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Antibacterial plant extracts inhibit the beta-lactamase of *Escherichia coli* clinical isolates

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Abstract
Beta-lactamase production is the main resistance mechanism to beta-lactam antibiotics by *Enterobacteriaceae* including *Escherichia coli*. In this study, we selected *Terminalia superba*, *Annona senegalensis*, *Ipomoea batatas* and *Psidium guajava*, which antibacterial properties have been proved with the aim to evaluate their beta-lactamase inhibitory activity. Production of β-lactamase was determined by modified tube acidimetric method using benzyl penicillin as substrate and phenol red in liquid medium. The tests were carried out on the ability of ethanolic plants extracts to reduce the hydrolysis of benzyl penicillin.
Three plant extracts showed beta-lactamase inhibitory activity, with the highest enzyme inhibition rate found with *Terminalia superba*. Plant extracts with beta-lactamase inhibiting potential can be associated with beta-lactam antibiotics which are no longer effective to overcome the growing resistance to these widely prescribed antibiotics. The finding of this study showed the potential of antibacterial plants in the search for new beta-lactamase inhibitors.

Keywords: *Escherichia coli*, beta-lactamase inhibitor, micro-acidimetric method, antibacterial plant extract

Introduction
In developing countries, infectious diseases are a public health problem because of their frequency and severity. Indeed, they are the cause of more than 17 million deaths per year in the world, more than half of which come from the African continent alone [1]. The discovery of penicillin (β-lactam) 2 was a major step forward in reducing mortality from infectious diseases [2]. Beta-lactam antibiotics are the most developed and widely prescribed antibacterial drugs due to their low toxicity and broad spectrum.
Several mechanisms are used by bacteria to resist beta-lactams. The production of inactivating enzymes, the beta-lactamases, is the most important resistance mechanism in *enterobacteria*. [3, 4]. With the widespread use of β-lactams, bacteria have evolved to diversify β-lactamases, extended their spectrum of activity, and spread among many species of *enterobacteria* [5]. They hydrolyze beta-lactams responsible for the antibacterial activity, causing treatment failure as many bacteria become resistant to most clinically used drugs. In addition, in recent years, there has been a significant decrease in the discovery of new antibiotics. The rapid emergence of antibiotic resistance is reducing the clinical life span of these drugs [6]. A way to overcome this resistance issue can be to search for compounds capable of inhibiting beta-lactamases which can be associated with already used antibiotics [7].
The present study was undertaken to evaluate TEM β lactamase production of *E. coli* strains isolated from urinary tract infections. The inhibition of these beta-lactamases with four plant extracts which have shown antibacterial activities in previous studies reported by many authors was determined.

Material and Methods
Plants material
Leaves of three plants *Annona senegalensis*, (*Annonaceae*), *Ipomoea batatas* (*Convolvovulaceae*), *Psidium guajava* (*Myrtaceae*) and *Terminalia superba* (*Combretaceae*) the stem bark of were harvested in Abomey-Calavi, Benin.

A voucher specimen for each species *T. superba* (N° YH623/HNB), *I. batatas* (N° YH624/HNB), *P. guajava* (N° YH625/HNB), *A. senegalensis* (N° YH626/HNB), were deposited at the Benin national herbarium, University of Abomey-Calavi. The plant materials were dried at 25±2 °C for two weeks. They were subsequently mashed using a grinder (Retsch type SM 2000/1430/Upm/Smf, Haan, Germany).

Bacterial strains

Twenty beta-lactamase producing bacterial strains of *Escherichia coli* from our laboratory microorganism collection were used. They have been isolated from patients affected by urinary tract infections at national university hospital Hubert Koutoukou Maga (HKM) in Cotonou, Benin. TEM type beta-lactamase genes have been previously detected in all the strains [8].

Preparation of plant extracts

To process the ethanolic plant extracts, 500 ml of 96° ethanol was added to 100 g of each plant powder. The mixtures were subjected to continuous stirring for 72 hours. The obtained extracts were filtered thrice using Whatman filter paper. The filtrates were evaporated in a rotary evaporator (IKA HB10S40, Germany). The resulting concentrated extracts were dried at 50 °C in the oven and kept at 4 °C in closed glass bottles until further use. All experiments were carried out thrice and the results were calculated as mean.

