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Crude extracts from *Diospyros gillettii* stem bark attenuate *Shigella flexneri*-induced diarrhea in mice

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ABSTRACT

Objective: To evaluate the anti-shigellosis activity of the hydroethanol extract of *Diospyros gillettii* (*D. gillettii*) stem bark in *Shigella flexneri* (*S. flexneri*)-induced diarrheal mice.

Methods: The hydroethanolic extract was obtained by maceration of *D. gillettii* stem bark in 70% hydroethanol (water:ethanol; 30:70, v/v) solution. Then, mice pretreated with cyclophosphamide for immunosuppression were administered orally with an inoculum containing *S. flexneri*, and subsequently treated with 100, 200, and 400 mg/kg of the hydroethanol extracts for 10 days. The bacterial colonies were enumerated and hematological and biochemical parameters were determined. Serum pro-inflammatory mediators including IL-1 β , IL-18, and TNF- α , and nitric oxide levels were quantified by ELISA. Histological analyses of the kidney, liver, and colon were also conducted.

Results: Treatment with 200 and 400 mg/kg of the hydroethanolic extracts markedly inhibited the growth of *S. flexneri*. Moreover, treatment with *D. gillettii* extract downregulated the levels of IL-1 β , IL-18, and TNF- α , and restored hematological and biochemical parameters as well as histological architecture of the colon, liver, and kidneys. Additionally, the oral administration of 2000 mg/kg *D. gillettii* extract did not induce any sign of toxicity, with a median lethal dose greater than 2000 mg/kg.

Conclusions: *D. gillettii* extract demonstrates the anti-shigellosis effects in *S. flexneri*-induced diarrheal mice, supporting the traditional use of this plant in treating diarrhea.

KEYWORDS: *Diospyros gillettii*; *Shigella flexneri*; Diarrhea; Pro-

inflammatory cytokines; Acute toxicity; Shigellosis

1. Introduction

Shigellosis is a gastrointestinal disease caused by bacteria of the genus *Shigella* and transmitted through consumption of spoiled

Summary

Question: Does *Diospyros gillettii* stem bark extract exhibit antidiarrheal activity in *Shigella flexneri*-induced diarrheal mice?

Findings: The crude extract from *Diospyros gillettii* stem bark attenuated *Shigella flexneri*-induced diarrhea in mice by improving hematological and biochemical parameters, reducing inflammation as well as mitigating histological changes in colonic, hepatic, and renal tissues.

Meaning: This study provides evidence supporting the ethnopharmacological use of *Diospyros gillettii* in treating diarrhea conditions.

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food, poor hygiene, or *via* contact from one individual to another[1]. These pathogens include four main species *viz.* *Shigella flexneri* (*S. flexneri*), *Shigella dysenteriae*, *Shigella sonnei* and *Shigella boydii*[2]. Among these bacteria, *S. flexneri* and *Shigella sonnei* are the principal germs responsible for infectious diarrhea in developing and high-income countries, respectively[3]. *S. flexneri*-caused diarrhea is among the leading causes of death in children under 5 years old[4]. In fact, *Shigella*-caused diarrhea accounted for approximately 212 000 deaths and about 13% of all diarrhea-associated deaths in 2016[1]. The treatment of *Shigella*-induced diarrhea relies on the use of an antimicrobial agent coupled with the addition of zinc in rehydration solutions that improves the effect of water and electrolytes and reinforces the immune response of the intestinal mucosa[5]. An assortment of antibiotics is used as the first (ciprofloxacin) and second (ceftriaxone and azithromycin) line treatments for shigellosis[6]. However, cases of arthropathy (joint toxicity) were reported in children treated with ciprofloxacin[7]. Moreover, a number of reports have pointed out the existence of a high rate of resistance of *Shigella* species to ceftriaxone, azithromycin, and other antibiotics[8]. The development of multi-resistant microbial strains, coupled with the toxicity of these antibiotics, justifies an urgent need to search for alternative medications against *Shigella*-induced diarrhea.

In ethnomedicine, the use of medicinal plants for the treatment of diarrheal conditions is undeniable[9]. For instance, different parts of *Diospyros gillettii* (*D. gillettii*) are ethnobotanically used to cure several diseases, including diarrheal infections[10]. Other species of the genus *Diospyros* have also been utilized in traditional medicine to overcome diarrhea[10]. For example, indigenous people from the Central African Region cure diarrhea or dysentery by using *Diospyros mespiliformis* (leaves and bark) decoctions[11,12]. Accumulated evidence has shown that a number of African and Asian countries use *Diospyros ferrea* fruits, *Diospyros mafiensis*, and *Diospyros rotundifolia* roots in their respective decoction forms to relieve diarrheal conditions[13,14]. Previous phytochemical studies of *D. gillettii* allowed for the isolation and characterization of a number of compound classes, including coumarins (bergenin and norbergenin), naphthoquinones, terpenoids, and flavonoids, and others[15–17]. Tameye[18] identified numerous compounds from *D. gillettii*, including seven coumarins [11-*O*-(*E*)-cinnamoylnorbergenin (leaves), norbergenin, 4-*O*-galloylnorbergenin and 4-*O*-*p*-hydroxybenzoylnorbergenin (leaves, stem bark, and twigs), perallylated derivative of 4-*O*-*p*-hydroxybenzoylnorbergenin and peracetylated derivative of 4-*O*-*p*-hydroxybenzoylnorbergenin (stem bark), and 11-*O*-*p*-hydroxybenzoylnorbergenin (twigs)]; three pentacyclic triterpene acids [ursolic acid and ursolic acid 28-allyle (leaves), and corosolic acid (leaves and twigs)] and five pentacyclic triterpenoids [oleanolic (leaves) and rotundic (twigs) acids, betulin (leaves and twigs), betulinic acid (leaves and stem bark), and lupeol (leaves, stem bark and twigs)].

In a recently published paper, we demonstrated the *in vitro* anti-

Shigella effects of methanol, ethanol and hydroethanolic extracts on four *Shigella* species, with hydroethanolic extract being the most potent and less toxic antibacterial extract[10]. The antibacterial mechanism of action was also elucidated against the most susceptible strain (*S. flexneri*)[10].

In our continuous efforts to search for alternative therapies against infectious diarrhea, this research sought to investigate the effects of the hydroethanolic extract prepared from the stem bark of *D. gillettii* on *S. flexneri*-induced diarrhea in mice.

2. Materials and methods

2.1. Plant extraction

The hydroethanolic extract was obtained by maceration of *D. gillettii* stem barks as previously described by Nguelo Talla *et al.*[10]. The obtained extract was kept at 4 °C until use.

2.2. Animals and ethical statement

Inbred mice (Balb/c; *Mus musculus*) of both sexes, aged from 1 to 2 months and weighing between 20 to 25 g were used in the *in vivo* experiments. They were reared (by using standard animal pellets and water *ad libitum*) at the Animal House of the Department of Animal Physiology, Faculty of Science and University of Yaounde 1. An authorization for animal studies was acquired from the National Animal Ethics Committee, Government of Cameroon (reference number: FW-IRB00001954, dated 15th July 2017). Mice were arbitrarily grouped into 6 animals each and acclimatized under normal experimental settings for further *in vivo* tests.

