Available online at www.sciencedirect.com
ScienceDirect
Chinese Journal of Natural Medicines 2013, **11**(5): 0488−0493
doi: 10.3724/SP.J.1009.2013.00488
Chinese Journal of Natural Medicines

In vivo anti-hyperglycemic activity of saliva extract from the tropical leech *Hirudinaria manillensis*

Abdualkader, Abdualrahman Mohammed[1*], Ghawi, Abbas Mohammad[2], Alaama, Mohamed[1], Awang, Mohamed[1], Merzouk, Ahmed[3]

[1]*Pharmaceutical Chemistry Department, Faculty of Pharmacy, International Islamic University Malaysia, 25200 Kuantan, Pahang, Malaysia;*
[2]*Basic Medical Science Department, Faculty of Pharmacy, International Islamic University Malaysia, 25200 Kuantan, Pahang, Malaysia;*
[3]*Biopep Solutions Inc., 235-11590 Cambie Road, Richmond, BC V6X 3Z5, Canada*

Available online 20 Sept. 2013

[ABSTRACT] The anticoagulant effect of leech saliva was traditionally employed in the treatment of diabetes mellitus complications such as peripheral vascular complications. This study was carried out to examine the effect of leech saliva extract (LSE) on blood glucose levels in alloxan-induced diabetic rats. First, LSE was collected from leeches which were fed on a phagostimulatory solution. Second, total protein concentration was estimated using the Bradford assay. Third, diabetic rats were injected subcutaneously (sc) with LSE at doses of 500 and 1 000 μg·kg^{-1} body weight (bw). Other diabetic rats were injected sc with insulin at doses of 10 and 20 U·kg^{-1} bw. Another group was injected simultaneously with LSE (250 μg·kg^{-1} bw) and insulin (10 U·kg^{-1} bw). Fasting blood glucose (FBG) concentrations were monitored during a study period of eight hours at regular intervals. Findings showed that both doses of LSE resulted in a significant and gradual decrease in FBG starting from 10%−18% downfall after two hours of injection reaching the maximal reduction activity of 58% after eight hours. Remarkably, LSE was sufficient to bring the rats to a near norm-glycemic state. The high dose of insulin induced a severe hypoglycemic condition after 2−4 h of injection. The lower dose was able to decline FBG for 2−6 h in rats which became diabetic again after 8 h. On the other hand, the concurrent injection of low doses of LSE and insulin produced a hypoglycemic effect with all rats showing normal FBG levels. Taken together, these findings indicated that the subcutaneous injection of LSE of the medicinal Malaysian leech was able to provide better glycemic control compared with insulin. Moreover, the synergism between LSE and insulin suggests that LSE could be utilized as an adjuvant medication in order to reduce insulin dosage or to achieve better control of blood glucose.

[KEY WORDS] Alloxan; Diabetes mellitus; Insulin; Leech; *Hirudinaria manillensis*

[CLC Number] R965 **[Document code]** A **[Article ID]** 1672-3651(2013)05-0488-06

1 Introduction

Amongst haematophagous animals, leeches have been known as familiar therapeutic tools since the ancient ages until the present day. The word leech is etymologically derived from the Anglo-Saxon word for physician '*laece*' which clearly represents the importance of leeches in medicine [1]. Traditionally, leech application was used for many human body disorders starting from the conventional usage of leeches for bloodletting. Moreover, many reports mentioned the usage of leeches in skin diseases, nervous system abnormalities like brain congestion, urinary and reproductive system problems (nephritis, vaginitis). In addition, ocular inflammation, dental problems, and hemorrhoids were also treated by leech therapy [1-3]. On the other hand, there was no scientific documentation of the medical importance of leeches until 1884, when John B. Haycraft (1857−1922) outlined the existence of a potent anticoagulant agent, hirudin, in

[Received on] 10-Aug.-2012
[Research funding] This project was supported by the grant (No. EDW B 10-0393) from Research-Management Centre, International Islamic University Malaysia.
[*Corresponding author] Abdualkader, Abdualrahman Mohammed: E-mail: armak1983@gmail.com
These authors have no any conflict of interest to declare.

leech saliva which explained why blood oozing from a leech wound coagulated slower than an ordinary wound [4]. Henceforth, many extensive studies have been undertaken on leech products, especially leech saliva. Consequently, a large number of peptides and proteins with substantial clinical applications have been identified and characterized [5]. Recently, leeching has established itself in microsurgery, reconstructive operations [6], metastasis [7] and cancer [8].

