

Original research article

Evaluation of anti-biofilm, anti-quorum, anti-dysenteric potential of designed polyherbal formulation: *in vitro* and *in vivo* study

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Abstract

Bacillary dysentery (shigellosis) continues to cause havoc worldwide, with a high infectivity rate. It causes bloody diarrhea, and around 99% of bacillary dysentery cases occur in developing countries. The objective of this study is to develop a polyherbal formulation with the scientific rationale in treating infectious bacillary dysentery disease. The anti-bacterial activity, the minimum inhibitory concentration of the formulation against bacillary dysentery, causing microbes like *Shigella flexneri* (MTCC 1457), *Escherichia coli* (MTCC 1687), and *Salmonella enterica* (MTCC 98), was analysed by well-diffusion method and broth dilution method, respectively. The biofilm inhibition activity was determined on 96 well polystyrene plates and anti-quorum sensing activity by *Chromobacterium violaceum* CV026. The cytotoxicity was examined by acute oral toxicity. Excreta and organ bacterial load were analyzed by serial dilution method. The formulation efficacy was determined by analyzing the blood sample of rats. The antimicrobial efficacy of the developed formulation was calculated by measuring the zone of inhibition which was found to be 24 mm, 25 mm, and 25 mm, and the MIC values of 1.5 mg/ml, 1.5 mg/ml, and 2.0 mg/ml against *S. flexneri*, *S. enterica*, *E. coli*, respectively. The results show that the polyherbal formulation significantly reduced biofilm formation and has anti-quorum sensing activity. The formulation also effectively decreases the bacterial load and increases the K⁺, Na⁺, and Ca⁺⁺ ions in animals treated with the formulation. The developed formulation was found to be non-toxic and effective against bacillary dysentery; thus, it can be used for treating bacillary dysentery and related complications.

Keywords: Anti-biofilm; Anti-dysenteric; Anti-quorum; Cytotoxicity; Herbal formulation

Highlights:

- The designed herbal formulation shows anti-dysenteric activity.
- The formulation effectively reduces the *S. flexneri*, *E. coli*, *S. enterica* biofilm.
- The *in vivo* cytotoxicity study proves that the composition is safe.
- Experimental evidence proves the therapeutic efficacy and suggests that the formulation can be used to treat bacillary dysentery.

Introduction

Dysentery, also known as bloody diarrhea, is a condition that is identified by the repeated passing of watery bloody stools containing visible red blood cells and mucus. Bacillary dysentery (BD) occurs due to the failure of one or more functions of the alimentary canal (Bhattachaya et al., 2012). It is caused by a wide range of pathogens, most commonly *Shigella* species. Others may include *Salmonella* and *E. coli*. In humans, it is mainly spread by water and food, primarily via the feco-oral route, and has an incubation period of 1 to 4 days (Niyogi, 2005). The clinical symptoms include bloody stool, fever, and

stomach cramps. In both developing and developed countries, bacillary dysentery still remains a public health concern (Kotloff et al., 1999). There are an estimated 165 million reported cases of bacillary dysentery and approximately 1 million annual deaths worldwide – mostly in developing countries (Carayol and Tran Van Nhieu, 2013). Countries including India, Iran, Indonesia, Bangladesh, China, U.S., and Vietnam have reported the emergence of *Shigella* MDR strains. *S. flexneri* shows 74.1% resistance to nalidixic acid, and 45.6% resistance to ciprofloxacin has consistently been reported (Niyogi, 2005). In 2004, *S. flexneri* was found to be resistant to fluoroquinolone, thus this drug was no longer preferred for the treatment. The widespread resistance of *Shigella* to nearly all current types of

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drugs in use is a matter of concern. In recent years, due to excessive use of antibiotics, antimicrobial resistance among *Shigella* species has been increasingly restricting the possibility of suitable antibiotic treatment (Kotloff et al., 1999).

Several studies confirm that the compound inhibiting biofilm will also inhibit quorum-sensing (QS) (Singh et al., 2021). Currently, there is a greater global interest in non-synthetic, natural drugs derived from herbal sources, owing to better tolerance and minimum adverse drug effects. Hence, there is a requirement for identifying new anti-biofilm and anti-QS inhibitors of natural origin in order to minimize the adverse effects, toxicity, and resistance.

