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Botanical from the bark of *Zizyphus jujuba* Mill. (Rhamnaceae) had weak anti-Klebsiella activity, but strongly potentiated the effects of antibiotics against multidrug-resistant phenotypes

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Abstract

Background: *Klebsiella pneumoniae* is medically the most important species of this genus. *Klebsiella oxytoca* also cause infections in human but to a much lesser degree than *K. pneumoniae*. In this work, the antibacterial potential of the methanol extract from the bark of *Zizyphus jujuba* (ZJB) was evaluated against the multidrug-resistant (MDR) clinical isolates of *Klebsiella pneumoniae* and *Klebsiella oxytoca* overexpressing AcrAB-TolC efflux pumps.

Methods: The broth microdilution method combined with the rapid para-iodonitrotetrazolium chloride (INT) colorimetric technique was used to determine the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) of ZJB alone, in the presence of an efflux pump inhibitor (EPI) phenylalanine-arginine *β*-naphthylamide (PAβN), or in the presence of antibiotics. The phytochemical screening of ZJB was evaluated using standard methods.

Results: ZJB displayed weak antibacterial activities with MIC values above 625 µg/mL in all the 14 tested *Klebsiella* species. In the presence of PAβN, the activity of ZJB increased by 4- to more than 128-fold on all the tested bacteria. At MIC/2 and MIC/4, ZJB 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. ZJB contains alkaloids, flavonoids, triterpenes, saponins, phenols, and anthocyanins.

Conclusion: This study has demonstrated that ZJB could be used as an antibacterial agent if it is combined with an efflux pump inhibitor or with antibiotics against MDR bacteria over-expressing active efflux pumps.

Keywords: Antibacterial activity; antibiotics; efflux pumps; *Klebsiella*; multidrug resistance; *Zizyphus jujuba*

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Background

Klebsiella pneumoniae is medically the most important species of this genus. *Klebsiella oxytoca* also causes infections in humans but to a much lesser degree than *K. pneumoniae* [1]. *K. pneumoniae* is involved in diseases such as community-acquired pneumonia where alcoholics constitute the main patient population at risk [2, 3], rhinoscleroma and ozena [4], and nosocomial infections, causing 8% of all hospital-acquired infections [1]. Hospital-acquired bacterial infections caused by *Klebsiella* spp. include urinary tract infections, pneumonia, septicemia, wound infections, nosocomial infections in intensive care unit patients, and neonatal septicemia [1]. High resistance of *Klebsiella* sp. including *K. pneumoniae* to ceftriaxone (CRO), gentamicin (GEN), chloramphenicol (CHL), ciprofloxacin (CIP), and doxycycline (DOX) has been reported significantly higher in human immunodeficiency virus (HIV) patients [5, 6]. *K. oxytoca* caused hospital-acquired infection in adults and has multidrug resistance to commonly used antibiotics such as imipenem (IMI), meropenem (MEM), GEN, amikacin (AMI), CRO, CIP, aztreonam (ATM) among others [7]. In diabetic patients, uropathogenic *K. pneumoniae* strains have also been very resistant to antibiotics such as CRO, cefixime (CFM), ceftazidime (CAZ), cefotaxime (CTX), cefepime (FEP), and CIP [8]. The World Health Organization (WHO) reveals that third-generation cephalosporin-resistant *K. pneumoniae* is associated with 77% of deaths in Africa [9]. Considering the potential of the plant kingdom as a source of bioactive products, botanicals should be deeply explored to discover novel drugs to combat bacterial infections, especially those expressing resistant phenotypes [10]. Several African medicinal plants have shown good efficacy against multidrug-resistant (MDR) strains of *Klebsiella* species. Some of these plants include *Eucalyptus robusta* [11], *Beilschmiedia obscura* [12], *Fagara tessmannii* [13], *Fagara macrophylla* [14], *Beilschmiedia acuta, Clausena anisata, Newbouldia laevis,* and *Polyscias fulva* [15], *Erythrina sigmoidea* [16], *Harungana madagascariensis* [17], *Adansonnia digitata* [18], *Capsicum frutescens* [19], *Dioscorea bulbifera* [20], *Allanblackia gabonensis* [21], *Fagara leprieuri* [22], and *Myristica fragrans* [23]. The present study was planned to evaluate the anti-Klebsiella activity of another African medicinal plant, *Zizyphus jujuba* Mill. (Rhamnaceae). *Ziziphus jujuba* is native to China and is commonly known as jujube, red date, Chinese date, and Chinese jujube. The plant has been traditionally used to treat various diseases such as respiratory system diseases (asthma, cough, and laryngitis), gastrointestinal problems (constipation, colitis, and liver diseases), and cardiovascular and genitourinary system diseases [24]. The fruit is recognized as an emollient and laxative; it can purify blood and improve blood circulation, relieve internal heat, and reduce inflammation [24].

