In vitro anti-biofilm effects of *Loxostylis alata* extracts and isolated 5-demethyl sinensetin on selected foodborne bacteria

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Abstract

Bacterial biofilms pose health challenges both in clinical environments and the food industry. Major foodborne bacterial pathogens form biofilms on surfaces and persist, causing infections in humans that may be difficult to treat. Conventional use of antibiotics is fast becoming ineffective due to emerging resistance of pathogens to antibiotics. Previous studies have demonstrated the antimicrobial potential of *Loxostylis alata* A. Spreng. ex Rchb. extracts against a range of bacterial pathogens. The inhibitory effects of methanol and aqueous extracts of *L. alata* and an antibacterial

compound (5-demethyl sinensetin) isolated from the leaves, on the growth and development of microbial biofilms was investigated against clinical isolates of *Enterobacter cloacae, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis* and reference strains of *Bacillus cereus, Salmonella* Enteritidis and *Salmonella* Typhimurium using the crystal violet (CV) assay. The Kirby-Bauer disk diffusion method was utilized to determine antibiotic susceptibility and resistance profiles of the four clinical and three reference isolates. *Enterobacter cloacae* was resistant to tetracycline and doxycycline and *P. mirabilis* was resistant to colistin while *E. coli* and *K. pneumoniae* were pan-susceptible to all the antibiotics screened. The hot water extract reduced biofilm adhesion from 7% to >50% for the clinical isolates and the ATCC strains, except for *S.* Typhimurium. The methanol extract inhibited the growth of preformed biofilms of *S.* Enteritidis and *S.* Typhimurium by >50% and also reduced the biofilm biomass in *S.* Enteritidis and *E. coli* by >50%. The plant extracts and compound were able to reduce initial cell attachment and biofilm biomass, although inhibition of growth in a preformed biofilm was not attained.

Keywords: Biofilm; Anti-biofilm; *Loxostylis alata*; Foodborne pathogens.

1. Introduction

Globally, there has been an increase in the incidence of human disease outbreaks associated with foodborne pathogens of both food animals such as poultry, cattle, swine or sheep and contaminated fresh produce (Berger et al., 2010). It has been estimated that 20% of contamination occurs on the farm during outbreaks, while the remaining contamination may occur as a result of mishandling after leaving the farm (Yaron and Römling, 2014, Buscaroli et al., 2021). Adherence, attachment,

colonization and survival of foodborne pathogens on food produce are essential for successful contamination (Yaron and Römling, 2014, Bai et al., 2021). There is documented evidence that many pathogenic bacteria which are implicated in a large number of foodborne disease outbreaks can adhere to and form biofilms on various surfaces (Berger et al., 2010, Winkelströter et al., 2014, Bridier et al., 2015, Chakroun et al., 2018, Rather et al., 2021), leading to persistence and resistance to treatment (Collignon and Korsten, 2010, Yaron and Römling, 2014, Uruén et al., 2020). It has been speculated that approximately 60% of human infections result from biofilm formation on the human mucosa (Sandasi et al., 2010).

Biofilms are described as complex communities of microorganisms in which cells are attached to a surface and to each other, and are embedded in a self-produced matrix of extracellular polymeric substances (EPS) (Costerton et al., 1999, Iliadis et al., 2018). Biofilms can be formed by mono or polymicrobial species (Crull et al., 2011) and are predominantly made up of water. Other components such as viable and dead bacteria, extracellular DNA, proteins, polysaccharides, lipids and lipopolysaccharides (LPS) (Crull et al., 2011, Bridier et al., 2015, Iliadis et al., 2018). Biofilms form part of bacteria's architecture (Saxena et al., 2019). They serve to capture nutrients, aid persistence and proliferation, and provide a physical barrier for bacteria against environmental effects such as desiccation, disinfection, UV radiation, antimicrobial substances and also against host immune cells (Lapidot et al., 2006, Giaouris et al., 2012, Abebe, 2020, Bi et al., 2021). Biofilm formation aids bacterial survival, persistence and recurrent cross-contamination throughout the food production process (Winkelströter et al., 2014, Rather et al., 2021). Most of the pathogens that cause foodborne diseases (such as *Bacillus cereus*, *Escherichia coli*, *Salmonella* species and *Staphylococcus* species, among others) can adhere to and form biofilms

