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Antibacterial, Antibiofilm Activities and Toxicity of *Uvaria chamae* P. Beauv (Annonaceae)

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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: Bacteria biofilms are a serious global health concern. The rapid increase of antimicrobial resistance in diarrheagenic bacteria due to biofilm formation has limited the clinical usefulness of some antibiotics in circulation.
Objectives: *Uvaria chamae* has shown broad spectrum antibacterial activity, hence the need to study its antibiofilm activity against enteroaggregative *Escherichia coli* (EAEC) strains implicated in paediatric diarrhoea.
Methods: Samples of authenticated *U. chamae* root, stem and leaf were collected, air-dried, ground and extracted by cold maceration in dichloromethane and methanol separately. The EAEC strains tested were; O42, DH5α, MN5DE, D25D and D28I. The plant extracts were subjected to quantitative and qualitative phytochemical screening and the 50% lethality (LC_{50}) brine shrimp assay carried out. Extracts were screened for antibacterial activity using agar diffusion method, while agar dilution and broth dilution methods were used to determine minimum inhibitory and bactericidal concentrations, respectively. Biofilm inhibition of the active extracts was investigated by crystal violet method.
Results: All the EAEC strains were multi-drug resistant, but susceptible to gentamicin and azithromycin. Dichloromethane leaf extract (DLE) and methanol leaf extract (MLE) inhibited the growth of the tested EAEC strains with the MIC of MLE D28I being MIC 3.75 mg/mL. The percentage biofilm inhibition by MLE against EAEC strains O42, MND5E and D25D were 72%, 74.5%, and 63%, respectively. Alkaloids were the most abundant in the methanol leaf extract of *U. chamae* (MLE). The extracts had LC_{50} >1000 µg/mL.
Conclusion: *Uvaria chamae* is non-toxic and possesses antibiofilm potential that could be further developed as a natural remedy for diarrhoea.

Keywords: Biofilm, Diarrhoea, Enteroaggregative *Escherichia coli*, *Uvaria chamae*

INTRODUCTION

Bacteria cells exhibit two predominant modes of growth: planktonic free-floating cells and sessile aggregated biofilm modes (Jamal *et al.*, 2015). Bacteria biofilm is a complex structure of microbiome made up of different bacteria colonies or single type of bacteria cells in a group encased in an extracellular polymeric matrix, which is composed of exopolysaccharides, extracellular DNA (e-DNA), proteins, amyloidogenic proteins and polysaccharides (Divakar *et al*., 2019). Notably, a significant number of bacterial cells, 40% - 80% on earth are biofilm formers (Flemming and Wuertz, 2019).

It has been reported that 65% and 80% of all microbial and chronic infections, respectively are associated with biofilm formation (Lewis, 2001; Jamal *et al*., 2018). These infections may occur on abiotic surfaces; on or within indwelling medical devices and in the host system. Biofilm formation is associated with 2% of infections on breast implants, also 2% of joint prostheses infections; 4% of infections on the mechanical heart valves, as well as the pacemakers and defibrillators; 10% of infections on ventricular shunts and about 40% of infections on ventricular-assisted devices (Jamal *et al*., 2018).

Bacteria biofilms are of serious global health concern because of their resistance to antibiotics, host immune system and other external stresses. Bacteria cells in biofilms have shown 10 - 1000 times more antibiotic resistance than the free-floating bacteria cells (Mah, 2012). Biofilm allows bacteria to exist in a wide range of physiological states, enabling them survive unpredictable environmental stressors such as temperature changes, desiccation, ultraviolet radiation, cleansing agents and so on. It blocks the bacteria from host's immune cells and antibiotics and this makes the bacteria resistant to antibiotics, leading to multi drug resistant, extensively drug resistant and totally drug resistant bacteria (Netsanet *et al.*, 2017).

