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Research Article

Biofilm inhibitory activity of selected medicinal plants against enterotoxigenic *Escherichia coli* ATCC 35401 strain

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Abstract
Enterotoxigenic *Escherichia coli* (ETEC), a pathotype of *E. coli*, accounts for a significant episode of diarrhea among under five years children. In infectious diarrhea, biofilms contribute to treatment failures. Using crystal violet-based biofilm inhibition assay, we investigated chloroform and acetone extracts of nine selected medicinal plants for biofilm inhibitory activity against ETEC. Extracts that inhibit biofilm without affecting planktonic bacterial growth were further profiled by phytochemical screening and infrared spectroscopic analysis. Of the eighteen extracts investigated, only two chloroform extracts, namely; *Psidium guajava* and *Nauclea latifolia*, gave reproducible biofilm inhibitory activity of 44% and 30%, respectively, against ETEC while inhibiting growth by less than 10%. The two extracts, found to be rich in phytochemicals, showed relatively good antibiofilm activity against ETEC. *Psidium guajava* and *Nauclea latifolia* could be sources of lead compounds for development of drugs to control infectious diarrhea caused by ETEC with less selection pressure on the pathogen.

Key Words: Biofilm inhibition, enterotoxigenic *Escherichia coli, Psidium guajava, Nauclea latifolia*

INTRODUCTION

Diarrhea is among the leading cause of illness among children under five years of age worldwide, with about 1.7 billion cases and 525,000 deaths annually (Hartman *et al*., 2023). Diarrheal deaths have been decreasing over time in developed countries on account of various advancements comprising improved sanitation, hygiene, nutrition and vaccines. However, this is less so in many low-and middle-income countries (LMICs) as approximately 90% of diarrheal deaths occur in sub-Saharan Africa and South Asia (Demissie *et al*., 2021; Hartman *et al*., 2023; Okeke, 2009; Troeger *et al*., 2018).

Enterotoxigenic *Escherichia coli* (ETEC), a pathotype of E. coil, was revealed by The Global Enteric Multicentre Study as one of the leading infectious pathogens causing moderate-to-severe diarrhea in under-fives children in LMICs; accounting for over 200 million diarrhea episodes annually (Kotloff *et al*., 2013; Toledo *et al*., 2023). The ETEC is also one of the commonest aetiologies of diarrhea in travelers to LMICs, foodborne outbreaks in developed countries, and is implicated in persistent diarrheal diseases (Beatty *et al*., 2006; Fleckenstein, 2021).

Infectious diarrhea often requires the use of antibiotics as part of overall therapeutic management. Biofilms formation by some diarrheagenic pathogens is reported to be one of the contributory factors undermining therapy with antibiotics. Biofilms are known to be complex, polymicrobial communities embedded in a protective extracellular matrix of organic polymers which impedes permeability to antimicrobial agents. Thus, infections associated with biofilms are usually difficult to treat (Cleaver & Garnett, 2023; Fleckenstein & Kuhlmann, 2019; Okeke, 2009). While there are growing efforts to develop vaccines to control enteric pathogens, the quest for an approach that reduces resistant selection pressure such as inhibition of biofilm formation represents a noteworthy alternative approach (Fleckenstein, 2021; Kwasi *et al*., 2022).

A number of medicinal plants are used in folk medicine to treat diarrheal diseases, many of which have been shown to have antibacterial properties against many diarrheagenic pathogens (Dubreuil, 2013; Haudecoeur *et al*., 2018; Rawat *et al*., 2017). Based on these reported ethnomedicinal uses and available documentation in the literature, some medicinal plants were selected for this study. Here, we report on the potential antibiofilm activity of eighteen solvent extracts obtained from nine selected medicinal plants against ETEC ATCC 35401.

MATERIALS AND METHODS

Plant materials: Fresh leaves of *Psidium guajava* L, *Vernonia amygdalina* Del., *Gmelina arborea* Roxb., *Nauclea latifolia* Sm, *N. diderrichii* (De wild.) Merr., *Morinda yucatanensis* Greenm., and *Vitellaria paradoxa* C. F. Gaertn. Roscoe were collected around the University of Ibadan, South-West, Nigeria. The leaves were air-dried at ambient temperature (26-30°C) for four weeks and milled into coarse powder using a laboratory blender. *Garcinia kola* Heckel seeds and *Zingiber officinale* Roscoe rhizomes were procured from Ojoo market in Ibadan. The seeds and the rhizomes were chopped into pieces, air-dried at ambient temperature (26-30°C) for eight weeks, and milled into coarse powder. All plant samples were identified, authenticated and specimens deposited at the Forestry Research Institute of Nigeria Jericho, Ibadan herbarium.

