Electric Power Systems Research 190 (2021) 106720

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

α-Amylase and α-glucosidase inhibitors from *Zanthoxylum chalybeum* Engl. root bark

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ARTICLE INFO

Keywords:
Zanthoxylum chalybeum
Amide alkaloids
α-Amylase and α-glucosidase inhibitors

ABSTRACT

A systematic analysis of the root bark of *Zanthoxylum chalybeum* was conducted to establish the antidiabetic potential of isolated compounds based on its ethnomedicinal use to manage diabetes. Chromatographic separation of alkaloid extracts led to isolation of three undescribed amides, chaylbemide A (**1**), chalybemide B (**2**) and chalybemide C (**3**) alongside the known fagaramide (**4**); four known benzophenanthridine alkaloids skimmianine (**5**), norchelerythrine (**6**), 6-acetonyldihydrochelerythrine (**7**) and 6-hydroxy-*N*-methyl decarine (**8**). The alkaloid free extracts yielded three known lignans, ailanthoidol (**9**), 2,3-epoxy-6,7-methylenedioxy coniferyl alcohol (**10**), sesamine (**11**), together with five known triterpenoids, lupeol (**12**), lupanone (**13**), 3α,20-dihydroxy-28-lupanoic acid (**14**), 20-hydroxy-3-oxo-28-lupanoic acid (**15**) and 3α,20,28-trihydroxylupane (**16**). The structures of the compounds were established based on 1D and 2D NMR spectroscopic and mass spectrometric experiments. Compounds **1**–**8** displayed inhibitory activities against both α-amylase and α-glycosidase in the range of IC_{50} = 43.22–49.36 μM which showed no significant ($P > 0.05$) difference to the positive control acarbose (IC_{50} = 42.67; 44.88 μM). The results confirmed anti-hyperglycemic potential of alkaloids from *Z. chalybeum* which lends credence to its use towards management of diabetes susceptibilities.

1. Introduction

The antihyperglycemic effects of plants extracts are attributed to their ability to restore the function of pancreatic tissues by causing an increase in insulin output or inhibition of the two intestinal digestive enzymes that regulate the amount of glucose available for absorption into the bloodstream [1]. However, such traditional knowledge, derived empirically, has to be supported by scientific validation. For instance, a number of plants have great reputation in traditional medicine, for a majority, the pharmacology and efficacy has not been studied using scientific methods. Ethno-medicinal applications of *Zanthoxylum chalybeum* Engl. (Rutaceae) towards management of several diseases such as malaria, diabetes, fevers, sickle cell diseases [2,3] has been noted and investigated to some levels. The root and stem bark decoctions of *Zanthoxylum chalybeum* reported for management of diabetes, has since been investigated to possess relatively significant antihyperglycemic activities against streptozotocin and alloxan-induced diabetic rat [4,5,6]. Despite earlier establishment of the metabolites of this plants including skimmianine [7], chelerythrine, nitidine and methyl canadine [8], fagaramide [9], 2,3-epoxy-6,7-methylenedioxyconiferyl and dihydrochelerythrine [10], the potential active antihyperglycemic compounds have never been established. In anticipation for discovering new antidiabetic compounds, a systematic in vitro evaluation of the active antihyperglycemic compounds from the root bark of the *Z. chalybeum* was conducted.

2. Experimental

2.1. General instrumentation

IR spectra were obtained from a Bruker Tensor 27 spectrometer with KBr pellets. UV data were generated from a Shimadzu UV2401PC spectrophotometer. NMR spectra were recorded on Bruker AV-600 spectrometers operating at 600 and 150 MHz, for ^{1}H and ^{13}C NMR spectra, respectively. Coupling constants were expressed in Hz and chemical shifts are given on a ppm scale with reference to the solvent signals. HREIMS was recorded on an on a Waters Auto Spec Premier P776 mass spectrometer. Column chromatography (CC) was performed

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https://doi.org/10.1016/j.fitote.2020.104719
Received 28 July 2020; Received in revised form 29 August 2020; Accepted 30 August 2020
Available online 12 September 2020

with silica gel Merck 60G, 70–230 mesh) and Reverse phase LiChroprep Rp-18 gel (40–63 μm, Merck). TLC was carried out on silica gel H-precoated plates (Machenry-Nagel Co. Germany). Spots were detected by spraying with Dragendorff's reagent for alkaloid fractions and *p*-anisaldehyde for alkaloid free fractions (Kobian, Kenya and Sigma-Aldrich Chemical Co. USA). Other reagents included organic solvents such as *n*-hexane, dichloromethane, ethyl acetate, 95% methanol ethanol; glacial acetic acid, and dimethylsulfoxide (DMSO). A-amylase and α-Glucosidase (from *Saccharomyces cerevisiae*), *p*-nitrophenyl-α, *D*-glucopyranoside and acarbose were purchased from Sigma-Aldrich Chemical Company. Enzymatic activity (fluorescence) was recorded using a SpectraMax GeminiXS Spectrofluorometer (Molecular Devices, Sunnyvale, CA).

2.2. Plant materials

The root bark of *Z. chalybeum* were collected from Homa Hills, Homa Bay County, Kenya (0° 23′S, 34° 30′E) in September 2018. Identification and authentication of the plant samples was done by Dr. Philip Onyango a taxonomist at Maseno University, Department of botany where a voucher specimen (DWN/MSN/01/2018) was deposited at the Maseno University herbarium.