Methods

Plant material and extraction

The bark of *Zizyphus jujuba* Mill. (Rhamnaceae) was harvested in Garoua, North Region of Cameroon, in December 2022. The plant was identified at the National Herbarium of Cameroon (HNC) under the identification number 34519/HNC. The bark was air-dried and then ground. The powder obtained was macerated in 95% methanol in the proportion 1/3 (m/v) 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 dried in an oven at 40°C for complete evaporation of the residual solvent. The obtained crude extract (botanical, ZJB) was stored 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 plant extract. 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 bacteria, 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 bacteria [25-27].

Bacterial species

The bacteria used included twelve *Klebsiella pneumoniae* (ATCC11296, KP80, KP55, KP24, KP58, K2, K22, KP96, KP46, KP63, KP44, and KP42) and two *Klebsiella oxytoca* (KO126 and KO107) strains or clinical isolates. Their bacterial features were reported earlier [27, 28].

Determination of minimal inhibitory and bactericidal concentrations

The minimal inhibitory concentrations (MIC) and the minimal bactericidal concentrations (MBC) of ZJB and antibiotics alone, in the presence of PAβN (EPI) were determined using the rapid INT colorimetric method as previously described in similar experimental conditions [19-21, 25, 29-37]. Each experiment was repeated three times in triplicate.

Determination of the antibiotic-potentiating effects of ZJB

The effects of the association of ZJB 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 combined microbroth dilution and the rapid INT colorimetric method as previously described in the similar experimental conditions [38, 39]. 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 [40].

Phytochemical screening of the botanical

The phytochemical screening of the ZJB was done following the standard methods described for alkaloids, anthocyanins, flavonoids, phenols, saponins, tannins, and triterpenes [41, 42].

Interpretation of antibacterial data

The general interpretation of the antibacterial activity of the botanical was performed according to the cutoff values established by Kuete [43] as follows: significant activity (MIC <100 µg/mL), moderate (100 <MIC ≤ 625 µg/mL), and weak (MIC> 625 µg/mL). For Enterobacteria such as Klebsiella species, 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) [44]. Bactericidal activities are considered when the ratios MBC/MIC are below or equal to 2; MBC/MIC ratios above 2 define the bacteriostatic activities [45-48].

Results

The crude extract from the bark of Zizyphus jujuba displays weak antibacterial effects

The MIC and MBC of ZJB are shown in Table 1. It appears that ZJB displayed weak antibacterial activities with MIC values above 625 µg/mL in all the 14 tested *Klebsiella* species. No MBC value was detected at up to 2048 µg/mL. In contrast, the MIC values of the reference antibiotic, IMI, varied from 4 to 8 µg/mL. This drug generally displayed bactericidal effects with the MBC/MIC ratio of 1 and 2 against most tested bacteria.

PAβN enhances the activity of the crude extract from the bark of Zizyphus jujuba

To check whether the bacterial efflux pumps are involved in weak effects observed with ZJB, the MICs of the botanical were determined in the presence of PAβN. The results are shown in Table 2. In the presence of PAβN, the activity of ZJB increased by 4- to more than 128-fold on 100% (11/11) of the tested bacteria. The highest increase of >128-fold was obtained when ZJB was combined with EPI against KP55, KP24, ATCC11296, KP96, KP63, and KP42. The increase in the antibacterial activities of ZJB in the presence of PAβN indicates that the constituents of the ZJB are substrates of the bacterial efflux pumps.

The crude extract from the bark of Zizyphus jujuba potentiated the activity of antibiotics

ZJB at MIC/2 and MIC/4 was combined with antibiotics and tested against eight *K. pneumoniae* and two *K. oxytoca* strains. The results are summarized in Table 3. It appears that the antibacterial activities of the antibiotics increased in the presence of ZJB at MIC/2 and MIC/4, with the AMF ranging from 2 to 128 in most of the cases. At MIC/2 and MIC/4, ZJB potentiated the activity of DOX, LEV, IMI, CIP, CRO, and TET against at least 80% of the MDR bacterial strains tested.

