Microbial Pathogenesis 95 (2016) 133–141

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Anti-hemolytic, hemagglutination inhibition and bacterial membrane disruptive properties of selected herbal extracts attenuate virulence of Carbapenem Resistant *Escherichia coli*

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ARTICLE INFO

Article history:
Received 12 March 2016
Accepted 1 April 2016
Available online 4 April 2016

Keywords:
Hemolysis
Hemagglutination
Membrane disruption
Natural plant products
Carbapenem Resistant *Escherichia coli*

ABSTRACT

Expression of a multitude of virulence factors by multi-drug resistant microbial strains, *e.g.*, Carbapenem Resistant *Escherichia coli* (Family: Enterobacteriaceae; Class: Gammaproteobacteria), is responsible for resistance against beta-lactam antibiotics. Hemolysin production and induction of hemagglutination by bacterial surface receptors inflicts direct cytotoxicity by destroying host phagocytic and epithelial cells. We have previously reported that *Berberis aristata*, *Camellia sinensis*, *Cyperus rotundus Holarrhena antidysenterica* and *Andrographis paniculata* are promising herbal leads for targeting Carbapenem resistant *Escherichia coli*. These herbal leads were analyzed for their anti-hemolytic potential by employing spectrophotometric assay of hemoglobin liberation. Anti-hemagglutination potential of the extracts was assessed by employing qualitative assay of visible RBC aggregate formation. *Camellia sinensis* (PTRC-31911-A) exhibited anti-hemolytic potential of 73.97 ± 0.03%, followed by *Holarrhena antidysenterica* (PTRC-8111-A) *i.e.*, 68.32 ± 0.05%, *Berberis aristata* (PTRC-2111-A) *i.e.*, 60.26 ± 0.05% and *Cyperus rotundus* (PTRC-31811-A) *i.e.*, 53.76 ± 0.03%. Comprehensive, visual analysis of hemagglutination inhibition revealed that only *Berberis aristata* (PTRC-2111-A) and *Camellia sinensis* (PTRC-31911-A) exhibited anti-hemagglutination activity. However, *Andrographis paniculata* (PTRC-11611-A) exhibited none of the inhibitory activities. Furthermore, the pair wise correlation analysis of the tested activities with quantitative phytochemical descriptors revealed that an increased content of alkaloid; flavonoids; polyphenols, and decreased content of saponins supported both the activities. Additionally, flow cytometry revealed that cell membrane structures of CRE were damaged by extracts of *Berberis aristata* (PTRC-2111-A) and *Camellia sinensis* (PTRC-31911-A) at their respective Minimum Inhibitory Concentrations, thereby confirming noteworthy antibacterial potential of both these extracts targeting bacterial membrane; hemolysin and bacterial hemagglutination.

1. Introduction

Hemolysins, extracellular toxic proteins produced by many gram-negative bacteria, such as *Escherichia coli*, *Serratia* spp. *Proteus* spp., *Vibrio* spp., *Pseudomonas aeruginosa* and gram-positive bacteria, *e.g. Streptococcus* spp. *Staphylococcus aureus*, *Bacillus cereus*, *Clostridium tetani* are considered as one of the most important bacterial virulence factors [1]. Hemolysins cause lysis of erythrocytes and other mammalian cells by inducing cytolysis mediated membrane pore formation [2]. Additionally, many pathogenic bacteria like Carbapenem resistant *Escherichia coli* has the ability to agglutinate erythrocytes by virtue of their surface receptors (*e.g.*, mannose receptors, pilli, fimbriae), thereby leading to easy bacterial dissemination across mucosal barriers; breach of immunity checkpoints; and easy sequestering of host nutrients and iron reserves [3].

Furthermore, hemolysis and hemagglutination are direct

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http://dx.doi.org/10.1016/j.micpath.2016.04.005
0882-4010/

indicators of free radical damage which might be counteracted by antioxidants [4,5]. Several herbal secondary metabolites such as flavonoids and polyphenols have anti-oxidant properties to stabilize erythrocyte membrane by scavenging free radicals, thereby protecting host cells from oxidative damage caused by hemolysis and hemagglutination [6]. On the contrary, saponin, a steroid or tri-terpenoid aglycone (sapogenin) is an herbal secondary metabolite which induces hemolysis and hemagglutination [7]. This definitely suggests that herbal extract rich in antioxidants *i.e.*, polyphenols, flavonoids and deficient in saponins might serve as a potential candidate for hemolysis and hemagglutination inhibition.

Moreover, bacterial cell membranes based efflux pumps and secretory systems play an important role in bacterial pathogenicity as well as in imparting antimicrobial resistance [8].

We have recently reported that *Berberis aristata* and *Camellia sinensis* possess anti-adhesion, anti-quorum sensing and anti-biofilm activity, thereby attenuating the virulence of Carbapenem Resistant *Escherichia coli* (CRE) [9]. The present study was further undertaken to elucidate the mechanism of action of promising Natural Plant Products (NPPs) against indispensable virulence determinants of CRE, *i.e.* hemolysin, hemagglutination and bacterial membrane. Furthermore, present day hemolysin and hemagglutinin inhibitors are fraught with limitations such as exorbitant cost, limited action, non-absolute treatment, organ toxicity *etc.* Hence, it becomes imperative to search for novel antibacterial moieties targeting such enteric virulence factors.

