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Antibacterial and antibiotic-potentiation activities of methanol extracts from *Blighia sapida* K. D. Koenig (Sapindaceae) against Gram-negative multidrug-resistant bacteria overexpressing efflux pumps

Ancela W. B. Yendze[1], Larissa Mpude[1], Eric Cadet[1], Derick J. Assonfack[1], Valaire Y. Matieta[1], Jenifer R. N. Kuete[1], Junior F. Megaptche[1], Idrios N. Bonsou[1], Michael F. Kengne[1], Armelle T. Mbaveng[1*], and Victor Kuete[1**]

Abstract

Background: The emergence and spread of multidrug-resistant (MDR) Gram-negative bacteria, particularly those overexpressing efflux pumps, make treatment difficult using conventional antibiotics. This study aims to assess the antibacterial activity of methanol extracts (botanicals) from the leaves (BSL) and bark (BSB) of *Blighia sapida* against a panel of MDR bacteria that overexpress efflux pumps.

Methods: Phytochemical screening of botanicals was carried out using standard qualitative assays. The antibacterial activity and the association of the botanicals with an efflux pump inhibitor (EPI), phenylalanine-arginine β-naphthylamide (PAβN), and antibiotics were evaluated using broth microdilution methods meanwhile the mode of action of BSL was investigated on proton pumps H+/ATPases assays.

Results: The secondary metabolites such as phenols, anthocyanins, saponins, alkaloids, and flavonoids were found in BSL and BSB. BSL and BSB showed activity against 93.33% and 60% of the isolates/strains, with minimum inhibitory concentrations (MICs) ranging from 16-1024 µg/mL. The addition of EPI increased the antibacterial activity of the botanicals against all tested bacteria. The botanicals also boosted the effectiveness of antibiotics at half MIC and quarter MIC, enhancing the activity of at least 57.14% of tetracycline (TET), ciprofloxacin (CIP), and imipenem (IMI) against the bacteria. When evaluated for its effects on the H+/ATPases proton pumps of *Providencia stuartii* ATCC29916, BSL showed potential in inhibiting the activity of this enzyme.

Conclusion: These results suggest that *Blighia sapida* contains secondary metabolites that can act alone or in combination with antibiotics to treat bacterial infections caused by MDR Gram-negative bacteria that overexpress efflux pumps.

Keywords: Antibacterial activity; antibiotics; *Blighia sapida*; efflux pumps; multidrug resistance; Sapindaceae.

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

[1]Department of Biochemistry, Faculty of Science, University of Dschang, Dschang, Cameroon

Other authors E-mails:
whitneyancela13@gmail.com (Ancela W. B. Yendze); larissampude5@yahoo.com (Larissa Mpude); derickassonfack7@gmail.com (Derick J. Assonfack); ericcadet19@gmail.com (Eric Cadet) ; yvmatieta@yahoo.com (Valaire Y. Matieta); jeniferkuete@gmail.com (Jenifer R. N. Kuete); bonichrist89@yahoo.com (Idrios N. Bonsou); megapfabrice@gmail.com (Junior F. Megaptche); fmkengne@yahoo.com (Michael F. Kengne)

