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Biofilm-inhibition activities of fractions of *Senna Siamea* (LAM) Irwin & Barneby leaf against *Escherichia coli*

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Escherichia coli **has carved its niche in the urinary tract with the formation of a formidable matrix called biofilm. This biofilm is not only recalcitrant to the body's immune system but also resistant to antibacterial agents*. Senna siamea* (Lam) Irwin and Barneby is a medicinal plant with established antibacterial effect against planktonic cells of many bacteria. An attempt was made herein to evaluate the effect of its leaf extract and fractions on biofilm of *E. coli* isolates. Crude extracts of leaf, stem bark and root of this plant were prepared using ethanol as the solvent for the cold extraction. Phytochemical screening was carried out on the three extracts. Two *E. coli* strains from different antenatal patients attending General Hospital, Kafanchan, Kaduna were donated to us by a researcher from Ahmadu Bello University, Zaria and the reference strain, *E. coli*, WDCM 00013 (from Germany) were tested for biofilm production using the Congo red method. Antimicrobial susceptibility testing of the crude extracts against the isolates was carried out using the agar diffusion method. The Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) were determined for the leaf extract of the plant using micro broth dilution and agar diffusion methods respectively. In order to establish the antibiofilm activities of the leaf extract of the plant, sub-inhibitory concentrations (sub-MIC) were used against the test isolates in the remaining assays in the work. Column chromatography backed by thin layer chromatography (TLC) was used to fractionate leaf extract (having the best antibacterial activity) of the plant, using different ratios of a combination of hexane, ethyl acetate and n-butanol as fractionating solvents. MIC and MBC of the leaf extract were and 50 mg/ml respectively. High values of percentage biofilm inhibition were observed against all the bacterial isolates from the antibiofilm assay. Combination of solvents in the increasing order of polarity enhanced the antibiofilm activity of the various fractions of the leaf extract of Senna siamea. In conclusion, further fractionation of Senna siamea leaf extract increases its antibiofilm activities.**

Key words: Biofilm, *Senna siamea,* column chromatography, thin layer chromatography, *Escherichia coli.*

INTRODUCTION

Biofilms are densely packed communities of microbial cells that grow on living or inert surfaces and surround themselves with secreted polymers. Many bacterial species form biofilms, and their study has revealed them to be complex and diverse. The structural and physiological complexity of biofilms have led to the idea

that they are coordinated and cooperative groups, analogous to multicellular organisms (Nadell et al., 2008). Researchers have estimated that 60-80% of microbial infections in the body are caused by bacteria growing as a biofilm, as opposed to planktonic (free-floating) bacteria. Some external biofilm, namely chronic wounds and dental plaque, can be manually removed. Because of their inaccessibility and heightened resistance to certain antibiotic combinations and dosages, internal biofilms are more difficult to eradicate (Anderson et al., 2003).

Escherichia coli bacteria in urinary tract infection present several virulence factors that allow them to colonize host mucosal uroepithelium, injure and invade host tissues, overcome host defence mechanisms, incite a host inflammatory response and eventually proceed from the lower urinary tract to the renal cavities and tissues. Several surface determinants involved in *E. coli* biofilms are flagella and motility, Fimbriae, Fimbriae, Autotransporter proteins, Curli, F conjugative pilus and Exopolysaccharide production

Senna siamea which belongs to the sub-family fabaceae (Caesalpinioideae) of family leguminosae has its leaf being used as vegetables in Thailand (Otimenyin et al., 2007). Also, Aliyu (2006) found that *S. siamea* is ethno medicinally used as laxative, blood cleaning agent, cure for digestive system, urinogenitory disorders, herpes and rhinitis.

However, despite a vast literature search, we are yet to come by a single published work on the antibiofilm activities of *S. siamea* plant which is the premise on which this work is undertaken. It is the aim of this research to determine the antibiofilm activities of *S. siamea* plant leaf, stem bark and root extracts against biofilm-forming *E. coli* clinical isolates while using the reference strain *E. coli* WDCM 00013 (obtained from Sigma Aldrich, Germany) as standard for measuring the activities of the clinical isolates. This will be done by comparing the minimum inhibitory concentrations (MICs) of the three extracts so as to pick the most active of the extracts. It is to be borne in mind that lower (than the MIC) concentrations are to be used for the antibiofilm assay since the target for disruption is the bacterial biofilms and not the bacteria themselves. It is also the aim of this research to further fractionate the most active extract for the in situ production of phytochemicals, tannin and flavonoids. The choice of these two metabolites is based on the fact that they are polar and that their standards are easily obtainable. The method to be employed is column chromatography backed with thin layer chromatography (TLC) while the use of standard tannin and flavonoids serves to locate the R_f values of the phytochemicals in the extract .The biofilms of the bacterial isolates will then be subjected to treatment with the *in situ* produced phytochemicals (Figure 1).