2.3. Bacterial strain

The *in vivo* antibacterial activity was performed using the bacterial strain *S. flexneri* NR 518, which was acquired from the Biodefense and Emerging Infections Research Resources Repository (BEI Resources, Rockville, United States of America). The bacterium was sustained in an uninterrupted culture in test tubes on Mueller Hinton Agar slope (4 °C) at the Laboratory for Phytobiochemistry and Medicinal Plant Studies, Faculty of Science and the University of Yaounde 1, Cameroon.

2.4. *In vivo* antibacterial tests

2.4.1. Immunosuppression of mice

To prevent the immune system from disease intervention in *S. flexneri*-induced diarrheal mice, immunosuppression of the animals was performed two days before infection through an intraperitoneal administration of 30 mg/kg of cyclophosphamide. During this period, the mice were also deparasitized with streptomycin (200 mg/kg/day)

to further enhance colonization of *S. flexneri* within the colon.

2.4.2. Infection and treatment

Shigellosis was induced in mice *via* an oral administration of *S. flexneri* inoculum (0.25 mL), adjusted to 4.0 McFarland Standard by colony transfer of bacteria cultivated on Mueller Hinton Agar (containing approximately 1.2×10^9 CFU/mL).

Thirty-six mice, which were allocated into six groups of 6 mice each, were treated for 10 days from Day 3 post infection as follows: Group I received only distilled water (10 mL/kg) (normal control group). Groups II-VI were infected with *S. flexneri* as described above, and then administered with 2% dimethyl sulfoxide (DMSO) in water (negative control), ciprofloxacin (30 mg/kg) (positive control), and 100, 200, and 400 mg/kg of hydroethanolic extracts, respectively. The animals were observed during 4 hours post-treatment. During this period, changes in mobility, volume of stools, presence or absence of mucus in the stools, and number of *Shigella* colonies were recorded in the experimental animals. During the treatment period (10 d), stools were collected daily for the evaluation of the bacterial load.

2.4.3. Quantification of the bacterial load in the stools

To evaluate the density of *S. flexneri*, 0.1 g of stool was collected from each mouse and dissolved in 5 mL of normal saline, followed by a series of five dilutions using the dilution factor 100 000. From the as-prepared stool solutions, 100 μ L were transferred onto Petri dishes containing SSA (*Salmonella-Shigella* Agar) medium. After 24 h of incubation, the bacterial load was determined by direct counting of the *S. flexneri* colonies. The aspect (feature) of the collected fresh stools was also classified according to several criteria, such as consistency (0: normal or compact; 1: liquid), presence of mucus (0: absence of mucus; 1: presence of mucus), and color (0: normal or dark, 1: brownish and 2: yellowish)[19].

2.4.4. Sacrifice of the animals

At the end of the experimental period, the treated mice were fasted overnight and then sacrificed using anesthetic chloroform. Afterward, the blood was collected from the retro-orbital plexus in EDTA tubes for the determination of the hematological parameters and in normal tubes (free of anticoagulants) for serum preparation, which was further preserved at low temperature (-80°C) for biochemical analyses. Next, the colon, spleen, liver, and kidneys were collected from mice, defatted, weighed, and kept (in 10% formalin) for histological analyses.

2.4.5. Hematological analysis

Hematological parameters, such as levels of blood (red and white) cells, hemoglobin, and hematocrit, as well as lymphocytes, granulocytes, monocytes and platelets were determined by standard clinical procedures using an automatic hematological analyzer.

2.4.6. Serum biochemistry

To obtain the serum, the blood samples, which were collected in tubes without anticoagulants, were subjected to centrifugation (4000 rpm) at 4°C for 10 min. Analysis of biochemical parameters, such as aspartate transaminase (AST), creatinine, alanine transaminase (ALT), cholesterol, nitric oxide (NO), total bilirubin, urea, and proteins was conducted using respective reagent kits. Moreover, the levels of the cytokines including TNF- α , IL-1 β , and IL-18 were estimated with enzyme-linked immunosorbent assay (ELISA) kits and a spectrophotometer for the reading.

2.4.7. Sample preparation for histopathology

The colon, liver, and kidneys were collected from treated mice and preserved in 10% formalin, followed by sample dehydration using graded concentrations of ethanol. Afterward, the tissue preparations were sequentially embedded in parafilm. Then, the thick sections (4-5 μ m) were stained using hematoxylin-eosin staining solution. The as-prepared sections were further examined under a light microscope (Olympus microscope; Shinjuku City, Tokyo, Japan) connected to a computer. Microscopic photographs of arbitrary observations were taken up at $400\times$ using the software Minisee version 1.0.

2.5. Acute toxicity

The hydroethanol extract of *D. gillettii* was suspended in 2% DMSO in distilled water and a single dose of 2000 mg/kg was administered orally to a group of female mice (5 mice) (group 1) according to the guidelines (number 420) established by the Organization for Economic Co-operation and Development, whereas the vehicle control group (group 2; 5 mice) received no extract treatment. Afterward, the mice were observed for mortality, and changes in posture, mobility, piloerection, and respiratory pattern during the first 12 hours post-treatment. The animals were weighed every other day and observed for 2 weeks following treatment. Next, the LD₅₀ was calculated as the minimum dose required to kill half of the test animals.

2.6. Statistical analysis

The results were presented as mean \pm standard deviation (mean \pm SD). Statistical analysis was carried out between control and experimental groups by one-way analysis of variance (ANOVA) followed by the Dunnett test using Graph Pad Prism 8.0.1 software. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of *D. gillettii* extract on the body weights of mice

The oral administration of *S. flexneri* suspension (1.2×10^9 CFU/mL)

to balb/c mice induced a significant reduction of body weights in infected mice. However, the oral treatment of mice with the hydroethanol extract (200 and 400 mg/kg) restored the body weights to normal from day 4 and day 7, respectively (Supplementary Figure 1). However, the infected animals that did not receive extract or drug treatment experienced diarrheal conditions until completion of the experiment with a drastic decrease in their body weights.

3.2. Effects of the hydroethanolic extract of *D. gillettii* on the relative weights of mouse organs

There were no statistically significant differences ($P > 0.05$) in the relative organ weights of mice challenged with *S. flexneri* infection and administered with different *D. gillettii* extracts (100, 200, and 400 mg/kg), compared to the group that only received distilled water (negative control) (Supplementary Figure 2).

3.3. Effects of the extract from *D. gillettii* stem bark on the length of the colon

Figure 1 indicates the effects of the hydroethanolic extract of *D. gillettii* on the colon length of mice challenged orally with *S. flexneri* suspension and further treated with the plant extract. The hydroethanolic extract of *D. gillettii* prevented the shortening of the colon compared with the untreated negative control group, and the significant effect was observed at 200 mg/kg.

3.4. Influence of the hydroethanolic extract of *D. gillettii* on the fecal incontinence

Figure 2 exemplifies the effects of the plant extract on the aspect of the feces after treatment of *S. flexneri*-infected mice for 10 d. The oral administration of *S. flexneri* to mice induced severe diarrhea with a mean score of 6. Treatment with the hydroethanolic extract of *D. gillettii* stem bark induced a dose-dependent reduction in the frequency of diarrhea in *S. flexneri*-infected mice (Figure 2). Mice that received extract treatment at 100, 200, and 400 mg/kg were completely cured of diarrhea after 10, 8, and 7 days of treatment, respectively.