Enteroaggregative *Escherichia coli* is the second most common cause of traveler's diarrhea and a common cause of acute diarrhea in children living in developing and developed countries, adults and people with HIV infection living in developing countries (Okeke and Nataro, 2001). EAEC has a great public health impact because it causes growth retardation and reduced intellectual development in malnourished children from developing countries with poor unsanitary and drinking water conditions (Estrada-Garcia and Navarro-Garcia, 2012). Globally, diarrhea is the second leading cause of child mortality, morbidity and malnutrition in children under the age of 5 and it accounts for about 525,000 deaths in children especially in developing countries (WHO, 2017). In 2019, it accounted for approximately 9% of all deaths in children under the age of 5 (UNICEF, 2022).

The rapid increase of antimicrobial resistance in diarrheagenic bacteria due to biofilm formation poses a great threat to public health because some of the clinically available treatments are not effective against the pathogens. The discovery and development of alternative antibacterial agents that are safe and effective for the treatment of infections caused by these bacteria cannot be over emphasized. In this study, the plant used has shown antibacterial and antibiofilm activities against a wide range of bacteria hence, the need to study its activity against enteroaggregative *Escherichia coli* isolates and their antibiofilm.

METHODOLOGY

Plant Collection, Preparation, Extraction and Phytochemical Analysis

The root bark, stem bark and leaves of *Uvaria chamae* were all collected and authenticated at Forest Herbarium (FHI) with a voucher number FHI 107901. The fresh leaves were spread in open air under a shade and away from direct sunlight. The stems and roots were size reduced with a knife and allowed to dry in the laboratory. Pulverized plant parts were separately weighed (610 g) and successively extracted with sufficient quantity of methanol, dichloromethane and ethyl acetate by maceration. The extracts were subsequently concentrated using rotary evaporator at 40^{o}c. Qualitative phytochemical analysis of the various extracts was carried out following the methods outlined by Oloche *et al*. (2022)**.**

Quantitative analysis of the phytochemical constituents of *Uvaria chamae* was carried by standard methods. The alkaline gravimetric method was used to quantitatively analyse alkaloids, flavonoids and saponins (Harborne, 1973), the Follin-Dennis spectrophotometric method was employed for tannins (Pearson, 1976), while the total phenols by the method described by Hayata *et al*. (2020).

Bacterial cultures

Four strains of enteroaggregative *Escherichia coli* (O42, MND5E, D25D and D28I) and a control *E coli* strain, DH5α were obtained from the Molecular and Biotechnology laboratory of the Department of Pharmaceutical Microbiology, Faculty of Pharmacy, University of Ibadan, Nigeria and used for the study.

Susceptibility Testing of bacterial isolates to plant extracts

This was done using agar well diffusion method. 0.1 mL of the 0.5 MacFarland standardized bacteria culture was seeded into 20 mL sterile Mueller Hinton agar at 50º C in McCartney bottle which was then aseptically poured into the plates and allowed to set. Wells were aseptically made on the inoculated agar using a 6mm in diameter cork borer. Graded concentrations of the plant extracts were measured into designated wells, while 50% DMSO used as the solvent and 10µg of Gentamicin served as negative and positive controls, respectively. The plates were left for about 45 minutes for the extracts to diffuse into the agar, after which they were incubated overnight at 37ºC. This procedure was carried out for all the EAEC strains and in duplicates. The zones of inhibitions were measured and recorded.

Determination of Minimum Inhibitory Concentration

Minimum inhibitory concentration of the active extracts against the EAEC isolates was carried out using the agar and modification of broth dilution methods. One milliliter of graded concentration (125 mg/mL to 6.25 mg/mL) of each extract was diluted with 19 mL Mueller Hinton agar mixed thoroughly, poured into sterile Petri dish and allowed to set. The agar surface was then streaked with the standardized test culture. Again, gentamicin was used as drug control. The plates were incubated at 37°C overnight and the plates were observed for presence or absence of growth. All plates were made in duplicates. The lowest concentration preventing visible growth was taken as minimum inhibitory concentration (MIC). Again, the EAEC isolates were grown overnight in a nutrient broth, adjusted to 0.5 MacFarland standard and used to inoculate tryptone soy broth dispensed into each of the wells of 96-well microtiter plates. Hundred microlitres (100 µL) of the different concentration of the extracts were added to the wells in triplicates, while 100 µL of 10 µg of gentamicin served as the positive control. The plates were incubated at 37 ºC overnight after which iodonitrotetrazolium (INT) dye was added to view the inhibitory effects of the phytochemicals.

