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Antidiabetic Activity of *Phyllanthus amarus* Schum and Thonn (Euphorbiaceae) on Alloxan Induced Diabetes in Male Wistar Rats

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Abstract: This study was undertaken to investigate the antidiabetic effect of aqueous and hydroalcoholic extract of *Phyllanthus amarus* Schum and Thonn, a medicinal plant used in Togo for treating diabetes and many others diseases. Diabetes was induced in fasted rats (12 h) by a single intraperitoneal injection of 120 mg kg⁻¹ of alloxan monohydrate. Two doses (500 and 1000 mg kg⁻¹) of the both extracts of *Phyllanthus amarus* were administered orally to diabetic rats. The normal control group receives distilled water only. After 15 days treatment, body weight gain, blood glucose level, serum insulin, total cholesterol, triglycerides and malondialdehyde were evaluated. At the doses tested, aqueous and hydroalcoholic extract of *P. amarus* decrease significantly blood glucose level after 15 days of administration. Aqueous extract reduce body weight gain contrary to hydroalcoholic extract. Serum insulin increases in group treated with extracts in compared to diabetic control group. The hydroalcoholic extract reduce the malondialdehyde concentration in the serum. Values of total cholesterol and triglycerides are similar in all the groups. This study demonstrated the potential antidiabetic property of aqueous and hydroalcoholic extract of *Phyllanthus amarus* thus justifying its traditional usage.

Key words: *Phyllanthus amarus*, alloxan, antidiabetic, antioxidant

INTRODUCTION

Diabetes is the world's largest endocrine disease involving metabolic disorders of carbohydrate, fat and protein. The number of diabetic patients is gradually increasing worldwide. Currently there are over 150 million diabetics worldwide and this is likely to increase to 300 million or more by the year 2025 (IDF, 2003).

Diabetes mellitus is characterized by high blood glucose levels due to absolute or relative deficiency of circulating insulin levels. The increased blood glucose level generates glucose auto oxidation and auto oxidative glycosylation of proteins which leads to oxidative stress by increasing the reactive oxygen species. Reactive oxygen species contribute to the development of diabetic complications (Wiernsperger, 2003).

Though different types of oral hypoglycemic agents are available along with insulin for the treatment of diabetes mellitus, there is a growing interest in herbal remedies, due to the side effects associated with these therapeutic agents (Proks *et al.*, 2002). More than 400 species have been reported to present antidiabetic activity (Yeh *et al.*, 2003).

Phyllanthus amarus Schum and Thonn is a small shrub which belongs to Euphorbiaceae family. The plant is distributed in tropical and subtropical countries and is used for the treatment of various diseases and disorders, such as jaundice, constipation, diarrhea, kidney ailments. Phytochemistry of this species is well known and reveals the presence of gallotannins, lignans like phyllanthin and hypophyllanthin and alkaloids such as isobubbialine and epibubbialine (Joseph and Raj, 2011; Annamalai and Lakshmi, 2009). Compounds isolated from *Phyllanthus* were found to possess anti-viral property against hepatitis B virus *in vitro* (Huang *et al.*, 2003). Hydroalcoholic extract of *Phyllanthus amarus* induced liver regenerative effect against liver cell injury (Chattopadhyay *et al.*, 2006). The extract also possessed anti-tumour, anti-carcinogenic and anti-inflammatory properties (Kassuya *et al.*, 2006; Kiemer *et al.*, 2003). Gallotannin and ellagitannins (geraniin and corilagin) were shown to be the most potent mediators of the antiviral HIV activity (Notka *et al.*, 2003). Studies revealed the hypoglycemic activity of the plant (Raphael *et al.*, 2002; Adeneye *et al.*, 2006) but no studies have been conducted to show the effect of the plant on serum

insulin level (hormone involved in regulating blood glucose). This study was undertaken to evaluate the antidiabetic effect (effect on blood glucose level, on serum insulin, serum triglyceride and cholesterol) of *Phyllanthus amarus* in alloxan induced diabetic rats.

MATERIALS AND METHODS

Chemicals: Alloxan monohydrate, DPPH (2, 2-diphenyl-1-picrylhydrazyl) and AAPH (2, 2'-azobis 2 amidinopropanedihydrochloride) were purchased from sigma France.

