The Journal of Phytopharmacology 2021; 10(5):289-293
Online at: www.phytopharmajournal.com

Research Article

ISSN 2320-480X
JPHYTO 2021; 10(5): 289-293
September- October
Received: 04-07-2021
Accepted: 21-07-2021

doi: 10.31254/phyto.2021.10503

Ashok Singh Rathor
Department of Veterinary Clinical Medicine, Ethics and Jurisprudence, College of Veterinary and Animal Science, RAJUVAS, Bikaner-334001, Rajasthan, India

Taruna Bhati
Department of Veterinary Microbiology and Biotechnology, College of Veterinary and Animal Science, RAJUVAS, Bikaner-334001, Rajasthan, India

AP Singh
Department of Veterinary Clinical Medicine, Ethics and Jurisprudence, College of Veterinary and Animal Science, RAJUVAS, Bikaner-334001, Rajasthan, India

Correspondence:
Dr. Ashok Singh Rathor
Department of Veterinary Clinical Medicine, Ethics and Jurisprudence, College of Veterinary and Animal Science, RAJUVAS, Bikaner-334001, Rajasthan, India
Email: vetasr@yahoo.com

In vitro antibacterial activity of extracts of *Dalbergia sissoo* and *Aegle marmelos* against Enterotoxigenic *Escherichia coli* from calves

Ashok Singh Rathor, Taruna Bhati, AP Singh

ABSTRACT

Neonatal calf diarrhoea (NCD) is the most common cause of morbidity and mortality in pre-weaned dairy calves, causing huge economic and productivity losses to dairy industry worldwide. Enterotoxigenic *E. coli* (ETEC) is one of the important causes of neonatal diarrhoea and high mortality in very young calves. Shisham (*Dalbergia sissoo*) leaves and Bael (*Aegle marmelous*) unripe fruit have been used in diarrhoea and dysentery with very good results. Hence the present study was conducted to evaluate the *in vitro* antibacterial activity of the aqueous and methanolic extract of *Dalbergia sissoo* and *Aegle marmelos* against 16 *E. coli* isolated from colibacillosis affected diarrhoeic calves in Bikaner district of Rajasthan. The average zones of inhibitions of aqueous extract of *Aegle marmelos* and methanolic extract of *Dalbergia sissoo* against *E. coli* were 23.75±0.34 mm, 18.75±0.32 mm, 16.5±0.24 mm, 11.58 ±0.14 mm and 7.88±0.24 mm at concentrations of 1000 mg/ml, 500 mg/ml, 250 mg/ml 125 mg/ml and 62.5 mg/ml, respectively. The average zones of inhibition of aqueous extract of *Dalbergia sissoo* and methanolic extract of *Aegle marmelos* against *E. coli* were 24.75±0.20 mm, 19.87±0.34 mm, 17.25±0.24mm, 12.86±0.13 mm and 8.50±0.14 mm at concentrations of 1000 mg/ml, 500 mg/ml, 250 mg/ml 125 mg/ml and 62.5 mg/ml, respectively. The maximum zone of inhibition was reported by aqueous extract of *Dalbergia sissoo* and methanolic extract of *Aegle marmelos* (>24 mm of diameter) at concentration of 1000 mg/ml. Based on average zone of inhibition, the *in vitro* antibacterial activity of aqueous extract of *Dalbergia sissoo* and methanolic extract of *Aegle marmelos* was found to be more against *E. coli* as compared to aqueous extract of *Aegle marmelos* and methanolic extract of *Dalbergia sissoo* at varying concentrations. In conclusion, both combination of aqueous and methanolic extract of *Dalbergia sissoo* and *Aegle marmelos* showed *in vitro* antibacterial properties against *E. coli*.

Keywords: Neonatal calf diarrhoea, *Escherichia coli*, Colibacillosis, *Dalbergia sissoo*, *Aegle marmelos*, Antibacterial.

