Bioorganic & Medicinal Chemistry 19 (2011) 5225–5230

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

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

Tylosema esculentum extractives and their bioactivity

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ARTICLE INFO

Article history:
Received 4 April 2011
Revised 29 June 2011
Accepted 4 July 2011
Available online 14 July 2011

Keywords:
Tylosema esculentum
Antioxidant
Antimicrobial
Cytotoxicity
Fatty acids

ABSTRACT

The investigation of *Tylosema esculentum* (Morama) husks, cotyledons, and tuber yielded griffonilide **2**, compound **1**, griffonin **3**, gallic acid **4**, protocatechuic acid **5**, β-sitosterol **6**, behenic acid **7**, oleic acid **8**, sucrose **9**, 2-*O*-ethyl-α-D-glucopyranoside **10**, kaempferol **11** and kaempferol-3-*O*-β-D-glucopyranoside **12**. The structures of the isolates were determined by NMR, HR-TOF EIMS, IR and UV–vis spectroscopy, and by comparison with literature data. The husk EtOAc and *n*-butanol extracts demonstrated >90% DPPH radical scavenging activity at concentrations of 25, 50 and 250 μg/mL. Furthermore the husk extracts showed higher total phenolic content (233 mg GAE/g). The extractives exhibited minimum inhibitory quantities of 50–100 μg or no activity against *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Candida albicans*. The tuber extracts were inactive against Caco-2 and Hela cell lines, while the husk extracts showed low activity against Caco-2 and Vero cell line with IC_{50} values >400 μg/mL. The GC–MS analysis showed the beans and tuber non-polar (*n*-hexane) extracts major constituents as fatty acids.

1. Introduction

Morama [*Tylosema esculentum* (Burch.) Schreiber; Fabaceae: Caesalpiniaceae] bean and tuber are gathered from the wild and eaten by the indigenous people of the Kalahari Desert of Botswana, spreading to Namibia and South Africa. The bean oil is reported to be rich in mono- and di-unsaturated fatty acids and contains no cholesterol.[1,2] It is also a good source of calcium, iron, zinc, phosphate, magnesium and B-vitamins. Morama bean is an excellent source of good quality proteins. The protein content is 32–45%, while oil content is 30–42%.[1–3]

The much publicized healthy living concept has embraced the consumption of fresh fruit, tea, herbs and vegetables, alongside physical exercises. The increased intake of dietary antioxidant helps to maintain a balance between antioxidants and oxidants in living organisms, to alleviate oxidative stress. The role of reactive oxygen species/and or oxidants in many diseases including malaria, acquired immunodeficiency syndrome, heart disease, diabetes, cancer,[4] asthma, inflammatory joint disease and immune system decline are well documented.[5,6] The resurgence of interest in the natural control of phytopathogens and increasing consumer demand for effective, safe and natural products means that quantitative data on plant extracts are required.[7–9] Prior to this work, documented research on Morama focused mostly on nutritional compositional levels and domestication.[3,10,11] *T. esculentum* seed coat (husk) ethanolic extracts (0.01–0.001 mg/mL), cotyledon ethanolic extracts (⩾0.1 mg/mL) and tuber water extracts (0.1–0.01 mg/mL) are reported to exhibit high in vitro inhibition of rotaviruses which are a major cause of diarrhoea among infants and immunocompromised people.[12] The seed coat is reported to have a higher concentration of total phenolic acids and total flavonoids than the cotyledon, where levels were determined using reversed-phase high performance liquid chromatography.[13] Some of the detected phenolics in the cotyledon and seed coat were myricetic, kaempeferol, rutin, gallic acid, protocatechuic acid and sinapic acid.[13] The Literature[12,13] has recommended the isolation phytochemicals of *T. esculentum* extracts and identification of the responsible bioactive compounds, as isolations have not been reported.[13] The objective of the study was to investigate the phytochemical constituents, antioxidant potential of Morama extracts and phenolic compounds as well as to establish the composition of the non-polar extracts using GC–MS analysis. The cell growth inhibition and antimicrobial activity were also purposed to be carried out to determine the antimicrobials.

2. Results and discussion

2.1. Phytochemistry

The phytochemical work-up of *T. esculentum* (Morama or Marama) husks, cotyledons and tuber yielded twelve secondary

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doi:10.1016/j.bmc.2011.07.006

metabolites. The chemical structures (Fig. 1) of the compounds were identified based on spectral data analyses (NMR, MS, IR, UV and [α]) D and comparison with the cited Literature data. The cyanogenic glucoside, griffonin (3),[14] and the phenolic compounds gallic acid (4), protocatechuic acid (5),[15] kaempferol (11) and kaempferol-3-*O*-β-D-glucopyranoside (12),[16] were isolated from the non edible part, which are the husks (very hard seed coat). The Morama edible parts are the dehulled beans (cotyledons) and tubers. The dehulled beans afforded β-sitosterol (6),[17] oleic acid (8),[18] sucrose (9)[19] and 2-*O*-ethyl-α-D-glucopyranoside (10).[20] Griffonilide (1),[14] compound (2) and behenic acid (7)[21] were obtained from the tuber. It was of significance importance that the cytotoxic compound 3 was not detected from the edible parts of Morama bean.