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Background

Infectious diseases, caused by pathogenic microorganisms like viruses, parasites, fungi, and bacteria, are a serious concern, especially in tropical countries where they account for at least 50% of all deaths in the 21st century [1]. Shockingly, in 2017 in developing nations, 70% of child deaths are due to infectious diseases, with an estimated 560,000 out of 2.7 million annual neonatal deaths attributed to these diseases. Since 2000, neonatal deaths have decreased by 44%, according to the World Health Organization (WHO). In 2022, almost half (47%) of all deaths in children under 5 occurred during the first 28 days of life, which is a highly vulnerable period requiring high-quality intrapartum and newborn care. Sub-Saharan Africa accounted for 57% (2.8 (2.5–3.3) million) of total under-5 deaths but only 30% of global live births in 2022. The region also had the highest neonatal mortality rate in the world at 27 deaths per 1000 live births, followed by central and southern Asia with a neonatal mortality rate of 21 deaths per 1000 live births [2]. The discovery of the first antibiotic in 1928 marked a turning point in the fight against these deadly diseases [3]. Yet, the misuse of antibiotics has given rise to the dangerous threat of multidrug resistance, causing millions of deaths worldwide. In 2019, the global death toll due to antimicrobial resistance was an estimated 4.95 million, with 1.27 million of these deaths resulting from infectious diseases caused by pathogenic bacteria [4, 5]. Efflux pumps are transporter proteins that forcefully eject antibiotics from inside bacterial cells to the exterior, thereby preventing the antibiotics from reaching their target and significantly contributing to drug resistance. These powerful pumps can expel a wide range of antibiotic families into the external environment, posing a substantial challenge to treatment [6, 7]. There are five main efflux pump families, with the resistance nodulation cell division (RND) family of efflux pumps playing a dominant role in conferring resistance to various antibiotic classes in Gram-negative bacteria. For instance, AcrAB-TolC pumps are prevalent in Enterobacteriaceae bacteria, while MexAB-OprM pumps are prominent in *Pseudomonas aeruginosa* [4, 8, 9]. The rise of MDR bacteria is a growing concern. The absence of new antibiotics underscores the need to seek new effective antibacterial agents with medicinal plants [10-12]. The African flora, particularly in Cameroon, is rich in medicinal plants that have demonstrated their ability to control various human diseases [10, 13-23]. Exploring this flora is a promising strategy for discovering new antimicrobial agents [24-34]. Numerous studies conducted in Cameroon have conclusively demonstrated the antibacterial activities of plants against MDR Gram-negative bacteria [35-38]. *Blighia sapida* K. D. Koenig (Sapindaceae) is a well-known plant in traditional Cameroonian medicine. It is used to treat various diseases such as diarrhea, conjunctivitis, fever, internal hemorrhage, dysentery, skin infections, constipation, and backache [39]. Research by Peace et al. [40] and Ologundudu et al. [39] have shown that this plant has antibacterial properties. The current study aims to determine the antibacterial activity of the methanol extracts from the leaves and bark against MDR Gram-negative bacteria. The study also aims to investigate the effect of the leaf extract on H+/ATPases in the most susceptible Gram-negative bacterial strain. Additionally, the effects of combining the plant extracts with an efflux pump inhibitor (EPI), phenylalanine-arginine β-naphthylamide (PAβN), and antibiotics against Gram-negative bacterial strains was assessed.

Methods

Plant material and extraction

Blighia sapida, commonly known as Ackee, was harvested in the South-west region of Cameroon in September 2023. The parts used for this work were its leaves and bark. Samples of this plant were identified in the Cameroon National Herbarium under the code 2733 SRF/Cam by the botanist Eric Ngansop Tchatchouang. The leaves and bark were harvested, dried away from the sun, and then crushed to obtain a powder. The powder was macerated in methanol in a ratio of 1:3 (weight to volume) for 48 hours at room temperature. The mixture was mixed 3 to 4 times a day to maximize the yield. After maceration, the mixture was filtered with Whatman filter no.1. The filtrate was evaporated using a rotary evaporator at 65°C. The crude extracts from the leaves (BSL) and bark (BSB) referred to as botanicals were dried in an oven to remove any remaining solvents and then stored at 4°C for future use.

Chemicals and culture media

Dimethyl sulfoxide (DMSO) served to solubilize plant extracts. Antibiotics used included β-lactams: ampicillin (AMP), penicillin (PEN), carbapenem imipenem (IMI), the cephalosporins cefixime (CFX), and ceftriaxone (CTX); fluoroquinolones: ciprofloxacin (CIP) and levofloxacin (LEV), and the tetracycline (TET). Mueller Hinton Agar (MHA) was used for the activation of bacteria; Mueller Hinton Broth (MHB) was used for microdilution as a nutrient medium for bacteria. Cetrimide agar was a selective culture medium used for identifying *Pseudomonas aeruginosa*. MacConkey agar was used to differentiate and isolate Gram-negative bacteria. Eosin Methylene Blue (EMB) agar was used to inhibit Gram-positive bacteria while promoting the growth of Gram-negative bacteria. para-Iodonitrotetrazolium chloride ≥ 97% (INT) was used as the bacterial growth indicator. The efflux pump inhibitor (EPI), phenylalanine-arginine *β*-naphthylamide (PAβN) at 0.2% was used. All chemicals were purchased from Sigma-Aldrich (St. Quentin Fallavier, France).

