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Resistance modulation studies of vernolide from *Vernonia colorata* (Drake) on ciprofloxacin, amoxicillin, tetracycline and erythromycin

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ABSTRACT

Resistance modulatory activities of natural products on traditional antibiotics have gained scientific interest in recent years. This is aimed at maximizing antimicrobial potency with major strides in curbing microbial resistance and leading to potential drug discovery. The antimicrobial activity of vernolide, a compound isolated from *Vernonia colorata* was investigated following its folklore use as antibiotic. The compound Vernolide was isolated from the leaves of *Vernonia colorata* and the identity was confirmed by appearance, melting point, ¹H and ¹³C NMR as well as UV-Vis and IR spectroscopy. The antimicrobial activity of the isolate was evaluated with MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide) assay using micro broth dilution and 96 well microtiter plates against standardized bacteria suspensions of selected inoculum. Vernolide showed activity against *Escherichia coli*, *Staphylococcus aureus* and *Enterococcus faecalis* with minimum inhibitory concentrations (MICs) of 0.3, 0.3 and 0.4 mg/ml respectively. The MIC of the selected antibiotics, namely on ciprofloxacin, amoxicillin, tetracycline and erythromycin were subsequently determined as a range between 0.1µg/ml to 16 µg/ml. Modulation studies between vernolide and the selected antibiotics showed that all antibiotics were able to prevent growth of the microorganisms at a concentration of 0.2 mg/mL vernolide and varying concentrations of the standard antibiotics in the range of 4.00 µg/ml to 0.0005 µg/ml which are far well below their observed MICs. Vernolide has therefore been shown to possess strong resistance modulatory activity on ciprofloxacin, amoxicillin, tetracycline and erythromycin against *Escherichia coli*, *Staphylococcus aureus* and *Enterococcus faecalis*.

Keywords: *Vernonia colorata*, Vernolide, Minimum inhibitory concentration (MIC), Resistance modulation.

INTRODUCTION

Phyto medicine has gained increasing traction over the years as an area of research. This is due to the increasing use of herbals as sources of remedies in our traditional and non-traditional settlements. According to the World Health Organization (WHO), up to 65% of the world's population employ plants or plant extracts as medicine for human therapy^[1]. Crude extracts and isolates from several plants have demonstrated significant pharmacological benefits ranging from antimicrobial, anti-inflammatory, anti-malarial, anthelmintic, analgesic and antidiabetic effects. Orthodox traditional antimicrobial medicines, however, face increasing challenge of resistance against microbes. Research has shown that about 90-95% of *Staphylococcus aureus* strains are now resistant to penicillin, a drug which was not so long ago regarded as first line therapy^[2]. As a result of these developments, newer areas of research have opened up where synergism of natural products and conventional antibiotics are being tested to establish possible candidate for further drug discovery and development^[3-6]. Currently, treatment protocols for resistant microbes are expensive and some completely non-existent. However, development of newer antibiotics has also shown to be expensive and time consuming^[7].

Vernonia colorata (Willd) Drake (common in Ghana with the Akan name “mpena-sere” translated as ‘concubine laugh’, Fig. 1, is a shrub which grows up to 8m high. The species is very similar to *Vernonia amygdalina* in appearance. The leaves of the plant are extremely bitter but taken locally as a decoction for the treatment of malaria and as antibiotic in liver, stomach and skin conditions^[8].

This study reports the resistance modulatory activity of vernolide isolated from the extracts of the leaves of *Vernonia colorata* on ciprofloxacin, amoxicillin, tetracycline and erythromycin against *Escherichia coli*, *Staphylococcus aureus* and *Enterococcus faecalis*.



Figure 1: The leaves of *Vernonia colorata*

MATERIALS AND METHODS

MATERIALS

The reagents hexane, petroleum ether, chloroform, methanol and ethanol used for this work was obtained from Fisher Scientific, UK. Ethyl acetate was obtained from Chemiphase, UK. *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923) and *Enterococcus faecalis* (ATCC 29212) were obtained from the Department of Microbiology, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST.

METHODS

Sample Collection and Extraction

Fresh leaves of the plant were collected the University, KNUST in October, 2016 and authenticated by the Department of Herbal Medicine in the Faculty of Pharmacy and Pharmaceutical Sciences, KNUST. Voucher specimen number of KNUST/HM1/2017/L004 was assigned and deposited in the department's herbarium.

The fresh leaves were air dried and pulverized to obtain the course powdered form. The powder was extracted using cold maceration with 96% ethanol for 4 days. The ethanolic extract obtained was filtered through No. 1 Whatman's filter paper and the filtrate evaporated to dryness using a rotary evaporator at reduced temperature of about 50 °C and a rotational speed of 5 revolutions per minute (rpm) to obtain the dried crude extract.

