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A moderately active crude methanol extract from the whole plant of *Kalanchoe crenata* (Andrews) Haw. (Crassulaceae) strongly potentiated the effect of antibiotics against multidrug-resistant Gram-negative bacteria

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Abstract

Background: Resistant bacteria develop a high level of resistance to multiple drugs, limiting treatment options and increasing morbidity and mortality. This work was planned to evaluate the antibacterial potential of the methanol extract from the whole plant of *Kalanchoe crenata* (KCW) against multidrug-resistant (MDR) Gram-negative bacteria.

Methods: The minimal inhibitory concentrations (MIC) and the minimal bactericidal concentrations (MBC) of KCW alone, in the presence of an efflux pump inhibitor (EPI) phenylalanine-arginine *β*-naphthylamide (PAβN), or in the presence of antibiotics were assessed using the broth microdilution method combined with the rapid para-iodonitrotetrazolium chloride (INT) colorimetric technique.

Results: KCW displayed weak antibacterial activities with MIC values ranging from 128 to 1024 µg/mL against 10 of the 15 tested bacterial strains. Moderate antibacterial activities with MIC values ranging from 128-625 µg/mL were recorded against some strains belonging to *Klebsiella pneumoniae*, *Escherichia coli*, and *Providencia stuartii*. PAβN does not significantly enhance the activity KCW. At MIC/2, KCW potentiated the activity of doxycycline (DOX), levofloxacin (LEV), imipenem (IMI), ciprofloxacin (CIP), ceftriaxone (CRO), and tetracycline (TET) against at least 80% of the MDR bacterial strains tested.

Conclusion: The present study demonstrated that KCW is a moderately active antibacterial agent, but a good efflux pump inhibitor that could potentiate the activity of antibiotics against MDR bacteria over-expressing active efflux pumps.

Keywords: Antibacterial; antibiotic-potentiation; Crassulaceae; efflux pumps; *Kalanchoe crenata*; multidrug resistance.

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Background

Bacterial resistance to antibiotics remains a serious health concern globally [1]. Resistant bacteria develop a high level of resistance to multiple drugs, limiting treatment options and increasing morbidity and mortality. The WHO report attributes 45% of deaths in Africa to multidrug-resistant (MDR) bacteria [1]. It is estimated that between 2015 and 2050, bacterial infections and antimicrobial resistance will have caused approximately 1.3 million deaths in Europe [1]. More than 2.8 million people in the United States die from antibiotic-resistant infections each year [1]. Despite the great diversity of antibiotics used clinically, resistance of bacteria to all classes has been observed to date. This resistance causes a huge financial burden in all countries. For example, the Center for Diseases Control and Prevention (CDC) determined that treating six alarming antibiotic-resistance threats accounts for more than $4.6 billion USD in healthcare costs annually. These bacteria include vancomycin-resistant *Enterococcus*, carbapenem-resistant *Acinetobacter*, methicillin-resistant *Staphylococcus aureus* (MRSA), carbapenem- and extended-spectrum cephalosporin-resistant *Enterobacteria*, and MDR *Pseudomonas aeruginosa* [1, 2]. The continuous search for new drugs capable of counteracting various forms of resistance remains therefore a priority for researchers around the world. These new antimicrobials should also provide an advantage related to their lower toxicity compared to existing ones. Medicinal plants are an undeniable source of low-toxicity drugs that can help in the fight against human diseases [3-8]. Their pharmacological activities against resistant phenotypes of bacteria, parasites, or even cancer cells have now been demonstrated [9-25]. Numerous scientific publications have demonstrated over the past two decades the strong capacity of African medicinal plants and their constituents to hinder the growth of these cell forms that are harmful to animals and humans [26-29]. The intensification of this research will make it possible to afford a good number of bioactive substances that can satisfactorily pass the various clinical phases to have new drugs that are more effective and less harmful. Thus, we were interested in this work, to determine the antibacterial potential of *Kalanchoe crenata* (Andrews) Haw. (Crassulaceae) on MDR phenotypes. It is a succulent flowering plant native to Madagascar and is commonly known as Kalanchoe, Mother of millions, Never die, Dog's liver, Orange forest kalanchoe. *Kalanchoe crenata* is present in tropical Africa, from Kenya to Tanzania, Uganda, Burundi, Central African Republic, Rwanda, Zaire, Guinea, Sierra Leone, Angola, but also in South Africa [30]. The plant is traditionally used for the treatment of headache, general debility, dysentery, smallpox and convulsion, earache, wounds and sores, but also as a sedative and a remedy for chronic cough [31]. The plant previously displayed cytotoxic activity towards PC212 human mesothelioma cells, A549 human non-small cell lung cancer (NSCLC) cells, HepG2 hepatocarcinoma cells, MCF-7 breast adenocarcinoma cells, and DLD-1 colorectal adenocarcinoma cell lines [32], analgesic and anticonvulsant effects [33], antihyperglycemic activity [34], and nephroprotective effects [35]. The antibacterial potential of this plant has previously been documented on *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Bacillus subtilis*, *Shigella flexneri*, *Escherichia coli*, and *Staphylococcus aureus* [36].

