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Antimicrobial resistance of Gram-negative bacilli isolates from inpatients and outpatients at Yaounde Central Hospital, Cameroon

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Summary *Objective:* To determine and compare antimicrobial susceptibility patterns of pathogenic bacteria from inpatients and outpatients at a university teaching hospital in Yaounde, Cameroon.

Methods: Gram-negative bacilli isolates ($n = 522$), obtained from a wide range of clinical specimens (urine, pus and blood) from inpatients and outpatients at Yaounde Central Hospital between March 1995 and April 1998, were evaluated for resistance to antibiotics (amoxicillin, amoxicillin/clavulanate, piperacillin, cefazolin, cefoxitin, cefotaxime, ceftazidime, aztreonam, imipenem, gentamicin, tobramicin, ofloxacin and trimethoprim/sulfamethoxazole).

Results: Of the 522 isolates recorded, 80.3% were Enterobacteriaceae. A high incidence of resistance to amoxicillin (85%), piperacillin (75%) and trimethoprim/sulfamethoxazole (71%) was observed. The proportion of antimicrobial-resistant isolates from inpatients was significantly higher than that from outpatients ($P < 0.05$), except for piperacillin, tobramicin and trimethoprim/sulfamethoxazole. The combinations of antimicrobial and organism showed that the percentage of ceftazidime-resistant *Pseudomonas aeruginosa* and ceftazidime-resistant *Enterobacter cloacae* were 26.8% and 24% respectively. The rate of antimicrobial resistance in isolates from inpatients was not significantly higher than that in isolates from outpatients for all the antimicrobial/organism combinations, except for ceftazidime-resistant *Escherichia coli*, which was exclusively found in isolates from inpatients. Among Enterobacteriaceae, high and low level penicillinase (mostly in *E. coli* (13.6% and 11% respectively) and *Klebsiella* spp. (9% and 8% respectively) were the most important β-lactam resistance phenotypes (31.2% and 23.6%, respectively). Wild type (exclusively observed in *E. coli*, *Proteus mirabilis* and *Salmonella* spp.) and low level penicillinase

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were higher in outpatient than inpatient isolates (wild type—17.9% vs 10.8% and low level penicillinase—29.4% vs 20.5%, respectively; $P < 0.05$). However, extended spectrum β-lactamase strains (*Klebsiella* spp. (3.5%), *E. coli* (2.6%), *Citrobacter* spp. (0.7%), *Enterobacter* spp. (0.4%) and *P. mirabilis* (0.2%)) were exclusively recovered from inpatients. Penicillinase and high level cephalosporinase resistance phenotypes were frequently observed in non-fermenter Gram-negative bacilli (46.6% and 29.1% respectively). However, there were no significant differences in penicillinase and cephalosporinase resistance between inpatient and outpatient isolates.

Conclusion: As the incidence of antimicrobial resistance is substantially higher in isolates from inpatient than outpatient pathogens, more resources should be allocated within the hospital to encourage good antibiotic practices and good hospital hygiene.

Introduction

Antimicrobial-resistant bacteria are the cause of numerous clinical problems worldwide. The development and the increase of antimicrobial resistance among microbial pathogens causing nosocomial and community-acquired infections is known to be associated with the level of antibiotic use.[1] Most studies have found a higher prevalence of antimicrobial resistance in hospitals than in the community.[2–4] The strategy for the control of antimicrobial resistance lies mainly in the implementation of effective infection control measures and antibiotic auditing. For these measures, it is important to have data on the identification and resistance patterns of clinical bacteria, and to know the magnitude of antimicrobial resistance in hospitals compared with that in the community. These data may help to establish preventive and therapeutic guidelines for clinicians when appropriate.[5,6]

This study was designated to evaluate and to compare the resistance of Gram-negative bacilli causing nosocomial and community-acquired infections in Yaounde Central Hospital, Cameroon.

Materials and methods

Study design and patients

To monitor the antimicrobial resistance patterns of nosocomial and community-acquired infections, relevant data from Yaounde Central Hospital (YCH) concerning Gram-negative bacilli isolated from inpatients and outpatients were analysed. The study was carried out prospectively between March 1995 and April 1998. YCH is a university teaching hospital with about 500 beds containing several departments, including: medicine, surgery, intensive care, obstetrics/gynaecology, paediatrics and emergency. Patients included in this study were registered on a study investigation form with details of name, age, sex, hospital department, date of admission, diagnosis on admission, antibiotic prophylaxis or treatment and clinical specimens. This form was completed with the results of laboratory diagnosis and data of antimicrobial susceptibility tests. After laboratory diagnosis, we excluded patients without Gram-negative bacilli.

Bacterial isolates

A total of 522 Gram-negative bacilli isolates isolated between 1995 and 1998 were obtained from a wide range of clinical specimens including urine, pus and blood from inpatients and outpatients. Only one isolate per patient was studied. Organisms were identified by conventional methods[7] and confirmed by API 20E (bioMérieux France). Nosocomial infections were diagnosed on the basis of clinical and laboratory data. Isolates recovered from patients at least 48 hours after admission were considered nosocomial. Community-acquired infections were diagnosed on admission and from ambulatory care. The isolates studied included *Escherichia coli*, *Klebsiella pneumoniae*, *K. oxytoca*, *Proteus mirabilis*, indole positive *Proteus* spp., *Providencia* spp., *Salmonella* spp., *Shigella* spp., *Pseudomonas* spp., *Acinetobacter baumannii* and *Flavobacterium meningosepticum*.

Susceptibility testing

Antimicrobial susceptibilities were determined by Kirby—Bauer disk diffusion following the definition of National Committee of Clinical Laboratory Standards (NCCLS) for agar diffusion tests.[8] The antibiotics tested were amoxicillin, amoxicillin/clavulanate, piperacillin, imipenem, cefazolin, cefoxitin, cefotaxime, ceftazidime, gentamicin, tobramycin, ofloxacin, and trimethoprim/sulfamethoxazole.

E. coli American Type Culture Collection (ATCC) 25922 and *P. aeruginosa* ATCC 27853 were tested along with the isolates for quality control purposes. Test results were only accepted when inhibition zone diameters of the control strains were within performance ranges.[8] The data were stratified by all inpatients and all outpatients.

Antimicrobial/organism combinations and β-lactam resistance phenotype

We studied the following antimicrobial/organism combinations, taking into account their current and potential clinical importance based on the results of antimicrobial susceptibility tests. These combinations included: ceftazidime/*E. coli*; ceftazidime/*Klebsiella* spp.; ceftazidime/*Enterobacter cloacae*; ceftazidime/*Pseudomonas aeruginosa*; imipenem/*P. aeruginosa*.

The β-lactam resistance phenotypes were determined using the results of antimicrobial susceptibility tests.[9,10] Resistance phenotypes were defined as follows:

For Enterobacteriaceae:

- Wild type: strain susceptible to all β-lactams used.
- Penicillinase phenotype: strains resistant to amoxicillin and piperacillin and moderately resistant to cefazolin.
- High level penicillinase: strains resistant to amoxicillin, amoxicillin/clavulanate, piperacillin and cefazolin.
- Inhibitor-resistant TEM phenotype: strains resistant to amoxicillin, amoxicillin/clavulanate and piperacillin.
- Low level cephalosporinase: strains resistant to amoxicillin, amoxicillin/clavulanate, cefazolin, and cefoxitin.
- High level cephalosporinase: strains resistant to amoxicillin, amoxicillin/clavulanate, piperacillin, cefazolin, cefoxitin, moderately resistant to cefotaxime, ceftazidime and aztreonam.
- Extended spectrum β-lactamase: strains resistant to amoxicillin, amoxicillin/clavulanate, piperacillin, cefazolin, cefotaxime, ceftazidime and aztreonam. We confirmed this phenotype by the double disk synergy test.[11]

For non-fermenter Gram-negative bacilli:

- Penicillinase: strains resistant or moderately resistant to piperacillin, cefotaxime and aztreonam.
- High level cephalosporinase: strains resistant to piperacillin, cefotaxime, aztreonam and ceftazidime.
- Low level cephalosporinase: strains resistant to cefotaxime.

As with the data of susceptibility tests, the data of β-lactam resistance phenotype were stratified by all inpatients and all outpatients. The β-lactam resistance phenotype of organisms from hospitalised patients was compared with that of isolates from outpatients.

Statistics

Data were analysed using Whonet 4 (World Health Organisation, Geneva, Switzerland) and Epi Info version 6.04c (Centers for Disease Control and Prevention, Atlanta, GA, USA). The Mantel-Haenszel chi-squared test was used and the 2-tailed Fisher's exact test was used when an expected value was less than 5. A P value of less than 0.05 was considered significant.

Results

Enterobacteriaceae constituted 80.3% of the 522 isolates. The distribution of bacterial species by clinical specimens and origin is presented in Table 1. *E. coli* was the predominant pathogen isolated from both inpatients and outpatients, representing 25.9% and 43.4% of all isolates, respectively. The majority of isolates (67.4%) were nosocomial. From inpatients, pathogens were mostly isolated from pus (50.9%) while the outpatient strains were mostly from urine (56.5%).

The results of susceptibility to antibiotics of different strains showed that the highest rates of resistance (resistant plus intermediate) were to amoxicillin (85%), piperacillin (73%) and trimethoprim/sulfamethoxazole (71%) (Table 2).

Except for piperacillin, tobramicin and trimethoprim/sulfamethoxazole, the occurrence of resistance in isolates from inpatients was significantly higher than that in isolates from outpatients; for trimethoprim/sulfamethoxazole, the occurrence of resistance in isolates from outpatients was higher than that in isolates from inpatients (Table 3).

In all, 419 isolates of Enterobacteriaceae were collected (80.3%). The incidence of resistance to amoxicillin, amoxicillin/clavulanate, piperacillin, cefazolin and trimethoprim/sulfamethoxazole were 85%, 62%, 73%, 69% and 71% respectively (Table 2). More than 85% were susceptible to third

Table 1 Distribution (%) of bacterial species by clinical specimen and origin.

Bacterial species ($n = 522$) (%)	Inpatient $n = 352$ (67.4)	Outpatient $n = 170$ (32.6)	Clinical specimens					
			Urine		Pus		Blood	
			Inpatient $n = 145$ (41.2)	Outpatient $n = 96$ (56.5)	Inpatient $n = 179$ (50.9)	Outpatient $n = 68$ (40.0)	Inpatient $n = 28$ (8.0)	Outpatient $n = 6$ (3.5)
Escherichia coli	25.9	43.4	37.9	53.1	19.0	30.9	7.1	0.0
Klebsiella spp.	18.2	19.1	18.6	16.7	15.6	20.6	32.1	50.0
Proteus spp.	15.9	12.7	14.5	12.5	19.0	14.7	3.6	0.0
Enterobacter spp.	8.8	5.2	8.3	5.2	8.9	4.4	10.7	16.7
Citrobacter spp.	4.8	5.2	6.2	4.2	4.5	7.4	0.0	0.0
Pseudomonas spp.	17.9	8.7	9.0	3.1	25.7	14.7	14.3	33.3
Acinetobacter baumannii	5.7	2.3	2.8	2.1	6.1	2.9	17.9	0.0
Others[a]	2.8	3.5	2.8	3.0	1.2	4.4	14.3	0.0

n: number of strains.
[a] *Serratia* spp., *Salmonella* spp., *Shigella* spp. and *Flavobacterium meningosepticum*.

Table 2 Antimicrobial susceptibility (%) of 522 strains of Gram-negative bacilli.

Antibiotics	Breakpoints	Number of isolates tested	Susceptibility (%)		
			Resistant	Intermediate	Susceptible
Amoxicillin[a]	14–17	419	83	2	15
Amoxicillin/clavulanate[a]	14–17	419	30	32	38
Piperacillin	18–20	522	65	8	27
Cefazolin[a]	15–17	419	53	16	31
Cefoxitin[a]	15–17	419	20	6	74
Cefotaxime	15–22	522	15	18	67
Ceftazidime	15–17	522	9	5	86
Aztreonam	16–21	522	16	11	73
Imipenem	14–15	522	1	1	98
Gentamicin	13–14	522	30	3	67
Tobramycin[b]	13–14	96	42	2	56
Ofloxacin	13–15	522	9	2	89
Trimethoprim/sulfamethoxazole[a]	11–15	419	69	2	29

[a] Only tested against Enterobacteriaceae.
[b] Only tested against non-fermenter Gram-negative bacilli.

generation cephalosporins, ofloxacin and imipenem. As for Gram-negative bacilli, the inpatients' isolates presented a significantly higher rate of antimicrobial resistance than the outpatients' isolates, except for imipenem and trimethoprim/sulfamethoxazole (1.9% vs 0%, 70.9% vs 72.8%, respectively). For non-fermenter Gram-negative bacilli, a total of 103 isolates were collected (19.7%). High rates of resistance were observed to cefotaxime, aztreonam and piperacillin (95%, 77% and 64%, respectively). A high proportion of isolates were susceptible to imipenem (95%) whereas only 70% were susceptible to ofloxacin and 63% to ceftazidime. However, the proportion of antimicrobial resistance in isolates from inpatients was not significantly higher than that in isolates from outpatients except cefotaxime (96.4% vs 10.5%, respectively, $p < 0.001$).

