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Antibacterial activity of phytochemicals from *Bauhinia thonningii* significantly increased in the presence of the efflux pump inhibitor, phenylalanine-arginine-*β*-naphthylamide towards multidrug-resistant phenotypes

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Abstract

Background: View the rapid spread of multidrug-resistant bacteria worldwide; alternatives to antibiotics are necessary for handling various bacterial infections. This study aimed to investigate the antibacterial activity of eight flavonoids isolated from the leaves methanol extract of *Bauhinia thonningii* against multi-drug resistant (MDR) bacteria expressing efflux pumps.

Methods: The antibacterial activity of the phytochemicals alone and in the presence of phenylalanine-arginine-*ß*-naphthylamide (PAβN) was carried out by the broth micro-dilution method. Spectrophotometric methods were used to evaluate the effects of quercetin-3-*O*-L-rhamnopyranoside (**5**) on bacterial growth curve and bacteriolysis, whereas its effect on the bacteria H^+-ATPase pumps was monitored by pH measurement.

Methods: The antibacterial activity of the phytochemicals alone and in the presence of phenylalanine-arginine-*ß*-naphthylamide (PAβN) was carried out by the broth micro-dilution method. Spectrophotometric methods were used to evaluate the effects of quercetin-3-*O*-$_L$-rhamnopyranoside (**5**) on bacterial growth curve and bacteriolysis, whereas its effect on the bacteria H^+-ATPase pumps was monitored by pH measurement.

Results: The tested phytochemicals had selective antibacterial activity with minimal inhibitory concentration (MIC) ranging from 8 to 128 µg/mL. Compounds **5, 6,** and **8** were active against all the tested bacteria, and compound **8** showed the lowest MIC value (8 µg/mL), especially against *E. coli* AG102, *S. aureus* MRSA3, and *P. stuartii* NEA16. Compound 5 presented good activity (8 < MIC ≤ 32 µg/mL) against 50% of the tested bacteria. Moreover, compounds **5** and **6** have displayed excellent activity (4 < MIC ≤ 32 µg/mL) against *P. aeruginosa* PA124. In the presence of PAβN, the antibacterial activity of all the tested compounds increased 2 to >128 folds on at least one of the tested bacteria. Quercetin-3-*O*-L-rhamnopyranoside (**5**) inhibited bacterial growth in the exponential phase, induced bacteriolysis, and inhibited the H+-ATPase pumps in *P. stuartii* NEA16.

Conclusion: Compounds **5, 6,** and **8** isolated from the leaves of *Bauhinia thonningii* deserve further investigations as potential drugs to fight infections involving MDR bacteria expressing efflux pumps.

Keywords: *Bauhinia thonningii*; antibacterial activity; quercetin-3-*O*-$_L$-rhamnopyranoside; modes of action; multidrug resistance; efflux pumps.

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Background

Multidrug-resistant (MDR) bacteria are a major threat to modern medicine, causing life-threatening infections. Based on the Centers for Disease Control and Prevention (CDC), at least 44,000 people die each year in the United States as a direct result of these antibiotic-resistant infections [1]. The indiscriminate use and overuse of antibiotics are some of the most essential causes underlying the emergence of MDR bacteria [2 - 4]. In the hospital setting, some MDR bacteria are of greatest concern. These bacteria are MDR Gram-positive bacteria such as *Methicillin-Resistant Staphylococcus aureus (MRSA), vancomycin-resistant enterococci*, drug-resistant *Streptococcus pneumoniae*, and the MDR gam-negative bacteria including *Pseudomonas spp., Klebsiella pneumonia, Acinetobacter spp., Enterobacter spp.*, and others [5]. View the spread of multidrug-resistant; alternatives to antibiotics are necessary for handling various bacterial infections. Therefore, medicinal plants that are used to treat various health problems including infections, and which are known for their richness in bioactive secondary metabolites are an alternative [6, 7]. *Bauhinia thonningii* Schum. *(*Synonym: *Piliostigma thonningii* (Schum) Miline-Redhead) belongs to the family Caseaphinaceae and it is found in most parts of the world including Africa and Asia. It is a plant with diverse ethnomedical applications [8]. The leaves and bark of *B. thonningii* are used for the treatment of many ailments including diarrhea, malaria, ulcers, wounds, heart pain, arthritis, pyrexia, leprosy, sore throat, toothache, gingivitis, bronchitis, and cough [9, 10]. Moreover, its roots and twigs are used to treat dysentery, fever, skin diseases, wound infections, and cough [11]. Some studies revealed the presence of flavonoids, tannins, kaurane diterpenes, alkaloids, carbohydrates, saponins, terpenes, and volatile oils in *B. thoningii* [12-17]. Previous works have demonstrated that extracts/compounds from *B. thonningii* possess antilipidemic [10], anthelminthic [11], anti-inflammatory [12], antibacterial [17, 18], antioxidant [19], gastroprotective [20], aphrodisiac [21] and antiprotozoal [22] activities. This study aimed to investigate the antibacterial activity of eight flavonoids named 6-C-methylquercetin-3, 4'-dimethyl ether (**1**); 6-*C*-methylquercetin-3,7-dimethyl ether (**2**); 6-C-methylquercetin 3,7,3'-trimethyl ether (**3**); quercetin-3-*O*-$_L$-rhamnopyranoside (**5**); quercetin-3-*O*-$_L$-rhamnopyranoside (**6**); 6,8-*C*-dimethylkaempferol 3,7-dimethyl ether (**7**); 6,8-*C*-dimethylkaempferol-3-methyl ether (**8**), and 6,8-*C*-dimethylquercetin-3-methyl ether (**9**) from the leaves methanol of *Bauhinia thonningii* against multi-drug resistant (MDR) bacteria expressing active efflux pumps. Moreover, the mechanisms of action of one of the most active compounds, quercetin-3-*O*-$_L$-rhamnopyranoside (**5**), were further investigated in this study.

