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The Ethanolic Leaf Extracts of *Dissotis multiflora* (Sm) Triana and *Paullinia pinnata* Linn Exert Inhibitory Effect on *Escherichia coli* Through Membrane Permeabilization, Loss of Intracellular Material, and DNA Fragmentation

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Abstract

Background: *Dissotis multiflora* (Sm) Triana and *Paullinia pinnata* Linn are widely used in Cameroonian traditional medicine to treat infectious diseases. These plants were found to be a reservoir of antioxidant and antimicrobial agents and have the potential to be used in clinic. Objective: To determine the mechanism of action of the ethanolic leaves extracts of *Dissotis multiflora* and *Paullinia pinnata* on *Escherichia coli.* Methodology: The microbroth dilution method was used to determine the minimum inhibitory concentrations (MICs) and the minimum bactericidal concentrations (MBCs) of *D. multiflora* and *P. pinnata* ethanolic leaves extracts. The above samples were tested for their rate of killing of *E. coli* cells at 1 MIC and 2 MICs. Sorbitol protection, outer membrane permeability, loss of 260-nm-absorbing material, fluorescence microscopy, and DNA degradation assay were used to examine the ultrastructural changes in bacteria induced by the extracts. Results: *D. multiflora* and *P. pinnata* extracts inhibited bacterial growth with MICs of 390.62 and 781.25 µg/mL respectively, while the MBCs values were found to be 781.25 and 1562.5 µg/mL respectively. Treatment with extracts had shorter kill-time in a time-dependent manner with effect most pronounced at 2 MICs than 1 MIC. The MIC of *D. multiflora* increased 4x from 390.62 µg/mL after 24 h of incubation to 1562.5 µg/mL after 7 days in the presence of an osmoprotectant indicating the inhibition of synthesis of cell wall constituents. *P. pinnata* had no effect on cell wall. Both extracts exhibited the greatest leakage and release of DNA materials at 30, 60, 90, and 120 min in concentration-dependent manner. Treated groups had higher values than control. At low concentrations (1/2 MIC and 1 MIC), these extracts effectively permeate the intact outer membrane of Gram-negative bacteria. Both extract were implicated in DNA fragmentation. Moreover, fluorescent cells observed further confirmed its inhibitory effect against the tested pathogen. The antibacterial action involved disruption of membrane potential, increase of membrane permeabilization, leakage of cellular material, and death suggesting them to be an alternative to antibiotics. Conclusion: These findings contribute to the understanding of the antibacterial inhibitory effect of *D. multiflora* and *P. pinnata.*

Keywords: *D. multiflora, P. pinnata, Escherichia coli,* Antibacterial activity, Membrane permeabilization, DNA Fragmentation, Ethanolic leaf extracts.

INTRODUCTION

Infectious diseases remain one of the major public health problems that lead to human morbidity and mortality [1]. *Escherichia coli* is one of the microorganisms that cause significant health threat and responsible for a number of human illness conditions especially in developing countries [2-4]. Currently, a wide range of antimicrobials have shown efficacy against bacterial infections. However, most of them have shown several undesirable side effects [5]. Furthermore, pathogen resistance due to over prescription and overuse of drugs add to the existing causes [6,7]. It is therefore urgent to find novel and efficient agents to control emerging pathogens [8].

The mechanisms by which antimicrobials act are mediated through their ability to inhibit the synthesis of the cell wall and proteins, chromosome replication, and modify cell membrane permeability [9]. The cell wall provides shape and cell integrity. Therefore, the disturbance of cell wall and membrane integrity, and its disruption can lead to metabolic dysfunction and cell death [10,11]. The mode of action of plant extracts is similar to that of antibiotics for it has been shown that they disturb cell structures, increase their permeability, and lead to release of intracellular material [12-14]. The intensive search and employment of innovative research strategies to explore newer, safe, and effective antimicrobial agents which are now being sourced from bioactive components of medicinal plants are increasing [6,15-18]. Plant extracts are used as alternative to antibiotics to prevent and control various diseases, especially in the developing countries because it is relatively cheaper and easily accessible [16,19,20].

The leaves of *Dissotis multiflora* (Sm) Triana (Melastomataceae) are widely used in the Cameroon traditional medicine to treat infectious diseases. In our previous findings, we reported the presence of phytochemicals, antibacterial, antioxidant, cytotoxicity, genotoxicity, and effect of the ethanolic leaf extract of *D.*

multiflora in infectious diarrhea [21-23]. *Paullinia pinnata* Linn (Sapindaceae) family is an important medicinal plant used in many indigenous medical systems for the treatment of infectious diseases [24]. Several studies revealed antibacterial and antidiarrheal activities of extracts and isolated compounds from *P. pinnata* [25-30]. *D. multiflora* and *P. pinnata* have shown promising antibacterial activities. However, the information available regarding their mode of action is not yet reported. Therefore, it is necessary to explore the target sites of these two extracts towards *E. coli*. The present study is a follow up on the previously reported antibacterial activity of the two extracts. It was designed to determine the mechanisms of action of *D. multiflora* and *P. pinnata* in antimicrobial potential against *E. coli* through kill-time analysis, interferences in the synthesis of cell wall, the permeability of cell membrane, the release of cell constituents, and the DNA degradation assay, in order to better understand their antibacterial activity.

