

RESEARCH ARTICLE

# *Catharanthus roseus* extract attenuates *E. coli* toxin-induced short circuit current in isolated jejunal epithelium of goat and buffalo

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## ABSTRACT

Enterotoxigenic *E. coli*-derived heat-stable enterotoxin not only alters intestinal barrier functions and produces secretory diarrhea but also causes production losses in livestock. This study aimed to elucidate the ameliorative effect of *Catharanthus roseus* extract on Cl<sup>-</sup> secretion in an in vitro model. Isolated jejunal epithelia from goats (N = 10) and buffaloes (N = 4) were divided into four groups: A) control; B) 1.5% *C. roseus* extract; C) 10% *E. coli* toxin, and D) 10% *E. coli* toxin and 1.5% *C. roseus* extract. The jejunal epithelia were mounted on Ussing chambers with an exposed surface area of 0.95 cm<sup>2</sup> and treated with *E. coli* toxin on the mucosal side to invoke Cl<sup>-</sup> channels. Transepithelial potential difference (Pd<sub>t</sub>), tissue conductance (Gt), and short circuit current (Isc) were determined under voltage-clamped conditions. *E. coli* toxin produced a significant increase in the Pd<sub>t</sub>, Gt, and Isc for both goat and buffalo jejunal epithelia, whereas *C. roseus* extract on the mucosal side alone or followed by the *E. coli* toxin decreased these parameters, which were comparable with the control. The change in Isc was more pronounced in goats than buffaloes ( $\Delta\text{Isc}_{\text{goat}} > \Delta\text{Isc}_{\text{buff}}$ ). In experiments with the goat jejunal epithelia, replacement of Cl<sup>-</sup> from the mucosal buffer decreased Isc, similar to a decrease observed for the *C. roseus* extract groups. In conclusion, the results of this study suggest that *C. roseus* extract has a potential to attenuate *E. coli*-induced Cl<sup>-</sup> secretion in isolated jejunal epithelia of goats and buffaloes.

**Keywords:** *Catharanthus roseus* extract; Electrophysiology; Secretory diarrhea; Small intestine; Ruminants

## INTRODUCTION

Diarrhea remains a leading global health problem in livestock, with a high morbidity rate (Yitagesu et al., 2021; Demil et al., 2021) and a considerable production loss (Muskens et al., 2012; Gale et al., 2022). Antimicrobials are commonly used not only to counter the deleterious effects of infectious diarrhea (Uyama et al., 2020) but also as growth promoters (Aarestrup, 2000). However, indiscriminate use of antimicrobials results in antimicrobial resistance (AMR); hence, the use of antimicrobials as a growth promoter in livestock has been banned in various countries to prevent the generation of the resistant strain. Since the restricted use of antimicrobials may jeopardize livestock yield, alternative approaches are needed for sustainability in the growth and performance of livestock.

Enterotoxigenic *E. coli* is one of the most common causes of secretory diarrhea in farm animals, characterized by increased fluid secretion. *E. coli* toxin increases the Cl<sup>-</sup> secretion via cystic fibrosis transmembrane conductance regulator (CFTR) primarily through the cyclic AMP pathway (Huang et al., 2015; Longo et al., 2016) or by increasing cGMP production and thus reduction in fluid uptake (Evans Jr and Evans, 1996). Cl<sup>-</sup> secretion in an isolated epithelium using the Ussing chamber set-up is known to increase the short circuit current (Argenzio and Whipp, 1983; Guandalini et al., 1982) that can be attenuated by various plant extracts (Gabriel et al., 1999; Hör et al., 1995).

Medicinal plants are loaded with bioactive phytochemicals with antimicrobial (Hemeg et al., 2020), antioxidant (Yıldız

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Received: 31 January 2023; Accepted: 14 September 2023

et al., 2021) and anti-inflammatory properties (Pradeep and Kuttan, 2004). Recently, there has been an increased focus on alternative health promoters to counter the harmful effects of AMR (Lodemann et al., 2008; Marquardt and Li, 2018). Studies have shown that many medicinal plants are employed in ethnoveterinary practices in pigs and cattle (Xiong and Long, 2020) that can improve gut motility, stimulate absorption, and reduce the secretion of electrolytes (Noubissi et al., 2019; Gunawardana and Jayasuriya, 2019; Shirinda et al., 2019). *Catharanthus roseus* (*C. roseus*), commonly known as bright eyes, belongs to the Apocynaceae family and is an important medicinal plant harboring multiple chemical compounds with antimicrobial, antioxidant, antidiabetic and antidiarrheal activities (Gajalakshmi et al., 2013). *C. roseus* extract has a potential to treat wounds and sheep-biting louse in rats (Parihar et al., 2022), while lumpy skin disease in cattle and buffalo (Malabadi et al.). It is not only considered useful for growth (Yasmin et al., 2022) but a common herbal remedy for diarrhea in South Asia.

