*).

Blood was obtained by snipping tail of rat with the help of sharp needles and the blood glucose levels were monitored at two hours interval (2, 4 and 6 hours) after administration of extract orally and continue monitored on the following day until day five by using a single strip touch glucometer. The animals were sacrificed under chloroform anesthesia after day five. Blood was collected from the dorsal aorta. Whole blood was then centrifuged using refrigerated centrifuge at 4 °C to remove the red blood cells and recover serum. Serums were separated and collected using dry autoclaved micropipette and store in –80 °C for analyses. The analyses were completed within 24 hours upon sample collection. All experiments were carried out in accordance to methodology for hypoglycemic activity of the extracts in rats published by Pranakhon (2011)

Biochemistry test

A clinical biochemistry experiment was performed on samples of blood to determine major toxic effects in tissues and specifically on kidney and liver. Blood was

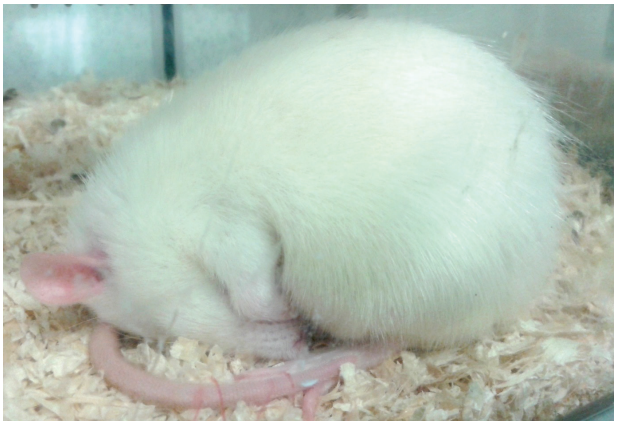

Image 1. Diabetic *Sprague-Dawley* Male Rats

collected in the blood tubes containing potassium oxalate and sodium fluoride as anticoagulants and serum were immediately separated by centrifugation at 3,500 g for 10 minutes within 2 hours after blood collection. Estimation of serum total protein levels, creatinine and urea were evaluated using automated clinical chemistry analyser (TRX 7010, Biorex Mannheim, Germany) for kidney function, whilst liver function of rats were evaluated by measuring the blood serum level, signified by the level of these parameters: Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total protein (TP), total bilirubin (TBIL), albumin (ALB) and globulin (GLOB).

Statistical analysis

All results were expressed as mean ± standard error of the mean (S.E.M) analysed by SPSS® version 15.0 (SPSS Inc., 2008). Differences between groups were analysed by one-way analysis of variance (ANOVA) by Tukey's test and the value of $p < 0.05$ was considered statistically significant. All antidiabetic assays and animal test were performed in six replicates.

Results and discussion

Blood glucose effects

Overproduction of glucose in bloodstream through excessive hepatic glycogenolysis and gluconeogenesis is one of the fundamental causes of hyperglycemia and diabetes mellitus. Increase in blood glucose concentration is an important characteristic in diabetic studies. The effects of oral extracts administered on blood glucose level from day 1 until day 5 in STZ-induced diabetic rats are depicted in *Figure 1*. The initial blood glucose levels of diabetic rats selected for the study were in the range of 7.0 to 12.0mmol/L. The experimentally induced diabetes had significantly increased ($p < 0.05$) the fasting blood glucose level by 3-fold from initial reading.

The results showed that treatment with aqueous and methanolic *A. malaccensis* extracts for five days could reduce the fasting blood glucose to normal level, demonstrating good glycemic control that characterised antidiabetic properties (*Figure 1*). Treatment with methanolic and aqueous leaf extracts of *A. malaccensis* at the dosage of 500 mg/kg body weight for five days exhibited a significantly decreased ($p < 0.05$) in blood glucose of STZ-induced diabetic rats as compared to untreated diabetic and normal control groups. Blood glucose level in induced diabetic rats dropped on day one after oral administration of *A. malaccensis* extracts and continued to decrease until day five, the result of which comparable to metformin.

