Research Square

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Screening of β-Lactamase Inhibitors Using Phytochemicals with recombinant β-Lactamase

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Abstract

The emergence and rapid spread of β-lactamase-mediated antibiotic resistance poses a significant challenge to the effectiveness of β-lactam antibiotics, highlighting the need for exploring β-lactamase inhibitory agents. The present study aimed to develop a recombinant TEM β-lactamase and evaluate natural plant extracts for their β-lactamase inhibitory potential. A conserved *blaTEM* sequence (DQ849322.1) was codon-optimized, cloned into the pET-28a (+) expression vector and over expressed in *Escherichia coli* BL21 (DE3). A specific activity of 211 IU/mg was observed for the purified recombinant enzyme. The purified enzyme was used for evaluating the inhibitory potential of 60 herbal and spices aqueous extracts through nitrocefin assay and microbial inhibition assay. Extracts from Custard apple (*Annona squamosa)* seeds, Java Plum (*Syzygium cumini)* fruit, Dry lemon zest (*Citrus aurantifolia)*, Clove (*Syzygium aromaticum)*, Winter cherry (*Withania somnifera)* roots, Cinchona leaves (*Cinchona pubescens)*, and Carom seeds (*Trachyspermum ammi)* exhibited higher β-lactamase inhibition. The selected extracts had an inhibition percentage of 78.60 ± 0.19%, 77.89 ± 0.16%, 77.80 ± 0.20%, 75.31 ± 0.15%, 71.31 ± 0.30%, 67.32 ± 0.17% and 66.87 ± 0.11%, respectively ($p < 0.0001$). Amoxicillin resistant *E. coli*, improved sensitivity, when tested along with inhibitors. The results suggest that plant-derived bioactive phytochemical constituents possess potential β-lactamase inhibitory activity and may serve as valuable sources for antibiotic resistance-modifying agents.

INTRODUCTION

The increasing emergence of antimicrobial resistance (AMR) is undermining the effectiveness of existing antimicrobial treatments, posing a serious threat to effective infection management. Antibiotic-resistant bacteria contribute substantially to health-care associated infections, accounting for more than 25% of nosocomial infections [1]. If effective interventions are not implemented, antimicrobial resistance could result in nearly 10 million deaths annually by the year 2050 [2]. Bacterial resistance is mediated through several mechanisms to evade the action of antibiotics. The misuse and abuse of antibiotics, particularly at sub-lethal doses, promote the emergence of antibiotic-resistant bacteria by facilitating genetic adaptations and the selection of resistance determinants. Such practices are commonly associated with the indiscriminate use of antibiotics in livestock production and agriculture, contributing to environmental contamination with antibiotics and antimicrobial resistance genes [6]. Moreover, prolonged and inappropriate antibiotic exposure disrupts the normal gut microbiota, promoting the development of collective resistome genes [7]. Enzymatic inactivation is the most common mechanism, which involves the hydrolysis or chemical modification of antibiotics by bacterial enzymes, preventing the drugs from exerting their antimicrobial effects [4]. Furthermore, antimicrobial resistance genes can be disseminated through horizontal gene transfer, contributing to the rapid spread of resistance across the environmental bacterial populations [5].

Among the different antimicrobial resistance mechanisms, resistance to β-lactam antibiotics remains one of the clinical concerns because β-lactam antibiotics are the most widely prescribed class of antibiotics worldwide. A major contributor to this resistance is the production of β-lactamases, the

enzymes that are responsible for inactivation of β-lactam antibiotics through cleavage of the β-lactam ring. On the basis of their functional profiling, β-lactamases are categorized into various groups and subgroups [8]. Extended-spectrum β -lactamases (ESBLs) have also become prevalent in society and represent a major challenge to health care management systems. These enzymes are capable of hydrolysing and inactivating a broader spectrum of antibiotics [9]. Among the various ESBLs, TEM-derived β-lactamases have undergone successive mutations, leading to the evolution of variants with expanded substrate specificity [10]. TEM-derived enzymes are among the most widely reported and documented in extremely drug-resistant bacteria [11]. Their widespread occurrence highlights need of developing β-lactamase inhibitors.

Several strategies have been explored to address β-lactamase-mediated resistance. Among these, employment of β-lactamase inhibitors has emerged as an effective approach for overcoming β-lactam resistance. The most commonly used β-lactamase inhibitors are Clavulanic acid, sulbactam, and tazobactam [13]. The clinical efficacy has been increasingly compromised by the emergence of new β-lactam-resistant pathogens [14]. Moreover, despite their clinical use, these inhibitors are associated with several side effects, including gastrointestinal disturbances and hypersensitivity reactions, emphasizing the need to identify novel β-lactamase inhibitors with improved safety profiles from natural sources [15–16].

The approaches have been employed for developing novel β-lactamase inhibitors with potential efficacy against ESBLs [17]. Nanoparticles have demonstrated significant antimicrobial activity against antibiotic-resistant bacteria in *in vitro* studies, although their potential cytotoxicity at higher concentrations remains as a major limitation [18]. Phage therapy has emerged as an effective alternative to antibiotic treatment because of its potential activity against antimicrobial-resistant. bacteria. However, it is yet to be studied for human employment. Furthermore, certain phages may facilitate the transfer virulence factors, highlighting the need for further research before its routine use in humans [19]. However, further studies are required to optimize their efficacy and support their clinical application [20]. [21] Jacobowski suggested that innovative strategies could be developed from the research on peptides, phages nanotechnology, quorum-sensing inference, and CRISPR-Cas systems to combat antimicrobial resistance.

Deployment of natural inhibitors against β- lactamases could be a promising therapeutic outcome to counteract antibiotic-resistant bacteria. Natural products have attracted considerable attention as potential sources of novel antimicrobial and resistance-modifying agents because of their structural diversity and diverse biological activities [22]. The selective inhibition of β-lactamase enzymes offers a promising approach to restore the efficacy of β-lactam antibiotics while minimizing cytotoxicity to host cells, making it a potential therapeutic agent for human health and use [23, 24].

The present study aimed to identify β-lactamase inhibitory potential of selected herbs and spices.

MATERIALS AND METHODS

Materials

The *E. coli* BL21 (DE3) expression host used in this study was obtained from Department of Microbiology, Osmania University, Hyderabad, India. The pET-28a (+) expression vector, NdeI and XhoI restriction enzymes were Fast Digestive from Fermentor. T4 DNA ligase, isopropyl β-D-1-thiogalactopyranoside (IPTG), nitrocefin substrate and other molecular biology-grade reagents and chemicals were obtained from Merck Life Science. Nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography resin was procured from Qiagen.

The *bla*TEM gene sequence with accession number DQ849322.1 was retrieved from the NCBI database. The codon-optimized synthetic gene was commercially synthesized and purchased from Biotech Desk Pvt. Ltd.