on most materials and under almost all the environmental conditions in food production plants (Jan et al., 2011, Bridier et al., 2015, Buscaroli et al., 2021). Likewise, in the health sector, biofilm-associated infections (BAI) are known to trigger chronic conditions such as endocarditis, osteomyelitis, cystic fibrosis and chronic otitis media (Hall-Stoodley et al., 2012, Vestby et al., 2020, Kolpen et al., 2022) that may be difficult to treat.

Infections with biofilm-growing pathogens are highly resistant to both antibiotics and host immune defences (Sun et al., 2013, Uruén et al., 2020), which constitutes a public health concern. One of the widely accepted reasons for the emergence of antibiotic resistance is the inappropriate and excessive use of antibiotics for treatment and prevention and the use of antibiotics as animal growth promoters (AGPs) (Phillips et al., 2004, Tang et al., 2017). The use of AGPs began in the mid-1950s with the addition of sub-therapeutic doses of procaine penicillin and tetracycline to animal feed to enhance the feed-to-weight ratio for poultry, swine and beef cattle and has been practiced to date (Marshall and Levy, 2011). Over time, however, the negative impacts of this practice have been linked to the emergence of antibiotic-resistant bacterial strains (Martinez, 2009, Landers et al., 2012, Van Boeckel et al., 2015). Studies have shown the possibility of transferring these resistant zoonotic bacteria to humans (Létourneau et al., 2010, Tadesse et al., 2012, Liu et al., 2016).

The huge challenge posed by antibiotic resistance can be addressed firstly by preserving the efficacy of existing antibiotics via regulating use, or identifying new antibiotics with modes of action different from the current mechanisms to which resistance persists (Wright, 2017). Finally, we need to explore natural products as alternatives to conventional anti-infective strategies. Plants have been fundamental in the drug discovery process (Taylor, 2013), and may provide safer alternatives to

allopathic medicines (Mohsenipour and Hassanshahian, 2015). In previous studies, leaf extracts of *Loxostylis alata* A. Spreng. ex Rchb. were reported to have antibacterial activity against several bacterial and fungal pathogens (Suleiman et al., 2010, Suleiman et al., 2012, Gado et al., 2017). Further isolation and characterization of the methanol leaf extract of *L. alata* yielded flavones (Gado et al., 2018), among other metabolites. The crude extract has been reported to have some degree of cytotoxicity against Vero kidney cells, with LC_{50} = 0.20 mg/mL (Gado et al., 2021). A dose of 300 mg/kg of the extract fed to chickens induced some toxicity, detected by decreased feed intake and weight loss (Suleiman et al., 2012). However, lower concentrations of 100 and 200 mg/kg significantly reduced clinical disease (aspergillosis), with the extract showing similar efficacy as the positive control, ketoconazole, which was dosed at 60 mg/kg (Suleiman et al., 2012). This study aimed to determine the antimicrobial activity of the methanol and hot water extracts and the purified compounds against selected biofilm-forming food pathogens. The antibiotic susceptibility and resistance patterns of the bacterial isolates used in this study were also determined.

