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Synergistic antimicrobial action of trans-cinnamaldehyde with last resort antibiotics against *Escherichia coli*

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Abstract
Antibiotic resistance emergence in bacterial pathogens is a global issue and to control it, government enforce strict drug usage regulations and researchers trying to search new antibiotics and explore the paradigm of combinational therapy with other antimicrobials and herbal drugs. Trans-cinnamaldehyde (TC) is the main component of cinnamon oil, which has antimicrobial properties and used as herbal medicine. The present investigation was carried out to explore the interaction of TC with few last resort antibiotic drugs for its synergistic potential. The antibiotic resistance pattern of selected antibiotics against *E. coli* was also determined to access the level of antimicrobial resistance among different species of animals. A total 430 isolates of *Escherichia coli* were tested for antibiotic resistance and their MICs were calculated. Checkerboard assay and FICIs were used to determine the synergy between TC and imipenem. Resistant was observed in 20.9%, 3.25%, 8.13%, 5.81% and 9.06% to colistin (CL), imipenem (IPM), nitrofurantoin (F/M), polymixin B (PB) and TC, respectively in *E. coli* isolates (430), while all isolates were sensitive for tigecycline (TGC). Further, 80 *E. coli* isolates revealed synergy between TC and any of the five selected antibiotics. TC showed synergistic antimicrobial effect with IPM in 72 *E. coli* isolates, with CL in 39 isolates, and with F/M in 28 isolates. While no synergistic action was observed with TGC and PB. Present work showed the potential of synergistic effect of the TC with antibiotics in combating the antibiotic resistance by combinational therapy.

Keywords: Trans-cinnamaldehyde, antibiotic resistance, synergy, herbal drugs, *E. coli*

Introduction
The rampant and indiscriminate use of antibiotics has increased bacterial drug resistance worldwide and made it a global health concern. India is currently at highest point in terms of antimicrobial resistance (AMR) among humans as well as food animals (Taneja *et al*., 2019) [19]. The use of antibiotics in animals as antimicrobial growth promoters influences the prevalence of resistance in animal bacteria like *Escherichia coli (E. coli), Campylobacter* spp., *Salmonella* spp. and enterococci. *E. coli* is an efficient exchanger of genetic material with other *E. coli* strains and more pathogenic bacteria like *Salmonella* sp., *Shigella* sp., *Proteus* sp. etc. (Alam *et al*., 2013) [1], which enable it to transfer the antibiotic resistance genes to them and hence worsen the global problem of antibiotic resistance.
Cinnamomum zeylanicum, a natural spice and remedy to combat bacterial infection, belongs to the family *Lauraceae* and its active components are trans-cinnamaldehyde (TC), cinnamaldehyde, ethyl cinnamate, eugenol, β-caryophyllene, linalool and methyl chavicol (Brackman *et al*., 2011) [5]. TC has received a significant attention in recent years because of its substantial antimicrobial and medicinal properties (Doyle *et al*., 2019; Unlu *et al*., 2010) [6, 20]. The emergence and spread of resistance to carbapenems among food-producing and companion animals constitute a major public health concern. Imipenem (IPM) although is considered as one of best drugs to treat MDR bacteria but due to its higher cost, risk of seizures and its dose-dependent gastrointestinal side effects as compared with other carbapenems, its use has been limited.

Similarly, nitrofurantoin (F/M) is an effective drug but may cause nephrotoxicity at therapeutic doses. Other antibiotics, colistin (CL) and polymixin B (PB) are Other antibiotics, colistin (CL) and polymixin B (PB) are considered as a last line of treatment for some MDR bacteria but recently resistance against both has been reported (Nordmann *et al*., 2016) [13]. Some recent antibiotics like tigecycline (TGC) is used for most complicated intra-abdominal, skin and soft tissue infections due to its activity against most carbapenem-resistant *E. coli* (Falagas *et al*., 2011) [7]. However, tigecycline use alone remains a concern in blood, urine, respiratory or other serious infections and there have been several reports of development of resistance due to low concentration level (Soren *et al*., 2015) [16]. Therefore, an alternative approach may be to use combination therapy which will reduce the dose and toxicity.

Synergism is defined as the interaction of two or more agents to produce a combined effect greater than the sum of their individual effects. The complex synergistic interactions with herbal drugs and antibacterial agents are believed to enhance the bioavailability of active components, reduce toxicity, minimize adverse effects and promote therapeutic effects. There are reports of use of combined antibiotic therapy such as beta-lactams and aminoglycosides with fluoroquinolones against Gram-negative bacteria (Tamma *et al*., 2012) [18]. To control antibiotic resistance governments, try to enforce strict drug usage regulations and researchers are trying to find new antibiotics and explore the paradigm of combinational therapy with other antimicrobials and herbal drugs. In this investigation, we explored the antibacterial activity of TC and its interaction with antibiotics in *E. coli* isolates.

