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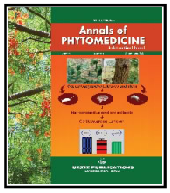

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Therapeutic efficacy of *Dalbergia sissoo* Roxb. and *Aegle marmelos* (L.) Correa. in combination in diarrhoeic calves with altered haematological parameters

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Abstract

Neonatal calf diarrhoea is one of the important causes of morbidity and mortality in dairy calves, thereby causing economic losses to the farmers. The leaves of *Dalbergia sissoo* Roxb. and dried fruit pulps of *Aegle marmelos* (L.) Correa. have astringent property and reduce irritation in the digestive tract, showing antidiarrheal activity against many microbial agents. Hence, the present study was conducted to evaluate two therapeutic regimens for their efficacies on altered haematological parameters in calf diarrhoea caused by *E. coli*. Each of the drug regimens was tried separately on a group of 8 diarrhoeic calves with one group (Group II) calves being administered combination of methanolic extract of *D. sissoo* @ 1000 mg/kg b. wt. and aqueous extract of *A. marmelos* @ 200 mg/kg b. wt. and second group (Group III) calves being administered combination of methanolic extract of *A. marmelos* @ 200 mg/kg b. wt. and aqueous extract of *D. sissoo* @ 1000 mg/kg b. wt. till recovery or maximum of 5 days with parenteral fluid therapy. Efficacy of therapeutic trials was evaluated on the basis of improvement in the clinical profile and return of altered haematological parameters towards normalcy. The treatment Groups (II and III) had Mean ± SE values of Hb, PCV, TEC, TLC, neutrophils significantly lower (p<0.05) after treatment (6 day) than the pre-treatment (0 day) values with the corresponding groups. It was concluded that the combination of *D. sissoo* and *A. marmelos* extracts was an effective therapy in the treatment of neonatal calf diarrhea and the combination also aided in the improvement in haematological parameters of diarrheic calves.

1. Introduction

Neonatal calf diarrhea (NCD) or calf scours is a serious multi-factorial disease, causing huge morbidity and mortality in new born calves leading to both productivity and economic loss to the dairy industry worldwide (Mousa *et al*., 2021). The etiology includes both infectious and non-infectious factors (the health and immune status of the calf, its metabolism, veterinary treatment, management of the herd, and environmental factors) (Wei *et al*., 2021). Among these factors, infectious agents are the leading cause of death which includes viruses (Rotavirus, Coronavirus, Bovine viral diarrhea virus), bacteria (*Escherichia coli*, *Clostridium perfringens* Type C, *Salmonella* spp.) and parasites (*Cryptosporidia and Coccidia*) (Constable *et al*., 2017; Wei *et al*., 2021). Enterotoxigenic *E. coli* (ETEC) is one of the main bacterial causes of calf diarrhea which is characterized by the presence of specific adhesins and enterotoxins.

Clinical findings reveal weakness in 1-4 day old newborn calves, diarrhea and progressive dehydration, high temperature, foul-smelling white or yellowish to tarry black coloured faeces. As a result, the affected calves revealed higher mean values of haemoglobin, packed cell volume, total leukocyte count, and total erythrocyte count. There was increase in total protein and globulin, whereas albumin and A/G ratio are decreased. Increased blood urea nitrogen and a decrease in serum sodium and hyperkalemia are the major consequence of diarrhea (Asati *et al*., 2008; Kumar *et al*., 2010).

