

Article

Anti-Shigellosis Activity and Mechanisms of Action of Extracts from *Diospyros gillettii* Stem Bark

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Abstract: Shigellosis is a pathological condition that affects the digestive system and possibly causes diarrhoea. *Shigella* species, which are responsible for this disease, are highly contagious and spread through contaminated food and water. The increasing development of resistance by *Shigella* species necessitates the urgent need to search for new therapies against diarrhoea-causing shigellosis. The scientific validation of medicinal plants, such as *Diospyros gillettii*, which is used for the traditional treatment of diarrhoeal conditions is worthwhile. The present study aims to investigate the antibacterial activity of extracts from *D. gillettii* against selected *Shigella* species. Extracts from *D. gillettii* stem bark were prepared by maceration using various solvents. The antibacterial activity of *D. gillettii* extracts was evaluated in *Shigella dysenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei* using a microdilution method, whereas a cytotoxicity test was performed on Vero and Raw cells using resazurin-based colorimetric assays. Bacterial membrane-permeability studies were evaluated using propidium iodide (PI)- and 1-N-phenyl-naphthylamine (NPN)-uptake assays, whereas inhibition and eradication tests on bacterial biofilms were carried out by spectrophotometry. As a result, methanol, ethanol and hydroethanol (water: ethanol; 30:70, v/v) extracts of *D. gillettii* inhibited the growth of *S. boydii*, *S. flexneri* and *S. sonnei*, with minimum inhibitory concentration (MIC) values ranging from 125 to 500 µg/mL, without toxicity to Vero and Raw cells. Time-kill kinetics revealed bactericidal orientation at 2 MIC and 4 MIC and a bacteriostatic outcome at 1/2 MIC. The mechanistic basis of antibacterial action revealed that *D. gillettii* extracts inhibited and eradicated *Shigella* biofilms and promoted the accumulation of NPN and PI within the inner and outer membranes of bacteria to increase membrane permeability, thereby causing membrane damage. This novel contribution toward the antibacterial mechanisms of action of *D. gillettii* extracts against *Shigella* species substantiates the use of this plant in the traditional treatment of infectious diarrhoea.

Keywords: *Diospyros gillettii*; diarrhoea; anti-shigellosis activity; cytotoxicity; mechanisms of action



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1. Introduction

Shigella spp. are Gram-negative and facultative anaerobic bacteria that can easily spread from one person to another and cause an enteric infection called shigellosis or bacillary dysentery [1]. Species of *Shigella* that are responsible for pathogenic diarrhoea include *Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii* and *Shigella sonnei* [2]. Infectious diarrhoea has been the cause of high morbidity and mortality in developing countries, especially in

sub-Saharan Africa and South Asia [2,3]. Recent estimates pointed out about 188 million cases of *Shigella* infection per year with 1 million deaths. In fact, there are nearly 1.7 billion cases of childhood diarrhoeal disease every year, with approximately 525,000 deaths of those under five [4]. Even children who survive diarrhoeal diseases face the likelihood of repeated infections and subsequent health issues over the rest of their lives. The 2015 estimates noted 30,000,000 severe cases of diarrhoea in Africa with 330,000 deaths [5]. In a study conducted by Njunda et al. [6], 4.5% of the cases of *Shigella* infection were recorded among children under 15 years old after stool examination in the Buea Health District, Buea, and the Southwest Region of Cameroon. It has also been reported that infantile diarrhoea is among the main causes of death in children under 5 years old in Cameroon, together with malaria, measles, and respiratory tract disease [7]. Humans are the main reservoir of *Shigella* bacteria since there is well-established human-to-human transmission by a fecal–oral route or the ingestion of contaminated food and/or water [8]. When *Shigella* species enter the digestive system, they are able to resist the acidity of the stomach to reach the small intestine and multiply. Next, *Shigella* migrates to the small intestine and produces enterotoxins and serotype toxin 1, which are responsible for watery and bloody diarrhoea. It takes only 12 h to 3 days to observe the first clinical symptoms following contact with *Shigella* species [8]. Common manifestations of *Shigella* infection include vomiting, fever, tenesmus, headache, abdominal pain, and bloody mucoid diarrhoea [9,10]. Current treatments for infectious diarrhoea include the use of antibiotics, such as ciprofloxacin, ceftriaxone, and azithromycin [11]. However, the development of resistance by *Shigella* species toward these antibiotics has resulted in their inefficiency [12]. In addition to drug resistance, the problem of toxicity has rendered these drugs useless. Thus, there is an urgent need to search for safe and effective anti-shigellosis drugs. Research on the use of plants to treat infectious diseases has significantly contributed to the discovery of a number of anti-infective agents, including colchicine, emetine, quinine, berberine, and so on. These essential medicines, which are powered by plants, have prompted many scientists to work on plants with the intention of developing safe therapies. For example, oregano oil encompasses the two most powerful antibacterial and antifungal compounds, namely carvacrol and thymol [13]. Numerous active antimicrobial principles (reserpine, opium, menthol, and allicin), which were isolated, respectively, from plants, such as *Vinca minor*, *Papaver somniferum*, *Mentha piperita*, and *Allium cepa*, were found to inhibit the growth of a number of bacterial and fungal strains.

Based on these considerations, the search for safe antimicrobial agents might be inspired by active secondary metabolites from plants. One such plant is *Diospyros gillettii*, which contains several compounds that were found to inhibit the growth of Gram-positive (*Bacillus subtilis* DSMZ 704, *Micrococcus luteus* DSMZ 1605, *Staphylococcus warneri* DSMZ 20036) and Gram-negative (*Escherichia coli* DSMZ 1058 and *Pseudomonas agarici* DSMZ 11810) bacteria [14]. As a matter of fact, the chemistry of *Diospyros gillettii* and other *Diospyros* species has been studied and reported by several authors [14–18]. The research by Tameye [17], which detailed the extraction and isolation of pure compounds in various parts (leaves, stem bark, and twigs) of *Diospyros gillettii*, led to the identification of seven coumarins: 11-*O*-(*E*)-cinnamoylnorbergenin (1) (leaves), 4-*O*-*p*-hydroxybenzoylnorbergenin (2), 4-*O*-galloylnorbergenin (3), norbergenin (4) (leaves, stem bark, and twigs), a per-acetylated derivative of 4-*O*-*p*-hydroxybenzoylnorbergenin (5), a per-allylated derivative of 4-*O*-*p*-hydroxybenzoylnorbergenin (6) (stem bark), and 11-*O*-*p*-hydroxybenzoylnorbergenin (7) (twigs) (Figure S1, Supplementary Materials); three pentacyclic triterpene acids: ursolic acid (8), ursolic acid 28-allyle (9) (leaves), and corosolic acid (10) (leaves and twigs); and five pentacyclic triterpenoids: lupeol (11) (leaves, stem bark, and twigs), betulin (12) (leaves and twigs), betulinic acid (13) (leaves and stem bark), oleanolic acid (14) (leaves), and ro-tundic acid (15) (twigs) (Figure S2, Supplementary Materials). Compounds 8, 10, 11, 13, and 14 were also identified in *Diospyros fragrans* by the same research group [18]. Furthermore, previous studies by Mallavadhani et al. [19] have shown that *Diospyros* plants afford an incredible amount of naphthoquinones, which can be used as a baseline for taxonomic studies of this plant. As summarized and discussed in Maridass's research paper [20], the

chemistry of *Diospyros* plants has led to the identification of numerous classes of compounds, such as flavonoids, terpenoids, fatty acids, coumarins, and naphthoquinones, among others. More recently, Ribeiro et al. [21] documented the existence of triterpenoids, naphthoquinone, fatty acids, flavonoids, phenols, monoterpenoids, diterpenoids, and others in seventeen *Diospyros* species [15,16,21]. Therefore, there is no denying that *Diospyros gillettii* contains a myriad of secondary metabolites that have been identified and reported thus far. Among these compounds, 4-O-galloylnorbergenin (2), norbergenin (4), ursolic acid (8), corosolic acid (10), lupeol (11), betulin (12), and betulinic acid (13) were reported to exhibit antibacterial activity against bacteria other than *Shigella* species. Indeed, a few authors revealed that extracts and compounds from *D. gillettii* possess antioxidant and antibacterial activities, without cytotoxicity on KB-3-1 (human epithelial carcinoma cells) and HT-29 (human colorectal adenocarcinoma cells with epithelial morphology) cell lines [14,17]. Although little is documented on the ethnomedicinal uses of *D. gillettii*, various organs (leaves, fruits, stem, and bark) of other *Diospyros* species were recorded as being used to treat malaria, headache, dysentery, fever, and diarrhoea [22,23]. The decoction of *D. mafiensis* and *D. rotundifolia* roots and *D. ferrea* fruits are used for the traditional treatment of diarrhoea in Mozambique [24], South Africa [25], and India [26], respectively. The leaf and bark decoction of *D. mespiliformis* is used in the Central African Region to treat malaria, diarrhoea, and dysentery, whereas the roots are used as chewing sticks for oral hygiene [27–29]. In Cameroon, *Diospyros gillettii* is used by the Pygmées Baka people to treat respiratory tract diseases [30].

