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Biofilm Inhibitory Activity of *Nauclea latifolia*, *Ocimum gratissimum* and *Garcinia kola* Extracts Against Enteroaggregative *Escherichia coli*

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article.

Abstract

Background: Enteroaggregative *Escherichia coli* (EAEC), a biofilm-forming *E. coli* pathotype, is recognized as one of foremost causes of diarrhea in children under five years old, traveller's diarrhea and persistent diarrhea. Biofilm complicates treatment as it enhances the pathogen's ability to undermine host immune responses, often resulting in emergence of resistant mutant.

Objectives: In search for potential therapeutic leads against EAEC with less selection pressure, the study was designed to investigate selected medicinal plants for biofilm inhibitory activity against EAEC.

Material and Methods: Pulverised dry plant materials of *Nauclea latifolia* (leaf), *Ocimum gratissimum* (leaf) and *Garcinia kola* (seed) were macerated in acetone for 24 hrs. The supernatants were concentrated at reduced pressure and dried *in vacuo*. Thereafter, varying concentrations (0.31-5.0 mg/mL) of the extracts were evaluated for growth and biofilm inhibitory activities against EAEC 042 strain using the crystal violet-based biofilm assay method. Biofilm inhibitory data were analysed using 2-way ANOVA, while the most bioactive of the extracts was profiled by phytochemical analysis.

Results: *Nauclea latifolia* reproducibly inhibited biofilm formation by greater than 30% (defined biofilm inhibition cut-off) while inhibiting growth by under 10% (defined growth inhibition cut-off). The other two plants showed relatively weak biofilm inhibitory activities. Phytochemical evaluation revealed the presence of alkaloids, tannins, steroids, saponins, flavonoids, and cardiac glycosides.

Conclusions: *Nauclea latifolia* exhibited good biofilm inhibitory activity against EAEC 042 strain with less selection pressure. The plant represents a potential source of novel antibiofilm compounds against enteroaggregative *E. coli*.

Keywords: Antibiofilm; *Nauclea latifolia*; Secondary metabolites, Diarrhea

INTRODUCTION

Diarrhea accounts for a significant cause of illness among people of all ages, particularly in low- and middle-income countries. It is a major contributor to malnutrition and growth impairment in young children; and a foremost cause of death among under-five 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 among the commonest pathogenic causes. Of the diarrhoeagenic *E. coli* pathotypes, enteroaggregative *Escherichia coli* (EAEC), is a predominant cause of diarrhea in infants in developing countries, traveller's diarrhea, and

persistent diarrhea in patients with HIV infection for which antibiotics are often required (Hartman et al., 2023; Kwasi et al., 2022; Nataro et al., 2006).

Enteroaggregative *E. coli* 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; Kwasi et al., 2022). 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). Furthermore, antimicrobial resistance has been reported for all classes of diarrheagenic *E. coli* including EAEC these past few decades (Abishad et al., 2021; Okeke, 2009). Inhibition of biofilm formation process without inhibiting growth is a welcome alternative and non-bactericidal approach in the management of infectious diarrheal diseases as such agents may exert less selection pressure for antimicrobial resistance and helps to conserve antimicrobials for those cases where there are no alternatives (Ghosh et al., 2020; Kwasi et al., 2022).

In Africa and many developing countries, a number of medicinal plants are used as phytomedicine in the management and treatment of many ailments including diarrheal diseases (Onohuean & Igere, 2023; Rawat et al., 2017) . Some of the plants reportedly used ethnomedicinally in the treatment of dysentery and diarrhea include *Nauclea latifolia* Sm., *Ocimum gratissimum* Linn., and *Garcinia kola* Heckel. *Nauclea latifolia*, an evergreen, multi-stemmed shrub, or tree, is used traditionally as a remedy for dysentery and diarrhea (Haudecoeur et al., 2018). *Ocimum gratissimum*, known commonly as scent leaf, is used in the treatment of diarrhea, 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 (Tauchen et al., 2023).

