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Mechanisms of action of roots crude extract and adianthifolioside GS1 from *Albizia adianthifolia* (Fabaceae) against MDR Gram-negative enteric bacteria

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Abstract

Background: The Cameroonian pharmacopoeia is full of many plants used amongst population to treat various infections. *Albizia adianthifolia (Schum*.) is one of these plants and therefore several previous studies have reported the antibacterial activities of the crude methanol extract of the roots of this plant as well as those of some of its isolated compounds. In this study, the methanol root extract, and its isolated compound "adianthifolioside GS1" were evaluated on bacterial growth kinetics, bacterial membrane, biofilms, and sheep erythrocytes. Adianthifolioside GS1 was tested for its antibiotic stimulation activity against multidrug resistant (MDR) Gram-negative bacteria.
Methods: The antibacterial activities of adianthifolioside GS1 in combination with the usual antibiotics were evaluated using the modified p-iodonitrotetrazolium chloride (INT) rapid colorimetric test. Determination of the effect of "crude extract and adianthifolioside GS1" samples on bacterial growth kinetics, bacterial membrane, biofilms, and sheep erythrocytes was carried out using spectrophotometric methods.
Results: In combination with erythromycin (ERY), gentamicin (GEN), streptomycin (STR) and tetracycline (TET), adianthifolioside GS1 showed the most significant synergistic effects on all bacteria tested. Adianthifolioside GS1 and the crude root extract of *Albizia adianthifolia* showed a bacteriostatic effect on the strains tested and caused membrane lysis in *Klebsiella pneumoniae* KP55 and *Escherichia coli* AG102 strains at the concentrations tested. *Albizia adianthifolia* root extract showed significant inhibition of the biofilms of the strains tested, unlike adianthifolioside GS1. The crude extract and its isolated compound (adianthifolioside GS1) exhibited low toxicity to red blood cells.
Conclusion: All the results obtained in the framework provide important information on the crude root extract of *Albizia adianthifolia* and its isolated compound "adianthifolioside GS1" as good candidates for the elaboration of phytomedicines and drugs respectively, and the association of adianthifolioside GS1 with the usual antibiotics that can be used to potentiate the action of the latter.

Keywords: *Albizia adianthifolia*; adianthifolioside GS1; Mechanisms of action; Fabaceae; multi-drug resistance; enteric bacteria.

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Background

The treatment of bacterial infections currently poses a worrying public health problem, especially due to the emergence of multi-resistant pathogenic microorganisms. Indeed, these infections are responsible for 70% of the mortality cases caused by pathogenic microorganisms [1]. Pathogenic microorganisms affect all age groups and the World Health Organization (WHO) has estimated the global number of neonatal deaths at 2.8 million in 2015, 47.6% of which are due to bacterial infections [2]. To date, despite the existence of several strategies to combat bacterial infections, antibiotic therapy remains the most important one. However, despite the fact that the use of antibiotics has considerably improved the state of health, the often abusive and inappropriate use of antibiotics has subsequently favoured the emergence of a natural adaptation phenomenon: resistance [1, 3, 4, 5] leading to the emergence and re-emergence of many diseases due to the reduction or even loss of efficacy of antibacterial agents. This has a direct impact on the increase in the length of hospitalisation, the cost of care and the mortality rate [6]. In addition, the emergence of MDR bacterial strains appears to be the main cause of treatment failure [7]. Among the known resistance mechanisms, active efflux via resistance-nodulation cell division (RND) pumps is one of the most common systems in Gram-negative bacterial strains [8]. This phenomenon has led to the inactivation of several classes of antibiotics, raising fears worldwide that the pre-antibiotic era is returning [9]. Consequently, the search for new molecules capable of combating this resistance most effectively has become an absolute necessity. The medicinal plants of Cameroon's plant flora, which abound in numerous bioactive substances, have proven their ability to inhibit the growth of most strains of Gram-negative MDR bacteria [10, 11, 12] and also to potentiate the activity of common antibiotics [13, 14, 15]. In our various approaches to combat MDR bacteria by identifying antibacterial agents from the traditional pharmacopoeia in the fight against bacterial infections, we have focused more on *Albizia adianthifolia* (Schum.) (Fabaceae) which is a plant used in traditional medicine for the treatment of various infections [16, 17]. The antibacterial activity of the methanol extract from the roots of this plant as well as that of its isolated compound "adianthifolioside GS1" has been previously reported [18, 19].

In the present study, the activity of the crude methanol extract from the roots of this plant and its isolated compound "adianthifolioside GS1" were studied against some MDR bacteria and on sheep erythrocytes. And the combination effect of adianthifolioside GS1 on common antibiotics was evaluated.

Methods

General procedure

For compound characterization, ElectroSpray ionization mass spectrometry (ESI-MS), nuclear magnetic resonance (NMR) spectra (1 H, 13C, HSQC, HMBC and COSY), column chromatography (CC), thin layer chromatography (TLC), IR spectra, UV spectra and Alpha D values were performed according to previously described protocols [19].

Plant material, extraction and purification of bioactive compound

The description of the plant material, the extraction protocol, as well as the process of isolation and purification of adianthifolioside GS1 or 3-O-{α-L-o-arabipyranosyl-(1→6)-β-D-glucopyranosyl}oleanolic acid from the crude methanol extract of the roots of the *Albizia adianthifolia* plant has been described above by [19]. The elucidation of the chemical structure of this compound using NMR (1H and 13C) data, in comparison with the literature, was reported by [19] (Figure 1).

Chemicals for antibacterial assays

The microbial growth indicator used in this study was p-iodonitrotrazolium chloride ≥97% (INT, Sigma-Aldrich). The reference antibiotics used were : a β-lactamine " Penicillin (PEN) ", three aminosides " Kanamycin (KAN), Streptomycin (STR) and Gentamycin (GEN) ", a cycline " Tetracycline (TET) ", two quinolones "Ciprofloxacin (CIP) and Norfloxacin (NOR)", a phenolic "Chloramphenicol (CHL)", a polypeptide "polymycin B (Poly B)" and a macrolide "Erythromycin (ERY)". Triton X 1% solution and posphate buffer were used as positive and negative controls, respectively, for erythrocyte haemolysis. The developer of the biofilm formation was the purple crystal. A 2.5% dimethylsulphoxide (DMSO) surfactant was used to dissolve our test samples and Bradford's protein reagent. These molecules and reagents were obtained from Sigma-Aldrich (St. Quentin Fallavier, France).

Bacterial strains and culture media

A total of 7 Gram-negative MDR bacteria (including clinical strains and those from the American Type Culture Collection "ATCC") were investigated in this work. They included resistant strains of *Escherichia coli, Enterobacter aerogenes, Klebsiella pneumoniae* and *Pseudomonas aeruginosa.* The characteristics of these bacteria were previously reported (Supplementary File 1; Table S1) [20]. Prior to testing, the bacteria were grown on Mueller Hinton agar (MHA; Sigma), while Mueller Hinton broth (MHB; Sigma) was used for the antibacterial test [21].

Erythrocyte cells

The erythrocytes used in this study were of animal (sheep) origin and were used immediately after their isolation from the blood instantaneously collected at the time of animal sacrifice.

