



Antibacterial Activity of Traditionally Used Plants against the Resistant Enteropathogenic *Escherichia coli*

Nitika Sharma, Anil Kumar Mishra, Ashok Kumar, Ashish Srivastava¹,
Kumarsen Gururaj, Desh Deepak Singh², Tarun Pal Singh, R.V.S. Pawaiya

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ABSTRACT

Background: It is well known that the herbs have therapeutic efficacy against infectious agents including bacterial ones. In the current study, the antibacterial activities of methanolic extracts of nine herbs were evaluated against the resistant enteropathogenic *E. coli* (EPEC) using disc diffusion method.

Methods: Isolation and identification of *E. coli* and EPEC was done by standard bacteriological methods and PCR assays. Sensitivity of the EPEC isolates to antibiotics was determined by disc diffusion method. The methanolic extracts of the herbs were prepared and growth inhibition test was done by disc diffusion method. Minimum inhibitory concentration and minimum bactericidal concentration were determined by agar microdilution method.

Result: Out of 150 faecal samples, a total of 135 *E. coli* isolates were isolated and among them, a total of 36 isolates were identified as EPEC. EPEC isolates showed resistance to multiple antibiotics. The maximum mean inhibition zone of antibacterial effect against the resistant clinical isolates of EPEC was demonstrated by the methanolic extract of *Holarrhaena antidysenterica* (19.16±0.76 mm) followed by *Acacia nilotica* (18.00±1.00 mm) and *Punica granatum* (16.67±1.53 mm). This study validates that the said plants can be used as therapy against colibacillosis in neonatal goat kids.

Key words: *Acacia nilotica*, Colibacillosis, *E. coli*, Goat-kids, *Holarrhaena antidysenterica*, Neonatal diarrhoea, *Punica granatum*.

INTRODUCTION

Colibacillosis, commonly referred as *E. coli* scours, is the principal cause of neonatal diarrhoea in kids leading to heavy mortality (Smith and Sherman, 2009). Colibacillosis is caused by various pathotypes of *E. coli* and the most commonly associated pathotype is EPEC (Mishra *et al.*, 2019).

For treatment of colibacillosis, various antimicrobial agents are routinely used (Constable, 2004). *E. coli* is rapidly gaining resistance against routinely used antibiotics due to their indiscriminate use (Voravuthikunchai *et al.*, 2004). The latest generation of antibiotics are becoming out of reach for the poor goat keepers due to their high cost. Further, prolonged use of oral antibiotic therapy is potentially harmful to the intestinal mucosa of the neonatal goat kids resulting in malabsorption and diarrhoea (Farnsworth and Bunyapraphatsara, 1992). Associated side effects, development of antibiotic resistance and non-availability in rural and hilly regions are the other deterrence to use of antibiotics (Ma *et al.*, 2021).

Plants have been used since time immemorial as growth promoters to treat a variety of diseases, ailments and disorders (Chakrabarti *et al.*, 2021; Maqbool *et al.*, 2021). Traditionally, rural and pastoral livestock owners of India have been using various plants to treat diarrhoea in ruminant neonates as non-specific antidiarrheal agents (Srivastava and Mondal, 2016; Laloo and Hemlatha, 2011; Srinivasan *et al.*, 2001). The present study was designed to evaluate the antibacterial activity of nine plants namely *Holarrhaena antidysenterica*, *Acacia nilotica*, *Punica granatum*, *Gloriosa*

Division of Animal Health, ICAR-Central Institute for Research on Goats, Makhdoom, Farah-281 122, Mathura, Uttar Pradesh, India.

¹Department of Veterinary Clinical Medicine, Ethics and Jurisprudence, U.P. Pandit Deen Dayal Upadhyaya Veterinary University, Mathura-281 001, Uttar Pradesh, India.

²Department of Veterinary Pathology, College of Veterinary Sciences, Acharya Narendra Deva University of Agriculture and Technology, Ayodhya-224 229, Uttar Pradesh, India.

Corresponding Author: Anil Kumar Mishra, Division of Animal Health, ICAR-Central Institute for Research on Goats, Makhdoom, Farah-281 122, Mathura, Uttar Pradesh, India.
Email: anilmishradr@gmail.com

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suberba, *Aegle marmelos*, *Cassia tora*, *Bambusa arundinacea*, *Psidium guajava* and *Murraya koenigii*, which are commonly used by local livestock owners to treat diarrhoea in animals including goat-kids.

