

## Inhibition of Beta-Lactamase by 1,4-Naphthalenedione from the Plant *Holoptelea integrifolia*

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**Abstract** The most important mechanism of the beta-lactam antibiotic resistance is the destruction of the antibiotics by the enzyme beta-lactamase. Use of beta-lactamase inhibitors in combination with antibiotics is one of the successful antibacterial strategies. The inhibitory effect of a phytochemical, 1,4-naphthalenedione, isolated from the plant *Holoptelea integrifolia* on beta-lactamase is reported here. This compound was found to have a synergistic effect with the antibiotic amoxicillin against a resistant strain of *Staphylococcus aureus*. The enzyme was purified from the organism and incubated with the compound. An assay showed that the compound can inhibit the enzymatic activity of beta-lactamase. Modeling and molecular docking studies indicated that the compound can fit into the active site of beta-lactamase. Hence, the compound can serve as a potential lead compound for the development of effective beta-lactamase inhibitor that can be used against beta-lactam-resistant microbial strains.

**Keywords** Beta-lactamase · *Staphylococcus aureus* · Amoxicillin · *Holoptelea integrifolia* · Phytochemicals · 1,4-Naphthalenedione

### Introduction

Beta-lactam antibiotics are the most important antimicrobial agents used in the treatment of a variety of infections [1]. Characterized by a four-membered, beta-lactam ring, these agents interfere with the cell wall biosynthesis. The beta-lactam antibiotics have structural similarities with bacterial substrates and, hence, enable them to bind to and inactivate the transpeptidases involved in bacterial cell wall synthesis [2].

Antibiotic resistance is becoming an increasing problem for clinicians worldwide, in both hospital and community settings [3]. A major mechanism of the beta-lactam antibiotic resistance is the production of beta-lactamase, an enzyme that hydrolyzes the amide bond of

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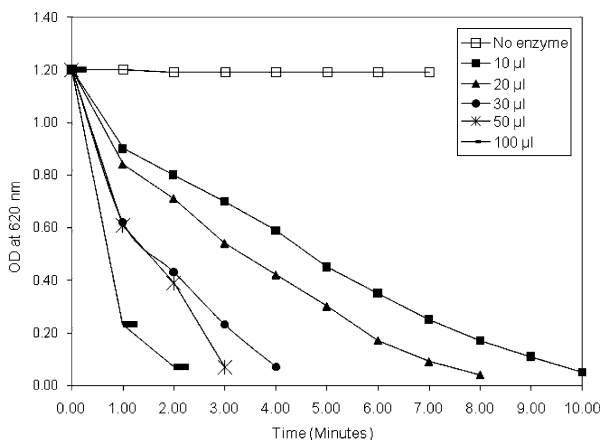
the characteristic beta-lactam ring. As a result, the antibiotics can no longer inhibit bacterial cell wall synthesis. Beta-lactamases are produced by a wide range of prokaryotes [4]. Staphylococcal beta-lactamase was among the first bacterial enzymes recognized as capable of destroying penicillin. The enzyme produced by strains of *Staphylococcus aureus* was long considered a single and unique extracellular enzyme directed primarily against penicillin. Later, the location of Staphylococcal beta-lactamases was determined as pericellular rather than as extracellular [5].

Beta-lactamase inhibitors can be combined with a susceptible antibiotic to restore its activity against beta-lactamase-producing organisms. This has been proven to be an effective strategy for overcoming this particular mechanism of resistance [6]. However, various aspects, such as matching of pharmacokinetic properties of the two partners, duration of the effect of the inhibitor, and antibiotic reactivity under the very different conditions, are to be considered and optimized in order to produce an effective combination [7].

The use of plant extracts and phytochemicals having antimicrobial properties can be of great use in therapeutic treatments. The synergistic effects of these compounds in association with some antibiotics had been established [8]. The main objective of the present study was to isolate and characterize plant-based beta-lactamase inhibitors, which can be used against the resistant strains of *S. aureus*. The inhibitory effects of 1,4-naphthalenedione isolated from the plant *Holoptelea integrifolia* on the beta-lactamase purified from *S. aureus* are reported here. The compound can bind to the active site of beta-lactamase, as shown by the modeling and docking studies.