A total of 60 natural herbs and spices were selected for the screening of potential β-lactamase inhibitors. The materials were taxonomically identified by Dr. Vijay Bhaskar Reddy, Taxonomist, Department of Botany, Osmania University, Hyderabad.

Escherichia coli K-12 strain ATCC 10798, which is susceptible to β-lactam antibiotics was used as the test organism for evaluating the inhibitory activity against β-lactamase of the test samples.

E. coli Amoxicillin resistant strain isolated from sewage sample was used for synergistic activity of selected herbs and spices with antibiotics.

Methods

Selection of β-Lactamase Gene Sequence

TEM-type β-lactamase is a predominant enzyme responsible for β-lactamase mediated antibiotic resistance among Gram-negative bacteria [25, 26, 27]. The *bla*TEM gene is the specific genetic sequence that encodes this enzyme. Hence, *bla*TEM gene was selected for recombinant cloning. A total of 150 *bla*TEM gene sequences were retrieved from NCBI database and analyzed by multiple sequence alignment using ClustalW to find the similarity among the sequences retrieved. Conserved domain analysis was performed using NCBI Conserved-Domain (CD) search. The sequence exhibiting highest degree of conserved regions was selected for subsequent codon optimization and synthetic gene synthesis.

Codon Optimization

The *bla*TEM gene sequence exhibiting the highest degree of conserved regions with accession number DQ849322.1 was subjected to codon optimization using DNA 2.0 software for maximum expression of β-lactamase in *E. coli*. Codon optimization was carried out according to the standard *E. coli* usage

frequency table [28], using above 15% threshold as per the codon usage frequency to ensure high-level protein expression.

Synthetic Gene Synthesis and Subcloning

The codon-optimized *bla*TEM gene was synthesized by Biotech Desk Pvt. Ltd and provided in pUC19 vector. The gene sequence was subcloned into the pET-28a (+) expression vector. Restriction digestion of both the *bla*TEM gene and pET-28a (+) vector was performed using NdeI and XhoI restriction enzymes, after which the insert was ligated into the vector with T4 DNA ligase. In pET-28a (+), the gene was incorporated with N-terminal 6x His Tag to facilitate purification of expressed protein and T7 promoter for efficient expression of recombinant β-lactamase. The pET-28a (+) vector contained a kanamycin resistance gene that served as a selection marker for screening recombinant transformants.

Transformation

E. coli BL21 (DE3) was used as the host strain for transformation and expression studies. Transformation of recombinant pET-28a (+) plasmid carrying *bla*TEM gene into *E. coli* BL21 (DE3) cells was carried out by electroporation. *E. coli* BL21 (DE3) and pET-28a (+) *bla*TEM recombinant plasmid were taken in 1:0.01 and 1:0.02 ratios for transformation. Electroporation was carried out at 1.6volts for 9s. Transformed cells were spreaded on sterile nutrient agar containing 25µg/mL Kanamycin and incubated at 37 ℃ for 24 h to screen for recombinant cells. After incubation, positive recombinant colonies were selected.

Confirmation of Recombinants

The selected colonies were subsequently confirmed by restriction digestion and polymerase chain reaction (PCR).

The recombinant plasmid was subjected to restriction digestion with NdeI and XhoI restriction enzymes to release the expected 861bp band, followed by analysis using agarose gel electrophoresis.

PCR amplification was performed using the primers: Forward Primer: 5’-GTGCACGAGTGGGTTACATC-3’ and Reverse Primer: 5’-GAATAGTGTATGCGGCGACC-3’. The PCR mixture was prepared at final volume of 25µL containing 2 µL of plasmid DNA template, 2.5µL of 10× PCR buffer, 0.5µL deoxynucleotide triphosphates (10mM), 1µL $MgCl_2$ (25mM), 0.5µL each of the forward and reverse primers (10µM), 1µL of Taq DNA polymerase (1U/µL), and nuclease-free water to adjust the final reaction volume. Amplification was carried out with an initial denaturation at 95 ℃ for 5 min, followed by 30 cycles of denaturation at 95 ℃ for 1 min, primer annealing at 57 ℃ for 1 min and an extension at 72 ℃ for 1 min. The reaction concluded with a final extension at 72 ℃ for 5 min, after which the PCR products were analyzed by agarose gel electrophoresis.

Protein Expression, Purification and Activity Studies

Expression

An overnight culture of recombinant *E. coli* BL21 (DE3) was grown in LB broth containing kanamycin (25µg/mL) and incubated at 37°C and shaking at 200 rpm. The culture was subsequently shifted into fresh LB broth and grown until the mid-log phase of $OD_{600} \approx 0.6$ was reached. Protein expression was then induced by adding 1M IPTG stock solution to 100mL culture to achieve a final IPTG concentration of 0.5mM. The induced culture was incubated at 37°C for 8 h.

Purification

Following induction, the cultured cells were harvested by centrifugation at 8000rpm for 10 min. The bacterial cells were lysed using TNE, TNE-SDS and small glass beads. The cell lysate was centrifuged at 10,000rpm for 10 min, and the supernatant containing recombinant protein was collected. Purification of the recombinant protein was carried out by subjecting the supernatant to Ni-NTA affinity chromatography. The column was equilibrated with 10 column volumes of Buffer A (50 mM NaH_2PO_4, 300 mM NaCl, 10mM imidazole, pH 8.0). The supernatant was loaded onto the column at a slow flow rate, followed by washing with Buffer A to remove unbound proteins. The recombinant protein was eluted using a stepwise imidazole gradient (25-250mM) prepared in Buffer A, and the final elution was carried out by addition of Buffer B (250mM imidazole). The eluted fractions were subjected to SDS-PAGE to confirm the purified recombinant β-lactamase protein. The fraction band corresponding to expected molecular weight of β-lactamase (29kDa) was selected for His-tag removal. The His-tag was removed using enterokinase, and the cleaved protein solution was passed through a Ni-NTA affinity column at a flow rate of 1 mL/min. The flow-through contained purified recombinant β-lactamase enzyme was collected for subsequent studies.

β-lactamase Activity Assay: The enzyme activity of recombinant β-lactamase was determined by nitrocefin assay. A 10 mg/mL nitrocefin stock solution was prepared in dimethyl sulfoxide (DMSO) and stored under dark conditions until use. The reaction mixture contained 5mL of 100 mM phosphate-buffered saline (PBS; pH 7.0), 50µL of nitrocefin working solution and 100µL of purified recombinant β-lactamase. The hydrolysis of nitrocefin by recombinant β-lactamase was determined by measuring the absorbance at 486nm.

Samples of Herbs and Spices

A total of 60 medicinal herbs and spices were selected based on their reported anti β-lactamase and antimicrobial properties and traditional medicinal uses. Different plant parts were used depending on the plant species and literature as given below.

