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Antibacterial potential and modes of action of botanicals from *Momordica foetida* (Cucurbitaceae) against multidrug-resistant bacteria expressing active efflux pumps

Cedric F. Tchinda[1], Olive Monique F. Demgne[2], Armelle T. Mbaveng[3*], Veronique P. Beng[2], Victor Kuete[3**]

Abstract

Background: *Momordica foetida* (cucurbitaceae) known as a bitter cucumber in Cameroon is used as a medicinal plant. This plant is widely used in the treatment of microbial infections, diabetes, hypertension, stomach, and intestinal irritation. The leaves are also used to treat malaria, earache, and toothache. This study aimed at investigating the antibacterial activity of the methanol extract of *M. foetida* seeds against multidrug-resistant bacteria expressing active efflux pumps and to characterize the mode of action of this extract.

Methods: The antibacterial activity of the crude extract of *M. foetida* in the presence and absence of an efflux pump inhibitor, phenylalanine-arginine *β*-naphthylamide (PAβN), was evaluated by the serial micro-dilution method. The determination of the effect of this sample on bacterial H^+-ATPase proton pumps was performed by a standard method using a pH meter and the study of the effect of the same sample on cell growth kinetics, bacterial membrane, and biofilms was performed by a spectrophotometric method.

Results: The phytochemical composition of the crude extract of *M. foetida*, evaluated using standard qualitative methods, showed a rather selective distribution of secondary metabolites (presence of polyphenols, flavonoids, alkaloids, triterpenes, sterols, and saponins). The crude extract of this plant virtually inhibited the growth of 90.90% (20/22) of the bacteria studied with minimal inhibitory concentrations (MIC) ranging from 64 to 1024 µg/mL. The lowest MIC of 64 µg/mL was observed against *Escherichia coli* (AG102, MC4100, W3110), *Klebsiella pneumoniae* (KP55 and ATCC 11296), *Providencia stuartii* (NEA16, ATCC 29916 and PS2636), *Pseudomonas aeruginosa* (PA124) and *Staphylococcus aureus* (MRSA9) strains. In the presence of PAβN, the activity of the extract was increased against all strains and isolates used. The crude extract caused a prolongation of the lag phase and a general decrease in the growth of *Escherichia coli* AG102 bacteria due to the blocking of the H^+-ATPase proton pumps of this bacterium, resulting in a decrease in the acidity of the medium. This sample destroyed the bacterial membrane and showed significant activity on the biofilms of this strain tested.

Conclusion: The results obtained in the present work provide important and significant data that could allow the probable use of *Momordica foetida* in the control of bacterial infections.

Keywords: Bacteria; Cucurbitaceae; modes of action; efflux pumps; *Momordica foetida*; multidrug resistance.

*Correspondence: *Tel.: +237 676542386; E-mail address: armbatsa@yahoo.fr; ORCID: https://orcid.org/0000-0003-4178-4967 (Armelle T. Mbaveng); **Tel.: +237 677355927; E-mail address: kuetevictor@yahoo.fr; ORCID: http://orcid.org/0000-0002-1070-1236 (Victor Kuete)*

[1]Centre for Research on Medicinal Plants and Traditional Medicine, Institute of Medical Research and Medicinal Plants Studies (IMPM), Ministry of Scientific Research and Innovation, Yaoundé, Cameroon; [2]Department of Biochemistry, Faculty of Science, University of Yaoundé I, Yaoundé, Cameroon; [3]Department of Biochemistry, Faculty of Science, University of Dschang, Dschang, Cameroon

Other authors:
E-mail: tchindafossicedric@yahoo.fr (Cedric F. Tchinda) ; E-mail: princessdemleur@yahoo.fr (Olive Monique F. Demgne); E-mail: v.penlap@yahoo.fr (Veronique P. Beng)

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Background

Infectious diseases are currently a major public health concern. They are responsible for 17 million deaths worldwide each year, accounting for approximately 30% of global mortality, particularly in developing countries such as South Asia and Sub-Saharan Africa. Bacterial infection is responsible for 560 000 of the 2.7 million neonatal deaths recorded each year [1].

Despite the existence of several strategies for bacterial infection control, antibiotic therapy is still the most important. Despite the impact of antibiotic therapy on human health, the population's inappropriate and excessive use of antibiotics has resulted in selection pressure by bacteria [2, 3, 4], resulting in the emergence and re-emergence of many diseases that are responsible for increased hospitalization time, cost of care, and mortality rate [5]. In addition, the emergence of MDR bacterial strains is the primary cause of therapeutic failure [6]. Active efflux via the RND (Resistance-Nodulation-Division) and MFS (Major Facilitator Superfamily) pumps for Gram-negative and Gram-positive bacteria, respectively [7], which support different antibiotic families, is one of the known resistance mechanisms. In view of this increasingly worrying situation due to the growth of these resistances throughout the world, the search for new molecules capable of combating these resistances more effectively has become an absolute necessity. Medicinal and edible plants rich in bioactive substances, particularly those from Cameroon, have proven to inhibit the growth of most Gram-positive and Gram-negative MDR bacteria [8-21]. Most of these plants, including *M. foetida* (cucurbitaceae), a plant of the Cameroonian pharmacopoeia, are traditionally used to treat many infectious diseases caused by resistant micro-organisms [22]. In the present study, the activity of the crude methanol extract of this plant was investigated against some MDR bacteria in the presence of phenylalanine-arginine-β-naphthylamide (PAβN) and the effects of the latter on growth kinetics, bacterial membrane, proton pumps, and biofilms was evaluated.

