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Antibacterial potential and modes of action of methanol extracts of flowers and leaves of *Vernonia glabra* (Steetz) Vatke (Asteraceae) against multidrug-resistant Gram-negative bacteria overexpressing efflux pumps

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Abstract

Background: Bacterial infections caused by multidrug-resistant (MDR) Gram-negative bacteria remain a public health problem and have contributed to a reduction in the range of antibiotics available for antibiotic therapy. The search for new antibacterial substances is becoming increasingly important, and plants represent an important reservoir of therapeutic molecules. In the present study, the antibacterial activity, and modes of action of *Vernonia glabra* against Gram-negative and multi-resistant bacteria were evaluated.

Methods: The antibacterial activity of *Vernonia glabra* extracts was assessed using the broth microdilution method, and the effects of the flower extract on bacterial growth kinetics and on the H^+-ATPase proton pumps of *Providencia stuartii* ATCC29916 were carried out using standard experimental protocols; qualitative reference methods were used to identify the secondary metabolites present in the extracts.

Results: Phytochemical screening of *Vernonia glabra* flower and leaf extracts revealed the presence of alkaloids, phenols, flavonoids, tannins, triterpenes, saponins, and anthocyanins. The flower and leaf extracts showed antibacterial spectra of 100% and 93.33% respectively against the bacteria tested. With minimal inhibitory concentrations (MIC) ranging from 32 µg/mL to 2048 µg/mL, the flower extract showed excellent activity against *Escherichia coli* AG100 and *Providencia stuartii* ATCC29916 with a MIC of 32 µg/mL, while the leaf extract showed good activity against *Klebsiella pneumoniae* ATCC11296 and *Providencia stuartii* PS2636 with a MIC of 256 µg/mL. The flower extract inhibited the growth of *Providencia stuartii* ATCC22916 at the exponential phase and inhibited its H+-ATPase proton pumps. In the presence of the efflux pump inhibitor, phenylalanine-arginine *β*-naphthylamide (PAβN), the activity of the leaf extract increased in 90.90% of bacteria tested. With activity enhancement factors ranging from 2- to 128-fold, both extracts potentiated the activity of antibiotics (imipenem, ampicillin, levofloxacin, tetracycline, vancomycin, ceftriaxone, ciprofloxacin, and doxycycline) against at least 70% of the bacteria tested.

Conclusion: The results obtained in the present work show that *Vernonia glabra* is a source of antibacterial molecules that can be used against MDR Gram-negative bacteria.

Keywords: Antibacterial; Asteraceae; efflux pumps; modes of action; multidrug resistance; *Vernonia glabra*.

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Background

Infectious diseases represent an important cause of morbidity and mortality worldwide and are currently a major public health concern [1]. Infectious diseases are responsible for 17 million deaths worldwide every year, accounting for around 30% of global mortality, particularly in developing countries [2, 3]. According to the World Health Organization, among the 2.7 million neonatal deaths are recorded each year worldwide, due to infectious diseases, with pathogenic bacteria responsible for 560,000 cases [3]. The discovery of antibiotics has helped to dramatically reduce the death rate from bacterial infections worldwide; unfortunately, the abusive and inappropriate use of these antibiotics has led to the development of bacterial resistance that has evolved into multidrug resistance to many antibiotics. Multidrug-resistant (MDR) bacteria are responsible for numerous therapeutic failures [4]. The resistance mechanisms of pathogenic bacteria mainly include the overexpression of efflux pumps, the modification of the antibiotic target, and the impermeability of the cell wall [4]. Clinically, the most frequent MDR phenotypes in several classes of antibiotics are Gram-negative bacteria such as Enterobacteriaceae and some bacterial species belonging to the genus *Pseudomonas* [5-7]. The discovery of new effective antibacterial molecules capable of circumventing this phenomenon is now an absolute necessity [8-12]. Several previous studies [13-20] have demonstrated that botanicals from food and medicinal plants are a source of active substances that can overcome bacterial multidrug resistance by acting alone or in synergy with antibiotics. In the present work, we targeted *Vernonia glabra* (Asteraceae), a plant traditionally used to treat wound infections, diarrhea, cough, pneumonia, and stomach ailments [21, 22]. The antibacterial activity of methanol extracts of the leaves and flowers of *Vernonia glabra* against MDR Gram-negative bacteria was evaluated as well as the phytochemical screening of the botanicals; The modes of action of the botanical from *V. glabra* flowers on *Providencia stuartii* was also assessed. Finally, the effects of the combination of extracts from the leaves and flowers of *Vernonia glabra* with an efflux pump inhibitor, phenylalanine arginine-β-naphthylamide (PAβN), and antibiotics on MDR bacteria were determined.

Methods

Plant material and extraction

The leaves and flowers of *Vernonia glabra* (Steetz) Vatke were harvested in Dschang (Western Region of Cameroon) in December 2022. The identification of this plant was made by the botanists of the Cameroon National Herbarium (HNC) (Yaoundé-Cameroon) from reference samples with voucher code 50075/HNC. The plant parts (flower and leaf) were air-dried in the shade and then ground. The obtained powders were macerated in methanol at 95°C (1: 3 m/v) at room temperature for 48 hours. At the end of the maceration, filtration was carried out using Whatman N°1, and the filtrate obtained was evaporated using a rotary evaporator at 65°C. The crude extract was covered in a sterile bottle and then dried in an oven at 40°C to eliminate the residual solvent, then stored at 4°C for subsequent use.