Meanwhile, a detailed evaluation of the antibacterial activity of *Diospyros gillettii* stem bark against *Shigella* species—the pathogens responsible for infectious diarrhoea—is yet to be unveiled. Thus, the present study aims to investigate the antibacterial activity of *Diospyros gillettii* extracts against a number of *Shigella* species, including *Shigella flexneri*, *Shigella boydii*, *Shigella dysenteriae*, and *Shigella sonnei*. The mechanistic basis of the antibacterial efficacy of *D. gillettii* extracts is also evaluated through biofilm inhibition and eradication and membrane-permeabilization assays.

2. Results

2.1. Yield of Extraction

The yields of extraction were found to be 5.6%, 7.61% and 8.27% for ethanol, methanol and hydroethanol (water/ethanol; 30:70, v/v) extracts, respectively.

2.2. Antibacterial Activity

2.2.1. Minimum Inhibitory Concentrations (MICs)

Table 1 shows the MIC values corresponding to the lowest concentrations of the extracts for which there was no observed growth of *Shigella* spp. The in vitro incubation of *Shigella* species with different plant extracts led to a decrease in the bacterial load as the minimum inhibitory concentrations varied from 125 to 1000 µg/mL. Methanol and hydroethanol extracts were found to be the most active extracts, yielding MIC values of 125 and 250 µg/mL in *Shigella boydii* and *Shigella sonnei*, respectively. *Shigella boydii* (MIC < 250 µg/mL) was found to be the most susceptible strain, whereas *Shigella dysenteriae* (MIC: 1000 µg/mL) was the most resistant strain. Ciprofloxacin, the standard antibiotic compound, presented MIC values varying from 0.010 to 0.039 µg/mL.

Table 1. Minimum inhibitory concentration of extracts in *Shigella* spp.

Sample	Minimum Inhibitory Concentrations (µg/mL) ^a			
	SF NR 518	SB NR 521	SD CPC	SO NR 519
DGsM	250	125	1000	250
DGsE	250	250	1000	250
DGsHE	500	125	1000	250
Ciprofloxacin (PC)	0.010	0.019	0.010	0.039

^a: Values are means of triplicate determination (n = 3); DGsE: Ethanol extract of *Diospyros gillettii*; DGsHE: Hydroethanol extract of *Diospyros gillettii*; DGsM: Methanol extract of *Diospyros gillettii*; PC: Positive control; SF: *Shigella flexneri*; SB: *Shigella boydii*; SD: *Shigella dysenteriae*; CPC: Centre Pasteur of Cameroon; SO: *Shigella sonnei*.

2.2.2. Time-Kill Kinetics

The time-kill kinetics of methanol, ethanol and hydroethanol extracts were evaluated on the most susceptible strains, viz. *S. flexneri* NR 518, *S. boydii* NR 521, and *S. sonnei* NR 519. Figures 1–3 show the curves obtained by plotting optical densities (values that are proportional to the bacterial population) versus the time of incubation of bacteria with methanol, ethanol, and hydroethanol extracts, respectively. After two hours of bacterial incubation with the methanol extract, there was a decreasing trend in the curves, especially at MIC, 2 MIC, and 4 MIC, irrespective of the *Shigella* strains considered (Figure 1). After 10 h of incubation, at MIC, 2 MIC, and 4 MIC, almost all the curves overlapped with the X axis (except for *S. boydii*), suggesting that the bacterial population was cleared, irrespective of the strain considered. This bacterial clearance was later confirmed, as beyond 10 h of incubation (10–24 h), there was no resurgence of the curves, suggesting the bactericidal orientation of the methanol extract at MIC, 2 MIC, and 4 MIC. Treatment with ciprofloxacin showed the same trend, confirming the bactericidal trend of this drug vis-à-vis *Shigella* species. At a concentration less than the MIC (1/2 MIC), there was an increased inclination of the curve from 8 h of incubation time onwards, suggesting a bacteriostatic orientation. By contrast, the curve obtained from the untreated group of bacteria showed an increased trend from time 0 to 24 h, suggesting no inhibition and the exponential growth of bacteria.

Figure 2 shows the growth kinetics of *Shigella* species after incubation with the ethanol extract of *Diospyros gillettii*. Irrespective of the concentration of extract considered (MIC, 2 MIC and 4 MIC), there was a decreased trend in the curves, suggesting the inhibition of the bacterial population. After 10 h of incubation at MIC, 2 MIC, and 4 MIC, all the curves overlapped with the X axis, suggesting the clearance of the bacterial population, irrespective of the strain considered. This bacterial clearance was later confirmed, since beyond 10 h of incubation (10–24 h), the bacteria did not resume growth, suggesting the bactericidal orientation of the ethanol extract at MIC, 2 MIC, and 4 MIC.

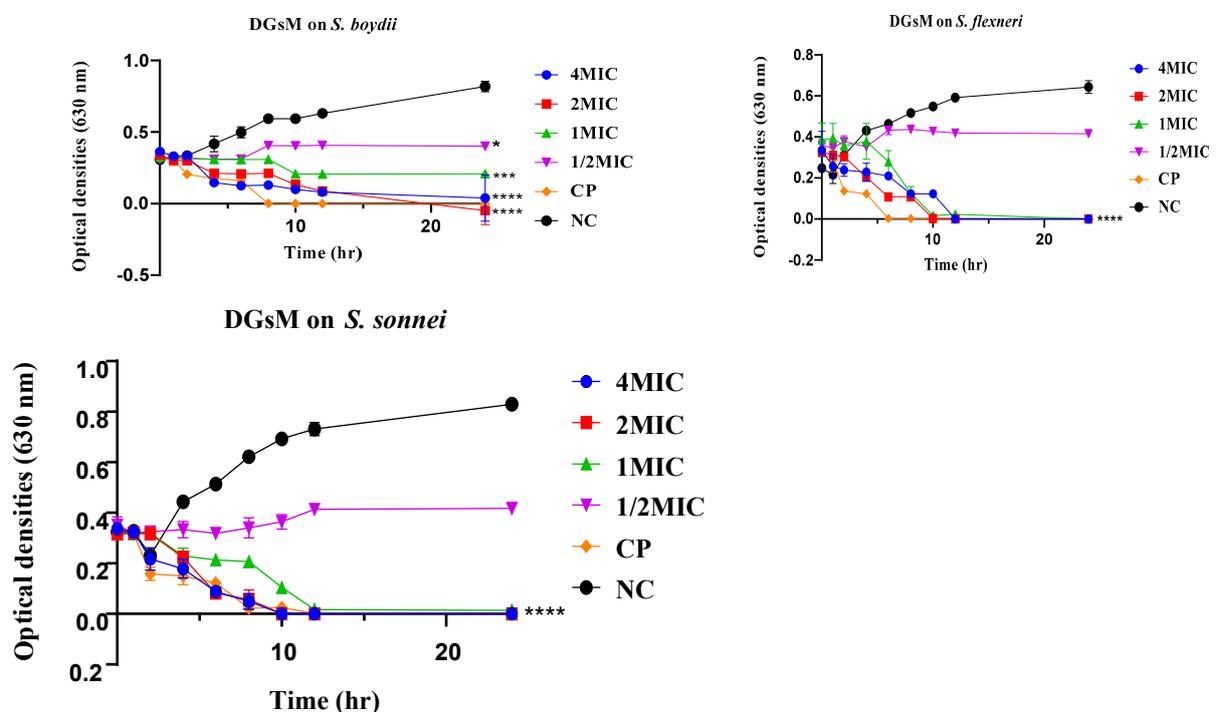


Figure 1. Effects of DGs *Diospyros gillettii* methanolic extracts on the growth kinetics of *S. boydii*, *S. flexneri*, and *S. sonnei*. DGsM: Methanol extract of *Diospyros gillettii*; MIC: Minimum inhibitory concentration; CP: Ciprofloxacin; NC: Negative control; *S. boydii*: *Shigella boydii*; *S. flexneri*: *Shigella flexneri*; *S. sonnei*: *Shigella sonnei*; Data are represented as mean \pm standard deviation; $p < 0.05$ (*), $p \leq 0.001$ (**), $p \leq 0.0001$ (****); Significant difference compared with negative control (Dunnett's test).