Metformin was used in this experiment because it is safe, widely available, inexpensive and has considerable use in various different clinical settings (Bergmark et al. 2019). It has potential use in early stage of type 2 diabetes mellitus (T2DM) compared to other drugs such as insulin in late T2DM, and sulfonylureas are only useful in patients with fewer β-cells (Lorenzati et al. 2010). Metformin also has been the drug of choice for the treatment due to lesser side effects (gastrointestinal side effects) produced from its use.

As shown in *Figure 1*, there are significant differences ($p < 0.05$) between blood glucose level in the healthy normal male rats and diabetic control ($p < 0.05$). In diabetic control groups, the animals were not treated with any extracts, fed with standard diet and allowed to drink 10% glucose solution similar to treated group. According to *Figure 1*, there was significant reduction in blood glucose level of treated rats on day five after treatment with 500mg/kg of methanolic and aqueous *A. malaccensis* compared to untreated diabetic group (5.12 + 0.23 and 5.23 + 0.32 vs. 16.27 + 0.29 mmol/L, respectively, $p \leq 0.05$). Similarly, it was observed that the blood glucose level was significantly decreased with the treatment of metformin (4.15 + 0.21 mmol/L). One of the possible reasons for the increase in blood glucose level with the passage of time is the metabolism of active ingredients contained in the extract, which have been responsible for the hypoglycemic effects.

It was found through this study that the methanolic extract demonstrated a slightly higher antidiabetic activities than the aqueous extract with the percentage of blood glucose lowering effect of 57.08% and 55.48% respectively; the results were similar to that of metformin, 68.79%. According to Dieu-Hien et al. (2019) efficiency of extraction favours the highly polar solvent where methanol has high polarity compared to aqueous solvent indicating that methanol extract contained more bioactive compounds which posseses antidiabetic activity.

Therefore, from these findings, both methanolic and aqueous *A. malaccensis* extracts are able to restore the blood glucose levels of untreated diabetic group (at a dosage of 500 mg/kg) to a healthy level, which is comparable to the normal control group. Furthermore, we found that both methanolic and aqueous extracts of *A. malaccensis* are as efficient as commercial drug metformin in lowering blood glucose levels in STZ-induced diabetic rats, with a slightly better effect observed in the methanolic extract.

