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Chemical constituents of *Palisota ambigua* (Commelinaceae) with their antibacterial activities

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Abstract

Background: The development and spread of resistance by bacteria to existing antibiotics are becoming more common nowadays. Considering the ethnopharmacological relevance of *Palisota ambigua* (Commelinaceae), this study was designed to investigate the antibacterial activities of chemical constituents of *P. ambigua* against two Gram-positive (*Staphylococcus aureus* ATCC 25923 and *Streptococcus pneumoniae* ATCC 49619) and two Gram-negative (*Escherichia coli* ATCC 8739 and *Klebsiella pneumoniae* 109) bacteria.

Methods: The plant extracts were prepared by maceration in organic solvents. Chromatography techniques were used for isolation and purification of compounds. Their structures were determined through spectroscopic and spectrometric data, as well as by comparison with literature data. The broth microdilution method was used for antibacterial assays through the determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

Results: The methanolic extract, the ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH) fractions from the aerial parts of *P. ambigua* exhibited significant to moderate antibacterial activity (MIC = 32 – 512 µg/ml). The chemical investigation of fractions led to the isolation of twelve known compounds namely, *N*-benzoyl-$_L$-phenylalanyl-$_L$-phenylalaninol acetate (aurantiamide acetate) (**1**), 20-hydroxyecdysone (**2**), rubrosterone (**3**), *β*-sitosterol 3-*O*-*β*-D-glucopyranoside (**4**), 3*β*-hydroxystigmast-5-en-7-one (**5**)**,** lupeol (**6**), betulinic acid (**7**), bis(2-ethylhexyl) terephthalate (**8**)**,** 2,3-di-*O*-dodecanoyl-*sn*-glycerol 1-*O*-(6-*O*-*α*-D-galactopyranosyl)-*β*-D-galactopyranoside (**9**), docosanoic acid (**10**), pallidol (**11**), and apigenin (**12**). All isolates displayed antibacterial activity varying from weak to moderate against the tested pathogenic bacteria. Among the tested compounds, 20-hydroxyecdysone-2,3,22-triacetate (**2a**) was the most active with a MIC value of 32 µg/mL against *Klebsiella pneumoniae* and *Staphylococcus aureus*.

Conclusion: The results of the present study indicate that *P. ambigua* contains antibacterial isolates, therefore confirming some of its uses in traditional medicine.

Keywords: *Palisota ambigua*; Commelinaceae; chemical constituents; antibacterial activity.

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Background

Despite recent advances in drug discovery, infectious diseases still represent a source of morbidity and mortality worldwide. This is due not only to the phenomenon of antibiotic resistance but also to the emergence of new deadly pathogens causing pandemics. There is a very urgent need to find new drugs that can be used to effectively combat these microorganisms. This problem can be solved by research into medicinal plants, as they have widely been used to treat diseases, including microbial infections, in traditional medicine. *Palisota ambigua* is a robust herb of the genus *Palisota*, the most predominant genus in the Commelinaceae family. This grass is over 2 m high and is found in lowland rainforests [1]. Named «Iguewe-nowee» in Nigeria, «Kundu kimbimba» in West Cameroon, this plant is less reported for its uses in folk medicine. However, the ethnobotany surveys conducted in the South-West region revealed that *P. ambigua* is used by the local population for the treatment of diarrhea. The phytochemical screenings revealed that Commelinaceae family produces diverse classes of secondary metabolites like alkaloids, steroids, saponins, fatty acids, and tannins [2]. Ecdysteroids and flavonoids have been also isolated from this family of plants [3,4]. Knowing that there is a taxonomic distribution of secondary metabolites between species of the same family, we have investigated the chemical constituents of the aerial part of *P. ambigua*, and the evaluation of their antibacterial activities in order to valorize plants of the genus *Palisota*. The present study is the first report on the isolation of chemical constituents from *P. ambigua*.

Methods

General procedures

In this study, ^{1}H NMR, ^{13}C NMR, HSQC, ^{1}H-^{1}H COSY, and HMBC spectra were recorded on Bruker AVANCE 600 spectrometer (Bruker, Germany) in deuterated solvents with TMS as internal standard. Agilent 6545 QTOF-MS spectrometer was used to perform ESI high-resolution (HR) mass spectra. Melting points were recorded on a Stuart SMP20 digital melting point apparatus. Column chromatography was performed using 70–230 and 230–400 mesh silica gel 60 (Merck), and Sephadex LH-20. Thin Layer Chromatography was achieved on percolated silica gel 60 F254 (Merck) plates. Compounds were revealed by spraying TLC plates with 10% sulphuric acid followed by warming at 90 °C and / or UV–Visible lamp multiband UV-54/365 nm (Model UVGL-58 Upland CA 91786, USA).

Plant material

The aerial part of *Palisota ambigua* was collected in September 2019 in Buea, South-West region of Cameroon. A sample has been identified at the National Herbarium of Cameroon under the voucher number N ° 11044SRFCam.