Extraction yield

The extraction yield (y) was obtained by calculating the ratio of the dried extract of plant to the dried weight of the plant. It is expressed as a percentage (%) and calculated according to the following formula:

$$y = \frac{ME \times 100}{MP}$$

Y: extraction yield (%); EM: extract mass (g); PM: powder mass (g).

Phytochemical analysis

The main secondary metabolites were researched in plants using conventional characterization methods. The presence of tannins and phenolic compounds were identified by the $FeCl_3$ test and Stiasny reagent. Flavonoids were detected by the reaction to cyanidine, saponoids by the foam test, triterpene and steroids by test of Liebermann-Burchard. The detection of mucilages was carried out by the ethyl ether test. Alkaloids were revealed by the test of Mayer and Dragendorf and reducing compounds by the Fehling reagent test. Coumarins were identified by their property to present a clear fluorescence to UV rays. Cyanogenetic compounds were revealed by the Born Trager reaction and free anthracenes were detected using diluted ammonia [9].

Biochemical detection of β-lactamase by the acidimetric method

The production of β-lactamase was determined by the acidimetric method using benzyl penicillin as substrate [10, 11]. The hydrolysis of benzyl penicillin (penicillin G) leads to penicilloate formation (Figure 1).

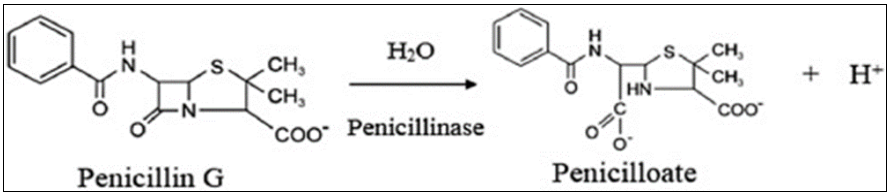

Fig 1: Hydrolysis of benzyl penicillin by beta-lactamases.

The violet colour of phenol red changes to yellow due to the acidification of the reaction medium. The bacterial inoculum was obtained from an overnight culture of the conserved *E. coli* strains on Luria Bertani agar plate. Four small colonies were picked up from the agar plate and suspended in 500 µl of 9% NaCl solution to give an opacity of a 0.5 McFarland standard.

To prepare the indicator solution, 1.000.000 units of benzyl penicillin were dissolved in 800 µl of sterile distilled water, to which 100 µl of 1N NaOH solution was added to adjust the pH to 8. The mixture turned to fuchsia colour after mixing 300 µl of 0.3% phenol red. Then 150 µl of the prepared indicator solution was added to the bacterial inoculum. The colour development was observed every 10 min within 1hour. A colour change to orange or yellow should occur depending of the level of the amount of enzyme present in the inoculum. The strains selected for this study were clinical isolates of penicillinase and extended spectrum beta-lactamase producing *Escherichia coli*. Their antibiotic resistance pattern is summarised in table1. *E. coli* ATCC 25922 strain was used as negative control.

Table 1: Resistance pattern of the used *Escherichia coli* strains

Antibiotics	ESBLs		Penicillinases			Control (*E. coli* ATCC 25922)
	EB1	EB2	EP1	EP2	EP3	
Amoxicillin	R	R	R	R	R	S
Amoxicillin + clavulanic acid	R	I	R	I	S	S
Cefalotin	R	R	I	I	S	S
Ceftriaxone	R	R	S	S	S	S
Cefotaxime	R	R	S	S	S	S
Aztreonam	R	R	S	S	S	S
Imipenem	S	S	S	S	S	S
Doxycycline	R	R	R	R	R	S
Netilmicin	R	R	S	S	S	S
Amoxicillin	R	R	S	S	S	S

Gentamicin	S	I	S	R	S	S
Ciprofloxacin	S	I	S	R	S	S
Ofloxacin	S	I	S	R	S	S
Nalidixic acid	R	R	R	R	S	S
trimethoprim/sulfamethoxazole	R	R	R	R	R	S

ESBLs: extended spectrum beta-lactamases; R: resistant; I: intermediate; S: sensitive

Beta-lactamase inhibition by ethanolic plant extracts

The detection of beta-lactamase is performed with the acidimetric method described above using microtiter plates. The hydrolysis of the benzyl penicillin was compared with the reaction in presence of the plant extracts. A control without the substrate was included to evaluate the effect of the plant extracts colour at the reaction medium.

