Preliminary phytochemical screening, antioxidant and anti-hyperglycaemic activity of *Moringa oleifera* leaf extracts

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Abstract: *Moringa oleifera* plant has been widely used for a vast number of folkloric medicinal purposes. The research aimed to investigate the antioxidant and antihyperglycaemic activity of *Moringa oleifera* leaf extracts obtained using different solvent systems for extraction. The solvent extracts of *Moringa oleifera* were: water extract (100% MoWE), 50% Methanolic extract (50% MoME), 100% Methanolic extract (100% MoME), 50% Ethanolic extract (50% MoEE), and 100% Ethanolic extract (100% MoEE). The *in vitro* antioxidant activity was evaluated by the use of the 1,1-diphenyl-2-picryl hydrazyl (DPPH) scavenging assay which showed the hydro-alcoholic extracts to have the highest reducing power, though lower than that of the standard, vitamin C. The hypoglyceamic activity was evaluated for the extracts at graded doses of 200mg/kg, 300mg/kg and 400mg/kg in wistar rats. Relative to the positive control, all treatment groups showed a significant statistical decrease in blood glucose levels. The decrease trends as metformin (84.14%) ≥50% MoEE 300mg/kg (83.72%) >MoWE 300mg/kg (82.42%) ≥ 50% MoEE 200mg/kg (82.32%) >100% MoEE 400mg/kg (81.96%) >50% MoME (80.69%) >100% MoME 300mg/kg (78.47%) >50% MoME 200mg/kg (66.34%). Overall, the 50% MoEE at a dose of 300mg/kg showed superior antioxidant properties, weight restorative and pronounced hypoglyceamic effects. The weight restorative effect of high dose alcoholic extract of *Moringa oleifera* was also observed in the study. This study establishes novel and foundational considerations for further isolation and characterization studies for the hypoglyceamic compounds in the plant.

Keywords: Diabetes, Metformin**,** *Moringa oleifera*, hypoglyceamia, antioxidant.

INTRODUCTION

Diabetes mellitus is a metabolic disorder that alters the body's ability to use insulin, a pancreatic hormone, to aid the conversion of glucose to glucagon. This disorder is clinically characterized by hyperglycemia, glycosuria, hunger, thirst and gradual loss of weight. In developing countries, diabetes has been recognized as one of the prime causes of death owing to its high prevalence attributed to lack of modern diagnostic facilities for the early detection of the disease (Ogbonnia *et al*., 2010) and increased consumption of staple foods implicated in the disease progression. Globally, diabetes mellitus has an estimated prevalence of 4.4% by the year 2030 such that the incidence worldwide is expected to rise to 366 million in 2030 from the 171 million statistics of 2000 (Ruangwatcharin *et al*., 2007).

Among the many aetiologies of diabetes, excessive oxidative stress has been linked to the pathogenesis of diabetes mellitus and its complications. Oxidative stress and damage to the tissue are common effects of chronic diseases like diabetes, rheumatoid arthritis and atherosclerosis (Kangralkar *et al*., 2010). In studies involving experimental diabetes, alloxan has been widely used to destroy pancreatic beta-cells with specific selectivity (Singh and Maheshwari, 1983).

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Medicinal plants have been reported to be sources of potential therapeutic agents against various diseases owing to their biodiversity and presence of a wide array of bioactive secondary metabolites (Farombi, 2003). *Moringa oleifera L.* (Moringaceae) is a multifunctional plant with vast economic, nutritional and health potentials (Ogbunuga for *et al*., 2011). Traditionally, the plant is used for the treatment of ailments such spasm, diarrhoea, as diuretic and stimulant in paralytic affliction, epilepsy and hysteria (Quisumbing, 1978).

Due to the vast ethnomedicinal uses and research on *Moringa oleifera*, this study aims to evaluate the effects of different commonly used extraction solvents on the hypoglyceamic potential of *M. oleifera* and thereafter establish the best solvent system for future studies.

MATERIALS AND METHODS

Chemicals and reagents

Alloxan monohydrate (Sigma-Aldrich Co. UK), 1,1-diphenyl-2-picryl hydrazyl (DPPH), ACCU-CHEK glucometer test kit (Diagnostic kit).