Methods

Plant mateial and extraction

The whole plant of *Kalanchoe crenata* was collected in Dschang, West Region of Cameroon, in December 2022. The plant was identified at the National Herbarium of Cameroon (HNC) under the identification number 35196/HNC. The whole plant was air-dried and then ground. The powder obtained was macerated in 95% methanol for 48 hours, then the mixture was filtered using Whatman N°1. The resulting filtrate was concentrated under a vacuum (BÜCHI R-200) at 65°C at reduced pressure. The crude extract was completely dried in an oven at 40°C. The obtained crude extract (botanical, KCW) was kept at 4°C till further use.

Chemicals and culture media

The efflux pump inhibitor (EPI), the antibiotics, and bacterial growth revelator among others were purchased from Sigma-Aldrich (St. Quentin Fallavier, France). The EPI used was phenylalanine-arginine *β*-naphthylamide (PAβN). para-iodonitrotetrazolium chloride ≥ 97% (INT) was used as the bacterial growth indicator. Dimethyl sulfoxide (DMSO) served to solubilize the botanical. The antibiotics used include doxycycline (DOX), levofloxacin (LEV), imipenem (IMI), ciprofloxacin (CIP), ampicillin (AMP), ceftriaxone (CRO), tetracycline (TET), and vancomycin (VAN). The culture media used were Mueller Hinton Agar (MHA), for the activation of bacterial strains and isolates, Mueller Hinton Broth (MHB), for antibacterial testing, and Eosin methylene blue (EMB), used as a specific and differential culture medium to confirm the purity of bacterial strains [37-39].

Bacterial species

The Gram-negative bacteria tested included both reference strains and clinical isolates of *Escherichia coli (*ATCC10536, AG102, and AG100), *Klebsiella pneumoniae (*ATCC11296, KP55, and K2), *Pseudomonas aeruginosa* (PA01 and PA124), *Enterobacter aerogenes* (EA3, EA298, and EA27), and *Providencia stuartii* (ATCC29916, PS2636, and NEA16). Their bacterial features were previously reported [37, 39-51]. *Escherichia coli (*AG102, and AG100), *Klebsiella pneumoniae (*KP55, and K2), *Enterobacter aerogenes* (EA3, EA298, and EA27), and *Providencia stuartii* (PS2636, and NEA16) are clinical bacterial strains over-expressing AcrAB-TolC efflux pumps while *Pseudomonas aeruginosa* PA124 over-expressed MexAB-OprM pumps [51-54].

Determination of minimal inhibitory and bactericidal concentrations

The minimal inhibitory concentrations (MIC) and the minimal bactericidal concentrations (MBC) of KCW and antibiotics alone, in the presence of PAβN (EPI) were determined using the combined microbroth dilution and rapid INT colorimetric method as previously described in similar experimental conditions [37, 50, 52, 54-60]. Each experiment was repeated three times in triplicate.

Determination of the antibiotic-potentiating effects of KCW

The effects of the association of KCW at the sub-inhibitory concentrations of MIC/2 and MIC/4 with antibiotics (DOX, LEV, IMI, CIP, AMP, CRO, TET, and VAN) were determined against the MDR bacteria using the rapid INT colorimetric method as previously described in the similar experimental conditions [37-39,

43]. Antibiotic-resistance modulating 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 [61].

Interpretation of antibacterial data

The General interpretation of the antibacterial activity of botanicals was performed according to the cutoff values established by Kuete [62] as follows: significant activity (MIC <100 µg/mL), moderate (100 <MIC ≤ 625 µg/mL), and weak (MIC> 625 µg/mL). For Enterobacteria, the following specific cutoff points were used: 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) [63]. For *Pseudomonas aeruginosa*, the following scales were applied: outstanding activity when MIC ≤ 32 µg/mL; excellent activity when 32 < MIC ≤ 128 µg/mL; very good activity when 128 < MIC ≤ 256 µg/mL; good activity when 256 < MIC ≤ 512 µg/mL, average activity when 512 < MIC ≤ 1024 µg/mL, weak activity or not active when MIC values >1024 µg/mL [64]. Bactericidal activities are considered when the ratios MBC/MIC are below or equal to 2; MBC/MIC ratios above 2 define the bacteriostatic activities [65-68].