2. Materials and Methods

2.1 Culture of *E. coli* isolates: During the period of 2015 to 2017, total 402 fecal samples of clinically sick as well as healthy animals of different species (cattle, sheep, goats, pigs and poultry) were cultured from Bareilly, Uttar Pradesh, India. Briefly, the fecal samples were inoculated into MacConkey broth for 6-8 hrs, then streaked onto MacConkey agar (MA) and Eosin methylene blue agar (EMB) plates and were incubated at 37 °C for 24 hrs. The lactose fermenter colonies on MA further screened for metallic sheen on EMB (Fig.1A). The bacterial colonies with metallic sheen were confirmed for *E. coli* by performing biochemical tests. The characterized *E. coli* cultures were stored in 20% glycerol broth and nutrient agar slants for further testing.

2.2 Preparation of isolates for sensitivity testing: The test culture of *E. coli* was grown overnight in Luria Bertani (LB) broth at 37°C. The bacteria were inoculated on Muller Hinton agar (MHA) and incubated for 24 hrs. A single colony was inoculated into Muller Hinton broth (MHB) and the concentration was adjusted to 0.5 McFarland standard to contain approximately 1×10^4 cfu/ml.

2.3 Antimicrobial agents: Imipenem (IPM-10 µg), Nitrofurantoin (F/M-300 µg), Colistin (CL-10 µg), Tigecycline (TGC-15 µg) and Polymixin B (PB-300U) discs were obtained from Becton, Dickinson and Company (BD, Difco). Discs of TC (1µg) were prepared by soaking 4 mm diameter discs of Whatman filter paper no. 3 in 4% TC (Sigma) in methanol.

2.4 Disc diffusion assay: The disc diffusion assay was done to determine antibiotic resistance and for primary screening method of synergy between TC and antibiotics. TC disc was placed in the center of the agar plate and other antibiotic discs at 15 mm apart on the periphery. The key-hole formation between growth inhibition zones of TC and any of the antibiotics tested was considered as a positive indicator of synergy between the two. The absence of keyhole formation was considered as control for the respective *E. coli* isolate.

2.5 Minimum inhibitory concentration (mic): Antimicrobial activity of TC and IPM was assessed by determination of MIC in accordance with CLSI guidelines (NCCLS, 2014). The MIC for TC and IPM was determined using a two-fold broth dilution method. The lowest concentration, at which the drug inhibited the growth of *E. coli*, was breakpoint to adjust the MIC for respective *E. coli* isolate. Two tubes were kept as control in broth dilution method. In the first tube, MHB was added along with antibiotic only and in second tube MHB was added with culture only.

2.6 Checkerboard assay: Evaluation of synergism and antagonism between different combinations for TC and IPM was performed by a checkerboard assay. For the checkerboard assay, growth of *E. coli* was adjusted to 10^5cfu/ml in MHB broth. Holes of 6 mm diameter were made in MHA plate and the *E. coli* culture was inoculated and incubated for 8 hours. The IPM dilutions were added in a vertical direction and the TC dilutions in a horizontal direction. In each well, 25 µl of both drugs were added except in controls where only one drug was added along with 25 µl of DMSO to make it 50 µl. The concentration of both the drugs added in wells was decided by MIC values of drugs. The plates were kept in an upright position for 6 hours and then inverted, and incubated for 24 hours at 37 °C. After 24 hours of incubation, the test culture was observed for clear areas around wells.

2.7 Fractional inhibitory concentration indices (FICI): The isolates showing synergy were tested for quantification of synergy by determining FICI. The MIC value for individual antibiotic was taken from broth dilution method for individual drug. The MIC in combination was provided by checkerboard assay. Both values were used to calculate fractional inhibitory concentration (FIC) as mentioned by (Orhan *et al*., 2005) [14]. For determining the effect of different antibiotics on isolates of diverse origin Chi-square test (χ^2) statistics was used.