MATERIALS AND METHODS

Reagents and chemicals

2,3,5-triphenyltetrazolium Chloride (TTC) (Himedia, India), Dimethylsulfoxide (DMSO), sorbitol (0.8 M) (Srichem, India), agarose (Himedia, India), Ethidium bromide (Himedia, India), Bromophenol blue (Himedia, India), xylene cyanol FF (Himedia, India), glycerol (Himedia, India), Tris, base (Himedia, India), Tris-Hcl (Himedia, India), Ethylenediamine teraacetic acid (EDTA) dihydrate (Fischer Scientific), Mueller-Hinton agar (Himedia, India), Mueller-Hinton broth (Himedia, India), Genomic DNA extraction kit (GeneiTM, India), DNA ladder (1 Kb, Sigma, India), Whatman No. 1 filter paper (Whatman GE Healthcare Company). All the other chemicals of analytical grade were purchased from Sigma-Aldrich India Limited, Hi Media, India and Merck, India.

Microorganism and Culture

A clinical strain of *E. coli* was used in this study and provided by the Department of Bioscience and Biotechnology, Banasthali Vidyapith, Banasthali, India and was stored at -80°C in 15% glycerol stocks to be used in the study. Colonies grown on a Mueller-Hinton agar plate incubated overnight at 37°C were inoculated in 10 mL of Mueller-Hinton broth, and incubated at 37°C under shaking and used.

Plant Material and extraction

D. multiflora and *P. pinnata* leaves were collected and treated as previously described [23,30]. The leaves of *D. multiflora* and *P. pinnata* were air-dried for two weeks and ground separately. Then, fine dried material powder was soaked for 48 h in 95% ethanol with frequent stirring. The mixture was filtered with Whatman No. 1 filter paper and concentrated using a Rotavapor (Buchi) at 55°C. Then, each extract was collected in Eppendorf tube and preserved in a refrigerator at 4°C for further use. Sample was diluted ten-fold in 1% (v/v) dimethylsulfoxide (DMSO) and used in the assays.

Determination of the Minimum Inhibitory Concentrations (MICs) and the Minimum Bactericidal Concentrations (MBCs)

The MICs and the MBCs of the crude extracts to *E. coli* were determined by microbroth dilution method in 96 plates as previously described [31]. Briefly, each well was filled with 100 µL of Mueller Hinton broth. A series of two-fold dilution of each crude extract was made with final concentrations ranged from 25 x 10^3 to 24 µg/mL and from 25 to 0.048 µg/mL for ciprofloxacin used as reference. A 100 µL standardized suspension of *E. coli* (1.5 x 10^6 CFU/mL) was mixed with the above samples. After 24 h incubation at 37°C, MICs were determined after the addition of 40 µL of 2,3,5-triphenyltetrazolium chloride (TTC) at 0.01% in sterile distilled water and incubation at 37°C for 30 min. Wells that contained only extract and nutrient broth were used as negative control. The MIC was defined as the lowest concentrations those inhibited visible bacterial growth. The MBCs were determined by picking 50 µL aliquots from each well having no growth in MICs assay plate to new plate with each 100 µL of Mueller-Hinton broth, and the lowest concentration of the well which did not show colour change by addition of TTC.

Bacterial Growth Curve

The effects of the crude extracts on *E. coli* growth were evaluated following a previous report by Babii et al. [32]. Briefly, a volume of 150 µL from an overnight preculture of *E. coli* containing 1.5 x 10^6 CFU/mL was added in 15 mL of Mueller Hinton Broth with 1/2 MIC, MIC, and 2 MIC values as final concentrations of *D. multiflora* and *P. pinnata*. Treated samples were incubated on an orbital shaker (190 rev/min, 37°C) for 10 h. Growth rates were determined by measuring OD600 at regular intervals using UV-vis spectrophotometer. 1% DMSO was supplemented to control.

Sorbitol Protection Assay

The effects of sorbitol on antibacterial activity of the crude extracts were evaluated by microbroth dilution method according to the previous report by Frost et al. [33] with slight modifications. Briefly, duplicate plates (96 wells) containing test samples (100 µL) were prepared; one containing two-fold dilutions of test extracts and the other containing test extracts plus 0.8 M sorbitol (Srichem, India) as an osmoprotectant. 100 µL of cell suspension (0.5 McFarland) was added in all the wells, plates were then incubated at 37°C (Cooling Incubator, REMI). The plates were read at 2 and 7 days.

Release of 260 nm-Cellular Absorbing Materials

The measurement of the release of 260 nm-absorbing-materials from *E. coli* cell was performed as previously described by Bennis et al.[34] with slight modifications. Briefly, a suspension of *E. coli* (2.5 x 10^7 CFU/mL) grown in Mueller Hinton Broth (Himedia, India) at 37°C for 48 h was treated with *D. multiflora* and *P. pinnata* extracts at 1/2 MIC, 1 MIC, 2 MIC for 1 h or a fixed concentration (1 MIC and 2 MIC) for different time intervals (30, 60, 90, and 120 min). Suspension without extract was run as negative control. After treatment, samples were centrifuged at 10,000 x g for 10 min (Cooling centrifuge, REMI), and the absorbance of supernatant was read at 260 nm using UV-vis spectrophotometer.

