Mal J Nutr 22(2): 267 - 278, 2016

Glucose Lowering Effect of Horseradish Tree (*Moringa oleifera* Lam. Leaf Decoction in Alloxan-Induced Diabetic Mice

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ABSTRACT

Introduction: The study was conducted to determine the glucose lowering effect of horseradish tree leaf decoction (HTLD) in alloxan-induced diabetic mice. **Methods:** The leaves of the Institute of Plant Breeding Moringa 3 (IPBM3) strain of horseradish tree were used in this study. The median effective dose (ED_{50}) of IPBM3 HTLD was determined using normal Institute of Cancer Research (ICR) mice for 14 days and the computed ED_{50} was administered once, twice and thrice per day for 28 days via gavage in alloxan-induced diabetic mice. Fasting blood glucose level (FBGL), body weight, feed intake and water intake were determined weekly. Thin layer chromatography and *in vitro* glucose lowering activity experiments were also done to determine the major polyphenol found in IPBM3 HTL with glucose lowering activity. **Results:** Administration of the ED_{50}, 417 mg/20 mL/kgbw, once, twice and thrice per day for 28 days significantly reduced the mean FBGL of diabetic mice by more than 50% and the reduction was statistically comparable with the metformin-treated group. Mean body weights and feed intakes were normal and statistically comparable for all groups. Mean water intakes were slightly higher than in the non-diabetic control group. The major polyphenol in IPBM3 HTLD was found to be quercetin. The *in vitro* glucose lowering activity experiment showed that both petroleum ether and water extracts of IPBM3 HTL have glucose lowering activity similar to standard quercetin. **Conclusion:** The present results show that IPBM3 HTLD possesses glucose lowering activity and offers potential for use in the management of diabetes mellitus.

Key words: Blood glucose, diabetes mellitus, horseradish tree leaves, phenolics, quercetin

INTRODUCTION

Diabetes mellitus is an endocrine and metabolic disorder characterised by high glucose levels in the blood as a result of defects in the secretion and or action of insulin. Insulin is a hormone needed for the utilisation and storage of glucose in the body (Mahan & Escott-Stump, 2008). According to the World Health Organization (WHO, 2015), diabetes mellitus caused 1.5 million deaths in 2012 and more than 80% of diabetes mellitus deaths occur in low- and middle-income countries. Diabetes mellitus is also projected to be the leading cause of death in 2030. In the Philippines, it is the 8th leading

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cause of death and accounted for 20,239 deaths in 2011 (Department of Health [DOH], 2011). Moreover, the 8th National Nutrition Survey (NNS) conducted by the Food and Nutrition Research Institute of the Department of Science and Technology (FNRI-DOST) revealed an increase in the prevalence of diabetes mellitus among adults to 5.4% as compared to 4.8% in 2008 (FNRI-DOST, 2013).

Good management of diabetes mellitus rests on the use of oral hypoglycemic drugs or insulin, proper diet and exercise. In developing countries, oral anti-diabetic agents are costly and are not readily accessible to diabetic patients (Rao *et al*., 2010). In addition, there are also documented side effects with the use of these synthetic hypoglycemic drugs. Gastro-intestinal side effects, lactic acidosis (Inzucchi *et al.,* 2012), and vitamin B12 malabsorption (Mahajan & Gupta, 2010) have been observed with the use of biguanides, particularly metformin; bloating, flatulence, and abdominal discomfort have been observed with the use of α-glucosidase inhibitors (Cheng & Fantus, 2005); and hypoglycemia and weight gain with the use of sulfonylureas (Cheng &Fantus, 2005; Inzucchi *et al.,* 2012).

