View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by AJOL - African Journals Online

FRIEDELIN: A BACTERIAL RESISTANCE MODULATOR FROM PAULLINIA PINNATA L.

K. Annan[1], F. Adu[2] and S.Y. Gbedema[2]
[1]Department of Pharmacognosy,
Kwame Nkrumah University of Science and Technology, Kumasi, Ghana
[2]Department of Pharmaceutics,
Kwame Nkrumah University of Science and Technology, Kumasi, Ghana

ABSTRACT
As part of the project to identify plant natural products which modulate bacterial multidrug resistance (MDR), bioassay-guided isolation of the methanol extract of Paullinia pinnata L. roots yielded four known compounds namely friedelin, β-sitosterol, β-sitosterol acetate and β-sitosterol D-glucoside. The structures were established by spectroscopic methods and comparison with published data. These compounds were tested for in vitro antibacterial and resistance modifying activities against strains of Staphylococcus aureus; SA1199B, RN4220 and XU212 possessing the Tet(K), Msr (A), and Nor(A) multidrug resistance efflux mechanisms respectively. At 10µg/ml, none of the compounds displayed any antibacterial action but in combination with tetracycline, erythromycin and norfloxacin, friedelin displayed a 2-fold, 4-fold and 16-fold potentiation of activities of these antibiotics against XU212, SA1199B and RN4220 possessing the Tet(K) [tetracycline resistant], Nor(A) [norfloxacin resistant] and Msr(A) [macrolide resistant] transporters respectively.

Keywords: *Paullinia pinnata*, resistance modulators, friedelin, *Staphyloccocus aureus.*

INTRODUCTION
The effectiveness of the arsenal of antibiotics now in use, including methicillin, is decreasing in the face of emerging bacterial resistance and has been the subject of various reviews and research. During the last two decades, new infectious diseases have appeared e.g Ebola and Buruli ulcer, and some old ones previously thought to be controlled e.g gonorrhoea, have re-emerged (Casell and Mekalonos, 2001). Furthermore, the usefulness of existing antimicrobial agents is rapidly fading, tipping the balance in favour of multidrug resistant pathogens, including MRSA, and there appears to be few, if any, new classes of drugs currently in use to fight against the multidrug resistant pathogens. Multidrug-resistance (MDR) exhibited by many bacterial species is a major problem in treating both hospital and community acquired infections. Many strains of MRSA possess efflux pumps such as the specific TetK and MsrA transporters which export or extrude certain tetracyclines and macrolides, and the

multidrug resistance proteins NorA and QacA which confer resistance to a wide range of structurally unrelated antibiotics (Koyama, 2006). The inhibition of efflux pumps or mechanism in a bacterial cell could therefore be a means of reversing resistance.

A bacterial resistance modifying agent therefore, reduces the minimum inhibitory concentration (MIC) for an antibiotic to which resistance has already occurred. This could be of great benefit in combinatory therapy, perhaps facilitating the reintroduction of antibiotics that are no longer effective due to resistance (Gibbons, 2005). An example of synergistic combination in commercial antibacterial chemotherapy in the treatment of various infections is Augmentin^{TM}, comprising β-lactamase inhibitor, clavulanic acid with amoxicillin. As part of an ongoing study, the antibacterial activity of *Paullinia pinnata* L. against three resistant strains of *Staphylococcus aureus*; *SA1199B, RN4220, XU212* had been assessed by Annan *et al*. (2005). We here report the effect of the isolates from the methanolic extract on the resistant *Staphylococcus* strains and also in combination with standard antibiotics tetracycline, erythromycin and norfloxacin respectively. These were tested against the bacteria strains to which they are resistant.

MATERIALS AND METHODS

Plant materials

Plant materials were collected in June 2005 and authenticated at the Centre for Scientific Research into Plant Medicine (CSRPM), Akwapim-Mampong, Ghana, where voucher specimen 13/05/013 has been deposited.

Preparation of extracts

50 gm of the dried powdered roots was packed into a cellulose thimble (28x100 mm) and soxhlet -extracted with 400ml methanol (Sigma) over 48 hours until the material was exhausted. The extract was concentrated under reduced pressure using the rotary evaporator and dried over nitrogen to give a yield of 3.21gm.

