

Original Research Article

Anti-diabetic activity-guided screening of aqueous-ethanol *Moringa oleifera* extracts and fractions: Identification of marker compounds

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Abstract

Purpose: To explore the anti-diabetic effects of *Moringa oleifera* extracts and fractions, and to identify their active/marker compounds.

Methods: Five different aqueous ethanol extracts (95, 75, 50, 25 %v/v and 100 % water) of *Moringa oleifera* were given orally to normal rats to assess their hypoglycemic activities and effect on intraperitoneal glucose tolerance test (IPGTT) data. Rats with streptozotocin-induced diabetes were used to assess acute and sub-chronic anti-hyperglycemic activities. The most active extract was further subjected to liquid-liquid fractionation into hexane, chloroform, ethyl acetate, butanol, and water; these fractions were screened for anti-diabetic activities. The most active extract, and fractions thereof, were then subjected to qualitative and quantitative phytochemical analysis. Standardization was achieved via thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC), and used to identify marker compounds.

Results: Of all the extracts and fractions, 95 % (v/v) ethanol extract (at 1,000 mg/kg) and the butanol fraction thereof (at 500 mg/kg) were the most active, reducing blood glucose concentration after one-time (acute) administration to diabetic rats ($p < 0.01$). No significant hypoglycemic activity was apparent, and the materials had no effect on IPGTT performance by normal rats. TLC and HPLC identified quercetin 3- β -D-glucoside, kaempferol-3-O-glucoside, and cryptochlorogenic acid.

Conclusion: An *M. oleifera* leaf extract exhibited anti-hyperglycaemic activity in diabetic rats only. This effect was likely attributable to cryptochlorogenic acid, quercetin 3- β -D-glucoside, and kaempferol 3-O-glucoside.

Keywords: Anti-diabetic, *Moringa oleifera*, Cryptochlorogenic acid, Quercetin 3- β -D-glucoside, Kaempferol 3-O-glucoside, Streptozotocin

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INTRODUCTION

Glucose homeostasis is the balance between glucose production and utilisation by peripheral tissues. Insulin promotes glucose uptake by cells [1]. An insulin deficiency combined with insulin

resistance is responsible for diabetes mellitus [2]. The World Health Organization has observed that diabetes has become a major problem, and the number of cases is expected to rise through the year 2035 [3]. The prevalence of diabetes is

increasing among Malaysians, reaching 23.8 % in recent years [4].

Natural products and their derivatives constitute ca. 50 % of all anti-diabetics. The rate is higher when related synthetic materials are considered [5], but these materials are costly [6]. Generally, various extracts of natural materials are subjected to primary screening and the best extract is selected [7].

Marker compounds therein are identified using standard techniques [8]. High-performance liquid chromatography (HPLC) and thin layer chromatography (TLC) are valuable chromatographic techniques [9] employed to identify molecules responsible for therapeutic activities [10].

Recently, advances in the isolation and identification of active compounds in medicinal plants have featured fractionation-guided targeting.

Therefore, we identified active anti-diabetic compounds in extracts and fractions of *Moringa oleifera*. Our findings will aid future drug discovery/extraction from natural sources.

EXPERIMENTAL

Apparatus, chemicals, and drugs

A rotary evaporator (Buchi Labortechnik, CH-9230 Flawil Switzerland), a WinCATS Planar Chromatography Manager (Merck, Germany), streptozotocin (Sigma Aldrich Chemical Co, USA), acetonitrile and formic acid (Merck, Germany), quercetin 3- β -D-glucoside (Sigma Aldrich, USA), kaempferol 3-O-glucoside (480-10-4, Extrasynthese, Genay Cedex), cryptochlorogenic acid (Wuhan ChemFaces Biochemical Co. Ltd), glibenclamide 5 mg (Daonil) and metformine (500 mg; Glucophage; Merck, Germany) were used in this study.

Authentication of the plant and preparation of extracts

Plant material was purchased from Harbagus Sdn. Bhd., Pulau Pinang, Malaysia, and was authenticated by Dr Rahmad Zakaria. A voucher specimen has been placed in the herbarium of School of botany, Universiti Sains Malaysia (no. 11626). *M. oleifera* leaves were dried in the shade, ground to a powder, and extracted with 95, 75, 50, and 25 % (v/v) ethanol and 100 % water via maceration (Table 1). All extracts were concentrated under reduced pressure in a rotary evaporator and dried in an oven at 45 °C.

Experimental animals

Healthy male adult Sprague Dawley (SD) rats aged 8 - 9 weeks (230 \pm 30 g body weight) were used in the experimental studies. The animals were obtained from the Animal Research and Service Centre (ARASC), Universiti Sains Malaysia and allowed to adapt for seven days in the transit room of the School of Pharmaceutical Sciences at a temperature of 25 \pm 3 °C under a 12/12-h dark/light cycle. The rats had *ad libitum* access to rat pellets and water. The Animal Ethics Committee of Universiti Sains Malaysia approved the work (approval no. AEA/2015/612) and was performed in compliance with international guidelines (NIH) for the care and use of animals [11].

