Isolation and characterization of the alkaloid Nitidine responsible for the traditional use of *Phyllanthus muellerianus* (Kuntze) Excell stem bark against bacterial infections

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Abstract

Phyllanthus muellerianus (Kuntze) Excell (family *Euphorbiaceae*) stem bark methanol extract inhibited the growth of *Clostridium sporogenes* and *Streptococcus pyogenes*, responsible for gas gangrene and suppurative and non suppurative diseases, respectively. After the HPLC fingerprint acquisition a bioguided fractionation of the defatted methanol extract allowed the isolation of six fractions whose activity was evaluated against the two pathogen bacteria. A further purification of the most active fraction afforded a pure compound responsible for the very interesting inhibitory activity against *Clostridium sporogenes* and *Streptococcus pyogenes* (MIC 0.91µM, MIC 3.64µM). ^{1}H-NMR and MS analytical techniques allowed the identification of the bioactive specie as the quaternary ammonium alkaloid Nitidine, whose presence in the genus *Phyllanthus* is here observed for the first time. A study on the counter ion of this quaternary ammonium alkaloid, performed using energy dispersive spectroscopy (EDS) coupled with scanning electron microscopy (SEM) was also carried out.

Keywords: *Phyllanthus muellerianus, Clostridium sporogenes, Streptococcus pyogenes,* bioguided fractionation, Nitidine.

1. Introduction

The species of the genus *Phyllanthus (Euphorbiaceae)* are widely distributed in most tropical and subtropical areas and have long been used in folk medicine to treat several diseases [1]. For example, the decoction of *Phyllanthus muellerianus* stem bark is used in Cameroon by Pygmies Baka as a remedy for tetanus [2]. This traditional use has been validated in our previous study [3] where the interesting activity of *Phyllanthus muellerianus* stem bark methanol extract against *Clostridium sporogenes* ATCC 3584 and *Streptococcus pyogenes* ATCC 19615 is described. More recently the essential oil, obtained from the same stem bark and fully characterized, was found to have an interesting activity against the same bacteria, suggesting that this activity could be rationale for the use of this plant in pygmies traditional medicine, mainly for the treatment of tetanus and wound infections [4].

To complete the research on this plant, here we report a bio-guided fractionation of the active methanol extract with the final aim to isolate the compound/s responsible for the biological activity described in our previous publications. Particularly, we described the purification procedures that allowed to isolate the bioactive compound and the analytical techniques needed to identify it as the known quaternary ammonium alkaloid Nitidine (Figure 1), never found before in the genus *Phyllanthus*.

Nitidine salts were firstly isolated in 1959 from *Zanthoxylum nitidum* (Roxb.) (Rutaceae) [5] by melting point and elemental analyses and identified as acetate, chloride, iodide, ψ-Cyanide by melting point and elemental analyses; later Nitidine

nitrate and Nitidine chloride were identified by means of TLC and UV and IR spectroscopies in *Zanthoxylum Myriacanthum* [6] and *Zanthoxylum parvifoliolum* [7], respectively. In 1995 Nitidine was isolated from *Toddalia asiatica* [8] and in 2007 from *Broussonetia papyrifera* [9] fruits and identified by comparison of its chromatographic and spectroscopic properties (MS, IR, NMR) to those of an authentic sample but no data are available concerning the nature of the counter ion.

Here we reported for the first time, the isolation and identification of Nitidine, by means of HPLC, LC-MS and NMR analytical techniques, in *Phyllanthus muellerianus*, ever observed in any plant of the genus *Phyllanthus* before, , and a study on the counter ion of this quaternary ammonium alkaloid, performed using energy dispersive spectroscopy (EDS) coupled with scanning electron microscopy (SEM). Moreover, although Nitidine is already known for its biological activities, such as regulation of inflammatory diseases [10], antimalarial [11], anticancer [12], antioxidant, antimicrobial and bacteriostatic [13-15], its activity against *Clostridium* bacteria is here described for the first time.

2. Experimental

2.1. Plant Material

The stem bark of *Phyllanthus muellerianus* was collected in Cameroon in July 2009 in the camps of Abing. The plant was identified at the National Herbarium of Yaoundé by the Cameroonian botanist Mr Nana. A voucher specimen (no. BWPV03) is deposited at the Department of Drug Sciences of the University of Pavia. The bark was dried for 15 days in a dark and ventilate room at 25-30° C, then grounded and the powder (780g) stored at -20° C.

