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Further antibacterial compounds from *Myristica fragrans*

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Abstract

Background: *Myristica fragrans* Houtt is a medicinal food plant of the family Myristicaceae. Previous phytochemical investigation of its seed kernel known as nutmeg led to the isolation of antibacterial flavonoid, 3',4',7-trihydroxyflavone. The present study aimed at identifying further antibacterials of the crude methanol extract from nutmeg.

Methods: The modified rapid *p*-iodonitrotetrazolium chloride (INT) colorimetric assay was used to determine the Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) on the tested bacteria. Column chromatography was used for the purification of the seed kernel extract whilst the structures of compounds were determined by MS and NMR analysis.

Results: The chemical study of the methanol extract led to the identification of already described saponin (**1**), mixture of succinic (**2A**) and fumaric (**2B**) acids and tetramethylhexestrol (**3**). Among the isolated compounds, the mixture of acids **2A** and **2B** showed the most interesting activity with the lowest MIC of 8 µg/mL against *E. coli* ATCC8739.

Conclusions: Nutmeg contains various phytochemicals that can be used to combat bacterial infections including multidrug resistant (MDR) phenotypes.

Keywords: Antibacterial; Compounds; multidrug resistant; *Myristica fragrans*; Myristicaceae.

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Background

Myristica is a genus of trees in the family Myristicaceae. There are about 150 species distributed from Asia to North Australia and the Western Pacific [1]. With various common names; mace, muscadier, *Myristica*, nutmeg, nutmeg tree, *M. fragrans* is a spreading aromatic evergreen tree usually growing to around 5 to 13 meters [1]. The fruits are fleshy, drooping, yellow, smooth, 6 to 9 cm long with a longitudinal ridge. When ripe, the succulent yellow fruit coat splits into 2 valves revealing a purplish-brown, shiny seed (nutmeg) surrounded by a red aril (mace). Seed (nutmegs) are broadly ovoid (2 to 3 cm long), firm, fleshy, whitish and transverse by red-brown veins [1]. *Myristica fragrans* is native to the Banda Islands in Eastern Indonesia (Moluccas) and is cultivated in the Banda Islands, Grenada, the Caribbean, South India, Sri Lanka, Malaysia, Sumatra, Brasil, tropical Southeast Asia and Australia [2]. In Cameroun, nutmegs are one of the products brought in from abroad and so, widely sprayed in many supermarket everywhere in the Country. Nevertheless, some privates, due to the use in food industry (bakery), cultivated the species. *Myristica fragrans* deposited at the botanical garden of Limbe, South-West Region of Cameroon. Elsewhere in the World, nutmegs are used for dental caries, spice, leucorrhoea and local stimulant for gastro-intestinal tract [3]. The commonly known groups of compounds from *M. fragrans* are lignans, fatty acids and essential oils or flavor substances [4]. Previous phytochemical investigation of its seed kernel known as nutmeg led to the isolation of antibacterial flavonoid, 3',4',7-trihydroxyflavone [5]. As part of our contribution to the studies of antibacterial plants from the Cameroonian folk medicine [6-8], we report herein further antibacterial compounds from *Myristica fragrans*.

Methods

General experimental procedure

Mass spectral data [ElectroSpray Ionization Mass Spectrometry (ESI-MS)] were measured on a Waters Synapt HDMS spectrometer. NMR Spectra (^{1}H, ^{13}C, HSQC, HMBC and COSY) were recorded with an Agillent DD2 NMR (400 MHz) spectrometer. Column chromatography was performed on silica gel Merck 60 F_{254} [(0.2-0.5 mm) and (0.2-0.063 mm)] 70-230 and 230-400 mesh (Darmstadt, Germany) or Sephadex LH-20. Pre-coated silica gel 60 F_{254} Thin Layer Chromatography (TLC) plates (Merck, Germany) were used for monitoring fractions and spots were detected with UV light (254 and 365 nm) and then sprayed with 50% sulphuric acid (H_2SO_4) followed by heating at 100°C.

