Analysis of Inhibitory Propensity and Percentage Inhibition of *Tecoma stans* and *Plectranthus vettiveroides* Leaves against *Escherichia coli* ESBL Enzyme in Namakkal District Broilers, Tamil Nadu, India

S. Kameshwaran¹, K. Abhenaya², R. Manivannan³, N. Elavarasan⁴, D. S. Asok Kumar⁴, J. Priya² and V. Priyanka⁴

¹. Department of Pharmacology, Excel College of Pharmacy, Namakkal, Tamil Nadu 637303, India
². Department of Pharmaceutical Chemistry, JKKM Institute of Medical Sciences College of Pharmacy, Gobi, Erode, Tamil Nadu 638506, India
³. Department of Pharmaceutics, Excel College of Pharmacy, Namakkal, Tamil Nadu 637303, India
⁴. Department of Pharmaceutical Chemistry, Excel College of Pharmacy, Namakkal, Tamil Nadu 637303, India

Abstract: On samples phenotypically positive for extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli*, the inhibitory potential and percent inhibition of fresh juice from *Tecoma stans* and *Plectranthus vettiveroides* leaves on beta-lactamase enzyme of cecal samples of healthy broilers were tested. Two hundred (200) samples of cecal treated for ESBL-producing *E. coli* were obtained from 38 Namakkal poultry selling outlets. The impacts of *T. stans* and *P. vettiveroides* leaves were interpreted as chromogenic substrate by colorimetric assay with CENTA and nitrocefin. The mean absorbance value of the inhibitory potential was inversely proportional. *Tecoma stans* exhibited 29% and 31% of CENTA and nitrocefin inhibition, respectively. The mean absorbance value of *Plectranthus vettiveroides* and the 10.0% inhibition of CENTA and nitrocefin in both (*p > 0.05*) showed no significant difference in CENTA and nitrocefin activity. As normal regulation, tazobactum (100 μM) exhibited a 99.89% and 98.31% inhibition against CENTA and nitrocefin, respectively. The combination of *Plectranthus vettiveroides* and *Tecoma stans* showed 14% to 15% inhibition, respectively, with CENTA and nitrocefin. Results showed that beta-lactamase enzyme activity which inhibited with *T. stans* was higher than *P. vettiveroides* leaf juice and the combination of the two herbs showed no substantial difference in activity.

Key words: CENTA, colorimetric assay, *Escherichia coli*, ESBL, nitrocefin, *Plectranthus vettiveroides*, *Tecoma stans*.

1. Introduction

Poultry farmers face a gigantic antimicrobial resistance crisis. Intense poultry rearing is nowadays very popular. The use of antibiotics by poultry farmers at low doses as growth promoters to the entire flock contributes to high demand for antibiotic selection. This also results in a higher proportion of resistant bacteria in poultry fecal flora. *Escherichia coli* mainly produces resistant phenotypes in the poultry population and, thus, commensally *E. coli* serves as an indicator bacterium in the Gram-negative species group [1]. The first experimental study of stretched-spectrum beta-lactamase (ESBL) was conducted in 1989. *E. coli* land was obtained from ear discharge from Munich (Germany) of a very young child aged 4 months. CTX-M-1 was known; here, CTX means cefotaximase and M means Munich. Another cefotaximase called MEN-1 was obtained from an Italian patient in France, similar to CTX-M-1 [2, 3]. CTX-M-2 was collected in Argentina from *Salmonella typhimurium* isolated from patients suffering from meningitis, septicemia and enteritis. Kumar *et al.*, in 2012, reported 55.55% ESBL, including 66.67% from blood, 65% from aspirate, 57.14% from human waste, 55% from wound and...
54.67% from urine in various hospitals. In the USA, ESBL-producing *E. coli* ranged from 0 to 25%. Japan’s prevalence of ESBL-producing *E. coli* was 0.1% [4, 5]. In India, the number of ESBL producers registered ranged from 22% to 75% [2-4].

In 2013, Shailesh et al. [6] also documented the incidence of TEM, SHV, and OXA genes isolated from diabetic patients’ ulcerative feet. These studies further demonstrated the therapeutic importance of enzyme-producing ESBL bacteria in humans as well [6].

As indicator bacteria are present in the animal community, the incidence of resistance is best studied in *E. coli*, which is an indicator bacterium [7]. Gram-negative bacilli-producing enzyme ESBL emerged as a major hazard over a 2-year period due to the clonal expansion of producer bacteria. The principal explanation for this expansion is the lateral transfer of ESBL genes to plasmids [5, 8, 9].