METHODOLOGY

Leaves, stem bark and root parts of *S. siamea* tree behind Ahmadu Bello University Fire Service were collected while the plant was identified at the herbarium section of the Biological Science Department, Ahmadu Bello University, Zaria, with the voucher number 613. They were shade dried, homogenised and extracted with ethanol through cold extraction for three days. After decantation, the filtrates were concentrated and dried in a water bath at temperature of 40°C. The dry extracts were subjected to phytochemical screening according to the standard methods of Trease and Evans (2009). The pellets of standard strain *E. coli* WDCM 00013 (from Sigma Aldrich, Germany) were propagated as provided by the American Type Culture Collection (2014). The test isolates, *E. coli* B27 and C1 from the urine of antenatal patients attending General Hospital, Kafanchan, Kaduna (donated by a co-researcher) were purified by regularly subculturing them on tryptic soy agar slant at 37°C for 24 h and subsequently subjected to congo red assay for detection of biofilm-forming capacity as described by Freeman et al. (1989).

The isolates were then subjected to antibacterial susceptibility testing using agar well diffusion method according to Clinical Laboratory Standards Institute (CLSI) guidelines. Broth dilution method was adopted to determine the minimum inhibitory concentrations (MIC) of the most active extract based on zones of inhibition of bacterial growth. Minimum bactericidal concentration (MBC) was also determined for the extract using the agar dilution method.

The antibiofilm activity of the most active plant extract was determined by the method described by Filoche et al. (2005). Overnight culture of all the test organisms was grown to provide pre-formed biofilms. One hundred microlitre (100 µl) of each of these cultures was put into a 96-well microtitre plate. The plate was then incubated for further 4 h at 37°C to allow cells' attachment. Next, 100 µl of the plant extract at sub-inhibitory concentrations (20, 10, 5.0, 2.5, 1.25 and 0.625 mg/ml) was added to the wells. Each test was provided in two wells as duplicate. Growth control (cells + broth), media control (only broth) and blank control (broth + extract) were included. The plate was then incubated at 37°C for further 24 h. Following 24 h incubation, the supernatant was removed and each well was rinsed with sterile saline three times. The modified crystal violet assay was then used to assess the biomass of the attached cells as follows: biofilms formed by adherent cells in plate were stained with 0.1% crystal violet and incubated at the room temperature for 20 min. Excess stain was rinsed off by thorough washing with deionized water and plates were fixed with 200 µl of 96% ethanol. Optical densities (OD) of stained adherent bacteria were measured at 630 nm using an ELISA microplate reader (Stepanovic et al., 2007). The percentage of biofilm inhibition was calculated using the following formula: [(OD growth control – OD sample) / OD growth control] × 100.

Fractionation of the most active extract of the plant

Twenty gram of ethanolic extract of the plant was subjected to column chromatography on silica gel (120 mesh) packed and eluted successively starting with 100% n-hexane, then ethyl acetate and then methanol. This was carried out by increasing the solvent

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Figure 1. *S. siamea* plant.

polarity in the following ratio of the solvents; 90:10, 80:20, 70:30, solvents were also tested. Several pools of elutes were collected in different beakers and some representative samples were subjected to thin layer chromatography (TLC). The TLC was developed in a TLC tank with silica gel 60 using n-hexane: ethyl acetate: methanol in ratio 2:7:1 as the developing solvent system and R_f value values were calculated using the formula: R_f value = Distance moved by the molecule/ Distance moved by the mobile phase

Visualisation was carried out by dipping the plate in vanillin sulphuric acid (1%). Elutes with similar R_f values were combined as a single fraction. Those with different R_f values were grouped. Fractions were then subjected to qualitative analysis with flavonoids and tannins being the two phytochemicals tested for. Fractions most positive for flavonoids were combined and a sample from this was run on TLC plates alongside standard flavonoids, quercetin to confirm the presence of flavonoids in the fraction. Similarly, the fractions most positive for tannins were run on TLC plates alongside a standard tannin to confirm the presence of tannin in the fraction.

Antibiofilm activities of the fractions

The flavonoid-rich fraction and the tannin-rich fraction were tested against the test bacterial isolates for their antibiofilm activities using the modified crystal violet method.