Despite the scarce research on using leeches in the treatment of diabetes mellitus (DM), it is being used by traditional practitioners because of the powerful effects of the anticoagulants-containing leech saliva to alleviate DM complications. For instance, some clinicians use leech therapy to improve blood circulation of extremities in order to avoid gangrene and amputation [9]. The current research was designed to evaluate the antihyperglycemic activity of leech saliva obtained from *Hirudinaria manillensis*, along with examining its ability to prevent the experimental induction of DM in an animal model.

2 Materials and Methodology

2.1 *Chemicals, reagents and instrument*

Sodium chloride and arginine hydrochloride were the products of Merck (Germany). Bradford reagent kit was procured from Amresco (Canada). Parafilm membrane was obtained from American Can Company (USA). Anhydrous glucose was the product of Fisher Scientific (Germany). Alloxan monohydrate and bovine serum albumin and insulin from bovine pancreas ($\geqslant$ 27 U·kg^{-1}) were purchased from Sigma Aldrich (Germany).

Centrifugation was done using a Universal 32R centrifuge produced by Hettich Zentrifugen (Germany). Microplate reader model Infinite M200, NanoQuant TECAN was the product of Tecan (USA). Lyophilization was performed using a Christ freeze-drier model Alpha 1-4LD (Germany). One Touch Ultra glucometer and test strips used for the determination of blood glucose concentration were the product of LifeScan Inc., USA. Microscopic examination was done using a Nikon Eclipse 80i microscope (Japan).

2.2 *Leech sampling, saliva collection and total protein estimation*

Leeches, *Hirudinaria manillensis* (Lesson, 1842), were collected from the natural lake, Cheneh, located in Terengganu, Malaysia. They were maintained in well-aerated plastic containers filled with un-chlorinated tap water. Water was regularly changed every 2−3 d. Leech feeding and saliva extract collection were executed as previously described using a phagostimulatory solution (PHS) of 0.001 mol·L^{-1} arginine in 0.15 mol·L^{-1} sodium chloride [8]. Thereafter, LSE was lyophilized and the resulting powder was dissolved in an appropriate volume of distilled water to be concentrated ten-fold. The resultant solution was termed as the ten-time concentrated leech saliva extract (10 × LSE) which was used during the following procedures.

The total protein estimation of LSE was performed according the standard protocols using Amresco Bradford reagent kit [10]. Bovine serum albumin (BSA) was used as a standard protein. The PHS was considered as a blank.

2.3 *Animals and induction of diabetes mellitus*

Male Sprague Dawley rats (80−110 g) were obtained from the Faculty of Veterinary Medicine, University Putra Malaysia. Rats were kept in a well air-conditioned animal room under 12 h/12 h dark and light cycle at room temperature. They were acclimatized for a period of one week prior to the experiment. During the acclimatization period, rats were housed in polypropylene cages lined with pine wood husk, changed every two days, and they were given free access to tap water and commercial dry pellet diet, Gold Coin. All experimental protocols were approved by the Ethics Committee Meeting (No. 1/2011 on 22nd April, 2011) of Kulliyyah of Medicine, IIUM (Ref. No. IIUM/305/20/4/ 10).

Experimental diabetes was induced by inter-peritoneal (ip) injection of alloxan to overnight fasted animals at a dose of 160 mg·kg^{-1} body weight [11]. In order to prevent fatal alloxan-induced hypoglycemia, rats were administered with 20% glucose solution intraperitoneally followed by 5% glucose solution orally for the next 12 h [12]. The diabetic state of the rats was assessed via fasting blood glucose level (FBG) of fresh capillary blood taken from a tail vein puncture. All measurements were made using a Touch Ultra glucometer. Rats showing FBG more than 11.1 mmol·L^{-1} were considered diabetic [11] and selected for the study.

2.4 *Experimental procedures*

Forty male rats were divided randomly into eight groups, each comprising five rats as detailed below:

Control groups:

Negative control: normal rats.

Positive control: induced-diabetic untreated rats.