The leaves of Coriander (*Coriandrum sativum*), Turmeric (*Curcuma longa*), Drumstick (*Moringa oleifera*), Hibiscus (*Hibiscus rosa-sinensis*), Rosy Periwinkle (*Catharanthus roseus*), Garden sage (*Salvia officinalis*),

Sweet leaf (*Stevia rebaudiana*), Cinchona (*Cinchona pubscens*), Custard apple (*Annona squamosa*), Papaya (*Carica papaya*), Lemon (*Citrus aurantifolia*), Rose (*Rosa indica*), Henna (*Lawsonia inermis*), Mango (*Mangifera indica*), Indian beech (*Pongamia pinnata*), Sacred fig (*Ficus religiosa*), Coromandel ebony (*Diospyros melanoxylon*), Wild mint (*Mentha arvensis*), Rosemary (*Rosmarinus officinalis*), Bay leaf (*Cinnamomum tamala*), Curry leaves (*Murraya koenigii*), Pomegranate (*Punica granatum*), Neem (*Azadirachta indica*), Holy basil (Ocimum tenuiflorum), Beleric myrobalan (*Terminalia bellirica*), Aloe vera (*Aloe barbadensis Miller*), Curry plant (*Helichrysum italicum*) and dried leaves of Fenugreek (*Trigonella foenum-graecum*) were used.

The seeds of Fenugreek (*Trigonella foenum-graecum*), Black mustard (*Brassica nigra*), Fennel (*Foeniculum vulgare*), Custard apple (*Annona squamosa*), Mango (*Mangifera indica*), Nutmeg (*Myristica fragrans*), Carom seeds (*Trachyspermumammi*), Clearing nut (*Strychnos potatorum*), Cardamom (*Elettaria cardamomum*), Black cardamom (*Amomum subulatum*), Custard apple (*Annona squamosa*) and dried seeds of Pomegranate (*Punica granatum*) were used.

Other plant materials include rhizomes of Ginger (*Zingiber officinale*) and White turmeric (*Curcuma zedoaria*), bark of cinnamon (*Cinnamomum verum*), the bulbs of Onion (*Allium cepa*) and Garlic (*Allium sativum*), Sorghum (*Sorghum bicolor*) and Barley (*Hordeum vulgare*) grains, roots of Winter cherry (*Withania somnifera*) ,dried unripe fruit of Black pepper (*Piper nigrum*), dried fruit of Cumin (*Cuminum cyminum*), flower bud of Cloves (*Syzygium aromaticum*), blades of Lemon grass (*Cymbopogon citratus*), inner pith of Tapioca pearls (*Manihot esculenta*), zest of Dry lemon (*Citrus aurantifolia*), Psyllium husk (*Plantago ovata*) husk, Acacia gum (*Acacia senegal*) granules, Java plum fruit (*Syzygium cumini*), extract of Catechu (*Acacia catechu*), Castor (*Ricinus communis*) oil and Fuller's earth(*Solum fullonum*).

Extract preparation of samples

Aqueous extracts of selected herbs and spices were prepared by grinding the samples and then suspending 10g of each grinded sample in 50mL of phosphate buffer and mixing thoroughly to obtain uniform suspension. The mixtures were heated on a water bath at 60 ℃ for 10 min to facilitate the extraction of bioactive compounds. The extracts were cooled, filtered through Whatman No.1 filter paper, and stored at 4 ℃ until further use in β-lactamase inhibition assays.

β-lactamase inhibition assays

Nitrocefin assay:

β-Lactamase inhibitory activity was determined using nitrocefin as the chromogenic substrate according to Toppi et al [29] with minor modifications. 100 µL of purified β-lactamase enzyme (10 mg/mL) and 100 µL of test sample extracts were dispensed into sterile 1.5mL eppendorf's and incubated at 37^0C for 10 min. Subsequently, 25 µL of nitrocefin substrate (0.5 mg/mL) was added to initiate the reaction and incubated for an additional 10 min at 37°C. Nitrocefin alone and the enzyme- nitrocefin mixture served as controls. The hydrolysis of nitrocefin by recombinant β-lactamase was determined by measuring absorbance at 486 nm.

Microbial inhibition assay:

The microbial inhibition assay was performed by the agar well diffusion method with slight modifications according to Sohail et al.,[30]. Sterile double-strength Luria agar (LA) plates were prepared for the assay. 100 µL of Penicillin sensitive *E. coli* K12 culture was uniformly spread onto the LA plates. Wells of 60 µL capacity were made using a sterile dweller. For the assay, 25 µL of purified recombinant β-lactamase (10 mg/mL), was mixed with 20 µL of each plant extract and incubated at room temperature for 15 min to allow enzyme–inhibitor interaction. Following incubation, 10 µL of Penicillin G (10,000 U/mL) antibiotic was added to the reaction mixture. These reaction mixtures were then dispensed into the wells. Antibiotic alone and the antibiotic–enzyme mixture served as controls. The plates were incubated at 37°C for 24 h, after which the zones of inhibition were measured in millimetres (mm). Restoration of the inhibition zone compared with the ampicillin–enzyme control indicated β-lactamase inhibitory activity of the test samples.

Synergistic Assay with Resistant E. coli:

Antimicrobial Susceptibility Test

Synergistic activity of Amoxicillin and selected plant extracts against Amoxicillin resistant *E. coli* was evaluated using the agar well diffusion method. Nutrient agar plates were prepared and inoculated with resistant *E. coli* culture using spread plate method under sterile conditions. Wells were made on the agar plate using a sterile well borer. Amoxicillin (10mg/ml) was mixed with selected plant extracts of *Annona squamosa* seeds, *Syzygium cumini* fruit, *Citrus aurantifolia* zest, *Syzygium aromaticum, Withania somnifera* roots, *Cinchona pubescens* leaves, and *Trachyspermum ammi* at the ratio of 1:1, and 20 µL of prepared mixture was loaded into wells. Amoxicillin alone (10 mg/ml) was loaded into a separate well as control. These plates were then incubated at 37°C for 24 h. Following incubation, the antimicrobial activity was evaluated by observing and measuring the zone of inhibitions around the wells.

Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration values of Amoxicillin-plant extracts were assessed by the broth microdilution method. An active culture of resistant *E. coli* was diluted 1:100 in the fresh Luria broth (L.B) and grown until the log phase of growth (OD 600 ≈ 0.6) was achieved. Amoxicillin was mixed with selected plant extracts of *Annona squamosa* seeds, *Syzygium cumini* fruit, *Citrus aurantifolia* zest, *Syzygium aromaticum, Withania somnifera*, *Cinchona pubescens* leaves, and *Trachyspermum ammi* at the ratio of 1:1. A series of dilutions of these mixtures were prepared and then dispensed into sterile 96-well microtiter plate containing L.B to obtain final concentrations of 0.25, 0.5, 0.75, 1.0, 1.25 and 1.5 mg/mL, with final volume of 300µL per well. Wells were then inoculated with the bacterial suspension at 1:100 (v/v) ratio. Plates were incubated at 37°C for 24 h. Different concentrations of Amoxicillin alone (0.25, 0.5, 0.75, 1.0, 1.25 and 1.5 mg/mL) was dispensed in wells to compare the activity. Following incubation, wells were examined for visible turbidity indicative of bacterial growth.