Chemicals and culture media

para-Iodonitrotetrazolium chloride ≥ 97% (INT) was used as the bacterial growth indicator. The efflux pump inhibitor, phenylalanine-arginine *β*-naphthylamide (PAβN) was used. Dimethyl sulfoxide (DMSO) served to solubilize plant extracts. Eight antibiotics from four families, namely ampicillin (AMP), ceftriaxone (CRO), imipenem (IMI), tetracycline (TET), doxycycline (DOX), vancomycin (VAN), levofloxacin (LEV), and ciprofloxacin (CIP) were used. Mueller Hinton Agar (MHA) was used for the activation of bacteria; Mueller Hinton Broth (MHB) was used for microdilution as a nutrient medium for bacteria; Eosin Methylene Blue (EMB), Mac Conkey and cetrimide agars were used to ensure the purity of strains and isolates of *Escherichia coli, Klebsiella pneumonia*, and *Pseudomonas aeruginosa*, respectively. All chemicals were purchased from Sigma-Aldrich (St. Quentin Fallavier, France).

Tested bacteria

The Gram-negative bacteria tested included both reference strains and clinical isolates of *Escherichia coli (*ATCC10536, AG102, and AG100), *Klebsiella pneumoniae (*ATCC11296, KP55, and K2), *Pseudomonas aeruginosa* (PA01 and PA124), *Enterobacter aerogenes* (EA3, EA298, and EA27), and *Providencia stuartii* (ATCC29916, PS2636, and NEA16). Their bacterial features were previously reported [6, 23-33]. *Escherichia coli (*AG102, and AG100), *Klebsiella pneumoniae (*KP55, and K2), *Enterobacter aerogenes* (EA3, EA298, and EA27), and *Providencia stuartii* (PS2636, and NEA16) are clinical bacterial strains overexpressing AcrAB-TolC efflux pumps while *Pseudomonas aeruginosa* PA124 overexpressed MexAB-OprM pumps [34-37].

Determination of minimal inhibitory and bactericidal concentrations

The bacterial inoculum was prepared as previously described [38-44] in comparison to the turbidity of a standard McFarland 0.5 ($1.5x10^8$ CFU/mL). The various plant extracts and the reference drug (imipenem) were dissolved in DMSO-MHB. Plant extracts were prepared at 8192 µg/mL, and antibiotics at 1024 µg/mL. PAβN was prepared at the concentration of 100 µg/mL. Botanicals were tested alone, then in the presence of PAβN (EPI). The combination of plant extracts with EPI was intended to evaluate the function of efflux pumps in bacterial resistance to the test extracts [23, 24, 43, 45]. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of test extracts alone were determined using a 96-well broth microdilution method combined with the rapid INT colorimetric method [45-47]. Imipenem was used as a positive antibacterial control, whereas DMSO 2.5%+MHB and MHB alone were used as negative controls. MIC was considered the lowest concentration of plant extract which produced complete inhibition of bacterial growth after 18 to 24 hours of incubation at 37°C, whereas MBC was considered the lowest concentration of a sample that did not induce a color change with the addition of INT following additional 48 hours of incubation [48-50]. Each experiment was repeated three times in triplicate.

Evaluation of the effect of V. glabra flower extract on the kinetics of bacterial growth

To evaluate the effect of the methanol extract of the flowers of *V. glabra* on the kinetics of bacterial growth, the optical densities (OD) were measured following the earlier reported protocol [33, 39, 51]. The *P. stuartii* ATCC29916 strain was activated onto MHA at 37°C for 18 h. Subsequently, a few colonies of this bacterial culture were removed to prepare a suspension with turbidity corresponding to Mc Farland 0.5 ($1.5×10^8$ CFU/mL). With MHB, 20 mL of inoculum solution was prepared at a concentration of 10^6 CFU/mL. These inocula were treated with the botanicals at MIC/2, MIC, and 2×MIC,

and the whole was incubated with stirring at a speed of 130 rpm using a magnetic stirrer to allow good dispersion of these. A positive control contained CIP at MIC while the negative control was MHB + the bacterial suspension. After incubation times of 0 min, 1 h, 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, 14 h, 16 h, 18 h, and 20 h, 200 µL of each solution were introduced into the wells of flat-bottomed microplates and the OD were read at 600 nm. Each test was repeated 3 times.

Evaluation of the effect of the methanol extract of the flowers of V. glabra on the H+-ATPase proton pumps

The effects of leaf methanol extract *of the flowers of V. glabra* were assessed on the H^+-ATPase-mediated proton pumping of *K. pneumoniae* ATCC11296 at 0.5×MIC, MIC, and 2×MIC as earlier described [44]. The action on H^+-ATPase-mediated proton pumping was done by controlling the acidification of the bacterial growth medium over 60 min following the procedures previously described [52, 53].

Determination of the antibiotic-potentiating effects of the botanicals

The effects of the association of the botanicals with antibiotics were determined against the MDR bacteria. At first, extracts were used at the sub-inhibitory concentrations of MIC/2, MIC/4, MIC/8, and MIC/16 for a preliminary assay on *Escherichia coli* AG102, which then allowed the selection of appropriate sub-inhibitory concentrations of MIC/2 and MIC/4 for further combination testing (Data not shown). Antibiotic-resistance modulating factor (AMF) was calculated as the ratio of the MIC of the antibiotic alone versus MIC in combination with the plant extract. The potentiation effect was considered for AMF ≥ 2 [54].

Phytochemical screening of flower and leaf extracts of V. glabra

Phytochemical screening was done following the standard methods described for alkaloids, anthocyanins, flavonoids (Shinoda test), phenols, saponins, tannins, and triterpenes (Liebermann-Burchard test) [10, 55].