Herbal formulations are a standardized composition of herbs comprising a mixture of one or more herbal plants used for the management of various diseases, such as asthma, Alzheimer's, inflammatory arthritis, and microbial infection (Prior and Cao, 2000). In this study, a polyherbal formulation consisting of *Camellia sinensis* (Leaf), *Citrus lemon* (Fruit), *Terminalia chebula* (Fruit) extract along with *Cinnamon*, and *Thyme* oil was developed for the first time, and investigated for its anti-dysenteric, anti-quorum, and anti-biofilm activities.

Materials and methods

Collection of plant extracts and reagents

Cinnamon oil, *Thyme* oil, Hexanoyl Homoserine Lactone (HHL), and Kanamycin was purchased from Sigma-Aldrich Inc. (Bengaluru, India). Standardized extracts (*Camellia sinensis* (Batch no. KP/TC/001/16), *Terminalia chebula* (Batch no. CS0010416), *Citrus lemon* (Batch no. NBT/1705576)) were purchased from K. Patel Phyto Extractions Pvt. Ltd. (Gujarat, India) and Saamir international Pvt. Ltd. (Delhi, India) respectively. A detailed description of the extracts and oils can be found in Table 1. The prepared stock was kept at -20°C until further use.

Bacterial strain and culture condition

Bacterial strain CV026 (*C. violaceum*) was used to detect quorum sensing and was procured from ATCC (ATCC 31352), CTET Spain. Other strains, including *Escherichia coli* (MTCC

1687), *Shigella flexneri* (MTCC 1457), *Salmonella enterica* (MTCC 98) were obtained from MTCC, IMTECH (Chandigarh, India). Except for CV026, which was grown at 28°C rest, all bacterial strains were grown in LB (Luria-Bertani) media at 37°C , pH 7.0.

Experimental animals

Male young, healthy Wistar albino rats (180–210 g) were kept in polypropylene cages at the animal house of the United Institute of Pharmacy, Allahabad, under a standard condition of 12/12-h light and dark cycle. The rats were fed with a standard pellet diet (Amrut, India) and water *ad libitum*. The entire test was performed during the period of light (0800:1600 hours).

Preparation of polyherbal formulation

The desired amount of powdered extracts of different plant parts viz. *C. sinensis* 19.45% (leaf), *C. lemon* 21.61% (fruit), *T. chebula* 17.3% (fruit) were mixed by a mechanical stirrer, and *Cinnamon* oil 9.09% and *Thyme* oil 32.55% were mixed in a sterile container and was mixed with the extract by agitation process to obtain a homogeneous mixture. The obtained formulation was kept at -4°C and used in an aqueous medium along with 0.1% tween during the study. The formulation was designed on the basis of the screening results (MIC, anti-bacterial activity, etc.) of individual extracts and oils. A description of the extracts and oil used in the formulation can be seen in Table 1.

Minimum inhibitory concentration analysis

The minimum inhibitory concentration of formulation against *E. coli*, *S. flexneri*, *S. enterica* was calculated by following the NCCLS guidelines (CLSI, 2006). In brief, an overnight *E. coli*, *S. flexneri*, *S. enterica* culture ($\text{OD } 600 \text{ nm} = 1$) was grown with or without the formulations (concentrations varied from 0.1 mg/ml to 3.5 mg/ml) in LB medium for 24 h at 37°C , in 96 well plates. Afterward, the concentration at which the growth of *S. flexneri*, *S. enterica*, *E. coli* was found inhibited was noted as the MIC for the designed formulation, and further experiments were carried out at sub-MIC concentration (Singh and Agarwal, 2021a).

Table 1. Description of the extracts and oil used in the formulation

Sample	Company	Appearance	Active compounds	Activity	References
<i>C. sinensis</i>	K. Patel Phyto Extractions Pvt. Ltd	Greenish brown	Epigallocatechin-3-gallate, epicatechin, epigallocatechin gallate, kaempferol, theanine, quercetin, myricetin	Anti-oxidant, anti-bacterial, anti-inflammatory, anticancer	Koch et al., 2019
<i>T. chebula</i>	K. Patel Phyto Extractions Pvt. Ltd	Dark brown	Tannic acid, gallic acid, ethyl gallate, ellagic acid, chebulic acid, ascorbic acid, chebulagic acid, mannitol, corilagin	Anti-bacterial, antioxidant, anticarcinogenic, anti-inflammatory	Chang and Lin, 2012
<i>C. lemon</i>	Saamir international Pvt. Ltd.	Light brown	Limonene, hesperidin, eriocitrin, neoeriocitrin, eriodiktyol, limocitrin, apigenin, diosmin	Antimicrobial, anti-inflammatory, antiparasitic, anticancer activities.	Klimek-Szczykutowicz et al., 2020
<i>Cinnamon</i> oil	Sigma Aldrich	Dark yellow	Cinnamaldehyde	Anti-fungal, antidiabetic, antimicrobial, antioxidant activities	Prabuseenivasan et al., 2006
<i>Thyme</i> oil	Sigma Aldrich	Colorless	Thymol	Antiseptic, anti-inflammatory, antispasmodic, antimicrobial, antiviral, anti-fungal	Ocana and Reglero, 2012