In recent years, there has been renewed interest in the use of herbal plants in the treatment of different diseases including diabetes mellitus. One of the plants that has been used in ayuverdic medicine for common ailments is horseradish tree (*Moringa oleifera*). It is a fast growing, perennial tree indigenous to the Himalayan foothills of South Asia from north-eastern Pakistan to northern West Bengal State in India and north-eastern Bangladesh (Roloff *et al.*,2009). Horseradish tree leaves in particular, have been demonstrated to possess anticancer (Khalafalla *et al.,* 2010), hepato-protective (Buraimoh, Bako & Ibrahim, 2011), hypolipidaemic (Atsukwei *et al.,* 2014), nephro-protective (Adeyemi & Elebiyo, 2014), and many other beneficial effects. However, studies on the glucose lowering activity of horseradish tree leaves are limited. Therefore, this study was conducted to determine the glucose lowering effect of the Institute of Plant Breeding Moringa 3 (IPBM3) strain of horseradish tree leaf tea decoction (HTLD) in alloxan-induced diabetic mice.

METHODS

Identification and selection of horseradish tree leaves (HTL)

The IPBM3 HTL from the Institute of Plant Breeding (IPB), University of the Philippines Los Baños (UPLB) was used in this study. The sample was selected and used in this study because it contains the highest total phenolic content among the different IPB strains of HTL, based on the analytical data of the Institute. The scientific name of the sample was validated by the Museum of Natural History, UPLB.

Preparation of powdered HTL

Fresh IPBM3 HTL sample leaves weighing 1650 g were washed with distilled water, oven dried at 40ºC (Miean & Mohamed, 2001) for 24 h using a convection oven, ground into powder using a milling machine, and sieved using 80 mesh to obtain 429 g powered IPBM3 HTL. The powdered sample was packed and sealed in laminated foils to ensure freshness. The moisture content of the powdered leaves was found to be 2.45% using the oven method.

Median effective dose (ED_{50}) determination

Twenty (20) healthy, 6- to 8-week-old male Institute of Cancer Research (ICR) mice purchased from the Food and Drug Administration (FDA) in Alabang, Muntinlupa City were used for the determination of the median effective dose (ED_{50}) of IPBM3 horseradish tree leaf decoction (HTLD). Animals were

randomly divided into four groups namely: control group (n=5), given distilled water; treatment group 1 (n=5), received 360 mg/20 mL/kgbw IPBM3 HTLD, low dose; treatment group 2 (n=5), given 720 mg/20 mL/kgbw IPBM3 HTLD, medium dose; and treatment group 3 (n=5), given 1440 mg/20 mL/kg bw IPBM3 HTLD, high dose.The three doses were arbitrarily chosen. The medium dose was chosen based on a preparation of horseradish tree leaf tea in humans. This was then translated into a dose for mice using the formula for dose translation based on body surface area by Reagan-Shaw, Nihal & Ahmad (2007). Low dose was determined by dividing the medium dose by two while high dose was twice the value of the medium dose.

Mice were housed individually in a polycarbonate cage with a stainless steel top at the small laboratory animal experimental room, Department of Basic Veterinary Sciences, College of Veterinary Medicine, University of the Philippines Los Banos under 12h:12h light/dark cycle with lights on at 7:00 A.M. and off at 7:00 P.M., 24±2ºC room temperature and 40-60% humidity. Commercial feeds and distilled water were provided *ad libitum*.

The IPBM3 HTLD at different concentrations of 360 mg/20 mL, 720 mg/ 20 mL, and 1440 mg/ 20 mL was prepared by placing the pre-weighed IPBM3 HTL powder in tea bags in 20 mL of hot water, brewed for 5 min at 70°C and allowed to cool down at room temperature. All treatments and distilled water for the control group were given once a day for 14 days via gavage. Fasting blood glucose levels (FBGL) of mice in all treatment groups were measured on day 0, 7 and 14. Observation was done daily and all animals were sacrificed after 14 days.

All mice-related procedures undertaken were approved by the Institutional Animal Care and Use Committee (IACUC) of the College of Veterinary Medicine, University of the Philippines Los Banos (Protocol Number: 2013-36).