Phytochemical Screening and Isolation

Phytochemical screening was conducted on crude ethanolic extract using methods and procedures adopted from Trease and Evans [9]. The crude extract stock solution was prepared by dissolving in ethanol.

Column chromatography using silica gel as stationary phase was used to isolate vernolide.

The column was eluted using a gradient system of hexane, chloroform, ethyl acetate and methanol guided by previous isolation [10]. The final crystals were further purified by recrystallizing in chloroform: ethylacetate (9:1).

Isolate Confirmation

The identity of the isolate was confirmed with melting point, IR and UV-Vis spectrophotometry as well as proton and carbon-13 NMR.

The melting point of the isolate was determined in triplicate using the melting point apparatus obtained from Stuart, UK with serial number of R0001105350.

A spectrum of the isolate was obtained for the isolate concentration of 2mg/mL. The UV spectrum was obtained at wavelength scale from 200-500nm and absorbance range from 0-2 using methanol as reference solvent. The UV-Vis spectrometer used was obtained from Jenway, United Kingdom with model number of 7313.

The IR spectrum of the isolate was obtained with the PerkinElmer Fourier Transform (FT) IR spectrometer obtained from Waltham, USA with model number A/0626/15. The sample was run at wavenumber from 4000 to 400 cm⁻¹.

The proton and carbon-13 nuclear magnetic resonance spectra were obtained using Bruker Biospin NMR spectrometer. The sample was dissolved in deuterated chloroform (CDCl₃) and the NMR was obtained at frequency of 500MHz at a temperature of 298K. The NMR spectrometer used was obtained from Billerica, USA with model number of F/NMR/A175.

Antimicrobial Analysis

The antimicrobial activity of the isolated compound was assessed by determining the minimum inhibitory concentration (MIC) against the microorganisms used.

The three (3) microorganisms were selected based on their implication in most infections. Microbial strains were obtained from the Kwame Nkrumah University of Science and Technology precisely the microbiology section at the Faculty of Pharmacy and Pharmaceutical Science. The bacterial strains were Gram-positive and Gram-negative bacteria, namely *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212 and *Escherichia coli* ATCC 25922. The microbial stains were sub-cultured 24 hours prior to experiment in a nutrient broth at 37 °C. Standardized suspensions of microorganisms were prepared from the overnight broth cultures. Standardization was by serial dilution of the culture in sterile normal saline to achieve a suspension of equal turbidity using 0.5 Mc. Farland standards by visual comparison to achieve a dilution approximately 10⁵ CFU/ml.

The MIC of the isolate was obtained with micro broth dilution method using the 96 well micro titer plates. A stock concentration of 10mg/ml vernolide was prepared by dissolving 50mg of the compound in 5ml of methanol. This was used as stock concentration to prepare well concentrations ranging from 0.9mg/ml to 0.05mg/ml. The 96-well sterile plates were prepared by dispensing into each well 100µl of double strength nutrient broth, 80 µl of the test samples and 20 µl of the inoculums of standardized suspensions of the

various cultures of test organisms [11]. Cells 11 and 12 of the plate were employed as negative and positive control respectively. The negative control was performed by adding only nutrient broth and sterile water. The positive control was performed by adding only the nutrient broth and inoculum to the well. The plate was incubated in an incubator at 37 °C for 24 hours. After incubation, the activity in each well was detected with MTT. 20 µl of the MTT was added into each well and the results read after 30 minutes.

The same procedure was used to determine the MIC of the selected antimicrobials; ciprofloxacin, amoxicillin, tetracycline and erythromycin. The antimicrobials were selected because to their different mechanisms of action.

Resistance-Modulation Studies

The stock concentration of vernolide which was 1 mg/ml was used to prepare 10 different concentrations of the wells such that the concentrations obtained were below the MIC of the antibiotics. Moreover, each well concentration was prepared such that a single well of 200 µl contained 0.2 mg/ml of vernolide. Concurrently, the concentrations of 4, 2, 1, 0.5, 0.25, 0.1, 0.05, 0.025, 0.001 and 0.0005 µg/ml of the antibiotics were prepared from their stock solutions. The well plates were prepared by dispensing into each well 100 µl of double strength nutrient broth, 80 µl of the test samples and 20 µl of the inoculums of standardized suspensions of the various cultures of test organisms. Cells 11 and 12 of the plate were employed as negative and positive controls respectively. The plate was incubated at 37 °C for 24 hours [12].

RESULTS

Phytochemical Screening

The phytochemical screening results obtained from the extract is as indicated in Table 1.