MATERIALS AND METHODS

The research work was conducted at ICAR-Central Institute for Research on Goats (ICAR-CIRG), Makhdoom, Farah, Mathura, UP, India during 2018-2020. Faecal samples were collected from 150 neonatal goat kids suffering from

colibacillosis. Isolation and identification of *E. coli* was done as per the standard bacteriological method (Mishra *et al.*, 2020; Mishra *et al.*, 2017). The identification of EPEC was done by pathotype specific PCR targeting its bundle forming pilus A (bfpA) gene as used by Lopez *et al.* (2003). Sensitivity of all isolates of EPEC to the antibiotics was determined by disc diffusion method of Kirby *et al.* (1968). The details of the antibiotics used in the study are given in Table 1.

The existing scientific literature on the herbs, indigenous traditional knowledge (ITK), availability of the herbs in the particular area and traditional beliefs of the rural livestock farmers are taken into consideration for the selection and identification of the medicinal herbs (Srivastava and Mondal, 2016). On this basis, nine herbs namely bark of *Holarrhena antidysenterica*, bark of *Acacia nilotica*, rind of *Punica granatum*, leaves of *Gloriosa suberba*, semi ripe fruit of *Aegle marmelos*, leaves of *Cassia tora*, leaves of *Bambusa arundinacea*, leaves of *Murraya koenigii* and leaves of *Psidium guajava* were selected for the study. The plants were authenticated on taxonomical basis by the Faculty of Department of Botany, Babu Shivnath Aggarwal (BSA) College, Mathura, Uttar-Pradesh, India. The methanolic extracts of the plants were prepared as per the method recommended by Paech and Tracy (1956).

A standard disc diffusion method described by Bauer *et al.*, (1966) was used for assessing the antibacterial efficacy of the selected plant materials against the resistant EPEC. Methanolic extracts of the herbal plants were screened for antibacterial property and compared with ciprofloxacin (HiMedia® discs, 6 mm) as a standard antibiotic against the resistant EPEC as described by Prasanth *et al.*, (2001). Sterile filter paper discs of 6 mm diameter (Hi Media®) were soaked with 20 µl of extract diluted in 1 % Tween-20 solution in three different concentrations (100, 200 and 400 mg/ml). Three discs were impregnated with 2 mg, 4 mg and 8 mg of the each plant extract, respectively and their antibacterial activity was evaluated by measuring the diameter of inhibition zone as per the method described by Srivastava and Mondal (2016).

Minimum inhibitory concentration (MIC) of the methanolic herbal extract revealing the highest antibacterial activity during *in vitro* screening was measured using broth dilution method (Doughari, 2006). Further, the minimum bactericidal concentration (MBC) of the methanolic herbal extract was assessed as per the method described by Doughari (2006).

Statistical analysis of the data obtained was done by one way analysis of variance (ANOVA) at 95% level of significance and results are displayed as Mean±S.E (Snedecor and Cochran, 1994).

RESULTS AND DISCUSSION

Isolation, characterisation and confirmatory identification of enteropathogenic *E. coli*

A total of 135 isolates of *E. coli* were recovered from 150 faecal samples from diarrhoeic neonatal goat-kids (Fig 1).

Pathotype specific PCR targeting bfpA resulted in to a single amplicon of 324 bp, which indicates confirmatory identification of EPEC as illustrated in Fig 2. In the current study, EPEC was selected as target pathogen, because it is the most common cause of colibacillosis in neonatal goat-kids as reported by Mishra *et al.* (2019). The pattern of antibiotic resistance shown by EPEC isolates indicated their multi-antibiotic resistant status (Table 1).

