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## Potential antibacterial activity of berberine against multi drug resistant enterovirulent *Escherichia coli* isolated from yaks (*Poepagus grunniens*) with haemorrhagic diarrhoea

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### ABSTRACT

**Objective:** To evaluate the antimicrobial efficacy of berberine, a plant alkaloid. **Methods:** Five multi-drug resistant (MDR) STEC/EPEC and five MDR ETEC isolates from yaks with haemorrhagic diarrhoea were selected for the study. Antibacterial activity of berberine was evaluated by broth dilution and disc diffusion methods. The binding kinetics of berberine to DNA and protein was also enumerated. **Results:** For both categories of enterovirulent *Escherichia coli* (*E. coli*) isolates, berberine displayed the antibacterial effect in a dose dependent manner. The MIC<sub>50</sub> of berberine chloride for STEC/EPEC isolates varied from 2.07  $\mu$  M to 3.6  $\mu$  M with a mean of (2.95  $\pm$  0.33)  $\mu$  M where as for ETEC strains it varied from 1.75 to 1.96  $\mu$  M with a mean of (1.87  $\pm$  0.03)  $\mu$  M. Berberine bind more tightly with double helix DNA with Bmax and Kd of (24.68 $\pm$ 2.62) and (357.8 $\pm$  57.8), respectively. Berberine reacted with protein in comparatively loose manner with Bmax and Kd of (18.9 $\pm$ 3.83) and (286.2 $\pm$ 113.6), respectively. **Conclusions:** The results indicate clearly that berberine may serve as a good antibacterial against multi drug resistant *E. coli*.

## 1. Introduction

Diarrhoea is probably the most common problem encountered by the farm animals. Other than the dietary factors, bacterial, viral or parasitic infection is the main causes of diarrhoea in livestock. However, diarrhoeagenic *Escherichia coli* (*E. coli*) are probably the most common pathogen to affect the animals due to its ubiquitous existence, easy transmission and its rapidly emerging drug resistance property. Our recent reports suggested that yak is a potential reservoirs of multidrug resistant STEC and EPEC and it is of great public health significance as they were isolated from alimentary tract of diarrhoeic

yak[2] as well as from raw yak milk and milk products[3] frequently consumed by the tribal highlanders. STEC and ETEC have also been isolated from faecal samples diarrhoeic lambs[4] from the same region, showing their existence in various animal species. Very limited studies were conducted on the antimicrobial resistance of the diarrhoeagenic *E. coli* in India. Our previous studies indicated that many strains of *E. coli* from diarrhoeic yaks exhibited resistance against gentamicin, tetracycline, ampicillin, furazolidone, Co-trimoxazole, colistin, cephalixin, and nalidixic acid. The better antimicrobial agents in terms of sensitivity were norfloxacin and cephalaxime[1]. However, antimicrobial resistance of STE/EPEC or ETEC may be transferred via plasmid transfer to other sensitive bacteria and they can enter in human food chain. Report on rapid emergence of resistance

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against the frontline and conventional remedies<sup>[1, 5]</sup> among other pathogens in yaks like *Moraxella bovis*, *Neisseria*, *Mycobacterium* is a grave concern now–a–days. Therefore, a safe, novel, cost–effective, alternative therapeutic strategy especially from herbal origin is the need of the hour. Some of the plant derived products were recorded to be effective against many of the multi–drug resistant bacterial pathogens in yaks<sup>[6]</sup>. However, such trials were not made against enterotoxigenic or Shiga toxin producing *E. coli* strains despite their resistance against various antimicrobials. Berberine is a plant derived quaternary ammonium salt from the protoberberine group of isoquinoline alkaloids, with a long history of medicinal use in both Ayurvedic and Chinese medicine. It is generally found in the roots, rhizomes, and stem bark of varieties of medicinal plants like *Berberis aquifolium* (Oregon grape), *Berberis vulgaris* (Barberry), and *Berberis aristata* (Tree Turmeric), *Hydrastis canadensis* (Goldenseal), *Phellodendron amurense* (Amur Cork Tree), *Coptis chinensis* (Chinese Goldthread), *Tinospora cordifolia* (Guduchi)<sup>[7–8]</sup>. Literatures regarding the antimicrobial efficacy of berberine are scarcely available. Therefore, the present study was conducted to determine the antimicrobial potency of berberine against drug resistant *E. coli*.

