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Original research paper

Acalypha wilkesiana 'Java white': Identification of some bioactive compounds by GC-MS and their effects on key enzymes linked to type 2 diabetes

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In this study, we identified bioactive compounds from the ethanolic extracts of the leaves, stem bark and root bark of *Acalypha wilkesiana* through GC-MS analysis and investigated the effects of these extracts on some of the enzymes linked to type 2 diabetes. Plant parts were extracted sequentially with ethyl acetate, ethanol and water. GC-MS analysis revealed the presence of long-chain alkyl acids, esters, ketones and alcohols including phytol and phytol acetate along with some secondary metabolites such as xanthone, vitamin E and various types of sterols including stigmasterol, campesterol and sitosterol. Ethanolic extracts of all the parts showed a dose-dependent inhibition of α-glucosidase and α-amylase activity. The extracts also demonstrated anti-lipase activity. The ethanolic extract of root bark showed the highest inhibition of enzymes compared to other extracts. The EC_{50} values (concentrations for 50 % inhibition) of α-glucosidase, α-amylase and lipase inhibition were 35.75 ± 1.95, 6.25 ± 1.05 and 101.33 ± 5.21 μg mL^{-1}, resp. The study suggests that *A. wilkesiana* ethanolic extracts have the ability to inhibit the activity of enzymes linked to type 2 diabetes. Further studies are needed to confirm the responsible bioactive compounds in this regard.

Keywords: Acalypha wilkesiana, α-glucosidase, α-amylase, lipase, type 2 diabetes, ethanolic extract

Diabetes has gradually become a major health concern in both developed and developing countries, while controlling hyperglycemia is one of the major challenges in the management of this disease (1). Apart from hyperglycemia, almost 50 % of diabetic patients suffer from at least one or two diabetic complications such as diabetic retinopathy, cardiomyopathy, nephropathy, neuropathy and lower limb amputations, which are more noticeable in elderly patients (2). Approximately 108 million people were diagnosed with diabetes in 1980, which has increased to 422 million in 2014, with a projected increase to 642 million by the year 2030 (2).

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Diabetes is a metabolic disorder caused by deficiency of insulin secretion by the pancr amstaff alan eatic beta-cells (type 1), insulin insensitivity, and partial pancreatic beta-cell malfunction (type 2) (3). Type 2 diabetes (T2D) is the most prevalent form of diabetes and occurs due to the insensitivity of insulin to postprandial glucose overload which causes persistent hyperglycemia (4). The spread of this disease has been linked to societal and cultural changes in diet and lifestyle that could lead to obesity, a major risk factor for the rapidly rising prevalence of T2D (5). Obesity as a result of abdominal fat deposit may lead to sustained insulin resistance in obesity related T2D (6). Controlling hyperglycemia is a major challenge in the management of T2D.

A number of conventional drugs such as sulfonylureas, biguanides and α-glucosidase inhibitors are currently being used for the management of hyperglycemia as well as T2D. α-glucosidase is linked to the breakdown of disaccharides and starch into glucose while α-amylase breaks down long-chain carbohydrates into glucose (7). Inhibition of α-glucosidase and α-amylase is one of the key targets in the discovery of potential drugs for the treatment of T2D *via* reducing small intestinal glucose absorption (8). However, high cost and numerous side effects of these synthetic drugs have led to the exploration of alternative therapies in alleviating T2D (9). In addition, side effects including nausea, diarrhea (commonly seen in patients on metformin), hypoglycemia, weight gain (sulfonylureas), increased risk of heart failures and bone fractures (thiazolidinediones) (10) led to the search for alternative therapies, particularly from medicinal plants. Medicinal plants have been reported to be rich in bioactive phenolics, which can be used to treat diverse diseases (11) including T2D.

Acalypha wilkesiana 'Java white' is a member of the spurge family (*Euphorbiaceae*). It is a tropical and subtropical evergreen shrub with greenish yellow to white dotted green leaves mottled with irregular green spots. It grows in various parts of Africa but is native to South Pacific islands. In western Nigeria, a boiled decoction of the leaves of *A. wilkesiana* Müll. Arg. is used in the treatment of fungal infections seen in newborns, and also against hypertension and diabetes mellitus in adults (12). The leaves of this plant also possess anti-inflammatory, anti-microbial and anti-pyretic activities (13). Although some hypoglycemic, anti-diabetic and hypolipidemic effects of *A. wilkesiana* Müll. Arg. of leaves and roots have been reported in some recent studies (12, 14, 15), the underlying mechanisms behind these effects and phytochemical constituents of different parts of this plant are not fully understood.