Antimicrobial resistance in *E. coli* has been a significant issue in the treatment and control of diarrheal diseases and exposure to antimicrobial agents amplifies the resistance in the *E. coli* population, inhabiting animal and human intestinal tracts. The emergence of multidrug-resistant pathogenic *E. coli* strains poses a growing threat to the successful management of calf scours (Donaldson *et al*., 2006). Apart from creating drug resistant strains, the use of synthetic drugs leads to serious side effects in animals. Traditional ethnoveterinary medicines are a less expensive, easier and longer-lasting alternative to manufactured drugs and pharmaceuticals (Xiong and Long, 2020) and these plant-based drugs are natural products, non-narcotic, easily biodegradable, cause minimum environmental hazards, have no adverse side effects and are easily available at affordable prices (Sharma, 2021; Nayak *et al*., 2021). Because of the increased need for plant-based veterinary treatment that does not have detrimental effects on animal health, the herbal product market is expected to develop in the near future (Parveen *et al*., 2020).

The unripe fruit of *Aegle marmelos* (L.) Correa (Bael), a traditional Indian medicinal plant, has astringent properties that minimise gastrointestinal irritation and is a very useful remedy for diarrhoea and dysentery (Brijesh *et al*., 2009). The efficacy of *A. marmelos* fruit in the treatment of diarrhoea and dysentery has led to its inclusion in the British Pharmacopoeia (Chopra, 1982). Methanol extracts of the root bark of *A. marmelos* have showed high inhibition of *E. coli*

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over other organisms (Priyadharshini *et al*., 2019). Though ,*Dalbergia sissoo* Roxb. or North Indian Rose wood or Shisham is among the most important Indian timber tree but various parts of *D. sissoo* plant like its bark has astringent property and traditionally used for treating diarrhea and dysentery in animals and humans (Wankhade *et al*., 2019). Hence, the present study was conducted to determine the therapeutic efficacy of combination of alcoholic and aqueous extracts of *A. marmelos* and *D. sisso* in *E. coli* infected calf diarrhea cases.

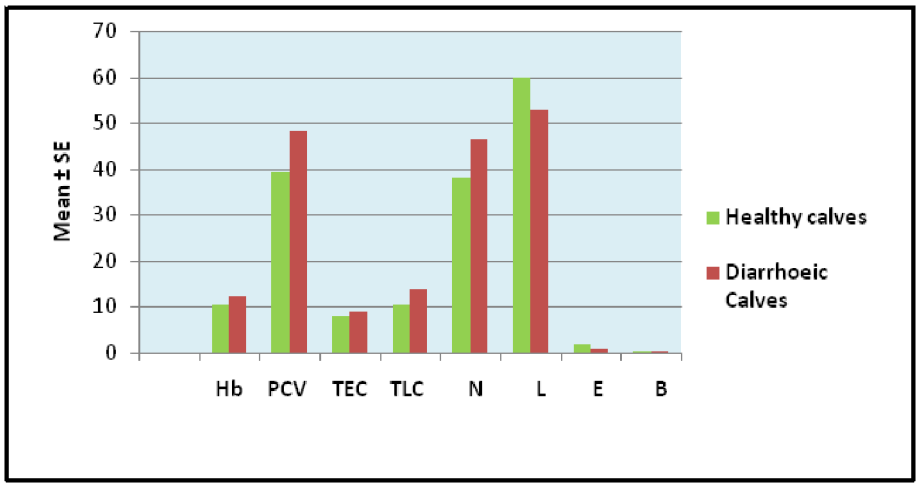

Figure 1: Mean ± SE values of haematological parameters of control and colibacillosis affected calves.

Table 1: Treatment groups and therapeutic dosages

Groups (8 cattle calves each group)	***Dalbergia sissoo* leaves**	***Aegle marmelos* unripe fruit**	**Parenteral fluid therapy (5 days)**
Group l (Healthy calves)	-	-	-
Group II	Methanolic extract (@ 1000 mg/kg b. wt. P.O.)	Aqueous extract (@ 200 mg/kg b. wt. P.O.)	Ringers lactate DNS along with 1.3% sodium bicarbonate I.V.
Group III	Aqueous extract (@ 1000 mg/kg b. wt. P.O.)	Methanolic extract (@ 200 mg/kg b. wt. P.O.)	Ringers lactate DNS along with 1.3% sodium bicarbonate I.V.

