

Original Article

THE INHIBITORY EFFECT OF METHANOLIC AND AQUEOUS EXTRACTS OF *TERMINALIA CHEBULA* DRIED FRUITS ON METALLOBETALACTAMASES OF *ACINETOBACTER BAUMANNII*

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ABSTRACT

Objective: to evaluate Metallo-beta-lactamase (MBL) inhibitory effect and antimicrobial activity against *Acinetobacter baumannii* of methanolic and aqueous extracts of *Terminalia chebula* dried fruits.

Methods: effect of EDTA (Ethylenediaminetetraacetic acid), methanolic and aqueous extracts of dried fruits of *T. chebula* on Metallo-beta-lactamase activity was studied *in vitro* using cephaloridin as substrate. The degree of inhibition was determined by IC_{50} and the antimicrobial activities of both extracts against *Acinetobacter baumannii* were qualitatively and quantitatively assessed by the presence or absence of inhibition zones, MIC and MBC values.

Results: EDTA, methanolic and aqueous extracts inhibit metallo-beta-lactamases activity with IC_{50} (3.98 mM, 18.45 and 19.68 μ g/ml), respectively. Polyphenols in aqueous and methanolic extracts are 513.094 \pm 3.061 and 496 \pm 2.856 μ g GAE/mg, respectively.

Conclusion: These results indicate that both extracts of *T. chebula* are rich in polyphenols and have a strong inhibitory effect on metallo-beta-lactamases in a competitive manner compared to EDTA. In addition, both extracts of *T. chebula* were also screened for their remarkable antibacterial and bactericidal activity against *Acinetobacter baumannii* confirmed by low minimum inhibitory and bactericidal concentration (MIC and MBC).

Keywords: Metallo-beta-lactamases, *Acinetobacter baumannii*, *Terminalia chebula*, Cephaloridin, EDTA.

INTRODUCTION

Bacteria have developed several resistance mechanisms against beta-lactam antibiotics. Among them, generation of beta-lactamases, which hydrolyze the amide bond in beta-lactam rings to inactivate beta-lactam antibiotics. Beta-Lactamases can be grouped into four classes according to their sequence homology [1]. Class A, C, and D enzymes, generally called serine beta-lactamases, use a serine residue in their active site to hydrolyze beta-lactam antibiotics [2]. Class B enzymes, also called Metallo-Beta-Lactamases (MBLs), and are characterized by one or two zinc ions (Zn) that are indispensable for their activity [3]. The clinical inhibitors for these enzymes are not known [4]. EDTA is the only known inhibitor used for its detection [5]. The MBLs are further divided into B1, B2, and B3 subclasses [6]. Both B1 and B3 subclasses have a broad-spectrum substrate profile including penicillins, cephalosporins and carbapenems. In contrast, the B2 subclass enzymes primarily hydrolyze carbapenems [7].

Medicinal plants have long been utilized as a source of therapeutic agents. Plants are known to produce different secondary metabolites which are naturally toxic to bacteria. Lin et al proposed the combination of antibiotics and flavonoids as a potential new strategy for development of new therapies in the future, for infections caused by extended spectrum of beta-lactamases (ESBLs) producing bacteria [8]. *Terminalia chebula* Retz indigenous to Pakistan and India, is a member of the Combretaceae family.

The dried fruit of *T. chebula* (*Chebulae Fructus*), commonly known as black myrobalan in English and Xi-Qin-Ge or Zhang-Qin-Ge in China, have traditionally been used as a popular folk medicine [9] for alternative, astringent, denrifice, purgative, stomachic, tonic, antiseptic, cardiogenic and laxative purposes. This fruit is also useful for burns, digestive disorders, diabetes, eye diseases, weak eye sight, fever, skin diseases and kidney dysfunction along with other herbs [10]. In efforts to find new bioactive beta-lactamase inhibitors, which can be used against the resistance strain *Acinetobacter baumannii*, this study investigated the aqueous and methanolic extracts of

Terminalia chebula dried fruits, which were evaluated for their inhibitory effect on metallo-beta-lactamases and antibacterial activity against *Acinetobacter baumannii* producing MBLs; where the kinetics parameters were investigated.