Plant material: Whole plant of *P. amarus* was collected in July 2007 from Tsevie (at 36 km North-East of Lome, Togo). A voucher specimen was identified and kept in the herbarium of the Laboratory of Botany and Plant Ecology (Faculty of Science/University of Lome). Plant was cleaned out with water and cut into small pieces, dried and extracted with water or ethanol/water (1:1 v/v) as following. Plant material (200 g) was soaked in water (2 L) and heated in a water bath at 80°C for 1 h for the aqueous extract. The same quantity was macerated 72 h in ethanol/water solution (2 L) for hydroalcoholic extract. The crude extracts were filtered with Whatman paper and evaporated in vacuo at 40°C using a rotary evaporator (Buchi, France). The yield of the preparation was 16% (w/w) for Aqueous Extract (AE) and 13% (w/w) for Hydroalcoholic Extract (HAE).

Animals: Male Wistar rats (200-250 g) were obtained from DEPRE animal center (France) and were fed with conventional chow (toxins-free according to EU regulation) and tap water ad libitum. Rats were acclimatized for 7 days under standard environmental conditions of temperature, relative humidity and dark/light cycle. The animals were divided randomly into different groups for the experiment and were deprived of food except water 16 h prior to the experiments. All animal experiments were conducted according to the law set by European and national authorities and following the agreement of the ethical working group at the University Bordeaux 2.

Induction of diabetes in rats: Diabetes was induced in fasted rats (12 h) by a single intraperitoneal injection of 120 mg kg⁻¹ of alloxan mono hydrate. Alloxan was freshly dissolved in distilled water and the injection volume was 20 mL kg⁻¹. The diabetic state was assessed by measuring the non-fasting blood glucose level 10 days after alloxan injection. Rats with blood glucose level in a range of

200-500 mg dL⁻¹ with polyuria and glucosuria were selected for the experiment. Blood glucose levels were measured with a glucometer (Optium Sense, France) on the tail vein.

Experimental design: Rats were divided into seven groups of seven rats:

- **Group 1:** Normal control rats received distilled water alone
- **Group 2:** Diabetic control rats received distilled water alone
- **Group 3:** Diabetic rats, treated with 500 mg kg⁻¹ AE
- **Group 4:** Diabetic rats, treated with 1000 mg kg⁻¹ AE
- **Group 5:** Diabetic rats, treated with 500 mg kg⁻¹ HAE
- **Group 6:** Diabetic rats, treated with 1000 mg kg⁻¹ HAE
- **Group 7:** Diabetic rats, treated with 100 mg kg⁻¹ metformin (standard oral hypoglycemic agent)

The plant extract dissolved in distilled water was fed orally by gastric intubation during 15 days. All the drugs were given as a single dose in the morning. Body weight and blood glucose levels were estimated on days 1, 7 and 15. After the last dose, animals were anaesthetized with ether. Blood samples collected by the orbital sinus puncture were centrifuged at 500× g and serums were kept at -20°C. Total Cholesterol (TC) and Triglyceride (TG) were estimated by enzymatic method using reagent kit (BioMérieux, France); insulin concentration was determined using commercially available enzyme-linked immunoassay kit (ELISA kit, USA). Malondialdehyde concentration was measured spectrophotometrically.

Antioxidant activities: The free radical scavenging activity of the extracts was determined by DPPH (2, 2-diphenyl-1-picrylhydrazyl) test and AAPH (2, 2'-azobis 2 amidinopropanedihydrochloride) test.

The free radical scavenging activity of the extracts was measured *in vitro* by the stable 2, 2-diphenyl-1-picrylhydrazyl free radical assay. Briefly, plant extract (0.25 mL) was added to 1.5 mL of methanolic solution of DPPH. Absorbance was determined at 517 nm after 30 min of incubation at room temperature. Quercetin was used as standard controls.

The percentage inhibition activity was calculated from:

$$\frac{(Ac-Ae)}{Ac} \times 100$$

where, Ac is the absorbance of the control and Ae is the absorbance of the extract/standard.

IC₅₀ value denotes the concentration of sample, which is required to scavenge 50% of DPPH free radicals.

AAPH is a free radical generator inducing hemolysis in RBC (red blood count). The free radical scavenging activity of the extracts was determined by the method described by Sekiya *et al.* (2002).