2. Materials and Methods

2.1 Preparation of plant extracts

Leaves of *L. alata* were collected in the summer months of 2016 from the Manie van der Schijff Botanic Garden, University of Pretoria. Voucher specimens of the plants were identified and deposited at the H.G.W.J. Schweickerdt Herbarium of the University of Pretoria, South Africa (voucher no: PRU 124357). The ground dried leaves of the plant were extracted separately with methanol (Minema, South Africa) and hot water in a ratio of 1:10 ground dried leaf material to extractant. Ten (10) grams

of ground leaves were extracted with 100 mL of 100% methanol (Minema, South Africa) and hot water. The resulting suspensions were left to stand for 24 h at room temperature, and supernatants were decanted into pre-weighed glass containers after filtering through Whatman No. 1 filter paper (Sigma-Aldrich, United States) and concentrated to dryness under a stream of cold air (Air dryer,Heraeus; Germany). The extraction was repeated twice on the marc. The dried extracts were stored at 4°C in tightly stoppered glass vials (Lasec, South Africa) until use. The extracts were reconstituted to 10 mg/mL with 50% acetone (Minema, South Africa) for the methanol extract and sterile distilled water for the water extract.

The pure compound was isolated using column chromatography as previously described (Gado et al., 2021).

2.2 Screening for antibiotic susceptibility and resistance profiles of the clinical bacterial species

Antimicrobial susceptibility testing for antibiotics, including amikacin (AK) 30 µg, amoxicillin/clavulanic acid (AMC) 30 µg, ampicillin (AMP) 10 µg, ceftiofur (EFT) 30 µg, chloramphenicol (C) 30 µg, colistin sulphate (CT) 10 µg, doxycycline (DO) 30 µg, gentamycin (CN) 10 µg, tetracycline (TE) 30 µg and sulphamethoxazole/trimethoprim (SXT) 25 µg (ThermoScientific, South Africa), was carried out using the Kirby-Bauer disk diffusion method (Hudzicki, 2009) against four clinical isolates obtained from hen's eggs (Jambalang et al., 2017), namely *Escherichia coli, Enterobacter cloacae*, *Klebsiella pneumoniae* and *Proteus mirabilis*. The antibiotic disks were selected according to the 2015 standard regulations of the Clinical Laboratory Standards Institute (CLSI) for bacteria isolated from animals (Papich, 2015). Briefly, 0.5 McFarland equivalent bacterial culture suspension was prepared by picking a colony (of each of the four clinical isolates) from the Mueller-Hinton agar plate (Merck, South

Africa) after overnight incubation (IncoTherm; Labotec) at 37°C and re-suspending in a sterile 14 mL Falcon tube (ThermoScientific, South Africa) containing 5 mL normal saline (Merck, South Africa) by vortexing (VM-300 vortex mixer, Gemmy; Taiwan). A sterile cotton swab (Lasec, South Africa) was dipped into the bacterial suspension and excess fluid on the swab was removed by pressing against the bottleneck. The surface of the Mueller Hinton agar plate (ThermoScientific, South Africa) was swabbed to obtain confluent growth. The antibiotic disk sticks (Oxoid, England), were dispensed on the inoculated MH agar plates. The plates were incubated at 37ºC (IncoTherm incubator, Labotec, South Africa) for 18 h. The zones of inhibition were measured with a Vernier caliper (Lasec, South Africa) and interpreted according to the CLSI guidelines (Clinical Laboratory Standards Institute, 2015).

2.3 Bacterial strains, growth conditions and inoculum preparation for biofilm assay

Three strains from the American Type Culture Collection, namely *Bacillus cereus* (ATCC 21366), *Salmonella* Enteritidis (ATCC 13076) and *Salmonella* Typhimurium (ATCC 14028) and clinical isolates *Escherichia coli, Enterobacter cloacae*, *Klebsiella pneumoniae* and *Proteus mirabilis* isolated from hen's eggs (Jambalang et al., 2017) were included in this study. Precultures of all the bacterial strains were prepared from the original culture, stored at −80°C on ceramic beads in cryoprotective media (Pro-Lab diagnostics Microbank@ 20) by dropping one bead for each strain in 10 mL of Tryptone Soya Broth (TSB) (Merck, South Africa) in sterile 50 mL Falcon tubes (ThermoScientific, South Africa) and incubating in an MRC orbital shaker incubator (United Scientific, South Africa) at 37°C between 18 h to 20 h. Working cultures were produced by inoculating bacteria from each preculture into 10 mL of fresh TSB (Merck, South Africa) and incubating (MRC orbital shaker incubator; United Scientific, South