The anti-hemolytic and anti-hemagglutination activity of selected aquo-ethanolic extracts of *Berberis aristata, Camellia sinensis, Cyperus rotundus, Holarrhena antidysenterica* and *Andrographis paniculata*, were evaluated at *in vitro* level against the targeted pathogen (CRE).

Membrane disruption potential of the test extracts was tested using flow cytometry wherein significant changes in cell membrane permeability of bacteria, following treatment with herbal extracts/standard antibiotics were investigated. The estimated values of various mechanistic activities were then subjected to a correlation analysis with phytochemical descriptors *i.e.*, presence/absence of polyphenols, flavonoids and saponins.

2. Materials and methods

2.1. Bacterial strain and culture conditions

Pathogenic microorganism(s) tested in this study *i.e.*, Carbapenem Resistant *Escherichia coli* (CRE) were isolated from biological fluids *i.e.*, blood and urine. These were collected from a registered pathological center *i.e.*, National Centre for Disease Control [NCDC], New Delhi, India. Pathogenic bacteria were grown in nutrient broth (HiMedia) at 37 °C for 24 h, followed by isolation on Mac Conkey agar plates. The bacterial isolates were identified following their morphology and biochemical characteristics by using standard microbiological techniques, as described earlier in our previous studies [9]. Furthermore, the clinical isolate of CRE was preserved and tested for its antibiotic sensitivity profile (Table 1). Also the BLAST analysis of its gene sequence (not shown here) exhibited 99% homology with 7F strain of NDM-1 *Escherichia coli* (Gene Accession Number: KJ131191, GI: 602624949).

2.2. Plant material and extract preparation

Stem bark of *Berberis aristata*, leaves of *Camellia sinensis*, roots of *Cyperus rotundus*, stem of *Holarrhena antidysenterica* and leaves of *Andrographis paniculata* were collected in the month of December 2013 from the fields of Uttarakhand, India. The identity and authenticity of the plant material was confirmed by an ethno botanist and voucher specimens were deposited in the repository at Institute of Nuclear Medicine and Allied Sciences. Plant material(s) was air dried and powdered, followed by extraction with water and ethanol (1:1, v/v) for 4–5 h (temperature ~ 55° C) on soxhlet apparatus (Buchi Labortechnik, Switzerland) separately [10]. The residual solvent of concentrated extract was then evaporated under reduced pressure in rotary evaporator (Cole Parmer, India). The crude extract obtained was lyophilized (80–200 mTorr; Temp: −30 to −80 °C) in Lyophilizer (FTS System, USA), followed by spray drying so as to develop amorphous powder (muddy brown in color). Extracts were designated as PTRC-2111-A (*Berberis aristata*), PTRC-31911-A (*Camellia sinensis*), PTRC-31811-A (*Cyperus rotundus*), PTRC-8111-A (*Holarrhena antidysenterica*) and PTRC-11611-A (*Andrographis paniculata*). Quality Control indices phytochemical fingerprint of all these extracts have already been published (Table 2) [9].

2.3. Liquid chromatography mass spectrometry analysis

Herbal extracts, dissolved in a mixture of ethanol and water (1:1 v/v) were analyzed on Shimadzu LC-6A HPLC machine utilizing Waters Novapak C18 column (250 × 4.6 mm, 4 μm) with auto-injector (SIL-10 AD VP) and diode array detector (SPD M-10 A). Mobile phase and flow rate of elution were standardized according to each herbal extract. Equilibration time was nearly 45 min with a 9-step gradient of geometric progression. LC-MS analysis was performed using a LCQ MS mass spectrometer (Thermo-Finnigan, India). The cone gas and desolvation gas flow rate were set at 80 and 510 L/Hr. Ion spray voltage was set at 4500 V. The voltage at the orifice plates focusing extractor and Channel Electron Multiplier (CEM) was adjusted at 2–5 V and 650 V respectively. Positive ions were scanned in the range 60–700 Dalton (Da) using 10 ms dwell time and a 0.5 Da step size during scans [11]. The comparative analysis of eluted peak data with respect to mass spectral data of reference compound(s) was used to identify known compounds in the respective extracts.

2.4. Anti-hemolytic assay

The erythrocytes from rat blood (Sprague dawley rat, Male, 6 weeks old) were separated by centrifugation and diluted with isotonic sodium phosphate buffer (0.2 M, pH 7.4) to yield a 4% suspension. Volume of extract(s) corresponding to their respective Minimum Inhibitory Concentration (MIC) was added to 2 mL of the suspension of erythrocytes with final volume adjusted to 3.5 mL by adding phosphate buffer. The reaction mixture was pre-incubated for 120 min at room temperature and then 0.5 mL Hydrogen peroxide (free radical inducer) was added. Incubation was concluded afterwards by centrifugation at 10,000 g for 5 min. Extent of hemolysis was determined by measurement of the absorbance at 540 nm corresponding to hemoglobin liberation [12]. Rutin treated sample was considered as positive control. The sample containing only erythrocyte suspension without any herbal or standard was considered as maximum level of hemolysis (Negative Control). Anti-hemolytic activity was expressed as the inhibition percentage and was calculated using the following formula: