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Antibiotic-potentiation activities of three animal species extracts, *Bitis arietans, Helix aspersa,* and *Aristaeomorpha foliacea* and mode of action against MDR Gram-negative bacteria phenotypes

Michel-Gael F. Guefack[1], Simplice B. Tankeo[1], Carine M.N. Ngaffo[1], Paul Nayim[1], Brice E.N. Wamba[1], Idrios N. Bonsou[1], Victor Kuete[1], Armelle T. Mbaveng[1*]

Abstract

Background: In recent years, drug resistance to human pathogenic bacteria has been commonly reported from all over the world. As antimicrobial activities of most medicinal plants and antibiotics have been already explored, it is more important to make investigations on animal species mainly invertebrates which could constitute an efficient source of antimicrobial molecules. This work was aimed at contributing to the fight against microbial resistance through the study of antibacterial potential of three animal species (*Helix aspersa, Bitis arietans, Aristaeomorpha foliacea*) on several multidrug-resistant (MDR) Gram-negative strains overexpressing efflux pumps including *Escherichia coli*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*.

Methods: The microdilution technique was used to evaluate the antibacterial activities of the tested samples by determining their minimal inhibitory concentrations (MICs), as well as the effect of their combination with antibiotics. Studies on the mechanisms of action of the most active sample, dried *Bitis arietans* extract, was carried out using standard methods for evaluating the effects of this extract on bacterial H^+-ATPases-mediated proton pumps and on bacterial growth kinetics. In this latter case, the optical density was read spectrophotometrically.

Results: Zoochemical screening indicated the presence of protein constituents and alkaloids and the absence of other metabolites in all tested extracts. Dried *B. arietans* showed the best antibacterial activity by inhibiting the growth of 90% of studied bacterial strains with MICs ranging from 128 to 2048 µg/ml. Moreover, this extract presented a significant activity (100≤MIC≤512 µg/ml) against 35% of bacteria that are *E. coli* (ATCC8739, AG100A_{Tet}, MC4100), *E. aerogenes* EA27, *K. pneumoniae* ATCC11296, *P. aeruginosa* (PA01, PA124) and a moderate activity (512<MIC≤2048 µg/ml) against 55% of studied bacteria. It was followed by fresh *B. arietans* which inhibited the growth of 65% of bacteria with significant activity on three bacteria (*E. coli* ATCC8739, *E. aerogenes* ATCC13048 and *K. pneumoniae* ATCC11296. These two extracts showed bactericidal effects on many strains. The other extracts samples selectively exhibited an antibacterial activity against less than 40% of strains. All samples potentiated the activity of at least 56% of used antibiotics against at least 70% of studied bacterial strains. *B. arietans* extracts at MIC/2 and MIC/4 mostly improved the activities of more than 78% of antibiotics on at least 70% of bacteria with improvement activity factors (IAF) ranging from 2 – 128 suggesting that this animal contains bioactive compounds which could act as efflux pumps inhibitors. Bacterial growth kinetic study showed that when treated with dried *B. arietans* extract (the most active sample) at different concentrations MIC/2, MIC and 2xMIC, the growth of tested bacteria (*E. coli* ATCC8739) decreased respectively when the concentrations increased. Furthermore, this extract inhibited the H^+-ATPase-mediated proton pumps of this bacterium increasing the pH values.

Conclusion: Results obtained in the present work provide interesting data for the use of dried *B. arietans* extract and invertebrates in general in the traditional therapy for the treatment of bacterial infections involving multidrug-resistant phenotypes.

Keywords: Gram-negative bacteria; multidrug resistance; efflux pumps; infectious diseases; animal species; secondary metabolites.

*Correspondence: Tel.: +237 675468927; E-mail address: armbatsa@yahoo.fr; ORCID: https://orcid.org/0000-0003-4178-4967 (Prof. Dr A.T. Mbaveng)
[1]Department of Biochemistry, Faculty of Science, University of Dschang, Dschang, Cameroon.

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Background

Infectious diseases are the main cause of mortality in the world with about 17 million of victims each year, with higher prevalence in Sub-Saharan Africa [1]. According to the World Health Organization, infectious diseases are the leading cause of death among children and young adults worldwide, with a higher prevalence in developing countries [2]. Infections caused by resistant microorganisms often fail to respond to the standard treatment, resulting in prolonged illness and greater risk of death. Antimicrobial resistance hampers the control of infectious diseases and increases the costs of health care [3]. Many cases of resistance determinants have been described with the emergence of more and more resistant bacteria like *Escherichia coli*, *Pseudomonas aeruginosa, Klebsiella pneumoniae or Staphylococcus aureus* [4]. Such bacteria are becoming a serious clinical problem throughout the world. The impact of multidrug resistance is clinically and economically important in terms of morbidity and mortality as well as in terms of hospitalization costs. The proliferation of resistant bacteria and bacterial infections is nowadays a major public health problem despite the abundance of antibiotics developed to limit their incidence. This resistance reduces the efficacy of the commonly used antibiotics leading to an increase in the number of infected persons as well as deaths. Amongst the different mechanisms used by bacteria to resist or escape the action of antibiotics, active efflux is one of the most important which is mainly found on Gram-negative bacteria [5,6,7]. RND (resistance-nodulation cell division) family of efflux pumps represents the main cause of multidrug resistance by efflux in Gram-negative bacteria. Faced with this worrying situation, the search for new natural effective and available antibacterial compounds, but also substances capable of potentiating the activity of commonly used antibiotics, is therefore becoming essential. Several studies reported in the literature demonstrated their capacity to improve the antimicrobial activities of conventional antibiotics [8,9,10,11]. Naturally, animals are rich in many substances including proteins, polypeptides or glycopeptides and certain secondary metabolites which can have a pharmacological interest or display an antimicrobial activity [12,13]. Many peptides are considered as the main element of inner immune system of the multicellular organisms. Their structure confers to them some essential characteristics for their antimicrobial activities [14,15]. Animals like *Helix aspersa, Bitis arietans, Aristaeomorpha foliacea* are traditionally used in the treatment of some illness (Table 1). To date, only a few studies have been made on the antimicrobial activities of animal species. The present study was carried out to investigate the antibacterial potential of the methanolic extracts of the above three animals, their capacity to enhance the activity of some conventional used antibiotics as well as the mechanisms of action of the most active extract.

Methods

Animal's collection

Tested samples were constituted of three adult vertebrate and invertebrate animal species namely *Helix aspersa, Bitis arietans, Aristaeomorpha foliacea*. They were collected in Dschang (West Region of Cameroon) in October and December 2018 and were identified in the laboratory of animal biology, University of Dschang-Cameroon where a sample was deposited. Table 1 shows studied samples and some information about their traditional use as well as their previous biological activities.

Microorganisms

Twenty multidrug resistant Gram-negative bacteria were used. These microorganisms constituted of reference strains and clinical isolates included *Escherichia coli, Enterobacter aerogenes, Klebsiella pneumoniae, Providencia stuartii* and *Pseudomonas aeruginosa* strains. They were provided from American Type Culture Collection (ATCC) and laboratory of UMR-MD1 of the University of Mediterranean, Marseille, France. Table 2 shows studied bacteria with their characteristics.

Chemicals and culture media

Nine conventional antibiotics including Ciprofloxacin (CIP), Erythromycin (ERY), Oxaciclin (OXA), Thiamphenicol (THI), Flucloxacillin (FLC), Ofloxacin (OFL), Azithromycin (AZT), Doxycycline (DOX), Gentamycin (GEN) were used. *p*-Iodonitrotetrazolium chloride (INT) was used for colorimetric detection of living bacteria and dimethylsulfoxide (DMSO) for extracts and antibiotics dissolution. All these substances provided from Sigma-Aldrich (St. Quentin Fallavier, France). The Mueller Hinton Agar and Mueller Hinton Broth were used as culture media respectively for bacterial activation and for the determination of antibacterial activity parameters: minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs).

Animal extraction

Animals were firstly washed with water, then flesh were isolated from shells, scales and internal organs as guts, pancreas, lungs, heart and other parts different from the flesh. These fleshes were then washed with a phosphate buffer, pH 7.2 to avoid loss of proteins and water and were dried at room temperature sheltered from sun. Dried samples were crushed, and the obtained powders were macerated in methanol solvent in the proportions 1/3 m/v for 48 hrs shaking three times per days. After filtration using Whatman n°1 filter paper, the filtrates obtained were concentrated under reduced pressure (at 65°C) in a rotary evaporator to give the crude extracts which were dried at room temperature for complete evaporation of solvent. These crude extracts were kept at 4°C until further use. Extractive yields (Table 1) of each sample were obtained by calculating the (crude extract weight / powder weight) x100.