## 2. Materials and methods

### 2.1. Chemicals

All the chemicals & reagents used in the present study were obtained from Sigma Chemicals, USA, E–Merk, India, Hi–Media, Mumbai, India and SRL, India.

### 2.2. Experimental design

In order to carry out the antibacterial effect of berberine, five multi–drug resistant (MDR) STEC/EPEC and five MDR ETEC strains isolated from yaks with haemorrhagic diarrhoea were selected. All these enterovirulent *E. coli* isolates were resistant to more than six drugs investigated in the present study and representatives of diverse serogroups.

### 2.3. Determination of antibacterial activity by broth dilution method

Broth dilution assay was performed as stated earlier by the author<sup>[5]</sup>. Briefly, sterile tubes containing 10 mL of trypticase soy broth or MH broth (Hi Media, Mumbai) were supplemented with different concentrations of serially diluted berberine chloride (0–10  $\mu$  M). The tubes were inoculated separately with multi–drug resistant strains of enterovirulent *E. coli* isolates at concentrations of  $1.5 \times 10^6$ /mL –  $3 \times 10^6$ /mL respectively. The initial OD (optical density) was observed at 650 nm. Following incubation for 24 h, again

the OD<sub>650</sub> was observed. The viability of the organisms was calculated in terms of turbidity and the difference between final OD<sub>650</sub> and the initial OD<sub>650</sub> (Specific OD<sub>650</sub>) is interpreted as the growth of the bacteria. The viability of bacteria at any specific concentration was calculated as the percentage of specific OD<sub>650</sub> at that concentration versus the specific OD<sub>650</sub> of the tube without berberine supplementation (negative control). The calculation can be shown as follows

Percent viable =

$$\frac{\text{Specific OD}_{650} (\text{Final OD}_{650} - \text{Initial OD}_{650}) \text{ for any concentration of berberine} \times 100}{\text{Specific OD}_{650} (\text{Final OD}_{650} - \text{Initial OD}_{650}) \text{ of the negative control (berberine)}}$$

Specific OD<sub>650</sub> (Final OD<sub>650</sub>–Initial OD<sub>650</sub>) of the negative control (berberine)

For each concentration of berberine, equal amount of DMSO was added to each tube to exclude out the bactericidal effect of the vehicle, if any.

### 2.4. Determination of antibacterial activity by disc diffusion method

Disc diffusion method was employed following the recommendations of the CLSI, 2008 as described elsewhere<sup>[5]</sup>. Sterile disc (Hi Media, Mumbai) were impregnated with each of the berberine solution (200 mg/mL) to get the desired concentration (0–10  $\mu$  M), and dried in laminar flow cabinet. Following spreading the bacterial inoculums (Resistant strains of enterovirulent *E. coli* as stated above) evenly over the surface of Muller Hinton agar plates, the impregnated discs were positioned and incubated at 37 °C for 24 h. Sterile disc was used as negative control. The antibacterial activity was interpreted from the size of the zone of inhibition.

