

In vitro antibiofilm activity of tormentic acid against *Pseudomonas aeruginosa*

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Research Article

Keywords: Callistemon citrinus, antibacterial, biofilms, tormentic acid, extracellular polysaccharide formation

Posted Date: July 18th, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-3143105/v1>

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Additional Declarations: No competing interests reported.

Abstract

Background: Biofilm formation is the cause for the prevalence of nosocomial infections. *Pseudomonas aeruginosa* is one of the leading cause of nosocomial infections and affects more than 2 million patients. Infections caused by *P. aeruginosa* can be very serious and life-threatening. *P. aeruginosa* can adhere to biotic and abiotic surfaces through the organisation of bacterial biofilms known to confer drug resistance. Consequently, the logical and strategic intervention against bacterial biofilms is the use of multi-targeted combinational therapies. The present study reports the antibacterial and antibiofilm effect of tormentic acid and *Callistemon citrinus* extracts on *P. aeruginosa*.

Methods: Tormentic acid, hydroethanolic extract, and DCM: methanol extract from *C. citrinus* were studied for their inhibitory effects on the growth of *P. aeruginosa* using the microbroth dilution method. Their effect on biofilms was studied using a crystal violet.

Results: These compounds did not effectively inhibit growth because the minimum inhibitory concentration (MIC) was greater than 100 µg/ml. The standard drug, ciprofloxacin inhibited growth with an MIC of 0.25 µg/ml. Ciprofloxacin had a minimum biofilm inhibitory concentration (MBIC) greater than 1 µg/ml. Tormentic acid and the two extracts were able to significantly inhibit the adhesion of *P. aeruginosa* cells, which led to an indirect reduction of biofilms formed at 100 µg/ml. The result of the biofilm detachment assay showed that tormentic acid and DCM: methanol extract had a significant effect on biofilm disruption while the hydroethanolic extract had no effect on biofilm detachment. Capsular polysaccharide analysis showed that the test samples tormentic acid, hydroethanolic, and DCM: methanol extracts significantly reduced the amount of capsular polysaccharide content at 100 µg/ml. Ciprofloxacin at 0.25 µg/ml (MIC) did not reduce the amount of extracellular polysaccharide formed.

Conclusions: Tormentic acid and extracts significantly reduced amount of extracellular polysaccharide content, which are an important requirement in cell adhesion process resulting in reduced amount of biofilms formed. Tormentic acid can be considered as a candidate compound for the development of antibiofilm drugs.

Introduction

Antimicrobial resistance as a result of biofilms is one of the biggest threats to human and animal health today [1]. Biofilm-related infections have been found to be the most difficult to eradicate, as there is an increase in antimicrobial tolerance in biofilms compared to planktonic cells [2, 3]. It is estimated that more than 99% of all bacteria exist as biofilms and 60% of all human infections are a result of biofilms. [4]. *Pseudomonas aeruginosa* has been identified as one of the most common pathogens found in biofilms in a healthcare setting [5]. It is an opportunistic pathogen belonging to the Pseudomonadaceae family [6]. *P. aeruginosa* is a ubiquitous gram-negative bacterium that can grow easily in wide range environments [7]. It causes more serious disease in immune-compromised people and is common in

causing nosocomial infections [8]. Nosocomial infections are those that do not present at the time of hospital admission, but usually develop during the stay in the hospital [9]. Bacteria cell of *P. aeruginosa* are found mainly as biofilms rather than planktonic cells in rich medium [10]. The biofilm matrix of *P. aeruginosa* is made up of various interconnected polysaccharides, proteins, and DNA molecules that are collectively termed extracellular polymeric substance (EPS) [11].

The human respiratory tract presents an advantageous environment to which *P. aeruginosa* has become particularly well adapted. *P. aeruginosa* has the formidable ability to cause both chronic infections in the lung of CF patients and acute nosocomial pneumonia [12]. Biofilm formation is a primary characteristic of chronic infections of *P. aeruginosa* [13]. Biofilms have been shown to be more resistant to antimicrobial agents and capable of allowing bacteria to evade host immune response. [14]. Therefore, infections caused by biofilm-forming *P. aeruginosa*, such as in cystic fibrosis of the lung, are almost impossible to eradicate [15]. *P. aeruginosa* is commonly found to be the first or second major pathogen causing ventilator-associated pneumonia (VAP) [16].

Studies focused on the intensive care unit (ICU) have found that 8% of all hospital-associated infections were due to, *P. aeruginosa* causing, central line-associated bloodstream infections, surgical site infections, of catheter-associated urinary tract infection and of ventilator-associated pneumonia (VAP) infections [16]. *P. aeruginosa* is among the five leading bacterial pathogens including, *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and Enterococcus species, causing nosocomial bacteremia and regularly leading to sepsis [17]. Despite the introduction of effective anti-pseudomonal β -lactams and the related reduction in mortality rates, the bacteremia of *P. aeruginosa* is still one of the most feared nosocomial infections. These infections are largely associated with higher mortality than with other infecting pathogens, and persistence, particularly related to device-related bacteremia, continues to plague patients [17]. *P. aeruginosa* infections can be treated by aminoglycosides such as gentamicin and tobramycin [18]. However, prolonged use is known to impair kidney function and cause damage to auditory nerves that leads to deafness [18], therefore, the use of aminoglycosides has been restricted due to their side effects. One of the great challenges of treating any *P. aeruginosa* biofilm infection is the intrinsic antibiotic resistance of this pathogen. *P. aeruginosa* encodes for multiple MDR efflux pumps and antibiotic inactivating enzymes [19].