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78 *2.2 Bioassay-guided fractionation, isolation and identification of active compound*

79 *2.2.1 Plant extraction and HPLC fingerprint analysis*

80 The stem bark dried powder (128 g) was suspended in n-Hexane (500 ml) in a round
81 bottom flask equipped with a condenser. The mixture was sonicated for 10 minutes,
82 then refluxed for 2 h, filtered, re-suspended in fresh n-Hexane (500 ml) and refluxed for
83 further 2 h. After filtration, the filtrate was evaporated to dryness under *vacuum*. The
84 procedure was repeated up to a constant weight of the dry extract (yield 0.8 % on a dry
85 mass basis). Further extractions on the same plant material were carried out with
86 CH_2Cl_2 and MeOH following the same procedure described above. The solvent removal
87 afforded 0.6% and 3.8% of dried extracts on a dry mass basis, respectively. HPLC was
88 used to analyse the phytochemical profile of the extracts. Fingerprint analyses were
89 carried out on a HP 1100 series system Agilent Technologies) equipped with a manual
90 injector, a 20 µl sample loop and a quaternary gradient pump along with pulse
91 dampener, using a Chromsystems C18 column (Chromsystems) (5 µm, 250 x 4.6 mm
92 i.d.). The mobile phase was a combination of solvent A (0.08% TFA in water) and
93 solvent B (0.08% TFA in ACN) . The gradient program was: 0-55 min linear gradient to
94 100% of solvent B; 5 min, hold at 100% solvent B. The injection volume was 10 µl.
95 The elution flow rate was 1 ml/min and the detection wavelength was set at 225 nm.
96 A Biotage KP-C18 scaling column (55 µm, 250 x 4.6 mm i.d.) was used for
97 development, in order to obtain a good separation on the next preparative steps. Solvent
98 systems as for the analytical column; flow rate: 3 ml/min and the detection wavelength
99 was set at 225 nm and 366 nm.

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2.2.2 Fractionation of the defatted MeOH extract

The defatted methanol extract (DME, 2g) was subjected to flash chromatography using a Biotage Isolera Prime system (Snap 120g KP-C18-HS cartridge). A stepwise gradient H_2O-CH_3CN with 0.08% of TFA was used. Gradient elution: 95/5→90/10 in 2 CV (column volume= 150 ml, each achieved in 3.75 min), 90/10→88/12 in 3 CV, 88/12 in 5 CV, 88/12→85/15 in 15 CV, 85/15→72/28 in 13 CV, 72/28 in 10 CV, 72/28→50/50 in 5 CV, 50/50→30/70 in 5 CV, 30/70→100 in 7 CV, at a flow rate of 40 ml/min. The elution was monitored at 225 and 366 nm. Six fractions were collected, namely F1, F2, F3, F4, F5 and F6.

2.2.3 Fractionation of F4

Based on the biological activities, F4 (0.013 g) was further chromatographed on a discovery reversed-phase SPE cartridge (2g/12ml, Supelco). Separation was carried out by stepwise elution H_2O-CH_3CN, with 0.08% of TFA. Gradient elution: 90/10 for 4 Column Volume (CV = 6 ml,), 80/20 for 4 CV, 75/25 for 4 CV, 70/30 for 4 CV, 100% CH_3CN for 4 CV, at a flow rate of 3 ml/min. Fractions obtained using 20% and 25% of CH_3CN (test tubes 2-17), after solvent evaporation, afforded bright yellow needle-shaped crystals with the same HPLC profile. Thus, the crystals were collected yielding 10 mg (76,9%).

2.4. Chemical characterization of crystals

The chemical characterization of crystals was achieved by NMR, MS analyses and energy dispersive spectroscopy EDS coupled with scanning electron microscopy SEM.