Extraction and isolation

The aerial part of *P. ambigua* was collected, cut into small pieces, dried, and then ground. The resulting powder (3.5 Kg) was extracted by maceration three times (24 h each time) in 25 L of MeOH. Filtration followed by evaporation under reduced pressure led to 149.5 g of methanolic extract. An amount of 146.6 g of this extract was first suspended in distilled water (600 mL), then extracted with EtOAc (1 L) and *n*-BuOH (650 mL) to yield after evaporation to dryness 45.2 g and 30.9 g, respectively. The EtOAc fraction (40.2 g) was chromatographed using silica gel 60 (0.063–0.200 mm) as stationary phase, eluted with Hexane-EtOAc with increasing polarity (from hexane-EtOAc 10% to EtOAc 100%) then, EtOAc-MeOH with increasing polarity (from EtOAc 100% to EtOAc-MeOH 20%) to afford seven sub-fractions (from A to G). A mass of 25.9 g of the *n*-BuOH fraction was subjected to column chromatography using silica gel 60 (0.063–0.200 mm) as stationary phase and EtOAc-MeOH with the increasing amount of methanol (from EtOAc 100% to EtOAc -MeOH 40%) as eluent to afford five sub-fractions ranging from I to M. The sub-fraction E (1.8 g) was purified using a silica gel column eluted with hexane-EtOAc (30:70) to yield compound **1** (100 mg). The sub-fraction K (2.1 g) was chromatographed on silica gel column using EtOAc as mobile phase to yield compound **2** (150 mg) and compound **3** (15.3 mg). The filtration of sub-fraction I yielded compound **4** (175.6 mg). The sub-fraction F (5.0 g) was chromatographed on a silica gel column eluted with hexane-EtOAc followed by repeated column chromatography on Sephadex LH-20 gel eluted with MeOH to afford compounds **5** (8.1 mg) and **8** (10 mg). The sub-fraction C (3.3 g) was subjected to repeated column chromatography on silica gel using hexane-EtOAc (10:90) to yield compounds **6** (160.3 mg) and **7** (5.4 mg). Successive column chromatography carried out on sub-fraction L (2.0 g) using the binary and ternary systems (EtOAc-MeOH and EtOAc-MeOH-H_20 with increased polarity) led to the isolation of compound **9** (20 mg). The filtration of sub-fraction B (10.4 g) afforded compound **10** (180 mg). The chromatography of the sub-fraction G (6.1 g) on a silica gel column eluted with hexane-EtOAc (40:60) yielded compounds **11** (5.1 mg) and **12** (20.3 mg).

*Acetylation of 20-hydroxyecdysone (**2**)*

Compound **2** (80 mg, 0.166 mmol) was dissolved in pyridine (4 mL), then acetic anhydride (82.7 µL, 0.175 mmol) was added and the mixture was allowed to stir at room temperature [5]. The reaction was monitored by TLC until the disappearance of the substrate (after 4 hr). Distilled water (25 mL) was added to the reaction medium and the resulting mixture was extracted with EtOAc (50 mL). The organic phase was evaporated to dryness under reduced pressure to afford a residue which was purified using silica gel column chromatography eluted with hexane-EtOAc (30 %) to yield **2a** (20.6 mg, 0.034 mmol) characterized as 20-hydroxyecdysone-2,3,22-triacetate.

Antibacterial activity

The antibacterial activity of the methanolic extract, EtOAc and *n*-BuOH fractions as well as some isolated compounds, was assessed by determining the minimum inhibitory concentration (MIC) using the liquid microdilution method [6,7] on two Gram-positive (*Staphylococcus aureus* ATCC 25923 and *Streptococcus pneumoniae* ATCC 49619) and two Gram-negative (*Escherichia coli* ATCC 8739 and *Klebsiella pneumoniae* 109 (clinical isolate)) bacteria. *K. pneumoniae* 109 is a multiresistant clinical isolate to minocycline, nalidixic acid, imipenem, and nitrofurantoine [8]. All these bacteria were taken in the Research Unit of Microbiology and Antimicrobial Substances. The bacterial species were kept at 4 °C and activated on BBL® nutrient agar (NA, Conda, Madrid, Spain) for 24 hr before the antibacterial test. Bacterial suspensions were prepared from 24-hour-old cultures. Two colonies of each bacterium were then collected and separately diluted with sterile

0.9% NaCl solution to turbidity comparable to 0.5 on the McFarland scale, which corresponding to approximately 1.5 x 10^8 CFU/mL. This suspension was again diluted to 1/100, then absorbance was adjusted to 0.1 at 600 nm (spectrophotometer JENWAY 6105). These dilutions, corresponding to a cell's concentration of approximately 10^6 CFU/mL, were used in the antibacterial analyses.

For the determination of MIC values, aqueous solutions of the samples were prepared in 10% aqueous dimethyl sulfoxide (DMSO, Fisher Chemicals, Strasbourg, France) at a concentration of 4096 µg/mL. Serial dilutions of these stock solutions were made in 2 parts in Mueller-Hinton broth (MHB). A sterility test (10%, v/v aqueous DMSO and medium), a negative control (10%, v/v aqueous DMSO, medium and inoculum), and a positive control (10%, v/v aqueous DMSO, medium, inoculum and water-soluble antibiotic) were included for each experiment. 100 µL of each concentration was added to a well of 96-well microtiter plate containing 90 µL of MHB and 10 µL of inoculum and completed to a range of concentration going from 2048 to 0.125 µg/mL (2048 to 8 µg/mL for the extracts and 256 to 0.125 µg/mL for pure compounds). The concentration range for standard reference antibiotic (ciprofloxacin, Sigma Aldrich, Sternheim, Germany) was 256 µg/mL to 0.125 µg/mL. Plates were covered and incubated at 37°C for 24 hr on a shaker (Flow Laboratories) at 300 rpm. At the end of different incubation times, minimum inhibitory concentrations (MICs) were considered as the lowest concentrations of agents at which we did not obtain macroscopic growth due to the turbidity of the well. Minimum bactericidal concentrations were obtained by subculturing 10 µL of the contents of wells where growth was not visible to the naked eye with Mueller-Hinton Agar (MHA) medium. The lowest concentrations that did not cause colonies or appeared only on subculture plates were considered minimal bactericidal concentrations (MBC). Triplicates were performed per test solution and concentration.