Despite the antidiarrhoeal effects of *C. roseus* on the livestock, it is not yet clear whether *C. roseus* can impart any changes in the jejunal absorptive or secretory capacity. This research is aimed to evaluate the effect of *C. roseus* extract on the electrophysiological parameters of isolated jejunum of goat and buffalo pretreated with *E. coli* toxin.

## MATERIAL AND METHODS

### Experimental animals and study design

Jejunal tissue from hey-fed goats (N=10) of age 12±2 months, BW 25±2 Kg, and buffalo (N=4) of age between 24±2 months, BW 450±5 kg, were divided into four groups, namely: Group A (Control); Group B (1.5% *C. roseus* extract); Group C (10% *E. coli* toxin), and Group D (10% *E. coli* toxin and 1.5% *C. roseus* extract). For each set of experiments, sufficient epithelia from each animal were available. All the procedures related to the housing and slaughtering of the animals were approved by the UVAS Ethical committee No. DR/75.

### Preparation of tissue

Following slaughter, deskinning, and evisceration, the middle part of jejunum (~ 1.0 m length) was collected and rinsed with normal saline (38°C). The muscular layer was stripped off, and the luminal contents were removed. The epithelial tissues were then transported in a pre-warmed (38°C) buffer solution (Buffer 1, see composition below) pre-gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub> to the electrophysiology lab, Department of Physiology, UVAS, Lahore, Pakistan (Geiger et al., 2020).

### Plant collection and extracts preparation

*C. roseus* (L.) G. Don leaves were collected from the University of Veterinary and Animal Sciences, Lahore, and were authenticated (Voucher number: GC. Herb. Bot. 3478). The extract was prepared as described previously (Hassan et al., 2011). Briefly, the leaves were cleaned, shade dried, and ground with a blender (Miller III, Ms 223- China) to obtain 50g of dried powder. Ethanolic extract (~4g) from this powder was obtained using Soxhlet apparatus (Quickfit Pyrex, Staffordshire, UK) and rotary evaporator (Bibby Sterlin Ltd Model RE100, Staffordshire, UK). The extract was kept at 4°C for later use.

### *E. coli* toxin preparation

Enteropathogenic *E. coli* strain O127 was kindly provided by the Institute of Microbiology, UVAS, Lahore, which was cultured in L broth and incubated at 37°C for 20 hours with vigorous shaking. The cells were centrifuged at 17000 x g for 15 min, disrupted by sonication (Uesaka et al., 1994), and were centrifuged again for 30 min at 5000 rpm to collect the supernatant, which was then stored in Eppendorf tubes at -40° C until used.

### Ussing chamber experiments

The jejunal epithelia were cut into pieces of 4×4 cm and mounted between the two halves of the Ussing Chamber to give an exposed surface area of 0.95 cm<sup>2</sup> (Rabbani et al., 2011). Mounted tissues were continuously bathed on each side with 16ml buffer solution (Buffer 1) consisting of: NaCl 115 mM, NaHCO<sub>3</sub> 25 mM, NaH<sub>2</sub>PO<sub>4</sub> 0.4 mM, Na<sub>2</sub>HPO<sub>4</sub> 2.4 mM, KCl 5 mM, Glucose 5 mM, CaCl<sub>2</sub> 1.2 mM, and MgCl<sub>2</sub> 1.2 mM. The buffer solution was continuously gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, while the pH of the buffer was maintained at 7.4 (38°C), and the osmolarity was set at 280 mosmol/kg. For the experiments with Cl-free buffer (Buffer 2), an equimolar of Na-gluconate was added to maintain the osmolarity. At the beginning of the experiment, the epithelia were incubated under open-circuit conditions (20 minutes) for equilibration and were then voltage clamped by fixing the voltage at 0mV (short circuit). Electrical measurements, trans-epithelial tissue conductance (Gt), potential difference (Pd), and short circuit current (Isc) were continuously monitored with an automatic computer-controlled voltage-clamp device (Mussler, Aachen, Germany). The toxin (10%) and the extract (1.5%) were added on the mucosal side as per the groups to observe changes in the electrophysiological parameters. The experiments lasted for approximately 1.5 hours.