Vehicle control: induced-diabetic rats injected subcutaneously (sc) with the PHS at a dose of 20 mL·kg^{-1} body weight (bw).

Comparative control: induced-diabetic rats injected sc with the standard dose of insulin of 20 U·kg^{-1} bw [13].

The hypoglycemic activity of LSE:

Induced-diabetic rats injected sc with LSE at a dose of 500 μg·kg^{-1} bw.

Induced-diabetic rats injected sc with LSE at a dose of 1 000 μg·kg^{-1} bw.

LSE in combination with insulin:

Induced-diabetic rats injected sc with a half dose of insulin, 10 U·kg^{-1} bw bovine pancreas insulin suspension in distilled water.

Induced-diabetic rats injected sc with 250 μg·kg^{-1} LSE + 10 U·kg^{-1} bw bovine pancreas insulin.

The antihyperglycemic activity of LSE was assessed by the fall in FBG values within eight hours measured at two-hour intervals. The percentage decrease in fasting blood

glucose concentration was calculated from the following equation [14]:

Percentage decrease in FBG after x hour =

$$\frac{FBG(0, hour) - FBG(x, hour)}{FBG(0, hour)}$$

2.5 *Toxicity and histopathological evaluation*

All rats were kept under close observation during the trial period of 8 hours. For the whole experimental period, general behavior and mentality patterns (physical activity, weakness, aggressiveness, awareness, motor activity, food refusal, diarrhea, noisy breathing, writhing, etc.), and mortality were monitored [15-16]. After the experimental regimen, LSE-treated rats were anesthetized with ether and sacrificed by cervical dislocation. Specimens from their pancreas and liver were fixed in 10% neutral buffered formalin, prepared and stained by hematoxylin–eosin for light microscopic examination [17].

2.6 *Statistical analysis*

The results were expressed as the mean of triplicates ± the standard error of the mean (SEM). All data were evaluated with Statistical Package for Social Science (SPSS 18.0) software using general linear model GLM repeated measurement ANOVA followed by Dunnett's (2-sided) post-hoc test considering the positive control group as a reference. Values were considered statistically significant with $P < 0.05$.

3 Results and Discussion

3.1 *LSE collection and total protein estimation*

The total volume of the colorless fluids collected from twenty starved leeches was 190 mL (pH 6.45), meaning that each leech can produce 9.5 mL of LSE. Protein estimation (Fig. 1) revealed that the collected LSE contained a total protein concentration of (54.29 ± 2.58) μg·mL^{-1}. Lyophilization of LSE yielded a white fine powder which was later dissolved in a suitable volume of distilled water immediately prior to use to give the 10 × LSE. This indicates that the protein concentration of 10 × LSE was 542.99 μg·mL^{-1}.

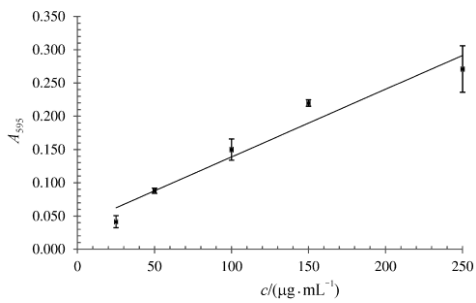

Fig. 1 Standard curve for total protein estimation in leech saliva extract (LSE). Assay was carried out according to the standard protocols of Bradford method. Bovine serum albumin (BAS) was considered as the standard protein. The phagostimulatory solution (PHS) was used as blank. Y = 0.001 X + 0.028, R^2 = 0.953: Y = absorbance A_{595} and X = BSA concentration μg·mL^{-1}

A comparison between the volume of LSE (9.5 mL) given by one leech with the total protein [(54.229 ± 2.589) μg·mL^{-1}] revealed that each leech can give a total protein of about 500μ corresponding the minimum dose by which the experimental rats were injected. Consequently, the rats were injected with saliva at doses of 500 and 1 000 μg·kg^{-1} bw as if they had been bitten by one and two leeches, respectively.

