REVERSION OF ANTIBIOTIC RESISTANCE WITH BETALACTAMASE INHIBITOR FROM MEDICINAL PLANTS

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ABSTRACT

Objective: Screening of medicinal plants for the presence of beta-lactamase inhibitor identified three plants; Terminalia chebula, Terminalia bellirica, and Ocimum tenuiflorum, extracts of which inhibit beta-lactamase enzyme in vitro. The objective of this study was to evaluate and compare beta-lactamase inhibiting potential of these plant extracts.

Methods: Extracts of these plants were prepared with 6 solvents of different polarity. Beta-lactamase inhibition study was performed using antibiotic-resistant bacterial strains in bioassay and by micro-iodometric assay. Multidrug-resistant clinical strains of Escherichia coli and laboratory strain with plasmid carrying beta-lactamase gene as positive control were used.

Results: Our results from bioassay, as well as micro-iodometric assay for enzyme activity, confirmed the presence of beta-lactamase inhibitor in these plant extracts. Among the extracts made by different solvents, hexane and ethyl acetate extract of T. chebula, hexane extract of T. bellirica, and all extracts of O. tenuiflorum except dichloromethane, possessed beta-lactamase inhibitor. Multidrug-resistant clinical isolate of E. coli AIIMS-1 could be reverted by applying 50 µg/ml of each of the medicinal plants. The micro-iodometric result showed highest beta-lactamase inhibition with O. tenuiflorum extracts. Comparative evaluation of the O. tenuiflorum extracts with increasing concentration of inhibitor suggests that ethyl acetate extract of O. tenuiflorum contains the highest inhibition potential, which is comparable with clavulanic acid.

Conclusion: The results demonstrated that the ethyl acetate extract of O. tenuiflorum contain the highest level of beta-lactamase inhibitor, which in the future can be used as an alternative to synthetic beta-lactamase inhibitors that are presently being used to control beta-lactam antibiotic resistance.

Keywords: Beta-lactamase inhibitor, Micro-iodometric assay, Ocimum tenuiflorum Terminalia chebula, Terminalia bellirica.

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INTRODUCTION

Bacteria are increasingly acquiring resistance to beta-lactam antibiotics and impose challenges to the treatments [1]. Bacterial resistance to beta-lactam antibiotics can be due to any of the 3 reasons; the production of beta-lactam-hydrolyzing beta-lactamase enzymes, the utilization of beta-lactam-insensitive cell wall transpeptidases, and the active expulsion of beta-lactam molecules from Gram-negative cells with the help of efflux pumps [2].

Escherichia coli is a common cause of infections and bacteremia in humans. Different types of infection such as urinary tract infection, pulmonary, and gastrointestinal infections are the most common ones encountered worldwide [3,4]. E. coli becomes frequently resistant to aminopenicillins, such as amoxicillin or ampicillin, and narrow-spectrum cephalosporin [5,6]. Through the acquisition of extended-spectrum beta-lactamases (ESBLs), E. coli strains often develop resistance to 3rd generation cephalosporin and monobactams (i.e., aztreonam) [7,8]. A priority list was announced by the WHO, in which Enterobacteriaceae and ESBLs-producing pathogens were considered to be the most critical ones, against which new antibiotics are required [9]. In the recent years, there has been an increased incidence of ESBLs that hydrolyze and cause resistance to oxymiminocephalosporins and aztreonam among Gram-negative bacteria. Such enzymes are mainly plasmid encoded in E. coli, Klebsiella pneumoniae, Enterobacter, Pseudomonas, and Shigella [10,11]. The presence of betalactamase imparts resistance to penicillin, extended-spectrum cephalosporin, monobactams, and carbapenams. To overcome this resistance pattern, clavulanate, sulbactam, and tazobactam (beta-lactamase inhibitors) came into clinical practice. These inhibitors itself have very little antibacterial property but enhance the activity when combined with beta-lactams (amoxicillin, ampicillin, piperacillin, and ticarcillin) in the treatment of serious microbial infections. However, in this era, resistant pathogens have emerged where these combinations were also not effective [12]. Considering the present scenario of synthetic antibiotic resistance in bacteria, there is an urgent need to isolate herbal beta-lactamase inhibitor with fewer side effects such that antibiotics to which the bacterial isolates turned resistance can be used again along with the isolated beta-lactamase inhibitors.