Compound **2** was isolated as a mixture (1:3) with 1 and was recognized from NMR data as the minor peaks in ^{1}H and ^{13}C NMR spectrum of the mixture (Table 1). The ^{13}C NMR spectra of 1 exhibited signals for three oxygenated methine carbons (C-6, 7, 7a), three methine carbons (C-3, 4, 5), a quaternary carbon (C-3a) and a carbonyl carbon (C-2). The ^{1}H NMR spectra of compound **1** exhibited six signals, each integrating for one proton and analysis of the HSQC spectra revealed that the protons were all attached to methine carbons. Three protons resonated at δ_H 3.54 (1H, dd, J = 2.4, 8.1 Hz, H-7), 4.33 (1H, *dt*, J = 2.4, 2.4, 8.4 Hz, H-6) and 4.90 (1H, dd, J = 2.1, 10.8 Hz, H-7a). Two *cis*-vicinally coupled (^3J) protons resonated at δ_H 6.27 (1H, dd, J = 2.1, 9.9 Hz, H-4) and 6.62 (1H, dd, J = 2.4, 9.9 Hz, H-5) and an olefinic proton signal was observed at δ_H 5.89 (1H, br s, H-3). The IR absorption bands for the butenolide moiety were observed at 1726 and 1649 cm^{-1}.[22] The IR and NMR spectral data of 1 indicated the presence of α,β,γ, δ-unsaturated carbonyl system. The HR-TOF EIMS spectra molecular ion at m/z 170.0576 was used to establish the molecular formula as $C_8H_{10}O_4$. The five degrees of unsaturation in the molecule were accounted for by a bicyclic structure with one carbonyl group and two double bonds.

The spectral data of **1** was similar to literature data.[14] The ^{13}C NMR spectral pattern of **2** was almost identical to that of **1** showing eight signals. The difference was in the presence of two downfield ^{13}C resonances at δ 35.4 (3-CH_2) and 37.5 (3a-CH) in the spectra of 2. The absence of the methine signal at δ_C 111.6 (δ_H 5.90, *d*, J = 1.8 Hz, H-3) and the quaternary carbon signal at δ_C 164.8 (C-3a) found in the spectra of **1** were key observations. These observations indicated that **2** had the same basic structure as **1**, but have a reduced C-3/C-3a double bond. The ^{1}H NMR spectra of 2 exhibited germinally (J = 9.3, 17.4 Hz) coupled methylene proton signals resonating up-field at δ_H 2.37 (1H, dd, J = 8.7, 17.4 Hz, H-3a) and 2.75 (1H, dd, J = 9.3, 17.7 Hz, H-3b). The signals were assigned to C-3 (δ_C 35.4) using HMQC spectrum correlations.

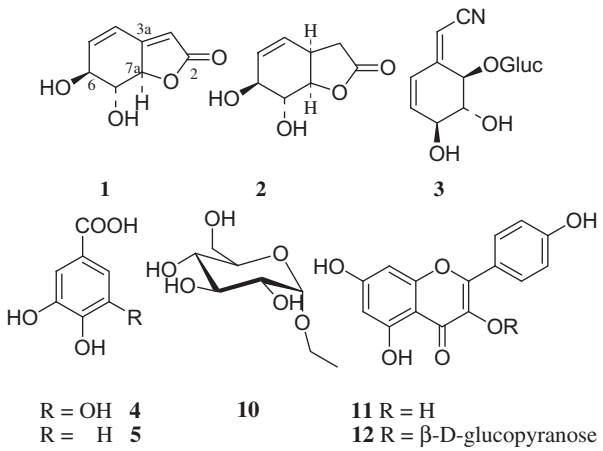

Figure 1. Chemical structure of compounds isolated from *T. Esculentum*.

Table 1
^{1}H and ^{13}C NMR data for compounds 1 and 2

H/C	δ_H[a] (J) δ_C **1**	δ_H[a] (J) δ_C **2**
2	–	–
	175.8	178.2
3	5.90, *d* (1.8)	2.37 dd (8.7, 17.4) 2.75 dd (9.3, 17.7)
	111.6	35.4
3a	–	3.54 dd (2.4, 8.1)
	164.8	37.5
4	6.27 dd (2.1, 9.9)	5.72 m
	120.6	131.8
5	6.62 dd (2.4, 9.9)	5.72 m
	144.2	126.6
6	4.33 *dt* (2.4,2.4, 7.8)	4.06 *dt* (2.4, 8.1)
	73.6	71.2
7	3.54 dd (2.4, 8.1)	4.33 dd (2.4, 7.8)
	80.0	74.7
7a	4.90 dd (2.1, 10.8)	4.54 dd (1.2, 8.1)
	85.2	83.3

[a] MeOD, (x) proton multiplicity in Hz.