Study of the inhibition of efflux pumps: potentiation of the action of antibiotics by adianthifolioside GS1

A preliminary trial was carried out on strain PA124 (see Supplementary File 1; Table S2) by testing the combination of adianthifolioside GS1 at its different sub-inhibitory concentrations with certain antibiotics, which allowed us to select the appropriate sub-inhibitory concentrations in order to evaluate the potentiating effect of adianthifolioside GS1. Following this test, MIC/2 and MIC/4 were selected and subsequently used for the

sample-antibiotic combination against the selected MDR microorganisms [14, 18].

Fractional inhibitory concentrations (FICs) were calculated as the ratio of the MIC of the antibiotic in the combination to that of the antibiotic alone ($MIC\text{-}_{antibiotic\ in\ combination}/MIC\text{-}_{antibiotic\ alone}$) and interpreted as synergistic (≤ 0.5), indifferent (1-4) or antagonistic (>4) [22, 23].

Antimicrobial mechanisms of action

Effect of test samples on bacterial growth

Evaluation of the effect of the raw root extract of *Albizia adianthifolia* and adianthifolioside GS1 on bacterial growth was carried out using the spectrophotometric method described in [24] with some modifications. It was carried out with the following bacteria: *E. coli* AG102 and *K. pneumoniae* KP55 whose bacterial suspensions were prepared at a concentration of 108 CFU/mL in the corresponding vials. The substances of interest were incorporated into the corresponding vials to obtain the concentrations 0.5 × MIC, MIC and 2 × MIC. For the different tests, a growth control for each strain was included in a vial containing no substances of interest, and a negative control (growth medium without drug or organism). Chloramphenicol was used as the reference antibiotic molecule and positive control. The strains of each of these bacteria were used to calibrate the optical density measurements against the number of viable cells [25]. The vials were incubated at 37°C on an orbital shaker (Orbital Shaking Incubator, REMI) at 17g and samples were then taken aseptically at different time intervals (0 min, 1 h, 2 h, 4 h, 8 h and 12 h). The absorbances were read using a spectrophotometer from THERMO SCIENTIFIC (Langenselbold, Germany) at a wavelength of 600 nm. Subsequently, the evolution of the microbial load was adapted to the Baranyi model using the DMFIT software "Dnamic Modeling Fit" version 3.5, which made it possible to determine the kinetic parameters "Speed and latency time" (Supplementary File S3).

The experiment was repeated 3 times and the results obtained were used to plot the bacterial growth curves [DO = f (time)] using GraphPad Prism version 6.00 software. (Figure 2a, 2b, 3a and 3b).

Effect of test samples on the bacterial membrane

The evaluation of the effect on the bacterial membrane by our test samples is done using the protocol described in [26]. In each vial, the bacterial suspensions (*E. coli* AG102 and *K. pneumoniae* KP55) were adjusted to 108 CFU/mL and then the test substances were added at concentrations of 0.5 × MIC, MIC and 2 × MIC. The different mixtures were incubated at 37 °C and the samples collected at 0 min, 2 h, 4 h, 8 h and 12 h treatment in the test and control vials. Polymycin B was used here as a positive control and the growth medium without test substances or microorganisms as a negative control. The various debris present in the samples were removed by centrifugation (Compact Cooling Centriguge, REMI) at 33540g for 15 min. The various metabolites with low molecular weights (nucleic acids, metabolites, etc.) resulting from bacterial lysis were present in the supernatant and the proportions in relation to the negative and positive controls were determined by colorimetric determination using Bradford's reagent followed by an absorbance reading at 595 nm using a THERMO SCIENTIFIC spectrophotometer (Langenselbold, Germany). Calibration curve of the assay by the Bradford method is shown in the supplementary file (S4). The test was repeated three times and the data obtained from the OD reading was used to plot the histograms [Protein Amount = f (time)] using Microsoft Office Excel 2016. (Figure 4 and 5).

Determination of the Effect of Test Samples on Biofilms

Inhibition of biofilm formation

Anti-biofilm activity was assessed in 96-well flat bottom plates using a method adapted by [27] with some modifications. The bacterial strains of *E. coli* (AG102), *K. pneumoniae* (KP55) and *E. aerogenes* (EA27) are were treated in microdilution plates with adianthifolioside GS1 and crude extract of *Albizia adianthifolia* at varying concentrations between 128 µg/mL - 4 µg/mL and 4096 µg/mL - 128 µg/mL and 4096 µg/mL - 128 µg/mL respectively, then incubated at 37°C for 18 hours under orbital shaking (Orbital Shaking Incubator, REMI) at 7g.The planktonic cells were then removed and the plates rinsed with sterile distilled water and stained for 15 minutes by incorporating 200 µL of purple crystal into each well. Discolouration with acetic acid (30%), followed by measurement of the optical density (OD) of the violet crystal solution at 595 nm was carried out after the biofilms were stained and rinsed with sterile distilled water. The biofilm formation inhibition curves allowing to deduce the Minimum Biofilm Inhibitory Concentrations 50% ($MBIC_{50}$) are represented in the supplementary file (S5). The tests were repeated three times and the Minimum Biofilm Inhibitory Concentrations 50% ($MBIC_{50}$) were 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 specimen.

Effect on established biofilm

The effect of samples tested on established biofilm was studied using the method previously described by [27] with slight modifications. Bacterial cultures of strains of *E. coli* (AG102), *K. pneumoniae* (KP55) and *E. aerogenes* (EA27) were previously prepared in liquid medium (MHB) and incubated at 37°C for 18 hours under orbital shaking Incubator, REMI at 7g. After biofilm formation, the planktonic cells were removed and the plates were treated with adianthifolioside GS1 and crude root extract of *Albizia adianthifolia* at varying concentrations between 128 µg/mL - 4 µg/mL and 4096 µg/mL - 128 µg/mL respectively. Subsequently, the plates were reincubated again at 37°C for 18 hours under Orbital Shaking Incubator, REMI (7g) and rinsed with sterile distilled water, then stained for 15 minutes by incorporating 200 µL of purple crystal into each

well. Discolouration 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 specimen.

Erythrocyte hemolysis tests

Isolation of erythrocytes

Isolation of erythrocyte cells from sheep blood was carried out according to the method described in [28]. This protocol is carried out while respecting the animal's welfare conditions. A volume of 10 mL of sheep blood was collected in an EDTA tube and then centrifuged (Compact Cooling Centriguge, REMI) at 301g for 5 minutes. After centrifugation, the plasma was removed, normal saline solution (0.9% NaCl) was inserted and washed. Then we introduced a saline phosphate buffer solution (100 mM, pH=7.4).

Haemolysis test

The evaluation of the haemolytic activity of our test substances was done using the protocol described in [28] with some modifications. The concentration of erythrocyte cells in the blood of the sheep was determined using an automatic blood cell counter of the brand ERMA INC. PCE-210N (Becton-Dickinson, Germany) (supplementary file S6). The red blood cells were subsequently washed, diluted 50 times and then treated with the substances of interest at final concentrations 256 µg/mL, 128 µg/mL, 128 µg/mL, 64 µg/mL, 64 µg/mL, 32 µg/mL for the adianthifolioside GS1; and 2560 µg/mL, 1280 µg/mL, 512 µg/mL, 256 µg/mL for the crude extract. As our substances of interest were dissolved in DMSO, a 2.5% DMSO solution and a PBS solution were the negative controls while the 1% Triton X solution was the positive control. The treated cells were incubated at 37°C for 60 minutes under gentle agitation. At the end of the incubation, the samples were kept on ice for 5 minutes and then centrifuged (Compact Cooling Centriguge, REMI) at 301g for 15 min. The haemoglobin released by the treated erythrocytes was determined by measuring the absorbance of the supernatant at 450 nm using a THERMO Multiscan EX microplate reader (Langenselbold, Germany). Each test was performed three times and the percentage of haemolysis was calculated according to the following formula:

% haemolysis = [OD (sample) - OD (negative control) / OD (positive control) - OD (negative control)].