Africa) at 37°C for another 18 h. The overnight cultures were standardised to yield a concentration of 1.0 x 10^6 colony-forming unit (CFU)/mL before biofilm formation. This was achieved by diluting the overnight cultures with TSB (Merck, South Africa) to obtain an absorbance (OD_{590} nm) of 0.02 using a spectrophotometer (Epoch microplate reader: BioTek, United States). In a concomitant study, the purity of each preculture was confirmed by streaking for pure colonies on Tryptone Soy Agar (TSA); (Merck, South Africa) plates and confirming homogenous morphology of the developed colonies following incubation at 37°C (IncoTherm; Labotec, South Africa) for 24 h.

2.4 Inhibition of biofilm formation

The biofilm inhibition assay was done according to Sandasi *et al*. (2011). In this study, three stages of biofilm development were investigated, namely: (i) no attachment/planktonic (T0), (ii) initial attachment (T4) and (iii) irreversible attachment (T24). Briefly, to prevent initial cell attachment, 100 µL of plant extracts were aliquoted into the wells of a sterile 96-well microtitre plate (Lasec, South Africa). A 100 µL aliquot of standardized concentration of cultures (OD_{590} = 0.02 or 1.0 x 10^6 CFU/mL) of *B. cereus* (ATCC 21366), *E. coli, E. cloacae*, *K. pneumoniae*, *P. mirabilis, S.* Enteritidis (ATCC 13076) and *S.* Typhimurium (ATCC 14028), was added into the wells except wells designated for medium blank, samples' blank and positive control. Microtitre plate (Lasec, South Africa) were incubated (IncoTherm; Labotec, South Africa) for 24 h at 37°C without shaking (Sandasi et al., 2008). The final concentration of the extracts in the wells was 5 mg/mL, while that of the compound was 1 mg/mL. Gentamicin (Virbac, South Africa) and tetracycline (Sigma-Aldrich, United States) at a concentration of 2 mg/mL (final concentration of 1 mg/mL) were used as the positive controls. A 50% acetone (Minema, South Africa) mix and sterile distilled water with

culture served as negative controls. After incubation, the biofilm biomass was assayed using the modified crystal violet (CV) staining assay (Sandasi et al., 2011) and the percentage inhibition was determined using the following equation:

$$\% \text{ Inhibition} = \left(\frac{(\text{OD negative control} - \text{OD media control}) - (\text{OD test} - \text{OD extract control})}{(\text{OD negative control} - \text{OD media control})}\right) \times 100$$

To determine the ability of the extracts to prevent biofilm development, biofilms were pre-formed in 96-well microtitre plates (Lasec, South Africa) by dispensing 100 µL of standardized bacterial cultures (1.0×10^6 CFU/mL) into the wells and incubating (IncoTherm; Labotec, South Africa) for 4 h and a duplicate set of plates was incubated for 24 h at 37°C. Following incubation, 100 µL of each plant extract and compound was added to a final concentration of 1 mg/mL in the wells. Controls were included as previously described. The plates were further incubated for 24 h (IncoTherm; Labotec) at 37°C without shaking (Sandasi et al., 2008). After incubation with the extracts, the crystal violet assay was performed to assay for biofilm biomass.