Statistical Analysis

All experiments were conducted thrice in triplicates (n = 9) and the values are presented as Mean ± Standard Deviation. Differences among test extracts and experimental controls were determined using one-way Analysis of Variance (ANOVA) followed by Dunnett's post-hoc multiple comparison test. The $p < 0.001$ and $p < 0.05$ were considered statistically significant.

RESULTS

Multiple Sequence Alignment and Conserved Domain Analysis of blaTEM Gene Sequences

Multiple sequence alignment of 150 *bla*TEM gene sequences retrieved from NCBI database using ClustalW, revealed a high degree of sequence conservation, exhibiting greater than 99% sequence similarity among the aligned sequences, particularly in the active site and conserved catalytic regions responsible for antibiotic hydrolysis. Minor nucleotide variations were observed, with comparatively higher sequence variability occurring around the 830bp region. Among the analyzed sequences, the *bla*TEM gene sequence with accession number DQ849322.1 showed a highly conserved β-lactamase domain and was selected for subsequent cloning, expression, and inhibition studies. The analysis showed a significant domain alignment with a bit score of 605.6945968 and an E-value of 0, confirming the presence of a highly conserved β-lactamase domain.

Construction of Recombinant pET-28a (+)- bla TEM Expression Vector

The selected *bla*TEM gene sequence was codon optimized, synthesized and cloned into the pET-28a (+) expression vector to construct the recombinant pET-28a-*bla*TEM plasmid. The b*la*TEM-1 gene was placed between the NdeI and XhoI restriction sites, with the T7 promoter positioned upstream of the gene and the 6×His tag positioned downstream of the T7 promoter. The recombinant vector construct containing the *bla*TEM gene insert along with the essential vector components is represented in Fig. 1.

Confirmation of Recombinants

Recombinant pET-28a (+) clones were confirmed by antibiotic selection, PCR amplification, restriction digestion, and agarose gel electrophoresis. The transformed clones were screened on kanamycin-containing selective media, confirming the presence of kanamycin resistance marker in the recombinant construct.

PCR amplification of the recombinant clones produced an expected *bla*TEM amplicon of 861 bp (Figure.2B), confirming the presence of the target gene insert. Comparative analysis of plasmid sizes showed that the pET-28a (+) vector resolved at 5.3 kbp (Figure.2C), whereas the recombinant pET-28a (+) exhibited an increase in size of 6.1 kbp band (Figure.2D). Restriction digestion using NdeI and XhoI of the recombinant construct released fragments of 5.3 kbp and 861 bp, corresponding to the vector backbone and *bla*TEM insert, respectively (Figure.2E).

Protein Expression, Purification and Activity assessment

IPTG induction resulted in successful expression of recombinant β-lactamase in *E. coli* BL21 (DE3), which was confirmed by SDS-PAGE analysis. SDS-PAGE analysis revealed an intense protein band of ∼ 30 kDa in the IPTG-induced recombinant cells (Figure.3B), indicating overexpression of recombinant β-lactamase. Following Ni-NTA affinity chromatography, the purified enzyme appeared as a distinct band at ∼30 kDa (Figure.3C), confirming successful purification of the recombinant enzyme.

Enzyme activity of purified β-lactamase was assessed using the nitrocefin assay. The specific activity of the enzyme increased progressively during purification. The crude lysate exhibited a specific activity of 48 IU/mg, which increased to 120 IU/mg following Ni-NTA affinity chromatography and further to 211 IU/mg after His-tag removal. The progressive increase in specific activity indicates successful enrichment of the recombinant β-lactamase and improved enzyme purity, making it suitable for subsequent inhibitor screening assays.

β-lactamase inhibitory assays

Nitrocefin Assay:

The nitrocefin hydrolysis assay was employed to assess the inhibitory activity of the selected plant extracts against recombinant β-lactamase. The absorbance values obtained at 486 nm for the selected plant extracts and controls are presented in Table 1.

Table.1 Inhibitory effects of selected plant extracts against recombinant β-lactamase determined by the nitrocefin assay

Samples (Sample Extract + β-lactamase + Nitrocefin Substrate)	Nitrocefin Assay (absorbance measured at 486nm)	Percentage of Inhibition (%)
Nitrocefin	0.015 ± 0.012	100.00
Nitrocefin + Beta-lactamase	1.141 ± 0.014	100.00
Amoxicillin Potassium Clavunate	0.015 ± 0.014	100.00
Zingiber officinal	0.742 ± 0.0051	35.44 ± 0.45
Piper nigrum seeds	0.815 ± 0.0044	28.95 ± 0.39
Rosmarinus officinalis leaves	0.432 ± 0.0035	62.97 ± 0.31
Murraya koenigii leaves	0.685 ± 0.0033	40.50 ± 0.29
Cinnamomum verum inner bark	0.866 ± 0.0037	24.42 ± 0.33
Brassica nigra seeds	0.885 ± 0.0038	22.74 ± 0.34
Syzygium aromaticum bud	**0.293 ± 0.0017**	**75.31 ± 0.15**
Rosa indica leaves	0.985 ± 0.0037	13.85 ± 0.33
Ficus religiosa leaves	0.996 ± 0.0084	12.88 ± 0.75
Annona squamosa seeds	**0.256 ± 0.0021**	**78.60 ± 0.19**
Curcuma longa leaves	0.822 ± 0.0036	28.33 ± 0.32
Cinchona pubscens leaves	**0.383 ± 0.0019**	**67.32 ± 0.17**
Lawsonia inermis leaves	0.842 ± 0.0054	26.55 ± 0.48
Punica granatum dried seeds	0.651 ± 0.0045	43.52 ± 0.40
Acacia catechu	0.953 ± 0.0042	16.70 ± 0.37
Withania somnifera roots	**0.338 ± 0.0034**	**71.31 ± 0.30**
Citrus aurantifolia zest	**0.265 ± 0.0022**	**77.80 ± 0.20**
Trachyspermum ammi seed	**0.388 ± 0.0012**	**66.87 ± 0.11**
Syzygium cumini fruit	**0.264 ± 0.0018**	**77.89 ± 0.16**
Cinnamomum tamala leaf	0.914 ± 0.0095	20.16 ± 0.84