Results and discussion

Phytochemical investigation

The phytochemical study of the methanolic extract of *P. ambigua* led to the isolation of twelve known compounds. They were identified either by spectroscopic and spectrometric analysis (1D and 2D NMR) followed by comparison with published data or by measurement of their melting points. These compounds included *N*-benzoyl-$_L$-phenylalanyl-$_L$-phenylalaninol acetate (aurantiamide acetate) (**1**) [9], 20-hydroxyecdysone (**2**) [10], rubrosterone (**3**) [11], *β*-sitosterol 3-*O*-*β*-D-glucopyranoside (**4**) [12], 3*β*-hydroxystigmast-5-en-7-one (**5**) [13], lupeol (**6**) [14], betulinic acid (**7**) [15], bis(2-ethylhexyl) terephthalate (**8**) [16], 2,3-di-*O*-dodecanoyl-*sn*-glycerol-1-*O*-(6-*O*-*α*-D-galactopyranosyl-*β*-D-galactopyranoside (**9**) [17], docosanoic acid (**10**) [18], pallidol (**11**) [19], and apigenin (**12**) [20] (Figure 1). All these secondary metabolites are isolated from *P. ambigua* for the first time. However, 20-hydroxyecdysone (**2**) has already been obtained from *P. hirsuta* and many genera of the Commelinaceae family [2,10]. This compound belongs to the class of ecdysteroids considered as the chemotaxonomic markers of plants of the family Commelinaceae [21]. In addition to 20-hydroxyecdysone (**2**), rubrosterone (**3**) is another ecdysteroid isolated from *P. ambigua* during this work. Furthermore, the presence of secondary metabolites like *β*-sitosterol 3-*O*-*β*-D-glucopyranoside (**4**), 3*β*-hydroxystigmast-5-en-7-one (**5**), docosanoic acid (**10**), and apigenin (**12**) in *P. ambigua* is not surprising since saponins, steroids, fatty acids, and flavonoids were reported in plants of the family Commelinaceae [2,4].

Although phthalates such as bis(2-ethylhexyl) terephthalate (**8**) are known to be used in polymers as plasticizers, there is evidence indicating the presence of these compounds in secondary metabolites of organisms including plants, animals, and microorganisms [22].

*N-Benzoyl-$_L$-phenylalanyl-$_L$-phenylalaninol acetate (**1**):* Yellow powder, HRESI (+): m/z 445.2116 $[M+H]^+$ ($C_{27}H_{28}N_2O_4$); ^{1}H NMR (600 MHz, CD_3OD) *δ* (ppm), *J* (Hz): 7.26 (*m*, H-2, H-6), 7.19 (*m*, H-3, H-5), 7.19 (*m*, H-4), 3.15 (*dd*, *J* = 13.7, 6.8, H-7a), 3.01 (*dd*, *J* =13.7, 8.4, H-7b), 4.79 (*dd*, *J* = 8.3, 6.9, H-8), 7.19 (*m*, H-2' , H-6'), 7.26 (*m*, H-3' , H-5'), 7.11 (*m*, H-4'), 2.87 (*dd*, *J* = 13.8, 6.7, H-7' a), 2.86 (*dd*, *J* = 3.8, 7.9, H-7' b), 4.31 (*m*, H-8'), 3.98 (*dd*, *J* = 11.2, 4.4, H-9' a), 3.91 (*dd*, *J* = 11.2, 6.1, H-9' b), 2.0 (*s*, H-11'), 7.71 (*m*, H-2" , H-6"), 7.44 (*t*, *J* = 7.7, H-3" , H-5"), 7.53 (*dd*, *J* =10.6, 4.3, H-4"); ^{13}C NMR (150 MHz, CD_3OD) *δ* (ppm): 138.6 (C-1), 129.6 (C-2, C-6), 130.5 (C-3, C-5), 129.7 (C-4), 39.1 (C-7), 56.8 (C-8), 173.4 (C-9), 139.1 (C-1'), 127.9 (C-2', C-6'), 130.4 (C-3', C-5'), 127.6 (C-4'), 38.1 (C-7'), 51.3 (C-8'), 66.3 (C-9'), 172.7 (C-10'), 20.7 (C-11'), 135.4 (C-1"), 128.6 (C-2", C-6' '), 127.7 (C-3", C-5"), 133.0 (C-4"), 170.1 (C-7").