Methods

Chemicals

Ciprofloxacin (CIP) was used as a reference antibiotic (RA). Dimethyl sulfoxide (Sigma-Aldrich) was used to dissolve tested compounds. *p*-Iodonitrotetrazolium chloride (INT; Sigma-Aldrich) and phenylalanine-arginine-ß-naphthylamide (PAβN; Sigma-Aldrich) were used as microbial growth indicator and efflux pump inhibitor (EPI), respectively. The major fractions (fractions A-B) of the leaves' extract of *B. thonningii* afforded eleven (11) compounds identified as 6-C-methylquercetin-3, 4'-dimethyl ether (**1**); 6-C-methylquercetin-3,7-dimethyl ether (**2**); 6-*C*-methylquercetin 3,7,3'-trimethyl ether (**3**); quercetin (**4**); quercetin-3-*O*-$_L$-rhamnopyranoside (**5**); quercetin-3-*O*-L-rhamnopyranoside (**6**); 6,8-*C*-dimethylkaempferol 3,7-dimethyl ether **(7)**; 6,8-*C*-dimethylkaempferol-3-methyl ether **(8)**; 6,8-*C*-dimethylquercetin-3-methyl ether **(9),** ursolic acid (**10**) and 3-*O*-*β*-$_D$-glucopyranoside of *β*-sistosterol (**11**) (Figure 1). The experimental procedures for extraction, isolation, and identification as well as the structural elucidation procedure of these compounds were described (Figure S1-S25; Supplementary material). Among these phytochemicals, compounds **1-3**, and **5-9** were evaluated for their antibacterial activity in the absence and presence of PAβN.

Bacteria strains and growth conditions

Eight Gram-negative and two Gram-positive bacteria were used in this study. Gram-negative bacteria consisted of MDR isolates (laboratory collection) and reference strains of *Escherichia coli* (ATCC 8739, and AG102), *Klebsiella pneumoniae* (ATCC 11296, and KP55), *Providencia stuartii* (PS2636 and NEA16) and *Pseudomonas aeruginosa* (PA01 and PA124). The clinical Gram-negative strains were laboratory collection from UMR-MD1, University of Marseille, France whereas the reference strains were American-type culture collection (ATCC). The Gram-positive bacteria were methicillin-resistant *Staphylococcus aureus* (MRSA) isolates (MRSA3 and MRSA6) from the culture collection of the Laboratory of Microbiology, Graduate School of Pharmaceutical Sciences, University of Tokyo, Japan, which were kindly provided by Pr. Dzoyem, University of Dschang. Further details on the antibiotic-resistance profiles of the MDR isolates were previously reported [23-28]. Before any antibacterial assay, bacteria strains were maintained at 4°C and sub-cultured for 18h on Mueller Hinton Agar (MHA). Mueller Hinton broth (MHB) was used as a culture medium for the antibacterial assays.

Antibacterial assays

The antibacterial activity of phytochemicals was evaluated through the determination of MIC and MBC by the broth micro-dilution methods using *p*-Iodonitrotetrazolium chloride (INT) colorimetric as previously described [29, 30]. Briefly, the samples were dissolved in 10% DMSO/MHB and serially diluted two-fold in a 96-well microplate. Then, 100 µL of inoculum (2×10^6 CFU/mL) prepared in MHB was added to each well. Wells containing the vehicle (DMSO 2.5%) were used as growth control whereas ciprofloxacin was used as a reference antibacterial drug. The final concentration ranges were 1 to 128 µg/mL for the tested compounds and 0.5 to 64 µg/mL for the reference antibacterial drug (CIP). The plates were then covered with a sterile plate sealer, gently shaken, and incubated at 37°C for 18 h. The MIC defined as the lowest sample concentration that completely inhibited bacteria growth was detected following the addition of 40 µL INT (0.2 mg/mL) and incubation at 37°C for 30 min. Viable bacteria reduced the yellow dye to pink. For the minimal bactericidal concentrations (MBCs) determination, 150 µL of MHB were introduced in a new 96-well microplate, following the addition of 50 µL from the microplate contents (which did not receive INT during the MIC determination) and where no microbial growth was observed. Then, the microplate was incubated for 48 h at 37°C and the MBC of each sample was determined by adding INT as previously described. The MBC was regarded as the lowest concentration of samples, which did not produce a color change after the addition of INT. Each experiment was performed in duplicate and repeated thrice.