Results

The crude extract from the whole plant of Kalanchoe crenata displays moderate to weak antibacterial effects

The MIC and MBC were determined on a panel of 15 bacterial strains and the results are shown in Table 1. It appears that KCW displayed moderate to weak antibacterial activities with MIC values ranging from 128 to 1024 µg/mL against 10 of the 15 tested bacterial strains. Moderate antibacterial activities of KCW with MIC values ranging from 128-625 µg/mL were recorded against *Klebsiella pneumoniae* K2 and ATCC 11296, *Escherichia coli* ATCC10536, and *Providencia stuartii* PS2636 and ATCC 29916. The MBC values were generally above 1024 µg/mL. The MIC values of the reference antibiotic, IMI, varied from <4 to 128 µg/mL. This drug generally displayed bacteriostatic effects against the tested bacteria, with most of the MBC/MIC ratios above 2.

PAβN did not enhance the activity of the crude extract from the whole plant of Kalanchoe crenata

To check whether the bacterial efflux pumps are involved in the resistance of the tested bacteria to KCW, the MIC values of the botanical were determined in the presence of the EPI (PAβN). The results are shown in Table 2. In the presence of PAβN, the activity of KCW generally did not change, and only a 2-fold increase was observed on 18% (2/11) of the tested bacteria. This is a clear indication that KCW and its constituents are not the substrates of the bacterial efflux pumps.

The crude extract from the whole plant of Kalanchoe crenata significantly potentiated the activity of antibiotics

KCW at MIC/2 and MIC/4, was combined with antibiotics, and tested against ten bacterial species, including *E. aerogenes* EA3 and EA27, *P. stuartii* PS2636 and NAE16, *E. coli* ATCC10536 and AG100, *P. aeruginosa* PA01 and PA121, and *K. pneumoniae* KP2 and KP55. The results are shown in Table 3. It appears that the antibacterial activities of the antibiotics increased in the presence of the extracts of KCW at MIC/2 and MIC/4, with the AMF ranging from 2- to 128-fold in most of the cases. At MIC/2, KCW potentiated the activity of LEV, IMI, DOX, CIP, VAN, CRO, and TET against at least 80% of the MDR bacterial strains tested. At MIC/4, KCW also potentiated the activity of DOX, CIP, VAN, and CRO against at least 70% of the MDR bacterial strains tested.

Discussion

African medicinal plants have been shown to be efficient sources of antibacterial agents against both drug sensitive and MDR phenotypes [50, 56, 69-84]. The search for novel antibacterial agents should involve the use of MDR strains. In the present study, several MDR bacteria have been used. This could be evidenced by the increase in the antibacterial activity of the reference drug, IMI in the presence of PAβN (Table 2). In effect, PAβN has been identified as the efflux pumps inhibitor of AcrAB-TolC in Enterobacteria and MexAB-OprM in *Pseudomonas aeruginosa* [40]. The botanical from *Kalanchoe crenata* (KCW) displayed moderate to weak antibacterial activity according to Kuete [62]. However, in Enterobacteria very good activity (64 < MIC ≤128 µg/mL) [63] of KCW was obtained against *K. pneumoniae* ATCC11296 while average activity (256 < MIC ≤512 µg/mL) [63] was recorded against *K. pneumoniae* K2, *Escherichia coli* ATCC10536, and *Providencia stuartii* PS2636 and ATCC29916 (Table 1). Average antibacterial activity (512 < MIC ≤ 1024 µg/mL) [64] was obtained against *Pseudomanas aeruginosa* PA124. The inhibitory activities of KCW against the other bacterial strains were rather weak or considered not active. The poor antimicrobial activity of this plant has previously been reported against *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Bacillus subtilis* (MIC of 8 mg/mL), *Shigella flexneri* (MIC of 32 mg/mL), *Escherichia coli* (MIC of 64 mg/mL), and *Staphylococcus aureus* (MIC of 128 mg/mL) [36]. These data corroborate the results obtained in the present study, confirming that this plant is a moderately active antibacterial agent, as the overall activity was rather moderate.

It has been suggested that if an antibacterial agent potentiates the activity of at least 70% of antibiotics against at least 70% of the tested bacteria over-expressing active efflux pumps, it should be considered a potential EPI [85, 86]. In the present study, KCW at MIC/2, potentiated the activity of 7/8 (88%) antibiotics (LEV, IMI, DOX, CIP, VAN, CRO, and TET) against at least 80% of the MDR bacterial strains tested (Table 2). Therefore, this botanical is an an EPI.