Qualitative zoochemical screening

The detection of peptides in crude extracts was carried out using the ninhydrin reaction [16]. In presence of excess and hot ninhydrin, amino acids, peptides, or proteins undergo an oxidative deamination and decarboxylation. Ammonia condenses with two molecules of ninhydrin to form a purple complex. The detection of main classes of secondary metabolites including alkaloids, flavonoids, tannins, saponins, steroids, phenols, terpenes, anthraquinones and anthocyanins (Table 3) was carried out using previously described methods [17].

Antibacterial essays

Determination of minimal inhibitory and bactericidal concentrations

The minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBC) were determined using

microdilution method as described by [18] with some modifications [19]. In a 96-wells plate containing 100 µl of MHB culture medium, 100 µl of animal extracts dissolved in DMSO 2.5 % was added to first wells and then serially distributed to other wells. Then 100 µl of bacterial suspension ($2x10^6$ UFC/ml) were added to all wells to afford 200 µl. DMSO 2.5 % and Ciprofloxacin, the reference antibiotic tested at 256 µg/mL final concentration, were respectively used as negative and positive controls. Plates were then covered and incubated at 37°C for 18 hrs after which 40 µl of INT 0.2 % were introduced and plates were reincubated at 37°C for 30 min. The INT (yellow colour) is reduced by viable bacteria to yield pink colour. The MIC was defined as the lowest concentration that prevented the change of this colour and which resulted in the complete inhibition of bacterial growth. Moreover, the minimal bactericidal concentrations (MBCs) were determined by adding 50 µl from the previous wells content that did not received INT and that correspond to MICs values, to 150 µl of MHB contained in news plates. After incubation at 37°C for 48 hrs and addition of 40 µl of INT 0.2 %, MBCs of each sample were determined as described above. Each assay was performed in triplicate and two independent times (Table 4).

Plant extract was considered to have strong activity if MIC<100 µg/ml, significant activity if 100≤MIC≤512 µg/ml, moderate activity if 512<MIC≤2048 µg/ml and weak activity if MIC>2048 µg/ml. Moreover, plant extract was considered to bactericidal effect if MBC/MIC≤4 and bacteriostatic effect if MBC/MIC>4 [20].

Determination of minimal inhibitory concentrations of the combination

Plant extracts were combined to conventional antibiotics (tested at 256 µg/ml final concentration) to evaluate the effect of their association against tested bacteria. This was carried according to the method described by [19]. After serially diluted antibiotic solutions with concentrations varying from 0.5 to 256 µg/mL, 50 µl of extract solution followed by 50 µl of bacterial inoculum ($4x10^6$ UFC/ml) were then added and the microplates were then coved and incubated at 37°C for 18h. MICs of the combination extract-antibiotics were determined by introducing 40 µl of INT 0.2 % as described above. Preliminary tests were performed against *Pseudomonas aeruginosa* PA124 strain which was the most resistant bacteria and extracts were tested at MCI/2, MIC/4, MIC/8 and MIC/16(results are summarized in Table 1 of additional file). From the obtained results, two concentrations of extracts (MCI/2 and MIC/4) were chose to be tested against the other studied bacteria including *E. coli* (ATCC8739 and AG102), *E. aerogenes* (ATCC13048 and CM64), *K. pneumoniae* (ATCC11296 and KP55), *P. aeruginosa* (PA01 and PA124), *P. stuartii* (ATCC29916 and NEA16). The effects of combination were estimatited by calculating the improvement activity factors (IAF) of each combination using the following formulation: MIC of antibiotic alone / MIC of combination (Tables 5-10). Each assay was also performed in triplicate and two independent times. Extract and antibiotic were considered to have synergistic, indifferent, or antagonistic effects if IAF≥2, IAF=1 or IAF≤0.5 respectively [21].

Antibacterial mechanisms of action

The mechanisms of action of dried *Bitis arietans* which showed the best antibacterial activity, were investigated against the bacterial strain *Escherichia coli* ATCC8739.

Effect of tested extract on bacterial growth kinetic

Bacterial growth kinetic study was carried out using a spectrophotometer at 600 nm wavelength [22] and the sample was tested at concentrations of MIC/2, MIC and 2xMIC. Firstly, 500 µl of bacterial suspension ($1.5x10^8$ UFC/ml) from preculture were added to 450 mL of MHB (1/100 v/v dilution) and incubated at 37°C for 18 hrs 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) and DMSO (2.5% v/v) constituted the negative control. At regular interval times of 2 hrs from 0 to 18 hrs, aliquots of 1 mL from the preparation were deducted and introduced in a spectrophotometric tab and then, the optical density was read at a wavelength of 600 nm. From the obtained results, bacterial growth curves [DO = f (times)] were plotted using Microsoft excel software (Figure 1).

Effect of tested extract on bacterial H^+-ATPase-mediated proton pumps

It was done following the acidification of the external environment of the bacteria with the use of a pH-electrode [23]. A fresh bacterial colony was dissolved in 20 mL of MHB culture medium and incubated at 37°C under magnetic agitation for 18 hrs. Aliquots of 1 mL from this bacterial preculture were deducted and added to MHB to afford 100 mL final volume (1/100 v/v dilution), and then re-incubated at 37°C for 18 hrs under magnetic agitation. One hundred millilitre from this bacterial culture was centrifuged at 4000 rpm for 30 min at 4°C. Recuperated gut was washed with sterile distilled water then with KCl 50 mM and was dissolved in 50 mL KCl 50 mM. Obtained bacterial suspension was conserved at 4°C for 18 hrs (for glucose starvation), after which the pH was adjusted to 6.48 by adding HCl or NaOH solution. Then, 0.5 mL of tested sample was added to 4 mL of this bacterial culture ($1.5x10^8$ UFC/ml) and the mixture was incubated at 37°C for 10 min, after which, 0.5 mL of glucose 20% was added in order to initiate the acidification of the environment. DMSO (2.5% v/v) constituted the negative control. The pH values of tested samples were read at room temperature (25°C) every 10 min for 70 min, using a pH-meter. The curves [pH = f (times)] were labelled using Microsoft Excel software (Figure 2).

Results

Zoochemical composition

Extracts samples were submitted to a qualitative chemical screening to detect bioactive components in each animal extract as shown in Table 3. In fact, this table indicated that protein, amino acids, or peptides as well as alkaloids were present in all extracts. Meanwhile, the other analysed bioactive compounds were absent in all samples.

Antibacterial activity of plants extracts alone

Table 4 shows antibacterial activities of studied samples against some strains. This table indicates that all samples inhibited the activity of at least one bacterial strain with MICs ranging from 128 to 2048 µg/ml. *Bitis arietans* extract was the most active sample as they showed antibacterial activities on 90% (18/20) and 65% (13/20) of strains for dried and fresh extracts respectively. Moreover, dried one showed significant activity (100<MIC≤512 µg/ml) against 35% of bacteria with MIC= 128 µg/ml on two

Escherichia coli strains (ATCC8739 and AG100A$_{Tet}$) and a moderate activity (512<MIC≤2048 µg/ml) against 55% (8/20) of studied bacteria. Meanwhile, fresh one showed moderate activity against 65% of bacteria and weak antibacterial activity against the remaining strains. Furthermore, these two extracts showed bactericidal effects (MBC/MIC≤4) against many bacteria. This effect was most observed on *E. coli* and *Pseudomonas aeruginosa* strains. *Helix aspersa* and *Aristaeomorpha foliacea* extracts selectively inhibited the growth of less than 40% of studied bacteria with moderate or weak activities and did not show any MBC against all bacteria. For each plant sample, fresh crude extract was less active than dried one. Ciprofloxacin, the reference antibiotic used as positive control, inhibited the growth of all bacteria and presented a bactericidal effect against 80% of strains, whereas DMSO 2.5% used as negative control does not showed any antibacterial activity against all studied strains. *E. coli* ATCC8739 was the most susceptible bacterium whereas *P. aeruginosa* PA124 was the most resistant strain.