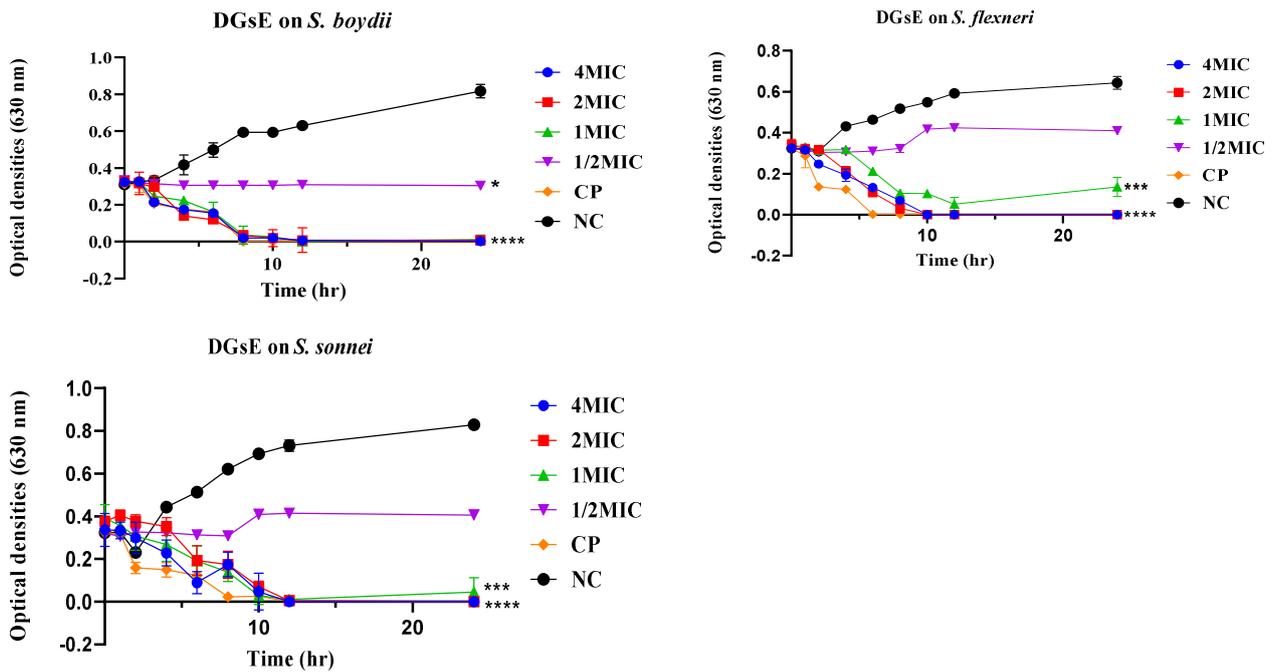


Figure 2. Effects of *Diospyros gillettii* ethanolic extracts on the growth kinetics of *S. boydii*, *S. flexneri*, and *S. sonnei*. DGSE: Ethanol extract of *Diospyros gillettii*; MIC: Minimum inhibitory concentration; CP: Ciprofloxacin; NC: Negative control; *S. boydii*: *Shigella boydii*; *S. flexneri*: *Shigella flexneri*; *S. sonnei*: *Shigella sonnei*. Data are represented as mean \pm standard deviation; $p < 0.05$ (*), $p \leq 0.001$ (**), $p \leq 0.0001$ (****); Significant difference compared with negative control (Dunnett’s test).

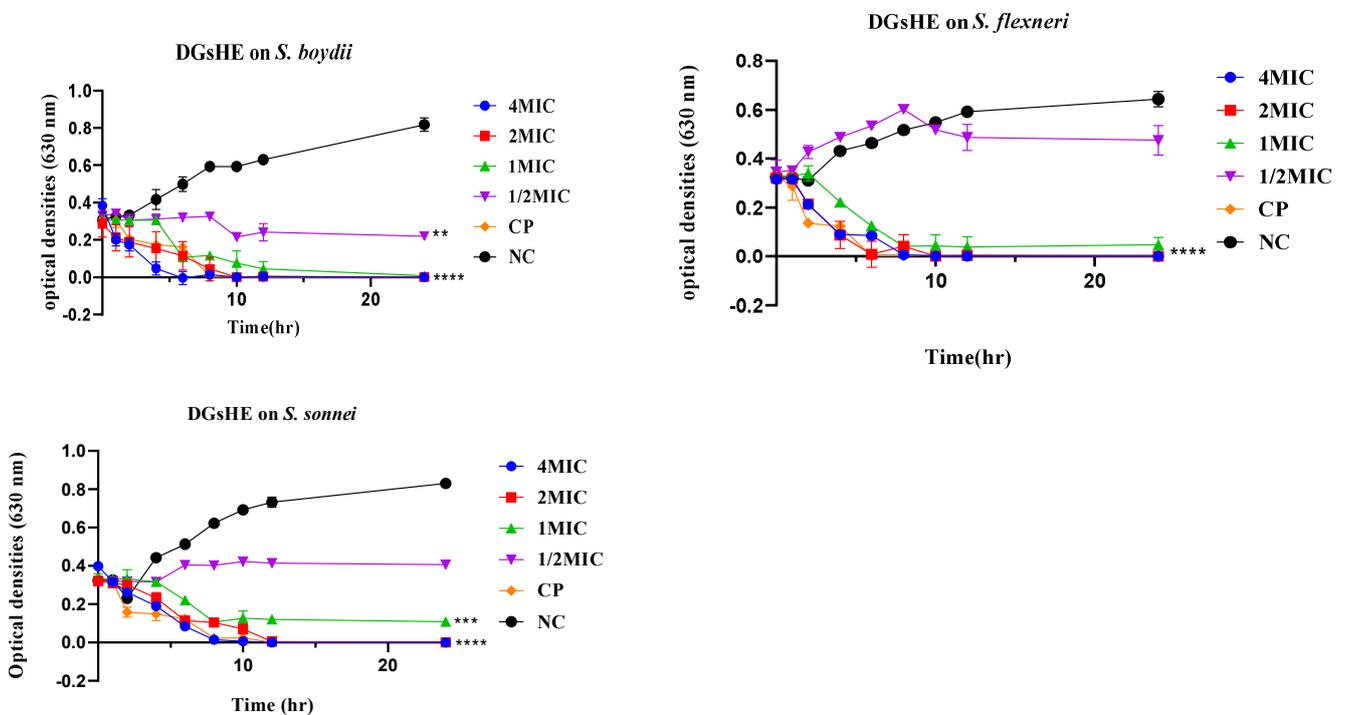


Figure 3. Effects of the hydroethanol extract of *Diospyros gillettii* on the growth kinetics of *S. boydii*, *S. flexneri* and *S. sonnei*. DGsHE: Hydroethanol extract of *Diospyros gillettii*; MIC: Minimum inhibitory concentration; CP: Ciprofloxacin; NC: Negative control; *S. boydii*: *Shigella boydii*; *S. flexneri*: *Shigella flexneri*; *S. sonnei*: *Shigella sonnei*. Data are represented as mean \pm standard deviation; $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****); Significant difference compared with negative control (Dunnett’s test).

Figure 3 reveals the growth kinetics of *Shigella* species after incubation with the hydroethanol extract of *Diospyros gilletii*. At MIC, 2 MIC, and 4 MIC, there was a decreased trend of the curves after 2 h of incubation time, suggesting the inhibition of the bacterial population. After 8 h of incubation, all the curves overlapped with the X axis, suggesting the clearance of the bacterial population. This bacterial clearance was later confirmed, since beyond 8 h of incubation (8–24 h), *Shigella* did not resume growth, suggesting the bactericidal inclination of the hydroethanol extract at MIC, 2 MIC, and 4 MIC.

2.2.3. Plausible Antibacterial Mechanisms of Action

Effect of Extracts on N-phenyl-naphthylamine Uptake by Bacteria

The outer-membrane permeability of the susceptible bacterial species (*S. boydii*, *S. flexneri*, and *S. sonnei*) was evaluated by measuring their 1-N-phenyl-naphthylamine's uptake upon treatment with different extracts of *Diospyros gilletii* stem bark. Figure 4A–C reveals the effect of various concentrations of extracts (1/2 MIC, MIC, 2 MIC, and 4 MIC) on the uptake of 1-N-phenyl-naphthylamine in *S. boydii*, *S. flexneri*, and *S. sonnei*. At 2 MIC and 4 MIC, there was a significant increase in the relative fluorescence intensity from t0 to t90 min, suggesting a higher intake of 1-N-phenyl-naphthylamine by bacteria at these concentrations. A similar trend was observed when the bacterial strains were treated with ethylene diamine tetraacetic acid (EDTA), a compound that was used as a positive control. By contrast, the curves, which were obtained from the group of bacteria that did not receive any treatment with extracts, remained unchanged throughout the experiment (from t0 to t90 min), inferring that the untreated bacteria did not uptake 1-N-phenyl-naphthylamine.

