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Original Article

Medicinal Plant Extracts as Potential β-Lactamase Inhibitors: Integrated Evaluation of β-Lactamase Enzyme Inhibition and *in vitro* Cytotoxicity

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ABSTRACT

Background: The increasing prevalence of β-lactamase-mediated antimicrobial resistance has substantially reduced the effectiveness of β-lactam antibiotics, highlighting the need to identify novel β-lactamase inhibitors. Medicinal plants are rich sources of structurally diverse phytochemicals and represent promising candidates for the discovery of natural antibiotic adjuvants capable of overcoming bacterial resistance mechanisms. **Aim:** This study aimed to evaluate the β-lactamase inhibitory activity and *in vitro* cytotoxicity of selected medicinal plant extracts to identify promising candidates for subsequent phytochemical investigation. **Materials and Methods:** Aqueous, methanolic, and ethyl acetate extracts prepared from *Azadirachta indica* A. Juss., *Phyllanthus emblica* Linn., *Phyllanthus niruri* Linn., *Ocimum tenuiflorum* Linn., *Solanum nigrum* Linn., *Terminalia bellirica* Gaertn. Roxb., and *Terminalia chebula* Retz. were screened for β-lactamase inhibitory activity using a commercial kit of β-lactamase enzyme assay. Cytotoxicity was assessed against human dermal fibroblast adult (HDF-a) cells using a resazurin-based cell viability assay. The findings were subsequently interpreted in the context of LC-MS-guided phytochemical investigations. **Results:** Methanolic extracts of *A. indica* and *P. emblica* exhibited the greatest β-lactamase inhibitory activity, whereas *T. bellirica* demonstrated little or no detectable inhibition. All extracts except the methanolic extract of *S. nigrum* were non-toxic toward HDF-a cells at 300 µg/mL. **Conclusion:** The selected medicinal plant extracts possess β-lactamase inhibitory activity under the experimental conditions evaluated. These findings identify medicinal plant extracts as potential sources of bioactive compounds capable of targeting β-lactamase-mediated antimicrobial resistance and provide a strong foundation for subsequent phytochemical isolation, functional characterisation, and validation of individual β-lactamase inhibitors. Collectively, this work supports the continued exploration of plant-derived natural products as antibiotic adjuvants to restore the efficacy of β-lactam antibiotics against resistant bacterial pathogens.

Keywords: Antibiotic adjuvants, Antimicrobial resistance, Cytotoxicity, Medicinal plants, Natural product discovery, Phytochemicals, β-Lactamase inhibition.

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INTRODUCTION

Antimicrobial resistance (AMR) has become one of the most pressing global public health challenges, threatening the effective prevention and treatment of bacterial infections and contributing to increased morbidity, mortality, and healthcare costs. Among currently available antimicrobial agents, β-lactam antibiotics remain the most widely prescribed class because of their broad-spectrum activity, favourable safety profile, and clinical effectiveness.[1,2] However, the rapid emergence and dissemination of β-lactamase enzymes, including extended-spectrum β-lactamases (ESBLs) and carbapenemases, have substantially compromised the therapeutic efficacy of these antibiotics by catalysing hydrolysis of the β-lactam ring. Although clinically approved β-lactamase inhibitors have improved treatment outcomes, the continued evolution of resistant enzymes has reduced their long-term effectiveness, reinforcing the urgent need to identify novel β-lactamase inhibitors capable of restoring the activity of existing β-lactam antibiotics.[1-4]

Natural products have historically provided an invaluable source of therapeutic agents, with medicinal plants representing an extensive reservoir of structurally diverse secondary metabolites that continue to inspire modern drug discovery. Phytochemicals, including flavonoids, hydrolysable tannins, phenolic acids, terpenoids, and related polyphenolic compounds

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have demonstrated a broad spectrum of biological activities, including antibacterial, antioxidant, anti-inflammatory, and enzyme-modulating properties. Increasing evidence indicates that some plant-derived metabolites can enhance antibiotic efficacy by targeting bacterial resistance mechanisms, including β-lactamase enzymes, thereby functioning as antibiotic adjuvants rather than conventional antibacterial agents alone. Despite this growing interest, relatively few studies have systematically investigated medicinal plant extracts for direct β-lactamase inhibitory activity, limiting the discovery of naturally derived inhibitors with potential to combat β-lactam-mediated resistance.[2-5]

Medicinal plants remain attractive candidates for β-lactamase inhibitor discovery because they contain chemically complex mixtures of bioactive metabolites that may act individually or collectively to modulate bacterial resistance mechanisms. The seven medicinal plants investigated in the present study (*Azadirachta indica, Phyllanthus emblica, Phyllanthus niruri, Ocimum tenuiflorum, Solanum nigrum, Terminalia bellirica, Terminalia chebula*) have been previously been reported to possess antibacterial and other pharmacological activities that are largely attributed to their rich phytochemical composition, particularly flavonoids, hydrolysable tannins, phenolic acids, and related polyphenolic constituents.[6-18] Consistent with these reports, a previous investigation by our group demonstrated that these medicinal plants possess noteworthy antibacterial activity against clinically relevant bacterial pathogens.[19] However, comparatively little information is available regarding the β-lactamase inhibitory potential and mammalian cell compatibility of these medicinal plant extracts.