Methods

Plant material and extraction

This medicinal plant used in the present work was collected from Mberenka (Southwest region, Cameroon) and was identified at the National Herbarium (Yaounde, Cameroon) where a voucher specimen was deposited under the following reference number 8114/SRF/Cam/K.

This plant sample was air-dried at laboratory temperature (22 ± 2ºC) and then powdered. The resulting powder was extracted with methanol (1:3 w/v) for 48 h at room temperature. The extract was then concentrated under reduced pressure at about 40ºC, to give a residue that constituted the crude extract. The extract was then stored at 4ºC until further use.

Chemicals for antimicrobial assay

The chemicals used in this study consisted of the reference antibiotics (RA) and the microbial growth indicator. *p*-iodonitrotrazolium chloride ≥97% (INT, Sigma-Aldrich) and phenylalanine-arginine-*β*-naphthylamide (PAβN; Sigma-Aldrich) were used as microbial growth indicator and efflux pump inhibitor (EPI), respectively. The reference antibiotics used were a Chloramphenicol (CHL), ciprofloxacin (CIP) and Polymyxin B (Poly B). The developer of the biofilm formation was the purple crystal. A 2.5% dimethyl sulfoxide (DMSO) was used to dissolve our test samples. These molecules and reagents were obtained from Sigma Aldrich (St. Quentin Fallavier, France).

Bacteria strains and culture media

The microorganisms used were multidrug-resistant (MDR) bacteria, phenotypes involved in microbial infections, and expressing efflux pumps. These bacteria consisted of a panel of 22 bacteria (18 Gram-negatives and 4 Gram-positives). They included resistant strains of *Escherichia coli, Enterobacter aerogenes, Klebsiella pneumoniae, Providencia stuartii, Pseudomonas aeruginosa* and *Staphylococcus aureus.* These bacterial strains were provided by the American Type Culture Collection (ATCC) and the laboratory of UMR-MD1 of the University of Mediterranean, Marseille, France. The characteristics of these bacteria were previously reported [17, 23]. Bacterial strains were maintained on agar plates at 4°C and subcultured onto appropriate fresh agar plates 24 hours before any antibacterial testing. Mueller Hinton agar (MHA; Sigma) was used for bacterial activation and Mueller Hinton broth (MHB; Sigma) was used as a liquid medium for antibacterial assays.

Antibacterial testing

The respective minimal inhibitory concentrations (MICs) of samples on the studied bacteria were determined using rapid INT colorimetric assay described by Eloff [24] with some modifications [25]. Briefly, the test samples were first dissolved in DMSO/MHB mixture. The solution obtained was then added to MHB and serially diluted two-fold (in a 96-well microplate). One hundred microlitres (100 µL) of inoculum (1.5×10^6 CFU/mL) prepared in MHB was then added. The plates were covered with a sterile plate sealer, then agitated to mix the contents of the wells using a shaker and incubated at 37°C for 18 h. The final concentration of DMSO was less than 2.5% and does not affect microbial growth. Wells containing MHB, 100 µL of inoculum, and DMSO at a final concentration of 2.5% served as a negative control. Chloramphenicol and Ciprofloxacin were used as reference antibiotics for Gram-negative and Gram-positive bacteria respectively. The MICs of samples were determined after 18 h of incubation at 37°C, following the addition of (40 µL) of 0.2 mg/mL INT and incubation at 37°C for 30 minutes [26]. Viable bacteria reduced the colorless dye to pink. MIC was defined as the lowest sample concentration that prevented this change and exhibited complete inhibition of microbial growth. For the minimal bactericidal concentrations (MBCs) determination, a volume of 150 µL of MHB has been introduced in a new 96-well microplate, following the addition of 50 µL of the previous well microplate contents where no microbial growth was observed, which did not receive an INT (during the reading of MICs). After 48 h incubation, at 37°C, the MBC of each sample was determined and defined by adding 40 µL of 0.2 mg/mL INT as previously described. It should be noted that samples were tested alone and then, in the presence of PAβN, an efflux pump inhibitor, at 30 mg/L final concentration. In this last case, the activity improvement factors (AIFs) were determined to qualify the potentiation level of sample activity by this inhibitor, using the $MIC_{sample\ alone}/MIC_{sample\text{-}PA\beta N}$ combination ratio. All assays were performed in triplicate and repeated thrice.

Phytochemical screening

The presence of the major classes of secondary metabolites, namely alkaloids, polyphenols, flavonoids, triterpenes, sterols, and

saponins was determined using the standard phytochemical methods described by Harborne [27].