% hemolysis = Percentage hemolysis; OD (sample) = Optical density of the test substance; OD (negative control) = Optical density of phosphate buffer in the presence of blood; OD (positive control) = Optical density of Triton X (1%) in the presence of blood.

Results

Effects of the association of the adianthifolioside GS1 with antibiotics

Following a preliminary study carried out on *Pseudomonas aeruginosa* PA124, the sub-inhibitory concentrations of CMI/2 and CMI/4 were chosen for further studies based on the results obtained. Adianthifolioside GS1 was combined separately with seven antibiotics (CIP, ERY, GEN, KAN, NOR, STR and TET) to evaluate its potential potentiating effect. Table 1 summarises the results indicating that synergistic effects were found with all samples tested with most of the antibiotics tested. The synergistic effects ranged from 28, 57% to 100% on the different microorganisms with the compound. In combination with the antibiotics ERY and STR, adianthifolioside GS1 showed the most significant synergistic effects (100%) at their different sub-inhibitory concentrations (CMI/2 and CMI/4) against all strains tested. Furthermore, this same compound, in combination with KAN, showed the lowest synergistic effects, ranging from 28.5% to 71.5% compared to the other antibiotics in the panel used. The synergistic effect was also noted (100%) with adianthifolioside GS1 in combination with Gentamycin (GEN) against the bacteria tested; this was also the case when the compound (at MIC/2) was combined with NOR (Table 1). No antagonistic effects were noted when adianthifolioside GS1 was combined with antibiotics. However, indifference effects were observed in some cases.

Antibacterial mechanisms of action

Effect of test samples on bacterial growth

An analysis of the results presented in Figures 2 and 3 shows in general that in the absence of the test substances (inoculum alone), the growth curves of the *E. coli* AG102 and *K. pneumoniae* KP55 strains show a regular pattern of bacterial growth. *K. pneumoniae* KP55 shows a distinct latent phase, an accelerated phase, an exponential phase, and a stationary phase. In *E. coli* AG102, however, the bacterial growth curve shows two (02) phases: a latent phase and an exponential phase. The latency phase lasts about 1.57 hours for *E. coli* AG102 and 3.76 hours for *K. pneumoniae* KP55 after adaptation to the Baramyi model and at the end of this phase, the active multiplication of the bacterial strains begins. The presence of 2.5% DMSO does not affect the bacterial growth whatever the strain used. The reference molecule here is chloramphenicol (CHL) at the MIC and has shown an inhibitory effect on the growth of these bacterial strains throughout the experiment.

Figure 2a shows the growth curve of the *E. coli* AG102 strain in the presence of the crude extract. At MIC and 2 × MIC concentrations of the crude extract of *Albizia adianthifolia* roots, the growth curves are almost parallel to the x-axis, indicating that the crude extract at these concentrations completely inhibited the growth of the bacteria. At the 0.5 × MIC concentration, the crude extract exerted a growth-inhibiting effect in the exponential phase, during which the slope observed is less steep at this concentration of the extract. Furthermore, at the same concentration (0.5 × MIC), the latency phase remained almost identical to that of the negative control with a duration of 1.61 hours.

Figure 2b also shows that at the concentrations (2 × MIC, MIC and 0.5 × MIC), adianthifolioside GS1 exerted a concentration-dependent effect on the growth curve of the bacterium. This effect is observed in the exponential growth phase, which shows less and less steep slopes as the concentration of the compound increases. At these concentrations, adianthifolioside GS1 caused a prolongation of the latency phase up to about 3.273 hours. After the 4th hour, this compound, at a concentration of 0.5 × MIC caused a prolongation of the stationary phase until the end of the experiment, whereas at concentrations of 2 × MIC and MIC, and still at the 4th hour, adianthifolioside GS1 induced a decrease in the growth of the bacteria until the 12th hour of the experiment, characterised by a phase of decline.

Figure 3a shows the growth curve of *K. pneumoniae* strain KP55 in the presence of the crude extract. From this figure it can be seen that at MIC and 2 × MIC concentrations of the crude extract of the roots of *Albizia adianthifolia*, the growth curves remained almost parallel to the x-axis, indicating that the crude extract at these concentrations completely inhibited the growth of the bacterium. At a concentration of 0.5 × MIC, the crude extract had an inhibitory effect on the growth of the bacterium (from the 8th hour until the end of the experiment) characterised by a phase of decline. Similarly, at this concentration (0.5 × MIC), the crude extract caused a lengthening of the latency phase to approximately 3.90 hours.

Figure 3b shows the growth curve of the *K. pneumoniae* KP55 strain in the presence of the adianthifolioside GS1. This figure shows that when this compound at MIC and 2 × MIC concentrations is added to the culture medium, growth is zero (almost straight and horizontal curve). At a concentration of 0.5 × MIC, this compound caused a prolongation of the latency phase to about 3.88 hours. After the 4th hour, compound 8 at the 0.5 × MIC concentration caused a lengthening of the exponential phase until the end of the experiment with a significant decrease in the microorganism concentration of 48.06% ($p < 0.001$) and 39.40% ($p < 0.001$) respectively at the 8th hour and 12th hour compared to the negative control (KP55).

Effect of test samples on the bacterial membrane

The analysis of the results presented in Figures 4 and 5, shows that independently of time and concentration, the quantities of protein substances dosed are significantly different from those observed in suspensions containing no test substance and depending on the strain (negative controls). Adianthifolioside GS1 induced a significant increase ($p < 0.05$) in the amount of total protein in *E. coli* AG102 cell cultures at the 2nd and 4th hour of treatment at concentrations of 0.5 × MIC, MIC and 2 × MIC, compared to the values obtained in the negative control (AG102). The latter also induced a significant ($p < 0.05$) increase in protein in *K. pneumoniae* KP55 cell cultures at 0.5 × MIC, MIC and 2 × MIC at the 2nd hour, compared to the values obtained in the negative control (KP55) (Figure 5).

Albizia adianthifolia root extract induced a significant increase in the amount of protein in *K. pneumoniae* KP55 cell cultures ($p < 0.05$) at 12 hours at 0.5 × MIC, MIC and 2 × MIC, compared with the negative control (KP55) (Figure 4). In addition, this extract also showed a significant high protein level ($p < 0.05$) in *E. coli* AG102 at hour 4 and again at concentrations of 0.5× MIC, MIC and 2× MIC when compared to the values obtained in the negative control (AG102).

However, we noticed significant increases in the amount of protein in the cultures with our test substances compared to the bacterial strains tested were less than that of polymycin B (positive control) at the different tets concentrations.