2. Materials and Methods

2.1 Ethical approval

This study was conducted following approval by the research committee and Institutional Animal Ethics Committee (Reg. No.-2044/GO/Re/SL/18/CPCSEA), Rajasthan University of Veterinary and Animal Sciences, under permission number CVAS/IAEC/2021-22/11/04.

2.2 Collection, processing and formulation of plant materials

Dried powder of *D. sissoo* leaves and unripe fruit of *A. marmelos* were procured from local market, and identification was validated by a botanical expert at the Botanical Survey of India's Arid Zone Regional Centre (AZRC) in Jodhpur. Using distilled water and 99.9% methanol as solvents, the dried powder was extracted for 24 h in a Soxhlet apparatus using the Soxhlet extraction (continuous hot extraction) method as per Redfern *et al*. (2014). The solvents were removed in rotary evaporator and the crude extracts were dried at room temperature in a steady air current. The dried aqueous and methanolic extracts were then stored in air tight jars at 4ºC till further use.

2.3 Therapeutic study

2.3.1 Selection of animals

In the present investigation, a total of 24 calves below one month of age were included and divided into three groups. These animals were housed at private dairy farms as well as individual holdings in Bikaner district of Rajasthan, India. Out of 24 calves, eight apparently healthy calves were taken as control and were kept in Group l to study normal parameters. The rest 16 calves showing classical clinical signs of diarrhoea irrespective of sex and breed were further divided into two groups. On the basis of 16S rRNA and K99 gene based PCR, these diarrhoeic calves were confirmed to be suffering from colibacillosis as described earlier by Rathor *et al*. (2021). These 16 calves were classified as Group II and Group III comprising of eight calves in each group for therapeutic trials of various regimens of *D. sissoo* leaves and *A. marmelos* unripe fruit.

2.3.2 Haematological study

Blood samples were taken from all 24 calves in the study on day 0 (pre-treatment) and day 6 (post-treatment) from jugular vein in EDTA

coated sterile vials for the estimation of haematological parameters, *viz*, haemoglobin (Hb), packed cell volume (PCV), total leukocyte count (TLC), total erythrocyte count (TEC), differential leucocyte count (DLC) as per the standard methods (Brar *et al*., 2004).

2.4 Calculation of dose of herbal extract

The dose of herb was calculated on the following observations:

When administered as a crude powder, the herb's dose was taken as four times the dose of herbal pure extract, for safety reasons. The maximum dose of a chemical should not be more than 1/10 of the 50% lethal dose (LD_{50}) dose of that particular chemical (Wynn and Fougere, 2007).

LD_{50} dose of *A. marmelos* pure methanolic extract and aqueous extract was found to be 1318 mg/kg body weight and 1549 mg/kg body weight, respectively (Veerappan *et al*., 2007) Thus, to be on the safe side, its dose was taken as 1/10 of the LD_{50}, *i.e*., 200 mg/kg body weight. Similarly, the safe dose of *D. sissoo* was reported to be 1000 mg/kg body weight by Govindula (2017).

2.5 Treatment groups

The effect of *D. sissoo* leaves and *A. marmelos* unripe fruit was studied in calves of Group lI and Group lII affected with colibacillosis as mentioned in Table 1. Estimation of the degree of dehydration and base deficit was calculated by the method given by Constable *et al*. (2017) for diarrhoeic calves under field conditions.

2.6 Statistical analysis

The data obtained in the research work undertaken were statistically analyzed and compared using standard formulas given for mean, standard error, one-way analysis of variance (F-test) and t- test as per the procedures explained by Snedecor and Cochran (1956) and significance of mean differences were tested by least significance difference (LSD).