Many phenotypes of resistance to β-lactams (wild type, low level penicillinase, high level penicillinase, inhibitor-resistant TEM, low level cephalosporinase, high level cephalosporinase and extended spectrum β-lactamase) were identified. For Enterobacteriaceae, high and low

Table 3 Antimicrobial resistance in Gram-negative bacilli isolates from inpatients vs outpatients.

Antibiotics	Number of resistant isolates/total of number of isolates tested (%)		P value
	Inpatients	Outpatients	
Amoxicillin	239/268 (89.2)	120/151 (79.5)	<0.01
Amoxicillin/clavulanate	182/268 (67.9)	79/151 (52.3)	<0.01
Piperacillin	264/352 (75.0)	116/170 (68.2)	0.10[a]
Cefazolin	201/268 (75.0)	88/151 (58.2)	<0.01
Cefoxitin	86/268 (32.1)	26/151 (17.2)	<0.01
Cefotaxime	141/352 (40.0)	32/170 (18.8)	<0.01
Ceftazidime	63/352 (17.9)	14/170 (8.2)	<0.01
Aztreonam	120/352 (34.1)	21/170 (12.3)	<0.01
Imipenem	11/352 (3.1)	0/170 (0.0)	0.01
Gentamicin	137/352 (38.9)	32/170 (18.8)	<0.01
Tobramycin	36/77 (46.7)	5/19 (26.3)	0.10[a]
Ofloxacin	53/352 (15.0)	7/170 (4.1)	<0.01
Trimethoprim/sulfamethoxazole	190/268 (70.9)	110/151 (72.8)	0.67[a]

[a] Not significant.

Table 4 Resistance to specific antimicrobials in isolates from inpatients vs outpatients for antimicrobial/pathogen combinations.

Antimicrobial/pathogen combination	Number of resistant isolates/total of number of isolates tested (%)		P value
	Inpatients	Outpatients	
Ceftazidime/*Escherichia coli*	9/91 (9.9)	0/72 (0.0)	<0.01
Ceftazidime/*Klebsiella pneumoniae*	9/64 (14.1)	2/30 (6.7)	0.92[a]
Ceftazidime/*Enterobacter cloacae*	5/22 (22.7)	1/3 (33.3)	0.58[a]
Ceftazidime/*Pseudomonas aeruginosa*	16/56 (28.6)	2/11 (18.2)	0.73[a]
Imipenem/*Pseudomonas aeruginosa*	4/56 (7.1)	0/11 (0.0)	0.82[a]

[a] Not significant.

level penicillinase, mostly observed in *E. coli* (13.6% and 11%), and *Klebsiella* spp. (9% and 8%) were the most important resistance phenotypes (31.2% and 23.6% respectively). Wild-type (only observed in *E. coli* (6%), *P. mirabilis* (6%) and *Salmonella* spp. (1%)), and low level penicillinase phenotypes were recovered in significantly higher numbers from outpatients' than from inpatients' isolates (wild type—17.9% vs 10.8% and low level penicillinase 29.1% vs 20.5%, respectively $p < 0.05$). However, extended spectrum β-lactamase-producing strains (*Klebsiella* spp. (3.5%), *E. coli* (2.6%), *Citrobacter* spp. (0.7%), *Enterobacter* spp (0.4%) and *P. mirabilis* (0.2%)) were exclusively recovered from inpatients (11.9%, $p < 0.001$).

For non-fermenter Gram-negative bacilli, penicillinase (*Pseudomonas* spp. (37.8%), *Acinetobacter baumannii* (8.7%)) and high level cephalosporinase (*Pseudomonas* spp. (15.5%) and *A. baumannii* (13.6%)) were the most frequent resistance phenotypes (46.6% and 29.1% respectively). Low level cephalosporinase (observed in *Pseudomonas* spp. — 18.4% of isolates) was more frequently observed in outpatients' than inpatients' isolates (26.3% vs 17.8%) as was high level cephalosporinase (31.6% vs 28.6%) while penicillinase was more frequently found in isolates from inpatients than outpatients (48.8% vs 42.1%). However, the difference between these isolates was not significant.

For antimicrobial/organism combinations the proportion of ceftazidime-resistant *P. aeruginosa* and ceftazidime-resistant *E. cloacae* were 26.8% and 24% respectively. The proportion of antimicrobial resistance in isolates from inpatients was not significantly higher than that in isolates from outpatients for all of the antimicrobial/organism combinations except for ceftazidime/*E. coli*. The imipenem-resistant *P. aeruginosa* and ceftazidime-resistant *E. coli* isolates were observed exclusively in isolates from inpatients (Table 4).

Discussion

The study provides insights into the problem of resistance in bacterial Gram-negative enteric pathogens in inpatients and outpatients in YCH, Cameroon. To our knowledge this is the first study in Cameroon determining antimicrobial susceptibility patterns of nosocomial and community-acquired Gram-negative bacilli. Results have demonstrated that in general, Gram-negative bacilli have high rates of resistance to the commonly used antibiotics. A high incidence of resistance was expressed by all bacilli to the penicillins, first generation cephalosporins and trimethoprim/sulfamethoxazole. Many multi-resistant strains were detected. The rates of antimicrobial resistance reported in this study were much higher than those published in developed countries but are similar to those reported in other developing countries.[9,12–17]

Many factors have contributed to such high rates of resistance, including misuse of antibiotics by health professionals, unskilled practitioners and laypersons, misuse of antibiotics by the public (antibiotics can be purchased without prescription), poor drug quality, unhygienic conditions accounting for the spread of resistant bacteria, and inadequate surveillance (lack of information from routine antimicrobial susceptibility testing of bacterial isolates and surveillance testing of bacterial isolates and surveillance of antibiotic resistance, all of which are crucial for good clinical practice and for rational policies against antibiotic resistance).[18]

Our analysis suggests that the rate of resistance in nosocomial pathogens to a variety of antimicrobials, such as amoxicillin, amoxicillin/clavulanate, cefazolin, cefoxitin, cefotaxime and gentamicin, commonly used to treat nosocomial infections, is significantly higher in the hospital setting than the outpatient setting. The high rate of antimicrobial resistance in pathogens isolated in the hospital could possibly be explained by the selective

effect of treatment with multiple antimicrobials for a single patient, which may result in the amplification of antimicrobial resistance in some organisms.[2,19]

This study has demonstrated that the hospital is the focus of antimicrobial resistance. However, there were exceptions. The increasing percentage of trimethoprim/sulfamethozaxole-resistant Enterobacteriaceae; ceftazidime-resistant non-fermenter Gram-negative bacilli; inhibitor-resistant TEM phenotype-resistant Enterobacteriaceae; high level cephalosporinase phenotype-resistant non-fermenter Gram-negative bacilli, and ceftazidime-resistant *E. cloacae* among isolates from outpatients suggest that some form of selection effect on these isolates exists for the outpatients of this hospital.[2]

Some authors have observed in the hospital settings a strong correlation between ceftazidime usage and occurrence of ceftazidime-resistant *P. aeruginosa* and *E. cloacae* isolates.[20–24] In this study, the incidence of ceftazidime-resistant *P. aeruginosa* and *E. clocae* isolates was high. However, information about ceftazidime usage in YCH was not available.

The combination of trimethoprim-sulfamethoxazole is extensively used in Africa owing to its antimicrobial spectrum of activity and its low cost. In addition, resistant bacteria have been isolated from the stool flora of infants who have never been exposed to antibiotics, in contrast to the situation in well developed countries.[25] This reflects the role of poor sanitation in the emergence and dissemination of resistant strains.

The results confirm previously reported findings about the occurrence of high rates of resistance of Gram-negative bacilli to β-lactam antibiotics and trimethoprim/sulfamethoxazole in developing countries, which are much higher than those observed in developed countries. As the incidence of antimicrobial resistance is higher in inpatients' than outpatients' pathogens and because infection control measures may be difficult to implement, more resources should be allocated in the Cameroon to stem possible problems within hospitals, including good antibiotic practices and improvements in hygiene.

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Emergence of CTX-M-15-Producing Enterobacteria in Cameroon and Characterization of a *bla*$_{\text{CTX-M-15}}$-Carrying Element

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CTX-M-15-producing *Klebsiella pneumoniae* and *Escherichia coli* emerged recently in Cameroon. CTX-M-15 was encoded by two different multiresistance plasmids, of which one carried an IS*Ecp1*-*bla*$_{\text{CTX-M-15}}$ element flanked by a 5-bp target site duplication and inserted within a Tn*2*-derived sequence. A truncated form of this element in the second plasmid was identified.

Extended-spectrum β-lactamase (ESBL)-positive enterobacteria are frequently isolated in hospitals in Cameroon. Up to 1999, SHV-12 and SHV-2a were the dominant ESBLs (J. Gangoue-Pieboji, B. Bedenic, S. Koula-Shiro, et al., Program Abstr. 9th Int. Congr. Infect. Dis., abstr. 15419, 2000). In a PCR-based screening for *bla* types applied to enterobacteria collected during July and August 2002 in Yaounde Central Hospital, it was found that 14 out of 17 ESBL-positive isolates produced SHV ESBLs, confirming previous findings. The remaining isolates (one *Klebsiella pneumoniae* isolate and two *Escherichia coli* isolates), however, were *bla*$_{\text{CTX-M}}$ positive. CTX-M is a rapidly growing family of ESBLs that preferentially hydrolyze cefotaxime. The *bla*$_{\text{CTX-M}}$ genes are commonly found in plasmids carried by enterobacteria. CTX-M ESBLs have been reported worldwide, the highest prevalence being observed in Latin America, Eastern Europe, and the Far East (3, 16). We report here on the emergence of CTX-M producers also in Cameroon.

The three clinical isolates studied (*K. pneumoniae* YC-17 and *E. coli* YC-5b and YC-14) had been derived from patients with urinary tract infection acquired during hospitalization. The isolates were resistant to amoxicillin, amoxicillin-clavulanate, piperacillin, cefotaxime, ceftazidime, cefepime, and aztreonam, as determined by the agar dilution method. Activity of cefotaxime and ceftazidime was restored by clavulanic acid. MICs of piperacillin-tazobactam, cefoxitin, and imipenem were within the susceptibility range. Isolates were also resistant to various non-β-lactam antibiotics by a disk diffusion assay (Table 1).

β-Lactamases were extracted by ultrasonic treatment and characterized by isoelectric focusing. Isolates produced β-lactamases with apparent isoelectric points (pIs) equal to 7.3 and 8.8. *E. coli* YC-5b produced an additional β-lactamase focusing at 5.4. Isolates were positive in a PCR specific for *bla*$_{\text{CTX-M-3}}$-related genes (6). Sequencing the PCR products showed 100% homology with *bla*$_{\text{CTX-M-15}}$ (accession no. AY044436) (6). CTX-M-15 corresponded to the β-lactamase with a pI of 8.8. Also, by PCR with *bla*$_{\text{TEM}}$- and *bla*$_{\text{OXA}}$-specific primers (1, 15) and the sequencing of the amplicons the β-lactamases with pIs of 7.3 and 5.4 were identified as OXA-30 and TEM-1. Therefore, oxyimino-β-lactam resistance was mainly due to CTX-M-15.

In conjugation experiments performed in liquid media *E. coli* YC-14 and *K. pneumoniae* YC-17 transferred resistance to oxyimino-β-lactams and aminoglycosides to an *E. coli* K-12 host (Table 1). Plasmid analysis indicated transfer of 90-kb plasmids that produced similar PstI restriction patterns. Additionally, in both preparations, PstI fragments equal in size (5.3 kb) hybridized with a digoxigenin-labeled *bla*$_{\text{CTX-M-15}}$ probe, suggesting spread of a single plasmid (pYC-14). *E. coli* YC-5b harbored a 50-kb plasmid (pYC-5b) that was used to transform *E. coli* DH5α. Transformants exhibited the resistance phenotype of *E. coli* YC-5b (Table 1). The PstI-generated restriction pattern of pYC-5b was different from that of pYC-14. Hybridization of the *bla*$_{\text{CTX-M-15}}$ probe occurred on a 3.4-kb PstI fragment of pYC-5b. Isoelectric focusing and PCR experiments showed that pYC-5b and pYC-14 coded also for the penicillinases produced by the respective clinical isolates.

