Abstract

Enteroaggregative *Escherichia coli* (EAEC), a biofilm-forming diarrheagenic pathogen, is a major cause of persistent diarrhea. Biofilm complicates treatment as it enhances the pathogen's ability to undermine host immune responses including antibiotics, resulting in emergence of resistant mutant. In search for biofilm inhibitors without selection pressure, this study investigated the acetone extracts of *Nauclea latifolia* (leaf), *Ocimum gratissimum* (leaf) and *Garcinia kola* (seed) for their biofilm inhibitory activities. Pulverised dry plant materials were macerated in acetone for 24 hrs and varying concentrations (0.31-5.0 mg/mL) of the crude extracts thereof evaluated for growth and biofilm inhibitory activities against EAEC042 using the crystal violet-based biofilm assay method. The biofilm inhibitory data were analysed using One-way ANOVA. The most bioactive of the extract was profiled by phytochemical analysis. Of the three extracts investigated, only *N. latifolia* inhibited biofilm formation by greater than 30% (biofilm inhibition cut-off) while inhibiting growth by under 10% (growth inhibition cut-off). The others showed weak biofilm inhibitory activities. Phytochemical evaluation revealed the presence of alkaloids, tannins, steroids, saponins, flavonoids, and cardiac glycosides.

Keywords: Antibiofilm; *Nauclea latifolia*; Secondary metabolites

Introduction

Diarrhea account for a significant cause of illness among people of all ages, particularly in low- and middle-income countries (Jensen et al., 2014; Troeger et al., 2018; Walker et al., 2010). It is a major contributor to malnutrition and growth impairment in young children; and a foremost cause of death among under-fives children with sub-Saharan Africa and South Asia accounting for about 90 per cent of such deaths (Okeke, 2009; Troeger et al., 2018). While diarrheal disease is caused by a wide array of pathogens from contaminated food and water sources, rotavirus and multiple pathotypes of diarrhoeagenic *Escherichia coli*, are the two commonest pathogenic causes (Hartman et al., 2023; Okeke, 2009). Among the various pathotypes of diarrhoeagenic *E. coli*, enteroaggregative *Escherichia coli* (EAEC), is a predominant cause of diarrheal disease globally and a major cause of persistent diarrhea for which antibiotics are often required (Kwasi et al., 2022; Nataro et al., 2006).

Medicinal plants are known established repository of bioactive compounds (Clark, 1996). In Africa, and other low-income countries, they are used as phytomedicine in the management and treatment of many ailments including diarrheal disease. *Nauclea latifolia* Sm., a spreading, evergreen, multi-stemmed shrub, or tree, is used traditionally as a remedy for dysentery and diarrhea (Haudecoeur et al., 2018). *Ocimum gratissimum* Linn., known commonly as scent leaf, is used in the treatment of diarrhea, headache, fever, skin disease and pneumonia (Prabhu et al., 2009). *Garcinia kola* Heckel is a perennial crop that is distributed throughout West and Central Africa forest. The seeds are chewed as an aphrodisiac, and use in treatment of cough, chest colds, laryngitis, bronchitis, liver disorders, as well as dysentery and diarrhea (Farombi & Owoeye, 2011; Tauchen et al., 2023).

EAEC forms copious biofilm which is a significant pathogenicity trait and is known to be contributory to its persistent colonization and transmission of diarrheal disease (Aijuka et al., 2018; Jensen et al., 2014; Kwasi et al., 2022; Nataro et al., 2006). Biofilm formation, a rather complex and multifactorial process, enhances the ability of pathogens to thwart host defence mechanisms and the action of antimicrobial agents (Ghosh et al., 2020; Hall-stoodley et al., 2004; Ito et al., 2009). Thus, inhibition of the biofilm formation process without inhibiting growth is envisaged as a welcome approach in the management of infectious diarrheal diseases as such agents may exert

less selection pressure for antimicrobial resistance (Ghosh et al., 2020; Kwasi et al., 2022). In search for potential biofilm inhibitors of natural sources that could disrupt or inhibit EAEC adherence to host cells without inhibiting growth, this study investigated the acetone extracts of *N. latifolia*, *O. gratissimum* and *G. kola* for their potential EAEC biofilm inhibition activities.