Tested bacteria

The Gram-negative bacteria tested included both reference strains and clinical isolates of *Escherichia coli (*ATCC10536, AG102, and AG100), *Klebsiella pneumoniae (*ATCC11296, KP55, and KP63), *Pseudomonas aeruginosa* (PA01, PA124, and PA0100), *Enterobacter aerogenes* (EA3, EA282, and EA27), and *Providencia stuartii* (ATCC29916, PS2636, and NEA16). Their bacterial features were previously reported [28, 34, 41-50]. *Escherichia coli (*AG102, and AG100), *Klebsiella pneumoniae (*KP55), *Enterobacter aerogenes* (EA3, EA282, and EA27), and *Providencia stuartii* (PS2636, and NEA16) are clinical bacterial strains overexpressing AcrAB-TolC efflux pumps while *Pseudomonas aeruginosa* PA124 overexpressed MexAB-OprM pumps [36, 51-54].

Determination of minimal inhibitory and bactericidal concentrations

The bacterial inoculum was prepared as previously described by comparing it to the turbidity of a standard McFarland 0.5 ($1.5x10^8$ CFU/mL) [24, 30, 55-59]. The various plant extracts and the reference drug (IMI) were dissolved in DMSO-MHB. Plant extracts were prepared at 8192 µg/mL, and antibiotics at 512 µg/mL and 4096 µg/mL (in some cases). PAβN was prepared at a

concentration of 100 µg/mL. The botanicals were tested alone and then in the presence of PAβN (EPI). The combination of plant extracts with EPI was intended to evaluate the function of efflux pumps in bacterial resistance to the test extracts [41, 42, 58, 60]. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of test samples alone were determined using a 96-well broth microdilution method combined with the rapid INT colorimetric method [60-62] . IMI was used as a positive antibacterial control, whereas DMSO 2.5%+MHB and MHB alone were used as negative controls. MIC was considered the lowest concentration of plant extract which produced complete inhibition of bacterial growth after 18 to 24 hours of incubation at 37°C, whereas MBC was considered the lowest concentration of a sample that did not induce a color change with the addition of INT following additional 48 hours of incubation [31, 63, 64]. Each experiment was repeated three times in triplicate.

Evaluation of the effect of BSL on the functioning of H+/ATPases proton dependent pumps of P. stuartii ATCC29916

The effects of BSL were assessed on the H^+-ATPase-mediated proton pumping of *P. stuartii ATCC29916* at 0.5×MIC, MIC, and 2×MIC as earlier described [59]. The action on H^+-ATPase-mediated proton pumping was done by controlling the acidification of the bacterial growth medium over 60 min following the procedures previously described [65-68].

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

Botanicals and IMI were also tested in the presence of PAβN (30 µg/mL) as previously described [41]. The ratio $MIC_{(sample\ alone)}/MIC_{(sample\ +PA\beta N)}$ referred to as the activity improvement factor (AIF) was used to determine the fold increase of the antibacterial activity of the samples in the presence of PAβN. The bacteria tested included *E. coli* (ATCC10536 and AG102), *K. pneumoniae* (ATCC11296, and KP55), *E. aerogenes* EA282, *P. stuartii* PS2636, and *P. aeruginosa* PA01 and PA124. IMI at concentrations ranging from 1 to 128 µg/ml to serve as a reference. Each assay was repeated thrice.

Determination of the antibiotic-potentiating effects of the botanicals

The effects of the association of the botanicals with antibiotics were determined against the MDR bacteria using the broth microdilution method as previously described [26, 34]. The tested antibiotics included CTX, AMP, PEN, CFX, LEV, CIP, TET, and IMI. The tested bacteria were *E. coli* ATCC10536 and AG102, *P. aeruginosa* PA124, *K. pneumoniae* (KP55 and ATCC11296), *P. stuartii* PS2636, and *E. aerogenes* EA282. At first, extracts were used at the sub-inhibitory concentrations of MIC/2, MIC/4, MIC/8, and MIC/16 for a preliminary assay on PA01, which then allowed the selection of appropriate sub-inhibitory concentrations of MIC/2 and MIC/4 for further combination testing (Data not shown). Activity modulation factor (AMF) was calculated as the ratio of the MIC of the antibiotic alone versus MIC in combination with the plant extract. The potentiation effect was considered for AMF ≥ 2 [69].

Phytochemical screening of botanicals

Phytochemical screening was done following the standard methods described for alkaloids, anthocyanins, flavonoids (Shinoda test), phenols, saponins, and triterpenes (Liebermann-Burchard test) [10, 70].