### Statistical analysis

Statistical analyses were performed with SPSS (Version 20, Chicago, USA). The Kolmogorov-Smirnov test assessed

the normal distribution of data. Data were presented as mean  $\pm$  SE and analyzed by one-way analysis of variance (ANOVA) followed by Tukey's tests. An independent t-test was used to compare two treatment groups where required. The level of significance was kept at  $p < 0.05$ . Graphical representation of data was carried out using Sigma plot (Version 12, London, UK).

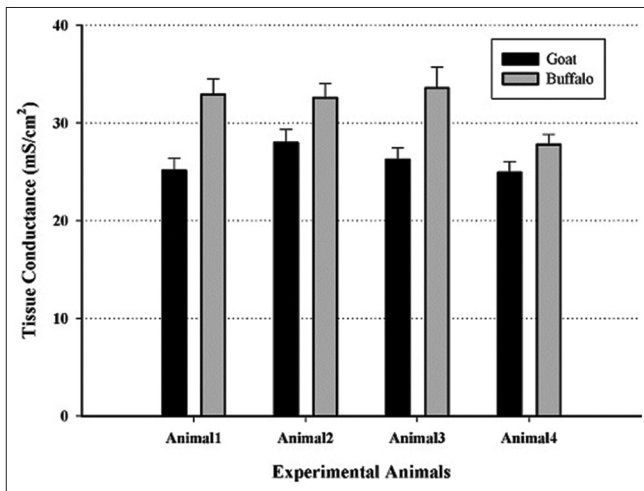
## RESULTS

### Epithelial viability and standardization

To ascertain the epithelial viability, the tissue conductance (Gt) from both goats and buffaloes was determined before the addition of toxin or extract after mounting the epithelia on Ussing Chambers. The average Gt of goats and buffaloes were  $26.07 \pm 1.24$  mS/cm<sup>2</sup> and  $31.73 \pm 1.55$  mS/cm<sup>2</sup> respectively, which was not significantly different among the experimental animals from the same species ( $p > 0.05$ ).

### Effect of treatment on electrophysiology

During the course of the experiments (50 to 60 min), the changes in I<sub>sc</sub> following the treatment reflected a trend that was more pronounced in goats compared to the buffaloes (Fig. 2).



AQ3 Fig 1. ???

The addition of toxin to the mucosal side resulted in a significant rise in the short circuit current (I<sub>sc</sub>) from  $12.71 \pm 0.08$  to  $21.18 \pm 0.15$   $\mu$ A/cm<sup>2</sup> in goats and from  $6.50 \pm 0.08$  to  $12.33 \pm 0.19$   $\mu$ A/cm<sup>2</sup> in buffalo ( $p < 0.05$ ). Adding plant extract dropped the I<sub>sc</sub> in buffalo compared to the control; however, this decrease was insignificant for goats. The addition of *E. coli* toxin, followed by adding plant extract on the mucosal side of the same tissue, resulted in a drop in I<sub>sc</sub> that was not significantly different from the control group (Tables 1 and 2).

The change in I<sub>sc</sub> ( $\Delta$ I<sub>sc</sub>) for group C vs. group D was significantly different ( $p < 0.05$ ) for both goats and buffaloes, respectively (Independent T-Test).

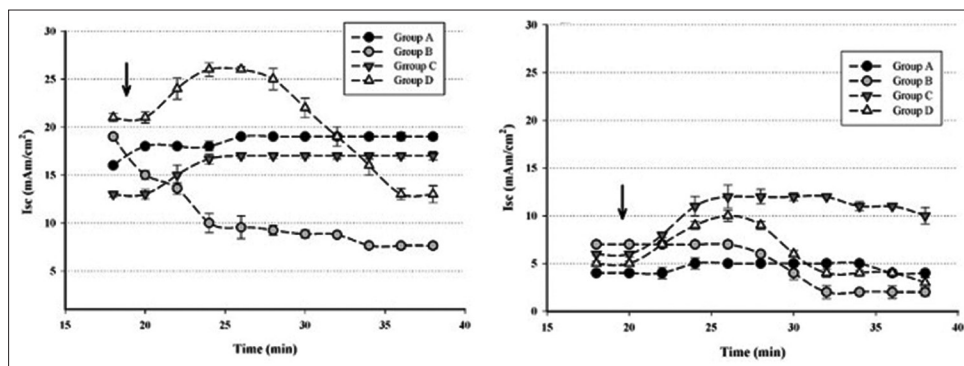
### Effect of Cl<sup>-</sup> replacement on the short circuit current