Odoh *et al.* (14) and Olukunle *et al.* (12) reported the antidiabetic and hypoglycemic as well as hypolipidemic activities of root and leaf extracts, respectively, in alloxan-induced diabetic rats; however, stembark was not used in their studies and Odoh *et al.* (14) only qualitatively determined the phytochemical constituents in root extracts. Furthermore, Igwe *et al.* (16) only analyzed the leaf ethanolic extract to identify its bioactive compounds by using GC-MS while other parts of the plant were not analyzed. On the other hand, although aqueous and ethanolic extracts of the leaves and root bark of this plant have been shown to reduce blood glucose levels in alloxan-induced diabetic animals (12, 14), the mechanisms behind these effects are still unknown.

Hence, this study was designed not only to analyze the phytochemical constituents of the ethanolic extracts of leaf, root bark and stem bark of *A. wilkesiana* "Java white" *via* GC-MS analysis but also to examine the effects of various extracts from the different parts of the plant on the activity of enzymes linked to T2D, such as α-glucosidase, α-amylase and lipase, to understand the mechanisms behind their reported hypoglycemic and antidiabetic effects (12, 14).

EXPERIMENTAL

Chemicals and equipment

Absolute ethanol, dinitrosalicylic acid (DNS), ethyl acetate, *n*-hexane and starch were purchased from Merck (Germany). Acarbose, ethylenediaminetetraacetic acid (EDTA), morpholinepropanesulphonic acid (MOPS), orlistat (tetrahydrolipstatin), porcine pancreatic amylase, *p*-nitrophenyl butyrate (*p*NPB), *p*-nitrophenyl-α-D-glucopyranoside (*p*NPG) and yeast α-glucosidase were obtained from Sigma-Aldrich (Germany).

A Büchi Rotavapor II (Büchi, Switzerland) and a Synergy HTX Multi-mode Reader (BioTek Instruments Inc, USA) were used.

Plant materials

Leaves, stem bark and root bark of *A. wilkesiana* 'Java white' were collected from Ibadan, Nigeria, during October 2015. These plant parts were then washed and air-dried to constant mass, then blended to fine powder using a miller and stored in air-tight containers.

Extraction of crude extracts

One hundred grams of powdered plant sample from each plant part were initially defatted with *n*-hexane, then extracted in a sequential manner with ethyl acetate, ethanol and water by soaking in 200 mL of the relevant solvent for 48 h. After filtering through a Whatman filter paper No. 1, the extracts were concentrated *in vacuo* using a rotary evaporator. Aqueous extracts were concentrated to dryness by placing them in a water bath set at a temperature of 50 °C until completely dried.

Gas chromatography-mass spectroscopic (GC-MS) analysis

Ethanolic extracts of the leaves, stem bark and root bark of *A. wilkesiana* were subjected to GC-MS analysis. GC-MS analysis was carried out on an Agilent Technologies 6890 Series GC coupled with an Agilent 5973 mass selective detector equipped with Agilent Chemstation software (Agilent, USA). A HP-5MS capillary column was used (30 m × 0.25 mm ID, 0.25 µm film thickness, 5 % phenylmethylsiloxane). Helium was used as the carrier gas at a flow rate of 1.0 mL min^{-1} and linear velocity of 37 cm s^{-1}. Injector temperature was set at 250 °C. Oven temperature was programmed to reach 280 °C from the initial 60 °C at a rate of 10 °C min^{-1}. One-µL injections were made in the split mode with a split ratio of 20:1. The MS was operated in the electron ionization mode at 70 eV and electron multiplier voltage of 1859 V. The operating parameters for the instrument were: ion source temp. 230 °C, quadrupole temp. 150 °C, solvent delay 4 min and scan range 50–70 amu. Compounds were identified using the NIST library by direct comparison of the mass spectral data of known compounds.

Inhibitory activity of plant extracts

α-glucosidase. – α-glucosidase inhibitory activity was measured according to a published method (17) with slight modifications. Briefly, 500 µL of 1.0 U mL^{-1} of the

α-glucosidase enzyme dissolved in phosphate buffer (100 mmol L^{-1}, pH 6.8) was incubated for 20 min at 37 °C with 250 µL of extract or acarbose (standard). *p*-nitrophenyl-α-D--glucopyranoside (pNPG) solution (of 5 mmol L^{-1}, 250 µL) prepared in the same phosphate buffer was then added to the reaction mixture and incubated for another 30 min at 37 °C. This caused a release of *p*-nitrophenol, whose absorbance was subsequently recorded at 405 nm. The inhibitory activity was recorded as the proportion of the control lacking inhibitor. The experiment was performed three times.

α-amylase. – α-amylase inhibitory activity was determined by a previously published method (18) with slight modifications. Briefly, 250 µL (1 mg mL^{-1} stock) of the extracts at different concentrations (50–250 µg mL^{-1}) or acarbose was incubated for 20 min at 37 °C with 500 µL of the enzyme (porcine pancreatic amylase 2 U mL^{-1}) in phosphate buffer (100 mmol L^{-1}, pH 6.8). Then, 250 µL of 1 % starch prepared in the same buffer was added to the premixed solution and incubated further for 1 h at 37 °C. One mL of the color reagent, dinitrosalicylate (DNS), was subsequently added and the mixture was boiled for 10 min. The absorbance was taken at 540 nm and the activity of inhibition was recorded as the proportion of the control without extract. All experiments were carried out in triplicate.