### 2.5. Determination of binding kinetics of berberine to DNA and protein

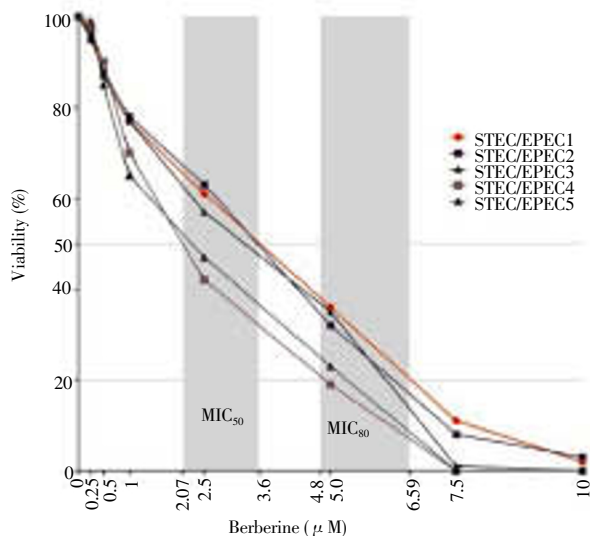
The binding kinetics of berberine was determined by the earlier described protocol of Jian–ling et al. with little modification<sup>[9]</sup>. Briefly, 50 mg of Salmon sperm DNA, and BSA (Procured from Sigma, USA) was dissolved in 500  $\mu$  L PBS buffer (0.02 mol/L, pH 7.0), containing various concentrations of berberine (25, 50, 75, 100, 150, 200, 250, 300, 400, and 500  $\mu$  g/mL). The control group contained only berberine for each concentration. The mixture was incubated at 37 °C at room temperature for 1 h. Following this 1.2 volumes of isopropanol and 1.2 volumes of 80% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> were added into the tubes containing DNA and BSA respectively, and stored at 4 °C for 1 h. Finally the mixture and control tubes were centrifuged at 13 000 r/min for 30 min. The supernatants were transferred into new tubes, and the deposits were collected and dried. Afterwards, the absorbance of the supernatants was measured by UV–Spectrophotometer (Shimadzu) at 370 nM, and defined as Ad (the remainder berberine). The A<sub>370</sub> of the control group was defined as Ac. The amount of bound berberine (Ab)

was calculated by the equation:  $Ab = Ac \times Ad$ . The binding kinetic curves were plotted using the software GraphPad Prism 5, with the X axis of berberine concentration and Y axis of the absorbance value. The kinetic parameters were given by one site binding (hyperbola) equation of the same software and Bmax and Kd were determined accordingly.

### 3. Results

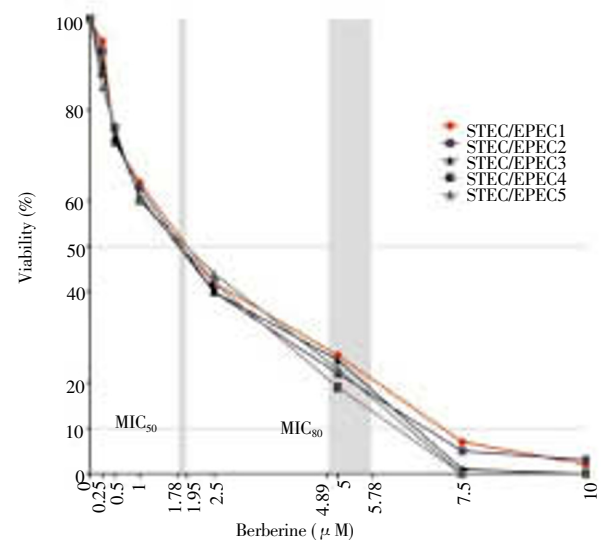
#### 3.1. Determination of minimal inhibitory concentration by broth dilution method