*20-Hydroxyecdysone (**2**):* White amorphous powder, HRESI (+): m/z 481.3164 $[M+H]^+$, ($C_{27}H_{44}O_7$); ^{1}H NMR (600 MHz, CD_3OD) *δ* (ppm), *J* (Hz): 1.45 (*m*, H-1a), 1.81 (*m*, H-1b), 3.86 (*ddd*, *J* =12.1, 4.4, 3.1, H-2), 3.97 (*q*, *J* = 3.0, H-3), 1.76 (*m*, H-4a), 1.72(*m*, H-4b), 2.39 (*d*, *J* = 3.9, H-5), 5.82 (*d*, *J* = 2.6, H-7), 3.17 (*m*, H-9), 1.83 (*m*, H-11a), 1.74 (*m*, H-11b), 1.90 (*ddd*, *J* = 12.7, 5.1, 2.2, H-12a), 2.15 (*td*, *J* = 13.1, 4.9, H-12b), 1.97 (*d*, *J* = 5.7, H-15a), 1.61 (*m*, H-15b), 1.82 (*m*, H-16a), 2.00 (*m*, H-16b), 2.41 (*d*, *J* = 3.9, H-17), 0.90 (*s*, H-18), 0.99 (s, H-19), 1.22 (*m*, H-21), 3.34 (*m*, H-22), 1.31 (*m*, H-23a), 1.29 (*m*, H-23b), 1.45 (*m*, H-24a), 1.82 (*m*, H-24b), 1.22 (*m*, H-26), 1.23 (*m*, H-27); ^{13}C NMR (150 MHz, CD_3OD) *δ* (ppm): 35.9 (C-1), 67.3 (C-2), 67.1 (C-3), 31.5 (C-4), 50.4(C-5), 204.5 (C-6), 120.6 (C-7), 166.5 (C-8), 33.7 (C-9), 37.9 (C-10), 20.1 (C-11), 31.1 (C-12), 49.2 (C-13), 83.8 (C-14), 30.4 (C-15), 20.0 (C-16), 49.2 (C-17), 16.7 (C-18), 23.0 (C-19), 76.5 (C-20), 19.6 (C-21), 77.0 (C22), 25.9 (C-23), 41.0 (C-24), 69.9 (C-25), 28.3 (C-26), 27.5 (C-27).

*20-Hydroxyecdysone-2,3,22-triacetate (**2a**):* White amorphous powder, ESIMS (+): *m/z* 652.3 $[M+2Na]^+$; ^{1}H NMR (600 MHz, CD_3OD) *δ* (ppm), *J* (Hz): 1.97 (*d*, *J* = 4.5, H-1a), 1.57 (*m*, H-1b), 5.12 (*dt*, H-2), 5.37 (*dt*, H-3), 1.93 (*m*, H-4a), 1.79 (*m*, H-4b), 2.34 (*dd*, *J* = 13.5, 4.1, H-5), 5.87 (*d*, *J* = 2.6, H-7), 3.25 (*ddd*, *J* = 10.4, 7.2, 2.5, H-9), 1.87 (*m*, H-11a), 1.73 (*m*, H-11b), 2.19 (*m*, H-12a), 1.90 (*m*, H-12b), 2.01 (*m*, H-15a), 1.65 (*m*, H-15b), 2.00 (*m* ; H-16a), 1.83 (*m*, H-16b), 2.41 (*t*, *J* = 9.0, H-17), 0.91 (*s*, H-18), 0.99 (s, H-19), 1.32 (*m*, H-21), 4.90 (*m*, H-22), 1.78 (*m*, H-23a), 1.51 (*m*, H-23b), 1.46 (*m*, H-24a), 1.41 (*m*, H-24b), 1.22 (*m*, H-26), 1.23 (*m*, H-27), 2.13 (s, 2-MeCO), 2.01 (s, 3-MeCO), 2.11 (s, 22-MeCO); ^{13}C NMR (150 MHz, CD_3OD) *δ* (ppm): 33.5 (C-1), 68.7 (C-2), 67.3 (C-3), 28.7 (C-4), 51.1 (C-5), 203.2 (C-6), 120.7 (C-7), 166.2 (C-8), 33.6 (C-9), 37.9 (C-10), 20.2 (C-11), 31.1 (C-12), 47.3 (C-13), 83.7 (C-14), 30.4 (C-15), 19.1 (C-16), 49.6 (C-17), 16.7 (C-18), 22.8 (C-19), 76.1 (C-20), 20.2 (C-21), 79.1 (C22), 24.8 (C-23), 40.2 (C-24), 69.6 (C-25), 28.1 (C-26), 27.5 (C-27), 170.6 (2,3-COO), 203.2 (22-COO), 19.5 (2,3-MeCOO), 19.8 (22-MeCOO).

*Rubrosterone (**3**):* Orange oil, ^{1}H NMR (600 MHz, CD_3OD) *δ* (ppm), *J* (Hz): 1.47 (*dd*, *J* = 13.5, 12.1, H-1a), 1.82 (*dd*, *J* =13.5, 4.5, H-1b), 3.85 (*ddd*, *J* = 12.1, 4.5, 3.1, H-2), 3.97 (*q*, *J* = 3.1, H-3), 1.75 (*dd*, *J* = 5.0, 3.3, H-4a, H-4b), 2.45 (*dd*, *J* = 12.3, 5.1, H-5), 5.92 (*d*, *J* = 2.6, H-7), 3.20 (*m*, H-9), 1.68 (*m*, H-11a), 1.90 (*d*, *J* = 1.2, H-11b), 1.60 (*ddd*, *J* = 12.9, 5.0, 2.1, H-12a), 2.15

(*td*, *J* = 13.1, 4.8, H-12b), 2.31 (*m*, H-15a), 2.04 (*m*, H-15b), 2.53 (*ddd*, *J* = 18.3, 9.4, 1.4, H-,16a), 2.38 (*m*, H-16b), 0.9 (*s*, H-18), 1.01 (*s*, H-19); ^{13}C NMR (150 MHz, CD_3OD) *δ* (ppm): 35.7 (C-1), 67.2 (C-2), 67.0 (C-3), 31.5 (C-4), 50.6 (C-5), 204.5 (C-6), 121.0 (C-7), 163.3 (C-8), 34.4 (C-9), 37.7 (C-10), 19.3 (C-11), 23.6 (C-12), 52.7 (C-13), 79.1 (C-14), 27.1 (C-15), 32.6 (C-16), 218.0 (C-17), 16.2 (C-18), 23.2 (C-19).