Effects of extract-antibiotic combination

Samples were then combined to commonly used antibiotics to improve their activities and the results are shown in Tables 5-10. The numbers in parentheses which represent the improvement activity factors (IAF) mean the close relationship and type of effect (synergism, antagonism, or indifference) between extract sample and antibiotic. Notice that in most cases, synergistic effects were obtained for each combination, as the MICs of the combination are less than those of antibiotic when tested alone. IAF values varied from 2 to 128 and did not change at MIC/2 and MIC/4 in most cases. Moreover, at MIC/2 and MIC/4, dried and fresh *Bitis arietans* extracts improved the activities of 78% (7/9) and 67% (6/9) of antibiotics (Oxacillin, Gentamicin, Ciprofloxacin, Doxocyclin, Flucloxacillin, Ofloxacin, Azithromycin) respectively against more than 70% of studied bacterial strains (Tables 5 and 6). With dried *Aristaeomorpha foliacea* at MIC/2 and MIC/4, the activities of 78% and 33% of antibiotics respectively were improved against more than 70% of bacteria. Meanwhile, the fresh portion of the same animal extract, at MIC/2 as well as at MIC/4, enhanced the antibacterial potential of 56% of tested antibiotics against more than 80% of strains (Tables 7 and 8). Almost similar situations were obtained with *Helix aspersa* animal as at MIC/2, its dried extract increased the activity of 56% of antibiotics and 33% of antibiotics at MIC/4 against at least 70% of studied bacteria. Instead at MIC/2 and MIC/4 of its fresh extract, the antibacterial potential of 56% of tested antibiotics improved on more than 70% of strains (Tables 9 and 10). Notice that all animal extracts improved the activity of all antibiotics on at least 40% of bacteria, except dried *A. foliacea* and dried *H. aspersa* which enhanced the activities of 20% (Thiamphenicol and Erythromycin) and 30% (Oxacillin, Thiamphenicol and Erythromycin) respectively against less than 30% of bacteria. Ciprofloxacin and Doxocyclin were antibiotics which mostly presented synergistic effects (IAF≥2) with all extracts at the two sub-inhibitory concentrations. Indifferent effects (IAF=1) were mostly obtained against *P. aeruginosa* PA124. Furthermore, antagonistic effects (IAF≤0.5) were selectively obtained in some few cases. Higher values of IAF (16 – 128) were mostly obtained combining extracts with Gentamicin, Ciprofloxacin, Doxocyclin, Ofloxacin and Azithromycin.

Effect of dried B. arietans on bacterial growth kinetic

Figure 1 shows bacterial (*E. coli* ATCC8739) growth curves in absence and presence of dried *B. arietans* at different concentrations (MIC/2, MIC and 2xMIC) and ciprofloxacin (at MIC) during the time. Growth kinetic was followed-up during 18 hrs and the optical density (OD) values were measured each 2 hrs at 600 nm wavelength. After lag-phase which lasted for 2 hrs, bacteria grow exponentially in absence of treatment (inoculum alone) and in presence of DMSO 2.5% used as negative control till 18 hrs. This bacterial growth decreases in presence of tested extract sample when his concentrations increase. At MIC/2, bacteria started to grow after 8 hrs and the number remains constant after 2 hrs. At MIC and 2xMIC of this extract, the number of bacteria in the medium is very low after 18 hrs of incubation and the lag-phase is prolonged till 12 hrs for MIC and 14 hrs for 2xMIC. Meanwhile, when treated with reference antibiotic ciprofloxacin used as positive control, no bacterial growth was not observed after 18 hrs of the experiment. The growth of the bacterial strain in presence of *B. arietans* at 2xMIC is almost the same as those in presence of ciprofloxacin, the reference antibiotic.

Effect of dried B. arietans on bacterial H^+-ATPase-mediated proton pumps

Figure 2 shows the effect of dried *B. arietans* at MIC on *E. coli* ATCC8739 H^+-ATPase-mediated proton pumps. During period of the experiment, the pH values decreased from 6.4 to 4.8 in presence of DMSO 2.5% used as negative control. Whereas, when the inoculum was treated with tested extract, pH values increased from 6.4 to 7.7. Notice that a decrease of pH values is favourable for the growth and multiplication of studied bacterial, whereas increase of pH values (basic condition of the environment) is unfavourable for its growth.

Discussion

This work was carried out to evaluate the antibacterial potential of the extracts from some animal species including *Bitis arietans, Helix aspersa and Aristaeomorpha foliacea*. The antimicrobial activity of a natural substance depends on bioactive components contained in this substance. A natural substance is considered to have strong activity against one or more bacterial strain if MIC≤100 µg/ml, significant activity if 100<MIC≤512 µg/ml, moderate activity if 512<MIC≤2048 µg/ml and weak activity if MIC>2048 µg/ml [20]. Based on this classification scale, dried *B. arietans* extract which showed significant activity against 35% of bacteria with lowest MICs (128 µg/ml) against two *E. coli* (ATCC8739 and AG100A$_{Tet}$) and moderate activity against other studied bacteria was the most active sample. It had been followed by fresh *B. arietans* which showed significant and moderate activities against 15% and 50% of bacteria, respectively. To the best of our knowledge, no antimicrobial activity of *B. arietans* extracts against MDR phenotypes has not been reported. But several previous studies on the same specie or family animals have been investigated. Oils from some *Bitis* gender have been shown to have antibacterial properties against *E. coli* and *P. aeruginosas* [24]. Venoms from *Bothrops jararaca* and *Cerastes cerastes* snakes showed bacteriostatic and bactericidal effects against some bacterial strains [25]. This can justify the bactericidal effects obtained with *B. arietans* extracts against some studied strains in this work. This activity could be related to the presence of bioactive molecules such as L-amino acid oxidase and glycopeptides. Previous studies reported the antibacterial activities of these molecules and antimicrobial peptides which could act either on the structural integrity of membrane through the formation of pores, canals or

micelles, on intracellular targets [25,26,27,28]. Now, it is fully documented that the snake venoms have several cytotoxic factors along with potent killing on bacteria as well as viruses. Moreover, moderate antibacterial activity of aqueous and methanol extracts from *Helix aspersa* against *Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* strains have been reported [28]. This corroborates results obtained in the present work. *H. aspersa* flesh was shown to be rich in antimicrobial peptides, proteins, water and poor in fats. Also, some minerals and oligo-elements including calcium, magnesium, phosphor, ion, copper, and vitamins (B6, B12 et C) present in large quantity in flesh snail could participate in biological activity of antimicrobial peptides [29]. Achacin, a glycopeptides present in snail mucus, showed a bactericidal effect breaking down bacterial cellular membrane. Recently, several studies carried out on snail secretion composition have confirmed that the

H. aspersa mucus contains a good number of natural substances with beneficial and therapeutic properties for human skin, such as allantoin and glycolic acid [30,31]. *Aristaeomorpha foliacea* in this work also showed moderate antibacterial activity against some studied strains. Until now, no antimicrobial study of this animal species had not been reported. The present work constitutes the first investigation concerning the antimicrobial potential of this animal sample. Nevertheless, antioxidant properties from extract of *A. foliacea* have been reported. These properties are due to the presence of phenol and carotenoid bioactive components in this extract [32]. Peptides and proteins as well as alkaloids were detected in all tested samples. The different antibacterial activities of tested extracts observed could be due to the quantity of each of these bioactive components in each extract. It had been shown that antimicrobial potential of a bioactive component in a natural substance is also influenced by its concentration or proportion [33]. This difference of activity could also be due to other elements present that could negatively or positively participate to the activity of extract, acting in antagonistic or synergistic manner, respectively.

To be very efficient, different classes of antibiotics are used to be combined for the treatment of infections caused by pathogenic bacteria. Nowadays, several studies are oriented to the combination of natural bioactive compounds and commonly used antibiotics to evaluate the potentiation effects of the activity of antibiotics by these compounds [8,9,11,34,35,36]. Extracts were tested at the sub-inhibitory concentrations MIC/2 and MIC/4 and the calculated values of the improvement activity factors (IAF) indicated the degree of correlation between antibiotic and bioactive components of extract. All tested samples selectively improved the activity of used antibiotics suggesting a synergistic effect between them, as IAF values are ≥2. This effect can be explained to the fact that bioactive components of extracts and antibiotic might act by different mechanisms on different target sites of the bacteria [37]. Among these mechanisms, one of the most important is the inhibition of efflux pumps which is the main mechanism used by studied bacteria to resist to many classes of antibiotics [5,6,7,38]. It has been shown that an antibacterial substance which potentiates the activity of around 70% of antibiotics against 70% of bacteria could be considered an efflux pumps inhibitor (EPI) [39]. In the present work, *B. arietans* and *A. foliacea* extracts which have significantly increased the activities of the majority of antibiotics against most bacteria could contain bioactive compounds acting as efflux pumps inhibitors. As antibiotics are actively effluxed, inhibition of these pumps or their genes expression by these bioactive compounds increases the intracellular concentrations of antibiotics allowing them to efficiently act upon on their targets [30,40,41]. It had been reported that baicalein a trihydroxy flavone isolated from *Thymus vulgaris* plant, exhibited an inhibiting effect of EPs in methicillin-resistant *S. aureus* restoring the activity of some antibiotics [42]. Moreover, indifferent effects (IAF=1) obtained in some cases indicate that extract has no inhibitory effect against bacteria and could not influence the antibacterial activity of antibiotic.