3.2 *The anti-hyperglycemic activity of leech saliva extract (LSE)*

After three days of intraperitoneally alloxan injection (160 mg·kg^{-1} bw), results revealed that alloxan caused a significant increase ($P < 0.001$) in blood glucose concentration in the positive control group when compared with the negative control. The FBG levels were significantly reduced ($P < 0.001$) after subcutaneous injection of LSE at both doses of 1 000 and 500 μg·kg^{-1} bw. In addition, a significant reduction in FBG ($P < 0.05$) occurred two hours after injection of LSE, and a more significant decline was noticed after four, six and eight hours ($P < 0.001$). Furthermore, all of the insulin-injected rats experienced a sharp, significant decrease ($P < 0.001$) in FBG after two hours of injection. Rats which received the lower dose of insulin (10 U·kg^{-1} bw) returned to the diabetic state, with a rapid increase in FBG values, after 8 hours of treatment ($P = 0.090$). Whereas, the rats which received insulin (10 U·kg^{-1}) and LSE (250 μg·kg^{-1}) exhibited a significant drop in FBG during the whole eight hour study period ($P < 0.001$). In contrast, the diabetic rats injected with the phagostimulatory solution showed no significant reduction in FBG compared with the positive control rats. Results are presented in Table 1.

Fig. 2 illustrates that the dose of 1 000 μg·kg^{-1} bw gradually decreased FBG by about 18% after two hours of subcutaneous administration compared with a 10% reduction caused by the dose 500 μg·kg^{-1} bw. After four to six hours of injection, the biological activity of the injected LSE went through a steady state stage, because both doses exhibited approximate blood glucose lowering activity ranging from 40%−50%. Thereafter, the maximum antihyperglycemic activity of LSE (56.5% reduction in FBG) was found after eight hours of injection. Noteworthy, all of the LSE-injected rats exhibited FBG values less than the diabetic limit of 11.1 mmol·L^{-1} [11] after eight hours of injection, meaning that the eight hour treatment session was sufficient to bring the diabetic rats to the normal state.

On the other hand, the subcutaneous injection of insulin at a dose of 20 U·kg^{-1} bw induced a sharp reduction in FBG of about 80%−90% within two to six hours of dosage. During this period, rats which were injected with this dose of insulin experienced a hypoglycemic condition with low FBG values which eventually resulted in the death of one rat. After eight hours of injection, the blood glucose concentrations started to

rise but less than the diabetic range. In contrast, a lower dose of insulin (10 U·kg^{-1} bw) resulted in a dramatic decline in FBG of about 80% during the first four hours of administration and the hypoglycemic activity was reduced slightly after six hours of injection. After eight hours of injection of the lower dose of insulin, all experimental animals tended to become diabetic again showing FBG values of (22.4 ± 4.7) mmol·L^{-1}.

Interestingly, the concurrent administration of both LSE (250 μg·kg^{-1} bw) and insulin (10 U·kg^{-1} bw) produced a sharp decline in the fasting blood glucose of approximately 93% during the first four hours of injection. After six to eight hours, blood glucose concentrations displayed a marginal increment with a hyperglycemic lowering activity of the LSE-insulin mixture effectively sufficient to keep the rats at the normal state.

Table 1 The effect of different doses of leech saliva extract (LSE) and insulin on fasting blood glucose (FBG) in alloxan-induced diabetic rats at various time intervals (hours) (mean ± SEM, *n* = 5)

Groups/doses	FBG (mmol·L^{-1})				
	0 h	2 h	4 h	6 h	8 h
Negative control [α]	4.6 ± 0.4**	5.1 ± 0.2**	4.7 ± 0.2**	5.4 ± 0.2**	5.1 ± 0.2**
Vehicle control	22.8 ± 3.6	22.0 ± 3.6	22.8 ± 3.3	19.9 ± 3.5*	22.4 ± 4.7**
Comparative control [α]	26.4 ± 1.7	4.2 ± 1.3**	2.7 ± 0.6**	5.3 ± 0.5**	8.4 ± 0.9**
Diabetic + LSE 1 000 μg·kg^{-1} [α]	21.0 ± 2.8	17.0 ± 2.7*	11.9 ± 2.7**	10.8 ± 3.0**	8.8 ± 3.1**
Diabetic + LSE 500 μg·kg^{-1} [α]	21.3 ± 3.2	19.0 ± 2.3*	12.6 ± 1.9**	11.4 ± 1.9**	8.0 ± 1.5**
Diabetic + insulin 10 U·kg^{-1} [α]	24.9 ± 1.3	4.9 ± 0.4**	5.1 ± 0.4**	8.1 ± 0.4**	21.3 ± 3.2
Diabetic + LSE 250 μg·kg^{-1} + insulin 10 U·kg^{-1} [α]	23.2 ± 1.2	2.4 ± 0.3**	1.6 ± 0.2**	3.9 ± 0.4**	4.6 ± 0.2**
Positive control	27.3 ± 2.1	27.6 ± 2.2	26.96 ± 1.1	25.6 ± 1.2	27.5 ± 1.7