Interpretation of antibacterial data

Several cutoff points are available for the interpretation of the antibacterial activity of plant products including extracts from edible plants [8, 56]. According to Kuete [8], the following threshold values are applied to botanicals: significant activity (MIC <100 µg/mL), moderate (100 <MIC ≤ 625 µg/mL), and low or negligible (MIC> 625 µg/mL). According to Tamokou et al. [56], the cutoff point for the antibacterial activity of botanicals from edible plants are as follows: highly active (MIC below 100 µg/mL), significantly active (100 ≤ MIC ≤ 512 µg/mL), moderately active (512 < MIC ≤ 2048 µg/mL), low activity (MIC > 2048 µg/mL), and considered not active (MIC > 10 mg/mL). However, updated and rationally defined cutoff points of the antibacterial botanicals have been defined, considering the various bacterial species [57-60]. For Enterobacteria: outstanding activity (MIC ≤8 µg/mL), excellent activity (8 < MIC ≤64 µg/mL), very good activity (64 < MIC ≤128 µg/mL), good activity (128 < MIC ≤256 µg/mL), average activity (256 < MIC ≤512 µg/mL), weak activity (512 < MIC ≤1024 µg/mL), and not active (MIC values >1024 µg/mL) [57]. For *P. aeruginosa:* outstanding activity (MIC ≤ 32 µg/mL), excellent activity (32 < MIC ≤ 128 µg/mL), very good activity (128 < MIC ≤ 256 µg/mL), good activity (256 < MIC ≤ 512 µg/mL), average activity (512 < MIC ≤ 1024 µg/mL), weak activity or not active (MIC values >1024 µg/mL) [58]. Bactericidal activities are considered when the ratios MBC/MIC are below or equal to 2 [49, 50, 61, 62]. The above appreciation criteria will be used to discuss the antibacterial activities of the studied samples.

Results

Antibacterial activity of the botanicals from V. glabra

The antibacterial activities of the botanicals from *V. glabra* are shown in Table 1. It appears that the methanol extract of the flowers had a spectrum of antibacterial activity of 100% (15/15) against the tested bacteria with the MIC values ranging from 32 to 256 µg/mL. This extract had excellent activity against the Enterobacteriaceae *E. coli* AG100 and *P. stuartii* ATCC29916 with a MIC value of 32 µg/mL as well as against the strains *K. pneumoniae* K2, *E. coli* ATCC10536, *P. stuartii* (PS2636, NEA16) and *E. aerogenes* (EA3, EA298) with a MIC of 64 µg/mL. Against *P. aeruginosa* (PA01 and PA121), this extract displayed excellent activity with a MIC value of 128 µg/mL; against *P. aeruginosa* PA124, the extract was very active with a MIC value of 256 µg/mL. Bactericidal activity has been recorded with botanical from the flowers against *P. aeruginosa* PA124, *P. stuartii* NEA16, and *E. aerogenes* (EA27 and EA298). The Botanical from the leaves of *V. glabra* had an antibacterial activity spectrum of 93.33% (14/15) with MIC values ranging from 256 to 2048 µg/mL. This extract had good activity against the *K. pneumoniae* ATCC11296 and *P. stuartii* PS2636 with a MIC value of 256 µg/mL. Against *P. aeruginosa* PA01, this extract had good activity with a MIC value of 512 µg/mL. Bactericidal activity was recorded with this *V. glabra* leaf extract against *P. aeruginosa* PA01, *E. coli* (AG100 and ATCC10536) and *E. aerogenes* EA298.

The EPI, PAβN enhanced the activity of botanicals from V. glabra

The effect of the combination of leaf and flower extracts of *V. glabra* in the presence of an efflux pump inhibitor (PAβN) was evaluated by determining the MIC values of these extracts in the presence of the EPI vis-à-vis 11 bacteria (Table 2). It appears that in the presence of PAβN the activity of the extract of the leaves of *V. glabra* was enhanced in 90.90% (10/11) of the bacteria tested with an increase of 64-fold against *P. aeruginosa* PA121 and *E. aerogenes* EA3. It can also be noted that the activity of the extract from the flowers of *V. glabra* was improved on 72.72% (8/11) of the strains and isolates tested with an increase of 16-fold on *E. coli* AG102 and *E. aerogenes* EA27. The improvement in the antibacterial activities of extracts of leaves and flowers of *V. glabra* in the presence of PAβN indicates that constituents of these botanicals are substrates for bacterial efflux pumps.

Effect of V. glabra flower extract on the kinetics of bacterial growth

To determine at which phase of bacterial growth *V. glabra* exerts its antibacterial effect, the growth kinetics of *P. stuartii* in the presence of the most active methanol extract of *V. glabra* (flowers) was performed. Figure 1 represents the growth kinetics of *P. stuartii* ATCC29916 in the absence and in the presence of the extract and of CIP at MIC/2, MIC, and 2MIC. It was found that the growth curve of *P. stuartii* ATCC29916 in the absence or in the presence of the extract at MIC/2 had all the phases of bacterial growth except the last phase: a phase of latency (0 h – 2h), an exponential phase (2 h – 10 h) and a stationary phase (10 h – 20 h). In the presence of the extract at MIC and 2MIC, a decrease in

the exponential phase was noted between 2 h and 8 h, and a prolongation of the stationary phase was observed between 8 h and 20 h. It is worth noting that CIP at MIC caused a shortening of the exponential phase and prolongation of the stationary phase between 6 h and 20 h.