Plant material

The dried seeds of *M. fragrans* were purchased in March 2015 at Douala central market, Littoral Region of Cameroon. The species was identified at the Cameroon National Herbarium (Yaoundé), by comparison with a voucher specimen and deposited under the reference No. 60342 HNC (YA).

Extraction and purification

The powder obtained from the seeds of *M. frangrans* (1500 g) were macerated in methanol (MeOH, 5 L) for 48 h at room temperature. The extract was then concentrated under reduced pressure to give a semi-solid reddish brown fatty residue, (370 g). The extract was kept at 4°C until further use. Part of the crude extract (350 g) was subjected to silica gel column chromatography eluted with gradients of CH_2Cl_2-EtOAc and EtOAc-CH_3OH. Seventy fractions of 400 mL each were collected using mixtures of CH_2Cl_2-EtOAc 85:15, 70:30 and 30:70, evaporated under reduced pressure and combined on the basis of their TLC profiles into five main fractions coded A-E (A: 1-6; B: 7-32; C: 33-51; D: 52-60; E: 61-70). Before further separation, the obtained fractions were screened for antibacterial activity against the selected bacterial strains. Regarding to the obtained results, fractions D (10 g) and E (5 g) were not further investigated due to their low activity. Fraction A (150 g), with the most considerable antibacterial profile, was not further investigated because it contained mostly fatty materials. Fraction B (10 g) was separated by a column chromatography over silica gel using a gradient of CH_2Cl_2-EtOAc (100:0, 95:5, 90:10, 85:15, 80:20, 75:25 and 70:30) to afford seven sub-fractions (FrB1-FrB7). Following their TLC profiles, subfractions FrB2 and FrB3 were retained for further purifications over silica gel, for FrB2, with CH_2Cl_2-EtOAc to afford sitosterol 3-*O*-*β*-D-glucopyranoside (10 mg). Similarly, fraction FrB3 was purified by Sephadex LH-20 column chromatography with isocratic CH_2Cl_2-MeOH (90:10) to give the mixture of fumaric (**2A**) and succinic (**2B**) acids (7 mg). Fraction C (7 g) was subjected to column chromatography over silica gel eluted with CH_2Cl_2-EtOAc (90:10, 85:15, 80:20, 75:25 and 70:30) to afford five sub-fractions (FrC1-FrC5). As for the fraction B, only the sub-fractions FrC3 and FrC4 were retained, on the basis of their TLC profiles, for further purifications. Thus, FrC3 was chromatographed over Sephadex LH-20 column chromatography with isocratic CH_2Cl_2-MeOH (1:1) to give tetramethylhexestrol (**3**) (15 mg) while subfraction FrC4 was purified by silica gel column

chromatography with isocratic CH_2Cl_2-EtOAc (2:98) to give compound **1** (5 mg).

Antibacterial assays
Chemicals for antibacterial assays

Chloramphenicol (CHL) (Sigma-Aldrich, St. Quentin Fallavier, France) was used as reference antibiotic while *p*-iodonitrotetrazolium chloride ≥ 97% (INT, Sigma-Aldrich) was used as microbial growth indicator.

Bacterial strains and culture media

MDR isolates and reference strains of *Escherichia coli*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Providencia stuartii* and *Pseudomonas aeruginosa* were used in this work; their resistance profiles were previously reported [9]. They were conserved at 4°C and were grown on a fresh appropriate agar plates for 24 h before Minimal Inhibitory Concentration (MIC) testing. Mueller Hinton Agar (Sigma) was used to activate the microorganisms whilst Mueller Hinton broth (MHB; Sigma) was used for antibacterial assays [10,11].