2.3. Extraction and isolation

The root bark of *Z. chalybeum* were chopped into small pieces separately, air-dried at room temperature under shade for 21 days and ground into fine powder using an electric pulverizer. The powdered root bark (0.8 kg) were exhaustively extracted with 95% aqueous methanol (4 × 1.5 L) and filtered. The successive filtrates were combined and concentrated in vacuo using a rotary evaporator to yield (45 g) 5.6% crude extract.

Total alkaloid extraction: A portion (30 g) of the crude extracts suspended in 200 mL of 2% 0.1 M H_2SO_4 in ethyl acetate under agitation for 40 min, replicated four times and the extracts were combined, followed by three times extraction of the non-polar compounds using 300 mL *n*-hexane using a separatory funnel. The acidic polar fractions was neutralized using 0.1 M ammonia hydroxide solution, followed by further extraction using 200 mL dichloromethane (CH_2Cl_2), until a negative Dragendorff's test was observed. The organic fraction (CH_2Cl_2 extracts) was dried with anhydrous sodium sulphate, followed by further concentration in *vacuo* to yield 10.5 g (1.31% of the crude extract).

The alkaloid extract (9 g) was chromatographed on a normal phase silica gel 60 column eluting with CH_2Cl_2, using gradient polarity (*v/v*) of CH_2Cl_2: CH_3OH 10:0 (500 mL), 9:1 (500 mL), 8:2 (500 mL), 7:3 (500 mL), 1:1 (500 mL) and final 100% MeOH (300 mL). The process resulted into 34 fractions which were combined into 10 fractions following their TLC profiles that were monitored using Dragendorff's reagent spray on TLC plates. Fractions 2 (40 mg) and 3 (67 mg) eluted with 1% CH_3OH in CH_2Cl_2 were further combined and separated using preparative thin layer chromatography (PTLC) developing with 8% CH_3OH in CH_2Cl_2. Bands related to each isolated compound were scrapped, dissolved in DCM then filtered to afford two UV active compounds, norchelerythrine (**6**) and 6-acetonyldihydrochelerythrine (**7**). Fraction 4 (209 mg) and 5 (162 mg) that were eluted with CH_2Cl_2: CH_3OH 8:2 *v*/v were combined and subjected to column (60 cm × 3.0 cm, SiO_2) chromatography eluting with CH_2Cl_2: CH_3OH gradient polarity of 9:1, 8:2 and 7:3 to afford 8 fractions (25 mL) which were combined into three fractions according to their TLC profiles. Compound **5** (skimmianine, 30 mg), Compound **6** (50 mg) and **7** (80 mg) were obtained by recrystallization with CH_2Cl_2: *n*-hexane of sub-fraction one, two and three, respectively of Fraction 4 and 5. Fraction 6 (410 mg) and fraction 7 (296 mg) eluted with 7:3 *v*/v CH_2Cl_2:CH_3OH mixtures were combined and subjected to column (60 cm × 4.0 cm, SiO_2) chromatography, eluting with CH_2Cl_2: CH_3OH gradient polarity of 8:2, 7:3 and 1:1 to afford 12 fractions (25 ml each) which were combined into four fractions according to their TLC profiles. Sub-fraction one of fraction 6 and 7 yielded compound **4** (fagaramide 40 mg) as the first crystalline compound from CH_2Cl_2:*n*-hexane mixture whereas the mother liquor produced a precipitate of compound **1** {6-Benzo[1,3]dioxol-5-yl-hexa-2,5-dienoic acid isobutylamide} (chaylbemide A), 76 mg. Sub-fraction three of fraction 6 and 7 yielded white crystalline solids of compound **2** {4-Methoxy-*N*-(2-methoxyphenyl)-*N*-methyl-benzamide (chalybemide B), 77 mg} from CH_2Cl_2:*n*-hexane mixture. Finally fractions 8, 9 and 10 (158, 214 and 176 mg, respectively) eluted with 1:1 *v/v* CH_2Cl_2: CH_3OH were combined and re-chromatographed on CC (60 cm and 4.0 cm, SiO_2), gradient elution with CH_2Cl_2: CH_3OH (7,3 1,1 and 3,7 *v/v*). Four major fractions were achieved, from which sub-fraction two yielded compound **3** {*N*-(2-Hydroxy-2-methyl-propyl)-3-phenyl-acrylamide (chalybemide C), 30 mg} following CH_2Cl_2: CH_3OH crystallization. Sub-fraction three yielded additional compound **2** (47 mg) and compound **8** (6-hydroxy-*N*-methyl decarine, 61 mg), following fractional re-crystallizations in CH_2Cl_2: *n*-hexane.

Alkaloid-free extracts: The aqueous non-alkaloid fraction was concentrated in vacuo to dryness and afforded 10.9 g (1.36% of crude extracts) which was then suspended in 200 mL distilled water followed by sequential solvent extractions beginning with 3 × 300 mL *n*-hexane, followed by 3 × 300 mLethyl acetate, and finally 3 × 300 mL *n*-butanol (C_4H_9OH) using a separatory funnel. The combined decanted organic layer extracts, were evaporated in *vacuo* to yield 2.05 g (0.13%), 5.01 g (0.63%) and 3.1 g (0.39%), respectively.