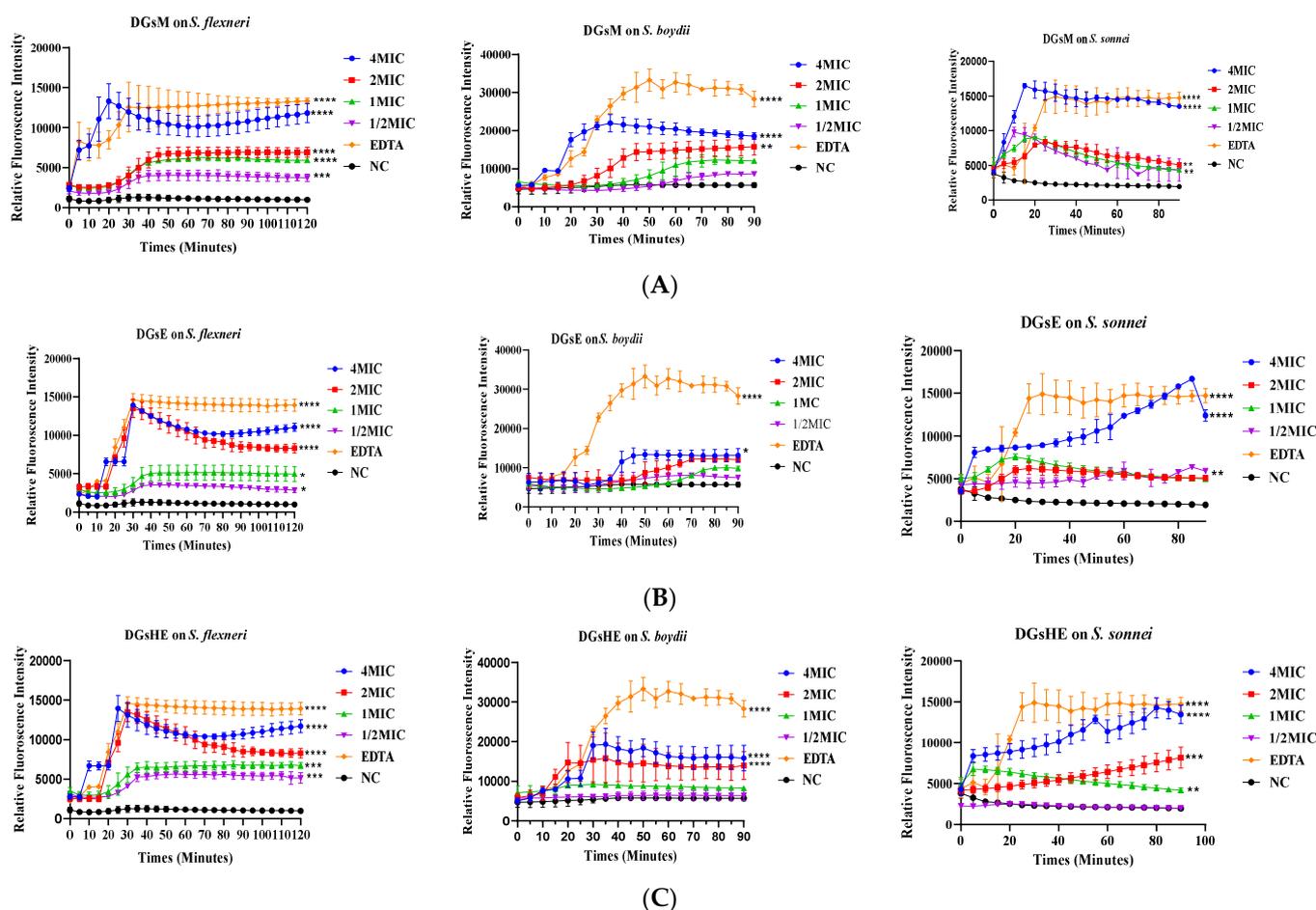


Figure 4. Effect of various concentrations of methanol (A), ethanol (B) and hydroethanol (C), and extracts on the uptake of N-phenyl-naphthylamine by *S. boydii*, *S. flexneri*, and *S. sonnei*. DGsE: Ethanol

extract of *Diospyros gillettii*; DGsHE: Hydroethanol extract of *Diospyros gillettii*; DGsM: Methanol extract of *Diospyros gillettii*; EDTA: Ethylene diamine tetraacetic acid; MIC: Minimum inhibitory concentration; NC: Negative control; CP: Ciprofloxacin; *S. boydii*: *Shigella boydii*; *S. flexneri*: *Shigella flexneri*; *S. sonnei*: *Shigella sonnei*. Data are represented as mean \pm standard deviation; $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****); Significant difference compared with negative control (Dunnett's test).

Propidium Iodide Uptake Assay

Following the graphs displayed in Figure 5A–C, it can be seen that the fluorescence intensity, which was measured as a function of time, increased continuously for all treatments with extracts at 2 MIC and 4 MIC, vis-à-vis *S. sonnei* and *S. boydii*, suggesting propidium iodide (PI) uptake by these bacteria. Moreover, the curves obtained from treatments against *S. flexneri* remained unchanged throughout the experiment (0–70 min). Overall, methanol, ethanol, and hydroethanol extracts triggered the rapid permeability of the bacterial membrane in *S. sonnei* and *S. boydii*, and propidium iodide fluorescence started increasing from 10 min at 4 MIC and 2 MIC; thereafter, it became constant, while at 1 MIC and 1/2 MIC, there was no increase in fluorescence in *S. boydii* and *S. sonnei* (Figure 5A–C). Normally, it is known that for extracts that work by propidium iodide uptake, the fluorescence intensity increases continuously. This shows that the antibacterial mode of action of the extracts is not achieved in *S. flexneri* through propidium iodide uptake. Nonetheless, this mode of action was verified in *S. sonnei* and *S. boydii*.

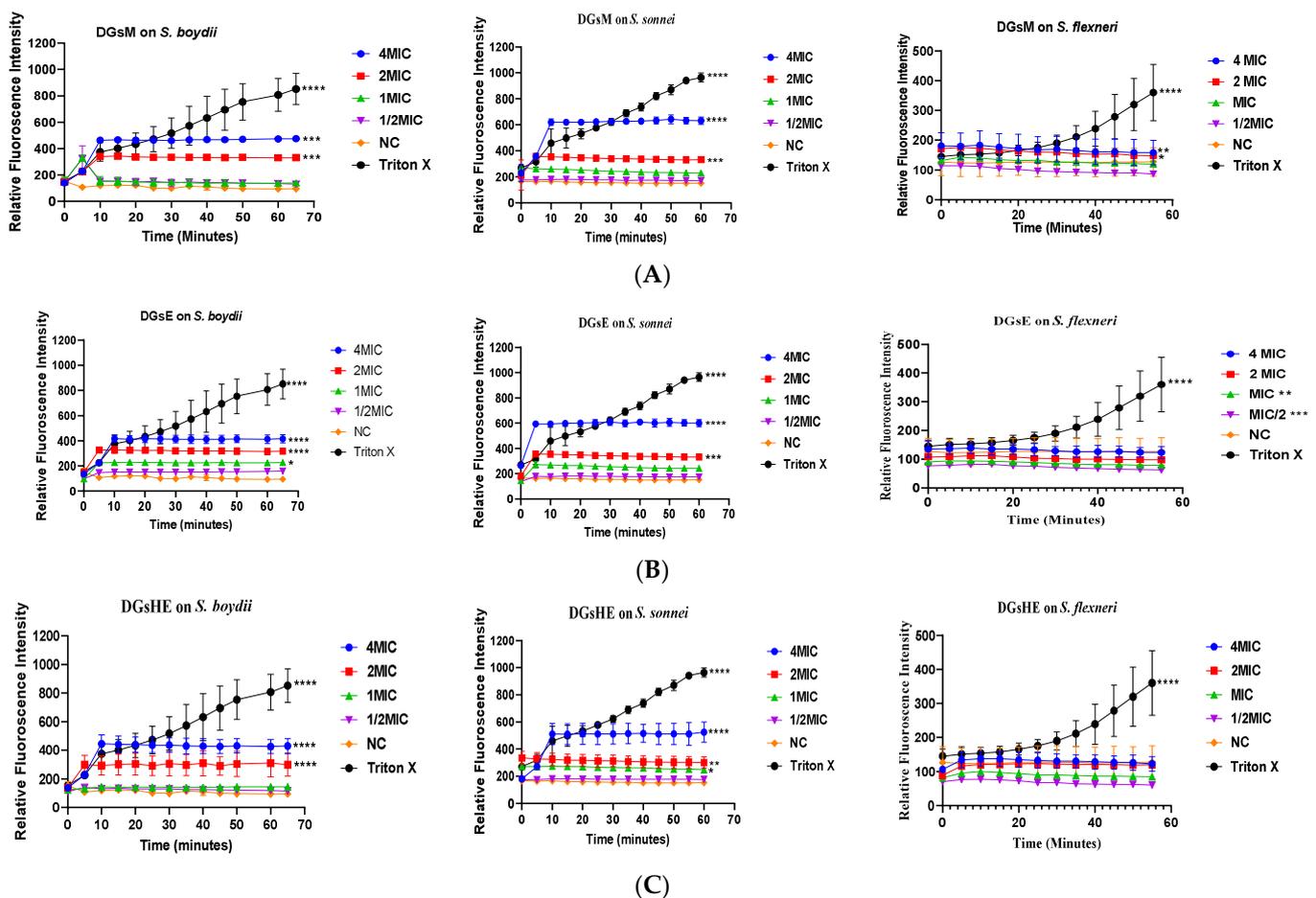


Figure 5. Effect of various concentrations of methanol (A), ethanol (B) and hydroethanol (C), and extracts on the uptake of propidium iodide by *S. boydii*, *S. flexneri*, and *S. sonnei*. DGsE: Ethanol extract

of *Diospyros gillettii*; DGsHE: Hydroethanol extract of *Diospyros gillettii*; DGsM: Methanol extract of *Diospyros gillettii*; MIC: Minimum inhibitory concentration; NC: Negative control; CP: Ciprofloxacin; *S. boydii*; *Shigella boydii*; *S. flexneri*; *Shigella flexneri*; *S. sonnei*; *Shigella sonnei*. Data are represented as mean \pm standard deviation; $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****); Significant difference compared with negative control (Dunnett's test).