MATERIALS AND METHODS

Plant material

The dried fruits were purchased from a local herbal market in Setif, Algeria, in February 2013 and were identified as fruit of *Terminalia chebula* (Family: Combretaceae) by Prof. LAOUERH, Department of Ecology and Vegetal Biology, University Setif-1; then it was powdered using a coffee grinder (Moulinex, Paris, France), and utilized in our studies.



Bacterial strain

The *A. baumannii* strain was collected from a hospitalized patient at Setif University Hospital in 2013. It was identified with a series of 20 ready biochemical compounds (API-20 E gallery, Biomérieux - France).

Chemicals

All chemicals used were purchased from Sigma Aldrich (Germany) and all solvents were distilled before use.

Preparation of extracts

Methanolic extract was prepared by maceration of 30 g of dried fruit powder of *T. chebula* in 80% methanol for 48 h, with continuous

stirring at room temperature; after filtration, the filtrate was concentrated under reduced pressure at 40 °C and centrifuged. Aqueous extract was prepared by decoction of 30 g of dried fruit powder of *T. chebulain* 300 ml of distilled water at 100°C for 20 min; the decoction obtained was filtered with a Whatman paper (pore size 3 µm) then centrifuged.

- Both extracts were lyophilized then stored at -20°C and used in the present study.

Estimation of total phenolic content

Total phenolic content of aqueous and methanolic extracts was determined by the Folin-Ciocalteu method [11].

Preparation of β-lactamases

A. baumannii strain was grown in 1.5 ml of brain heart infusion (BHI) at 37°C with 250 µg of imipenem under vigorous agitation for 24 h. The cells were concentrated by centrifugation at 18000 × g for 30 min, and crude enzymatic preparations were made by sonicating (vibracell-USA) for 12 seconds (two cycles) with 12 seconds cooling in between sonications; this was repeated seven times. The lysates were centrifuged at 4°C, 20 000 × g for 30 min. Nucleic acids were precipitated by the addition of 2% streptomycin sulfate and centrifuged at 18000 × g for 30 min. The supernatant was dialyzed overnight and concentrated with polyethylene glycol (PEG) against phosphate buffer (pH 7, 0.5M) and used for further analysis.

Characterization of betalactamases

Acinetobacter baumannii was confirmed to be MBLs producing isolates by the imipenem-EDTA combined disc test (CDT), substrate profile, and their sensitivity to EDTA and clavulanic acid.

Imipenem-EDTA combined disc test (CDT)

The MBL phenotypic detection method employed was the imipenem-EDTA combined disc test (CDT). The *Acinetobacter baumannii* strain was adjusted to the McFarland 0.5 standard and were used to inoculate Mueller-Hinton agar plate, Two 10 µg imipenem discs were placed on the surface of the agar plate and EDTA (0.5M, pH8) solution was added to one of them to obtain a concentration of 1900 µg. The inhibition zones of imipenem and imipenem-EDTA discs were compared after 16-18 h of incubation at 35°C.

Determination of β-lactamases activity

All reactions were performed at 30°C in 0.65 ml of assay buffer (50 mM HEPES; pH 7.2; 50 µM ZnSO₄; 150 mM NaCl and 0.005 mg BSA/ml) and 10 µl semi-purified extract of metallo-betalactamases.

Different classes of betalactams antibiotics (penicillin G [$\epsilon_{235 \text{ nm}} = 560 \text{ M}^{-1} \cdot \text{cm}^{-1}$], cephaloridin [$\epsilon_{260 \text{ nm}} = 1000 \text{ M}^{-1} \cdot \text{cm}^{-1}$], imipenem [$\epsilon_{299 \text{ nm}} = 9000 \text{ M}^{-1} \cdot \text{cm}^{-1}$] and aztreonam [$\epsilon_{320 \text{ nm}} = 700 \text{ M}^{-1} \cdot \text{cm}^{-1}$]) were used as substrate to determine the kinetic parameters K_m and V_{max} . The assays were performed using a UV-1800 spectrophotometer (shimadzu, JAPAN) by observing changes in absorption resulting from the opening of the β-lactam ring at specific wavelengths for each antibiotic evaluated. The results are presented as the average ± standard deviation based on four independent measurements. The protein concentration of semi-purified extract were determined using the Bradford assay [12]; the Coomassie Plus protein assay reagent and bovine serum albumin as a standard. Kinetic parameters were derived from the initial velocity using SIGMAPLOT 12.0.