2.0 Methods

2.1 Plant materials

Nauclea latifolia (leaf), *Ocimum gratissimum* (leaf) and *Garcinia kola* (seed) were collected, dried and processed into powdered materials as previously described (Aderibigbe et al., 2022; Aderibigbe & Anowai, 2020). The materials (200 g each) were extracted twice with acetone (1 L) by maceration for 24 h at room temperature. The supernatants were filtered, concentrated using rotary evaporator, and dried *in vacuo* at 40 °C for 48 hours.

2.2 Preparation of extracts concentrations

Varying lower concentrations (0.31 – 5 mg/mL) of the extracts in doubling dilutions downwards, guided by reported MICs in literature, were prepared in DMSO, stored at -20°C, then thawed prior to each experiment (Prabhu et al., 2009).

2.3 Bacterial strain and culture conditions

Enteroaggregative *Escherichia coli* reference strain 042 (EAEC 042), obtained from the Molecular Biology Laboratory, Department of Pharmaceutical Microbiology, Faculty of Pharmacy, University of Ibadan, Nigeria, was sub-cultured on Tryptone Soya Agar (TSA) and grown at 37°C in an incubator overnight. Thereafter, a pure colony of 042 was grown in a Luria Bertani broth (LB broth) at 37°C with shaking overnight.

2.4 Biofilm inhibition assays

Biofilm inhibition assay was set up in triplicates, using the methods of Kwasi et al., 2022. Briefly, 5 µL of varying concentrations of extract solutions (A-C) were dispensed into a sterile 96-well polystyrene plate, while using 2 µL of DMSO to serve as control to compensate for the solvent in which the extracts were dissolved. After these, each 96-well plate received 195 and 192 µL of Dulbecco's Modified Eagle's Medium (DMEM) in the control and test wells, respectively. 3 µL of the overnight culture of 042 was thereafter dispensed into the wells and the plate was swirled carefully. Plates were incubated at 37°C for 8 hours, and optical densities were measured at 595

nm in a Microplate reader (UV spectrophotometer) to determine planktonic cell growth. The used media was carefully aspirated, and the wells of the 96-well plates were washed thoroughly (up to three times in a Microplate washer), then dried. After this, the wells received 200 µL of 75% methanol for 10 minutes to fix the biofilms, then the plates were dried. Plates were subsequently stained with 0.5% crystal violet for 5 minutes, washed thoroughly and then dried. 200 µL of 95% ethanol was dispensed into the wells and allowed to stand for 20 minutes to elute biofilms. Eluted crystal violet was measured at an optical density of 570 nm to quantify biofilm. Gentamycin (0.25 µg/mL) was used as the positive control. Growth and biofilm inhibitions were determined from the average of three replicates using the formulas:

$$\% \text{ Growth inhibition} = \frac{\text{Optical density at 595 nm of control - Optical density at 595 nm of test}}{\text{Optical density at 595 nm of control}}$$

$$\% \text{ Biofilm inhibition: } \frac{\text{Optical density at 570 nm of control - Optical density at 570 nm test}}{\text{Optical density at 570 nm of control}}$$

2.5 Phytochemical evaluation

Following the biofilm inhibition assays, the extract that gave positive biofilm inhibitory activity was subjected to phytochemical evaluation to determine its secondary metabolites composition following standard test procedures (Sofowora, 1993).

2.6 Statistical analysis

All experiments were replicated twice and values expressed as Mean ± SEM (Standard Error of the Mean). Statistical analyses were conducted using the GraphPad Prism Software 7 (GraphPad Software Inc., California, USA). Data from biofilm inhibition were analysed using 2-way analysis of variance (2way ANOVA) along with Tukey's post-hoc test for multiple comparisons.

3.0 RESULTS

The details of the selected plants species and the extraction yield are presented in Table 1.

The results of growth and biofilm inhibitory effects of the various acetone extracts of the plant species on EAEC 042 strain are presented in Fig 1 and 2, respectively. No inhibition of growth

and biofilm were observed with the negative control. With gentamycin positive control, no growth was observed at 0.25µg/mL, while it gave a biofilm inhibition value of 37.9%. The extracts gave varying degree of growth inhibition against EAEC with least effect being observed with *N. latifolia*. In addition, for the biofilm inhibition, only *N. latifolia* extract exhibited significant biofilm inhibitory activity against EAEC, while the other two extracts, namely, *G. kola* and *O. gratissimum*, did not. Post ANOVA Tukey's multiple comparisons test revealed that the effect of concentrations 5, 2.5 and 1.25 mg/mL of *N. latifolia* extract on EAEC 042 strain were not significantly different from one another ($p > 0.05$), while they significantly differ from 0.61 and 0.31 mg/mL ($p < 0.0001$, Table 2).