3.5. Effects of the hydroethanolic extract of *D. gillettii* on the bacterial load in *S. flexneri*-induced diarrheal mice

A significant increase in the bacterial load was observed in *S. flexneri*-infected mice. However, the oral administration of the hydroethanol extract from *D. gillettii* stem bark caused a substantial decrease in the bacterial load in *S. flexneri*-induced diarrheal mice.

Twenty-four hours were more than sufficient to obtain a bacterial population of approximately 28×10^6 CFU/mL in mice. Thereafter, 10 days' treatment of infected animals with three doses (100–400 mg/kg) of *D. gillettii* extract led to a significant decrease in the bacterial population

with complete elimination of the bacterial growth on day 10 and day 7 post-infection for 200 and 400 mg/kg, respectively (Figure 3).

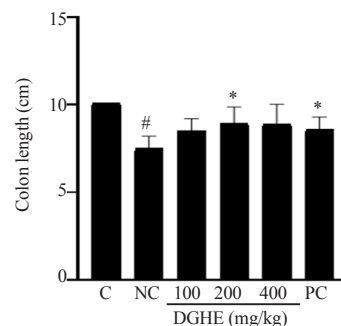


Figure 1. Effects of the hydroethanolic extract from *Diospyros gillettii* (*D. gillettii*) on colon length of *Shigella flexneri* (*S. flexneri*)-induced diarrheal mice. The results are presented as mean \pm standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA) followed by the Dunnett test. # $P < 0.05$ compared with the normal control; * $P < 0.05$ compared with the negative control. DGHE: hydroethanol extract of *D. gillettii*; C: normal control; NC: negative control (untreated *S. flexneri*-infected mice); PC: positive control [ciprofloxacin (30 mg/kg)].

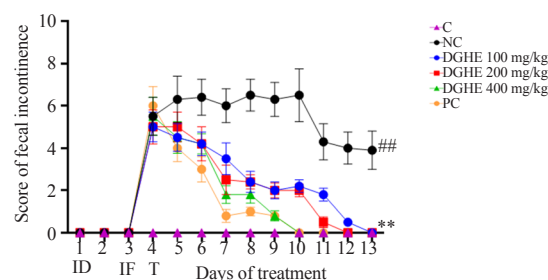


Figure 2. Effect of the hydroethanolic extract of *D. gillettii* on fecal incontinence in *Shigella*-induced diarrheal mice. The results are presented as mean \pm SD and analyzed by ANOVA followed by the Dunnett test. ## $P < 0.001$ compared with the normal control; ** $P < 0.001$ compared with the negative control. ID: immunodepression; IF: infection; T: the date of initiating extract treatment.

3.6. Influence of the hydroethanolic extract of *D. gillettii* on hematological parameters of mice

Table 1 summarizes the hematological parameters of animals challenged with *S. flexneri* infection and administered with different doses of *D. gillettii*. Upon infection with *S. flexneri*, a significant decrease in the number of blood (red and white) cells, lymphocytes, monocytes, granulocytes, and platelets, as well as hematocrit and hemoglobin levels was noteworthy. Treatment with *D. gillettii* extract (100–400 mg/kg) augmented the levels of hemoglobin, hematocrit, lymphocytes, monocytes, granulocytes, and platelets (Table 1).

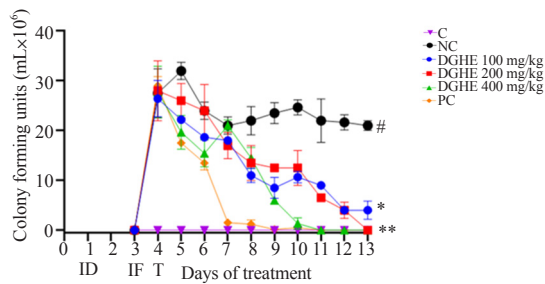


Figure 3. Effects of *D. gillettii* hydroethanolic extract on bacterial population in *S. flexneri*-induced diarrheal mice. The results are presented as mean±SD and analyzed by ANOVA followed by the Dunnett test. # $P < 0.05$ compared with the normal control; * $P < 0.05$, ** $P < 0.001$ compared with the negative control.

3.7. Effects of the hydroethanolic extract of *D. gillettii* stem bark on biochemical parameters of mice

A significant augmentation in the activity of serum ALT and AST was noted in *S. flexneri*-induced diarrheal mice. Treatment with the hydroethanolic extract induced a significant ($P < 0.001$) decline in AST and ALT levels of infected mice (Supplementary Figure 3). The level of bilirubin was significantly increased in infected mice ($P < 0.05$), however, this trend was reversed ($P < 0.05$) by the administration of the hydroethanolic extract (Supplementary Figure 4).

3.8. Effects of the hydroethanolic extract on urea and creatinine levels in mice infected with *S. flexneri*

A substantial increase in urea and creatinine levels was observed in infected mice. Treatment with 400 mg/kg of extract significantly ($P < 0.05$) reduced urea level. Moreover, 200 and 400 mg/kg of plant extract induced a decline in the creatinine level in *S. flexneri*-infected mice (Supplementary Figure 4).

3.9. Effects of the hydroethanolic extract on serum protein levels in mice infected with *S. flexneri*

Mice challenged with *S. flexneri* showed significantly decreased protein levels. The administration of the hydroethanolic extract at all doses reversed the reduced protein level in infected mice, with 100 mg/kg showing the best effect ($P < 0.05$) (Supplementary Figure 5).

3.10. Impact of the hydroethanolic extract on serum total cholesterol

Cholesterol level was markedly enhanced in *S. flexneri*-infected mice ($P < 0.05$). All doses of *D. gillettii* extract significantly ($P < 0.05$) decreased cholesterol levels (Supplementary Figure 5).

3.11. Effects of the hydroethanolic extract of *D. gillettii* on pro-inflammatory cytokines

A rise in IL-1 β , IL-18, and TNF- α was found in infected mice. The hydroethanolic extract of *D. gillettii* (400 mg/kg) significantly ($P < 0.05$) reduced the levels of IL-1 β and TNF- α in *S. flexneri*-induced diarrheal mice (Figure 4).

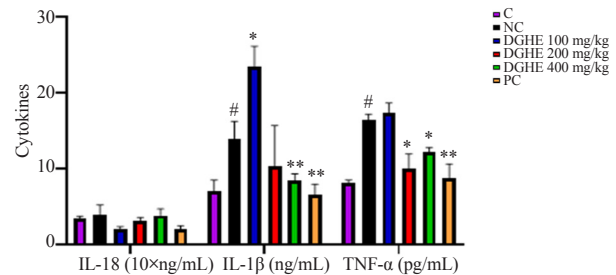


Figure 4. Effects of the hydroethanolic extract of *D. gillettii* on the serum level of IL-18, IL-1 β , and TNF- α in *S. flexneri*-induced diarrheal mice. The results are presented as mean±SD and analyzed by ANOVA followed by the Dunnett test. # $P < 0.05$ compared with the normal control; * $P < 0.05$, ** $P < 0.001$ compared with the negative control.