Anti-biofilm activity

In this study, we investigated the ability of adianthifolioside GS1 and the crude root extract of *Albizia adianthifolia* on the inhibition of biofilm formation and eradication of preformed biofilm by strains of *E. coli* AG102, *K. pneumoniae* KP55 and *E. aerogenes* EA27.

For the inhibition of biofilm formation, the results show that our test substances exerted a concentration-dependent inhibition of biofilm formation. However, adianthifolioside GS1 did not show significant activity against biofilms produced by all the strains of panel bacteria used (Figure 6). From the different levels of inhibition obtained with each concentration, we deduced the Minimum Biofilm Inhibitory Concentrations 50% ($MBIC_{50}$). The overall results are shown in Table 2. According to the results presented in this Table, the crude extract showed a better activity compared to the compound. All values inhibiting 50% of biofilms ranged from 407 µg/mL to 1023 µg/mL. The biofilms formed by *E. aerogenes* EA27 were the least sensitive (biofilm inhibitory values are much higher than planktonic cell inhibitory concentrations). Adianthifolioside GS1 did not significantly inhibit biofilm formation, with maximum inhibition percentages of 42.87%, 41.79% and 15.84% against *K. pneumoniae* KP55, *E. aerogenes* EA27 and *E. coli* AG102 strains respectively at the highest tested concentration of 128 µg/mL (Table 2).

The effect of our test substances on the destruction of preformed biofilms by the strains used was also investigated and the results obtained from this test show that, depending on the concentration used, the test substances effectively destroy the preformed biofilms. However, these test substances did not show significant activity against the biofilms formed by the different strains of panel bacteria used, as the different percentages of sample destruction were less than 95% (Figure 7). The values of the biofilm eradicating concentrations (BEC) were not determined with respect to these different percentages of sample destruction (<95%) for all bacterial strains tested.

Anti-hemolytic activity

The haemolytic activity of our test substances in this context concerned red blood cells isolated from sheep blood. The results obtained from this test showed total haemolysis with Triton X-100 1% (positive control) (Figure 8a and 8b). The Blood Formula Count (BFC) of the sheep blood sample shows a red blood cell concentration of 5.31 x 106/µL (see Supplementary File S6).

The effect of the crude root extract of *Albizia adianthifolia* (Figure 8a) observed on the erythrocyte membrane induced significant ($p < 0.05$) increases in haemolytic activity at concentrations 2560 µg/mL (12.25%) and 1280 µg/mL (9.62%); this compared to the negative control (PBS + Blood). However, this haemolysis does not lead to a critical decrease in the number of red blood cells, since for a normal blood count, the red blood cell count should vary from 4.5 to 6 x 1012/l in men and from 4 to 5.4 x 1012/l in women [29]. In this particular case, however, the red blood cell counts obtained after haemolysis are 4.66 x 1012/l and 4.80 x 1012/l respectively at haemolysis rates of 12.25% and 9.62% are included in the ranges of variation of red blood cell counts in men and women as mentioned above.

Adianthifolioside GS1 (Figure 8b) showed significant increases ($p < 0.05$) in the percentages of haemolysis of 10.94% and 6.62% respectively at concentrations of 256 µg/mL and 128 µg/mL compared to the negative control. However, as mentioned above, these haemolysis rates do not also result in a critical decrease in the number of erythrocytes present in sheep blood. Statistical analysis of the haemolysis percentages of 64 µg/mL (2.07%) and 32 µg/mL (0.82%) show that they are not significantly different compared to the negative control.

Discussion

Effects of association of adianthifolioside GS1 with antibiotics

Synergistic effects due to the combination of Adianthifolioside GS1 with ERY, STR, as well as KAN in relation to all tested bacteria were noted. Synergistic or compound-modulating effects with other antibiotics were found in more than 70% of the bacteria tested in several cases, with FIC values mostly ranging from 0.5 to 0.007. These results suggest that this compound could be considered as a potential efflux inhibitor [23]. The antibiotic-modulating effect of adianthifolioside GS1 against resistant Gram-negative bacteria is reported here for the first time. This study also provides more information on the ability of adianthifolioside GS1 to potentiate the activity of antibiotics against MDR bacteria expressing active efflux.

Antibacterial mode of action

The crude root extract of *Albizia adianthifolia* and its isolated compound "adianthifolioside GS1" were tested to evaluate their effects on the growth kinetics of *E. coli* AG102 and *K. pneumoniae* KP55 strains. During the growth of the strains in the absence of test substances, the latency time of the *E. coli* AG102 and *K. pneumoniae* KP55 strains was 1.57 hours and 3.76 hours respectively for untreated cells after adaptation to the Baramyi model. During this period, the bacteria adapt to their environment and synthesize enzymes necessary for the degradation metabolism of the substrates present in the medium. Thus, any increase in the duration of the latency time could be directly correlated either to the inactivation of these enzymes, or simply to the inhibition of their synthesis. The work [30] shows that in the presence of certain substances such as phenols, the latency time increases in proportion to the concentration of the sample introduced into the medium, which explains the results obtained with the crude extract of *Albizia adianthifolia* [Figures 2(a) and 3(a)], in which several classes of phenolic compounds were detected [18]. Analysis of the curves in Figures 2 and 3 shows that the crude extract and the adianthifolioside GS1 exerted their inhibitory activities at various stages of bacterial growth. The course of the latency phase would be disrupted when the microorganisms are in contact with these substances, as an extension of this phase is observed at a concentration of 0.5 x MIC where applicable. The raw root extract of *Albizia adianthifolia* and adianthifolioside GS1 at MIC and 2 x MIC concentrations affected the smooth running of the exponential phase. During this phase, rapid assimilation of the various growth factors occurs; our test substances could therefore inhibit the activity of an important enzyme in the production of energy, or inhibit the absorption of the various nutrients for the bacterium. The curves of the bacterial kinetics at concentrations of 0.5 x MIC, MIC and 2 x MIC corroborate the results obtained when evaluating the impact of the test substances on the bacterial membrane (Figures 4 and 5).

The significant variations in the extracellular quantities (concentrations) of the protein substances show the alteration of the membrane of the bacteria treated at the different concentrations tested; and thus the bacteriostatic effect of the substances tested. The effect of our extract and its product (adianthifolioside GS1) (Figures 4 and 5) on the different strains (*K. pneumoniae* KP55 and *E. coli* AG102) resulted in an increase in the proteins in the different suspensions. Indeed, this significant increase ($p < 0.05$) of proteins in the medium compared to the negative control indicates membrane lysis induced by these test substances. The study carried out by [31] on the mechanisms of action of some essential oil constituents showed that citronellol and citronellal (monoterpenes) have a strong activity with regard to the lysis of the bacterial membrane, especially in Gram-negative bacteria. Thus, the results obtained with the extract on *K. pneumoniae* KP55 (12th hour) and *E. coli* AG102 (4th hour) strains at concentrations of 0.5 x MIC, MIC and 2 x MIC could be justified by the presence of terpene and phenolic compounds present in the crude methanol extract of *Albizia adianthifolia* roots which would be responsible for its lytic effect on the bacterial cell membrane (Figure 4).
Some bacteria have the ability to prevent or delay the entry of antibiotics by secreting a matrix of exopolysaccharides commonly known as biofilm. The formation of biofilms is a phenomenon responsible for therapeutic failures and the emergence of new resistant strains [32]. Biofilm formation by *E. coli* AG102, *E. aerogenes* EA27 and *K. pneumoniae* KP55 strains has been inhibited by the extract and adianthifolioside GS1 from the roots of *A. adianthifolia* (Figure 6). The crude extract showed better biofilm inhibitory activity but did not eradicate biofilms in all strains