INTRODUCTION

Neonatal diseases and mortality among the cattle and buffalo calves are the major cause of economic losses in livestock production [1]. Calf diarrhoea or calf scours accounts for approximately 75% of the mortality of dairy calves under three weeks of age. Neonatal calf diarrhoea (NCD) is a multifactorial and multifaceted disease complex that can be triggered by both infectious and non-infectious causes that are related to the animal, environment and the management factors [2]. Because of the multifactorial nature of NCD, it is difficult to be controlled effectively [3]. Multiple enteric pathogens (*e.g.*, viruses, bacteria, and protozoa) are involved in the development of calf diarrhoea [4].

NCD due to *E. coli* is the main cause of mortality of new born calves in dairy herds all over the world and reports from India indicate that this disease is prevalent even on organized dairy farms throughout the country [5]. Enterotoxigenic *E. coli* (ETEC) infection is the most common type of colibacillosis and neonatal calves are most susceptible to ETEC infection during first 4 days after birth and develop watery diarrhoea if infected.

Antibiotic therapy is frequently used to treat different infectious diseases in animals, including NCD. Administration of antibiotics may result in mortality rate reduction in some cases but the indiscriminate use of antibiotics has been accompanied by an increase in bacterial resistance, generating important public health issues and economic losses in production industries in recent years [6].

Herbal medicine remains one of the most common forms of therapy widely available throughout the world population [7-9]. WHO has also emphasized the need to integrate traditional indigenous health care system with modern facilities [10]. Shisham (*Dalbergia sissoo*) leaves and Bael (*Aegle marmelous*) unripe fruit were reported to be used in diarrhoea and dysentery with very good results. Studies have

showed that *Dalbergia sissoo* also have anti-inflammatory, antipyretic, analgesic, anti-oxidant, anti-diabetic properties [11-13]. Aqueous and methanolic extracts of *Dalbergia sissoo* (Shisham) leaves against *E. coli* isolates showed good *in vitro* antibacterial activity [14]. The unripe fruit pulp of *Aegle marmelos* affected the bacterial colonization to gut epithelium, production and action of certain enterotoxins and inhibiting the intestinal motility and secretion [15]. The half ripened Bael fruit has astringent property that reduces irritation in the digestive tract and is excellent treatment for diarrhoea and dysentery [16].

Hence the present study was conducted to evaluate the *in vitro* antibacterial activity of the aqueous and methanolic extract of *Dalbergia sissoo* and *Aegle marmelos* against *E. coli* isolated from diarrhoeic calves in Bikaner district of Rajasthan.

MATERIAL AND METHODS

Ethical approval: This study was conducted as per approval of research committee and Institutional animal ethics committee guidelines.

Collection of Faecal Samples: A total of 25 faecal samples were collected on 0-day pre-treatment in sterilized test tubes for ruling out parasitic infestation if any and from rectum using transport swab for bacterial culture examination for isolation and identification of *E. coli* bacteria.

Isolation and Identification of bacteria: The faecal samples were inoculated on MacConkey agar and incubated for 24 h at 37ºC under aerobic conditions. Lactose fermenting colonies were further streaked on Eosin Methylene Blue Agar (EMB) for isolation of *E. coli.* The organisms were isolated and identified as described by [17] and [18]. The confirmation of *E. coli* isolates was done by using biochemical test kits (Hi Enterobacteriaceae identification kit KB003).

Molecular identification of *E. coli:* DNA was extracted using commercially available DNA Sure Blood Mini Kit NP-61107Genetix Biotech Asia Pvt. Ltd (New Delhi). Genotypic confirmation of *E. coli* was done by 16S rRNA ribotyping [19] using forward Primer 1 (5' GCT TGA CAC TGA ACA TTG AG 3') and reverse Primer 2 (5' GCA CTT ATC TCT TCC GCA TT 3'). The 25.0 µl reaction mixture consisted of 5.0 µl 5X Go Taq® Flexi buffer, 3.0 µl $MgCl_2$ (25mM),1.0 µl dNTP mix (25mM each), 1.0 µl Forward Primer (10 pM/µl), 1.0 µl Reverse Primer (10 pM/µl), 0.2 µl *Taq* DNA polymerase (5U/µl), 3.0 µl DNA template (30 ng/µl) and 10.8 µl nuclease free water to make 25.0 µl. Amplification was carried out in a Veriti thermal cycler (Applied biosystem) using parameters as mentioned in table 1. The PCR products were resolved on 1.2% agarose gels prepared in 1.0X TBE buffer containing 0.5 µg/ml of ethidium bromide and 100 bp DNA ladder was used as molecular marker. The amplification products were electrophoresed for 50-60 min at 100 Volts. The gels were then visualized under gel documentation system (ENDURO GDS).