Interpretation of antibacterial activity of botanicals

Updated and rationally defined cutoff points of the antibacterial botanicals have been defined for Enterobacteria as follows: outstanding activity (MIC ≤8 µg/mL), excellent activity (8 < MIC ≤64 µg/mL), very good activity (64 < MIC ≤128 µg/mL), good activity (128 < MIC ≤256 µg/mL), average activity (256 < MIC ≤512 µg/mL), weak activity (512 < MIC ≤1024 µg/mL), and not active (MIC values >1024 µg/mL) [71]. For *P. aeruginosa* these cutoff points were defined as follows*:* outstanding activity (MIC ≤ 32 µg/mL), excellent activity (32 < MIC ≤ 128 µg/mL), very good activity (128 < MIC ≤ 256 µg/mL), good activity (256 < MIC ≤ 512 µg/mL), average activity (512 < MIC ≤ 1024 µg/mL), weak activity or not active (MIC values >1024 µg/mL) [19]. Bactericidal activities are considered when the ratios MBC/MIC are below or equal to 2 [63, 64, 72, 73]. These appreciation criteria will be used to discuss the antibacterial activities of the studied samples.

Results

In vitro antibacterial activity of botanicals

The antibacterial activity of MDR Gram-negative bacteria was determined in vitro by evaluating the MICs and MBCs of each extract. The MBC/MIC ratios of the extracts were calculated to determine each bacteriostatic and bactericidal activity and their values are recorded in Table 1. It appears BSL and BSB had MIC values ranging from 16 to 2048 µg/mL. BSL and BSB displayed an inhibition spectrum of 93.33% and 60% against the tested bacterial strains respectively. An excellent activity was shown against *P. stuartii* ATCC29916 with a MIC value of 16 µg/mL and NEA16 with a MIC value of 64 µg/mL. Very good activity was also recorded for the MDR strains of *E. coli* AG100, *K. pneumoniae* KP55, *E. aerogenes* EA27, *P. stuartii* PS2636, and *P. aeruginosa* PA0100 (MIC value of 256 µg/mL). BSL was bactericidal against *E. coli* ATCC11296, *K. pneumoniae* ATCC11296, KP63, *E. aerogenes* EA282, *P. stuartii* NEA16, and *P. aeruginosa* PA01, PA124, and PA0100. BSB showed excellent activity for *P. stuartii* ATCC29916 (MIC value of 16 µg/mL), very good activity for *K. pneumoniae* KP55 (MIC value of 128 µg/mL), and good activity for *P. stuartii* PS2636 (MIC value of 256 µg/mL). BSB had a bactericidal effect against *E. coli* AG100, *E. aerogenes* EA27, *P. stuartii* ATCC29916, and *P. aeruginosa* PA01, PA124 and PA0100.

Effect of BSL on H+ proton pumps/ATPases

The capacity of BSL to inhibit the functioning of proton pumps of the strain of *P. stuartii* ATCC29916 was evaluated by measuring at different time intervals the pH of the environment containing the bacteria strain in the presence of the extract. Figure 1 shows the different graphs plotted indicating the pH evolution against time in the presence as well as in the absence of our extract at MIC/2, MIC, and 2MIC. This study observed changes in pH over time in a medium containing the strain *P. stuartii* ATCC29916 with and without the addition of BSL at MIC/2, MIC, and 2MIC. The negative control with no extract demonstrated a gradual decrease in pH, indicating normal activity of the proton pumps that acidify the surrounding environment. However, BSL at MIC/2 induced a slight decrease, suggesting that the extract was effectively inhibiting the activity of the proton pumps. The graph of the MIC indicated an increase in pH over time, which suggests a slightly strong inhibition of the proton pump activity. On the other hand, the graph at 2MIC

showed a slight decrease in pH initially but increased to up to 6.9 later. This suggests a possible inhibition or slight inhibition of the proton pumps by the extract.

PAβN enhanced the activity of botanicals and reference antibiotic

To ascertain this, the MIC values of botanicals with PAβN were determined and the results are summarized in Table 2. In the presence of the EPI, BSL and BSB showed improved activity against all tested MDR Gram-negative bacteria. PAβN increased the antibacterial activity of BSL, with enhancement factors, AIF, ranging from 16 to 128. The highest AIF of 128 was observed against *E. coli* ATCC10536 and *P. aeruginosa* PA01. Similarly, BSB's antibacterial activity was enhanced with AIF ranging from 2 to 256, with the highest significant value of 256 against *P. aeruginosa* PA01 and PA124, and *E. aerogenes* EA282.