Determination of Minimum Bactericidal Concentration

Minimum bactericidal concentration of active plant extracts was determined by a modification of the method of Aibinu *et al*. (2006). To a 0.5 mL of each test organism (obtained from 24 hour overnight broth culture) was added to 0.5 mL of extract at different concentration as used in the MIC that showed no visible growth on the agar plates. The set up were incubated at 37°C overnight and the culture again streaked out onto the surface of sterile extract-free agar in Petri dishes and incubated at 37 ºC for 24 hours. The lowest concentration for each extract that prevented bacterial recovery on extract-free agar after 24 hours at 37°C of incubation were recorded as the minimum bactericidal concentration (MBC).

Antibiofilm test

Biofilm assay was carried out by the methods of Pratt and Kolter (1998) with some modifications. Sub-MIC concentration (1.56 mg/ mL) of the plant extracts were made in a labeled 96-well plate from stock solution. Subsequently, standardized overnight culture of the test bacterial was added and the set up incubated at 37°C for 24 hours. The set up were made in triplicates with positive and negative controls containing gentamicin (5 µg/ mL) and untreated overnight culture of the test bacterial, respectively. Planktonic cell growth was determined by quantifying optical density of the culture above the biofilm at 595 nm. Plates were washed with phosphate buffered saline using a microplate washer to remove non-adhering cells, then air-dried and fixed with 75% ethanol for 10 minutes. The methanol fixed biofilms were stained with 0.5% crystal violet (CV) for 5 minutes and the excess CV discarded and washed with water. The plates were again air dried and the CV eluted with 95%. Biofilm was quantified by determining the optical density of the eluted crystal violet at 570 nm using a multiscan microplate spectrophotometer. The biofilm inhibitory effect of each extract was computed from the mean of the three replicates.

Brine shrimp lethality assay

Brine shrimp lethality assay was carried out as previously reported by Abiodun *et al*., 2022. *Artemia Salina* (brine shrimp) eggs (Artemix^{R}) was manufactured by Dohse Aquaristik Gdilute co. KG, Germany. The eggs were hatched in a tank containing natural seawater at room temperature and a part of the tank was exposed to light. After 48 hours, ten active nauplii (brine shrimps) were introduced into the graded concentration of the extracts (5.0- 0.31 mg/mL) in a 96-well plate. Cyclophosphamide was used as the positive control, while seawater served as the negative control. The extracts were tested in triplicate. After 24 hours, the number of surviving nauplii was counted. The percentage mortality was calculated for each concentration using equation 1.

Subsequently, the LC_{50} of each extract was determined on Graph Pad Prism.

$$\% \text{ mortality} = \frac{Number\ of\ surviving\ nauplii\ in\ negative\ control - surviving\ nauplii\ in\ test\ well}{Number\ of\ surviving\ nauplii\ in\ negative\ control} * 10 \quad \ldots\ldots (1)$$

RESULTS

Percentage Extract Yield of Plant

The different plant parts yielded variable extracts relative to the extraction solvent. The yield ranged from 1.0 to 8.3% for both dichloromethane and methanol, respectively. The leaves in dichloromethane gave the highest yield (8.3%) while the extract from the same plant part with methanol yielded 6.4% as shown in Table 1.

Table 1: Percentage Yield of Extracts

Plant part	Solvent	Yield of extract (g)	Percentage yield (%)
Leaves	Dichloromethane	50.75	8.3
	Methanol	39.31	6.4
Stem bark	Dichloromethane	10.04	1.6
	Methanol	13.74	2.3
Root bark	Dichloromethane	6.12	1.0
	Methanol	17.66	2.9

Initial weight of pulverized plant parts= 610g

Qualitative and Quantitative Analysis of Phytochemical Constituents

The phytochemicals detected in the extracts are presented in Table 2. The phytochemical groups, alkaloids, anthraquinones, cardiac glycosides, flavonoids and terpenoids, but not saponins, steroids and tannins were detected in varying amount in all the extracts of *Uvaria chamae*. Notably, steroids were only detected in abundance in the methanol leaf extract. Table 3 shows the quantities of the phytochemicals present in the extracts of the various plant parts. The quantities of the phytochemicals determined varied ranging from 1% of saponins in dichloromethane leaf extract to 70% flavonoids in methanol stem extract. The quantity of the flavonoids in the methanol and dichloromethane extracts of the leaf, stem and root were significantly higher ($p>0.5$) compared to the other phytochemicals.