Plant sample collection and identification

The *Moringa oleifera* leaves were collected from the suburb of Tanke premises in Ilorin, Kwara State in June, 2013. The plant was authenticated by a certified taxonomist in the herbarium unit of the Department of Botany, Faculty of Science, University of Lagos, Nigeria, and was assigned a voucher number LUH 6077. The

leaves were air-dried at room temperature for one week until a constant weight was obtained and then pulverized using a high capacity blending machine (Master Chef$^{(R)}$). The total weight of *Moringa oleifera* powdered sample obtained was 1158g

Plant extraction

The pulverized *M. oleifera* leave sample was divided into 5 portions, and each portion was cold-macerated at 25°C for 6 days according to a method described by (Naznin, *et al*., 2008) using five different solvent systems: 100% Methanol, 100% Ethanol, Water-Methanol (50/50), Water-Ethanol (50/50) and 100% Water to obtain the different extracts labelled respectively as 100% MoME, 100% MoEE, 50% MoME, 50% MoEE and 100% MoWE. The water portion was cold-macerated for 24 hours. At the end of each maceration, filtration was carried out on each portion and the filtrate concentrated using a rotary evaporator. The concentrate of each solvent system was then freeze-dried. The weights of the extracts were obtained and the percentage yields were calculated as 100% MoME (2.49%), 100% MoEE (3.17%), 50% MoME (9.42%), 50% MoEE (8.52%) and 100% MoWE (6.47%).

Phytochemical screening of the plant extracts

The presence of different phytoconstituents was tested for by using standard procedures as described by (Trease, 2002; Sofowora, 1993; Siddiqui and Ali, 1997). Prior to each screening, a small portion of the freeze dried extracts were dissolved with a little quantity of distilled water.

Experimental animals

Seventy healthy male and female wistar rats (12 weeks old) of average weight of 100g, were sourced from the Animal Facility of the Veterinary Medicine Department, University of Ibadan, Oyo State. The animals were kept in well ventilated plastic cages, in the Animal Facility of the College of Medicine, University of Lagos, under standard atmospheric conditions, 12-12 hours light and dark cycle, to acclimatized for two months. they were fed with Grower's Mash (Aliment croissance) manufactured by livestock feeds Plc, Ikeja and clean drinking water. They were sub-grouped into 4-5 rats/group.

Determination of antioxidant activity of the M. oleifera extracts against DPPH (Free radical scavenging activity)

The free radical scavenging activity was assayed by a slightly modified method of (Brand-Williams *et al*., 1995; Braca *et al*., 2002). Vitamin C was used as standard and the antioxidant activities of the plant extracts were assessed on the basis of their radical scavenging effect of the DPPH radical. Serial dilutions of the extracts were prepared to obtain 100, 150, 200, 250 and 300µg/ml. A stock solution of vitamin C was also prepared from which 20, 40, 60, 80 and 100µg/mL dilutions were prepared in methanol. 2mL methanol and 0.5mL of 1mM DPPH solution was added into the standard and extract concentration samples. The readings were determined by measuring the UV/Visible absorbance of the different concentrations at 517nm and triplicate readings were obtained. A blank solution was prepared and measured containing 2mL of methanol and 0.5 ml of a 1mM DPPH. The radical scavenging activity was calculated using the formula:

$$\% \text{ Inhibition} = \frac{(AB - AA)}{AB} \times 100$$

where A_B is the absorption of the blank sample and A_A is the absorption of the test extract solutions.

Induction of diabetes and screening for diabetic rats

Experimental diabetes was induced by administering a single intra-peritoneal dose of freshly prepared alloxan solution (150mg/kg) prepared in 0.9% (w/v) normal saline solution to a group of rats fasted overnight (Lino Cde *et al*., 2004). Following three days post-alloxan administration, the fasting blood glucose levels (BGLs) of all the rats were determined and rats with BGLs greater than 180mg/dL were considered diabetic according to (Stanley, 2001).