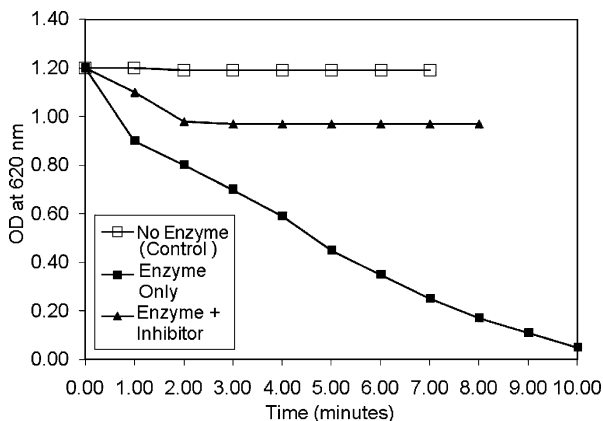
## Results

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) indicated that the molecular weight of the protein is approximately 30 KDa. In microiodimetric assay with amoxicillin as the substrate, the enzyme showed a sharp decrease in the OD with respect to time. The result is given in Fig. 1. The rate of decrease in OD is proportional to the amount of enzyme present in the reaction mixture. The concentration-dependent rate of change of OD confirms the presence of active beta-lactamase in the reaction mixture. At the same



**Fig. 1** Microiodimetric assay for the beta-lactamase activity with amoxicillin as substrate. Rate of decrease in OD is proportional to the amount of enzyme solution (10, 20, 30, 50, and 100 µl) added

**Fig. 2** Microiodimetric assay showing the effect of 1,4-naphthalenedione on beta-lactamase. The enzyme incubated with the compound did not show any considerable decrease in OD with respect to time



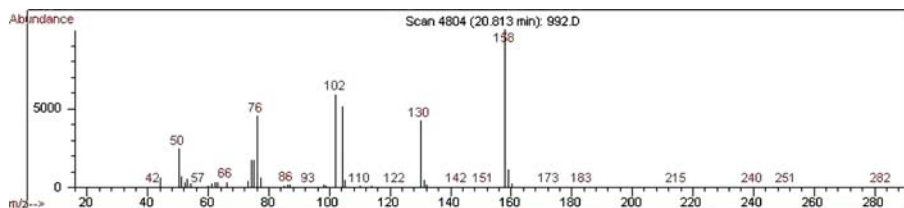
time, the enzyme preincubated with the compound showed no considerable decrease in the OD as illustrated in Fig. 2. This shows that the compound can effectively inhibit the enzymatic activity of beta-lactamase.

The active compound from the plant *H. integrifolia* was identified as 1,4-naphthalenedione by gas chromatograph-mass spectrometry (GC-MS) and Fourier transform-infrared (FT-IR). The GC-MS spectrum is given in Fig. 3. The identification was based on comparison of their mass spectra with WILEY 275.L database. The structure of the compound was confirmed by FT-IR spectrum (Fig. 4).

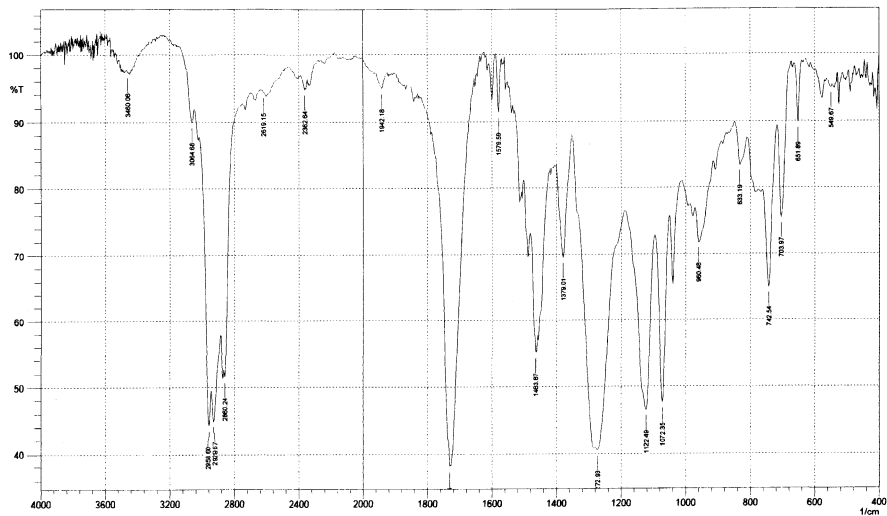
The 1,4-naphthalenedione docked in the active site of beta-lactamase is shown in Fig. 5. The docked structure was selected based on the lowest binding energy and possibility of making hydrogen bonds (Table 1). The binding energy was found to be  $-24.17$  kcal/mol. Protein residues Ser 70 and Lys 73 are making hydrogen bonds with the carbonyl oxygen of the ligand. The number of van der Waal's interactions ( $\leq 4$  Å) between the ligand and the enzyme is 118. This contributes to the stability of the complex.