The phytochemical screening of the only extract, *N. latifolia*, that gave positive biofilm inhibition against EAEC 042 strain revealed the presence of alkaloids, steroids, saponins, flavonoids, tannins and cardiac glycosides.

4. DISCUSSION AND CONCLUSION

Discussion

The three selected plants are reportedly used ethnomedicinally in various places in the treatment of dysentery and diarrhea. In addition, a number of previous studies have confirmed the antimicrobial activities of these plants against many diarrhea-causing pathogens including *E. coli* (Deeni & Hussain, 1991; Enemchukwu et al., 2019; Kin et al., 2018). Thus, in screening for biofilm inhibitory activity, we chose acetone as the extracting solvent since it is an organic and medium polar solvent with high solvent strength. Thus, it could extract compounds across wide polarity ranges (Aderibigbe et al., 2022; Eloff, 1998).

While there are different mechanisms of action for antidiarrheal activity exhibited by medicinal plants, antimicrobial effect is considered a key mechanism with infectious diarrheal disease (Rawat et al., 2017). With close to 90% of diarrhea-associated deaths in LMICs attributable to pathogens spread through contaminated water and food, the principal means of management include rehydration as well as hygiene and nutritional measures (Hartman et al., 2023). Infectious diarrhea (acute or persistent), however, often require the use of antibiotics in the therapeutic management. The advent of antimicrobial resistance, which incidentally has been reported for all classes of diarrheagenic *E. coli* including EAEC these past few decades often complicate treatment (Abishad et al., 2021; Okeke, 2009). EAEC forms copious and distinct biofilms which are contributory to

its establishment of infection and upsetting treatments with antibiotics (Croxen & Finlay, 2010). Biofilm-grown bacterial population have been found to be insensitive to many stressors, including antibiotics and host immune responses. Thus, targeting EAEC biofilms is a welcome alternative and non-bactericidal strategy for diarrheal disease management as it helps to conserve antimicrobials for those cases where there are no alternatives and reduce selection pressure for the emergence of resistant mutants (Kwasi et al., 2022). To this end, small molecules that will minimally affect the growth of the organism while inhibiting or disrupting biofilm forming process are desirable. So, in this study, we adopted the criteria used by Kwasi et al. (2022) - Growth inhibition $\leq$ 10%, and Biofilm inhibition $\geq$ 30%. This approach was a clear departure from the general drive to 'kill the diarrhea-causing pathogens' approach underlining antimicrobial studies.

The result, for *G. kola*, showed that none of the investigated concentrations met the 10% maximum growth inhibition cut-off (20.5-10.7%) and their biofilm inhibitory effects were rather weak and fell below the defined cut-off (highest was 22.0% at 5 mg/mL, as against the $\geq$ 30% cut-off). Varying aqueous and non-aqueous extracts and isolated compounds from *G. kola* seeds have been reported to have antimicrobial activities against *E. coli* and many other organisms (Enemchukwu et al., 2019; Tauchen et al., 2023). In a recent study, ethanol extract from *G. kola* seed showed antimicrobial activity against diarrheagenic *E. coli* multi-drug resistant isolates, while the aqueous extract did not (Enemchukwu et al., 2019). In another study, it was reported that the seed methanol extract and fractions showed antidiarrheal activity via anti-motility and anti-secretory effects (Okoronkwo et al., 2022). In the current study, the growth inhibitions (above 10%) observed with *G. kola* extract indicated that it possessed antimicrobial activity against EAEC 042. This, along with the weak biofilm inhibitory activity disqualifies the extract from been considered a good candidate for biofilm inhibitory activity. With *O. gratissimum*, at two of the concentrations investigated, the 10% maximum growth inhibition cut-off was met. Yet, as was observed with *G. kola* extract, none of the concentrations investigated met the cut-off of 30% minimum biofilm inhibition. Thus, as with *G. kola*, *O. gratissimum* extract exhibited weak biofilm inhibitory activity against EAEC 042. There are however a number of reports in literature that indicated that extracts from *O. gratissimum* possessed good antimicrobial activity against gut pathogens including *E. coli* (Amengialue et al., 2013; Kin et al., 2018). On the other hand, *N. latifolia* extract showed good biofilm inhibitory effects of 64.0, 51.9 and 49.9% at 5.0, 2.5 and 1.25 mg/mL, respectively, and the growth inhibitory effects were all below the 10% cut-off. However, these results are less potent

when compared with that of gentamycin at 0.25 µg/mL, a reference pure molecule. *N. latifolia* is traditionally used in treating various infectious diseases and alcoholic extracts thereof shown to exhibit antimicrobial activity against *E. coli*, and other pathogens (Haudecoeur et al., 2018). Based on ethnomedicinal usage as antidiarrheal, alcoholic and aqueous extracts of the root back were investigated and found to exhibit anti-amoebic activity, significantly decrease diarrhea frequency, and inhibited small intestinal motility in mice (Owolabi et al., 2010; Tonal et al., 2000).