Experimental design

A 21-day pilot study was carried out on overnight fasted rats to determine the effective doses that can be used for the experiment. The screened diabetic rats (48) were divided into 10 groups of 4-5 rats per group.

Group 1: Negative control which received animal feed and water only

Group 2: Alloxan-induced but not treated

Group 3: Alloxan-induced + treated with Metformin 21.4mg/kg/bw (Comparative control)

Group 4: Alloxan-induced + treated with 300mg/kg/bw of *M. oleifera* water extract (MoWE)

Group 5: Alloxan-induced + treated with 200mg/kg/bw of *M. oleifera* 50% methanolic extract (50% MoME)

Group 6: Alloxan-induced + treated with 300 mg/kg/bw of *M. oleifera* 50% methanolic extract (50% MoME)

Group 7: Alloxan-induced + treated with 300 mg/kg/bw of *M. oleifera* 100% methanolic extract (100% MoME)

Group 8: Alloxan-induced + treated with 200 mg/kg/bw of *M. oleifera* 50% Ethanolic extract (50% MoEE)

Group 9: Alloxan-induced + treated with 300 mg/kg/bw of *M. oleifera* 50% Ethanolic extract (50% MoEE)

Group 10: Alloxan-induced + treated with 400 mg/kg/bw of *M. oleifera* 100% Ethanolic extract (100% MoEE)

The extracts were administered orally based on body weights, once daily for 24 days.

Assessment of antidiabetic activity in diabetic rats

With the aid of an auto analyzer ACCU-CHEK glucometer test kit, baseline blood glucose levels, post-induction blood glucose levels and pre-treatment blood glucose levels were determined. However, the progression of glucose change was monitored every 5 days till the end of the study. Blood samples were collected by the tail vein

puncture using blood lancets into the test strips of the glucometer.

STATISTICAL ANALYSIS

GraphPad Prism 5.0 was used for all statistical analyses. Data were presented as Mean ± SEM and analyzed by student's paired t-test for any statistical significant difference in the pre- and post-treatment group data. This was followed by a One-Way ANOVA across the groups using the Dunnett's multiple Comparison test (post test). Statistical significant differences were considered at $p<0.05$, $p<0.01$ and $p<0.001$.

RESULTS

The different solvent extracts of *M. oleifera* showed the presence of all the tested phytoconstituents as shown in table 1, suggesting that the presence of the phytoconstituents is independent of the extracting solvent.

Table 2 presents antioxidant effects of the different solvent extracts of *M. oleifera* as measured by the percentage inhibitions of radicals generated by DPPH. At the highest concentrations of the solvent extracts, 100% methanolic extract showed the highest inhibition while the 50% ethanolic extract showed the least.

At the end of the study, the percentage change in the body weights of the experimental rats (as shown in table 3) was determined to evaluate the accompanying effects of the standard and test treatments. The percentage change was calculated as the percentage deviation from the pre-induction weights and followed by the comments.

Values are expressed as Mean ± SEM, analyzed using Graphpad prism 5.0 student paired t-test between the post-induction blood glucose levels and the post-treatment blood glucose levels; where ns=Not significant; *= P<0.05; **=p<0.01; ***=p<0.001. One Way ANOVA comparing all groups with the negative control, $^{a}p<0.05$; Comparing all groups with the positive control, $^{b}p<0.001$; Comparing all groups with the comparative control, $^{c}p<0.05$.

During the course of the study, the blood glucose levels were determined at pre-determined times, and the percentage change was calculated as the percentage deviation from the mean post-induction glucose levels of the different treatments as seen in table 4.

Values are expressed as Mean ± SEM, analyzed using Graphpad prism 5.0 student paired t-test between the post-induction blood glucose levels and the post-treatment blood glucose levels where ns=Not significant; *= P<0.05; **=p<0.01; ***=p<0.001. One Way ANOVA comparing all groups with the negative control, $^{a}p<0.001$; Comparing all groups with the positive control, $^{b}p<0.001$; Comparing all groups with the comparative control, $^{c}p<0.001$.