Evaluation of the effect of efflux pumps on the antibacterial activity of the samples

Phytochemicals and CIP were also tested in the presence of PAβN (30 µg/mL) against ten bacteria strains including eight MDR phenotypes (*E. coli* AG102, *K. pneumoniae* KP55, *P. stuartii* PS2636, NEA16, *P. aeruginosa* PA01 and PA124, and *S. aureus* MRSA3 and MRSA3) as previously described [23, 31]. The ratio $MIC_{(sample\ alone)}/MIC_{(sample\ +PA\beta N)}$ was used to determine the fold increase of the antibacterial activity of the samples in the presence of PAβN. Each assay was repeated thrice.

*Evaluation of the effect of compound **5** on Providencia stuartii NEA16 growth curve*

The effect of compound **5** on *P. stuartii* NEA16 growth curve was determined as previously described [32] with slight modifications. A suspension (1.5 $\times 10^8$ CFU/mL) was prepared from 18 h bacteria cultures and diluted in MHB to obtain an inoculum (10^6 UFC/mL). Then, a mixture consisting of 22 mL of inoculum, 0.5 mL of the tested samples (at concentrations of MIC/2, MIC, and 2×MIC) or MHB were done into test tubes, which were incubated at 37°C for 0, 1, 2, 4, 6, 10, 12, 14, 16, and 18 h under agitation at 150 rpm. After incubation, 500 µL of each tube content was collected and its absorbance was measured at 600 nm using a spectrophotometer. A tube made up of inoculum and DMSO was used as control. Each assay was carried out in triplicate.

Evaluation of the membrane integrity of Providencia stuartii NEA16

The membrane integrity of *P. stuartii* NEA16 was assessed by measuring the absorbance at 260 nm (A260) of the released materials into the suspension as previously described [33], with some modifications. Briefly, suspensions of bacteria (adjusted at 10^6 CFU/mL) were prepared from the fresh culture on the MHA medium. The bacterial cells were then treated or not with HRB or compound **5** (at 1/2MIC, MIC, and 2 × MIC), and incubated at 37°C for 12 h. Samples were centrifuged, and the absorbance of the supernatant was estimated at 260 nm for control and treated cells using a spectrophotometer. Tubes containing inoculum and DMSO were used as controls. Each assay was done in triplicate.

H^+-ATPase-mediated pump assay

The effect of compound **5** on the H+-ATPase pumps of *P. stuartii* NEA16 was assessed by monitoring the acidification of the bacterial growth medium as previously described [34, 35]. Briefly, a suspension of *P. stuartii* NEA16 was cultured in MHB for 18 h at 37°C and the resulting culture (100 mL) was centrifuged at 3000 rpm for 10 min at 4°C. The resulting pellet was washed twice in distilled water, then in 50 mM KCl, and suspended in 50 mL of 50 mM KCl. The resulting suspension was incubated overnight at 4°C. In 4.0 mL of that suspension, the tested samples were added (at MIC/2, MIC, and 2×MIC), and the pH was adjusted to 6.8 with 1 M HCl or 0.1M NaOH. Upon 10 min pre-incubation at 37°C, the acidification of the medium was initiated by adding 20% glucose, followed by pH measurement every 10 min for 60 min using a pH meter. Control was made up of tubes containing inoculum and DMSO. Each essay was performed in triplicate.

Results

Antibacteril activity of the isolated compounds

Table 1 presents the antibacterial activity of compounds isolated from *B. thonningii* against the tested bacteria. These compounds presented MICs ranging from 8 to 128 µg/mL. Compounds 5, 6, and 8 were active against all the tested bacteria (100%). In addition to its broad spectrum of activity, compound 8 showed the best activity (MIC = 8 µg/mL), especially against *E. coli* AG102, *S. aureus* MRSA3, and *P. stuartii* NEA16. It is followed by compounds 5, 7, 6, and 3 with a MIC = 16 µg/mL against 4/10 (*E. coli* AG102, P. aeruginosa PA124 and S. aureus MRSA3 and MRSA6), and 3/10 (*E. coli* AG102, *P. aeruginosa* PA124, and *S. aureus* MRSA3), 1/10 (*P. stuartii* PS2636), and 1/10 (*S. aureus* MRA3), respectively. The reference antibiotic, ciprofloxacin presented MICs ranging from 1 to 128 µg/mL. Furthermore, it can be noted that compounds 3, 5, 6, 7, and 8 were more active (8 ≤ MIC ≤ 128 µg/mL) on the Methicillin-Resistant *Staphylococcus aureus* isolates (MRSA3 and MRSA6) than the reference antibiotic (MIC = 128 µg/mL). In general, bactericidal effects (MBC/MIC ≤ 4) were recorded with compound 5 and ciprofloxacin on 60% (6/10) of the bacteria tested, whereas compound 8 has presented MBC/MIC > 4 on 30% (3/10) of these bacteria.