Plasmids pYC-5b and pYC-14 were partially digested with Sau3A, and the fragments were ligated into pBCSK(+) (Stratagene). Recombinant plasmids were used to transform *E. coli* DH5a. Selection was performed in media containing either cefotaxime or ampicillin. Colony hybridization with a *bla*$_{\text{CTX-M}}$ probe was also applied to facilitate selection. Nucleotide sequences of overlapping fragments were determined with an ABI 377 sequencer (Applied Biosystems).

In pYC-5b, an IS*EcpI* insertion sequence, comprising an intact *tnpA* gene and two 30-bp imperfect inverted repeats (IRL and IRR) characteristic of this element (accession no. AJ242809) (9), was located 48 bp upstream of *bla*$_{\text{CTX-M-15}}$. The promoter driving *bla*$_{\text{CTX-M}}$ transcription was identified within the 3′ noncoding sequence of IS*EcpI* (13). An 18-bp sequence corresponding to the external part of IRR of IS*EcpI* (putative IRR) was found 373 bp downstream of *bla*$_{\text{CTX-M-15}}$. The intervening 373-bp sequence had 55% homology with the respective chromosomal region of *Kluyvera cryocrescens* (from nucleotide [nt] 3304 to 3677 in the sequence with accession no. AY026417) (4). The IS*EcpI*-*bla*$_{\text{CTX-M-15}}$-containing sequence was flanked by 5-bp direct repeats and inserted within *tnpA* (*tnpA*Δ*1*, 214 nt from the 5′ end; *tnpA*Δ*2*, 2,246 nt) of a Tn*2*-

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TABLE 1. Antibiotic susceptibility of CTX-M-15-producing strains

Strain	MICs (μg/ml) of[a]:												Other resistance markers[b]
	AMX	AMC	PIP	TZP	FOX	CTX	CTX+	CAZ	CAZ+	ATM	FEP	IMI	
E. coli YC-14	≥256	32	≥256	32	8	≥256	0.5	32	1	64	64	0.25	Gm, Tb, Sul, Tmp, Cm
E. coli K-12(pYC-14)	≥256	32	≥256	16	8	≥256	0.25	32	0.5	32	32	0.12	Gm, Tb
K. pneumoniae YC-17	≥256	64	≥256	32	16	≥256	1	128	2	128	128	0.5	Gm, Tb, Sul, Tmp, Cm
E. coli K-12(pYC-17)	≥256	32	≥256	16	8	≥256	0.25	32	0.5	32	32	0.12	Gm, Tb
E. coli YC-5b	≥256	32	≥256	32	16	≥256	0.5	64	1	64	64	0.12	Gm, Tb, Sul, Tmp
E. coli DH5α(pYC-5b)	≥256	32	≥256	8	4	≥256	0.12	32	0.5	32	16	≤0.06	Gm, Tb, Sul, Tmp
E. coli K-12	4	2	1	1	4	≤0.06	—[c]	0.25	—	≤0.06	≤0.06	≤0.06	
E. coli DH5α	2	2	1	1	4	≤0.06	—	0.12	—	≤0.06	≤0.06	≤0.06	

[a] AMX, amoxicillin; AMC, amoxicillin-clavulanic acid (2:1); PIP, piperacillin; TZP, piperacillin plus tazobactam (4 μg/ml); Fox, cefoxitin; Ctx, cefotaxime; CTX+, cefotaxime plus clavulanic acid (4 μg/ml); CAZ, ceftazidime; CAZ+, ceftazidime plus clavulanic acid (4 μg/ml); ATM, aztreonam; IMI, imipenem.
[b] Gm, gentamicin; Tb, tobramycin; Sul, sulfonamides; Tmp, trimethoprim; Cm, chloramphenicol.
[c] —, not done.

derived sequence. The latter also contained part of the respective *tnpR* (*tnpR*Δ; 173 nt from the 5′ end) and was flanked by directly repeated IS*26* elements (Fig. 1A). The truncated forms of transposase and resolvase of Tn*2* were, most likely, not functional. A homologous segment, extending from the 3′ end of the *tnpA* gene of IS*Ecp1* up to the IS*26* of the right end, was carried by the self-transferable plasmid pYC-14. This sequence was preceded by IS*26* (Fig. 1B).

Since its first description in 2001, CTX-M-15 has been identified in multiple locations in Asia and Europe (2, 5–8, 10–12, 17). This study documents for the first time the emergence of CTX-M-15-producing enterobacteria in an African country. CTX-M-15 differs from CTX-M-3 by an Asp-240→Gly substitution that increases activity against ceftazidime (14). The enhanced substrate spectrum of CTX-M-15 is probably a factor contributing to its spread.

IS*Ecp1*-like sequences have been associated with various $bla_{\text{CTX-M}}$ genes of the three major evolutionary groups (3). The presence of a 5-bp duplication at the boundaries of the IS*Ecp1*-$bla_{\text{CTX-M-15}}$ element and the resemblance of its right end to the IRR of IS*Ecp* are indicative of transposition. Similar sequence characteristics in the recently described IS*Ecp1B*-$bla_{\text{CTX-M-19}}$ element led to the hypothesis that IS*Ecp1* mediates a regular transposition process (13). However, the putative IRRs of these elements had less than 60% homology with the corresponding region of IRR and also differ from each other by 9 nt (50% homology). Therefore, the possibility for a one-ended transposition mechanism cannot be definitely excluded (P. D. Stapleton, Abstr. 39th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1457, 1999).

Geographical and temporal clusters of identical $bla_{\text{CTX-M}}$ genes carried by apparently different plasmids have also been reported in previous studies (reviewed in reference 3). Notably, the sequence homology of the CTX-M-encoding loci in pYC-5b and pYC-14 extends beyond IS*EcpI*-$bla_{\text{CTX-M-15}}$, including parts of the Tn*2* flanking segments. Recently, Lartigue et al. described plasmids carrying IS*Ecp1*-$bla_{\text{CTX-M-15}}$ elements inserted within *tnpA* of a Tn*2*-like transposon harbored by *E. coli* isolates from France and India (8). Furthermore, a GenBank search revealed a plasmid from *E. coli* isolated in Canada (pC15-1a) that also contained a Tn*2*-inserted IS*Ecp1*-$bla_{\text{CTX-M-15}}$ (from nt 17077 to 23482 in the sequence with accession no.

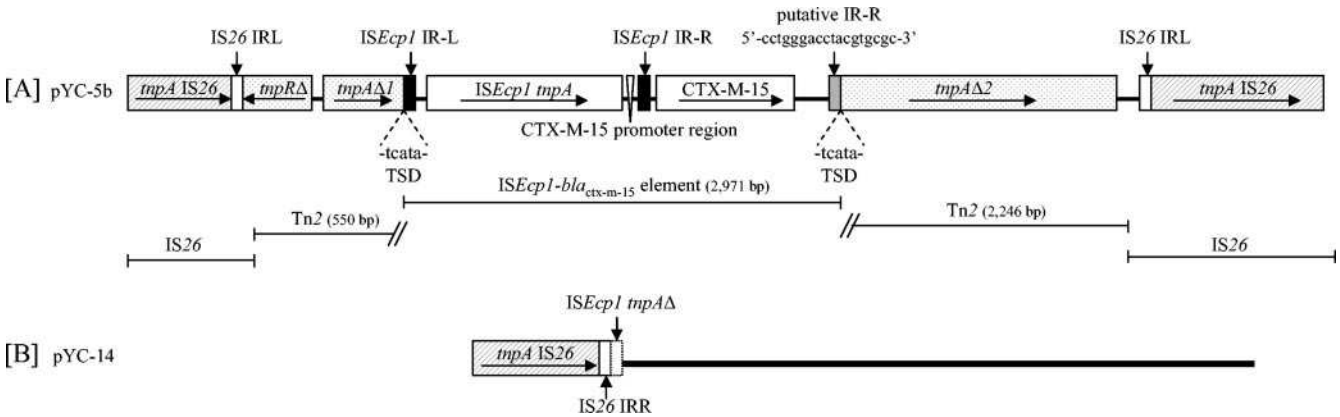

FIG. 1. Schematic representation of the IS*Ecp1*-$bla_{\text{CTX-M-15}}$-containing sequences in plasmids pYC-5b (A) and pYC-14 (B). Inverted repeat sequences (IR) and target site duplications (TSD) are shown. Arrows indicate direction of transcription. The thick line (B) denotes homology with the sequence in panel A.

AY458016 [M. R. Mulvey et al., unpublished data]). This sequence was homologous to that found in pYC-5b except that it lacked the left-hand IS*26*. Also, in silico restriction analysis of pC15-1a indicated patterns different from that of pYC-5b. Since IS*Ecp1* does not exhibit marked target site selectivity, it can be hypothesized either that the CTX-M-15-encoding plasmids discussed here diverged from an ancestral IS*Ecp1-bla*$_{\text{CTX-M}}$-carrying plasmid or that the IS*Ecp1-bla*$_{\text{CTX-M-15}}$ sequence was independently acquired as part of a larger mobile element.

Nucleotide sequence accession numbers. The described sequences have been assigned GenBank accession numbers AY604721 and AY604722.

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Extended-Spectrum-β-Lactamase-Producing *Enterobacteriaceae* in Yaounde, Cameroon

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Organisms producing extended-spectrum β-lactamases (ESBLs) have been reported in many countries, but there is no information on the prevalence of ESBL-producing members of the family *Enterobacteriaceae* in Cameroon. A total of 259 *Enterobacteriaceae* strains were isolated between 1995 and 1998 from patients at the Yaounde Central Hospital in Cameroon. Enterobacterial isolates resistant to extended-spectrum cephalosporin and monobactam were screened for ESBL production by the double-disk (DD) synergy test. Thirty-one (12%) of these *Enterobacteriaceae* strains were shown to be positive by the DD synergy test, suggesting the presence of ESBLs. Resistance to oxyimino-cephalosporins and monobactams of 12 (38.7%) of the 31 strains—i.e., 6 *Klebsiella pneumoniae*, 4 *Escherichia coli*, 1 *Citrobacter freundii*, and 1 *Enterobacter cloacae* strain—was transferred to *E. coli* HK-225 by conjugation. Resistance to gentamicin, gentamicin plus trimethoprim-sulfamethoxazole, or trimethoprim-sulfamethoxazole was cotransferred into 6, 2, and 1 of these transconjugants, respectively. All 12 transconjugants were resistant to amoxicillin, piperacillin, all of the cephalosporins, and aztreonam but remained susceptible to cefoxitin and imipenem. Crude extracts of β-lactamase-producing transconjugants were able to reduce the diameters of inhibition zones around disks containing penicillins, narrow- to expanded-spectrum cephalosporins or monobactams when tested against a fully susceptible *E. coli* strain but had no effect on such zones around cefoxitin, imipenem, and amoxicillin-clavulanate disks. The β-lactamases produced by the 12 tranconjugants turned out to be SHV-12 by DNA sequencing. Therefore, the ESBL SHV-12 is described for the first time in Cameroon.

Many extended-spectrum β-lactamases (ESBLs) are plasmid-mediated derivatives from TEM- and SHV-type enzymes and cause resistance to expanded-spectrum cephalosporins. They belong to Bush group 2be (6). Since their initial description in Germany in 1983 (13), ESBLs have diversified and spread worldwide. Several ESBLs appear to be particularly widely disseminated, being found in many countries, whereas others seem to occur more commonly in one or few countries (4). The various national patterns of antibiotic consumption in hospitals probably account for the differences in distribution of these enzymes. In an attempt to detect and study the dissemination of ESBLs in a central African country (Cameroon), we collected and characterized producers of such enzymes among clinical isolates of *Enterobacteriaceae* at Yaounde Central Hospital between 1995 and 1998. The ESBL SHV-12 was found in several species of *Enterobacteriaceae* for the first time in Cameroon.

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MATERIALS AND METHODS

Bacterial strains. A total of 259 isolates, members of the *Enterobacteriaceae* family were collected from patients in Yaounde Central Hospital (Table 1). Isolates were collected over 3-year period (April 1995 to March 1998) from urine, pus, and blood. The isolates were identified by conventional techniques (9) and were confirmed by the API 20 E (bioMérieux, France). There were no replicate strains isolated from any patient in the present study. *Escherichia coli* HK 225 (12), which is resistant to rifampin and streptomycin, was used as a recipient strain for transfer experiments by conjugation. *E. coli* ATCC 25922 was used as a control strain for antimicrobial susceptibility testing and as a negative control for PCR experiments. *E. coli* K12R111 (provided by Danielle Sirot) encoding TEM-1 and plasmid pMPA encoding SHV-2A were used as positive controls for bla_{TEM} and bla_{SHV} genes, respectively.