Botanical from V. glabra flower inhibits the H+-ATPases proton pumps

To verify the ability of the flower extract of *V. glabra* to hinder the functioning of the H^+-ATPase proton pumps in *P. stuartii* ATCC29916, the pH of the medium containing *P. stuartii* ATCC29916 was measured (Figure 2). It appears that the pH of the medium containing *P. stuartii* ATCC29916 in the absence of the extract decreases over time. However, the pH of the medium containing *P. stuartii* ATCC29916 in the absence and in the presence of the extract at the MIC/2 had a higher decrease and reach the lowest pH values. They show a decrease in pH from 6.4 to 4.25 in the absence of the extract and 4.45 in the presence of the extract at MIC/2. At MIC the pH decreases to 4.8 while at 2MIC it decreases to 5.15. This is an indication the botanical inhibits the H^+-ATPase proton pumps.

Botanicals potentiated the activity of antibiotics

To evaluate the activity of the crude extracts in association with antibiotics, a preliminary test was carried out against the MDR strain of *Escherichia coli* AG102, to select the extracts which better potentiated the action of antibiotics vis-à-vis this bacterium, and to determine the sub-inhibitory concentrations of appropriate extracts to be used in combination with antibiotics. A preliminary assay of botanicals from *V. glabra* leaf and flower at sub-inhibitory concentrations MIC/2, MIC/4, MIC/8, and MIC/16 was performed; Extracts at MIC/2 and MIC/4 had better antibiotic-potentiating activities (Data not shown). Botanicals were further combined with antibiotics at MIC/2 and MIC/4 and the results are shown in Tables 3 and 4. It appears that the activity of each antibiotic increased in the presence of the extracts of leaves and flowers vis-à-vis at least one bacterium tested with AMF ranging from 2- to 128-fold. The extract from the leaves at MIC/2 potentiated the activity of DOX on 100% (10/10) of the bacteria tested; also, the activities of CIP, VAN, and CRO increased on 90% (9/10) of tested bacteria; the activities of AMP, TET, LEV, and IMI increased vis-à-vis 80% (8/10) tested bacteria. At MIC/4, this extract potentiated the activity of DOX, CIP, and VAN against 90% (9/10) of the bacteria tested; the activity of TET increased against 80% (8/10) of the bacteria tested; the activities of LEV, AMP, and CRO were improved on 70% (7/10) of the bacteria tested. IMI had an improvement in activity on 60% (6/10) of the bacteria tested. With the extract of the flowers of *V. glabra*, a potentiation of the activity of CRO at the MIC/2 vis-à-vis 100% (10/10) of bacteria tested was noted. The activities of IMI, DOX, CIP, and VAN increased on 90% (10/10) of bacteria tested; The activity of TET, LEV, and AMP increased vis-à-vis 80% (8/10), 70% (7/10), and 60% (6/10) of the bacteria tested, respectively. At the MIC/4, the activities of CIP, VAN, and CRO increased on 90% (9/10) of the bacteria tested; That of DOX increased against 70% (7/10) of bacteria tested. The remaining antibiotics were potentiated by this extract with potentiation percentages varying from 50 to 60%.

Phytochemical composition of the botanicals

The phytochemical composition of methanol extracts from the flowers and leaves of *V. glabra* is shown in Table 5. It can be noted that the extracts of flowers and leaves of *V. glabra* contain alkaloids, flavonoids, triterpenes, saponins, tannins, phenols, and anthocyanins.

Discussion

The importance of medicinal plants as a source of potential medicine to fight bacterial, fungal, parasitic, and viral infections, as well as MDR cancer phenotypes, has been largely demonstrated [63-86]. In the present study, the ability of the botanicals from *V. glabra* against MDR bacteria expressing active efflux pumps was determined. According to the established classification scale for the antibacterial activities of plant extracts against Enterobacteriaceae [57], the methanol extract of *V. glabra* flowers showed excellent activity against *E. coli* AG100, *P. stuartii* ATCC29916, *K. pneumoniae* K2, E. coli ATCC10536, *P. stuartii* (PS2636, NEA16), and *E. aerogenes* (EA3, EA298); extract of the leaves had good activity against *K. pneumoniae* ATCC11296 and *P. stuartii* PS2636. According to the classification standards against *P. aeruginosa* [58], the methanol extract of *V. glabra* flowers had excellent activity against *P. aeruginosa* PA01 and PA121. As *V. glabra* leaves are edible, it should be noted that the botanical had significant effects on many tested bacteria [56].