Compound **1** molecular formula $C_8H_8O_4$ was established by HR-TOF EIMS $[M]^+$ = 168.0420. The HR-TOF EIMS spectra molecular ion at m/z 170.0576 was used to establish the molecular formula of **2** as $C_8H_{10}O_4$. The four degrees of unsaturation in the molecule were accounted for by a bicyclic structure with one carbonyl group and one double bond. Based on the NMR, MS data and comparison with data of **1** in Table 1 and published data,[14] compound **2** was identified as 3,3a,7,7a-tetrahydro-6,7-dihydroxy-2(6*H*)-benzofura-none. It is also important to mention that compound **1** was also obtained from the hydrolysis of compound **3** using trifluroacetic acid.

The relative stereochemistry of **1** was established by the NOE correlations observed in a NOESY experiment. In the NOESY spectrum, H-7a showed NOE interactions with H-6 and none with H-7, suggesting the same orientation for H-6 and H-7a protons. The NOE correlations and the small coupling constants (J = 2.1–2.4 Hz) between H-6 and H-7a corroborated an α-orientation for H-6 and H-7a in **1**. The small coupling constants (J = 2.1–2.4 Hz) between H-6 and H-7a occurs presumably due to the '*W*-coupling', where the H–C–C–C–H system lies in the same plane.[23] The same coupling constants and the negative sign of optical rotation similar to those of compound **1**, were used to assign the α-orientation for H-6 and H-7a and β-orientation for H-7 in **2**.

2.2. Total phenolic content and antioxidant activity

The total phenolic content (TPC) of Morama extracts were estimated using gallic acid as a standard and was expressed as milligrammes of gallic acid equivalents (GAE) per gramme dry extract (mg GAE/g) (Table 2). The EtOAc and *n*-butanol extracts (Table 2) exhibited the highest total phenolic content (>233 mg GAE/g),

Table 2
Total phenolic content (TPC) of *T. esculentum* extracts

Extract	TPC (mg GAE/g)
Fresh milk	0.41 ± 0.07
Dried milk	1.54 ± 0.02
EtOAc T	91.94 ± 0.12
70% MeOH T	92.36 ± 0.23
$CHCl_3$	5.81 ± 0.05
EtOAc H	233.42 ± 2.50
n-butanol H	235.11 ± 3.00
70% EtOH de-hulled bean	39.99 ± 0.08

Values are average of three replications. T-tuber, H-husk.

while morama milk showed low detection of phenolics (0.41 mg GAE/g).

Morama extracts and compounds **1**, **3**, **4**, **5**, **11** and **12** were assayed for DPPH radical scavenging activity. The Morama husk EtOAc extract (EC_{50} = 4.93 μg/mg) and *n*-butanol extract (EC_{50} = 3.47 μg/mL) exhibited higher DPPH free radical scavenging activities than the Morama tuber extracts (EC_{50} = 91.76 μg/mL), morama milk (EC_{50} >1000 μg/mL) and cotyledons EtOH (EC_{50} = 282.81 μg/mL) extracts. Morama milk poor DPPH radical scavenging activity was attributed to its high content of oils, 30–42%[2,3] and low phenolic content. Compounds **4**, **5**, **11**, **12** showed activities comparable to the standard (ascorbic acid), with compound **4**, after 30 min of reaction time showing EC_{50} of 1.85 μg/mL compared to that of the reference compound, ascorbic acid (EC_{50} = 41.08 μg/mL). The quantitative DPPH radical scavenging activity was also shown as % of DPPH radical scavenged (after 3 h) in Figure 2. The husk extracts scavenged 94% of the DPPH radical at concentration of 25 μg/mL, this was attributed to the presence of phenolic compounds 4–5 and 11–12 in the extracts. Morama husk extracts could, thus, have an antioxidant potential.-after 3 h of reaction time. Values are means of triplicate determinations, SD ± 0.02–0.05. H-Husks, T-Tuber.

A positive linear correlation between antioxidant activity and total phenolic content for extracts at 5, 25, 50 and 250 μg/mL (correlation coefficient r = 0.9395 and 0.9543, 0.9328 and 0.8804 respectively) was observed. These correlations suggested that the phenolic compounds contributed significantly to the antioxidant capacity of the investigated plant extracts. These results were consistent with the trends in literature reporting such positive correlation between total phenolic content and antioxidant activity.[24,25]

The effect of Morama extracts and gallic acid on scavenging of hydrogen peroxide is shown in Figure. 3.