Resistance to antimicrobials has made scientists think of plant use as an alternative to antibiotics. Zaichang et al. [10] screened several Chinese herbal and medicinal ethanol extracts for beta-lactamase inhibitor activity using the colorimetric enzyme assay process. Solanki and Selvanayagam [11] also recorded the inhibitory ability of *Plectranthus vettiveroides*, *Punica granatum*, *Tecoma stans*, *Glycyrrhiza glabra*, *Piper longum*, *Zingiber officinalis* and 15 other ESBL enzyme plant extracts using chromogenic CENTA substrates. The nitrocefin assay method [12] was used to test beta-lactamase enzyme inhibitors from the Chinese herbal extracts. Nevertheless, the fair assessment of certain herbs was hindered by the extract solution’s brown or yellow color.

2. Materials and Methods

2.1 Sample Collection

In the present research, 200 cecal swab samples were collected and screened for the presence of ESBL-producing *E. coli* from freshly slaughtered broilers from 38 poultry sales outlets of Namakkal, Tamilnadu, India.

2.2 Phenotypic Screening of Samples

After preferential enrichment of samples in buffered peptone water and Mueller-Hinton broth (cefotaxime 2 µg/mL and aztreonam 4 µg/mL), double-disk synergy screening, combined disk check procedure (cefotaxime (30 µg) and cefotaxime + clavulanic acid (30 µg + 10 µg)), and minimum inhibitory concentration enzyme (MIC) strips.

2.3 Isolation of Beta-Lactamase Enzyme

The beta-lactamase enzyme was isolated from positive samples and subjected to further study of the two herbs’ inhibitory potential. Overnight bacteria cultures were freshly inoculated in a rotary shaker, holding a temperature of 35 °C, for 2-h production, supplemented inducer (penicillin G 400 µg/mL), with additional 4 h incubation. Cell pellets were poured by centrifugation, suspended again, and eroded at 4 °C with potassium phosphate buffer (0.05 M, pH 7.0). The bacteria were again centrifuged and suspended in the 10-fold concentrated phosphate buffer. Later, sample sonication was performed in an ice bath sonicator for 5 min. Cellular debris was discarded at 4 °C for 20 min with centrifugation at a speed of 40,000 rpm. The resulting supernatants were kept at -20 °C before further use.

2.4 Grounding of the Herbs

New leaf juice of *T. stans* & *P. vettiveroides*—Fresh *P. vettiveroides* leaves were collected from Rasipuram (Namakkal district), Tamil Nadu during the month of May 2019. A plant herbarium specimen had been deposited at the Pharmacognosy Section. The plant has been described by Dr. G. V. S. Murthy, Joint Director of India’s Botanical Survey, Southern Circle, TNAU Campus, Coimbatore, who has authenticated the plant from literature information available. The leaves were grinded in water, and lyophilized to obtain dry powder that was later used at a concentration of 10 mg/mL (50 µL/well volume).
2.5 Colorimetric Assay

For the present study, CENTA and Nitrocefin (Nitrocefin-Calbiochem make 5 mg) (CENTA-Calbiochem make 25 mg) were used as chromogenic substrates. At a final concentration of 0.4 mmol/L, nitrocefin (98% pure) was dissolved in dimethyl sulfoxide. CENTA was dissolved to a final concentration of 0.4 mmol/L in distilled water. Colorimetric assay was performed in an EX 200-240 V, 50/60 Hz Thermo Scientific Multiskan, wavelength range 400-750 nm, with Ascent Software (Bengaluru, Karnataka, India). The enzyme extract’s explicit behavior was read out in a 100-μL reaction mixture. In short, 8 μL of the enzyme was initially stabilized at 25 °C with the potassium phosphate buffer (100 mmol/L) at pH 7.0 for 10-15 min. Fresh leaves of T. stans for each respective well 50 µL of tazobactum (make Sigma Aldrich) as normal beta-lactamase inhibitor in 100 µmol concentration. Add T. stans and P. vettiveroides (10 mg/mL concentration). Five microliters (5 μL) of the substratum was inserted into the wells after 25 min and incubated again at 25 °C for 20 min; potassium phosphate buffer was added to make the final volume 100 µL. The plate was again incubated at 25 °C for 20-25 min, after the desired color development of the incubation period was analyzed for CENTA and nitrocefin in the form of absorbance at 405 nm and 486 nm wavelength. Potassium phosphate buffer was used in blank 95 μL along with the substrate [13, 14].

2.6 Preparation of Test Solution

CENTA and nitrocefin stock solution was taken; nitrocefin was dissolved in dimethylsulfoxide, resulting in a final concentration of 4 μmol/mL, which was further diluted to create a double dilution of 2, 4, 8, 12, 16, 20 and 24 nmol/mL to prepare a standard curve. CENTA was dissolved in distilled water and, like nitrocefin, a double dilution of 2, 4, 8, 16, 20 and 24 nmol/ml was further diluted in order to create a normal curve from the 4 μmol/mL stock solution.