β-Sitosterol 3-O-β-D-glucopyranoside (***4***)*:* White amorphous powder, mp = 289-291 ˚C (Lit: 290-292 ˚C [12].

3β-Hydroxystigmast-5-en-7-one (***5***)*:* White oil; ^{1}H NMR (600 MHz, CD_3OD) *δ* (ppm), *J* (Hz): 2.02 (*dt*, *J* = 13.7, 3.5, H-1a), 1.65 (*m*, H-1b), 1.92 (*m*, H-2a), 1.64 (*m,* H-2b), 3.58 (*tt*, *J* = 11.2, 4.6, H-3), 2.42 (*t*, *J* = 2.2, H-4a), 2.49 (*dd*, *J* = 4.9, 2.4, H-4b), 5.67 (*d*, *J* = 1.8, H-6), 2.34 (*d*, *J* = 2.4, H-8), 1.53 (*m*, H-9), 1.67 (*dd*, *J* = 7.0, 2.6, H-11), 2.09 (*dt*, *J* = 12.8, 3.4, H-12a), 1.18 (*m*, H-12-b), 1.34 (*m*, H-14), 1.31 (*m*, H-15), 1.14 (*m*, H-17), 0.75 (*s*, H-18), 1.26 (*s*, H-19), 1.41 (*m*, H-20), 0.98 (*d*, *J* = 6.6 Hz, H-21), 1.42 (*m*, H-22a), 1.09 (*m*, H-22-b), 1.23 (*m*, H-23), 0.99 (*d*, *J* = 6.6, H-24), 1.91 (*m*, H-25), 0.84 – 0.87 (*m* ; H-26, H-27), 1.35 (*m*, H-28), 0.89 (*m*, H-29); ^{13}C NMR (150 MHz, CD_3OD) *δ* (ppm): 36.1 (C-1), 30.5 (C-2), 69.8 (C-3), 41.3 (C-4), 167.6 (C-5), 124.9 (C-6), 203.3 (C-7), 45.2 (C-8), 50.0 (C-9), 38.3 (C-10), 20.8 (C-11), 38.6 (C-12), 42.9 (C-13), 50.0 (C-14), 28.8 (C-15), 45.2 (C-16), 54.6 (C-17), 10.9 (C-18), 16.3 (C-19), 35.9 (C-20), 18.0 (C-21), 33.7 (C-22), 26.1 (C-23), 45.9 (C-24), 30.5 (C-25), 18.8 (C-26), 18.0 (C-27), 22.7 (C-28), 10.9 (C-25).

Lupeol (***6***)*:* White amorphous powder, mp = 210-214 ˚C (Lit: 210-212 °C [14].

Betulinic acid (***7***)*:* White amorphous powder mp = 303-305 ˚C (Lit:301-303 °C [15].

Bis (2-ethylhexyl) terephthalate (***8***): Greenish oil; HRESIMS (+): m/z 391.2846 $[M+H]^+$ ($C_{24}H_{39}O_4$); ^{1}H NMR (600 MHz, CD_3OD) *δ* (ppm), *J* (Hz): 8.14 (*s*, H-3), 4.31 (*dd*, *J* = 5.7, 2.8, H-1'), 1.77 (*m*, H-2'), 1.46 (*m*, H-3'), 1.39 (*m*, H-4'), 1.38 (*m*, H-5'), 0.95 (*t*, H-6'), 1.51 (*m*, H-7'), 1.00 (*t*, H-8'); ^{13}C NMR (150 MHz, CD_3OD) *δ* (ppm): 165.7 (C-1), 134.2 (C-2), 129.2 (C-3), 67.3 (C-1'), 38.9 (C-2'), 30.3 (C-3'), 28.8 (C-4'), 22.6 (C-5'), 22.37 (C-1), 13.0 (C-6'), 23.7 (C-7'), 10.1 (C-8').

2,3-di-O-hexadecanoyl-sn-glycerol-1-O-(6-O-α-D-galactopyranosyl-β-D-galactopyranoside) (***9***): white oil; HRESIMS (+): *m/z* 677.3719 $[M+Na\text{-}C_{16}H_{31}O]^+$, ($C_{47}H_{88}O_{15}$); ^{1}H NMR (600 MHz, CD_3OD) *δ* (ppm), *J* (Hz): 4.45 (*dd*, 12.3, 2.9, H-1a), 4.25 (*dd*, 12.3, 6.7, H-1b), 5.26 (*m*, H-2), 3.96 (*m*, H-3a), 3.75 (*m*, H-3-b), 4.27 (*d*, 7.2, H-1'), 3.53 (*d*, *J* = 2.5, H-2'), 3.51(*d*, *J* = 4.7, H-3'), 3.76 (*m*, H-4'), 3.75 (*m*, H-5'), 3.69 (*d*, *J* = 3.9, H-6' a), 3.92 (*m*, H-6' b), 4.89 (*d*, 4.89, H-1''), 3.80 (*d*, *J* = 3.7, H-2''), 3.53 (*d*, *J* = 2.5, H-3''), 3.75 (*m*, H-4''), 3.87 (*m*, *J* = 6.1, H-5''), 3.73 (*d*, *J* = 2.2, H-6''a, H-6''b), 2.35–1.30 (*m*, 28H, CH_2 alkyl chains), 0.92 (*t*, 6H, CH_3 alkyl chains), ^{13}C NMR (150 MHz, CD_3OD) *δ* (ppm): 62.5 (C-1), 70.4 (C-2), 67.9 (C-3), 104.2 (C-1'), 71.0 (C-2'), 73.3 (C-3'), 70.1 (C-4'), 73.2 (C-5'), 66.4 (C-6'), 99.2 (C-1''), 70.1 (C-2''), 71 (C-3''), 70.1 (C-4''), 71.2 (C-5''), 61.4 (C-6''), 175.8, 175.7 ($2COC_{11}H_{23}$), 33.7 - 22.4 (CH_2 alkyl chains), 13.1(CH_3 alkyl chains).