Antimicrobial susceptibility testing and detection of ESBL producers. Antimicrobial susceptibility was determined by disk diffusion tests according to the methods of the Clinical and Laboratory Standards Institute (CLSI; formerly National Committee for Clinical Laboratory Standards) (16) by using disks from BBL Microbiology Systems (Cockeysville, Md.). The tested antibiotics were amoxicillin, amoxicillin-clavulanate, piperacillin, cefazolin, cefoxitin, cefotaxime, ceftazidime, aztreonam, imipenem, gentamicin, ciprofloxacin, and trimethoprim-sulfamethoxazole.

The double-disk (DD) synergy test (11) was used for detection of ESBLs in clinical and transconjugant strains. A central disk of amoxicillin-clavulanate was surrounded by disks with cefotaxime, ceftriaxone, ceftazidime, and aztreonam at a distance of ca. 19 mm (center to center) on a Mueller-Hinton agar plate (Difco Laboratories, Detroit, MI) inoculated according to the standard procedures (16). Distortion of the peripheral inhibition zones of surrounding antibiotics toward the central disk with clavulanate was indicative for an ESBL. The tests were repeated with a disk spacing of 15 mm (center to center).

TABLE 1. Occurrence of ESBL-producing phenotypes among members of the family *Enterobacteriaceae*

Organism	Total isolates analyzed	No. of isolates with ESBL phenotypes (%)
Escherichia coli	91	13 (14.3)
Klebsiella spp.	64	12 (18.8)
Proteus spp.	56	1 (1.8)
Enterobacter spp.	31	2 (6.5)
Citrobacter spp.	17	3 (17.6)
Total	259	31 (12)

MICs for positive DD synergy test strains were determined by using the E-test (AB Biodisk, Solna, Sweden) according to the instructions of the manufacturer.

Conjugation experiments. Transfer of resistance phenotypes was performed by a liquid mating method (19) in brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.). Culture mixtures were incubated overnight at 37°C with clinical isolates as donors and *E. coli* HK 225 as the recipient. After conjugation, bacterial suspensions were plated onto agar containing 50 μg of ampicillin, 10 μg of ceftazidime, 2 μg of cefotaxime, 100 μg of rifampin, and 2,000 μg of streptomycin per ml. The resulting transconjugants were purified and identified with API 20 E strips. The frequencies of transfer were determined per input donor cell.

Characterization of β-lactamases. Transconjugant strains were grown at 37°C with shaking in brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.) containing ceftazidime (10 μg/ml), cefotaxime (2 μg/liter), and ampicillin (50 μg/liter). The cells were harvested during late log phase (8) by centrifugation at 3,000 × *g* at 4°C, washed twice in 0.1 M phosphate buffer (pH 7.0), and resuspended in 1/50 of the original volume. After cell rupture by sonication on ice, the cellular debris was removed by two centrifugations at 5,000 × *g*, each for 30 min at 4°C. Both times, the supernatant was carefully removed without solid particles. The crude extracts were stored at −20°C.

The extracts' ability to hydrolyze β-lactam antibiotics (amoxicillin, amoxicillin-clavulanate, piperacillin, cefazolin, cefoxitin, ceftriaxone, cefotaxin, ceftazidime, aztreonam, and imipenem) was evaluated by a microbiological method (15). Disk diffusion test were performed according to the methods of the CLSI (16), and antimicrobial disks were immediately impregnated with 10 μl of crude extract after their application. The inhibition zones produced against *E. coli* HK 225 were compared to those observed by untreated disks. A reduction of the inhibition zone diameter as a consequence of the applied extract was considered evidence for β-lactamase activity.

Molecular analysis techniques. DNA of transconjugants strains was extracted with phenol and chloroform, precipitated with isopropanol, and resuspended in TE buffer (3). *bla* genes were amplified by PCR with the following oligonucleotide primers: TEM OT-1-F (5′-TTGGGTGCACGAGTGGGTTA-3′) and OT-2-R (5′-TAATTGTTGCCGGGAAGCTA-3′), which amplified a 465-bp fragment of the bla_{TEM} gene (2), and SHV-F (5′-CGC CGG GTT ATT CTT ATT TGT CGC-3′) and SHV-R (5′-TCT TTC CGA TGC CGC CGC CAG TCA-3′), which amplified a 1,017-bp fragment of the bla_{SHV} gene (17). The cycle conditions were as follows: 30 cycles, with 1 cycle consisting of denaturation at 95°C for 30 s, annealing at 52°C (TEM) or 68°C (SHV) for 30 s, and extension at 72°C for 1 min. Each PCR program was preceded by a denaturation step of 94°C for 5 min and followed by a final denaturation at 72°C for 10 min. The SHV PCR products were digested with the restriction enzyme NheI (17).

Sequencing of the PCR products was performed by using the ABI Prism BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA) according to the recommendations of the manufacturer in an ABI Prism 310 genetic analyzer. Sequence data were processed and analyzed with the version 9.0 GCG Sequence Analysis Software Package (Genetics Computer Group, Madison, WI).

Sequence accession number. The nucleotide sequence of bla_{SHV-12} from *C. freundii* 09 is filed in the GenBank database under the accession number AY940490.

RESULTS

Detection of ESBL-producing isolates. A total of 259 isolates (124 urine isolates, 120 isolates from pus, and 15 isolates from blood) belonging to the family *Enterobacteriaceae* were studied, among which 31 (12%) potential ESBL producers were identified by the DD synergy test. Characteristic clavulanate-induced distortions of inhibition zones that were indicative for ESBL production were found in 86, 90, 62, and 97% of the strains around the disks containing cefotaxime, ceftriaxone, ceftazidime, and aztreonam, respectively. The ESBL producer's isolates were investigated, and the ESBL-producing phenotype was found most frequently among *Klebsiella* species (18.8%; *K. pneumoniae* [$n = 11$] and *K. oxytoca* [$n = 1$]), followed by *Citrobacter* species (17.6%; *C. freundii* [$n = 3$]) and *E. coli* (14.3%) (Table 1). Of the ESBL-producing strains, 15 were from urine, 11 were from pus, and 5 were from blood; isolates were mainly from patients in the intensive care unit (ICU) (38.7%) and the surgical (45.1%) ward (Table 2).

MICs of extended-spectrum cephalosporins exhibited a notable variability among different isolates, but MICs of CAZ were always ≥2 μg/ml (Table 2). All of the isolates were resistant to amoxicillin, piperacillin, and cephalothin, and most of them were also resistant to amoxicillin-clavulanate, gentamicin, and trimethoprim-sulfamethoxazole. All of the isolates were susceptible to imipenem, and most of them were also susceptible to cefoxitin, ciprofloxacin, and amikacin (Table 2).

Transferability of ESBL genes. Among potential ESBL producers, 12 (38.7%) were transferred oxyimino-cephalosporin resistance to *E. coli* HK 225. The transfer frequencies were between 9×10^{-8} and 6.7×10^{-4} per input donor. The isolates included six of *K. pneumoniae*, four of *E. coli*, one of *E. cloacae*, and one of *C. freundii*. Resistance determinants against the following non-β-lactam antibiotics were cotransferred to *E. coli* HK 225: gentamicin (six cases), gentamicin and trimethoprim-sulfamethoxazole (two cases), and trimethoprim-sulfamethoxazole (one case). In contrast, resistance to cefoxitin or to other antibiotic classes was not transferred (Table 3).

β-Lactamase type. Supernatants containing crude β-lactamase extracts from transconjugants did not affect the inhibition zones of *E. coli* HK 225 around cefoxitin, imipenem, and amoxicillin-clavulanate disks. The supernatants did, however, significantly reduce the inhibition zones around disks containing amoxicillin, piperacillin, cefazolin, cefotaxime, ceftriaxone, ceftazidime, and aztreonam (data not shown). These results were consistent with the presence of the detected ESBLs.

All 12 transconjugants were subjected to molecular detection and characterization procedures aimed at bla_{TEM} and bla_{SHV} genes. From all 12 strains, no PCR product was obtained with TEM specific primers, whereas a characteristic 1,017-bp amplimer was synthesized, which proved to be degradable by the restriction enzyme NheI into two fragments of 770 and 247 bp in length. This positive PCR/NheI test indicated that an SHV-ESBL was produced by all transconjugants and hence by the 12 corresponding donor clinical isolates (Table 3).

Nucleotide sequencing revealed that all 12 amplimers were 100% homologous to each other along their entire length. This also confirmed the PCR/NheI results and showed that the deduced amino acid sequences differed in the following positions (Ambler numbering [1]) from the SHV-1 standard (4): leucine 35→glutamine, glycine 238→serine, and glutamic acid 240→lysine. Thus, SHV-12 (http://www.lahey.org/studies/webt.htm) was identified in all of the 12 strains (Table 3). More-

TABLE 2. Distribution, clinical features, antimicrobial susceptibility test, and resistance transfer of *Enterobateriaceae* with ESBL phenotype

Organism	Isolate	Ward	Specimen	MIC (μg/ml)[a]									DD synergy test	Other resistance marker(s)[b]	Transfer of ESBL gene
				Amx	Amc	Pip	Cfz	Fox	Ctx	Cro	Caz	Imp			
Escherichia coli	15/98	Surgery	Pus	>256	32	>256	>256	32	16	32	6	0.19	+	Sxt	−
	J51	Surgery	Pus	>256	>256	>256	>256	8	3	4	8	0.125	+		−
	J55	Surgery	Pus	>256	>256	>256	>256	>256	>256	>256	24	0.19	+	Gm, Sxt	+
	H302	ICU	Blood	>256	16	>256	>256	3	4	3	4	0.125	+	Gm, Cip, Sxt	−
	301/98	ICU	Pus	>256	12	>256	>256	12	12	16	12	0.25	+	Gm, Cip, Sxt	−
	424/97	Obstetrics/ Genecology	Urine	>256	>256	>256	>256	>256	>256	>256	>256	1.5	+	Gm, Amk, Sxt	−
	J6	Surgery	Urine	>256	16	>256	>256	3	2	3	32	0.125	+	Sxt	+
	268/98	ICU	Urine	>256	24	>256	>256	12	12	16	32	0.125	+	Gm, Cip, Sxt	+
	288/98	ICU	Urine	>256	8	>256	>256	6	8	12	64	0.125	+	Cip, Sxt	+
	H304	ICU	Blood	>256	24	>256	>256	4	4	4	32	0.19	+	Gm, Cip, Sxt, Amk	−
	U349	ICU	Urine	>256	12	>256	>256	3	48	48	96	0.125	+	Gm, Cip, Sxt	−
	508	Surgery	Pus	>256	8	>256	>256	3	4	2	24	0.19	+	Gm, Sxt	−
	H281	ICU	Blood	>256	8	>256	>256	1.5	3	2	16	0.19	+	Sxt	−
Klebsiella pneumoniae	U229	ICU	Urine	>256	16	>256	>256	3	48	32	>256	0.125	+	Gm, Sxt	+
	U271	Surgery	Urine	>256	32	>256	>256	2	256	256	96	0.19	+	Gm, Sxt	+
	U284	Surgery	Urine	>256	32	>256	>256	2	>256	>256	>256	0.19	+	Gm, Amk, Sxt	+
	J55	Surgery	Pus	>256	16	>256	>256	6	16	12	64	0.19	+	Gm	+
	J51	Surgery	Pus	>256	16	>256	>256	3	3	6	64	0.25	+	Gm, Sxt	−
	203cp	Urology	Urine	>256	24	>256	48	3	1	2	12	0.25	+	Gm, Sxt	−
	176cp	ICU	Blood	>256	24	>256	>256	4	2	3	12	0.125	+	Gm, Sxt	−
	J21	Urology	Urine	>256	3	8	12	3	1	1.5	4	0.19	+	Sxt	−
	083	ICU	Blood	>256	12	>256	>256	3	2	2	3	0.125	+	Gm, Sxt	−
	48/42	Surgery	Urine	>256	16	>256	>256	96	24	32	32	0.125	+	Sxt	+
	508	Surgery	Pus	>256	24	>256	>256	3	16	32	6	0.19	+	Gm, Sxt	+
Klebsiella oxytoca	103	Urology	Urine	>256	16	>256	>256	4	4	4	12	0.125	+	Gm, Sxt	−
Enterobacter aerogenes	1086	Surgery	Pus	>256	24	>256	>256	96	1.5	3	16	0.19	+	Gm, Sxt	−
Enterobacter cloacae	189	Surgery	Urine	>256	>256	>256	>256	>256	>256	>256	>256	0.25	+	Gm, Sxt	+
Citrobacter freundii	09/98	ICU	Pus	>256	48	>256	>256	>256	8	16	96	0.19	+	Gm, Sxt	+
	J13	Urology	Urine	>256	64	>256	>256	>256	12	16	32	0.19	+	Gm, Cip, Sxt	−
	U271	Surgery	Urine	>256	>256	>256	>256	93	96	96	64	0.125	+	Gm, Sxt	−
Proteus mirabilis	294	ICU	Pus	>256	8	>256	>256	1.5	4	8	8	0.125	+	Gm, Sxt	−

[a] Amx, amoxicillin; Amc, amoxicillin-clavulanic acid (2:1); Pip, piperacillin; Cfz, cefazolin; Fox, cefoxitin; Ctx, cefotaxime; Cro, ceftriaxone; Caz, ceftazidime; Atm, aztreonam; Imp, imipenem.
[b] Amk, amikacin; Gm, gentamicin; Cip, ciprofloxacin; Sxt, trimethoprim-sulfamethozaxole.