Effect of the test sample on bacterial growth kinetic

The bacterial growth kinetic study of the test sample was realized through the spectrophotometric method at a wavelength of 600 nm [28]. The bacterium used for this study was a reference strain *Escherichia coli* AG102 and the sample was tested at the concentrations of 0.5 x MIC, MIC, and 2 x MIC. Firstly, 500 µL of bacterial suspension ($1.5x10^8$ UFC/mL) from preculture was added to 450 mL MHB (1/100 v/v dilution) and incubated at 37°C for 18 h under magnetic agitation and in the presence of tested sample at different concentrations. Reference antibiotic, chloramphenicol was used as positive control whereas, inoculum ($1.5x10^8$ UFC/mL)/DMSO (2.5% v/v) mixture constituted the negative control. At 0, 0.5, 1, and 2 h followed by regular interval times of 2 h from 2 to 24 h, aliquots of 1 mL from the preparation were deducted and introduced in a spectrophotometer (THERMO SCIENTIFIC, Langenselbold, Germany) tab and then, the optical density was read at 600 nm wavelength. From the obtained results, bacterial growth curves [OD = f (times)] were established using Microsoft Excel software (Figure 1).

Effect of the test sample on the bacterial membrane

The effect of the extract on the membrane was carried out by the method described by Leejae et al. [29] with some modifications. Briefly, a bacterial suspension with an optical density (OD) of 2.0 was prepared from a fresh 18 h culture. Then, the bacterial cells were separated from the medium by centrifugation at 400 g, for 15 min, washed twice in PBS buffer (pH 7.4), and re-suspended in the same buffer. The suspensions were treated with the extract at concentrations of 0.5 x MIC, MIC, and 2 x MIC. Polymyxin B was used as a positive control. The culture was incubated at 37°C for 60 min under agitation. Samples taken at times 0, 30, and 60 min of the experiment were centrifuged at 13400 g for 15 min. At each time and for each concentration of extract, the optical density was measured at 260 nm with a spectrophotometer (THERMO SCIENTIFIC, Langenselbold, Germany) (Figure 2).

Effect of the test sample on bacterial H^+ -ATPase- dependent proton pumps

The ability of the tested sample to inhibit the H^+-ATPase-mediated proton pumps of *Escherichia coli* AG102 was evaluated by monitoring the acidification of the bacterial growth medium as previously described by Manavathu et al. [30] with slight modifications. Briefly, 100 mL of bacteria suspension was cultured in MHB for 18 h at 37°C. The resulting culture was centrifuged at 3000 tr/min for 10 min at 4°C. The pellet was first washed twice in distilled water, then in 50 mM KCl, and suspended in 50 mL of 50 mM KCl. Then, the cell suspension was incubated overnight (18 h) at 4 ◦C for glucose starvation. In 4.0 mL of the cell medium, 0.5 mL of the tested sample at different concentrations (0.5 x MIC, MIC, and 2 × MIC) were added, and pH was adjusted to 6.8 with 1 M HCl or 0.1M NaOH. During 10 min pre-incubation at 37°C, medium acidification was initiated by the addition of 0.5 mL of 20% glucose, followed by pH measurement every 10 min for 1 h using a pH meter. The curves [pH = f (times)] were labelled using Microsoft Excel software (Figure 3). The tube containing MHB, inoculum, and DMSO was used as a negative control. Each essay was performed in triplicates.

Effect of the test sample on the Inhibition of biofilm formation

Anti-biofilm activity was assessed in 96-well flat bottom plates using a method adapted by Chaib et al. [31] with some modifications. The *Escherichia coli* bacterial strain (*Escherichia coli* AG102) was treated in microdilution plates with the tested sample at concentrations of 1024 µg/mL – 8 µg/mL, and then incubated at 37°C for 18 h under orbital shaking (REMI) at 7 g. Next, the planktonic cells were removed, and the plates were rinsed with sterile distilled water and stained for 15 minutes by incorporating 200 µL of crystal violet into each well. Leaching was performed with the addition of acetic acid (30%). The wells were then rinsed with sterile distilled water followed by measurement of the absorbance of the crystal violet solution at 595 nm.
The tests were repeated three times and the Minimum Biofilm Inhibitory Concentration 50% (MBIC50) was deduced using the biofilm inhibition percentages for each test calculated in relation to the untreated control values, according to the following formula:

% inhibition = [OD (untreated value) - OD (treated value) / OD (untreated value)] ×100
% inhibition = Percentage inhibition; Untreated value = Optical density of the biofilm alone; Treated value = Optical density of the biofilm in the presence of the test sample.

Effect of the test sample on formed biofilm

The effect of the sample tested on formed biofilm was studied using the method previously described by Chaib et al. [21] with slight modifications. The bacterial culture of a strain of *Escherichia coli* AG102 was previously prepared in liquid medium (MHB) and incubated at 37°C for 18 hours under an orbital shaking Incubator, REMI at 7 g. After biofilm formation, the planktonic cells were removed, and the plates were treated with a sample tested at varying concentrations between 1024 µg/mL - 8µg/mL. Subsequently, the plates were re-incubated at 37°C for 18 hours under Orbital Shaking Incubator, REMI (7 g) and rinsed with sterile distilled water, then stained for 15 minutes by incorporating 200 µL of crystal violet into each well. Discoloration with acetic acid (30%), followed by measurement of absorbance of the violet crystal solution at 595 nm was carried out after the biofilms were stained and rinsed with sterile distilled water. The tests were repeated three times and the Biofilm Eradication Concentrations (BEC) were deduced using the biofilm eradication percentages for each test which were calculated in relation to the untreated control values, according to the following formula:

% eradication = [OD (untreated value) - OD (treated value) / OD (untreated value)] ×100
Eradication % = Eradication percentage; Untreated value = Optical density of the preformed biofilm; Treated value = Optical density of the preformed biofilm in the presence of the test sample.