Outer Membrane Permeability

The outer membrane permeability was determined according to Hao et al. [35] with slight modifications as described by Da Silva et al. [36]. Briefly, a 18 h *E. coli* culture (5 x 10^7 cells/mL) was inoculated into Mueller-Hinton broth containing extract at 0 MIC, 1 MIC, and 2 MICs on the sterilized test tubes. The mixture was incubated at 37°C for 24 h. *E. coli* growth was measured at 450 nm using UV-vis spectrophotometer.

Fluorescent Microscopic Analysis

The bacterial membrane integrity was appreciated by capturing confocal photomicrographs of the uptake of the fluorescent nuclear dye ethidium bromide for *E. coli* as

previously described by Lambert et al. [37] with slight modifications. Exponential phase cells were centrifuged at 4000 x g for 5 min (Cooling centrifuge, REMI, India), washed several times and suspended in phosphate buffer saline (PBS, pH-7.4). 10 µg/mL of ethidium bromide was added to 200 µL aliquots of these cells, then extracts were added for final concentrations equal to 1 MIC and 2 MICs values of DMSO. During the 37°C incubation, samples of treated and untreated cells were taken at 2 h and the fluorescent cells were observed using a Leica Confocal Laser Scanning Microscope (Leica DM 1000). At least three random, independent images were captured per sample. The percentage of fluorescent cells was determined by calculating the ratio between fluorescent cells and total cells visualized with differential interference contrast (DIC).

DNA degradation assay

The effect of *D. multiflora* and *P. pinnata* on DNA integrity was evaluated as previously reported by Babii et al. [32]. The genomic DNA was isolated after 3 h treatment of *E. coli* with extracts or DMSO using a ZR Bacterial DNA MiniPrep kit (Zymo Research, Reiburg, Germany). The DNA samples were electrophoresed in 1% agarose gel at 120 V until sufficient resolution was obtained. A 1 kb ladder (New England Biolabs, Ipswich, MA) was used as standard size marker. The bands were visualized by ethidium bromide (EB) in gel staining under UV light (GeNei™, UVITEC, Cambridge).

Statistical Analysis

Data are expressed as mean ± standard deviation of triplicate determinations. Microsoft Excel was used to enter and capture data from which graphs and tables were extracted. Differences between groups were assessed by One-way analysis of variance (ANOVA) with post hoc comparisons. All analyses were carried out using GraphPad Prism 7. Significant differences were considered at $P < 0.05$.

RESULTS

MIC and MBC

The antibacterial activity of *D. multiflora* and *P. pinnata* leaves extracts was performed using clinical isolate of *E. coli*. Both plant exhibited potent inhibitory effect as shown by their various MIC and MBC values. In this assay, the MIC values of *D. multiflora* and *P. pinnata* against the tested pathogen were found to be 390.62 and 781.25 µg/mL respectively, while the MBC values were found to be 781.25 and 1562.5 µg/mL respectively. Ciprofloxacin exhibited a MIC and MBC value of 1.62 µg/mL. A bactericidal activity of all the extracts was recorded.

Bacterial Growth Curve

D. multiflora and *P. pinnata* leaves extracts inhibited the growth of *E. coli* at different concentrations used with immediate decrease of bacterial load 1 h after incubation. Treatment with *D. multiflora* extract showed a decrease in bacterial load at different times in concentration dependent manner (Figure 1A). As from 6 h, an increase of bacterial load was noted with 1/2 MIC. *P. pinnata* extract showed a decrease in bacterial load from 0 to 2 h of incubation (Figure 1B). However, an increase in bacterial load was noted from 2 to 6 h with 1 MIC and 2 MICs.

