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Research Article

PROTEOMIC ANALYSIS OF *ESCHERICHIA COLI* IN RESPONSE TO CATECHINS RICH FRACTION

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ABSTRACT

Objective: Multi drug resistance is a major medical concern. Medicinal plants have been accepted as potential reserviour of lead compounds for drug design and development. However, molecular mechanism of antimicrobial action of phytoconstituents with medicinal properties, still needs to be explored. Therfore, proteomic analysis technique was carried to trace molecular mechanism of action of catechin rich fraction.

Methods: Catechin rich fraction from geen tea was prepared. Comparative proteomic analysis of *E.coli* incubated with sub-MIC concentration of cahechin rich fraction was carried out to explore the mode of action of catechins and to identify the cellular targets.

Results: Minimum inhibitory concentration of catechin rich fraction against *E. coli* was found to be 0.5 mg/mL. Proteomics analysis reveals that catechins rich fraction down-regulate a number of proteins important for bacterial survival such as PstC, NADH dehydrogenase, succinyl-CoA synthetase α-subunit, glyceraldehyde-3-phosphate dehydrogenase and iron-containing superoxide dismutase.

Conclusion: Catechins rich fraction down-regulate expression of several important proteins/ targets to inactivate or kill the bacteria. Furthermore, such fractions needs to be standardized for further use alone or in combination with antibiotics for effective treatment of the multi-drug resistant bacteria.

INTRODUCTION

From millions of years, microorganism's survival has been possible due to their ability to adapt against antimicrobial agents by the help of spontaneous mutation or by DNA transfer and differential protein expression. This process facilitates the resistance towards the action of certain antibiotics, making the antibiotic action ineffective [1]. During severe infections, microbial resistance results in effectiveness towards routine antibiotic therapy is not beneficial in the treatment. Gram-negative bacteria include *Pseudomonas aeruginosa, K. pneumoniae*, *Salmonella typhi*, *Escherichia coli. E. coli* is most common medically relevant pathogens and also involved in nosocomial infections [2-4].

The disease causing ability of gram-negative bacteria is often related to its surface components [3, 5, 6]. *E. coli* is an opportunistic bacterium that can cause diarrhea and various infections in the gastrointestinal tract and urinary tract. To overcome the multidrug resistant (MDR) stress, researches have been performed in the field of medical sciences to develop novel antibiotics but these bacteria find new pathways and develop drug-resistant due to some integral abilities [7]. Drug resistance developed by pathogenic microorganisms draws much attention towards the use of plant extracts and its biologically active compounds in the form of herbal medicine. Plants with medicinal properties represent an alternate to synthetic antibiotics for the treatment of several infectious diseases and extensively been explored for finding new active biomolecules that triggers an effective antimicrobial partway and with no strain resistance [8]. About 20% of the plants found worldwide has been tested pharmacologically or biologically and a substantial number of new antibiotics introduced in the market are based on the natural or semi-synthetic resources [9].

Plants are able to synthesize a wide range of secondary metabolites (organic compounds) that are not involved in the organism's growth, development or reproduction [10, 11]. They vary structurally and most of them are distributed among a very limited number of plant species [12]. Secondary metabolites help in the plant defense system against herbivores and other defenses related to interspecies [13]. Humans use secondary metabolites in the form of herbal and recreational drugs as well as medicines. In recent years, the use of some secondary metabolites as an alternative to conventional antibiotics has generated the interest in human health research. More than 13,000 secondary metabolites have been isolated from the medicinal plants which are less than 10% of the total [14]. In many cases, these secondary metabolites serve as plant defense or perform specialized mechanisms. These secondary metabolites were found to be endowed with medicinal properties including antimicrobial activity. Based on their biosynthesis, secondary metabolites of plant can be differentiated into three groups: (1) terpenoids, (2) flavonoids and allied phenolic and polyphenolic compounds and (3) nitrogen-containing alkaloids and sulphur-containing compounds. Flavonoids are the group of phenolic secondary metabolites in plants that are widespread in nature. Catechins are the well-known flavonoids known of antimicrobial activity and also used for the symptomatic treatment of several gastrointestinal, respiratory and vascular diseases [15]. Catechins are essential components in foods as well as in herbal medicines. These are well reported in *S. asoca*, an important and most legendary medicinal plant, known to possess antimicrobial activity [15-19]. In 2008, Puhl and Treutter reported the anti-infective activity of catechins [20]. A correlative study was carried out by our and concluded that CA levels increases in the re-generated bark and leaves which shows their importance in the prevention of infection [16]. The richest source of catechins is Green tea leaves are reported to have antimicrobial activity [21]. However the antimicrobial molecular mechanism of action of catechins was not completely understood. Large-scale studies in the field of proteomics and metabolomics, successfully exploring the differences in gene expression, protein and metabolite abundance, modification of post-translational protein thus maping the biochemical regulations and processes occur in cells. Proteomics and likewise technologies are very helpful to explore molecular mechanism of antimicrobial compound. In order to achieve a complete analysis of the biological response of a complex system, it is important to monitor the response of an organism to a conditional difficulty at the transcriptome, proteome and metabolome levels [22-23]. In the present study, proteome analysis was carried out to explore the effect of catechins rich fraction on *E. coli*. 2-Diamentional gel electrophoresis profiles were used to identify the significantly up/down regulated proteins in catechin treated bacterial culture.