A stepwise strategy that combines biological screening with phytochemical investigation is required to prioritise promising medicinal plants before undertaking detailed compound isolation and mechanistic studies. As part of our ongoing research project, comprehensive LCMS profiling of these same medicinal plants subsequently identified 174 putative phytochemicals spanning multiple classes of secondary metabolites, thereby providing a phytochemical framework for targeted biological validation and functional investigation of individual compounds.[20] Establishing the β-lactamase inhibitory activity and cytotoxicity profiles of the crude plant extracts therefore represents a critical first step in identifying promising candidates for subsequent phytochemical investigation.

The present study aimed to systematically evaluate the β-lactamase inhibitory activity and *in vitro* cytotoxicity of the selected medicinal plant extracts using a commercial β-lactamase inhibition assay kit and human dermal fibroblast adult (HDF-a) cells, respectively. By identifying medicinal plant extracts with promising β-lactamase inhibitory activity and acceptable cellular compatibility, this study establishes the foundation for an LCMS-guided natural product discovery plan aimed at identifying plant-derived β-lactamase inhibitor candidates. Together with subsequent phytochemical investigations, this integrated approach contributes to the development of evidence-based strategies for discovering natural antibiotic adjuvants capable of restoring the effectiveness of β-lactam antibiotics against resistant bacterial pathogens.[20]

MATERIALS AND METHODS

Plant Materials

Seven medicinal plants were selected for this study: *Azadirachta indica* A. Juss., *Phyllanthus emblica* Linn., *Phyllanthus niruri* Linn., *Ocimum tenuiflorum* Linn., *Solanum nigrum* Linn., *Terminalia bellirica* (Gaertn.) Roxb., and *Terminalia chebula* Retz. All plant materials were purchased in powdered form from Australian online suppliers.

Leaf powders were used for *A. indica, P. niruri, O. tenuiflorum*, and *S. nigrum*, whereas dried fruit powders were used for *P. emblica, T. bellirica*, and *T. chebula*. The *P. emblica* and *T. chebula* fruit powders were manufactured by Aarshaveda and purchased from Sattvic Australia. The *T. bellirica* fruit powder and the leaf powders of *O. tenuiflorum* and *S. nigrum* were purchased from Navafresh Australia. The identity, authenticity, and quality of the plant materials were verified by the respective suppliers. All powders were stored at -20ºC at the School of Pharmacy and Medical Sciences, Griffith University, Gold Coast, Queensland, Australia.

Preparation of plant extracts

Aqueous and methanolic extracts were prepared separately from all seven plant materials. Ethyl acetate extracts were additionally prepared from *T. bellirica* and *T. chebula* for toxicity analysis. For each extraction, 1 g of plant powder was transferred into separate sterile 50 mL Falcon tubes and sterile deionised water or analytical-reagent-grade methanol was added to a final volume of 50 mL.[18,19] For the two Terminalia species, an additional extraction was performed using analytical-reagent-grade ethyl acetate. Methanol and ethyl acetate were supplied by ChemSupply (Gillman, South Australia, Australia).

The plant-solvent mixtures were mixed at room temperature for 24 hr and then vacuum-filtered through Whatman No. 54 filter paper (Sigma-Aldrich, Melbourne, Victoria, Australia) into pre-weighed tubes. The aqueous extracts were lyophilised for 72 hr using an Alpha 1-4 LSC plus benchtop freeze dryer (Martin Christ, Osterode am Harz, Germany). The methanolic and ethyl acetate extracts were evaporated in water bath at 40ºC until completely dry.

The dried extracts were weighed to determine their extraction yields and then reconstituted in 10 mL of 1% Dimethyl Sulfoxide (DMSO; Merck, Macquarie Park, New South Wales, Australia). The solutions were sterilised using 0.22 µm syringe filters

(Sarstedt, Mawson Lakes, South Australia, Australia) and stored at -20°C until required for analysis.