The effect of dried *B. arietans* on *E. coli* ATCC8739 growth kinetic was investigated (Figure 1). Microbial growth results in increase in number or size of micro-organism. Bacterial growth kinetic is mainly constituted of a lag-phase followed by an exponential-phase and a stationary-phase as well as a decline-phase [43,44]. In normal growth conditions, the duration of the lag-phase is approximately 2 hrs during which bacteria adapt themselves to their new environment and produce enzymes for substrates metabolism [45]. During exponential-phase, the bacteria cell rapidly multiply themselves and their number considerably increases before going down at the stationary-phase as the number of deaths is equal to the number of living bacteria. This is due to the arrest of bacterial growth because of the reduction of nutrients in the medium. Decline phase corresponds to completely lack of nutrients and toxic wastes accumulation in the medium conducting to death of higher number of bacteria [44]. In the present study, curves obtained with negative controls (inoculum alone and DMSO) showed normal growth of studied bacteria with respect to the above descriptions. Meanwhile in presence of tested sample, bacterial growth is very low suggesting the inhibitory effect of this sample on this bacterial multiplication. Notice that lag-phase was prolonged till 8 hrs for MIC/2, 12 hrs for MIC and 14 hrs for 2xMIC of extract justifying that the higher the concentration, the lower the maximum specific growth rate. This explains the bactericidal effect of this dried *B. arietans* against tested bacteria (Table 4). Similar results against the same studied bacterial strain were previously reported [46].

The capacity of dried *B. arietans* to inhibit bacterial H^+-ATPase-mediated proton pumps of plasma membrane was also evaluated (Figure 2). The role of these proton pumps in cytoplasmic pH regulation has been reported [47]. Previous studies showed that cytoplasmic pH of *E. coli* is modulated by the alkalization and acidification of the environment. This acidification is conducted by a proton impulse via a Na^+/H^+ (or K^+/H^+) systems which is mediated by a membrane potential generated by the respiratory chain and during which protons H^+ migrate from cell cytoplasm to the external environment allowing the entry sodium or potassium into the cytoplasm. Such systems are coupled to the production of energy in form of ATP required for good functioning of bacteria [47,48]. The inhibition of these H^+-ATPase-mediated proton pumps leads to the decrease of extracellular H^+ protons and to the increase of pH. It is reported that the minimum pH supporting bacterial proliferation for an *Escherichia coli* strain is 4.4 [49]. Furthermore, this bacterium is supplied by a multiple complex of pH-dependent on many strategies of acid tolerance allowing it to survive in stomach acidic pH conditions [49,50]. In the present work, tested sample provoked an increase of pH (from 6.4 to 7.7) compared to negative control whose pH decreased (from 6.4 to 4.8) during the time of experiment. This indicates that tested extract induced an inhibition of H^+-ATPase-mediated proton pumps of studied bacterial strain, *E. coli* ATCC8739 and suggests that this mechanism could constitute one of the ways by which this extract exerts its antibacterial activity.

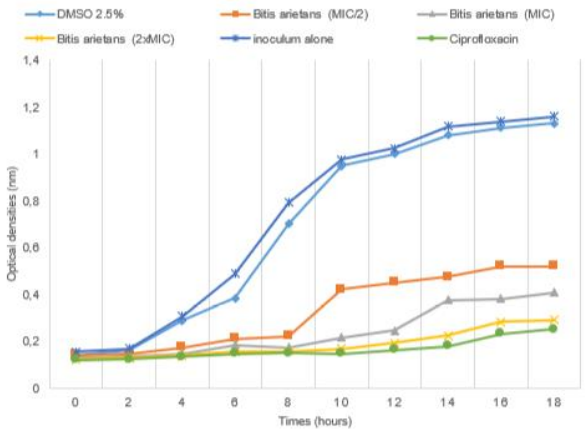

Figure 1. Effect of dried *Bitis arietans* extract at different concentrations on growth kinetic of *Escherichia coli* ATCC8739

These results show that DMSO at 2.5% concentration used to dissolve tested samples, did not inhibit the growth of studied bacterial strains and did not influent the activity of these samples

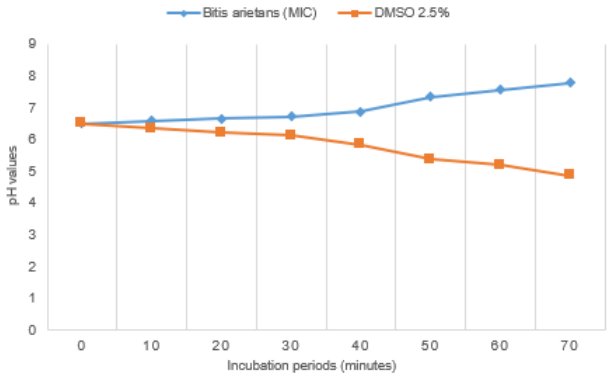

Figure 2. Effect of dried *Bitis arietans* extract on *Escherichia coli* ATCC8739 H^+-ATPase-mediated proton pumps

Increase of pH values or acidic conditions of the medium favourites the growth of tested bacterial meanwhile, decrease of pH values or basic conditions allows his growth inhibition

Table 1. Animal extracts, their extractive yields, traditional use and biological activities

Animal extracts	Family	Extractive yields (%)	Traditional use	Biological activities
Helix aspersa	*Helicidae*	11.96	Treat itch, cutaneous infections and cardiovascular diseases; it is used in the treatment of dermatological disorders and viral infections caused by papilloma viruses [51,52]	Anti-proliferative, antioxidant and antibacterial activities; proteins from snails active against soil borne pathogens, *Se* and *Pa* [28,51,52]
Aristaeomorpha foliacea	*Aristeidae*	10.61	Used to treat food poisoning and constipation [53]	No Biological activities
Bitis arietans	*Viperidae*	10.36	It is used in the treatment of venereal and bacterial infections, poison [12,24,54]	Snake venoms are platelet aggregation inhibitors and have bactericidal, antiviral and cytotoxic activities; its essential oil showed antimicrobial activities [54,55]

Extractive yield of each sample was obtained by calculating the crude extract weight / powder weight *Se:* Staphylococcus epidermidis *Pa: Pseudomonas aeruginosa*

Table 2. Characteristics of the studied bacterial strains

Species	Types	Characteristics	References
Escherichia coli	ATCC8739	Reference strain	[56]
	AG100A	*E. coli* K-12 expressing △acrAB: KAN^r	[57]
	AG102	△acrAB mutant AG100, owing acrF gene markedly over expressed;TET^r	[58]
	$AG100A_{Tet}$	△acrAB mutant AG100, with over-expressing *acrF* gene; TET^r	[59]
	W3110	Wild type *E. coli* K-12	[60]
	MC4100	Wild type *E. coli* expressed ABC pumps KAN^r	
Enterobacter aerogenes	ATCC13048	Reference strain	[56]
	EA27	Clinical MDR isolate exhibiting energy-dependent norfloxacin and chloramphenicol efflux with KAN^r, AMP^r, NAL^r, STR^r, TET^r	[61,62]
	EA289	KAN sensitive derivative of EA27	[61]
	EA294	EA289 expressing acrA: KAN^r	
	EA298	EA289 expressing *tolC* : KAN^r	[61,63]
Klebsiella pneumoniae	ATCC11296	Reference strain	
	Kp55	Clinical MDR isolate, TET^r , AMP^r, ATM^r, CEF^r	[56]
	Kp63	Clinical MDR isolate, TET^r, CHL^r, AMP^r, ATM^r	[64]
Providencia stuartii	NEA16	Clinical MDR isolate, AcrAB-TolC	[56]
	PS299645	Clinical MDR isolate, AcrAB-TolC associated to types OMPF and OMPC porines	
Pseudomonas aeruginosa	PA 01	Reference strain	[56]
	PA 124	Clinical MDR isolate, expressing *MexAB-OprM*	[57]

KAN^r, TET^r, AMP^r, NAL^r, STR^r, ATM^r, CEF^r, CHL^r : resistant (r) to kanamycin, tetracycline, ampicillin, nalidixic acid, streptomycin, aztreonam, cefepime, chloramphenicol, respectively; MDR : Multidrug-resistant ;. AcrAB-TolC, AcrAB and TolC are efflux pumps.