DISCUSSION

The aim of the study was to investigate the effects of different extraction solvent systems on the anti-oxidant and hypoglyceamic effects of *M. oleifera.* table 1 shows the phytoconstituents present in the different extracts of *M. oleifera* leaves. Earlier reports by (Srinivasa *et al*., 2011) revealed that phytochemical investigation of *M. oleifera* contains phytoconstituents such as carbohydrates, tannins, flavonoids, triterpenoids, and alkaloids in chloroform and methanol extracts. The report of (Shahriar *et al*., 2012) is consistent with findings from our study that the preliminary phytochemical screening of the methanolic ethanolic crude extracts of leaf of *M. oleifera* revealed the presence of different phytochemicals as phenols, flavonoids, tannin, saponins, alkaloids, glycosides, carbohydrate and terpenoids. Our result further establishes that hydro-alcoholic solvent systems of low-molecular weight alcohols like methanol and ethanol revealed the presence of reducing sugars, alkaloidal salts, steroids and terpeniods, saponins, flavonoids, phenolics, cardiac glycosides, coumarin glycosides and anthraquinones.

The marked presence of some of the secondary metabolites can be explained by their existence mainly in the salt forms. We can validate from this study the effect of solvent polarity on the different phytoconstituents in *M. oleifera* suggesting that many of the phytoconstituents in the plant leaves are polar. The highly polar phytoconstituents can be suggested agents responsible for the anti-oxidant and the hypoglyceamic activities of *M. oleifera* leaves.

Oxidative stress caused by the generation of free radicals has been largely implicated in diseases with the pathophysiology being oxidative damage to normal cells. Hence, antioxidant properties of some plants have also been used as a free radical scavenger to mop up these free radicals. Certain plant secondary metabolites like phenolics, flavonoids and tannins possess primary antioxidant properties which show promising activity than ascorbic acid (Adesina *et al*., 2011)

The DPPH test shows the ability of the test compound to act as a free radical scavenger. DPPH is a free radical and it gives a strong absorption band at 517 nm in the visible region of the electromagnetic radiation, imparting on it a deep violet colour. This absorption diminishes as the electron is paired off (Ayoola *et al*., 2008)

The percentage inhibitions were concentration-dependent and at the highest concentration of 300µg/mL, the percentage inhibitions show a trend as 100% MoME (33.19%) > 50% MoME (10.85%) >100% MoEE (10.73

Table 1: The Phytochemical profile of the different *M. oleifera* extracts

Phytoconstituents	MoME (100%)	MoME (50%)	MoEE (100%)	MoEE (50%)	MoWE
Reducing sugars	+	+	+	+	+
Alkaloids	+	+	+	+	+
Steroids	+	+	+	+	+
Terpenoids	+	+	+	+	+
Saponins	+	+	+	+	+
Flavonoids	+	+	+	+	+
Phenolics	+	+	+	+	+
Cardiac glycosides	+	+	+	+	+
Coumarin glycosides	+	+	+	+	+
Anthraquinones	+	+	+	+	+

Key: (+) = Present; (-) = Absent

Table 2: The results of the percentage Inhibitions of the different solvent system extracts

Concentration (µg/mL)	Percentage Inhibitions				
	100% ME	50% ME	100% EE	50% EE	WE
100	3.67	-1.99	3.47	1.84	3.39
150	5.74	3.51	3.49	2.66	3.86
200	9.91	0.24	5.16	6.15	2.89
250	18.67	4.76	6.78	8.33	4.43
300	33.19	10.85	10.73	8.49	10.25