Antibiotic-activity modulation effects of BSL

An initial test was performed to assess the antibacterial impact of BSL and BSB when combined with antibiotics. The MDR *P. aeruginosa* PA01 was used, and the assay sought to determine the most effective sub-inhibitory concentrations of these extracts that could enhance the efficacy of standard antibiotics. The study found that when BSL and BSB were used at half and quarter of the minimum inhibitory concentration (MIC/2 and MIC/4), they increased the effectiveness of the antibiotics more than when used at MIC/8 and MIC/16 during the combined treatment. The AMF ranged between 2 and 256 (Data not shown). Because of their significant enhancement of antibiotics, these two plant parts were selected for further testing. They were then tested on 7 bacterial strains and isolates, and the results are documented in Tables 3 and 4. The recorded AMF ranged from 0.03 to 256. BSL demonstrated potent potentiation activity against most of the tested strains when combined with IMI (85.71% for MIC/2 and 57.14% for MIC/4). Additionally, TET, CFX, and PEN potentiated the activity of 71.42% antibiotics at MIC/2 and MIC/4 against the tested bacteria (Table 3). BSL also potentiated the activity of AMP and CIP on 57.14% of antibiotics at MIC/2 and 42.85% and 71.42% respectively at MIC/4. The activity of CTX was potentiated by 42.85% and 28.57% at MIC/2 and MIC/4 of BSL, respectively; that of LEV was potentiated by 14.28% at MIC/2 and MIC/4 (Table 3). BSB exhibited remarkable potentiating activity, enhancing the activity of PEN by 100% at MIC/2 and 42.85% at MIC/4 against the various bacterial strains tested (Table 4). The activity of TET was potentiated in 85.71% and 57.14% of the cases at MIC/2 and MIC/4, respectively. The activities of CIP, IMI, CTX, AMP, and CFX were potentiated in 57.14% of the cases at MIC/2 meanwhile that of CIP was potentiated in 71.42% of the cases. LEV showed the lowest potentiation when combined with BSB (28.57%) at MIC/2 and MIC/4 (Table 4).

Phytochemistry

Phytochemical screening of BSL and BSB revealed the presence of phenols, terpenoids, saponins, flavonoids, and anthocyanins in the two plant extracts; BSB additionally contained alkaloids.

Discussion

The rise of antibiotic-resistant microorganisms poses a significant global health threat, causing millions of deaths annually [1]. Numerous research studies have emphasized the vital role that medicinal plants can play in addressing human ailments [23, 28, 34, 46, 74-90]. African flora, and traditional Cameroonian pharmacopeia in particular, have shown promise in inhibiting the growth of a wide range of MDR Gram-negative bacteria and enhancing the effectiveness of common antibiotics [34, 91]. In our study, we assessed the effectiveness of methanol extracts from *B. sapida* in inhibiting the growth of MDR Gram-negative bacteria. The antibacterial activity of the extracts was evaluated based on the recent criteria established for Enterobacteriaceae and *P. aeruginosa* [19, 71]. Previous studies by Peace et al. [40] and Ologundudu et al. [39] on *B. sapida* demonstrated that the leaves and bark of this plant exhibited antibacterial activities against the tested bacterial strains. However, our current research study yielded significantly higher activities compared to these previous studies, which could be attributed to differences in the geographic locations of the harvested samples. Bacterial proton pumps are proteins embedded in the cell membrane that use energy from ATP hydrolysis to move protons (H+) against their concentration gradient, essentially pushing them out of the cell [92-94]. This research aimed to understand how BSL affects the H+/ATPase proton pumps in *P. stuartii* ATCC29916. It was found that the BSL inhibited the activity of proton pumps, leading to a rise in pH at 2MIC, indicating a slightly strong inhibition effect on the proton activity. These results are like those of Mapie et al. [50], who demonstrated that botanicals from the Cameroonian flora were able to inhibit the functioning of the proton pumps.