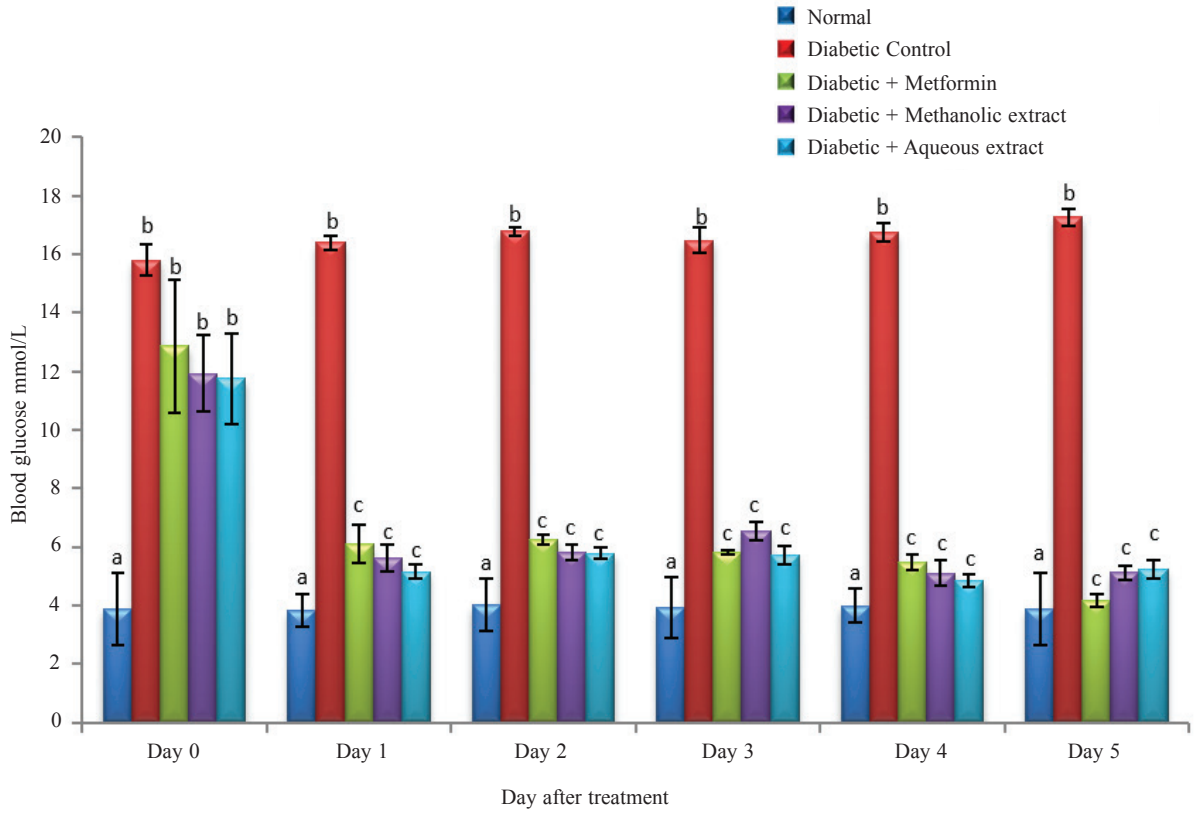

Figure 1. Effects of A. malaccensis aqueous and methanolic extracts on blood glucose of the STZ-induced rats after five days treatment. Values expressed were mean ± S.E.M. A P-value less than 0.05 ($p \leq 0.05$) was considered as significant difference compared with diabetic control using one-way ANOVA by Tukey's post-test. (n = 6 rats in each group)

Kidney profile

Kidney is the major excretory organ, which function is profiled to examine the safety of tested substance (Sun et al. 2012). Kidney damages are indicated by measuring the level of three metabolites excreted from kidney to bloodstream: urea, creatinine and uric acid. Insulin deficiency and consequent inability of glucose to reach hepatic tissues stimulate gluconeogenesis as an alternative route for glucose supply (Dale et al. 2002). This route is sustained by increased proteolysis which releases free glycogenic amino acids into the plasma, which would then deaminated in the liver, resulting in consequential increase of urea in the blood. Creatinine derived from the breakdown of muscle creatine and its concentration in serum is proportional to the body muscle mass. The amount of creatinine is usually constant and thus, easily excreted by the kidney; elevation in the level of creatinine indicates diminished renal function (Sushrut et al. 2009). Meanwhile, uric acid is the major metabolic product of purine metabolism and its elevation in the serum signifies kidney impairment.

Creatinine, urea and uric acid were identified parameters in assessment of kidney profile in STZ-induced diabetic rats as outlined in *Table 1*. The results show that the serum urea, creatinine, and uric acid concentrations of untreated diabetic rats were significantly higher (p <0.05). These are indications of kidney damage, which was probably due to reduced functional capacity in kidney as reflected by the increased levels of serum calcium ion, creatinine, urea and uric acid in STZ-induced diabetic rats. Administration of *A. malaccensis* extracts had a positive impact on kidney function index of STZ-induced diabetic rats by significantly reduced the levels of creatinine, urea and uric acid as compared to normal group, thereby conferring protection against impairment due to diabetes. However, such differences in values were minor when comparing extracts and species, and the values remained within the normal range.

Liver profile

Diabetes causes increased protein catabolism, with amino acids flowing into the liver to feed gluconeogenesis (Marion 2000). This accelerated proteolysis of uncontrolled diabetes occurs due to insulin deficiency. Similar phenomenon might have accounted for the decrease in total protein content in untreated STZ-induced diabetic rats. Administration of *A. malaccensis* extracts to diabetic rats had significantly inhibited proteolysis caused by insulin deficiency and thus, increased the level of total proteins to almost similar level rendered by treatment with metformin.