Phytochemical composition of the botanicals

The phytochemical assessment of ZJB revealed the presence of alkaloids, flavonoids, triterpenes, saponins, phenols, and anthocyanins.

Discussion

The screening of antibacterial agents from African medicinal plants has been very successful in the last two decades with a good panel of efficient botanicals and phytochemicals being documented against MDR as well as drug susceptible phenotypes [29, 31, 49-64]. The use of MDR bacteria and especially those expressing active efflux pumps in the search for novel drugs is an attractive strategy. In the present study, the MDR *Klebsiella* isolate used active over-expressed active efflux pumps of the resistance-nodulation cell division family (RND), AcrAB-TolC. The expression of AcrAB-TolC pumps could be evidenced by the increase in the antibacterial activity of IMI by 4- to more 16-fold in the presence of PAβN against most of the tested bacteria (Table 2). In effect, In effect, PAβN is a well-known inhibitor of AcrAB-TolC pumps in Enterobacteria [65]. The activity of ZJB also increased significantly in the presence of the PAβN (8- to >128-fold), clearly indicating that the antibacterial phytochemicals from ZJB are the substrate of the efflux pumps, and that the combination with an EPI will be necessary if the botanical is to be used to fight bacterial *Klebsiella* infections. However, the extract without an EPI could not be considered active [43, 44]. Botanicals inhibiting at least 70% of antibiotics against at least 70% of the tested bacteria over-expressing active efflux pumps should be considered as a potential EPI [66, 67]. In this study, ZJB at MIC/2 and MIC/5 potentiated the activity of 6/8 (75%) tested antibiotics (DOX, LEV, IMI, CIP, CRO, and TET) against at least 80% of the MDR bacterial strains tested (Table 3). ZJB could therefore be considered an EPI. Phytochemicals classes detected in the ZJB such as alkaloids, flavonoids, triterpenes, saponins, phenols, and anthocyanins contain several antibacterial compounds that could be responsible of the of observed inhibitory activities [68, 69].

Table 1. Antibacterial activities of crude methanol extract from the bark of *Zizyphus jujuba*.

Bacterial strains	**Tested samples, MIC and MBC (MIC and MBC in µg/mL), and their ratio**					
	Botanical			**ATB**		
	ZJB			**IMI**		
	MIC	MBC	R	MIC	MBC	R
Klebsiella pneumoniae						
ATCC11296	2048	-	nd	4	8	2
KP80	2048	-	nd	4	4	1
KP55	2048	-	nd	8	16	2
KP24	2048	-	nd	4	16	4
KP58	2048	-	nd	4	4	1
K2	512	-	nd	4	4	1
K22	2048	-	nd	4	8	2
KP96	2048	-	nd	4	4	1
KP46	2048	-	nd	4	4	1
KP63	2048	-	nd	4	4	1
KP44	2048	-	nd	8	8	1
KP42	2048	-	nd	4	16	4
Klebsiella oxytoca						
KO126	1024	-	nd	4	16	4
KO107	2048	-	nd	4	4	1

ZJB: crude methanol extract from the bark of *Zizyphus jujuba*; 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 extract from the bark of *Zizyphus jujuba* 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**		
	ZJB			**IMI**		
	MIC alone	**MIC+PAβN**	**R**	**MIC alone**	**MIC+PAβN**	R
Klebsiella pneumoniae						
KP55	2048	<16	**>128**	8	1/2	**16**
KP24	2048	<16	**>128**	4	1	**4**
KP58	2048	16	**128**	4	1	**4**
ATCC11296	2048	<16	**>128**	4	1	**4**
KP96	2048	<16	**>128**	4	1	**4**
KP46	2048	256	**8**	4	1/2	**8**
KP63	2048	<16	**>128**	4	<1/4	**>16**
KP44	2048	128	**16**	8	8	**1**
KP42	2048	<16	**>128**	4	1/2	**8**
Klebsiella oxytoca						
KO107	2048	256	**8**	4	1	**4**
KO126	1024	256	**4**	4	2	**2**

ZJB: crude methanol extract from the bark of *Zizyphus jujuba*; 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 in the absence and presence of the crude extract from the bark of *Zizyphus jujuba*.