Bacteria possess efflux pumps, which act like tiny pumps that expel antibiotics out of the cell thus reducing their effectiveness [95]. *B. sapida* contains various phytochemicals with antibacterial potential including alkaloids, terpenoids, saponins, flavonoids, anthocyanins, and phenols and these compounds could potentially inhibit the action of efflux pumps, thereby increasing the intracellular concentration of antibiotics and thus improving their effectiveness. Research studies carried out by Fankam et al. [35] have shown that there was a significant rise in antibacterial activity of the extracts of *Dichrostachys glomerata* when combined with PAβN against resistant strains of *E. coli, K. pneumoniae*, and *P. stuartii*. The results obtained are similar to those obtained in this work and thus conclude that the presence of PAβN an efflux pump inhibitor enhanced the antibacterial activity of the extracts of *B. sapida*. The association of antibiotics with the methanol extracts of *B. sapida* can be a valuable strategy for combating antimicrobial resistance. In effect, it was demonstrated in the present study that botanicals from *B. sapida* exhibited remarkable synergistic effects when combined with a range of antibiotics. These synergistic effects might result from the simultaneous or combined action of the plant-derived compounds and antibiotics at different target sites within the bacterial cells.

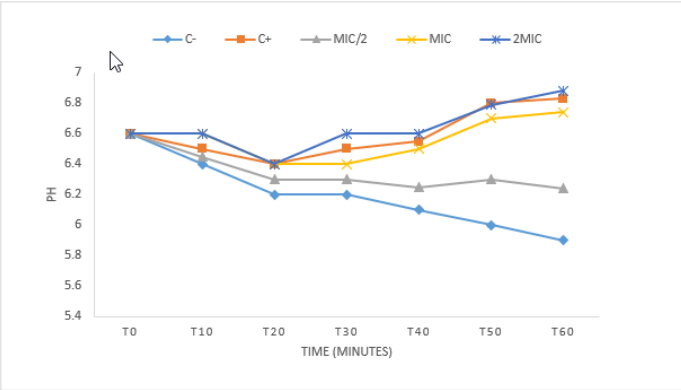

Figure 1. Effect of *B. sapida* leaves extract (BSL) on *P. stuartii* ATCC29916 H+/ATPase proton pumps

Table 1. Minimal inhibitory and bactericidal concentrations of the leave (BSL) and bark (BSB) extracts of *Blighia sapida*, and imipenem against the tested bacteria.

Tested bacteria		**Samples, MIC and MBC (in µg/mL), and MBC/MIC ratios**								
		***B. sapida* leaves**			***B. sapida* bark**			**Imipenem**		
		MIC	**MCB**	**R**	**MIC**	**MCB**	**R**	**MIC**	**MBC**	**R**
Escherischia coli	ATCC10536	2048	>2048	Nd	2048	2048	Nd	32	16	2
	AG100	128	1024	8	1024	2048	2	32	256	8
	AG102	256	2048	8	512	2048	4	32	256	8
Klebsiella pneumoniae	ATCC 11296	512	1024	2	512	2048	4	32	128	4
	KP55	256	2048	8	128	2048	16	⩾128	512	4
	KP63	128	256	2	2048	2048	Nd	16	128	8
Enterobacter aerogenes	EA3	256	2048	8	128	2048	Nd	8	64	8
	EA27	128	2048	16	1024	2048	2	4	4	1
	EA282	512	1024	2	2048	2048	Nd	128	2048	16
Providencia stuartii	ATCC29916	16	1024	64	16	16	1	16	256	16
	PS2636	128	1024	8	256	2048	8	32	256	8
	NEA16	64	64	1	512	2048	4	32	2048	64
Psedomonas aeruginosa	PA01	1024	2048	2	2048	2048	1	⩾128	2048	16
	PA124	512	512	1	2048	2048	1	⩾128	2048	16
	PA0100	128	256	2	2048	2048	1	32	32	1

MIC: Minimal Inhibitory Concentration, MBC: Minimum Bactericidal Concentration, R: ratio of MBC/MIC

Table 2. Minimum inhibitory concentrations of the botanicals and imipenem alone and in the presence of PAβN.

Tested bacteria		***B. sapida* leaves**			***B. sapida* bark**			**Imipenem**		
		MIC alone	**+PAβN**	**R**	**MIC alone**	**+PAβN**	**R**	**MIC alone**	**+PAβN**	**R**
E. coli	ATCC10536	1024	<8	128	2048	1024	2	32	<1	32
	AG102	256	<8	32	512	<8	64	32	<1	32
K. pneumoniae	ATCC11296	512	<8	64	512	<8	64	32	<1	32
	KP55	256	<8	32	128	<8	16	⩾128	<1	128
E. aerogenes	EA282	512	<8	64	2048	<8	256	⩾128	<1	>128
P. stuartii	PS2636	128	<8	16	256	<8	32	32	<1	32
P. aeruginosa	PA01	1024	<8	128	2048	<8	256	⩾128	<1	128
	PA124	512	<8	64	2048	<8	256	⩾128	<1	128

MIC alone: Minimum inhibitory concentration in the absence of the inhibitor, +PAßN: Minimum inhibitory concentration in the presence of the inhibitor, R or AIF (activity improvement factor): MIC/+PAβN ratio, nd: not determined.