TABLE 3. Characteristics of transconjugant strains

Transconjugant *E. coli* strains	Antibiotic resistance pattern[a]	*bla* gene		β-Lactamase type
		TEM	SHV	
Hk-225-U288	ESBL	−	+	SHV-12
Hk-225-J55E	ESBL, Gm	−	+	SHV-12
Hk-225-J6	ESBL	−	+	SHV-12
Hk-225-U268	ESBL, Gm	−	+	SHV-12
Hk-225-508K	ESBL, Gm	−	+	SHV-12
Hk-225-U229	ESBL, Sxt	−	+	SHV-12
Hk-225-48/42	ESBL	−	+	SHV-12
Hk-225-U271	ESBL, Gm, Sxt	−	+	SHV-12
Hk-225-U284	ESBL, Gm, Sxt	−	+	SHV-12
Hk-225-J55K	ESBL, Gm	−	+	SHV-12
Hk-225-U189	ESBL, Gm	−	+	SHV-12
Hk-225-09	ESBL, Gm	−	+	SHV-12
Hk-225		−	−	−

[a] Gm, gentamicin; Sxt, trimethoprim-sulfamethozaxole.

over, two silent mutations at codons 138 and 268 were the same as those described in the original sequence of bla_{SHV-12} (EMBL accession no. X98105 [18]), namely, CTG and ACG, as opposed to CTA and ACC, respectively, in the standard bla_{SHV-1} sequence. However, one additional G-to-C transversion immediately after the TAA stop codon was detected (position 935 in X98105).

The bla_{SHV-12} sequence and flanking regions of *C. freundii* 09 were deposited in GenBank (accession no. AY940490) as the representative for the 12 isolates listed in Table 4.

The 12 SHV-12-producing isolates exhibited a relatively narrow clinical distribution. They were mainly obtained from the surgical ward between 1996 and 1998 (Table 4).

DISCUSSION

The aim of this study was to determine the prevalence and characterize ESBL producers among members of the family *Enterobacteriaceae* in Yaounde Central Hospital. We found isolates that expressed an ESBL producer phenotype among every genus that was tested. ESBLs were present in 31 (12%) among the 259 *Enterobacteriaceae* isolates evaluated here. The prevalence among genera is varied, with rates of 18.8% for *Klebsiella* spp. and 1.8 to 17.6% for all other genera. Of 31 ESBL producer isolates, 12 (38.7%) transferred the ESBL gene to *E. coli* HK 225 and were also found to produce SHV-12 ESBL. These data represent the first report of the prevalence of ESBL producers among *Enterobacteriaceae* in Cameroon.

TABLE 4. Distribution of SHV-12-producing clinical isolates of *Enterobacteriaceae*

Species	Isolate	Date (day/mo/yr)	Ward
Escherichia coli	J6	14/02/1997	Surgery
	J55E	19/08/1997	Surgery
	U268	13/03/1998	ICU
	U288	17/03/1998	ICU
Klebsiella pneumoniae	508K	26/07/1996	Surgery
	J55K	19/08/1997	Surgery
	U229	04/03/1998	ICU
	48/42	04/03/1998	Surgery
	U271	13/03/1998	Surgery
	U284	16/03/1998	Surgery
Enterobacter cloacae	U189	24/02/1998	Surgery
Citrobacter freundii	09	16/02/1998	ICU

In the present study, most ESBL producers were collected from patients in the surgical ward and the ICU. In these wards, isolates are exposed to great antibiotic pressure. Furthermore, many of these patients are particularly vulnerable to infection because they are immunocompromised or have an easy avenue of access for bacteria (23).

The susceptibility test data showed that the ESBL producers which were resistant to most β-lactams and non-β-lactams such as gentamicin and trimethoprim-sulfamethoxazole were multidrug-resistant strains. The ESBL producers usually carry a multiresistant plasmid, the genes conferring resistance to β-lactam and non-β-lactam antibiotics (10, 23). All of the ESBL producers were susceptible to imipenem, and most were also susceptible to cefoxitin, amikacin, and ciprofloxacin. If the patients are infected by ESBL producers, carbapenem may be used (5, 14).

The results of the present study are evidence of an ongoing outbreak during the sampling period from 1996 to 1998 at Yaoundé Central Hospital of ESBL-producing organisms attributable to at least four species of *Enterobacteriaceae*: *K. pneumoniae*, *E. coli*, *C. freundii*, and *E. cloacae*. Although the bla_{SHV-12} gene was cotransferable along with other resistance determinants upon conjugation and was found in several species, it seemed possible that the determinant is located on a large low-copy broad-host-range plasmid, as is usually the case (10, 21). In the present study, we focused on determinants that were easily transferable by conjugation in vitro (more than a third of the ESBL isolates from Yaounde Central Hospital) because these are the most clinically relevant factors with regard to the speed of dissemination.

Within the collection of 31 strains that yielded positive DD results, ceftazidime and aztreonam showed the lowest (62%) and highest (96%) rates of detection, respectively. These results indicate that at maximum sensitivity, when looking for ESBLs, several oxyimino-cephalosporins and aztreonam should be used simultaneously for DD testing. This is in agreement with the recommendations by Coudron et al. (7). Moreover, this finding underlines the fact that synergy tests and other physiological tests in general are of limited sensitivity in detecting ESBLs, a fact that has been stated already in 1995 (14) and confirmed by careful studies involving site-directed mutagenesis, as well as different copy number cloning vectors in isogenic systems (20).

ESBLs are now a problem for hospitalized patients worldwide. The rates of ESBL producers among *Klebsiella* sp. and *E. coli* at our center are 18.8 and 14.3%, respectively. This prevalence is lower for *Klebsiella* sp. and higher for *E. coli* than that reported by the SENTRY worldwide surveillance program, in which the ESBL prevalences in *K. pneumoniae* and *E. coli* were 45 and 8.5% (Latin America), 25 and 7.9% (Western Pacific), or 23 and 5.3% (Europe), respectively (22).

In conclusion, the present study emphasizes the importance of screening for ESBLs even in countries where such enzymes

have not been reported previously. The plasmid-mediated ESBLs have been already disseminated to four different species of *Enterobacteriaceae* and escalated into a multiclinic outbreak at Yaounde Central Hospital by the time they were discovered.

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The in-vitro antimicrobial activities of some medicinal plants from Cameroon

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The antimicrobial activities of 10 plant species (*Voacanga africana, Crepis cameroonica, Plagiostyles africana, Crotalaria retusa, Mammea africana, Lophira lanceolata, Ochna afzelii, Ouratea elongata, Ou. flava and Ou. sulcata*), each of which is currently used in the traditional medicine of Cameroon, were investigated *in vitro*. The activities of a methanol extract of each plant were tested, in disc-diffusion assays, against 37 reference or laboratory strains of seven species of microorganism (*Staphylococcus aureus, S. epidermidis, Enterococcus hirae, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa and Candida albicans*). The minimal inhibitory concentrations of each extract were then estimated, against each of the more susceptible microorganisms (i.e. those giving an inhibition zone measuring at least 9 mm in diameter in the disc-diffusion assays), by agar dilution. Although, in the disc-diffusion assays, each of the 10 methanol extracts investigated displayed some degree of antimicrobial activity against at least one species of microorganism, no activity against the Gram-negative bacteria (*Es. coli, K. pneumoniae* and *Ps. aeruginosa*) was observed. The extract with the greatest antimicrobial activity was that of *Pl. africana* (Euphorbiaceae).

Medicinal plants have long been the subject of human curiosity and need. In many places in Cameroon there is a rich tradition of the use of herbal medicine in the treatment of various infectious and other diseases, inflammations and injuries (Adjanohoun *et al.*, 1996). Plant-derived products are present in 14 of the 15 therapeutic categories of pharmaceutical preparations that are currently recommended by medical practitioners, and they form an important part of the health-care system in the western world (Phillipson and Anderson, 1989). Although more than 250,000 species of higher plants are currently recognized, only about 5%–10% of them have ever been chemically investigated (Nahrstedt, 1996). The growing problem of resistance in pathogenic microorganisms, against many of the antibiotics in routine use, combined with the existing problem of the adverse effects of many antibiotic treatments, has recently increased research on the antimicrobial activities of various extracts and compounds isolated from the plant species used in herbal medicine (Nostro *et al.*, 2000; Kokoska *et al.*, 2002).

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Plant-based antimicrobials represent a vast and largely untapped source of medicines with enormous therapeutic potential. Some of these compounds will almost certainly be effective in the treatment of infectious diseases while causing few, if any, of the side-effects that are often associated with synthetic antimicrobials (Cowan, 1999; Iwu *et al.*, 1999). The aim of the present study was to explore, *in vitro*, the antimicrobial properties of methanol extracts of 10 plant species used in folk medicine in Cameroon.

MATERIALS AND METHODS

Plant Material

The seeds, stems or leaves (whichever parts of the plant are used in traditional medicine) of 10 plant species (*Voacanga africana, Crepis cameroonica, Plagiostyles africana, Crotalaria retusa, Mammea africana*, *Lophira lanceolata*, *Ochna afzelii*, *Ouratea elongata,* *Ou. flava* and *Ou. sulcata*) were collected from various places in Cameroon in September–November 2003 (Table 1). Samples were identified at the National Herbarium of Cameroon, where a voucher specimen of each species was deposited.

Preparation of Extracts

Each batch of plant material was air dried and powdered. Between 15 and 60 g of each powder were then extracted with 500 ml methanol, at room temperature and with constant shaking, for 24 h. Each resultant extract was filtered and then concentrated to dryness under reduced pressure.

Microorganisms

The antimicrobial activity of each methanolic extract was measured against 37 microbial cultures, representing six species of bacteria and one of yeast (see Table 2). The cultures were either of reference strains,

TABLE 1. *Details of the 10 species of medicinal plant that were investigated*

Family	Species	Voucher number	Site of collection	Part used	Uses in traditional medicine
Apocynaceae	*Voacanga africana* Stapf ex S. Elliot	9227/SRF/CAM	Obala	Seed	Orchitis, tooth decay, gonorrhoea
Compositae	*Crepis cameroonica* Babc. ex Hutch. et Dalz	22072/SRF/CAM	Obili (Yaoundé)	leaf	Nose and ocular infections, diarrhoea
Euphorbiaceae	*Plagiostyles africana* Prain ex De Wild	5723/SRF/CAM	Mont Cameroun	Leaf	Ocular infection, chest complaints
Fabaceae	*Crotalaria retusa* Linn.	23781/SRF/CAM	Obili (Yaoundé)	Leaf	Eczema
Guttifereae	*Mammea africana* G. Don	17276/SRF/CAM	Yaoundé	Stem	Scabies, constipation, abortion, syphilis, gonorrhoea,
Ochnaceae	*Lophira lanceolata* Van Tiegh ex Keay	3512/SRFK/CAM	Balamba (Bafia)	Leaf	Tooth ache, dermatosis, wounds, conjunctivitis
	Ochna afzelii R. Br. ex Oliv	8493, H.N.C. Yde	Nkolafamba	Stem	Tooth ache, respiratory tract infection
	Ouratea elongata C. Sastre	56132, H.N.C., Yde	Mont Kala	Leaf	Gastritis, rheumatism
	Ouratea flava Hutchinson et Dalziel ex Stapf	27056, H.N.C., Yde	Mont Elonden	Leaf	Gastritis, rheumatism
	Ouratea sulcata (Tiegh.) Keay	10133/SRF/CAM	Kribi	Leaf	Gastritis, rheumatism

and acquired from the American Type Culture Collection (Manassas, VA), or of clinical isolates from the laboratory collection held at the Institute of Medical Research and Medicinal Plant Studies in Yaounde, Cameroon. Each was routinely sub-cultured, at 37°C, on tryptic-soy agar (bioMérieux, Marcy l'Etoile, France; bacteria) or Sabouraud dextrose agar (bioMérieux; *Candida albicans*).