Blood glucose measurement

Blood samples (single drops) were collected by nicking the tail vein, and blood glucose (BG) levels were determined using a glucometer (Roche, ACCU-CHEK Performa no. 55404068967).

Anti-diabetic studies

Hypoglycaemia in normoglycaemic rats

Forty-two male SD rats were used to measure hypoglycaemic activity. The negative control (NC) and positive control (PC) groups were given distilled water (10 mL/kg) and water containing glibenclamide (10 mg/kg), respectively. The treated groups were given 95, 75, 50 and 25 % (v/v) ethanol and 100 % water extracts (1,000 mg/kg orally). After 14 h overnight fasting, fasting blood glucose (FBG) levels were determined; the treatments were then applied, and glucose levels were measured 1, 3, 5 and 7 h later.

Intraperitoneal glucose tolerance test in normoglycaemic rats

Normal rats were divided randomly into eight groups. One hour after oral administration of all treatments, the rats were injected intraperitoneally with a glucose load (500 mg/kg body weight). BG levels were measured at 0, 15, 30, 45, 60, 90, and 120 min thereafter.

Anti-hyperglycaemic activity in streptozotocin-treated rats: single-dose study

Diabetes was induced by intraperitoneal injection of streptozotocin (45 mg/kg body weight) after overnight fasting. Glucose was added to the drinking water (to 5 %, w/v) for 12 h to reduce mortality caused by hypoglycaemia. BG levels

were measured 72 h later. Rats with FBG concentrations > 15 mmol/L were considered to be diabetic and were used in further studies.

To measure anti-hyperglycaemic activity, rats were divided into seven groups. The experimental protocol was as described above, except that the PC group received metformin (500 mg/kg).

Anti-hyperglycaemic activity in streptozotocin-treated rats: multiple-dose study

The groups formed were the same as those created for the single-dose study described above. Diabetic rats were treated with *M. oleifera* aqueous ethanol extracts (1,000 mg/kg) once daily for 14 days. BG levels were determined on day-0, 7 and 14 after overnight fasting.

Fractionation (Liquid-liquid extraction)

The most active extract (the 95 % [v/v] ethanol) of *M. oleifera* was subjected to further fractionation. The extract was first partitioned with distilled water, and then (serially) with hexane, chloroform, ethyl acetate, and butanol, in a separating funnel. All five fractions (hexane, chloroform, ethyl acetate, butanol, and water) were concentrated separately under reduced pressure in rotary evaporator and dried in an oven at 45 °C.

Anti-hyperglycaemic screening of *M. oleifera* fractions in the rat streptozotocin model

Seven groups (six rats per group) were used. All groups underwent treatment with fractions (500 mg/kg) suspended in 10 % (v/v) Tween 80. FBG levels were determined after administration of various fractions and a standard drug at 1, 3, 5, and 7 h.

Phytochemical screening of extracts and fractions

For qualitative phytochemical screening, samples (extracts and fractions) were dissolved in dimethyl sulfoxide. Tannins, phenols, saponins, flavonoids, alkaloids, terpenoids, and glycosides were assayed using standard tests [12,13].

Thin layer chromatography

Qualitative screening of active compounds was performed on 20 × 20-cm silica gel TLC plates (Merck). The samples (the 95 % ethanol extract and the butanol fraction) were dissolved to 5 mg/mL and the standards (cryptochlorogenic

acid, kaempferol-3-O-glucoside, and quercetin 3-β-D-glucoside) were dissolved to 1 mg/mL. The TLC plates were developed in a TLC chamber (10 × 10 cm); the mobile phase was ethyl acetate: methanol: Acetic acid: water (110: 13.5: 8: 8 by volume). The plates were sprayed with the natural product (NP) reagent and viewed under ultraviolet light at 254 and 366 nm with the aid of a winCATS Planar Chromatography Manager. Rf values were calculated using equation 1:

$$R_f = \text{distance travelled by compound} / \text{distance travelled by solvent front} \dots\dots\dots (1)$$

HPLC development and validation

A 95 % (v/v) ethanol extract, the butanol fraction thereof (1,000 ppm), and standard compound (2,000 ppm) stock solutions were prepared in methanol, filtered through syringe filter (0.45-μm pore size), and analysed on an 1100/1200 HPLC platform (Agilent Technologies, USA) fitted with a column thermostat, a quaternary pump, a diode array detector, and an agilent 1200 autosampler (SL 1). We used a Hypersil Gold C4 column with a particle size of 5.0 μm and dimensions of 250.0 × 4.6 mm (Thermo-Scientific, USA). The solvent systems were 0.1 % (v/v) formic acid (B) and acetonitrile (C), and elution employed the following gradient: 0 - 5 min, B (97 %) and C (3 %); 5 - 14.5 min, B (80 %) and C (20 %); 14.5 - 16.5 min, B (97 %) and C (3 %). The injection volume was 20 μL, the column flow rate was 1.2 mL/min, and the run time was 18 min. All chromatographic signals were detected at 344 nm.