RESULTS

Biofilm production of the bacterial isolates

These are the images of the biofilms of *E. coli* C1, B27 and the standard strain *E. coli* WDCM 00013. Appearance of dark colonies in Figures 2 to 4 indicates the formation of strong biofilms by the isolates. Though all the three extracts of *S. siamea* plant did not show a good antibacterial activity, the leaf extract was chosen for the rest part of this study since it was the only extract with antibacterial activity in at least two concentrations or more in all the test isolates used in this study (Table 1).

Minimum inhibitory concentration (MIC) of the leaf extract was determined to be 25 mg/ml. The minimum bacteriocidal concentration (MBC) of the extract was found to be 100 mg/ml. Consequently, a sub-MIC concentration of 20, 10, 5, 2.5, 1.25 and 0.625 mg/ml were adopted for the extract in the biofilm inhibition assay (Tables 2 and 3).

Optical Densities of the Escherichia isolates biofilms

Escherichia coli isolates B27, C1 and WDCM 00013 had the following after being treated with Senna siamea leaf, stem bark and root extracts as shown in Appendix Table 1, 2 and 3 respectively.

The use of standard tannin and flavonoids (tannic acid and quercetin) during the thin layer chromatography confirmed the presence of tannin and flavonoid respectively in the extracts (Figure 5).

Antibiofilm activities of the phytoconstituents

Appendix Tables 4 and 5 show the optical densities (OD) obtained when the phytoconstituents were used against the Escherichia coli isolates. The percentage biofilm inhibition due to the anti-adhesive activity of the ethanolic extract of *S. siamea* leaf against test biofilm-forming *E. coli* isolates at the sub-inhibitory concentrations used is as shown in Tables 4 and 5.

Figure 2. *Escherichia coli* C1 isolate Biofilm.

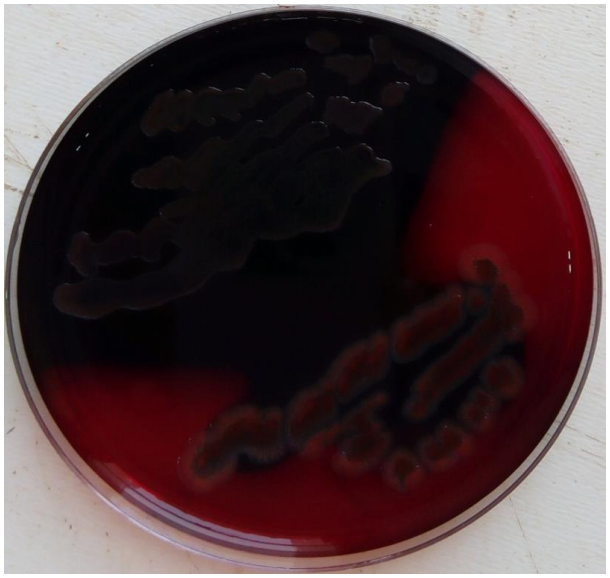

Figure 3. *E. coli* B27 isolate biofilm.

Figure 4. ***E. coli*** WDCM 00013 standard isolate biofilm.

DISCUSSION

S. siamea plant was reported to possess antimicrobial activities by Mohammed et al. (2013). However, in this study, *S. siamea* plant parts have not shown tangible antibacterial activities. Lack of significant antibacterial activity by the extracts of this plant may be explained by the fact the local usage of the plant for the treatment of typhoid is usually prolonged, perhaps to achieve the needed effective dosage level. This is in tandem with the study carried out by Doughari and Okafor (2008) in which the aqueous leaf extract of *S. siamea* only sparingly inhibited the growth of *Salmonella typhi* even at the highest concentration used in the study.

Interestingly, *S. siamea* leaf extract showed remarkable disruption of biofilms of the test organisms. This is evident in the high percentage biofilm inhibition observed when this extract was tested against strong biofilm-forming *E. coli* isolates; as high as 79.5% against the *E. coli* C1 in Table 2.

It is noteworthy to mention that in this work, the antibiofilm activities by the various extracts of *S. siamea* were best at lower concentrations as it can be seen in Table 2 that the *S. siamea* leaf extract had no activity at all against the standard *E. coli* WDCM 00013 at 20 mg/ml, only for this extract's activity to increase as the concentration decreases. This observation is similar to that of Gislene et al. (2000) where it was reported that plant extracts like *Syzygyum joabolanum* inhibited the growth of resistant bacteria at lower concentration. Supporting this fact is the work of Artini et al. (2018) where it was reported that different essential oils extracted from mediterranean plants were able to destabilize biofilm at very low concentration without impairing bacterial viability.