Table 1. Effects of the hydroethanolic extract of *D. gillettii* on hematological parameters of infected mice.

Parameters	Normal control	Negative control	Positive control	DGHE 100 mg/kg	DGHE 200 mg/kg	DGHE 400 mg/kg
RBC ($10^3/\mu\text{L}$)	9.03±0.22	6.35±0.31 [#]	8.59±0.32 [*]	8.50±0.16 [*]	8.84±0.29 [*]	8.94±0.29 [*]
HGB (g/dL)	15.90±0.29	10.25±0.32 ^{##}	15.03±0.75 [*]	14.38±0.91 [*]	14.33±0.64 [*]	15.50±0.78 [*]
HCT (%)	43.08±0.13	38.10±1.71 ^{##}	42.45±1.34 [*]	42.43±0.80 [*]	43.37±0.14 [*]	43.60±0.98 [*]
WBC ($10^3/\mu\text{L}$)	9.20±0.90	7.12±0.62 [#]	7.93±0.65	8.55±0.31	8.97±0.28	8.43±0.12
LYM ($10^3/\mu\text{L}$)	3.13±0.85	1.60±0.66 ^{##}	2.83±0.46 [*]	2.20±0.57 [*]	2.63±0.46 [*]	2.63±0.49 [*]
MON ($10^3/\mu\text{L}$)	1.07±0.21	0.48±0.12 ^{##}	0.67±0.23 [*]	0.73±0.28 [*]	0.78±0.15 [*]	1.03±0.34 [*]
GRA ($10^3/\mu\text{L}$)	4.67±0.93	2.78±0.61 [#]	4.30±0.37 [*]	4.37±0.42 [*]	3.93±0.70 [*]	3.93±0.50 [*]
PLA ($10^3/\mu\text{L}$)	422.00±21.57	235.00±33.78 ^{##}	456.50±25.09 ^{**}	433.60±24.09 ^{**}	458.67±1.41 ^{**}	432.00±23.26 ^{**}

The results were presented as mean ± standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA) followed by the Dunnett test. # $P < 0.05$ and ## $P < 0.001$ compared with the normal control group; * $P < 0.05$ and ** $P < 0.001$ compared with the negative control. RBC: red blood cells; HGB: hemoglobin; HCT: hematocrit; WBC: white blood cells; LYM: lymphocytes; MON: monocytes; GRA: granulocytes; PLA: platelets.

3.12. Influence of the hydroethanolic extract of *D. gillettii* on NO levels

The oral gavage of a suspension containing 1.2×10^9 CFU/mL of *S. flexneri* to mice led to a significant ($P < 0.001$) elevation in NO levels. After treatment of the infected mice with the hydroethanolic extract of *D. gillettii* (200 and 400 mg/kg), the levels of NO were significantly decreased (Figure 5).

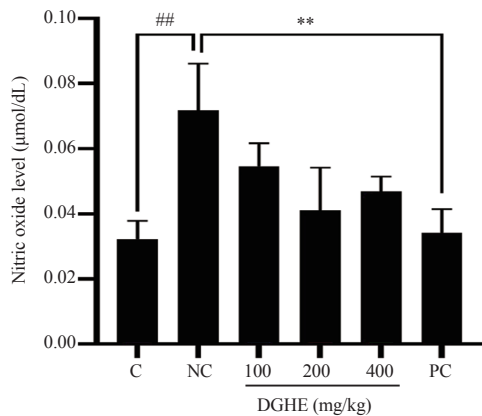


Figure 5. Effects of the hydroethanolic extract of *D. gillettii* on the levels of nitric oxide in *S. flexneri*-induced diarrheal mice. $###P < 0.001$ compared with the normal control; $**P < 0.001$ compared with the negative control.

3.13. Effects of hydroethanolic extract of *D. gillettii* on the architecture of different organs of mice infected with *S. flexneri*

3.13.1. Influence of the hydroethanolic extract on the histology of mouse liver

As shown in Supplementary Figure 6B, vascular congestion of the hepatic central vein, intense inflammation of hepatic cells, and leukocyte infiltration were observed in *S. flexneri*-infected mice. However, treatment with 100, 200, and 400 mg/kg of the extract alleviated these abnormal changes (Supplementary Figure 6D-F).

3.13.2. Effect of extract on the histology of the kidneys

Supplementary Figure 7A presents normal renal parenchyma, distinct glomerula, clear Bowman's or urinary space, and well-differentiated proximal and distal bypass tubes. However, mice infected with *S. flexneri* showed narrowing of the urinary space, cell degeneration and swelling, and undifferentiated proximal and distal bypass tubes (Supplementary Figure 7B). Infected mice treated with plant extract at 100 (Supplementary Figure 7D), 200 (Supplementary Figure 7E), and 400 (Supplementary Figure 7F) mg/kg showed a normal architecture of the renal parenchyma.

3.13.3. Effect of extract on the histology of the colon

The normal mice showed normal architecture of the intestinal tissue with three main clear layers, *viz.* mucosa, submucosa, and the

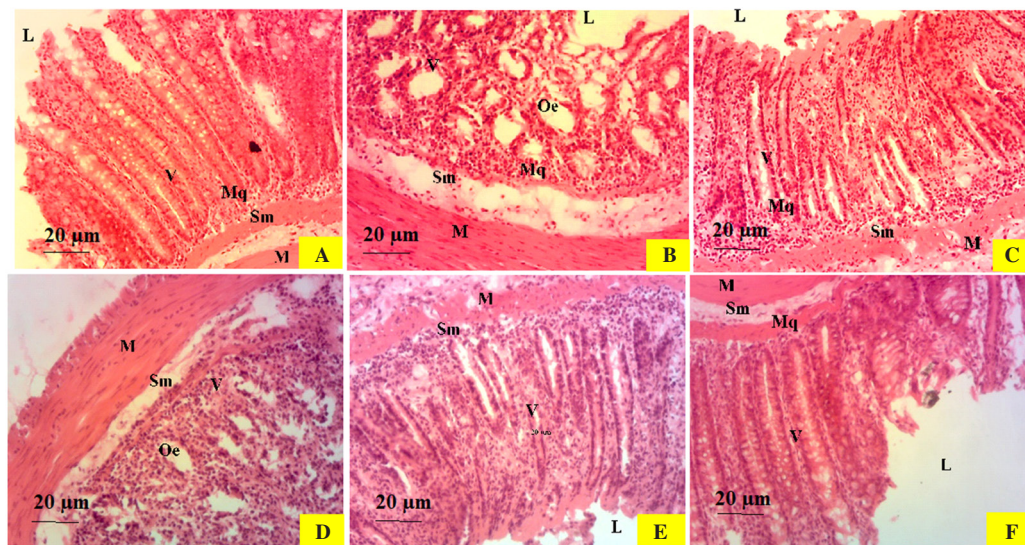


Figure 6. Microphotographs of the colon of mice challenged with *S. flexneri* and treated with the hydroethanol extract of *D. gillettii* (Hematoxylin-eosin $\times 100$; scale bar=20 μm). A: Uninfected mice that received only distilled water (normal control); B: Mice challenged with *S. flexneri* infection and left untreated (negative control); C: Mice infected with *S. flexneri* and treated with ciprofloxacin (30 mg/kg); D, E and F: Mice challenged with *S. flexneri* infection and treated using 100, 200 and 400 mg/kg of hydroethanol extract of *D. gillettii*. L: intestinal lumen; M: muscular layer; Mq: mucosa; Oe: oedema due to degeneration of villi; Sm: submucosa; V: intestinal villi.

muscular layer.