^{1}H and ^{13}C NMR were recorded on a Varian (Palo Alto, CA, USA) Inova 500 spectrometer (499.87 and 125.70 MHz, respectively) in CD_3OD with TMS as internal standard. Complete assignment was performed on the basis of 2D experiments. MS data were obtained using a Thermo Finnigan LTQ-DECA (San Jose, CA; USA) ion trap mass spectrometer equipped with a Finnigan electrospray interface over the mass range from m/z 250 to 1500. Thermo Finnigan Xcalibur software (version 1.4) was used for data acquisition. The mass spectrometer was operated in the positive ion mode. The samples were dissolved in H_2O:CH_3CN (50:50) and introduced into the ESI source by continuous infusion at a rate of 5 µl/min by a syringe pump. The capillary voltage and temperature were set to 30 V and 250 °C respectively. Nitrogen was used as the sheath gas.

Concerning SEM and EDS analyses, small amounts of the dried samples were placed on a bi-adhesive carbon slide, fixed on the aluminum sample-holder. The typical gold-coating for SEM preparations was avoided in order to allow the quantitative determination of the elementary composition of the samples. The SEM analysis was performed using an EvoMA10 microscope from Zeiss. The experiments were carried out using a LaB_6 filament, with the tension and the current of the beam set to 20 kV and 30 pA respectively. EDS measurements were performed using an INCA Energy 350 X Max detector from Oxford Instruments, equipped with a Be window. Cobalt standard was used for the calibration of the quantitative elementary analysis. Several determinations were made for each sample at different points in the powders grains.

147 *2.3. Evaluation of the antimicrobial activity*

148 The following strains were used for testing the antimicrobial activity of the crude
149 extracts: *Clostridium sporogenes* ATCC 3584 and *Streptococcus pyogenes* ATCC
150 19615. Bacteria were cultured in Tryptone Soya Broth (TSB, Oxoid, Basingstoke, UK)
151 at 37 °C, under anaerobic atmosphere (80% N_2, 15% CO_2 and 5% H_2) in an anaerobic
152 jar (Oxoid, Basingstoke, UK) for *Clostridium sporogenes* [3].
153
154
155 *2.4. Evaluation of the minimum inhibitory concentration (MIC) and the minimum*
156 *bactericidal concentration (MBC).*
157 Solutions of *P. muellerianus* extracts were obtained in distilled water and filtered
158 through Millex GP membrane (0.22 μm; Millipore Corp., Billerica, MA). The
159 antimicrobial activity of the extracts was determined with the macrodilution broth
160 method, according to Clinical and Laboratory Standards Institute [16], with some
161 modifications reported in this paragraph. The desired concentration was achieved
162 through the addition of appropriate *P. muellerianus* extract volume to 1 ml of Iso-
163 Sensitest broth (ISB, Oxoid) in 15X 100 mm test tubes. Bacterial suspensions were
164 added to the test tubes to bring inoculum size to 10^7 – 10^8 colony-forming units
165 (CFU)/ml. The minimum inhibitory concentration (MIC) was evaluated after a 24 h
166 incubation at 37 °C, as the lowest concentration that completely inhibited the formation
167 of visible microbial growth. Control test tubes containing broth without plant extracts
168 for each organism tested were used (negative control). Even bactericidal activity was
169 determined by macrodilution method. Minimum bactericidal concentration (MBC) was
170 evaluated by inoculating aliquots of culture medium in which the inhibition of bacterial

proliferation was observed. MBC was the lowest concentration capable of killing the microbial cells [17]. Incubating temperature was 37° C. All the experiments were performed in triplicate and bacteria-free broth was included as culture control. The *P. muellerianus* extracts were tested in the concentrations range 10–2000 µg/ml. Stock standard solution of ampicillin was used as positive control.

2.5 Cell toxicity: MTS assay

In order to complete the study on the above mentioned Nitidine salts , two cell toxicity tests were performed. Two tumor cell lines (A549, lung cancer; CaKi-2, kidney carcinoma) and one endothelial cell line (HUVEC) were used. A cell viability test was performed to assess the effect of Nitidine on cell growth. Two tumor cell lines (A549, lung cancer; CaKi-2, kidney carcinoma) and one endothelial cell line (HUVEC) were used[G1]. The tumor cells were grown at 37°C in a humidified atmosphere added of 5% CO_2. When at confluence, the cells were split and counted by a Burker chamber using trypan blue as a dye (trypan blue exclusion test). After a proper dilution, cells were plated in a 96-well flat-bottom microplate at a density of $3x10^3$ cells in 100 µl of growth medium. After 12 hours, growth medium was replaced by the PBS or Nitidine containing medium at 5, 10, 25, 50 µM concentration. After 18 h incubation the medium was replaced and 20 µl of MTS reagent (Promega) were added to each well. After 2 h of incubation the absorbance was measured at 490 nm wavelength using a microplate reader. Five wells for each experimental point were used and each experiment was performed at least twice.