Antibacterial efficacy of the selected plants against the resistant EPEC

Among the 9 methanolic extracts evaluated for their antibacterial activity, only extracts of *Holarrhena*

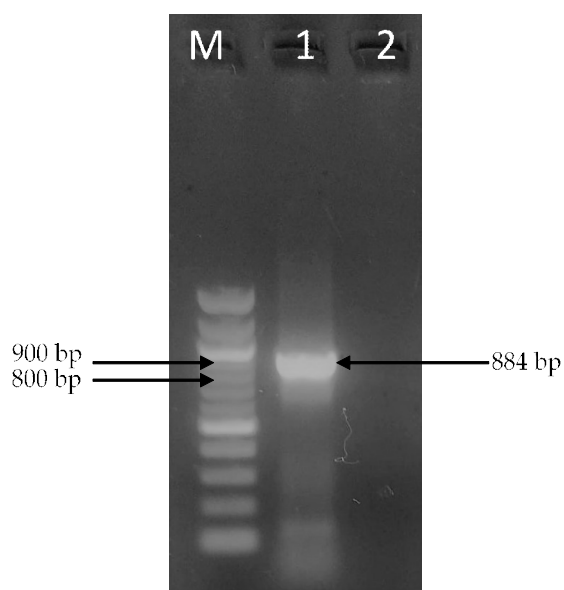


Fig 1: Detection of *E. coli* by PCR.

Lane M: Molecular weight marker

Lane 1: Amplified PCR products

Lane 2: Negative sample

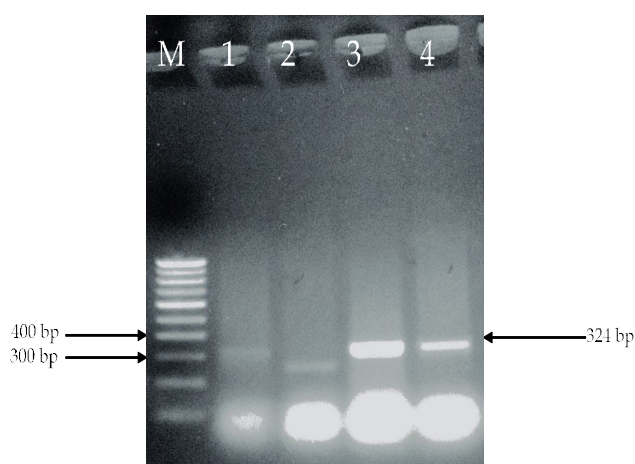


Fig 2: Detection of EPEC by PCR.

Lane M: Molecular weight marker.

Lane 1, 3, 4: Amplified PCR products.

Lane 2: Negative sample.

antidysenterica (bark), *Acacia nilotica* (bark), *Punica granatum* (rind), *Gloriosa superba* (leaves) and *Aegle marmelos* (fruit) showed antibacterial activity against antibiotic resistant clinical isolates of EPEC (Table 2). The leaves of other 4 medicinal plants viz. *Cassia tora*, *Bambusa arundinacea*, *Murraya koenigii* and *Psidium guajava* did not exhibit any antibacterial potential against the resistant EPEC isolates (Table 2). The maximum mean inhibition zone of antibacterial effect was demonstrated by the methanolic extract of *Holarrhena antidysenterica* (19.16 ± 0.76 mm) followed by *Acacia nilotica* (18.00 ± 1.00 mm) and *Punica granatum* (16.67 ± 1.53 mm) (Table 2). Standard antibiotic, i.e., ciprofloxacin produced consistent inhibition zone of 32 mm and served as positive control, whereas discs impregnated with aqueous tween-20 solution (1%) didn't exhibit any antibacterial activity in the form of inhibition zone and served as negative control.

The antibacterial property of plant extracts varies with type of solvents used (Rani and Khullar, 2004). Methanolic or acetone extracts of plants possessing antimicrobial potential exhibited much more pronounced activity in comparison to ethanol or aqueous extract against a variety of pathogenic microbes like *E. coli* (Eloff, 1998). Taking these studies into the consideration, the methanolic extracts of the herbs were used for evaluation of their antimicrobial properties.

During *in vitro* screening of plant extracts for antibacterial property, *Holarrhena antidysenterica* exhibited maximum zone of inhibition and therefore, it was considered

for further evaluation in terms of MIC and MBC. After 24 hr of incubation, turbidity appeared in tubes containing the extract @ 0.5 and 1.0 mg/ml, whereas no growth was observed in all the other tubes (containing *Holarrhena antidysenterica* extract @ 2,3,4,5,6,7,8,9 and 10 mg/ml) on visual inspection. Lush growth was observed in the tube not containing any amount of the extract. On the basis of the above finding, the MIC of *Holarrhena antidysenterica* extract was considered to be 2 mg/ml. During MBC trial, good growth was observed on nutrient agar plates streaked with the inoculum taken from MIC tubes having *Holarrhena antidysenterica* extract @ 0.5 and 1.0 mg/ml, whereas significant reduction in bacterial growth was seen at 2 mg/ml (MIC value). No bacterial growth occurred at concentrations of 3-10 mg/ml. Hence, MBC of *Holarrhena antidysenterica* extract was considered to be 3 mg/ml.