Generally, phytochemical screening of a plant extract provides clues to putative class of secondary metabolites present in the extract. Incidentally, regarding the phytochemical found to be present in *N. latifolia* leaf, a similar result was earlier reported by Aderibigbe and Anowai (2020). The only exception was that cardiac glycosides were detected in the current study on account of exhaustive extraction process. *Nauclea latifolia* has been reported to be rich in indole alkaloids such as strictosamide, vincosamide, angustoline, and angustine (Aderibigbe et al., 2021; Haudecoeur et al., 2018). Some of these secondary metabolites present in the leaf extracts could be responsible for the observed activity and will warranty further investigation.

Conclusion

Of the acetone extracts of the three plant species evaluated for biofilm inhibitory activity against EAEC 042 strain, only *Nauclea latifolia* showed good biofilm inhibitory activity. Further studies will include evaluation of antibiofilm activity across a broad spectrum of genome-sequenced EAEC strains to decipher the mechanism of antibiofilm activity and to isolate the bioactive compounds.

Table 1: Percentage yields and plant details

Plant species	Family names	Common names	Part used	Voucher number	Extracts yield (% w/w)
***Nauclea latifolia* Sm.**	Rubiaceae	African peach	Leaf	110021	6.1
***Garcinia kola* Heckel**	Guttiferae	Bitter kola	Seed	110593	4.2
***Ocimum gratissimum* Linn.**	Lamiaceae	Scent leaf	Leaf	111995	5.7

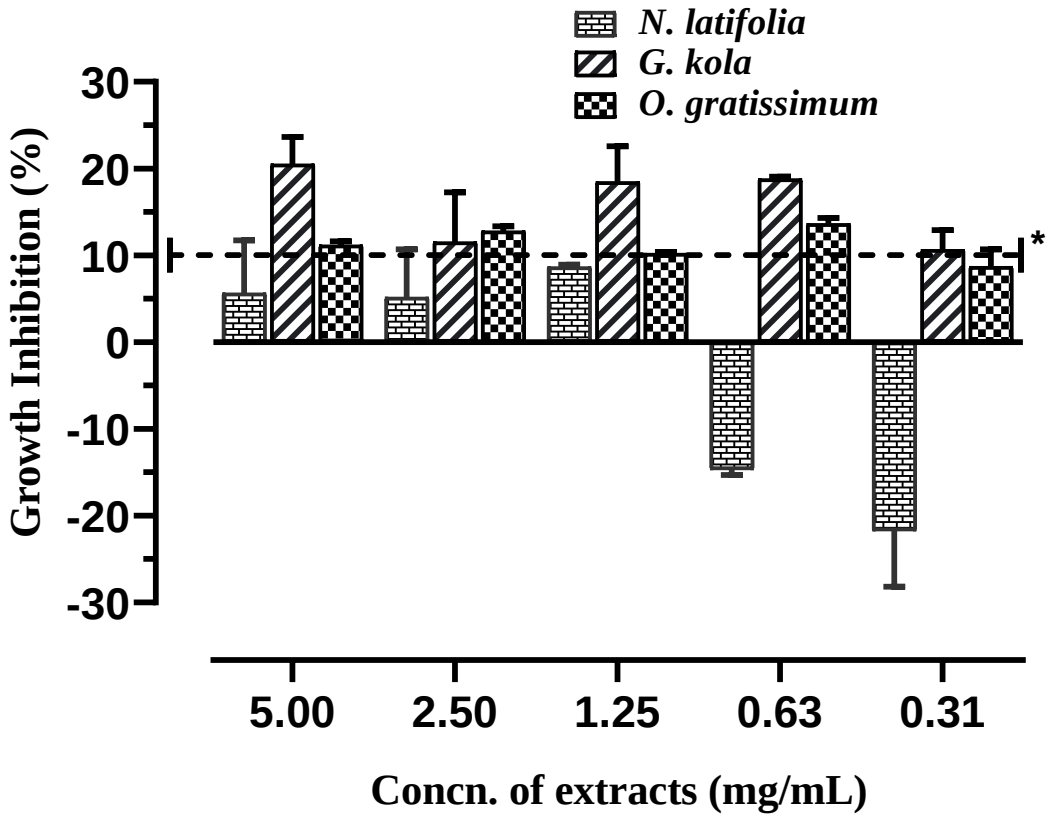

Fig.1 Percent growth inhibition of varying sub-mic concentration of extracts of the three plant species against enteroaggregative *Escherichia coli*. Each value represents three replicates of two independent experiments.