3. Results

3.1 Haematological parameters in colibacillosis

The mean ± SE values of haematological parameters of healthy calves and colibacillosis affected calves are presented in Table 2, Figure 1. Mean values of Hb (g/dl), PCV (%) and TEC (x106/µl) in healthy calves were 10.48 ± 0.22, 39.35 ± 0.96 and 7.99 ± 0.17, whereas in colibacillosis were 12.38 ± 0.22, 48.33 ± 1.13 and 8.82 ± 0.31, respectively. The mean ± SE values of TLC (x103/µl) in healthy calves were 10.25 ± 0.14 and 13.78 ± 0.45 in diarrhoeic calves.

Table 2: Mean ± SE values of haematological parameters of healthy control and colibacillosis affected calves

S. No.	Parameters	Healthy calves (n = 8)	Diarrhoeic calves (n =16)	Statistical analysis (T- test)
1	Haemoglobin (g/dl)	10.48 ± 0.22	12.38 ± 0.22	**
	(Range)	(9.8 - 11.4)	(11.2 - 13.2)	
2	PCV (%)	39.35 ± 0.96	48.33 ± 1.13	**
	(Range)	(35 - 43)	(45 - 55.2)	
3	TEC (10^6/µl)	7.99 ± 0.17	8.82 ± 0.31	*
	(Range)	(7.31 - 8.61)	(7.61 - 12.98)	
4	TLC (10^3/µl)	10.25 ± 0.14	13.78 ± 0.45	**
	(Range)	(9.56 - 10.62)	(12.24 - 16.87)	
	DLC			
	1. Neutrophils (%)	38.13 ± 0.91	46.25 ± 0.82	**
	(Range)	(34 – 42)	(41 – 49)	
	2. Lymphocytes (%)	60.00 ± 1.12	52.75 ± 0.92	**
	(Range)	(56 – 64)	(49 – 58)	
	3. Eosinophils (%)	1.75 ± 0.31	0.75 ± 0.31	NS
	(Range)	(1 – 3)	(0 – 2)	
	4. Basophils (%)	0.25 ± 0.16	0.25 ± 0.16	NS
	(Range)	(0 – 1)	(0 – 1)	

* = Significant ($p \leq 0.05$)
** Highly significant ($p \leq 0.01$)
NS = Non-significant ($p > 0.05$)

The differential leucocyte count (DLC) revealed mean values of neutrophils (%) in the healthy control and colibacillosis as 38.13± 0.91 and 46.25 ± 0.82, respectively. The mean value of neutrophils in colibacillosis was significantly higher than healthy calves. The mean lymphocyte (%) in diarrhoeic calves (60.00 ± 1.12) was significantly lower than healthy calves (52.75 ± 0.92).

3.2 Evaluation of therapeutic regimens

Total two therapeutic regimens were evaluated for their efficacies in calf diarrhoea caused by *E. coli* as given in Table 1. The drug trial (s) showing faster recovery (after treatment) in all the altered values of clinical and haemato-biochemical parameters was established as the best therapeutic regimen.The haematological parameters, *viz*., Hb, PCV, TEC, TLC and DLC were determined for evaluation of therapeutic efficacy of various therapeutic regimens of *D. sissoo* and *A. marmelos*. For this, the pre-treatment parameters (day 0) were compared with post-treatment (day 6) parameters.

The pre-treatment (day 0) and post-treatment (day 6) mean ± SE values of haemoglobin (g/dl), PCV (per cent), TEC (106/µl) and TLC (103/µl) of healthy calves of Group I and colibacillosis affected diarrhoeic calves of Group II and Group III are presented in Table 3 and data are depicted in Figure. 2. In the treatment Groups (II and III), Mean ± SE values of haemoglobin, PCV, TEC and TLC were significantly lower after treatment than the pre-treatment values with the corresponding groups.