In search for potential biofilm inhibitors of natural sources that could disrupt or inhibit EAEC adherence to host cells without inhibiting growth, this study was designed to investigate acetone extracts of *N. latifolia*, *O. gratissimum* and *G. kola* for their potential EAEC biofilm inhibition activities.

METHODOLOGY

Plant Material

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 dried plant materials (200 g each) were macerated twice in acetone (1 L) for 24 h at room temperature. The supernatants were filtered, concentrated using rotary evaporator, and dried *in vacuo* at 40 °C for 48 hours.

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).

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 fresh colony of 042 was grown in a Luria Bertani broth (LB broth) at 37°C with shaking overnight.

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 plates, 2 µL of DMSO served as control to compensate for the solvent in which the extracts were dissolved. After these, 195 and 192 µL of Dulbecco's Modified Eagle's Medium (DMEM) each were placed in the control and test wells, respectively. Three microliters (3 µL) of the overnight culture of 042 was thereafter dispensed into the wells and the plates swirled carefully. The 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 media were then carefully aspirated, while the plates' wells were washed thoroughly (up to three times in a Microplate washer), and dried. After this, 200 µL 75 % methanol was placed in each well for 10 minutes to fix the biofilms, and the plates dried. The plates were subsequently stained with 0.5% crystal violet for 5 minutes, washed thoroughly and then dried. Ethanol (95 %, 200 µL each) 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 equations (1) and (2).

Phytochemical evaluation

Following the biofilm inhibition assays, the extract with positive biofilm inhibitory activity was subjected to phytochemical evaluation to determine the composition of its secondary metabolites (Sofowora, 1993).

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 (2-way ANOVA) along with Tukey's post-hoc test for multiple comparisons.

$$\% \textbf{ Growth inhibition} = \frac{\textit{Optical density at } 595 \textit{ nm of control} - \textit{Optical density at } 595 \textit{ nm of test}}{\textit{Optical density at } 595 \textit{ nm of control}} \quad \textbf{……..(1)}$$

$$\% \textbf{ Biofilm Inhibition} = \frac{\textit{Optical density at } 570 \textit{ nm of control} - \textit{Optical density at } 570 \textit{ nm of test}}{\textit{Optical density at } 570 \textit{ nm of control}} \quad \textbf{…….(2)}$$

RESULTS

The extraction yields obtained from the maceration of the powdered plant materials in acetone and other details about the selected plants species are presented in Table 1.

The solvent extracts of the plant species were investigated for biofilm inhibitory activity against EAEC 042 strain. For our assessment, we adopted the criteria used by Kwasi et al. (2022) - extract must demonstrate at least 30% biofilm inhibition and under 10% growth inhibition at the concentration investigated. The growth and biofilm inhibitory effects of the extracts 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 the biofilm inhibition value was 37.9 %. Of the plants' extracts, varying degree of growth inhibitions against EAEC were recorded with the least effect been from *N. latifolia*. For the biofilm inhibition, only *N. latifolia* extract exhibited significant biofilm inhibitory activity against EAEC 042. For *G. kola* extract, none of the investigated concentrations met the 10% maximum growth inhibition cut-off (20.5-10.7%), their biofilm inhibitory effects were weak and fell below the defined cut-off (highest was 22.0% at 5 mg/mL, as against the ≥ 30% cut-off). 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 ($p < 0.0001$).

The following secondary metabolites – alkaloids, steroids, saponins, flavonoids, tannins and cardiac glycosides – were found to be present in *N. latifolia*, the only extract with positive biofilm inhibitory activity against EAEC 042.

Table 1: Percentage yields of the acetone extracts and other details on the plant species

