REVERSION OF ANTIBIOTIC RESISTANCE WITH BETA-LACTAMASE INHIBITOR FROM MEDICINAL PLANTS

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Received: 01 June 2017, Revised and Accepted: 23 June 2017

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 amoxicillin, 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 oximino-cephalosporins 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 beta-lactamase 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 themselves 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); ATCC-729 and ATCC-3; and standard strain microbial type culture collection (MTCC-279) (E. coli obtained from MTCC and Gene Bank; produces beta-lactamase) and DJ12 (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 (1.5×10^8 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
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 PO₄⁻-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 inhibitor(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, AIIMS-1, and MTCC-729 show decolorization by hydrolyzing penicillin G converting it into penicilloic acid and hospital isolates HI-2, HI-7, and AIIMS-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, AIIMS-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 amoxicillin resistance in *E. coli* by plant extracts

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

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**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><em>E. coli</em></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>AIIMS-1</td>
<td>-  -  +++  ++++</td>
</tr>
<tr>
<td>AIIMS-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) AIIMS-1, (8) AIIMS-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. tenuiforum*. 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. tenuiforum*, 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 penicilloic 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, AIIMS-1, DJ1.2, 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. tenuiforum* had shown weak beta-lactamase inhibition against AIIMS-1 whereas strong inhibition against DJ1.2, HI-13, and MTCC-729.

**Enzyme inhibition kinetics using extracts of *O. tenuiforum***

From the above study, *O. tenuiforum* 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. tenuiforum* 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. tenuiforum*. 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 inhibition by *T. chebula* and *T. bellirica* extracts combined with different

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**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 together (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)**

**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 ciprofl oxacin) 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 *O. 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 *O. 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 *T. 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.

**ACKNOWLEDGMENT**

We acknowledge the Department of Biotechnology (DBT), Ministry of Science and Technology (Grant No. BT/25/4/NE/TBP/2011) for financial support.

**REFERENCES**


9. WHO’s Certified. Global Priority List of Antibiotic-Resistant Bacteria