Pancreatic lipase. – The pancreatic lipase inhibitory activity was determined by a previously reported method (19). The enzyme containing buffer was prepared by adding a solution of porcine pancreatic lipase (2.5 mg mL^{-1}) in 10 mmol L^{-1} MOPS and 1 mmol L^{-1} EDTA, pH 6.8. Tris buffer (100 mmol L^{-1} Tris-HCl and 5 mmol L^{-1} $CaCl_2$, pH 7.0; 169 µL) was added to 100 µL of extract (50–250 µg mL^{-1}), or orlistat; then 20 µL of enzyme buffer was added and incubated for 15 min at 37 °C. Afterwards, 5 µL of substrate solution (10 mmol L^{-1} *p*NPB in dimethyl formamide) was added and incubated for 30 min at 37 °C. The lipase activity was subsequently determined by measuring the breakdown of *p*NPB to *p*-nitrophenol at 405 nm. All assays were carried out in triplicate.

Statistical analysis

Data are presented as mean ± SD. Data were analyzed using a statistical software package (SPSS for Windows, version 23, IBM Corporation, USA) using Tukey's-HSD multiple range *post-hoc* test. Values were considered significantly different at $p < 0.05$.

RESULTS AND DISCUSSION

GC-MS analysis

Ethanolic extracts of the leaves, stem bark and root bark of *A. wilkesiana* 'Java white' were subjected to GC-MS analysis to identify some major bioactive compounds.

According to the data presented (Figs. 1, 2, 3 and Tables I, II and III), 25 phytochemicals were identified from this plant, which included four major classes of compounds: fatty acids and fatty acid esters, phytols, xanthones and sterols, many of which have been reported to have various medicinal effects including α-amylase and α-glucosidase inhibitory activities (20–26). In addition, EC_{50}, or the minimum concentration required for 50 % inhibition of enzyme activity, was also calculated for different extracts from each part of

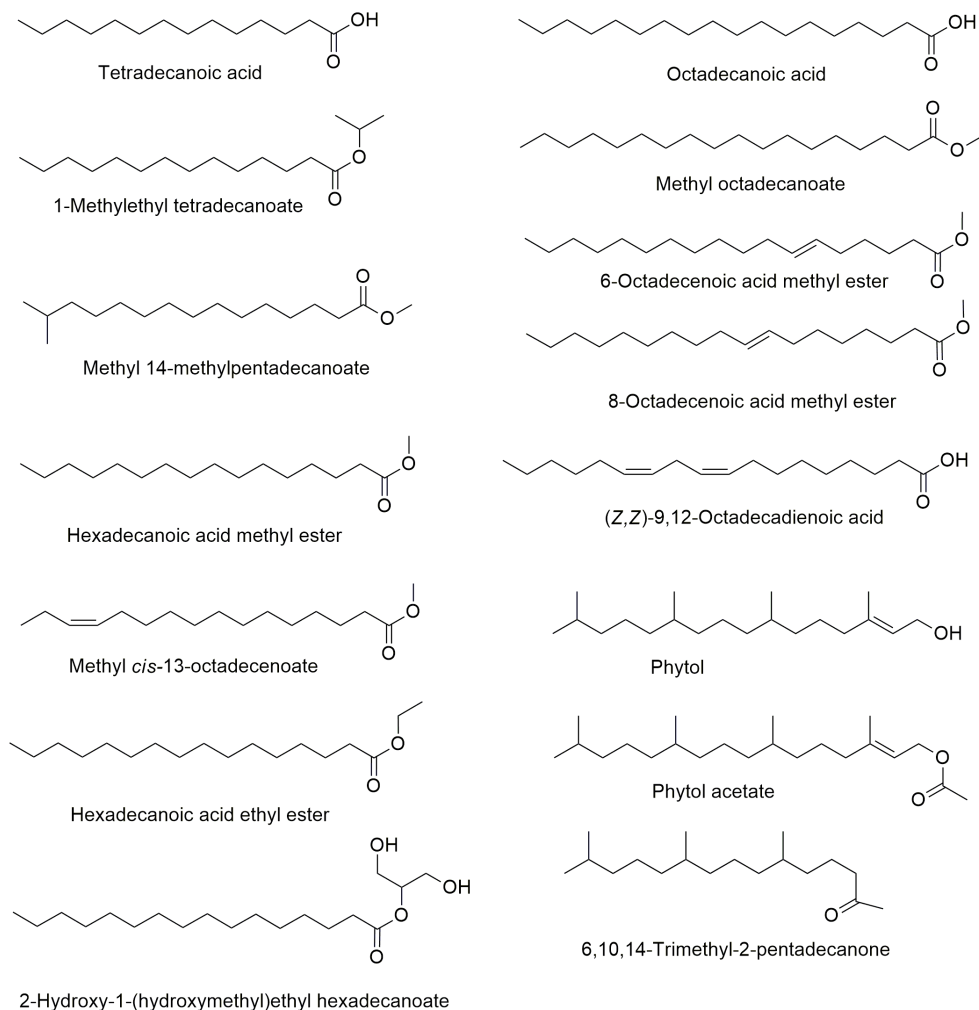

Fig. 1. Long-chain alkyl acids, esters, ketones and alcohols identified from the ethanolic extracts of *A. wilkesiana* leaves, stem bark and root bark.