Results

Antibacterial activity

The antibacterial susceptibility of the tested sample was evaluated by determining the MICs and MBCs of each sample on the bacteria under study (Table 1). The MBC/MIC ratio was used to calculate the bactericidal or bacteriostatic effects of each sample on a bacterial strain. These findings show that crude extract *M. Foetida* had antibacterial activity on 90,90% (20/22) of the strains tested,

with MICs ranging from 64 à 1024 µg/mL. The lowest MIC of 64 µg/mL was obtained against *Escherichia coli* (AG102, MC4100, W3110), *Klebsiella pneumoniae* (KP55 and ATCC 11296), *Providencia stuartii* (NEA16, ATCC 29916 and PS2636), *Pseudomonas aeruginosa* (PA124) and *Staphylococcus aureus* MRSA9. According to the MBC/MIC values, the effects of the studied extract were mainly bacteriostatic (MBC/MIC > 4). The extract showed bactericidal activity against the *Providencia stuartii* strain (NEA16). For Gram-negative and Gram-positive bacteria, CHL and CIP were used as reference antibiotics, respectively. CHL inhibited 94.4% of Gram-negative bacteria tested and CIP inhibited 100% of Gram-positive bacteria, with MICs ranging from 8 µg/mL to 256 µg/mL and 0.5 µg/mL to 2 µg/mL, respectively.

Minimal inhibitory concentrations of sample in association with PAβN

Looking at the results of our sample's antibacterial activity, which are transcribed in Table 2, we notice a total potentiation of our tested substances (AIF ≥ 2). Indeed, we notice a clear improvement in the antibacterial activity of our tested substance when associated with PAβN. It also appears from this table that PAβN tested at a concentration less than or equal to 128 µg/mL does not present any growth-inhibiting effect on the selected bacteria. In general, the activity obtained with our extract in combination with the efflux pump inhibitor was clearly better than that with Chloramphenicol associated with the inhibitor. This being the case, the Activity improvement factor (AIF) determined for our extract was 90% greater than or equal to that of chloramphenicol. All other strains were more than sensitive to the combination of the test substance with PaβN. That said, the test substances had improved activity in the presence of PaβN with AIFs ranging from 4 to 32. CHL activity selectively increased, with AIFs ranging from 2 to 32, but *Klebsiella pneumoniae* strains (ATCC11296 and KP55) showed no improvement in susceptibility to the combination of CHL and PaβN. Of all the strains tested, ATCC25923 showed almost constant sensitivity to the action of the efflux pump inhibitor, this is justified by the achievement of an activity enhancement factor with a value of 16-32 in 100% of the extract.

Phytochemical screening

The results of Table 3 reporting the qualitative phytochemical analysis indicate that the classes of secondary metabolites highlighted in this work were all detected in the raw extract tested.

Effect of the test sample on bacterial growth kinetic

Curves presented in Figure 1 show bacterial growth in the absence and presence of test samples at different concentrations (0.5 x MIC, MIC, and 2 x MIC) as a function of time. The studied bacterial strain was *Escherichia coli* AG102. Growth kinetic was followed during 24 h and the optical density values were measured each 2 h at 600 nm wavelength. Figure 1 shows three stages of bacterial growth for the crude extract of *M. foetida* and negative controls (lag, exponential and stationary stages). At 0.5 x MIC, we see a decrease in the slope of the exponential phase while the lag phase remains unchanged compared to the control growth curve. At the 16th hour, the growth curve of *Escherichia coli* AG 102 in the presence of *M. foetida* extract joins that described by the control. After this time, the curve shows a decline in growth and stabilizes from the 22nd hour until the end of the experiment. At MIC, we see a prolongation of the lag phase until the 8th hour, after which we have a weak growth phase from the 8th hour to the 14th hour. This is followed by a stationary phase that extends to the end of the experiment. At 2 x MIC, we have a curve that is almost linear and almost parallel to the abscissa axis, indicating that the growth of the bacteria is inhibited. Furthermore, the number of living bacteria in the presence of tested samples was significantly lower than in the presence of negative controls (inoculum and DMSO 2.5%).

Effect of the test sample on the bacterial membrane

The absorbance of the filtrate at 260 nm was used to assess the leakage of intracellular material from *Escherichia coli* AG102 after exposure to different concentrations of *M. foetida* extract. The presence of cytoplasmic elements in the culture medium could explain irreversible damage to the bacterial membrane. The results obtained after treatment of *Escherichia coli* AG102 bacterial cells with our different sample concentrations (2 x MIC, MIC, and 0.5 x MIC/2) and the determination of the proteins in the culture media are shown in Figure 2. The results show a concentration-dependent increase in optical density compared to the control after 12 hours. The observation was more remarkable at higher concentrations. The activity *of M. foetida* extract at 0.5 x MIC, MIC and 2 x MIC was greater than that of polymixin B.