used (Figure 7). This inhibitory activity could be due to the presence of terpene compounds (stigmasterol and β-sitosterol; β-sitosterol 3-O-β-D-glucopyranoside) in the crude extract tested. In fact, [33] reported the activity of terpenes in relation to the biofilms formed by the methicillin-resistant and methicillin-sensitive Gram-negative and Gram-positive strains. The phytochemical tests previously highlighted by [18] highlight the presence of bioactive phenolic compounds in the methanol extract of A. adianthifolia roots. These molecules would act by diffusing through the exopolysaccharide layer in order to exert antimicrobial effects on sessile cells. Moreover, certain phenolic compounds inhibit biofilm production by mimicking the functioning of Quorum sensing molecules [34]. Indeed, Quorum sensing is a system of production of molecular signals correlated to the microbial population in order to induce or reduce growth. Moreover, the flavonoids present in the crude extract are believed to be involved in the antibiotic film activity, as they have the potential to disturb the stability of the preformed exopolysaccharide layer, leading to a reduction in the metabolic activity of the biofilm [35]. The results obtained with the adianthifolioside GS1 highlight the low capacity of this molecule to inhibit the formation of biofilms and to destroy them. This could be justified by the fact that triterpene saponins have a low antibiotic film activity.

All molecules eligible for research into new drugs must have no or low toxic properties; it is with this in mind that the erythrocyte haemolysis test was carried out in this context. Treatment of erythrocytes with high concentrations of methanol extract from the roots of *Albizia adianthifolia* (2560 µg/mL) and adianthifolioside GS1 (250 µg/mL) resulted in the detection of low levels of haemoglobin in the extracellular medium. This corresponded to a percentage of erythrocyte haemolysis of 12.25% and 10.94% for the crude extract and adianthifolioside GS1 respectively. The standards issued by the manufacturer of the automatic blood cell counter used for the quantification of red blood cells range from 3.5x106 to 5.6x106/µL. Since a decrease of the above percentage does not result in a significant decrease in red blood cell count, it is possible that the samples tested are low toxicity subject to further toxicological testing. In addition, [36] have shown that 50-100% of red blood cells are lysed in the presence of amphotericin B at concentrations between 30 and 100 µg/mL, while chloramphenicol at a concentration of 4 mg/mL results in haemolysis of 14-15% of red blood cells.

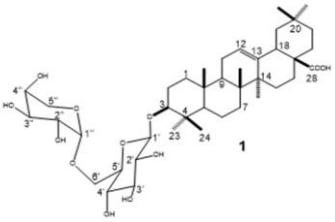

Figure 1. Chemical structure of adianthifilioside GS1 isolated from the roots of *Albizia adianthifolia*

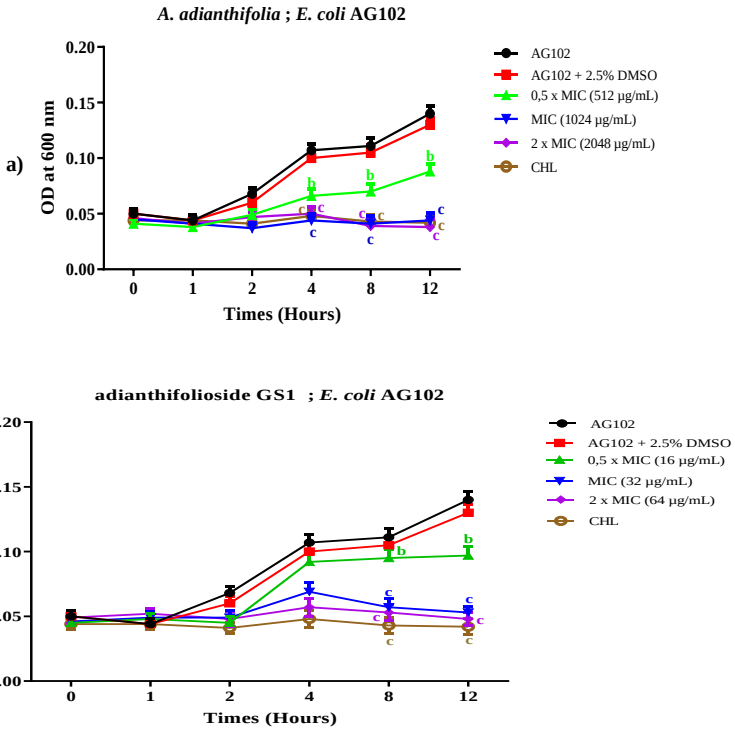

Figure 2. Growth curve of *E. coli* AG102 in the presence of *Albizia adianthifolia* root extract (a) and adianthifolioside GS1 (b)

Each point represents the mean ± ESM; n = 3 (number of repetitions). [b]p < 0.01 significant difference from negative control (AG102); [c]p < 0.001 significant difference from negative control (AG102); AG102 = strain *E. coli* ; 2.5% DMSO = 2.5% (v/v) Dimethylsulfoxide; 0.5 x MIC = Minimum Inhibitory Concentration multiplied by one demi; MIC = Minimum Inhibitory Concentration; 2 x MIC = Minimum Inhibitory Concentration multiplied by two; CHL = Chloramphenicol.

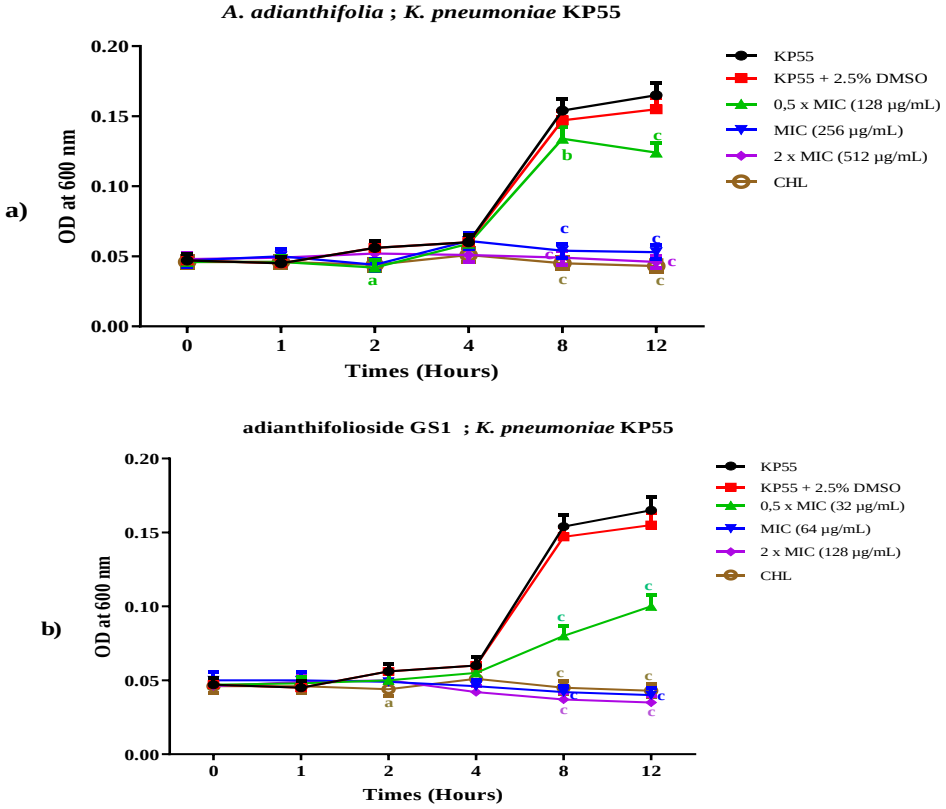

Figure 3. Growth curve of *K. pneumoniae* KP55 in the presence of *Albizia adianthifolia* root extract (a) and adianthifolioside GS1 (b)