MATERIALS & METHODS

Chemicals

Resazurin dye, commassie stain, bromophenol blue, urea, CHAPS, DTT, iodoacetamide, trypsin, Luria broth, acetone of analytical grade were used. Double distilled water (DDW) was used throughout the experiments.

Bacteria

Microbial Type Culture Collection (MTCC) registered bacterial isolates of *E. coli* (MTCC 433) was obtained from Institute of Microbial Technology, Chandigarh.

Collection of catechins rich fraction

For collection of catechins rich fraction, 20 gms green tea were washed and crushed. The crushed material was mixed with equal quantity of deionized water (Direct-Q, Millipore) and incubated with continuous shaking overnight at 80°C. The samples were centrifuged at 10000 g for 10 minutes and filtered through 0.22µ filters (Hi-media). The extract was lyophilized using lyophilizer (Freezone 4.5 Labconco, CA, USA) and stored at -80 °C till further use. The filtrate (catechins rich fraction) was used for antimicrobial screening and bacterial incubation at sub-MIC.

Antimicrobial evaluation of cathechin rich fraction

Antibacterial screening of cathechin rich fraction was done by Resazurin based Microtitre Dilution Assay as reported earlier [24-26]. Resazurin dye (300 mg) was dissolved in 40 ml sterile water. Vortex was used to homogenize the solution**.** This solution was then referred as Resazurin dye solution. Under aseptic conditions, the first row of 96 well microtiter plate (Tarson) was filled with 100 µl of test materials in 10% (v/v) sterile water. All the wells of microtitre plates were filled with 100 µl of nutrient broth. Two fold serial dilution (through out the column) was achieved by starting transferring 100 µl test material from first row to the subsequent wells in the next row of the same column and so that each well has 100 µl of test material in serially descending concentrations. 10 µl of resazurin solution as indicator was added to each well. Finally, a volume of 10 µl was taken from bacterial suspension and then added to each well to achieve a final concentration of 5×10^6 CFU/ml. To avoid the dehydration of bacterial culture, each plate was wrapped loosely with cling film to ensure that bacteria did not become dehydrated. Each microtitre plate had a set of 3 controls: (a) a column with Streptomycin as positive control, (b) a column with all solutions with the exception of the test extract and (c) a column with all solutions except bacterial solution replaced by 10 µl of nutrient broth. The plates were incubated in temperature controlled incubator at 37°C for 24 h. The color change in the well was then observed visually. Any color change observed from purple to pink or colorless was taken as positive. The lowest concentration of catechins rich fraction at which color change occurred was recorded as the MIC value. All the experiments were performed in triplicates. The average values were calculated for the MIC of test material.

Incubation of Bacterial Culture with catechins rich fraction

Microorganism used for the study was *E. coli*. The culture medium used in the experiments was LB (Lauria Bertini) medium. Selection experiment was carried out in the LB agar medium (LB medium with 1.5% agar) and experiments for determining growth parameters were performed in LB broth medium. Bacteria were grown in LB broth overnight to early stationary phase. Incubation at 37°C till O.D became 0.6. CRF was added according to sub MIC. Seven hours of incubation at 37°C was given followed by the centrifugation at 10,000 g for 20 min at 4°C. Distribute pellet and supernatant in different falcon tubes.