Assay of Metallo-betalactamases inhibitory activity

To examine the inhibition effect of EDTA, clavulanic acid, aqueous and methanolic extracts, an enzyme assay was performed by determining the IC₅₀, which is defined as the concentration of inhibitor necessary to inhibit 50% MBLs activity. The assay was performed under previously described conditions after incubating the reaction mixture for 10 min with varied concentrations of EDTA (1 µM to 5 mM), clavulanic acid (1 µM to 7 mM), aqueous and methanolic extracts (1 µg/ml to 1 mg/ml); respectively, before initiating the reaction with cephaloridin. Percentage of Inhibition of beta-lactamase was calculated as follows:

$$\text{Inhibition \%} = 100 \times (A_c - A_e) / A_c$$

Where, A_c: metallo-betalactamase activity without inhibitors, and A_e: metallo-betalactamase activity in the presence of plant extracts or inhibitors.

Antibacterial activity

The antibacterial effect of methanolic and aqueous extracts of *Terminalia chebula* dried fruits was tested against *Acinetobacter baumannii* using disc diffusion assay described by Genç *et al* [13]. Briefly, 20 ml of Muller-Hinton (MH) agar were poured in sterile Petri plates and allowed to solidify. Then, 100 µl of overnight test *A. baumannii* cultures were spread on the surface of the dried MH agar. Sterile antibiotic discs (6 mm diameter) were placed on the surface of the inoculated plate and filled with 1500 and 3000 µg/ml of the extracts. Gentamycin (10 mg/disc) was used as a reference antibiotic control, whereas, sterile distilled water was the negative control. After incubation at 37°C for 24 hours the growth of inhibition zones formed around the disc were measured with a transparent ruler in millimeter and documented. The solvent used was tested and did not show any inhibition of bacterial growth. The results are averages of triplicate tests ± standard deviation.

Determination of MIC and MBC

Minimum inhibitory concentration (MIC) was done by the method described by Verporte *et al.* [14]. Briefly, the extracts were incorporated into Mueller-Hinton broth at concentration ranging from 0.01-1 mg/ml. A control tube containing the growth medium and *A. baumannii* was set-up. The mixtures were incubated at appropriate temperature of 37°C for 24 h. The minimum inhibitory concentration (MIC) of the extracts was regarded as the lowest concentration of the extract that did not permit turbidity or growth of *A. baumannii*.

Minimum bactericidal concentration (MBC) value was determined by sub culturing the test dilution [which showed no visible turbidity] on to freshly prepared nutrient agar media. The plates were incubated further for 24 h at 37°C. The highest dilution that yielded no single bacterial colony on the nutrient agar plates was taken as MBC.

RESULTS

Total phenolic contents

The determination of total phenolic contents of aqueous and methanolic extracts of *Terminalia chebula* dried fruits was conducted using Folin-Ciocalteu, it was expressed as µg gallic acid equivalent per mg dried extract (µg GAE/ mg E). Polyphenols in aqueous and methanolic extracts are 513.094 ± 3.061 and 496 ± 2.856 µg GAE/mg dried extract, respectively. These values showed that the both extracts are rich in phenolic compounds.

Confirmatory test

In this study, the imipenem-EDTA combined disc test (CDT) was used as a phenotypic confirmatory method for metallo-betalactamases production. An increase in the inhibition zone of EDTA+IMP in comparison with IMP was interpreted as a positive result for metallo-betalactamases production. Our study showed that a disk with imipenem plus 1900 µg of EDTA increase the mean inhibition zone diameter by 17 mm (8 mm with IMP; 25 mm with EDTA+IMP), However, this test was positive for *A. baumannii* imipenem resistant isolate, indicating metallo-betalactamases production (Figure 1).



Fig. 1: It Shows MBL combined disc test positive of *Acinetobacter baumannii*

Beta-lactamases activity

The kinetic parameters of the semi-purified metallo-beta-lactamase, including K_m and V_m , were determined using different β -lactam class. Under the adopted experimental conditions, this enzyme is able to hydrolyse all tested β -lactam antibiotics including benzylpenicillin, cephaloridin and imipenem except aztreonam (monobactam). From these results, the semi-purified metallo-beta-lactamase is cephalosporinase showing the highest activity with cephaloridine as substrate ($V_{max} / K_m = 8.80 \pm 0.014$). The enzyme showed low activity and less affinity for imipenem than other substrate (Table 1).