The antibacterial effect of berberine on different MDR STEC/EPEC and ETEC isolates (Table 1) is depicted in Figure 1 and 2. For both categories of enterovirulent *E. coli* isolates, berberine displayed the antibacterial effect in a dose dependent manner. There was no distinct variability among these strains in terms of their susceptibility to berberine. At a concentration of 1  $\mu$  M, berberine decreased the viability of the MDR STEC/EPEC strains to 65%–77% and at 5  $\mu$  M the viability was decreased to 19%–36%. At a concentration of 7.5  $\mu$  M viability of the MDR STEC/EPEC strains was decreased significantly to 0–11%. The viability pattern of the MDR ETEC strains is illustrated in Figure 2. At 1  $\mu$  M concentration of berberine hydrochloride, the viability of the ETEC strains were decreased to 60%–64% and it was further reduced to 19%–26% and 0–7% at 5  $\mu$  M and 7.5  $\mu$  M respectively. The MIC<sub>50</sub> of berberine chloride for STEC/EPEC isolates varied from 2.07  $\mu$  M to 3.6  $\mu$  M with a mean of (2.95  $\pm$  0.33)  $\mu$  M where as for ETEC strains it varied from 1.75  $\mu$  M to 1.96  $\mu$  M with a mean of (1.87  $\pm$  0.03)  $\mu$  M. The MIC<sub>80</sub> of berberine chloride for STEC/EPEC and ETEC strains were (5.82  $\pm$  0.32)  $\mu$  M and (5.36  $\pm$  0.14)  $\mu$  M, respectively.



**Figure 1.** Graphical illustration of antibacterial activity of berberine against multi-drug resistant STEC/EPEC isolates from yaks as displayed by broth dilution method.

Y axis shows the viability of the bacteria tested against different concentrations of berberine plotted on X axis.



**Figure 2.** Graphical illustration of antibacterial activity of berberine against multi-drug resistant ETEC isolates from yaks as displayed by broth dilution method.

Y axis shows the viability of the bacteria tested against different concentrations of berberine plotted on X axis.

#### 3.2. Determination of antibacterial activity by disc diffusion method

The disc diffusion assay revealed comparable zones of inhibition with berberine at 24h of incubation. Antibacterial activity of berberine against multi-drug resistant enterovirulent *E. coli* isolate from yaks showed the isolate displayed a clear and large zone of inhibition against berberine impregnated disc (B; 10  $\mu$  M). However, it was completely resistant to Norfloxacin (Nx; 10  $\mu$  G). A sterile disc impregnated with same amount of dimethyl sulphoxide (S) was also kept as vehicle control.

Vehicle control discs or antibiotic control discs had no observable effect on the bacterial growth. Against both categories of MDR isolates berberine reduced the growth conspicuously. However, distinct variation was observed between the susceptibility of STEC/EPEC and ETEC. At a concentration of 2.5  $\mu$  M zone of inhibition of the STEC/EPEC isolates was (2.2  $\pm$  0.25)  $\mu$  M. At the same concentration, the zone of inhibition was markedly higher (3.9  $\pm$  0.43)  $\mu$  M in ETEC isolates. Similarly at higher concentrations of berberine i.e. 5 and 10  $\mu$  M, the zones of inhibition of ETEC isolates (6.6  $\pm$  0.4 mM; 9.6  $\pm$  0.8 mM) were much higher than that of the STEC/EPEC isolates (7.3  $\pm$  0.46 mM; 15.2  $\pm$  0.9 mM). The results were quite in parallel with that of the broth dilution methods where, MDR ETEC isolates showed more sensitivity than that of the STEC/EPEC isolates to berberine hydrochloride. Nevertheless, in both the methods berberine displayed clear antibacterial activity against MDR STEC/EPEC and ETEC isolates.

**Table 1**

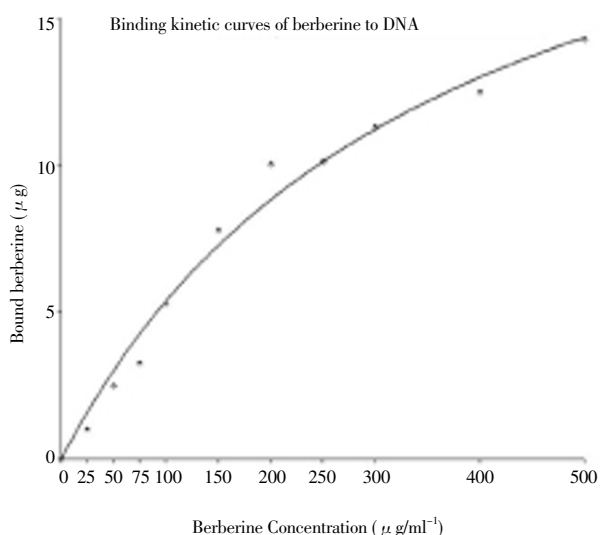
Details of antimicrobial resistance profile of multi–drug resistant STEC/EPEC and ETEC isolates from yaks.