Agar well diffusion method

The bacterial inoculums were spread briefly over the prepared agar plate. Then, aseptically, a well was punched with a sterile tip. A volume (100 μ l) of the prepared formulation was poured into the created well and incubated at 37 °C for 24 h. The diameter of the clear halo zone was measured in mm (Singh and Agarwal, 2021b; Vattam et al., 2007).

Quantification of biofilm

Bacterial biofilms were allowed to form under the absence or at sub-MIC concentrations of formulation in 96-well microtiter plates. In brief, 100 μ l of TSB (Trypticase soy broth) media was added to U-shaped 96 well plates, along with formulation in all wells except control well. Further, 1 μ l of 0.5 OD bacteria culture was added to each well and left for 24 h of incubation at 37 °C. After incubation, non-adhere cells were discarded, and the plate was washed with PBS, and each well was stained by adding 100 μ l of 0.5% Crystal Violet (CV) and left for 15 min. After 15 min, CV stain was discarded, and wells were again washed by using PBS. DMSO was added to solubilize the CV, and the plates were left for 6–8 min, and absorbance was taken at 590 nm in a microplate reader (Dohare et al., 2021; Kalia et al., 2020).

Screening for anti-QS activity

100 μ l of an overnight grown culture (of OD 1 at 600 nm) was spread on the LB agar plates (having a hexanoyl homoserine lactone (HHL) 0.125 μ g/ml and Kanamycin 20 μ g/ml) to form a uniform lawn. After 5 minutes of incubation, wells were punched aseptically with the help of a sterile tip or cork borer. Formulation dissolved in an aqueous solvent was put into the wells and left for incubation at 28 °C for 24 h. The plates were analyzed for the halo zone formed along with the wells with purple backgrounds (McClellan et al., 1997).

Acute oral toxicity studies

The lethal median dose (LD₅₀) determination was done in albino rats by adopting revised OECD guidelines number 423 (OECD, 2001). A single dose of the formulation (5 mg/kg, 50 mg/kg, 300 mg/kg, and 2000 mg/kg) was given orally by oral gavage to each animal's group (three each). The volume of each administered oral dose did not exceed 1 ml for each rat. The animals in each group were allowed free access to food as well as water. However, the animals of different groups were deprived of food for 2 hrs before and 4 h after dosing. Each animal was monitored every hour for the first 12 h for any sign of toxicity (restlessness, irritability, touch response). Later, they were monitored twice a day for any abnormal changes, throughout the 14-day study period (Lipnick et al., 1995).

In vivo anti-dysentery activity

Experimental design

Four groups of animals were used to study the effect of polyherbal formulation. Each group consisted of five rats. Dysentery was induced by a single intraperitoneal (i.p.) injection of (10⁸ cells of *S. flexneri*) in all the groups except the control group (Sharma et al., 2017).

- **Group I:** Normal control (NC) rats received physiological saline solution.
- **Group II:** Dysenteric control (DC) rats received normal saline solution.
- **Group III:** Dysenteric rats were treated with herbal treated (HT) 250 mg/kg b.w.
- **Group IV:** Dysenteric rats were treated with standard drug norfloxacin (AT) 20 mg/kg b.w.

All of the animals were observed for 7 days for common physiological parameters, physical activity, presence of blood (mucus) in the feces, and death rate. The test drug and standard drug were administered through an oral route by using an oral gavage tube. Fecal samples were collected at a different time interval, homogenized in PBS followed by dilution, and spread on SS (Salmonella Shigella) agar plates to confirm the presence of *Shigella flexneri*. For this purpose, 0.5 g faeces were collected and homogenized in sterile PBS (4.5 ml), followed by serial dilutions. From each dilution, 500 μ l was taken and seeded over agar plates. After 24 h of incubation at 37 °C, the cell number was counted (Kouitchou et al., 2013).