Each point represents the mean ± ESM; n = 3 (number of repetitions). [a]p < 0.05 significant difference from negative control (KP55); [b]p < 0.01 significant difference from negative control (KP55); [c]p < 0.001 significant difference from negative control (KP55); KP55 = strain *K. pneumoniae*; 2.5% DMSO = 2.5% (v/v) Dimethylsulfoxide; 0.5 x MIC = Minimum Inhibitory Concentration multiplied by one demi; MIC = Minimum Inhibitory Concentration; 2 x MIC = Minimum Inhibitory Concentration multiplied by two; CHL = Chloramphenicol.

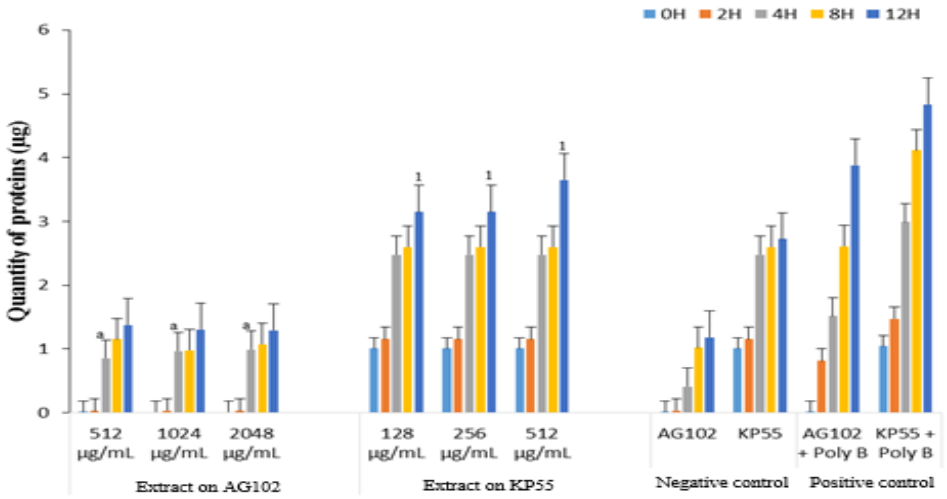

Figure 4. Protein levels in media inoculated with *E. coli* AG102 and *K. pneumoniae* KP55 strains and treated with crude *A. adianthifolia* root extract

Each bar represents the mean ± ESM; n = 3 (number of repetitions). [a]p < 0.05 significant difference from negative control (AG102); [1]p < 0.05 significant difference from negative control (KP55); 512 µg/mL, 1024 µg/mL and 2048 µg/mL represent the values of 0.5 x MIC, MIC and 2 x MIC of the extract on strain E. coli AG102, respectively; 128 µg/mL, 256 µg/mL and 512 µg/mL represent the values of 0.5 x MIC, MIC and 2 x MIC of the extract on K. pneumoniae KP55, respectively; AG102 + Poly B and KP55 + Poly B correspond to solutions of E. coli AG102 and K. pneumoniae KP55 treated with polymycin B, respectively.

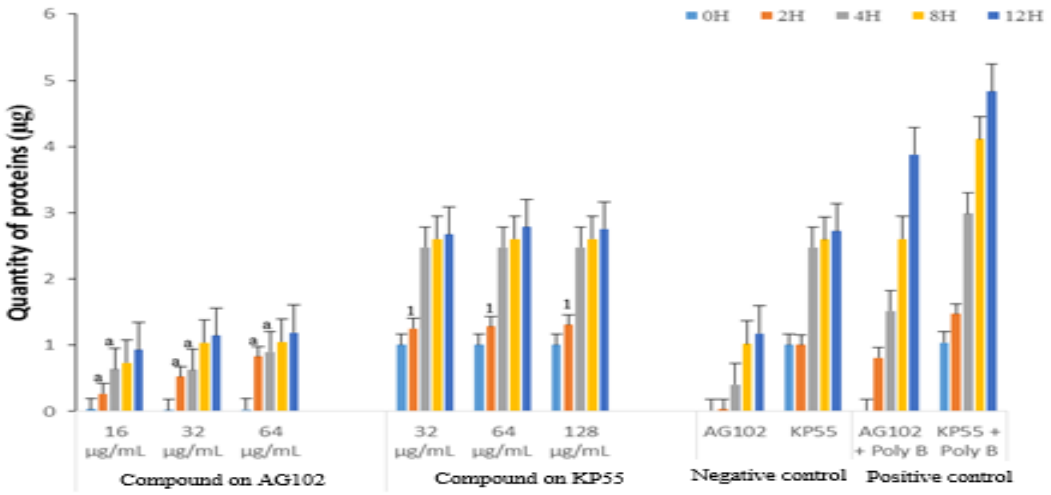

Figure 5. Protein quantities in media inoculated with *E. coli* AG102 and *K. pneumoniae* KP55 strains and treated with adianthifolioside GS1
Each bar represents the mean ± ESM; n = 3 (number of repetitions). [a]p < 0.05 significant difference from negative control (AG102); [1]p < 0.05 significant difference from negative control (KP55); 16 µg/mL, 32 µg/mL and 64 µg/mL represent the values of 0.5 x MIC, MIC and 2 x MIC of adianthifolioside GS1 on strain E. coli AG102, respectively; 32 µg/mL, 64 µg/mL and 128 µg/mL represent the values of 0.5 x MIC, MIC and 2 x MIC of adianthifolioside GS1 on K. pneumoniae KP55, respectively; AG102 + Poly B and KP55 + Poly B correspond to solutions of E. coli AG102 and K. pneumoniae KP55 treated with polymycin B, respectively.

