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Bioactive Phenylethanoids from the Seeds of *Manilkara zapota*

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Authors' contributions

This whole work was carried out in collaboration between all authors. Authors FAAT and ABN collected the plant and made the extraction. Authors FAAT, MF and ABN wrote the protocol and performed the phytochemical analysis. Authors JCN, AFKW and JDW performed the biological tests in collaboration and the spectroscopic analysis. Authors FAAT, AFKW and JDW wrote the first draft of the manuscript. Author JDW managed the supervision of the study. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Discovering new lead compounds against cancer and bacterial infections is a crucial step to ensuring a sustainable global pipeline for new effective drugs. These study focuses on the isolation of secondary metabolites of methanol extract from the seeds of *Manilkara zapota* (Sapotaceae) a Cameroonian medicinal plant.
Study Design: According to the literature, plants of the genus *Manilkara* are potential sources of antibacterial and anticancer secondary metabolites.
Methodology: The air-dried and powdered seeds (1.8 kg) of *M. zapota* was extracted at room temperature for 72 h with a mixture of CH_2Cl_2/methanol (1/1). The extract was concentrated to dryness under vacuum and the residue was subjected to repeated column chromatographic separation. The structures of the isolates were established by means of spectroscopic methods.

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These compounds were screened *in vitro* for their free radical scavenging activity using DPPH.
Results: New phenylethanoid, 2-(4-hydroxyphenethyl) tetratriacontanoate (**1**), together with twelve known compounds were isolated from the CH_2Cl_2/methanol (1/1) extract from the seeds of *Manilkara zapota*. The structures of all compounds were determined by comprehensive analysis of their 1D and 2D NMR, mass spectral (EI and ESI) data, chemical reactions and comparison with previously known analogs. The radical scavenging activity using DPPH assay gave significantly high antioxidant values for the crude extract with IC_{50}8.50 µg/mL, and moderate activity for the phenylethanoid compounds **1**-**6** with IC_{50} 62.52- 70.20 mM compared to the phenolic synthetic antioxidant standard BHA with IC_{50} 44.20 mM.
Conclusion: Six phenylethanoids with moderate antioxidant activity were isolated from the seeds of *Manilkara zapota*.

Keywords: Manilkara zapota; seeds; Sapotaceae; phenylethanoyl; antioxidantactivity.

1. INTRODUCTION

Manilkara zapota (Sapotaceae), known commonly as “Sapodilla”, is an evergreen tree up to 10 m high. Native to Mexico and Central America, it is cultivated in tropical areas including Cameroon [1]. The fruit has a rusty brown skin and a yellowish-brown or orange pulp with a sweet pleasant flavour and a mild aroma when ripe. Sapodilla fruit is often eaten fresh, but the pulp is also incorporated into sherbets, milkshakes and ice cream [2].

The seeds of *M. zapota* are applied traditionally as aperients, diuretic tonic and febrifuge, while the stem bark is used as astringent and febrifuge [3]. In addition, leaves and bark are taken against cough, cold, dysentery and diarrhoea [3]. Previous phytochemical investigations revealed triterpenoids, saponins, polyphenols, some of these compounds exhibiting potent α-amylase and α-glucosidase inhibition, as well as antimicrobial, antioxidant and cytotoxic activity [4,5]. Widespread traditional medicinal use and significant biological activities of compounds investigated so far justified continued investigation of *M. zapota*. This paper reports the isolation and structure elucidation of a new phenylethanoid (**1**), together with antioxidant activity of isolated compounds.