Effect of PAβN on the antibacterial activity of the isolated compounds

The role of efflux pumps of the MDR bacteria tested on the antibacterial activity of the isolated compounds was evaluated in the presence of PAβN, an efflux pump inhibitor (Table 2). As a result, the antibacterial activity of all the tested compounds was improved 2 to >128 times on at least one of the tested MDR bacteria in the presence of PAβN. The activity of compounds **2, 5, 6, 7,** and **8** was significantly improved on 80% and more of the tested MDR bacteria. The highest fold increase (128-folds) was observed with compounds **6** and **8** (against *K. pneumoniae* KP55 and *P. aeruginosa* PA01), and compound **7** (*E. coli* ATCC10536 and AG102, *K. pneumoniae* ATCC11296 and KP55, and *P. aeruginosa* PA01). Similarly, the activity of ciprofloxacin was significantly improved (4 to 32-fold) against all the tested bacteria.

*Effect of compound **5** on Providencia stuartii* NEA16 *growth curve*

Figure 2 shows the growth curve of *P. stuartii* NEA16 in the presence and absence of compound **5** and ciprofloxacin. In the absence of the tested samples (Control), the *P. stuartii* NEA16 growth curve showed three different phases namely lag phase, exponential phase, and stationary phase. Tested samples have globally affected the growth of *P. stuartii* NEA16 compared to control, this in a concentration-dependent manner by reducing the bacteria number (decreasing absorbance). At its MIC/2, compound **5** affected only the stationary growth phase by reducing the bacteria number (decreasing absorbance), whereas at its MIC and 2×MIC, it reduced the duration of the exponential phase from 8 to 6 h and prolonged that of the stationary phase to about 2 h. The reference antibacterial, ciprofloxacin has considerably inhibited bacterial growth, prolonged lag phase from 2 to 10 h, and reduced the exponential phase and stationary from 8h to 4 h.

*Effect of compound **5** on the membrane integrity of Providencia stuartii* NEA16

The leakage of intracellular material of *P. stuartii* NEA16 was evaluated after 12 h of its exposure to different concentrations of compound **5**. Compound **5** has induced a concentration-dependent (MIC/2, MIC, and 2×MIC) increase of 260 nm absorbing materials in *P. stuartii* NEA16 compared to the control. Polymycin B, used as reference antibiotic also induced the liberation of 260 nm absorbing materials in *P. stuartii* NEA16 compared to the control. Its effect was most pronounced compared ayato that of compound 5 at all its tested concentrations (Figure 3). These results indicate that compound 5 disturbs the membrane integrity of *Providencia stuartii* NEA16.

*Effect of compound **5** on H^+-ATPase pumps of Providencia stuartii* NEA16

The effects of compound 5 and ciprofloxacin on the inhibition of *H^+-ATPase pumps* of *P. stuartii* NEA16 are depicted in Figure 4. It appears that a time-dependent decrease of the pH (acidification) was observed in all bacteria cultures. Therefore, a huge time-dependent acidification was observed for the control culture until the end of the experiment (60 min) compared to the culture treated with compound **5** and ciprofloxacin, where the acidification was mainly observed between 0 and 30 min. Therefore, between 30-60 min only a slight decrease of pH was observed for compound 5 (MIC and 2 × MIC) and ciprofloxacin compared to the control culture, indicating that compound 5 induces a dose-dependent inhibition of the H+/ATPase pumps of *P. stuartii* NEA16.