β-Lactamase inhibition screening

The ten selected plant extracts were tested for β-lactamase inhibition activity using a commercially supplied β-lactamase assay kit (Sigma-Aldrich, Bayswater, Australia). Each sample was prepared at a total volume of 200 µL for duplicate testing. The final concentrations in the assay wells were 100 µg/mL for plant extracts. DMSO percentages were calculated to ensure they remained between 0.01% in the wells. To achieve the desired final concentrations, 5×concentrated working solutions of each extract and positive control were prepared. For extracts with a target final concentration of 100 µg/mL in the assay wells, 5×concentrated working solutions were prepared at 500 µg/mL.

The extracts selected for study were *Azadirachta indica* (AzI-Aq, MeOH), *Phyllanthus emblica* (EO-Aq, MeOH), *Phyllanthus niruri* (PN-Aq, MeOH), *Terminalia bellerica* (TB-Aq, MeOH), and *Terminalia chebula* (TCh-Aq, MeOH). A volume of 20 µL of test components were dispensed into duplicate wells of a 96-well plate. In the assay, the "sample inhibitor" refers to the sample (plant extract) being tested. The experimental setup included: (i) sample inhibitor (5× inhibitor solution), (ii) sample blank (5× inhibitor solution without enzyme), (iii) enzyme control (β-lactamase assay buffer), and (iv) control wells containing either the reference inhibitor (clavulanic acid) or vehicle (DMSO).

For the inhibition reaction, enzyme was diluted 1 in 25 in the assay buffer. The mixture was added to wells containing sample inhibitors, enzyme controls, inhibitor controls, or vehicle controls. In the case of sample blanks or vehicle blanks, 50 µL of β-lactamase assay buffer alone was used. Each well therefore received 50 µL of the appropriate inhibition reaction mixture. The plate was mixed either by gentle pipetting, or by horizontal shaker and then incubated at 25°C for 10 min, protected from light by covering it with aluminium foil. The enzymatic reaction mixture was prepared by combining 29 µL of β-lactamase assay buffer with 1 µL of nitrocefin, yielding 30 µL per well. This mixture was added to each well and mixed thoroughly.

The enzymatic activity was monitored by measuring absorbances at 490 nm using a microplate reader in kinetic mode for 30 min, with measurements recorded each minute. Data collection and analysis were performed using the Tecan i-control 2.0 software on an Infinite 200 Pro system. Two-time intervals [time intervals of 5-10 min (T1), followed by time intervals of 11-15 min (T2)] were selected from linear window of 5 min to 15 min for the calculation of % relative inhibition.

The sample inhibitor tested the inhibitory effect of each sample on β-lactamase activity. The sample blank contained the same sample without the enzyme and was used to account for any background absorbance from sample readings. The enzyme control contained only β-lactamase and buffer (without inhibitor) and represented 100% enzyme activity, serving as the baseline for inhibition calculations. The inhibitor control included a β-lactamase inhibitor (clavulanic acid, Merck Life Science Pvt. Ltd., Australia) to confirm that the assay effectively detected enzyme inhibition.

To calculate the percentage of relative inhibition (% RI), the absorbance (ABS) for each well was plotted over time. Two time points (T_1 and T_2) within the linear range (T_1 = 5-10 min; T2 = 11-15 min) of the curve were selected to determine the slope for each well between T_1 and T_2. The corresponding absorbance values (ABS_1 and ABS_2) were used to calculate the reaction rate (ABS/minute). The slope of the sample blank was subtracted from that of the corresponding sample to obtain the slope of corrected measurement (Slope CM), which was then used to calculate the % relative inhibition.

$$\text{Slope} = (ABS_2 - ABS1)/ (T_2 - T_1) = \Delta ABS/\text{minute}$$

$$\text{Slope CM} = (\text{Slope Extract} + \text{Enzyme}) - (\text{Slope Extract only})$$

$$\%\ \text{RI} = \text{Slope EC} - \text{Slope CM} / \text{Slope EC} \times 100$$

The Slope of the Corrected Measurement (Slope CM) represents the rate obtained from the sample inhibitor, whilst the Slope of the Enzyme Control (Slope EC) corresponds to the β-lactamase activity. Irreversible inhibitors, such as clavulanic acid, completely suppress β-lactamase activity, resulting in ΔABS = 0 and a % relative inhibition of 100%. Negative values were adjusted to 0%, and results exceeding 100% were set to 100%. Notably, these values are relative to the measured absorbance and do not represent absolute inhibition.