Docosanoic acid (***10***)*:* White amorphous powder; ^{1}H NMR (600 MHz, $CDCl_3$) *δ* (ppm), *J* (Hz): 2.37 (*t*, 7.5, H-2), 1.66 (*m*, H-3), 1.26 - 1.37 (H-4 - H-20), 1.31 (*m*, H-21), 0.90 (*t*, 7.0, H-22). ^{13}C NMR (150 MHz, $CDCl_3$) *δ* (ppm): 179.2 (C-1), 33.8 (C-2), 24.7 (C-3).

Pallidol (***11***) *:* White oil; ^{1}H NMR (600 MHz, CD_3OD) *δ* (ppm), *J* (Hz): 3.74 (s, H-6, H-12), 4.48 (s, H-5, H-11), 6.12 (d, J=2.1, H-l, H-7), 6.54 (*d*, *J* = 2.0, H-3, H-9), 6.67 (d, J = 8.5, H-3', H-5', H-3', H-5''), 6.90 (*d, J* = 8.5, H-2', H-6', H-2'', H-6''); ^{13}C NMR (150 MHz, CD_3OD) *δ* (ppm): 101.1 (C-1, C-7), 154.1(C-2, C-8), 101.9 (C-3, C-9), 157.9 (C-4, C-10), 122.4 (C-4a, C-10a), 53.3(C-5, C-11), 59.4 (C-6, C-12), 149.5 (C-6a, C-12a), 137.1(C-1', C-1''), 127.8 (C-2', C-6', C-2'', C-6''), 114.5 (C-3', C-5', C-3'', C-5''), 154.6 (C-4', C-4'').

Apigenin (***12***)*:* White amorphous powder; ^{1}H NMR (600 MHz, CD_3OD) *δ* (ppm), *J* (Hz): 6.89 (*s*, H-3), 6.46 (*d*, *J* = 2.2, H-6), 6.87 (*d*, J = 2.2, H-8), 7.97 (*d*, *J* = 8.8, H-2', H-6'), 6.97 (*d*, *J* = 8.8, H-3', H-5') ; ^{13}C NMR (150 MHz, CD_3OD) *δ* (ppm): 164.8 (C-2), 105.8 (C-3), 182.5 (C-4), 161.6 (C-5), 99.9 (C-6), 163.9 (C-7), 95.6 (C-8), 157.4 (C-9), 105.7 (C-10), 121.4 (C-1'), 129.1 (C-2', C-6'), 116.5 (C-3', C-5'), 161.9 (C-4).

Antibacterial activity

The antibacterial activity of the extract, fractions, and isolated compounds was performed, and the results are presented in Table 1. All the samples displayed antibacterial activity against the tested bacteria. According to antimicrobial cut-off points of the plant extracts and pure compounds [23, 24], the methanolic extract showed excellent activity against *Staphylococcus aureus* ATCC 29213 (MIC = 32 µg/mL) whereas the EtOAc fraction displayed good activity (128 ≤ MIC ≥ 256 µg/mL) against all the microorganisms tested. The *n*-BuOH fraction exhibited very good activity (MIC = 128 µg/mL) against *Klebsiella pneumoniae* 109 and excellent activity against *Escherichia coli* ATCC 8739 (MIC = 32 µg/mL), *Streptococcus pneumoniae* ATCC 49619 (MIC = 64 µg/mL), *Staphylococcus aureus* (29213) (MIC = 64 µg/mL). Concerning the isolated compounds, 2,3-di-*O*-dodecanoyl-*sn*-glycerol-1-*O*-(6-*O*-*α*-D-galactopyranosyl-*β*-D-galactopyranoside (**9**) was averagely active against *Klebsiella pneumoniae* 109 (MIC = 64 µg/mL), while aurantiamide acetate (**1**) and pallidol (**11**) exhibited good activity against *Escherichia coli* ATCC 8739 (MIC = 32 µg/mL). Rubrosterone (**3**) and betulinic acid (**7**) showed average activity against *Streptococcus pneumoniae* ATCC 49619 (MIC = 64 µg/mL). The acetylation of 20-hydroxyecdysone (**2**) relatively increased the antimicrobial activity (MIC from 128 to 32 µg/mL) against *Klebsiella pneumoniae* 109 and *Staphylococcus aureus* ATCC 29213. The presence of acetyl groups in this compound did not influence the growth of *Streptococcus pneumoniae* ATCC 49619 and decreased the activity against *Escherichia coli* ATCC 8739. However, according to the results obtained by Shirshova and co-workers, 20-hydroxyecdysone 2,3,22-triacetate (**2a**) completely inhibited the growth of *Staphylococcus aureus* and *Escherichia coli* isolates [5]. This difference in activity could be explained by the genetic differences between the microorganisms. To the best of our knowledge rubrosterone (**3**) has not yet been reported for its antibacterial activity. The ratio MBC/MIC allows us to conclude that most of the test samples had bactericidal effect [25-27] depending on the sensitive bacteria. The isolated compounds could be responsible of the antibacterial activity of the methanolic extract of *P. ambigua*, a medicinal plant traditionally used to cure diarrhea caused by the tested bacteria.

