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Biotyping, molecular characterization and screening for antibacterial phytochemicals against shiga toxin producing *E.coli* from cattle

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ABSTRACT

Shiga toxin producing E.coli (STEC) is an emerging pathogen. These STEC strains are grouped as O157 and non O157 group. The ability of STEC to cause serious disease in human is related to the production of one or more shiga- like toxins (stx1, stx2 or both). This study was designed to investigate the prevalence and characterization of Shiga toxin producing strains of E.coli (STEC) in cattle of Coimbatore. A total of 150 cattle samples were collected from different slaughter houses and transported to the laboratory. The different serogroups isolated are known for certain life threatening disease in humans in other parts of the world. Stx1 and Stx2 genes were identified by PCR. Antibiotic resistance is increasing among these STEC and usage of antibiotics for treatment of such strains result in induction of toxin production. Hence, phytochemicals from Punica granatum peels and Psidium guajava leaf extracts were studied as an alternative to antibiotics.

Key words: Shiga toxin producing *E.coli, Punica granatum, Psidium guajava, Stx*1 and *Stx*2.

INTRODUCTION

Enterohemorrhagic *E.coli* (EHEC): EHEC produces toxins, known as verotoxins (VT) or Shiga-like toxins(ST) because of their similarity to the toxins produced by *Shigella dysenteriae*. There is a lack of surveillance for this organism due to the difficulty in isolating STEC. However there are only a few reports of isolation of STEC[1] from Tamil Nadu. Thus keeping in view, the above facts, a proposed study was planned to detect and characterize the field isolates of STEC from cattle, in Coimbatore by biochemical and molecular methods and also to find a bioactive phytochemical as an anti shiga toxin agent.

MATERIALS AND METHODS

Sample Collection and Identification of *E.coli*

A total of 150 faecal samples from cattle (100 diarrhoeal and 50 healthy) were collected from different areas in Coimbatore. *Escherichia coli* from the samples were confirmed by standard procedures [2] . *E.coli* MTCC 730 was used as a control.

Bio typing of the isolates

Fermentation reaction of salicin, raffinose and sucrose was studied. [3]. Based on carbohydrate fermentation of 3 sugars viz. raffinose, salicin and sucrose, all the *E. coli* isolates were bio typed into 7 different combinations.

Antimicrobial Susceptibility testing

The bacterial isolates were subjected to *in vitro* antibiotic susceptibility test. The isolates were tested against commonly used antibiotics like amikacin (AK-30mcg), aztreonam (AO-30mcg), cefazolin (CZ-30mcg), ceftazidime (CA-30mcg), ampicillin (A-30mcg), ciprofloxacin (CF-5mcg), gentamicin (G-30mcg), kanamycin (K-30mcg), nalidixic acid (NA-30mcg), trimethoprim (TR-30mcg), cefixime (CFX-5mcg) and tetracycline (T-30mcg). *E.coli* MTCC 443 was used as a control [4]. The Pearson chi-square statistical test was used to determine whether significant differences exist between different parameters of the present study.

Molecular characterization of the isolates

PCR reaction for detection of toxigenic genes

Shiga toxin producing isolates were confirmed by the presence of stx1 (BGRIU- TCAACGAAAAATAACTTCGCT and BGR1D - CAGTTAATGTGGTTGCGAAGG) and stx2 (BGRD2U- ATGAAGTGTATATTATTTAAA and BGRD2D – TCAGTCATTATTAAACTGCAC) genes. The PCR was performed following the method of Lee *et al*.[5].

Screening for bioactive phytochemicals against Shiga toxin producing *E.coli*

Preparation of plant extracts and testing of antibacterial activity

Dried leaves of *Psidium guajava* and peels of *Punica granatum* were powdered and extracted by soxhlet apparatus by increasing order of polarity with petroleum ether, benzene, chloroform, ethanol and methanol for 48 hours. The various extracts obtained were concentrated and dissolved in Dimethyl sulphoxide (DMSO). The prepared extracts were tested for antibacterial activity against shiga toxin producing *E.coli* by agar well diffusion method. Phytochemical screening for flavonoids, alkaloids, tannins, saponins and terpenoids were done following standard methods as described by Harborne, Trease and Evans[6,7].

Screening for bioactive phytochemicals

Purification of quercetin from *Psidium guajava* was done following the method of Meena and Patni[8]. The residues were subjected to TLC and HPLC. The TLC plates were developed with n-butanol: acetic acid: water (4:1:5 upper layer) for *Psidium guajava* [9] and water:acetic acid solvent system (3:2) for *Punica granatum extracts*. quercetin, ellagic acid and the antibiotic ampicillin were used as controls. The crude methanol extract of *Punica granatum* was subjected to column chromatography using silica gel and eluted with ethyl acetate. Antibacterial activity of the ethyl acetate fraction was confirmed by agar well diffusion method. This ethyl acetate fraction of the *Punica granatum* rind was taken for GC-MS analysis. Interpretation of mass spectrum GC-MS was done using the database of National Institute Standard and Technique (NIST 11.L).

RESULTS

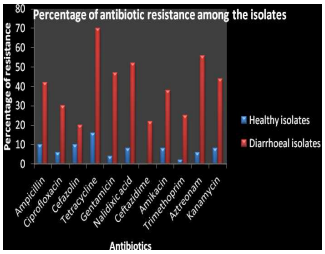
A total of 150 *Escherichia coli* strains were isolated. *Escherichia coli* was identified as Gram negative, oxidase negative, motile, indole positive, methyl red positive, voges proskauer test negative, citrate utilisation test negative, urease test negative and catalase positive, following the standard biochemical procedures [2].