Discussion

Antibiotic resistance continues to evolve and spread beyond all boundaries and as a result, infectious diseases have become more challenging, leading to an increase in morbidity and mortality [36]. Consequently, alternative strategies to combat infections caused by multidrug-resistant bacteria are needed. Plants have a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids, and flavonoids found abundantly in plants, and have been reported to possess a range of biological activity including antibacterial properties [6, 37-40]. Possible use of plants including those from Africa to fight antimicrobial resistance was demonstrated [41-48]. In this study, we have evaluated the antibacterial activity of eight flavonoids isolated from *B. thonningii* leave methanol extract against multi-drug resistant (MDR) bacteria expressing active efflux pumps in the absence and presence of PAβN, a known efflux pump inhibitor (EPI). According to the cut-off values of MICs of the phytochemical against enterobacteria [49], Gram-positive bacteria [50], and *Pseudomonas* spp. [51], compound 5 displayed good activity (8 < MIC ≤ 32 µg/mL) against *E. coli* ATCC10536 and AG102*, P. stuartii* PS2636 and NEA16 and *S. aureus* MRSA3 and MRSA6*.* Compound 6 also showed good activity (8 < MIC ≤ 32 µg/mL) against *E. coli* AG102*, K. pneumonia* ATCC11296*, P. stuartii* PS2636, and NEA16 and *S. aureus* MRSA3*.* Compound 8 displayed very good activity (4 < MIC ≤ 8 µg/mL*)* against *E. coli* AG102*, P. stuartii* NEA16, and *S. aureus* MRSA3*;* and good activity (8 < MIC ≤ 32 µg/mL) against *K. pneumonia* ATCC11296 and *P. stuartii* PS2636. Moreover, compounds **5**, **6**, **7** and **9** had excellent activity (4 < MIC ≤ 32 µg/mL) against *P. aeruginosa* PA124. The tested phytochemicals were flavonoids and mainly quercetin and kaempferol derivatives. Natural flavonoids have been reported to possess various pharmacological properties including their good antibacterial activity against both Gram-positive and Gram-negative isolates [52, 53]. For instance, quercetin and kaempferol have shown antimicrobial activity against Gram-negative and Gram-positive bacteria [54, 55]. In a recent study, quercetin aglycones showed strong antimicrobial activity against *S. aureus* (MIC = 6.25 µg/mL) and *S. haemolyticus* (MIC = 50 µg/mL) [56]. In this study, the tested flavonoids named; quercetin-3-*O*-$_L$-rhamnopyranoside (**5**), quercetin-3-*O*-$_L$-rhamnopyranoside (**6**), and 6,8-*C*-dimethylkaempferol-3-methyl ether (**8**) presented comparative antibacterial activity, with MICs ranging from 8 to 128 µg/mL. Moreover, we have demonstrated in our previous study that 6-C-methylquercetin-3,4'-dimethyl ether possessed interesting antibacterial activity [17]. All these data indicate that phytochemicals **5**, **6,** and **8** can be considered good candidates to tackle infections due to MDR bacteria.

Drug efflux is one of the key mechanisms of resistance in bacteria, mainly Gram-negative bacteria. The efflux pump systems allow the microorganisms to regulate their internal environment by removing toxic substances, including antimicrobial agents, metabolites, and quorum-sensing signal molecules [57]. Efflux pumps can be blocked by substances known as efflux pump inhibitors (EPIs), thereby restoring the activity of antibiotics and/or extracts or phytochemicals [58]. The role of efflux pumps on the tested compounds was assessed in the presence of PAβN, an EPI. In the presence of PAβN, the antibacterial activity of all the tested compounds was improved 2 to 128 folds on at least one of the bacteria tested, indicating that the tested compounds and mainly compounds **6**, **7**, and **8** are substrates of the efflux pumps of the tested MDR bacteria. Moreover, these data confirm that the tested MDR bacteria overexpressed efflux pumps as a main resistant mechanism.

The studied modes of action of antimicrobial agents are related to a large variety of bacterial targets and processes, including the inhibition of bacterial cell-wall biosynthesis and cell membrane destruction, inhibition of bacterial protein biosynthesis, DNA replication and repair, and metabolic pathways, among others [59, 60]. The antibacterial activities of flavonoids are commonly attributed to their ability to inhibit DNA gyrase, cell membrane function, and bacterial energy metabolism [56, 61]. Flavonoids are also proven as inhibitors of quorum sensing and biofilm formation [62]. In this study, the effect of quercetin-3-*O*-L-rhamnopyranoside (**5**) has been evaluated on *P. stuartii* NEA16 growth curve, lysis, and energy production (H+-ATPase pumps).

The bacterial growth rate during the exponentially growing phase is the most important parameter as it represents the genetic and environmental influences on the bacterial growth dynamics [63]. Compound **5** at its MIC and 2×MIC has shortened the exponential growth phase of *P. stuartii* NEA16, indicating a possible denaturation of proteins by compound **5** leading to the bacteria death. It is known that flavonoids as quercetin and as well as its derivatives, interact with DNA helicases, proteins essential for DNA replication, repair, and recombination [64, 65].

Nucleic acids and derivatives appear as one of the main cellular components. Therefore, an increase of the absorption at 260 nm in the medium containing bacteria may indicate the presence of nucleic acids, and consequently reflects the loss of the bacterial membrane integrity [66]. In this study, compound **5** showed a concentration-dependent increase in 260 nm absorbing materials in treated bacteria compared to control, suggesting a lysis of the cytoplasmic membrane of *P. stuartii* NEA16 by the compound.

Bacteria must maintain a cytoplasmic pH that is compatible with optimal functional and structural integrity of the

cytoplasmic proteins that support growth [67]. This is done by the plasma membrane H^+-ATPases (H^+-pumps), which are the primary active transporters that translocate protons to the outside of each cell, providing the electrical and chemical energy that drives solute transport [68]. Thus, inhibition of H^+-ATPase pumps may lead to death or the inhibition of the growth of the bacteria [69]. In this study, compound 5 has induced a dose-dependent inhibition of the H+/ATPase pumps of *P. stuartii* NEA16, suggesting that it prevents the production of energy which is necessary for the metabolism and transport of the solutes.