METHODS

Bacterial strains and preparation of bacterial inoculums

Bacterial strains used in the study were beta-lactam antibiotic resistant E. coli hospital isolates - 2, 7, and 13 (HI-2; HI-7, and HI-13); AIIMS-1 and AIIMS-2, and standard strain microbial type culture collection (MTCC-729) (E. coli obtained from MTCC and Gene Bank; produces beta-lactamase) and DJ1.2 (DH5α transformed with pJET1.2 plasmid containing Amp’ gene; positive control for beta-lactamase). All the organisms were maintained on Luria-Bertani agar plates. In the autoclaved test tubes, 2 ml of Luria-Bertani broth containing (50 µg/ml ampicillin) was taken and the single colony of bacteria was inoculated into the broth. The broth was vortexed thoroughly and incubated overnight in the shaker at 37°C. Turbidity was then checked and adjusted to that of 0.5 McFarland (1.5×10⁶ cells/ml) in each experiment.

Plants collection and preparation of plant extracts

The dried fruits of Terminalia chebula, Terminalia bellirica, and dried aerial parts of Ocimum tenuiflorum were collected from the local market (Delhi). Samples were identified at the National Institute of Science...
Communication and Information Resources, Raw material Herbarium and Museum, Delhi (RHMD), where voucher specimen of each species was deposited with voucher numbers (T. chebula - 3047-74-5, T. bellirica - 3047-74-4, and O. tenuiflorum - 3047-74-7). Plant extracts were prepared as described previously [13]. Plant material was weighed and pulverized into the coarse powder using mortar-pestle. Powdered plant material was soaked in 40 ml solvent and incubated overnight at 37°C for 24 hrs at 90 rpm. The content was then filtered with Whatman No. 1 filter paper. The extract was collected in a sterile glass vial and dried under vacuum and finally reconstituted to 1 ml and stored at 4°C. The extracts were prepared in different solvents according to their polarity and the stock concentration was 50 µg/µl.

Agar cup assay to check the presence of beta-lactamase inhibitor in plant extracts

A volume of 100 µl (turbidity adjusted to that of 0.5 McFarland = 1.5 × 10^6 cells/ml) from the overnight grown bacterial inoculum (AIHIMS-1) was aseptically transferred on the Luria-Bertani agar plates and spread evenly using sterile glass spreader. Using sterile cork borer wells were punched in the agar plate and loaded separately with 50 µl of plant extract, 50 µl solvent controls (any one solvent based on the prepared extract - chloroform/ethanol/methanol/hexane), and 10 µl of substrate (ampicillin). One well was loaded with 50 µl of plant extract and 50 µl of ampicillin together. A known beta-lactamase inhibitor clavulanic acid (CA): amoxicillin (2:1, 100 µg/µl) was used as positive control in the assay system. The Petriplates were incubated overnight at 37°C and zone of inhibition of the samples was recorded. Each experiment was performed in triplicates.

Preparation of crude beta-lactamase enzyme

E. coli culture was grown from a single colony in the presence of ampicillin (20 µg/ml). These cells were harvested by centrifugation (4000 rpm, 15 minutes at 4°C) and washed twice in phosphate buffer (0.01 M, pH 7.0). The cells were disrupted using an ultrasonic disintegrator, with 3 minutes sonic disintegration at 4°C. Cell debris was first removed by ultracentrifugation (40,000 g, 40 minutes at 4°C). The enzyme unit was calculated spectrophotometrically at 630 nm and found to be 0.4 U/ml. The crude beta-lactamase enzyme was stored at −20°C [14].