Protein Extraction (Secretory Protein)

Equal volumes of supernatant plus methanol were taken and kept overnight at -20°C. The sample was centrifuged at 20,000 g for 20 minutes at 4°C. Supernatant discarded and the pellet was washed twice by suspending in acetone containing 1% DTT, kept at -20°C for 1 hour and centrifuged at 20,000 g for 20 minutes at 4°C. The vacuum dried pellet was directly dissolved in Iso-Electric Focusing (IEF) buffer comprising 8 M urea, 20 mM DTT, 4% CHAPS and 2% ampholyte (pH 3-10) by vortexing for 1 h at 20°C. This solution was centrifuged at 20,000 g for 20 min at 20°C. The supernatant was collected and the residue re-extracted with iso-electric focusing buffer. The combined supernatants were centrifuged and supernatant taken. Estimation of total protein content for each sample was done according to the method described by BiCinchoninic Acid (BCA) assay, using Bovine Serum Albumin (BSA) as the standard [27].

Protein extraction (Cell Pellate)

Pellate washed again with PBS buffer solution and bacterial cell pellets were suspended in 500 ul of 10 mM Tris, 1mM EDTA containing 0.5 mg/ml lysozyme (Sigma L-6876, St. Louis, MO), 15 µM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) (Sigma, St. Louis, MO) and 50 U/ml benzonase (Qiagen, Valencia, CA) and mixed at 4°C for 1 hour. Protein extraction was performed by adding 1 ml of (1% w/v C7 (Sigma C-0856), 2M thiourea, 7M urea and 40mM Tris base) solution to 500 µl of treated samples from above and placing this mixture in tubes. Cells were sonicated, cycles for 5 min at an interval of 1 min each. Then 5 ml of acetone containing 10% TCA (w/v) and 1% DTT (w/v) was added. The samples were kept overnight at -20°C. The sample was centrifuged at 20,000 g for 20 min at 4°C. The pellet was washed twice by suspending in acetone containing 1% DTT, kept at -20° C for 1 h, and centrifuged at 20,000 g for 20 min at 4°C. The vacuum dried pellet was directly dissolved in IEF buffer comprising 8 M urea, 20 mM DTT, 4% CHAPS, and 2% ampholyte (pH 3-10) by vortexing for 1 h at 20°C. This solution was centrifuged at 25,000 g for 20 min at 20°C. The supernatant was collected and the residue re-extracted with iso-electric focusing buffer. The combined supernatants were centrifuged and supernatant taken. Estimation of total protein content for each sample was done according to the method described by BCA assay, using BSA as the standard [27].

2-Dimensional Gel Electrophoresis (IEF/SDS- PAGE)

Equal quantities of protein (secretory & cellular) were taken and diluted to a final volume of 450 µl in rehydration buffer (8 M urea, 20 mM DTT, 4% CHAPS, 2% ampholyte (pH 3–10) and 0.01% bromophenol blue) and subsequently applied on Immobiline Dry Strip, 24cm length pH 3–10 (GE Healthcare) for rehydration at 20°C for 20 h. IEF (Iso-Electric Focusing) was performed using a Multiphor II horizontal electrophoresis system (Amersham Biosciences, Uppsala, Sweden) as follows: 500 V for 2 h, 1000 V for 1 h followed by a linear increase from 1000 V to 8000 V for 4 h and finally 8000 V to give a total of 50 kVh. Equilibration was done using buffer (50 mM Tris–HCl, pH 8.8, 6 M urea, 20% (v/v) glycerol, 2% (w/v) SDS) plus 20 mm DTT for 15 min and equilibration buffer plus 125 mM iodoacetamide for another 15 min. After equilibration IPG strips were placed on top of vertical slabs of acrylamide gel (12.5%). The stacking gel was formed by a layer of 1% (w/v) agarose. Electrophoretic migration along the second dimension was performed using the SDS-PAGE running buffer (Towbin) at 20°C in Hoofer six gel electrophoresis system at 15 mA/gel for 1 h, followed by 25 mA/gel for 8 h.

Stain and Analysis of 2-D Gels

After the electrophoresis, gels were silver stained according to the protocol described by Rabilloud et al (1992) [28]. Computerized 2-D gel analysis, including protein spot detection and quantification were performed using Image Master Platinum 4 software (GE Healthcare). Three replicate gels were run for each different sample.