Determination of bacterial susceptibility

The MIC determination on the tested bacteria were conducted using rapid INT colorimetric assay according to previously described methods [12] with some modifications [13, 14]. The test samples and RA were dissolved in dimethylsulfoxide (DMSO)/MHB. The final concentration of DMSO was lower than 2.5% and does not affect the microbial growth. The solution obtained was then added to MHB, and serially diluted two fold (in a 96- wells microplate). The bacterial concentration was 1.5 x 10^6 CFU/mL. The plates were incubated at 37°C for 18 h. The assay was repeated thrice. Wells containing MHB, 100 µL of inoculum and DMSO to a final concentration of 2.5% served as negative control. The MIC of samples was detected after 18 h incubation at 37°C, following addition (40 µL) of 0.2 mg/mL of INT and incubation at 37°C for 30 minutes. Viable bacteria reduced the yellow dye to pink. MIC was then defined as the sample concentration that prevented the color change of the medium and exhibited complete inhibition of microbial growth [12]. The MBC was determined by adding 50 µL aliquots of the preparations, which did not show any growth after incubation during MIC assays, to 150 µL of adequate broth. These preparations were incubated at 37°C for 48 h. The MBC was regarded as the lowest concentration of the sample, which did not produce a color change after addition of INT as mentioned above [15].

Results and discussion

The MeOH extract from the seeds of nutmegs was repeatedly chromatographed on silica and Sephadex LH-20 gel. Three compounds (Figure 1) were isolated including a saponin (**1**), succinic (**2A**) and fumaric (**2B**) acids and tetramethylhexestrol (**3**). The structures of the compounds (Figure 1) were determined by the interpretation of HRMS and 1D&2D NMR spectra as well as IR and UV methods.

Compound **1** was obtained as a white amorphous powder in EtOAc. Its molecular formula $C_{48}H_{72}O_7$ was deduced from its ESI-MS spectra which showed the pseudo-molecular ion $[M+HCOO]^-$ at *m/z* 831.6 and $[M+Na]^+$ at *m/z* 809.6. Its Infrared spectrum exhibited bands due to one hydroxyl groups centered at 3409 cm^{-1}, a carbonyl at 1660 cm^{-1} and an olefinic at 1637 cm^{-1}. The ^1H and ^{13}C NMR spectra of **1** showed signals of a sitosterol skeleton at δ_H 0.55 (s, 3H, H-18), 0.82 (d, 3H, *J* = 6.3 Hz, H-21), 0.86 (s, 3H, H-19), 3.47 (m, 1H, H-3) and 5.23 (bs, 1H, H-6) for protons and at δ_C 140.4 (C-5), 121.5 (C-6) and 78.2 (C-3) for carbons [16]. This skeleton was also confirmed by interactions (Fig. 2) observed from H-19 at δ_H 0.86 to the carbon at δ_C 140.4 (C-5); from H-18 at δ_H 0.55 to the carbons at δ_C 55.9 (C-17) and 42.2 (C-13) and from H-21 at δ_H 0.82 to the carbon at δ_C 55.9 (C-17) on the HMBC spectrum. In addition, the ^{13}C NMR spectrum displayed signals of a fatty acid moiety at δ_C 173.1 (C-1''), 34.2 (C-2'') and 14.2 (C-14''). The resonance of protons of this last carbon split into a triplet at δ_H 0.73 (t, 3H, *J* = 7.4 Hz, H-14'') on its ^1H NMR spectrum. The aliphatic chain was deduced to be linked on a sugar through an ester bridge with the sugar carbon at δ_C 64.3 (C-6') in agreement with the correlations observed in the HMBC spectrum of **1** from H-6' at δ_C 4.38 (m, 1H, H-6'α) and 4.19 (dd, 1H, *J* = 11.5 and 8.0 Hz, H-6'β) to the carbonyl C-1''. The sugar was identified as β-D-glucose by comparison of its carbon signals with those of the β-D-glucose in the litterature. The HMBC interaction from H-3 at δ_H 3.47 to the anomeric carbon C-1' at δ_C 101.8 supports the linkage of the sugar moiety and the sitosterol via C-3-O-C-1' ether connection (Figure 2). Base on the evidence above, the structure of **1** was fully assigned as sitosterol 3-*O*-[β-D-glucopyranos-6'-yl tetradecanoate].