Nitrocefin alone exhibited an absorbance of 0.015 ± 0.012, and Amoxicillin Potassium Clavunate showed absorbance of 0.015 ± 0.014, indicating no hydrolysis of nitrocefin. Whereas the nitrocefin-β-lactamase control recorded an absorbance of 1.141 ± 0.014, indicating efficient hydrolysis of nitrocefin by recombinant β-lactamase enzyme. Among the tested extracts, Custard apple seeds (*Annona squamosa*) exhibited the highest inhibition of β-lactamase of 78.60 ± 0.19%, followed by Java plum fruit (*Syzygium cumini*) with 77.89 ± 0.16%, Dry lemon zest (*Citrus aurantifolia*) with 77.80 ± 0.20%, Clove (*Syzygium*

aromaticum) with 75.31 ± 0.15%, Winter cherry roots (*Withania somnifera*) with 71.31 ± 0.30%, Cinchona (*Cinchona pubescens*) leaves with 67.32 ± 0.17%, Carom seeds (*Trachyspermum ammi*) with 66.87 ± 0.11%, indicating strong inhibition of nitrocefin hydrolysis. Rosemary leaves (*Rosmarinus officinalis*) leaves, Ginger (*Zingiber officinale*), Curry leaf (*Murraya koenigii*), Pomegranate (*Punica granatum*), Black pepper (*Piper nigrum*), turmeric (*Curcuma longa*), henna (*Lawsonia inermis*), cinnamon (*Cinnamomum verum*), Black mustard (*Brassica nigra*) demonstrated moderate inhibition. In contrast, comparatively higher absorbance values were recorded for Sacred fig (*Ficus religiosa*) leaves, Rose leaves (*Rosa indica*), Catechu (*Acacia catechu*) and Bay leaf (*Cinnamomum tamala*), indicating comparatively lower β-lactamase inhibitory activity among evaluated extracts. All tested plant extracts demonstrated a statistically significant reduction in nitrocefin hydrolysis compared to the control ($p < 0.0001$).

Microbial Inhibition Assay:

The microbial inhibition assay was performed using *E. coli* K12 to evaluate the restoration of Penicillin G activity following β-lactamase inhibition by the selected plant extracts. The corresponding zones of inhibition and restoration of Penicillin G activity (%) are presented in Table 2. Penicillin G and Amoxicillin Potassium Clavunate exhibited the highest zone of inhibition (29 ± 0.12 and 29 ± 0.14 mm), corresponding to 100% activity, whereas the Penicillin G + β-lactamase control showed a markedly reduced zone of 4.5 ± 0.15 mm, representing 0% restoration. Among the tested extracts, *Annona squamosa* seeds showed the highest restoration of Penicillin G activity (75.51 ± 0.70%), followed by, *Syzygium cumini* fruit (67.35 ± 0.62%), *Syzygium aromaticum* buds (67.35 ± 0.69%) and *Citrus aurantifolia* zest (63.27 ± 0.54%). Moderate restoration of Penicillin G was observed with *Cinchona pubescens, Withania somnifera*, *Trachyspermum ammi*, *Murraya koenigii*, *Zingiber officinale*, *Punica granatum*, *Rosmarinus officinalis*, *Piper nigrum*, *Cinnamomum verum*, *Lawsonia inermis*, *Curcuma longa*. Comparatively lower restoration of Penicillin G activity was observed against β-lactamase was recorded for *Rosa indica*, and *Ficus religiosa*, *Brassica nigra*, *Cinnamomum tamala* and *Acacia catechu*. The addition of plant extracts significantly restored the zone of inhibition compared to the Penicillin G + β-lactamase control ($p < 0.0001$)

Table.2 Restoration of Penicillin G activity by selected plant extracts against recombinant β-lactamase determined by microbial inhibition assay

Samples (Sample Extract + β-lactamase +Penicillin G)	Zone of inhibition (mm)	Percentage restoration of Penicillin G activity (%)
Standard Penicillin G Alone	29 ± 0.12	100.00
Penicillin G + Beta-lactamase	4.5 ± 0.15	0.00
Amoxicillin Potassium Clavunate	29 ± 0.14	100.00
Zingiber officinal	18 ± 0.05	55.10 ± 0.48
Piper nigrum seeds	14 ± 0.08	38.78 ± 0.45
Rosmarinus officinalis leaves	16 ± 0.04	46.94 ± 0.40
Murraya koenigii leaves	18 ± 0.05	55.10 ± 0.48
Cinnamomum verum inner bark	14 ± 0.09	38.78 ± 0.45
Brassica nigra seeds	12 ± 0.02	30.61 ± 0.25
Syzygium aromaticum bud	**21 ± 0.11**	**67.35 ± 0.69**
Rosa indica leaves	11 ± 0.03	26.53 ± 0.24
Ficus religiosa leaves	11 ± 0.01	26.53 ± 0.21
Annona squamosa seeds	**23 ± 0.09**	**75.51 ± 0.70**
Curcuma longa leaves	14 ± 0.02	38.78 ± 0.31
Cinchona pubscens leaves	**19 ± 0.05**	**59.18 ± 0.51**
Lawsonia inermis leaves	14 ± 0.04	38.78 ± 0.35
Punica granatum dried seeds	15 ± 0.02	42.86 ± 0.35
Acacia catechu	13 ± 0.03	34.69 ± 0.30
Withania somnifera roots	**19 ± 0.01**	**59.18 ± 0.47**
Citrus aurantifolia zest	**20 ± 0.05**	**63.27 ± 0.54**
Trachyspermum ammi seed	**18 ± 0.02**	**55.10 ± 0.44**
Syzygium cumini fruit	**21 ± 0.08**	**67.35 ± 0.62**
Cinnamomum tamala leaf	12 ± 0.05	30.61 ± 0.32

Synergistic Assay with Resistant E. coli:

Antimicrobial Susceptibility Test

The antibacterial activity of Amoxicillin in combination with selected plant extracts was evaluated against Amoxicillin-resistant *E. coli* using the agar well diffusion method and the respective zone of inhibitions are presented in Table.3. Amoxicillin alone exhibited a zone of inhibition of 4 ± 0.03 mm, indicating resistance of *E. coli* strain to Amoxicillin. The highest activity was observed with Amoxicillin + *Annona squamosa* seeds (25 ± 0.05 mm), followed by *Citrus aurantifolia* zest (23 ± 0.08 mm) and *Syzygium cumini* fruit (22 ± 0.15 mm). Combinations with *Syzygium aromaticum* bud and *Withania somnifera* produced zones of 21 ± 0.02 and 21 ± 0.11 mm, respectively, while *Cinchona pubescens* leaves and *Trachyspermum ammi* seeds showed zones of 19 ± 0.03 and 18 ± 0.04 mm, respectively. Overall, the combinations demonstrated enhanced antibacterial activity and are statistically significant when compared with amoxicillin alone ($p < 0.0001$)

Table.3 Antimicrobial activity of selected plant extracts against resistant *E. coli*.