the plant for different enzymes, as presented in Table IV. However, it should be kept in mind that due to the difference in the nature of samples, namely, extracts (as mixtures of various compounds) and standards (pure compounds), their inhibitory values cannot be directly compared.

Although Odoh *et al.* (14) determined qualitatively the presence of alkaloids, terpenoids, flavonoids, saponins, steroids and tannins in the root methanolic extract and Igwe *et al.* (16) identified 12 compounds in the leaf ethanolic extract, in our study, 25 major bioactive compounds were identified in ethanolic extracts of various parts of the plant (Figs. 1–3 and Ta-

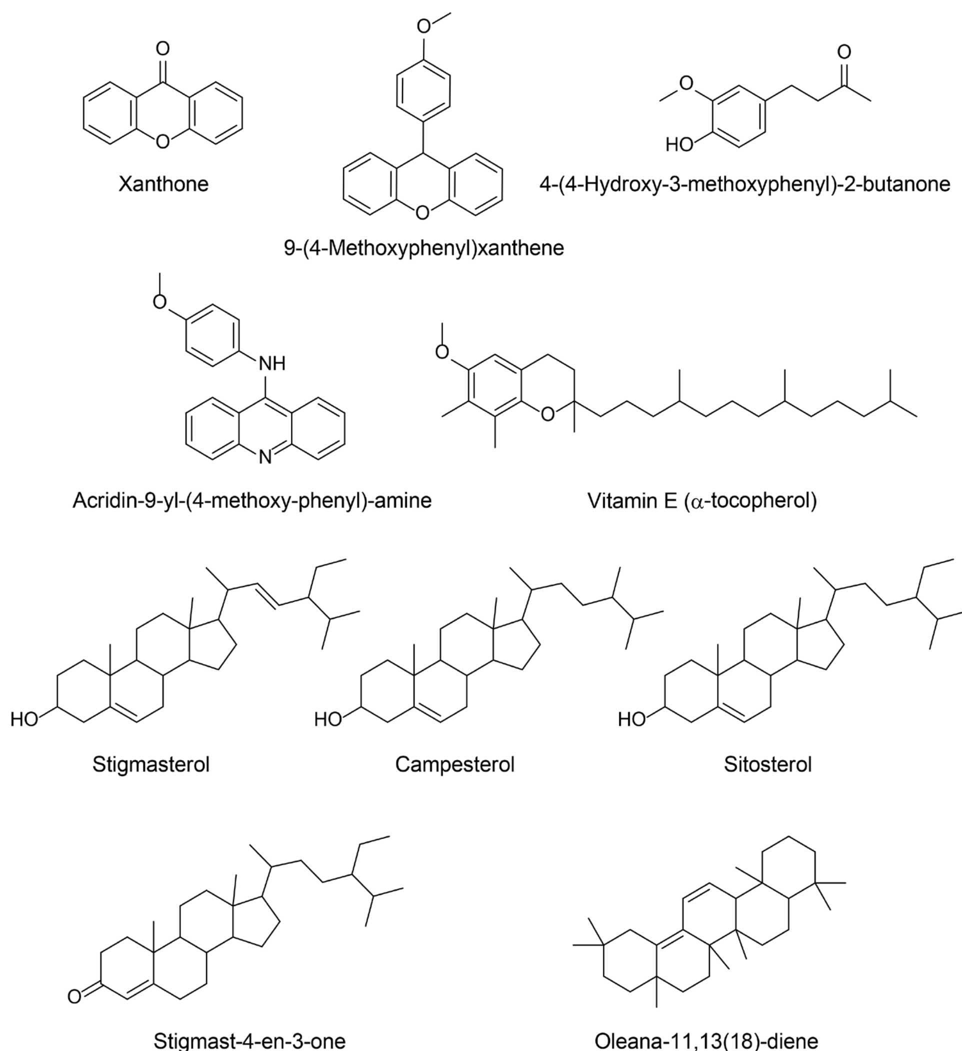

Fig. 2. Aromatics, sterols and triterpenes identified in the ethanolic extracts of *A. wilkesiana* leaves, stem and root bark.

bles I–III). Igwe *et al.* (16) identified predominantly terpenes, followed by long-chain fatty acids, long-chain fatty acyl alcohols, esters and their derivatives, whereas we additionally identified phytol, phytol acetate, vitamin E and various types of sterols such as campesterol, stigmasterol and sitosterol (Table I, Figs. 1–3). Apart from the leaf, the ethanolic extracts of root bark and stem bark were also analyzed by GC-MS in our study although no additional types of compounds were identified (Figs. 1–3 and Tables II and III) except xanthone and an aromatic amine in the ethanolic extract of root bark (Fig. 2 and Table III).