Table 3. Chemical composition of animal extracts

Bioactive compounds	**Extracts samples**					
	Helix aspersa		***Aristaeomorpha foliacea***		***Bitis arietans***	
	Dried	**fresh**	**Dried**	**fresh**	**Dried**	**fresh**
Alkaloids	+	+	+	+	+	+
Polyphenols	-	-	-	-	-	-
Flavonoids	-	-	-	-	-	-
Tannins	-	-	-	-	-	-
Steroids	-	-	-	-	-	-
Anthocyanins	-	-	-	-	-	-
Anthraquinones	-	-	-	-	-	-
Saponins	-	-	-	-	-	-
amino acids/ Peptides /Proteins	+	+	+	+	+	+

[-]: absence of metabolites; [-]: presence of metabolites; Each extract contains at least one secondary metabolites

Table 4. Minimal inhibitory and bactericidal concentrations of crude extracts and Ciprofloxacin against bacterial strains

Bacterial strains	**Extracts samples**										**Ciprofloxacin**		
	Helix aspersa		***Aristaeomorpha foliacea***		***Bitis arietans***								
	Dried	**Fresh**	**Dried**	**Fresh**	**Dried**			**Fresh**					
	MIC	**MIC**	**MIC**	**MIC**	**MIC**	**MBC**	**R**	**MIC**	**MBC**	**R**	**MIC**	**MBC**	**R**
Escherichia coli													
ATCC8739	2048	1024	512	2048	128	256	2	512	1024	2	1	4	4
AG100A	-	-	-	-	1024	-	>2	-	nt	nd	2	8	4
AG102	1024	-	-	1024	2048	-	>1	2048	-	>1	4	8	2
AG100ATet	1024	1024	-	2048	128	512	4	1024	2048	2	16	32	2
MC4100	-	-	-	-	512	2048	4	2048	-	>1	2	4	2
W3110	-	-	2048	-	2048	-	>1	2048	-	>1	4	16	4
Enterobacter aerogenes													
ATCC13048	1024	-	-	-	1024	-	>2	512	1024	2	1	8	8
EA27	2048	-	2048	-	512	1024	2	2048	-	>1	1	16	16
EA289	-	-	-	-	-	nt	nd	-	nt	nd	2	16	8
EA294	-	-	-	-	2048	-	>1	-	nt	nd	2	8	4
EA 298	-	-	-	-	-	nt	nd	-	nt	nd	16	64	4
CM64	-	-	1048	-	1024	-	>2	2048	-	>1	32	128	4
Klebsiella pneumoniae													
ATCC11296	-	-	-	-	512	1024	2	512	-	>4	2	4	2
KP55	-	-	-	-	2048	-	>1	1024	-	>1	32	128	4
KP63	-	-	2048	-	2048	-	>1	2048	-	>1	16	16	1
Providencia stuartii													
ATCC29916	2048	-	-	2048	2048	-	>1	-	nt	nd	4	32	8
PS2636	-	-	-	-	2048	-	>1	-	nt	nd	16	64	4
NEA16	-	-	-	-	2048	-	>1	2048	-	>1	16	64	4
Pseudomonas aeruginosa													
PA01	2048	-	512	2048	256	1024	4	2048	-	>1	8	32	4
PA124	-	-	2048	-	512	1024	2	-	nt	nd	32	256	8
PSBS	35	10	35	25	90			65			100		

MIC : minimal inhibitory concentration; MBC : minimal bactericidal concentration; R : MBC / MIC ratio (a sample is considered as bacteriostatic or bactericidal when this ratio is >4 or ≤4 respectively); (-) : MIC or MBC > 2048 µg/mL for crude extracts and > 256 µg/mL for ciprofloxacin; nt : not tested; nd : not determined (as no MIC and MBC values were not observed till 2048 µg/mL); PSBS : percentage of susceptible bacteria to substances; Fresh and dried *Helix aspersa* and *Aristaeomorpha foliacea* extracts did not showed any MBC against all bacteria; DMSO 2.5% used as negative control does not showed inhibitory effect against all bacteria

Table 5. MICs of antibiotics in combination with dried *Bitis arietans*

Antibiotics	MICs of extract	Bacterial strains and concentrations of antibiotics										PBS (%)
		E. coli		*E. aerogenes*		*K. pneumoniae*		*P. aeruginosa*		*P. stuartii*		
		ATCC8739	AG102	ATCC13048	CM64	ATCC11296	KP55	PA01	PA124	ATCC29916	NEA16	
Oxacillin	0	32	32	64	64	64	2	64	32	64	64	
	MIC/2	8(4)	64(0.5)	16(4)	64(1)	16(4)	0.25(8)	32(2)	4(4)	8(8)	16(4)	**70**
	MIC/4	16(2)	64(0.5)	16(4)	64(1)	32(2)	1(2)	32(2)	4(4)	32(2)	32(2)	**70**
Thiamphenicol	0	2	2	4	4	32	16	2	4	16	8	
	MIC/2	4(0.5)	1(2)	4(1)	0.5(8)	32(1)	8(2)	2(1)	2(2)	16(1)	1(8)	40
	MIC/4	4(0.5)	1(2)	4(1)	1(4)	32(1)	16(1)	2(1)	2(2)	16(1)	1(8)	30
Gentamicin	0	2	1	16	8	1	2	16	4	16	8	
	MIC/2	0.25(8)	0.125(8)	2(8)	2(4)	0.125(8)	0.5(4)	16(1)	0.25(16)	2(8)	1(8)	**90**
	MIC/4	0.5(4)	0.25(4)	4(4)	2(4)	0.5(2)	1(2)	16(1)	0.25(16)	8(2)	2(4)	**90**
Erythromycin	0	1	2	16	16	1	8	16	2	16	16	
	MIC/2	0.5(2)	0.125(16)	16(1)	8(2)	2(0.5)	16(0.5)	16(1)	2(1)	1(16)	0.25(64)	50
	MIC/4	1(1)	1(2)	16(1)	8(2)	2(0.5)	16(0.5)	16(1)	2(1)	2(8)	0.5(32)	40
Ciprofloxacin	0	1	4	1	32	2	32	8	32	4	16	
	MIC/2	0.125(8)	2(2)	0.25(4)	2(16)	2(1)	2(16)	0.5(16)	1(32)	2(2)	1(16)	**90**
	MIC/4	0.25(4)	2(2)	0.5(2)	4(8)	2(1)	4(8)	1(8)	4(8)	4(1)	1(16)	**80**
Doxycycline	0	2	8	2	2	16	8	4	16	2	16	
	MIC/2	0.125(8)	0.125(64)	0.125(16)	0.125(16)	0.5(32)	1(8)	1(4)	1(16)	0.5(4)	2(16)	**100**
	MIC/4	0.25(4)	0.25(32)	0.25(8)	0.125(16)	0.5(32)	2(4)	1(4)	2(8)	2(1)	2(8)	**90**
Flucloxacillin	0	16	2	32	4	32	8	4	32	2	32	
	MIC/2	0.5(32)	2(1)	32(1)	0.5(8)	4(8)	0.5(16)	0.5(8)	4(8)	0.5(4)	32(1)	**70**
	MIC/4	1(16)	2(1)	32(1)	0.5(8)	4(8)	0.5(16)	0.5(8)	8(4)	1(2)	32(1)	**70**
Ofloxacin	0	2	2	32	16	8	2	4	2	2	16	
	MIC/2	0.5(4)	2(1)	4(8)	0.25(64)	0.5(16)	0.25(8)	0.5(8)	0.125(16)	0.25(8)	16(1)	**80**
	MIC/4	0.5(4)	2(1)	8(4)	0.5(32)	1(8)	0.5(4)	0.5(8)	0.5(4)	0.5(4)	16(1)	**80**
Azithromycin	0	4	16	16	16	4	4	4	4	4	16	
	MIC/2	4(1)	8(2)	16(1)	0.25(64)	0.125(32)	4(1)	0.25(16)	0.125(32)	0.5(8)	2(8)	**70**
	MIC/4	4(1)	_8(2)	16(1)	0.5(32)	0.25(16)	4(1)	0.25(16)	0.5(8)	1(4)	4(4)	**70**

PBS : percentage of bacterial susceptibility; The numbers in parenthesis represent the improvement activity factors (IAF) [there is synergism when IAF≥2, indifference when IAF=1 and antagonism when IAF≤0.5]; IAF values were obtained by calculating the MIC of antibiotic alone over MIC of the combination; 0: MICs values of antibiotics tested alone; The MIC of extract sample is those showed in Table 4; *E. coli: Escherichia coli; E. aerogenes: Enterobacter aerogenes; K. pneumoniae: Klebsiella pneumoniae; P. aeruginosa: Pseudomonas aeruginosa; P. stuartii: Providencia stuartii*