The results of the antibacterial activity of the extracts of the leaves of *V. glabra* obtained in this study against *E. coli* and *P. aeruginosa* corroborate the results of the research work of Ngonda et al. [21] who showed that acetone extract of *V. glabra* leaves had excellent activity on *E. coli* and on *P. aeruginosa* with MIC values of 0.625 mg/mL and 0.313 mg/mL, respectively. According to these authors, the antibacterial activity of *V. glabra* is due to the presence of flavonoids which can complex with extracellular and soluble proteins and other components of the bacterial wall. Besides, the work of Kitonde et al. [22] on the antibacterial activity of the dichloromethane-methanol extract of the leaves of *V. glabra* against *E. coli* showed that this extract had a very good activity with a MIC value of 100 µg/mL. This result is in line with ours, although it should be noted that according to the results of the work of Kitonde et al. [22] carried out in Kenyan samples, the flower extract was not active against *E. coli*; this difference with the data recorded herein on the same part of the plant could be due to the difference in the extraction solvents used and/or to the pedoclimatic conditions which influence the qualitative and quantitative chemical composition in secondary metabolites of the plant. From the work of Banda et al. [87] on the dichloromethane extract of the roots of *V. glabra* against *Mycobacterium tuberculosis*, this extract showed exceptional activity with a MIC value of 4.88 µg/mL. This investigation is a confirmation of the interesting antibacterial property of *V. glabra*. *V. glabra* flower extract had bactericidal effects against *P. aeruginosa* PA124, *E. aerogenes* (EA27, EA298) and *P. stuartii* NEA16, and bacteriostatic on the rest of the bacteria. The phytochemical screening revealed the presence of alkaloids, phenols, flavonoids, tannins, triterpenes, saponins, and anthocyanins. These results are in agreement with the work of de Ngonda et al. [21] and Kitonde et al. [22] who showed that extracts from the leaves, flowers, and root of *V. glabra* contained alkaloids, terpenoids, quinones, flavonoids, saponins, steroids, phytosterols, and phenolic compounds. Indeed, it is well established that the antibacterial activity of a plant is linked to its composition in secondary metabolites [9, 88].

The ability of the crude extract of *V. glabra* flowers to influence one or more phases of the growth of *P. stuartii* ATCC29916 was determined (Figure 1). During the lag phase,

which lasts less than 2 hours under normal conditions, bacteria synthesize enzymes that metabolize nutrient substrates for growth and multiplication [89]. During the exponential phase, the bacterial cell multiplies rapidly, in the stationary phase the number of dead is equal to the number of living bacteria. The decline phase corresponds to a total lack of nutrients and an accumulation of toxic waste in the environment leading to the death of a greater number of bacteria [90]. In the present study, the growth curve of *P. stuartii* ATCC29916 in the absence of extract shows all these phases of normal bacterial growth. In the presence of the extract of the flowers of *V. glabra*, a shortening of the exponential phase is observed, suggesting an inhibition of the growth of *P. stuartii* ATCC29916 in its exponential growth phase. This inhibition could be due to the presence of alkaloids present in the flowers of *V. glabra*. Indeed, according to Di Somma et al. [91], alkaloids can block all bacterial division by inhibiting the temperature-sensitive filamentous protein Z (FTsZ) responsible for bacterial division.

The extract from the flowers of *V. glabra* was also tested on the H^+-ATPase-dependent proton pumps of *P. stuartii*. Indeed, the energy necessary for the development of the metabolic reactions of bacteria depends on the proper functioning of these H^+-ATPase-dependent proton pumps, which are protein enzymes necessary for the formation of a large electrochemical gradient of protons and the maintenance of the Intracellular cytoplasmic pH [92]. The inhibition of these pumps by a substance leads to the reduction of H^+/protons in the extracellular medium which will become less and less acid indicating the inactivation of the H+-ATPase pumps, compromising the survival of the bacterium which will die because of the lack of energy [92]. From the data obtained in the present study, the extract of the flowers of *V. glabra* tested induced an inhibition of the H^+-ATPase-dependent proton pumps in *P. stuartii* ATCC29916.

In this work, the MICs of the extracts of leaves and flowers of *V. glabra* and of imipenem in the presence of an inhibitor of efflux pumps (PAβN) increased in 72.72% and 90.90% (10/11) and (8/11) bacteria tested, respectively. These results corroborate the work carried out by Kuete et al. [24] who showed that in the presence of PAβN, the activity of the natural products increases if they are substrate of EPI. This is therefore a confirmation that the overexpression of the efflux pumps is the mode of resistance of the tested MDR bacteria.

The combination of antibiotics and plant extracts is an important means of mitigating antimicrobial resistance [93]. In this study, the results obtained showed that in the presence of extracts of flowers and leaves of *V. glabra*, the activities of DOX and CRO increased vis-à-vis 100% bacteria tested, while that of all eight antibiotics (LEV, IMI, AMP, TET, DOX, CIP, VAN and CRO) increased against at least 70% of the bacteria tested with AMF ranging from 2 to 128. The modulating effects of the extracts of the leaves and flowers of *V. glabra* might be due to the inhibition of the efflux pumps or the expression of their genes by the bioactive substances contained in the extracts with the ability to increase intracellular concentrations of antibiotics allowing them to act effectively on bacterial targets [94]. Also, phenolics such as flavonoids detected in the flowers and leaves of *V. glabra* can inhibit bacterial resistance mediated by the production of beta-lactamases [95]. Based on the work of Braga et al. [96, 97], substances capable of potentiating the activity of antibiotics on at least 70% of a panel of MDR bacteria by overexpression of efflux pumps are potential efflux pump inhibitors. The extracts of leaves and flowers of *V. glabra* could therefore be potential sources of efflux pump inhibitors.

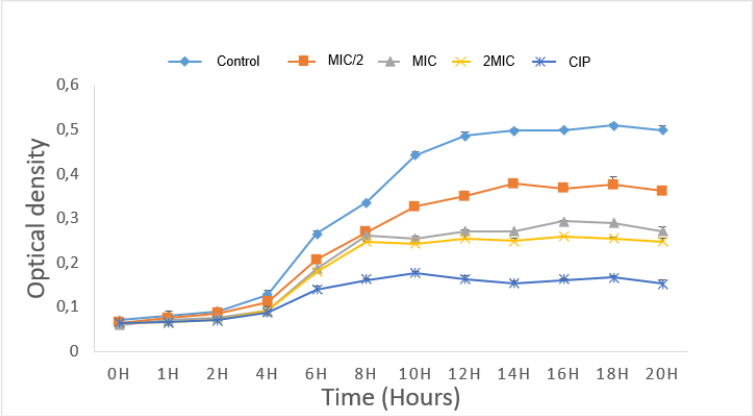

Figure 1. Effect of methanol extract of *V. glabra* flowers on growth kinetics of *P. stuartii* ATCC29916.