Isolation of compounds

Column chromatography was used to isolate compounds from the active chloroform fraction. 2.0gm of material was packed over 150g silica gel [(0.040-0.060mm) 230-400 mesh ASTM] using the wet method. Gradient elution was employed, starting with 1% ethyl acetate in petroleum ether (40-60^{o}C) and ending with 100% ethyl acetate. 30 x 200ml aliquots were collected (1-30) and concentrated under reduced pressure. Based upon the TLC (thin layer chromatography) analysis of the thirty different aliquots, 1-4 were bulked and further purified to yield **1** (24.3mg). Aliquots 5-9 were bulked and further crystallized in chloroform to yield **2** (33.4mg). Aliquots 10-13 were bulked, subjected to further column chromatography to yield **3** (425mg). Aliquots 16-30 were bulked, subjected to further column chromatography and crystallized out of methanol as 4 (45.6mg).

General methods

The NMR (both ^{1}H and ^{13}C) spectra were determined with the assistance of the Chemistry Department, King's College, London. ^{1}H and ^{13}C-NMR were recorded on a Bruker DPX 600 spectrometer (ppm, *J* in Hz) using TMS as internal standard. DEPT and 2D-NMR experiments were also carried out on the same instrument.

Bacteria used

The resistant bacteria used for the tests were a kind donation from Prof. Simon Gibbons of Centre for Pharmacognosy and Phytotherapy, The School of Pharmacy, University of London and included resistant strains of *Staphylococcus aureus SA1199B, RN4220, XU212.*

Antibacterial assay

Inocula of the bacteria were prepared from the 24 h Mueller-Hinton broth (Sigma) cultures and suspensions were adjusted to 10^{5}CFU/ml. Isolates were dissolved in 2% dimethylsulphoxide (DMSO). MIC values of the isolates were determined based on a micro-well dilution method (Eloff, 1998). The 96-well sterile plates were pre-

pared by dispensing 180 µl of the inoculated broth plus a 20 µl aliquot of the isolate made up in broth or 20 µl broth in the case of negative control in each well. Tetracycline (Sigma) was included as positive control. Plates were incubated for 24 h at 37°C. Bacterial growth was determined after addition of 50µl p-iodonitrotetrazolium blue (0.2 mg/ml, Sigma).

Bacterial resistance modulation assay

The modulation assay was performed using 10µg/ml of the isolated compounds to find the effect of combining them with standard antibiotics norfloxacin, tetracycline and erythromycin on resistant strains of *Staph. aureus SA1199B* (NorA), *XU212* (tetK) and *RN4220* (MsrA) respectively following the method of Oluwatuyi *et al*. (2004). 100µl of Muller Hinton Broth (MHB) was placed in each well except wells in column 1. 200µl of antibiotic was placed in wells of column 1 and serially diluted 2-fold to give a dilution range 512 -1µg/ml. To the wells in first three rows were added 100µl of the compound (made so as to bring the net concentration in each well to 10µg/ml) except those in columns 11 and 12. To the wells in rows four to six were added 100µl of reserpine (solution made so as to bring the net concentration in well to 10µg/ml). Wells in rows seven and eight were however, maintained free from both the isolate and reserpine. Wells in columns 11 and 12 were used as general and sterile controls respectively. Plates were incubated at 37°C for 24 hours, after which 20µl of MTT was added to each well and incubated for further 30 minutes. Inhibition of bacterial growth was visible as a clear well and the presence of growth detected by the presence of a blue colour in the well. All experiments were performed in triplicate under aseptic conditions.