Jejunal epithelia from goats were initially incubated with Buffer 1 and equilibrated under short circuit conditions for 12-15 minutes. The I<sub>sc</sub> during this period averaged  $16.46 \pm 0.26$   $\mu$ Am/cm<sup>2</sup>. Replacement of Cl<sup>-</sup> with Cl<sup>-</sup>-free buffer (Buffer 2) on the mucosal side resulted in a sharp decline in I<sub>sc</sub> within 5 minutes to an average of  $10.86 \pm 0.17$   $\mu$ Am/cm<sup>2</sup>. This decline continued for 10 minutes; however, it became less steep and dropped to  $6.32 \pm 0.21$   $\mu$ Am/cm<sup>2</sup> (Fig. 4, filled circles).

## DISCUSSION

Gastrointestinal disorders result in significant economic losses in the livestock industry worldwide. Antimicrobials are discouraged due to antimicrobial resistance and withdrawal time in food animals (Aarestrup, 2000; Muskens et al., 2012), and alternates of antimicrobials are extensively explored (El-Seedi et al., 2013; Gunawardana and Jayasuriya, 2019). The present study demonstrates an antidiarrheal potential of a common medicinal plant of South Asia, *C. roseus*, in an in vitro model.

As the Ussing chamber is considered a gold standard for determining the epithelial barrier functions (Clarke, 2009), and since our experiments involved *E. coli* toxin,



AQ3 Fig 2. ???

**Table 1: Effect of treatment on electrophysiological parameters across isolated jejunal epithelium of goat**

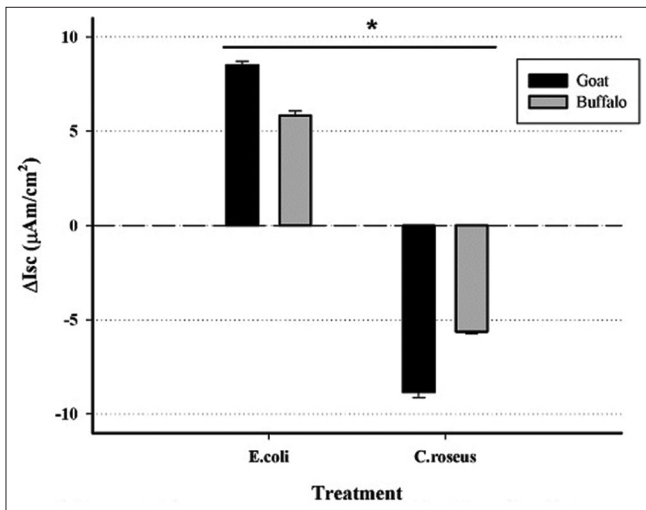
Groups	Gt (mS/cm <sup>2</sup> )	Pdt (mV)	Isc (μA/cm <sup>2</sup> )	N/n
A	30.67±0.18 <sup>a</sup>	0.10±0.01 <sup>a</sup>	12.71±0.08 <sup>a</sup>	10/16
B	32.58±0.26 <sup>b</sup>	0.24±0.02 <sup>b</sup>	8.96±0.32 <sup>b</sup>	10/16
C	28.06±0.34 <sup>c</sup>	0.20±0.01 <sup>c</sup>	21.18±0.15 <sup>c</sup>	10/16
D	29.42±0.39 <sup>d</sup>	0.27±0.01 <sup>b</sup>	12.36±0.17 <sup>a</sup>	10/16

Data are presented as mean ± SE; N = number of animals; n = number of epithelia for each treatment. Values in the same column bearing different superscripts are considered significantly different at p < 0.05 (One way ANOVA)