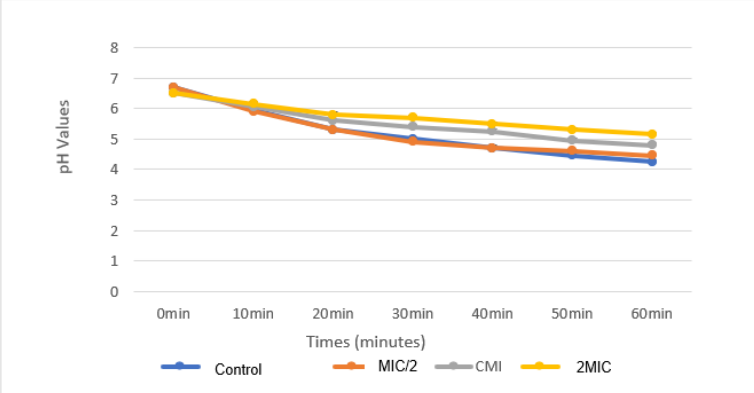

Figure 2. Effect of methanol extract of *V. glabra* flowers on H^+/ATPase proton pumps of *P. stuartii* ATCC29916.

Table 1. Minimal inhibitory and bactericidal concentrations of *V. glabra* flower and leaf extracts (µg/mL).

Bacterial species		*Tested samples and MIC values*								
		V. glabra leaf			*V. glabra* flower			Imipenem		
		MIC	MBC	R	MIC	MBC	R	MIC	MBC	R
Pseudomonas aeruginosa	PA01	512	2048	4	128	-	nd	16	64	4
	PA121	1024	-	nd	128	1024	8	16	128	8
	PA124	1024	-	nd	256	1024	4	4	32	8
Klebsiella pneumoniae	K2	512	-	nd	64	512	8	<4	64	16
	KP55	1024	-	nd	256	2048	8	<4	64	16
	ATCC11296	256	2048	8	128	2048	16	<4	<4	1
Escherichia coli	AG100	512	1024	2	32	1024	32	8	64	8
	AG102	>2048	-	nd	256	-	nd	16	64	4
	ATCC10536	512	2048	4	64	512	8	8	64	8
Providencia stuartii	PS2636	256	-	nd	64	1024	16	8	64	8
	NEA16	512	1024	nd	64	256	4	8	64	8
	ATCC29916	512	-	nd	32	-	nd	<4	16	4
Enterobacter aerogenes	EA3	1024	-	nd	64	-	nd	8	64	8
	EA27	2048	-	nd	256	512	2	8	16	2
	EA298	512	2048	4	64	256	4	8	8	1

MIC: Minimal inhibitory Concentrations; MBC: Minimum Bactericidal Concentration; R: MBC/MIC ratio; nd or (-): not determined.

Table 2. Minimal inhibitory concentrations of *V. glabra* extracts in the absence and in the presence of PAβN.

Bacterial species		*Tested samples and MIC values*								
		V. glabra leaf			*V. glabra* flower			Imipenem		
		MIC alone	+PAβN	R	MIC alone	+PAβN	R	MIC alone	+PAβN	R
Pseudomonas aeruginosa	PA01	512	<16	>32	128	<16	>8	16	16	1
	PA121	1024	16	64	128	16	8	16	2	8
Klebsiella pneumoniae	K2	512	16	32	64	16	4	<4	1	<4
	KP55	1024	512	2	256	128	2	<4	4	<4
Escherischia coli	AG100	512	32	16	32	<16	2	8	2	4
	AG102	>2048	512	>4	256	16	16	16	2	8
	ATCC10536	512	16	32	64	<16	>4	8	1	8
Providencia stuartii	PS2636	256	16	16	64	64	1	8	4	2
	NEA16	512	512	1	64	64	1	8	8	1
Enterobacter aerogenes	EA3	1024	<16	>64	64	64	1	8	4	2
	EA27	2048	512	4	256	16	16	8	<1	>8

MIC alone: Minimal inhibitory concentration in the absence of the inhibitor, +PaβN: MIC in the presence of the inhibitor, R: MIC alone *vs* MIC with PAβN ratio, nd: not determined.

Table 3. MICs (µg/mL) of antibiotics in the absence and presence of *V. glabra* leaf extract.