Table 6. MICs of antibiotics in combination with fresh *Bitis arietans*

Antibiotics	MICs of extract	Bacterial strains and concentrations of antibiotics										PBS (%)
		E. coli		*E. aerogenes*		*K. pneumoniae*		*P. aeruginosa*		*P. stuartii*		
		ATCC8739	AG102	ATCC13048	CM64	ATCC11296	KP55	PA01	PA124	ATCC29916	NEA16	
Oxacillin	0	32	32	64	64	64	2	64	32	64	64	
	MIC/2	8(4)	4(8)	4(16)	32(2)	1(64)	0.25(8)	1(64)	8(4)	64(1)	1(64)	**90**
	MIC/4	16(2)	8(4)	16(4)	32(2)	2(32)	1(2)	1(64)	32(1)	64(1)	1(64)	**80**
Thiamphenicol	0	2	2	4	4	32	16	2	4	16	8	
	MIC/2	2(1)	1(2)	2(2)	2(2)	1(32)	16(1)	0.25(8)	4(1)	0.5(32)	8(1)	60
	MIC/4	2(1)	2(1)	2(2)	2(2)	1(32)	16(1)	1(2)	4(1)	1(16)	8(1)	50
Gentamicin	0	2	1	16	8	1	2	16	4	16	8	
	MIC/2	0.125(16)	0.125(8)	4(4)	1(8)	0.5(2)	2(1)	0.5(32)	0.125(32)	4(4)	8(1)	**80**
	MIC/4	0.25(8)	0.125(8)	4(4)	2(4)	1(1)	2(1)	1(16)	0.125(32)	8(2)	8(1)	**70**
Erythromycin	0	1	2	16	16	1	8	16	2	16	16	
	MIC/2	1(1)	2(1)	16(1)	2(8)	1(1)	8(1)	0.5(32)	2(1)	1(16)	1(16)	40
	MIC/4	1(1)	2(1)	16(1)	2(8)	1(1)	8(1)	1(16)	2(1)	2(8)	1(16)	40
Ciprofloxacin	0	1	4	1	32	2	32	8	32	4	16	
	MIC/2	1(4)	1(4)	0.25(4)	0.5(64)	0.125(16)	16(2)	0.125(64)	8(4)	0.125(32)	0.25(64)	**100**
	MIC/4	1(4)	2(2)	0.25(4)	1(32)	0.25(8)	32(1)	0.125(64)	16(2)	0.125(32)	0.5(32)	**90**
Doxycycline	0	2	8	2	2	16	8	4	16	2	16	
	MIC/2	0.125(16)	0.125(64)	0.25(8)	0.125(16)	0.25(64)	8(1)	0.125(32)	8(2)	1(2)	4(4)	**90**
	MIC/4	0.25(8)	0.25(32)	0.25(8)	0.125(16)	0.5(32)	8(1)	0.25(16)	8(2)	2(1)	8(2)	**80**
Flucloxacillin	0	16	2	32	4	32	8	4	32	2	32	
	MIC/2	16(1)	0.25(8)	32(1)	0.5(8)	0.5(64)	8(1)	0.25(16)	32(1)	0.5(4)	32(1)	50
	MIC/4	16(1)	0.25(8)	32(1)	0.5(8)	1(32)	8(1)	0.25(16)	32(1)	0.5(4)	32(1)	50
Ofloxacin	0	2	2	32	16	8	2	4	2	2	16	
	MIC/2	0.25(8)	0.125(16)	16(2)	0.25(64)	0.25(32)	1(2)	0.25(16)	0.5(4)	0.25(8)	0.5(32)	**100**
	MIC/4	1(2)	0.25(8)	16(2)	0.5(32)	0.5(16)	1(2)	0.25(16)	0.5(4)	0.5(4)	1(16)	**100**
Azithromycin	0	4	16	16	16	4	4	4	4	4	16	
	MIC/2	0.5(8)	1(16)	1(16)	0.125(128)	0.125(32)	1(4)	0.25(16)	0.5(8)	0.5(8)	16(1)	**90**
	MIC/4	1(4)	2(8)	1(16)	0.25(64)	0.25(16)	4(1)	0.5(8)	1(4)	0.5(8)	16(1)	**80**

PBS : percentage of bacterial susceptibility; The numbers in parenthesis represent the improvement activity factors (IAF) [there is synergism when IAF≥2, indifference when IAF=1 and antagonism when IAF≤0.5]; IAF values were obtained by calculating the MIC of antibiotic alone over MIC of the combination; 0: MICs values of antibiotics tested alone; The MIC of extract sample is those showed in Table 4; *E. coli: Escherichia coli; E. aerogenes: Enterobacter aerogenes; K. pneumoniae: Klebsiella pneumoniae; P. aeruginosa: Pseudomonas aeruginosa; P. stuartii: Providencia stuartii*

Table 7. MICs of antibiotics in combination with dried *Aristaeomorpha foliacea*

Antibiotics	MICs of extract	Bacterial strains and concentrations of antibiotics										PBS (%)
		E. coli		*E. aerogenes*		*K. pneumoniae*		*P. aeruginosa*		*P. stuartii*		
		ATCC8739	AG102	ATCC13048	CM64	ATCC11296	KP55	PA01	PA124	ATCC29916	NEA16	
Oxacillin	0	32	32	64	64	64	2	64	32	64	64	
	MIC/2	8(4)	8(4)	2(32)	16(4)	64(1)	1(2)	64(1)	8(4)	16(4)	32(2)	**80**
	MIC/4	16(2)	32(1)	8(8)	32(2)	64(1)	1(2)	64(1)	16(2)	64(1)	32(2)	60
Thiamphenicol	0	2	2	4	4	32	16	2	4	16	8	
	MIC/2	2(1)	2(1)	1(4)	4(1)	32(1)	1(2)	2(1)	1(4)	16(1)	8(1)	30
	MIC/4	2(1)	2(1)	2(2)	4(1)	32(1)	2(1)	2(1)	1(4)	16(1)	8(1)	20
Gentamicin	0	2	1	16	8	1	2	16	4	16	8	
	MIC/2	0.125(16)	0.125(8)	0.25(64)	4(2)	0.25(4)	0.5(4)	32(0.5)	0.5(8)	4(4)	2(4)	**90**
	MIC/4	0.25(8)	0.25(4)	2(8)	8(1)	0.5(2)	1(2)	32(0.5)	1(4)	8(2)	8(1)	**70**
Erythromycin	0	1	2	16	16	1	8	16	2	16	16	
	MIC/2	1(1)	2(1)	32(0.5)	16(1)	1(1)	16(0.5)	16(1)	1(2)	4(4)	8(2)	20
	MIC/4	1(1)	2(1)	32(0.5)	16(1)	1(1)	16(0.5)	16(1)	1(2)	8(2)	8(2)	20
Ciprofloxacin	0	1	4	1	32	2	32	8	32	4	16	
	MIC/2	0.125(8)	0.125(32)	<0.125(>8)	0.25(128)	0.25(32)	1(2)	8(1)	16(2)	0.125(32)	2(8)	**80**
	MIC/4	0.25(4)	0.25(16)	0.125(8)	0.25(64)	0.25(32)	1(2)	8(1)	16(2)	0.25(16)	4(4)	**80**
Doxycycline	0	2	8	2	2	16	8	4	16	2	16	
	MIC/2	0.125(16)	0.125(64)	0.25(8)	0.125(16)	0.5(32)	1(8)	2(2)	4(4)	0.5(4)	4(4)	**100**
	MIC/4	0.25(4)	0.25(32)	1(2)	0.125(16)	1(16)	2(4)	2(2)	4(4)	2(1)	16(1)	**80**
Flucloxacillin	0	16	2	32	4	32	8	4	32	2	32	
	MIC/2	8(2)	0.25(8)	32(1)	0.5(8)	4(8)	0.5(16)	0.5(8)	32(1)	1(2)	32(1)	**70**
	MIC/4	8(2)	0.25(8)	32(1)	1(4)	4(8)	0.5(16)	1(4)	32(1)	2(1)	32(1)	60
Ofloxacin	0	2	2	32	16	8	2	4	2	2	16	
	MIC/2	0.5(4)	0.25(8)	2(16)	0.25(64)	0.5(16)	0.25(8)	0.5(8)	2(1)	0.5(4)	16(1)	**80**
	MIC/4	0.5(4)	0.25(8)	2(16)	0.25(64)	1(8)	0.25(8)	1(4)	2(1)	1(2)	16(1)	**80**
Azithromycin	0	4	16	16	16	4	4	4	4	4	16	
	MIC/2	0.5(8)	16(1)	1(16)	0.25(64)	0.25(16)	4(1)	0.25(16)	2(2)	2(2)	8(2)	**80**
	MIC/4	2(2)	16(1)	1(16)	0.5(32)	0.25(16)	4(1)	0.5(8)	4(1)	2(2)	16(1)	60