Effect of the test sample on bacterial H^+ -ATPase- dependent proton pumps

Figure 3 shows the evolution of cell culture pH as a function of incubation time in the presence of the test sample at 0.5 x MIC, MIC, and 2 x MIC. *Escherichia coli* AG102 was used as the bacterial strain and 2.5% DMSO as the negative control. The curves obtained showed a decrease in pH values after 60 minutes in the presence of *M. foetida* crude extract (from 6.68 to 6.31), whereas the pH values did not decrease significantly in the presence of DMSO (from 7.6 to 6.9). It should be noted that a basic pH is unfavorable to the growth of the bacteria studied.

Anti-biofilm activity

According to the results, our extract appears to have reduced or even inhibited the formation of biofilms by the AG102 strain. The optical density values in the absence of our test substance are much higher than those reported by the strain in the presence of the active substance. The absorbances obtained in the presence of the AG102 strain with decreasing extract concentrations reflect inhibition, the percentages of which are summarized in Figure 4. Our extract appears to have inhibited biofilm formation effectively, with inhibition percentages ranging from 83% to 90.82%. It can be seen from the results depicted in Figure 5 that our substance has remarkable destructive activities on the already-formed biofilm. In general, we can see that the eradication percentages obtained from the control wells (containing formed biofilms) are lower than those obtained from the test wells (containing our test substance). Indeed, with eradication percentages ranging from 64.94% to 80.93%, our extract effectively eradicated the formed biofilms.

Discussion

The search for new and more effective ways to combat MDR bacteria remains a serious public health concern. The antibacterial activity of several African medicinal plants has been well documented [32-38]. The presence of secondary metabolites in the plant extract used in this study may explain the observed antibacterial activity. According to Kuete's classification [39], a plant extract was considered significantly active when the MIC was

100 µg/mL, moderately active when the MIC was 624 g/mL, and weakly active when the MIC was greater than 2048 µg/mL. However, the activity spectrum and level of resistance of the strains studied should be considered. As a result, the antibacterial activity observed in this study varies from one bacterial strain to another. These findings show that crude extract *M. Foetida* had antibacterial activity on 90,90% (20/22) of the strains tested, with MICs ranging from 64 à 1024 µg/mL. The lowest MIC of 64 µg/mL was observed against *Escherichia coli* (AG102, MC4100, W3110), *Klebsiella pneumoniae* (KP55 and ATCC 11296), *Providencia stuartii* (NEA16, ATCC 29916 and PS2636), *Pseudomonas aeruginosa* (PA124) and *Staphylococcus aureus* MRSA9. According to the MBC/MIC values, the effects of the studied extract were mainly bacteriostatic (MBC/MIC > 4). The extract showed bactericidal activity against the *Providencia stuartii* strain (NEA16). The presence of secondary metabolites within the plant would justify the activity observed with our used cucurbit extract. On the other hand, many studies have shown the antioxidant, anti-diabetic, and antimicrobial properties of the leaves of this plant [40-41]. In addition, the leaves and roots of this plant are used in traditional medicine for the treatment of many diseases and pain. Thus, the presence of certain metabolites could be justified by soil composition, temperature, and light intensity. Indeed, not only was this extract active on 20 of the 22 strains tested, but we also noted MICs=64 µg/mL on 10 of the strains, including one strain of *Staphylococcus aureus* (MRSA9), *Pseudomonas aeruginosa* (PA124), three strains of *Providencia stuartii* (NEA16, ATCC29916, and PS2636) and *Escherichia coli* (AG102, MC4100, and MC4100) and two strains of *Klebsiella pneumoniae* (KP55 and ATCC11296). The differences in susceptibility found for the same extract with different strains could be explained by intrinsic differences in the chemical composition of the bacterial wall and/or in genetic elements of resistance that may or may not be transferable between strains such as plasmids or transposons [42].

The updated cut-off point for the classification of the antibacterial botanicals against Enterobacteria such as *Escherichia coli, Klebsiella pneumoniae, Providencia stuartii*, and *Enterobacter aerogenes,* have been established as follows [43]: outstanding activity is observed when MIC ≤ 8 µg/mL, excellent activity when 8 < MIC ≤ 64 µg/mL, very good activity when 64 < MIC ≤ 128 µg/mL, good activity when 128 < MIC ≤ 256 µg/mL, average activity when 256 < MIC ≤ 512 µg/mL, weak activity when 512 < MIC ≤ 1024 µg/mL, and not active MIC > 1024 µg/mL. Consequently, Excellent antibacterial activities were achieved with the crude extracts against *Escherichia coli* AG102, MC4100, and W3110 strains, *Klebsiella pneumoniae* ATCC11296 and KP55 strains, and *Providencia stuartii* ATCC29916, NEA16 and PS2636 (Table 1). Concerning Gram-positive bacteria such as *Staphylococcus aureus*, the updated cut-off points of activity of botanicals have been also defined as follows [44]: outstanding activity when MIC ≤ 8 µg/mL, excellent activity when 8 < MIC ≤ 40 µg/mL, very good activity when 40 < MIC ≤ 128 µg/mL, good activity when 128 < MIC ≤ 320 µg/mL, average activity when 320 < MIC ≤ 625 µg/mL, weak activity when 625 < MIC ≤ 1024 µg/mL, not active when MIC values> 1024 µg/mL. Consequently, very good activity was observed for the crude extract against *Staphylococcus aureus* strains MRSA9 (Table 1). In *Pseudomonas aeruginosa*, the thresholds of activities of botanicals have been updated as follows [45]: outstanding activity when MIC ≤ 32 µg/mL, excellent activity when 32 < MIC ≤ 128 µg/mL, very good activity when 128 < MIC ≤ 256 µg/mL, good activity when 256 < MIC ≤ 512 µg/mL, average activity when 512 < MIC ≤ 1024 µg/mL, weak activity or not active when MIC values> 1024 µg/mL. Therefore, excellent activities were obtained with the crude extract in both PA01 and PA124 strains (Table 1).