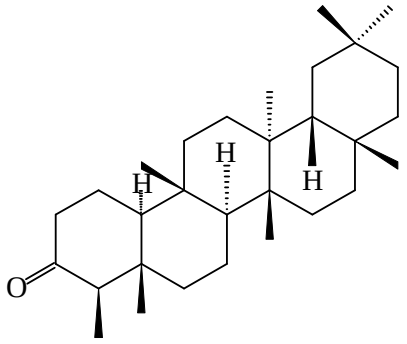
RESULTS AND DISCUSSION

Compound [1] was crystallized out of chloroform as fine needle-like crystals with melting point, 258-259°C (uncorrected). It showed positive Liebermann's test and IR absorption peaks at (KBr) ν_{max}: 2946, 2887, 2868, 1720, 1464, 1384, 1360 cm^{-1}. MS molecular peak at m/z 426 and other fragments at 384, 302, 273, 203, 123, 95, 55; characteristic of 3-keto-triterpenoid compounds (Budzikiewicz *et al*., 1964). The ^{13}C-NMR spectrum displayed a total of 30 carbon resonances, including a carbonyl carbon at δ 213.1. The DEPT experiment indicated that 23 out of the 30 carbon atoms in [1] were attached to protons, exhibiting signals for 8 methyls, 11 methylenes, 4 methines and 7 quarternary carbons. The ^{1}H-NMR spectrum showed 7 three proton singlets at δ 0.71, 0.87, 0.98, 1.00, 1.00, 1.03, 1.18 and a doublet centred at δ 0.87. These were attributed to H-24, H-25, H-29, H-26, H-30, H-27, H-28 and H -23. On the basis of the above spectral features, [1] was characterized as friedelin [friedelan-3-one], the identity of which was further substantiated by comparison of its spectroscopic data with published values (Rahman *et al*., 2005). Friedelin [1] has been reported in several species from different genera. However, this is being reported for the first time in the genus *Paullinia*.

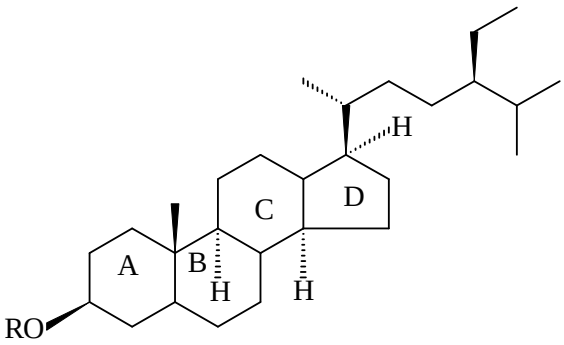
On crystallization in chloroform, compound [2] was obtained as white needle crystals with melting point 138-139°C (uncorrected). IR (KBr) νmax: 3440, 2900-2850, 1430, 1370, 1615, 1051, 960 cm^{-1}. LR-EI-MS, *m/z* (rel. int., %) 414 (90), 396 (40), 351 (11), 303 (22), 255(33), 213 (22), 159 (20), 1449 (22), 107 (22), 55 (18). The EI-MS of [2] exhibited a maximum peak at *m/z* 414, corresponding to the molecular formula $C_{29}H_{50}O$. This is also in close agreement with the literature values of β-sitosterol (El Deeb et al., 2003). The ^{13}C-NMR of [2] also compared with the literature (Kovganko *et al*., 1999). The unequivocal identification of [2] was confirmed by direct TLC comparison with the reference commercial β-sitosterol (Sigma-Aldrich, USA, batch number 47C-0262). Co-chromatography of [2] and the reference sample gave identical R_f values and chromogenic reaction to spray reagents. β-sitosterol has been isolated from numerous species and different families, including the leaves of *P. pinnata* (Miemanang *et al*., 2006).

On crystallization in methanol, [3] was obtained as white amorphous powder with melting point 118-120^{o}C (uncorrected). The EI-MS of [3] exhibited a maximum peak at *m/z* 456, corresponding to the molecular formula $C_{31}H_{52}O_2$. The ^{13}C-NMR of [3] also compared with the literature data for β-sitosterol acetate (Kovganko *et al*., 1999). The chemical shifts of carbon atoms in rings B, C and D and the side chains of [3] and β-sitosterol [2], were identical. The differences in the chemical shifts of the atoms in ring A were as a result of the conversion of OH, attached to C-3 in β-sitosterol, to the acetate. Thus the signal for C-3 in the spectrum of the acetate shifted to downfield by ~2 ppm owing to the α-effect of the acetoxy group, compared with its position in the spectrum of OH in β-sitosterol [2]. The signal for C-2 and C-4 in the spectrum of the acetate shift to upfield due to the β-effect of the acetoxy group.