In vivo evaluation of IPBM3 horseradish tree leaf decoction (HTLD) glucose lowering activity

Induction of diabetes mellitus in mice

A total of 110, 6- to 8-week-old, male, healthy ICR mice were injected once intra-peritoneally with 200 mg/kgbw of freshly prepared alloxan monohydrate (Sigma-Aldrich, St. Louis, MO, USA) using 1 mL syringe with 26 gauge needle to induce diabetes mellitus. Two weeks after administration of alloxan monohydrate, mice were fasted for 12 h and their FBGL was measured using a portable glucometer (Galaxy G-26®, S&S Enterprises, USA). Blood samples were collected in the tail vein. To further confirm successful induction of diabetes mellitus, alloxan monohydrate injected-mice with FBGL of ≥180 mg/dL were subjected to oral glucose tolerance test (OGTT). Fasting blood glucose level was measured before (0 min) and 30, 60, and 120 min post-gavaging of 20% glucose solution using a portable glucometer. Mice with FBGL of ≥ 200 mg/dL following OGTT were considered diabetic, and were used for the glucose lowering effect determination of IPBM3 HTLD.

Glucose lowering effect of IPBM3 HTLD

For *in vivo* glucose lowering activity in diabetic mice, the computed ED_{50} (417 mg HTL/20 mL distilled water/kg bw) using linear regression was used.

A total of 10 healthy non-diabetic and 50 alloxan-induced diabetic male ICR mice were randomly divided into six groups: Group 1 (n=10), non-diabetic mice given distilled water; Group 2 (n=10), diabetic mice given distilled water; Group 3 (n=10), diabetic mice given metformin (Merck, Germany) at a dose of 500 mg/kg bw;

Group 4 (n=10), diabetic mice given IPBM3 HTLD once a day at a dose of 417 mg/20 mL/kg bw; Group 5 (n=10), diabetic mice given IPBM3 HTLD twice a day at a dose of 417 mg/20 mL/kg bw and; Group 6 (n=10), diabetic mice given IPBM3 HTLD three times a day at a dose of 417 mg/20 mL/kg bw. All treatments were given via gavage for 28 days. Fasting blood glucose level was measured weekly. Feed and water intakes were measured daily while body weight was measured weekly. All animals were sacrificed after the 28-day experimental period.

Determination of major phenolic component of IPBM3 HTL using thin layer chromatography

The IPBM3 HTL sample was subjected to thin layer chromatography (TLC) for identification of major phenolic components following the method of Malbaša, Lončar & Kolarov (2004). Extrac-tion was done by placing 50 g of powdered IPBM3 HTL in 500 mL of hot distilled water; brewed for 5 min at 70°C, and then 15 mL of the water extracted IPBM3 HTL and 15 mL of petroleum ether were mixed in a separatory funnel for 5 min. After separation, the 15 mL petroleum ether layer was vaporised to dryness and then the dried sample was dissolved in 0.5 mL petroleum ether. Solutions for standard phenolic compounds like cathechin, chlorogenic acid, gallic acid, pyrogallicacid, and quercetin were prepared by dissolving 10 mg of each standard in 1 mL of methanol. Thin layer chromatography was performed using silica gel 60 F_{254} as stationary phase and chloroform: ethyl-acetate:formic acid (5:4:1) as mobile phase. Plates were sprayed with $FeCI_3$ (2% in ethanol). Water extract obtained from the first extraction was also subjected to TLC following the same procedure done in petroleum ether extract.

The retention factor (Rf) values of the standards and the spots observed from the two extracts were calculated using the formula below:

$$\frac{\text{distance traveled by the sample}}{\text{distance traveled by the solvent}}$$