The results show that Morama extracts had an effective H_2O_2 scavenging activity in a concentration dependant manner. All the tested extracts and controls (gallic acid and ascorbic acid) demonstrated greater than 50% scavenging activity at all concentrations tested [12.5, 25, 50, 125 and 250 μg/mL]. The husk EtOAc extract demonstrated the highest H_2O_2 inhibition values (64–83%) while the tuber methanol extract exhibit the lowest H_2O_2 inhibition values at 50–71%.

2.3. Antimicrobial studies

The preliminary antimicrobial activities of the extractives were evaluated using the TLC auto-bioautography method. The results indicated very weak antimicrobial activities against *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Candida albicans*. The extracts and compounds 1, 3–10 exhibited minimum inhibitory quantities of 50–100 μg or no activity compared to the reference compound, chloramphenicol (0.001 μg).

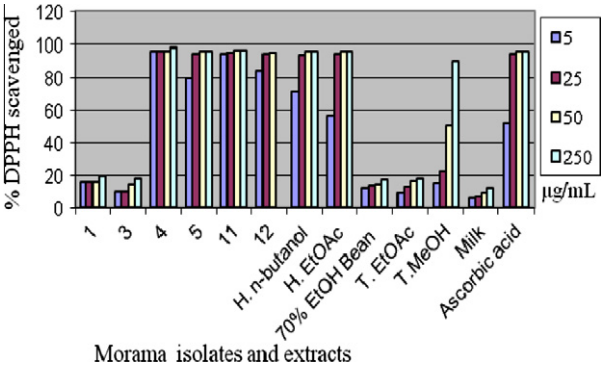

Figure 2. *T. esculentum* extractives DPPH radical scavenging activity (%) after 3 h of reaction time.

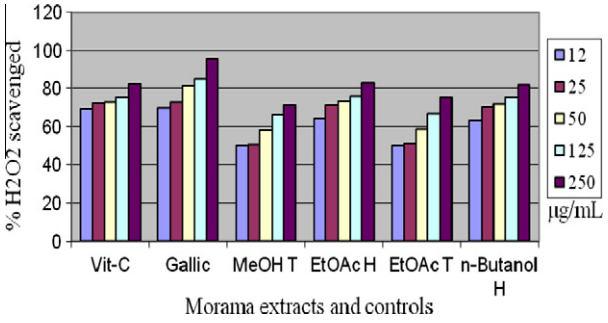

Figure 3. Scavenging of hydrogen peroxide by *T. esculentum* extracts.

2.4. Cell growth inhibition

The Caco-2 (human colon adenocarcinoma cells), HeLa (cervical cancer cells) cells and normal cells derived from African green monkey kidney cells (Vero) were used for the cytotoxicity tests in this study. The cytotoxicity of the tuber and husk extracts against the cell lines are summarized in Table 3. The result show that tuber extracts had no cytotoxicity against Caco-2 and Hela cell lines, but these extracts showed lower activity against Vero cell lines with values of IC_{50} >400 μg/mL. The *n*-butanol extract of the husk showed stimulation of cell proliferation in Hela cells with ED_{50} values of 430 μg/mL. Epidemiological evidence suggest that polyphenolic phytochemicals posses cancer chemopreventive properties,[26] but in this study the husk extracts which have shown good antioxidant properties show high IC_{50} values in cell growth inhibition. There was a negative correlation between the DPPH and H_2O_2 scavenging properties and cell growth inhibition test.

2.5. GC–MS analysis

The IR spectrum of the non-polar extracts of both the tuber and dehulled bean (cotyledons) displayed the strong bands at 2960 and 2873 cm^{-1} (C–H stretching of methylene groups), 1736 cm^{-1} (carbonyl groups), 1471 (C–H bending) and 1184 cm^{-1} (C–O stretching). These bands were indicative of the fatty acid nature of the non-polar extracts.[27]

The non-polar extracts from the edible parts (tuber and cotyledons) were analyzed using GC–MS. A total of thirteen compounds were identified in the sample non-polar extracts and these accounted for 99.36% of cotyledons and 85.48% of tuber extract content. The main components are listed in Table 4. The cotyledons extract main constituents were oleic acid (50.7%) and palmitic acid (23.9%), while the tuber extract main constituents were palmitic acid (55.1%) and oleic acid (26.8%). Saturated fatty acids accounted for 27.9% in the

Table 3
Cytotoxic activities of Morama extracts

Extract	Cell types		
	Caco-2	HeLa	Vero
MeOH T	Inactive[a]	Inactive[a]	Active[b]
EtOAc T	Inactive[a]	Inactive[a]	Active[b]
EtOAc H	Active[b]	Active[c]	Active[b]
n-butanol H	Active[b]	stimulate[d]	Active[b]

[a] IC_{50} >1000 μg/mL.
[b] 400 μg/mL < IC_{50} < 1000 μg/mL.
[c] IC_{50} = 120 μg/mL
[d] ED_{50} = 430 μg/mL. Data shown represent the mean ± SD of three independent determinations. T-tuber, H-husk.