The antibacterial activity of plant extracts can be attributed to the presence of broad-spectrum antimicrobial compounds in them (Marjorie, 1999). Methanolic extracts of *Holarrhena antidysenterica*, *Acacia nilotica*, *Punica granatum* and *Gloriosa superba* possess high amount of tannins and phenolic compounds (Senthilkumar, 2013; Kavitha and Niranjali, 2009; Misar *et al.*, 2007; Prasanth *et al.*, 2001). Tannins possess antimicrobial potential owing to their astringent activity and capability to inactivate the microbial adhesins, enzymes, cell envelope transport proteins etc. (Cowan, 1999). Like synthetic phenols compounds, tannins adversely affect the integrity of bacterial cell membranes. Tannins chelate the metal ions such as

Table 1: Antibiotic Susceptibility of the *E. coli* isolates (n=36) obtained from the diarrhoeic neonatal goat-kids.

Antimicrobial agent	Concentration (μ g)	Extent of the susceptibility		
		Susceptible (%)	Intermediate (%)	Resistant (%)
Amikacin	10	94.44 (34/36)	2.77 (1/36)	2.77 (1/36)
Ampicillin	10	16.6 (6/36)	13.88 (5/36)	69.44 (25/36)
Amoxicillin	10	30.55 (11/36)	2.77 (1/36)	66.66 (24/36)
Amoxy-Clavulinic acid	10	8.33 (3/36)	19.44 (7/36)	63.88 (23/36)
Colistin	50	83.33 (30/36)	16.66 (6/36)	0 (0/36)
Ceftriaxone	10	55.55 (20/36)	13.88 (5/36)	30.55 (11/36)
Cephadroxil	30	61.66 (22/36)	13.88 (5/36)	25 (9/36)
Cephalexin	30	66.66 (24/36)	8.33 (3/36)	25 (9/36)
Chloramphenicol	10	50 (18/36)	30.55 (11/36)	19.44 (7/36)
Ciprofloxacin	10	41.66 (15/36)	30.55 (11/36)	27.77 (10/36)
Co-Trimoxazole	25	5.55 (2/36)	5.55 (2/36)	88.88 (32/36)
Enrofloxacin	10	36.11 (13/36)	11.11 (4/36)	52.55 (19/36)
Erythromycin	10	27.77 (10/36)	33.33 (12/36)	38.88 (14/36)
Furazolidone	100	100 (36/36)	0 (0/36)	0 (0/36)
Gentamicin	10	52.77 (19/36)	19.44 (7/36)	27.77 (10/36)
Norfloxacin	10	52.77 (19/36)	11.11 (4/36)	36.11 (13/36)
Novobiocin	5	50 (18/36)	13.88 (5/36)	36.11 (13/36)
Oxytetracycline	30	38.88 (14/36)	5.55 (2/36)	55.55 (20/36)
Streptomycin	10	16.66 (6/36)	16.66 (6/36)	66.66 (24/36)
Sulfadiazine	100	2.77 (1/36)	5.55 (2/36)	91.66 (33/36)
Tobramycin	10	47.22 (17/36)	16.66 (6/36)	36.11 (13/36)

Table 2: *In vitro* antibacterial activity of the plant extracts against enteropathogenic *E. coli*.