Antimicrobial Assays

DISC DIFFUSION

Each dried extract was dissolved in a 10% dimethyl sulphoxide/0.5% Tween-20 solution, to give 100 mg/ml, and then sterilized by filtration through a 0.22-µm-pore filter (Millipore, Billerica, MA). The antimicrobial activities of each diluted extract were then investigated by disc diffusion, following the methods recommended by the National Committee for Clinical and Laboratory Standards in the U.S.A. (Anon., 1999) and using, for each assay plate, 100 µl saline containing 10^7 colony-forming units (cfu) of a bacterial species [on Mueller–Hinton agar (bioMérieux)] or 10^5 cfu of *Ca. albicans* (on Sabouraud dextrose agar). The discs (6 mm in diameter) were each impregnated with 15 µl of a diluted extract (giving 1.5 mg dried extact/disc) before being placed on a pre-inoculated agar plate. Negative controls were prepared using the solvent employed to dissolve the plant extracts.

TABLE 2. *The antimicrobial activities of the medicinal-plant extracts, as measured, against six species of bacteria strains and* Candida albicans*, in disc-diffusion assays*

	Diameter (mm) of zone of inhibition around test disc impregnated with:*										
Microbe	Cc	Cr	Ll	Ma	Oa	Oe	Of	Os	Pa	Va	NC
Staphylococcus aureus											
Sa1	–	–	–	10	–	–	–	13	9	–	–
Sa2	8	–	–	13	–	–	–	12	12	7	–
Sa3	–	–	–	10	–	–	–	10	12	–	–
Sa4	9	10	9	13	9	–	–	11	12	–	–
ATCC 25923	–	9	–	11	–	–	–	11	12	–	–
Staphylococcus epidermidis	10	10	8	12	9	–	–	12	13	–	–
Enterococcus hirae ATCC 9790	–	–	NT	12	–	–	–	–	–	11	–
Escherichia coli (12 strains)	–	–	–	–	–	–	–	–	–	–	–
Klebsiella pneumoniae (four strains)	–	–	–	–	–	–	–	–	–	–	–
Pseudomonas aeruginosa (four strains)	–	–	–	–	–	–	–	–	–	–	–
Candida albicans											
Ca1	9	10	9	9	8	10	10	NT	12	7	–
Ca2	8	10	8	–	11	–	13	NT	10	10	–
Ca3	–	8	9	7	10	8	8	NT	14	9	–
Ca4	10	10	8	–	10	8	10	NT	14	–	–
Ca5	7	9	–	9	9	–	8	NT	16	–	–
Ca6	10	10	10	11	10	–	11	NT	15	–	–
Ca7	10	8	9	8	8	7	8	NT	16	–	–
Ca8	–	–	8	–	8	–	7	NT	13	8	–
Ca9	–	–	8	8	8	7	8	NT	11	8	–
Ca10	9	9	8	8	9	8	9	NT	16	–	–

*Methanolic extract of *Crepis cameroonica* (Cc), *Crotalaria retusa* (Cr), *Lophira lanceolata* (Ll), *Mammea africana* (Ma), *Ochna afzelii* (Oa), *Ouratea elongata* (Oe), *Ouratea flava* (Of), *Ouratea sulcata* (Os), *Plagiostyles africana* (Pa) or *Voacanga africana* (Va) or the extract-free diluent used as the negative control (NC).
NT, Not tested.

The plates were then incubated at 37°C for 24 h (bacteria) or 48 h (yeast) before the diameter of any zone of inhibition around each disc was measured. Each assay was repeated three times.

DETERMINATION OF MINIMAL INHIBITORY CONCENTRATIONS

The minimal inhibitory concentrations (MIC) of each extract, against each microbial species that gave an inhibition zone of at least 9 mm with the extract in the disc-diffusion assays, were then determined using an agar-dilution method (Anon., 1999).

Each target species of microorganism was cultured overnight on tryptic-soy agar and then suspended in sterile saline to give 10^4 cfu/ml. Each dried plant extract was dissolved in 10% dimethyl sulphoxide/0.5% Tween-20 solution, to give 100 mg/ml, before being sterilized by filtration through a 0.22-µm-pore filter. Each extract was then serially diluted with sterile distilled water before being mixed with sterile molten Mueller–Hinton agar, to give final concentrations between 2 and 1024 µg dried extract/ml. Each agar solution was vortexed and then immediately poured into a Petri dish. A suspension of one of the test organisms (at 10^4 cfu/ml) was spotted on the cold agar with a micropipette. The inoculated plates were incubated at 37°C for 24 h (bacteria) or 48 h (yeasts). At the end of the incubation period, the plates were evaluated for the presence or absence or microbial growth. The lowest concentration of an extract at which there was no visible growth was taken as the MIC for the extract–microbe combination being considered. As controls, the MIC of penicillin G against each bacterial species and econazole against the *Ca. albicans* were similarly determined.

Phytochemical Screening

Standard phytochemical methods (Harborne, 1973) were used to check each plant extract investigated for the presence of alkaloids, coumarins, flavonoids, sterols and triterpenes.

RESULTS AND DISCUSSION

The results of the disc-diffusion assays are summarized in Table 2 and the estimated MIC are given in Table 3. Although, in the disc-diffusion assays, each of the 10 plant extracts tested inhibited the growth of at least one of the species of Gram-positive bacteria investigated (*Staphylococcus* spp. and *En. hirae*) and/or at least one strain of *Ca. albicans*, no activity against the Gram-negative bacteria used in the assays (i.e. *Es. coli*, *K. pneumoniae* and *Ps. aeruginosa*) was observed (Table 2).

In the disc-diffusion assays, the extracts from *M. africana*, *Ou. sulcata* and *Pl. africana* showed good activity against *Staphylococcus* spp., giving relatively wide inhibition zones (of 10–13, 10–13 and 9–12 mm, respectively; Table 2). The extract from *M. africana* was also active against *En. hirae* (giving a 12-mm inhibition zone). In the agar-dilution assays, however, the extracts from *M. africana* and *Ou. sulcata* gave MIC above the highest concentration tested (1024 µg/ml; Table 3).

In the disc-diffusion assays, the strains of *Ca. albicans* were found to be most sensitive to extracts from *Cre. cameroonica*, *Cro. retusa*, *L. lanceolata*, *Oc. afzelii*, *Ou. flava* and *Pl. africana*. The extract from *Pl. africana* showed the highest activities against these microbes, with inhibition zones of 10–20 mm (Table 2), although the corresponding MIC were only 1024 or >1024 µg/ml (Table 3).

There appear to have been no previous reports on the antimicrobial activities, or the chemical natures of the potentially antimicrobial compounds, of the 10 plant species investigated in the present study. The results of the phytochemical tests showed the presence of flavonoids in all 10 extracts, whereas terpenes, alkaloids, coumarins and

TABLE 3. *Minimal inhibitory concentrations of each of the plant extracts, as measured in the agar-dilution assays*

	Minimal inhibitory concentration (μg/ml) of:*											
Microbe	Cc	Cr	Ll	Ma	Oa	Oe	Of	Os	Pa	Va	PG	Eco
Staphylococcus aureus												
Sa1	NT_{la}	NT_{la}	NT_{la}	>1024	NT_{la}	NT_{la}	NT_{la}	>1024	512	NT_{la}	2	NT
Sa2	NT_{la}	NT_{la}	NT_{la}	>1024	NT_{la}	NT_{la}	NT_{la}	>1024	512	NT_{la}	<0.25	NT
Sa3	NT_{la}	NT_{la}	NT_{la}	>1024	NT_{la}	NT_{la}	NT_{la}	>1024	128	NT_{la}	4	NT
Sa4	>1024	NT_{la}	>1024	>1024	>1024	NT_{la}	NT_{la}	>1024	64	NT_{la}	0.25	NT
ATCC 25923	NT_{la}	>1024	NT_{la}	>1024	NT_{la}	NT_{la}	NT_{la}	>1024	>1024	NT_{la}	2	NT
Staphylococcus epidermidis	>1024	>1024	NT_{la}	>1024	>1024	NT_{la}	NT_{la}	>1024	64	NT_{la}	0.25	NT
Enterococcus hirae ATCC 9790	NT_{la}	NT_{la}	NT	>1024	NT_{la}	NT_{la}	NT_{la}	NT_{la}	NT_{la}	>1024	1	NT
Candida albicans												
Ca1	>1024	>1024	>1024	>1024	NT_{la}	>1024	>1024	NT	>1024	NT_{la}	NT	>1024
Ca2	NT_{la}	NT_{la}	NT_{la}	NT_{la}	>1024	NT_{la}	>1024	NT	>1024	1024	NT	256
Ca3	NT_{la}	NT_{la}	>1024	NT_{la}	>1024	NT_{la}	NT_{la}	NT	>1024	1024	NT	256
Ca4	>1024	NT_{la}	NT_{la}	NT_{la}	>1024	NT_{la}	>1024	NT	>1024	NT_{la}	NT	NT_{la}
Ca5	NT_{la}	>1024	NT_{la}	>1024	>1024	NT_{la}	NT_{la}	NT	>1024	NT_{la}	NT	>1024
Ca6	>1024	>1024	>1024	>1024	>1024	NT_{la}	>1024	NT	>1024	NT_{la}	NT	>1024
Ca7	>1024	NT_{la}	>1024	NT_{la}	NT_{la}	NT_{la}	NT_{la}	NT	1024	NT_{la}	NT	>1024
Ca8	NT	NT	NT_{la}	NT	NT_{la}	NT	NT_{la}	NT	>1024	NT_{la}	NT	>1024
Ca9	NT	NT	NT_{la}	NT_{la}	NT_{la}	NT_{la}	NT_{la}	NT	>1024	NT_{la}	NT	>1024
Ca10	>1024	>1024	NT_{la}	NT_{la}	>1024	NT_{la}	>1024	NT	1024	NT_{la}	NT	1024

*Methanolic extract of *Crepis cameroonica* (Cc), *Crotalaria retusa* (Cr), *Lophira lanceolata* (Ll), *Mammea africana* (Ma), *Ochna afzelii* (Oa), *Ouratea elongata* (Oe), *Ouratea flava* (Of), *Ouratea sulcata* (Os), *Plagiostyles africana* (Pa) or *Voacanga africana* (Va) or, as positive controls, penicillin G (PG) or econazole (Eco).
NT_{la}, Not tested because the results of the disc-diffusion assays indicated low activity (with an inhibition zone measuring <9 mm in diameter); NT; not tested.

steroids were detected in eight, three, three and one of them, respectively (Table 4). It is probable that it is some of these compounds, alone or in combination, that are responsible for the observed antibacterial activities (Cowan, 1999). Little is known about the potentially antifungal compounds of the plants assayed; the presence of flavonoids and terpenes and a certain degree of lipophicity may determine the observed

TABLE 4. *The results of the phytochemical screening of the 10 plant extracts*

	Component				
Plant	Alkaloids	Coumarins	Flavonoids	Steroids	Terpenes
Crepis cameroonica	+	−	+	−	+
Crotalaria retusa	+	−	+	−	+
Lophira lanceolata	−	−	+	−	+
Mammea africana	−	+	+	−	−
Ochna afzelii	−	+	+	−	+
Ouratea elongata	−	−	+	−	+
Ouratea flava	−	−	+	−	+
Ouratea sulcata	−	−	+	−	+
Plagiostyles africana	−	+	+	+	−
Voacanga africana	+	−	+	−	+

toxicity towards *Ca. albicans*, via interactions with constituents of the yeast-cell membrane (Tomas-Barberan *et al.*, 1990).

Oral use of *Cro. retusa* is very dangerous, since this plant is known to be very toxic (Nuhu *et al.*, 2000; Nobre *et al.*, 2005). Nothing is known of the human toxicities of the other plants studied.

Curiously, there was no uniform response to an extract between species of bacteria, bacterial strains of the same species, or the *Ca. albicans* isolates investigated, perhaps reflecting intraspecific differences in cell-wall composition. Gram-negative bacteria have an outer phospholipidic membrane with structural lipopolysaccharide components. This composition makes the cell wall impermeable to lipophilic solutes, and the porins in the cell wall form a selective barrier to hydrophilic solutes, with an exclusion limit of about 600 Da (Nikaido and Vaara, 1985). As seen in the present study, Gram-positive bacteria are generally more susceptible to antimicrobial compounds because they have only an outer peptidoglycan layer, which is not an effective permeability barrier (Scherrer and Gerhardt, 1971).

The present results support the traditional use of many of the plants investigated. The plant giving the methanolic extract with the greatest antimicrobial activity (the only one to give MIC of <1024 μg/ml) was *Pl. africana*. The bioassay-guided isolation and identification of the active principles of this plant are now in progress.