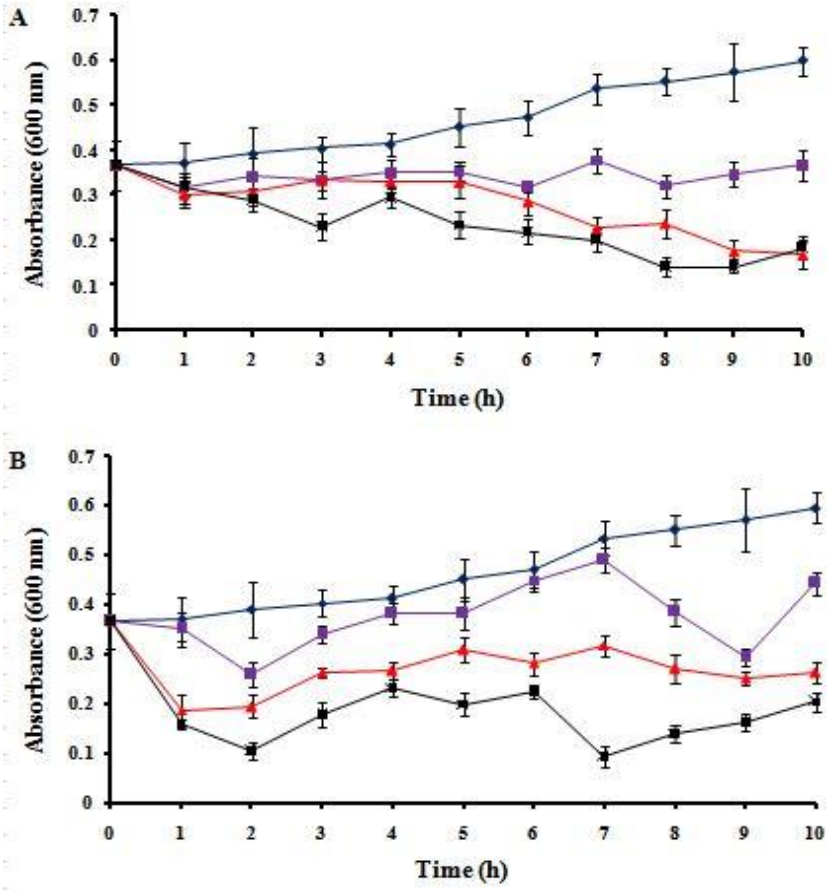

Figure 1: The growth curves of *E. coli* cells exposed to different concentrations (1/2 MIC, 1 MIC, and 2 MICs) of extracts. ♦ Control; ■ 1/2 MIC; ▲ 1 MIC; ■ 2 MICs. (A): *D. multiflora;* (B): *P. pinnata.* Values are mean ± standard deviation of independent triplicate measurements. Values in the same column with different superscripts are significantly different according to Post hoc multiple comparison test ($P < 0.05$).

Sorbitol Protection Assay

The effect of the ethanolic extracts of *D. multiflora* and *P. pinnata* on bacterial cell wall was determined in the presence and absence of sorbitol (Table 1). The antibacterial activity of *D. multiflora* decreased in the presence of sorbitol, an osmoprotectant with increasing MIC observed. The MIC of *D. multiflora* increased 4x as from day-7 with MIC varying from 390.62 µg/mL after 48 h of incubation to 1562.5 µg/mL after 7 days. However, the MIC of *P. pinnata* remained the same in the presence and absence of sorbitol. These results showed that *D. multiflora* extract was implicated in the inhibition of the synthesis of cell wall constituents of *E. coli* while *P. pinnata* had no effect on the cell wall.

Table 1: Effect of the ethanolic extracts of *D. multiflora* and *P. pinnata* on the cell wall of *E. coli* in the presence and absence of sorbitol

Test sample	Mimimum inhibitory concentration (µg/mL)			
	Day-2		Day-7	
	-Sorbitol	+Sorbitol	-Sorbitol	+Sorbitol
D. multiflora	390.62[a]	1562.5[b]	781.25[c]	1562.5[b]
P. pinnata	1562.5[b]	1562.5[b]	3125[d]	3125[d]

Values are mean ± standard deviation of independent triplicate measurements. Values in the same column with different superscripts are significantly different according to Post hoc multiple comparison test ($P < 0.05$).

Release of 260nm-Cellular Absorbing Material

The OD260 values of the filtrates from *E. coli* control suspension remained approximately the same after 30, 60, 90, and 120 min. Upon treatment with the 1/2 MIC, 1 MIC, and 2 MICs of *D. multiflora* and *P. pinnata*, the OD values of the filtrates from *E. coli* suspensions increased after 1 h in concentration-dependent manner. The OD values from suspensions treated by the extracts were greater than values from control (Figure 2).

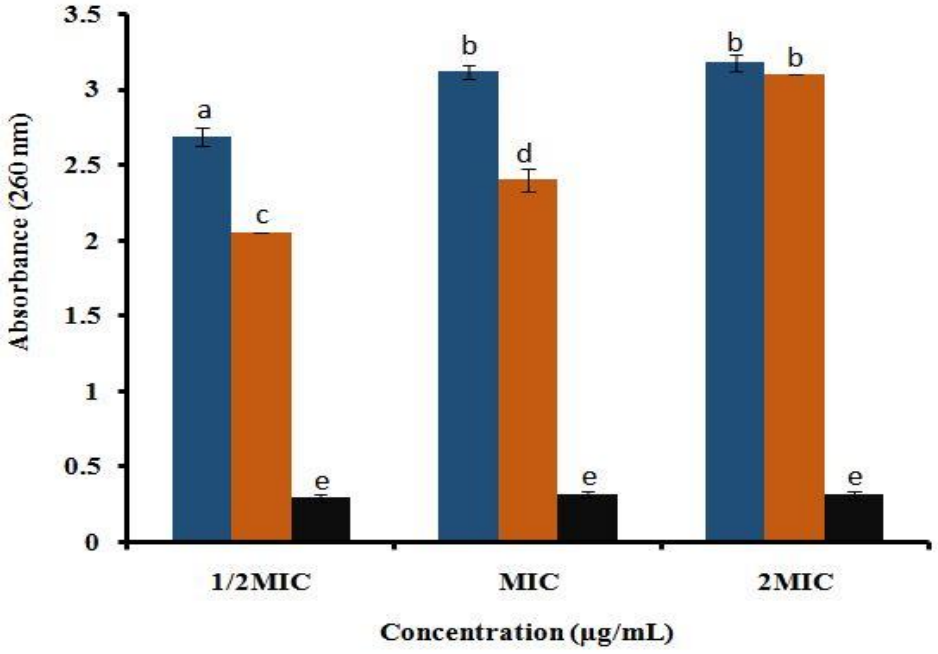

Figure 2: Release of cellular material of *E. coli* absorbing at 260 nm following treatment with *D. multiflora* and *P. pinnata* extracts for 60 minutes. ■ *D. multiflora*; ■ *P. pinnata*; ■ Control. Values are mean ± standard deviation of independent triplicate measurements. Values in the same column with different superscripts are significantly different according to Post hoc multiple comparison test ($P < 0.05$).