Fig. 3. GC-MS chromatograms of ethanolic extracts of *A. wilkesiana*: a) leaves, b), stem bark and c) root bark.

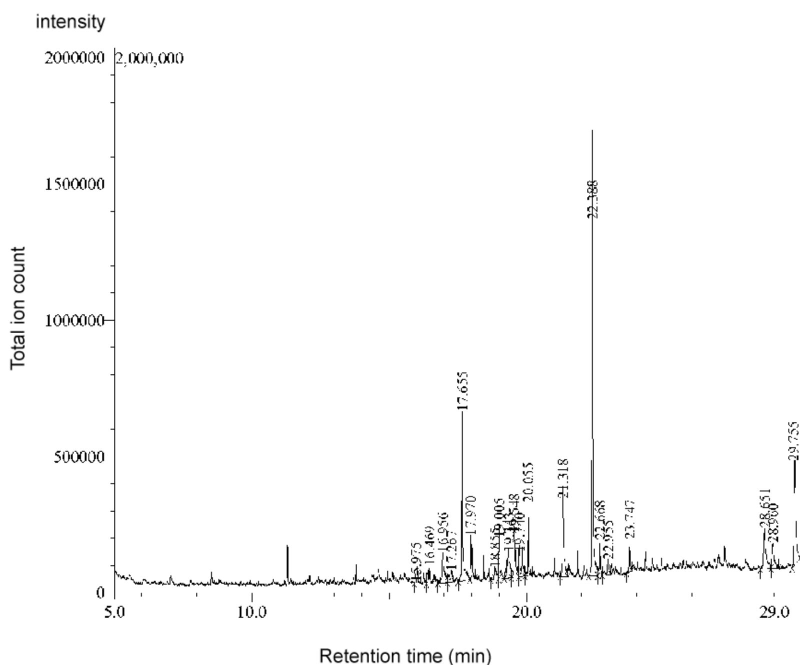

Fig. 3. continued..

Table I. Compounds identified in the ethanolic extract of the leaves of A. wilkesiana *by GC-MS*

Compound	Molecular formula	M_r	t_R (min)	Relative abundance (%)
Phytol acetate	$C_{22}H_{42}O_2$	338.57	16.42	5.76
6-Octadecenoic acid methyl ester	$C_{19}H_{36}O_2$	296.49	19.00	0.57
Phytol	$C_{20}H_{40}O$	296.53	19.10	2.00
Octadecanoic acid	$C_{18}H_{36}O_2$	284.48	19.55	2.56
9-(4-Methoxyphenyl)xanthene	$C_{20}H_{16}O_2$	288.39	22.39	2.89
2-Hydroxy-1-(hydroxymethyl)ethyl hexadecanoate	$C_{19}H_{38}O_4$	330.50	22.52	1.76
Acridin-9-yl-(4-methoxy-phenyl)-amine	$C_{20}H_{16}N_2O$	300.35	24.33	0.38
Oleana-11,13(18)-diene	$C_{30}H_{48}$	408.70	27.22	1.48
Vitamin E (α-tocopherol)	$C_{29}H_{50}O_2$	430.71	27.37	1.16
Campesterol	$C_{28}H_{48}O$	400.68	28.64	1.77
Stigmasterol	$C_{29}H_{48}O$	412.69	28.96	2.32
Sitosterol	$C_{29}H_{50}O$	414.71	29.77	1.81

The compounds listed in the table matched the same compounds in the NIST library.

Table II. Compounds identified in the ethanolic extract of A. wilkesiana *stem bark by GC-MS*

Compound	Molecular formula	M_r	t_R (min)	Relative abundance (%)
Tetradecanoic acid	$C_{14}H_{28}O_2$	228.37	15.56	0.39
1-Methylethyl tetradecanoate	$C_{17}H_{34}O_2$	270.45	16.25	0.31
Phytol acetate	$C_{22}H_{42}O_2$	338.57	16.41	2.00
Ethyl hexadecanoate	$C_{18}H_{36}O_2$	284.48	17.97	2.60
Methyl *cis*-13-octadecenoate	$C_{19}H_{36}O_2$	296.43	18.99	0.43
Phytol	$C_{20}H_{40}O$	296.53	19.10	1.05
9-(4-Methoxyphenyl)xanthene	$C_{20}H_{16}O_2$	288.34	22.39	5.01
Stigmast-4-ene-3-one	$C_{29}H_{48}O$	412.69	23.24	1.82
Campesterol	$C_{28}H_{48}O$	400.68	28.64	3.08
Stigmasterol	$C_{29}H_{48}O$	412.69	28.96	1.56
Sitosterol	$C_{29}H_{50}O$	414.71	29.75	7.57

The compounds listed in the table matched the same compounds in the NIST library.