Inhibition and Eradication of Bacterial Biofilms

a. Inhibition of biofilm formation

According to Figure 6, the biofilm formation [of *S. boydii* (A) and *S. sonnei* (B)] was significantly inhibited ($p \leq 0.05$) by methanol, ethanol, and hydroethanol extracts of *D. gillettii* stem bark (at MIC, 2 MIC and 4 MIC) after 24 h of incubation. The highest percentages of the inhibition of biofilm formation were observed with 4 MIC. Moreover, ciprofloxacin, the positive control used, showed more than a 100% inhibition of biofilms.

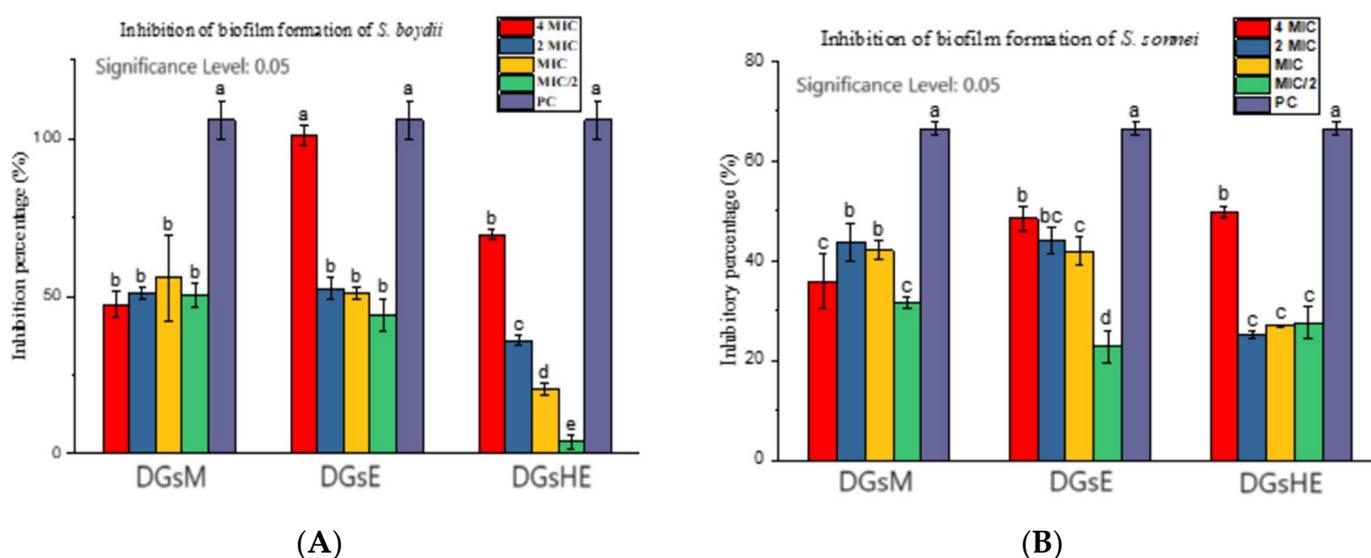


Figure 6. Inhibition of biofilms from *S. boydii* (A) and *S. sonnei* (B) by methanol, ethanol and hydroethanol extracts. DGsM: Methanol extract of *Diospyros gillettii*; DGsE: Ethanol extract of *Diospyros gillettii*; DGsHE: Hydroethanol extract of *Diospyros gillettii*; MIC: Minimum inhibitory concentration. Data are represented as mean \pm standard deviation. ^a $p \leq 0.05$, ^b $p \leq 0.01$, ^c $p \leq 0.001$, ^d and ^e: The values are not significantly different ($p > 0.05$) (Dunnett's test); Values are significantly different compared to the value of negative control (no biofilm inhibition).

b. Eradication of biofilms

According to Figure 7, more than 50% of biofilms formed by *S. boydii* (within 24 h of incubation) (Figure 7A) were eradicated by methanol, ethanol, and hydroethanol extracts at MIC, 2 MIC, and 4 MIC after 24 h of incubation. Moreover, the biofilms formed by *S. sonnei* (Figure 7B) were also eradicated (<50%) by all the extracts (except for the methanol extract, which showed 80% inhibition at 4 MIC) at 4 MIC, 2 MIC, and MIC.

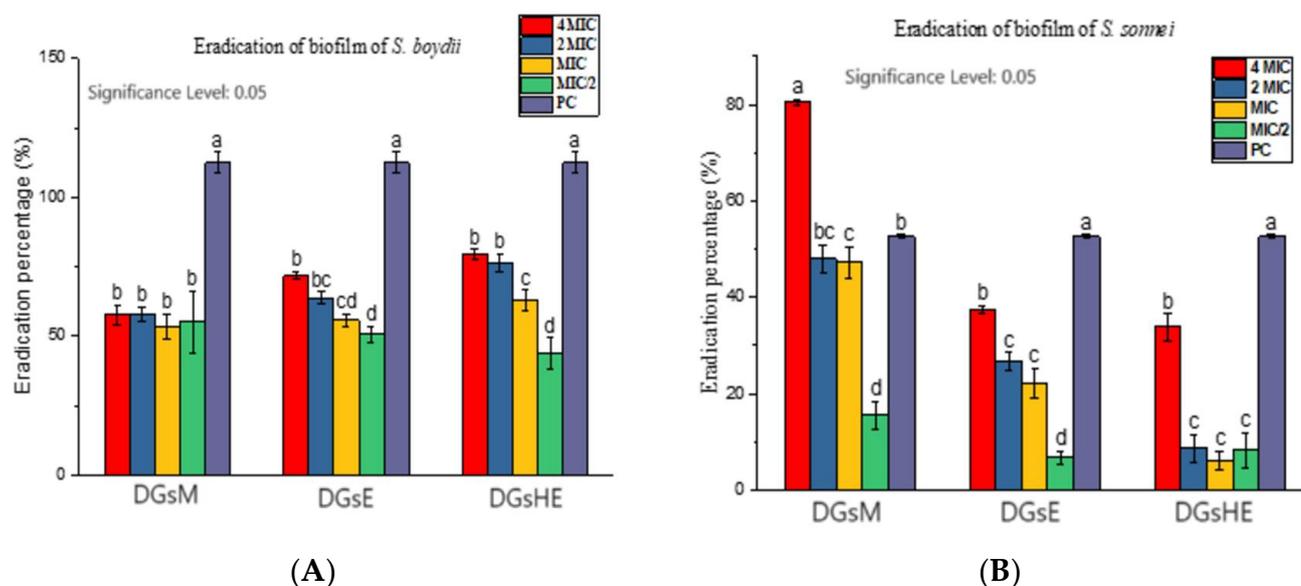


Figure 7. Eradication of biofilms formed by *S. boydii* (A) and *S. sonnei* (B) using methanol, ethanol and hydroethanol extracts. DGsM: Methanol extract of *Diospyros gillettii*; DGsE: Ethanol extract of *Diospyros gillettii*; DGsHE: Hydroethanol extract of *Diospyros gillettii*; MIC: Minimum inhibitory concentration. Data are represented as mean \pm standard deviation. ^a $p \leq 0.05$, ^b $p \leq 0.01$, ^c $p \leq 0.001$, ^d: The values are not significantly different ($p > 0.05$) (Dunnett's test); Values are significantly different compared to the value of negative control (no biofilm eradication).

2.2.4. Cytotoxicity Studies

Table 2 summarizes the 50% cytotoxic concentration of methanol (45.24 and 52.00 $\mu\text{g}/\text{mL}$), ethanol (54.52 and 65.30 $\mu\text{g}/\text{mL}$), and hydroethanol (39.94 and 47.15 $\mu\text{g}/\text{mL}$) extracts against Vero and Raw cells, respectively, vs. dimethylsulfoxide (DMSO, a reference cytotoxic agent) (1.40 and 0.95 $\mu\text{g}/\text{mL}$). According to the National Cancer Institute classification, compounds with $\text{CC}_{50} > 20 \mu\text{g}/\text{mL}$ are poorly cytotoxic or non-cytotoxic [31]. Compared to DMSO, which showed CC_{50} values less than 2 $\mu\text{g}/\text{mL}$, methanol, ethanol, and hydroethanol extracts can be considered non-cytotoxic extracts.

Table 2. Median cytotoxic concentrations of the extracts.

Sample	Median Cytotoxic Concentrations ($\mu\text{g}/\text{mL}$) ^a	
	Vero ATCC CRL 1586	Raw 264.7
DGsM	45.245 \pm 4.51	52 \pm 0.0565
DGsE	54.525 \pm 2.73	65.3 \pm 5.529
DGsHE	39.94 \pm 1.73	47.15 \pm 10.38
DMSO	1.40 \pm 0.21	0.95 \pm 0.022

^a: Values are means of triplicate determination ($n = 3$); DGsM: Methanol extract of *Diospyros gillettii*, DGsE: Ethanol extract of *Diospyros gillettii*; DGsHE: Hydroethanol extract of *Diospyros gillettii*; DMSO: Dimethylsulfoxide. Data are represented as mean \pm standard deviation.