Dry *n*-hexane non-alkaloid extract (2 g) was loaded on silica gel in column (60 × 3 cm, SiO_2) and eluted stepwise with *n*-hexane adjusting the polarity with ethyl acetate as (1:0, 99:1, 95:5, 9:1, 4:1, 3:1, 2:1). Out of the 35 eluant fraction (25 mL each), five fractions were pooled together based on TLC profiles monitored using *p*-anisaldehyde-sulphuric acid reagent. The third sub-fraction formed a white precipitate which gave one purple-blue spot on TLC at 4:1 (*n*-hexane-ethyl acetate, R_f = 0.36), that was confirmed to be lupeol (**12)** based on TLC comparison with an authentic sample. The fourth (87 mg) and firth (112 mg) sub-fraction were combined and subject to flash chromatography (30 × 1 cm, SiO_2) eluted with *n*-hexane: ethyl acetate (95:5, 9:1, 4:1) and two fractions were obtained which gave a white crystallized solid (58 mg) identified as lupanone (**12**). Ethyl acetate extract (6 g) was loaded on silica gel column (120 × 60 mm, SiO_2), eluting with n-hexane-EtOAc (1:0 to 7:3, 5% gradient, 500 mL of each eluent) to yield a total of 55 fractions which were then combined into five fractions. The first two fractions afforded more of lupeol **11** (20 mg) and lupenone **12** (30 mg). The third fraction crystallized in methanol to form platy-like white crystals (90 mg) of 2,3-epoxy-6,7-methylenedioxy coniferyl alcohol (**10)**. The fourth fraction showing single purple-blue spot on TLC with p-anisaldehyde reagent crystalized to form white crystals (45 mg) of 3α,20-dihydroxy-28-lupanoic acid (**14**). The fifth fraction yielded white solid (62 mg) which crystallized in *n*-hexane: ethyl acetate (9:1) to give 20-hydroxy-3-oxo-28-lupanoic acid (**15**). Further purification of the sixth fraction using silica gel column (120 × 60 mm, SiO_2) eluting with *n*-hexane: ethyl acetate (9:1, 4:1) gave more of the white solid of compound **15** (25 mg) and compound **16** (3α,20,28-trihydroxylupane, 80 mg). A portion of n-C_4H_9OH fraction was (2 g) also chromatographed on reverse (C_{18} reverse phase SiO_2, 40–63 μm) flash chromatography, eluting with H_2O- CH_3OH (9.1); CH_3OH; CH_3OH - CH_2Cl_2 [1] and finally CH_2Cl_2. A total of 30 fractions (each 50 ml) which were further combined to 3 fractions according to their TLC profiles were obtained. The first fraction yielded a white amorphous solid that was confirmed to be sesamine (**11,** 103 mg), the second fraction yielded few mixtures of sesamine (**11**) and ailanthoidol (**9**) that crystalized in CH_2Cl_2; *n*-hexane as white crystalline solid (62 mg).

Table 1
^{1}H and ^{13}C NMR (600 and 150 MHz, δ ppm, $CDCl_3$) Spectroscopic Data for **1**, **2** and **3.**

Compound **1**			Compound **2**			Compound **3**		
Position	δ_H, mult (J, Hz)	δ_C	Position	δ_H, mult (J, Hz)	δ_C	Position	δ_H, mult (J, Hz)	δ_C
1		169.3(C=O)	1		166.5(C=O)	1		168.1(C)
2	5.74 d (15.2)	123.8 (CH)	2	6.64 d (15.5)	119.8(CH)	2		122.2(C)
3	6.05 dd (15.2, 7)	146.8 (CH)	3	7.73 d (15.5)	141.6 (CH)	3		159.8(C)
4	2.39 m	35.3 (CH_2)	4		134.9 (C)	4	6.89 dd (8.3, 1.2)	112.2(CH)
5	6.24 dd (15.2, 7)	129.2 (CH)	5	7.53 m	126.2 (CH)	5	7.52 m	132.4(CH)
6	6.77 d (15.2)	129.5 (CH)	6	7.36 m	128.4 (CH)	6	7.05 t (7.7)	122.5(CH)
7		132.5 (C)	7	7.47 m	127.7 (CH)	7	7.86 dd (8.3, 1.8)	132.1(CH)
8	6.89 d (1.2)	107.9 (CH)	8	7.36 m	128.4 (CH)	1’		131.6(C)
9		147.9(C–O)	9	7.53 m	126.2 (CH)	2′	7.15 d (7.2)	127.1(CH)
10		148.6(C–O)	1’	3.47 br s	58.8 (CH_2)	3’	6.86 d (7.2)	113.2(C)
11	6.73 d [8]	109.1 (CH)	2’	–	70.1 (C–O)	4′		158.2(C–O)
12	6.83 dd (8, 1.2)	120.2 (CH)	3′	1.19 br s	27.2 (CH_3)	5′	6.86 d (7.2)	113.2(CH)
1′	3.13 t [6]	49.5 (CH_2)	4′	1.19 br s	27.2 (CH_3)	6′	7.15 d (7.2)	127.1(CH)
2′	1.78 m	29.3 (CH)				7’	3.91 br s	57.1(CH_2)
3′	0.91 d [6]	19.3 (CH_3)				OCH_3	3.73 br s	56.4 (CH_3)
4′	0.94 d [6]	19.3 (CH_3)				OCH_3	3.72 br s	56.6 (CH_3)
OCH_2O	5.92 br s	99.4 (CH_2)						

Assignments were aided by 2D NMR; COSY, HMQC and HMBC for ^{1}H NMR whereas DEPT, HMQC and HMBC aided ^{13}C NMR.