Quantification of identified compounds

To quantify cryptochlorogenic acid, quercetin 3-β-D-glucoside, and kaempferol-3-O-glucoside in the active 95 % (v/v) ethanol extract and the butanol fraction thereof, the peak heights of samples were compared with those of standards.

Evaluation of linearity, accuracy and precision

Linearity was checked by evaluating the peak heights (in mAU) of standards (12.5 - 200 μg/mL). Accuracy was evaluated by checking agreement between the amounts applied and the amounts measured by HPLC. We also evaluated the repeatability (precision) of the method. We injected three sets of standard mixtures three times daily over 3 consecutive days and measured intra-day and inter-day accuracy and precision.

Statistical analysis

The results are presented as mean \pm standard deviations and were analyzed by one-way analysis of variance with the aid of IBM SPSS software, followed by Dunnett's post hoc test. Differences between means were considered to be statistically significant at $p < 0.05$, < 0.01 , or < 0.001 . For graphical presentation, GraphPad Prism (version 6.0) software was used.

RESULTS

Effect of extracts of *Moringa oleifera* in normal rats

BG concentrations rose after administration of all extracts (95, 75, 50, 25 % [v/v] ethanol and water) for up to 3 h (compared with FBG levels), but declined over the next 4 h (Table 2). No significant reduction in glucose concentration was observed, except in the glibenclamide group (31.81 %).

Effect of different extracts on IPGTT in normoglycemic rat

Glibenclamide greatly lowered the BG concentration (by 35.52 %) 15 min after glucose loading, compared with the NC. Similarly, the treated groups exhibited some improvements in glucose tolerance (GT) at 15 min, but these differences were not significant. The 95 and 50

% (v/v) ethanol extracts afforded only 12.41 % improvement in GT. GTs were 5.4, 10.64, and 11.73 % after administration of 75 and 25 % (v/v) ethanol and aqueous extracts, respectively (Table 3).

Effects of multiple-dose *M. oleifera* extracts in diabetic rats

The ethanol (95, 75, 50, and 25 % [v/v]) and aqueous extracts, metformin, significantly reduced the BG levels on days 7 and 14 after overnight fasting ($p < 0.001$; Figure 1). On day 7, the 50 and 25 % (v/v) extracts reduced the BG concentration by 55.35 and 54.57 %, whereas the reduction afforded by the 95 % (v/v) extract was 64.07 % compared with the control. Likewise, the aqueous extract afforded a 60.06 % reduction in BG level compared with the initial value (19.28 ± 0.36 mmol/L).

Effects of single-dose *M. oleifera* extract in diabetic rats

Metformin afforded 42.87 % reduction in hyperglycaemia, compared with the NC, 7 h after administration (Table 4). The 95 and 75 % (v/v) ethanol extracts lowered the BG level by 30.28 and 18.05 %, respectively. The 50 and 25 % (v/v) ethanol extracts did not markedly reduce the BG level, whereas the aqueous extract lowered the BG level significantly to only 1 h after administration ($p < 0.05$).

Table 1: *M. oleifera* aqueous-ethanol extracts

Extracts (V/V) Ethanol (95%): water (mL)	Weight of dried leaves powder (g)	Weight of extract (g)	Physical appearance	Yield (%)
95% (1000:0)	800	184.45	Black-greenish and sticky and oily	23
75% (789: 211)	800	214.96	Dark brown and sticky	26.87
50% (526: 474)	800	192.76	Dark brown and sticky	24.01
25% (263: 737)	800	253.81	Dark brown and sticky	31.72
Water (0:1000)	800	217.75	Dark brown	27.22

Table 2: Effect of *M. oleifera* extracts in normal rats (n=6)

Sample/extract	Dose/kg	Blood glucose levels after treatment (mmol/L)				
		0 (FBG)	1	3	5	7
NC	10mL	5.1 \pm 0.46	4.98 \pm 0.60	5.01 \pm 0.68	5.06 \pm 0.44	5.13 \pm 0.58
G	10mg	4.96 \pm 0.33	3.81 \pm 0.94 ^a	3.75 \pm 0.77 ^b	3.45 \pm 0.19 ^c	4.1 \pm 0.43 ^a
E 95%	1000mg	4.95 \pm 0.48	5.38 \pm 0.61	5.67 \pm 0.44	5.22 \pm 0.44	5.11 \pm 0.65
E 75%	1000mg	5.15 \pm 0.48	5.52 \pm 0.52	5.62 \pm 0.53	5.28 \pm 0.55	4.75 \pm 0.74
E 50%	1000mg	5.15 \pm 0.50	5.33 \pm 0.36	5.43 \pm 0.43	5.15 \pm 0.23	5.01 \pm 0.51
E 25%	1000mg	4.88 \pm 0.44	5.4 \pm 0.76	5.83 \pm 0.66	4.95 \pm 0.82	5.07 \pm 0.48
AE	1000mg	5.13 \pm 0.54	5.97 \pm 0.30	5.83 \pm 0.32	5.4 \pm 0.52	5.06 \pm 0.83