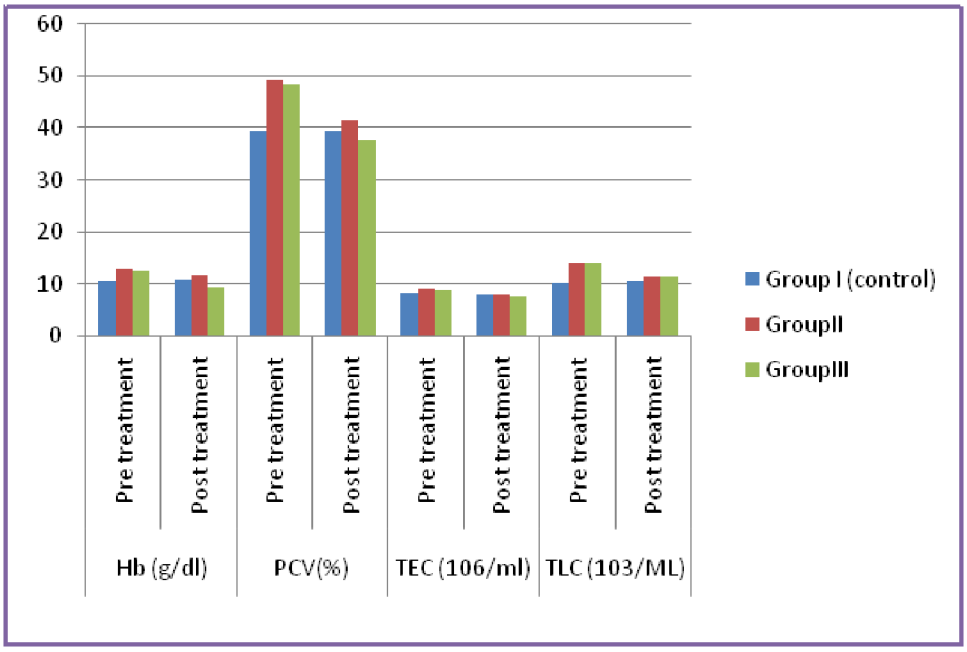

Figure 2: Pre and post-treatment Mean ± SE values of haemoglobin, PCV, TEC and TLC of colibacillosis affected and control calves.

Table 3: Pre and Post-treatment Mean ± SE values of haemoglobin, PCV, TEC and TLC of control and colibacillosis affected calves

Parameter	Hb (g/dl)		PCV(%)		TEC (10^6/ml)		TLC (10^3/ml)	
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
Group I (control)	$10.48^{ax} \pm 0.22$	$10.58^{ax} \pm 0.20$	$39.35^{ax} \pm 0.96$	$39.38^{ax} \pm 1.11$	$7.99^{ax} \pm 0.17$	$7.97^{ax} \pm 0.17$	$10.25^{ax} \pm 0.14$	$10.30^{ax} \pm 0.11$
Group II	$12.65^{bx} \pm 0.12$	$11.45^{aby} \pm 0.31$	$49.26^{bx} \pm 0.91$	$41.59^{aby} \pm 1.40$	$8.94^{bx} \pm 0.58$	$7.94^{aby} \pm 0.37$	$13.90^{bx} \pm 0.72$	$11.42^{bx} \pm 0.66$
Group III	$12.38^{bx} \pm 0.22$	$9.31^{aby} \pm 0.21$	$48.33^{bx} \pm 1.13$	$37.71^{aby} \pm 1.17$	$8.82^{bx} \pm 0.31$	$7.46^{aby} \pm 0.49$	$13.78^{bx} \pm 0.45$	$11.23^{aby} \pm 0.37$
Means having different superscript in a row (x,y) differ significantly (p<.05) Means having different superscript in a column (a,b) differ significantly (p<.05)								

For differential leucocyte parameters, the pre-treatment (day 0) and post-treatment (day 6) Mean ± SE values of neutrophils (per cent), lymphocytes (per cent), eosinophills (per cent) and basophills (per cent) of healthy calves of Group I and colibacillosis affected diarrhoeic calves of Group II and Group III are presented in Table 4. In the treatment Groups (II and III), Mean ± SE values of neutrophils were significantly lower while that of lymphocytes were significantly higher after treatment than the pre-treatment values with the corresponding groups (Figure 3).