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Antimicrobial activity against gram negative bacilli from Yaounde Central Hospital, Cameroon

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Abstract

Background: Antimicrobial resistance among bacteria pathogens is a world-wide issue. The antimicrobial susceptibility patterns of common pathogenic bacteria are essential to guide empirical and pathogen-specific therapy; unfortunately, these data are scarse in Cameroon.
Objective: To determine the antimicrobial susceptibility patterns of Gram-negative bacilli isolated in Yaounde Central Hospital Laboratory of Bacteriology.
Methods: Gram-negative bacilli isolates (n = 505), obtained from a wide range of clinical specimens (urine, pus and blood) in Yaoundé Central Hospital Laboratory of Bacteriology between March 1995 and April 1998, were evaluated for resistance to antibiotics (amoxicillin, amoxicillin/clavulanate, piperacillin, cefazolin, cefoxitin, cefotaxime, ceftazidime, aztreonam, imipenem, gentamicin, tobramicin, ofloxacin and trimethoprim/sulfamethoxazole) by the Kirby-Bauer disk diffusion method.
Results: High rates of resistance were found in most of the bacteria studied. Resistance to all isolates was mostly observed for amoxicillin (87%), piperacillin (74%) and trimethoprim/sulfamethoxazole (73%). Susceptibilities to third generation cephalosporins (cefotaxime, ceftazidime) and monobactame (aztreonam) were = 91% for *Escherichia coli*, = 71% for *Klebsiella* spp., = 98% for *Proteus mirabilis*, = 50% for *Enterobacter* spp. and *Citrobacter* spp. *Pseudomonas aeruginosa* was less susceptible to cefotaxime (2%) and aztreonam (33%), and highly susceptible to ceftazidime (72%) whereas *Acinetobacter baumannii* was highly resistant to aztreonam (100%), to cefotaxime (96%) and cetazidime (62%). Imipenem (98%) was the most active antibiotic followed by the ofloxacine (88%). Susceptibility of all isolates to gentamicin was 67%.
Conclusion: These results indicate that surveillance to antimicrobial resistance in Cameroon is necessary to monitor microbial trends, antimicrobial resistance pattern, and provide information for choosing empirical or direct therapy to physicians.
Key words: antimicrobial agents, resistance, Gram-negative bacilli, bacteria susceptibility testing, Cameroon.
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Introduction

Gram-negative bacilli are the most important bacterial pathogen, and are generally resistant to antibiotics [1, 2, 3]. Monitoring for antimicrobial resistance in this group is important because resistance has been reported to be associated with increased patient morbidity and mortality, and contributed to escalating healthcare cost [4]. Antimicrobial resistance is increasing in many bacteria and is a worldwide problem [5, 6].

The antimicrobial susceptibility patterns of common pathogenic bacteria are essential to guide empirical and pathogen-specific therapy. This information is also important for rational policies against antimicrobial resistance. Unfortunately, in many developing countries, these data are scarce because of dwindling resources. In Cameroon, data on antimicrobial resistance among bacterial pathogens are sparse. In an effort to determine the extent of antimicrobial resistance amongst Gram-negative bacilli isolated in Yaounde Central Hospital Laboratory of Bacteriology, we conducted a survey of 505 Gram-negative bacilli and measured their in vitro susceptibility to antimicrobial agents.

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Materials and Methods

Between April 1995 and March 1998, all the aerobic Gram-negative bacilli isolated in the laboratory of bacteriology of Yaounde Central Hospital were collected prospectively. The isolates studied were confined to unrelated first isolates from different patients, and did

not include multiple isolates from the same patient. Isolates were recovered from urine, pus and blood cultures and identified by standard laboratory technique methods [7] and confirmed by Api 20E (BioMerieux, France).

The antimicrobial susceptibility test was determined by the Kirby-Bauer disk diffusion method following the National Committee of Clinical Laboratory Standards (NCCLS) for agar diffusion tests [8]. The antibiotics tested were amoxicillin (30μg), amoxicillin/clavulanate (20/10 μg), piperacillin (100 μg), imipenem (10 μg), cefazolin (30 μg), cefoxitin (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), aztreonam (30 μg), gentamicin (10 μg), tobramycin (10 μg), ofloxacin (5 μg) and trimethoprim/sulfamethoxazole (1.25/23.75 μg). The following American Type Culture Collection (ATCC) microorganisms were tested each time susceptibility testing was performed: *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. Test results were only validated in the cases where inhibition zone diameters of the control strains were within performance ranges [8].

Data were analyzed using Whonet 4 (World Health Organization, Geneva, Switzerland) and resistance included combined, intermediary and resistance results.

Results

A total of 505 aerobic isolates were collected, identified, and tested. Enterobacteriaceae (*E. coli*, *Klebsiella* spp., *Proteus* spp., *Enterobacter* spp., and *Citrobacter* spp.) represented 79.8% of the isolated strains. Non fermentative Gram negative bacilli (*Pseudomonas* spp. and *Acinetobacter baumannii*) represented 20.2%. The sources of the isolates are shown in table 1.

Table 2 summarizes the results of susceptibility tests of Gram-negative bacilli studied against antimicrobial agents. Imipenem (98% susceptible (S)) was the most active agent against all pathogens tested followed by ofloxacine (88% S) and ceftazidime (86% S). Amoxicillin (13% S), piperacillin (26% S), trimethoprim/sulfamethoxazole (27% S), cefazolin (30% S) and amoxicillin/clavulanate (37% S) were the least active agents.

Against *E. coli*, susceptibility rates range from 15% (amoxicillin, piperacillin) to 99% (imipenem). The least active agents against *Klebsiella* spp. were amoxicillin (0% S) followed by piperacillin (10% S) and trimethoprim/sulfamethoxazole (15% S). The most active agents were imipenem (93% S) and ofloxacin (98% S). *Proteus mirabilis* was the most sensitive pathogen of all microorganisms. The susceptibility rate ranged from 40 % (amoxicillin) to 100% (imipenem, ceftazidime and aztreonam). Concerning *Enterobacter* spp., only two antimicrobial agents had activity rate >80% ((ofloxacin (98%) and imipenem (97%)). For *Citrobacter* spp. the most active agents were imipenem (96% S) followed by ceftazidime (76% S) and ofloxacin (73% S). Only 94% of *P. aeruginosa* isolates were susceptible to imipenem, as compared to 100% observed for *A. baumannii*. However, 72% of *P. aeruginosa* were susceptible to ceftazidime as compared to only 38 % of the *A. baumannii*. All the *A. baumannii* isolates tested were resistant to aztreonam.

Table 1. Distribution (%) of bacterial species by clinical specimen

Organism	Clinical specimen		
	Pus	Urine	Blood
Escherichia coli	33.7	65.0	1.3
Klebsiella spp.	43.3	44.3	12.4
Proteus mirabilis	58.0	42.0	0.0
Enterobacter spp.	47.5	42.5	10.0
Citrobacter spp.	50.0	50.0	0.0
Indole positif *Proteus* spp.	50.0	43.8	6.2
Pseudomonas aeruginosa.	71.6	22.4	6.0
Acinetobacter baumannii	54.2	25.0	20.8
Pseudomonas spp.	70.0	10.0	20.0

Table 2. Susceptibility (%) of Gram negative bacilli studied

	All isolates	*E. coli*	*Klebsiella* spp.	*P. mirabilis*	*Enterobacter* spp.	*Citrobacter* spp.	Indole positif *Proteus* spp.	*P. aeruginosa*	*A. baumannii*	*Pseudomonas* spp.
	n=505	n=163	n=97	n=62	n=40	n=26	n=16	n=67	n=24	n=10
Isolates										
Antibiotic	%S	%S	%S	%S	%S	%S	%S	%S	%S	%S
Amoxicillin	13	15	0	40	0	0	0	/	/	/
Amoxicillin / clavulanate	37	43	35	69	0	40	0	/	/	/
Piperacillin	26	15	10	53	35	19	56	46	4	50
Cefazolin	30	41	24	48	0	4	0	/	/	/
Cefoxitin	74	91	84	97	5	8	31	/	/	/
Cefotaxime	67	91	71	98	60	50	94	2	4	30
Ceftazidime	86	95	88	100	80	76	94	72	38	70
Aztreonam	73	92	75	100	65	69	88	33	0	20
Imipenem	98	99	99	100	97	96	94	94	100	100
Gentamicin	67	84	65	68	60	58	50	49	58	50
Tobramycin	57	/[a]	/	/	/	/	/	56	69	50
Ofloxacine	88	91	98	98	98	73	82	66	70	90
Trimethoprim /sulfamethoxazole	27	22	15	55	43	19	6	/	/	/

[a] not tested

Discussion

This study represents an extensive examination of the susceptibility patterns of Gram negative bacilli isolated in the laboratory of bacteriology of Yaounde Central Hospital. Our isolates represente both nosocomial and community acquired-pathogens, and were collected from April 1995 to March 1998. The results of this study show that in general, high rate of resistance occurs among Gram-negative bacilli to commonly used antibiotics (penicillins, first generation cephalosporin and trimethoprim/sulfamethoxazole).

For *E. coli* and *Klebsiella* spp., the rate of resistance to third generation cephalosporins and other â-lactam antibiotics can be explained by the high production of penicillinase and the production of extended spectrum â-lactamase [3, 9]. However, the rate of this resistance is high compared to those reported in developed countries [10, 11].

Enterobacter spp. and *Citrobacter* spp. were highly resistant to ceftazidime, cefotaxime and aztreonam. Similar results were observed in Cairo (Egypt) by El Kholy *et al.*, 2003 [12] and in developed countries [10, 13]. This resistance could be explained by the high production of cephalosporinase and the production of extended spectrum â-lactamase by these strains [3, 9].

Our data showed that *P. mirabilis* is the most sensitive species. The susceptibility rates of this species to all antibiotics tested are compared to those observed in developed countries [10, 11].

Non-â-lactam antibiotics resistance rates among the species of the *Enterobacteriaceae* family studied were comparable to the reported rates in other parts of developing countries [14, 15], but higher than those reported in developed countries [10, 13]. The combination of trimethoprime/sulfamethoxazole is extensively used in Africa owing to its antimicrobial spectrum of activity, and its low cost [16]. In addition, extended spectrum â-lactamase production is usually associated with resistance to non-â-lactam antibiotics such as aminoglycosides, fluoroquinolones and trimethoprim/sulfamethoxazole [17].

The susceptibility rates of isolates of *P. aeruginosa* and *A. baumannii* in this study to all antibiotics tested except for imipenem, were low compared to those reported in developed countries [10, 18, 19] and similar to those observed in Egypt [12] and in West Africa [15].

In conclusion, our study suggests the high rates of antimicrobial resistance among Gram-negative bacilli. The presence of *E. coli* and *Klebsiella* spp. isolates resistant to third generation cephalosporin suggests the importance of monitoring this phenotype. Particularly alarming is the appearance of low level imipenem resistance among different species of Gram negative bacilli studied. The results of this study indicate that more resources should be allocated to encourage good antibiotics utilization and practice. In addition, to provide information for choosing either empirical or direct therapy to physicians, surveillance to antimicrobial resistance is necessary.

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SHORT COMMUNICATION

Screening of Some Medicinal Plants from Cameroon for β-Lactamase Inhibitory Activity

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In efforts to find new bioactive β-lactamase inhibitors, this study investigated 16 Cameroonian plants belonging to 10 families which were evaluated for anti-β-lactamase activity. The investigation showed that extracts 2, 6, 3 and 5 of the 16 plants investigated presented interesting *in vitro* β-lactamase inhibition (over 90%), respectively, of the β-lactamases TEM-1, OXA-10, IMP-1 and P99. These extracts were from *Mammea africana* (all β-lactamases), *Garcinia lucida*, *G. kola* (OXA-10, IMP-1 and P99), *Bridelia micrantha* (OXA-10, P99), *Ochna afzelii* (OXA-10, P99), *Prunus africana* (IMP-1) and *Adenia lobata* (TEM-1). After elimination of tannins (according to the European Pharmacopoeia) the extracts from *B. micrantha*, *G. lucida* and *M. africana* were tested further for their anti-β-lactamase activity. The extracts from *B. micrantha* and *G. lucida* exhibited potent inhibitory activity, respectively, of β-lactamase OXA-10 (IC_{50} = 0.02 mg/mL) and P99 (IC_{50} = 0.01 mg/mL). The anti-β-lactamase activity of *M. africana* extract was weak. The isolation and the structural elucidation of the active constituents of *G. lucida* and *B. micrantha* will provide useful leads in the development of β-lactamase inhibitors.

Keywords: medicinal plants; β-lactamase; β-lactamase inhibitor; inhibitory activity; Cameroon.

INTRODUCTION

Traditional healing plays an integral part in black African culture and according to the World Health Organization's estimates, it provides for the primary heath care needs for a large majority (80%) of the population in Africa (WHO, 2002). In many places in Cameroon, there is a rich tradition in the use of herbal medicine for the treatment of various infectious diseases, inflammations, injuries and other diseases (Adjanohoun *et al*., 1996).