Table III. Compounds identified in the ethanolic extract of A. wilkesiana *root bark by GC-MS*

Compound	Molecular formula	M_r	t_R (min)	Relative abundance (%)
4-(4-Hydroxy-3-methoxyphenyl)-2-butanone	$C_{11}H_{14}O_3$	194.23	14.37	0.63
Phytol acetate	$C_{23}H_{46}O$	338.57	16.41	0.46
6,10,14-Trimethyl-2-pentadecanone	$C_{18}H_{36}O$	268.48	16.46	0.89
Xanthone	$C_{13}H_8O_2$	196.20	16.95	1.68
Hexadecanoic acid methyl ester	$C_{17}H_{34}O_2$	270.45	17.30	0.43
Hexadecanoic acid ethyl ester	$C_{18}H_{36}O_2$	284.48	17.97	1.54
8-Octadecenoic acid methyl ester	$C_{19}H_{36}O_2$	296.49	18.99	1.91
(*Z,Z*)-9,12-octadecadienoic acid	$C_{18}H_{32}O_2$	280.45	19.28	0.70
9-(4-Methoxyphenyl)xanthene	$C_{20}H_{16}O_2$	288.34	22.38	21.02
Acridin-9-yl-(4-methoxy-phenyl)-amine	$C_{20}H_{16}N_2O$	300.35	24.55	0.36
Campesterol	$C_{28}H_{48}O$	400.68	28.64	3.71
Stigmasterol	$C_{29}H_{48}O$	412.69	28.96	2.08
Sitosterol	$C_{29}H_{50}O$	414.71	29.75	9.60

The compounds listed in the table matched the same compounds in the NIST library.

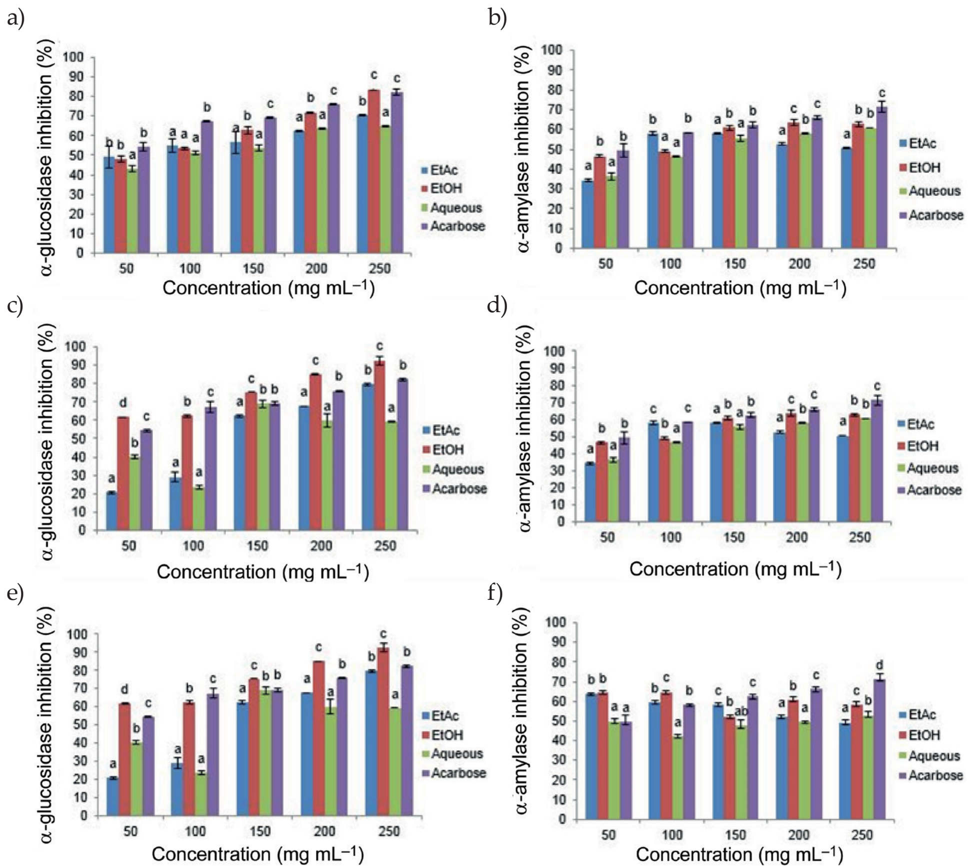

Fig. 4. α-glucosidase and α-amylase inhibitory activities of leaves (a and b), stem bark (c and d) and root bark (e and f) extracts of *A. wilkesiana.* Data are presented as mean ± SD of triplicate determinations. $^{a-c}$ Different letters over the bars for a given concentration are significantly different from each other ($p < 0.05$). EtAc – ethyl acetate, EtOH – ethanol.