Table 4
GC–MS analysis of *T. esculentum* non-polar extracts from the bean and tuber

Components	RI[a]	(%) Composition[b]
Cotyledons		
Palmitic acid	1960	23.9
Oleic acid (9*E*)	2102	40.3
Linoleic	2161	18.1
Oleic acid (9*Z*)	2175	10.4
Stearic acid	2184	4.0
Icosanoic acid	2463	1.08
Tuber		
4-Methyldodecane	1200	2.06
Pentadecanoic acid	1870	0.22
Palmitic acid	1960	55.07
14-Methylpentadecanoic	2099	1.08
Oleic acid (9*E*)	2102	15.76
Oleic acid (9*Z*)	2175	11.04
Stearic acid	2184	0.23

[a] Retention indices (RI) relative to C_9-C_{24} *n*-alkanes on the HP 5MS column.
[b] % composition based on peak areas calculated in GC on HP 5MS column.

cotyledons and 56.6% in the tuber non-polar extract, while monounsaturated fatty acids composition was 51.8% and 26.8% in the cotyledons and tuber extracts respectively. The polyunsaturated fatty acids were detected only in the cotyledons extract at 18.1%. These results are in agreement with the published data,[3,13] which reports 27.6% saturated fatty acids, 50.2% monounsaturated fatty acids and 21.2% poly unsaturated fatty acids in the dehulled beans.

In conclusion, the phytochemical work-up of Morama husks, cotyledons and tuber yielded twelve compounds. The results established that the non-polar extracts of the edible parts (tuber and dehulled bean) of Morama were constituted by fatty acids while the polar extracts of the non-edible husks showed good antioxidant properties but weak antimicrobial activity and cytotoxicities. The known high content of amino acids, fatty acid in Morama and its long storage period[13] and now the isolation of the secondary metabolites from the different parts of *T. esculentum*, which showed the absence of cynanogenic glucoside from the cotyledons, add to the widely held view that Morama cotyledons has potential as a food source, while the husk extracts antioxidant potential need to be investigated further.

3. Experimental

3.1. General

Merck (Darmstadt, Germany) silica gel 60 (size 0.040–0.063 mm) was used for column chromatography. TLC was carried out on 0.25 mm layer of Merck silica gel 60 F_{254} pre-coated on aluminium sheets. Merck silica gel 60 $HF_{254\,+\,366\ nm}$ coated on 20 x 20 cm glass plates (0.5 mm thickness) were used for preparative TLC (PTLC). The UV light (λ_{max} 254 and 366 nm) and 1% vanillin-sulphuric acid spray were used for visualization. The ^{1}H NMR, ^{13}C NMR, DEPT-135, COSY, HMQC, HSQC, HMBC and TOCSY were acquired on Bruker Avance DPX 300, and DRX 600 MHz. HR-MS were obtained on a GCT Premier Mass Spectrometer (Waters) in EI Mode. UV-Vis spectra were recorded on Shimadzu (UV-2101 PC) UV-Vis spectrometer (Kyoto, Japan). IR spectra were measured on a Shimadzu Hyper FT-IR 8700 (Kyoto, Japan). Melting point was recorded (uncorrected) on Stuart Scientific melting point apparatus SMP1 (UK). Specific rotation $[\alpha]_D$ was determined through Autopol IV (Rudolph Research Analytical) Automatic Polarimeter at λ = 589 nm.

3.2. Chemicals

Hydrogen peroxide, 1,1-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Aldrich (Munich, Germany) and Folin-Ciocalteu reagent from Rochelle Chemicals (South Africa). All reagents were of analytical grade or better.

3.3. Plant Material

The Morama bean and tuber samples were supplied in April 2007 by Thusano Lefatsheng agency, in Mmankgodi village, in the South District of Botswana. A voucher specimen (voucher # MB4kg1990) has been deposited at the University of Botswana Herbarium.