Furthermore, Lui (2003) reported that natural phytochemicals at the low levels present in fruit and vegetables offer health benefits, but these compounds may not be effective or safe when consumed at higher doses, even in a pure dietary supplement form. Though flavonoids and tannins were not isolated from the fractions of the plant's extract, the qualitative analysis conducted on the fractions showed that they contained these phytochemicals. This was further confirmed by the R_f values obtained from the thin layer chromatography as compared with those of the standards used. It can be

Table 1. Phytochemical constituents in the three extracts.

Sample Test	Ethanolic leaf	Ethanolic stem bark	Ethanolic root
Test for Carbohydrate	+	—	+
Test for anthraquinone	—	+	+
Test for unsat. steroid and tri terpenes	+	+	+
Test for cardiac glycoside	+	+	+
Test for saponin	+	+	-
Test for Tannins	+	+	+
Test for flavonoids	+	+	+
Test for alkaloids	+	+	+

: + = Present, — **=** Absent.

Table 2. Antibiofilm activity of *S. siamea* leaf extract on the bacterial isolates.

Concentration (mg/mL) / Isolate	Percentage biofilm inhibition (%)					
	20	**10**	**5**	**2.5**	**1.25**	**0.625**
B27	76.4	72.7	75.5	78.2	74.1	76.8
WDCM	0	18.6	39.5	61.8	68.2	65.5
C1	77.3	77.3	79.5	79.5	77.3	79.5

Table 3. Phytochemical compounds in various fractions.

Fraction type	Flavonoids	Tannins	Phenols
2:7:1 Hex-Eth.Acet-MeOH	**+++**	—	—
7:2:1 Hex-Eth.Acet-MeOH	—	**+++**	—

Hex-Eth.Acet-MeOH = Hexane-Ethyl acetate-Methanol; + = Detected, - = Not detected, ++ = more detected,+++ = very much detected **.**

Table 4. Antibiofilm activity of flavonoids-rich extract on the *E.coli* isolates.

Concentration (mg/mL) / Isolate	Percentage biofilm inhibition (%)					
	20	**10**	**5**	**2.5**	**1.25**	**0.625**
B27	83.3	77.7	66.7	50	66.7	66.7
C1	72.2	61.1	44	5.5	83.3	77.6
WDCM	77.7	72.2	72.2	72.2	77.7	44

seen that the flavonoids-containing fraction has higher antibiofilm activities than the tannin-containing fraction. According to Tsuchiya and Linuma (2000), antibacterial flavonoids might be having multiple cellular targets, rather than one specific site of action. One of their molecular actions is to form complex with proteins through nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation. This when compared with tannin's affinity for forming polymerization with extracting solvent, according to Naima et al. (2015), it will be understood why the flavonoid-rich fraction had more antibiofilm activities than