Samples (Sample Extract + 10 mg/mL Amoxicillin)	Zone of inhibition (mm)
Standard Amoxicillin (10mg/mL)	4 ± 0.03
Amoxicillin Potassium Clavunate	28 ± 0.12
Annona squamosa seeds	25 ± 0.05
Syzygium cumini fruit	22 ± 0.15
Citrus aurantifolia zest	23 ± 0.08
Syzygium aromaticum bud	21 ± 0.02
Withania somnifera roots	21 ± 0.11
Cinchona pubscens leaves	19 ± 0.03
Trachyspermum ammi seeds	18 ± 0.04

Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of Amoxicillin in combination with the selected plant extracts was additionally evaluated against resistant *E. coli* using the broth microdilution method. The MIC values are presented in Table.4. The *E. coli* showed resistance to Amoxicillin, exhibiting an MIC of 1.25 mg/mL. Among the tested combinations, Amoxicillin with *Annona squamosa* seeds and *Syzygium cumini* fruit showed the greatest reduction in MIC, with an MIC of 0.25 mg/mL, representing a five-fold reduction compared with Amoxicillin alone. Combinations with *Citrus aurantifolia* zest and *Withania somnifera* roots exhibited MIC values of 0.5 mg/mL, while *Syzygium aromaticum* bud, *Cinchona pubescens* leaves and *Trachyspermum ammi* seeds showed MIC values of 0.75 mg/mL. These findings indicate an enhanced antibacterial effect of Amoxicillin when combined with the selected plant extracts against the resistant *E. coli* strain ($p < 0.0001$).

Table 4
Minimum inhibitory concentration (MIC) of Amoxicillin–plant extract combinations against resistant *E. coli*.

Samples (Sample Extract + Amoxicillin)	MIC Value (mg/mL)
Standard Amoxicillin Control	1.25
Amoxicillin Potassium Clavunate	0.25
Annona squamosa seeds	0.25
Syzygium cumini fruit	0.25
Citrus aurantifolia zest	0.5
Syzygium aromaticum bud	0.75
Withania somnifera roots	0.5
Cinchona pubscens leaves	0.75
Trachyspermum ammi seeds	0.75

DISCUSSION

The increasing emergence of β-lactamase-mediated antimicrobial resistance, together with limitations of existing synthetic β-lactamase inhibitors, emphasizes the need for novel therapeutic alternatives [31]. Natural phytochemicals have attracted considerable attention as alternative β-lactamase inhibitors because of their structural diversity and multiple biological activities.

This study focused on the identifying potential natural β-lactamase inhibitors through recombinant TEM-β-lactamase. TEM is the most prevalent β-lactamases in Gram-negative pathogens and serves as the evolutionary precursor of numerous extended-spectrum β-lactamase (ESBL) variants [26, 27, 32]. Owing to its widespread occurrence, well-characterized catalytic mechanism, and clinical relevance, the *bla*TEM gene was selected as the target for inhibitor screening.

The highly conserved *bla*TEM (DQ849322.1) was successfully codon optimized, cloned, and expressed, demonstrating the suitability of the employed expression strategy for recombinant β-lactamase production. Codon optimization is a widely adopted approach to improve heterologous protein production by adapting codon usage to the preferred codon bias of the expression host, thereby enhancing translational efficiency, mRNA stability, and overall protein yield [33, 34]. Recombinant clones harbouring pET-28a (+)-*bla*TEM construct were successfully transformed into *E. coli* BL21(DE3), selected by using kanamycin resistance marker and confirmed by PCR amplification and restriction digestion as employed by Al-Muhanna et al., [35]. Recombinant protein expression was subsequently induced using IPTG, resulting in enhanced expression of the target protein [36, 37]. Li et al. [38] also reported successful construction, expression, and purification of multiple clinically relevant recombinant β-lactamases, including TEM-1 and SHV-1, using the pET-28a and *E. coli* BL21(DE3) expression system.

The purified recombinant β-lactamase was used for screening 60 medicinal herbs and spices using nitrocefin hydrolysis assay and microbial inhibition assays to investigate their inhibitory activity. Among the tested extracts, Custard apple seeds (*Annona squamosa*), Java plum fruit (*Syzygium cumini*), Dry lemon zest (*Citrus aurantifolia*), Clove (*Syzygium aromaticum)*, Winter cherry roots (*Withania somnifera*), Cinchona leaves (*Cinchona pubescens*), and Carom seeds (*Trachyspermum ammi*), exhibited superior inhibitory activity. The observed inhibition may be associated with bioactive phytoconstituents present in plant extracts, having potential β-lactamase inhibitory properties.

The present study employed aqueous extracts of selected herbs and spices, whereas Anago et al. [39] and Sohail et al. [30] utilized ethanolic and methanolic extracts, respectively, to evaluate β-lactamase inhibitory activity. Although organic solvents efficiently extract a wide range of phytochemicals, aqueous extraction offers a simple, non-toxic, cost-effective, and environmentally preferable alternative while closely resembling the traditional use of medicinal plants [40]. The present extracts were prepared in an aqueous medium with the aim of facilitating the future use of these herbs and spices as adjuncts alongside with antibiotics. Sohail et al. [30] reported *Terminalia bellirica*, *Terminalia chebula*, *Bacopa monnieri*, *Zingiber officinale*, *Cymbopogon citratus*, *Allium sativum*, and *Capsicum annuum* as potent β-lactamase inhibitors using methanolic extracts and iodometric and bioassay methods. While, the present study employed recombinant β-lactamase with nitrocefin and microbial inhibition assays, leading to the identification of plant extracts with significant β-lactamase inhibitory activity.

The β-lactamase inhibitory activity observed in this study is supported by previous investigations on several medicinal plants. Irawan et al. [41] revealed that *A. squamosa* leaf extract effectively inhibited the growth of *Staphylococcus aureus* owing to the presence of antimicrobial phytochemicals such as acetogenins, phenolics, flavonoids. Likewise, *Syzygium cumini* has been extensively reported to possess antimicrobial and antioxidant activities owing to its abundance of phenolic compounds, flavonoids, ellagic acid, and quercetin, which have also been implicated in β-lactamase inhibition [42, 43]. *Citrus aurantifolia* zest and *Syzygium aromaticum* buds also demonstrated promising inhibitory activity, which may be attributed to flavonoids, phenolics, terpenoids, and eugenol, respectively, contributing to antimicrobial and resistance-modulating effects [44, 45]. The inhibitory activity observed can be attributed to phytochemicals, including flavonoids, phenolic compounds, alkaloids, terpenoids, tannins, and essential oils, many of which have been previously reported to possess antimicrobial and enzyme inhibitory activities [46, 47].

However, several extracts exhibited only moderate or minimal β-lactamase inhibition, suggesting that although they may possess antimicrobial properties, their bioactive constituents may not specifically target TEM-type- β-lactamase. Variations in phytochemical composition and extraction efficiency may also have contributed to the observed differences in inhibitory activity. Although Kongkham et al. [49] identified that plant secondary metabolites from *Ficus religiosa* had a potential β-lactamase inhibitory activity using both in vitro and computational approaches, the same plant exhibited comparatively low inhibitory activity under the experimental conditions of the present study.