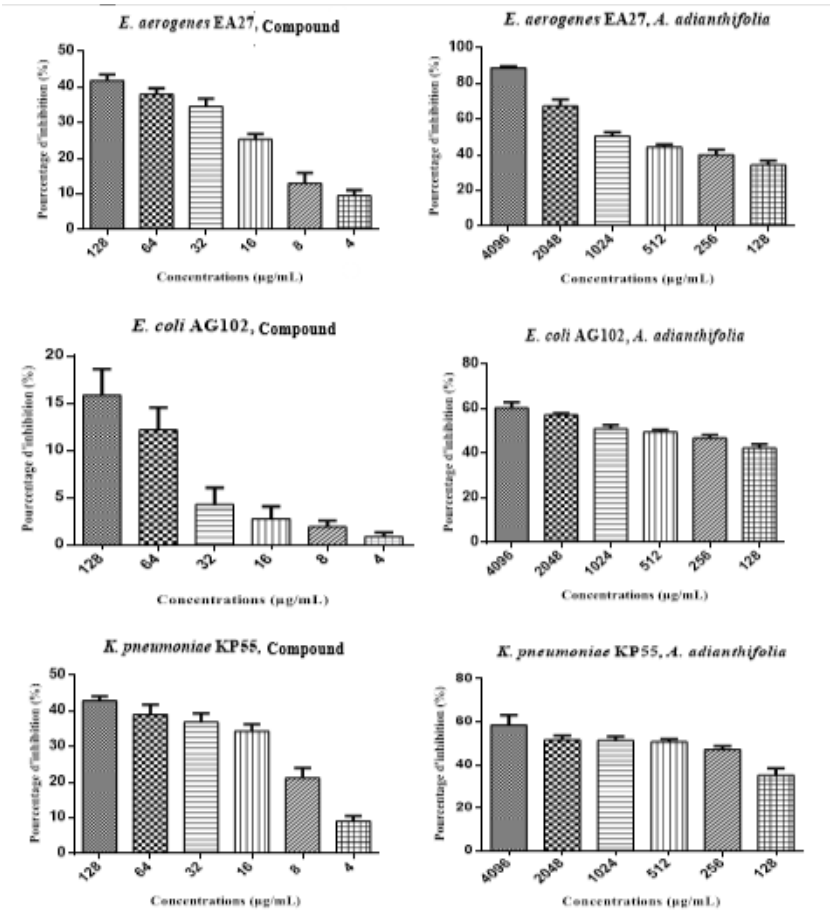

Figure 6. Inhibition of biofilm formation by adianthifolioside GS1 and crude root extract of *Albizia adianthifolia*
Each bar represents the average ± ESM; n = 3 (number of repetitions).

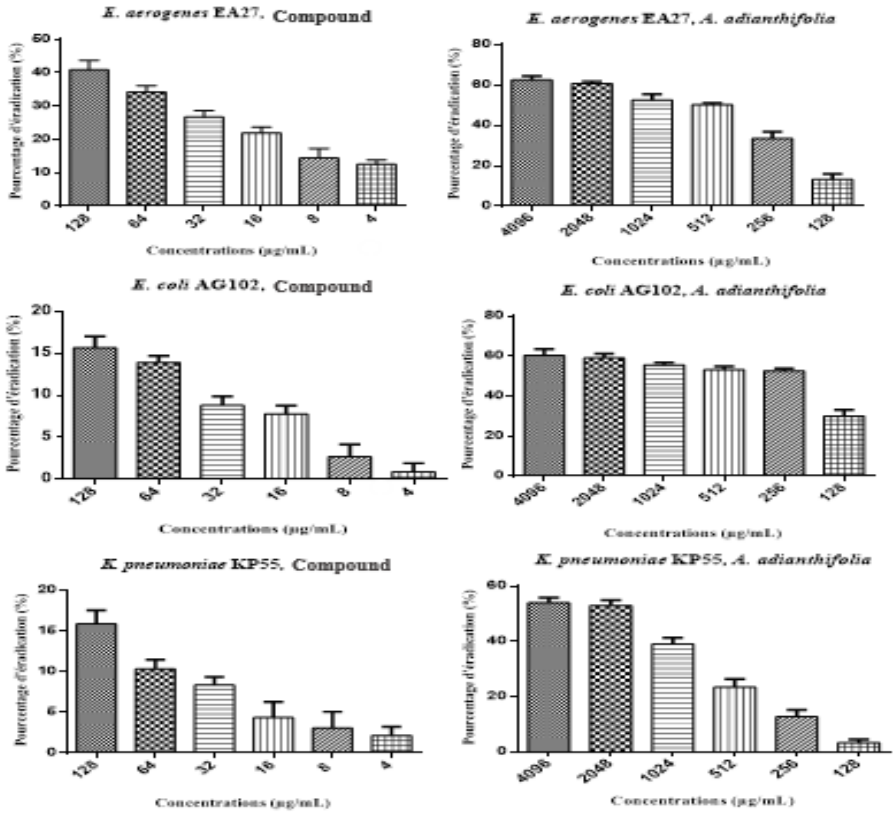

Figure 7. Effect of the root extract of *Albizia adianthifolia* and adianthifolioside GS1 roots on the biofilms formed.
Each bar represents the average ± ESM; n = 3 (number of repetitions).

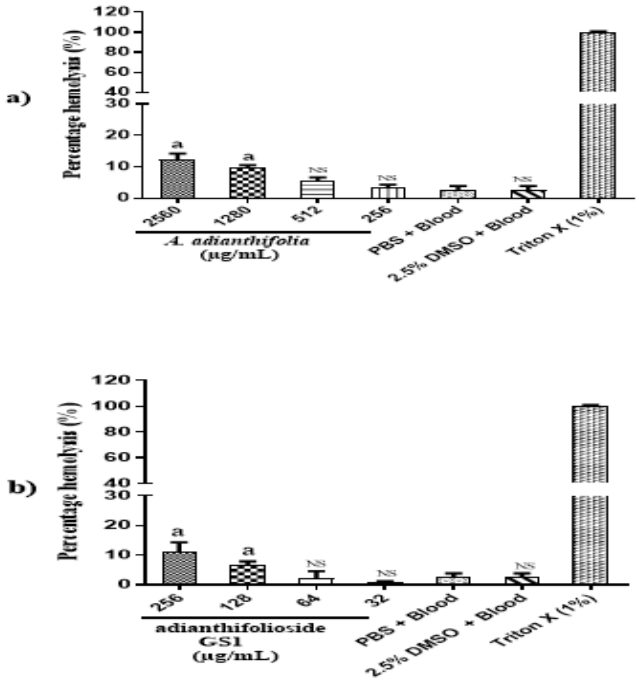

Figure 8. Hemolytic activity of the root extract of *Albizia adianthifolia* (a) and adianthifolioside GS1 (b)
Each bar represents the mean ± ESM; n = 3 (number of repetitions). [a]p < 0.05 Significant difference from negative control (PBS + Blood); NS = Not Significant from negative control (PBS + Blood).

Table 1. MIC of antibiotics after the association with adianthifolioside GS1 at MIC/2 and MIC/4 against seven MDR bacteria strains