In mice infected with *S. flexneri*, the architecture of the colon was disorganized with edema, and the integrity of their intestinal villi was damaged (Figure 6B). After treatment of *S. flexneri*-infected mice with 200 (Figure 6E) and 400 (Figure 6F) mg/kg of *D. gillettii* extract, the abnormalities or changes caused by the infection were restored.

3.14. Acute toxicity

No death or adverse effect was observed in treated and control groups of animals after 14 days of observation, and the LD₅₀ of the hydroethanol extract of *D. gillettii* was considered to be more than 2000 mg/kg.

4. Discussion

The emergence of germs, such as *Shigella* species that are resistant to a number of antibiotics poses a serious threat to humankind. This alarming situation has compelled several researchers to search for alternative therapies against *Shigella*-caused diarrhea. Since medicinal plants represent a good source of active compounds that could be prospected as potential leads for the discovery of effective antibiotics, our previous report highlighted the inhibitory effects of crude extracts from *D. gillettii* stem bark against a panel of *Shigella* species and their potential mechanisms of action, thus substantiating the ethnopharmacological use of this plant as a remedy for diarrheal conditions[10]. In brief, methanol, ethanol, and hydroethanolic extracts, which were prepared from *D. gillettii* stem bark were evaluated for *in vitro* antibacterial activity against four *Shigella* species, including *S. flexneri* NR 518, *Shigella boydii* NR 521, *Shigella sonnei* NR 519 and *Shigella dysenteriae* CPC[10]. Although the degree of the antibacterial activity of *D. gillettii* methanol extract was similar to that of hydroethanol extract, the latter was chosen for *in vivo* studies due to palm wine extraction (maceration, decoction, etc.) that is mostly preferred traditionally to extract plant secondary metabolites over other alcoholic solvents. Based on these considerations, this research sought to investigate the *in vivo* anti-shigellosis activity of *D. gillettii* stem bark in *Shigella*-induced diarrheal mice. Accordingly, the hydroethanolic extract of *D. gillettii* stem bark, which was found to be the most potent and less toxic extract upon *in vitro* anti-*Shigella* screening (100<minimum inhibitory concentrations <512 µg/mL) in our previous report[10], was selected for *in vivo* studies in *S. flexneri*-infected mice. Noteworthy, a number of recently published papers demonstrated the susceptibility of *Shigella* species to botanicals from plant species, such as *Thymus vulgaris* and *Ocimum basilicum*[20], *Cardiospermum grandiflorum*, and *Blighia welwitschii*[21], and even *D. gillettii*[10].

In this study, the oral gavage of 1.2×10^9 CFU/mL of *S. flexneri* to mice caused a number of diarrheal symptoms, including weight loss, shortening of the colon length, excessive secretion of mucous, and watery and bloody stools. Moreover, increased bacterial load was identified in the feces of infected mice. Further, oral administration of different doses of the hydroethanol extract of *D. gillettii* stem bark (100-400 mg/kg) for 10 d induced a significant decline in the fecal bacterial load. Indeed, the animals that received 200 and 400 mg/kg of extract were completely recovered from diarrhea after 10 and 7 days of treatment, respectively. The antidiarrheal activity of this plant may be linked to its antibacterial effects on *S. flexneri*. Moreover, there was a slight variation of the bacterial load (from day 0 and day 10 post-infection) in infected and untreated animals; however, this difference might be consistent with the involvement of the immune system in the disease condition. Accumulated evidence has shown that *D. gillettii* contains a number of secondary metabolites, including terpenoids (lupeol and betulinic acid), coumarins (4-*O*-galloylnorbergenin and norbergenin), pentacyclic triterpenes (corosolic and ursolic acid) and sterols (cholesterol and β -sitosterol)[10,18,22]. These metabolites might have contributed to the anti-shigellosis effects displayed by *D. gillettii* extract. Moreover, the antibacterial activity of coumarins, such as bergenin and its derivatives has already been described across the literature[18,23]. The mechanistic basis of the antibacterial action of bergenin and its congeners includes free radical scavenging and lipid peroxidation inhibitory activities[10,24], apoptosis and cell cycle arrest, and cell death[25]. Nyemb *et al.*[25] demonstrated that bergenin can easily traverse the highly asymmetric complex lipid bilayer of the outer membrane of bacteria to cause lysis and cell death[25].

Once ingested, *Shigella* initiates an anti-inflammatory cascade following invasion into the small intestine, colonic epithelium, and lamina propria, where it generates enterotoxins and other toxin types to cause infectious diarrhea.

The oral administration of *S. flexneri* to mice induced a significant reduction in the levels of hematological (blood cells, hemoglobin, platelets, and hematocrit) parameters. This observation might be attributed to severe anemia caused by bloody diarrhea[26]. However, these parameters returned to normal after treatment with the hydroethanol extract (100, 200, and 400 mg/kg). The levels of serum ALT, AST, and bilirubin were significantly increased in mice infected orally with *S. flexneri*, indicative of liver damage by the pathogen[27]. However, the oral gavage of infected animals with various doses of *D. gillettii* extract (100-400 mg/kg) reversed these changes. Additionally, failure of creatinine and urea levels to decrease in the blood is the best indicator of kidney damage[28]. Kidneys filter creatinine from the blood to eliminate it through the urine; however, the increase of creatinine in the blood indicates kidney dysfunction[29]. There is accumulated evidence showing that as *Shigella* enters the peritoneal cavity, it inserts a range of effector

proteins *via* type three secretion system (T3SS) to alter and colonize the muscular part of the colon, and further invade the lamina propria, then the lumen[30,31]. The infection of mice with *S. flexneri* induced an increase in serum cholesterol and a decrease in total proteins; however, treatment with *D. gilletti* extract normalized the levels of cholesterol and total proteins. Gururaja *et al.*[32] have previously demonstrated the efficacy of plant extracts in lowering the level of serum cholesterol in small animal models[32].

In addition, the release of pro-inflammatory cytokines has intricately been incriminated in the pathogenesis of several human infections, such as bacterial diarrhea[33,34]. In this study, the levels of IL-1 β , IL-18, and TNF- α were increased in *S. flexneri*-infected animals in response to the activation of macrophages, endothelial, dendritic, and other immune cells. The oral treatment of *S. flexneri*-infected mice with *D. gilletti* incited a decline in the levels of TNF- α , IL-1 β , and IL-18. This result shows that the hydroethanol extract of *D. gilletti* might have improved the immune system functions by downregulation of TNF- α , IL-1 β , and IL-18 levels as previously demonstrated in a report by Gasmi *et al.*[35]. Accumulated evidence highlights the role of secondary metabolites, such as steroids[36], flavonoids (naringenin)[37], coumarins[38], phenolics[39], *etc.* in the downregulation of certain pro-inflammatory cytokines[40]. Since *D. gilletti* is well known to contain most of these compounds[16–18], it is reasonable to postulate that these metabolites might have aided in the reduction of the levels of cytokines. The reduction of the NO level following the oral gavage of infected mice with *D. gilletti* extract is consistent with the involvement of this plant extract in the regulation of the immune system[41].