[α] significant compared to the positive control group; * $P < 0.05$ and ** $P < 0.001$

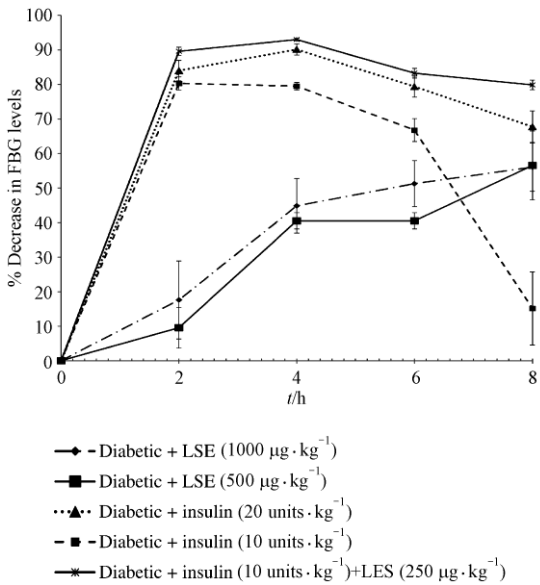

Fig. 2 Percentage decrease in fasting blood glucose in alloxan-induced rats treated with different doses of leech saliva extract (LSE) and insulin (mean ± SEM, *n* = 5). Fasting blood glucose levels (FBG) were taken at two-hour intervals. The percentage decrease values in FBG were calculated according to the equation mentioned in materials and methodology section.

Taken together, these findings indicate that the subcutaneous injection of the salivary gland secretion (LSE) collected from the medicinal Malaysian leech, *H. manillensis*, showed hypoglycemic activity which provided better glycemic control than that of insulin alone, with no fatal hypoglycemic cases during the eight hour study period. Moreover, it was found that LSE and insulin at lower doses had a synergistic antihyperglycemic activity. This type of synergism suggests that LSE could be utilized as an adjuvant medication in order to reduce insulin dosage or to achieve acceptable control of blood glucose concentration instead of using mixtures of short- and long-acting insulin. In fact, LSE antidiabetic activity may be due to the presence of one or more antihyperglycemic factors, thought to be peptides or proteins, and/or their synergistic properties. The mechanism by which LSE exhibited its blood glucose lowering activity still needs to be determined but many mechanisms could be hypothesized. Alloxan is a selective cytotoxic agent to the pancreatic *β*-cells by accelerating their necrotic cell death by forming reactive oxygen species (ROS), resulting in decreased endogenous insulin release (insulinopenia) and a consequent insulin-dependent type 1 diabetes syndrome [12]. In case that most *β*-cells have been damaged, leech saliva extract (LSE) could have potential extrapanreatic effects. One suggested mechanism could be termed as an "insulin mimic", that insulin-like proteins in LSE bind the *α*-subunit of insulin receptors in the tissue cells, which results in conformational changes of the *β*-subunit of the receptors and eventually receptor activation. Consequently, phosphorylation cascades will be triggered within the cell leading to multiple effects, involving glucose uptake by muscle cells due to glucose receptor translocation and more hepatic glycogen formation due to glycogen synthase activation [18].

In contrast, because it is already known that the majority of *β*-cells were destroyed by alloxanisation, other mechanisms that may reduce the blood glucose levels depending on

the presence of circulating insulin and intact β-cells, such as the direct stimulation of insulin secretion via closing ATP-sensitive potassium channels of pancreatic islet β-cells, improving insulin receptor sensitivity, etc. are not applicable [19]. Hence, more studies are required to determine the active principle and its actual mechanism of action.