Treatment (n=3)	<i>Holarrhena antidysenterica</i>	<i>Acacia nilotica</i>	<i>Punica granatum</i>	<i>Gloriosa superba</i>	<i>Aegle marmelos</i>	<i>Cassia tora</i>	<i>Bambusa arundinacea</i>	<i>Murraya koenigii</i>	<i>Psidium guajava</i>
2 mg/disc	15.53±0.55 ^{aDE*}	13.50±0.50 ^{aC}	14.13±1.03 ^{aC}	11.33±1.53 ^{aB}	9.00±1.00 ^{aB}	-	-	-	-
4 mg/disc	16.93±1.01 ^{abD}	16.17±1.04 ^{bd}	15.50±1.32 ^{aCD}	13.97±1.62 ^{aC}	11.00±1.00 ^{bb}	-	-	-	-
8 mg/disc	19.16±0.76 ^{be}	18.00±1.00 ^{ce}	16.67±1.53 ^{ad}	14.00±2.00 ^{aCD}	11.37±0.78 ^{bb}	-	-	-	-

*Mean with different superscripts in a row (capital letters) and column (small letters) differs significantly (p<0.05).

iron and form a complex, which results in antimicrobial activity through iron depletion (Scalbert, 1991). Alkaloids present in the methanolic extract of *Holarrhena antidysenterica* interfere and reduce the initial bacterial adhesion to intact epithelial cells and it may exert an anti-adherence effect against the pathogenesis of enteropathogenic *E. coli* in the host epithelial cells (Kavitha and Niranjali, 2009).

In the present study, the methanolic extract of semi-ripe fruit of *Aegle marmelos* showed antibacterial activity against the multi-drug resistant EPEC. Similar results have also been reported regarding the antibacterial potential of *Aegle marmelos* against a variety of enteropathogenic bacteria in previous studies (Mazumder *et al.*, 2006). In our study, the methanolic extract of the leaves of *Psidium guajava* did not exhibit any antibacterial potential against multi-drug resistant EPEC isolates. However, in the previous reports, the antimicrobial potential of *Psidium guajava* against enteropathogenic *E. coli* isolates from calves was confirmed (Srivastava and Mondal, 2016). This variation could be due to differences of genotypes amongst EPEC isolates and herbal plants.

It has been reported earlier that ethanolic extract of *Holarrhena antidysenterica* bark had MIC value of 2 mg/ml and MBC value of 3 mg/ml against *E. coli* (Singh *et al.*, 2016). In the present study, methanolic extract of *Holarrhena antidysenterica* bark exhibited a MIC value of 2 mg/ml and a MBC value of 3 mg/ml against the multi-drug resistant EPEC. Similarities in the results of present study and previous studies may be due to several factors. The most important one is that the methodology as well as the pathogen chosen for the study is same *viz.* diarrhoeagenic *E. coli*.

Similar to our results, exploratory studies carried out by Misar *et al.*, (2007) in mice also revealed presence of significant antidiarrheal activity in the methanolic extract of *Acacia nilotica*. Its aqueous extract was found effective in treating biofilm forming and multidrug resistant uropathogenic *E. coli* by Elamary *et al.*, (2020). Several studies support the ethnomedicinal use of *Acacia nilotica* bark for the treatment of diarrhoea and other ailments (Misar *et al.*, 2007).

Though, various reports have linked the antimicrobial potential of plants to the presence of tannins in them (Girard and Bee, 2020) but in the present study, no antimicrobial activity was observed in the methanolic extracts of *Bambusa arundinacea* and *Psidium guajava* leaves, which contain significant amount of tannins. Hence, further detailed

phytochemical evaluation of plant extracts exhibiting significant antibacterial potential is warranted.

It is quite obvious from the existing scientific literature, antibacterial potential of different plants tested, could not be endorsed entirely to presence of any singular chemical constituent (Ozcelik *et al.*, 2006; Marjorie, 1999). Differences in the efficacy and specificity exist among the different types of phytochemicals present in a plant or its extract. These differences are attributed to the concentration of phytochemicals and chemical structure. Further investigations are needed to determine the optimal combinations of phytochemicals and the most suitable and cost-effective manner to deliver them (Girard and Bee, 2020). Certain disagreements between *in vitro* and *in vivo* results could be explained by the metabolism of phytochemicals along the intestinal tract. Whether and which phytochemicals and their metabolites remain active in the intestine warrant further research. There is a necessity to further explore different phytochemical components of the plant extract in terms of their mode of action and possible interactions responsible for their antibacterial potential.

CONCLUSION

This study validates the use of *Holarrhena antidysenterica*, *Acacia nilotica*, *Punica granatum*, *Gloriosa superba* and *Aegle marmelos* as sole or adjunct therapy against colibacillosis in neonatal goat-kids.

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Conflict of interest: None.

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