The enhanced antibacterial activity observed for the Amoxicillin–plant extract combinations against resistant *E. coli* further supports their potential as antibiotic adjuvants. In the present study, *Annona squamosa* seeds and *Syzygium cumini* fruit showed the greatest enhancement, reducing the Amoxicillin MIC from 1.25 to 0.25 mg/mL. Similarly, Eumkeb et al. [50] reported synergistic activity of Amoxicillin with Galangin, Kaempferide and Kaempferide-3-*O*-β-D-glucoside against Amoxicillin-resistant *E. coli*, with MICs ranging from 0.5 to > 1.0 mg/mL and evidence of Penicillinase inhibition. Another study by Eumkeb et al. [50] reported synergistic activity of Amoxicillin with Luteolin, with Amoxicillin and Luteolin showing MICs of > 1.0 and ≥ 0.2 mg/mL, respectively, along with Penicillinase inhibition. These findings are consistent with the enhanced antibacterial activity and reduced Amoxicillin MIC observed with the selected plant extracts in the present study.

Wong et al. [51] observed that phytochemicals exhibit synergistic antimicrobial activity when combined with conventional antibiotics.. Newman and Cragg [52] emphasized that natural products continue to serve as one of the most important sources of lead compounds for drug discovery, while Atanasov et al. [53] highlighted that their unique chemical diversity provides valuable scaffolds that are often difficult to reproduce synthetically. Although computational approaches, including molecular docking and in silico screening have accelerated the identification of potential inhibitors [54], experimental validation remains essential. The present findings provide a foundation for future bioassay-guided isolation and β-lactamase assays of active phytoconstituents to facilitate the development of effective plant-derived β-lactamase inhibitors.

CONCLUSION

The present study successfully established a recombinant TEM-1 β-lactamase expression and purification system for the evaluation of potential natural β-lactamase inhibitors. The findings concluded that the aqueous extracts of *Annona squamosa* seeds, *Syzygium cumini*, *Citrus aurantifolia*, *Syzygium aromaticum, Withania somnifera*, *Cinchona pubescens*, and *Trachyspermum ammi* exhibited a promising β-lactamase inhibitory effect. Furthermore, the selected plant extracts enhanced the antibacterial activity of Amoxicillin against the resistant *E. coli* strain, with *Annona squamosa* and *Syzygium cumini* showing the greatest improvement and reduction in MIC value. Further identification and characterization of the active phytoconstituents, supported by in silico and pharmacological studies, may contribute to the development of novel β-lactamase inhibitor strategies to overcome antibiotic resistance.

Abbreviations

AMR Antimicrobial resistance

ESBLs Extended spectrum β -lactamases

CRISPR Clustered regulatory interspaced palindromic repeats

Ni-NTA Nickel-nitrilotriacetic acid

NCBI National Centre for Biotechnology Centre

ATCC American type culture collection

CD Clustered domain

PCR Polymerase chain reaction

IPTG Isopropyl β-D-1-thiogalactopyranoside

MIC Minimum inhibitory concentration

ANOVA Analysis of variance

Declarations

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Authors contribution

Syeda Sumaiyya Fatima: Investigation, Methodology, Data curation, and Writing – original draft.

S. Asha: Conceptualization, Supervision, Project administration, and Writing – review and editing.
Chand Pasha: Conceptualization, Supervision, Project administration, and Writing – review and editing.

Conflict of interest Authors declare no conflict of interest.

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Data availability All data generated or analysed during this study are included in this research article

The authors declare no competing interest

Ethical Approval This Study did not involve the use of human participants or animals. Therefore, ethical approval from a human or animal ethics committee is not required for this research.