2.6 Cell toxicity: LDH assay

194 A Lactate dehydrogenase (LDH) release test was performed to evaluate whether the
195 Nitidine induced decrease of cell viability was due to a direct cytotoxic effect. The cells
196 were treated as described above and, at the end of the Nitidine treatment, the medium
197 was used for the colorimetric LDH activity assay (Promega). Five wells for each
198 experimental point were used and each experiment was performed at least twice.
199
200 **3. Results and discussion**
201 As reported in our recent work [3], methanol and defatted methanol extracts showed a
202 good bacteriostatic activity against *C. sporogenes and S. pyogenes.* These results allow
203 to assume a similar effect on other Clostridia species, such as *C. tetani* and *C.*
204 *perfrigens*. Even if both extracts showed significant antibacterial activity, only the
205 defatted methanol extract was further chemically investigated, due to its higher
206 solubility in water. After the HPLC fingerprinting f, (Figure 2, defatted MeOH extract),
207 DME was purified by reverse phase flash chromatography in order to isolate and
208 identify the active components. The experimental conditions were optimized using a
209 Biotage KP-C18-HS scaling column, then the method was efficiently transferred to a
210 flash chromatography Biotage system. Six fractions (F1-F6) were collected (Figure 2),
211 and their biological properties compared to the whole DME. F4 and F5 exhibited the
212 most significant antimicrobial activity against *C. sporogenes* and *S. pyogens* with MIC
213 values of 25 µg/ml and 50 µg/ml (F4), and 37.2 µg/ml and 56 µg/ml (F5), respectively
214 (Table 1). Although F4 and F5 showed a comparable activity, the most abundant F4
215 fraction was further purified by SPE (see Experimental section) affording bright yellow
216 crystals.

217 *3.1 Identification of the bioactive compound and study on the counter ion*

218 The crystals were analysed by direct infusion electrospray ionization mass spectrometry
219 (ESI-MS). ESI-MS total ion current trace (TIC), in the positive ion mode, revealed the
220 presence of a single ion signal at m/z 348 (Figure 3). The isolated compound was
221 unambiguously identified by 1D and 2D NMR experiments as Nitidine, a
222 benzophenanthridine quaternary ammonium alkaloid naturally occurring in *Rutaceae*
223 species, especially in the genus *Zanthoxylum,* but never found before either in the genus
224 *Phyllanthus* and in the *Euphorbiaceae* species. NMR spectral data were in accordance
225 with literature data [18] as well as with MS^2 spectra [19].
226 Since Nitidine chloride is very likely the natural product , the TFA used in the
227 bioguided fractionation procedure induced an ion exchange displacement giving rise to
228 Nitidine trifluoroacetate. Thus, the sample was analysed by elemental analysis through
229 EDS-SEM in order to confirm this hypothesis and asses the nature of the counter ion.
230 As expected, a massive amount of fluorine was detected (Figure 4).
231 All available literature data [20-22] on the characterization of Nitidine chloride report
232 the isolation of the quaternary ammonium alkaloid by treatment with HCl thus the
233 counter ion is of course chloride, but to our knowledge there is no scientific evidence
234 concerning the natural counter ion.
235 Several attempts have been carried out, both in direct and reverse phase
236 chromatography, to obtain pure Nitidine avoiding the ion exchange displacement. In
237 the absence of bases (direct phase) and acid (reverse phase), no purification occurred;
238 on the other hand, with bases and acids, the natural counter ion is always displaced;
239 even the weaker formic acid, used instead of TFA, led to Nitidine formate, confirming
240 that the relative acid proportion in the mobile phase is so large that it will displace the