To confirm the bacterial colonization in intraperitoneally challenged rats, infected colons were collected and homogenized in PBS and were plated on *Shigella* agar. Bodyweight variation, feed intake, water intake, and stool frequency was monitored at different intervals for all the experimental animal groups. The frequency of faeces was calculated for 7 consecutive days. At the end of the treatment, all animals were euthanized on the 7th day by intraperitoneal injection of 200 mg/kg pentobarbital euthanasia solution, and important organs viz. colon (large intestine), liver, and kidney were isolated for further analysis (Longanga Otshudi et al., 2000).

Biochemical analysis

At the end (7th day) of the experiment, the blood from all the experimental animal groups was collected in EDTA tubes to analyze the RBCs, Platelet, and Hemoglobin. For ion (sodium, potassium, and calcium) measurement, the blood samples were collected in heparinized tubes (Parasuraman et al., 2010).

Ethics

The research protocol was approved by the Institutional Animal Ethics Committee (IAEC), United Institute of Pharmacy, Allahabad, with approval number (UIP/IAEC/Nov.-2019/06).

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 3.03 software for Windows, GraphPad Software, Inc. CA, USA. The values are presented as mean \pm SEM (Standard Mean Error). The data were analyzed by one way ANOVA and two way ANOVA; only values $p < 0.05$ were considered significant.

Results

Minimum inhibitory concentration

MIC of the developed polyherbal formulation was assessed against different bacillary dysentery, causing microbes, and the MIC values were found to be 1.5 mg/ml against *S. flexneri*, 1.5 mg/ml against *S. enterica*, and 2.0 mg/ml against *E. coli*. The MIC value of norfloxacin were found to be 0.05 mg/ml against *S. flexneri*.

Zone of inhibition

The polyherbal formulation was screened against bacillary dysentery, causing microbes, i.e., *Shigella flexneri*, *Salmonella enterica*, *E. coli*, and the inhibitory zone (I.Z.) of 24 mm \pm 0.57, 25 mm \pm 1.15, and 25 mm \pm 1.54 was observed, respectively.

Anti-biofilm activity analysis

The anti-biofilm activity of the formulation was analyzed against *S. flexneri*, *S. enterica*, and *E. coli*. The anti-biofilm

activity analysis of the formulation was done below MIC value, and the percentage of biofilm inhibition was observed to be decreased by 67.94%, 65.56%, and 51.94% in the presence

of 0.8 mg/ml formulation against *S. flexneri*, *S. enterica*, and 0.9 mg/ml against *E. coli* when compared to the control (as shown in Fig. 1).

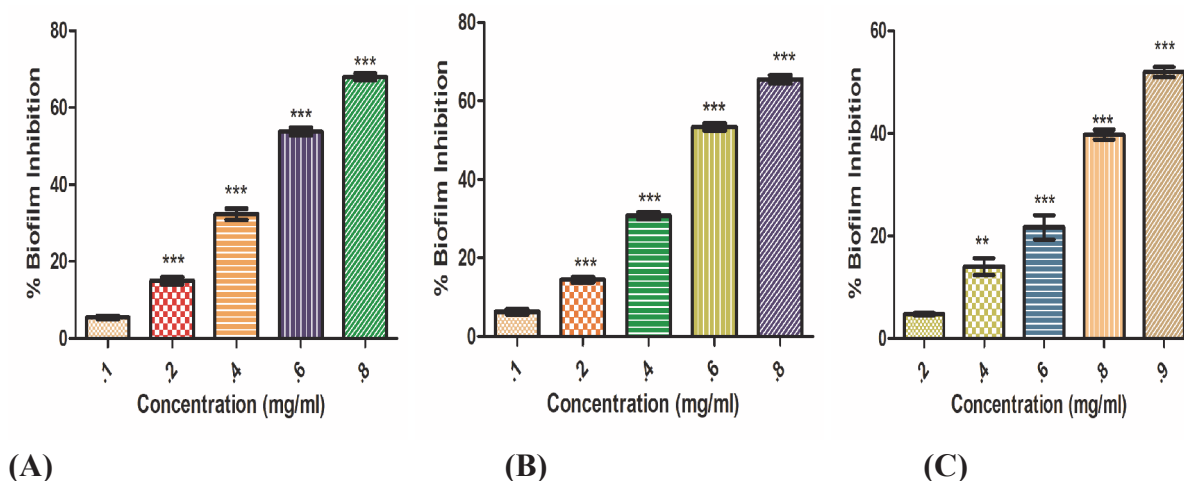


Fig. 1. The % biofilm inhibition of (A) *S. flexneri*, (B) *S. enterica*, (C) *E. coli* by designed formulation. Error bars indicate the \pm standard deviations of three measurements. *** $P < 0.001$ and ** $P < 0.01$ is considered as significant (One-way ANOVA).