*Sitosterol 3-O-[β-D-glucopyranos-6'-yl tetradecanoate] (**1**):* White amorphous powder; UV (MeOH), λ_{max} (log ε) : 257 (2.67); IR (KBr) ν_{max}: 3409, 1660, 1637, 1453, 1058, 1022 cm^{-1}; ^1H-NMR (C_5D_5N+DMSO, 400 MHz), δ 5.23 (1H, bs, H-6), 4.38 (1H, m, H-6'α), 4.37 (1H, d, *J* = 7.8 Hz, H-1'), 4.19 (1H, dd, *J* = 8.0 Hz and *J* = 11.5 Hz, H-6'β), 3.47 (1H, m, H-3), 2.36 (2H, dd, *J* = 4.0 Hz and *J* = 13.2 Hz, H-4), 2.21 (2H, m, H-2''), 0.86 (3H, s, H-19), 0.82 (3H, d, *J* = 6.3 Hz, H-21), 0.76 (3H, m, H-26), 0.73 (3H, t, *J* = 7.4 Hz, H-7''), 0.71 (3H, m, H-27), 0.55 (3H, s, H-

18). ^{13}C-NMR (C_5D_5N+DMSO, 100 MHz), *δ* 173.1 (C-1''), 140.7 (C-5), 121.5 (C-6), 101.8 (C-1'), 78.2 (C-3), 77.2 (C-3'), 74.1 (C-5'), 74.0 (C-2'), 71.1 (C-4''), 64.3 (C-6'), 56.6 (C-14), 55.9 (C-17), 50.1 (C-9), 45.5 (C-24), 42.2 (C-13), 40.2 (C-12), 38.9 (C-4), 37.3 (C-1), 36.6 (C-10), 35.9 (C-20), 34.2 (C-2''), 33.7 (C-22), 31.8 (C-7), 31.7 (C-8), 29.7-29.2 (C-3''-C-13''/C-2/C-25), 28.1 (C-16), 25.8 (C-15), 25.1 (C-23), 23.0 (C-28), 21.0 (C-11), 20.0 (C-26), 19.3 (C-19), 19.1 (C-27), 18.9 (C-21), 14.2 (C-14''), 12.0 (C-29), 11.9 (C-18) ; (-) ESIMS *m/z* 831.6 $[M+HCOO]^-$ and 809.6 $[M+Na]^+$.

*Succinic (**2A**) and fumaric (**2B**) acuds:* White amorphous powder; ^{1}H-NMR (C_5D_5N, 400 MHz), *δ* 11.16 (4H, bs, OH), 7.44 (2H, s, H-2'/3'), 3.01 (4H, s, H-2/3. ^{13}C-NMR (C_5D_5N, 100 MHz), *δ* 175.0 (C-1/4), 167.9 (C-1'/4'), 135.0 (C-2'/3'), 29.9 (C-2/3).

*Tetramethylhexestrol (**3**):* White amorphous powder; ^{1}H-NMR (DMSO, 400 MHz), *δ* 7.25 (4H, d, *J* = 8.0 Hz, H-2'/6'/2''/6''), 7.15 (4H, d, *J* = 8.0 Hz, H-3'/4'/3''/4''), 1.67 (6H, s, H-3/5), 1.56 (1H, s, H-4/6). ^{13}C-NMR (DMSO, 100 MHz), *δ* 152.1 (C-4'/4''), 148.9 (C-1'/1''), 127.9 (C-2'/6'/2''/6''), 120.3 (C-3'/5'/3''/5''), 42.5 (C-1/2), 30.9 (C-3/4/5/6).