β-Lactams constitute one of the most important families of antibiotics, but resistance to these drugs has emerged following their widespread utilization (and sometimes over utilization) on a worldwide scale as antimicrobial agents. Resistance to this antibiotic family can be attributed to several contributing factors. However, the production of β-lactamases (EC 3.5.2.6) is the most important determinant of resistance (Livermore, 1995). These enzymes which hydrolyse the β-lactam ring have been the subject of extensive microbiological, biochemical and genetic investigations. More than 270 β-lactamases have been described (http://www.lahey.org/studies/webt.htm) and divided into four molecular classes: A, B, C and D (Ambler, 1980).

β-Lactamases-related resistance can be approached using multiple therapeutic interventions including non β-lactams, β-lactamase-stable β-lactams or β-lactamase inhibitors. The most convenient clinical approach is the combination of a suicide inhibitor (clavulanate, sulbactam and tazobactam) with a number of penicillins. These combinations inhibit the growth of bacteria producing molecular class A, β-lactamases, such as the common TEM (Temoniera) and SHV (sulphydryl variable) β-lactamases often encoded by plasmids in Gram-negative bacteria. Because the approved inhibitors do not have useful activity against the Class C chromosomal serine cephalosporinases or the metallo-beta-lactamases that are now appearing more frequently on multidrug resistant plasmids, their utility is becoming more limited (Doi *et al*., 2004). In addition, resistance to this combination has emerged (Livermore, 1995). Although a highly specific inactivator with broad-spectrum inhibitory

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activity and good pharmacological properties is highly attractive, this agent currently remains elusive.

This paper investigates some Cameroonian medicinal plants used by the population to cure various diseases by screening them for their potential β-lactamase inhibitory activity.

MATERIAL AND METHODS

Plant material. The fruits, seeds, stems, leaves, barks, twigs or roots [whichever parts of the plant are used in traditional medicine (Adjanohoun *et al*., 1996; Betti, 2004)] of 16 plant species (*Eremomastax speciosa*, *Picralima nitida*, *Bridelia micrantha*, *Garcinia lucida*, *G. Kola*, *Mammea africana*, *Afzelia bipindensis*, *Dorstenia picta*, *D. tenera*, *D. convexa*, *D. barterii*, *Erythrina variegata*, *Ochna afzelii*, *Ouratea flava*, *Adenia lobata* and *Prunus africana*) were collected from various places in Cameroon from 1998 to 2002 (Table 1). Samples were identified at the National Herbarium of Cameroon, where a voucher specimen of each species was deposited.

Preparation of extracts. Each batch of plant material was air dried and powdered. Between 15 and 60 g of each powder was then extracted with 500 mL of a cold mixture of methylene chloride/methanol (1:1) or methanol (Table 1), at room temperature and with constant shaking, for 24 h. Each resultant extract was filtered and then concentrated to dryness under reduced pressure.

Enzymes. The following purified β-lactamases of classes A, B, C and D were used in this study: TEM-1 (Class A); IMP-1 (Class B); P99 (Class C); OXA-10 (Class D). The β-lactamases were produced and purified as described by Galleni and Frère (P99, 1988), Ledent *et al*. (OXA-10, also called PSE-2, 1993), Vanhove *et al.* (TEM-1, 1995) and Laraki *et al*. (IMP-1, 1999).

Elimination of tannins. The crude plant extracts were treated with hide powder (Sigma) in order to remove tannins according to the method recommended by the European pharmacopoeia.

A mixture made of 10 mg of crude plant extract, 100 mg of hide powder and 10 ml of distilled water was stirred for 1 h. It was then centrifuged for 10 min at 16 000 × **g** and filtered through a 0.45 µm Millipore filter. The filtrate was lyophilized and the powder weighed and dissolved in 50 mM sodium phosphate buffer, pH 7 for activity tests with the various enzymes.

Enzyme assays. Crude plant extracts at a single concentration (6 mg/mL) were tested for their ability to inhibit the hydrolysis of nitrocefin (Oxoid, Ltd) by β-lactamase.

Plant extract solutions were prepared by dissolving each crude extract in 50 mM sodium phosphate buffer (pH 7.0). Eighty microliters of each plant extract solution was dispensed into the wells of microdilution plates. Control wells contained phosphate buffer alone. Ten microliters of diluted β-lactamase was added to each well. Preincubation was performed for 30 min at room temperature. The assay was started by the manual addition of 10 µL of nitrocefin (500 µM) to duplicate wells containing inhibitor and to the control wells. Measurement of the activity of β-lactamase was done with the help of a plate reading spectrophotometer (Powerwave, Bio-tek, UK) at 482 nm for at least 30 min. The initial velocity of β-lactamase catalysed hydrolysis was determined in each well as the absorbance units per minute (AU/min). The residual activity of enzyme was calculated as follows:

$$\text{Residual activity (\%)} = \frac{\textit{initial velocity (sample)}}{\textit{initial velocity (blank)}} \times 100$$

and the percentage of inhibition of the sample could be calculated by using the following equation:

$$\text{Inhibition (\%)} = 100 - \textit{residual activity}.$$

RESULTS AND DISCUSSION

The 16 plant species investigated are reported in Table 1, with the parts used and medicinal uses. The inhibition activity of crude extracts from these plants against four classes of β-lactamases examined in the present study was assessed quantitatively by measuring the inhibition percentage. The results are given in Table 1 and show that of the 19 plant extracts studied, **7**, **5**, **4** and **10** presented a poor anti-β-lactamase activity (less than 50%) for TEM-1, OXA-10, IMP-1 and P99, respectively. Out of **12**, **14**, **15** and **9** extracts which are active at 50% and more, **2**, **6**, **3** and **5** presented strong inhibition properties, respectively, of TEM-1, OXA-10, IMP-1 and P99 with values around 90%. These plants belong to five plant families (Euphorbiaceae, Clusiaceae, Ochnaceae, Passifloraceae and Rosaceae). The plant family Clusiaceae was the most important with three plants (*Garcinia lucida*, *G. kola* and *Mammea africana*) revealing the highest inhibition activity on almost all the β-lactamases tested.

The extracts from *M. africana*, *G. lucida* and *G. kola* showed a strong inhibition activity against OXA-10, IMP-1 and P99 (95%–100%, Table 1). The activity of OXA-10 and P99 was also inhibited by extracts of *Bridelia micrantha* (99.2%; 92%) and *Ochna afzelii* (98% and 94%, respectively). Only extracts of *M. africana* and *Adenia lobata* showed high inhibition of TEM-1 β-lactamase with values 99% and 90%, respectively (Table 1).

Taking into account that there is no well known inhibitor of Ambler class B, D and C β-lactamases, plants extracts from *B. micrantha*, *G. lucida* and *M. africana* were chosen for further study. These extracts were first treated with hide powder in order to remove tannins and their inhibition of β-lactamases was tested at several concentrations.

An extract of *G. lucida* showed good inhibitory activity against P99. At 0.012 mg/mL, the percentage of inhibition was 57%. The 50% inhibitory concentration (IC_{50}) of the extract was determined graphically as 0.01 mg/mL. With this extract, the inhibition of IMP-1 and OXA-10 were 39% and 57%, respectively, at a concentration of 0.4 mg/mL. However, for TEM-1, this inhibitory activity was 61% at a concentration 4 mg/mL.

Bridelia micrantha extracts exhibited good inhibition of OXA-10. At 0.03 mg/mL, the anti-β-lactamase

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Table 1. Plants screened for the presence of β-lactamases inhibitors

Family name	Botanic name	Voucher number	Location[a]	Part used	Solvent[b]	Uses[c]	β-lactamases inhibition (%)			
							TEM-1	OXA-10	IMP-1	P99
Acanthaceae	*Eremomastax speciosa* (Hochst.) Cufod.	5452/SRF/CAM	Ya	Stem	M	gu, an	44.80	53.70	63.20	40.50
Apocynaceae	*Picralima nitida* (Stapf.) T. & H.Durand	26128/SFR/CAM	Mb	Seed	M	ht, fe, ma	66.80	58.60	54.50	72.20
				Bark			58.10	51.30	39.60	38.90
Euphorbiaceae	*Bridelia micrantha* (Hochst.) Baill.	3179/SRFK	Ya	Bark	MCM	cough atb, gu, ve, od	62.70	99.20	73.20	92.00
Fabaceae	*Afzelia bipindensis* Harms	1334/SRFK	Ya	Root	MCM	pu, ve	59.10	85.80	84.10	53.20
	Erythrina variegata L.	49229/HNC	Ya	Bark	M	dy, fe, as, co, lc, di, le	42.30	59.90	67.00	46.40
Clusiaseae	*Garcinia lucida* Vesque	27844/HNC	Ya	Fruit	MCM	gi, gu, fpw	81.60	99.80	98.30	99.90
	Garcinia Kola Heckel	9815/SRF/CAM	NM	Bark	MCM		66.40	99.70	97.60	99.60
	Mammea africana G.Don	17276/SRF/CAM	Ya	Bark	M	sc, pu, abt, sy, go	99.40	97.60	95.90	96.60
Moraceae	*Dorstenia picta* Bur.	32453/SRF/CAM	To	Leaf	M	dh, wo, ai, atb, oc	35.10	66.70	85.30	42.40
	Dorstenia tenera Bur.	32455/SRF/CAM		Leaf	M		49.20	18.60	61.50	31.10
	Dorstenia convexa De Wild	53450/H.N.C. Yde		Twigs	M		31.90	24.70	32.40	30.60
	Dorstenia barterii Bur.	28157/SRF/CAM		Twigs	M		65.70	50.70	49.80	23.40
Ochnaceae	*Ochna afzelii* R. Br. Ex Oliv	8493/H.N.C.	Nk	Bark	M	th, rti	64.30	97.90	89.90	94.20
	Ouratea flava Hutchinson & Dalziel ex Stapf	27056/H.N.C.	El	Leaf	M	gu, rh	35.90	48.40	61.40	33.00
Passifloraceae	*Adenia lobata* (Schum. & Thonn.)	5962/SRF/CAM	Ba	Stem	M	co	28.50	36.20	80.80	42.10
				Leaf			90.40	68.00	52.30	38.40
Rosaceae	*Prunus africanus* (Hook. F.) Kalkman	35612/H.N.C.	Bal	Leaf	M	di, pro, ap, pu	66.00	96.80	81.90	88.90
				Bark			73.90	48.60	85.00	75.20

[a] Ya, Yaounde; Mb, Mbalmayo (Centre province); NM, Ngok Mapoubi (Centre province); To, Tombel (south West province); Nk, Nkolbisson (Yaounde); El, Elounden (Yaounde); Ba, Baham (West province); Bal, Balembo (West Cameroon).

[b] M, methanol; MCM, methylene chloride/methanol (v/v).

[c] abt, abortion; ai, antiinflammatory; an, anaemia; ap, abdominal pain; as, asthmas; atb, antimicrobial; co, cough; col, colics; dh, diarrhea; di, dermatological infection; di, diabetes; dy, dysentery; fe, fever; fpw, fermentation of palm wine; gi, gynecological infection; go, gonorrhea; gu, gastric ulcer; ht, hypertension; lc, liver complaint; le, leucorrhoea; ma, malaria; od, ocular disease; pro, prostatitis; pu, purgative; rh, rheumatism; rti, respiratory track infection; sc, scabies; sy, syphilis; th, toothache; ve, vermifuge; wo, wounds.

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activity was 75% and the IC_{50} was 0.02 mg/mL. This plant extract also exhibited a poor inhibitory effect at 4 mg/mL on TEM-1 and P99 (53% and 56% inhibition, respectively).

The inhibition of P99 and OXA-10, respectively, by *G. lucida* and *B. micrantha* extracts could be due to only one product since the residual activity was hyperbolic vs the concentration of the extract (result not shown).

The anti-β-lactamase activity of *M. africana* extract was poor. The inhibition percentages of TEM-1 and IMP-1 were 50% (4 mg/mL) and 31% (0.4 mg/mL), respectively.

Phytochemical information about most of the plants tested is limited. There appear to have been no previous reports on the anti-β-lactamase activities, or the chemical natures of the potentially inhibitory compounds of the 16 plant species investigated in the present study. However, some studies revealed the presence of coumarins, flavonoids and xanthones in *M. africana* (Ouahouo *et al*., 2004; Gangoué-Piéboji *et al*., 2006), flavonoids and xanthones in *G. kola* (Terashima *et al*., 1999; Iwu *et al*., 2002), cycloartane derivatives, anthocyane, flavonoids, saponins and triterpenes (Nyemba *et al*., 1990; Guedje, 2002), friedelin, taraxerone, epifriedelinol, taraxerol in *B. micrantha* (Pegel and Rogers, 1968; Kouam *et al*., 2005). It is probable that some of these compounds are responsible for the observed anti β-lactamase activities. In addition, *B. micrantha* is known to have antimicrobial properties (Abo and Ashidi, 1999; Lin *et al.*, 2002; Samie *et al*., 2005).

The isolation and the structural elucidation of the active constituents of *G. lucida* and *B. micrantha* will provide useful leads in the development of β-lactamase inhibitors.

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