In vitro **determination of glucose lowering activity of IPBM3 HTL extracts**

Both water and petroleum ether extracts of IPBM3 HTL which based on TLC results contain quercetin, were tested for *in vitro* glucose lowering activity using the glucose oxidase method by Khan *et al.* (2005). Standard quercetin at 0.005 mg and 0.01 mg was also tested using the same method to check if it has comparable glucose lowering activity with the extracts. Glucose solution (0.05 mL) of concentration 20, 40, 60, 80 and 100 mg/dL were placed in different test tubes, respectively. Then, 0.01 mL, 0.05 mL, and 0.10 mL of water extract (5g powder/ 100 mLdistilled water) and petroleum ether extract of the IPBM3 HTL were each added in separate test tubes and kept for 4 h. Subsequently, 5 mL of glucose oxidase enzyme was added to all test tubes and kept for 30 min in the dark at room temperature. The calibrated spectrophotometer was adjusted at 546 nm wavelength and results were recorded. Glucose concentration was calculated using the formula below:

$$\text{Glucose Concentration} = \frac{Au}{Ak} \text{ X C}$$

where

Au = absorbance of unknown (extracts of plants)

Ak = absorbance of known (standard glucose)

C= concentration of standard glucose

Statistical analysis

Data obtained were analysed using analysis of variance (ANOVA) followed by post-hoc Tukey-Kramer multiple comparison test with $P<0.05$ (SAS version 9.1).

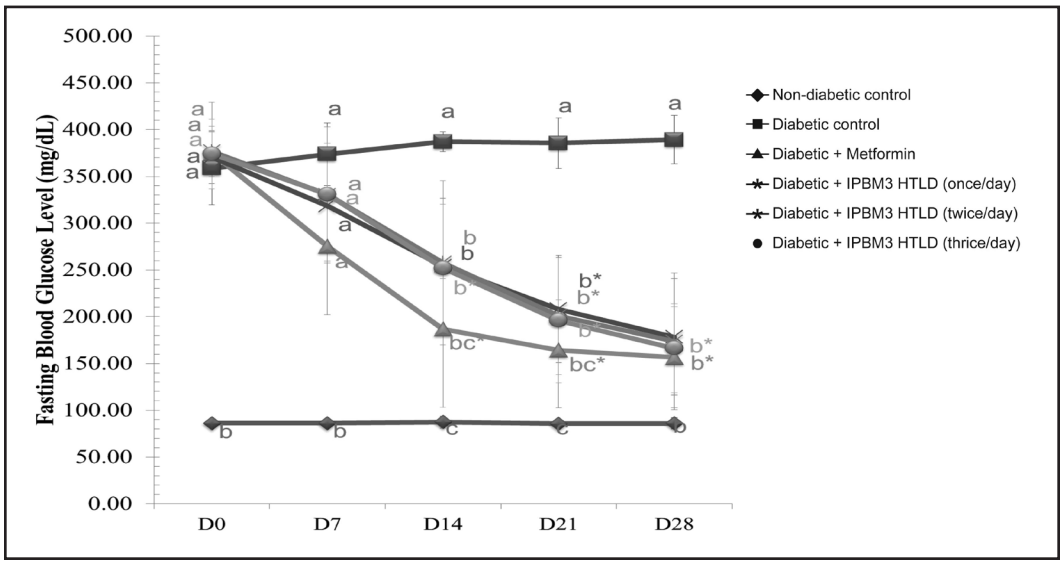

Figure 1. Fasting blood glucose level of non-diabetic control and alloxan-induced diabetic mice. Non-diabetic control given distilled water (20 mL/kgbw); Diabetic control group given distilled water (20 mL /kg bw); Diabetic group +standard metformin (500mg/ kg bw); Diabetic + HTLD (417 mg /20 mL/kg bw) once/day; Diabetic + HTLD (417 mg /20 mL/kg bw) twice/day; Diabetic +HTLD (417 mg/ 20 mL/kg bw) thrice/day. Values are mean ± (tab) (SEM). Values with dissimilar letters in each time point are significantly different, $p<0.05$. *Significantly different from day 0 in the same treatment, $p<0.05$

RESULTS

Determination of median effective dose of HTLD

After 14 days of HTLD administration, low, medium and high doses of HTLD were all effective in reducing the FBG levels of experimental mice. Using linear regression, the computed median effective dose was 417 mg HTL/20 mL distilled water/kg bw.