α-glucosidase and α-amylase inhibitory activity

The effects of various extracts on the two key enzymes, α-glucosidase and α-amylase, implicated in T2D are shown in Figs. 4a-f. All the extracts showed significant α-glucosidase ($p < 0.05$) inhibitory activity, in a dose dependent manner. The ethanolic extracts from stem bark and root bark, however, showed significantly ($p < 0.05$) higher activities than the others. This is also evident from their lower EC_{50} values compared to other extracts (Table IV). The ethanolic extract of root bark showed the presence of xanthone (Fig. 2, Table III). Xanthones are biologically active plant phenols that have lately received profound interest (23). Xanthones have been studied to possess, among others, anti-inflammatory, anti-cancer,

Table IV. EC_{50} *values of various extracts of* A. wilkesiana *parts in inhibiting α-glucosidase, α-amylase and lipase activities*

Sample	EC_{50} (μg mL^{-1})		
	α-glucosidase	α-amylase	Lipase
Leaves			
Ethyl acetate	74.63 ± 2.87^{b}	296.18 ± 37.18^{f}	384.55 ± 23.84^{e}
Ethanol	67.18 ± 7.65^{b}	75.35 ± 8.25^{c}	149.70 ± 8.34^{c}
Aqueous	88.35 ± 15.37^{c}	118.9 ± 19.3^{d,e}	203.71 ± 1.82^{d}
Stem bark			
Ethyl acetate	242.99 ± 41.83^{e}	274.55 ± 31.09^{f}	146.88 ± 19.84^{c}
Ethanol	37.10 ± 2.71^{a}	>1000^{g}	113.38 ± 18.23^{b}
Aqueous	267.94 ± 24.90^{e}	>1000^{g}	894.61 ± 36.28^{f}
Root bark			
Ethyl acetate	124.04 ± 16.72^{d}	98.65 ± 15.11^{d}	>1000^{f}
Ethanol	35.75 ± 1.95^{a}	6.25 ± 1.05^{a}	101.33 ± 5.21^{b}
Aqueous	127.25 ± 23.60^{d}	151.04 ± 12.59^{e}	>1000^{e}
Standard			
Acarbose	36.27 ± 1.84	53.77 ± 3.95	ND
Orlistat	ND	ND	0.03

Data are presented as mean ± SD values of triplicate determinations.
$^{a-g}$ Different superscripts in a column for a given parameter denote significant difference from each other; $p < 0.05$.
EC_{50} – the minimum extract concentration required for 50 % inhibition, ND – not determined.

anti-hyperglycemic and anti-hypertensive effects (24, 25). A study conducted in 2014 showed that xanthones extracted from the plant *Swertia mussotii* exerted α-glucosidase inhibitory activity (26). In a previous study, it was also reported that long-chain unsaturated fatty acids had significant α-glucosidase inhibitory activity (27). This implies that the α-glucosidase inhibitory activity of *A. wilkesiana*, especially the low EC_{50} values of the ethanolic root bark (Table IV), could be attributed to the xanthones and long-chain unsaturated fatty acids and their derivatives.

In vitro pancreatic α-amylase activity was also inhibited by all extracts of plant parts, with the most significant ($p < 0.05$) activity exhibited by the ethanolic extract of root bark, with the lowest EC_{50} value of 6.25 ± 1.05 μg mL^{-1}.

Ethanolic extracts of the various parts of *A. wilkesiana* have been reported to contain various long-chain fatty acids and fatty acid esters, *e.g.*, tetradecanoic acid, octadecanoic acid, octadecanoic acid methyl ester, phytol, phytol acetate, xanthone and various sterols including stigmasterol (Figs. 1, 2 and 3). In a number of recent studies, plant extracts containing these compounds have been shown to have α-amylase, α-glucosidase and lipase