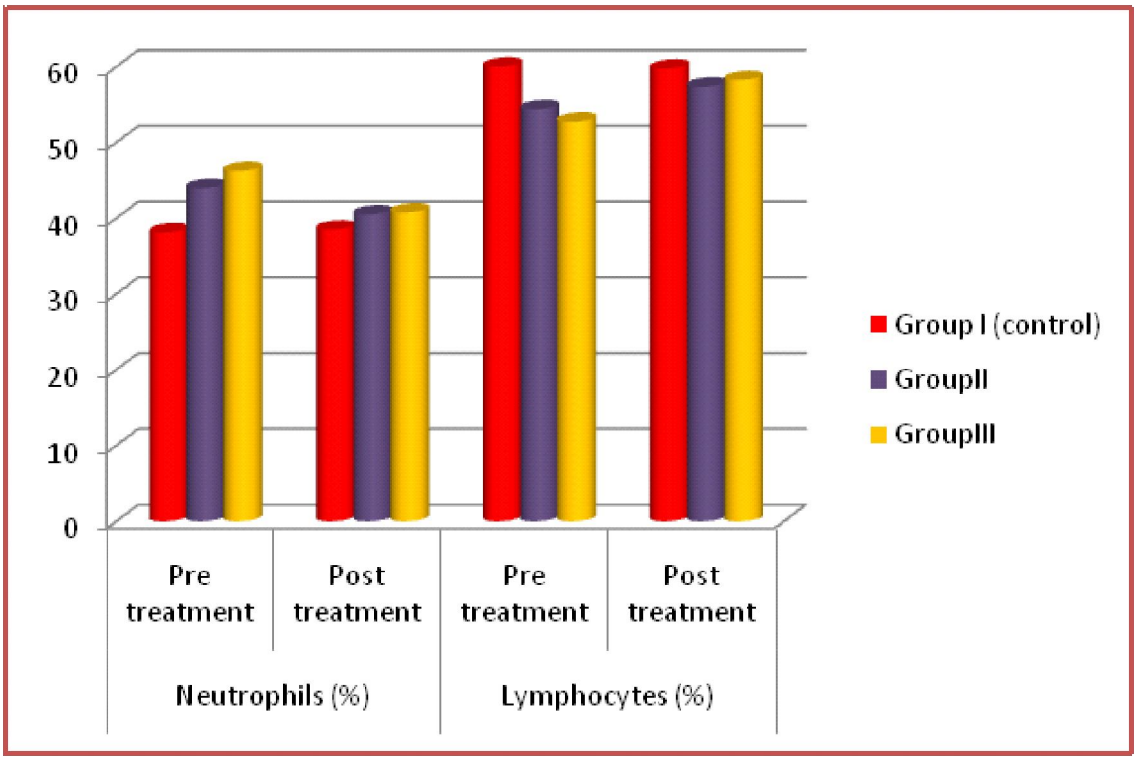

Figure 3: Pre and post-treatment Mean ± SE values of neutrophils and lymphocytes of colibacillosis affected and control calves.

Table 4: Pre and post-treatment Mean ± SE values of neutrophils, lymphocytes, eosinophils and basophils of control and colibacillosis affected calves

Parameter	Neutrophils (%)		Lymphocytes (%)		Eosinophils (%)		Basophils (%)	
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
Group I (control)	$38.13^{ax} \pm 0.91$	$38.50^{ax} \pm 0.91$	$60.00^{ax} \pm 1.12$	$59.75^{ax} \pm 1.11$	$1.75^{ax} \pm 0.31$	$1.63^{ax} \pm 0.26$	$0.25^{ax} \pm 0.16$	$0.25^{ax} \pm 0.16$
Group II	$44.00^{bx} \pm 0.96$	$40.50^{aby} \pm 0.78$	$54.38^{bx} \pm 1.15$	$57.38^{bx} \pm 0.96$	$1.00^{bx} \pm 0.33$	$1.38^{bx} \pm 0.32$	$0.63^{ax} \pm 0.18$	$0.75^{bx} \pm 0.16$
Group III	$46.25^{bx} \pm 0.82$	$40.75^{aby} \pm 1.03$	$52.75^{bx} \pm 0.92$	$58.25^{aby} \pm 1.00$	$0.75^{cx} \pm 0.31$	$0.75^{cx} \pm 0.31$	$0.25^{ax} \pm 0.46$	$0.25^{ax} \pm 0.16$