Sl No	Isolates used	Serogroups	Antimicrobial resistance*
1	STEC/EPEC1	O5	Ak, Am, CH, Cj, Co, E, Fz, G, K, N, Na, Nf, S
2	STEC/EPEC2	O28	Ak, AT, Ce, CH, Ci, Cj, CPT, CU, E, Fz, G,K, N, Nf, Nx, O, Pf, PT, S
3	STEC/EPEC3	O60	Ac, Ak, Am, AT, Ax, CA, Cj, E, Fz, K, Na, Nf, S
4	STEC/EPEC4	O147	Ac,Ax, AT,CA,Cf, CH,Cj, Do,E, Fz,G, K, Na,Nf, Nx,O, Pf
5	STEC/EPEC5	O172	AT, CH, E, Nf, S
6	ETEC1	O8	Ac, Ak, Am, AT, E, N, Na, Nf, O, S, T
7	ETEC2	O55	CH, Do, E, Fz, K, Na, Nf, T
8	ETEC3	O110	Am, AT, Ax, Cl, Co, Fz, K, N, Na, S
9	ETEC4	O141	Ak, Am, CA, Do, E, Fz, K
10	ETEC5	O159	AK, AT, Ce, CH, Cj, E, Fz, N, Na, Nf, O, S, T

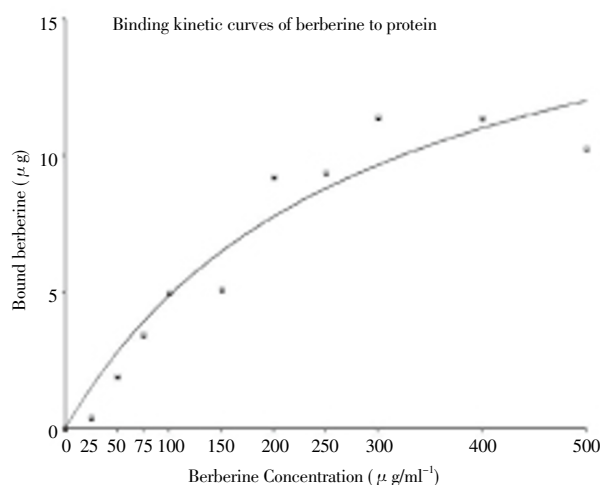
\*Ac (Amoxicillin+Clavulanic acid, 30  $\mu$ g), Ak (amikacin, 30  $\mu$ g), 10  $\mu$ g), Am (amoxicillin, 25  $\mu$ g), AT (azithromycin, 30  $\mu$ g), Ax (ampicillin+cloxacillin, CA (ceftazidime, 30  $\mu$ g), Ce (cephataxime, 30  $\mu$ g), Cf (ciprofloxacin, 5  $\mu$ g), CH (cephalothin, 30  $\mu$ g), Ci (ceftriaxone, 30  $\mu$ g), Cj (Cefaclor, 30  $\mu$ g), Cl (colistin, 10  $\mu$ g), Co (co–trimoxazole, 25  $\mu$ g), CPT (cefepime +tazobactam, 30+10  $\mu$ g), CU (Cefuroxime, 30  $\mu$ g), Do (doxycycline hydrochloride, 30  $\mu$ g), E (erythromycin, 10  $\mu$ g), Fz (furazolidone, 50  $\mu$ g), G (gentamicin, 10  $\mu$ g), K (kanamycin, 30  $\mu$ g), N (neomycin, 30  $\mu$ g), Na (nalidixic acid, 30  $\mu$ g), Nf (nitrofurantoin, 300  $\mu$ g), Nx (norfloxacin, 10  $\mu$ g), O (oxytetracycline, 30  $\mu$ g), Pf (pefloxacilin, 5  $\mu$ g), PT (piperacillin+tazobactam, 100+10  $\mu$ g), S (streptomycin, 30  $\mu$ g), T (tetracycline, 30  $\mu$ g).