Finally, the present study demonstrates the potential of African plants as a source of antibacterial agents to combat drug resistant phenotypes. The results corroborate several other studies in African medicinal plants demonstrating their potential as safe botanicals or phytochemicals to combat various human ailments including inflammatory diseases, cancers, as well as bacterial, fungal, and viral infections amongst others [70-114].

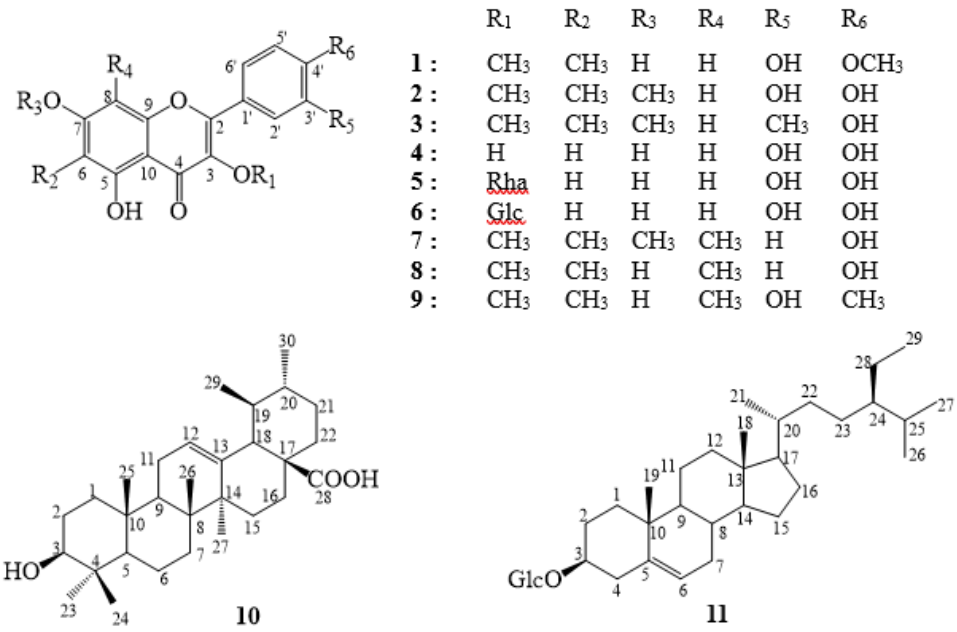

Figure 1. Phytochemicals isolated from the leaves of *B. thonningii.*

6-C-methylquercetin-3, 4'-dimethyl ether (**1**); 6-C-methylquercetin-3,7-dimethyl ether (**2**); 6-C-methylquercetin 3,7,3'-trimethyl ether (**3**); quercetin **(4)**; qeurcetine-3-*O*-$_L$-rhamnopyranoside **(5)**; quercetin-3-*O*-L-rhamnopyranoside (**6**); 6,8-*C*-dimethylkaempferol 3,7-dimethyl ether **(7)**; 6,8-*C*-dimethylkaempferol-3-methyl ether **(8)**; 6,8-*C*-dimethylquercetin-3-methyl ether **(9),** ursolic acid **(10)** and 3-*O*-β-$_D$-glucopyranoside of β-sistosterol **(11)**.

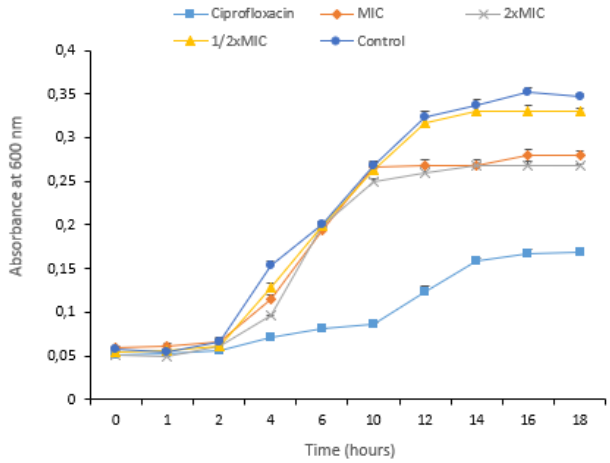

Figure 2. Effect of compound **5** on *Providencia stuartii* NEA16 growth curve.

Each point represents the mean ± SD (n= 3); MIC: Minimum inhibitory concentration. The MIC value of compound **5** was 32 µg/mL.

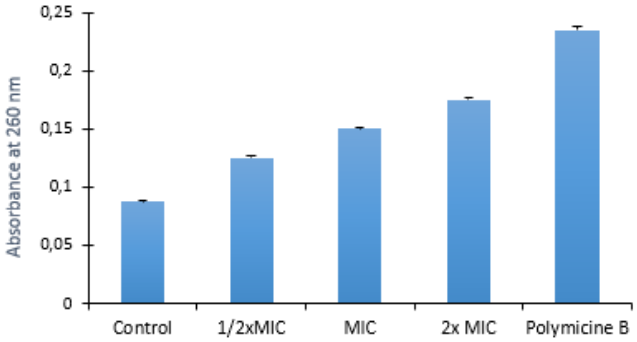

Figure 3. Leakage of 260 nm absorbing materials from *Providencia stuartii* NEA16 by compound 5.
Each bar graph represents the mean ± SD (n= 3); MIC: Minimum inhibitory concentration. The MIC value of compound **5** was 32 µg/mL.