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Bacterial strain: Enterotoxigenic *Escherichia coli* (ETEC) ATCC 35401 reference strain was retrieved from the Department of Microbiology, School of Life Sciences Federal University of Technology Akure and cultured in the Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmacy, University of Ibadan, Nigeria.

Chemicals and reagents: Luria Bertani broth (LB Merck, Germany), magnesium sulphate powder (RX Chemicals, India), L-arginine powder (Biotech USA), crystal violet powder (Sigma Aldrich England), Dimethyl sulphoxide (DMSO, MP, USA), distilled water, Dulbecco's Modified Eagles Medium (DMEM, ThermoFisher Scientific). Nitazoxanide tablet was sourced from a local Pharmacy store in Ibadan, Nigeria. All solvents were analytical grades, Sigma Aldrich, UK.

Solvent extraction: The pulverized plant materials (120 g each) were initially defatted using 500 ml of n-hexane. Afterward, samples were extracted by successive cold maceration in solvents of varying polarities for 24h in each instant: chloroform 500 mL (twice), the mark dried and then acetone 500 mL (twice). The supernatants were filtered, concentrated under reduced pressure at 30°C and later dried in-vacuo for 48h. The dried extracts were kept in a refrigerator until needed.

Growth and biofilm inhibition assays: The growth and biofilm inhibition assays were conducted with little modification as previously described (Ahmad *et al*., 2019; O'Toole, 2011). The ETEC strain ATCC 35401 was grown in Luria Bertani broth (LB) overnight. A 1:100 dilution of the standardized overnight culture was made into fresh sterilized medium, with magnesium sulphate and arginine as supplements. 100 µL of the diluted medium was transferred into 96-well plates, followed by transferring test concentrations (0.03 g of each extract in 1 mL of DMSO) into the wells except the control wells, and incubating for 24 h at 37 ºC. After incubation, the growth inhibition was determined by taking measurements in a micro plate reader at 550 nm. The media were then carefully aspirated, while the plates' wells were washed with sterile distilled water three times and dried. 125 µL of 0.1% solution of crystal violet was added per well and incubated at ambient temperature for 15 minutes. Afterward, the plates were removed, washed again three times with sterile distilled water and allowed to air dry. After drying, 30% acetic acid in water was added to each well to solubilize the content and the plates incubated for 15 minutes. 125 µL solubilized microplates content were then transferred to new flat-bottomed microplates and the absorbance determined at 550 nm. Diluted medium, without the test sample, serves as negative control and 0.02 g/mL of nitazoxanide as positive control. All experiments were run in triplicates. Data from growth and biofilm inhibition assays (average of three replicates) were analyzed by comparing percentage inhibitions for the chloroform and acetone extracts using Student's t-test with the aid of GraphPad Prism Software (GraphPad Software Inc., USA).

The percentage growth and biofilm inhibitory effects of each extract against the tested organism were computed using the formulae:

$$\% \text{ Inhibition} = \frac{\text{Average OD}_{\text{control wells}} - \text{Average OD}_{\text{treated wells}}}{\text{Average OD}_{\text{control}}} \times 100 \quad \text{.....Equation 1}$$

Where OD represents the optical density taking from the plate reader.

Phytochemical and structural profile of bioactive extracts: To document the secondary metabolites profile of the bioactive extracts that showed biofilm inhibitory effect (≥30%) while inhibiting growth by under 10% (Kwasi *et al*, 2022), the phytochemical composition was determined following standard procedures (Sofowora, 1993). In addition, 1 mg of the extracts were smeared on a diamond crystal, inserted into the FT-IR instrument, Perkin-ElmerR spectrum 2, and measured by attenuated total reflectance (ATR) method.