Toxicity in adult human dermal fibroblasts

Human Dermal Fibroblasts adult (HDF-a) (Merck Life Science Pvt. Ltd., Australia) at passage 5 were used following routine subculture to confirm healthy morphology. Cell lines were grown in complete medium (fibroblast growth medium; supplemented with low serum growth supplement; Merck Life Science Pvt. Ltd., Australia) purchased from Thermo Fisher Scientific Australia Pvt. Ltd., Cells were expanded and counted using a Countess 3 Automated Cell Counter (Thermo Fisher Scientific, Australia) and the suspension was adjusted to 50,000 cells/mL. A 100 µL volume of the cell suspension (5,000 cells/well) was dispensed into each well of a 96-well flat-bottom plate, followed by 40 µL of complete medium to reach 140 µL/well. Plates were incubated for 24 hr at 37°C in an atmosphere of 5% CO_2.

The final volume used in the assay was 200 µL/well, with final concentrations of DMSO in any well not exceeding 0.75%. Plant extracts (1 mg/mL), and controls (docetaxel 1 mM; DMSO 0.1%) (Merck Life Science Pvt. Ltd., Australia, purity ≥97% HPLC) were used. All selected plant extracts were tested at a final concentration of 300 µg/mL, using 1 mg/mL stocks prepared in 0.1% DMSO. All

plant extracts and DMSO dilutions were prepared in autoclaved ultrapure water. For dosing, 60 µL of each extract was added to the appropriate wells, whilst for the positive control, 20 µL of 1 mM docetaxel plus 40 µL of complete medium were added. Plates were incubated for a further 24 hr.

Following aspiration of culture medium (200 µL/well), 200 µL of a 44 mM resazurin solution (Merck Life Science Pvt. Ltd., Australia) was added to each well. Plates were incubated for 1 hour and absorbances immediately recorded using a Tecan Infinite 200 Pro fluorescence plate reader equipped with i-control 2.0 software for fluorescence and absorbance measurements. Recordings were taken every hour for 6 hr. Average signals were quantified relative to the appropriate controls (DMSO, media), converted to percent response as cell viability after correction with the appropriate controls, and summarised graphically. All conditions were run in technical quadruplicate, and the experiment repeated on two separate days (n=8).

Selected extracts were tested at 300 µg/mL (0.1% DMSO). These included (AzI-Aq, MeOH), (EO-Aq, MeOH, EtOAc), (PN-Aq, MeOH), (OT-Aq, MeOH), (SN-Aq, MeOH), (TB-Aq, MeOH, EtOAc), and (TCh-Aq, MeOH, EtOAc). The % cell viability could then be calculated using the following formula:

% Cell Viability for extracts = Extract absorbance/ Media control absorbance x 100

% Cell Viability for DMSO = DMSO vehicle control exposure absorbance/ Media control absorbance x 100

To evaluate DMSO cytotoxicity, the cell viability of the DMSO control was first expressed relative to the media control, and for samples (extracts) the corrected viability (%) was obtained by normalising the sample viability to the DMSO control viability, using the following formula:

% Corrected Cell Viability of samples = Viability % (samples)/ Viability % (DMSO) x 100

This approach eliminates the contribution of DMSO toxicity and reflects extracts-specific effects on cell viability. Corrected cell viability percentage of extracts are depicted in the form of bar graph, with mean±SEM from a minimum of quadruplicates from two independent experiments (n=8). Plant extracts with cell viability ≤ 50% were considered toxic, whereas those with cell viability > 50% were deemed non-toxic.

Statistical analysis

For the β-lactamase inhibition assay, measurements were obtained only from technical replicates within a single experimental run due to the limited availability of assay reagents, which were prioritised for testing selected plant extracts and phytochemicals. Results are presented as mean±Standard Deviation (SD), representing within-run variability and assay precision under identical experimental conditions. Therefore, the data were analysed descriptively only; no inferential statistical testing was performed, and statistical significance could not be determined. The results should therefore be interpreted as preliminary screening data.

For the HDF-a cytotoxicity assay, each treatment was evaluated in quadruplicate across two independent experiments conducted on separate days, providing eight well-level measurements per treatment (n=8). Corrected cell viability values are presented as the mean±Standard Error of the Mean (SEM). Statistical differences among treatment groups and the control were evaluated using one-way Analysis of Variance (ANOVA), with $p<0.05$ considered statistically significant.

RESULTS

β-Lactamase inhibition screening of extracts

The percentage Relative Inhibition (% RI) of selected plant extracts on β-lactamase activity were assessed over linear window of two-time intervals (which were 5 min each): 5-10 min (time interval 1) and 11-15 min (time interval 2).

