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Research Article

REVERSION OF ANTIBIOTIC RESISTANCE WITH BETA-LACTAMASE 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 bacteria 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/µl of extract of all 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 oxyimino-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 carbapenems. 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^{r} 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

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 = 1.5×10^8 cells/ml) from the overnight grown bacterial inoculum (AIIMS-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× g, 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 2.03 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 cells. The mixture was incubated for 30 minutes at room temperature, followed by addition of two drops of starch-iodine reagent. Then, the color change was recorded which was due to the reaction of iodine with starch. The persistent blue color for longer than 10 minutes is an indication of penicillin molecule had not undergone beta-lactam ring cleavage. On the other hand, rapid decolorization occurred on hydrolysis of penicillin molecules, this indicates beta-lactamase activity. Penicillin G solution without enzyme was included as negative control. DJ1.2 is a positive control for beta-lactamase activity.

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 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 ampicillin 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

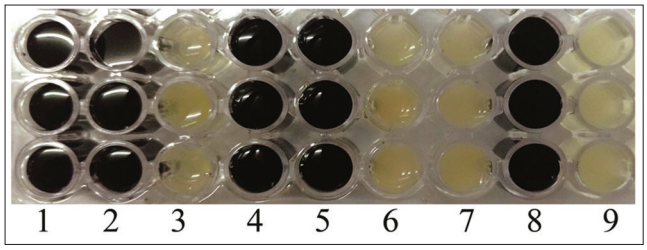

Fig. 1: Beta-lactamase inhibition assay using micro-iodometric method. (1 and 2) Negative control - containing penicillin G, (3) positive control - DJ1.2 for beta-lactamase, (4) HI-2, (5) HI-7, (6) HI-13, (7) AIIMS-1, (8) AIIMS-2, and (9) MTCC-729

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

Bacterial strains	Incubation time (min)			
E. coli	2	5	10	20
HI-2	-	-	-	-
HI-7	-	-	-	-
HI-13	-	-	++	+++
MTCC-729	-	-	-	+++
AIIMS-1	-	-	+++	++++
AIIMS-2	-	-	-	-
DJ1.2	-	-	+++	++++

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

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 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 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.

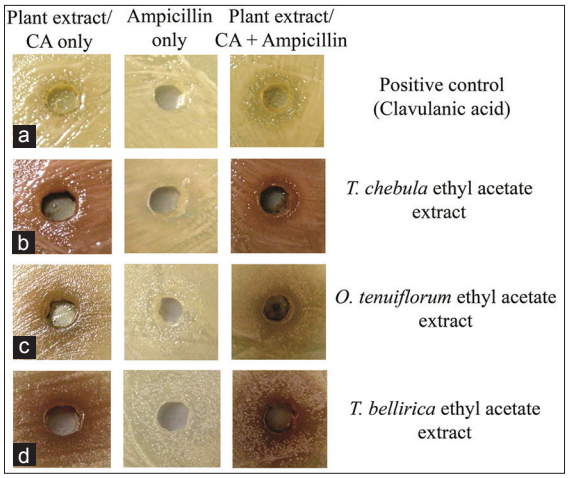

Fig. 2: Bioassay of beta-lactamase inhibition by plant extract. Growth inhibition study of beta- lactamase producing *E. coli* AIIMS-1 in the presence of only CA (100 µg/µl) or plant extract (50 µg/µl, left column), only ampicillin (10 µg/µl, middle column), and both together (right column). (a) Positive 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)

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 inhibition by *T. chebula* and *T. bellirica* extracts combined with different