Protein In-Gel Digestion

Tryptic digestion of differential protein spots were performed using the In-Gel Trypsin Digestion Kit (G Biosciences Ltd., UK) in accordance with supplier's instructions. In brief, excised protein spots were de-stained automatically, dehydrated, reduced with DTT, alkylated with iodoacetamide and digested with overnight incubation of samples at 37°C in the presence of trypsin. The resulting tryptic digests were analyzed by Q-TOF LC/MS (Agilent Technologies, USA).

Protein Identification

Peptide and protein identification were performed by the Spectrum Mill Software (Applied Biosystems). Each mass spectra was searched against the NCBInr database. The searches were run using the fixed modification of carboxymethylation labelled cysteine parameter enabled. Other parameters include MS spectral features (MH^+ 100 to

8000 Da, Extraction time range 0 to 300 min), Maximum ambiguous precursor charge 3, Precursor mass tolerence +/- 2.5 Da.

RESULTS

Antibacterial activity (Resazurin Microtitration Dilution Assay)

Catechins rich fraction was screened for their antibacterial potential and showed optimal good activity against *E.coli with a* MIC 0.5 mg/mL. Streptomycin is used in this study as positive control shows MICs values as 6.5μg/mL against the bacterial strains used in the study.

Protein extraction & estimation

The concentrations of cellular proteins were found to be 2.1 and 2.64 mg/mL in treated and control sample respectively. Extracellular protein concentrations were found to be 0.29 and 0.36 mg/ml in treated and control sample respectively.

Two-dimensional (2-D) gel electrophoresis based analysis and identification of secretory proteins

The 2-D gels were fixed for two hours in 7% acetic acid / 10% methanol and silver stained (Molecular Probes, Invitrogen Inc.). The destaining was performed with 7% acetic acid / 10% methanol and imaged (Bio-Rad). Image analysis of secretory proteins from two gels, derived from two separate shake flask cultures (Control and Treated) has been done using software from GE Healthcare Image master platinum 4. A number of protein spots appear to be larger and darker or smaller and lighter than corresponding spots in the control gels (Figure 1). Significantly high abundance 14 protein spots ($p<0.005$) were selected and were digested with trypsin using in gel digestion technique and their mass spectra were obtained using highly accurate Q-TOFMS instrument. Peptide sequences obtained from mass spectra were analyzed against non-reductant NCBI protein database by using Spectrum Mill software. Accuracy of protein identification was checked by using SPI score and percent amino acid coverage. On the basis of available information for all the 14 spots using Spectral Mill, two down-regulated proteins i.e. NADH dehydrogenase, PstC protein were identified.

Catechin rich fraction inhibited the synthesis of NADH dehydrogenase, which influences the respiratory chain in *E. coli*. Another protein PstC (membrane protein component of ABC phosphate transporter) related to membrane-associated phosphate-specific transporter (Pst) system [29] was found to be down-regulated. Pst is composed of four different proteins: PstS, PstC, PstA and PstB protein. The PstS component detects and binds Pi with high affinity; the PstA and PstC form transmembrane pores for the entry of Pi, while the energy is provided by PstB through ATP hydrolysis. Overall, Pst system participates in growth of cell, phosphate uptake and the expression of virulence-associated traits.

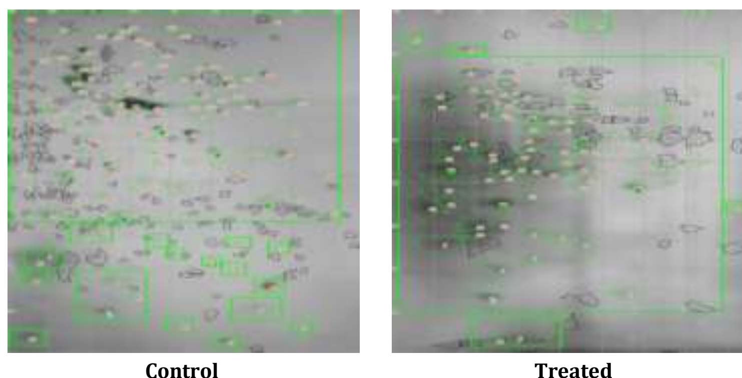

Fig. 1: Two-dimensional gel of secretory proteins in control & treated samples of *E. coli*.