Statistical analysis: All data were expressed as Mean±SEM. Statistical differences between treated groups and controls were determined by Analysis of Variance (ANOVA) followed by Fisher LSD test using Systat 10.0. Differences between groups were considered significant for p<0.05. Graphs were performed using Graph Pad Prism 4.

RESULTS

Effect of the extracts of *P. amarus* on body weight in diabetic rats: Fourteen days after the treatment, diabetic control showed significant reduction in their body weight compared to normal control (Table 1). The body weight gain was 15% in the diabetic control vs 40.8 % in the normal control. The aqueous extract (500 and 1000 mg kg⁻¹) reduced body weight while the hydroalcoholic extract caused body weight gain in diabetic rats.

Effect of the extracts of *P. amarus* on blood glucose level in diabetic rats: A marked rise in blood glucose level was observed in diabetic control compared to normal control rats (Table 2). At 500 and 1000 mg kg⁻¹, the aqueous extract causes a significant decrease (p<0.05) in blood glucose level on 15th day compared to diabetic control. The hydroalcoholic extract decrease significantly (p<0.001) blood glucose level at 1000 mg kg⁻¹.

Table 1: Effect of aqueous extracts (AE) and hydroalcoholic extract (HAE) on body weight gain (BWG)

Groups	Body weight (g)		BWG %
	Day 1	Day 15	
C	171.25±1.98	241.25±4.17	40.8
DC	219±14	251.83±17.97	15
AE500 mg kg ⁻¹	189±17.76	200.71±35.26	6.2
AE 1000 mg kg ⁻¹	224.28±10.78	238.71±11.66	6.4
HAE 500 mg kg ⁻¹	147±7.66	184.6±13.41	25.5
HAE 1000 mg kg ⁻¹	219.33±11.17	254.83±15.93	16.18
Met 100 mg kg ⁻¹	226.16±16	247±22.4	9.2

C: Normal control; DC: Diabetic control; AE 500, AE 1000 mg kg⁻¹: Diabetic rats fed with aqueous extract, respectively at 500 and 1000 mg kg⁻¹; HAE 500, HAE 1000 mg kg⁻¹: diabetic rats fed with hydroalcoholic extract, respectively at 500 and 1000 mg kg⁻¹; Met 100 mg kg⁻¹: Diabetic rats fed with metformin at 100 mg kg⁻¹. The body weight gain (BWG) % represent (D₁₅-D₁)/D₁×100. The data were expressed as Mean±SEM (n = 7) and evaluated by ANOVA followed by Fisher LSD test at 5%

Effect of the extracts of *P. amarus* on biochemical parameters: Table 3 shows the levels of serum insulin, total cholesterol, triglycerides and malondialdehyde concentration in normal and diabetic rats. In diabetic control, serum insulin level was significantly decreased (p<0.001) whereas serum malondialdehyde was significantly (p<0.05) increased in compared with the normal rats. Diabetic group treated with the extracts (500 and 1000 mg kg⁻¹) showed significant reduction in serum insulin level compared to control. Only the hydroalcoholic extract reduced significantly the concentration of serum malondialdehyde compared to control. Values of total cholesterol and triglycerides are similar in all the groups.

Antioxidant activities: Table 4 shows the free radical scavenging activity of the extracts determined by DPPH. Aqueous and hydroalcoholic extracts exhibited mild significant free radical scavenging activity compared to

Table 2: Effect of different doses of aqueous extracts (AE) and hydroalcoholic extract (HAE) on blood glucose level

Groups	Blood glucose level (mg dL ⁻¹)		
	Day 1	Day 7	Day 15
C	91.62±3.41	82.25±5.9	80.75±3.37
DC	327±32.56 ^{aaa}	311±37.23 ^{aaa}	374.5±34.27 ^{aaa}
AE 500 mg kg ⁻¹	337.14±33	235.28±38	210.28±39 ^b
AE 1000 mg kg ⁻¹	341.57±35.30	223.71±47.74	264.5±35.6 ^b
HAE 500 mg kg ⁻¹	306.8±35.5	364.8±77.17	441±50.56
HAE 1000 mg kg ⁻¹	282±37.72	257.6±44.4	187.6±20.3 ^{bbb}
Met 100 mg kg ⁻¹	299.5±18.34	336.5±56.24	225±35.67 ^b