PBS : percentage of bacterial susceptibility; The numbers in parenthesis represent the improvement activity factors (IAF) [there is synergism when IAF≥2, indifference when IAF=1 and antagonism when IAF≤0.5]; IAF values were obtained by calculating the MIC of antibiotic alone over MIC of the combination; 0: MICs values of antibiotics tested alone; The MIC of extract sample is those showed in Table 4; *E. coli: Escherichia coli; E. aerogenes: Enterobacter aerogenes; K. pneumoniae: Klebsiella pneumoniae; P. aeruginosa: Pseudomonas aeruginosa; P. stuartii: Providencia stuartii*

Table 8. MICs of antibiotics in combination with fresh *Aristaeomorpha foliacea*

Antibiotics	**MICs of extract**	Bacterial strains and concentrations of antibiotics										PBS (%)
		E. coli		***E. aerogenes***		***K. pneumoniae***		***P. aeruginosa***		***P. stuartii***		
		ATCC8739	**AG102**	**ATCC13048**	**CM64**	**ATCC11296**	**KP55**	**PA01**	**PA124**	**ATCC29916**	**NEA16**	
Oxacillin	0	32	32	64	64	64	2	64	32	64	64	
	MIC/2	0.5(64)	8(4)	2(32)	64(1)	1(64)	4(0.5)	2(32)	32(1)	64(1)	2(32)	60
	MIC/4	0.5(64)	32(1)	2(32)	64(1)	1(64)	4(0.5)	2(32)	32(1)	64(1)	2(32)	50
Thiamphenicol	0	2	2	4	4	32	16	2	4	16	8	
	MIC/2	1(2)	0.5(8)	0.5(8)	4(1)	32(1)	4(4)	0.25(8)	4(1)	16(1)	1(8)	60
	MIC/4	2(1)	1(2)	0.5(8)	4(1)	32(1)	16(1)	1(2)	4(1)	16(1)	1(8)	40
Gentamicin	0	2	1	16	8	1	2	16	4	16	8	
	MIC/2	0.125(16)	0.125(8)	16(1)	0.5(16)	0.25(4)	2(1)	1(16)	1(4)	1(16)	0.5(16)	**80**
	MIC/4	0.125(16)	0.125(8)	16(1)	1(8)	0.5(2)	2(1)	1(16)	1(4)	1(16)	1(8)	**80**
Erythromycin	0	1	2	16	16	1	8	16	2	16	16	
	MIC/2	0.125(8)	0.125(16)	1(16)	16(1)	1(1)	0.5(16)	1(16)	1(2)	0.5(32)	1(16)	**80**
	MIC/4	0.25(4)	0.125(16)	2(8)	16(1)	1(1)	1(8)	1(16)	1(2)	1(16)	1(16)	**80**
Ciprofloxacin	0	1	4	1	32	2	32	8	32	4	16	
	MIC/2	0.125(8)	0.125(32)	0.25(2)	0.5(8)	0.125(64)	0.25(128)	0.125(64)	8(4)	0.25(64)	1(16)	**100**
	MIC/4	0.25(4)	0.25(16)	0.5(1)	0.5(8)	0.125(64)	0.5(64)	0.25(32)	8(4)	0.25(64)	1(16)	**90**
Doxycycline	0	2	8	2	2	16	8	4	16	2	16	
	MIC/2	0.125(16)	1(8)	0.25(8)	0.25(8)	0.5(32)	0.125(64)	0.25(16)	2(8)	0.25(8)	16(1)	**90**
	MIC/4	0.5(4)	2(4)	0.25(8)	0.5(4)	0.5(32)	0.125(64)	0.5(32)	2(8)	1(2)	16(1)	**90**
Flucloxacillin	0	16	2	32	4	32	8	4	32	2	32	
	MIC/2	1(16)	0.125(16)	4(8)	1(4)	0.5(64)	8(1)	0.5(32)	32(1)	2(1)	32(1)	60
	MIC/4	2(8)	0.125(16)	8(4)	1(4)	1(32)	8(1)	0.5(32)	32(1)	2(1)	32(1)	60
Ofloxacin	0	2	2	32	16	8	2	4	2	2	16	
	MIC/2	0.125(16)	0.25(8)	1(32)	0.25(64)	0.5(16)	0.5(4)	0.5(32)	0.5(4)	1(2)	16(1)	**90**
	MIC/4	0.25(8)	0.25(8)	2(16)	0.25(64)	1(8)	0.5(4)	0.5(32)	0.5(4)	1(2)	16(1)	**90**
Azithromycin	0	4	16	16	16	4	4	4	4	4	16	
	MIC/2	0.25(16)	0.5(32)	16(1)	0.25(64)	0.25(16)	4(1)	1(4)	0.25(16)	4(1)	16(1)	60
	MIC/4	0.5(8)	0.5(32)	16(1)	0.25(64)	0.25(16)	4(1)	1(4)	0.5(8)	4(1)	16(1)	60

PBS : percentage of bacterial susceptibility; The numbers in parenthesis represent the improvement activity factors (IAF) [there is synergism when IAF≥2, indifference when IAF=1 and antagonism when IAF≤0.5]; IAF values were obtained by calculating the MIC of antibiotic alone over MIC of the combination; 0: MICs values of antibiotics tested alone; The MIC of extract sample is those showed in Table 4; *E. coli: Escherichia coli; E. aerogenes: Enterobacter aerogenes; K. pneumoniae: Klebsiella pneumoniae; P. aeruginosa: Pseudomonas aeruginosa; P. stuartii: Providencia stuartii*

Table 9. MICs of antibiotics in combination with dried *Helix aspersa*

Antibiotics	MICs of extract	Bacterial strains and concentrations of antibiotics										PBS (%)
		E. coli		*E. aerogenes*		*K. pneumoniae*		*P. aeruginosa*		*P. stuartii*		
		ATCC8739	AG102	ATCC13048	CM64	ATCC11296	KP55	PA01	PA124	ATCC29916	NEA16	
Oxacillin	0	32	32	64	64	64	2	64	32	64	64	
	MIC/2	8(4)	8(4)	64(1)	64(1)	64(1)	1(2)	64(1)	8(4)	64(1)	64(1)	40
	MIC/4	32(1)	32(1)	64(1)	64(1)	64(1)	1(2)	64(1)	16(2)	64(1)	64(1)	20
Thiamphenicol	0	2	2	4	4	32	16	2	4	16	8	
	MIC/2	0.5(4)	2(1)	4(1)	1(4)	32(1)	16(1)	2(1)	1(4)	2(8)	8(1)	30
	MIC/4	2(1)	2(1)	4(1)	2(2)	32(1)	16(1)	2(1)	1(4)	4(4)	8(1)	20
Gentamicin	0	2	1	16	8	1	2	16	4	16	8	
	MIC/2	1(2)	1(1)	0.25(64)	0.125(64)	0.5(2)	2(1)	16(1)	4(1)	0.25(64)	8(1)	50
	MIC/4	2(1)	1(1)	0.5(32)	0.25(32)	0.5(2)	2(1)	16(1)	4(1)	0.25(64)	8(1)	40
Erythromycin	0	1	2	16	16	1	8	16	2	16	16	
	MIC/2	0.125(8)	2(1)	16(1)	16(1)	1(1)	8(1)	16(1)	2(1)	0.5(32)	1(16)	30
	MIC/4	1(1)	2(1)	16(1)	16(1)	1(1)	8(1)	16(1)	2(1)	0.5(32)	2(8)	20
Ciprofloxacin	0	1	4	1	32	2	32	8	32	4	16	
	MIC/2	0.125(8)	2(1)	0.125(8)	0.5(8)	0.125(16)	2(1)	0.125(64)	8(4)	0.125(32)	0.25(16)	**80**
	MIC/4	0.25(4)	2(1)	0.25(4)	1(4)	0.125(16)	2(1)	0.25(32)	16(2)	0.5(8)	0.25(16)	**80**
Doxycycline	0	2	8	2	2	16	8	4	16	2	16	
	MIC/2	0.125(8)	0.5(16)	0.125(16)	0.25(8)	0.5(32)	8(1)	2(2)	4(4)	2(1)	16(1)	**70**
	MIC/4	0.25(4)	1(8)	0.125(16)	0.5(4)	0.5(32)	8(1)	2(2)	8(2)	2(1)	16(1)	**70**
Flucloxacillin	0	16	2	32	4	32	8	4	32	2	32	
	MIC/2	0.25(64)	0.25(8)	16(2)	1(4)	1(32)	8(1)	0.5(8)	32(1)	1(2)	32(1)	**70**
	MIC/4	0.5(32)	0.25(8)	32(1)	2(2)	1(32)	8(1)	1(4)	32(1)	1(2)	32(1)	60
Ofloxacin	0	2	2	32	16	8	2	4	2	2	16	
	MIC/2	0.25(8)	2(1)	0.5(64)	0.25(64)	0.25(32)	0.5(4)	0.5(8)	0.25(8)	1(2)	16(1)	**80**
	MIC/4	0.25(8)	2(1)	1(32)	0.25(64)	0.5(16)	1(2)	0.5(8)	0.5(4)	1(2)	16(1)	**80**
Azithromycin	0	4	16	16	16	4	4	4	4	4	16	
	MIC/2	0.5(8)	16(1)	1(16)	0.25(64)	0.5(8)	1(4)	0.5(8)	4(1)	1(4)	16(1)	**70**
	MIC/4	1(4)	16(1)	1(16)	0.5(32)	1(4)	4(1)	1(4)	4(1)	1(4)	16(1)	60