Protein profile in STZ-induced diabetic rats involved two major groups of protein: albumin and globulin, which are the mixtures of protein molecules that are useful for assessing the health of the liver. Albumin, which is manufactured in liver, is the major carrier protein that

circulates in the bloodstream while globulins are larger proteins responsible for immunologic responses (Amy 2006). The low concentration of serum albumin and globulin signifies chronic damage to the liver due to infection (Theodore et al. 2005). Therefore, reduction in serum albumin and globulin levels in untreated diabetic rats indicated diminished synthetic function of the liver. However, oral administration of methanolic and aqueous *A. malaccensis* extracts restored albumin and globulin levels to normal, yielding results that were nearly identical to metformin (standard drug used as a positive control) as shown in *Table 2*.

Bilirubin is the major product from the breakdown and destruction of old red blood cells. It is an important metabolic product with biological and diagnostic values (Amy 2006). It is removed from the body by the liver; hence, it is a good indicator to the health status of liver. Elevated serum bilirubin level observed in diabetic rats treated with methanolic and aqueous *A. malaccensis* extracts (*Table 2*) might be due to liver disease. Treatment with *A. malaccensis* extract was able to reverse this condition in diabetic rats by lowering the bilirubin level to normal condition. All data obtained in respect to liver function indices indicated the absence of any significant liver damage following the treatment with both methanolic and aqueous *A. malaccensis* extracts in diabetic rats

ALP is made mostly in the liver and bone with few made in intestine and kidney. The liver makes more ALP than other organs. ALP is often employed to assess the integrity of plasma membrane and endoplasmic reticulum (Shahjahan et al. 2004), while GGT is a membrane-localised enzyme that plays a major role in glutathione metabolism in the liver (Gjin et al. 2017). Damage of the liver is reflected by increased activity of these two enzymes in the serum, probably due to leakage in altered cell membrane structure. Therefore, increase in serum ALP in untreated diabetic rats signified damages in plasma membrane while decrease in its value in diabetic rats treated with *A. malaccensis* extracts signified the reverse effects which was almost similar to that of metformin (*Table 3*).

The transaminases (AST and ALT) are well-known enzymes used as biomarkers to predict possible toxicity effects on the liver (Rahman 2001). ALT is found mainly in the liver with smaller amount found in the kidney, heart, muscles and pancreas. ALT normally present at low concentration in blood. However, when the liver are damaged, it releases ALT into the bloodstream, which leads to significant increase in ALT levels. Meanwhile, AST is normally found in RBC, liver, heart, muscle tissues, pancreas and kidney. AST is also present at low concentration in blood under normal condition.

Table 1. Effects of oral administration of *A. malaccensis* aqueous and methanolic extracts on serum creatinine, urea and uric acid in normal and STZ-induced diabetic rat for kidney profile

Group	Serum level		
	Creatinine (μmol/L)	Urea (mmol/L)	Uric acid (μmol/L)
Normal range	17.68 – 70.72	5.35 – 7.497	147.75 – 356.88
Normal group	52.33 ± 1.67	5.87 ± 0.18	248.67 ± 9.65
Diabetic (control)	59.33 ± 3.21[a]	6.20 ± 0.44[a]	294.67 ± 11.94[a]
Diabetic + metformin	53.00 ± 3.04[b]	5.90 ± 0.18[b]	252.17 ± 14.34[b]
Diabetic + methanol extract	54.50 ± 2.97[b]	5.91 ± 0.47[b]	260.83 ± 8.96[b]
Diabetic + aqueous extract	53.50 ± 1.02[b]	5.90 ± 0.20[b]	255.17 ± 25.49[b]

Source of normal range: Giknis et al. (2008)

Values expressed were mean ± S.E.M. A P-value less than 0.05 ($p \leq 0.05$) was considered as significantly different compared with diabetic control group using one-way ANOVA by Tukey's test (n = 6 rats in each group).