Sensitivity to EDTA and clavulanic acid

Examination of the semi-purified metallo-beta-lactamase from *A. baumannii* in the presence of cephaloridin with and without EDTA

and clavulanic acid illustrated that the metallo- β -lactamase activity was inhibited in the presence of EDTA with $IC_{50} = 3.98$ mM in a competitive manner. The clavulanic acid had no effect on this semi-purified metallo-beta-lactamase.

The inhibitory effect of methanolic and aqueous extracts

The objective of this part of study was to evaluate the inhibitory effect of methanolic and aqueous extracts on the metallo-beta-lactamases of *Acinetobacter baumannii*. The enzyme activity was tested in the presence of increasing concentrations of both extracts of *Terminalia chebula* dried fruits. The IC_{50} of EDTA against this MBLs was 3.98 mM. Therefore, MBLs were more susceptible to methanolic and aqueous extracts of *T. chebula* dried fruits with IC_{50} values 18.45 and 19.68 μ g/ml, respectively, than EDTA.

Table 1: Kinetics parameters of metallo-beta-lactamases with beta-lactams antibiotics

beta-lactams class	substrate	nm	K_m (μ M)	¹ Specific activity	² Efficiency of hydrolysis
penem	Penicillin G	235	14.92 \pm 0.00888	80.64 \pm 1.147	5.40 \pm 0.036
cephem	cephaloridin	260	15.15 \pm 0.009	133.33 \pm 2.036	8.80 \pm 0.014
carbapenem	imipenem	299	47.62 \pm 0.012	52.57 \pm 0.973	1.10 \pm 0.002
monobactam	aztreonam	320	∞	0	0

¹ μ mol of substrate hydrolysed per mn and per mg of protein (μ mol. min^{-1} . mg^{-1}), ² μ mol. min^{-1} . mg^{-1}/K_m , The results are mean \pm SD (n=4)

Table 2: Antibacterial activity of methanol and aqueous extracts of Terminalia chebula dried fruits

organism	Zone of inhibition in (mm) at 1500 μ g/disc		Zone of inhibition in (mm) at 3000 μ g/disc	
	Methanolic extract	Aqueous extract	Methanolic extract	Aqueous extract
<i>A. baumannii</i>	17.5 \pm 1	18 \pm 0.5	22.5 \pm 0.5	24 \pm 0.25

The results are mean \pm SD (n=3)

Table 3: Antibacterial activity of methanol and aqueous extracts of Terminalia chebula dried fruits

organism	MIC (μ g/ml)		MBC (μ g/ml)		MBC/MIC
	Methanolic extract	Aqueous extract	Methanolic extract	Aqueous extract	Methanolic and aqueous extracts
<i>A. baumannii</i>	31.25 \pm 0.000	31.25 \pm 0.000	62.5 \pm 0.000	62.5 \pm 0.000	2 \pm 0.000

The results are mean \pm SD (n=3), MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration

Aqueous and methanolic extracts at different concentrations showed that they inhibit the enzyme activity in a dose dependent manner. The inhibition percentage was determined for each concentration, in the range of 10 to 40 μ g / ml, the inhibition increased from 23.52 \pm 0.0123% to 90.70 \pm 0.033% of the methanolic extract and 21.72 \pm 0.0265% to 87.96 \pm 0.098% of the aqueous extract.

Antibacterial activity

The antibacterial activity of methanol and aqueous extracts of dried fruits of *T. chebula* against *A. baumannii* were measured by measuring the zone of inhibition in disc diffusion method (Figure 2). Test sample per disc was about 1500 and 3000 μ g/disc. The zone of inhibition of methanolic and aqueous extracts is shown in Table 2. Negative control and Gentamycin did not show any inhibitory effect against *A. baumannii*.

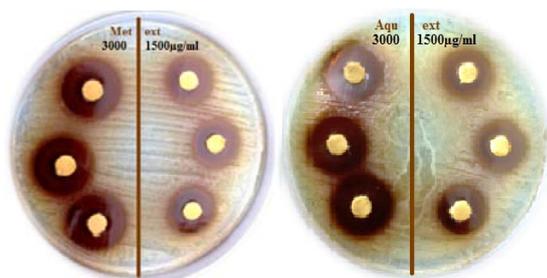


Fig. 2: It Shows the Antibacterial activity of methanolic and aqueous extracts of Terminalia chebula dried fruits at 1500 and 3000 μ g/ disc against Acinetobacter baumannii.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal (MBC)

MIC and MBC values obtained from the extracts of *T. chebula*, revealed that aqueous and methanolic extracts had the same MIC and MBC values: 31.25 \pm 0.000 and 62.5 \pm 0.000, respectively; MBC is twofold higher than MIC and the MIC/MBC ratio is 2 (Table 3).