Compound [4] was obtained as an off-white amorphous powder. The EI-MS exhibited a maximum peak at *m/z* 576, corresponding to the molecular formula $C_{35}H_{60}O_6$. The ^{13}C-NMR of [4] also compared with the literature data for β-sitosterol-D-glucoside (Pei-Wu *et al*., 1988). The chemical shifts of carbon atoms in B, C and D and the side chains of [4] and β-sitosterol [2], were almost identical. The differences in the chemical shifts of the atoms in ring A were as a result of the change from OH, attached to C-3 in β-sitosterol, to the O-glucoside. Glucosidation at position C-3 was concluded from the downfield chemical shift of carbon C-3 by ~5-12 ppm, compared with its position in the spectrum of β-sitosterol [2] (Agrawal, 1992). The signal for C-2 and C-4 in the spectrum of the glucoside shift to upfield, can also be ascribed to the β-effect of the glucoryl group. The location of the glucose at C-3 was further confirmed from the correlation of its H-1^{1} at δ 4.12 with C-3 at δ 78.1 in HMBC (Heteronuclear Multiple-bond Correlation) and COSY (Correlation spectroscopy) experiments. Acid hydrolysis of [4] proved β-sitosterol to be the aglycone moiety (co-chromatography with reference sample) and glucose was the sugar moiety (using butanol-acetic acid-water [5:1:4] in paper chromatography, using Whatman no. 4 paper (catalog no. 4001 150) and aniline hydrogen phthalate spray [aniline (0.93g) + phthalic acid (1.66g), dissolved in 100ml water-saturated butanol].



1



2 R=H

3 R=$COCH_3$

4 R=$C_6H_{11}O_6$

Antimicrobial activity

Friedelin [1] showed moderate antibacterial activity against the three resistant strains of *Staph aureus* with MICs ranging between 128 and 256 µg/ml (Table 1). The standard antibiotic, used as positive controls recorded low MIC values in all experiments except against bacterial strains to which they are resistant. Friedelin has previously been reported by Vasanth *et al*. (2001) to possess antimicrobial properties against Gram positive and Gram negative bacteria. β-sitosterol, β-sitosterol acetate and β-sitosterol-D-glucoside did not show antimicrobial actions within the concentration range tested.

The results of the bacterial resistance modulation assay using a sub-lethal concentration, i.e, 10µg/

Table 1: Antibacterial activities of isolated compounds expressed as minimum inhibitory concentrations (MIC) [µg/ml]

	Minimum inhibitory concentration [MIC] (µg/ml)						
Bacteria	**1**	**2**	**3**	**4**	**TET**	**ERY**	**NOR**
SA 1199B (NOR A)	256	>512	>512	512	8	8	32
XU 212 (TetK)	128	>512	>512	>512	128	4	4
RN 4220 (MsrA)	256	>512	>512	>512	8	256	4

Key; **1 –**friedelin ; **2 –***β*-sitosterol; **3 –***β*-sitosterol acetate; **4** -β-sitosterol D-glucoside; **TET**- Tetracycline; **NOR** – Norfloxacin; **ERY** - Erythromycin; n=3

Table 2: Antimicrobial susceptibility of test strains in the absence and presence of 10µg/ml of compounds and reserpine

Antimicrobial agent	**MIC (µg/ml) of test strain expressing the indicated efflux protein**		
	XU212 (TetK)	**RN4220 (MsrA)**	**SA1199B (NorA)**
Tetracycline	128	NT	NT
+ friedelin	64		
+β-sitosterol	128		
+β-sitosterol acetate	128		
+β-sitosterol glucoside	128		
+ reserpine	32		
Erythromycin	NT	256	NT
+ friedelin		16	
+β-sitosterol		256	
+β-sitosterol acetate		256	
+β-sitosterol glucoside		256	
+ reserpine		256	
Norfloxacin	NT	NT	32
+ friedelin			16
+β-sitosterol			32
+β-sitosterol acetate			32
+β-sitosterol glucoside			32
+ reserpine			8