In the histological analyses, the mice challenged with *S. flexneri* infection and subsequently administered with different doses (200 and 400 mg/kg) of hydroethanol extract of *D. gilletti* showed normal architecture of the liver and kidneys. These findings corroborate with the data obtained from analyses of serum biochemical markers of the liver (ALT, AST, and bilirubin) and kidneys (creatinine and urea) that returned to normalcy after treatment with *D. gilletti* extract. Moreover, the microphotographs of the colon of mice challenged with *S. flexneri* infection and administered with *D. gilletti* extract showed an improved and normal architecture of the colon.

Regarding the toxicity experiment, a single oral gavage of the hydroethanolic extract of *D. gilletti* at 2000 mg/kg revealed no mortality in mice, and the LD₅₀ was considered to be more than 2000 mg/kg. *D. gilletti* extract was considered a nontoxic extract because the dose of NOAEL (no observed adverse effects level) was found to be more than 2000 mg/kg.

Overall, this research demonstrates the *in vivo* anti-shigellosis effects of the hydroethanolic extract of *D. gilletti* stem bark in *S. flexneri*-induced diarrheal mice, as evidenced by improved biochemical and inflammatory parameters, and ameliorated histological changes in the architecture of the colon, liver and

kidney.

Nevertheless, there are some limitations in this study, such as the identification of active compounds of the bioactive extract, subacute and chronic toxicity studies of *D. gilletti* extract, and pharmacokinetic studies of the bioactive extract. Therefore, additional studies, including in-depth toxicity (sub-acute and chronic toxicities, *etc.*) and pharmacokinetic studies, as well as bio-guided fractionation of the most active anti-*Shigella* extract from *D. gilletti* are warranted to support the effective application of *D. gilletti* as a therapeutic for infectious diarrhea.

This research sought to evaluate the *in vivo* anti-*Shigella* effects of *D. gilletti* extract in *S. flexneri*-induced diarrheal mice. Balb/c albino mice, which were orally challenged with 1.2×10^9 CFU/mL of *S. flexneri* were subsequently administered with three doses (100, 200, and 400 mg/kg) of hydroethanolic extract of *D. gilletti* stem bark for 10 d. As a result, *D. gilletti* extract notably decreased the bacterial load in stools of treated mice by comparison with the negative control group. Moreover, the oral gavage of mice with 200 and 400 mg/kg of *D. gilletti* extract restored hematological and biochemical parameters, which were altered in untreated *S. flexneri*-infected mice. In mice challenged with *S. flexneri* infection and administered with *D. gilletti* extracts, the histological sections of the liver, kidneys, and colon showed normal architecture of these organs, whereas the untreated *S. flexneri*-infected mice presented disorganized organs with loss of integrity and edema. Mice challenged orally with *S. flexneri* suspension and treated with the extracts significantly reduced the levels of TNF- α , IL-1 β and IL-18, as well as NO, inferring that *D. gilletti* possesses immunomodulatory effects, which might have contributed to the antibacterial activity. The single oral gavage of hydroethanol extract of *D. gilletti* at 2000 mg/kg did not induce any mortality in mice, with an LD₅₀ value of >2000 mg/kg. These findings spotlight the anti-shigellosis potency of *D. gilletti* hydroethanol extract and recommend this plant as a promising alternative for shigellosis's treatment.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding authors upon request.

Authors' contributions

PKL, BPK and FFB designed the work, administered and supervised the project, and contributed to the final version of the manuscript, and approved the final version to be published. BLNK, JMTN, LMNN, TEN, VN and YKDM collected, analyzed and interpreted the data, and contributed to the final version of the manuscript, and approved the final version to be published.