3. Results and Discussion

3.1 Antimicrobial susceptibility testing of *E. coli* isolates

Antimicrobial resistance in *E. coli* has become an important issue in veterinary as well as in human medicine worldwide. Herbal drugs have good antimicrobial activity either alone or in combination with antibiotics but resistance of herbal drugs in bacteria is also reported (Bhardwaj *et al*., 2019) [3]. The high electronegative charge on TC affects the cell membrane permeability and hence interferes with synthesis of nucleic acid and protein in bacteria (Kwon *et al*., 2003). According to the biochemical profile (catalase, oxidase, Gram staining and colony morphology (Figure 1A)), 430 *E. coli* isolates were purified and identified. In the present

study TC inhibited the growth of 90.9% of *E. coli* isolates, revealing its excellent antibacterial potential. Surprisingly, resistance to TC was also observed in isolates of all species, specifically pig isolates showed more resistance. However, TC resistance in *E. coli* isolates has observed in the study also sparks the increasing concept of herbal drug resistance in past few years. Carbapenems are recently introduced antibiotics and emergence of resistance to them poses great threat to human as well as animal health as it is last resort drug in many disease conditions (Pesingi *et al*., 2019) [15].

Out of 430 *E. coli* isolates, 20.9%, 3.25%, 8.13% and 5.81% were found resistant to CL, IPM, F/M and PB, respectively, while all isolates were sensitive for TGC (Fig. 1B, 1C). It was observed that isolates from pig comparatively showed more resistance against IPM, CL and F/M while more PB resistance observed in isolates of cattle (Fig. 1D). Besides *E. coli,* the imipenem resistance was also reported in different species of bacteria viz. *Pseudomonas spp., Acinetobacter spp., Klebsiella spp., Citrobacter spp*. and *Proteus spp.* (Lee *et al*., 2019) [11]. The colistin and polymyxin B antibiotics are used as last line of antibiotic treatment for MDR infections. Detection of the colistin resistance gene *mcr-1*in food animals possesses a significant public health concern (Gupta *et al*., 2006) [8]. Similar to present study, CL resistance was found in gram-negative bacteria including *E. coli* isolates at the rate of 46.4% (Sun *et al*., 2017) [17]. F/M is an old antibiotic but it has again come in use because of uncontrollable rise in antimicrobial resistance. In case of TGC, all isolates were sensitive irrespective of source. But there are reports that TGC concentrates at sub lethal levels in blood, urine, respiratory infections, that leads to development of resistance (Falagas *et al*., 2011) [7]. So, the increasing resistance to these new generation antibiotic drugs is reported from the bacteria isolated from different animal species, although, use of these antibiotics are not permitted in animals. This may be due to transfer of the resistance genes to these bacteria through innate or acquired pathways. To combat the emerging antibacterial resistance, it is clear that there is need of use of antibiotics in combination with other antibiotic or herbal drugs.

3.2 Synergism between TC and selected antibiotics

Combination therapy strategies for antibiotic use along with herbal drugs can expand repertoire of antimicrobial treatments (Bhardwaj *et al*., 2016) [4]. Synergistic activity between CL and cinnamaldehyde at the rate of 10% has been reported in MDR *Pseudomonas aeruginosa* isolates (Utchariyakiat *et al*., 2016) [21]. In addition, synergistic activity between colistin and amikacin antibiotics has been reported against mcr-1 producing *E. coli* isolates (Zhou *et al*., 2017) [23]. In the present study, total 80/430 *E. coli* isolates showed synergy between TC and any of the five selected antibiotics (Fig. 2A, 2B). Seventy-two (90%) isolates showed synergy between TC and IPM, 39 (48.75%) between TC and CL, and 28 (35%) between TC and F/M (Fig. 2D). None of the isolates showed synergy of TC with TGC and PB (Fig. 2C, 2D). The synergistic interaction in *E. coli* isolates between TC and IPM was quantified. TC inhibited the growth of *E. coli* at concentrations of 80 -1280 µg/ml, similar to earlier reports (Zhang *et al*., 2015) [22] while MIC of IPM ranged from 2.5 - 40 µg/ml (Table 1). The results of FIC indices revealed that out of 72 *E. coli* isolates, 31 (43%) showed total synergy, 27 (37.5%) showed partial synergism and 14 (19%) showed indifference. In the synergistic combination, 320 µg/ml MIC of TC reduced the MIC of IPM from 40 µg/ml to 2.5 µg/ml (Fig. 2E). Reduction in MIC of IPM and cinnamon oil in combination treatment was reported earlier by Azza, *et al*. (2010) [2]. So, in the study combination of TC and IPM was used for expanding the antibacterial activity, reducing dose and decreasing the antimicrobial resistance during *in vitro* study.