Furthermore, the concomitant existence of antithrombotic [20], antioxidant [21] and hypoglycemic activities in the LSE of the medicinal Malaysian leech, *H. manillensis* provides an extraordinary protection from cardiovascular complications in diabetic patients who are at a high risk of developing heart diseases [22]. It was reported that the increased risk of cardiovascular complications in patients with diabetes mellitus, especially type 2, could be ascribed to many stress factors, such as hyperglycemia, hyperlipidemia, inflammation, coagulation and platelet adhesion disorders, hypertension and oxidative stress [23]. Some researchers argued that oxidative stress plays a pathogenic role in macro- and micro-events in diabetic patients due to hyperglycemia-induced glucose autoxidation and lipoprotein modifications such as glycation and oxidation [22].

3.3 *Toxicity and histopathology evaluation*

Neither mortality nor behavioral changes were observed among the saliva-injected animals during the study. All these animals exhibited typical locomotion and physical activity, with no signs of weakness or aggressiveness. No toxic reactions were observed for instance no anorexia, ataxia, piloerection, loss of weight, diarrhea, urination, breathing difficulty, or noisy breathing. On the other hand, injection of insulin at a dose of 20 U·kg^{-1} bw resulted in a hypoglycemic condition in all rats leading to the death of one rat. The other rats which survived showed less physical activity especially during the first two hours after injection. In contrast, injection of lower dose of insulin (10 U·kg^{-1} bw) led to hypoglycemic events which persisted for a shorter time of only two hours with no mortality. Additionally, the simultaneous administration of LSE and insulin induced hypoglycemic cases with no mortality.

Although the experiment took a short time, no toxic signs were observed when the tissue specimens taken from the treated animals by subcutaneous injection with 1 000 μg·kg^{-1} bw were compared with those taken from the normal control group. That means that both doses of LSE for such a period of time have no toxic effects. These observations were reinforced by the traditional leech therapy which did not document toxic events, even though leeches were directly applied to the patients. Nevertheless, there are some reports about post leeching infections [3].

4 Conclusions

To the best of our knowledge, this is the first demonstration that the salivary gland secretion of leeches (LSE) has an anti-hyperglycemic effect against type-1 like diabetes mellitus. It was shown that subcutaneous injection of LSE collected from the medicinal Malaysian leech, *H. manillensis*, was sufficient to obtain regular glycemic control, with no fatal post-injection hypoglycemic conditions during the study period. Moreover, it was found that both LSE and insulin at lower doses had a synergistic antihyperglycemic activity. This type of synergism suggests that LSE could be utilized as an adjuvant medication in order to reduce insulin dosage or to achieve acceptable control of blood glucose concentration instead of using mixtures of short- and long-acting insulin. The toxicity study presented in the current research suggested that leech saliva can be considered as a safe medication for diabetic patients. More studies of longer duration are needed to evaluate the effectiveness and toxicity of long-term usage. Moreover, the active compound(s) are still obscure which indicated the need for more researches aimed at isolating and identifying the anti-hyperglycemic agent(s) in leech saliva.

Acknowledgment

The authors gratefully thank Roy T. Sawyer and Fred O. P. Hechtel, Leeches Biopharm (UK) Ltd., for their highly appreciated assistance in leech taxonomy and identification.

References

[1] Whitaker IS, Izadi D, Oliver DW, *et al*. Hirudo medicinalis and the plastic surgeon [J]. *Brit J Plast Surg*, 2004, **57**(4): 348-353.

[2] Srivastava A, Sharma R. A brief review on applications of leech therapy [J]. *Arch Appl Sci Res*, 2010, **2**: 271-274.

[3] Porshinsky B, Saha S, Grossman M, *et al*. Clinical uses of the medicinal leech: A practical review [J]. *J Postgrad Med*, 2011, **57**(1): 65-71.

[4] Haycraft JB. On the action of a secretion obtained from the medicinal leech on the coagulation of the blood [J]. *Roy Soc of Lond*, 1884, **36**: 478-487.

[5] Corral-Rodríguez M, Macedo-Ribeiro S, Barbosa Pereira P, *et al*. Leech-derived thrombin inhibitors: From structures to mechanisms to clinical aspplications [J]. *J Med Chem*, 2010, **53**(10): 3847-3861.