3. Results and Discussion

The inhibitory ability and percent of T. stans inhibition were evaluated by CENTA and nitrocefin, chromogenic substrate in T. stans and P. vettiveroides leaves, an enzyme called beta-lactamase. T. stans had an inhibition rate of 25%-33% for CENTA and 26%-35% for nitrocefin inhibition and an inhibitory potential in the form of an absorption value of (0.4 ± 0.04)–(0.5 ± 0.05) and (0.36 ± 0.02)–(0.44 ± 0.04) for CENTA and nitrocefin, respectively, resulting in a non-significant difference in the activity of both substrates (Table 1). Absorption value (2.01 ± 0.04)–(2.07 ± 0.06) and 9.56%-10.24% CENTA inhibition and (1.76 ± 0.25)–(2.06 ± 0.04) and 9.4%-10.32%. P. vettiveroides leaves’ inhibition of nitrocefin enzyme activity was observed (Table 2).

Higher absorption value with leaves of P. vettiveroides suggests its low inhibitory ability and percent inhibition activity against enzyme β-lactamase compared with T. stans. The combination of two herbs produced an absorption value in the lower range of (1.69 ± 0.05)–(1.90 ± 0.08) and a 12%-14% inhibition of CENTA and nitrocefin, respectively, resulting in a non-significant difference between the inhibitory potential values and percent inhibition. T. stans and P. vettiveroides (1.81 ± 0.06 and 15.0% and 1.77 ± 0.05 and 14.54%) with CENTA and nitrocefin, respectively, further stating that the chromogenic properties of nitrocefin and CENTA can be used in easy and fast β-lactama detection assays.

A research on clove oil and rosemary essential oil demonstrated antibacterial activity against multidrug-resistant bacteria [8, 15]. Both of these
Table 1  Comparative study of inhibitory potential and percent inhibition of fresh leaf juice of *Tecoma stans* by colorimetric method using (CENTA and nitrocefin).

| Sample | Based on absorbance value | Percent inhibition | Based on absorbance value | Percent inhibition |
|--------|---------------------------|--------------------|---------------------------|--------------------|
|        | CENTA                     | Nitrocefin         | CENTA                     | Nitrocefin         |
| 1      | 0.4 ± 0.04                | 31                 | 0.42 ± 0.06               | 33                 |
| 2      | 0.4 ± 0.07                | 33                 | 0.44 ± 0.04               | 29                 |
| 3      | 0.5 ± 0.05                | 28                 | 0.43 ± 0.05               | 31                 |
| 4      | 0.5 ± 0.04                | 25                 | 0.48 ± 0.07               | 26                 |
| 5      | 0.4 ± 0.05                | 33                 | 0.41 ± 0.03               | 33                 |
| 6      | 0.4 ± 0.08                | 27                 | 0.36 ± 0.02               | 35                 |

\( n = 6; (P > 0.05) \)

Table 2  Comparative study of inhibitory potential and percent inhibition of fresh leaf juice of *Plectranthus vettiveroides* by colorimetric method using CENTA and nitrocefin.

| Sample | Based on absorbance value | Percent inhibition | Based on absorbance value | Percent inhibition |
|--------|---------------------------|--------------------|---------------------------|--------------------|
|        | CENTA                     | Nitrocefin         | CENTA                     | Nitrocefin         |
| 1      | 2.07 ± 0.06               | 9.56               | 1.76 ± 0.25               | 9.23               |
| 2      | 2.05 ± 0.06               | 10.24              | 2.06 ± 0.04               | 10.32              |
| 3      | 2.01 ± 0.04               | 9.54               | 2.01 ± 0.03               | 9.4                |
| 4      | 2.02 ± 0.04               | 10.16              | 2.03 ± 0.05               | 10.23              |
| 5      | 2.06 ± 0.06               | 10.25              | 2.02 ± 0.06               | 10.23              |
| 6      | 2.07 ± 0.04               | 10.22              | 2.03 ± 0.04               | 10.23              |

\( n = 6; (P > 0.05) \)

Table 3  Comparative study of inhibitory potential and percent inhibition of combination of fresh leaves juice of *Tecoma stans* and *Plectranthus vettiveroides* by colorimetric method using CENTA and nitrocefin.