The antibacterial activity of the isolated compounds is compiled in Table 1. The mixture **2A** and **2B** showed the most important activity with MIC values ranging from 8 to 256 µg/mL and inhibited the growth of 91.67% of the 12 tested Multi Drug Resistant (MDR) bacterial strains. Compounds **1** and **3** inhibited the growth of 75% and 41.67% of the 12 Gram-negative MDR tested bacteria respectively. According to the established criteria [17,18], the activity of compounds is significant when MIC<10 µg/mL, moderate when 10<MIC<100 µg/mL and low when MIC>100 µg/mL. On this basis, activities of compound were generally moderate and/or weak since their MIC values are ranging from 64-256 µg/mL. Nevertheless, the activity of the mixture **2A** / **2B** could be considered as significant against the strains of *E. coli* AG102 (MIC = 8 µg/mL). The overall activity of the tested compounds could be considered important if one considers that the bacterial strains used are multi-drug resistant phenotypes [19,20]. Therefore, they deserve further studies to develop potential antibacterial phytochemicals to combat MDR phenotypes.

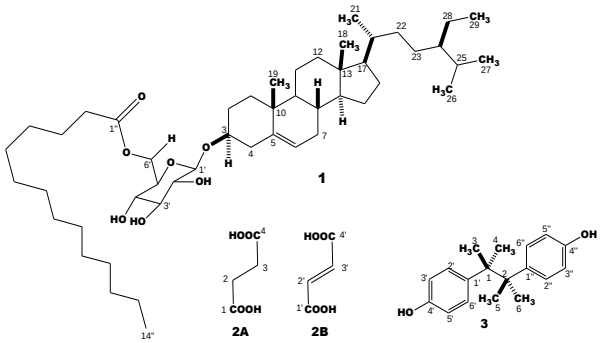

Figure 1. Structures of isolates from *M. fragrans*

Table 1. Minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) of compounds **1**, **2** and **3** from *Myristica fragrans* against bacterial strains

Bacterial strains	Substances (µg/mL)									Reference antibiotic		
	1			2			3			Chloramphenicol		
	MIC	MBC	R	MIC	MBC	R	MIC	MBC	R	MIC	MBC	R
Escherichia coli												
ATCC8739	128	-	-	8	128	16	128	-	-	4	16	4
AG100A$_{tet}$	256	-	-	16	64	4	-	-	-	16	64	4
AG102	128	-	-	64	-	-	16	256	16	64	256	4
Escherichia aerogenes												
ATCC13048	256	-	-	64	256	4	256	-	-	8	64	4
EA27	256	-	-	32	64	2	-	-	-	64	-	-
EA289	-	-	-	-	-	-	-	-	-	256	-	-
Klebsiellapneumoniae												
ATCC11296	256	-	-	32	128	4	-	-	-	16	128	8
KP63	-	-	-	128	-	-	-	-	-	64	-	-
Providenciastuartii												
ATCC299645	128	-	-	32	128	4	-	-	-	8	64	8
NAE16	256	-	-	16	256	16	64	256	4	32	256	8
Pseudomonas aeruginosa												
PA01	-	-	-	128	-	-	-	-	-	32	-	-
PA124	256	-	-	64	-	-	256	-	-	256	-	-

-: MIC or MBC superior to 256 µg/mL

Conclusions

The work highlighted the antibacterial activity of two known compounds with considerable MIC values.

The overall activity of the tested compounds could be considered important if one considers that the bacterial strains used are multi-drug resistant phenotypes. Therefore, they deserve further studies to develop potential antibacterial phytochemicals to combat MDR phenotypes.

Additional file

Supplementary file.docx. ^{1}H NMR, ^{13}C NMR spectra and chemical shifts of isolated compounds.[3,439 Ko]

Authors' Contribution

JKD, IKS and GTMB carried out the study; VK and PT designed the experiments. JKD, GTMB and VK wrote the manuscript; VK provided the bacterial strains; IC, VWK and SFE contributed to structural determination; PT and VK supervised the work; all authors read and approved the final manuscript.

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Conflict of interest

The authors declare that they have no competing interests.

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