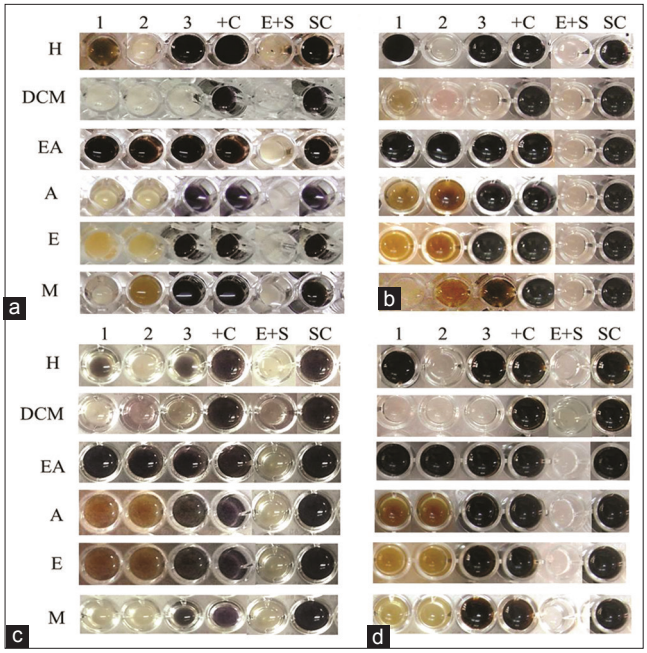

Fig. 3: Micro-iodometric assay result showing strong beta-lactamase inhibition (dark blue), weak inhibition (light blue), and no inhibition (decolourized) by (1) *Terminalia chebula*, (2) *Terminalia bellirica*, and (3) *Ocimum tenuiflorum,* +C = positive control, E+S = beta-lactamase enzyme + penicillin 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

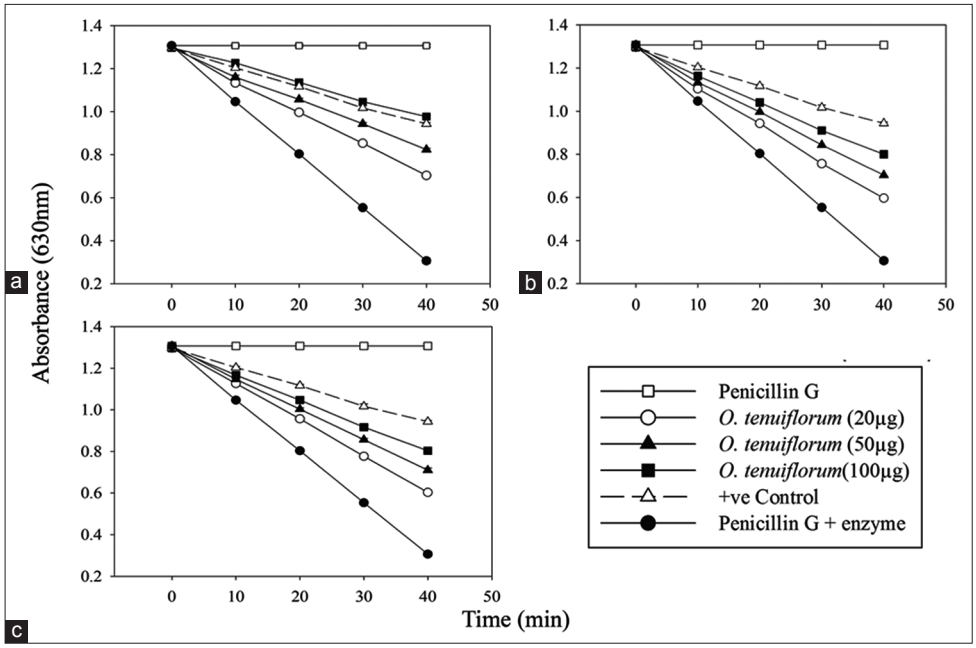

Fig. 4: Beta-lactamase inhibition kinetics with *Ocimum tenuiflorum.* Substrate (penicillin G) hydrolysis by enzyme with and without extracts of *Ocimum tenuiflorum.* (a) Ethyl acetate extract, (b) ethanol extract, and (c) acetone extract, *values (P<0.05) are mean ± SD of 3 readings

Table 2: Micro-iodometric assay result showing beta-lactamase inhibition by different extracts of *T. chebula*, *T. bellirica* and *O. tenuiflorum* against DJ1.2, HI-13, AIIMS-1 and MTCC-729 bacterial strains

Solvent/ pathogen	*T. chebula*				*T. bellirica*				*O. tenuiflorum*			
	DJ1.2	HI-13	AIIMS-1	MTCC-729	DJ1.2	HI-13	AIIMS-1	MTCC-729	DJ1.2	HI-13	AIIMS-1	MTCC-729
H	+	+++	+	+	-	-	-	-	+++	+++	+	+++
DCM	-	-	-	-	-	-	-	-	-	-	-	-
EA	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
A	-	-	-	-	-	-	-	-	+++	+++	+++	+++
E	-	-	-	-	-	-	-	-	+++	+++	+++	+++
M	-	-	-	-	-	-	-	-	+++	+++	+	+++

H: Hexane, DCM: Dichloromethane, EA: Ethyl acetate, A: Acetone, E: Ethanol, M: Methanol. Test result recorded as +++ strong inhibition, + weak inhibition and - no inhibition, *T. chebula: Terminalia chebula, T. bellirica: Terminalia bellirica, O. tenuiflorum: Ocimum tenuiflorum*

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 *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.

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