Table 3. Effects of the combination of antibiotics and BSL against MDR bacteria.

Antibiotics	BSL	Bacteria, MIC in µg/mL, and AMF (in bracket)							PSB (%)
		E. coli		*K. pneumoniae*		*E. aerogenes*	*P. stuartii*	*P. aeruginosa*	
		ATCC10536	AG102	ATCC11296	KP55	EA282	PS2636	PA124	
TET	0	2	8	4	8	32	>128	64	
	MIC/2	<1(2)	8(1)	<1(4)	<1(8)	4(8)	128(1)	32(2)	71.42%
	MIC/4	<1(2)	8(1)	128(0.O3)	<1(8)	4(8)	>128(1)	32(2)	57.14%
CIP	0	<1	1	32	4	8	8	4	
	MIC/2	<1(1)	<1(1)	<1(32)	<1(4)	1(8)	1(8)	<1(4)	71.42%
	MIC/4	<1(1)	<1(1)	<1(32)	<1(4)	8(1)	1(8)	<1(4)	57.14%
IMI	0	32	32	32	≥128	≥128	32	>128	
	MIC/2	<1(32)	2(16)	<1(32)	<8(16)	<4(32)	4(8)	128(1)	85.71%
	MIC/4	<1(32)	1(32)	<1(32)	256(0.5)	4(32)	1024(0.03)	>128(1)	57.14%
CFX	0	64	8	32	64	512	128	512	
	MIC/2	<8(8)	32(0.25)	<8(4)	<8(8)	512(1)	64(2)	32(16)	71.42%
	MIC/4	<8(8)	64(0.125)	<8(4)	<8(8)	512(1)	64(2)	128(4)	71.42%
AMP	0	>1024	1024	1024	1024	1024	1024	1024	
	MIC/2	<8(256)	1024(1)	1024(1)	1024(1)	128(8)	128(8)	4(256)	57.14%
	MIC/4	512(2)	1024(1)	1024(1)	1024(1)	128(8)	256(4)	1024(1)	42.85%
	0	<1	<1	2	<1	8	4	4	
LEV	MIC/2	<1(1)	<1(1)	<1(2)	<1(1)	8(1)	32(0.125)	4(1)	14.28%
	MIC/4	<1(1)	<1(1)	<1(2)	32(0.03)	32(0.25)	32(0.125)	4(1)	14.28%
	0	512	256	256	1024	256	1024	1024	
PEN	MIC/2	<8(64)	256(1)	<8(32)	<8(128)	<4(64)	512(2)	1024(1)	71.422%
	MIC/4	<8(64)	128(2)	<8(32)	<8(128)	256(1)	512(2)	1024(1)	71.42%
CTX	0	<8	<8	32	32	<4	8	512	
	MIC/2	<8(1)	<8(1)	<8(4)	16(2)	<4(1)	4(2)	512(1)	42.85%
	MIC/4	<8(1)	<8(1)	<8(4)	32(1)	<4(1)	<4(1)	256(2)	28.57%

MIC: Minimum Inhibitory Concentration; ampicillin (AMP), penicillin (PEN), carbapenem imipenem (IMI), the cephalosporins cefixime (CFX), and ceftriaxone (CTX); fluoroquinolones: ciprofloxacin (CIP) and levofloxacin (LEV), and the tetracycline (TET); (): AMF: Activity modulation factor; PBS: Percentage of bacteria where synergy is observed.

Table 4. Effects of the combination of antibiotics and BSB against MDR bacteria.