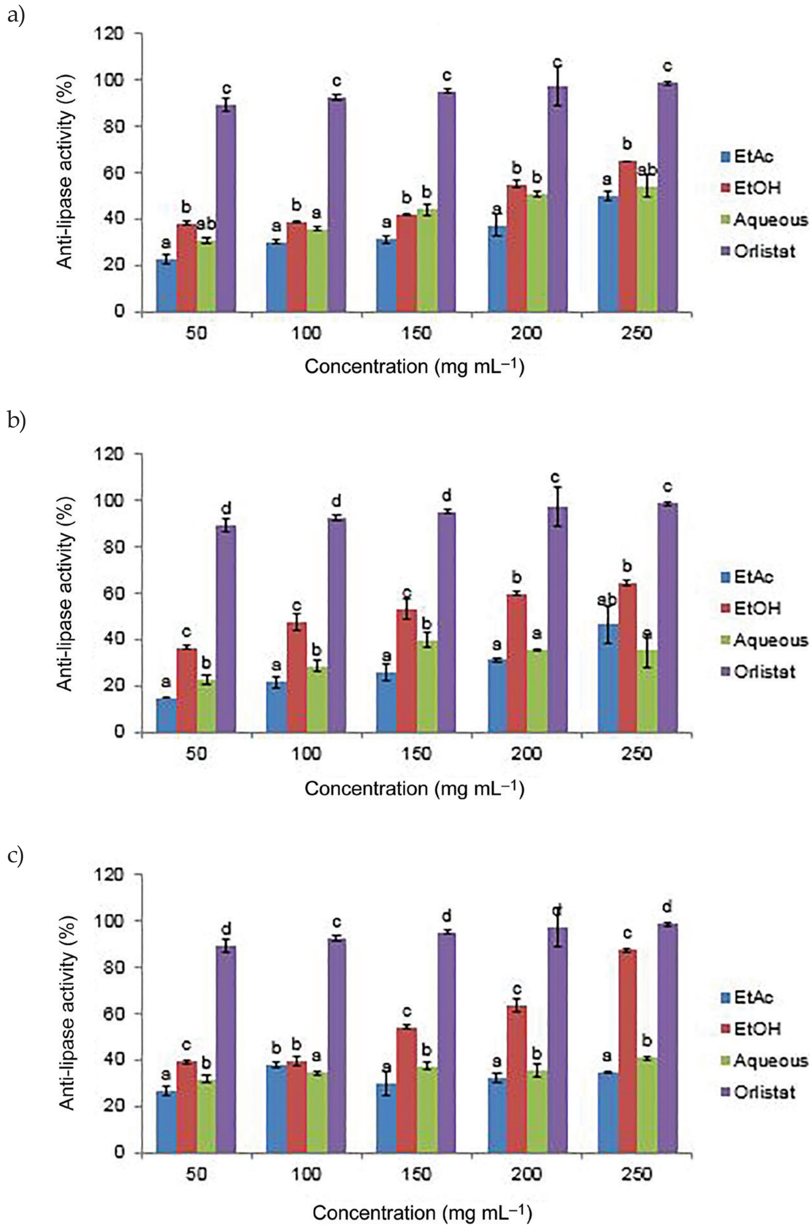

Fig. 5. Anti-lipase activity of: a) leaves, b) stem bark and c) root bark extracts of *A. wilkesiana.* Data are presented as mean ± SD of triplicate determinations.

[a-d] Different letters over the bars for a given concentration are significantly different from each other ($p < 0.05$). EtAc – ethyl acetate, EtOH – ethanol.

inhibitory activity (28–30). Hence, the enzyme inhibitory activity of this plant might be linked to some of these bioactive compounds.

We are inclined to believe that the inhibition of these enzymes by *A. wilkesiana* might indicate potential anti-hyperglycemic efficacy, which could corroborate its folkloric use as a medicinal plant for the treatment and management of T2D.

Pancreatic lipase inhibitory activity

Results given in Figs. 5a-c show the inhibitory potential of the extracts of all parts for pancreatic lipase activity, thus demonstrating potential anti-obesogenic activity, with the best activity exhibited by the ethanolic extract of root bark (EC_{50} = 101.33 ± 5.21 μg mL^{-1}).

Overweightness and obesity are responsible for causing insulin resistance, one of the two major ways of pathogenesis of T2D (31). On the other hand, the most popular screening method in the discovery of anti-obesogenic drugs is to identify potent lipase inhibitors (32). Hence, one of our objectives was to examine the ability of plant extracts to inhibit lipase. Ethanolic and aqueous extracts of the leaves have been reported to show serum triglyceride lowering abilities (33). This might be due to their reducing small intestinal absorption of dietary lipids *via* inhibiting lipase activity. The pancreatic lipase inhibitory activity displayed by the ethanolic extract of root bark (Fig. 5c) with considerably low EC_{50} values (Table IV) could further support the potential of *A. wilkesiana* for the management of T2D and its associated risk factors such as overweight and obesity.

CONCLUSIONS

The presence of potentially bioactive compounds in *A. wilkesiana* exerting carbohydrates and lipids digesting enzymes inhibitory effects is suggested in this study. These results indicate that no part of *A. wilkesiana* is a waste since it could contain potent inhibitory activity against some key enzymes linked to diabetes and obesity, which also gives antecedence to its use in folk medicine. These findings specifically point out the ethanolic extract of stem bark and root bark as more active extracts compared to leaf extracts. Although a number of bioactive compounds have been identified in *A. wilkesiana* extracts by GC-MS analysis, some additional techniques such as LC-MS or HPLC-PDA could expand the list of bioactive compounds, so further studies are warranted in this regard.

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