Table 2: Qualitative Phytochemical Screening of Extracts of Leaf, Stem and Root of *Uvaria chamae*

	Methanol			Dichloromethane		
Phytochemical	Leaf	Stem	Root	Leaf	Stem	Root
Alkaloids	++	+	++	+	++	+
Anthraquinones	+	+	+	+	++	++
Cardiac glycosides	+	++	+	+	+	+
Saponins	+	+	+	+	-	-
Flavonoids	++	++	++	++	++	++
Phenol	+	+	+	+	+	+
Terpenoids	+	+	+	++	+	++
Steroids	++	-	-	-	-	-
Tannins	+	+	+	+	+	-

Key: ++= Abundant, += Scanty, -= Absent

Table 3: Percentage Quantities of Phytochemicals present in Leaf, Stem and Root Extracts of *Uvaria chamae*

	Methanol (%)			Dichloromethane (%)		
Phytochemical	Leaf	Stem	Root	Leaf	Stem	Root
Alkaloids	31.2	23.3	38.4	11.0	27.0	14.0
Saponins	7.0	6.0	3.0	1.0	-	-
Flavonoids	58.0	70.0	67.0	43.0	45.0	32.0
Phenol	1.99	1.67	1.40	1.51	2.24	1.90
Terpenoids	12.0	7.0	9.0	24.0	8.0	32.0
Tannins	6.8	2.3	1.6	2.6	3.0	-

Resistance Profile of Experimental Bacterial Strains

The identity of all the laboratory enteroaggregative *E. coli* strains and the non-biofilm *E. coli* DH5α control strain was validated by morphological and biochemical tests. All the experimental strains presented as Gram-negatives with pink colonies and green metallic sheen on MacConkey agar and eosin blue, respectively. They were also indole positive and lactose fermenting. The result of the antibiotics resistance of the *E. coli* strains interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines and presented in Table 4. The isolates were all sensitive to gentamicin and azithromycin (Table 4).

Table 4: Antibiotics Resistance Profile of experimental *E. coli* strains

	Organism/ Strains				
Antibiotic	042	MND5E	D25D	D28I	DH5α
AS (20 µg)	R	R	R	R	R
BA (25 µg)	R	S	R	R	R
CF (30 µg)	R	R	R	R	R
PT (110 µg)	R	R	R	R	R
CL (30 µg)	I	I	S	S	S
CP (30 µg)	R	R	R	R	R
CR (30 µg)	R	S	R	I	R
TE (30 µg)	R	S	R	I	S
OF (5 µg)	S	R	S	S	S
GM (10 µg)	S	S	S	S	S
AT (15 µg)	S	S	S	S	S
LE (5 µg)	S	R	S	S	S

Key: S=Susceptible, I= Intermediate, R= Resistant, AS= Ampicillin/ Sulbactam, BA= Co-trimoxazole, CF= Cefotaxime, PT= Piperacillin/Tazobactam, CL= Chloramphenicol, CP= Ciprofloxacin, CR= Ceftriaxone, TE= Tetracycline, OF= Ofloxacin, GM= Gentamicin, AT= Azithromycin, LE= Levofloxacin

Antibacterial activity of Uvaria chamae extracts and phytochemical groups

Antibacterial activity of methanol and dichloromethane leaf, stem and roots extract of *U. chamae* measured as zones of bacteria growth inhibition is shown in Table 5. The antibacterial activity of the methanol leaf against the enteroaggregative *Escherichia coli* strain D25D and D28I measured 14 mm and was significantly higher ($p>0.05$) compared to other enteroaggregative *Escherichia coli* strains. However, this activity was significantly lower ($p>0.05$) than the control, gentamicin (D25D= 21 mm, D28I= 18 mm). The minimum inhibitory concentrations of sensitive strains ranged from 3.125 mg/mL to 6.25 mg/mL, while the minimum bactericidal concentrations were 6.25 mg/mL and 12.5 mg/mL. Interestingly, the phytochemicals; alkaloids, terpenoids, flavonoids and saponins did not show significant inhibition of bacterial growth at concentrations up to 2 mg/mL.