Table 1. Antibacterial activities of crude methanol extract from the whole plant of *Kalanchoe crenata.*

Bacterial strains	**Tested samples, MIC and MBC (MIC and MBC in µg/mL), and their ratio**					
	Botanical			ATB		
	KCW			IMI		
	MIC	MBC	**R**	MIC	MBC	**R**
Pseudomonas aeruginosa						
PA01	>2048	-	nd	16	64	4
PA121	>2048	-	nd	16	128	8
PA124	1024	-	nd	4	32	8
Klebsiella pneumoniae						
K2	512	-	nd	<4	64	16
KP55	>2048	-	nd	<4	64	16
ATCC 11296	128	-	nd	<4	<4	1
Escherichia coli						
AG100	1024	-	nd	8	64	8
AG102	>2048	-	nd	16	64	4
ATCC10536	512	-	nd	8	64	8
Providencia stuartii						
PS2636	512	1024	2	8	64	8
NEA16	2048	-	nd	8	64	8
ATCC 29916	512	-	nd	<4	16	4
Enterobacter aerogenes						
EA3	2048	-	nd	8	64	8
EA27	>2048	-	nd	8	16	2
EA298	2048	-	nd	8	8	1

KCW: crude methanol extract from the whole plant of *Kalanchoe crenata*; R: MBC/MIC ratio; (-): > 2048 or inactive; (nd): not determined; MIC: minimal inhibitory concentration; MBC: minimal bactericidal concentration; IMI: imipenem; ATB: Antibiotic.

Table 2. Minimal inhibitory concentrations of the crude methanol extract from the whole plant of *Kalanchoe crenata* in the presence of PAβN.

Bacterial strains	**Tested samples, MIC in the absence or presence of EPI (in µg/mL), and their ratio**					
	Botanical			**ATB**		
	KCW			**IMI**		
	MIC alone	**MIC+PAβN**	R	**MIC alone**	**MIC+PAβN**	R
Pseudomonas aeruginosa						
PA01	2048	2048	1	16	16	1
PA121	2048	2048	1	16	2	8
Klebsiella pneumoniae						
K2	512	256	2	<4	1	<4
KP55	2048	2048	1	<4	4	<4
Escherichia coli						
AG100	2048	2048	1	8	2	4
AG102	2048	2048	1	16	2	8
ATCC10536	2048	2048	1	8	1	8
Providencia stuartii						
PS2636	2048	2048	1	8	4	2
NEA16	2048	1024	2	8	8	1
Enterobacter aerogenes						
EA3	2048	2048	1	8	4	2
EA27	2048	2048	1	8	<1	>8

KCW: crude methanol extract from the whole plant of *Kalanchoe crenata*; IMI: imipenem; R: MIC alone *vs* MIC with PAβN ratio; MIC alone: Minimal Inhibitory Concentration of the sample alone; MIC+PAβN: Minimal Inhibitory Concentration of the sample in the presence of PAβN; ATB: Antibiotic

Table 3. MICs (µg/mL) of antibiotics of the crude methanol extract from the whole plant of *Kalanchoe crenata*.