*I**n vivo** glucose lowering activity of HTLD*
blood glucose lowering effect

Treatment of IPBM3 HTLDat 417 mg/20mL/kg bw, once, twice, and thrice per day for 28 days significantly reduced the mean FBGL of alloxan-induced diabetic mice by more than 50% mean reduction as compared to baseline levels (Figure 1). The reductions achieved by the three treatments were statistically comparable to the mean percent reduction achieved by standard metformin. It was also observed that the mean FBGL of all the HTLD-treated groups and the metformin-treated group were already statistically comparable to the mean FBGL of the non-diabetic control mice group at day 28.

Body weights, feed intake, water intake of IPBM3 HTLD-treated alloxan-induced diabetic mice

Figure 2A, 2B and 2C present the mean body weights, feed intake and water intake, respectively of HTLD-treated diabetic mice. The mean body weights of all groups of mice throughout the 28 days study-period were within the normal range. Slight insignificant reduction in the mean body weights were observed in the HTLD-

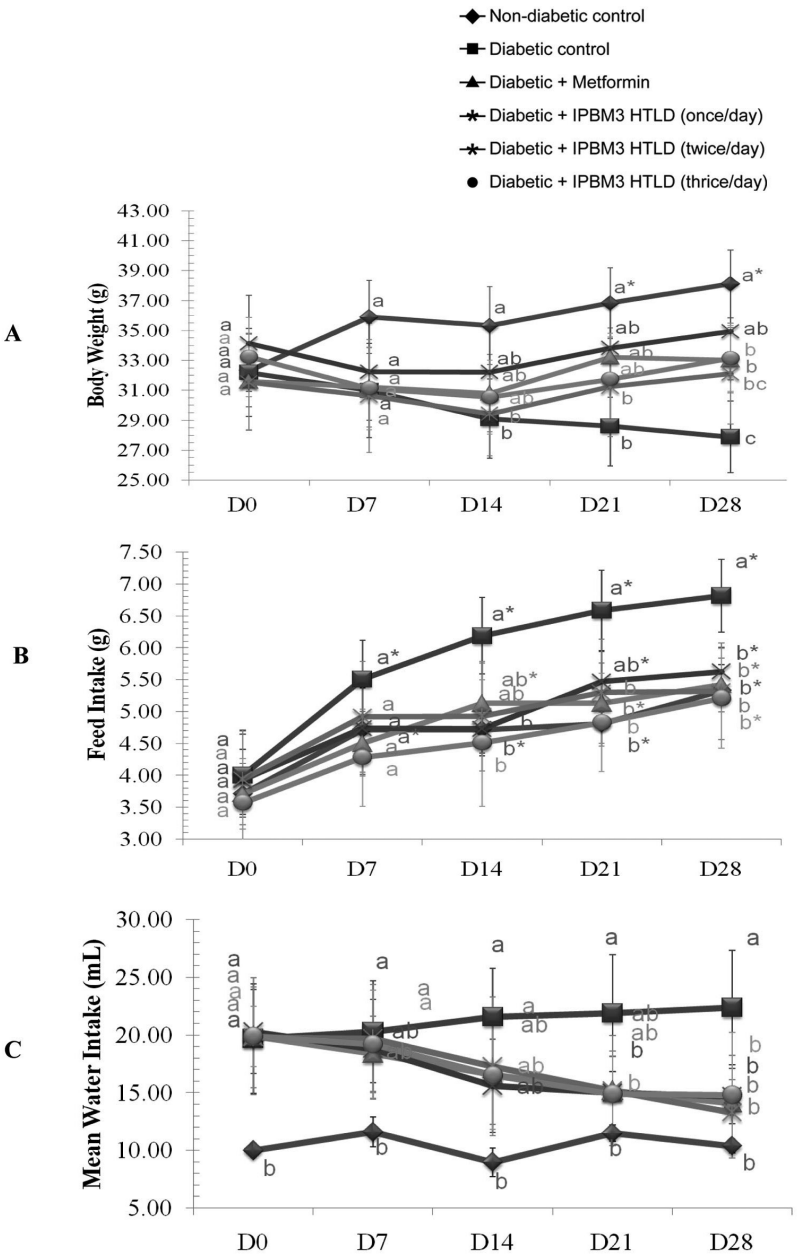

Figure 2. Body weight (A), feed intake (B) and water intake (C) of non-diabetic control and alloxan-induced diabetic mice. Non-diabetic control given distilled water (20 mL/kgbw); Diabetic control group given distilled water (20 mL /kgbw); Diabetic group +standard metformin (500mg/ kg bw); Diabetic + HTLD (417 mg /20 mL/kg bw) once/day; Diabetic + HTLD (417 mg /20 mL/kg bw) twice/day; Diabetic +HTLD (417 mg/ 20 mL/kg bw) thrice/day. Values are mean ± (tab) (SEM). Values with dissimilar letters in each time point are significantly different, $p<0.05$. *Significantly different from day 0 in the same treatment, $p<0.05$

treated groups and metformin-treated group until day 14, but improvement in the mean body weights was observed starting day 21.

With regard to feed intakes, all the treated groups had statistically comparable mean feed intake with that of the non-diabetic control group throughout the experiment period. On day 28, mean feed intakes of all the treated groups and the non-diabetic control group were significantly lower than that of the diabetic-control mice group.