All the antibiotics possess different mechanism of action. Imipenem inhibits transpeptidation of peptidoglycan layer after binding irreversibly to the active site of the PBPs, which in turn disrupts cell wall synthesis and finally cell death (Kapoor *et al*., 2017) [9]. On the other hand, CL acts on Gram-negative bacterial cell membrane through a detergent-like effect, leading to disruption of the outer membrane and loss of cellular contents, thus killing the bacterium. Therefore, disruption of cell envelope could have promoted permeability for both the drugs to reach at optimum concentration inside the bacterial cell. In this investigation, it has been observed that 35% of the isolates showed synergistic antimicrobial effect of TC with F/M, which may open a new window for use of F/M in combination with TC, at least for topical use. Although both IPM and CL are acting on the cell envelope, F/M inhibits several enzymes produced by bacteria involved in the synthesis of DNA and RNA. As we already know, TC also inhibits the nucleic acid synthesis, so it may possible that TC along with disrupting cell membrane, also aids F/M for doing its action more precisely.

The comparison of FIC for *E. coli* isolates from different sources revealed that isolates from cattle showed significantly higher levels of synergy than in goat ($p<0.01$), pig ($p<0.05$), poultry ($p<0.01$) and sheep ($p<0.01$) isolates. Similarly, isolates from pigs showed significantly ($p<0.01$) higher chances of synergy than isolates from poultry, sheep and goat. Synergy may be controlled by some universally occurring bacterial genes or due to genes present in a few selected strains or due to some specific targets on some strains of bacteria for combined effect of both drugs. It is hoped that a more profound understanding of the mechanisms of action and resistance will improve our ability to design and develop more potent and less toxic derivatives of IPM, CL and F/M. It is probable that there are multiple genes present in bacteria conferring resistance and if their expression or function is interrupted the bacteria may show sensitivity. However, to understand synergy between TC and antibiotics, more targeted studies are needed.

Table 1: MIC of TC and IPM against *E. coli*

MIC (µg/ml) value for TC	No. of isolates	MIC (µg/ml) value for IPM	No. of isolates
2560	-----	50	----
1280	3	40	3
640	12	20	13
320	22	10	31
160	24	5	20
80	11	2.5	5
40	------	1.25	-----
Total no. of isolates	72		72