RESULT

Table 1.
Details of medicinal plants and the percent yields of solvents extraction

S/ no.	Plant species	Family Name	Common name	Local name(s)	Part used	FHI no.	Yield (% w/w)	
							C*	A**
1	*Psidium guajava* L.	Myrtaceae	Guava	Guaba, Goba, Ugwoba	Leaf	114253	7.4	3.2
2	*Vernonia amygdalina* Del.	Asterales	Bitter leaf	Ewuro, Oriwo, Onugbu	Leaf	114254	7.4	1.9
3	*Gmelina arborea* Roxb.	Lamiaceae	White teak	Malaina	Leaf	114255	12.3	4.2
4	*Garcinia kola* Heckel	Clusiaceae	Bitter kola	Orogbo, Namijin Gworo	Seed	114258	5.1	3.9
5	*Nauclea latifolia* Sm.	Rubiaceae	African peach	Egbesi, Ubulu inu, Ttabashiya	Leaf	114256	3.0	2.7
6	*Morinda yucaata-nensis* Greenm.	Rubiaceae	Brimstone tree	-	Leaf	114257	7.1	1.9
7	*Vitellaria paradoxa* C. F. Gaertn	Sapotaceae	Shea-butter	K'awara, Emi-emi, Okwuma	Leaf	114260	4.8	3.9
8	*Zingiber officinale* Roscoe	Zingiberaceae	Ginger	Ata ile, Jinga, Chita	Rhizome	114259	6.5	1.8
9	*Nauclea diderrichii* (De wild.) Merr.	Rubiaceae	Opepe	Opepe, Uburu, Tafaashhya	Leaf	114261	4.9	2.7

*C – Chloroform; **A - Acetone

Extraction yield: The percentage yields from the successive extraction and other details about the selected plant species are presented in Table 1. The yields, which ranged from 12.3 – 1.8 %w/w, were relatively higher for the chloroform extracts when compared with the acetone extracts.

Bacterial growth and biofilm inhibitions: The results of growth and biofilm inhibitory effects on the ETEC ATCC 35401 strain are presented in Figure 1 and Figure 2, respectively. Growth inhibitions for the extract ranged from 51.8% (*G. arborea*, acetone extract) to 3.1% (*M. yucatanensis*, chloroform extract). For the biofilm inhibitions, the highest value was 44.3% (*P. guajava*, chloroform extract). Overall, only the chloroform extracts of *P. guajava* and *N. latifolia* showed biofilm inhibitory effects (≥30%) while inhibiting growth by less than 10%. No inhibition of growth and biofilm was observed with the negative control. Nitazoxanide, the positive control gave 25.8% (growth inhibition) and 48.3% (biofilm inhibition) at the concentration studied.

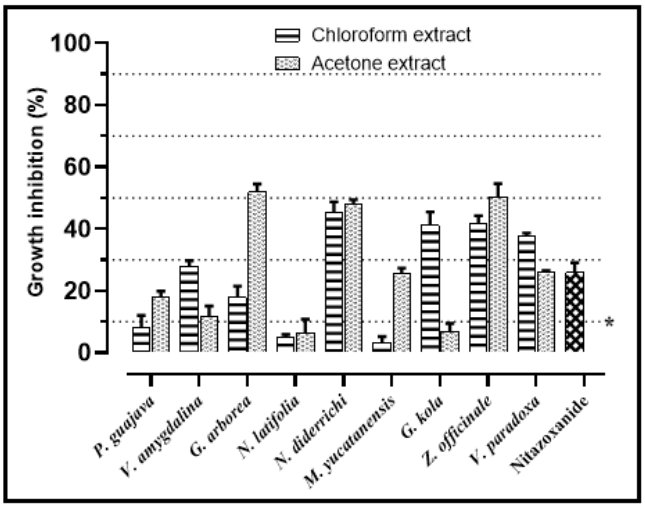

Figure 1.
Percent growth inhibition of the selected plants extracts against enterotoxigenic *Escherichia coli* ATCC35401 strain. Each value represents three replicates of three independent experiments after incubating for 24h at 37 ℃.
* - Represents maximum 10% cut-off for growth inhibitory activity.

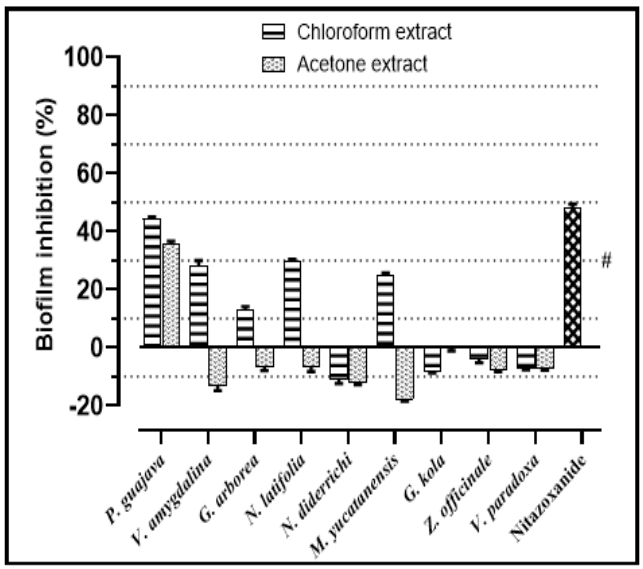

Figure 2.
Percent biofilm inhibition of the selected plants extracts against enterotoxigenic *Escherichia coli* ATCC35401 strain. Each value represents three replicates of three independent experiments after staining with crystal violet.
- Represents minimum 30% cut-off for biofilm inhibitory activity.