2.5 Crystal violet staining assay

The microtitre plates were washed thrice with sterile distilled water following incubation and oven-dried (Labcon 1030K, Labex, South Africa) at 60°C for 45 min. The wells were stained with 100 µL of 1% crystal violet (Sigma-Aldrich, United States) and incubated at room temperature (20°C ± 5°C) for 15 min. The plates were washed thrice with sterile distilled water to remove the unabsorbed stain. The semi-quantitative assessment of biofilm formation was done by adding 125 µL of 100% ethanol (Minema, South Africa) to destain the wells. A 100 µL aliquot of the destaining solution was transferred to a new 96-well microtitre plate (Lasec, South Africa) and the absorbance at 590 nm was measured using a microplate reader (Epoch, BioTek,

United States). The mean absorbance of the samples was determined and the percentage inhibition obtained using the following equation.

$$\% \text{ Inhibition} = \left(\frac{(\text{OD negative control} - \text{OD media control}) - (\text{OD test} - \text{OD extract control})}{(\text{OD negative control} - \text{OD media control})}\right) \times 100$$

3. Results

3.1 Antibiotic susceptibility screening

In this study, only the clinical isolates were subjected to antibiotic susceptibility screening to determine the antibiotic resistance profiles. Results obtained showed that *E. coli* and *K. pneumoniae* were susceptible to all the antibiotic drugs screened (with zones of inhibition between 12 mm and 25 mm) but *E. cloacae* and *P. mirabilis* were resistant to at least one antibiotic (Table 1). *E. cloacae* was resistant to the tetracycline class of antibiotics [doxycycline (12 mm) and tetracycline (8 mm)]..

3.2 Inhibition of biofilm formation

The results of biofilm inhibitory activity (BIA) of the crude methanol and hot water crude leaf extracts and a flavone active against the foodborne pathogens are presented in Table 2. Plant extracts or fractions with percentage inhibition values above 0 are considered to have “biofilm inhibition’. Biofilm inhibition values above 50% were considered to have good anti-biofilm activity (++), those with inhibition values between 0 and 50% indicated poor anti-biofilm activity (+), while values below 0% (-) were considered as having no biofilm inhibition or resulting in ‘enhancement of growth’ (Sandasi *et al*. 2008).

Table 1. Disk diffusion zones of inhibition by selected antibiotics against *Enterobacter cloacae, Escherichia coli, Klebsiella pneumoniae* and *Proteus mirabilis* isolates

Sample ID	Antibiogram																		
	Diameter (mm)																		
	AK30	**INT**	**AMP 10**	**INT**	**EFT30**	**INT**	**DO30**	**INT**	**CN10**	**INT**	**C30**	**INT**	**SXT25**	**INT**	**TE30**	**INT**	**AMC30**	**INT**	**CT10**	**INT**
Enterobacter cloacae	25	S	17	S	25	S	12	R	25	S	24	S	28	S	8	R	19	S	17	S
Escherichia coli	25	S	23	S	25	S	20	S	23	S	25	S	28	S	25	S	22	S	26	S
Klebsiella pneumoniae	24	S	12	S	25	S	19	S	23	S	24	S	15	I	22	S	25	S	17	S
Proteus mirabilis	22	S	25	S	30	S	21	S	22	S	21	S	20	S	20	S	25	S	0	R

INT = interpretation, R = Resistant, I= Intermediate, S= Susceptible, AK30 =Amikacin, AMP10 =Ampicillin, EFT30 =Ceftiofur, DO30 =Doxycycline, CN10=Gentamicin C30 =Chloramphenicol, SXT25 =Sulphamethoxazole/trimethoprim, TE30 =Tetracycline, AMC30 =Amoxicillin/clavulanic acid, CT10 =Colistin sulphate