The outcome revealed the presence of tanins, glycosides, saponins, alkaloids, flavonoids, cardiac glycosides and triterpenoids in the various samples. However anthracene glycosides was absent in all the samples screened.

Table 1: Results of phytochemical screening on ethanolic extract of *Vernonia colorata*

TEST	RESULTS
TANNINS	+
GLYCOSIDES(general)	+
SAPONINS	+
ALKALOIDS	+
FLAVONOIDS	+
STEROLS (terpenoid nucleus)	+
ANTRACENE GLYCOSIDES	-
CARDIAC GLYCOSIDES	+

Key: (+) = present, (-) absent

Melting point Determination

The melting point range of the pure compound was determined in triplicate and the mean value was calculated to be 171 – 173 °C.

UV-Vis spectrum

The UV-Vis scan of the isolated compound is shown in Fig. 2.

For the UV-Vis scan, methanol was used as reference solvent at qualitative cut off point of 205nm. A single peak obtained at 220nm can be attributed to the presence of the isolate.

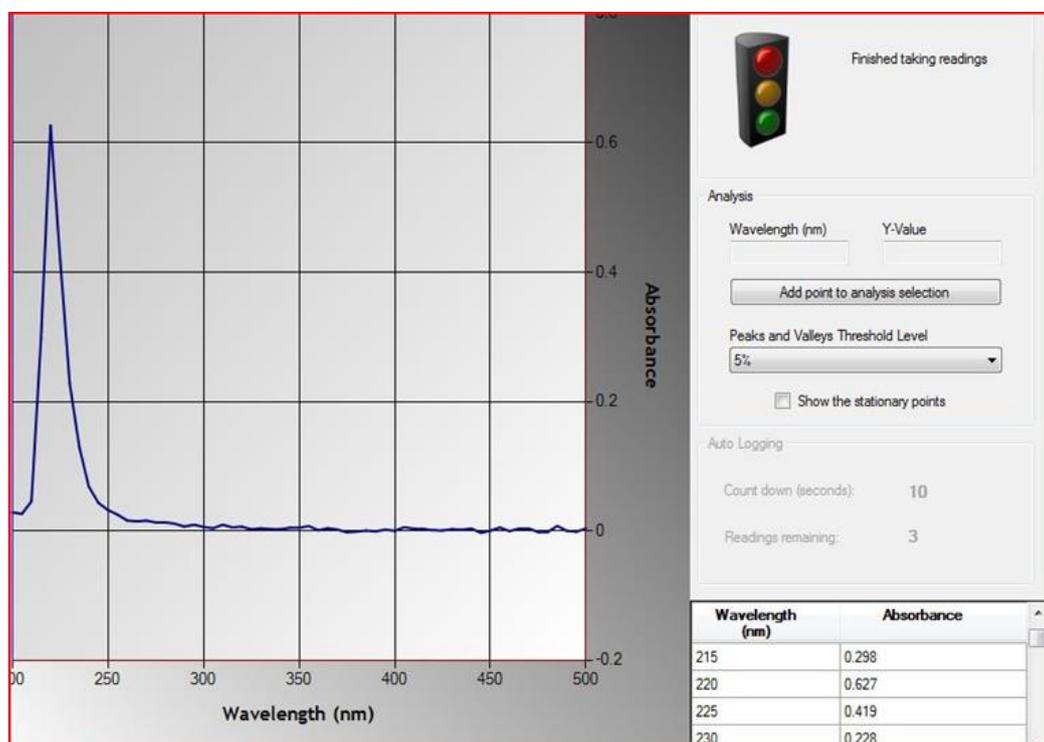


Figure 2: UV-Vis scan of a 2mg/mL concentration of the isolate

IR Spectrum

The IR scan and characteristics absorbances are shown in Fig. 3 and Table 2.

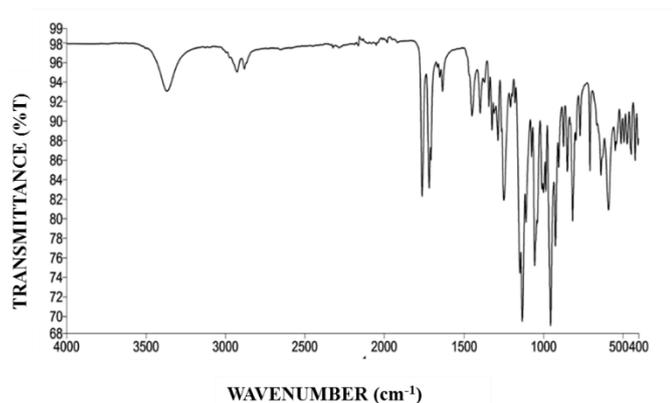


Figure 3: Infrared spectrum of the isolate vernolide

The list of the significant absorptions and their wavenumbers are summarized in Table 2.