Amplification of K99 gene: Highly conserved nucleotide sequence within the ETEC was targeted for amplification [20] using forward primer (5' TATTATCTTAGGTGGTATGG 3') and reverse primer (5' GGTATCCTTTAGCAGCAGTATTTC 3'). The reaction mixture for K99 gene (total volume 25.0 µl.) was prepared as mentioned for 16S rRNA gene based genotypic identification. The PCR was performed in Eppendorf Master Cycler Gradient using cycling parameters given in table 1. The PCR products were resolved on 1.2% agarose gels prepared in 1.0X TBE buffer containing 0.5 µg/ml of ethidium bromide and 1Kb DNA ladder was used as molecular marker.

Preparations of extracts of *Dalbergia sissoo* leaves and *Aegle marmelos* unripe fruit

Collection of plant materials: Dried powder of *Dalbergia sissoo* leaves and *Aegle marmelos* unripe fruit were purchased from the local market and identification was confirmed by botanical expert. The dried powder was stored in clean and air tight container till further use.

Soxhlet extraction: The extraction of *Dalbergia sissoo* leaves and *Aegle marmelos* unripe fruit was done by Soxhlet extraction (continuous hot extraction) method [21].

After the Soxhlet extraction, prepared crude extract was evaporated under reduced pressure by rotatory evaporator so that all the solvent was nearly evaporated. The concentrated extract thus obtained was left overnight at room temperature to evaporate any residual solvent. Finally, extract of a thick paste consistency was obtained which was stored in air-tight container at 4°C in refrigerator.

***In vitro* antibacterial activity of *Dalbergia sissoo* and *Aegle marmelos* extracts:** The antibacterial activity of aqueous and methanolic extracts was screened by agar cup method [22].

Test dilution of herbal extract: The different dilutions of herbal extracts were prepared and tested. For aqueous and methanolic extracts, dilution was prepared by dissolving prepared extract in triple glass distil water by serial dilution method to yield different concentration from 1000 mg/ml to 62.50 mg/ml.

Preparation and inoculation of agar plates: For testing *in vitro* antibacterial activity, standard agar cup method [22] using nutrient agar was used. Nutrient agar was prepared and autoclaved. About 25 ml of agar was poured in one petri-plate and allowed to solidify. Stock inoculums of test bacterium were swept over the agar plate using a sterile cotton swab. Plates were air dried for 5 minutes. Five equidistant wells of size 6 mm were cut into the agar. 100 µl of different concentration of extract was poured into different well. Plates were incubated at 37^0C for 24 h and zones of inhibition were measured. The final values were taken as mean ± S.E of the recorded observations.

RESULTS AND DISCUSSION

Prevalence of ETEC strains: In the present study, out of 25 faecal samples, 22 (88%) faecal samples were found positive for *Escherichia coli* as these produced pink colonies on MacConkey agar and characteristic green metallic sheen on EMB agar. These 22 isolates were further genotypically confirmed by 16S rRNA ribotyping and amplicons of 662 bp were obtained (Fig 1). Out of 22 *E. coli* isolates thus identified, 16 (72.7%) isolates were further confirmed as ETEC K99 strains by PCR amplification of K99 gene producing an amplicon of 314 bp (Fig 2). Diarrhoea in calves is commonly caused by ETEC [23]. A high prevalence of ETEC strains from cases of neonatal calf diarrhoea was reported in the present study. In contrast, a low prevalence with 16 samples (5.3%) positive for F5 (K99) fimbrial gene out of 268 *E. coli* isolated from 298 diarrheic neonatal calves at 1–30 days old has been also reported [24]. Similarly, 18.9% isolates with *K99* gene out of 37 *E. coli* from 82 diarrhoeic calves was

observed [25]. Mix infections were detected in diarrhoeic calves in studies from different parts of the world [3, 26].