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Figures

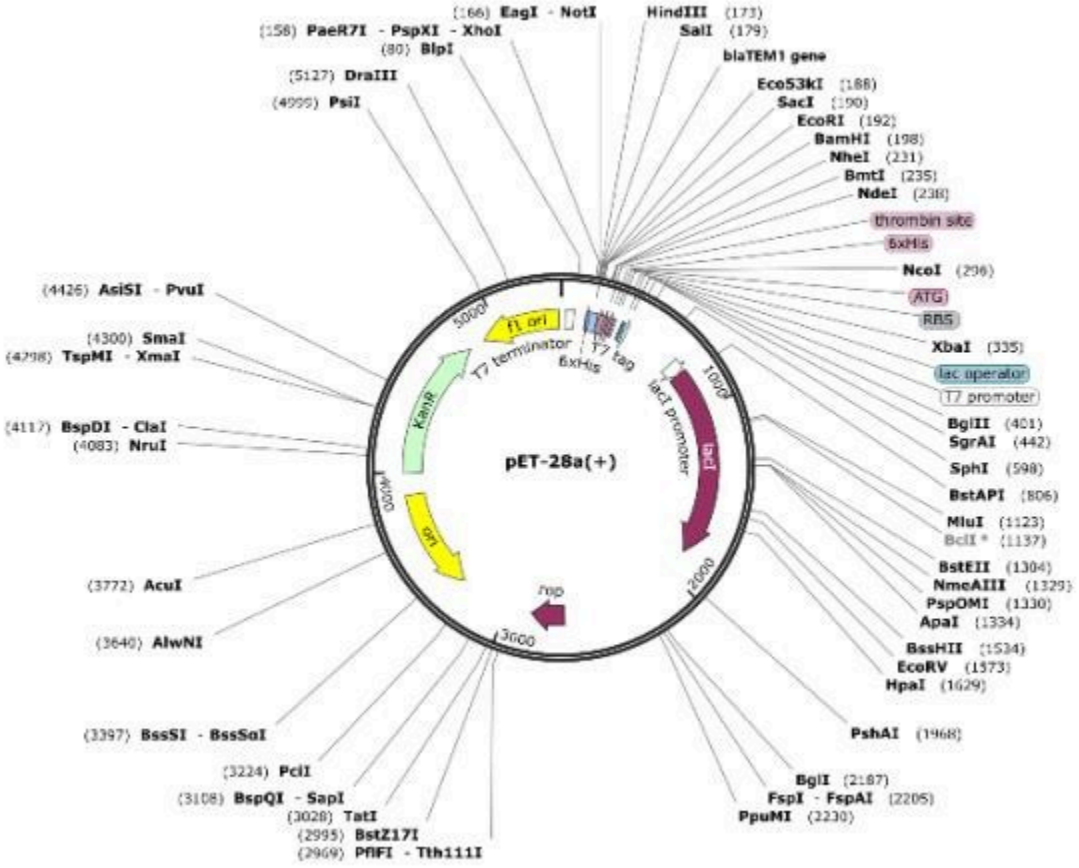


Figure 1

pET-28a (+) vector containing the *bla*TEM gene insert

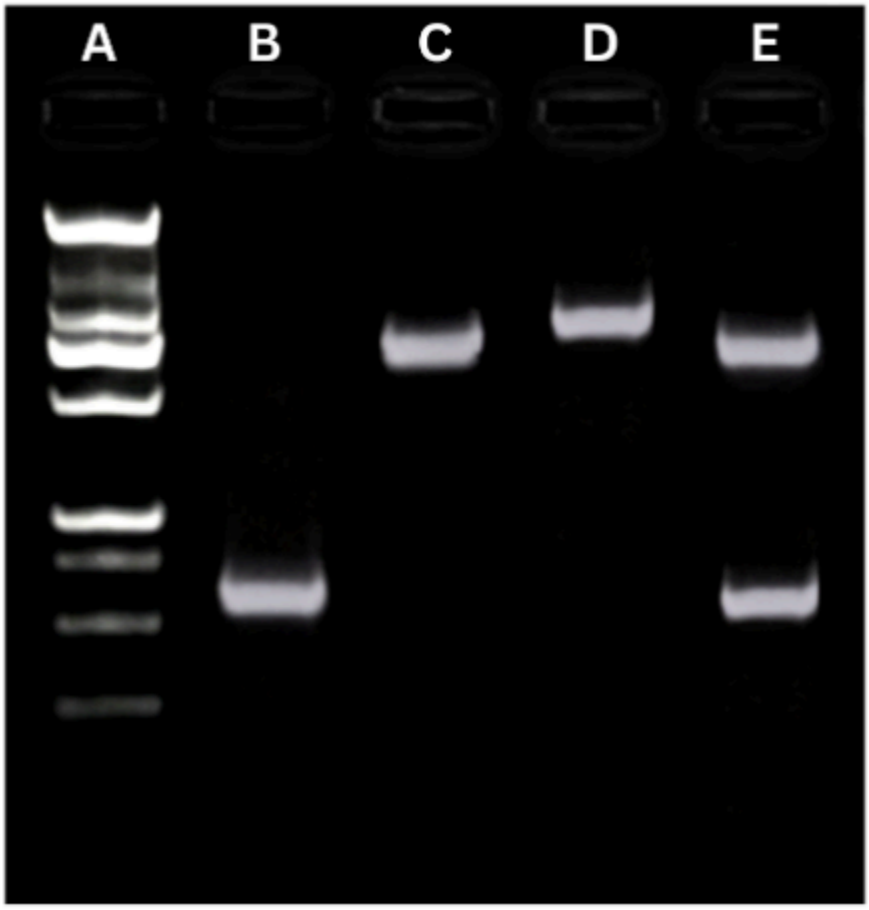


Figure 2

Confirmation of recombinant pET-28a (+) vector by PCR amplification and restriction digestion**;** (A)10kb DNA marker ladder (B) *bla*TEM PCR amplicon (C) pET-28a (+) vector (D) recombinant plasmid pET-28a (+) with *bla*TEM gene (E) Restriction digested recombinant pET-28a (+) vector

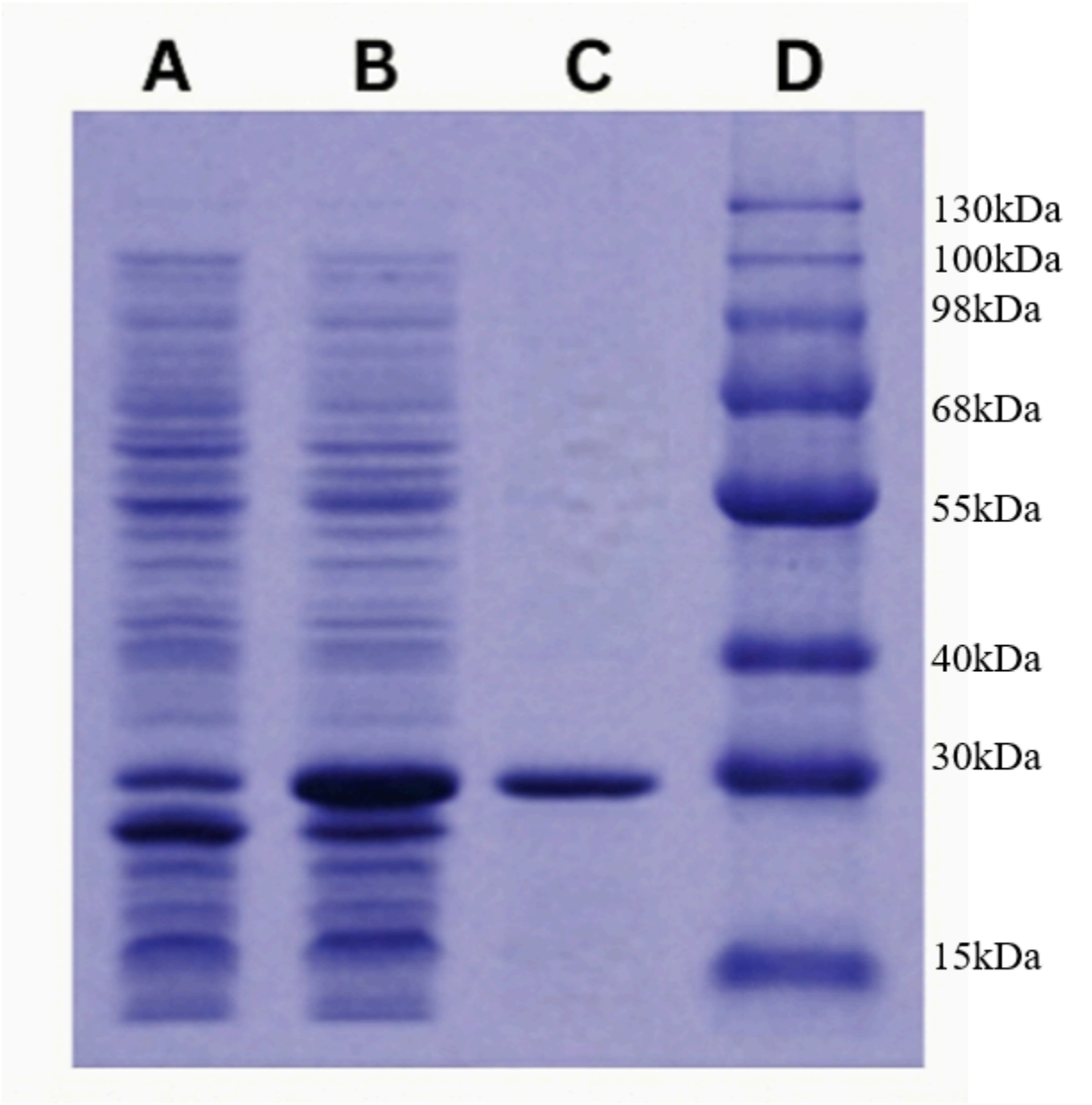


Figure 3

SDS-PAGE analysis of expressed and purified recombinant TEM β-lactamase; (A)Crude enzyme lysate (B) IPTG-induced recombinant β-lactamase (C) Purified recombinant β-lactamase (D) 130kDa to 15kDa Protein ladder