Analysis of anti-QS activity

The anti-QS activity of the designed herbal formulations was performed using a bioreporter strain CV026. The clear halo zone was found around the wells with the formulation, revealing the anti-QS activity of the designed formulation (Fig. 2). No bacterial inhibition zone was observed around the wells; only violacein production was inhibited.

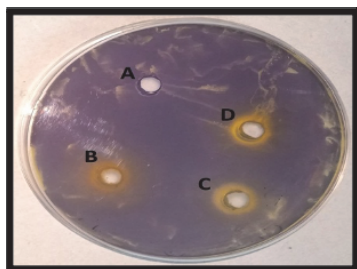


Fig. 2. A plate showing QS inhibition of *C. violaceum* CV026 by formulation, where **A**-control and **B** (0.2 mg/ml), **C** (0.3 mg/ml), and **D** (0.4 mg/ml) show the anti-QS activity of the designed formulation.

Acute toxicity study (LD₅₀)

Oral administration of polyherbal formulation in specified doses did not produce any significant changes in behavior, postural abnormalities, impairment in food and water intake, and loss or yellowing of hair. No mortality of animals was observed in herbal treated groups. The results indicate that the herbal formulation can be considered safe up to 2000 mg/kg. On the basis of LD₅₀ cut-off, 250 mg/kg dose has been selected for pharmacological activity.

Anti dysentery activity: Animal behavior, stool quality, and mortality

During the *in vivo* studies, no major change in behavior of group I (NC) rats, was observed, but in group II (DC) rats, wet faces, weight loss, and less activity was observed. Until the 3rd

day, less movement and activity in groups III (HT) and IV (AT) was observed, after which the activity and movement slowly began to increase. Weight loss in group III was also observed until the 3rd day, and in group IV, it was observed until the second day, after which the weight began to increase. Feed intake in group II was found to be decreased, and *vice versa* in group III and group IV. During the study period, no deaths were recorded with the formulation and norfloxacin (antibiotic)-treated groups, whereas deaths were noted on days 4, 5, 6 and 7 in group II (dysenteric control). The presence of wet faces was decreased in the formulation and norfloxacin (antibiotic)-treated groups (Table 2).

Table 2. Indicate the presence of dry faeces (denoted by “-” signs) and wet faeces (denoted by “+” signs) in different rat groups

S.No	Groups	Days				
		D0	D1	D3	D5	D7
1.	Group 1 (NC)	-	-	-	-	-
2.	Group 2 (DC)	-	+	+	+	+
3.	Group 3 (HT)	-	+	+	-	-
4.	Group 4 (AT)	-	+	+	-	-

Where NC: normal control, DC: diseased (dysenteric) control; HT: herbal treated (250 mg/kg); AT: antibiotic (20 mg/kg).

Stool bacterial density, frequency, and colon bacterial density

In the stools of group II animals, *Shigella* density increased significantly ($P < 0.05$) after dysentery appeared: 6.5×10^8 and 8×10^8 by the 1st and 3rd days, respectively, vs. 1×10^8 administered. In comparison with the negative control and the initial value (0 days), norfloxacin (a well-known antibiotic) significantly reduced *Shigella* density from the 2nd day (5.0×10^8) to the 7th day (0.5×10^8) after the start of treatment, which indicates its pharmacological significance. The polyherbal formulation also inhibited bacterial growth from the 2nd day

(5.5×10^8) to the 7th day (1.0×10^8) after the start of therapy, parallel to norfloxacin (Fig. 3).