PBS : percentage of bacterial susceptibility; The numbers in parenthesis represent the improvement activity factors (IAF) [there is synergism when IAF≥2, indifference when IAF=1 and antagonism when IAF≤0.5]; IAF values were obtained by calculating the MIC of antibiotic alone over MIC of the combination; 0: MICs values of antibiotics tested alone; The MIC of extract sample is those showed in Table 4; *E. coli: Escherichia coli; E. aerogenes: Enterobacter aerogenes; K. pneumoniae: Klebsiella pneumoniae; P. aeruginosa: Pseudomonas aeruginosa; P. stuartii: Providencia stuartii*

Table 10. MICs of antibiotics in combination with fresh *Helix aspersa*

Antibiotics	MICs of extract	Bacterial strains and concentrations of antibiotics										PBS (%)
		E. coli		*E. aerogenes*		*K. pneumoniae*		*P. aeruginosa*		*P. stuartii*		
		ATCC8739	AG102	ATCC13048	CM64	ATCC11296	KP55	PA01	PA124	ATCC29916	NEA16	
Oxacillin	0	32	32	64	64	64	2	64	32	64	64	
	MIC/2	4(8)	32(4)	2(32)	64(1)	1(64)	4(0.5)	2(32)	8(4)	64(1)	1(64)	60
	MIC/4	8(4)	32(1)	4(16)	64(1)	2(32)	4(0.5)	2(32)	8(4)	64(1)	2(32)	60
Thiamphenicol	0	2	2	4	4	32	16	2	4	16	8	
	MIC/2	1(2)	0.5(8)	0.5(8)	4(1)	32(1)	4(4)	1(2)	4(1)	16(1)	1(8)	60
	MIC/4	2(1)	1(2)	1(4)	4(1)	32(1)	16(1)	1(2)	4(1)	16(1)	1(8)	40
Gentamicin	0	2	1	16	8	1	2	16	4	16	8	
	MIC/2	0.125(16)	0.25(4)	16(1)	1(8)	0.25(4)	2(1)	0.5(32)	2(2)	1(16)	1(8)	**80**
	MIC/4	0.125(16)	0.5(2)	16(1)	1(8)	0.5(2)	2(1)	1(16)	2(2)	2(8)	1(8)	**80**
Erythromycin	0	1	2	16	16	1	8	16	2	16	16	
	MIC/2	0.5(2)	0.125(16)	1(16)	16(1)	1(1)	0.5(16)	1(16)	1(2)	1(16)	1(16)	**80**
	MIC/4	0.5(2)	0.25(8)	2(8)	16(1)	1(1)	1(8)	1(16)	1(2)	2(8)	1(16)	**80**
Ciprofloxacin	0	1	4	1	32	2	32	8	32	4	16	
	MIC/2	0.125(8)	0.125(16)	0.25(2)	0.5(8)	0.125(16)	2(16)	0.125(64)	32(1)	0.125(32)	1(16)	**90**
	MIC/4	0.25(4)	0.25(8)	0.5(1)	0.5(8)	0.125(16)	4(8)	0.25(32)	32(1)	0.25(16)	1(16)	**80**
Doxycycline	0	2	8	2	2	16	8	4	16	2	16	
	MIC/2	0.25(8)	0.5(16)	0.25(8)	0.125(16)	0.5(32)	0.125(64)	4(1)	8(2)	1(2)	16(1)	**80**
	MIC/4	1(2)	1(8)	0.25(8)	0.5(4)	0.5(32)	0.25(32)	4(1)	8(2)	1(2)	16(1)	**80**
Flucloxacillin	0	16	2	32	4	32	8	4	32	2	32	
	MIC/2	1(16)	0.5(4)	8(4)	1(4)	0.5(64)	8(1)	4(1)	64(0.5)	2(1)	32(1)	50
	MIC/4	1(16)	1(2)	8(4)	1(4)	1(32)	8(1)	4(1)	64(0.5)	2(1)	32(1)	50
Ofloxacin	0	2	2	32	16	8	2	4	2	2	16	
	MIC/2	0.25(8)	1(2)	1(32)	0.25(64)	0.5(16)	0.5(4)	4(1)	2(1)	1(2)	16(1)	**70**
	MIC/4	0.5(4)	1(2)	2(16)	0.25(64)	1(8)	0.5(4)	4(1)	2(1)	1(2)	16(1)	**70**
Azithromycin	0	4	16	16	16	4	4	4	4	4	16	
	MIC/2	0.25(16)	16(1)	16(1)	0.25(64)	0.25(16)	4(1)	4(1)	1(4)	4(1)	16(1)	40
	MIC/4	0.5(8)	16(1)	16(1)	0.25(64)	0.25(16)	4(1)	4(1)	2(2)	4(1)	16(1)	40

PBS : percentage of bacterial susceptibility; The numbers in parenthesis represent the improvement activity factors (IAF) [there is synergism when IAF≥2, indifference when IAF=1 and antagonism when IAF≤0.5]; IAF values were obtained by calculating the MIC of antibiotic alone over MIC of the combination; 0: MICs values of antibiotics tested alone; The MIC of extract sample is those showed in Table 4; *E. coli: Escherichia coli; E. aerogenes: Enterobacter aerogenes; K. pneumoniae: Klebsiella pneumoniae; P. aeruginosa: Pseudomonas aeruginosa; P. stuartii: Providencia stuartii*

Conclusion

It was concluded here that from tested samples, *B. arietans* was the most active extract and highly potentiated the activities of the majority of antibiotics against the majority of studied bacterial strains. Furthermore, it contains components which can inhibit efflux pumps as well as bacterial H^+-ATPase-mediated proton pumps. Meanwhile, other extract presented a moderate antibacterial activity. The overall results obtained indicate that *B. arietans* can be a suitable solution to fight against multidrug resistant bacteria expressing efflux pumps and that animal species can be fully exploited in the search of potentially active antimicrobial compounds as the antimicrobial of tested samples is reported in the present work for the first time.

Additional file

Table 1. MICs of antibiotics associated with animal extracts against *Pseudomonas aeruginosa* PA124; G1. Available online at: https://www.investchempharma.com/imcp48-guefack-et-al-supplementary-file/

Abbreviations

ATCC : American Type Culture Collection
MIC : Minimal inhibitory concentration
MBC : Minimal bactericidal concentration
DMSO : Dimethylsulfoxide
INT : *p*-Iodonitrotetrazolium chloride
MHA : Mueller Hinton agar
MHB : Mueller Hinton broth
OD : Optical density
RND : Resistance-nodulation-cell division
EPI : Efflux pumps inhibitor
IAF : Improvement activity factor
EY : Extractive yield
OXA : Oxacillin
THI : Thiamphenicol
ERY : Erythromycin
GEN : Gentamicin
CIP : Ciprofloxacin
DOX : Doxocyclin
AZI : Azithromycin
OFL : Ofloxacin
FLU : Flucloxacillin
PSBS : percentage of susceptible bacteria to substances
PBS : percentage of bacterial susceptibility

Authors' Contribution

MGFG performed the antibacterial activities of tested extracts alone and in combination with antibiotics. Zoochemical screening was done by CMNN. The mechanisms of action of most active sample were carried out by BENW, INB and PN. The manuscript was written by SBT and the work was supervised by VK and ATM.

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Conflict of interest

The authors declare no conflict of interest

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