All MICs were determined in triplicate. NT = not tested

ml, of isolated compounds in addition to different concentrations of standard antibiotics showed that friedelin [1] was able to reduce the MIC of norfloxacin from 32µg/ml to 16µg/ml (2-fold potentiation) against *SA1199B*. Friedelin also showed good modulation actions with erythromycin and tetracycline against *RN4220* and *XU212* respectively. Reserpine, which was used as control (Oluwatuyi *et al*., 2004) had 4-fold potentiation of tetracycline activity against *XU212* and norfloxacin activity against *SA1199B* (Table 2). However, reserpine had no effect on erythromycin against *RN4220* (MIC 256 µg/ml) whilst friedelin [1] showed a significant effect (16-fold potentiation).

Triplicate experiments were carried out but each experiment gave the same MIC for a particular extract tested.

Different classes of compounds of plant origin have been found to possess bacterial resistance modulatory actions (Gibbons, 2005). These include alkaloids (Brenwald *et al*., 1997), flavonoids and coumarins (Tsutomu *et al*., 2005), tannins and saponosides (Lee *et al*., 2000), diterpenoids, triterpenoids and steroids (Silvia *et al*., 1999).

Microbiological resistance mutations may be expressed in several ways. These include production of inactivating enzymes, changes in cell wall permeability, alterations to structural target, bypass of metabolic pathway, changes in efflux mechanisms and multiple resistance mechanisms (Wickens and Wade, 2005). Efflux pumps occur naturally in bacterial cells and are concerned with the removal of waste products (Poole, 2005). However, changes in their conformation can make them remove antimicrobials. Gram Positive organisms, including *Staph. aureus* can show resistance to tetracyclines by this mechanism (Miyamae *et al*., 2001). However, it is common for organisms to exhibit resistance by using a combination of two or more of the above mechanisms. Macrolide resistance in *Streptococci* may be due to a combination of increased efflux and ribosomal modification, a scenario commonly referred to as multi-drug resistance (Di Perri and Bonora, 2004).

In this study, the observed potentiation of the antimicrobial effects of the standard antibiotics used (tetracycline, erythromycin and norfloxacin) after combining them individually with 10 µg/ml of friedelin suggests it could modify the resistance mechanisms present. In the case of *SA1199B*, *RN4220* and *XU212* where the mechanisms of resistance have been confirmed to be by efflux (Gibbons, 2005), friedelin in low concentrations may act as substrates for the efflux proteins which the resistant bacteria overexpress thus allowing the respective antibiotics to enter the bacterial cell to cause toxicity.

CONCLUSIONS

Friedelin, isolated from *P. pinnata*, has been found to be a bacterial resistance modulator. This interesting finding is of significance especially in a developing country like Ghana, where antibiotics are often abused and taken concurrently with herbal medications. This may mean that the concurrent use of herbal extracts (which may contain modulatory principles) and clinical antibiotics, even at normal therapeutic levels, should be done with caution since minute quantities of some herbal extracts may act synergistically with the antibiotics to cause toxicity to the individual recipient.

AKNOWLEDGEMENT

The authors are grateful to the GETFUND for the financial support and also to the staff of the Chemistry Department, King's College London, for generating the spectral data of the isolated compounds.

REFERENCES

Agrawal, P.K. (1992). NMR spectroscopy in the structural elucidation of oligosaccharides and glycosides. *Phytochemistry* 31: 3307-3330.

Annan, K., Houghton, PJ., Hylands, PJ. and Gibbons, S. (2005). Antibacterial and Resistant

Modifying activity of *Paullinia pinnata*. *Journal of Pharmacy and Pharmacology* 57: S-74.

Brenwald, N.P., Gill, M.J. and Wise, R. (1997). The effect of reserpine, an inhibitor of multi-drug efflux pumps on the *in vitro* susceptibilities of fluoroquinolone-resistant strains of *Streptococcus pneumoniae* to norfloxacin. *Journal of Antimicrobial Chemotherapy* 40: 458-460.