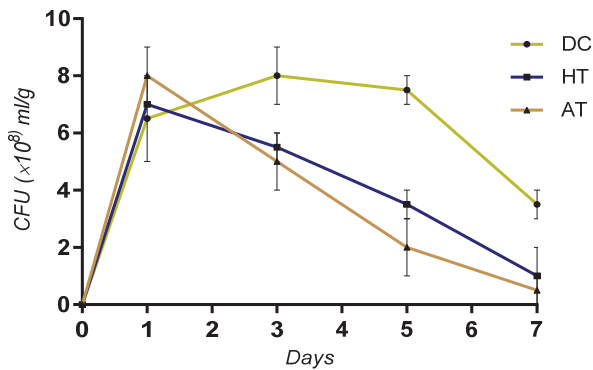


Fig. 3. CFU (colony forming unit) of different groups (DC = diseased control; HT = herbal treated and AT = antibiotic treated) excreta collected on different days D0, D1, D3, D5, and D7 respectively. Data $P < 0.05$ is considered as significant.

Total stool frequency was increasing in the dysenteric rats (groups II) from the 1st day of the experiment. In contrast, from the 2nd day of treatment, the total stool frequency started to significantly decrease in herbal formulation and antibiotic-treated rats (Fig. 4).

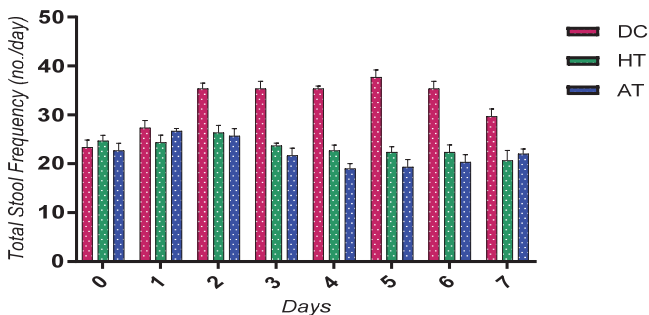


Fig. 4. Total stool frequency during the treatment of *S. flexneri* induced rats with the formulation and antibiotic groups (DC = diseased control; HT = herbal treated and AT = antibiotic treated). Each data column has the mean \pm S.D.

The *Shigella* density in the colon of herbal and antibiotic-treated groups was found to be significantly on the lower side ($P < 0.05$) when compared with the dysenteric groups (Fig. 5).

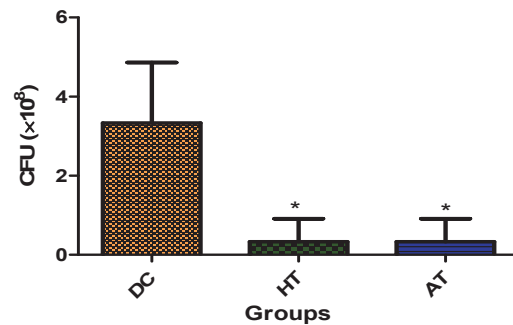


Fig. 5. CFU (colony forming unit) of different groups (DC = diseased control; HT = herbal treated and AT = antibiotic treated) colon. Error bars indicate the \pm standard deviations of three measurements. Data $P < 0.05$ is considered as significant (One-way ANOVA).

Both the treatment, polyherbal formulation, and norfloxacin noticeably reduced the dysenteric stool rate, stool frequency, and *Shigella* density in the colon.

Organ weight analysis

The isolated organs of the animals were weighed, as shown in Table 3.

Interestingly, it was found that the weight of different organs (liver, kidney, and colon) increased in comparison to the dysenteric control group organ weight.

Complete blood count and electrolyte chemistry analysis

Blood was collected from different groups of animals on the 7th day. It was found that both tests, as well as standard antibiotic-treated groups, show hematological data that is close to the control group. Approximately all the blood analysis values were found decreased in the dysenteric control (DC) group (Table 4).

A similar pattern was also observed in the electrolyte chemistry analysis (Fig. 6).