The release of 260 nm-cellular absorbing materials was assessed with extracts at concentrations corresponding to 1 MIC and 2 MICs. No increase of OD was observed from 30 to 90 min with 1 MIC and 2 MICs after exposure to *D. multiflora* (Figure 3A). However, an increase of OD was noted with increasing concentration of *P. pinnata* (Figure 3B).

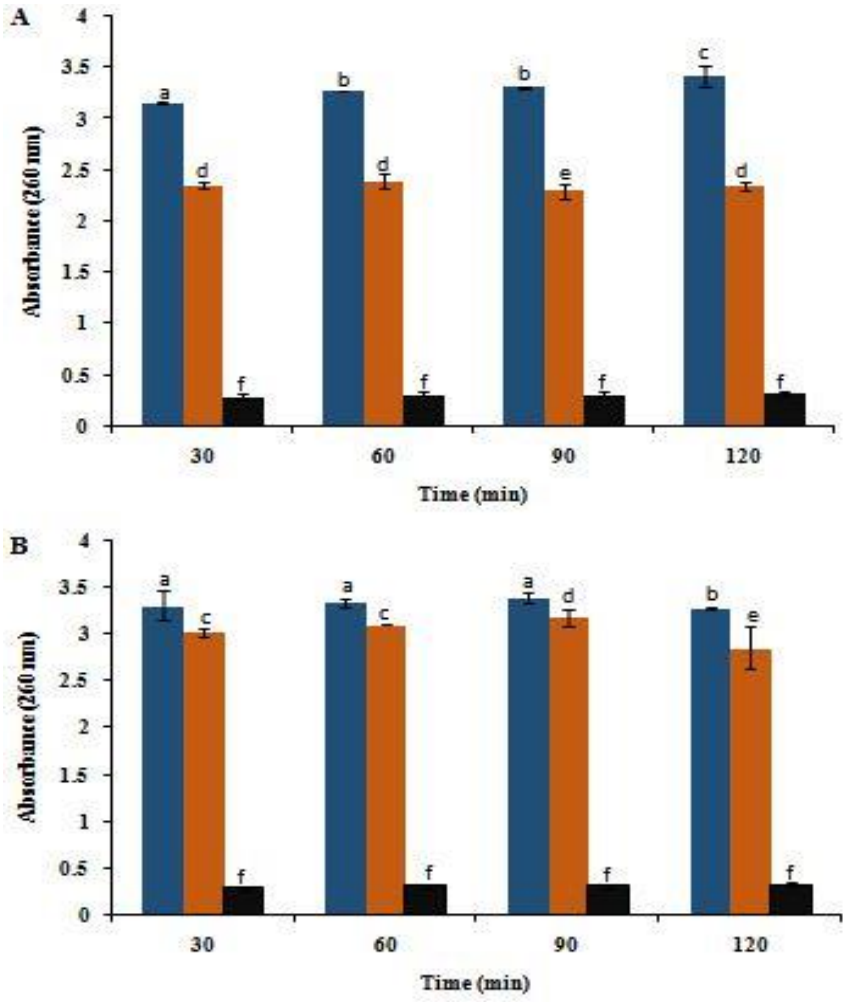

Figure 3: Release of 260 nm-absorbing material of *E. coli* following treatment with *D. multiflora* and *P. pinnata* extracts at different times. ■ *D. multiflora*; ■ *P. pinnata*; ■ Control. (A): 1 MIC; (B): 2 MICs. Values are mean ± standard deviation of independent triplicate measurements. Values in the same column with different superscripts are significantly different according to Post hoc multiple comparison test ($P < 0.05$).

Outer Membrane Permeability

The permeability of external membrane was determined at different concentrations (1/2 MIC, 1 MIC, and 2 MICs). A decrease of OD of external membrane constituents of *E. coli* exposed to *D. multiflora* and *P. pinnata* compared with control which showed constant absorbance (Figure 4). *D. multiflora* and *P. pinnata* increased membrane permeability of *E. coli* that led to the leakage and release of cellular material with activity most pronounced at 1/2 MIC and 1 MIC than 2 MICs.

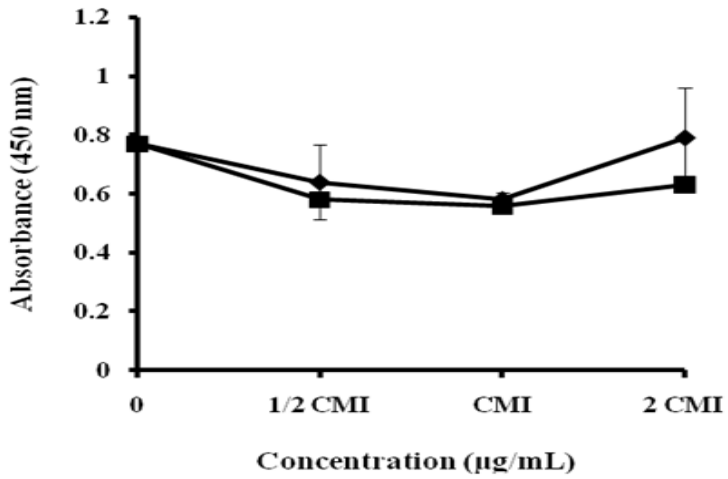

Figure 4: Outer membrane permeability of *E. coli* following treatment with *D. multiflora* and *P. pinnata* extracts at 1/2 MIC, 1 MIC, and 2 MICs. ♦ *D. multiflora*; ■ *P. pinnata*. Values are mean ± standard deviation of independent triplicate measurements.