FBG=fasting blood glucose, NC=negative control, G=glibenclamide, E=ethanol, AE=aqueous extract, ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$, treated groups were compared with NC

Table 3: Effect of *M. oleifera* extracts on IPGTT in normoglycaemic rats (n=6)

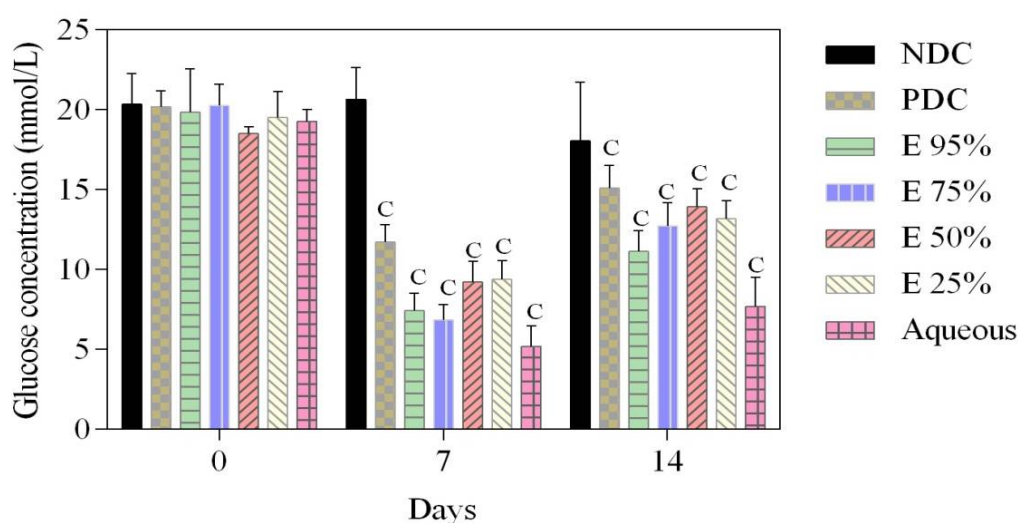
Treatments	Dose/kg body weight	Blood glucose levels after treatment (mmol/L)							
		FBG	0 min	15 min	30 min	45 min	60 min	90 min	120 min
NC	10mL		5.22±0.19	7.33±0.92	6.38±0.50	5.38±0.35	5.12±0.20	4.97±0.19	4.77±0.29
G	10mg		5.47±0.31	4.58±1.13 ^c	3.75±0.66 ^c	3.57±0.70 ^c	3.3±0.47 ^c	2.88±0.34 ^c	2.68±0.29 ^c
M	500mg		5.5±0.54	6.53±0.53	5.7±0.30	5.33±0.33	5.17±0.30	4.92±0.40	4.67±0.53
E 95%	1000mg		5.25±0.45	6.42±0.60	5.77±0.30	5.63±0.36	5.48±0.34	5.25±0.38	5.1±0.39
E 75%	1000mg		5.2±0.49	6.93±0.90	6.18±0.49	5.78±0.64	5.45±0.37	5.05±0.24	4.87±0.32
E 50%	1000mg		5.47±0.38	6.42±0.86	5.82±0.43	5.22±0.34	5.08±0.31	4.98±0.31	4.88±0.55
E 25%	1000mg		5.33±0.50	6.55±1.08	6.07±0.63	5.32±0.31	5.38±0.48	4.93±0.12	4.73±0.27
AE	1000mg		5.23±0.28	6.47±0.69	5.67±0.27	5.42±0.35	5.25±0.34	4.93±0.12	4.85±0.29

NC=negative control, G=glibenclamide, M=Metformine, E=ethanol, AE=aqueous extract, FBG=fasting blood glucose, Statistically compared with NC, ^a p < 0.05, ^b p < 0.01, ^c p < 0.001

Table 4: Effects of single dose *M. oleifera* extracts in diabetic rats (n = 6)