***10% maximum cut-off for growth inhibitory activity.**

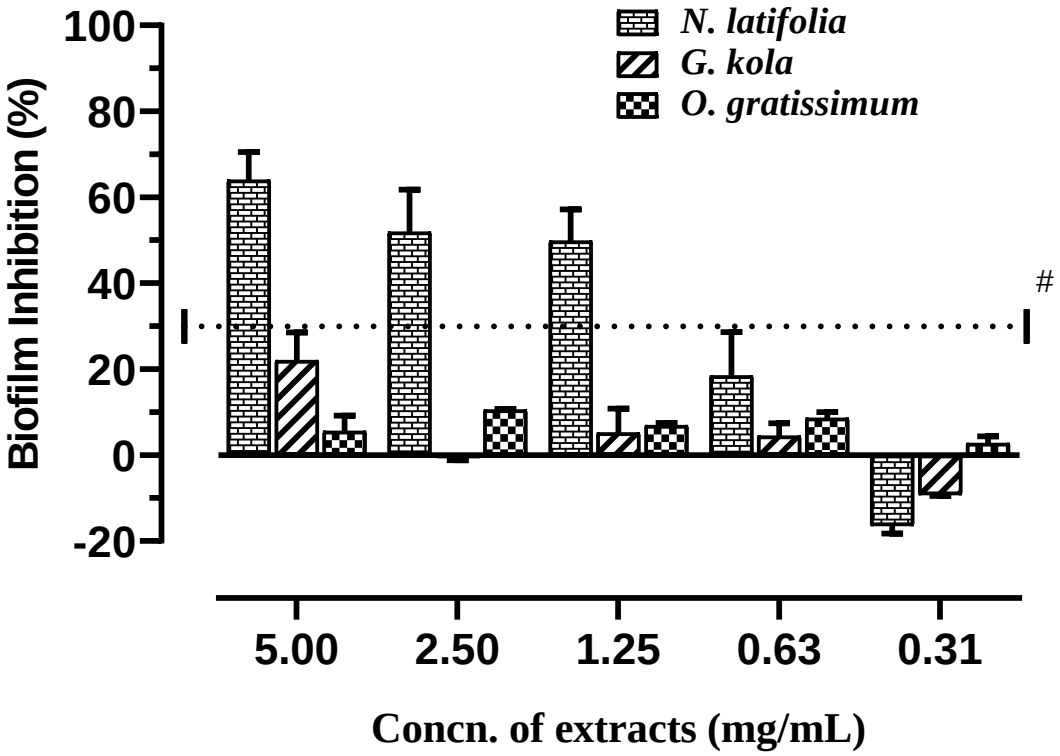

Fig.2 Percent biofilm inhibition of varying sub-mic concentration of extracts of the three plant species against enteroaggregative *Escherichia coli*. Each value represents three replicates of two independent experiments.

 # 30% minimum cut-off for biofilm inhibitory activity.

Table 2. Tukey's multiple comparisons test comparing the biofilm inhibitory effect of varying concentrations (mg/mL) of *N. latifolia* extract on EAEC 042 strain.

Comparisons	P-Value	Summary
5.00 vs. 2.50	0.1854	NS
5.00 vs. 1.25	0.0952	NS
5.00 vs. 0.63	<0.0001	ES
5.00 vs. 0.31	<0.0001	ES
2.50 vs. 1.25	0.9944	NS
2.50 vs. 0.63	<0.0001	ES
2.50 vs. 0.31	<0.0001	ES
1.25 vs. 0.63	0.0002	S
1.25 vs. 0.31	<0.0001	ES
0.63 vs. 0.31	<0.0001	ES

NS – Not Significant, ES – Extremely Significant, S – Significant

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SAA designed the experiment and wrote the initial draft of the manuscript, SAA and DAK carried out the experiment, analysed the results, read and approved the final manuscript.

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