DISCUSSION

Beta-lactamases can be divided into serine- β -lactamases and metallo- β -lactamases, which employ a serine and one or two zinc ions in the active site, respectively [15]. Except monobactams, the MBLs can degrade almost all classes of β -lactams including carbapenems, which are stable against these serine- β -lactamases. The currently clinical used beta-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam can poorly inhibit MBLs [16]. In this study, the positive result of CDT, inhibition by EDTA, insensitivity to clavulanic acid and the ability to hydrolyze all classes of beta-lactams except aztreonam establishes the enzyme as a metallo-beta-lactamase.

EDTA is a metallo-chelator known to potentiate the activities of some lactam antibiotics against MBL-producing microorganisms because the zinc-containing active site of MBL is inactivated by EDTA [17], [18]. In this study, we found that EDTA inhibit hydrolysis of cephaloridin by MBLs but with high concentration, indicating a stronger activity of MBLs in the extract of *A. baumannii*. Despite the toxicity of chemicals compounds as EDTA, which hinders its clinical use [19], research of natural MBLs inhibitors and alternative antibacterial drugs is an urgent need. The use of plant derived

natural compounds used as alternative sources of medicine continues to play major roles in the general wellness of people all over the world. The curative properties of medicinal plants are due to the presence of various complex chemical substances of different composition which occur as secondary metabolites such as phenolic compounds. Methanolic and aqueous extracts of *T. chebula* have been reported to exhibit a variety of biologic effects [20], and antimicrobial activity [21]. In the present study, we compared the inhibition effects of EDTA with methanolic and aqueous extracts of *T. chebula* dried fruits on MBLs of the extract of *A. baumannii*. Therefore, MBLs were more susceptible to methanolic and aqueous extracts of *T. chebula* dried fruits than EDTA. We were still curious if there is an effect on the *Acinetobacterbaumannii* strain as the inhibitory effect of the extracts of *Terminaliachebula* on the semi-purified extract of metallo beta-lactamase of the same strain, for this reason, the extracts of *Terminaliachebula* dried fruits were also screened for their antimicrobial activity against *Acinetobacterbaumannii* strain; The result showed that the methanolic and aqueous extracts of *T. chebula* dried fruits which have a strong MBLs inhibitory effect are potentially a rich source of antimicrobial agents (polyphenols) that confer a good antibacterial activity against *A. baumannii* with the same MIC and MBC; The MIC/MBC ratio indicated that extracts are bacteriostatic at lower concentration, while at higher concentration, are bactericidal. These effects could probably be due to phenolic compounds such tannin and flavonoids present in the *T. chebuladried* fruit extracts, which needs to be confirmed. Tannins act similar to EDTA so is capable to inhibit MBL which is an enzyme that requires Zn²⁺ for its mode of action and is known also to show the antibacterial activity by precipitation of the microbial proteins. Flavonoids are produced by the plants for the defense against the infection. So, the use of the methanolic and aqueous extract of this plant as an agent to decrease the resistance of *A. baumannii* to beta-lactams antibiotics needs further extensive research for their better economic and therapeutic utilization. The findings from this work may add to the overall value of the medicinal potential methanolic and aqueous extracts of *T. chebula*. Further phytochemical studies are required to determine the purified fractions/bioactive compounds responsible for the MBLs inhibitory and antibacterial activities of these species.

CONCLUSION

The results obtained in the present study can be concluded that *Acinetobacterbaumannii* resistance to beta-lactams antibiotics by production of Metallo-beta-lactamases. Aqueous and methanolic extracts of *Terminaliachebula* are rich in phenolic compounds; possess *in vitro* significant inhibitory effect on MBLs activity and have also a good antibacterial activity against *A. baumannii* confirmed by low minimum inhibitory and bactericidal concentration (MIC and MBC). *T. chebula* dried fruit can therefore be used as a natural antibacterial agent for the treatment of several infectious diseases caused by *Acinetobacterbaumannii*.

CONFLICT OF INTERESTS

Declared None

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