Group	Dose (per kg)	Blood glucose levels after treatment (mmol/L)				
		FBG	1 h	3 h	5 h	7 h
NDC	10mL	20.35±1.90	21.58±1.59	20.25±1.66	20.22±0.93	20.48±1.77
M	500mg	20.20±0.98	18.93±1.23 ^a	17.23±2.06 ^b	15.45±1.18 ^c	11.7±0.96 ^c (42.08%)
E 95%	1000mg	19.85±2.71	18.58±2.77 ^a	17.13±1.86 ^b	15.92±2.44 ^c	13.84±2.41 ^c (30.28%)
E 75%	1000mg	20.28±1.31	20.27±0.85	18.40±0.40	17.07±0.80 ^b	16.62±1.33 ^b (18.05%)
E 50%	1000mg	18.50±0.44	19.30±0.64	19.10±1.04	18.47±0.77	18.68±0.61
E 25%	1000mg	19.52±1.62	19.20±0.49	19.07±0.47	19.55±1.40	18.83±1.56 (3.53%)
AE	1000mg	19.28±0.73	18.63±2.32 ^a	18.33±1.87	18.22±0.89	18.03±0.57 (6.48%)

NDC (negative diabetic control), M (metformin treated positive control), E (ethanol) and AE (aqueous extract), ^a p < 0.05, ^b p < 0.01, ^c p < 0.001, FBG (fasting blood glucose). Statistically, the standard drug and extract treated groups were compared with NDC

**Figure 1:** Effects of multiple-dose *M. oleifera* extracts in diabetic rats. NDC-negative diabetic control, PDC-positive diabetic control, E-ethanol extract. The results of PDC and different aqueous-ethanol extracts were statistically compared with NDC, ^c p<0.001.

Effects of *M. oleifera* fractions in diabetic rats

Diabetic rats that received the butanol fraction developed significant ($p < 0.01$) acute anti-hyperglycaemic activity. No other fraction was effective in this regard (Table 5).

Phytochemical profile

The active butanol fraction and the 95 % (v/v) ethanol extract contained abundant flavonoids, glycosides, tannins, and phenols (Table 6). The aqueous extract lacked alkaloids and terpenoids, whereas hexane fraction contained only tannins and phenols.

Thin layer chromatogram

Figure 2 shows three prominent spots in the 95 % (v/v) ethanol extract and the butanol fraction. These were identified as quercetin 3- β -D-glucoside, kaempferol 3-O-glucoside, and cryptochlorogenic acid (Figure 3), with Rf values of 0.658, 0.711, and 0.316, respectively.

Identified compounds and validation results

HPLC chromatograms of the active 95 % (v/v) ethanol extract, the butanol fraction, and the standard mixture are shown in Figure 4. Compound identification was based on the absorption spectra, retention times, and peak

Table 5: Effects of *M. oleifera* fractions in diabetic rats

Group (n=6)	Dose per kg	FBG 0 h	Blood glucose levels after treatment (mmol/L)			
			1 h	3 h	5 h	7 h
NDC	10mL	20.92±2.55	21.02±2.28	20.08±1.73	19.10±1.90	20.68±2.98
PDC	500mg	18.40±2.28	15.77±1.58 ^b	13.93±1.96 ^c	12.85±1.94 ^b	10.22±1.49 ^c
HF	500mg	20.92±1.82	19.83±1.62	21.15±0.87	19.83±1.19	19.98±1.59
CF	500mg	18.30±2.55	21.15±2.49	20.17±1.65	19.22±1.77	18.42±2.09
EAF	500mg	18.13±0.67	19.58±1.38	18.90±1.50	19.65±2.89	18.33±0.95
BF	500mg	20.52±1.23	20.1±2.31	17.67±1.93	18.43±3.48	16.00±1.27 ^b
AF	500mg	19.50±3.81	20.85±2.81	19.70±2.78	18.37±3.41	18.12±1.93

NDC-negative diabetic control, PDC-positive diabetic control, HF-hexane fraction, CF-chloroform fraction, EAF-ethyl acetate fraction, BF-butanol fraction, AF-aqueous fraction, ^b ($p < 0.01$), ^c ($p < 0.001$), Groups were compared with NDC

Table 6: Phytochemical profile of *M. oleifera* extracts and fractions

Sample	Saponins	Tannins and phenols	Flavonoids	Alkaloids	Terpenoids	Glycosides
E 95%	+++	+++	++++	-	-	++
E 75%	+++	+++	+++	-	-	+++
E 50%	++	++	+++	-	-	+++
E 25%	++	++	++	-	-	+
W	+	+	+	-	-	++
100%						
Active 95% extract fraction						
HF	-	+++	-	-	-	-
CF	+	+++	-	-	-	++
EAF	-	++	+++	-	-	+++
BF	-	+	+++	-	-	+++
AF	-	+	++	-	-	++

'E'-ethanol, 'W'-water, , (+) sign indicate intensity of reaction, ++++ (very high), +++ (high), ++ (medium), + (low), (-) indicates absence of phytochemicals, HF-hexane fraction, CF-chloroform fraction, EAF-ethyl acetate fraction, BF-butanol fraction and AF-aqueous fraction