Means having different superscript in a row (x,y) differ significantly (p<.05)
Means having different superscript in a column (a,b) differ significantly (p<.05)

In case of comparison of Mean ± SE values of eosinophils and basophills between various treatment groups and between pre-treatment and post-treatment (6th day), there were significant differences (Table 4), but all data are within normal range and statistical differences do not have any significant importance.

4. Discussion

4.1 Haematological parameters in colibacillosis

There was significant increase in the mean values of Hb, PCV and TEC in colibacillosis affected calves as compared to healthy control group which is in agreement with earlier reports of Baber *et al.* (2000), Kumar and Mandial (2002), Kaur *et al.* (2006), Asati *et al.* (2008), Roy *et al.* (2009), Tikoo and Soodan (2009), Kumar *et al.* (2010), Mir *et al.* (2010) and Maharishi (2019). Increased Hb, PCV, and TEC levels in affected calves were caused by haemo-concentration due to dehydration and hypovolaemia, as diarrhoea causes excessive loss of intestinal fluid, culminating in severe dehydration (Eddy and Pinsent, 2004). The mean value of TLC in colibacillosis was significantly (p<0.01) higher than the control calves and similar to that reported by other workers (Fernandes *et al.*, 2009; Kumar *et al.*, 2010; Asati *et al.*, 2010; Mir *et al.*, 2010; Sharma, 2013; Bashir *et al.*, 2015; Shekhar *et al.*, 2017; Maharishi, 2019).

The most probable reason of increased TLC levels may be due to *Escherichia coli* infection. Leucocytosis in neonatal calf diarrhoea has been related to microbial infection and accompanying neutrophilia, according to Eddy and Pincent (2004) and Gerros *et al.* (1995). The occurrence of leukocytosis in white blood cells is a reliable indication of septicaemia, which is frequently associated with bacterial infection in the intestine (Jain, 1993). Bukhari (2002)

proposed that widespread tissue dehydration in neonatal calf diarrhoea was the cause of a rise in TLC values as well as other haematological parameters. Asati *et al.* (2008), on the other hand, found lower mean TLC values in colibacillosis, which they attribute to a transient leucopenic condition in diarrhoeic calves (Santos *et al.*, 2002).

There was marked neutrophilia and lymphopenia in diarrhoeic calves during our study. In neonatal calves suffering from diarrhoea, Brar *et al.* (2015) also observed neutrophilia with concomitant lymphopenia as a prominent characteristic of the circulating leukocyte profile. The considerable increase in circulating neutrophils associated with leucocytosis in the early stages of diarrhoea in calves, according to Malik *et al.* (2013), indicated pathogenic bacterial infection. Similar findings were also reported by Sharma (2013); Bashir *et al.* (2015) and Maharishi (2019).