Multidrug-resistant bacteria can over-express efflux pump systems, which expel antibacterial molecules from the cell and thus prevent them from performing their functions. The best-known method of combating this type of resistance today is by restoring bacterial susceptibility using efflux pump inhibitors (EPIs) to allow a threshold concentration of antibacterial agents capable of inducing bacterial cell death to be reached. PaβN is a commonly used efflux pump inhibitor that is thought to act on the RND pump family [45].

Concerning the results of the plant extract and their association with PAβN, we see an almost complete potentiation of its activity (AIF ≥ 2) (Table 2). This table also shows that PAβN tested at concentrations less than or equal to 128 µg/mL has no growth inhibitory effect on the selected bacteria. In general, the activity obtained with our sample in combination with the efflux pump inhibitor was clearly superior, at 90%, to that obtained with Chloramphenicol in combination with the inhibitor. These results indicate that the efflux pumps are partly responsible for the reduced activity of our plant extract. These results corroborate with previously published works [46-47] which demonstrated that efflux pumps decrease the intracellular concentration of chemicals and consequently their activity. However, the increased activity observed in multidrug-resistant phenotypes overexpressing efflux pumps suggests that the secondary metabolites responsible for the activity of *M. foetida* methanol extract constitute a substrate for efflux pumps and would have an intracellular site of action [48-49]. PAβN must have certainly blocked the efflux pumps, preventing the bioactive compounds in our sample from being extruded. As a result, the intracellular concentration of the bioactive substances contained in our tested extract would have increased because of this blockage.

The methanol extract of *M. foetida* was tested to evaluate its influence on the growth kinetics of *Escherichia coli* AG102. During the lag stage which lasts less than 2 hours under normal conditions, bacteria adjust to their new environment and synthesize enzymes that metabolize nutrient substrates for growth and multiplication. When compared to the negative control, the crude extract significantly inhibited bacterial growth by destroying enzymes, proteins, and transport systems that are required for bacterial survival [50]. This explains why there are so few multiplied bacteria during the exponential growth stage, which lasts 4 hours because there are so few metabolized substrates used by bacteria. The number of metabolized substrates decreased significantly during the stationary stage, resulting in the death of half-bacteria and, later, most of them due to the accumulation of toxic wastes in the medium [51].

The role of the methanol extract of *M. foetida* in the inhibition of plasma membrane-dependent H^+-ATP proton pumps was also studied. Bacterial proton pumps control cytoplasm pH and provide energy in the form of ATP, both of which are required for bacterial growth and survival. When these pumps are inhibited, the extracellular environment loses protons and becomes less acidic [52]. An increase in environmental pH in the presence of an antibacterial substance may result in this substance inhibiting H^+-ATPase-dependent proton pumps. This could jeopardize bacterial survival because the amount of energy produced will be insufficient for metabolism, growth, and multiplication. The minimum pH supporting bacterial proliferation for an *Escherichia coli* strain has been reported to be 4.4 [52]. Furthermore, this bacterium is supplied by a pH complex that is dependent on many acid tolerance strategies, allowing it to survive in stomach-acidic pH conditions [52, 53]. Various cations transport systems regulate the

bacterial cytoplasmic pH, which is close to neutral. In *Escherichia coli*, cytoplasmic pH is controlled by the expulsion of protons from the cell via the respiratory chain and the entry of acidic potassium ions into the cytoplasm [51, 54]. Moreover, when compared to the negative control (Figure 3), the crude extract tested induced a weak inhibition of H^+-ATPase-dependent proton pumps in *Escherichia coli* AG102, indicating that they are not the primary target of the crude extract's antibacterial action.

Plant phytochemicals' structural and chemical diversity allows them to have various targets on the bacterial cell structure, including the membrane, cell wall, metabolism, and/or molecular targets (proton pumps, proteins, DNA/RNA) via different modes of action. Active molecules targeting the bacterial wall must find complementary receptors for binding and action, whereas those acting within the cell must be able to cross the membrane as well as find target elements within the cell. The intracellular compartment contains nucleic acids and their derivatives, as well as the cell's main components, proteins. An increase in extracellular medium absorbance at 260 nm indicates the presence of nucleic acids and their derivatives and, as a result, a loss of membrane integrity [55 - 56]. Our findings revealed a concentration-dependent increase in absorbance at 260 nm when compared to the control, implying cytoplasmic membrane damage (Figure 2). This irreversible membrane alteration justifies our sample's bactericidal effect at high concentrations. As a result, the cell membrane is a preferred site of action for bactericidal substances [57].