C: Normal control; DC: Diabetic control; AE 500 mg kg⁻¹, AE 1000 mg kg⁻¹: Diabetic rats fed with aqueous extract, respectively at 500 and 1000 mg kg⁻¹; HAE 500, HAE 1000 mg kg⁻¹: Diabetic rats fed with hydroalcoholic extract respectively at 500 and 1000 mg kg⁻¹; Met 100 mg kg⁻¹: Diabetic rats fed with metformin at 100 mg kg⁻¹. The data were expressed as Mean±SEM (n = 7) and evaluated by ANOVA followed by Fisher LSD test at 5%. ^{aaa}p<0.001 vs Normal Control; ^bp<0.05, ^{bbb}p<0.01 and ^{bbb}p<0.001 vs Diabetic control

Table 3: Effect of aqueous extracts (AE) and hydroalcoholic extract (HAE) on serum insulin, MDA, triglyceride and total cholesterol

Groups	Insulin	MDA	TG	TC
	(ngmL ⁻¹)	(pmolmL ⁻¹)	(g L ⁻¹)	(g L ⁻¹)
C	4.5±0.55	6.7±0.31	1.38±0.2	0.76±0.046
DC	0.78±0.13 ^{aaa}	8±0.43 ^a	1.18±0.21	1.11±0.1
AE 500 mg kg ⁻¹	4.21±0.88 ^{bb}	6.8±0.66	1.54±0.16	0.93±0.07
AE 1000 mg kg ⁻¹	2.62±0.73 ^b	7.42±0.52	1.12±0.26	1.07±0.06
HAE 500 mg kg ⁻¹	2.64±0.83 ^b	6.2±0.58 ^b	1.17±0.12	1.06±0.04
HAE 1000 mg kg ⁻¹	2.55±0.53 ^b	6.6±0.4 ^b	0.95±0.11	0.86±0.062
Met 100 mg kg ⁻¹	1.34±0.47	6.2±0.58 ^b	1±0.16	0.93±0.09

C: Normal control; DC: Diabetic control; AE 500 mg kg⁻¹, AE 1000 mg kg⁻¹: Diabetic rats fed with aqueous extract, respectively at 500 and 1000 mg kg⁻¹; HAE 500, HAE 1000 mg kg⁻¹: diabetic rats fed with hydroalcoholic extract, respectively at 500 and 1000 mg kg⁻¹; Met 100 mg kg⁻¹: Diabetic rats fed with metformin at 100 mg kg⁻¹. The data were expressed as Mean±SEM (n = 7) and evaluated by ANOVA followed by Fisher LSD test at 5%. ^{aaa}p<0.001 vs Normal control; ^bp<0.05, ^{bb}p<0.01 vs Diabetic control

Table 4: Antioxidant activities of aqueous and hydroalcoholic extracts (AE and HAE) *in vitro*

	DPPH			AAPH		
	AE	EHA	QR	AE	HAE	AA
IC ₅₀ (µg mL ⁻¹)	514.58	447.92	21.8	0.13	>0.25	0.07

IC₅₀: Concentration which scavenge (*in vitro*) 50% of DPPH and AAPH free radicals. Quercetin (QR) and Ascorbic Acid (AA) were used as standard controls

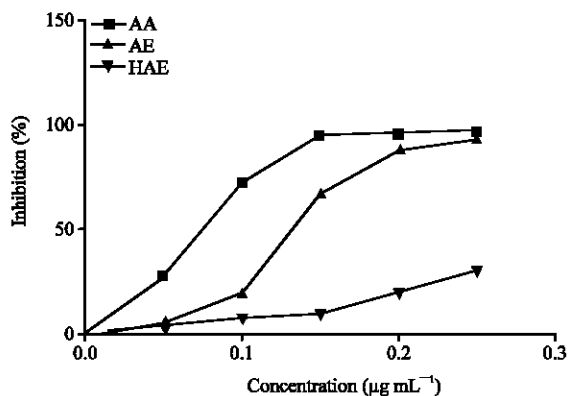


Fig. 1: Peroxyl radical scavenging effect of *P. amarus* extracts (AE and HAE) *in vitro*. Ascorbic Acid (AA) was used as positive control. Each point represent Mean±SEM (n = 4)

quercetin (standard control). IC₅₀ was respectively 514.58 and 447.92 µg mL⁻¹ for aqueous and hydroalcoholic extract.