3. Discussion

In the present study, extracts from the stem bark of *Diospyros gillettii* were evaluated for antibacterial activity against four species of *Shigella*, viz. *S. boydii*, *S. sonnei*, *S. flexneri* and *S. dysenteriae*. Moreover, the antibacterial mechanism of action of the most active extracts was investigated. Solvent extraction of the stem bark of *Diospyros gillettii* yielded methanol, ethanol, and hydroethanol extracts with yields of extraction of 5.6%, 7.61%, and 8.27%, respectively. The hydroethanol, composed of two solvents, i.e., ethanol and water, might have induced a leakage of more secondary metabolites from the plant, thereby increasing the yield of extraction (8.27%) [32]. The antibacterial test of the extracts

revealed MIC values ranging from 125 to 1000 $\mu\text{g}/\text{mL}$, with the most active extracts being the methanol and hydroethanol with MICs of 125 $\mu\text{g}/\text{mL}$ when tested in *S. boydii*. According to previous reports [33–35], the degree of antibacterial activity can be classified as very active ($<100 \mu\text{g}/\text{mL}$), active ($100 < \text{MIC} < 512 \mu\text{g}/\text{mL}$), moderately active ($512 < \text{MIC} < 2048 \mu\text{g}/\text{mL}$), and poorly active ($\text{MIC} > 2048 \mu\text{g}/\text{mL}$). Thus, the methanol extract was significantly active in *S. flexneri* ($100 < \text{MIC} < 512 \mu\text{g}/\text{mL}$), whereas ethanol and hydroethanol extracts were active ($100 < \text{MIC} < 512 \mu\text{g}/\text{mL}$) in all the bacterial species. All the extracts showed poor activity against *S. dysenteriae*. The observed antibacterial activity might be attributed to the presence of a number of secondary metabolites [pentacyclic triterpenes (corosolic acid and ursolic acid) and terpenoids (betulinic acid and lupeol), coumarins (norbergenin, 4-O-galloylnorbergenin), and the cholesterol derivative β -sitosterol] in *Diospyros gillettii* stem bark [14]. As already discussed, *Diospyros gillettii* contains bergenin and its derivatives, which are well known to possess antibacterial activity [14,17,36]. In a recently published review article by Salimo et al. [37], the antibacterial activity of bergenins was reported in 15 in vitro studies and 4 in vivo experiments [37]. Noteworthy, bergenin and its congeners participate in several mechanisms, such as lipid-peroxidation inhibitory activity and free-radical-scavenging activity [38], and initiate apoptosis and cell-cycle arrest in the G0/G1 phase [39]. Moreover, these compounds are able to easily cross the complex and multilayer lipopolysaccharide of bacteria cell walls, thereby causing lysis and cell death [40]. Thus, it is not unreasonable to speculate that these compounds might have contributed to the anti-shigellosis activity. The differences in the antibacterial action might be due to the diversity of the biochemical constituents and the genetic identity of bacteria, which potentially influenced the resistance in the tested bacteria [41]. Time-kill kinetic studies revealed that the bacterial inhibition was proportional to the concentration of the extracts. In fact, the extracts were found to be bactericidal at 2 MIC and 4 MIC when tested against *S. boydii*, *S. sonnei*, and *S. flexneri*. To exert their activity within the cell, almost all antibiotics have to cross the outer membrane of bacteria either by increasing membrane permeability or disruption. The measurement of the membrane permeability and disruption in bacteria might help in better understanding the mechanistic basis of the action [12]. The incubation of *S. boydii*, *S. sonnei*, and *S. flexneri* with various concentrations of extracts (4 MIC, 2 MIC, and MIC) promoted the uptake of N-phenyl-naphthylamine and propidium iodide by these bacteria, leading to an increase in the fluorescence [42]. The fluorescence of the NPN probe increases when this compound is incorporated into the hydrophobic core of the bacterial cell membrane, as compared with the fluorescence of a non-permeating bacteria cell. The incubation of bacteria with *D. gillettii* extracts induced a significant accumulation of NPN in the bacterial membrane, which was further inhibited by free divalent cations, such as Mg^{2+} and Ca^{2+} . These cations, which normally bind to negatively charged phosphate groups between lipopolysaccharide (LPS) molecules via ionic bridges, are crucial for the outer-membrane integrity of Gram-negative bacteria. Consequently, the chelation of these divalent ions by NPN would definitely disrupt the outer membrane's integrity and permeabilize the bacteria [43]. Propidium iodide, a dye that labels bacteria with a compromised inner membrane, binds to nucleic acids, increases its fluorescent signal, and constitutes a strong indicator of inner-membrane permeabilization. Methanol, ethanol, and hydroethanol extracts promoted the PI and NPN uptake in *S. boydii*, and *S. sonnei*, inferring that the test extracts permeabilized the inner and outer membranes of these bacteria [44,45]. Consequently, these extracts might have exerted antibacterial activity through bacterial membrane permeabilization. Extracts from *Diospyros gillettii* were further evaluated for antibiofilm formation using *S. boydii*, *S. sonnei*, and *S. flexneri*. Biofilm formation by bacteria is intricately involved in the mechanism of bacterial resistance as the biofilm matrix can slow antibiotic penetration through the membrane. To avoid the development of microbial biofilms, the inhibition of bacterial adhesion, the reduction of the initial bacterial growth, and the eradication of formed biofilms are noteworthy. Methanol, ethanol and hydroethanol extracts from *Diospyros gillettii* inhibited the formation of biofilm, with the percentage of inhibition ranging from 25% to 55% in *S. sonnei* and from 13% to

90% in *S. boydii*. The observed antibiofilm activity of the extracts might be attributed to the presence of a variety of secondary metabolites present in the plant [46]. Moreover, the extracts eradicated the biofilm formed by bacteria with percentages of inhibition varying from 8% to 80% in *S. sonnei* and from 48% to 78% in *S. boydii*. Antibiofilm activity has been attributed to the potential of active compounds to interfere with biofilm development, destabilize the biofilm, promote detachment, and sensitize biofilm cells [47]. In *S. boydii*, ethanol and hydroethanol extracts inhibited and eradicated the biofilms. All the extracts eradicated biofilms formed by *S. sonnei*. For ethanol and hydroethanol extracts, the MIC₅₀ was found to be 4 MIC, whereas the MEC₅₀ could not be observed in any of the extracts. To evaluate the cytotoxicity profile of the active extracts, their median cytotoxic concentrations (CC₅₀) were determined vis-à-vis human mammalian cells, such as Vero and Raw cells. The CC₅₀ values ranged from 39.94 ± 1.73 µg/mL to 54.525 ± 2.73 µg/mL on Vero cells and from 47.15 ± 10.38 µg/mL to 65.3 ± 5.52 µg/mL on Raw cells. As the CC₅₀ values of all the tested extracts were found to be more than 30 µg/mL, it can be speculated that none of the extracts were toxic to the normal cells, according to the criteria of cytotoxicity defined by the American Nation Cancer Institute [48,49].

To the best of our knowledge, no report has demonstrated the antibacterial activity and mechanisms of action of methanol, ethanol, and hydroethanol extracts from *D. gillettii* stem bark. The anti-*Shigella* activity of *D. gillettii* extracts, which is demonstrated in this study, substantiates the ethnopharmacological use of *D. gillettii* in the treatment of infectious diseases, such as infectious diarrhoea. However, antibacterial-guided fractionation of *D. gillettii* extracts should be performed to identify the active anti-*Shigella* principles of this plant. In addition, in vivo anti-*Shigella* and pharmacokinetic studies, as well as knowledge of detailed mechanisms of action, are required for the successful utilization of *D. gillettii* in the treatment of infectious diarrhoea.

4. Materials and Methods

4.1. Plant Collection and Identification

The stem bark of *Diospyros gillettii* De Wild (Figure 8) was collected in Badounga in the West Region of Cameroon in May 2022 and identified at the National Herbarium of Cameroon, where a voucher specimen of the species was deposited under number No 15418 HNC.



Figure 8. Photograph of *Diospyros gillettii* growing in Badounga village, Tonga sub-division, West Region, Cameroon (picture by B.-L.M.K.).

4.2. Plant Extraction

The stem bark was then cut into small pieces, air-dried for two weeks, and ground to obtain a powder. The crude extracts were prepared by the maceration of plant powder in three solvents, including ethanol, hydroethanol (water/ethanol; 30/70, *v/v*), and methanol. In brief, 10 g of dried powder was macerated in pure ethanol, methanol, and hydroethanol (30/70; *v/v*) (100 mL) for 72 h at room temperature with daily shaking. The resulting solution was then filtered using hydrophilic cotton, and the filtrate was further concentrated using a Buchi rotary evaporator at 55 °C to yield the crude extracts. The extraction yield was calculated using the following formula:

$$\text{Extraction yield (\%)} = \frac{\text{Mass of the crude extract (g)}}{\text{Mass of the plant material (g)}} \times 100$$

The crude extracts were kept in a refrigerator until further use.