In vitro antibacterial activity of extracts of *Dalbergia sissoo* and *Aegle marmelos*

The present study included evaluation of *in vitro* antibacterial activity of the aqueous and methanolic extract of *Dalbergia sissoo* and *Aegle marmelos* against *E. coli* isolated from colibacillosis affected diarrhoeic calves. Antibacterial activity was determined in terms of "zone of inhibition" diameter measured as the diameter of the clear zone around well in which there was no bacterial growth.

The *in vitro* antibacterial activity of combination of aqueous and methanolic extract of *Aegle marmelos* and *Dalbergia sissoo* in term of the zone of inhibition against *E. coli* is presented in Table 2. Based on average zone of inhibition, the *in vitro* antibacterial activity of aqueous extract of *Dalbergia sissoo* and methanolic extract of *Aegle marmelos* was found to be more against *E. coli* as compared to aqueous extract of *Aegle marmelos* and methanolic extract of *Dalbergia sissoo* at varying concentrations ranging from 62.5 mg/ml to 1000 mg/ml. The maximum zone of inhibition was reported by aqueous extract of *Dalbergia sissoo* and methanolic extract of *Aegle marmelos* (>24 mm of diameter) at concentration of 1000 mg/ml.

Similarly, many researchers conducted study on antimicrobial properties of *Dalbergia sissoo* against *E. coli* [27-35]. Chalcone [(*E*)-3-(3,4-dihydroxyphenyl)-1-(2,3,4-trihydroxyphenyl) prop-2-en-1-one] or okanin isolated from methanol extracts of *Dalbergia sissoo* exhibited good antibacterial activity towards *E. coli* [30]. While from the same study area, the *in vitro* antibacterial activity of methanolic extract of *Dalbergia sissoo* leaves was found higher as compared to respective concentrations of aqueous extract against *E. coli* isolates from diarrhoeic calves [14].

Table 1: PCR amplification parameters and conditions for 16S rRNA and *K99* gene amplification

Process	*E. coli (16SrRNA)*	*K99 gene*
Initial denaturation	95ºC for 2 min	95ºC for 2 min
Denaturation	94ºC for 45 sec	94ºC for 45 sec
Annealing	57ºC for 45 sec	53ºC for 60 sec
Elongation	72ºC for 45 sec	72ºC for 60 sec
Cycles	35	40
Final extension	72ºC for 7 min	72ºC for 7 min
Hold	4ºC	4ºC

Table 2: Mean ± SE values of zones of inhibition (mm) of aqueous and methanolic extract of *Aegle marmelos* and *Dalbergia sissoo* against *E. coli* at different concentrations

S. No.	Extract (Combination of each extract)	Zone of inhibition (Mean ± SE) (In mm)				
		1000 mg/ml	500 mg/ml	250 mg/ml	125 mg/ml	62.5 mg/ml
1.	Aqueous extract of *A. marmelos* and Methanolic extract of *D. sissoo*	23.75 ±0.34	18.75 ±0.32	16.5 ±0.24	11.58 ±0.14	7.88 ±0.24
2.	Aqueous extract of *D. sissoo* and Methanolic extract of *A. marmelos*	24.75 ±0.20	19.87 ±0.34	17.25 ±0.24	12.86 ±0.13	8.50 ±0.14