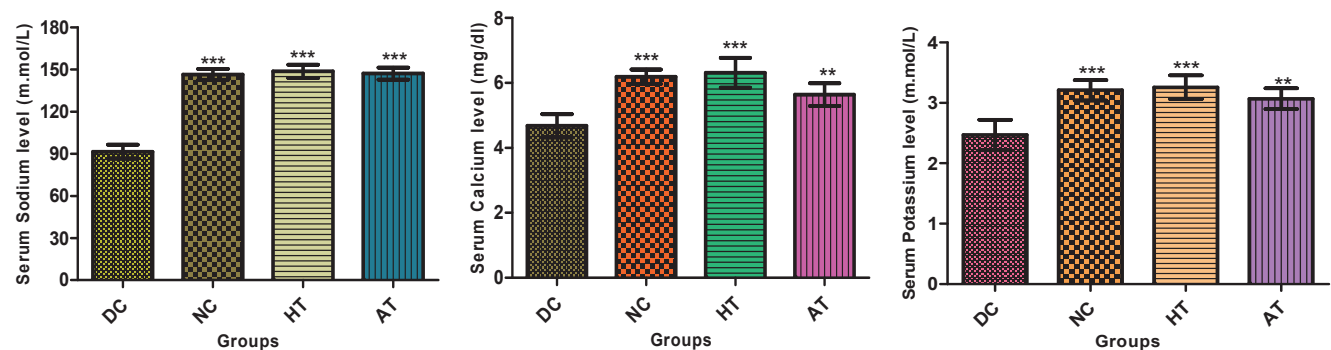


Fig. 6. Sodium, calcium, and potassium serum levels in *S. flexneri* infected rats after treatment with formulation and norfloxacin. The data represent the mean \pm standard deviation for five rats per group ($n = 5$). Value ** $p < 0.01$ and *** $p < 0.001$ is considered significant in comparison with the DC (disease control) (One-way ANOVA). HT: herbal treated (250 mg/kg); AT: antibiotic treated (20 mg/kg); NC: normal control; DC: diseased (dysenteric) control.

Table 3. Effect of designed formulation on the weight of rat's organs

Organ weight	Normal control (NC)	Diseased control (DC)	Herbal treated (HT)	Antibiotic treated (AT)
Liver	4.5 ± 0.37	3.6 ± 0.37	4.7 ± 0.41	4.8 ± 0.26
Kidney	1.7 ± 0.1	1.4 ± 0.15	1.8 ± 0.17*	1.83 ± 0.15
Colon	2.13 ± 0.25	1.76 ± 0.15	2.1 ± 0.2	2.36 ± 0.21

Formulation and antibiotics were administered to rats at a dose of 250 mg/kg and 20 mg/kg, respectively. The data represent the mean ± standard deviation. Value * $p < 0.05$, is considered significant.

Table 4. Effect of designed herbal formulation on blood cell in different rat groups

Parameters	Diseased control (DC)	Normal control (NC)	Herbal treated (HT)	Antibiotics treated (AT)
Haemoglobin (g/dl)	10.6 ± 0.39	13.5 ± 0.44***	12.9 ± 0.57***	12.05 ± 0.56**
TLC (Cells/cu.mm.)	4255 ± 121	6050 ± 218***	6150 ± 427***	6200 ± 223***
DLC (%)	Polymorphs	36.6 ± 2.3	46.4 ± 3.3***	40 ± 2.9 ^{ns}
	Lymphocytes	56.6 ± 2.8	44.5 ± 3.2***	49.6 ± 2.9*
	Eosinophils	2 ± 1	1.6 ± 0.8 ^{ns}	3 ± 1.2 ^{ns}
	Monocytes	6.4 ± 1.8	7.6 ± 1.6 ^{ns}	7.4 ± 1.1 ^{ns}
Platelet (Lacs/cu.mm)	6.0 ± 0.04	7.1 ± 0.10***	7.5 ± 0.33***	7.5 ± 0.05***
RBCs	4.8 ± 0.21	6.8 ± 0.37***	6.5 ± 0.12***	6.0 ± 0.30***

The data represent the mean ± standard deviation for five rats per group ($n = 5$). TLC: total leukocyte count; DLC: differential leukocyte count; RBC: red blood cell. Value * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ is considered significant in comparison with the DC (disease control) and ns is non-significant (One-way ANOVA).

Discussion

Bacillary dysentery is an infectious intestinal disease, frequent in rural communities. It has been determined as the main cause of mortality and morbidity among children, due to the increasing infection cases and the emergence of resistance among *Shigella* strains (Taneja and Mewara, 2016). Polyherbal formulations are a mixture of more than one plant species and will display a better therapeutic effect than single species (Sujatha and Shalin, 2012). Plant extracts and essential oil are natural candidates that attenuate microbial pathogenesis by interfering with the biofilm and quorum sensing system (Kalia et al., 2020). The disruption of the microbial cell to cell communication by oils and plant extracts is due to their various active compounds that mimic the quorum sensing signaling molecules, or may accelerate their degradation (Vattem et al., 2007). The formulation reduces the *S. flexneri* (67.94%), *S. enterica* (65.56%), *E. coli* (51.94%) biofilm formation in comparison with the control, and also possesses anti-QS and antimicrobial properties (Alibi et al., 2020; Koh and Tham, 2011; Singh et al., 2021).