4.3. Preparation of Stock Solutions

The stock solutions of the crude extracts were prepared at 100 mg/mL by dissolving 100 mg of each crude extract in 1 mL of dimethylsulfoxide 100% in 1.5 mL Eppendorf tubes and storing at 4 °C. Ciprofloxacin, which was used as the reference antibiotic, was prepared by dissolving 1 mg of the drug in 1 mL of 0.5 N HCL to yield a concentration of 1 mg/mL.

4.4. Antibacterial Activity

a. Bacterial strains

The in vitro antibacterial activity of extracts from *Diospyros gillettii* stem bark was evaluated in three reference bacterial strains (*Shigella flexneri* NR 518, *Shigella boydii* NR 521 and *Shigella sonnei* NR 519), which were obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI Resources, Rockville, MD, USA, 20,852), and one clinical isolate, *Shigella dysenteriae*, which was a kind gift from the Centre Pasteur of Cameroon. These bacterial strains were maintained in continuous culture in tubes on the Mueller Hinton Agar slope (4 °C) at the Laboratory for Phytobiochemistry and Medicinal Plant Studies of the Faculty of Science of the University of Yaounde 1, Cameroon.

b. Preparation of bacterial inoculum

The different bacterial inocula were prepared based on the 0.5 McFarland standard. Briefly, a colony from a 24 h culture on Müller Hinton agar (MHA) was removed using a platinum loop and introduced into a test tube containing 10 mL sterile physiological water (NaCl 0.9%); then, it was calibrated to a 0.5 McFarland standard using turbidity comparison to obtain 1.5×10^8 UFC/mL.

c. Determination of minimum inhibitory concentrations

The determination of the MIC was performed using the microdilution method in a liquid medium, as described by the Clinical Laboratory Standard Institute (CLSI), M07-A9 protocol, with slight modifications [50]. Briefly, 196 µL of Muller Hinton broth (MHB) was introduced into the first twelve wells of the microplate (CoStar, Washington, DC, USA), and 100 µL was placed in the rest of the wells of the microplate. Next, 4 µL of sterile solution containing different extracts concentrated at 100 mg/mL was introduced into the first twelve wells of column A, followed by five subsequent two-fold serial dilutions from line A to line F (concentration range: 250 µg/mL to 3.90625 µg/mL). Then, 100 µL of a bacterial suspension at 1×10^6 CFU/mL was introduced into all the wells, except for that of the sterility control. The negative control was composed of the culture medium and inoculum only, while the positive control (concentration range: 2.5–0.15625 µg/mL) was made up of the medium, inoculum, and ciprofloxacin. Noteworthy, the final concentration of the inoculum in the wells was 5×10^5 CFU/mL. The microplates were subsequently incubated at 37 °C for 24 h. At the end of the incubation period, 20 µL of a freshly prepared resazurin solution (0.15 mg/mL) was added to all the wells and further incubated for

30 min. The lowest concentration at which no color change from blue (resazurin) to pink (resorufin) was observed was considered as the minimum inhibitory concentration (MIC), corresponding to an absence of visible bacteria growth, and was expressed in $\mu\text{g}/\text{mL}$. The test was performed in triplicate and repeated twice. Subsequently, a time-kill kinetic assay was used to follow up the activity of plant extracts on bacterial growth (bactericidal or bacteriostatic activity) over time.

Time-Kill Kinetics

Time-kill kinetics were studied using a modified protocol described by [51]: notably, the use of opacimetry based on the turbidity of the cell suspensions as a function of the load rather than colonies counting on agar. In short, the assay was performed in sterile 96-well microplates at sub-inhibitory, inhibitory, and supra-inhibitory extract concentrations. Briefly, the extracts were diluted in MHB medium as previously described, in order to obtain $\frac{1}{2}$ MIC, MIC, 2 MIC, and 4 MIC. Ciprofloxacin ($2.5 \mu\text{g}/\text{mL}$) was used as a positive control. Then, $100 \mu\text{L}$ inoculum at 1×10^6 CFU/mL was introduced into each well to obtain a solution of 5×10^5 CFU/mL, except for the wells used for sterility control. The plates were then covered and incubated at 37°C for 24 h, during which the evolution of the bacterial load was followed by reading the optical densities at 630 nm of the preparations at different time intervals (0, 1, 2, 4, 6, 8, 10, 12, and 24 h) using a microplate reader (TECAN-Infinite M200, Tecan Austria GmbH, Salzburg, Austria). The assay was performed in triplicate and repeated twice. The bacterial growth curves were plotted using the optical densities against the incubation times. From these curves, we determined the minimum time at which the inhibitory effects started, the bactericidal and bacteriostatic effects of the extracts, and the re-emergence time of the bacterial strains.

Next, plausible mechanisms of the antibacterial action of the most active extracts were investigated using a membrane-permeabilization test, and we determined the inhibitory/eradication effect on bacterial biofilm formation.

4.5. Evaluation of Possible Mechanisms of Action

4.5.1. Membrane Permeabilization

The membrane permeabilization of the most promising extracts was evaluated using NPN (1-N-phenyl-naphthylamine) and propidium iodide (PI) uptake assays.

a. 1-N-phenyl-naphthylamine uptake assay

The outer membrane of Gram-negative bacteria provides a formidable barrier (an asymmetric bilayer that contains mainly lipopolysaccharide (LPS) in the outer leaflet and phospholipids in the inner leaflet) that should be crossed by antimicrobial agents to exert their action. NPN (1-N-phenyl-naphthylamine) is a neutral, hydrophobic fluorescent probe that cannot permeate the intact outer membrane of the bacteria. When the integrity of the bacteria outer membrane is destroyed, it will diffuse into the phospholipid bilayer and exhibit low-fluorescence quantum yield in an aqueous environment, and it becomes strongly fluorescent in a glycerophospholipid or hydrophobic environment [44]. Briefly, a 1-N-phenyl-naphthylamine (NPN) assay was performed to compare the effects of the plant extract on the permeabilization of the outer membranes using the modified protocol of [43,44]. Briefly, in sterile, 96-well, black flat-bottomed microplates, bacterial cells were grown overnight, washed with normal saline (0.9% NaCl), and centrifuged at 10,000 tr for 10 min in Eppendorfs tubes. The pellet was resuspended in normal saline and then adjusted to a 0.5 McFarland standard in tubes containing glucose with phosphate-buffered saline (50 mg of PBS in 50 mL of glucose). Next, $160 \mu\text{L}$ of cells was added to $20 \mu\text{L}$ NPN and $20 \mu\text{L}$ of extracts at various concentrations (4 MIC, 2 MIC, MIC, and $\frac{1}{2}$ MIC). The positive control consisted of ethylene diamine tetraacetate (EDTA) and NPN with bacteria suspension, whereas NPN was used as a negative control. Sterility control was composed of NPN with normal saline. The microplate was kept for 1 h 30 min in the dark, and then NPN fluorescence was read at 5 min intervals using a TECAN infinite M200 microplate

reader at 350 nm and 420 nm excitation and emission wavelengths, respectively. The assay was performed in triplicate and repeated twice.

The percentage of NPN uptake was calculated using the following equation:

$$\% \text{ NPN uptake} = \frac{(\text{Fobs} - \text{F0})}{(\text{F100} - \text{F0})} \times 100$$

where

Fobs = observed fluorescence at a given concentration of plant extract.

F0 = initial fluorescence of NPN with bacterial cells in the absence of plant extract

F100 = fluorescence of NPN with bacteria cells upon addition of 2 μL of EDTA.

b. Propidium iodide uptake assay

The effect of antibacterial crude extracts on the bacterial membrane integrity was assayed using a propidium iodide test. Propidium iodide (PI) is membrane-impermeable for intact cells and can only enter cells when the membrane permeability is augmented. Once in the cells, this fluorescent intercalating agent binds to nucleic acids (DNA, RNA) of necrotic cells, thereby discriminating between apoptotic and necrotic cells [45]. In this study, the PI uptake test was carried out according to a previously described protocol by Kwon et al. [44]. Briefly, bacterial cells were grown overnight in sterile, 96-well, black flat-bottomed microplates, washed with normal saline (0.9% NaCl), and then centrifuged at 10,000 tr/10 min in 1.5 mL Eppendorf tubes. The pellet was resuspended in normal saline and then adjusted to 10^6 cells/mL from a 0.5 McFarland standard, for a final volume of 64,000 μL in tubes containing 50 mg of PBS dissolved in 50 mL of glucose. Next, 20 μL of PI solution (1.3 mg of PI dissolved in 20 mL of PBS) was added to 160 μL of the bacterial suspension in a sterile, 96-well, black flat-bottomed microplate and incubated in the dark for 30 min. Afterward, 20 μL of various concentrations of plant extracts (4 MIC, 2 MIC, MIC, and $\frac{1}{2}$ MIC) were added, while triton X was used as the positive control. The negative control was composed of propidium iodide and the bacterial suspension, while propidium iodide plus PBS was used as a sterility control. Then, the microplate was incubated for 1 h without light exposure, and the optical densities were read using a TECAN infinite M200 microplate reader at 544 nm and 620 nm as excitation and emission wavelengths, respectively. The assay was performed in triplicate and repeated twice.