3.4. Extraction and Isolation

3.4.1. Husk

Powdered husk (4.5 kg) was first defatted with *n*-hexane and then extracted with 70% aqueous ethanol. The solvent was evaporated using rotary evaporator (Buchi R210, Switzerland) and the residual water was removed by freeze drying to give 300 g of dry extract. Part of the dry extract (178.8 g) was reconstituted in distilled water (200 mL) and extracted successively with ethyl acetate and *n*-butanol to yield the EtOAc (20.8 g) and *n*-butanol (14.7 g) extracts respectively. The *n*-butanol extract (14.7 g) was fractionated using silica gel column (500 g silica gel) eluting with *n*-hexane/chloroform (4:1) and increasing polarity to 100% chloroform and to 30% methanol in chloroform. Fractions (500 ml each) were collected and pooled according to similarity in TLC profiles to give fractions H_1 to H_7. White crystals identified as **6** came out of H_1 to H_3. H_4 (340 mg) was eluted through a Sephadex LH-20 (Sigma) column (MeOH/$CHCl_3$ 1:1) to give subfractions $H_{1'}$ to $H_{3'}$. $H_{2'}$ gave **4** as white crystals (70 mg), $H_{1'}$ gave **11** (10 mg), $H_{3'}$ afforded **5** (10 mg), H_5 yielded more **4** (1.2 g) after further purification using Sephadex LH-20 column. H_6 afforded **3** (430 mg) and H_7 yielded **12** (15 mg) on further purification on a Sephadex LH-20 column and repeated preparative TLC ($CHCl_3$/MeOH 4:1).

3.4.2. Cotyledons

The powdered cotyledons (952.7 g) were extracted with 70% ethanol which on evaporation and freeze drying gave 50.2 g extract, which was defatted with chloroform to give 4.8 g extract. In the fatty fraction **8** (9 mg) crystallized out. The mother liquor of this fraction was prepared for GC–MS analysis. The residual extract (40 g) was fractionated over silica gel (200 g) VLC column and eluted in increasing polarity from *n*-hexane/$CHCl_3$ (1:1) to pure chloroform, to which was added methanol increments until 30% methanol in chloroform. Seven fraction were collected and combined to give four main fractions B_1 (20.2 g), B_2 (8 g), B_3 (4 g) and B_4 (12.2 g). Part of B_1 (5 g) was fractionated over silica gel column using EtOAc/MeOH/H_2O (EMW) 7:2:1 ($B_{1'}$–$B_{4'}$), ethyl acetate/methanol/water (EMW) 6:3:1 ($B_{5'}$–$B_{6'}$) and 50% methanol ($B_{7'}$–$B_{8'}$). $B_{5'}$–$B_{6'}$ gave **9** (2 g), $B_{7'}$–$B_{8'}$ afforded **10** (3 g) while $B_{4'}$, B_2 and B_3 yielded more **9** (800 mg). B_4 was not interesting and was not worked on.

3.4.3. Tubers

The powdered tubers (1.35 kg) were extracted sequentially with EtOAc for three days and 70% MeOH for a further three days at 25 °C. Solvent evaporation yielded 8.21 g EtOAc and 19.4 g 70% MeOH extracts. Part of the EtOAc extract (7 g) was applied to VLC silica gel giving seven fractions which were combined to give main fractions T_1 (1.9 g), T_2 (3.0 g), T_3 (50 mg), T_4 (90 mg) and T_5 (0.9 g). T_1 was analyzed using GC–MS. T_2 gave white crystals (2.0 g) identified as behenic acid **7**. T_3 yielded crystals (30 mg) which are yet to be identified. T_4 gave a mixture (11.2 mg) of **1** and **2**. Repeated preparative TLC (EMW 7:2:1) yielded only **1** (7 mg).

The structures of all known compounds were identified based on spectral data analysis (NMR, MS, IR, UV and [α]) and comparison with literature data.

3.5. Griffonilide

Compound **1** was white solid; mp 180–182 °C; $[\alpha]_D = -11.0$ (*c* 0.98, MeOH); R_f: 0.51 (EMW, 7:2:1); UV/Vis (MeOH) λ_{max} nm (ε) 254 (4.5); IR (KBr) ν_{max} cm^{-1}: 3371, 3076, 1726, 1649, 1375, 1166, 1082, 1010 cm^{-1}. ^1H NMR (300 MHz, CD_3OD) and ^{13}C NMR (75.5 MHz, CD_3OD), ^1H and ^{13}C data in Table 1; HR-TOF EIMS *m/z* 168.0420 (calcd for $C_8H_8O_4$ 168.0423).

3.6. Compound 2

Compound **2** was mixed with **1** (minor peaks). Mixture $[\alpha]_D$ −14.0 (*c* 0.98, MeOH); R_f: 0.50 (EMW, 7:2:1). IR (KBr) ν_{max} cm^{-1}: 3371, 2914, 1736, 1649, 1375, 1166, 1082, 1010 cm^{-1}; ^1H and ^{13}C data in Table 1; HR-TOF EIMS *m/z* 170.0576 (calcd for $C_8H_{10}O_4$ 170.0579).