For the extracts (Figure 1), AzI-Aq inhibited β-lactamase activity by 31% at time interval 1 and 70% at time interval 2, whilst AzI-MeOH demonstrated strong activity with 92% and 85% inhibition at the respective intervals. This suggests that better inhibition response of AzI-MeOH at shorter exposure times. EO-Aq showed 23% inhibition at time interval 1 and 75% at time interval 2, whereas EO-MeOH achieved complete inhibition (100%) across both intervals. PN-Aq was inactive at time interval 1 but reached 25% inhibition at time interval 2, whilst PN-MeOH showed 8% initially and complete inhibition (100%) by time interval 2. Both TB-Aq and TB-MeOH exhibited no inhibition (0% at both intervals). TCh-Aq displayed no activity at time interval 1 but 90% inhibition at time interval 2, whilst TCh-MeOH showed 0% initially and 70% inhibition at time interval 2.

Toxicity

The toxicity of plant extracts was evaluated using human dermal fibroblasts adult (HDF-a) in cell viability assays. A cell viability of ≤50% was considered toxic, whereas values >50% indicated non-toxicity. At 300 µg/mL, all extracts, except the *Solanum nigrum* methanol extract, were non-toxic (Figure 2). Additionally, aqueous and methanol *Ocimum tenuiflorum*, and aqueous *Solanum nigrum* extracts increased cell viability levels above 100%.

DISCUSSION

Selected medicinal plant extracts exhibited markedly different capacities to inhibit β-lactamase activity, with the methanolic extracts of *A. indica* and *P. emblica* producing the strongest inhibitory effects. In contrast, *T. bellirica* showed little or no

detectable activity under the experimental conditions evaluated. This variation indicates that β-lactamase inhibition is influenced by both plant species and extraction method, reflecting differences in the recovery of bioactive constituents capable of interacting with the enzyme. Similar variability has been reported in previous studies, which demonstrate that medicinal plants are a valuable source of natural β-lactamase inhibitors, although their inhibitory potency differs considerably according to phytochemical composition and extraction methodology.[3-5] Plant-derived metabolites, particularly polyphenols and flavonoids, have been shown to interfere with β-lactamase activity through diverse inhibitory mechanisms, highlighting the potential of natural products as complementary approaches for overcoming enzyme-mediated β-lactam resistance.[2-4] Although the molecular interactions responsible for the inhibitory effects observed in the present study were not investigated, the extract-specific activity supports the presence of bioactive constituents with the potential to complement conventional β-lactam therapy. These findings provide the basis for examining how individual medicinal plant species and their phytochemical profiles contribute to the observed β-lactamase inhibitory activity.

A clear species- and extraction-dependent pattern of β-lactamase inhibition was observed among the selected medicinal plant extracts. Overall, the methanolic extracts of *A. indica* and *P. emblica* displayed the strongest inhibitory activity, whereas *P. niruri* exhibited a more gradual inhibitory response and the corresponding aqueous extracts generally showed lower or more variable activity under the experimental conditions. These differences are consistent with previous reports describing the diverse phytochemical composition of these species, including polyphenols, flavonoids, tannins, phenolic acids etc.[6-12] The greater activity observed in several methanolic extracts may reflect the improved recovery of bioactive constituents by organic solvents. However, the delayed inhibitory response of the methanolic extract of *P. niruri* suggests that extraction efficiency alone does not determine enzyme inhibition. This suggests that the chemical characteristics of the extracted metabolites may also influence their inhibitory behavior. This variation becomes particularly evident when comparing the two tannin-rich *Terminalia* species, which exhibited markedly different inhibitory profiles despite belonging to the same genus.

Although *Terminalia chebula* and *Terminalia bellirica* are both tannin-rich medicinal plants with well-documented antimicrobial properties, they exhibited markedly different β-lactamase inhibitory profiles in the present study. *Terminalia chebula* demonstrated substantial inhibition during the later stage of the assay, whereas *T. bellirica* showed little or no detectable β-lactamase inhibitory activity under the experimental conditions evaluated. This contrast indicates that broad-spectrum antibacterial activity does not necessarily correspond to direct inhibition of resistance enzymes. Whilst both species contain hydrolysable tannins, phenolic acids, flavonoids, and related polyphenolic compounds, differences in their chemical composition are likely to influence their capacity to interact with β-lactamase enzymes.[13-17] Consistent with our previous investigation, medicinal plant extracts may exert antibacterial