As for water intake, the baseline mean intake of all diabetic mice groups was significantly higher than the mean water intake of the non-diabetic control group. But on day 28, it was observed that the mean water intake of the HTLD-treated mice groups and the metformin-treated mice group was already statistically comparable to the mean water intake of the non-diabetic control group.

Major phenolic component of IPBM3 HTL using thin layer chromatography

The major polyphenol found in both petroleum ether and water extracts of powdered IPBM3 HTL based on TLC is shown in Figure 3. It can be seen that the major polyphenol in petroleum ether extract was quercetin, as indicated by its Rf value of 0.85 (Table 1) which is the same as that of standard quercetin. As for the water extracted, two spots were observed. One was quercetin (upper spot), as indicated also by the Rf value, and another spot (lower spot) was not identified due to limited standards available in the laboratory. The lower spot might be a polar substance found in HTLD that was not extracted by the non-polar petroleum ether.

***In vitro* glucose lowering activity of HTLextracts**

Based on glucose oxidase method, both the petroleum ether extract and water extract of IPBM3 HTL at 0.01 mL had a glucose lowering activity percentage that was statistically comparable with 0.01 mg standard quercetin (Table 2).

DISCUSSION

The present study determined the *in vivo* and *in vitro* glucose lowering activity of IPBM3 HTLD using alloxan-induced diabetic mice and glucose oxidase method, respectively. In addition, the major polyphenol present in the leaves was also identified.

The study used the IPBM3 strain of HTL because it contains the highest total

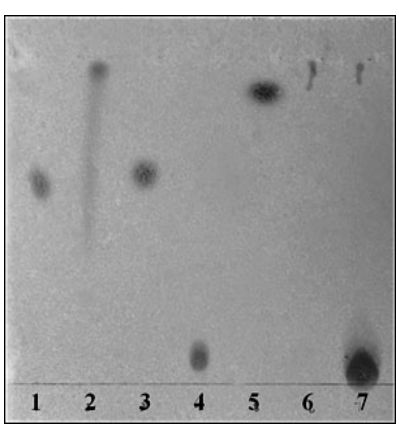

Figure 3. Thin layer chromatography of petroleum ether and water extracts of HTL. Standards: 1 (catechin), 2 (quercetin), 3(gallic acid),4 (chlorogenic acid), 5 (pyrogallic acid). Extracts: 6 (petroleum ether extract, 7 (water extract).

Table 1. Retention factor (Rf) values of standards and spots observed in HTL petroleum ether and water extracts.

Sample	*Rf value*
Standards	
1 (Catechin)	0.53
2 (Quercetin)	0.85
3 (Gallic acid)	0.56
4 (Chlorogenic acid)	0.06
5 (Pyrogallic acid)	0.79
Extracts	
6 (Petroleum ether)	0.85
7 (Water)	
Lower spot*	0.02
Upper spot *	0.85

*Spots from water extract found on the TLC plate (Figure 3)

Table 2. In vitro mean percent glucose lowering activity of horseradish tree leaf extracts and standard quercetin.