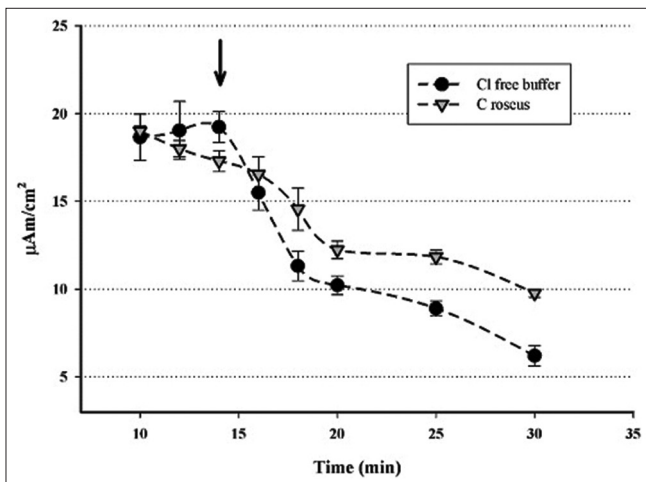
**Table 2: Effect of treatment on electrophysiological parameters across isolated jejunal epithelium of buffalo**

Groups	Gt (mS/cm <sup>2</sup> )	Pdt (mV)	Isc (μA/cm <sup>2</sup> )	N/n
A	23.37±0.37 <sup>a</sup>	0.12±0.01 <sup>a</sup>	6.50±0.08 <sup>a</sup>	4/8
B	28.51±0.41 <sup>b</sup>	0.18±0.01 <sup>a</sup>	6.80±0.24 <sup>a</sup>	4/8
C	23.38±0.59 <sup>b</sup>	1.41±0.03 <sup>b</sup>	12.33±0.19 <sup>b</sup>	4/8
D	32.68±0.30 <sup>c</sup>	0.36±0.01 <sup>c</sup>	6.71±0.15 <sup>a</sup>	4/8

Data are presented as mean ± SE; N = number of animals; n = number of epithelia for each treatment. Values in the same column bearing different superscripts are considered significantly different at p < 0.05 (One way ANOVA)



AQ3 Fig 3. ???



AQ3 Fig 4. ???

it was imperative to assess the tissue integrity before any intervention among animals within the same species ( $Gt_{\text{buffalo and goat}}; p > 0.05$ ). However, we did observe a significant rise in tissue conductance ( $p < 0.05$ ) after adding the *E. coli* toxin to the mucosal side. This outcome was in agreement with Field et al. (1972), who observed that when rabbit ileum was treated with *Vibrio cholera* toxin, the intestinal barrier function was compromised, and ion transport was altered. A similar trend was observed by Awad et al. (2015) for campylobacter infection of chicken gut. *E. coli* toxin in the rat proximal or distal colon also increased Isc, Pdt, and Gt, as reported by Charney et al. (2001). These are in line with the finding of our study for both buffalo and goats, whereby the tissue conductance, although differing significantly following toxin on the mucosal side, remained within the acceptable range of electrophysiology (not more than 25%). A possible reason for this increase could be because *E.coli* toxin may affect the intestinal mucosal permeability through disruption of tight junctions or zona occludens which was also reported by Fasano et al. (1991). For reasons unknown, *C. roseus* addition to the mucosal side also increased the tissue conductance ( $p < 0.05$ ), but this increment was lower than the toxin group.

Treatment of tissues with *C roseus* brought the Isc closer to the baseline suggesting an ameliorative effect on Cl secretion. This outcome can be linked to a reduction in secretory diarrhea where the active ingredients of *C roseus* extract; tannins, alkaloids, flavonoids, make intestinal mucosa more resistant and reduce secretion and peristaltic movements (Kyakulaga et al., 2011). This was also confirmed by (Paarakh et al., 2019) in a review where *C roseus* showed a dose dependent inhibition of castor oil induced diarrhea in rats. Similarly Patil et al. (2021) has described that *C roseus* extract has a potential to increase electrolyte absorption from the intestine.

## CONCLUSIONS

The present study reports an ameliorative potential of *C. roseus* on Cl secretion in an *in vitro* model with the isolated jejunal epithelia of goats and buffaloes evident from a concomitant decrease of short circuit current which was comparable with the Cl replacement. Our baseline data envisages an expanded study on the dose-dependent effect of *C. roseus* in various *in vivo* and *in vitro* models with a detailed investigation of the molecular levels.

## ACKNOWLEDGMENTS

This study was performed on the equipment provided by the German Academic Exchange Service (DAAD) for ‘German-Pakistani Research Collaboration.’

## Authors' contributions

Imtiaz Rabbani, Habib ur Rehman, Khalid Majeed, and Shahbaz Yousaf designed and supervised the experiments. Roomana Shams and Qurrat ul Ain performed the experiments and prepared the original draft. Aftab Anjum assisted in the plant extraction and *E. coli* toxin preparation. Zia ur Rehman and Imtiaz Rabbani reviewed and edited this manuscript.

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