The highest inhibition of cell attachment (BIA >100%) at T0 was obtained with the hot water extract against *K*. *pneumoniae*, followed by *S*. Enteritidis and *E*. *cloacae*. *Proteus mirabilis* was inhibited at just a little above 50% (57%) while *E. coli* and *B. cereus* were less strongly inhibited with BIA values <50%. Enhancement of cell attachment was however obtained with *S*. Typhimurium (BIA value ≤0%). The methanol extract only inhibited cell attachment against *E*. *cloacae* with a BIA value ≥100%, cell attachment of all six other bacteria (*B. cereus*, *E. coli, K. pneumoniae*, *P. mirabilis, S.* Enteritidis and *S.* Typhimurium) was unfortunately enhanced by the methanol extract of *L. alata*. The pure compound (5-demethyl sinensetin) had poor anti-adhesion activity against *B. cereus*, *E. coli, E. cloacae*, *K. pneumoniae* and *P. mirabilis* (BIA value = <50%) while the growth of *S.* Enteritidis and *S.* Typhimurium were rather enhanced. Similarly, enhancement of cell attachment was obtained with gentamicin (positive control) at the concentration used. Another positive control, tetracycline, however displayed inhibition of cell attachment against all pathogens ranging from 12% to >100%, with the least (12%) inhibition obtained against *E*. *cloacae*.

In contrast to the cell attachment inhibition observed for most of the bacteria, growth and development of the biofilm (T4) was observed for the extracts and compound against most of the isolates (*B. cereus*, *E. cloacae*, *K. pneumoniae* and *P. mirabilis*). Only the methanol extract showed good antibiofilm development against the preformed (T4) biofilm of *S*. Enteritidis and *S*. Typhimurium, while the hot water extract had poor inhibition against *S*. Enteritidis and *E.coli*. The compound 5-demethyl sinensetin poorly inhibited the preformed biofilm of *K. pneumoniae* and *E. coli*. Growth enhancement of preformed cells of *E. cloacae*, *K. pneumoniae*, *S*. Enteritidis and *S*.

Typhimurium was observed with tetracycline. In contrast, a good antibiofilm activity was observed for *K. pneumoniae*, *E. coli* and only poorly against *B. cereus*.

On investigating the developed biofilm biomass (T24), it was observed that disruption of established biofilm was obtained only with the methanol extract against *S*. Enteritidis, *P. mirabilis* and *E. coli*. In contrast, growth enhancement was observed for *B. cereus, E. cloacae*, *K. pneumoniae* and *S*. Typhimurium. Again, for both preformed and developed biofilms, gentamicin at the concentration used enhanced growth rather than inhibited development. Tetracycline however only had a good inhibition against *E. coli* but poorly inhibited the growth of *E. cloacae* and *B. cereus*. It was also observed to enhance the biofilm biomass of *P. mirabilis, K. pneumoniae*, *S*. Typhimurium and *S*. Enteritidis.

Table 2. Biofilm inhibition of *L. alata* extracts and 5-demethyl sinensetin against selected bacterial strains known to cause foodborne infections

Organisms	% inhibition														
	T_0					T_4					T_{24}				
	Samples			Positive controls		Samples			Positive controls		Samples			Positive controls	
	LAM	LAHW	DGLA	GEN	TET	LAM	LAHW	DGLA	GEN	TET	LAM	LAHW	DGLA	GEN	TET
Enterobacter cloacae	++	++	+	-	+	-	-	-	-	-	-	-	-	-	+
Proteus mirabilis,	-	+	+	-	+	-	-	-	-	-	++	-	-	-	-
Klebsiella pneumoniae	-	++	+	-	+	-	-	+	-	++	-	-	-	-	-
Salmonella Typhimurium	-	-	-	-	++	++	-	-	-	-	-	-	-	-	-
Salmonella Enteritidis	-	++	-	-	++	++	+	-	-	-	++	-	-	-	-
Escherichia coli	-	-	+	-	++	-	+	+	-	++	++	-	-	-	++
Bacillus cereus.	-	+	+	-	++	-	-	-	-	+	-	-	-	-	+

Good (++) BIA (> 50% inhibition); Poor (+) BIA (more than 0–50% inhibition); No (-) BIA (0 % or less); < 0%; LAM = *L. alata* methanol extract, LAHW= *L. alata* hot water extract, DGLA= compound (5-demethyl sinensetin), GEN= Gentamicin (positive control), TET= Tetracycline (positive control)