ATB	Extract concentration	MIC of antibiotics in the presence of extract and Antibiotic-resistance modulating factor (AMF)										PSP (%)
		E. aerogenes		*P. stuartii*		*E. coli*		*P. aeruginosa*		*K. pneumoniae*		
		EA3	EA27	PS2636	NEA16	ATCC10536	AG100	PA01	PA121	K2	KP55	
LEV	0	1	1/2	1/4	1/2	1/4	1/4	1/2	1/2	1/2	1/2	
	MIC/2	2 (0.5)	<1/16 (8)	<1/16 (4)	<1/16 (8)	<1/16 (4)	1/4 (1)	1/4 (2)	<1/16 (8)	1/8 (4)	<1/16 (8)	80
	MIC/4	4 (0.25)	<1/16 (8)	<1/16 (4)	<1/16 (8)	<1/16 (4)	1/4 (1)	1/2 (1)	1/4 (2)	1/8 (4)	<1/16 (8)	70
IMI	0	8	8	8	8	8	8	16	16	<4	4	
	MIC/2	2 (4)	1/16 (128)	4 (2)	8 (1)	<1/2 (16)	2 (4)	16 (1)	8 (2)	1 (4)	1/16 (64)	80
	MIC/4	4 (2)	1/2 (16)	8 (1)	8 (1)	<1/2 (16)	4 (2)	16 (1)	16 (1)	2 (2)	1/16 (64)	60
DOX	0	1	1/2	8	8	1	1/2	1/2	1	2	8	
	MIC/2	<1/16 (16)	<1/16 (8)	<1/16 (128)	1 (8)	<1/16 (16)	1/8 (4)	<1/16 (8)	1/8 (8)	1/8 (16)	1 (8)	100
	MIC/4	<1/16 (16)	<1/16 (8)	<1/16 (128)	1 (8)	<1/16 (16)	1/4 (2)	1/8 (4)	1 (1)	1/8 (16)	1 (8)	90
CIP	0	1	1/4	1/4	8	1/4	1/2	1	1/2	1/2	1/2	
	MIC/2	4 (0.25)	<1/16 (4)	<1/16 (4)	<1/16 (128)	<1/16 (4)	1/4 (2)	1/4 (4)	<1/16 (8)	1/8 (4)	<1/16 (8)	90
	MIC/4	4 (0.25)	<1/16 (4)	<1/16 (4)	<1/16 (128)	<1/16 (4)	1/4 (2)	1/4 (4)	1/8 (4)	1/8 (4)	<1/16 (8)	90
VAN	0	64	2	2	8	2	256	2	2	>64	>64	
	MIC/2	<2 (32)	1/2 (4)	<1/2 (4)	<1/2 (16)	<1/2 (4)	<2 (128)	1 (2)	<1/2 (4)	64 (1)	<1/2 (128)	90
	MIC/4	<2 (32)	1/2(4)	<1/2 (4)	<1/2 (16)	<1/2 (4)	8 (32)	1 (2)	<1/2 (4)	64 (1)	<1/2 (128)	90
AMP	0	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	
	MIC/2	4 (64)	<2 (128)	<2 (128)	<2 (128)	<2 (128)	32(8)	>256 (1)	16 (16)	>256 (1)	2 (128)	80
	MIC/4	4 (64)	<2 (128)	8 (32)	<2 (128)	4 (64)	>256(1)	>256 (1)	32 (8)	>256 (1)	2 (128)	70
TET	0	8	>8	1/4	>8	4	2	1/4	1	>8	>8	
	MIC/2	1/8 (64)	<1/16 (128)	<1/16 (4)	8(1)	<1/16 (64)	1/2 (4)	1/8 (2)	<1/16 (16)	>8 (1)	1 (8)	80
	MIC/4	1/8 (64)	<1/16 (128)	<1/16 (4)	8(1)	1/4 (16)	1/2 (4)	1/8 (2)	<1/16 (16)	>8 (1)	1(8)	80
CRO	0	32	32	64	256	8	32	8	16	8	64	
	MIC/2	8 (4)	<2 (16)	64 (1)	128 (2)	<2 (4)	2 (16)	4 (2)	<2 (8)	2 (4)	<2 (32)	90
	MIC/4	16 (2)	8 (4)	64 (1)	128 (2)	<2 (4)	4 (8)	8 (1)	4 (4)	8 (1)	<2 (32)	70

MIC: Minimal inhibitory Concentration; (): AMF (Activity modulating Factors); PSP (%): percentage of strain where potentiation effect was observed; ATB: Antibiotic; LEV: Levofloxacin; VAN: Vancomycin; CIP: Ciprofloxacin; TET: Tetracycline; DOX: Doxycycline; IMI: Imipenem; Ceftriaxone: CRO; AMP: Ampicillin.

Table 4. MICs (µg/mL) of antibiotics in the absence and presence of *V. glabra* flower extract