2. MATERIALS AND METHODS

2.1 General

The melting points were recorded on a Buchi 535 melting point apparatus and are uncorrected. Optical rotations were measured in $CHCl_3$ on a JASCO DIP-360 digital polarimeter using a 10 cm cell. CD (Circular Dichroism) spectra were measured on a JASCO J-810 spectropolarimeter. Infrared spectra were recorded on a JASCO 302-A spectrophotometer. ESI-HR (*Electrospray Ionisation High Resolution)* mass spectra were recorded on a Bruker FTICR 4.7 T mass spectrometer. EI-MS (*Electron Impact–Mass Spectrometry)* were recorded on a Finnigan MAT 95 spectrometer (70 eV) with perfluorokerosene as reference substance for EI-HR-MS (*Electron Impact- High Resolution-Mass Spectrometry)*. The ^{1}H- and ^{13}C-NMR (*Nuclear Magnetic Resonance)* spectra were recorded at 500 MHz and 125 MHz, respectively, on Bruker DRX 500 NMR spectrometers. Methyl, methylene and methine carbons were distinguished by DEPT (*Distorsionless Enhancement by Polarisation Transfer)* experiments. Homonuclear^{1}H connectivities were determined by using the COSY (*Correlation Spectroscopy)* experiment. One-bond ^{1}H-^{13}C connectivities were determined with HMQC (*Heteronuclear Multiple Quantum Coherence*) gradient pulse factor selection. Two- and three-bond ^{1}H-^{13}C connectivities were determined by HMBC (*Heteronuclear Multiple Bond Correlation*) experiments. Chemical shifts are reported in δ (ppm) using TMS (*Tetramethylsilane*) as internal standard, and coupling constants (*J*) were measured in Hz. Column Chromatography (CC) was carried out on silica gel (70-230 mesh, Merck). TLC (*Thin Layer Chromatography)* was performed on Merck precoated silica gel 60 F_{254} aluminium foil, and spots were detected using ceric sulfate spray reagent. Phenolic compounds were detected using $FeCl_3$ reagent. The purity of the compounds was investigated by means of ^{1}H-NMR and ESI-MS experiment. The degree of purity of the tested compounds was > 95%, and of the positive control BHA (3-*t*-butyl-4-hydroxyanisole) 99.9%. All other substances, if otherwise not specified, were purchased from Sigma-Aldrich (Germany). All reagents used were of analytical grade.

2.2 Plant Material

The seeds of *M. zapota* was collected at the Douala (Faculty of sciences) in the Littoral region of Cameroon in May 2013 and identified by Mr. Nana Victor, National Herbarium, Yaoundé, Cameroon. A voucher specimen (ref. 00007 FSUD/CAM) has been deposited at the Faculty of Sciences.

2.3 Extraction and Isolation

The air-dried and powdered seeds (1.8 kg) of *M. zapota* was macerated in methanol at room temperature for 72 h, then filtrated and evaporated under reduced pressure to obtain 13.5 g of the crude extract. A portion of 10.0 g was purified by column chromatography over silica gel 60 (230-400 mesh) and preparative TLC using a gradient system of hexane, ethyl acetate and MeOH. 125 sub-fractions (*ca*. 250 mL each) were collected and pooled on the basis of TLC analysis leading to three main fractions (A – C).

Fraction A (5.0 g, combined from sub-fractions 1-30) was chromatographed over a silica gel 60C column with a hexane-EtOAc gradient. 20 fractions of *ca*. 100 mL each was collected and combined based on TLC. Fractions 2-10 were further chromatographed over a silica gel 60H column with a mixture of hexane-EtOAc (5:1) for elution to yield lupeol (**9**) (10.5 mg) and *β*-amyrin (**7**) (5.3 mg). Fractions 11-20 were further chromatographed over a silica gel 60H column with a mixture of hexane-EtOAc (4:1) to yield a mixture of phenylethanoyls (**1**-**6**) (23.5 mg) and mixture of steroids (*β*-sitosterol + stigmasterol) (12.4 mg). Fraction B (2.5 g, combined from sub-fractions 21-45) was chromatographed over a silica gel 60C column with a hexane-EtOAc gradient. 15 fractions of *ca*. 100 mL each was collected and combined based on TLC. Fractions 1-5 were purified and yielded 3-oleanoic acid (**8**) (8.5 mg). Fraction C (1.3 g, combined from sub-fractions 46-65) was chromatographed over a silica gel 60C column with a hexane-EtOAc gradient. 10 fractions of *ca*. 100 mL each was collected and combined based on TLC. Fractions 1-7 were further chromatographed over a silica gel 60H column with a mixture of hexane-EtOAc (1:3) to yield betulinic acid (**10**) (11.5 mg).