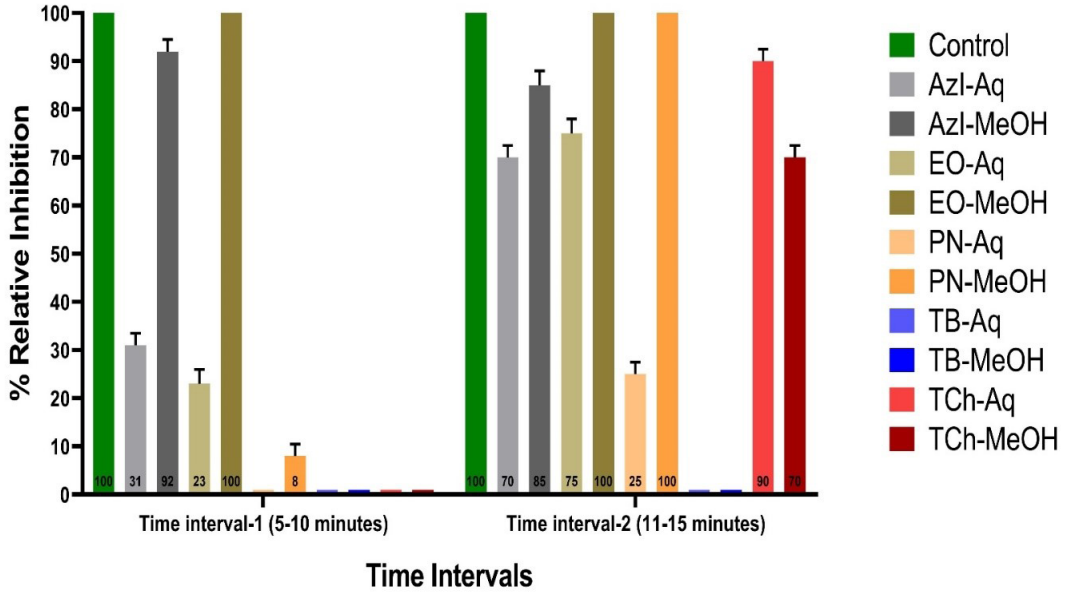


Figure 1: The percentage relative inhibition (%RI) of β-lactamase activity by plant extracts across two-time intervals of 5 min. AzI-Aq = aqueous *A. indica* extract; AzI-MeOH = methanol *A. indica* extract; EO-Aq = aqueous *P. emblica* extract; EO-MeOH = methanol *P. emblica* extract; PN-Aq = aqueous *P. niruri* extract; PN-MeOH = methanol *P. niruri* extract; TB-Aq = aqueous *T. bellirica* extract; TB-MeOH = methanol *T. bellirica* extract; TCh-Aq = aqueous *T. chebula* extract; and TCh-MeOH = methanol *T. chebula* extract. The positive control is the β-lactamase inhibitor, clavulanic acid. Bars represent the mean±SD of technical replicates from a single experimental run.