Beta-lactamase inhibition with plant extracts prepared in different solvents using micro-iodometric assay

The micro-iodometric assay was performed as described previously [15], for the detection of beta-lactamase enzyme in our E. coli bacterial strains. The iodine solution was freshly prepared by adding 20.3 g of iodine and 53.2 g of potassium iodide dissolved in 100 ml of distilled water. The substrate was freshly prepared by adding 10,000 U of penicillin G per ml of phosphate buffer (0.05 M, pH 7.0). The bacterial cultures for the test were picked up by using a sterile inoculating loop, suspended in penicillin solution to make a density of 10^8/ml. These cells were harvested by centrifugation (4000× g, 15 minutes at 4°C) and washed twice in phosphate buffer (0.05 M, pH 7.0). The pre-incubated sample containing beta-lactamase enzyme along with plant extract was added in the wells. The plate was incubated at 30°C for half an hour; two drops of starch and iodine solution were added to all the tested samples in the microtiter plate. Formation of blue color and its persistence for more than 10 min revealed the presence of beta-lactamase inhibitor.

Micro-iodometric assay used for the detection of beta-lactamase activity was modified for detection of beta-lactamase inhibition. In this assay, 0.4 U/ml of beta-lactamase enzyme was incubated with 50 µl of plant extract (50 µg/µl) for an hour at 30°C. Then, 50 µl of penicillin G (6 µg/µl) was added in the wells of microtiter plate. The pre-incubated sample containing beta-lactamase enzyme along with plant extract was added in the wells. The plate was incubated at 30°C for half an hour; two drops of starch and iodine solution were added to all the tested samples in the microtiter plate. Formation of blue color and its persistence for more than 10 min revealed the presence of beta-lactamase inhibitor.

Experiments were done in triplicates and CA - amoxicillin (Augmentin; 100 µg/µl) was used as positive control for beta-lactamase inhibitor throughout the experiment.

Enzyme inhibition kinetics using time versus different concentration of O. tenuiflorum

Enzyme inhibition kinetics was studied spectrophotometrically using time versus different concentrations of plant extracts. The reaction mixture included 50 µl of overnight grown E. coli culture (OD adjusted to 1), and plant extract of different concentrations 20, 50, and 100 µg/µl. Penicillin G (6 µg/µl) substrate was used in the reaction mixture and change in absorbance over a period of 40 minutes at a wavelength of 630 nm was recorded. The volume for measuring the absorbance was adjusted to 250 µl with 0.05 M PO_4^- buffer of pH-6. All the controls were included as described previously.

RESULTS

Detection of beta-lactamase activity

Beta-lactam antibiotics contain beta-lactam ring in their structure. Several antibiotic-resistant bacteria secrete beta-lactamase which hydrolyzes the beta-lactam ring. To identify new beta-lactam inhibitors(s), we screened medicinal plant using agar cup assay and micro-iodometric assay. The results are given in Fig. 1 where E. coli hospital isolates HI-13, AIHIMS-1, and MTCC-729 show decolorization by hydrolyzing penicillin G converting it into penicilloic acid and hospital isolates HI-2, HI-7, and AIHIMS-2 did not produce beta-lactamase, therefore, no color change was observed after incubation at 30°C for half an hour. The relative hydrolysis activities of beta-lactamase enzyme produced by different bacterial strains with respect to time are given in Table 1.

The results suggested that E. coli strains HI-13, MTCC-729, AIHIMS-1, and DJ1.2 produce beta-lactamase. These strains were used further to screen plant extracts for the presence of beta-lactamase inhibitors.

Reversal of ampicillin resistance in E. coli by plant extracts

The bioassay was performed with T. chebula, T. bellirica, and O. tenuiflorum extracts for the presence of beta-lactamase inhibition against resistant strain AIHIMS-1. However, the inhibition was seen

**Table 1:** Different Escherichia coli strains showing the relative extent of penicillin G hydrolysis, by the beta-lactamase enzyme

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Incubation time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>2  5  10  20</td>
</tr>
<tr>
<td>HI-2</td>
<td>-  -  -  -</td>
</tr>
<tr>
<td>HI-7</td>
<td>-  -  -  -</td>
</tr>
<tr>
<td>HI-13</td>
<td>-  -  ++  +++</td>
</tr>
<tr>
<td>MTCC-729</td>
<td>-  -  -  ++</td>
</tr>
<tr>
<td>AIHIMS-1</td>
<td>-  -  +++  ++++</td>
</tr>
<tr>
<td>AIHIMS-2</td>
<td>-  -  +++  ++++</td>
</tr>
<tr>
<td>DJ1.2</td>
<td>-  -  +++  ++++</td>
</tr>
</tbody>
</table>