Two-dimensional (2-D) gel analysis of cellular proteins

Image analysis of two gels from each set of cellular proteins, derived from two separate shake flask cultures (Control and Treated) has been done (Figure 2). The visual observations were confirmed by image analysis and quantification *(q* value). Significant high abundance 9 spots ($p<0.005$) were selected and subjected to digested with trypsin using in gel digestion technique and their mass spectra were obtained using highly accurate Q-TOFMS instrument. Peptide sequences obtained from mass spectra were analyzed against non-reductant NCBI protein database by using Spectrum Mill software. Accuracy of protein identification was checked by using SPI score and percent amino acid coverage. On the basis of available information for all the 9 spots using Spectral Mill, three down regulated proteins i.e. Succinyl-CoA synthetase α-subunit, glyceraldehydes-3-phospahte dehydrogenase and iron-containing superoxide dismutase were identified.

Catechin rich fraction significantly down regulates the succinyl-CoA synthetase α-subunit and glyceraldehyde-3-phosphate dehydrogenase proteins which are involved in the carbohydrates metabolism. Catechins rich fraction down regulated the periplasmic iron-containing superoxide dismutase, protein which is necessary for bacterial survival. It protects the bacteria by preventing the oxidative stress occurred due to the entry of toxic compounds into the bacteria [30].

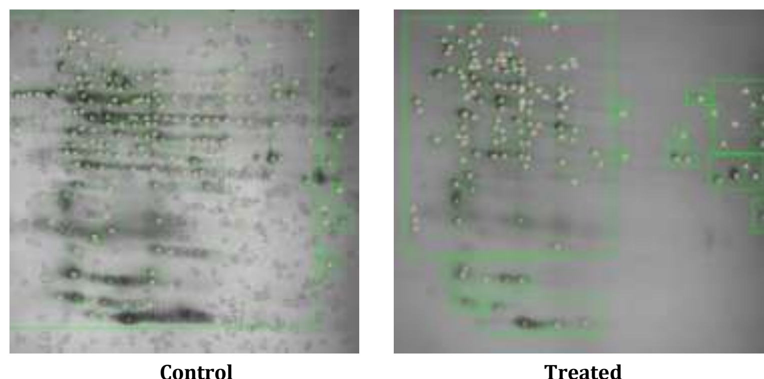

Fig. 2: Two-dimensional gel of cellular proteins in control & treated samples of *E. coli*.

E. coli secrete a number of proteins which help in biogenesis of organelles. Six highly conserved secretion systems are known to mediate protein export through the periplasmic space of gram-negative bacteria [31]. The protein analysis in the current study reveals that the water extract of Catechins rich fraction significantly down-regulate two secretary proteins i.e. NADH dehydrogenase, PstC protein and three cellular proteins i.e. Succinyl-CoA synthetase α-subunit, glyceraldehydes-3-phospahte dehydrogenase, iron-containing superoxide dismutase protein of *E. coli*.

CONCLUSION

Catechins are found to be associated with antimicrobial activity that induces change in the protein profile of bacteria. Catechin rich fraction induced cell death which is associated with the cellular processes that are unique to bacteria such as the respiratory chain. It affects NADH dehydrogenase due to which respiratory machinery is restricted in *E. coli*. It facilitates novel target for the development of antimicrobial drugs. Catechins rich fraction considerably affects PstC, succinyl-CoA synthetase α-subunit and glyceraldehyde-3-phosphate dehydrogenase proteins in *E. coli*, both of them are involved in the transportation and carbohydrates metabolism. However, catechins rich fraction down regulates succinyl-CoA synthetase α-subunit and glyceraldehyde-3-phosphate dehydrogenase by which starvation condition arises due to the inhibition of carbohydrates metabolism**.** Iron-containing superoxide dismutase is also known as a free radical scavenger that catalyzes the dismutation of superoxide which causes oxidative stress in bacteria. Due to the presence of a complex mixture of secondary metabolites in medicinal plants, their extracts show synergistic effects on the growth of bacteria. In the present study, treatment by catechins rich fraction down-regulate several proteins that can be used as target in antimicrobial drug development i.e. PstC, NADH dehydrogenase, succinyl-CoA synthetase α-subunit, glyceraldehyde-3-phosphate dehydrogenase and iron-containing superoxide dismutase.

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