Table 3: Effect of the different treatment groups on the body weight

Groups	Pre-induction weight (g)	Post treatment weight (g)	% Change; Comment
Control Group	112.5 ± 7.762	*123.3 ± 9.07 ^{b}	9.6%; Increase
Induced without treatment	122.7 ± 4.410	**71.67 ± 3.18 $^{a\ c}$	41.6%; Decrease
Standard Drug (Metformin)	135.4 ± 8.286	*130.0 ± 8.695 ^{b}	4.0%;Decrease
Water Extract 300mg/kg	135.3 ± 12.85	^{ns}132.5 ± 14.41 ^{b}	2.1%; Decrease
50% ME 200mg/kg	134.8 ± 9.876	^{ns}128.0 ± 6.55 ^{b}	5.0%; Decrease
50% ME 300 mg/kg	137.8 ± 11.84	^{ns}140.3 ± 11.35 ^{b}	1.8%; Increase
100% ME 300 mg/kg	149.0 ± 4.637	^{ns}152.0 ± 7.15 ^{b}	2.0%; Increase
50% EE 200 mg/kg	123.0 ± 2.302	^{ns}124.2 ± 3.09 ^{b}	0.97%; Increase
50% EE 300 mg/kg	153.0 ± 24.17	^{ns}159.3 ± 26.01 ^{b}	4.1%; Increase
100% EE 400 mg/kg	132.0 ± 7.556	^{ns} 136.0 ± 7.49 ^{b}	3.0%; Increase

Table 4: Effect of the different extracts on blood glucose levels (mg/dL) in alloxan-induced diabetic rats

Treatment Groups	Blood Glucose Levels (mg/dL)		% Change; Comment
	Post-Induction	Post-Treatment	
Control Group	75.50 ± 2.60	^{ns} 78.75 ± 2.32 ^{b}	4.30%; Increase
Induced without treatment	311.3 ± 30.28	*541.7 ± 10.73^{ac}	74.0%; Increase
Standard Drug (Metformin)	498.0 ± 65.24	**79.00 ± 3.27^{b}	84.14%; Decrease
Water Extract 300 mg/kg	591.6 ± 7.40	***104.0 ± 3.028^{b}	82.42%; Decrease
50% ME 200mg/kg	493.8 ± 76.08	**166.2 ± 25.59^{abc}	66.34%; Decrease
50% ME 300mg/kg	406.5 ± 40.51	**78.50 ± 7.79^{b}	80.69%; Decrease
100% ME 300mg/kg	535.6 ± 63.40	*115.3 ± 22.43^{b}	78.47%; Decrease
50% EE 200mg/kg	523.8 ± 46.05	***92.60 ± 6.19 ^{b}	82.32%; Decrease
50% EE 300mg/kg	469.8 ± 85.81	*76.50 ± 8.42 ^{b}	83.72%; Decrease
100% EE 400mg/kg	415.8 ± 79.84	*75.00 ± 5.21 ^{b}	81.96%; Decrease

%) >MoWE (10.25%) > 50% MoEE (8.49%), as seen in table 2. This trend signifies a better free radical scavenging activity of *Moringa oleifera* when extracted with absolute alcohols with very low molecular weights, 100% methanol in the case of our study. Overall, it can also be confirmed that the extracts of *Moringa oleifera*

obtained from the low-molecular weight alcohols showed better DPPH reduction than the hydro-alcoholic extracts in the reducing property of oxygenated species.

The study also assessed the progression of body weights of the rats following induction with alloxan and treatments with *M. oleifera* and Metformin relative to the control group. Results showed that induction with alloxan resulted in a statistically significant decrease in body weight accompanied with significant hyperglycemia. The 41.6% significant decrease in body weight of the induced diabetic rats (without treatment) following the administration of alloxan agrees with previous studies by (Rungby *et al*., 1992; Helal, 2000).

Regarding to the negative control and comparative (metformin) groups, a significant decrease ($p<0.05$) in body weights in the positive control group post-treatment with the metformin and *M. oleifera* is seen in table 3. It was also observed that relative to the positive control group, all groups showed a significant change ($p<0.05$ to $p<0.001$) in body weights at the end of the study. Appreciable weight loss was recorded in the induced but untreated group relative to their pre-induction weights, suggesting the weight-lowering effects of alloxan. However, no significant change was observed in the *M. oleifera* groups of both solvent systems. The highest weight appreciation was seen with the 300mg/kg of 50% methanolic *M. oleifera* extract.