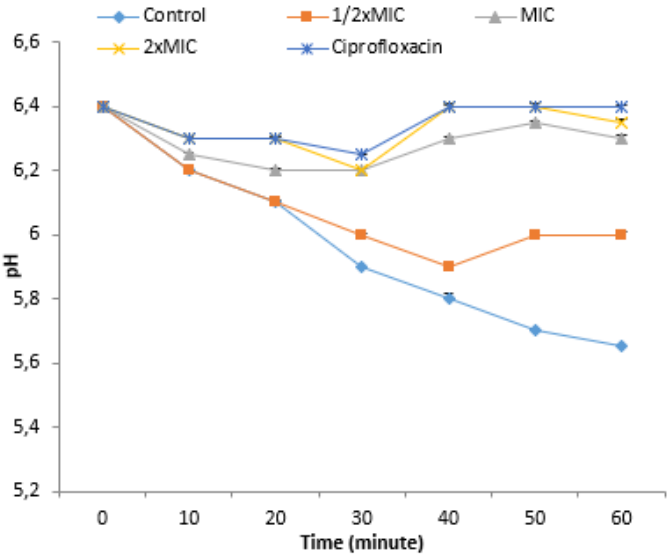

Figure 4. Effects of compound 5 on the proton-ATPase pumps of *Providencia stuartii* NEA16.
*Each point represents the mean ± SD (n= 3). MIC: Minimum inhibitory concentration. The MIC value of compound **5** was 32 µg/mL.*

Table 1. MICs and MBCs (µg/mL) of isolated compounds from the methanol leaves extract of B. thonningii and Ciprofloxacin against the tested bacteria.

Bacteria	Tested samples, MICs and MBCs (µg/mL), and MBC/MIC (in bracket)																	
	Compounds																Ciprofloxacin	
	1		2		3		5		6		7		8		9			
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E. coli																		
ATCC10536	128	/	>128	/	128	/	32	/	128	/	128	/	128	/	>128	/	8	8 (1)
AG102	>128	/	>128	/	128	/	16	64 (4)	64	/	16	/	8	64 (8)	64	/	2	8 (8)
K. pneumoniae																		
ATCC11296	64	/	>128	/	>128	/	64	/	32	/	128	/	16	64(4)	128	/	2	/
KP55	32	/	64	/	>128	/	64	/	128	/	>128	/	128	/	128	/	4	16 (4)
P. stuartii																		
PS2636	>128	/	64	/	32	/	32	64 (2)	16	128 (8)	128	/	16	64 (4)	32	/	2	8 (4)
NEA16	128	/	64	/	>128	/	32	/	32	/	32	/	8	128 (8)	>128	/	1	4 (4)
P. aeruginosa																		
PA01	128	/	>128	/	>128	/	32	128 (4)	128	/	128	/	128	/	>128	/	16	32 (2)
PA124	>128	/	>128	/	64	/	16	64 (4)	32	/	16	/	128	/	32	/	8	32 (4)
S. aureus																		
MRSA3	128	/	128	/	64	/	16	64 (4)	32	128 (4)	16	/	8	128 (8)	128	/	128	/
MRSA6	128	/	>128	/	16	/	16	64 (4)	128	/	64	/	64	/	32	/	128	/

MIC: Minimal inhibitory concentration; MBC: Minimal bactericidal concentration; **1**: 6-C-methylquercetin-3, 4'-dimethyl ether; **2**: 6-C-methylquercetin-3,7-dimethyl ether; **3**: 6-C-methylquercetin 3,7,3'-trimethyl ether; **5:** quercetin-3-*O*-L-rhamnopyranoside; **6**: quercetin-3-*O*-L-rhamnopyranoside; **7**: 6,8-C-dimethylkaempferol 3,7-dimethyl ether; **8**: 6,8-C-dimethylkaempferol-3-methyl ether; **9**: 6,8-C-dimethylquercetin-3-methyl ether.