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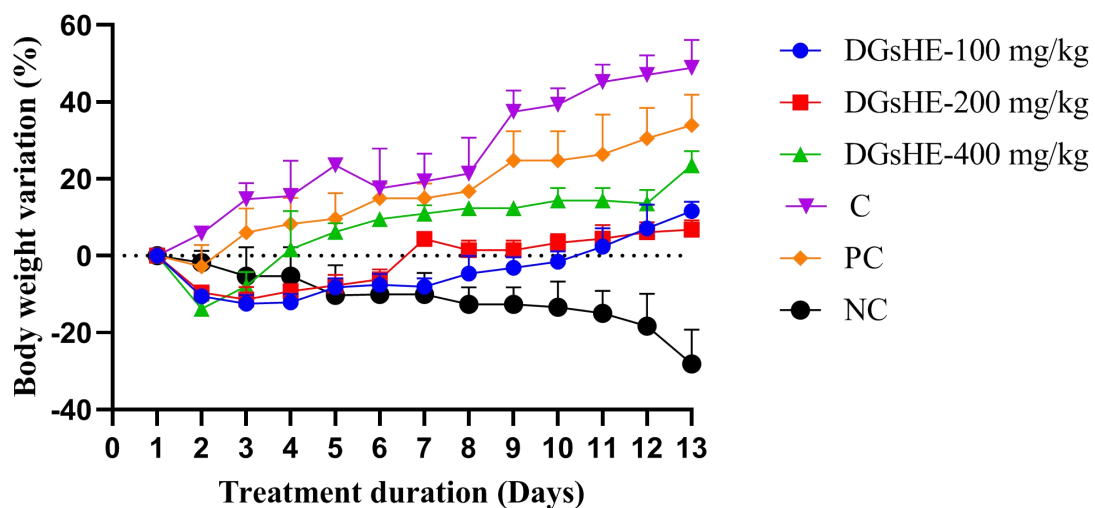
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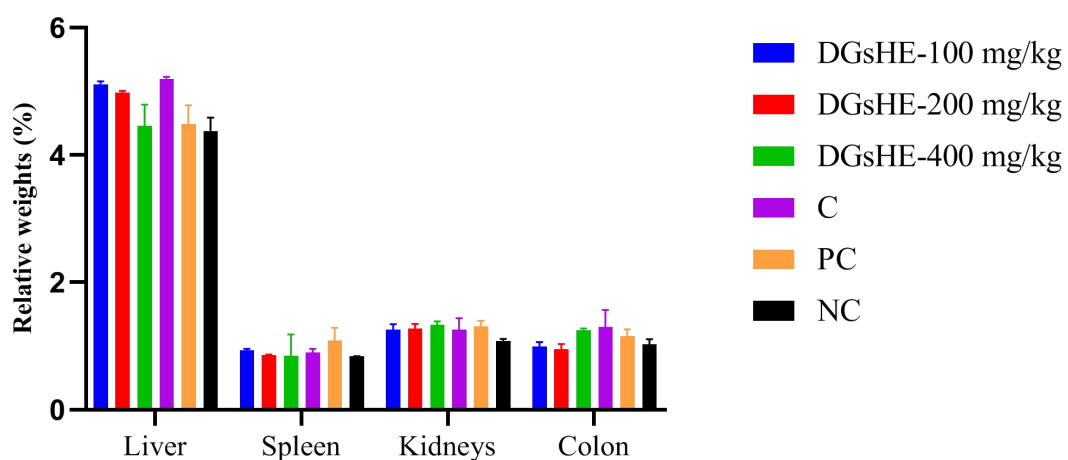
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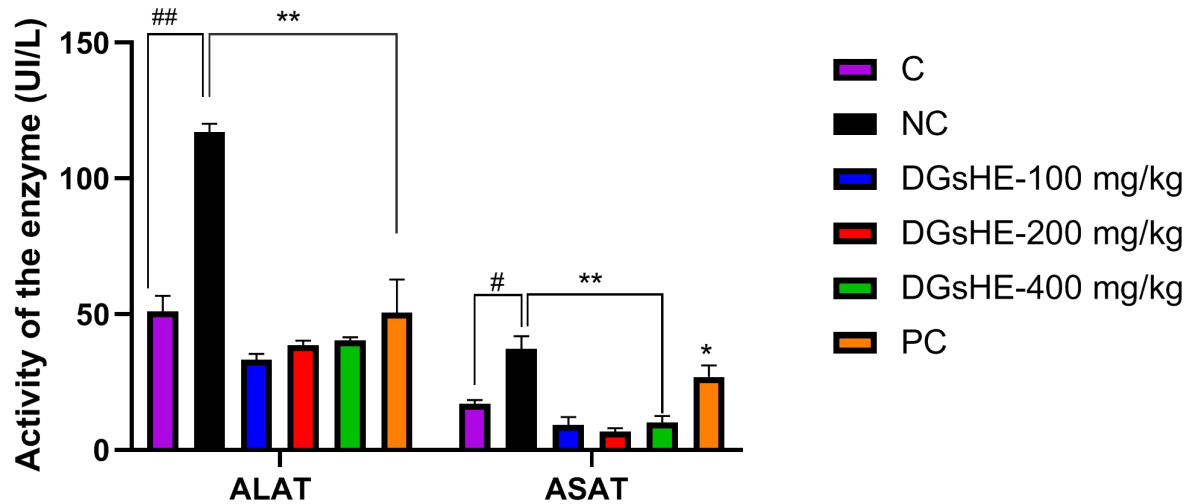
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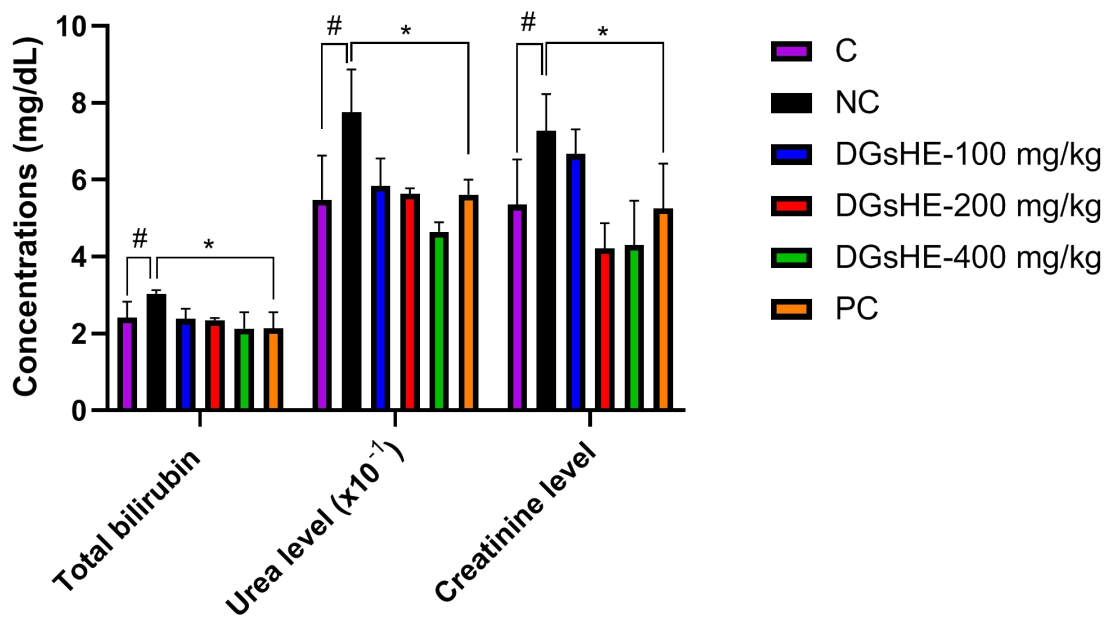
Supplementary Figure 1. Effects of the hydroethanolic extract of *D. gillettii* on the variation of body weights. **DGsHE-100 mg/kg:** Hydroethanol extract of *D. gillettii* at 100 mg/kg; **DGsHE-200 mg/kg:** Hydroethanol extract of *D. gillettii* at 200 mg/kg; **DGsHE-400 mg/kg:** Hydroethanol extract of *D. gillettii* at 400 mg/kg; **C:** Normal control; **NC:** Negative control; **PC:** Positive control.



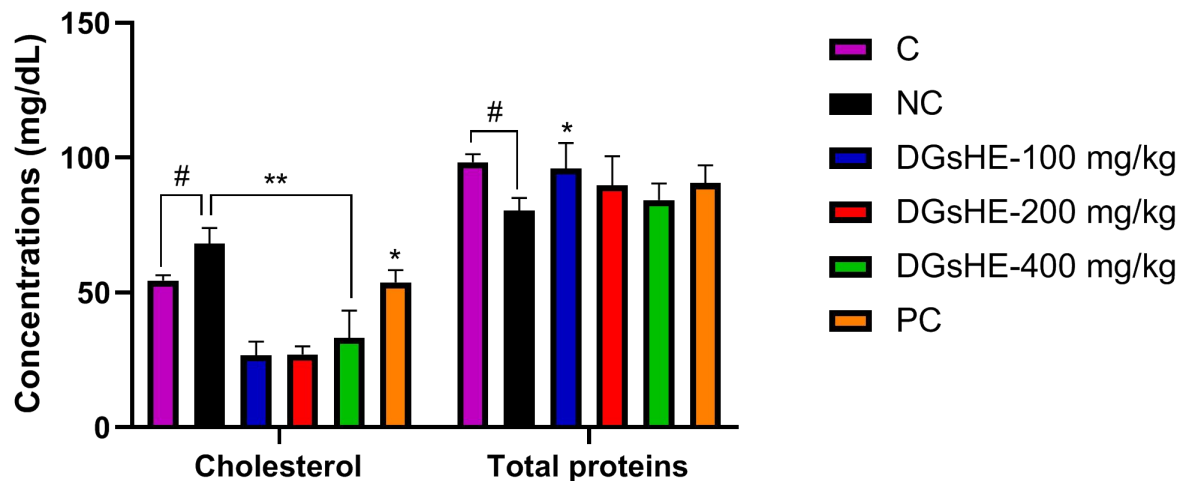
Supplementary Figure 2. Effects of the hydroethanolic extract from *Diospyros gillettii* (*D. gillettii*) stem bark on the relative weights of organs in *Shigella*-induced diarrheal mice. **DgsHE 100, 200 and 400 mg/kg:** hydroethanol extract of *D. gillettii* at 100, 200 and 400 mg/kg; **C:** Normal control; **NC:** negative control; **PC:** positive control.