4.2 Evaluation of therapeutic regimens

Diarrhoea causes an excessive loss of intestinal fluid, resulting in severe dehydration and hypovolaemia, resulting in haemo-concentration (Bukhari, 2002; Eddy and Pinsent, 2004) which leads to increase in the pre-treatment mean values of haematological parameters in diarrhoeic calves in comparison with healthy calves. Following remedial therapies, the haematological values consistently reverted to normal on day 6, presumably in response to restoration of water homeostasis and antidiarrhoeal properties of *D.sissoo* and *A. marmelos* which is in accordance to the earlier reports (Swaroop *et al.*, 2004; Mujumdar *et al.*, 2005; Brijesh *et al.*, 2006; Kalaskar *et al.*, 2010; Alam *et al.*, 2012; Al-Snafi, 2017). The antidiarrhoeal, antibacterial, astringent, antihelminthic, and anti-inflammatory properties documented in previous studies could represent the mechanism of action of the therapeutic combination of *D.sissoo* and *A. marmelos*. The astringent characteristic of the *A. marmelos* fruit (Bael) reduces gastrointestinal discomfort as reported by many workers (Qadri *et al.*, 2012; Dhankar *et al.*, 2011; Chopra, 1982). Several researchers have reported antidiarrhoeal activity and astringent property of *D. sissoo* (Kalaskar *et al.*, 2010; Amrutkar *et al.*, 2016).

Bacterial infection associated with neutrophilia, septicaemia and generalized tissue dehydration (Jain, 1993; Gerros *et al.*, 1995; Bukhari, 2002; Eddy and Pinsent, 2004) might be the most probable reasons of leucocytosis in young calves affected with diarrhoea on pre-treatment as compared with values of control group (healthy). Presumably, the potent antibacterial agents in Bael and Shisham (Brijesh *et al.*, 2009; Gupta, 2016; Prasad *et al.*, 2014; Parmar and Johari, 2014; Al-Snafi, 2016; Al-Snafi, 2017) blocked the rapid multiplication of *E. coli* in the present therapeutic trials supported by significant improvement in the values of leucocytes. Similarly, Wankhade *et al.* (2019) observed that the mean TLC and TEC was significantly (p<0.01) improved in diarrhoeic goats treated with polyherbal antidiarrhoeal preparations containing extracts of *A. marmelos*, *D. sissoo* and *Holarrhena antidysenterica*, indicated effectiveness of treatment. Certain phytochemicals including phenols, tannins and flavonoids present in the *D. sissoo* and *A. marmelos* are effective against various enteric pathogens (Maheshwari *et al.*, 2009; Gupta *et al.*, 2014; Prasad *et al.*, 2014; Maharishi, 2019).

In the present study, it was found that combination of methanolic extract of *A. marmelos* and aqueous extract of *D. sissoo* was most effective in the cases of diarrhoea associated with colibacillosis in cattle calves than the combination of methanolic extract of *D. sissoo* and aqueous extract of *A. marmelos*. The antibacterial activity of *A. marmelos* methanol extract has been documented (Phullan and Khullar, 2004). There are many previous workers (Duke *et al.*, 2000; Arora *et al.*, 2010; Mir *et al.*, 2010; Mehsere *et al.*, 2017; Ranaut *et al.*, 2018; Gupta *et al.*, 2020) who successfully used formulations of *D. sissoo* and *A. marmelos* alone or in combination with other polyherbal preparations in the treatment of calf diarrhoea.

Various other workers (Chattopadhyay *et al.*, 2009; Sujatha and Rajan, 2014; Nayak *et al.*, 2019) have also reported more potent antibacterial activity of alcoholic extracts of *A. marmelos* than the aqueous extract by *in vitro* and *in vivo* studies against various pathogenic bacteria of clinical origin including enteric pathogens. There is paucity of literature regarding use of *D. sissoo* (Shisham) and *A. marmelos* (Bael) for treatment of diarrhoea in calves.

5. Conclusion

The combination of methanolic extract of *A. marmelos* and aqueous extract of *D. sissoo* was most effective in improving and recovering from diarrhoea associated with colibacillosis in cattle calves in the current study. The combination was also found to be effective in restoring nearly normal values of haematological parameters in calves affected by diarrhoea. More research is needed in this area to isolate and identify the active components of *A. marmelos* and *D.sissoo* that mediate these effects.

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Conflict of interests

The authors declare no conflicts of interest relevant to this article.

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