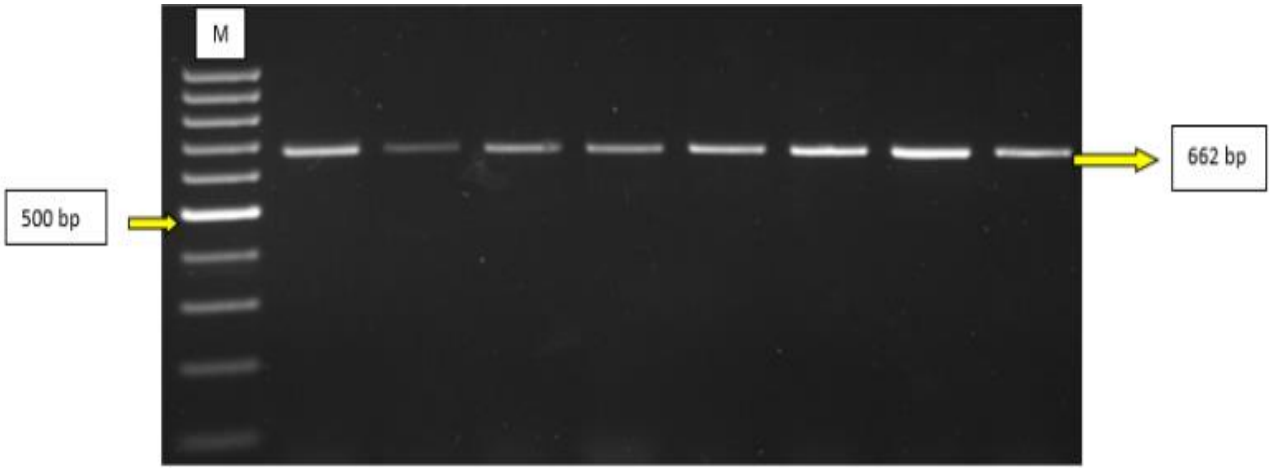

Figure 1: 16s rRNA ribotyping of *E. coli* isolates with species specific primer (M=Molecular marker 100bp)

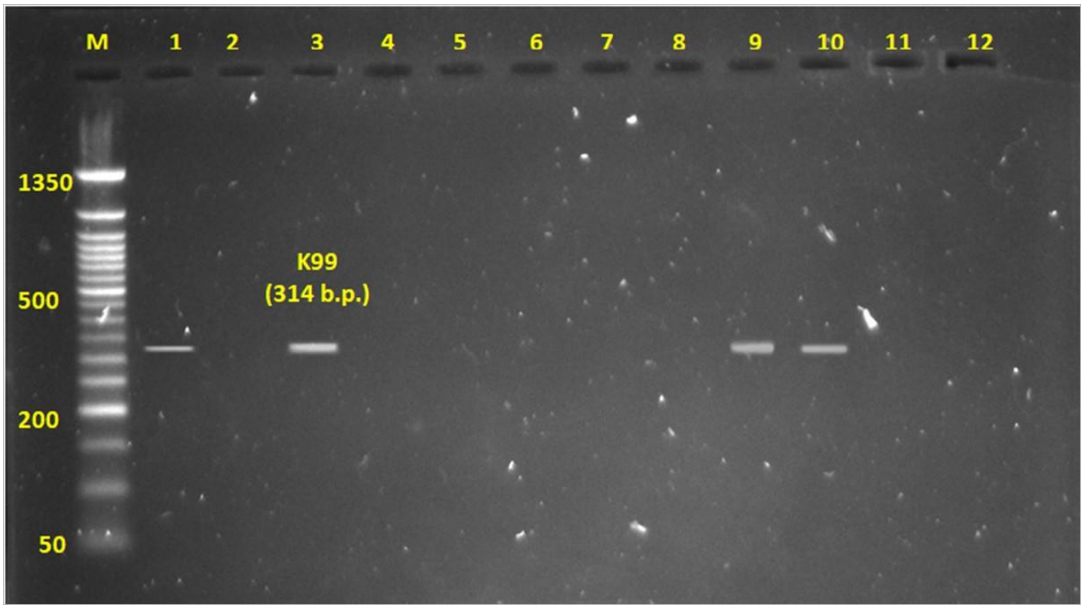

Figure 2: Agarose gel electrophoresis showing amplification of *K99* genes

CONCLUSION

Both combination of aqueous and methanolic extracts of *Dalbergia sissoo* and *Aegle marmelos* showed *in vitro* antibacterial properties against *E. coli*. Looking into the intricacy of neonatal calf diarrhoea and its economic impact on dairy industry, the role of herbal medicines may play a major remedy in traditional medical systems.