## Discussion and Conclusion

Molecular weight of the protein isolated is 30 KDa, as shown by the SDS-PAGE. The result matches the molecular weight of class-A beta-lactamase reported from *S. aureus* [9]. In microiodimetric assay, the OD was found to decrease with respect to time, and the rate of decrease is proportional to the amount of enzyme added to the reaction mixture. The result indicated the presence of active beta-lactamase enzyme in the reaction mixture. When the enzyme was preincubated with the phytochemical, it did not decrease the OD of the



**Fig. 3** GC-MS spectrum of the active principle isolated from *H. integrifolia*



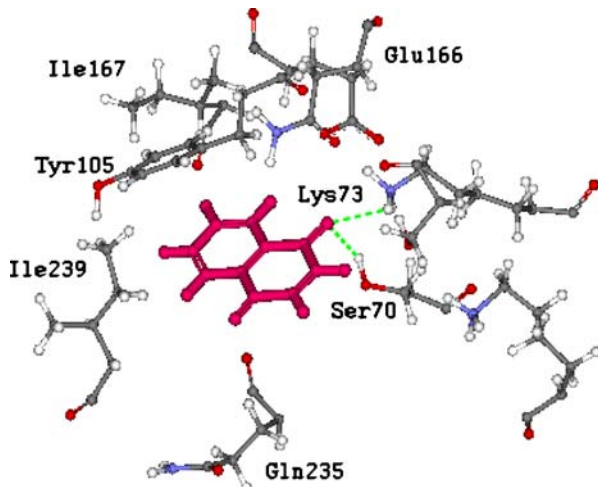
**Fig. 4** FT-IR spectrum of the active principle from *H. integrifolia*

reaction mixture. This indicated that the compound 1,4-naphthalenedione can inhibit the enzymatic activity of beta-lactamase.

Molecular docking studies were carried out in order to understand the mode of interaction between the enzyme and the inhibitor, 1,4-naphthalenedione. The compound is binding in the active site of the enzyme. One of its carbonyl oxygen is making hydrogen bonds with residues Ser 70 and Lys 73. A number of hydrophobic residues are surrounding the ligand. It seems the main stabilizing factor of the complex is van der Waals' interactions.

The studies indicate that the synergistic effect of the plant *H. integrifolia* extract with beta-lactam antibiotics is due to the presence of the compound 1,4-naphthalenedione. A number of 1,4-naphthalenedione derivatives have already been reported as having antibacterial activity [10, 11]. The compound 1,4-naphthalenedione has been reported as toxic to organisms. According to the International Occupational Safety and Health

**Fig. 5** The compound 1,4-naphthalenedione docked in the active site of beta-lactamase. Hydrogen bonds are shown as broken lines



**Table 1** Hydrogen bonds between beta-lactamase and the bound ligand 1,4-naphthalenedione.

Enzyme atom	Ligand atom	Bond length in Å
SER70 OG	O8	2.75
LYS73 NZ	O8	2.72

Information Centre (CIS) ([http://www.ilo.org/public/english/protection/safework/cis/products/icsc/dtasht/\\_icsc15/icsc1547.htm](http://www.ilo.org/public/english/protection/safework/cis/products/icsc/dtasht/_icsc15/icsc1547.htm)), the compound is labeled as UN Hazard Class: 6.1 and UN Pack Group: III. Symptoms like cough, sore throat, burning sensation, abdominal pain, nausea, and vomiting have been reported as a result of exposure to the compound.