Briefly, to 500 µl of the bacterial suspension comparable to 0,5 MacFarland, 50 µl of the ethanolic plant extract was added (50 mg/ml). Then the mixture was incubated at 37 °C for 1 hour. After incubation, 150 µl of benzyl penicillin reagent was added and the reaction medium was put at 37 °C for 1 hour. When compared with the test tube without plant extracts, changes of the reaction medium indicate varying degree of inhibition due to β-lactamase. The inhibitory activity of the plant extracts reduces or fully prevents the hydrolysis of benzyl penicillin.

Statistical analysis

The results of the plants extraction were expressed as mean values of the triplicate using Microsoft Excel 2016. Differences were analysed using the R software (Version 3.6.2). *P*-values less than 0.05 were considered significant.

Results

Extraction yield of the plant extracts

As showed in table 2, the extract of *Terminalia superba* have the highest yield (22%), following with *Psidium guajava* (16.3%) and *Annona senegalensis* (14.3%) whereas *Ipomoea batatas* having the lowest at 9.2% (Table2). All the differences between the extraction yields were statistically significant at 0.05.

Table 2: Comparison of the extraction yields

Botanical name	Used part	Weight of plant (g)	Weight of extract (g)	Extraction Yield (%)
Annona senegalensis	Leaves	100	14.35	14.35 [c]
Terminalia superba	Leaves	100	21.30	21.30 [a]
Ipomoea batatas	Leaves	100	9.04	9.04 [d]
Psidium Guajava	Stembarks	100	16.25	16.25 [b]

% represent percentage of extraction yield. Letters a, b, c, d: represent significant differences at $p \leq 0.05$. a > b > c>d.

Phytochemical composition of the used plant parts

The results of the qualitative phytochemical tests showed that the four plants contain various phytochemicals compounds. Leaves of the three plants *T. superba*, *A. senegalensis* and *I. batatas* and the stembark of *P. guajava* revealed the presence of alkaloids, flavonoids tannins and C-hétérosides. Catechins tannins were present in the four plants extracts whereas only *Annona senegalensis* do not contain gallic tannins. Quinone derivatives and cyanogenetic derivatives were not founded (Table 3).

Table 3: Phytochemical constituents of the used four plants extracts

Phytochemical tests	Results			
	A. senegalensis	*P. guajava*	*T. superba*	*I. Batatas*
Tannins	+	+	+	+
Gallic tannins	-	+	+	+
Catechin tannins	+	+	+	+
Antocyanins	-	-	-	+
Saponosids	+	+	-	-
Leucoanthocyanins	+	+	-	+
Flavonoids	+	+	+	+
Reducing compounds	-	+	-	-
Quinone derivatives	-	-	-	-
Cyanogenic derivates	-	-	-	-
Free anthracenics	-	+	+	-
Coumarins	+	+	+	-
Alkaloids	+	+	+	+
Mucilages	+	-	+	+
O-hétérosides	+	-	-	-
C-hétérosides	+	+	+	+
Triterpenoids	+	-	-	-

+: indicate the presence of constituent; - indicate the absence of constituent

Detection of beta-lactamase producing *E. coli* strains

Out of the tested twenty strains, beta-lactamase production was detected at varying levels corresponding to the different colour changes of the violet reaction medium as shown in figure 2.

Five strains exhibit high degree of substrate hydrolysis (+++) with turn of fuchsia colour of the reaction medium to yellow, nine strains showed mean degree of hydrolysis (++) since the reaction medium turned to deep orange, and six strains have low production of beta-lactamase (+) with colour change to deep orange. The control strain *E. coli* ATCC 25922 as expected has showed no colour change (-) (figure 2).

The five strains with higher production of beta-lactamase enzymes were used to evaluate the inhibitory potential of the plant extracts.