Table 2. MICs (µg/mL) of the isolated compounds in association with PAβN

Bacteria	Tested samples, MICs of samples in the presence and absence of PAβN (µg/mL), and AIF (in bracket)																	
	1	**1 + PAβN**	**2**	**2 + PAβN**	**3**	**3 + PAβN**	**5**	**5 + PAβN**	**6**	**6 + PAβN**	**7**	**7+ PAβN**	**8**	**8 + PAβN**	**9**	**9 + PAβN**	**CIP**	**CIP+ PAβN**
E. coli																		
ATCC10536	128	64 (2)	>128	16(**>8**)	128	128 (1)	32	1 (**32**)	128	8 (**16**)	128	1 (**128**)	128	128 (1)	>128	128(>1)	8	1 (**8**)
AG102	>128	64 (**>2**)	>128	16(**>8**)	128	128 (1)	16	4 (**4**)	64	8 (**8**)	16	1(**16**)	8	4 (2)	64	4 (**16**)	2	0.25 (**8**)
K. pneumoniae																		
ATCC11296	32	4 (**8**)	>128	32 (**>4**)	>128	32 (**>4**)	64	<1 (**>64**)	32	1 (**32**)	128	<1 (**>128**)	16	<1 (**<16**)	128	32 (**4**)	2	0.5 (**4**)
KP55	64	8 (**8**)	64	128 (1)	>128	16 (>**8**)	64	1 (**64**)	128	1 (**128**)	>128	1 (**>128**)	128	1 (**128**)	128	32 (**4**)	4	0.5 (**8**)
P. stuartii																		
PS2636	>128	128(>1)	64	4 (**16**)	32	32 (1)	32	8 (**4**)	16	2 (**8**)	128	1 (**128**)	16	2 (**8**)	32	16 (2)	2	0.5 (**4**)
NEA16	128	32 (**4**)	64	64 (1)	>128	8 (**>16**)	32	4 (**8**)	32	32 (1)	32	16 (2)	8	1 (**8**)	>128	8 (**>16**)	1	0.25 (**4**)
P. aeruginosa																		
PA01	128	32 (**4**)	>128	16 (**>8**)	>128	128 (>1)	32	<1 (**>32**)	128	1 (**128**)	128	1 (**128**)	128	1 (**128**)	>128	64 (**>2**)	16	2 (**8**)
PA124	>128	32 (**>4**)	>128	16 (**>8**)	64	16 (**4**)	16	16 (1)	32	32 (1)	16	16 (1)	128	16 (**8**)	32	16 (2)	8	0.5 (**16**)
S. aureus																		
MRA3	128	128 (1)	128	16 (**16**)	64	64 (1)	16	<1 (**>16**)	32	<1 (**>32**)	16	1 (**16**)	8	1 (**8**)	128	64 (2)	128	**8** (**16**)
MRA6	128	**8** (**16**)	>128	8 (**>16**)	16	16 (1)	16	<1 (**>16**)	128	2 (**64**)	64	(**16**)	64	16 (**4**)	32	16 (2)	128	**4** (**32**)

MIC: Minimal inhibitory concentration; CIP: Ciprofloxacin; MRSA: Methicillin- Resistant *Staphylococcus aureus;* PAβN: Phenylalanine-Arginine-β-Naphtylamide. Values in bold indicate significant AIF or fold increase of the sample activity in the presence of PAβN.

Conclusion

In this work, the antibacterial activity of eight flavonoids isolated from the leaves methanol extract of *Bauhinia thonningii* was investigated against multi-drug resistant (MDR) bacteria expressing efflux pumps. Among these phytochemicals, quercetin-3-O-$_L$-rhamnopyranoside (**5**), quercetin-3-*O*-L-rhamnopyranoside (**6**), and 6,8-*C*-dimethylkaempferol-3-methyl ether (**8**) displayed good to excellent activity against the tested bacteria. Quercetin-3-*O*-$_L$-rhamnopyranoside (**5**) inhibited bacterial growth in the exponential phase, induced bacteriolysis, and inhibited the H+-ATPase pumps in *P. stuartii* NEA16. All these data indicate that these compounds can be considered good candidates to tackle MDR bacterial infections. Their combinations with efflux pump inhibitors could be also envisaged to combat MDR bacteria expressing efflux pumps.

Additional file

Supplementary files SF1. Experimental procedure for extraction, purification and isolation; Supplementary files SF1. Procedure for the structural elucidation of the isolated compounds. Available at https://www.investchempharma.com/wp-content/uploads/2018/01/www.investchempharma.com-imcp92-supplementary-file.pdf

Abbreviations

ATCC: American-type culture collection
ATP: Adenosine triphosphate
CFU: Colony-forming unit
CIP: Ciprofloxacin
DMSO: Dimethyl sulfoxide
EPIs: Efflux pump inhibitors
INT: *p*-Iodonitrotetrazolium chloride
MBC: Minimal bactericidal concentration
MDR: Multidrug-resistant
MHA: Mueller–Hilton agar
MHB: Mueller–Hinton broth
MIC: Minimal inhibitory concentration
MRSA: Methicillin-resistant *Staphylococcus aureus*
PAβN: Phenylalanine arginine-β- naphthylamide

Authors' Contribution

VYM, FD, GRSM, JRNK, and FJM carried out the study; VYM, GRSM, AGF and IC prepared the data, AGF wrote the manuscript; VK provided the bacteria strains; AML, ATM, and VK supervised the work; all authors read and approved the final manuscript.

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Conflict of interest

The authors declare no conflict of interest.

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