Some bacteria can form structured communities that are surrounded by a polysaccharide matrix (biofilm) produced by the bacteria and attached to a support. The main cause of nosocomial infections is the formation of biofilms via matrix secretion (proteins, DNA, and exopolysaccharides) or adhesion to medical devices, implants, and damaged tissues. This matrix can prevent or delay antibiotic entry, thereby impairing antibiotic activity and causing therapeutic failures and the emergence of resistant phenotypes.

According to the findings of this study on the formation and eradication of biofilms, we discovered that our extract not only reduced or even inhibited the formation of biofilms by the *Escherichia coli* AG102 strain, but also destroyed the biofilms that had already formed (Figures 4 and 5). In general, when the percentages of inhibition and eradication in the control wells are compared to those in the test wells, the values in the control wells are lower. As a result, our test substance (methanol extract of *M. foetida*) effectively inhibited biofilm formation and eradicated existing ones. These findings lead us to believe that our extract is highly effective at inhibiting biofilm formation and capable of rapidly and effectively disrupting biofilm architecture. Because the biofilm layer is an impermeable barrier to many antibiotics, an antimicrobial agent's ability to penetrate and disperse it confirms its potency and efficacy [58]. This efficacy would be closely related to our extract's high concentration of secondary metabolites. Indeed, several authors [14, 59, 60] have demonstrated the effectiveness of terpenes, polyphenols, and flavonoids in inhibiting the formation of biofilms, their stability once formed, as well as the functioning of the molecules involved in Quorum Sensing.

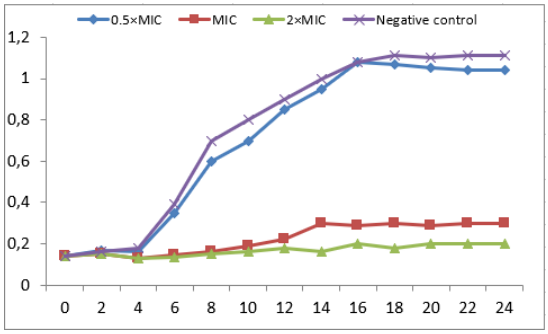

Figure 1. Effect of methanol extract of *M. foetida* at different concentrations on growth kinetic of *Escherichia coli* AG102

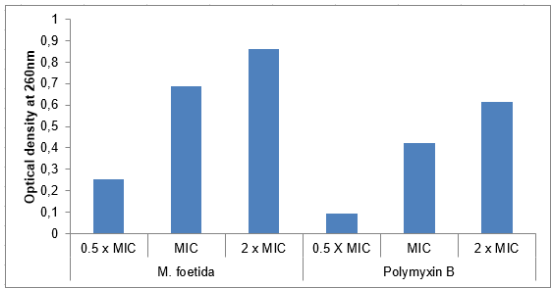

Figure 2. Optical density of the medium inoculated with *Escherichia coli* AG102 and treated with the methanol extract of *M. foetida*.

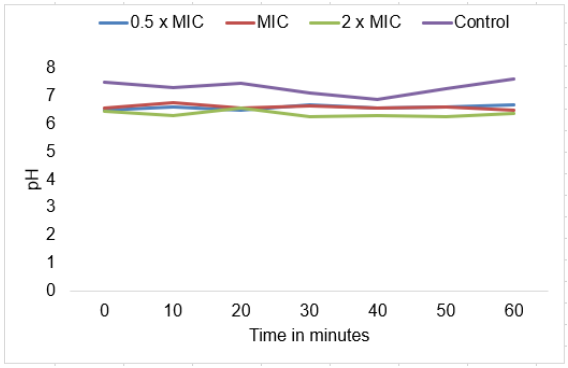

Figure 3. Effect of the methanol extract of *M. foetida* on *Escherichia coli* AG102 H^+-ATPase- dependent proton pumps

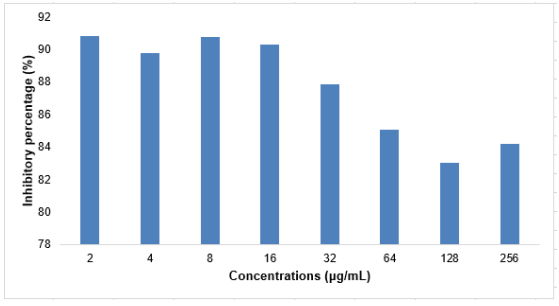

Figure 4. Inhibition in the formation of biofilm by *Escherichia coli* AG102 treated with methanol extract of *M. foetida*