The peroxyl radical scavenging effects were also examined. Both extracts showed dose-dependent antioxidant activity. The aqueous extract presented the strongest effect but this effect was lower than that of ascorbic acid used as standard control (Fig. 1).

DISCUSSION

In the present study, alloxan-induced diabetes in rat approximates non-insulin-dependent diabetic state with non-fasting blood glucose ranging between 200 and 500 mg dL⁻¹. Alloxan was described for the first time by Brugnalli in 1818. Several studies were conducted *in vitro* and *in vivo* to understand the cytotoxic effect of this drug, however, its precise mode of action remains a controversial subject (Janjic *et al.*, 1999). Alloxan specifically interacts with beta cells pancreatic generating oxygen free Radicals (ROS) responsible for its toxic effects (Weaver *et al.*, 1978; Boquist *et al.*, 1983). It is well reported that, partial destruction of beta cells causes a decrease in insulin secretion which is materialized by a chronic hyperglycemia (Ene *et al.*, 2008).

The effect of hydroalcoholic and aqueous extracts in diabetic rats compared to controls shows a significant decrease in diabetic blood glucose at 15th day of treatment. The hydroalcoholic extract was more effective at the end of the treatment. Alloxan caused body weight reduction, which was reversed by the hydroalcoholic extract at 500 mg kg⁻¹. Diabetic group treated with aqueous extract showed a severe body weight reduction compared to diabetic controls. The values found are similar to those of animals treated with metformin (standard reference). The effect of aqueous extract on body weight could be attributed to tannins which are more abundant in this extract than hydroalcoholic. Indeed, several studies have shown that tannins have antinutritional properties that occur in rodents by weight loss due to complexing proteins in the intestinal lumen (Butler, 1992; Carbonaro *et al.*, 2001) or by reduction in food intake (Oliveira *et al.*, 2005).

After 15 days of daily administration, insulin levels in the groups treated with aqueous extract and hydroalcoholic extract was significantly increased compared to untreated controls group. Our previous study reveals that aqueous and hydroalcoholic extract of *P. amarus* reduced significantly blood glucose level in glucose tolerance test but had no effect on fasted blood glucose level suggesting an extra pancreatic mechanism of the extract (Lawson-Evi *et al.*, 1997). Then, increased insulin levels can be attributed to glucose utilization in peripheral tissues. But it is possible that increased insulin concentration was due to beta cell regeneration as shown by Takasawa and Okamoto (2002). In the same way, increased insulin activity on liver cell regeneration by *Phyllanthus amarus* was reported by Chattopadhyay *et al.* (2007). At this time, no study has been conducted to show the action of the plant extract on beta cell regeneration but previous study in our laboratory had shown that low concentrations (<80 µg mL⁻¹) of aqueous and hydroalcoholic extract of *Phyllanthus amarus* led to 10-15% Caco-cell proliferation (Lawson-Evi *et al.*, 2008).

Abnormally high levels of free radicals in diabetic animals, cause membrane and tissue damage due to lipid peroxidation. Present result showed that the rate of malondialdehyde, a marker of lipid peroxidation, decreased significantly in the groups treated with hydroalcoholic extract compared to untreated controls. This confirms the antioxidant activity of extracts evaluated *in vitro* by induction of hemolysis rat erythrocytes and scavenging of free radical DPPH (2, 2-diphenyl-1-picrylhydrazyl). Antioxidant activity of *P. amarus* was confirmed by Shokunbi and Odetola (2008) and Kumaran and Karunakaran (2007). As a new strategy for alleviating the oxidative damage who leads to diabetes complications, interest has grown in the usage of natural antioxidants.

CONCLUSION

From the present study, it is concluded that *Phyllanthus amarus* extract exhibited antidiabetic activity by increasing serum insulin level in alloxanised rats. According to the antidiabetic and antioxidant activities, *Phyllanthus amarus* can help in the treatment of diabetes and in the prevention of its complications.

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