2.4. Physical and spectroscopic data of isolated of new compounds from Z. chalybeum root back

6-Benzo[1,3]dioxol-5-yl-hexa-2,5-dienoic acid isobutylamide (chaylbemide A, **1**): white powder; UV ($CHCl_3$) λ_{max} (log ε): 305 (0.2), 265 (0.47) nm; IR (KBr) νmax: 3457, 3316, 2943,1625, 1551, 1505, 1466, 1257 cm^{-1}; ^{1}H and ^{13}C NMR data (Table 1); HREIMS: *m/z* 287.1317 $[M]^+$ (calcd. for $C_{17}H_{21}NO_3$, 287.1312).

4-Methoxy-*N*-(2-methoxy-phenyl)-*N*-methyl-benzamide (chalybemide B, **2**): white crystalline; UV ($CHCl_3$) λ_{max} (log ε): 284 (0.76), 218 (0.48), 204 (0.24) nm; IR (KBr) νmax: 3424, 1736, 1649,1560, 1431, 1398, 1255, 1202, 1070 cm^{-1}; ^{1}H and ^{13}C NMR data (Table 1); HREIMS: *m/z* 219.1103 $[M]^+$ (calcd. for $C_{13}H_{17}NO_2$, 219.1105).

N-(2-Hydroxy-2-methyl-propyl)-3-phenyl-acrylamide (chalybemide C, **3**): white crystalline; UV ($CHCl_3$) λ_{max} (log ε): 284 (0.76), 218 (0.48), 204 (0.24) nm; IR (KBr) νmax: 3424, 1736, 1649,1560, 1431, 1398, 1255, 1202, 1070 cm^{-1}; ^{1}H and ^{13}C NMR data (Table 1); HREIMS: *m/z* 285.11373 $[M]^+$ (calcd. for $C_{17}H_{19}NO_3$, 285.1365).

2.5. α-Amylase inhibition assay

The antihyperglycemic activity of the crude extracts and the pure isolated compounds were assayed against porcine pancreas α-amylase. Individual compounds were dissolved in 5% DMSO in distilled water to produce an 8 mM stock solutions. Various dilutions of compounds were pre-incubated in 96-well plates for 30 min at 22–25 °C with 250 μU of α-amylase at a final concentration of 2.5 mU/mL. The incubation buffer consisted of 50 mM NaH_2PO_4, 50 mM NaCl, 0.5 mM $CaCl_2$ and 0.1% bovine serum, pH 6.0, and the final volume of the pre-incubation mixture was 75 μL. The final concentrations of the compounds (1–200 μM) together with acarbose (100 μM, positive control) were used against α-amylase enzymatic activity monitored by digestion of the DQ™ starch (25 μL of 20 μg/mL) substrate relative to a negative control of 5% DMSO concentrations. Fluorescence was measured using a SpectraMax GeminiXS Spectrofluorometer (Molecular Devices, Sunnyvale, CA) with excitation and emission wavelengths of 485 nm and 530 nm, respectively. The α-amylase inhibition rate was calculated relative to control as follows:

$$\%\text{Inhibition} = \frac{\text{Absorbance (Control)} - \text{Absorbance (Compound)}}{\text{Absorbance (Control)}} \times 100$$

2.6. α-Glucosidase inhibition assay

The antihyperglycemic activity of the crude extracts and the pure isolated compounds were assayed against α-glucosidase [11] pure compounds prepared as described in α-amylase assay above. The test compound and 2 mU of α-glucosidase was diluted to 97 μL in 0.1 M potassium phosphate buffer (pH 6.5) and pre-incubated in 96-well plates at 37 °C for 15 min. The reaction was initiated by adding 3 μL of 3 mM *p*-nitrophenyl-*α*, *D*-glucopyranoside (*p*-NPG) as substrate and incubated for an additional 15 min at 37 °C, followed by addition of 100 μL of 1 M Na_2CO_3 to stop the reaction. All test compounds were prepared in 5% DMSO in distilled water. The final concentrations of the compounds were between 5 and 200 μM while the final concentration of α-Glucosidase was 20 mU/mL. The enzyme activity was then determined by measuring the release of *p*-nitrophenol from the *p*-NPG substrate and the reaction monitored by change of absorbance at 410 nm using a SpectraMax 190 Spectrophotometer. The α-Glucosidase inhibition rate was calculated relative to control as follows:

$$\%\text{Inhibition} = \frac{\text{Absorbance (Control)} - \text{Absorbance (Compound)}}{\text{Absorbance (Control)}} \times 100$$

2.7. Data analysis

The concentration of the compounds that inhibits 50% of the enzyme activity (IC_{50}) was determined by logarithmic regression analysis from the mean percent inhibition. One-way analysis of variance (ANOVA, Tukey-Kramer HSD Post-Hoc) was used to determine differences in mean of IC_{50} of different groups. Values were considered significant at $p \leq 0.05$.