2-(4-Hydroxyphenethyl) tetratriacontanoate *(**1**)* White amorphouspowder. – UV (MeOH): λ_{max}(log ε) =275 (4.0), 240 (3.9) nm. – IR ($CHCl_3$): ν_{max} = 2900, 2825,1724, 1615, 1393 cm^{-1}. – ^{1}H NMR (500 MHz, $CDCl_3$): δ =0.89 (t, *J* = 7.0 Hz, 3H, CH_3), 1.27 (br s, 34H, $(CH_2)'_{17}$),1.62 (m, 2H, H-4''), 2.30 (t, *J* = 7.6 Hz, 2H, H-3''),2.87 (t,*J*= 6.9 Hz, 2H, H-2), 4.25 (t, *J* = 6.9 Hz, 2H, H-1), 6.78(d, *J* = 8.2 Hz, 2H, H-3' and H-5'), 7.10 (d, *J* = 8.2 Hz, 2H,H-2' and H-6'), 9.53 (br s, 1H, OH). – ^{13}C NMR (125 MHz,$CDCl_3$): δ = 13.1 (C-20''), 23.9 (C-3''), 30.9-28.0 (methylenes),33.2 (C-2''), 33.3 (C-2), 63.9 (C-1), 114.3 (C-3'and C-5'),129.0 (C-1'), 129.0(C-2' and C-6'), 157.2 (C-4'), 173.7(C-1''). – MS ((+)-ESI): *m/z* = 651 $[M+Na]^+$. – HRMS ((+)-ESI): *m*/*z* = 651.5689 (calcd. 651.5692 for $C_{42}H_{76}O_3Na$, $[M+Na]^+$).

2.3.1 Chemical derivatives

Transesterification: Compound **1** (3.0 mg) was refluxed at 70℃ in dry MeOH (5.0 ml) with NaOMe (5.0 ml) for 2 h. The reaction mixture was extracted successively with H_2O and $CHCl_3$. The chloroform phase was dried over Na_2SO_4 and evaporated to give 4-hydroxyphenylethanol. Addition of HCl (1 %) to the water phase followed by extraction with $CHCl_3$ afforded methyl tetra triacontanoate (1.1 mg).

2.4 Biological Activities

2.4.1 Determination of the radical scavenging activity

DMSO (5 µL) containing the test sample and 95 µL of DPPH (Sigma, 300 µM) in ethanol is given into a well of a 96-well microtiter plate (Molecular Devices, Germany) and incubated at 37℃ for 30 min. The absorbance is measured at 515 nm. The percentage of radical scavenging activity is determined by comparison with the negative control. Extract and and fractions (A-C) were tested at 20 mg/mL, compounds **1**-**10** at 1 mM, as well as the positive control BHA (3-*t*-butyl-4-hydroxyanisole) (Table 1).

Table 1. Free radical scavenging activity of crude extract and compounds 1-10 against DPPH (1,1-diphenyl-2-picrylhydrazyl)

Compounds	IC_{50}±(SEM)[a]
Extract	8.50±0.55[b]
1	70.20±1.72[c]
2-6	62.52±1.25[c]
7	201.14±4.53[c]
8	151.80±2.00[c]
9	223.11±5.64[c]
10	105.20±0.41[c]
[d]BHA	44.20±0.02[c]

*Extract was tested at 20 mg/mL, compounds **1**-**10** were tested at 1 mM.[a] Standard mean error. [b]IC_{50} in µg/mL [c]IC_{50} in mM, [d]BHA (3-t-butyl-4-hydroxyanisole)*

3. RESULTS AND DISCUSSION

The air-dried seeds of *M. zapota*was powdered and extracted with MeOH. The crude extract was separated by repeated column chromatography and preparative TLC (PTLC) to afford the one new phenylethanoid 1 and twelve known compounds (Fig. 1) identified as *2-(4-Hydroxyphenethyl)tetracosanoate* (2), *2-(4-Hydroxyphenethyl)docosanoate* (3), *2-(4-Hydroxyphenethyl)eicosanoate* (4), *2-(4-Hydroxyphenethyl)octadecanoate* (5), *2-(4-Hydroxyphenethyl)hexadecanoate* (6), β-amyrin (7), oleanolic acid (8), lupeol (9), betulinic acid (10), stigmasterol-3-*O*-β-D-glucopyranoside (11), stigmasterol andβ-sitosterol (12) [6-8].