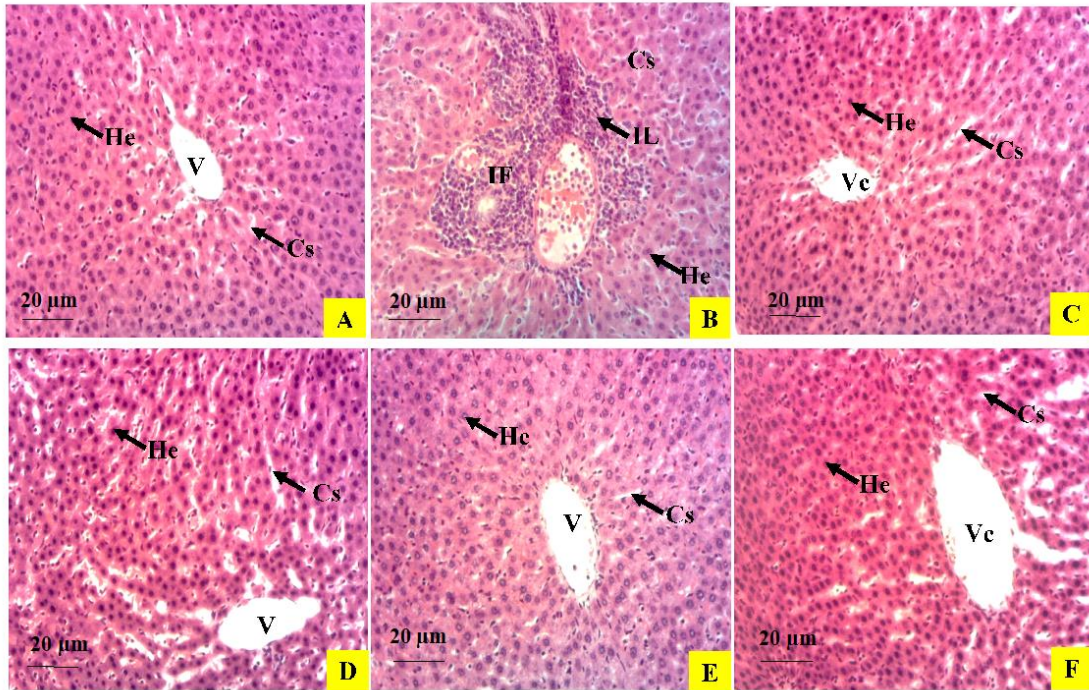
Supplementary Figure 3. Effects of the hydroethanolic extract of *D. gillettii* stem bark on the levels of alanine transaminase (ALAT) and aspartate transaminase (ASAT) in *Shigella flexneri*-induced diarrheal mice. #P<0.05, ##P<0.001 compared with the normal control; *P<0.05, **P<0.001 compared with the negative control.



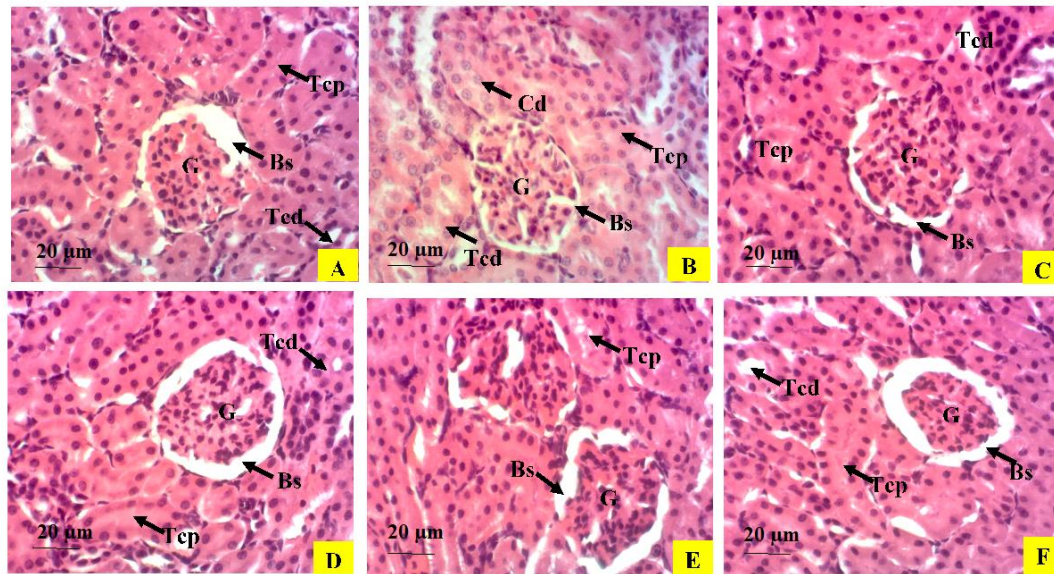
Supplementary Figure 4. Influence of the hydroethanolic extract of *D. gillettii* on the levels of bilirubin, urea and creatinine in mice infected with *Shigella flexneri*. #P<0.05 compared with the normal control; *P<0.05 compared with the negative control.



Supplementary Figure 5. Effects of the hydroethanolic extract of *D. gillettii* on the levels of serum cholesterol and total protein in mice infected with *Shigella flexneri*. #P<0.05 compared with the normal control; *P<0.05, **P<0.001 compared with the negative control.



Supplementary Figure 6. Microphotographs of the liver of mice challenged with *Shigella flexneri* infection and administered with the hydroethanol extract of *D. gilletti* (Hematoxylin-eosin $\times 100$; scale bar=20 μm). A: Uninfected mice that received distilled water (normal control); B: Mice infected with *Shigella flexneri* and left untreated (vehicle control); C: Mice infected with *Shigella flexneri* and subsequently treated with ciprofloxacin (30 mg/kg); D, E and F: Mice challenged with *Shigella flexneri* ingestion and administered with 100, 200, and 400 mg/kg of hydroethanolic extract of *D. gilletti*; Cs: sinusoidal capillaries; IF: inflammation; He: hepatocytes; IL: intense infiltration of leucocytes; V: intestinal villi; Vc: hepatic central vein.



Supplementary Figure 7. Microphotographs of the kidneys of mice challenged with *Shigella flexneri* infection and administered with the hydroethanolic extract of *D. gilletti* (Hematoxylin-eosin $\times 100$; scale bar=20 μm). A: Uninfected mice that received only distilled water (normal control); B: Mice challenged with *Shigella flexneri* infection and left untreated (vehicle control); C: Mice infected with *Shigella flexneri* and treated with ciprofloxacin (30 mg/kg); D, E and F: Mice challenged with *Shigella flexneri* infection and treated using 100, 200 and 400 mg/kg of hydroethanol extract of *D. gilletti*; Cd: cell degeneration ; Bs: Bowman's or urinary space; G: gomerula; Tcd: differentiated distal bypass tubes; Tcp: differentiated proximal bypass tubes.