3. Results and discussion

Systematic analysis of the alkaloids and alkaloid free extracts of root *Z. chalybeum* led to the characterization of three new aromatic amide alkaloids namely 6-Benzo[1,3]dioxol-5-yl-hexa-2,5-dienoic acid isobutylamide (chaylbemide A, **1**), 4-Methoxy-*N*-(2-methoxy-phenyl)-*N*-methyl-benzamide (chalybemide B, **2**) and *N*-(2-Hydroxy-2-methyl-propyl)-3-phenyl-acrylamide (chalybemide C, **3**) alongside a known amide alkaloid fagaramide (**4**) [9,12]; and four known benzophenanthridine alkaloids namely skimmianine (**5**) [7] norchelerythrine (**6**) [13], 6-acetonyldihydrochelerythrine (**7**)[14], 6-hydroxy-*N*-methyl decarine (**8**) [15]. The alkaloid free extracts yielded three known

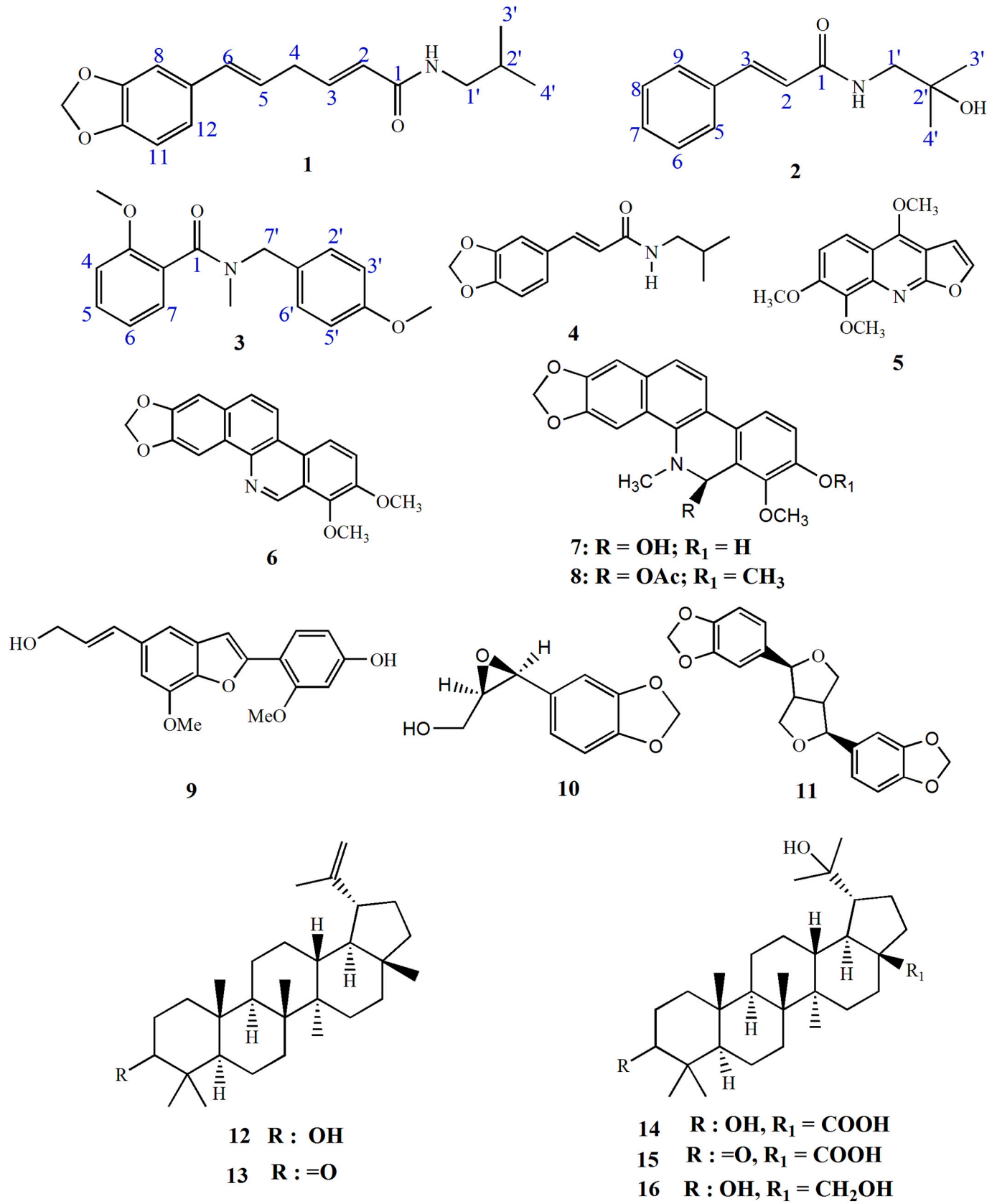

Fig. 1. Compounds isolated from the root bark of *Z. chalybeum.*

lignans; ailanthoidol (**9**) [16], 2,3-epoxy-6,7-methylenedioxy coniferyl alcohol (**10**) [10], sesamine (**11**) [17], together with five known triterpenoids; lupeol (**12**)[18], lupanone (**13**) [19], 3α,20-dihydroxy-28-lupanoic acid (**14**), 20-hydroxy-3-oxo-28-lupanoic acid (**15**) [20] and 3α,20,28-trihydroxylupane (**16**) [21] were isolated and identified (Fig. 1). The structures of compounds **4–16** were established based on