### 3.3. Determination of DNA and protein binding kinetics of berberine chloride

DNA and protein binding kinetics of berberine were computed in Graph pad prism (Figure 3, 4) and it clearly indicated that berberine chloride bind more tightly with double helix DNA with Bmax (maximum number of binding sites) and Kd (ligand concentration that binds to half the receptor sites at equilibrium) of  $[24.68 \pm 2.62] \mu$  M and  $[357.8 \pm 57.8] \mu$  M], respectively[R2; 0.98]. With protein berberine reacted comparatively in loose manner with Bmax and Kd of  $[18.9 \pm 3.83] \mu$  M and  $[286.2 \pm 113.6] \mu$  M], respectively[R2; 0.93].



**Figure 3.** Graphical illustration of DNA binding kinetics of berberine chloride.



**Figure 4.** Graphical illustration of protein binding kinetics of berberine chloride.

## 4. Discussion

The antibacterial activity of berberine as observed in the present study can be attributed to its strong binding to the nucleic acid. This is in corroboration with the previous findings of [6]. Berberine extracts and decoctions have demonstrated significant antimicrobial activity against a variety of organisms including bacteria, viruses, fungi, protozoans, helminths, and chlamydia [7–8]. Previously, antimicrobial property of berberine was reported in several experimental models [10–11]. Berberine containing plants – *Berberis vulgaris* and *Hydrastis canadensis* are used in homeopathic medicine for treatment of nephrolithiasis, back pain, urogenital infection and gastrointestinal problems. In traditional Indian Ayurveda, Chinese and Tibetan

medicine, berberine extract and decoction was known for its efficacy against recurrent and obstinate infection and fever<sup>[8, 12]</sup>. Current studies showed promising results using berberine in type II diabetes<sup>[13]</sup>, hypercholesterolemia, atherosclerosis<sup>[14]</sup>, congestive heart failure<sup>[8, 12, 15]</sup>, benign and malignant carcinoma<sup>[16]</sup> by clinical and experimental studies. Moreover, as a traditional medicine or dietary supplement, berberine has shown some activity against Alzheimer's disease<sup>[17]</sup>, methicillin resistant *Staphylococcus aureus*<sup>[11]</sup>, fungal infections, *Candida albicans*, yeast, parasites, and other bacterial/viral infections<sup>[9, 11]</sup>. The present investigation also indicated to its strong interaction to nucleic acid. Earlier also, potential nucleic acid-binding property of berberine was reported<sup>[18]</sup>. Anticancer and antibacterial activity of berberine might be probably due to its intercalation with nucleic acid inhibiting the multiplication of cells as also evident in this study by its DNA and protein binding kinetics. Use of berberine or other alternative drugs against STEC infection is more important and convincing as therapeutic application of conventional antibiotics like gentamicin, cefotaxime and ampicillin was reported to increase Shiga toxin release from the bacteria thus complicating the condition of the patients<sup>[19,20]</sup>.

Therefore, it may be concluded that berberine may be used as an effective antimicrobial agent against drug resistant *E. coli*, although a large-scale clinical trial is required to determine its therapeutic efficacy in vivo. Further, trials can be conducted to assess the antimicrobial efficacy of berberine hydrochloride against other multi drug resistant enteropathogens causing severe economic losses in animal husbandry.

### Conflict of interest statement

We declare that we have no conflict of interest.

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