3.7. Bioassays

3.7.1. TLC bioautography antimicrobial testing

The microorganisms used were: *S. aureus* (ATCC 9144), *E. coli* (ATCC 11229), *B. subtilis* (ATCC 6633), *P. aeruginosa* (NCTC 10332) and *C. albicans* (ATCC 10231). The microorganisms were obtained from the Department of Biological Sciences, University of Botswana. Chloramphenicol and miconazole (Aldrich, Germany) were used as standards for bacteria and fungi respectively.

The antimicrobial activities of extracts and compounds were evaluated using agar-overlay bioautography method. Stock solutions (10 mg/mL) of the test sample were prepared and serially-diluted to obtain concentrations of 5.0, 1.0, 0.05, 0.001 mg/mL. Aliquots of 10 μL of each concentration was spotted on Merck pre-coated Silica gel 60 HF_{254} TLC plates (0.25 mm thickness; 10 cm × 10 cm), corresponding to loading quantities of 100, 50, 10, 0.5 and 0.01 μg. The spots were of the same size and the solvent was allowed to evaporate in the fume hood. The seed layers were prepared by inoculating 10 mL aliquot of culture into 100 mL agar (Oxoid, UK: Sabouraud Dextrose) solution. 24-h old (37 °C) cultures were used. Using a sterile Pasteur pipette the TLC plates were overlaid with the agar. Plates were run in duplicates. After the medium congealed the TLC plates were incubated at 37 °C (*S. aureus*, *E. coli*) and 25 °C (*B. subtilis*, *P. aeruginosa*, *C. albicans*) for 24 h. The bioautograms were sprayed with an aqueous solution of thiazoyl blue (methylthiazolyltetrazolium bromide (Sigma); 200 mg in 100 mL distilled water) and further incubated for 4 h after which results were scored. White spots against purple background indicated inhibition zones. The minimum inhibitory quantity (MIQ in μg) was taken as the lowest loading quantity to exhibit an inhibition zone.

3.7.2. Growth inhibition assay

The Caco-2, HeLa cells and normal cells (Vero) were used. All cell lines were grown in advanced Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Grand Island, USA), supplemented with 5% foetal calf serum (Lonza, Basel, Switzerland), L-glutamine (2 mmol/L, Sigma), penicillin (100 U/mL, Sigma) and streptomycin (1 mg/mL, Fluka, Buchs, Switzerland). Cell lines were routinely grown in 25 cm^2 culture flasks (Corning, New York, USA) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air, until confluent monolayers were obtained. Culture medium was routinely changed. Before introduction of samples, the monolayers were washed twice with 100 μl DMEM without phenol red and supplements.

The inhibition study was performed when the cells grew up to 80–90% confluency and were seeded into 96-well plates at a density of 6 x 10^6 cells per well using method described in Agelis et al. 2007.[28] In assay 3-fold serial dilutions of each compound were tested. Then, the cells were incubated at 37 °C in the humidified incubator for 48 h. The plates were stained with Crystal Violet in ethanol, rinsed with water, and destained with 10% (v/v) acetic acid. The arbsorbance was measured at 590 nm, and the results were expressed, for each dilution, by the mean ratios (%, ±SD) of absorbances in treated wells to that in control wells.

The cell viability was determined by the following formula: % Cell viability = (Mean absorbance in test wells/Mean absorbance in control wells) × 100.

Dose–response curves between percentages of cell viability and concentrations of the extracts were constructed. The results are expressed to show the concentration necessary to show 50% inhibition. The 50% inhibitory concentration (IC_{50}), in the case of cell inhibition, or effective concentration (ED_{50}), in the case of cell stimulation, was determined from the plotted curve. Correlations were established using linear regression analysis, employing Microsoft Excel™ 2007 software.

3.7.3. DPPH free radical scavenging assay

Methanolic samples (2 mL) of various concentrations (0.005, 0.05, 0.1, 0.2 and 0.5) mg/mL were mixed with 2 mL of 2% DPPH. Morama milk was used as raw milk and 1 mL of the milk was diluted to 10 ml and treated as such. The absorbance of the mixture was measured at 517 nm using a spectrophotometer (UV 2101, Japan) at time intervals of 0.5, 1, 3, 6 and 24 h after incubation in the dark at room temperature. The% of the DPPH radical scavenged was calculated using the formula:% of DPPH scavenged = $(A_{blank}-A_{sample}/A_{blank}) \times 100$. Where, A_{blank} is the absorbance of DPPH solution with zero sample concentration and A_{sample} is the absorbance of DPPH and known sample concentration. Ascorbic acid was used as a reference compound. The experiment was performed in triplicate.