Antibiotics	Bacterial strains, MIC (µg/mL) of antibiotics in the absence and presence of adianthifolioside GS1								
	concentration	PA124	KP55	ATCC11296	EA27	ATCC13048	AG102	ATCC10536	PBSS (%)
CIP	**0**	**2**	**0.5**	**0.5**	**0.5**	**4**	**2**	**0.125**	
	CMI/2	**1 (0.5)S**	**0.5 (1)I**	**0.25 (0.5)S**	**0.125(0.25)S**	**0.5 (0.125)S**	**0.5(0.25)S**	**0.0625(0.5)S**	(6/7) 85.7%
	CMI/4	**2 (1)I**	**0.5 (1)I**	**0.5 (1)I**	**0.125(0.25)S**	**0.5 (0.125)S**	**0.5(0.25)S**	**0.125 (1)I**	**(3/7) 42.8%**
ERY	**0**	**>32**	**4**	**>32**	**>32**	**>32**	**16**	**16**	
	CMI/2	**32 (0.5)S**	**2(0.5)S**	**<0.25(<0.007)S**	**16(<0.5)S**	**8 (<0.25)S**	**4 (0.25)S**	**8 (0.5)S**	(7/7) 100%
	CMI/4	**32 (0.5)S**	**4 (1)I**	**<0.25(<0.007)S**	**16(<0.5)S**	**8 (<0.25)S**	**4 (0.25)S**	**8 (0.5)S**	(7/7) 100%
GEN	**0**	**>4**	**2**	**>4**	**4**	**4**	**>4**	**4**	
	CMI/2	**4 (0.5)S**	**1(0.5)S**	**1 (<0.25)S**	**1 (0.25)S**	**2 (0.5)S**	**2 (<0.5)S**	**0.5 (0.125)S**	(7/7) 100%
	CMI/4	**>4**	**1(0.5)S**	**1 (<0.25)S**	**1 (0.25)S**	**2 (0.5)S**	**2 (<0.5)S**	**2(0.5)S**	(6/6) 100%
KAN	**0**	**0.5**	**2**	**4**	**4**	**16**	**16**	**4**	
	CMI/2	**<0.125(0.25)S**	**1 (0.5)S**	**2 (0.5)S**	**4 (1)I**	**16 (1)I**	**8 (0.5)S**	**2 (0.5)S**	(5/7) 71.4%
	CMI/4	**<0.125(0.25)S**	**2 (1)I**	**4 (1)I**	**4 (1)I**	**16 (1)I**	**8 (0.5)S**	**4 (1)I**	**(2/7) 28.5%**
NOR	**0**	**>16**	**16**	**1**	**16**	**16**	**2**	**1**	
	CMI/2	**8(<0.5)S**	**2(0.125)S**	**0.125(0.125)S**	**4 (0.25)S**	**8 (0.5)S**	**0.5 (0.25)S**	**0.5 (0.5)S**	(7/7) 100%
	CMI/4	**8(<0.5)S**	**2(0.125)S**	**0.5 (0.5)S**	**4 (0.25)S**	**16 (1)I**	**2 (1)I**	**1 (1)I**	**(4/7) 57.1%**
STR	**0**	**>32**	**>32**	**>32**	**>32**	**>32**	**>32**	**>32**	
	CMI/2	**32 (0.5)S**	**32 (0.5)S**	**2 (<0.062)S**	**32 (0.5)S**	**32 (0.5)S**	**16 (<0.5)S**	**2 (<0.062)S**	(7/7) 100%
	CMI/4	**32 (0.5)S**	**32 (0.5)S**	**2 (<0.062)S**	**32 (0.5)S**	**32 (0.5)S**	**16 (<0.5)S**	**4 (<0.125)S**	(7/7) 100%
TET	**0**	**8**	**0.125**	**>16**	**>16**	**>16**	**>16**	**16**	
	CMI/2	**4 (0.5)S**	**0.125 (1)I**	**0.125(<0.007)S**	**4(<0.25)S**	**0.125(<0.007) S**	**0.5(<0.031)S**	**0.125(0.007)S**	(6/7) 85.7%
	CMI/4	**4 (0.5)S**	**0.125 (1)I**	**0.125(<0.007)S**	**4(<0.25)S**	**0.125(<0.007) S**	**1(<0.062)S**	**0.125(0.007)S**	(6/7) 85.7%

[a]Antibiotics [CIP: Ciprofloxacin, ERY: Erythromycin, GEN: Gentamycin, KAN : Kanamycin, NOR : Norfloxacin, STR: Streptomycin, TET : Tetracycline]. [b]Bacteria : Escherichia coli [ATCC10536, AG102], Pseudomonas aeruginosa [PA124], Enterobacter aerogenes [ATCC13048, EA27], Klebsiella pneumoniae [ATCC11296, KP55]. PBSS: Percentage of bacteria strain on which synergism has been observed; S: Synergy; I : Indifference ; ( ) : FIC (Fractional Inhibitory Concentration) of the antibiotics after association with compounds , 0 : MIC of the antibiotic also.

Table 2. Results of the biofilm sensitivity test for the crude extract and adianthifolioside GS1 from *Albizia adianthifolia* roots

Bacterial strains	*A. adianthifolia* (roots)		adianthifolioside GS1	
	CMI	**CIB_{50}**	**CMI**	**CIB_{50}**
E. coli AG102	**1024**	**537**	**32**	**-**
E. aerogenes EA27	**256**	**1023**	**128**	**-**
K. pneumoniae KP55	**256**	**407**	**64**	**-**

- : Inactive; MIC: Minimum Inhibitory Concentration; CIB_{50}: Minimum Inhibitory Concentration of 50% of the biofilm; these values are expressed in µg/mL.

Conclusion

This study presents results that would bode well for the use of substances derived from the roots of *Albizia adianthifolia* in the fight against infections caused by multi-resistant Gram-negative bacteria. Adianthifolioside GS1 could be used in combination with antibiotics to overcome bacterial resistance. More interestingly, the mechanisms of action of the raw root extract and one of its constituents are presented for the first time in this book. In order to make these results more specific in the development of new phytomedicines or drugs respectively, cytotoxicity and acute and sub-acute toxicity tests could be studied.

Additional files

Supplementary file 1: **Table S1.** Bacterial strains used and their characteristics. **Table S2.** Preliminary evaluation of the antibiotic resistance modulating activity of selected samples at sub-inhibitory concentrations against *Pseudomonnas aeruginosa* PA124. **S3.** Kinetics of strain evolution in the presence of the test samples

adapted to the Baranyi model and kinetic parameters. **S4.** Calibration curve of the assay by the Bradford method. **S5.** Biofilm formation inhibition curves. **S6.** Blood Formula Counts (BFC) result sheet. Available at: https://www.investchempharma.com/imcp46-supplementary-file-tchinda-et-al/

Abbreviations

A. adianthifolia : *Albizia adianthifolia* ; ATB: Antibiotic ; ATCC: American Type Culture Collection ; BEC: Biofilm Eradication Concentration ; CC: column chromatography; CFU: Colony Forming Unit; CHL: Chloramphenicol ; CIP: Ciprofloxacin ; DMSO: Dimethyl sulfoxide ; *E. aerogenes: Enterobacter aerogenes ; E. coli: Escherichia coli ;* ERY: Erythromycin ; GEN: Gentamycin ; INT: p-iodonitrotetrazolium chloride ≥ 97% (INT, Sigma-Aldrich) ; *K. pneumoniae: Klebsiella pneumoniae ;* KAN: Kanamycin ; $MBIC_{50}$: Minimum Biofilm Inhibitory Concentrations 50% ; MDR: Multidrug resistant ; MHB: Mueller Hinton Broth ; MIC: Minimal Inhibitory Concentration ; NMR: Nuclear Magnetic Resonance ; NOR: Norflocaxin ; OD: Optical Density ; PEN: Penicillin ; *P. aeruginosa: Pseudomonas aeruginosa ;* PBS : Phosphate Buffer Solution ; STR: Streptomycin; TET: Tetracyclin, TLC: Thin Layer Chromatography.

Authors' Contribution

CFT and IKV carried out the study and designed the experiments; CFT and VK wrote the manuscript; VK and VPB 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

Authors declare that there no conflict of interest.

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