Table 2: Table of most important absorptions in the IR spectrum of vernolide

PEAK	X (cm ⁻¹)	%T
1	3369.69	93.13
2	2928.33	95.1
3	1765.34	82.32
4	1720.41	83.16
5	1132.71	69.44

NMR Data

A summary of the NMR data obtained are showed in Table 3.

Table 3: Table showing the proton and carbon-13 NMR data for vernolide

CARBON	¹ H	¹³ C
1	2.72 (dd)	66.7
2	1.67 (m)	23.0
	2.29 (m)	
3	2.39 (m)	33.7
4	-	144.0
5	5.57 (d)	128.9
6	5.23 (t)	77.7
7	3.05 (m)	52.2
8	5.73 (s)	70.1
9	2.65 (d)	41.6
	1.38 (d)	
10	-	59.1
11	-	135.1
12	-	169.7

13	5.95 (d)	126.4
	6.35 (d)	
14	4.55 (s)	99.6
	4.59 (d)	
15	3.67 (d)	64.6
	-	
16	-	167.5
	-	
17	1.95 (s)	18.5
	6.16 (s)	
18	5.71 (d)	127.5
	-	

Antimicrobial Analysis

Table 4 shows the MIC of vernolide against *Escherichia coli*, *Staphylococcus aureus* and *Enterococcus faecalis*.

Table 4: Table showing the MIC (mg/ml) of vernolide against selected organisms

ORGANISM	MIC (mg/ml)
<i>Escherichia coli</i>	0.3
<i>Staphylococcus aureus</i>	0.3
<i>Enterococcus faecalis</i>	0.4

In the antimicrobial studies of standard antibiotics, the MIC of the antibiotics against the microorganisms are summarized in the Table 5.

Table 5: Table showing the MIC of the antibiotics against the selected organisms.

ANTIBIOTIC	MIC (µg/ml)		
	<i>E. coli</i>	<i>S. aureus</i>	<i>E. faecalis</i>
Ciprofloxacin	0.1	0.10	0.10
Amoxicillin	0.1	0.25	0.50
Tetracycline	16	1.00	0.25
Erythromycin	0.5	0.25	0.50

In the MIC determination of the standard antimicrobials, the values so obtained were consistent with range of literature values [13].

Resistance modulation studies

The modulation studies after 24 hours showed no growth at the negative control but growths at the positive control. There was no growth in concentrations ranging from 4 µg/ml to 0.0005 µg/ml after 24, 36 and 72 hours.

DISCUSSION

Results of phytochemical analysis corroborate similar works done on the leaves of *vernonia colorata* and *vernonia amygdalina* [14]. Ethanolic leaf extract of the plant showed the presence of tannins,

glycosides, saponins, alkaloids, flavonoids, terpenes and cardiac glycosides (Table 1). Terpenes have been reported to be the major constituent of the plant species responsible for its antimicrobial properties [15]. Alkaloids are a major source of pharmacologically active compounds in several plant genus and therefore their presence in the leaf extract should be investigated further. Anthracene glycosides were absent in the extract of the leaves [16]. Anthracene glycosides are pharmacologically responsible for purgative action of many plant extracts, as such the leaves will not be useful for conditions relating to constipation or piles. Flavonoids aside from their important antioxidant potential, are beneficial in conditions of anti-viral, anti-cancer, anti-inflammatory and some anti-allergic activities [17]. Saponins are known to help lower cholesterol levels in the blood which help reduce the incidence of cardiovascular diseases [18]. However, ethno medicinal review of the plant genus has not found any such use in orthodox or traditional medicine.

The melting point range of the pure compound was determined in triplicate and the mean value was calculated to be 171 – 173 °C. This is in close agreement to the melting point of vernolide in literature obtained as (171 – 172) °C. The melting point range obtained is sharp and undepressed which attest to the purity of the compound [10].

The UV-Vis scan (Fig. 2) confirms a single peak obtained at 220nm can be attributed to the presence of the isolate.

The IR spectrum (Fig. 3) showed the presence of absorptions at characteristic wavenumbers.