Antibiotics	BSL	Bacteria, MIC in µg/mL, and AMF (in bracket)							PSB (%)
		E. coli		*K. pneumoniae*		*E. aerogenes*	*P. stuartii*	*P. aeruginosa*	
		ATCC10536	AG102	ATCC11296	KP55	EA282	PS2636	PA124	
TET	0	2	8	4	8	32	>128	64	
	MIC/2	<1(2)	<1(8)	<1(4)	<1(8)	64(0.5)	32(4)	<1(64)	85.71%
	MIC/4	<1(2)	<1(8)	16(0.25)	128(0.06)	64(0.5)	32(4)	<1(64)	57.14%
CIP	0	<1	<1	32	4	8	8	4	
	MIC/2	<1(1)	<1(1)	<1(32)	<1(4)	<1(8)	<1(8)	<1(4)	71.42%
	MIC/4	<1(1)	<1(1)	<1(32)	<1(4)	4(2)	4(2)	<1(4)	71.42%
IMI	0	32	32	32	≥128	≥128	32	>128	
	MIC/2	<1(32)	32(1)	16(2)	128(1)	<1(128)	64(0.5)	<1(128)	57.14%
	MIC/4	<1(32)	32(1)	16(2)	256(0.5)	64(2)	64(1)	<1(128)	57.14%
CFX	0	64	64	32	64	512	128	>1024	
	MIC/2	<8(8)	<8(8)	32(1)	64(1)	512(1)	64(2)	<4(256)	57.14%
	MIC/4	<8(8)	<8(8)	128(0.25)	64(1)	512(1)	64(2)	1024(1)	42.85%
AMP	0	>1024	1024	1024	1024	1024	1024	1024	
	MIC/2	<8(128)	1024(1)	1024(1)	1024(1)	<4(256)	256(4)	<4(256)	57.14%
	MIC/4	<8(128)	1024(1)	1024(1)	1024(1)	1024(1)	256(4)	1024(1)	28.57%
	0	<1	<1	2	<1	8	4	4	
LEV	MIC/2	<1(1)	<1(1)	<1(2)	32(0.03)	16(0.5)	32(0.125)	<1(4)	28.57%
	MIC/4	<1(1)	<1(1)	<1(2)	32(0.03)	16(0.5)	32(0.125)	<1(4)	28.57%
	0	512	256	256	1024	256	1024	1024	
PEN	MIC/2	<8(64)	128(2)	<8(32)	<8(128)	128(2)	512(2)	<4(256)	100%
	MIC/4	<8(64)	128(2)	256(1)	1024(1)	256(1)	1024(1)	<4(256)	42.85%
CTX	0	<8	<8	32	32	<4	8	512	
	MIC/2	<8(1)	<8(1)	<8(4)	16(2)	8(0.5)	<4(2)	<4(128)	57.14%
	MIC/4	<8(1)	<8(1)	<8(4)	8(4)	8(0.5)	<4(2)	<4(128)	57.14%

MIC: Minimum Inhibitory Concentration; ampicillin (AMP), penicillin (PEN), carbapenem imipenem (IMI), the cephalosporins cefixime (CFX), and ceftriaxone (CTX); fluoroquinolones: ciprofloxacin (CIP) and levofloxacin (LEV), and the tetracycline (TET); (): AMF: Activity modulation factor; PBS: Percentage of bacteria where synergy is observed.

Conclusion

In the present study, it was demonstrated that extracts from both the leaves and bark of *B. sapida* had an arsenal of bioactive compounds and demonstrated significant antibacterial activity against a wide range of MDR Gram-negative bacteria. The leaf extract inhibited the activity of the bacterial proton pumps. Both leaf and bark extracts were substrates of bacterial efflux pumps on one hand, and on the other hand, potentiated the activity of antibiotics against the tested bacteria. Conclusively, the methanol extracts of the leaves and bark of *B. sapida* are potential sources of antibacterial compounds that could be used either alone or in association with efflux pump inhibitors or antibiotics to combat MDR bacteria.

Abbreviations

Abbreviations

AIF: activity improvement factors
AMF: Activity modulation factor
AMP: ampicillin
ATCC: American-type culture collection
CFU: Colony Forming Unit
CFX: cefixime
CIP: ciprofloxacin
CTX: ceftriaxone
DMSO: Dimethylsulfoxide
EPI: efflux pump inhibitor
IMI: imipenem
INT: Iodonitrotetrazolium chloride
LEV: levofloxacin
MBC: Minimum Bactericidal Concentration
MDR: Multidrug resistant
MHA: Mueller Hinton agar
MHB: Mueller Hinton broth
MIC: Minimal inhibitory Concentration
PAβN: phenylalanine arginine β-naphthylamide
PEN: penicillin
TET: tetracycline
WHO: World Health Organization

Authors' Contribution

AWBY, LM, DJA, EC, VYM, JRNK, INB, MFK, and JFM carried out the study; ATM and VK supervised the study; All authors read and approved the final version of the manuscript.

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Conflict of interest

The authors declare no conflict of interest.

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