Table 5: Diameter of Zones Bacterial Growth Inhibition by Leaf, Stem and Root Extracts of *Uvaria chamae*

	Methanol (mm)			Dichloromethane (mm)			
E. coli Strain	Leaf	Stem	Root	Leaf	Stem	Root	Control
O42	10	9	NZI	10	9	9	21
MND5E	10	NZI	8	9	NZI	NZI	19
D25D	14	NZI	NZI	12	11	NZI	21
D28I	14	NZI	NZI	10	7	8	18
DH5α	15	12	8	13	13	9	38

Key: Diameter of Cork borer= 6 mm, Control= Gentamicin 10 µg/ml, Concentration of extracts= 100 mg/mL, NZI= No Zone of Inhibition

Percentage biofilm inhibition by Methanol leaf extract of Uvaria chamae

Biofilm formation and inhibition by test bacteria was measured as optical density of eluted crystal violet. The percentage biofilm inhibition determined using the formulae previously stated is shown in Table 6. The percentage biofilm inhibition ranged from 41.8% in DH5α to 74.5% in MND5E. Biofilm formation by

EAEC strain O42 was significantly higher ($p>0.05$) compared to strain D25D, but not MND5E. However, inhibition of biofilm of *E. coli* strain O42 formation by methanol extract of *U. chamae* was not significantly different ($p<0.05$) from that of nitazoxanide (63%) a known O42 biofilm inhibitor that was used as the positive control.

Table 6: Percentage of Biofilm Inhibition

E. coli Strain	Mean biofilm of control	Mean biofilm of test	Percentage inhibition
O42	0.648	0.181	72.1
MND5E	0.548	0.140	74.5
D25D	0.373	0.149	63.0
DH5α	0.237	0.138	41.8

Key: Concentration of methanol leaf extract= 1.56 mg/ml, % Biofilm inhibition=$\frac{\text{OD570nm of control} - \text{OD570nm of test}}{\text{OD570nm of control}} * 100$

Lethality of *U. chamae* extracts to brine shrimp

The LC_{50} (µg/mL) of the extracts was determined to be all > 1000 µg/mL and range from 2600 to 5000 µg/mL. The 50% lethality concentrations of the extracts on brine shrimp were significantly higher ($p>0.05$) relative to cyclophosphamide a known cytotoxic agent (Table 7) that was used as the positive control.

Table 7: Fifty percentage (50%) Lethality Concentration of *Uvaria chamae* Extracts

EXTRACTS	LC_{50} (µg/mL)
Methanol roots	3400
Methanol Stem	2600
Methanol leaves	>5000
Dichloromethane root	>5000
Dichloromethane stem	2800
Dichloromethane leaves	>5000
Cyclophosphamide *	115.19

*standard drug/positive control, non-toxic (> 1000 µg/mL), weakly toxic (500-1000 µg/mL), moderately toxic (100-500 µg/mL), strongly toxic (0-100 µg/mL).