Fluorescent Microscopy

As *D. multiflora* and *P. pinnata* were able to reduce cultivable cell number of *E. coli*, it was of interest to examine whether they had an effect on biofilm viability. The ability of both the plant extracts to damage cellular membrane and make cellular nucleic acids accessible to fluorescent dye was determined after ethidium bromide staining. Intense fluorescent cells were observed after 2 h treatment with *D. multiflora* (Figure 5). The percentage of fluorescent cells increased with increasing concentration (1 MIC and 2 MICs) for *D. multiflora* (Figure 6). Similar results were observed after exposure with *P. pinnata* extract especially at 2 MICs (Figure 6). However, treatment with *P. pinnata* extract did not cause an intense increase in mean fluorescence percentage.

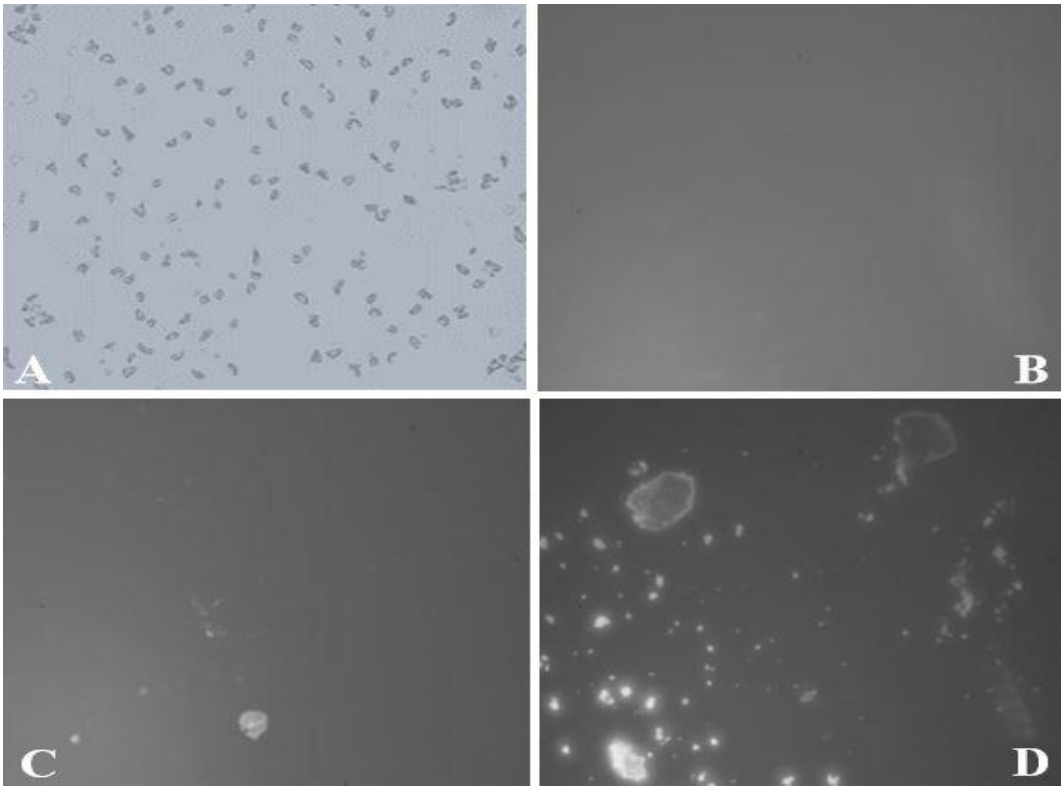

Figure 5: Inner membrane permeabilization induced by plant extracts. *E. coli* cells membrane disruption effect due to 2 h exposure to *D. multiflora*. Visualised by efflux of ethidium bromide (10 µg/mL), a nuclear fluorescent dye. Images are representative of typical results. (A): image of contrast of differential interference 1 min after exposure; (B): fluorescent image 1 min after exposure; (C): fluorescent image 2 h without exposure (Control); (D): fluorescent image 2 h after *D. multiflora* exposure; excitation line: 488 nm.