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

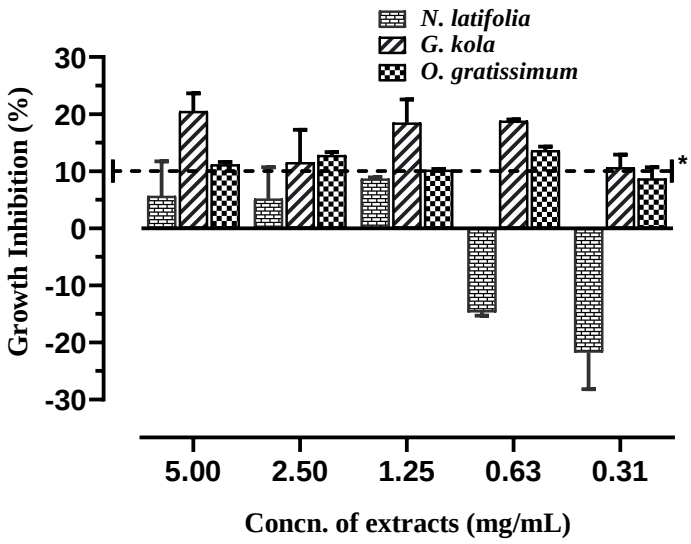

Figure 1. Percent growth inhibition of varying sub-mic concentration of extracts of the three plant species against enteroaggregative *Escherichia coli*. Each value represents three replicates of two independent experiments. *10% maximum cut-off for growth inhibitory activity.

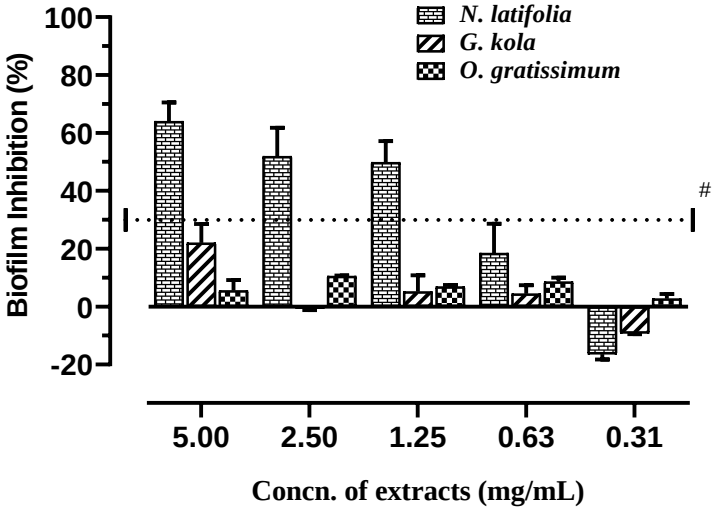

Figure 2. Percent biofilm inhibition of varying sub-mic concentration of extracts of the three plant species against enteroaggregative *Escherichia coli*. Each value represents three replicates of two independent experiments. # 30% minimum cut-off for biofilm inhibitory activity.

DISCUSSION

The increasing burden of infectious diarrhea in low- and medium-developing countries, foodborne outbreaks in industrialised nations as well as emergence of microbial resistance have necessitated the need to explore medicinal plants for possible leads that could serve as alternative therapeutic solutions (Hartman et al., 2023; Okeke & Nataro, 2001). This is more so since a number of medicinal plants are used in African Traditional Medicine to manage diarrheal diseases (Onohuean & Igere, 2023).

Aqueous, 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 an earlier 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 present study, the growth inhibitions (above 10%) observed with *G. kola* extract indicated that it possessed antimicrobial activity against EAEC 042 strain. This growth inhibition, however, 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 30% minimum biofilm inhibition cut-off. Thus, as with *G. kola*, *O. gratissimum* extract exhibited weak biofilm inhibitory activity against EAEC 042 strain. 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). This could suggest that *O. gratissimum* exhibit its antidiarrhea effect through antimicrobial activity.

Nauclea latifolia extract, unlike the other two extracts, 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, it must be noted that the biofilm inhibitory effect from *N. latifolia* is of lesser potency when compared with gentamycin, the reference molecule with 37.9% inhibition at 0.25 µg/mL. *Nauclea 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

In conclusion, of the three acetone extracts evaluated for biofilm inhibitory activity against EAEC 042 strain, only *Nauclea latifolia* showed good biofilm inhibitory activity. The plant could be a source of novel antibiofilm compounds against enteroaggregative *E. coli*. Further studies involving its evaluation against a broad spectrum of genome-sequenced EAEC strains as well as to isolate the bioactive compounds are ongoing.

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