DISCUSSION

This study evaluated the antibacterial and antibiofilm activities of the roots, stem bark and leaves of *Uvaria chamae* (Beauv) on Enteroaggregative *Escherichia coli* (EAEC). Two solvents, dichloromethane and methanol were used for the extraction to allow for the extraction of wide range of polar and non-polar components of the plant parts. Medicinal plants are rich in diverse secondary metabolites that exhibit different pharmacologic activities (Agbebi *et al*., 2024). However, the composition and quantity of these phytochemicals reported to be present in the plant significantly vary due to climatic and other conditions in which they grow (Samaniego *et al*., 2020), hence the need for analysis. Qualitative phytochemical screening carried out on the extracts of the plant parts validated the presence of alkaloids, flavonoids, anthraquinones, cardiac glycosides, phenol and terpenoids as earlier reported by Enin *et al*. (2021). The total phenolics content of the leaf and stem methanol and dichloromethane extracts were significantly lower compared to what was reported by Enin *et al*. (2021). The variation is suggestive of extractive solvent effects. While Enin and his collaborators (2021) used ethanol as the extractive solvent, methanol and dichloromethane were used in this research. This implies that if the phytochemical of interest are the phenolics, ethanol would be the preferred solvent over methanol or dichloromethane. However, if the preferred phytochemical are flavonoids, either methanol or ethanol could be used for extraction without significant impact of the quantity of the phytochemical.

Brine shrimp lethality test is a basic initial toxicity screening for additional research before using animal models (Wu, 2014) and does not require aseptic techniques. The assay is amenable for the use of small amount (2–20 mg or less) of the test substance (Quazi *et al*., 2017). The results obtained from this study suggest that dichloromethane root extract of *U. chamae* is the least toxic to brine shrimp and thus was used for further studies. The 50% lethality of extracts have been categorized as non-toxic (> 1000 µg/mL), weakly toxic (500-1000 µg/mL), moderately toxic (100-500 µg/mL), strongly toxic (0-100 µg/mL) [Ogbole *et al*., 2016]. The extracts had LC_{50} >1000 µg/mL suggesting that all the extracts of *Uvaria chamae* are non-toxic in brine shrimp lethality assay and thus safe. However, cyclophosphamide, a known

cytotoxic anticancer drug was 23-fold and 43-fold more toxic to brine shrimp compared to methanol stem and dichloromethane stem extracts, respectively.

The result of the antibiogram was used as a guide in the choice of the antibiotic that would serve as the positive control. The EAEC strains used for the research were multi-drug resistant, but sensitive to gentamicin and azithromycin. Antibacterial screening of *U. chamae* against test EAEC strains showed that at 100mg/mL the methanol leaf extract exhibited excellent growth inhibition against *E. coli* strain DH5α and EAEC strains D25D and D28I. The results obtained in this research is supported by the findings of Enabuele and Ifeka. (2022) and Oluremi *et al.* (2010) who previously showed that methanol leaf extracts of *Uvaria chamae* exhibited antibacterial activity against a wide range of bacteria like *E. coli, S. aureus, B. subtilis* and *P. aeruginosa*. Furthermore, Ochiabuto *et al*. (2022) reported that crude and diluted leaf and root methanol, hexane and aqueous of extracts of *Uvaria chamae* exhibited significant antimicrobial activity against bacterial pathogens including *S. aureus, B. subtilis, P. aeruginosa*, which further strengthens the fact that *U. chamae* possesses both Gram positive and Gram negative bacterial antimicrobial activity.

The biofilm inhibition assay was performed at concentration lower than the MICs of each test bacteria using the crystal violet method. The methanol leaf extract of *U. chamae* exhibited excellent antibiofilm activity against susceptible EAEC strains. Similarly, Madiba *et al*. (2023) reported the significant inhibition of *S. mutans* biofilm formation by 70% reduction in biofilm mass when exposed to dichloromethane extract of *U. chamae*. Interestingly, the ethanol leaf extract of *U. chamae* has also been reported (Nden *et al*., 2017) to significantly decrease preformed biofilm of *Clostridium difficile* and *Propionibacterium acne* strains which is an indication of good antibacterial activity against established infection by biofilm forming strains. Findings from this study and the collaborative data from other researchers suggest the broad spectrum antibacterial and antibiofilm activities of extracts of *Uvaria chamae*.

CONCLUSION

Uvaria chamae that is rich in the phytochemicals; flavonoids, terpenoids, anthraquinones and alkaloids inhibited growth and biofilm formation in susceptible enteroaggregative E. coli strains O42, MND5E and D25D. The plant could therefore be a potential source of effective anti-infective diarrheal agents against biofilm forming strains.

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