Acknowledgement

The authors are thankful to Dean, College of Veterinary and Animal Sciences Rajasthan University of Veterinary and Animal Sciences, Bikaner for providing financial and other necessary facilities to carry out this research work

REFERENCES

1. Singh DD, Kumar M, Choudhary PK, Singh HN. Neonatal calf mortality– an overview. Intas Polivet 2009; 10(2): 165-169.
2. Manzoor A, Nazir T, Untoo M, Fayaz A, Zaffer B, Afzal I, Akram T, Dar ZA. Calf scour: An obstacle in successful dairying: A review. Journal of Entomology and Zoology Studies; 2018; 6(5): 2001-2005.
3. Cho Y, Yoon KJ. An overview of calf diarrhea-infectious etiology, diagnosis, and intervention. J Vet Sci 2014; 15(1): 1-17.
4. Smith GW. Treatment of calf diarrhoea: oral fluid therapy. Vet. Clin. North. Am. Food Anim. Pract 2009; 25: 55-72.
5. Kumar S, Rajora VS, Arora N. Detection of enterotoxigenic *E. coli* in neonatal diarrhoeic calves using PCR technique. Journal of Entomology and Zoology Studies 2018; 6(6): 771-774
6. Umpiérrez A, Bado I, Oliver M, Acquistapace S, Etcheverría A, Padola LN, et al. Zoonotic potential and antibiotic resistance of *Escherichia coli* in neonatal calves in Uruguay. Microbes Environ 2017; 32: 275-82.
7. Gupta SS. Prospects and Perspectives of natural plant products in medicine. Ind. J Pharmacol 1994; 26: 1-12.
8. Vaidya AB, Antarkar VDS. New drugs from medicinal plants and approaches. J Assoc Phyc India 1994; 42(3): 221-222.
9. Shukla R, Sharma SB, Puri D, Prabhu KM, Murthy PS. Medicinal plants for treatment of diabetes mellitus. Indian J Clin Biochem 2000; 15(1):160-177.
10. Dalal KC. Present status and perspectives of medicinal plants. J. Res. Ind. Med. 1992, 15-20.
11. Sultana S, Khan MA, Ahmad M, Zafar M. Indigenous knowledge of folk herbal medicines by the women of district chakwal, Pakistan. Ethnobotanical Leaflets; 2006;10: 243-253.
12. Kumar N, Khurana SP. Bio Management of Wilting of a Valuable Timber and Medicinal Plant of Shisham (*Dalbergia sissoo* Roxb.)-A Review. Int. J. Curr. Microbiol. App. Sci. 2016; 5(1): 32-54.
13. Hassan W, Noreen H, Rehman S, Gul S, Amjad Kamal M, Paul Kamdem J, BT da Rocha J. Oxidative stress and antioxidant potential of one hundred medicinal plants. Current topics in medicinal chemistry 2017; 17(12): 1336-1370.
14. Nayak TC, Singh AP, Savita, Yadav R, Chahar A, Gupta SR, Kachhawa JP. *In vitro* antibacterial efficacy of aqueous and methanolic extracts of *Dalbergia sissoo* (Shisham) leaves against *E. coli* isolates from diarrhoeic calves. Haryana Vet. 2021; 60(Si): 41-43.
15. Liu ES, Cho CH. Relationship between ethanol- induced gastritis ulcer and gastric ulcer formation in rats. Digestion 2000; 62: 232.
16. Mir N. Rehydration and herbal therapy in calf diarrhoea. M.V.Sc. & A.H. Thesis (Veterinary Medicine) Jawaharlal Nehru Krishi Vishwa Vidyalaya, Jabalpur 2009.
17. Cowan ST, Steel KJ. Manual for the identification of Medical Bacteria. Cambridge University Press, Cambridge 1975.
18. Quinn PJ, Markey BK, Leonard FC, Hartigan P, Fanning S, Fitz-Patrick ES. Veterinary Microbiology and Microbial Disease. 2nd ed. Wiley Blackwell, Chichester, UK. 2011
19. Khaled A, Razik EI, Abdel R, Youssef F, Alaa M, Hazem A. Direct identification of major pathogen of bubaline subclinical mastitis in Egypt using PCR. American journal of science 2010; 6(10): 652-660.
20. Franck SM, Bosworth BT, Moon HW. Multiplex PCR for enterotoxigenic, attaching and effacing, and Shiga toxin-producing Escherichia coli strains from calves. J. clinical microbial 1998; 36(6): 1795-1797.
21. Redfern J, Kinninmonth M, Burdass D, Verran J. Using soxhlet ethanol extraction to produce and test plant material (essential oils) for their antimicrobial properties. Journal of Microbiology and Biology Education 2014; 15(1): 45-46.