Sample	*Glucose Lowering Activity (%)*				
	20 mg/dL	*40mg/dL*	*60mg/dL*	*80mg/dL*	*100mg/dL*
Standard Quercetin					
0.005 mg	7^{c}	29^{cd}	59^{c}	82^{d}	53^{bc}
0.01 mg	8^{c}	28^{d}	54^{cd}	82^{d}	53^{bc}
Water Extract					
0.01ml	9^{c}	29^{cd}	55^{cd}	94^{cd}	56^{b}
0.05ml	8^{c}	28^{cd}	60^{c}	98^{c}	51^{bc}
0.10ml	55^{a}	75^{a}	100^{a}	185^{a}	91^{a}
Pet. Ether Extract					
0.01ml	8^{c}	26^{d}	52^{d}	90^{cd}	49^{c}
0.05ml	7^{c}	33^{c}	52^{d}	96^{c}	42^{d}
0.10ml	36^{b}	67^{b}	88^{b}	172^{b}	86^{a}

Means with dissimilar superscripts in a column are significantly different, $p<0.05$.

phenols among the different IPBM3 strains. Polyphenols are of interest because their consumption is associated with lowering of diabetes rates and cardiovascular disease (De Bock, Derraik & Cutfield, 2012).

In vivo testing showed that IPBM3 HTLD significantly reduced the mean FBGL of alloxan-induced diabetic mice. The blood glucose lowering effect of HTL was also observed in the study of Divi, Bellamkonda & Dasireddy (2012), using aqueous extract; and Ndong *et al.* (2007) using leaf powder. Based on literature, polyphenols are responsible for the hypoglycemic activity of HTL. According to Ndong *et al*. (2007), horseradish tree (*Moringa oleifera*) leaf powder contains a high concentration of quercetin-3-glycoside (1494.2 µmol/100dry

weight (dw)), rutin (1446.6 µmol100 g/dw), kaempferol glycosides (394.4 µmol/100 gdw), and other polyphenols such as chlorogenic acid (134.5 µmol/100 gdw). The glucose lowering effect of the leaves might be due to its quercetin-3-glucoside and fibre content.

In this study, based on TLC results, the major polyphenol found in IPBM3 HTL petroleum ether and water extracts was quercetin. *In vitro* testing of both extracts also showed that their glucose lowering activity was comparable to that of standard quercetin.

Quercetin is a flavonoid that has been demonstrated to have many beneficial biological effects to health, including antioxidative and free radical scavenging activity (Zheng *et al.,* 2005). It has been reported to have anti-diabetic activity by bringing about regeneration of pancreatic islets and increasing insulin release in streptozotocin-induced diabetes. Also, it has been reported to stimulate Ca^{2+} uptake from isolated islet cells thus suggesting it to be effective even in non-insulin dependent diabetes (Sandhar *et al.,* 2011).

According to Kangralkar, Patil & Bandivadekar (2010), oxidative stress and oxidative damage to the tissue commonly lead to development of chronic diseases, such as diabetes. Oxidative stress is suggested to be the underlying mechanism for diabetes mellitus and its complications. Moreover, there is also increasing evidence that Ca^{2+} plays an important role in the regulation of insulin-generated signals and β-cell functions. Elevated or sustained levels of cytosolic calcium have been shown to diminish insulin sensitivity and might participate in the pathogenesis of insulin resistance in Type 2 diabetes mellitus and other related metabolic disturbances (Balasubramanyam *et al.,*2001).