ATB	**Extract concentration**	MIC of antibiotics in the presence of extract and Antibiotic-resistance modulating factor (AMF)										PSP (%)
		K. pneumoniae								***K. oxytoca***		
		KP24	**ATCC11296**	**KP46**	**KP44**	**KP42**	**KP63**	**KP58**	KP126	**KO107**	KO96	
DOX	0	1/8	4	8	1/2	1/2	8	1/4	4	1/2	8	
	MIC/2	< 1/16(2)	< 1/16(64)	1/16(128)	1/8(4)	1/8(4)	<1/16(128)	1/16(4)	1/4(2)	1/4(2)	1/4(32)	100%
	MIC/4	1/8(1)	1/8(32)	1/8(64)	1/(4)	1/8(4)	1/8(64)	1/16(4)	1/4 (2)	1/4(2)	1/8(64)	90%
LEV	0	1	1	4	1/4	4	8	1/2	1	1/4	1/2	
	MIC/2	1/2(2)	1/8(8)	1/16(64)	1/8(2)	1/16(64)	1/2(16)	1/8(4)	1/16(16)	1/8(2)	1/16(8)	100%
	MIC/4	1/2(2)	1/8(8)	1/16(64)	1/8(2)	1/16(64)	4(2)	1/4(2)	1/4(4)	1/8(2)	1/4(2)	100%
IMI	0	4	4	4	8	4	4	4	4	4	4	
	MIC/2	1/2(8)	1/2(8)	2(2)	4(2)	2(2)	1(4)	1/2(8)	1/8(32)	1(4)	< 1/16(64)	100%
	MIC/4	4(2)	2(4)	1/2(4)	1(2)	1(1)	2(4)	1/4(8)	1/4(16)	1/2(2)	1(8)	90%
CIP	0	1	1	2	1/8	4	4	1/2	2	1/4	2	
	MIC/2	1/2(2)	1/2(2)	1(2)	< 1/16(2)	< 1/16(64)	1/2(8)	1/8(4)	1/16(32)	1/8(2)	1/16(32)	100%
	MIC/4	1/2(2)	1/4(4)	1(2)	< 1/16(2)	< 1/16(64)	1 (4)	1/8(4)	1/16(32)	1/8(2)	1/16(32)	100%
AMP	0	>64	>64	>64	>64	>64	>64	256	>64	>64	256	
	MIC/2	>64(1)	>64(1)	>64(1)	>64(1)	>64(1)	>64(1)	32(8)	>64(1)	>64(1)	16(16)	20%
	MIC/4	>64(1)	>64(1)	>64(1)	>64(1)	>64(1)	>64(1)	32(8)	>64(1)	>64(1)	32(8)	20%
CRO	0	16	8	8	16	8	32	16	64	8	16	
	MIC/2	4(4)	2(4)	<1/2(16)	8(2)	<1/2(16)	<1/2(64)	1/2(32)	1(64)	<1/2(16)	1/2(32)	100%
	MIC/4	4(4)	4(2)	1(8)	4(4)	1(8)	<1/2(64)	1(16)	1(64)	1(8)	8(2)	100%
TET	0	2	1	8	8	1	1	1	1	2	8	
	MIC/2	2(1)	< 1/16(16)	8(1)	1(8)	1/8(8)	< 1/16(16)	1/16(16)	1/2(2)	1(2)	< 1/16(128)	80%
	MIC/4	2(1)	1/16(16)	8(1)	1(8)	1/8(8)	1/8(8)	1/8(8)	1/2(2)	1(2)	1/4(32)	80%
VAN	0	2	2	>64	1	>64	4	32	32	1	32	
	MIC/2	2(1)	2(1)	>64(1)	<1/2(2)	>64(1)	1/2(8)	32(1)	1/2(64)	<1/2(2)	1/2(64)	50%
	MIC/4	2(1)	2(1)	>64(1)	<1/2(2)	>64(1)	1(4)	64(0,5)	1/2(64)	<1/2(2)	1(32)	50%

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

This work has evidenced the weak antibacterial activity of the botanical from *Zizyphus jujuba*. The study has also shown that the crude extract from the bark of this plant could be used if it is combined with an efflux pump inhibitor or with antibiotics to tackle MDR bacteria over-expressing active efflux pumps. This plant deserved further investigations to identify its active constituents and to establish their safety.

Abbreviations

AMP: ampicillin
ATCC: American-Type Culture Collection
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
RND: resistance-nodulation cell division
TET: tetracycline
VAN: vancomycin
ZJB: methanol extract from the bark of *Zizyphus jujuba*

Authors' Contribution

GKF, GB, VYM, SMT, RN, 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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