ATB	**Extract concentration**	**Bacterial strains, MIC of antibiotics in the presence of extract and Antibiotic-resistance modulating factor (AMF)**										**PSP (%)**
		E. aerogenes		***P. stuartii***		***E. coli***		***P. aeruginosa***		***K. pneumoniae***		
		EA3	**EA27**	**PS2636**	**NEA16**	**ATCC10536**	**AG100**	**PA01**	**PA121**	**KP2**	**KP55**	
LEV	0	1	0.5	0.25	0.5	0.25	0.25	0.5	0.5	0.5	0.5	
	MIC/2	<0.0625 (16)	<0.0625 (8)	<0.0625 (4)	<0.0625 (8)	<0.0625(4)	0.25(1)	0.5(1)	0.25(2)	0.25 (2)	0.125(4)	80
	MIC/4	0.5(2)	<0.0625 (8)	<0.0625 (4)	<0.0625 (8)	0.125(2)	0.25(1)	1(1)	1(1)	0.5(1)	0.5(1)	50
IMI	0	8	8	8	8	8	8	16	16	<4	4	
	MIC/2	1/2 (16)	1 (8)	4 (2)	8(1)	<1/2 (16)	2 (4)	4 (4)	4 (4)	2 (2)	1/16 (64)	90
	MIC/4	8 (1)	2 (4)	8 (1)	8(1)	<1/2 (16)	2 (4)	16 (1)	8 (2)	8 (0.5)	1/16 (64)	50
DOX	0	1	0.5	8	8	1	0.5	0.5	1	2	8	
	MIC/2	0.125(8)	0.0625 (8)	<0.0625 (128)	0.5(16)	<0.0625(16)	0.25(2)	0.25(2)	1(1)	4(0.5)	1(8)	80
	MIC/4	0.125(8)	0.25(2)	<0.0625 (128)	1(8)	<0.0625(16)	0.25(2)	0.25(2)	1(1)	4(0.5)	2(4)	80
CIP	0	1	0.25	0.25	8	0.25	0.5	1	0.5	0.5	0.5	
	MIC/2	1(1)	0.5(0.5)	<0.0625 (4)	1(8)	<0.0625(4)	0.25(2)	0.25(4)	0.25(2)	0.5(1)	<0.0625 (8)	70
	MIC/4	1(1)	1(0.25)	0.125(2)	1(8)	<0.0625(4)	0.25(2)	0.5(2)	0.5(1)	0.5(1)	0.25(2)	60
VAN	0	64	2	2	8	2	256	2	2	>64	>64	
	MIC/2	2 (32)	1(2)	1(2)	8(1)	<0.5(4)	32(8)	1(2)	1 (2)	1(64)	<0.5 (128)	90
	MIC/4	4(16)	1(2)	1(2)	8(1)	1(2)	32(8)	2(1)	1 (2)	8(8)	<0.5 (128)	80
AMP	0	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	
	MIC/2	>256(1)	<2(128)	>256(1)	256(1)	>256(1)	>256(1)	>256(1)	32(8)	>256 (1)	256(1)	20
	MIC/4	>256(1)	8(32)	>256(1)	>256(1)	>256(1)	>256(1)	>256(1)	>256(1)	>256 (1)	>256(1)	10
TET	0	8	>8	0.25	>8	4	2	0.25	1	>8	>8	
	MIC/2	<0.0625 (128)	<0.0625 (128)	<0.0625 (4)	1(8)	0.5(8)	2(1)	0.125(2)	<0.0625 (16)	>8(1)	1(8)	80
	MIC/4	0.25(32)	<0.0625 (128)	<0.0625 (4)	8(1)	4(1)	2(1)	0.25(1)	<0.0625 (16)	>8(1)	8(1)	40
CRO	0	32	32	64	256	8	32	8	16	8	64	
	MIC/2	16(2)	<2(16)	64(1)	32(8)	<2 (4)	8(4)	4(2)	<2(8)	1(8)	16(4)	80
	MIC/4	32(1)	16(2)	64(1)	128(2)	<2 (4)	8(4)	8(1)	8(2)	2(4)	32(2)	70

MIC: Minimal Inhibitory Concentration; (): AMF: Antibiotic-resistance modulating Factor; PSP (%): percentage of strain where potentiation effect was observed; ATB: Antibiotic; LEV: Levofloxacin; VAN: Vancomycin; CIP: Ciprofloxacin; TET: Tetracycline; DOX: Doxycycline; IMI: Imipenem; CRO: Ceftriaxone; AMP: Ampicillin.

Conclusion

In the present study, the moderate antibacterial potential of the methanol extract from the whole plant of *Kalanchoe crenata* was demonstrated. It was also demonstrated that the botanical from this plant is an efflux pump inhibitor and therefore could potentiate the activity of antibiotics against MDR bacteria over-expressing active efflux pumps. Further study will be performed to identify phytochemicals responsible for the inhibition of the bacterial efflux pumps. Also, toxicological assessments of the botanical will be performed to evaluate its safety.

Abbreviations

AMP: ampicillin
ATCC: American-Type Culture Collection
CDC: Center for Diseases Control and Prevention
CIP: ciprofloxacin
CRO: ceftriaxone
DMSO: Dimethyl sulfoxide
DOX: doxycycline
EMB: Eosin methylene blue
EPI: efflux pump inhibitor
HNC: National Herbarium of Cameroon
IMI: imipenem
INT: para-Iodonitrotetrazolium chloride
LEV: levofloxacin
MBC: minimal bactericidal concentration
MDR: multidrug-resistant
MHA: Mueller Hinton Agar
MHB: Mueller Hinton
MIC: minimal inhibitory concentrations
PAβN: phenylalanine-arginine *β*-naphthylamide
TET: tetracycline
VAN: vancomycin
ZJB: methanol extract from the whole plant of *Kalanchoe crenata*

Authors' Contribution

FF, RN, VYM, GKF, SMT, JFM, and PN 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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