[6] Knobloch K. Leeches in microsurgery – An evidence-based approach. *In*: Manjunatha Kinj R, Clemetson KJ, Markland FS, *et al*. (eds). *Toxins and Hemostasis: from Bench to Bedside* [M]. Dordrecht, The Netherlands: Springer Science, 2011: 735-745.

[7] Schmied B, Hoeffken HW, Hornberger W, *et al*. *Peptides with anticoagulant activity* [P]. 1995-2-28. US5393873.

[8] Merzouk A, Ghawi AM, Abdualkader AM, *et al*. Anticancer effects of medical Malaysian leech saliva extract LSE [J]. *Pharm Anal Acta*, 2012, (S15): 1-5.

[9] Susanto S. Leeches used to treat diabetes patients [Online]. Indonesia: *The Jakarta Post*, Bantul. 2008. Available: http://www.thejakartapost.com/news/2008/04/30/leeches-used-treat-diabetes-patients.html [Accessed October 9th, 2011].

[10] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding [J]. *Anal Biochem*, 1976, **72** (1-2): 248-254.

[11] Abeeleh MA, Ismail ZB, Alzaben KR, *et al*. Induction of diabetes mellitus in rats using intraperitoneal streptozotocin: A comparison between two strains of rats [J]. *Eur J Sci Res*, 2009,

32(3): 398-402.

[12] Lenzen S. The mechanisms of alloxan- and streptozotocin-induced diabetes [J]. *Diabetologia*, 2008, **51**: 216-226.

[13] Booth DA, Brookover T. Hunger elicited in the rat by a single injection of bovine crystalline insulin [J]. *Physiol Behav*, 1968, **3**(3): 439-446.

[14] Madubunyi II, Onoja SO, Asuzu IU. *In vitro* antioxidant and *in vivo* antidiabetic potential of the methanol extract of *Ficus glumosa* Del (Moraceae) stem bark in alloxan-induced diabetic mice [J]. *Comp Clin Pathol*, 2012, **21**(4): 389-394.

[15] Gupta S, Sharma SB, Bansal SK, *et al*. Antihyperglycemic and hypolipidemic activity of aqueous extract of *Cassia auriculata* L. leaves in experimental diabetes [J]. *J Ethnopharmacol*, 2009, **123**(3): 499-503.

[16] Kumar GPS, Arulselvan P, Kumar DS, *et al*. Anti-diabtic activity of fruits of *Terminalia chebula* on streptozotocin induced diabetic rats [J]. *J Health sci*, 2006, **52**(3): 283-291.

[17] Rhiouani H, El-Hilaly J, Israili ZH, *et al*. Acute and sub-chronic toxicity of an aqueous extract of the leaves of *Herniaria glabra* in rodents [J]. *J Ethnopharmacol*, 2008, **118** (3): 378-386.

[18] Katzung BG, Masters SB, Trevor AJ. *Basic and Clinical Pharmacology* [M]. Beijing, China: McGraw Hill Co., 2009: 727-747.

[19] Bösenberg LH, Zyl DGV. The mechanism of action of oral antidiabetic drugs: A review of recent literature [J]. *JEMDSA*, 2008,**13**: 80-88.

[20] Abdualkader AM, Merzouk A, Ghawi AM, *et al*. *The anticoagulant activity of Malaysian leech saliva extract. In*: *the Abstracts of the 25th Scientific Meeting of the Malaysian Society of Pharmacology and Physiology* [C]. selangor, Malaysia: University Putra Malaysia, 2011

[21] Ghawi AM, Abdualkader AM, Merzouk A, *et al*. Free radical scavenging activity of the medicinal Malaysian leech saliva extract, *Hirudinaria manillensis* [J]. *J Bioequiv Bioavail*, 2012, **14**: 1-4.

[22] Jenkins AJ, Hill MA, Rowley KG. Diabetes and oxidant stress. *In*: Holtzman JL. (ed.) *Atherosclerosis and Oxidant Stress: A new Prospective* [M]. New York: Springer Science + Business Media, LLC. 2008:123-158

[23] Masharani U. *Diabetes mellitus and hypoglycemia*. *In*: McPhee SJ, Papadakis MA. (eds.) *Current medicnal treatment and diagnosis* (49th ed) [M]. USA: McGraw Hill Co., 2010.