Phytochemical and structural profile of bioactive extracts: The results of the phytochemical screening of the extracts that exhibited relatively good biofilm inhibitory effect are presented in Table 2, and their FT-IR spectra in Figures 3 and 4. Prominent in the spectra are the strong aliphatic C-H vibrational stretches (2919.71, 2850.00, and 2920.55, 2851.09 cm-1; possibly from terpenoids and steroids), weak O-H stretches (3342.00 and 3328.90 cm-1), as well as weak C=O stretches (1709.38 and 1686.90 cm-1) (Mayo *et al*., 2004). The secondary metabolites composition of these extracts showed they are mostly medium polar to non-polar compounds.

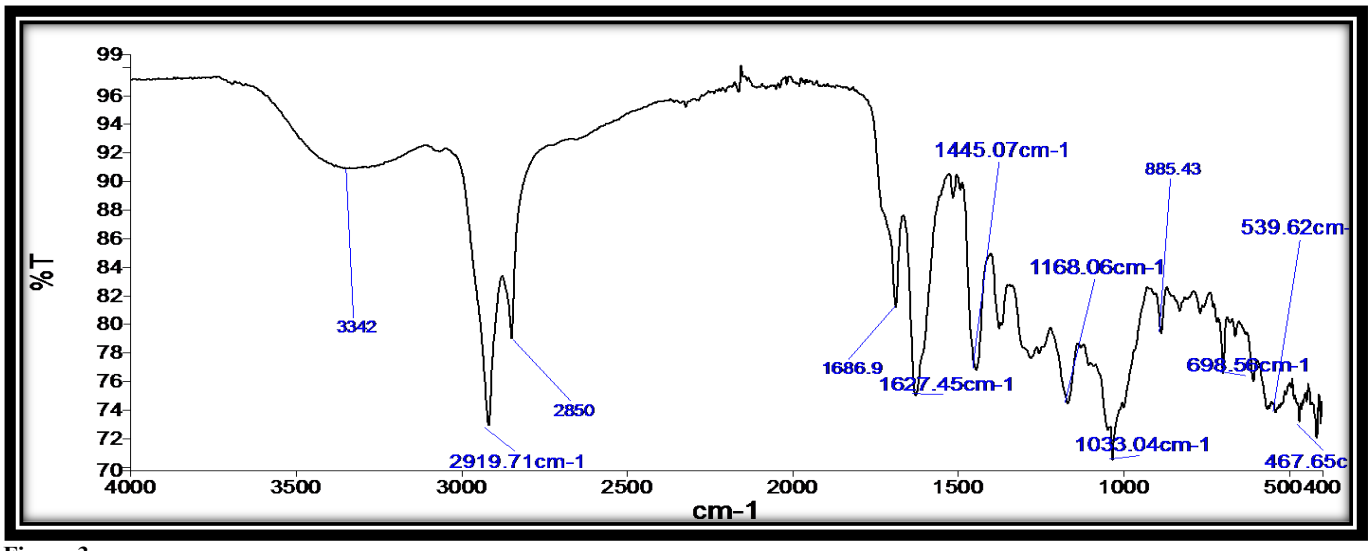

Figure 3.
FT-IR spectrum of *Psidium guajava* (leaf) chloroform extract showing the infrared absorption peak frequencies.