* Sign indicates increasing and – sign indicates no enzyme activity based on visible decolorization

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Fig. 1: Beta-lactamase inhibition assay using micro-iodometric method. (1 and 2) Negative control - containing penicillin G, (3) positive control - DJ1.2 for beta-lactamase, (4) HI-2, (5) HI-7, (6) HI-13, (7) AIHIMS-1, (8) AIHIMS-2, and (9) MTCC-729
only in 8 extracts which include hexane and ethyl acetate extracts of 
*T. chebula,* ethyl acetate extracts of *T. bellirica,* and all the extracts except 
dichloromethane of *O. tenuiflorum.* The results for ethyl acetate extract 
of all the three plants are given in Fig. 2, where ethyl acetate extract 
(50 µg/µl) combined with ampicillin (10 µg/µl) showed inhibition 
zones of 11.5 mm with *T. chebula,* 11 mm with *O. tenuiflorum,* and 
11 mm with *T. bellirica,* but plant extract and ampicillin alone were 
resistant to AIIMS-1. The positive control CA also exhibited same result 
as our plant extracts with 10.5 mm zone of inhibition.

**Beta-lactamase inhibition by plant extracts using micro-
iodometric method**

To inhibit beta-lactamase enzyme, plant extracts were screened for the 
presence of beta-lactamase inhibitor. Six different solvents were used 
in the study - hexane, dichloromethane, ethyl acetate, acetone, ethanol, 
and methanol. Plant extracts were pre-incubated with beta-lactamase of 
*E. coli* for 1 hr at 30°C for allowing the inhibitor to bind to the enzyme. 
This incubated reaction mixture was then added to the wells of microtiter 
plate containing substrate penicillin G prepared in the phosphate buffer 
and was further incubated for half an hour at 30°C. If the plant extract 
had potent beta-lactamase inhibitor, then hydrolysis would not take 
place, thereby decolorization, that is, oxidation of penicilllic acid by 
iodine would not take place as a result iodine would be free to bind with 
starch molecules and produce blue color indicating the presence of beta-
lactamase inhibitor in the plant extract. In contrast, the samples with no 
inhibitory activity would show decolorization.

The beta-lactamase inhibition activity from the crude plant extracts 
against the four beta-lactamase producing multidrug-resistant *E. coli* 
strains (HI-13, AIIMS-1, DJ1.2, and MTCC-729) was studied. The hexane 
extract of *T. chebula* had shown weak beta-lactamase inhibition against 
DJ1.2 and AIIMS-1 whereas strong inhibition was observed against 
HI-13 and MTCC-729 as given in Fig. 3 and Table 2. On the other hand, 
ethyl acetate extracts had shown strong inhibition against HI-13, 
AIIMS-1, DJ1.2, and MTCC-729 whereas dichloromethane, acetone, 
ethanol, and methanol extracts had shown no inhibition. The activity 
of DJ1.2, HI-13, AIIMS-1, and MTCC-729 was strongly inhibited by 
ethyl acetate extracts whereas no inhibition was noted in hexane, 
dichloromethane, acetone, ethanol, and methanol extracts of *T. bellirica.* 
The hexane and methanol extract of *O. tenuiflorum* had shown weak 
beta-lactamase inhibition against AIIMS-1 whereas strong inhibition 
against DJ1.2, HI-13, and MTCC-729.

![Fig. 2: Bioassay of beta-lactamase inhibition by plant extract. Growth inhibition study of beta-lactamase producing E. coli AIIMS-1 in the presence of only CA (100 µg/µl) or plant extract (50 µg/µl, left column), only ampicillin (10 µg/µl, middle column), and both (right column). (a) Positive control (CA) 10.5 mm, (b) Terminalia chebula ethyl acetate extract (11.5 mm), (c) Ocimum tenuiflorum ethyl acetate extract (11 mm), and (d) Terminalia bellirica ethyl acetate extract (11 mm) (Image 309x162 to 549x403)].