22. Cruickshank R, Duguid JP, Marmion BP, Swain RHA. Medicinal Microbiology. (2 Edn.), Vol. II. The English Language Book Society, Churchill, Livingston, Edinburgh, Scotland 1975.
23. Jay CM, Bhaskaran S, Rathore KS, Waghela SD. Enterotoxigenic K99^{+} *Escherichia coli* attachment to host cell receptors inhibited by recombinant pili protein. Vet. Microbiol 2004; 101: 153-160.
24. Shams Z, Tahamtan Y, Pourbakhsh A *et al.* Detection of enterotoxigenic K99 (F5) and F41 from fecal sample of calves by molecular and serological methods. Comp Clin Pathol 2012; 21: 475-478
25. Ok M, Güler L, Turgut K, Ok Ü, Şen I, Gündüz IK, Birdane MF, Güzelbekteş H. The Studies on the Aetiology of Diarrhoea in Neonatal Calves and Determination of Virulence Gene Markers of *Escherichia coli* Strains by Multiplex PCR. Zoonoses and Public Health; 2009; 56: 94-101.
26. Brunauer M, Roch FF, Conrady B. Prevalence of Worldwide Neonatal Calf Diarrhoea Caused by Bovine Rotavirus in Combination with Bovine Coronavirus, *Escherichia coli* K99 and *Cryptosporidium* spp.: A Meta-Analysis. Animals 2021; 11: 1014.
27. Brijesh S, Daswani PG, Tetali P, Antia NH, Birdi TJ. Studies on Dalbergia sissoo (Roxb.) leaves: possible mechanism of action in infectious diarrhoea. Indian Journal of Pharmacology 2006; 38(2): 120.
28. Malik S, Kumar A, Verma AK, Gupta MK, Sharma SD. Prevalence and antibiotic sensitivity pattern of coliform diarrhoea of calves in western Uttar Pradesh. In Proceedings of 29th ISVM convention and national symposium; 2011; 17: 5.
29. Aly HI, El-Sayed AB, Gohar YM, Salem MZ. The value-added uses of *Ficus retusa* and *Dalbergia sissoo* grown in Egypt: GC/MS analysis of extracts. J. Forest Prod. Ind 2013; 2(3): 34-41.
30. Behera PC, Verma SM, Kumar PM, Das NB, Mishra PM, Baliarsingh S. Anti-inflammatory and anti-microbial activity of chalcone from *Dalbergia Sissoo* Roxb. leaves. Am. J. Phytomed. Clin. Ther 2013; 1(2): 186-194.
31. Prasad N, Nandi D, Arora S, Pandey A. In vitro evaluation of antibacterial properties of *Moringa oleifera*, *Dalbergia sissoo* and *Alstonia scholaris*. In Vitro; 2014; 4(15): 54-55.
32. Parmar HS, Johari M. Antibacterial assay of leaf of *Dalbergia sissoo* roxb. Int. J. Pharm. Sci. Res 2014; 5(8): 501-507.
33. Al-Snafi AE. Antimicrobial effects of medicinal plants (part 3): Plant based review. IOSR J. Pharm; 2016; 6(10): 67-92.
34. Al-Snafi AE. Chemical constituents and pharmacological effects of *Dalbergia sissoo* - A review. IOSR J. Pharm; 2017; 7(2): 59-71.
35. Maharishi T. Therapeutic Study of *Aegle marmelos* in Calf Diarrhoea, M.V.Sc. Thesis submitted to RAJUVAS, Bikaner (Rajasthan). 2019

HOW TO CITE THIS ARTICLE
Rathor AS, Bhati T, Singh AP. *In vitro* antibacterial activity of extracts of *Dalbergia sissoo* and *Aegle marmelos* against Enterotoxigenic *Escherichia coli* from calves. J Phytopharmacol 2021; 10(5):289-293. doi: 10.31254/phyto.2021.10503