The oral acute toxicity of polyherbal formulation was performed according to the OECD 423 guidelines, and the result reveals that no hair loss, no behavioral change, no mortality, and no signs of toxicity were recorded during 14 days of observation (Singh and Agarwal, 2021a). The polyherbal formulation was devoid of any toxicity in rats, when given in doses of up to 2000 mg/kg.

In the dysenteric control group, *S. flexneri* induced dysentery was identified by soft bloody stools, emitting a foul odor, being less motile, and decreased body weight (Sharma et al., 2017; Singh and Agarwal, 2022). Two hours after bacterial inoculum, i.p. (intraperitoneal) administration, animals became calm, curled up, and showed less movement. Stools of

dysenteric group rats emitted a foul odor each time when the faeces appeared soft, molded, and lumpy, or had blood marks. Stools containing blood served as primary symptoms of bacillary dysentery (Sharma et al., 2017). Research studies have shown that the bacteria in the stool and colon were found to be increased in dysenteric groups, while a decrease in bacterial density was found in the case of treated groups (Kouitchou et al., 2013; Limsuwan et al., 2020; Singh and Agarwal, 2022; Yang et al., 2014).

An increase in bacterial (*Shigella*) load, as well as in the number of bloody stools was recorded in dysenteric control group rats; maybe due to bacterial overgrowth and, consequently, the production of Shiga toxin, and destruction of intestinal tissue and defense cells. All of these are typical symptoms of dysentery infection with invasive germs (Hodges and Gill, 2010). The produced Shiga toxin is responsible for the inflammation in the intestinal mucosa, the result of which includes alteration of electrolyte absorption and an increase in bacterial load. Consequently, this will explain the decrease noticed in Na^+ , K^+ , and Ca^{++} concentrations in non-treated (dysenteric rat) animals (Jorum et al., 2016). Dysenteric rats treated with norfloxacin or herbal formulation exhibit stools (without mucus or blood) and a reduced *Shigella* load. This decrease in *Shigella* bacterial load could be the result of a direct action of formulation bioactive compounds. Formulation protects dysenteric rats from weight loss. The formulation promotes weight gain, which may be due to the stimulation of the appetite, or due to the reduction of electrolyte and water losses (Noubissi et al., 2019; Singh and Agarwal, 2022).

Blood in stool is a sign of destruction or invasion of the host's intestine epithelial cells, which results in bleeding and ulceration. Therefore, an increase in weight loss, bacterial load, and also decrease in Na^+ , Ca^{++} , and K^+ ions in blood concentrations, along with a decrease in white blood (WBCs) and red blood cells (RBCs), is observed. A decrease in bacterial load and

increase in Na^+ , Ca^{++} , and K^+ ions, as well as RBCs and WBCs, were noticed in herbal treated animal groups, and may indicate that, like other plants and herbal formulation, the developed formulation also has immune-modulatory properties (Jorum et al., 2016). A possible mechanism is due to the chemical interaction of phytochemicals with the pathogen, or it may be because of the effect of different phytochemicals present in the formulation on the body's immune systems/homeostasis (Singh and Agarwal, 2022).

Conclusions

Due to overuse and misuse of antibiotics, antibiotic resistance in *S. flexneri* has increased. This increasing multi-drug resistance in *Shigella* spp. is a matter of concern. A polyherbal formulation comprising *Terminalia chebula* fruit, *Camellia sinensis* leaf, *Citrus lemon* fruit extract, and *Thyme*, and *Cinnamon* oil, was developed for the treatment of bacillary dysentery. The *in vitro* study results reveal that the developed formulation inhibits the bacterial growth, its biofilm, and were also have bactericidal effect. The *in vivo* toxicity analysis also proves that the composition is non-toxic and safe up to 2000 mg/kg.

Further, in an *in vivo* study, the formulation prevented infected animals from bloody stools, and effectively decreased the bacterial load in treated infected rats. Also, it increases the K^+ , Na^+ , and Ca^{++} ions in blood concentrations, along with increasing white blood and red blood cells. These findings support the use of the polyherbal formulation in the treatment of bacillary dysentery.

Ethical aspects and conflict of interests

The authors have no conflict of interests to declare.

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