Analysis of the IR spectrum reveals significant band characters (Table 2). The presence of broad and medium band at 3360 cm^{-1} consistent with the stretching vibrations of an O-H functional group. This functional group can be also found on carbon 13 of vernolide. Strong and sharp absorptions at 1760 cm^{-1} is indicative of -C=O , carbonyl functional group. The carbonyl functional groups can be found on carbon 12 and 15. At 1720 cm^{-1} there is the vibration due to C=C functional group of alkene compounds. These bonds are found between carbon 5 and 6 and also carbon 7 and 11 as well as carbons 16 and 17. Very strong absorptions in the region of 1100 cm^{-1} is characteristic of C-O stretching frequencies which can also be found at carbon 1 and the ring lactone in the structure of the isolate, Fig. 4.

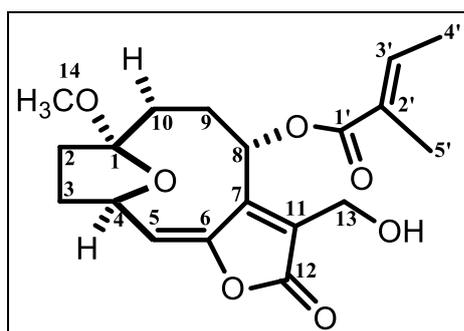


Figure 4: The structure of vernolide. [19]

The NMR data (Table 3) further reveals features characteristic of vernolide. Proton analysis of the compound gave chemical shifts with multiplicities of the peaks very similar to values in literature [19-21]. The integrals of the chemical shifts sum up to 22 hydrogens consistent with the molecular formulae $\text{C}_{19}\text{H}_{22}\text{O}_7$. Carbon-13 spectrum shows the presence of 19 carbons when the chemical shifts of the deuterated chloroform is accounted for. Again, this is consistent with the molecular formulae of vernolide [19].

The antimicrobial analysis (Tables 4 and 5) reveals vital properties of the isolated compound. There was growth in all the positive controls indicating that the nutrient broth is able to sustain the growth of the organisms. There was no growth at the negative controls meaning that the sterile water, environment and equipment used did not introduce any microorganisms into the nutrient broth. The compound was shown to be active against *E. coli*, *S. aureus* with MIC of 0.3 mg/ml. It was shown to be also active against *E. faecalis* with MIC of 0.4 mg/ml. These are consistent with previous MIC results done on vernolide using the agar well diffusion technique [10].

The modulation studies after 24 hours showed no growth at the negative controls but growths at the positive controls. Moreover, there was no growth in concentrations ranging from 4 $\mu\text{g/ml}$ to 0.0005 $\mu\text{g/ml}$ after 36 and 72 hours.

Sub inhibitory concentrations of the vernolide as well as the standard antibiotics were prepared to test the inhibition against the organisms. It is established that those concentrations alone ordinarily will not inhibit the growth of the organisms. The concentrations chosen were 0.2mg/ml of vernolide and concentration of 4 – 0.0005 $\mu\text{g/ml}$ of the standard antibiotics. Therefore, each well of inhibition contained 0.2 mg/ml of vernolide with concentrations varying from 4 $\mu\text{g/ml}$ of the standard antibiotic to 0.0005 $\mu\text{g/ml}$ against each microorganism, incubated for at least 24 hours.

After 24 hours, there was growth in all the positive controls indicating that the nutrient broth is able to sustain the growth of the organisms. There was no growth at the negative controls meaning that the sterile water, environment and instrument used did not introduce any microorganisms into the broth. Moreover, there was no growth at any level of concentration ranging from 4 – 0.0005 $\mu\text{g/ml}$. This is to suggest resistance modulatory effect of vernolide with the standard antimicrobials. Further, there was no growth in any well concentration after 72 hours of incubation, although this is not specific, it is possible that vernolide in combination with these standard antimicrobials have biocidal effect on the organisms.

However, vernolide has been established to be cytotoxic, limiting its potential to be used in further drug development and discovery [22]. Nonetheless, this activity has also been found to be structurally related to substitution effect at Carbon 13. The replacement of the hydroxyl functional group at that carbon with an acetate group to the sesquiterpene lactone reduces the cytotoxic activity to only marginal effects [22]. Therefore, this property of the compound can be exploited to enable the consideration of vernolide in further drug development.

CONCLUSION

Phytochemical analysis of the plant revealed the presence of phytochemicals including tannins, saponins, alkaloids, steroids and sterols. The identity of the compound was confirmed with its physical properties, UV, proton and carbon-13 NMR. The compound was found to be active against *Escherichia coli*, *Staphylococcus aureus* and *Enterococcus faecalis* with MIC of 0.3, 0.3 and 0.4 mg/ml respectively. The compound was shown to successfully modulate the effect of ciprofloxacin, amoxicillin, erythromycin and tetracycline even at concentrations ten times below the MIC of the drug. Therefore, combination therapy of vernolide and standard antimicrobials have the potential to be used in drug discovery and development.

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