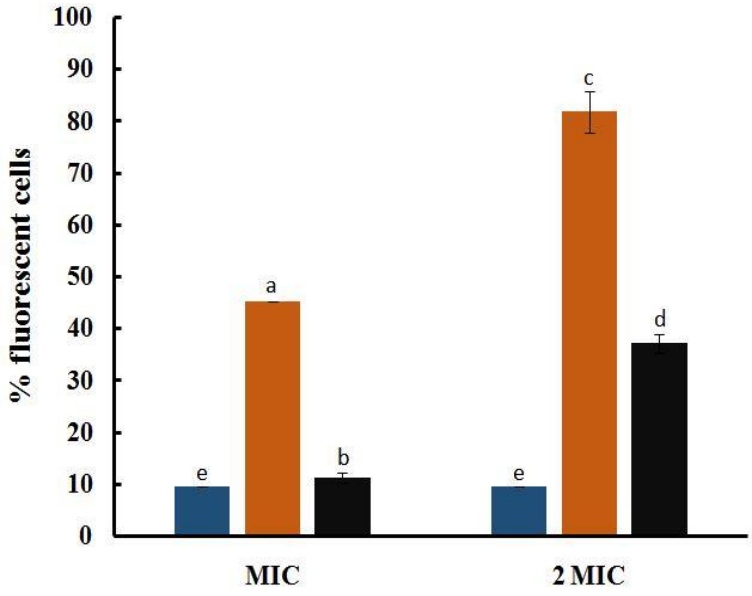

Figure 6: Mean fluorescence intensity in *E. coli* treated with different plant extracts. ■ Control; ■ *D. multiflora*; ■ *P. pinnata*. At least three random, independent images were captured per sample and the ratio between fluorescent cells and total cells visualized with differential interference contrast (DIC) was calculated as percentage. Values in the same column with different superscripts are significantly different according to Post hoc multiple comparison test ($P < 0.05$).

DNA degradation assay

This test was performed to detect damages of genomic DNA of *E. coli* by *D. multiflora* and *P. pinnata* extracts by observing bands on agarose gel after 3 h of exposure. The agarose gel electrophoresis of extraction products from treated samples showed no bands while that of DNA sample from *E. coli* not exposed to extracts showed bands similar to that of DNA ladder (Figure 7). These observations evidence the fact that both extracts maybe implicated in the hydrolysis of DNA.

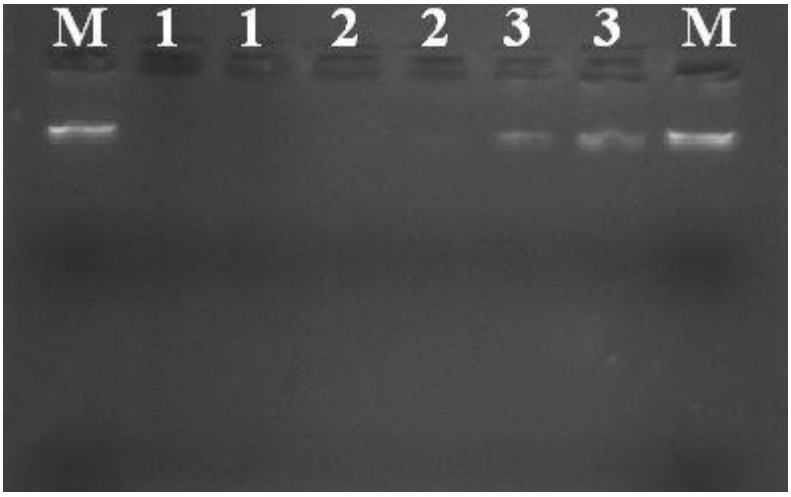

Figure 7: Agarose gel electrophoresis 1% of genomic DNA from *E. coli* after exposure with ethanolic extracts of *D. multiflora* and *P. pinnata.* M: DNA ladder (1 Kb); Lanes 1: DNA sample from *E. coli* treated with *D. multiflora* for 3 h; Lanes 2: DNA sample from *E. coli* treated with *P. pinnata* for 3 h; Lanes 3 : DNA from *E. coli* not exposed to extracts.

DISCUSSION

Despite diversity and aggressiveness of current antimicrobial treatment, bacterial infections still remain a public health concern in developing countries that face a lot of difficulties to solve the problem to which can be added resistance to current antibacterial agents used [38-39]. Natural products are reservoirs of novel efficient compounds with diverse biological properties that contribute significantly to the discovery of antibacterial substances [40-41]. *D. multiflora* and *P. pinnata* are used to treat diarrhea and other infectious diseases. To date, few studies revealed pharmacological properties of *D. multiflora* while many of them focussed on biological properties of *P. pinnata* [42-44]. However, no previous study was carried out to understand their mechanism of action in order to clarify their biological activity.

MIC and MBC

The antibacterial assay shows that *D. multiflora* and *P. pinnata* exhibited antibacterial activity with bactericidal effects. These results are in accordance with the similar reports that have confirmed their antibacterial efficacy [21,29].

Bacterial Growth Curve

In the present study, the bacterial growth curve of *E. coli* decreased with increasing concentrations and time of exposure to *D. multiflora* and *P. pinnata* leaves extracts. The results obtained revealed that both plant extracts exhibited bactericidal effects as shown by the immediate decrease of bacterial load 1 h after incubation. Hence, the ability of the plant extracts to inhibit the growth or cause death of bacteria is an indicator of their efficacy [32]. These observations are consistent with the previous report by Zhang et al. [4] who evidenced the shortening of kill-time following treatment of meat-borne *E. coli* with black pepper essential oil.

Sorbitol Protection Assay

The rigid cell wall of Gram-negative bacteria does not allow penetration of antibacterial agents contributing to resistance [45]. In order to determine the effect of the plant extracts on bacterial cell wall, an osmotic stabilizer, namely sorbitol was added in the medium. It has been shown that several antibacterial agents' action can be reduced in the medium containing an osmotic stabilizer [33]. The present study revealed a marginal increase of the MIC observed in the presence of *D. multiflora* extract and sorbitol signifying that the macromolecules and enzymes which ensure the synthesis of *E. coli* cell wall constituents are *D. multiflora* extract's targets [46]. The findings of this study are similar to the observation of Frost et al. [33] who reported the inhibitions of fungal cell wall synthesis and assembly. It has been demonstrated that flavonoids can complex soluble extracellular proteins and bacterial cell wall [20,47]. No evidence of inhibition of synthesis and cell wall assemblage was noted in the presence of *P. pinnata* extract and sorbitol. However, this extract causes membrane permeability suggesting that it should be its primary target site.