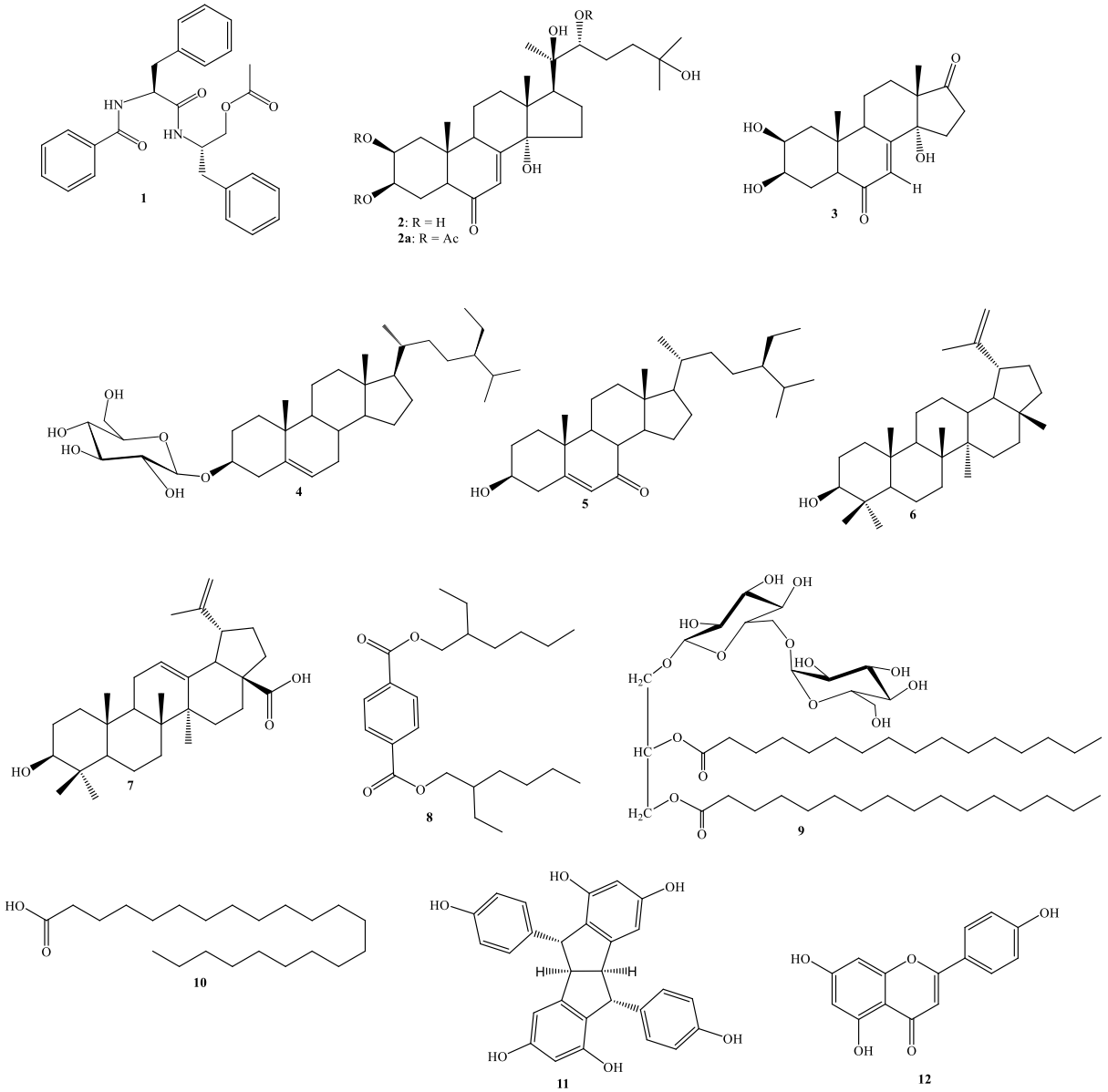

Figure 1. Structures of the isolated compounds from the aerial part of *P. ambigua*
1: Aurantiamide acetate, **2**: 20-hydroxyecdysone, **2a**: 20-hydroxyecdysone 2,3,22-triacetate, **3**: Rubrosterone, **4**: *β*-sitosterol 3-*O*-*β*-D-glucopyranoside, **5**: 3*β*-hydroxystigmast-5-en-7-one, **6**: Lupeol, **7**: Betulinic acid, **8**: Bis(2-ethylhexyl) terephthalate, **9**: 2,3-di-*O*-dodecanoyl-*sn*-glycerol 1-*O*-(6-*O*-*α*-D-galactopyranosyl)-*β*-D-galactopyranoside, **10**: Docosanoic acid, **11**: Pallidol, **12**: Apigenin