The fasting blood glucose level (fBGLs) test is a carbohydrate metabolic test which measures plasma or blood glucose level after a fast (usually 8-12 hr) by the release of glucose into the blood stream following the action of glucagon. Alloxan, like streptozocin, is used for induction of diabetes mellitus and has a destructive effect on the beta (β) cells of the pancreas (Bolaffi *et al*., 1986; Jelodar *et al*., 2003). Alloxan, being a toxic glucose analogue, inhibits glucose induced insulin secretion in the pancreatic β cells, thus selectively destroying the β cells by its accumulation in these cells via Glut 2 glucose transporters. The effect of this cellular accumulation is a cascade of free-radicals generating reactions such as superoxides, peroxides and hydroxyls (El-Missiry *et al*., 2000; Vinuthan *et al*., 2007). The necrotic action of these radicals on pancreatic beta cells is the mode of inducing insulin-dependent diabetes. In agreement with the study results, the significant 74.0% glucose rise in the induced but not treated group can be linked to the cytotoxicity of alloxan on the beta cells contrary to the meagre 4.3% increase in the negative control group - a rise which might be due to the food intake.

The study by (Manohar *et al*., 2012) established the hypoglycaemic effect of the aqueous extract of *M. oleifera* leaves (100, 200 and 300mg/kg) resulted in a highly significant reduction in blood glucose levels in fasted normal and alloxan-induced diabetic rabbits as compared with glibenclamide 0.5mg/kg two hours post-treatment with a maximum reduction seen in the 200mg/kg dose. This finding is consistent with our results that aqueous extract of *Moringa oleifera* (MoWE) will significantly reduce the blood glucose levels ($p<0.001$) in diabetic rats, but at 300mg/kg with no significant hypoglyceamic difference relative to Metformin (21.4mg/kg/body weight) in the present study.

Our results in table 4 clearly indicate the pronounced hypoglyceamic activity of *M. oleifera* leave extracts using different solvent systems in the diabetic rats. It can also be seen that the comparative control and the treatment groups showed a statistically significant decrease ($p<0.001$) in blood glucose levels relative to the induced but not treated group (positive control group). Despite the significance, the 50% MoME 200 mg/kg group showed the least drop (66.34%) in the glucose concentration. The 50% MoME 200mg/kg group showed a significant increase ($p<0.001$), a significant decrease ($p<0.001$) and a significant increase ($p<0.001$) in the glucose levels when compared with the negative control, positive control and the comparative control groups respectively. This implies that the hydro-methanolic (50:50) is not an effective extraction solvent system for the hypoglyceamic principles in *M. oleifera* leaves. Overall, the ethanolic and hydro-ethanolic solvent systems- 50% and 100% EE gave better glucose-lowering effects that favourably compares with metformin (84.14%), as seen in Table 4. These hypoglyceamic effects may be strongly suggestive of the ameliorative potentials of *M. oleifera* on the pancreatic beta-cells. Our findings established evidence that the hypoglyceamic effects of the treatment groups are dose-dependent and are further supported by the overall weight-restoring potential of the ethanolic solvent systems of *M. oleifera* (table 3).

CONCLUSION

Our study showed the effect of extraction solvent on the yields from *M. oleifera* leaves, with the (1:1) hydro-alcoholic solvents giving a higher yield - MoME (9.42%) and MoEE (8.52%). No variation was seen in the phytoconstituents of all extracts. However, the 100% methanolic *M. oleifera* extract showed the highest DPPH activity inhibition (33.19%) at 300µg/ml. It was also seen that *Moringa oleifera* leaves, extracted with ethanol showed very pronounced hypoglyceamic effects comparable to the standard drug, metformin. Relative to the positive control, all treatment groups showed a significant statistical decrease in blood glucose levels at the end of the study. The decrease trends as metformin (84.14%) ≥50% MoEE 300mg/kg (83.72%) >MoWE 300mg/kg (82.42%) ≥50% MoEE 200mg/kg (82.32%) > 100% MoEE 400mg/kg (81.96%) >50% MoME (80.69%) >100% MoME 300mg/kg (78.47%) >50% MoME 200mg/kg (66.34%). The alloxan-induced but not treated group showed a significant ($p<0.01$) loss in the body

weight while *Moringa oleifera* showed better ameliorative effects on the body weights of the animals than the standard drug of Metformin.

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