Cassell, G.H. and Mekalonos, J. (2001). Development of antimicrobial agents in the era of new and re-emerging infectious diseases and increasing antibiotic resistance. *Journal of American Medical Association* 285: 601-605.

Cielei, I. (1982). Methodology for analysis of vegetable drugs. Ministry of Chemical Industry, Bucharest, p.67.

Di Perri, G. and Bonora, S. (2004). Which agents should we use for the treatment of

Multi-drug resistant *Mycobacterium tuberculosis*? *Journal of Antimicrobial Chemotherapy* 54: 593-602.

El Deeb, K.S., Al-Haidari, R.A., Mossa, J.S. and Ateya, A. (2003). Phytochemical and pharmacological studies of *Maytenus forsskaoliana*. *Saudi Pharmaceutical Journal* 11: 184-191.

Eloff, J.N. (1998). A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta Medica* 64: 711-713.

Gibbons, S. (2005). Plants as a source of bacterial resistance modulators and anti-infective agents. *Phytochemistry Reviews*, 4: 63-78.

Koyama, J. (2006). Anti-infective quinone derivatives of recent patents. *Recent Patents on Anti-infective Drug Discovery*, 1: 113-125.

Kovganko, N.V., Kashkan, Z.N., Borisov, E.V. and Batura, E.V. (1999). ^{13}C-NMR spectra of β-sitosterol derivatives with oxidized rings A and B. *Chemistry of Natural Compounds* 35:646- 649.

Lee, C.J., Galazzao, J.L., Ianiro,T. and Warren, M.S. (2000). Novel bacterial efflux pump from microbial fermentation. In 40th Interscience Conference on Antimicrobial Agents and Chemotherapy, Toronto, Canada, American Society of Microbiology.

Miyamae, S., Ueda, O., Yoshimura, F., Tanaka, J. and Nikaido, H. (2001). A MATE family multidrug efflux transporter pumps fluoroquinolones in *Bacteroides thetaiotaomicron*. *Antimicrobial Agents and Chemotherapy* 45: 3341-3346.

Oluwatuyi, M., Kaatz, G.W. and Gibbons, S. (2004). Antibacterial and resistance modifying activity of *Rosemarinus officinalis*. *Phytochemistry* 65: 3249-3254.

Pei-Wu, G., Fukuyama, Y., Rei, W., Jinxian, B. and Nakagawa, K. (1988). An acylated sitosterol glucoside from *Alisma plantago-aquatica*. *Phytochemistry* 27: 1895-1898.

Poole, K. (2005). Efflux-mediated antimicrobial resistance. *Journal of Antimicrobial Chemotherapy* 56: 20-51.

Rahman, M.S., Chowdhury, R., Begum, B., Rahman, K.M. and Rashid, M.A. (2005). Phytochemical studies of *Amoora cucullata*. *Dhaka University Journal of Pharmaceutical Sciences* 4: 1-5.

Silvia, A.M., Gavit, N.E., Pere, W.D. and Hayashi, S.F. (1999). Identification of a tetracycline efflux resistance reverser UK-57, 562. in 39th Interscience Conference on Antimicrobial Agents and Chemotherapy. San Francisco, CA, American Society for Microbiology.

Tsutomu, H., Miwako, K., Kazutoshi, I., Tomoomo, O. and Sumiko, S. (2005). Effects of tannins and related polyphenols on methicillin-resistant *Staphylococcus aureus*. *Phytochemistry* 66: 2047-2055.

Vasanth, S., Govindarajan, S. and Raj, M.K. (2001). Antimicrobial activity of *Notania grandifolia* DC (Asteraceae). *Indian Journal of Pharmaceutical Sciences* 63: 244-245.

Wagner, H. and Bladt, S. (1996). Plant Drug A: A Thin Layer Chromatography Atlas, Second ed. Springer, New York.

Wickens, H. and Wade, P. (2005). Understanding antibiotic resistance. *The Pharmaceutical Journal* 274: 501-504.