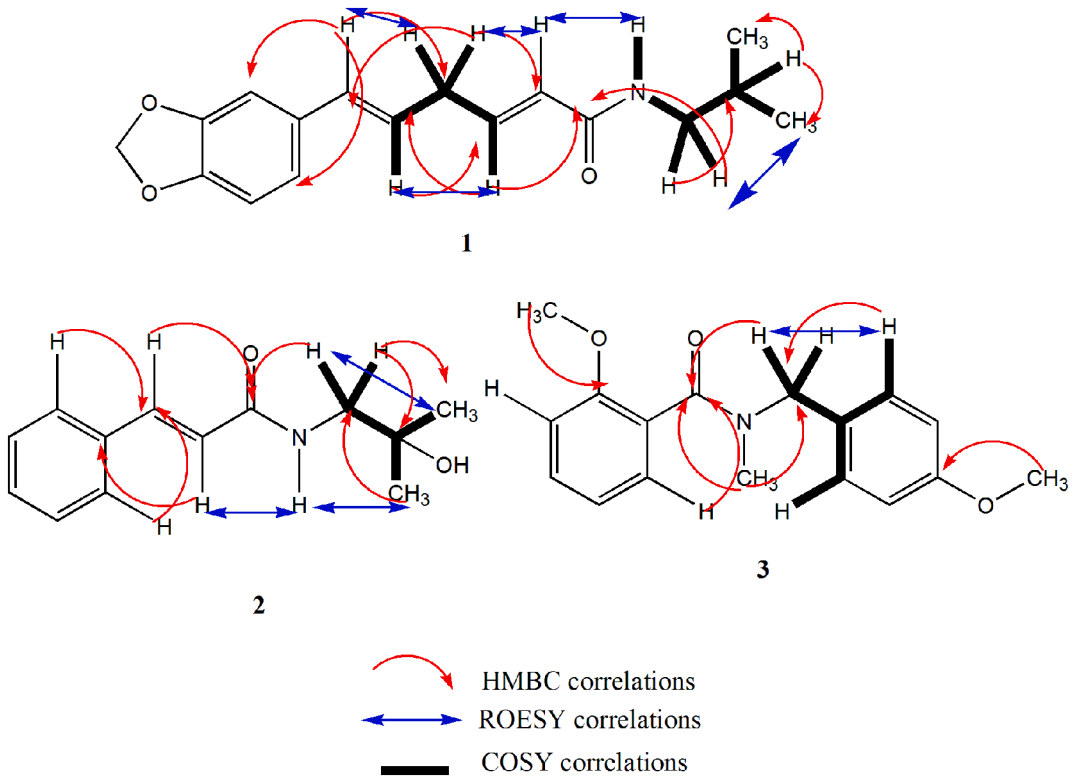

Fig. 2. Significant HMBC, COSY and ROESY correlations observed for compounds **1**, **2** and **3**.

1D NMR (^{1}H, ^{13}C and DEPT-135), 2D NMR (COSY, HMBC, HSQC) and MS experimental results which corresponded to the respective cited literature data reported previously.

Compound **1** was isolated as white powder, with a molecular formula of $C_{17}H_{21}NO_3$ as determined by HREIMS *m/z* 287.1317 $[M]^+$ calcd For $C_{17}H_{21}NO_3$ 287.1312). The IR spectrum showed an amide functionality, an aromatic and/or alkene functionality identified based on 3457 cm^{-1} and 1505–1447 cm^{-1} absorptions, respectively. Both ^{1}H and ^{13}C NMR spectra (Table 1) depicted characteristic signals for a benzo[1,3]dioxol unit based on δ_H 5.91 (br s, OCH_2O), 6.89 (d, J = 1.2 Hz, H-8), 6.73 (d, J = 8 Hz, H-11) and 6.75 (dd, J = 8.0, 1.2 Hz, H-12) and at δ_C 99.39, 107.87, 109.14, 120.15, 132.30, 147.87 and 148.56, respectively. The benzo[1,3]dioxol connectivity was supported by HMBC correlations (Fig. 2) observed as: from δ_H 5.91 (OCH_2O) to δ_C 147.9 (C-9) and 148.6 (C-10); a ^{3}J correlations from δ_H 6.87 (H-8) to 148.6 (C-10) and 120.2 (C-12); from δ_H 6.73 ($H-11$) to δ_C 132.5 (C-7) and 147.9 (C-9) and from δ_H 6.83 ($H-12$) to δ_C 107.8 (C-8) and 148.6 [10]. A second characteristic signal comprised two sets of olefinic protons at δ_H 5.74 (d, J = 15.2 Hz), 6.05 (dd, J = 15.2, 7 Hz), 6.24 (dd, J = 15.2, 7 Hz), 6.77(d, J = 15.2 Hz) and signals at δ_C 123.8, 146.8, 129.9 and 129.5 in the ^{1}H NMR, ^{13}C NMR (supported by HSQC DEPT) spectra, respectively. These set of signals indicated the presence of two *trans* configured C=C alkene groups. A signal at δ_H 2.39 (m, 2H) on ^{1}H NMR displayed COSY (Fig. 2) correlation with two of the olefinic protons (δ_H 5.74 and 6.24) together with HMBC correlations to carbon signals at δ_C 123.8 and 129.5 indicating a methylene link between the C=C hence the presences of penta-1,3-diene moiety. The ^{1}H NMR (Table 1) further displayed signals at δ_H 3.13 (t, J = 6 Hz, 2H) on δ_C 49.5 (HSQC) which showed COSY coupling with N−H and another proton at δ_H 1.78 (m, 1H) on δ_C 29.3, which in turn coupled to δ_H 0.98 (d, J = 6 Hz, 2-CH_3) indicated the presence of isobutylamide moiety [12]. The following HMBC (Fig. 2) correlations: from δ_H 3.13 (CH_2) to δ_C 169 (^{3}J, C=O) and δ_C 29.3 (^{2}J, C-2′); ^{2}J correlation between the methine proton δ_H 1.78 and methylene carbon (δ_C 49.5) and methyl carbons (δ_C 19.4) confirmed (C=O)$NHCH_2CH(CH_3)_2$ moiety, a common structural features of *Zanthylum* amide alkaloids. These NMR spectral characteristics established typical amide alkaloid except the presence of panta-1,3-diene moiety, from which one of the alkene proton at δ_H 6.77 (H-6) and δ_C 129.2 showed mutual HMBC correlation to the benzo[1,3]dioxol signal at δ_H 6.25 and δ_C 107.9, while another olefinic proton δ_H 5.74 (H-2) showed HMBC correlation to carbonyl carbon (δ_C 169.3) and the methylene carbon (δ_C 35.3) confirming penta-1,3-diene as the moiety joining the isobutylamide and the benzo [1,3]dioxol. The foregoing spectral evidences characterized compound **1** as 6-Benzo[1,3]dioxol-5-yl-hexa-2,5-dienoic acid isobutylamide obtained as a new natural product from *Z. chalybeum* and named as chalybemide A.