Alloxan induces diabetes by the action of reactive oxygen species with a simultaneous massive increase in cytosolic calcium concentration that causes rapid destruction of *β*-cells (Szkudelski, 2001). Antioxidants play an important part in the cell's defense mechanism against free radical damage (Abd El-Baky, 2011). The antioxidant property of quercetin that is present in HTLD and quercetin's ability to stimulate uptake of cytosolic calcium by the islets cells, as discussed above, might be the reasons for the glucose lowering activity of HTLD in alloxan-induced diabetic mice. According to Abd El-Baky (2011), quercetin is not only effective in generating insulin and inhibiting insulin resistance, but also in the protection of β-cells.

Slight insignificant reduction in the mean body weights of HTLD-treated mice followed by improvement in body weight was observed in this study. Similar observations were noted in the study of Shetti, Sanakal & Kaliwal (2012), where in alloxan-induced diabetes, the body weight of diabetic mice groups was reduced, and administration of ethanolic extract of black catnip (*Phyllanthus amarus*) ethanolic leaf with glucose lowering property led to improvement in body weight of diabetic-treated mice.

Weight loss is common in people with uncontrolled Type 1 diabetes mellitus despite the normal or even increased appetite. It is due to loss of body fluids which results from osmotic diuresis, and tissue loss because lack of insulin forces the body to use its fats and proteins stores as source of energy to provide the body's needs (Porth, 2006).

These might be the reasons why in spite of continued increase in the feed intakes (Figure 2B) of the diabetic groups, weight loss still occurred. The cause of slight continued weight gain of the diabetic-treated groups might be the result of blood glucose lowering effect of metformin (in the metformin-treated group) and HTLD (in the HTLD treated groups).

Similar to the findings of the present study, Hakkim *et al.*(2007) also reported

higher mean feed intake of diabetic control group using alloxan- induced diabetic rats compared to normal control and diabetic-treated rat groups with aqueous and ethanol extracts of tanners cassia (*Cassia auriculata* L.) flowers and glibenclamide. One of the three common symptoms of diabetes mellitus is polyphagia or excessive hunger, which results from cellular starvation and depletion of cellular stores of carbohydrates, proteins, and fats because cells cannot use glucose as source of energy (Porth, 2006). This might be the reason behind the findings of this study. The diabetic-control mice group consistently had high blood glucose levels (Figure 1) which indicate that the cells were unable to use glucose as energy source thus, cellular starvation occurred resulting in excessive hunger, and leading to increased feed intake.

The higher baseline water consumption of diabetic mice groups compared to non-diabetic control mice group and the reduction in the mean water consumption of the treated groups observed in this study are similar to the findings of Shan,Yang & Ren (2006) in their study using alloxan-induced diabetic mice. The authors observed that their diabetic treated groups with aqueous extract from the flower of elecampane (*Inula japonica* (IJ)) and its two fractions (IJR and IJP), had significant reduction in the mean water intake compared to the diabetic control group.

Another symptom of diabetes mellitus is excessive thirst or polydipsia due to water loss. When the levels of glucose, an osmotically active molecule, in the blood are high enough, the amount of glucose filtered by the glomeruli of the kidney exceeds the amount that the renal tubules can reabsorb. As a result, glycosuria occurs which is accompanied by losses of large amount of water in the urine. Thirst results from the intracellular dehydration that occurs when blood glucose levels increase and water is pulled out of body cells (Porth, 2006). This explains the higher water consumption of the diabetic groups compared to the non-diabetic control group. The reduction in the mean water intakes of the HTLD-fed mice groups and the metformin-treated mice groups might be attributed to the reduction in the mean fasting blood glucose level of the diabetic mice (Figure 1) as a result of treatment administration.

CONCLUSION

In conclusion, based on the present findings, IPBM3 HTLD possesses glucose lowering effects in alloxan-induced diabetic mice, and the effect might be due to its quercetin content. However, further studies need to be done to determine other phenolic components found in HTLD that might also have contributed to its glucose lowering activity.

Conflict of interest

The authors declare that there is no conflict of interest.

ACKNOWLEDGEMENTS

The authors are grateful to the DOST-ASTHRDP NSC for funding this research, to Prof Felicito Rodriguez for providing secondary data and for his guidance in the chemical analysis, and to Dr Rodel Maghirang for providing the samples.

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