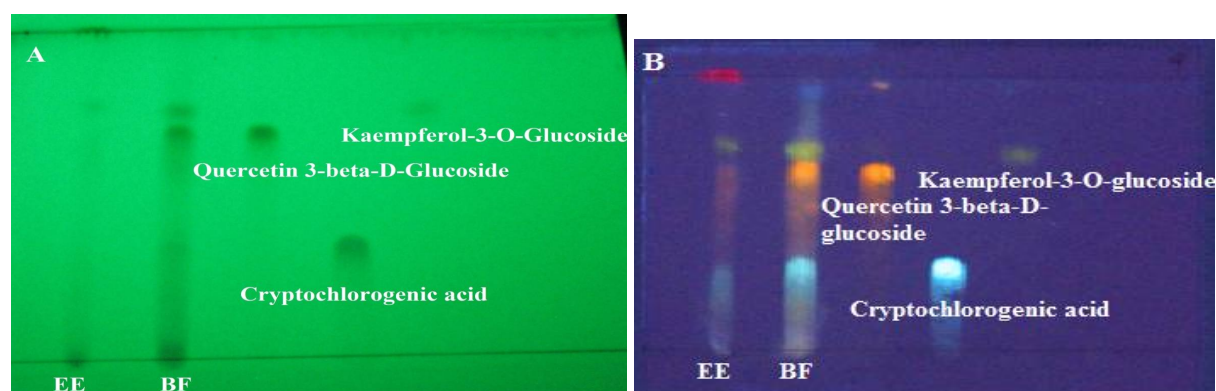


Figure 2: Chromatograms of *M. oleifera* extract, fraction, and identified compounds. EE-ethanol extract, BF-butanol fraction, TLC chromatogram at 254 (A) and at 366 nm (B).

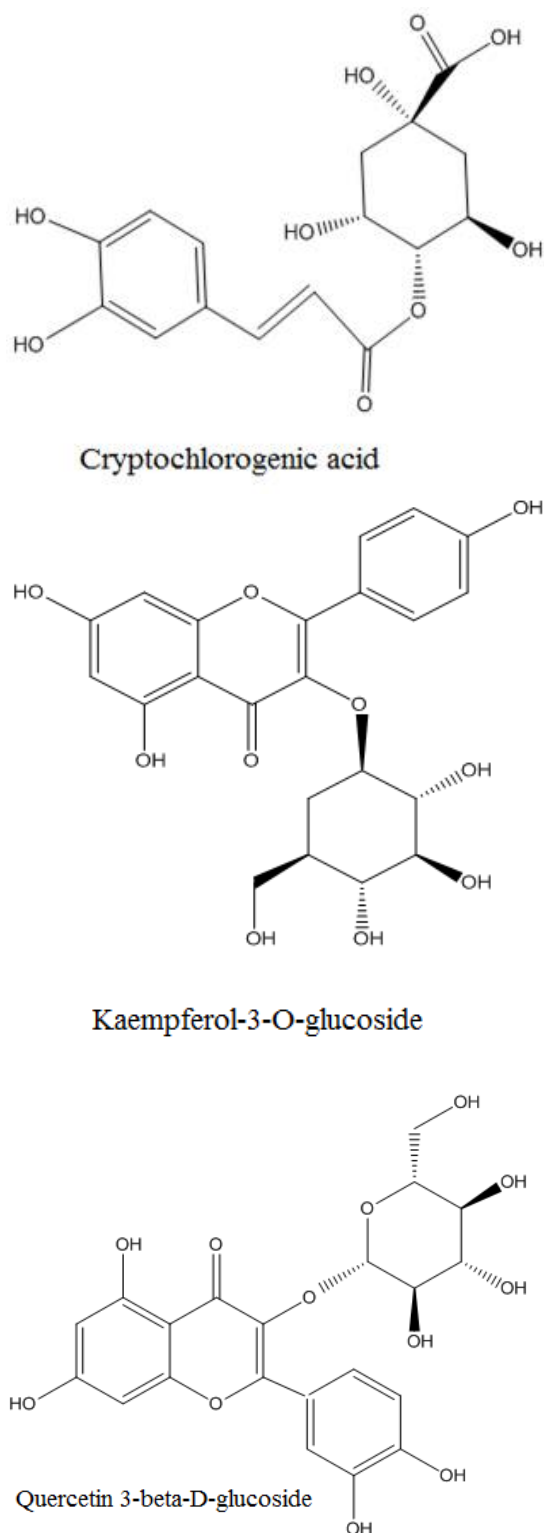


Figure 3: Chemical structures of identified compounds in *M. oleifera* extract and fraction

heights. Kaempferol-3-O-glucoside was present at high levels in the active 95 % (v/v) ethanol extract and the butanol fraction (56.58 and 216.69 $\mu\text{g/mL}$, respectively). The potential anti-diabetic cryptochlorogenic acid content of the

butanol fraction was 25.74 $\mu\text{g/mL}$ (Table 7). The limits of detection for cryptochlorogenic acid, quercetin 3- β -D-glucoside, and kaempferol-3-O-glucoside were 1.515, 1.816 and 1.862 $\mu\text{g/mL}$, respectively. The limits of quantification were 4.590, 5.503, and 5.642 $\mu\text{g/mL}$, respectively (Table 8).