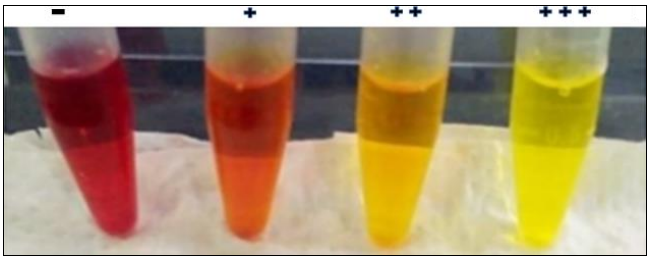

Fig 2: Tubes showing beta-lactamase detection using acidimetric test method

Inhibition of beta-lactamases by the tested plant extracts

The results of the beta-lactamases inhibition with the plant extracts is summarized in table 4. Out of the four extracts, three (*P. guajava, A. senegalensis* and *T. superba,*) have inhibited the hydrolysis of benzyl penicillin by the two extended spectrum beta-lactamases (EB1 and EB2) and the penicillinases (EP1,EP2 and EP3) from *E. coli* at varying degrees. Indeed, ethanolic leaves extract of *T. superba* has showed strong beta-lactamase inhibition activity. No inhibition of the tested beta-lactamases was observed with the extract of *I. batatas*. None of the plants extracts have an inhibitory action of the strain EP2 (table 4).

Table 4: Inhibition of the beta-lactamases by plant extracts

Plant ethanolic extracts	ESBLs *E. coli*		Penicillinases *E. coli*		
	EB1	EB2	EP1	EP2	EP3
A. senegalensis	++	+	+	-	-
T. superba	++	+	+	-	+
I. batatas	-	-	-	-	-
P. guajava	+	-	+	-	-

High inhibition of beta-lactamase (++); Low inhibition of beta-lactamase (+); no inhibition of beta-lactamase (-); EB: ESBL producing *E. coli*; EP: penicillinase producing *E. coli*; ESBLs: extended spectrum beta-lactamase.

An example of the acidimetric assay showing beta-lactamase inhibition with *Terminalia superba* and *Annona senegalensis* is depicted in figure 3. The first five lanes contained penicillinase strains (EP3, EP2 and EP1) and extended-spectrum beta-lactamase strains (EB1 and EB2). The mixtures of water (W) and substrate (S) are in the sixth lanes. The last lanes contained the negative control strain *E. coli* ATCC 25922 (NCS) and substrate (S).The wells of row A are controls without plant extract. Rows B and C witch contained *T. superba* and *A. senegalensis* respectively revealed various degrees of inhibition when compared with row A.Orange or deep orange colour indicate the presence of inhibition and yellow colour indicate no inhibition.

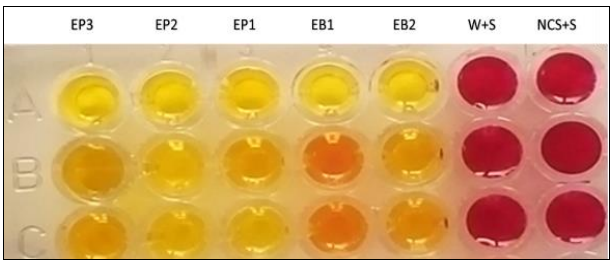

Fig 3: Beta-lactamase inhibition by plant extracts in a microtiter plate

Discussion

The yields of ethanolic extracts from the leaves differ from one plant to another. Several works have been reported about these four plants in the literature. The extraction yields values reported for *Annona senegalensis* [12] (7, 34%) and for *Ipomoea batatas* [36] (7.98%) were similar to the values of Loko for *Terminalia superba* leaves [14] and lower as the extraction yield of our study. This difference would generally be explained by the influence of the place of harvest, of the season, or the vegetative stage of the plant [15, 16].

Twenty strains with varying antibiotic profiles were used for the biochemical beta-lactamase detection by the acidimetric method using phenol red as colour indicator. This colorimetric method is less expensive and easy but can be influenced by the colour of the plant extract if the latter has a similar colour like the indicator used. For the detection of beta-lactamases production several tests were affordable. The detection of the beta-lactamase gene by PCR and sequencing are the most perform ant and expensive methods. Their results are not available quickly and cannot be often performed in a clinical laboratory due to special technical conditions. Among the biochemical detection methods, the use of the chromogenic beta-lactamase substrate nitrocefin is considered as the reference. The iodometric method which is based on the ability of penicilloic acid to destabilize the iodine-starch complex has been described by many authors. In this study we have chosen the acidimetric method using benzyl penicillin as substrate in association with phenol red as colour indicator. Both acidimetric and iodometric methods were simple and rapid to perform and affordable. When compared with the nitrocefin method, they showed similar or very close percentage of the detection of beta-lactamase producers. Indeed [17], reported percentages of respectively 79.9-83% for iodometric and 78-80.5% for acidimetric methods in comparison to 83.9% of beta-lactamase producing strains founded with the nitrocefin detection. This author pointed out that the iodometric method rather detected coagulase positive beta-lactamase producing staphylococci than coagulase negative. A study conducted in Nepal in 2014 has shown similar conclusions in detection of beta-lactamase producing staphylococci [18]. In contrast, Samant who investigated beta-lactamase production also in staphylococci reported similar results with acidimetric and iodometric methods [19]. No differences between the three biochemical methods were observed in tests performed with *Neisseria gonorrhoeae* and *Haemophilus influenzae* [10].