3.7.4. Total phenolic content

The procedure followed is in Yeboah & Majinda, 2009.[29] Briefly, 1 mg/mL solution in methanol was prepared for each extract. The fresh milk concentration was 1 mL in 10 mL of 90% methanol/water. Five different concentrations of gallic acid in 90% aqueous methanol ranging from 0.01 to 0.05 mg/mL were prepared. Aliquots of 0.5 mL test sample (standard or extract) and 0.5 mL of Folin-Cioucalteau reagent were added to a 10 mL screw cap test-tube. After 3 min 1 mL of 20% sodium carbonate solution was added. The mixture was shaken vigorously for 5 min and allowed to stand for 2 h. Finally, 5 mL of 80% aqueous methanol was added and the absorbance of the supernatant solution was determined at 725 nm using a Shimadzu UV-2101 spectrophotometer. The total phenolic content of the sample was determined by comparing the optical density of the sample with those of different concentrations of gallic acid, a standard phenolic compound. Analysis for each sample was performed in triplicate, and values for total phenolic content are expressed in milligrams of gallic acid equivalent (GAE) per gram of samples.

3.7.5. Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity of the plant extract was determined using methods described in Gulcin et al., 2004[30] and Ogunlana and Ogunlana., 2008.[31] Briefly, 32.7 mM solution of hydrogen peroxide was prepared in phosphate-buffer (PBS): pH 7.4. Hydrogen peroxide concentration was determined spectrophotometrically (UV-2101) from absorbance (A_0) at 230 nm using molar absorptivity of 81 M^{-1} cm^{-1}. 12.5–250 μg plant extract and standard (gallic acid and ascorbic acid) corresponding to 0.05, 0.1, 0.2, 0.5, 1 ml of 1 mg/mL plant extract stock solution were added to 0.6 mL hydrogen peroxide-PBS solution. The total volume of solution was 4 mL (topped-up using distilled water). Absorbance of hydrogen peroxide at 230 nm was determined after 30 min (A_1).

A blank solution containing phosphate buffer without hydrogen peroxide was prepared for background correction. The percentage of scavenged hydrogen peroxide of sample and standard compounds was calculated using the following equation:% Scavenged $[H_2O_2] = [(A_0-A_1)/A_0] \times 100$. Where A_0 was the absorbance of control and A_1 was the absorbance in the presence of the sample. The DPPH, TPC and H_2O_2 assays data were processed using Microsoft Excel™ 2007 software.

3.8. GC–MS analysis

3.8.1. Non-polar extract preparation

The chloroform cotyledons extract (4.6 g) and tuber EtOAc extract (Fraction T_1, 1.9 g) provided the non-polar extracts after being further extracted in a Soxhlet apparatus with *n*-hexane (2 h). The solvent was allowed to evaporate at room temperature. The cotyledons and tuber yielded 1.6 g (35%) and 0.25 g (13%) viscous non polar extracts respectively. The yield was expressed as weight per weight (w/w) based on the dry weight of extract. The non-polar extracts were dried over Na_2SO_4 and stored at 4 °C prior to qualitative analysis by GC–MS in the conditions described.

3.8.2. FT-IR of non polar extracts

The viscous non-polar extracts were subjected to FT-IR (Shimadzu Hyper FT-IR 8700 spectrometer, KBr disc, 0.6 mg) analysis for determination of their fatty acid nature.

3.8.3. GC–MS analysis

GC–MS analysis were performed using an HP-5 MS capillary column (25 m × 250 μm i.d., 0.25 μm film thickness, Agilent) in an Agilent 6890 gas chromatograph coupled to a Waters GCT Premier mass spectrometer. The carrier gas was helium with a constant flow rate of 1 mL/min. The oven temperature was initially kept at 50 °C for 6 min then ramped at 4 °C/min to 230 °C then gradually increased to 300 °C and held isothermally for 30 min. Solutions of the samples (100 ppm in chloroform) were injected manually at 250 °C. Injection volume was 1.0 μL in the split-less mode. Mass spectra were obtained by EI at electron energy of 70 eV.

3.8.4. Identification and quantification of constituents

The relative percent composition of the non-polar extract constituents was determined by computerized peak area measurements using internal normalization method. Identification of components was based on GC retention time on the HP-5MS capillary column, retention indices and by computer matching of the acquired mass spectra with those stored in the spectrometer data base using the NIST 05L Mass Spectral Library.[9,32] The identity of the spectra above 95% was considered for identification of constituents.

Acknowledgments

Mr. I. Morobe of Department of Biological Sciences, University of Botswana is thanked for his assistance during antimicrobial tests. This project was funded by the European Union under the Morama II project (EU FP-6 grant (FP6-2004-INCO-DEV-3-MARAMA II – 032059).

A. Supplementary data

Supplementary data (^1H and ^{13}C NMR spectras for compounds 1 and 2) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.07.006.

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