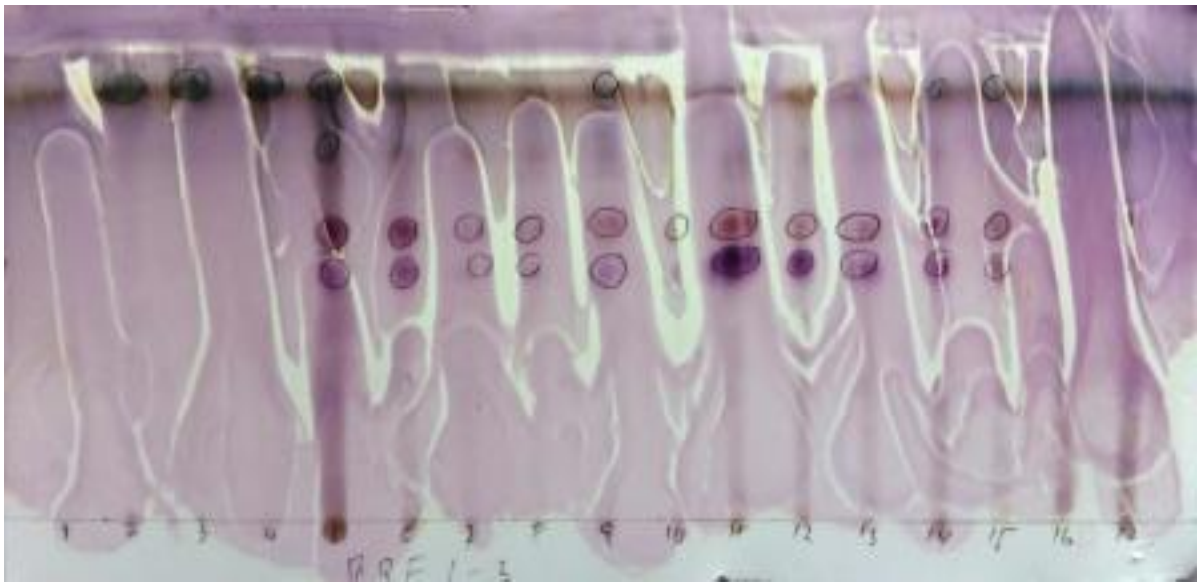

Figure 5. TLC plate showing the distance moved by tannin and flavonoids along with their standards from the baseline
NB: 5 and 6 are the starting points for tannin and its standard, tannic acid while 11 and 12 are the starting points for flavonoids and its standard, quercetin.

Table 5. Antibiofilm activity of tannin-rich extract on the *E. coli* isolates.

Concentration (mg/ml) / Isolate	Percentage biofilm inhibition (%)					
	20	**10**	**5**	**2.5**	**1.25**	**0.625**
B27	44.4	33.3	44.4	44.4	55.5	66.7
C1	22.2	11.1	44.4	55.5	88.9	88.9
WDCM	44.4	0	55.5	55.5	0	77.8

the tannin-rich fraction. Furthermore, the effect of mixing solvents in certain ratios for better extraction of plant's biomolecules has been demonstrated in this research as seen in the antibiofilm activities of 2:7:1 Hexane-Ethyl acetate-Metanol fraction.

In comparison, it could be seen that this work and our previous work, Usman et al. (2019) showed similar pattern of antibiofilm activities. However, it could be noted that while the minimum inhibitory concentration, MIC was 200 mg/ml in the previous work, it was 20 mg/ml in this work. Furthermore, the leaf extract of the plant in the previous work was not active enough for further assay whereas in this work, it was the best among the same set of extracts used in the previous work. This variation in activities of the same extracts of this plant might be due to change in time of collection of this plant's parts.

CONCLUSION

In this research, *S. siamea* leaf, ethanol extract had good antibiofilm activities against the *Escherichia coli* isolates used. Flavonoid-rich fraction had better biofilm disrupting activities against the test *Escherichia coli* isolates.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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APPENDICES

Appendix Table 1. Optical densities (O.D) from microtitre plate reader of ethanol leaf extract against the *E. coli* isolates.

Concentration (mg/mL) / Isolate	Average O.D at					
	20	**10**	**5**	**2.5**	**1.25**	**0.625**
B27	0.083	0.073	0.071	0.096	0.093	0.079
WDCM	0.102	0.081	0.080	0.065	0.066	0.076
C1	0.065	0.068	0.063	0.075	0.064	0.055

Appendix Table 2. Optical densities (O.D) from microtitre plate reader of ethanol stem bark extract against the *E. coli* isolates.

Concentration (mg/ml) / Isolate	Average O.D at					
	20	**10**	**5**	**2.5**	**1.25**	**0.625**
B27	0.032	0.041	0.034	0.036	0.072	0.032
WDCM	0.74	0.045	0.049	0.055	0.063	0.071
C1	0.062	0.074	0.061	0.049	0.069	0.059

Appendix Table 3. Optical densities (O.D) from microtitre plate reader of ethanol root extract against the *E. coli* isolates.

Concentration (mg/ml) / Isolate	Average O.D at					
	20	**10**	**5**	**2.5**	**1.25**	**0.625**
B27	0.091	0.073	0.085	0.047	0.058	0.069
WDCM	0.069	0.061	0.058	0.075	0.092	0.046
C1	0.065	0.078	0.083	0.055	0.054	0.055

Appendix Table 4. Optical densities (O.D) from microtitre plate reader of flavonoid-rich extract against the *E. coli* isolates.

Concentration (mg/ml) / Isolates	Percentage biofilm inhibition (%)					
	20	**10**	**5**	**2.5**	**1.25**	**0.625**
B27	0.096	0.081	0.085	0.074	0.098	0.093
C1	0.125	0.114	0.096	0.108	0.109	0.111
WDCM	0.108	0.097	0.113	0.117	0.119	0.084

Appendix Table 5. Optical densities (O.D) from microtitre plate reader of tannin-rich extract against the *E. coli* isolates.

Concentration (mg/ml) / Isolate	Percentage biofilm inhibition (%)					
	20	**10**	**5**	**2.5**	**1.25**	**0.625**
B27	0.095	0.062	0.085	0.108	0.098	0.093
C1	0.125	0.111	0.066	0.069	0.091	0.101
WDCM	0.079	0.087	0.103	0.087	0.109	0.084