Enzyme inhibition kinetics using extracts of *O. tenuiflorum*

From the above study, *O. tenuiflorum* has proved to be the best plant 
source for beta-lactamase inhibitor. Enzyme inhibition kinetic 
experiments were carried out to determine the efficacy of the 
*O. tenuiflorum* extracts made by different solvents. We used AIIMS-1 
strain as source of beta-lactamase, and enzyme inhibition kinetics 
was performed spectrophotometrically using time versus different 
concentration of *O. tenuiflorum.* The result showed dose-dependent 
inhibition and its rate of inhibition was plotted in the Fig. 4. Different 
concentration of ethyl acetate, ethanol, and acetone extracts used 
were 20, 50, and 100 µg/µl which were plotted in comparison with the 
positive control. In the experiment, penicillin G was hydrolyzed with 
time by the enzyme where the optical density of substrate was noted to 
be 1.30 at 630 nm. It was noted that the ethyl acetate had shown better 
inhibition when compared with the other extracts and comparable to 
well-known beta-lactamase inhibitor CA.

**DISCUSSION**

The research for new beta-lactamase inhibitors has become an urgent 
need due to the abrupt increase in antibiotic resistance among human 
pathogens [16]. In the previous study [17], more than 65% of *E. coli* 
isolates were resistant to newer quinolones such as ciprofloxacin and 
norfloxacin, whereas 75% of the isolates were resistant to nalidixic 
acid [18]. Interest in beta-lactamase inhibiting agents is largely focused 
on their combination with beta-lactam antibiotics for the treatment 
of infections caused by beta-lactamase producing bacteria [19]. To 
win this battle of antibiotic resistance among pathogenic bacteria, 
use of medicinal plants was encouraged as multiple compounds were 
available in herbal formulations [20]. The bioassay showed positive 
beta-lactamase inhibition by eight extracts against AIIMS-1. The 
results showed that AIIMS-1 exhibit resistance when plant extract and 
ampicillin were given alone but became susceptible when combined 
together. It was further confirmed by the micro-iodometric method. This 
assay was modified from a previous study [21], where it was shown that 
hibition by *T. chebula* and *T. bellirica* extracts combined with different

![Fig. 3: Micro-iodometric assay result showing strong beta-lactamase inhibition (dark blue), weak inhibition (light blue), and no inhibition (decolourized) by (1) Terminalia chebula, (2) Terminalia bellirica, and (3) Ocimum tenuiflorum, +C = positive control, E+S = beta-lactamase enzyme + penicillin G, SC: Solvent control H: Hexane, DCM: Dichloromethane, EA: Ethyl acetate, A: Acetone, E: Ethanol, and M: Methanol against, (a) DJ1.2, (b) HI-13, (c) AIIMS-1, and (d) MTCC-729].
antibiotics (tetracycline, chloramphenicol, streptomycin, nalidixic acid, and ciprofloxacin) against ESBL bacterial strains but found no synergistic interactions with any of the antibiotic except tetracycline.

The plant extracts were checked for the presence of beta-lactamase inhibitor using micro-iodometric assay and found that the extracts of *Ocimum tenuiflorum* had shown best beta-lactamase inhibition against all the four *E. coli* strains included in the present study. Out of which, AIIMS-1 being the resistant one was further selected for the dose-dependent enzyme inhibition kinetics study, in which ethyl acetate extract of *Ocimum tenuiflorum* 100 µg/µl had shown better inhibition than the other extracts. The results obtained from the present study provide evidence that extracts of *O. tenuiflorum* exhibit best beta-lactamase inhibition.

CONCLUSION

We conclude from this study that *Terminalia chebula*, *T. bellirica*, and *O. tenuiflorum* contains bioactive compound(s) which are capable of beta-lactamase inhibition. The compound(s) present in *O. tenuiflorum* has maximum enzyme inhibition activity. Further isolation and structure elucidation studies are required to develop potent beta-lactamase inhibitor.

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