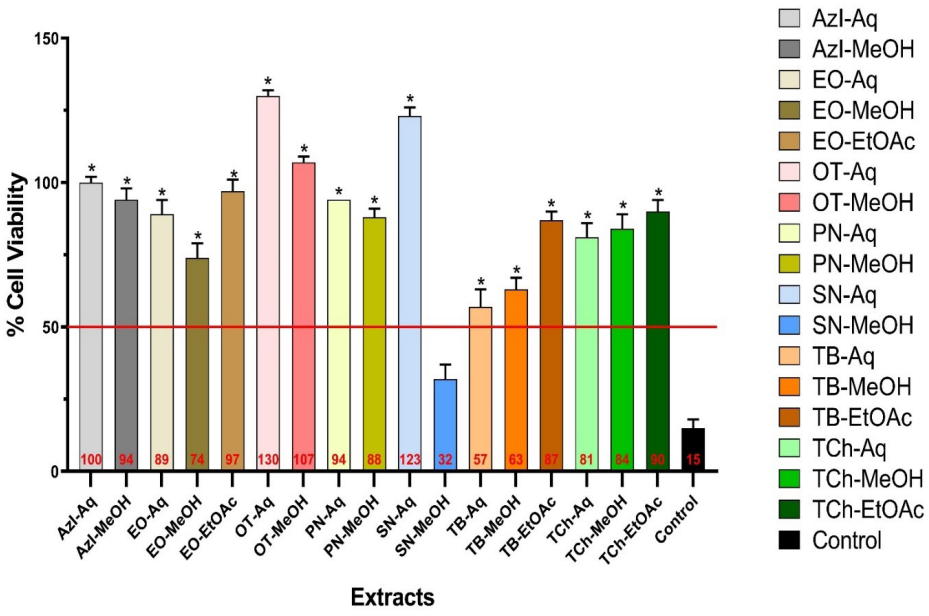


Figure 2: Cell viability percentage of plant extracts against human dermal fibroblasts adult (HDF-a). The results represent cell viability (%) after treatment with plant extracts at 300 µg/mL. Viability ≤50% was considered toxic represented by the red line, whereas >50% indicated non-toxicity. . AzI-Aq = aqueous *A. indica* extract; AzI-MeOH = methanol *A. indica* extract; EO-Aq = aqueous *P. emblica* extract; EO-MeOH = methanol *P. emblica* extract; PN-Aq = aqueous *P. niruri* extract; PN-MeOH = methanol *P. niruri* extract; TB-Aq = aqueous *T. bellirica* extract; TB-MeOH = methanol *T. bellirica* extract; TB-EtOAc = ethyl acetate *T. bellirica* extract; TCh-Aq = aqueous *T. chebula* extract; TCh-MeOH = methanol *T. chebula* extract and TCh-EtOAc = Ethyl acetate *T. chebula* extract. Positive control (docetaxel). Data are presented as mean±SEM from quadruplicate technical replicates across two independent experiments (n=8). Differences between treatment groups and the control were analysed using one-way ANOVA, with $p<0.05$ considered statistically significant and represent with*.

effects through multiple mechanisms beyond β-lactamase inhibition, including disruption of bacterial physiology and interference with essential cellular processes.[18] Therefore, the absence of detectable β-lactamase inhibition in *T. bellirica* should not be interpreted as a lack of antimicrobial potential, but rather as evidence that closely related medicinal plants can target different bacterial pathways. These findings emphasise that β-lactamase inhibitory activity cannot be predicted solely from taxonomic relatedness, traditional medicinal use, or reported antibacterial activity, highlighting the importance of identifying the specific phytochemicals responsible for enzyme inhibition in the most active extracts.

The variability in β-lactamase inhibition observed among the medicinal plant extracts indicated that further investigation at the phytochemical level was required. Although crude extracts are valuable for identifying biologically active plants, they cannot determine which individual phytochemicals are responsible for the observed β-lactamase inhibition. Therefore, evaluation of purified phytochemicals must be undertaken to identify the compounds contributing to the inhibitory activity and to improve our understanding of the relationship between chemical structure and enzyme inhibition. This approach also provides a foundation for identifying natural product scaffolds with potential as β-lactamase inhibitors or antibiotic adjuvants.

Building upon our previous investigations of the same seven medicinal plants, comprehensive LCMS profiling tentatively identified 174 phytochemicals, providing the phytochemical basis for subsequent biological evaluation.[19,20] Evaluation of the selected purified phytochemicals revealed considerable variation in antibacterial activity and only seven phytochemicals were selected for β-lactamase inhibition assay. Among the selected antibacterial phytochemicals evaluated, luteolin exhibited the strongest and most sustained inhibitory activity, maintaining complete inhibition throughout both kinetic intervals, while quercetin also showed consistently high inhibitory activity, reaching complete inhibition during the later phase of the assay. In contrast, gallic acid produced complete inhibition during the initial interval but declined markedly over time, whereas catechin displayed only transient partial inhibition. Corilagin, ellagic acid, and naringenin showed no detectable β-lactamase inhibitory activity despite demonstrating antibacterial activity in previous investigations.[20] The sustained inhibitory effects of luteolin and quercetin identify these flavonoids as the most promising β-lactamase inhibitors among the phytochemicals evaluated, whereas the variable responses of the remaining compounds highlight the functional diversity of structurally related natural products.

The marked differences in β-lactamase inhibitory activity among the phytochemicals suggest that subtle variations in chemical structure play an important role in determining their inhibitory potential. Flavonoids such as luteolin and quercetin possess multiple hydroxyl groups arranged on a rigid flavone or flavonol backbone, structural features that have been associated

with promising interactions within the active site of β-lactamase enzymes through hydrogen bonding and hydrophobic interactions.[21-29] Previous studies have reported that quercetin inhibits clinically important β-lactamases, including OXA-48 and CTX-M-15, and molecular docking analyses have demonstrated stable binding within the catalytic pocket of these enzymes.[30-32] Similarly, luteolin has been reported to exhibit inhibitory activity against β-lactamases and other bacterial enzymes, supporting its broad potential as a natural antimicrobial adjuvant.[23,27] In contrast, although gallic acid, catechin, corilagin, ellagic acid, and naringenin possess recognised antibacterial properties,[20-28] their limited or absent β-lactamase inhibitory activity in the previous study suggests that antibacterial efficacy alone does not necessarily translate into effective enzyme inhibition.