2-(4-Hydroxyphenethyl) tetratriacontanoate (**1**) was obtained as white amorphous powder, showing a positive reaction with $FeCl_3$ indicating their phenolic nature. Their UV spectrum exhibited two absorption maxima at 275 and 240 nm characteristic of phenylethanoids [6,9]. The presence of hydroxyl and ester functions was indicated by two IR bands at 2900 and 1724cm^{-1}, respectively. From the HR-ESIMS, the molecular composition was found to be $C_{28}H_{48}O_3Na$ by $[M+Na]^+$ at *m/z* = 455.3498 (calcd.455.3501). The ^{1}H NMR spectra of **3** showed the typical AA'XX' system of a *p*-disubstituted benzene ring at δ = 7.10 (d, *J* = 8.2 Hz, H-2'/H-6') and 6.78 (d, *J* = 8.2 Hz, H-3'/H-5'), the presence of a CH_2CH_2O unit at δ = 4.25 (t, *J* = 6.9 Hz, H-1), and 2.87 (t, *J* = 6.9 Hz, H-2), and a free hydroxyl group at δ = 9.53 (br s, OH-4_) exchangeable with D_2O. This inference was supported by the ^{13}C NMR and DEPT data, which showed characteristic signals of a *p*-disubstituted benzene ring at δ = 114.3 (C-3'/C-5'), 129.0 (C-1'), 129.0 (C-2'/C-6') and 155.2 (C-4'), and the CH_2CH_2O unit at δ = 63.9 (C-1) and 33.23 (C-2) [10]. Furthermore, in the ^{1}H NMR spectrum, a terminal methyl at δ = 0.89 (t, *J* = 7.90 Hz) and methylenes at δ = 2.30 (t, *J* = 7.6 Hz, -$\underline{CH_2}$-CO-), 1.62 (m, -$\underline{CH_2}$-CH_2-CO-), and 1.25 (br s, *n*H) were also observed. These data suggested the presence of a long chain linked to a 4-hydroxyphenylethanol moiety. The presence of a long chain was further confirmed by the ^{13}C NMR spectrum, which showed characteristic signals at δ = 173.7(C-1''), 33.2 (C-2''), 30.9-28.0 $(CH_2)_n$, 23.9 (C-3''), and 13.1 (CH_3) [11].

To determine the linkage between the long chain and the 4-hydroxyphenylethanol moiety, an HMBC experiment was used. In the HMBC spectrum, correlation of H-1 (δ = 4.25) with C-1'' (δ = 173.7), C-1' (δ = 129.0) and C-2 (δ = 33.3) suggested that the long chain is linked to the 4-hydroxyphenylethanol moiety by an ester function.

The methanolysis of **1** yielded methyl tetratriacontanoate (identified by EI-MS which indicated a molecular ion at *m/z* = 522 $[M]^+$, corresponding to a molecular formula $C_{35}H_{70}O_2$) and 4-hydroxyphenylethanol. The latter was identified by ^{1}H NMR and EI-MS. From these spectroscopic data, compound (**1**) was characterized as *2-(4-Hydroxyphenethyl)* tetratriacontanoate.

The radical scavenging activity using DPPH assay gave significantly high antioxidant values for the crude extract with IC_{50}8.50 µg/mL and moderate antioxidant for the phenylethanoid compounds **1**-**6** with IC_{50} 62.52-70.20 mM compared to the phenolic synthetic antioxidant standard BHA with IC_{50} 44.20 mM. However, the triterpenoid **7**-**10** delivered low antioxidant values (IC_{50} 103.20-223.11 mM).

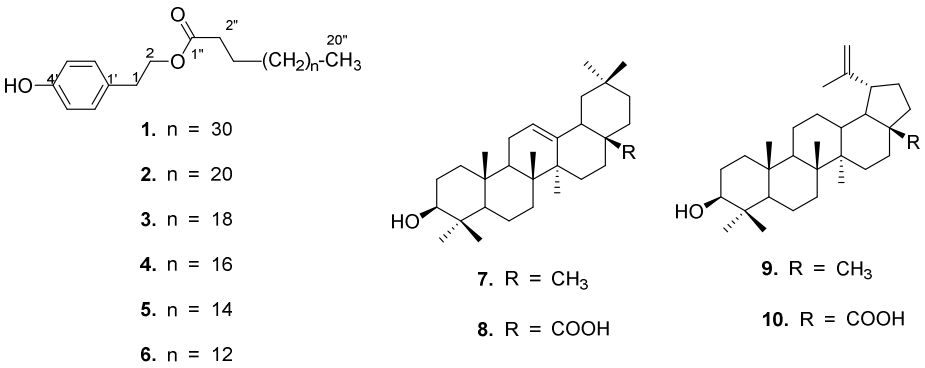

Fig. 1. Structures of some of the isolated compounds

4. CONCLUSION

One new phenylethanoid, 2-(4-Hydroxyphenethyl) tetratriacontanoate (**1**), together with twelve known compounds were isolated from the methanol extract from the seeds of *Manilkara zapota*. The radical scavenging activity using DPPH assay gave significantly high antioxidant values for the crude extract with IC_{50}8.50 µg/mL, and moderate activity for the pheneylethanoid compounds **1**-**6** with IC_{50} 62.52-70.20 mM compared to the phenolic synthetic antioxidant standard BHA with IC_{50} 44.20 mM. This result show that compounds in the plant may react by synergy effect.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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