Compound **2** was obtained as white crystalline solids with a molecular formula $C_{13}H_{15}NO_2$ deduced from its HREIMS at *m/z* 219.1103 $[M]^+$ (calcd. For $C_{13}H_{15}NO_2$, 219.1103). ^{13}C NMR (Table 1) supported the MS data by exposing thirteen carbons atoms characterized by DEPT as seven methines, one methylene and three quaternary carbons including one aromatic, one hydroxylated and one carbonyl carbons. In the ^{1}H NMR spectrum, aromatic signals were displayed as two broad doublets integrated for two protons each at δ_H 7.53 (d, J = 7.2 Hz) and δ_H 7.36 (d, J = 7.2 Hz) and a multiplet at δ_H 7.47 resonating for one proton, attributable to a monosubstituted aromatic ring. In addition, the presence of a *trans* configured alkene moiety was noted based on signals at δ_H 6.64 (1H, d, J = 15.5 Hz) and δ_H 7.73 (1H, d, J = 15.5 Hz), which showed independent HMBC correlation (Fig. 2) to carbonyl carbon and quaternary aromatic carbon at δ_C 166.5 (C=O) and 134.9 (C-4), respectively, attributable to a phenylethylene skeleton attached to carbonyl carbon. Furthermore, the ^{1}H NMR displayed signals at δ_H 3.47 (-CH_2, s, attached to δ_C 58.8 on HSQC) displaying COSY correlation with N−H and HMBC correlation to carbon at δ_C 70.1 (C-2′) and the carbonyl carbon δ_C 166.5 which suggested the methylene amide group. Additional HMBC correlation from the two mutually coupled methyls (δ_H 1.19) to carbon at δ_C 70.1 (C-OH) and 57.1 (C−H_2) confirmed the moiety as a 2-hydroxy-isobutyl amide [22]. Following the HMBC correlations of the ethylene protons and the methylene

protons to same carbonyl carbon, and the ROESY correlation which confirmed the spatial connectivity of compound **2** as *N*-(2-Hydroxy-2-methyl-propyl)-3-phenyl-acrylamide, isolated and characterized from *Zanthoxylum chalybeum* for the first time and named as chaylbemide B.

Compound **3** was isolated as white crystalline solids with a molecular formula of $C_{17}H_{19}NO_3$, as determined by the HREIMS at *m/z* 285.1373 $[M]^+$ (calcd. for $C_{17}H_{19}NO_3$, 285.1365), indicating nine degrees of unsaturation. The ^1H NMR (Table 1) spectrum displayed mutually coupled aromatic signals at δ_H 7.15 and 6.86 (each, 2H, d, J = 8.5 Hz,) typical *para*-substituted ring and an *ortho*-disubstituted benzene ring signaled by [δ_H 6.89 (1H, d, J = 8.3 Hz, H-3), 7.52 (1H, m, H4), 7.05 (1H, t, J = 7.7 Hz, H-5), and 7.86 (1H, dd, J = 7.7, 1.8 Hz, H-6)]. ^{13}C NMR (Table 1) spectrum showed carbon resonances which were classified by DEPT as one carbonyl group (δ_C 168.1), 12 aromatic carbons (δ_C 112.2–159.8), two methoxyls (δ_C 56.4 and 56.6), an *N*-Methyl (δ_C 34.9) and an aliphatic methylene (δ_C 57.1). The data of compound **3** were almost comparable to that of tessmamide previously isolated from *Z. tessmannii* [23] expect for the additional methylene group and the absence on methylenedioxy functionality on compound **3**. The overall connectivity and the planar structure of the compound **3** was favorably corroborated by the HMBC and COSY correlations (Fig. 2). For instance, the methoxy group at δ_H 3.73 showed ^3J correlation to 113.2 (C-3′/C-5′) and in turn δ_H 6.86 (H3′/5′) correlated to δ_C 56.4 (OCH_3) placing it at C-4′ ring B; the other methoxy group δ_H 3.72 correlated to δ_C 112.2 (C-1) and 122.2 (C-4). Moreover, a proton at δ_H 6.89 (H-3) correlated to δ_C 56.6 (OCH_3) placing it at C-3 of ring A. For the *N*-CH_3, its HMBC correlation to carbonyl δ_C 168.1 and methylene carbon δ_C 57.1 supported by the ^1H–^1H COSY off-diagonal peaks between *N*-CH_3 (δ_H 3.19) and CH_2 (3.91), which in turn coupled to δ_H 7.15 (H2′/6′) confirmed its connectivity and the *N,N*-methylenemethyl amide moiety. The aforementioned data characterized compound **3** as 4-Methoxy-*N*-(2-methoxy-phenyl)-*N*-methyl-benzamide isolated and characterized for first time from *Z. chalybeum* and named as chaylbemide C.