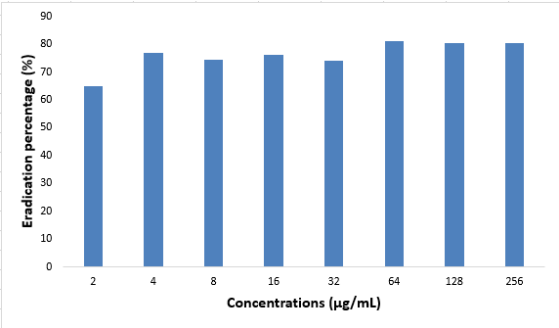

Figure 5. Eradication of biofilm formed by *Escherichia coli* AG102 and treated with methanol extract of *M. foetida*

Table 1. MICs and MBCs in µg/mL of methanol extract from the studied plant, chloramphenicol, and ciprofloxacin

Bacterial strains	Tested samples, MIC, and MBC (in bracket) values (µg/mL)	
	Momordica foetida	**ATB**
	Whole plant	CHL
Escherichia coli		
ATCC8739	128 (1024)	8(256)
ATCC10536	128(1024)	16(32)
AG100A$_{TET}$	128(-)	64 (128)
AG102	64 (512)	64(64)
MC4100	64 (-)	128(128)
W3110	64 (-)	64(128)
Enterobacter aerogenes		
ATCC13048	128 (1024)	8(32)
CM64	128 (-)	128(-)
EA27	128 (-)	-(-)
EA289	1024 (-)	256(-)
Klebsiella pneumoniae		
KP55	64 (1024)	32(128)
KP63	128 (-)	128(-)
ATCC11296	64 (512)	8(256)
Providencia stuartii		
NEA16	64 (128)	256(-)
ATCC29916	64 (1024)	16(32)
PS2636	64 (512)	32(32)
Pseudomonas aeruginosa		
PA01	128 (-)	128(-)
PA124	64 (-)	256(-)
Staphlococcus aureus		
ATCC25923	- (-)	<0,5(16)
MRSA3	- (-)	2(16)
MRSA8	1024(-)	2(8)
MRSA9	64 (512)	2(16)

– :>1024 (MIC) or not determined; the tested extracts were obtained from whole plant; ATB: Antibiotics; CHL: chloramphenicol; CIP: ciprofloxacin

Table 2. Antibacterial activity of methanol extract of *M. foetida* in absence and presence of PAßN

Bacterial strains	*M. foetida*		CHL		PAβN
	-	+	-	+	
Escherichia coli					
ATCC8739	128	4 (32)	8	2 (4)	>128
AG102	64	2 (32)	64	4 (16)	>128
Klebsiella pneumoniae					
ATCC11296	64	16 (4)	8	8 (1)	>128
KP55	64	16 (4)	32	32 (1)	>128
Enterobacter aerogenes					
ATCC13048	128	8 (16)	8	8 (8)	>128
CM64	128	4 (32)	128	4 (32)	>128
Providencia stuartii					
ATCC29916	64	4 (16)	16	8 (2)	>128
PS2636	64	2 (32)	32	4 (8)	>128
Staphylococcus aureus					
ATCC25923	256	8 (32)	<0,5	<0,125 (>4)	>128
MRSA3	256	16 (16)	2	<0,125 (>16)	>128

+: Presence of PAβN; - : Absence of PAβN ; / : No activity ; () : AIF « Activity improvement factor »

Table 3. Phytochemical composition of *Momordica foetida*

Phytochemicals	Inference
Polyphenols	+
Flavonoids	+
Alkaloids	+
Saponins	+
Steroids	+
Triterpens	+

(+): presence of metabolites; (-): absence of metabolites

Conclusion

The results obtained in the present work provide important and significant data that could allow the probable use of *Momordica foetida* in the control of bacterial infections. Most notably, the modes of action of the crude extract of this plant studied are presented in this work for the first time. Cytotoxicity, as well as acute and subacute toxicity tests, could be studied to make these results more specific in the development of a new Improved Traditional Medicine (ITM).

Abbreviations

AIF : Activity improvement factor ; ATCC: American Type Culture Collection ; BEC: Biofilm Eradication Concentration ; CFU: Colony Forming Unit ; CHL: Chloramphenicol ; DMSO: Dimethyl sulfoxide ; DNA: Deoxyribonucleic Acid ; INT: p-iodonitrotetrazolium chloride ≥ 97% (INT, Sigma-Aldrich) ; ITM: Improved Traditional Medicine ; MBIC50: Minimum Biofilm Inhibitory Concentrations 50% ; MDR: Multidrug-resistant ; MFS: Major Facilitator Superfamily; MHB: Mueller Hinton Broth ; MIC: Minimal Inhibitory Concentration ; *M. foetida* : *Momordica foetida* ; OD: Optical Density ; PAβN : Phenylalanine arginine-β- naphthylamide; PBS : Phosphate Buffer Solution ; PolyB: Polymixin B ; RA: Reference antibiotic; RNA: Ribonucleic acid ; RND: Résistance-Nodulation-Division ; SRF/Cam/ K : *Section des Réserves Forestières du Cameroun / Kamerun.*

Authors' Contribution

CFT and OMFD carried out the study; ATM and VK designed the experiments; CFT wrote the manuscript; ATM, VPB and VK supervised the work; VK provided the bacterial strains and facilities for antibacterial assays; all authors read and approved the final manuscript.

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Conflict of interest

The authors declare no conflict of interest.

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