Table 2. Effects of oral administration of *A. malaccensis* aqueous and methanolic extracts on blood serum in normal and STZ-induced diabetic for liver function profile

Group	Serum level			
	TBIL (μmol/L)	TP (g/L)	ALB (g/L)	GLOB (g/L)
Normal range	3.42 – 9.40	56 – 76	38 – 48	15 – 25
Normal Group	4.79 ± 0.11	72.90 ± 1.93	48.28 ± 1.63	25.05 ± 0.46
Diabetic (control)	5.20 ± 0.10[a]	60.18 ± 0.61[a]	36.88 ± 0.58[a]	22.72 ± 0.79[a]
Diabetic + Metformin	4.89 ± 0.06[b]	73.90 ± 1.20[b]	48.47 ± 0.45[b]	24.60 ± 0.80[b]
Diabetic + methanol extract	4.98 ± 0.08[b]	72.45 ± 2.57[b]	47.40 ± 0.53[b]	26.20 ± 0.73[b]
Diabetic + aqueous extract	4.96 ± 0.11[b]	73.12 ± 1.73[b]	47.75 ± 0.78[b]	26.18 ± 0.96[b]

Source of normal range: Giknis et al. (2008)

TBIL: total bilirubin, TP: total protein, ALB: albumin and GLOB: globulin. Values expressed were mean ± S.E.M. A P-value less than 0.05 ($p \leq 0.05$) was considered as significantly different compared with diabetic control group using one-way ANOVA by Tukey's post-test (n = 6 rats in each group).

Table 3. Effects of oral administration of *A. malaccensis* aqueous and methanolic extracts on enzymes in normal and STZ-induced diabetic rats for liver function profile

Group	Serum level		
	AST (U/L)	ALT (U/L)	ALP (U/L)
Normal range	74 – 143	18 – 78	62 – 230
Normal group	104.00 ± 5.82	55.62 ± 2.86	168.33 ± 9.54
Diabetic (control)	135.617 ± 4.81[a]	81.10 ± 5.51[a]	198.50 ± 22.43[a]
Diabetic + Metformin	105.83 ± 7.19[b]	56.63 ± 1.69[b]	176.17 ± 16.90[b]
Diabetic + methanol extract	110.83 ± 4.31[b]	57.45 ± 0.67[b]	185.33 ± 16.86[b]
Diabetic + aqueous extract	109.83 ± 5.04[b]	57.18 ± 2.41[b]	178.17 ± 17.62[b]

Source of normal range: Giknis et al. (2008)

AST: aspartate aminotransferase, ALT: alanine aminotransferase, ALP: alkaline phosphatase, LDH: lactate dehydrogenase, and CK: creatine kinase. Values expressed were mean ± S.E.M. A P-value less than 0.05 ($p \leq 0.05$) was considered as significantly different compared with diabetic control group using one-way ANOVA by Tukey's post-test (n = 6 rats in each group)

When body tissue and organ such as liver and heart is damaged, additional AST is secreted out into the bloodstream, resulting in increased AST level. Thus, the amount of AST in blood is proportional to the extent of the tissue damage.

Serum ALT and AST are useful indices for identifying inflammation and necrosis of the liver (Shivaraj et al. 2009). Elevation in serum activities of both transaminases as observed in diabetic rats indicated damage in the liver cells (Yakubu et al. 2003). According to *Table 3*, elevated AST and ALT levels in diabetic rats were significantly declined after treatment with methanolic and aqueous *A. malaccensis* extracts. Oral administration of *A. malaccensis* extracts attenuated the elevated activities of investigated enzymes in diabetic rats comparable to the effects rendered by metformin. This may be an indication of nontoxic nature and protective action of the extracts in reversing the damaging effects on liver due to diabetes.

Conclusion

A. malaccensis leaf extracts possesses potential dietary supplements that may be useful for allowing flexibility in meal planning and thus can be promising source for antidiabetic agent. For antidiabetic study in STZ-induced diabetic rats, the results showed that *A. malaccensis* aqueous and methanolic extracts gave a significant reduction in blood glucose level in a dose dependent manner. Furthermore, the methanolic extract more pronounce as antidiabetic activities than the aqueous extract. The assessment on diabetic rats treated with *A. malaccensis* extracts revealed that both extracts did not damage the proteins, implying that these extracts did not cause any significant damage to the internal organs; liver and kidney. Hence, this data suggests the antidiabetic properties of *A. malaccensis* are beneficial for correcting hyperglycaemia and alleviating the adverse effect of diabetes mellitus by enhancing antioxidant defences system, which could serve as an alternative medicine for treating diabetes. Therefore, further studies to identify the effective components of this extracts appear to be warranted.

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