3.1. α-Amylase and α-Glucosidase inhibition of pure isolates from Z. chalybeum

The dose dependent α-amylase inhibitory activities of the isolated compounds were processed on long probit analysis to establish the IC_{50} values (Table 2), from which the potential inhibitory effect of IC_{50} = 45.76, 43.22, 46.76, 47.36, 47.72, 46.48, 48.91, and 49.36 μM were observed from chalybemide A (**1**), chalybemide B (**2**), chaylbemide C (**3**), fagaramide (**4**), skimmianine (**5**), norchelerythrine (**6**), 6-acetonyldihydrochelerythrine (**7**), and 6-hydroxy-*N*-methyl decarine (**8**), respectively. There was no significant difference in the inhibitory activities ($P > 0.05$) between the eight alkaloids compared to the standard drug acarbose with IC_{50} = 42.67 μM. Three phenolic compounds, ailanthoidol (**9**), 2,3-epoxy-6,7-methylenedioxy coniferyl alcohol (**10**) and sesamine (**11**) displayed significantly ($P < 0.05$) low inhibitory activity (IC_{50} of 58.21, 58.21 and 54.67 μM, respectively) against α-amylase enzymes relative to acarbose.

The same pattern of activity was observed with α-glucosidase enzyme (Table 2) indicating that all the alkaloids had no significant activity ($P > 0.05$) compared to control acarbose while the three phenolics similarly showing no significant activity ($P > 0.05$) difference compared to the alkaloids but significantly ($P < 0.05$) different compared to the control which implied the phenolic displayed moderate to low inhibitory activity against α-glucosidase activities. The results obtained in this study inferred inhibitory activity of the plant *Z. chalybuem* to the alkaloids and slightly to the phenolic compounds thus supporting the previously established anti-hyperglycemic activities of *Z. chalybeum* stem and root extracts [4,5,6]. Inhibitor-enzymes hydrogen bonding interaction had previously been postulated as a possible inhibition mode involving active sites of α-amylase and glucosidases and the hydroxyl groups of molecules with phenolic entities [24], although energetics not proven, it can be inferred on the observed inhibition of the isolates of *Z. chalybeum*. However, the assertion cannot be authoritative until enzyme inhibition kinetics and structural inhibition energetics are established.

Table 2
In vitro IC_{50} values of compounds from *Z. chalybeum* against α-amylase and α-glucosidase inhibition compared to standard control acarbose.Inhibitor.

	α-amylase	α-glucosidase
	(μM)	(μM)
Acarbose	42.67	44.88
Chaylbemide A (**1**)	45.76	43.54
Chaylbemide B (**2**)	43.22	44.32
Chaylbemide C (**3**)	46.76	47.43
Trans-fagaramide (**4**)	47.36	47.23
Skimmianine (**5**)	47.72	47.63
Norchelerythrine (**6**)	46.49	48.70
6-Acetonyldihydrochelerythrine (**7**)	49.36	47.27
6-Hydroxy-*N*-methyl decarine (**8**)	48.91	49.20
Ailanthoidol (**9**)	58.21	57.11
2,3-Epoxy-6,7-methylenedioxy coniferyl (**10**)	48.34	47.45
Sesamine (**11**)	54.67	54.77
Lupeol (**12**)	116.19	90.86
Lupanone (**13**)	108.61	88.62
3α,20-Dihydroxy-28-lupanoic acid (**14**)	104.98	92.61
20-Hydroxy-3-oxo-28-lupanoic acid (**15**)	98.66	96.78
3α,20,28-Trihydroxylupane (**16**)	93.09	98.60
LSD ($P < 0.05$)	9.74	8.60
CV%	40	34

IC_{50} values, calculated from regression using five different concentrations (10.0, 20.0, 50.0, 100.0, and 150.0 μM) are expressed as mean of replicated experiments with percentage coefficient of variation (CV %) indicated, LSD ($P < 0.05$): Least Significant difference considered significant at $p \leq 0.05$.

4. Conclusion

The in *vitro* inhibition of compounds from root bark of *Z. chalybeum* against α-amylase and α-glucosidase enzymes confirmed the previously established in vivo antidiabetic properties of the plants crude extracts. Moreover, the realization of the some of the molecular structure of the alkaloids as the active compounds, is an indication of chemical entities which can be explored further through establishment their modes of inhibition for further steps in in drug discovery. The plant *Z. chalybeum* may be used to manage diabetes predisposing conditions such as excess accumulation of intestinal glucose levels before absorption into the blood systems.

Declaration of Competing Interest

There are no conflicts of interest among the authors.

Acknowledgement

The study was supported by the Third World Academy of Science TWAS, research Grant (RGA NO. 18-192 RG/CHE/AF/AC_G_FR3240303660 dated 24, September 2018). The authors wish to thank Dr. Philip Onyango taxonomist of the Department of Botany, Maseno University for the identification and collection of the plant materials.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fitote.2020.104719.

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