4.5.2. Antibiofilm Activity

Biofilms are structured microbial cells that occur as suspended aggregates attached to an inert or living surface to cause chronic infections [52]. Bacterial biofilms allow bacteria to resist and survive environmental pressure and enhance their resistance to antimicrobial agents [52].

a. Quantification of biofilm

Biofilm biomass measurement was performed by crystal violet staining according to a previously described protocol [53]. In brief, a single colony, which was isolated from an overnight bacteria culture on MHA, was suspended in normal saline and adjusted to 1×10^6 CFU/mL in MHB supplemented with 2% glucose and 0.4% bovine bile. Next, 200 μL aliquot of the bacterial suspension was dispensed into a 96-well, U-shaped sterile microplate, whereas the negative-control wells were filled with 200 μL MHB. The plates were then incubated for 24 h at 37 $^\circ\text{C}$. At the end of the incubation period, the microplate was gently emptied, and 200 μL of pure ethanol was added to each well and incubated for 15 min in order to fix the cells in the wells. Next, the wells were emptied and washed twice with sterile distilled water, followed by the addition of 200 μL crystal violet 0.1% to stain the biofilm formed. The plates were further incubated at 37 $^\circ\text{C}$ for 30 min, the remaining (residual) crystal violet was removed, and the wells were washed three times using sterile distilled water. Finally, 200 μL of a 33% acetic acid solution was introduced into the wells, and the absorbance was read at 590 nm using a TECAN (infinite M200) (Austria GmbH, Salzburg, Austria) plate reader.

- b. Determination of the minimum inhibitory concentration (MIC₅₀) and minimum eradication concentration (MEC₅₀) of biofilms

MIC₅₀ and MEC₅₀ were determined according to the microdilution method in a liquid medium. This assay was performed in triplicate in sterile, 96-well microplates at sub-inhibitory, inhibitory, and supra-inhibitory extract concentrations. Briefly, the extracts were diluted in MHB medium supplemented with 2% glucose and 0.4% bovine bile salt, as previously described, to obtain 4 MIC, 2 MIC, MIC, and ½ MIC. Next, a two-fold serial dilution of order 2 was carried out. Afterward, 100 µL of inoculum was added to all the wells, which were then incubated for 24 h at 37 °C. The final bacterial load in each well was 1 × 10⁶ CFU/mL. Ciprofloxacin (50 µg/mL) was used as a positive control. After incubation, non-adherent or planktonic cells were discarded, and 200 µL of ethanol was added to each well in order to fix the cells in the wells, and the microplates were incubated for an additional 15 min. Then, the plates were gently washed (two times) with distilled water and dried at room temperature. Next, each well was stained by adding 200 µL of 0.1% crystal violet and further incubated for 30 min, after which the stain was discarded and washed with distilled water so as to remove the excess stain. Finally, 200 µL of a 33% glacial acetic acid was introduced into the different wells, and the absorbance was read at 590 nm using a TECAN (infinite M200) plate reader. The experiment was performed in triplicate and repeated thrice. The MEC₅₀ was determined under the same conditions, with the only difference being that the biofilms were first formed in the culture medium (MHB medium supplemented with 2% glucose and 0.4% bovine bile salt) for 24 h at 25 °C, based on the strain. Next, the test samples were prepared as described above and introduced into the corresponding wells of the microplates, already containing the formed biofilms. The microplates were then re-incubated under the same conditions and treated as elucidated above. Finally, 200 µL of a 33% glacial acetic acid was introduced into the different wells, and the absorbance was read at 590 nm using a TECAN (infinite M200) plate reader. The experiment was performed in triplicate and repeated thrice. The optical densities, which were obtained from the spectrophotometer, were used to calculate the percentages of inhibition/eradication of biofilms using the following formula:

$$\% \text{ inhibition/eradication} = \frac{\text{OD negative control} - \text{OD test}}{\text{OD negative control}} \times 100$$

where OD is the optical density.

MIC₅₀ = minimal concentration that inhibited 50% biofilm formation, MEC₅₀ = minimal concentration that eradicated 50% of the biofilms formed.

Cytotoxicity studies of the most active extracts were carried out on Vero and Raw cells to evaluate their toxicity and selectivity.

4.6. Cytotoxicity Test

The extracts that inhibited the growth of the bacterial strains were evaluated against two selected human mammalian cells using a colorimetric method based on the reduction of resazurin to resorufin [54].

4.6.1. The Human Mammalian Cells

The *in vitro* cytotoxicity of the active extracts was evaluated on the mammalian African Green monkey Vero ATCC CRL 1586 and murine macrophage Raw cells 264.7 cell lines, provided by the Centre Pasteur of Cameroon and the Noguchi Memorial Institute for Medical Research, University of Ghana, respectively. The cells were maintained in a continuous culture in the laboratory and later used for the cytotoxicity test.

4.6.2. In Vitro Culture of the Vero and Raw Cell Lines

The Vero and Raw cells were maintained in complete DMEM (Dulbecco's Modified Eagle medium) (1% antibiotic and 10% FBS) and stored in a 25 cm³ cultured flask (T-Flask)

(CosStar, Washington, DC, USA) under standard conditions of 5% CO₂ at 37 °C. The culture medium was renewed every 72 h. Once cell confluence (70–90%) was reached, the cells were detached by treatment with 1 mL of trypsin-EDTA 0.05% for 5 min. After trypsin inactivation by adding 9 mL of complete DMEM, the detached cells were centrifuged at 1800 rpm for 5 min. The obtained pellet was then suspended in 1 mL of the medium, and the cell viability was evaluated using Trypan blue to calibrate the cell load on the Neubauer hemocytometer.

4.6.3. Determination of Median Cytotoxic Concentrations (CC₅₀)

As already discussed, the cytotoxicity of the most promising extracts was assessed according to the method described by Bowling et al. [54]. The assay was performed in duplicate in sterile 96-well microplates (CoStar, Washington, DC, USA). Briefly, 100 µL of cells' suspension containing 1×10^4 cells/well was introduced into all the wells of the plate and incubated for cell adhesion at 37 °C, 5% CO₂ for 18 h. Next, the cell culture medium was replaced with 90 µL of fresh medium, followed by the addition of 10 µL of test samples at different concentrations (final concentrations of the extracts: 2000, 1000, 500, 250, 125, and 62.5 µg/mL), in all wells except for those that served as the positive (DMSO, 20 µM) and negative (culture medium without test sample) controls. The plates were then incubated at 37 °C, 5% CO₂ for 48 h, after which 10 µL of a freshly prepared resazurin solution (0.15 mg/mL in PBS) was introduced into each well and incubated for 4 h at 37 °C under 5% CO₂. Next, the fluorescence of the obtained preparation was measured using a microtiter plate reader (TECAN-Infinite M200, Tecan Austria GmbH, Salzburg, Austria) at excitation and emission wavelengths of 530 and 590 nm, respectively. From the resulting values of optical densities, the percentage of cell viability was calculated with Microsoft Excel software (version 2013, Washington, DC, USA) using the following equation:

$$\% \text{Cell inhibition} = \frac{(Ac - At)}{(Ac)} \times 100$$

where At = Absorbance of test samples, Ac = Absorbance of negative control (cells without test samples).

A dose–response curve (cell viability versus concentration of the samples) was plotted using GraphPad Prism software to determine the median cytotoxic concentration (CC₅₀).

4.7. Statistical Analysis

The values were represented as the mean ± standard deviation. Statistical analysis was performed using one-way analysis of variance (ANOVA) using GraphPad Prism 8.0.1 software. The histograms were obtained using OriginPro Software (8.5.1). The percentages were calculated using Excel 2013 software. The differences between the means were compared using Dunnett's test with a 95% confidence level ($p \leq 0.05$).

5. Conclusions and Perspectives

The present study aimed to evaluate the antibacterial activity and mechanisms of action of extracts from *D. gilletti*. Upon in vitro antibacterial testing, extracts from *D. gilletti* afforded MIC values ranging from 125 to 1000 µg/mL, with the methanol extract being the most active as it had the lowest MIC values (125 µg/mL). Mechanistic studies of potent antibacterial extracts from *D. gilletti* revealed bactericidal orientation at 4 MIC and bacteriostatic tendency at 2 MIC. Furthermore, the bioactive extracts contributed to the destabilization of the inner and outer membranes of the *Shigella* species through 1-N-phenyl-naphthylamine and propidium iodide uptake by these membranes. In addition, *D. gilletti* extracts inhibited and eradicated biofilms formed by the shigellosis-causing microbes. *D. gilletti* extracts were also found to be non-cytotoxic on Vero and Raw cells. These results validate the ethnomedicinal uses of *D. gilletti* in the traditional treatment of infectious diseases such as diarrhoea. However, antibacterial-guided fractionation of *D. gilletti* extracts should be performed to identify the active principles of this plant. Moreover,

in vivo anti-shigellosis activity, pharmacokinetics, and in-depth mechanistic studies should be investigated for the successful utilization of *D. gilletti* in the treatment of diarrhoea caused by *Shigella* species.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ddc3010015/s1>, Figure S1: Coumarins isolated thus far from *Diospyros gilletii*, and Figure S2: Pentacyclic triterpenes (8–10) and triterpenoids (11–15) isolated thus far from *Diospyros gilletii*.

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