Table 1. Antibacterial activity of extract, fractions, and some isolated compounds from *P. ambigua*

Samples	Parameters (µg/mL)	Bacteria			
		Kp	*Ec*	*Sa*	*Sp*
MeOH extract	MIC	512	512	32	512
	MBC	1024	1024	64	1024
	MBC/MIC	2	2	2	2
EtOAc fraction	MIC	256	128	256	256
	MBC	512	256	512	512
	MBC/MIC	2	2	2	2
n-BuOH fraction	MIC	128	32	64	64
	MBC	256	64	128	128
	MBC/MIC	2	2	2	2
1	MIC	128	32	128	128
	MBC	-	64	-	-
	MBC/MIC	-	2	-	-
2	MIC	128	64	128	128
	MBC	-	128	-	-
	MBC/MIC	-	2	-	-
2a	MIC	32	128	32	128
	MBC	64	-	64	-
	MBC/MIC	2	-	2	-
3	MIC	128	128	32	64
	MBC	-	-	32	64
	MBC/MIC	-	-	1	1
5	MIC	128	64	128	128
	MBC	-	128	-	-
	MBC/MIC	-	2	-	-
6	MIC	128	64	128	128
	MBC	-	128	-	-
	MBC/MIC	-	2	-	-
7	MIC	128	64	32	64
	MBC	-	128	64	128
	MBC/MIC	-	2	2	2
8	MIC	128	128	64	128
	MBC	-	-	64	-
	MBC/MIC	-	-	1	-
9	MIC	64	64	64	128
	MBC	64	64	64	-
	MBC/MIC	1	1	1	-
10	MIC	128	64	64	128
	MBC	-	128	64	-
	MBC/MIC	-	2	1	-
11	MIC	128	32	64	128
	MBC	-	64	64	-
	MBC/MIC	-	2	1	-
12	MIC	128	128	64	128
	MBC	-	-	128	-
	MBC/MIC	-	-	2	-
Ciprofloxacin	MIC	16	8	8	16
	MBC	16	16	32	16
	MBC/MIC	1	2	4	1

Kp = *Klebsiella pneumoniae* 109, *Ec* = *Escherichia coli* ATCC 8739, *Sa* = *Staphylococcus aureus* ATCC 29213, *Sp* = *Streptococcus pneumoniae* ATCC 49619, - = >256 µg/mL, MIC = Minimum Inhibitory Concentration, MBC = Minimum Bactericidal Concentration, MeOH = Methanol, EtOAc = Ethyl acetate, *n*-BuOH = *n*-butanol, **1** = Aurantiamide acetate, **2** = 20-hydroxyecdysone, **2a** = 20-hydroxyecdysone 2,3,22-triacetate, **3** = Rubrosterone, **5** = 3β-hydroxystigmast-5-en-7-one, **6** = Lupeol, **7** = Betulinic acid, **8** = Bis(2-ethylhexyl) terephthalate, **9** = 2,3-di-*O*-dodecanoyl-sn-glycerol 1-*O*-(6-*O*-α-D-galactopyranosyl)-β-D-galactopyranoside, **10** = Docosanoic acid, **11** = Pallidol, **12** = Apigenin.

Conclusion

In this study, the chemical investigation of *P. ambigua* led to the isolation and characterization of aurantiamide acetate (**1**), 20-hydroxyecdysone (**2**), rubrosterone (**3**), *β*-sitosterol 3-*O*-*β*-D-glucopyranoside (**4**), 3*β*-hydroxystigmast-5-en-7-one (**5**), lupeol (**6**), betulinic acid (**7**), bis(2-ethylhexyl) terephthalate (**8**), 2,3-di-*O*-dodecanoyl-*sn*-glycerol 1-*O*-(6-*O*-*α*-D-galactopyranosyl)-*β*-D-galactopyranoside (**9**), docosanoic acid (**10**), pallidol (**11**), and apigenin (**12**). The methanolic extract, the EtOAc and *n*-BuOH fractions from the aerial parts of *P. ambigua* exhibited excellent to good antibacterial activity whereas the isolates displayed antibacterial activity varying from weak to good against the tested pathogenic bacteria; supporting the traditional use of *P. ambigua* to cure diarrhea caused by the tested bacteria.

Abbreviations

^{13}C-NMR: Carbon Thirteen Nuclear Magnetic Resonance; ^{1}H-NMR: Proton Nuclear Magnetic Resonance; 2D NMR: Two-dimension Nuclear Magnetic Resonance; ATCC: American Type Culture Collection; CC: Column Chromatography; DMSO: Dimethylsulfoxide; EtOAc: Ethyl acetate; MDR: Multi-Drug-Resistant; MeOH: Methanol; MHA: Mueller Hinton agar; MHB: Mueller Hinton broth; MIC: Minimum inhibitory concentration; MMC: Minimum Microbicidal Concentration; NA: Nutrient agar; IRAD: Institut de Recherche Agricole et de Développement; SDB: Sabouraud Dextrose Broth; TLC: Thin Layer Chromatography; $CDCl_3$: Deuterated chloroform; UV: Ultra-violet.

Authors' Contribution

SLKT, BTT, GMT and BT contributed to the investigation, methodology, and writing of the original draft. BKP helped in the structure elucidation. LAT, RBT and JDT supervised and revised the manuscript critically. All the authors read and approved the final manuscript.

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Conflict of interest

The authors declare no conflict of interest.

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