Intra-day accuracy (repeatability) values were from 98.08 - 100.37 % for cryptochlorogenic acid, 96.07 - 101.90 % for quercetin 3- β -D-glucoside, and 95.92 - 101.49 % for Kaempferol-3-O-glucoside. Intra-day precision values were 0.43 - 1.72 % for cryptochlorogenic acid, 0.88 - 1.68 % for quercetin 3- β -D-glucoside, and 1.27 - 1.91 % for kaempferol-3-O-glucoside. Inter-day accuracy values were 94.02 - 101.44 % for cryptochlorogenic acid, 94.11 - 101.72 % for quercetin 3- β -D-glucoside, and 95.06 - 101.77 % for kaempferol-3-O-glucoside. Similarly, the inter-day precision values were 0.44 - 2.09 % for cryptochlorogenic acid, 1.28 - 2.05 % for quercetin 3- β -D-glucoside, and 0.55 - 1.91 % for kaempferol-3-O-glucoside (Table 9).

DISCUSSION

Anti-diabetic herbal therapies are very important in the field of ethno-pharmacology. Currently, such therapies are urgently needed to replace oral hypoglycaemic drugs, which are increasingly unable to control all pathological symptoms of diabetes, and are often unavailable in remote areas [14]. We evaluated aqueous ethanol extracts of *M. oleifera* in normal and streptozotocin-induced diabetic rats to identify possibly relevant compounds.

The rises in BG levels of normal rats after administration of *M. oleifera* extracts may be attributed to the plant material's content of a high proportion of carbohydrates. Previous studies showed that the 90 % (v/v) ethanol extract contained 45.43 % carbohydrates [15].

The aqueous-ethanol extracts (95, 75, 50, 25 % [v/v] ethanol and 100 % water) did not exhibit a hypoglycaemic effect or affect intraperitoneal GT in normal rats. This lack of effect may be because homeostasis was intact; plant extracts did not interfere with the normal regulation of carbohydrate metabolism [16]. An earlier study [17] also showed no hypoglycaemic effect in normal Wister rats. Data from earlier studies differ somewhat from ours in terms of indications of a hypoglycaemic effect in normal rats; this discrepancy may be attributable to the use of different rat strains, climatic variation in *M. oleifera* species, and/or the doses given.

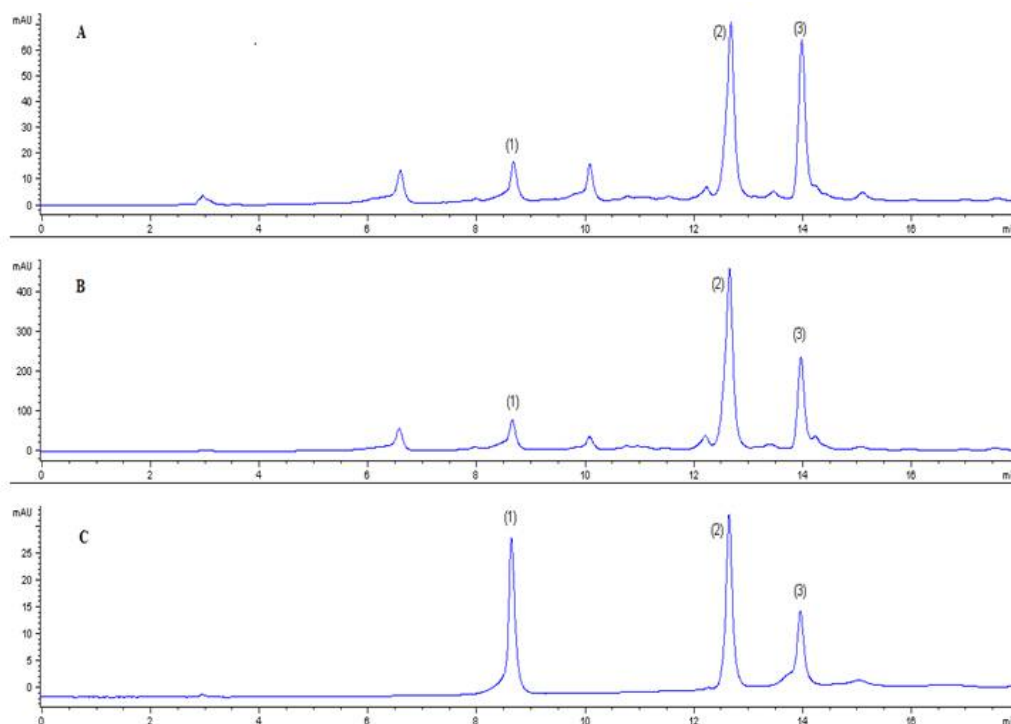


Figure 4: HPLC chromatograms of *M. oleifera* ethanol extract (A), butanol fraction (B) and standard compounds (C), Peaks of cryptochlorogenic acid (1), quercetin 3- β -D-glucoside (2) and kaempferol-3-O-glucoside (3)