However, since the compound can effectively bind to the active site of beta-lactamase, it can be used as an enzyme inhibitor. Hence, the compound can serve as a potential lead compound in the development of a more effective beta-lactamase inhibitor that can be used against beta-lactam-resistant microbial strains. The result also encourages the exploration of other plant-based inhibitor compounds against resistant strains of bacteria.

## Experimental

### Isolation, Purification, and Characterization of Beta-Lactamase from *S. aureus*

*Staphylococcus aureus* strains were obtained from the culture collection of the Institute of Microbial Technology, Chandigarh, India. The cells were found to be resistant to penicillin and chloramphenicol. They have been subcultured repeatedly in tryptone soy broth medium containing amoxicillin. The concentration of the antibiotic was gradually increased in the medium in order to scale up the resistance. The highest concentration of the antibiotics used is 5 µg/ml of broth. The antibiotic was purchased from Himedia (Mumbai, India).

*Staphylococcus aureus* cultures were grown overnight in tryptone soy broth in the presence of subminimal inhibitory concentration of amoxicillin at 37 °C, with continuous agitation. Subsequently, the cells were harvested by centrifugation for 15 min at 4,000×g, washed, and suspended in 0.1 M phosphate buffer of pH 7. Cells were broken using a lysopress at a pressure of 850 psi. Cell debris was removed from the lysopress extracts by centrifugation at 20,000×g for 30 min. The proteins in the supernatant were precipitated using ammonium sulfate at 70% saturation. The precipitate was isolated by centrifugation at 12,000×g for 10 min. The precipitate was redissolved and dialyzed against phosphate buffer. The protein was concentrated using ultrafiltration (A/G Technology Corporation, Needham, MA, USA) having a filter 10,000 NMWC, 1 mm, 16 cm<sup>2</sup>. The enzyme was purified by cation-exchange chromatography using carboxymethyl sephadex C-50 (Pharmacia Fine Chemicals, Uppsala, Sweden) in 10 mM phosphate buffer, pH 6.5, previously equilibrated with buffer. Elution was done with the same buffer containing a linear gradient of NaCl with 0.0 to 0.5 M range. The enzyme solution was dialyzed against a 10-mM phosphate buffer of pH 7.0. Protein concentration was determined using the method of Lowry et al. [12] with crystalline bovine serum albumin as the standard, and was found to be about 1 mg/ml. The molecular weight and purity of the purified enzyme was determined by SDS-PAGE [13].

Microiodimetric assay was carried out to determine the beta-lactamase activity of the purified enzyme with amoxicillin as the substrate. All assays were carried out in phosphate buffer (0.1 M, pH 7.0). Substrates were freshly prepared and kept on ice for the period of

the experiment. Hydrolyzed starch (0.2%) was dissolved in buffer by boiling gently for 2 to 3 min. Starch–iodine solution was made by adding 0.15 ml of iodine (0.08 M in 3.2 M potassium iodide) to 100 ml of this starch solution. A mixture of a 1-ml starch–iodine solution and a 1-ml substrate solution was taken in a glass cuvette and made up to 2.9 ml with phosphate buffer. The cuvette was left in the spectrophotometer for 5 min for temperature equilibration before the reaction was initiated by the addition of 0.1 ml of the enzyme solution. Absorbance at 620 nm was recorded at different time intervals using a Shimadzu Ultraviolet-Visible spectrophotometer against appropriate blanks. The assay was repeated with different volumes of enzyme solution, such as 0.05, 0.03, 0.02, and 0.01 ml. The OD-vs-time graph is given in Fig. 1. All experiments were performed at room temperature [14].

#### Extraction of Active Principle from Plant

*Holoptelea integrifolia* leaves were collected from the northern part of the state of Kerala, India, in the month of June. The plant was authenticated by an expert in the field of botany. A specimen was deposited in the Calicut University Herbarium. The leaves were crushed and extracted with hexane, diethyl ether, acetone, and water using a Soxhlet apparatus. The solvents were evaporated to get the fractions dry. The yields were found to be 0.8%, 1.0%, 1.7%, and 2.4%, respectively, for hexane, diethyl ether, acetone, and water, respectively.