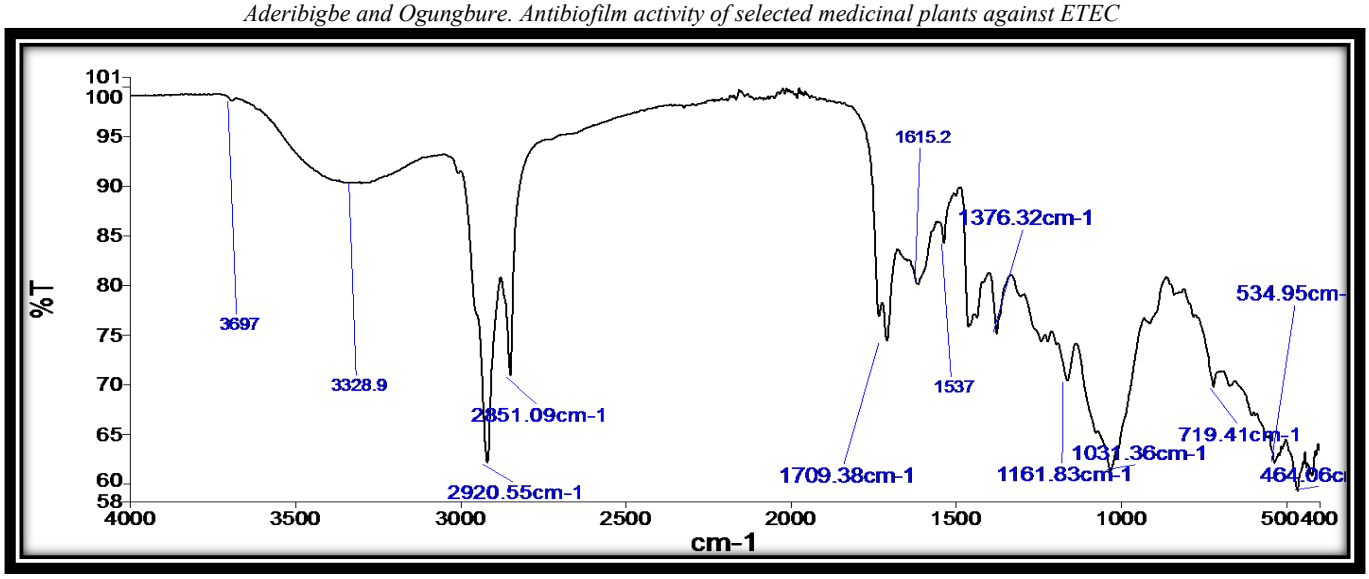

Figure 4.
FT-IR spectrum of *Nauclea latifolia* (leaf) chloroform extract showing the infrared absorption peak frequencies.

Table 2.
The secondary metabolites present in the chloroform extracts that showed satisfactory biofilm inhibition against enterotoxigenic *Escherichia coli* ATCC35401 strain

Secondary metabolites	Plant species (Chloroform extracts)	
	Psidium guajava	***Nauclea latifolia***
Alkaloids	+	+
Flavonoids	+	+
Tannins	-	-
Saponins	-	-
Cardiac glycosides	+	+
Steroids	+	+
Anthraquinones	+	+
Terpenoids	+	+

DISCUSSION

Medicinal plants still represent potential sources of bioactive molecules for therapeutic purposes. In this study, we explored nine selected medicinal plants for their potential antibiofilm activities. To maximise the extraction of the secondary metabolites' constituents of these plants, two extraction solvents of varying polarities were employed successively: chloroform, a non-polar solvent, to extract out the non-polar constituents; and acetone, a medium-polar solvent, to extract out the medium to polar constituents. Overall, higher yields were obtained for the chloroform extracts relative to the acetone extracts. The highest yield was obtained from *G. arborea* (chloroform extract, 12.3% w/w), while the least was from *Z. officinale* (acetone extract, 1.8% w/w). It is noted that although in traditional medicine, aqueous-alcoholic extracts were mostly used for extraction purpose, this does not preclude the use of other extraction solvents as adopted in this study. Acetone is a high extraction capacity solvent (solvent strength of 5.1 which is equivalent to that of methanol) with a polarized carbonyl group within the organic carbon framework. Thus, after chloroform, acetone facilitated the extraction of medium-polar and selected polar secondary metabolites by solvation through dipole–dipole interactions with the solute molecules.