Assessment of cytotoxicity alongside β-lactamase inhibition is essential during the early stages of natural product discovery, as candidate antibiotic adjuvants should demonstrate activity against bacterial targets, whilst maintaining acceptable compatibility with mammalian cells. In the present study, all medicinal plant extracts, except the methanolic extract of *S. nigrum*, were non-toxic towards HDF-a cells at 300 µg/mL under the investigated conditions, indicating encouraging *in vitro* biocompatibility for most of the extracts tested. In addition, the aqueous and methanolic extracts of *O. tenuiflorum* and the aqueous extract of *S. nigrum* produced cell viability values exceeding 100% in the resazurin assay. Although these values should be interpreted cautiously, they may reflect increased cellular metabolic activity or reducing capacity relative to the untreated control rather than a true increase in cell number and have been reported previously for certain plant-derived natural products in *in vitro* cell viability assays.[33-35] Our subsequent investigation of purified phytochemicals further demonstrated that the principal β-lactamase inhibitory compounds identified from these medicinal plants also exhibited favorable compatibility with HDF-a cells under the same experimental conditions.[20] Together, the crude extract and phytochemical findings indicate that β-lactamase inhibitory activity can be achieved whilst maintaining acceptable *in vitro* cellular compatibility at the concentrations evaluated. Nevertheless, these observations are limited to a single mammalian cell line and should not be interpreted as evidence of systemic safety. Further toxicological evaluation using additional mammalian cell models and *in vivo* studies are required to determine the therapeutic potential of these plant-derived β-lactamase inhibitor candidates.

The present findings highlight medicinal plants as valuable sources of bioactive compounds with potential to complement existing strategies for combating β-lactam-mediated antimicrobial resistance. By combining β-lactamase inhibition screening with cytotoxicity assessment, this study identified several medicinal plant extracts with promising biological activity, whilst establishing a foundation for subsequent phytochemical investigations. Together with our previous LCMS characterisation and biological evaluation of representative phytochemicals, these results demonstrate the value of integrating phytochemical profiling with functional validation to identify natural products with therapeutic potential.[19,20] Nevertheless, several limitations should be considered. The present investigation evaluated crude plant extracts using a single β-lactamase enzyme assay, whilst cytotoxicity was assessed in one mammalian cell line under defined experimental conditions. Future studies should therefore focus on bioassay-guided isolation of additional active constituents, detailed enzyme kinetic and molecular interaction studies, evaluation against clinically relevant β-lactamase families, including TEM, SHV, CTX-M, NDM, and OXA enzymes, assessment of combination therapy with β-lactam antibiotics, and validation in appropriate *in vivo* infection models. Collectively, these investigations will improve understanding of plant-derived β-lactamase inhibitors and support the development of natural antibiotic adjuvants capable of restoring the effectiveness of β-lactam antibiotics against multidrug-resistant bacterial pathogens.[36,37]

CONCLUSION

The present study demonstrated that several medicinal plant extracts possess β-lactamase inhibitory activity, although the magnitude of inhibition varied substantially according to plant species and extraction solvent. Among the extracts evaluated, methanolic extracts of *A. indica* and *P. emblica* exhibited the greatest β-lactamase inhibition. Cytotoxicity assessment further demonstrated that all extracts, except for the methanolic extract of *S. nigrum*, were non-toxic towards HDF-a cells at 300 µg/mL, indicating favourable *in vitro* biocompatibility for the majority of the medicinal plant extracts evaluated. Together, these findings support the potential of selected medicinal plants as sources of bioactive constituents capable of targeting β-lactamase-mediated antimicrobial resistance.

The present findings provide a strong foundation for the continued development of medicinal plant-derived β-lactamase inhibitors. Although further investigations are required to test additional active constituents, characterise their molecular mechanisms, evaluate activity against clinically relevant β-lactamase families, and validate efficacy and safety *in vivo*. Such compounds may contribute to future strategies aimed at restoring the effectiveness of β-lactam antibiotics and addressing the growing global challenge of antimicrobial resistance.

ABBREVIATIONS

AMR: Antimicrobial Resistance; **Aq:** Aqueous; **AzI:** *Azadirachta indica* A. Juss.; **CTX-M:** CTX-M-type β-lactamase; **DMSO:** Dimethyl Sulfoxide; **EO:** *Phyllanthus emblica* Linn.; **ESBL:** Extended-Spectrum β-lactamase; **EtOAc:** Ethyl Acetate; **MeOH:** Methanol; **NDM:** New Delhi metallo-β-lactamase; **OT:** *Ocimum*

tenuiflorum Linn.; **OXA:** Oxacillinase-type β-lactamase; **PN:** *Phyllanthus niruri* Linn.; **SD:** Standard Deviation; **SEM:** Standard Error of the Mean; **SHV:** SHV-type β-lactamase; **SN:** *Solanum nigrum* Linn.; **TB:** *Terminalia bellirica* (Gaertn.) Roxb.; **TCh:** *Terminalia chebula* Retz.; **TEM:** TEM-type β-lactamase; **ΔABS/minute:** Change in absorbance per minute.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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