Biotyping of the Isolates

Seven different biotypes were found. Strains fermenting raffinose were type 1, salicin were of type 2, sucrose were of type 3, strains that could ferment raffinose and salicin were grouped as type 4, salicin and sucrose were of type 5, sucrose and raffinose were of type 6 and that ferments all the three sugars were of type7. Biotype 6 was the predominant biotype found in both diarrhoeal and healthy cattle isolates(20%) and Biotype 7 was the next major type (19%).

Antibiotic Susceptibility testing

Drug resistance was observed in *E. coli* strains from diarrhoeal isolates. The statistical analysis of drug resistance among diarrhoeal and healthy isolates was found to be significant(P=24.263). The percentage of drug resistance of cattle diarrhoeal and healthy isolates were shown in fig.1.



Detection of toxigenic genes

Shiga toxin producing *E.coli* was confirmed by the presence of either *stx*1(300bp) or *stx*2 (450bp). Five cattle diarrhoeal isolates produced *stx*1 and *stx*2. The predominant virulent gene in diarrhoeal isolates was *stx*2. Thirty eight isolates were *stx*2 positive.

Screening for bioactive phytochemicals against Shiga toxin producing *E.coli*

The methanol extract of *Psidium guajava* and *Punica granatum* showed high antibacterial activity. The Phytochemical analysis of these extracts showed the presence of alkaloids, flavonoids, saponins and tannins.

Psidium guajava

TLC of *Psidium guajava* methanol extract separated into five spots with Rf values of 0.14cms, 0.21, 0.26, 0.64 and 0.98 that corresponds to quercetin. In bioautographic analysis, zone was observed in the spot that coincided with quercetin. When the purified fraction was subjected to HPLC, one peak was observed with RT of 2.5min (fig.2), which coincided with that of standard quercetin (fig.3). Thus the presence of the compound Quercetin was confirmed by TLC and HPLC.

Fig.2. HPLC Chromatogram of purified extract of *Psidium guajava*

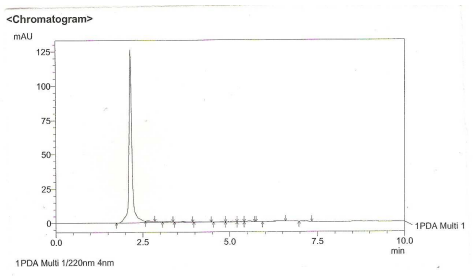
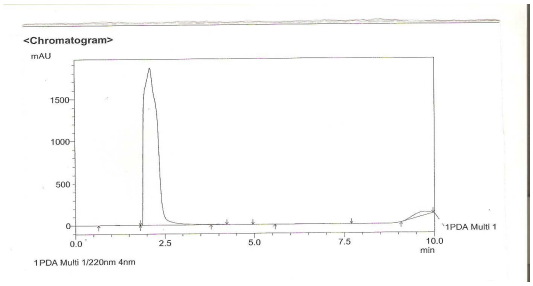
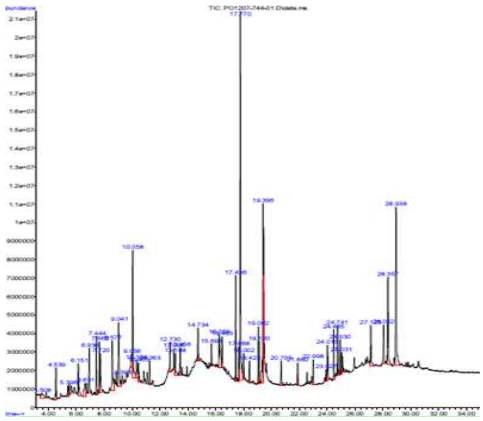


Fig. 3. HPLC Chromatogram of standard quercetin





Puncia granatum
In TLC, spots were observed at Rf values of 0.04, 0.07, 0.092, 0.23, 0.30 and 0.439. Ellagic acid and rifampin showed Rf value of 0.07. Bioautography showed a zone formation over the spots with Rf values of 0.07 and 0.439. GC-MS chromatogram is shown in fig.4. Thirty six compounds were identified in this study. n-Hexadecanoic acid (13.23%), 9,12-Octadecadienoic acid (Z,Z)- (8.47%), 3,4-Difluorobenzoic acid, 4-dodecyl ester (5.30%), Stigmasterol (4.64%) and 5-Hydroxymethylfurfural (4.18%) were the compounds showing higher area%.

DISCUSSION

Shiga toxin producing *E.coli* was found to be a major causative agent in diarrhea in Cattle. The same biotypes were predominant in both diarrhoeal and healthy cattle isolates. Thus biotyping alone cannot be used as as epidemiological tool to characterize strains. Emerging antibiotic resistance was observed by several authors in various studies [10-13]. All these studies show that although *E.coli* O157 is sensitive to most of the antibiotics, non O157 strains are resistant to multiple drugs. The same was observed in our study among the diarrhoeal cattle isolates. 38% were stx2 positive from diarrhoeal cattle samples. Only 5% were both stx1 and stx2 positive. TLC bioautographic analysis and HPLC analysis showed that Quercetin is one of the bioactive phytochemical from *Psidium guajava*. The extract of *Punica granatum*, on analysis by GC-MS, showed the presence of thirty six compounds. The compounds found in literature and in our results were Di hydroxyl pyridine [14], N-Nitroso-2-methyl-oxazolidine [15], 2,5-Furandicarboxaldehyde [16], Undecane [17], 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl [18], Catechol [19], Hesperetin [20] and Squalene [21]. Further the activity of each bioactive component should be investigated.

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