4. Discussion

4.1 Antibiotic susceptibility

The susceptibility of *E. coli* and *K. pneumoniae* to all the antibiotics screened in this study is encouraging, as this may be a result of good control practices in the use of antiobiotics in poultry farming in the areas where they were isolated. However, the resistance of *E. cloacae* and *P. mirabilis* to at least one of the screened antibiotics supports the risk of transferring these resistant strains to humans through contact or consumption, considering that these bacteria were isolated from commercial poultry eggs. There are reports of multidrug-resistant *E. cloacae* emerging globally (Wu *et al*., 2010, Mezzatesta *et al*., 2012, Liu *et al*., 2015) and in South Africa (Lowman *et al*., 2011, Brink *et al*., 2012, Osei Sekyere, 2016). Records support tetracycline's broad-spectrum properties, tolerability and clinical safety, and for these reasons, the tetracycline class of antibiotics is widely used for the treatment of bacterial infections and also as growth promoters in the livestock industry (Tao *et a*l., 2010, Marshall and Levy, 2011, Grossman, 2016). This widespread usage of tetracycline may be a significant factor responsible for the emergence of tetracycline-resistant bacteria and the transfer of tetracycline resistance genes between bacterial species (Tao *et al*., 2010). Resistance to tetracycline is dependent on approximately 38 acquired tetracycline resistance (*tet*) genes which code for different resistance mechanisms, including efflux proteins, ribosomal protection proteins and inactivating enzymes (Zhang *et al*., 2009). Resistance is said to be due to the acquisition of mobile genetic elements that carry tetracycline-specific resistance genes,as well as mutations within the ribosomal binding site, and/or chromosomal mutations which could lead to increased expression of intrinsic resistance mechanisms (Grossman, 2016). In this study, *P. mirabilis* was resistant to colistin sulphate, a drug listed in the WHO's list of

critically important antibiotics (WHO, 2017). Colistin resistance is an emerging global challenge, especially in the Enterobacteriaceae family isolated from retail meat, pigs and infected humans (Brink *et al*., 2013, Liu *et a*l., 2016, Schwarz and Johnson, 2016). Resistance to colistin was initially only associated with acquiring transferable colistin resistance genes, such as *mcr-1,* located on a conjugative plasmid (Schwarz and Johnson, 2016). Colistin resistance can be chromosomally located but plasmid located resistance can be more easily spread to other genera in the family (Adiguzel et al., 2021). However, there are currently eight *mcr-genes (mcr* 1-8*)* identified among members of the *Enterobacteriaceae* family (Wang et al., 2018). Production of a phosphoethanolamine transferase is a novel mechanism of colistin resistance described thus far in the literature (Liu et al., 2016). Reports of the prevalence of *mcr-1*-positive bacteria observed in animals and food of animal origin (Liu et al., 2016, Poirel and Nordmann, 2016) strongly suggest that these resistance genes have spread from the veterinary to the human domain (Nordmann and Poirel, 2016, Schwarz and Johnson, 2016).

Generally, the development of antibiotic resistance is associated with genetically transferable elements, including mutations and gene acquisition (Baquero et al., 2009). However, resistance could sometimes be phenotypic, achieved without any genetic modification (Levin and Rozen, 2006, Corona and Martinez, 2013). This is called phenotypic resistance and may be acquired due to selective pressure (Zhang et al., 2016), “drug indifference, the growth in biofilms and the phenomenon of persistence” (Corona and Martinez, 2013).