The dry fractions were made into a suspension using 10% dimethylsulfoxide (DMSO) in distilled water. The concentration of the material was made to 1 mg/ml. The disk diffusion assay was performed in order to detect the antibacterial effects of the extracts. The filter paper (Whatman No.1) disks of 6 mm in diameter were made and soaked in the extracts and dried in an incubator at 40 °C to remove the solvent. The concentration of antibiotics in the plate was about 4 µg/ml. The plates were inoculated with the bacterial cell culture with a concentration of  $10^8$  colony-forming units per milliliter, as described previously. Paper disks soaked in 10% DMSO and dried in an incubator at 40 °C were used as the control. For each extract, six separate disks, loaded with 50 µg per disk, were used, and the average value of the diameters of the inhibition zones was taken. Maximum resistance was shown by the ether extract, and it was further fractionated to identify the active compound.

Thin layer chromatography (TLC) was conducted to determine the most suitable solvent mixture for the separation and identify the purity of active compound. In order to separate different components of the extract, silica gel chromatography was performed. Mixtures of hexane and ethyl acetate of varying ratios were used to elute the fractions. A single band on the TLC plate was taken as an indicator of the purity of the components. Each fraction was tested for antibacterial activity using the disk diffusion method as described above.

#### Action of the Compound on Beta-Lactamase Enzyme

The dry fractions were made into a solution using 10% DMSO in distilled water. The concentration of the material was 1 mg/ml. The enzyme in 0.1 M phosphate buffer of pH 7.0 was incubated with equal volumes of the phytochemical solution for 15 min at 30 °C. The microiodimetric assay for the enzyme activity was repeated with the preincubated enzyme. The results are given in Fig. 2.

#### Identification of the Active Principle

GC-MS and FT-IR spectroscopic analysis were carried out to characterize the active principle. GC-MS analysis was carried out on a HP Chem gas chromatogram fitted with a

DB5 silica column interfaced with quadrupole mass selective detector operated by software. The oven temperature program was 60–260 °C at 3 °C/min; the carrier gas was helium, adjusted to a column velocity with a flow of 1.16 ml/min. The split ratio was 25:1, whereas the split flow was 1.4 ml/min. Interface temperature was 240 °C, mass scan range was 50–500 amu at 70 eV, scan velocity was 3.12, and resulting electromagnetic voltage was 1,200 V. One microliter of the sample (dissolved in hexane 100% volume to volume) was injected into the system. The identification of the compound was based on comparison of their mass spectra with the WILEY 275.L database. Also, the identification was assisted by comparison of their retention times and retention indices with authentic samples. The spectrum is shown in Fig. 3. The library database showed that the compound is 1,4-naphthalenedione. An FT-IR spectrum was taken on a Shimadzu spectrometer. The solid samples are milled with potassium bromide to form a very fine powder and compressed into a thin pellet for the analysis. The spectrum is shown in Fig. 4.

### Molecular Docking Studies

The protein coordinates were taken from the crystal structure of beta-lactamase of *S. aureus* with its inhibitor clavulanate from the Protein Data Bank (PDB ID: 1BLC). The residues that are important for the inhibitor binding were identified as Ser 70, Ser 130, Asn 132, Lys 234, and Gln 237. Atomic coordinates for the ligand 1,4-naphthalenedione were generated using the sketching option of Accelrys's Discovery Studio 2.0 (DS 2.0) (Discovery studio 2.0, Accelrys Inc. San Diego, CA, USA). The model was energy-minimized with MMFF [15] force field. Docking of the ligand to the active site beta-lactamase was carried out using Ligand Fit module of DS2. The nonbonded interaction cutoff distance was 10 Å to compute the energy grid for docking. Root mean square (RMS) threshold, site fusing RMS cutoff, rigid body standard deviation iterations, and rigid body BFGS [16] iterations of docking were set up with default values. After setting all the parameters, docking was carried out. All ligand poses were further optimized by BFGS rigid body minimization for 50 iterations. The docked structures were further minimized for 10,000 iterations by applying force field CHARMM [17]. The estimation of free energy of noncovalent complex formation from a receptor and ligand was calculated further using generalized Born with a simple switching implicit solvent model with a dielectric constant of 78.

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