While many medicinal plants have been reported to exhibit antibacterial activity, very few have been investigated for biofilm inhibitory activity against ETEC. In this study, we adopted two selection criteria, (1) bacterial cellular growth inhibition of <10%, and (2) biofilm inhibition of ≥30% (Kwasi *et al*., 2022; Maher *et al*., 2015). The end goal of this approach is to discover molecules from these extracts that will inhibit ETEC biofilm with minimal or no effect on the pathogen, thus minimising the emergence of resistant mutant. The bacterial growth inhibition results revealed that of the eighteen extracts investigated, only five of the extracts met the <10% growth inhibition criteria, namely; *P. guajava* (chloroform), *N. latifolia* (chloroform and acetone), *M. yucatanensis* (chloroform) and *G. kola* (acetone). For the biofilm inhibition activity, only two extracts gave biofilm inhibition of ≥30%, namely: *P. guajava* (chloroform and acetone), and *N. latifolia* (chloroform). A significant difference in biofilm inhibitory activities between the chloroform and acetone extracts was observed. Applying the two cut-offs, only two (2) of the screened extracts, comprising *P. guajava* and *N. latifolia* (coincidentally, both were chloroform extracts), gave reproducible biofilm inhibitory activity against the ETEC strain. *Psidium guajava* gave the highest biofilm inhibitory effect which was 1.5 times higher than *N. latifolia*. Regarding the two plants, there was an observable trend for both growth and biofilm inhibition: *P.* guajava > *N. latifolia*. It must also be noted that of the nine plants species, only *P. guajava* has its chloroform and acetone extracts showing positive biofilm inhibition. The acetone extract, however, did not meet the cut-off on account of higher growth inhibitory activity (>10%).

In a related study on biofilm inhibitory activity of some plant extracts against enteroaggregative *Escherichia coli* (EAEC), acetone extract of *N. latifolia* reproducibly inhibited EAEC biofilm formation with 64.0, 51.9 and 49.9% inhibitions at 5.0, 2.5 and 1.25 mg/mL, respectively, while growth inhibitions were all under 10% (Aderibigbe & Kwasi, 2024). Similarly, hexane and dichloromethane extracts of ripe fruit of *P. guajava* (at 100 µg/mL) inhibited *Pseudomonas aeruginosa* PAO1 biofilm formation (28% and 59%, respectively) without affecting the growth and viability of the pathogen. The polar ethanolic extract, unlike the other two non-polar extracts, did

not reduce biofilm formation significantly. Further study on the dichloromethane extract led to the isolation and identification of lycopene and β-sitosterol-β-D-glucoside as major anti-biofilm compounds (Mahavy *et al*., 2024). Similar to the present study, biofilm inhibitory effects were observed more with the non-polar extracts relative to the polar ethanol extract. In another study, Khan *et al*. (2023) reported that *P. guajava* (leaf) methanol extract inhibited biofilm development of *P. aeruginosa* PAO1 and *Chromobacterium violaceum* 12472 at 500 µg/mL (70.07%) and 250 µg/mL (59.22%), respectively (Khan *et al*., 2023). Some other reports on P. guajava leaf include: methanol extract exhibited inhibitory activity against *Salmonella* spp. *Shigella* spp. and enteropathogenic *E. coli* (EPEC) (Lin *et al*., 2002); ethanol extract showed antidiarrheal activity on evaluation in an EPEC induced diarrhoea rat model with a significant decline in the level of EPEC in treated rat stools after 4th hour of treatment at 200 mg/kg, p.o. (Hirudkar *et al*., 2020); and aqueous decoction was effective infectious diarrhoea with quercetin, a constituent of the decoction, inhibiting the invasion of enteroinvasive *E. coli* to HEp-2 cells (Birdi *et al*., 2010).

The phytochemical screening of the *P. guajava* and *N. latifolia* chloroform extracts revealed their secondary metabolites composition - mostly medium polar to non-polar compounds. This was corroborated by the infrared spectra which revealed strong aliphatic C-H vibrations (as the frequencies were below 3000 cm-1) and weak OH vibrations – which are structural motifs present in steroids, terpenoids, as well as alkaloids and flavonoids. While further study will involve the isolation and identification of the specific bioactive compounds in these extracts, there are reports in the literature of the presence of steroids, triterpenes, and monoterpene indole alkaloids isolated from *N. latifolia* (Haudecoeur *et al*., 2018) as well as ursolic acid, oleanolic acid, β-sitosterol glucoside and other triterpenoids isolated from the leaf extracts of *P. guajava* (Begum *et al*., 2002; Shao *et al*., 2012). Additionally, from the chloroform extract of Jeju guava (*P. cattleianum*) leaf, a closely related species of *P. guajava*, oleanolic, sesquiterpenes, ferulic acid and 3′, 4′, 5′ Trimethoxy flavone were reportedly isolated (Moon *et al*., 2011).

Conclusion

Psidium guajava and *Nauclea latifolia* leaves (chloroform extracts) showed relatively good biofilm inhibitory activity against enterotoxigenic *Escherichia coli* ATCC 35401 strain. They might be a potentially good source of new small molecules to control infectious diarrhea caused by enterotoxigenic *Escherichia coli*.

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Conflict of interest

None.

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