4.2 Antibiofilm activity

The inhibition of cell attachment observed against the two bacteria that showed resistance to at least one antibiotic in this study (*E. cloacae* and *P. mirabilis*) is

noteworthy. This shows the potential of *L. alata* in the development of anti-infective preparations with an alternative mechanism to direct bactericidal activity. The success of the *L. alata* extracts and compound in inhibiting cell attachment shown in this study is an indication of its potential for reducing microbial colonization on surfaces and epithelial mucosa, which could eventually lead to infections (Sandasi et al., 2010, MacKenzie et al., 2017). It was also interesting to note the activity (although poor) of the pure compound (5-demethyl sinensetin). The inhibition of cell attachment by the hot water extract observed for most tested bacteria is significant as water is commonly used traditionally to prepare medicinal decoctions. Although most biofilm studies use ciprofloxacin as the positive control, the possible explanation for the absence of antibiofilm activity observed with gentamicin for all stages of biofilm formation in this study, and with tetracycline for some stages/bacteria, suggests that biofilms are resistant to antimicrobial agents. It has been reported that tetracycline at a plasma concentration of 0.4 mg/mL showed negligible inhibition of biofilm growth, although it is considered the best inhibitor among several antibiotics tested for biofilm inhibition studies (Sandasi et al., 2008). The enhanced biofilm development that was shown upon treatment with extracts and compound in this study has been reported in other studies (Sandasi et al., 2008, Sandasi et al., 2010, Mohsenipour and Hassanshahian, 2015). The enhancement of attachment and growth of biofilm biomass by plant extracts has been speculated to be owing to the presence of certain compounds such as fibrinogen within the extracts that promote microbial adhesion (Sandasi et al., 2010).

Overall, inhibition of preformed biofilms and biofilm growth was less pronounced compared to inhibition of initial attachment. The absence of antibiofilm activity against most pathogens by the extracts and compound supports the theory that bacterial cells

in a biofilm are more resistant to antibiotics when compared to planktonic cells (Corona and Martinez, 2013, Bazargani and Rohloff, 2016). Inhibition of biofilm growth and development has thus been proven to be more difficult to achieve than inhibition of initial cell attachment. These results are consistent with previous reports (Sandasi et al., 2008, Mohsenipour and Hassanshahian, 2015, Bazargani and Rohloff, 2016).

Biofilms are complex structures that will obstruct the free diffusion of antibiotics or plant extracts (Corbin et al., 2011). Hence, extracts that can penetrate this barrier would most likely be successful antibiofilm compounds. The capability to diffuse into the biofilm will be dependent on the structure of the antibiotic or extracts (Singh et al., 2010). Thus it has been suggested that increasing the diffusion of antibiotics inside biofilms might thus help in enhancing their activity (Dong et al., 2012). Another possible cause of poor antibiofilm activity is the probable presence of elements within the biofilms that “bind antibiotics/compounds, sequestering them and hence reducing the freely available concentration of these compounds within the biofilm structure” (Corona and Martinez, 2013).

5. Conclusion

Challenges in the treatment of most microbial infections due to emerging resistance to conventional therapy have prompted research into identifying alternatives for treating infections. Biofilms are one of the major virulence factors of many pathogenic bacteria. Therefore, there is a growing interest in developing effective and safe antimicrobials, particularly from plant extracts and other biologically active compounds isolated from plants. Furthermore, the “One Health” concept, which unites clinical, environmental, veterinary antibiotic resistance surveillance and molecular epidemiological studies as a means to containing antibiotic resistance, is gaining ground. In the present study, the methanol and aqueous extracts and a derived

compound (5-demethyl sinensetin) from *L. alata* showed *in vitro* antibiofilm activity by inhibiting bacterial cell attachment and developing preformed biofilms of bacterial species implicated in foodborne infections. Future research endeavours should encompass the possible synergistic activity of 5-demethyl sinensetin and tetracycline against resistant *E. cloacae* and *P. mirabilis*. To our knowledge, this is the first report of the antibiofilm activity of the crude leaf extracts of *L. alata* and its isolated flavone compound (5-demethyl sinensetin) against biofilms formed by *Bacillus cereus, Enterobacter cloacae, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Salmonella* Enteritidis and *Salmonella* Typhimurium.

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