241 counter ion, even if a small amount of chlorine could be detected in the EDS-SEM
242 analysis (data not shown).
243 Chlorine was detected also in the EDS-SEM of Nitidine sample obtained by gel
244 filtration on Sephadex LH-20 [20] with 100% of MeOH as mobile phase but once again
245 the amount was lower than expected. However, it is to note that no other elements able
246 to act as counter ions were identified in this sample by EDS.
247 Thus, Nitidine chloride was purchased from Sigma Aldrich, when became commercially
248 available, and the EDS-SEM carried out on the commercial sample showed an higher
249 amount of chlorine (9%) compared to all the experimental samples.
250 The presence of chlorine only in the sample obtained by purification processes where no
251 acids and bases (gel filtration) or weak acid were used and the absence of other possible
252 counter ions, may support the hypothesis that nature produces Nitidine chloride;
253 however, the very poor amount of chloride anion detected in our sample, compared to
254 those in the standard, does not allow to clearly identify the natural counter ion (Figure
255 5).
256 On the other hand, all the quaternary ammonium alkaloids showed good antibacterial
257 activity even if only Nitidine chloride presented MIC values comparable to ampicillin
258 and resulted more active (0.91 μM) than control against *C. sporogenes*, as reported in
259 Table 2. Nitidine formate and TFA presented MIC values higher than ampicillin, with
260 consequently lower antimicrobial activity.
261
262 3.2 *Cell toxicity: MTS and LDH assays*
263 In order to investigate the biological properties of Nitidine salts isolated from
264 *Phyllanthus muellerianus,* two classical cell toxicity tests were performed, MTS and

LDH assays. Although the anticancer activity of Nitidine chloride is well documented in literature, no information are available on the side effects related to the traditional use of *Phyllanthus muellerianus;* moreover, d the biological activity of Nitidine TFA and formate on mammalian cells is unknown. Cancer and endothelial cell lines were selected to ascertain whether a general toxic effect towards all cell types or a specific anticancer activity could be observed.

All Nitidine salts, evaluated for their effect on cell growth, as described in the experimental section, showed a dose-dependent inhibition of cell viability in cancer cell lines while HUVEC were not affected by the treatment. These results are in agreement with literature data showing that Nitidine prevents cancer cells growth through the inhibition of transduction pathways like Src/FAK or AKT pathways [23]. When LDH release assays were performed, Nitidine TFA displayed a cytotoxic effect in A549 cells at 10 μM concentration while HUVEC were not affected by the treatment. Nitidine formate showed a cytotoxic effect only at 25 μM concentration.

The effect of Nitidine chloride on cell viability was not assessed since its inhibitory effects on cell growth are well documented in literature. Previous works reported that Nitidine chloride inhibitory effect on cell growth may be ascribed to the inhibitory effects on nuclear enzymes or intracellular pathways rather than to an aspecific cytotoxic effect leading to membrane rupture [23, 24]; our data suggest a similar mechanism for nitidine formate.

It is worthwhile to underline that the LDH release induced by Nitidine formate is observed at higher concentration (24 μM) compared to MIC (4,25 and 8,51 μM). On the other hand, Nitidine TFA induces LDH release at 10 μM, that is comparable with the

288 relative Nitidine TFA MIC values. We suggest that this might be ascribed to an
289 aspecific toxic effect due to TFA.
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292
293 **Conclusion**
294 The stem bark of PM showed an interesting activity against *C. sporogenes* and *S.*
295 *pyogenes* which supports the claimed traditional use. The bioguided fractionation of the
296 most active methanol extract allowed firstly the isolation of fractions responsible for the
297 activity and finally the isolation of a pure compound with antimicrobial activity. This
298 compound was unambiguously identified as the quaternary ammonium alkaloid Nitidine
299 by means of LC-MS and NMR analyses but all the experiments aimed to the elucidation
300 of the identity of natural counter ion, failed. However, results demonstrated that the
301 counter ion influences the biological activity only from the quantitative point of view
302 since all the salts showed an evident activity against the same bacteria with a greater
303 effect of the chloride salt.
304 Although Nitidine has been widely reported in literature, to the best of our knowledge it
305 has never been isolated and identified from *Phyllanthus* genus and Euphorbiaceae
306 family. Furthermore its antimicrobial activity against *Clostridia* is here reported for the
307 first time.
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309
310
311 **Acknowledgements**

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composition having bacteriostatic and emollient effect, and detergent and skin-care product prepared from the composition Assignee: Liuzhou LMZ Co., Ltd., Peop. Rep. China; LMZ (Yangzhou) Hotel Supplies Co., Ltd.; Anhui Liangmianzhen Fangcao Daily Chemical Co., Ltd.

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