| Sample | Based on absorbance value | Percent inhibition | Based on absorbance value | Percent inhibition |
|--------|---------------------------|--------------------|---------------------------|--------------------|
|        | CENTA                     | Nitrocefin         | CENTA                     | Nitrocefin         |
| 1      | 1.85 ± 0.09               | 14.21              | 1.78 ± 0.07               | 13.59              |
| 2      | 1.79 ± 0.06               | 14.99              | 1.75 ± 0.06               | 14.46              |
| 3      | 1.80 ± 0.07               | 13.27              | 1.79 ± 0.13               | 13.67              |
| 4      | 1.91 ± 0.11               | 15.95              | 1.93 ± 0.10               | 15.95              |
| 5      | 1.70 ± 0.06               | 14.21              | 1.62 ± 0.08               | 13.37              |
| 6      | 1.81 ± 0.06               | 15.03              | 1.77 ± 0.05               | 14.54              |

\( n = 6; (P > 0.05) \)

Substrates, which are chromogenic in nature, exhibit color change when they come into contact with β-lactamase enzyme; the color change is read as the absorption value in the microtiter plate assay. When the activity of the β-lactamase enzyme is inhibited, less color change occurs which, in effect, gives higher absorption value. In the present study, *T. stans* yielded lower absorption value in comparison to the control; this indicates a higher percent inhibition activity of the β-lactamase enzyme by the fresh leaves juice of *T. stans*.

Solanki and Selvanayagam [11] have reported the capacity for inhibitory *O. P. granatum, sanctum, S. aromaticum*, and *G. Glabra, P. longum, and Z. officinalis*, and 15 other plant extracts against ESBL enzyme by β-lactamase enzyme inhibition assay method using chromogenic substrate CENTA and percent inhibition of β-lactamase activity were seen on the
Table 4  Comparative study of inhibitory potential of tazobactum as standard control by colorimetric method using CENTA and nitrocefin.

| Sample | CENTA Percent inhibition | Nitrocefin Percent inhibition |
|--------|--------------------------|-----------------------------|
| 1      | 0.13 ± 0.003             | 99.88                        |
| 2      | 0.13 ± 0.02              | 99.89                        |
| 3      | 0.14 ± 0.02              | 99.88                        |
| 4      | 0.13 ± 0.02              | 99.89                        |
| 5      | 0.14 ± 0.003             | 92.19                        |
| 6      | 0.13 ± 0.003             | 99.89                        |

\(n=6; (P>0.05)\)

basis of optical density (OD) values. Rawat [16] also documented similar antibacterial activity of Tulsi, Neem, and Amla. Previously, nitrocefin had been used as a test substrate for studying interactions between the \(\beta\)-lactamase enzyme and its inhibitors [17-19]. In the past, specific inhibitor concentrations have been used to analyze a relative substrate affinity index based on a 5-min reaction between the \(\beta\)-lactamase enzyme, its receptor, and the substrate (nitrocefin). CENTA is a variable \(\beta\)-lactamase reagent of chromogenic cephalosporine. It transforms into chrome yellow from light yellow which is interrelated in the presence of water with the chemical breakdown of the \(\beta\)-lactam chain. CENTA can be used as an indicator component similar to other chromogens such as PADAC and nitrocefin to detect the isolates producing \(\beta\)-lactamase enzyme. It also exhibits antimicrobial activity against \textit{E. Coli}, \textit{Klebsiella} spp., \textit{Proteus mirabilis}, \textit{Staphylococcus aureus} and \textit{Streptococcus} spp. nonenterococci. Variations in percentage of herbal inhibition findings were also documented earlier with the nitrocefin competition assay, and some researchers have also documented the use of \(\beta\)-lactamase inhibitors screened from typical Chinese herbal extracts [12]. Comparing the properties of both the CENTA and nitrocefin substrates by frequently used microbiological media is quite unpretentious; this conclusion simulates the previous observation where CENTA was added to the regularly used microbiological broth media (Mueller-Hinton, brain-heart infusion, Schaedler, and Trypticase soy) and there was no noticeable general color change as observed by microplate read.

4. Conclusions

\textit{E. coli}-producing ESBL enzymes have been found to be prevalent in safe Namakkal broilers, with a prevalence of 33.5%, leading to a major cause of antibiotic resistance. Fresh leaf juice of \textit{T. stans} showed optimum and fresh leaf juice \textit{P. vettiveroides} showed limited potential for inhibition and percent inhibition of ESBL enzymes. Both herbs together showed greater activity than the individual herbs. The difference in the observations may be due to herbal color, which may have caused the impairment in the absorbance value, resulting in a low inhibition rate. A key benefit of a colorimetric approach is that color variations can be observed directly. Herbs alone or in combination for the poultry and other livestock sectors may be an effective replacement for the antibiotics. The phenotypic and genotypic features of these resistant genes are similar in humans and animals; thus the inhibitory effect of the herbs used in this study may be equally beneficial to humans as well [11].

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