ATB	Extract concentration	MIC of antibiotics in the presence of extract and Antibiotic-resistance modulating factor (AMF)										PSP (%)
		E. Aerogenes		*P. stuartii*		*E. coli*		*P. aeruginosa*		*K. pneumoniae*		
		EA3	EA27	PS2636	NEA16	ATCC10536	AG100	PA01	PA121	K2	KP55	
LEV	0	1	1/2	1/4	1/2	1/4	1/4	1/2	1/2	1/2	1/2	
	MIC/2	8 (0.125)	1/4 (2)	<1/16 (4)	<1/16 (8)	<1/16 (4)	1/4 (1)	1/4 (2)	1/8 (4)	1/2 (1)	1/8 (4)	70
	MIC/4	8 (0.125)	1/4 (2)	<1/16 (4)	<1/16 (8)	<1/16 (4)	1/4 (1)	1/2 (1)	1/2 (1)	1/2 (1)	1/8 (4)	50
IMI	0	8	8	8	8	8	8	16	16	<4	4	
	MIC/2	1/2 (16)	1 (8)	4 (2)	8(1)	<1/2 (16)	2 (4)	4 (4)	4 (4)	2 (2)	1/16 (64)	90
	MIC/4	8 (1)	2 (4)	8 (1)	8(1)	<1/2 (16)	2 (4)	16 (1)	8 (2)	8 (0,5)	1/16 (64)	50
DOX	0	1	1/2	8	8	1	1/2	1/2	1	2	8	
	MIC/2	<1/16 (16)	1/8 (4)	<1/16 (128)	1(8)	<1/16 (16)	1/8 (4)	1/4 (2)	1(1)	1/8(16)	1 (8)	90
	MIC/4	<1/16 (16)	1/4 (2)	<1/16 (128)	1 (8)	<1/16 (16)	1/8 (4)	1/4 (2)	1(1)	2(1)	8 (1)	70
CIP	0	1	1/4	1/4	8	1/4	1/2	1	1/2	1/2	1/2	
	MIC/2	1/2 (2)	1/4 (1)	<1/16 (4)	1 (8)	<1/16 (4)	1/4 (2)	1/8 (8)	<1/16 (8)	1/4(2)	<1/16 (8)	90
	MIC/4	1/2 (2)	1/4 (1)	<1/16 (4)	1 (8)	<1/16 (4)	1/4 (2)	1/4 (4)	1/4 (2)	1/4 (2)	<1/16 (8)	90
VAN	0	64	2	2	8	2	256	2	2	>64	>64	
	MIC/2	<2 (32)	<1/2 (4)	<1/2 (4)	<1/2 (16)	<1/2 (4)	<2 (128)	1 (2)	<1/2(4)	64 (1)	<1/2 (128)	90
	MIC/4	2 (32)	1 (2)	<1/2 (4)	1/2 (16)	<1/2 (4)	16 (16)	1 (2)	1 (2)	64 (1)	<1/2 (128)	90
AMP	0	>256	>2 56	>256	>256	>256	>256	>256	>256	>256	>256	
	MIC/2	<2 (128)	256 (1)	<2 (128)	<2 (128)	8 (32)	>256 (1)	32 (8)	8 (32)	>256 (1)	256 (1)	60
	MIC/4	8 (32)	>256 (1)	<2 (128)	<2 (128)	32 (8)	>256 (1)	32 (8)	>256 (1)	>256 (1)	256 (1)	50
TET	0	8	>8	1/4	>8	4	2	1/4	1	>8	>8	
	MIC/2	<1/16 (128)	1(8)	<1/16 (4)	8(1)	1/4 (16)	1/2 (4)	1/8 (2)	<1/16 (16)	>8 (1)	1(8)	80
	MIC/4	<1/16 (128)	1(8)	<1/16 (4)	8(1)	1/2 (8)	1 (2)	1/4 (1)	<1/16 (16)	>8 (1)	8(1)	60
CRO	0	32	32	64	256	8	32	8	16	8	64	
	MIC/2	16 (2)	16 (2)	<2 (32)	32 (8)	<2 (4)	4 (8)	4 (2)	<2 (8)	4 (2)	<2 (32)	100
	MIC/4	16 (2)	16 (2)	32 (2)	128 (2)	<2 (4)	4 (8)	8 (1)	<2 (8)	4 (2)	<2(32)	90

MIC: Minimal inhibitory Concentration; (): AMF (Activity modulating Factors); PSP (%): percentage of strain where potentiation effect was observed; ATB: Antibiotic; LEV: Levofloxacin; VAN: Vancomycin; CIP: Ciprofloxacin; TET: Tetracycline; DOX: Doxycycline; IMI: Imipenem; Ceftriaxone: CRO; AMP: Ampicillin.

Table 5. Qualitative phytochemical composition of flower and leaf extracts of *V. glabra*

Phytochemical classes	Botanical of *V. glabra*	
	Flower	Leaf
Alkaloids	+	+
Flavonoids	+	+
Triterpenes	+	+
Saponins	+	+
Tannins	+	+
Phenols	+	+
Anthocyanins	+	+

(+): present; (-): absent

Conclusion

In the present study, the antibacterial potential and the modes of action of extracts of *V. glabra* against MDR Gram-negative bacteria overexpressing the efflux pumps were determined. It was demonstrated that extracts of flowers and leaves of *V. glabra* had antibacterial activities against the tested MDR bacteria; They contain alkaloids, phenols, flavonoids, tannins, triterpenes, and saponins. The methanol extract of *V. glabra* flowers inhibited bacterial growth of *P. stuartii* ATCC29916 at the exponential phase and disrupted the functioning of H^+-ATPase-dependent proton pumps in this bacterium. Constituents from *V. glabra* flowers and leaves are substrates for bacterial efflux pumps. The botanicals from flowers and leaves improved the activity of imipenem,

ampicillin, tetracycline, doxycycline, ciprofloxacin, vancomycin ceftriaxone and levofloxacin against MDR bacteria tested. Finally, methanol extracts of the flowers and leaves of *V. glabra* are potential sources of antibacterial molecules effective alone and in combination with antibiotics against MDR Gram-negative bacteria.

Abbreviations

ATCC: American-type culture collection
MBC: Minimum Bactericidal Concentration
MIC: Minimal inhibitory Concentration
DMSO: Dimethylsulfoxide
EMB: Eosin Methylene Blue
AMF: Activity modulating Factor
HNC: National Herbarium of Cameroon
INT: Iodonitrotetrazolium chloride
MDR: Multidrug resistant
MHA: Mueller Hinton agar
MHB: Mueller Hinton broth
WHO: World Health Organization
PAβN: Phenylalanine Arginine-β-Naphthylamide
RND: Resistance nodulation-cell division
CFU: Colony Forming Unit

Authors' Contribution

RN, VYM, GKF, SMT, JFM, and PN carried out the study; ATM and VK supervised the study; All authors read and approved the final version of the manuscript.

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Conflict of interest

The authors declare no conflict of interest.

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