The inhibitory activity of four plant extracts which antibacterial activities have been already proved, was carried out using two ESBL and three penicillinases *Escherichia coli* strains.

Among the four tested plants extracts, none has inhibited the beta-lactamase of one penicillinase producing *E. coli* strain (EP2). This penicillinase producing strain EP2 was intermediate for amoxicillin + clavulanic acid, suggesting a low efficiency of inhibitors toward the secreted beta-lactamase. The ethanolic extract of *Ipomoea batatas* has no inhibitory effect on the tested *E. coli* strains. This finding could be explained with the different antibiotic resistance patterns of the used strains. In the same way, *Psidium guajava* which has not exhibited beta-lactamase inhibitory activity in a previous work performed in our laboratory [20], has shown in the present study inhibitory activity on two of the tested five strains. It should be remembered that the activity of a plant substance depends on several factors, including the extraction method, the concentration of the active compounds and the used bacterial strains [21].

Several authors have reported the antibacterial activity of *Ipomoea batatas* against various bacteria include *E. coli* [22, 23,

[24]. The antibacterial property of *Annona senegalensis* has been described in literature [37, 26]. Tests have shown the antibacterial activities of *Psidium guajava* [27, 28, 29]. The antibacterial activities of *Terminalia superba* have been demonstrated [30, 31, 20]. The inhibitory activity of *Annona senegalensis* and *Terminalia superba* on beta-lactamases in this study agrees with previous works performed in our laboratory in 2009 [20]. Considering the reported antibacterial activities of the used plant extracts, in addition to the results of the present study, one could evoke the synergistic coexistence of an antibiotic and a beta-lactamase inhibitor. The detection of clavulanic acid in the extract of *Rumex vesicarus* which was active against the beta-lactamase of *Pseudomonas aeruginosa* [32] supports this claim**.** Drug combination similar to amoxicillin+ clavulanic acid can occur in herbal remedies. Association of beta-lactamase inhibitors with beta-lactams are successfully used in the treatment of multidrug resistant bacteria**.**

All the four plants used contain flavonoids. Boussoualim has described beta-lactamase inhibitory activities of polyphenols and flavonoids. This property is due to the 4-oxo function of flavonoids which is similar to that of clavulanic acid and penicillin G [33]. Others authors have proved the beta-lactamase inhibitory activities of natural compounds extracted from *Spondias mombin* and *Rheum rhaponticum* [34, 35]. Some of the phytochemical compounds detected in the plant used in the present study may have been responsible for the beta-lactamase inhibitory activity on the tested *E. coli* strains.

Conclusion

This study was undertaken to determine beta-lactamase inhibitor activity of four plants with known antibacterial properties, using TEM beta-lactamase producing *Escherichia coli* strains issued of our laboratory microorganism collection. Ethanolic leaves extracts of *A. senegalensis*, *T. superba* and stembark of *P. guajava* revealed beta-lactamase inhibitory activity.

These plants may contain many phytochemicals compounds which are capable of beta-lactamase inhibitory activity.

Plant extracts with beta-lactamase inhibitory activity can be associated with antibiotics which are no longer effective in the treatment of infections due to beta-lactamase production. The findings of this study are interesting in insight of extending the spectrum of actually clinical affordable inhibitors like clavulanic acid, sulbactam and tazobactam. Further isolation of the active molecules of these plant extracts can be useful in the search for new beta-lactamase inhibitors.

Conflict of interests

None of the authors has declared any conflict of interests.

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