Release of 260nm-Cellular Absorbing Material

Exposure of *E. coli* to crude extracts of *D. multiflora* and *P. pinnata* led to rapid loss of 260 nm absorbing molecules in time and concentration-dependent manner. These results indicate eventual rupture of cellular membrane permeability control [48]. Consistent with these findings, the release of intracellular materials was reported by previous study [3]. In fact, the nucleic acids and proteins are essential constituents of bacteria cells. The loss of nucleic acids leads to dysfunction of DNA and proteins synthesis and inhibition of cellular growth. The disruption of cellular membrane releases DNA and RNA with high absorbing potential at 260 nm [3]. Furthermore, saponins found in these extracts can cause lysis of certain membrane and plasmatic proteins while tannins can inactivate microbial adhesion, enzymes, and proteins of cellular envelope [49,50]. The plants' extracts may have cause damage of membrane that lead to efflux of nucleotides and then a complete lysis of cell resulting to death [36,51,52]. It has been demonstrated that phenols and flavonoids possess detergent properties which exert action

on the destruction of cellular membrane [20]. Moreover, alkaloids were shown to intercalate between cellular membranes [53].

Outer Membrane Permeability

The outer membrane permeability of *E. coli* was evaluated in the presence of *D. multiflora* and *P. pinnata* extracts. The differential sensitivity of *E. coli* to antibiotics should be due to the fact that the peptidoglycan of Gram-negative bacteria is surrounded by external membrane that limits the diffusion across its covering lipopolysaccharides (LPS) that prevent penetration of numerous toxic compounds [54,55]. Phenolic compounds can extract LPS from bacterial membranes and intervene in their destruction [56]. The increase of membrane permeability of *E. coli* in the presence of *D. multiflora* and *P. pinnata* extracts was noted with 1/2 MIC and 1 MIC. Therefore, at low concentrations, these extracts effectively permeate the intact outer membrane of Gram-negative bacteria. This may be due to the presence of these bioactive secondary metabolites that facilitates the entry of antibacterial substances in the cell and affect the permeability and integrity of membrane followed by the loss of intracellular substances [57,58]. These results are similar with the previous report by Da Silva et al. [36] who showed that the hydroethanolic extract of *Piper umbellatum* increases permeabilization of outer membrane of *Shigella flexneri*, facilitating the entry of the antibiotics.

Fluorescent Microscopy

The effect of *D. multiflora* and *P. pinnata* extracts on membrane integrity was further confirmed by fluorescence microscopy using a fluorescent dye, ethidium bromide. Increased fluorescent was noted following treatment with the plant extracts. These results are consistent with the membrane disruption and bactericidal effects reported on *E. coli* in the present study. The fluorescent cells observed without exposure to the plant extracts may be due to natural death of *E. coli*. Moreover, the mean fluorescent intensity in *E. coli* treated with different plant extracts increased with increasing concentration and duration of exposure, and was most pronounced in the presence of *D. multiflora.* These results are similar with the previous results. [59] The fluorescent cells observed owed to plant extracts exposure indicated damage of cellular membrane which makes cellular nucleic acids accessible to the ethidium bromide [32].

DNA degradation assay

In our study, genomic DNA was extracted in order to determine the effect of *D. multiflora* and *P. pinnata* extracts on DNA degradation. It is evident from the results that both extracts were implicated in bacterial DNA degradation. This may be attributed to the phenolic components which can alter the structure and morphology of DNA [60].

The findings of this study suggests that the antimicrobial action of *D. multiflora* and *P. pinnata* could be due to their efficacy to alter cell membrane permeability parameters of the bacteria, and provoke leakage and release of cellular materials. These modifications of membrane permeability and the release of the cellular material lead to disorder, decomposition, and event death corresponding to a similar reduction in the time kill of viable bacteria. Due to the heterogeneous compositions of both extracts it is suggested that compounds from these plants products should be screened to obtain novel and effective antibacterial agents.

CONCLUSION

In summary, we report that the ethanolic leaf extracts of *D. multiflora* and *P. pinnata* exhibited antibacterial activity on *E. coli* with action in time and concentration dependent. This inhibitory activity of *D. multiflora* is through inhibition of cell wall constituents, while both the plant extracts act by permeabilization of cell membrane, release of intracellular materials, and DNA fragmentation.

LIMITATIONS OF THE STUDY

Only one strain (clinical isolate) of *E. coli* was used as test microorganism. Positive and negative control drugs were not included in the experiments as well as a membrane disturbing agent. In fact, this is a preliminary study which determined the susceptibility of the above strain in the presence and absence of the plant extracts. The upcoming conclusive study will include both the isolates and characterized strains, positive and negative control drugs, and bioactive isolated compounds.

AUTHOR CONTRIBUTIONS

AA, MN and FE designed the study. JP and SV supervised experiments. AA carried out the experiments, analyse data, and wrote the manuscript. JP critically revised the paper and intellectual content.

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CONFLICT OF INTEREST

The authors declare that there is no potential conflict of interest regarding the publication of this paper.

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