Table 7. Quantification of identified compounds in *M. oleifera* extract and fraction

Sample	Cryptochlorogenic acid ($\mu\text{g/mL}$)	Quercetin 3- β -D-glucoside ($\mu\text{g/mL}$)	Kaempferol 3-O-glucoside ($\mu\text{g/mL}$)
Active 95% ethanol extract	5.16	23.90	56.58
Active butanol fraction	25.74	159.81	216.69

Table 8: Showing linearity, regression values, retention time and LOD_LOQ

Standard	Linear range ($\mu\text{g/mL}$)	r^2	Retention time	LOD ($\mu\text{g/mL}$)	LOQ ($\mu\text{g/mL}$)
Cryptochlorogenic acid	12.5-200	0.9991	8.64	1.515	4.590
Quercetin 3- β -D-glucoside	12.5-200	0.9999	12.64	1.816	5.503
Kaempferol 3-O-glucoside	12.5-200	0.9998	13.95	1.862	5.642

Table 9: Inter-day (between day) and intra-day (within day) accuracy and precision of standards (cryptochlorogenic acid, quercetin 3- β -D-glucoside and kaempferol-3-O-glucoside)

Biological marker	Concentration ($\mu\text{g/mL}$)	Inter-day		Intra-day	
		Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
Cryptochlorogenic acid	12.5	94.02	1.66	98.08	0.85
	25	99.00	2.09	99.76	1.47
	50	101.44	1.61	100.37	1.72
	100	100.76	0.44	100.28	0.43
	200	99.76	0.15	99.92	0.78
Quercetin 3- β -D-glucoside	12.5	94.11	1.28	96.07	1.68
	25	99.82	1.90	97.47	1.18
	50	99.65	1.60	101.90	1.47
	100	101.72	1.41	100.60	1.63
	200	99.62	2.05	99.79	0.88
Kaempferol-3-O-glucoside	12.5	95.06	1.57	98.14	1.91
	25	97.58	1.61	95.92	1.27
	50	100.60	1.84	100.97	1.70
	100	101.77	1.91	101.49	1.65
	200	99.58	0.55	99.64	1.79

We found that the 95 % (v/v) ethanol extract and the butanol fraction thereof were the most active extract and fraction, respectively, exerting very significant acute anti-hyperglycaemic activities in diabetic rats. These effects may be attributable to the expression of glucose transporter-4 (GLUT-4) in the cell membrane, which promotes glucose uptake by skeletal muscle cells and inhibition of glucose synthesis in hepatic cells [18]; skeletal muscle cells constitute 70 % of the total body mass [19]. The results of multiple dosing were in agreement with those of a previous study [20] of the anti-diabetic potential of *M. oleifera* aqueous ethanol extracts ($p < 0.001$). The anti-hyperglycaemic effect was greater on day 7 than on day 14, possibly attributable to a reduction in appetite and induction of other anti-diabetic mechanisms.

Initially, we tested various isocratic and gradient systems in terms of optimum separation of compounds using a mobile phase (0.1 % [v/v] formic acid and acetonitrile). The isocratic method did not adequately separate phenolic compounds; only the gradient method produced well-resolved sharp peaks that absorbed at 344 nm.

Linearity was evident over the range of 12.5 - 200 µg/mL for cryptochlorogenic acid, quercetin 3-β-D-glucoside, and kaempferol-3-O-glucoside [$r^2 = 0.9991$ ($y = 3.1452x + 3.0369$), $r^2 = 0.9999$ ($y = 3.1485x - 1.8511$), and $r^2 = 0.9998$ ($y = 1.1045x + 0.0278$) respectively; time vs. peak height in mAU]. The data shown in Table 9 confirm the validity of the HPLC method in terms of intra-day and inter-day precision, accuracy, and repeatability. TLC also identified isoquercetin, astragaloside, and cryptochlorogenic acid in the 70 % [v/v] ethanol extract of *M. oleifera* leaves [21].

CONCLUSION

The anti-hyperglycaemic effect of the *M. oleifera* 95 % (v/v) ethanol extract and the butanol fraction thereof in diabetic rats may be attributable to cryptochlorogenic acid, quercetin 3-β-D-glucoside, and kaempferol-3-O-glucoside. This extract, and the compounds mentioned, may be alternative treatments for diabetes, and may prompt further drug discovery.

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DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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