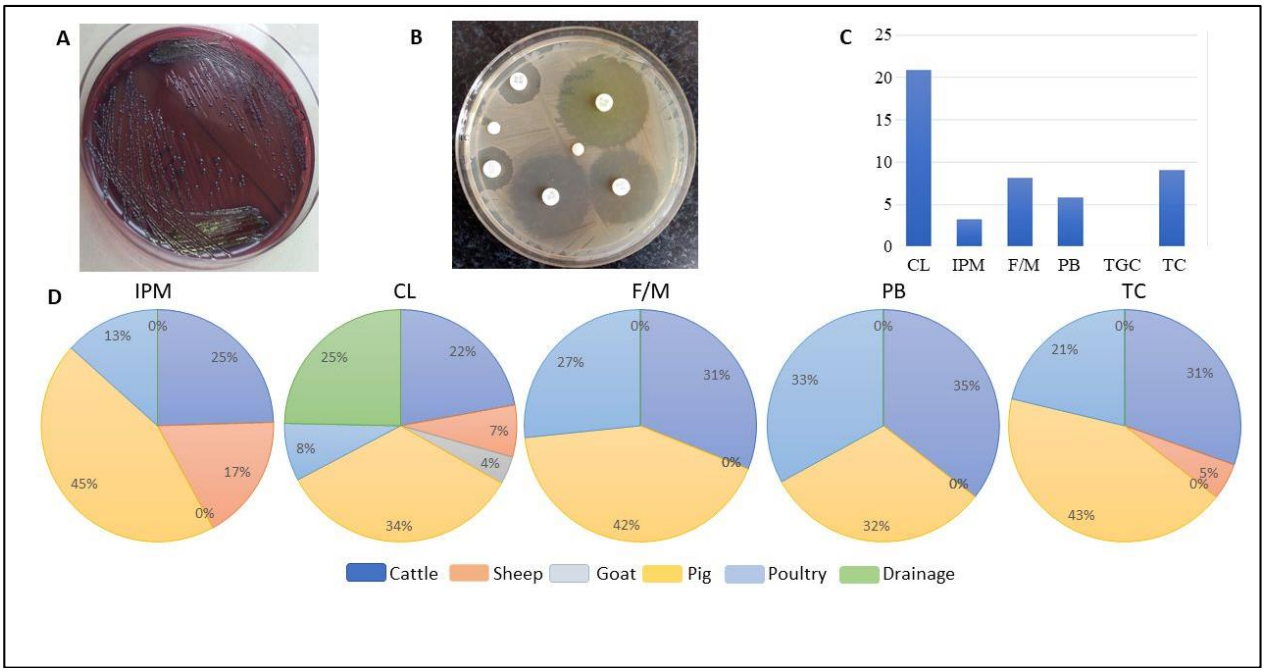

Fig 1: Antibiotic resistance of *E. coli* against IPM, CL, F/M, PB TGC and TC. (A) *E. coli* culture on the MHA agar; (B) sensitive *E. coli* isolate to IPM, TGC, F/M, CL and PB; (C) percentage of the isolates found resistant to different antibiotics (n-430); (D) distribution of the resistant isolates according to their source of origin.

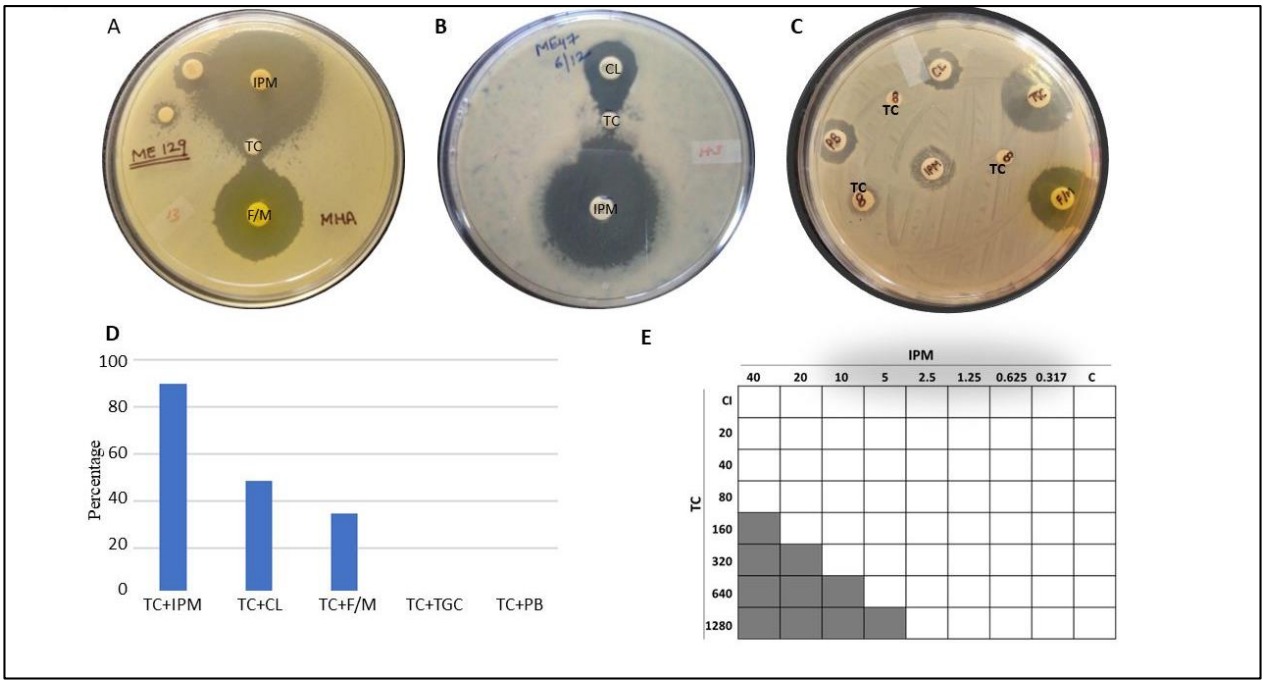

Fig 2: Synergy of TC with antibiotics in the *E. coli* isolate. (A) *E. coli* isolate showing synergy of TC with IPM and F/M; (B) synergy of TC with CL and IPM; (C) *E. coli* isolate not showing synergy with any of antibiotics; (D) graph depicting percentage of the isolate showing synergy of TC with antibiotics; (E) FIC index of TC with IPM

4. Conclusion

TC has a good antibacterial action against *E. coli* strains. Antimicrobial synergy was much better for TC and imipenem than for other combinations. The study indicated that there is a scope and need to explore the antimicrobial synergy of TC with other antibiotics in an *in vivo* model against MDR strains of bacteria for therapeutic purposes.

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6. Conflict of interest

The authors declare that they have no conflict of interest.

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