There are studies which have shown that phytochemicals provide some effective antimicrobial agents against some resistant pathogens [20, 21]. One of the plants commonly used in ethnomedicine is *Callistemon citrinus*, also known as *Callistemon lanceolatus* DC or commonly known as the crimson red bottle brush. *C. citrinus* is a flowering plant that belongs to the family *Myrtaceae* and it is indigenous to Australia [22]. It is also found widely in other parts of the world, including subtropical regions and tropical regions of South America and Asia [22]. *C. citrinus* has been commonly studied for its pharmacological effects. The leaves have exhibited calcium channel blocking and antispasmodic activities [23]. *C. citrinus* has also been used as folk medicine to treat conditions like gastrointestinal distress, pain and infections from bacteria, fungus and virus [24]. Tormentic acid is a triterpene found in several sources, including *C. citrinus*. The widely investigated property of tormentic acid is its anti-hepatotoxic effect, which can be an

attractive feature when governing highly concentrated levels of and adjuvant that could be regarded toxic in isolation [25]. The antimicrobial properties of the natural compound and its mechanism of action are not well documented. Bacterial biofilms from *P. aeruginosa* allow the pathogen to attach to medical devices and cause infections, especially in patients with in-dwelling medical devices [7]. Natural products which are similar in structure to tormentic acid, triterpenes, have shown anti-biofilm activity [26]. The aim of the study was to determine the effects of tormentic acid and *C. citrinus* extracts on the biofilm of *P. aeruginosa*.

Materials and Methods

Chemicals and reagents

All the chemicals used were obtained from Sigma Aldrich (Darmstadt, Germany). All solvents used were analytical reagent grade, these were acetone, methanol, n-hexane, ethanol, dichloromethane, 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT), potassium hydroxide (KOH), dimethyl sulfoxide (DMSO). Reagents used during the study including phenol, mannose, phosphate buffer saline, ciprofloxacin, dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide [MTT], Luria broth and Luria agar were also manufactured by Sigma Aldrich (Darmstadt, Germany). Tormentic acid used for the study was previously isolated by Bvumbi *et al.*, from *C. citrinus* [41], at the University of Zimbabwe (Department of Biochemistry, Faculty of Science).

Bacterial Strain and Growth Conditions

P. aeruginosa (ATCC 27853) used during the test was acquired from the Division of Microbiology, Department of Biological Sciences at the University of Botswana. Bacterial cells were stored by cryopreserving a 750 µl Luria broth overnight culture in 250 µl 50% (v/v) glycerol at -80°C. Bacterial cells were sub-cultured in Luria broth and plating the bacterial cells on Luria agar. Before to each assay, bacteria colonies were inoculated in fresh Luria broth and incubated overnight at 37°C in a Lab-Companion incubator (SI300 Incubated shaker, Jeiotech, Korea). Standardisation of the bacterial cultures was done using 0.5 McFarland standard.

Preparation of DCM: Methanol and Ethanol: Water Extracts

Extracts were prepared by grinding the leaves of *C. citrinus* into powder using a pestle and mortar. To each of 30 g of the *C. citrinus* leaf powder was added 100 ml of 50% methanol: 50% DCM as well as 50% ethanol: 50% water to extract the phytochemicals [27]. The mixture of the solvent and the leaf powder was filtered using Whatman's no 1 filter paper (Sigma-Aldrich, Taufkirchen, Germany) into sterile beakers. The filtrate was concentrated to dryness by evaporation at room temperature under a fan. The extracts were stored in 1.5 ml microtubes (Eppendorf™) and were kept at room temperature for future use.

Screening for antimicrobial activity

Antibacterial activity against *P. aeruginosa* was screened using ciprofloxacin, tormentic acid and DCM: methanol and ethanol: water extracts. The determination of antibacterial activity was performed using the broth microdilution method described by Eloff with modifications [28]. Ciprofloxacin (0.002 g) was dissolved in 1 ml of dimethylsulfoxide (DMSO). A concentration of 2 µg/ml of ciprofloxacin was prepared by mixing 0.2 ml of the dissolved drug with 9.8 ml of 3% DMSO (v/v) in Luria broth. Ten two-fold serial dilutions were prepared from the 2 µg/ml ciprofloxacin solution. Five, two-fold serial dilutions were carried out for each extract as well as tormentic acid starting from 200 µg/ml. A volume of 100 µl of each of the test samples was added separately to the wells of a 96-well microplate. *P. aeruginosa* cells were standardised to a concentration of 2×10^6 CFU/ml. A volume of 100 µl of bacterial cells were added to each well to give a final concentration of 1×10^6 CFU/ml. The final concentration of ciprofloxacin in the wells ranged from 0-100 µg/ml while the other test sample had final concentrations of 0, 12.5, 25, 50 and 100 µg/ml.

Pre-incubation readings of the cell densities at 590 nm were recorded using a Tecan microplate reader (TecanGenics-Pro, Grödig, Austria). Cell density was measured at 590 nm using the microplate reader (Tecan Genios-Pro microplate reader, Grödig, Austria) prior to overnight incubation at 37°C. Post-incubation readings of cell densities at 590 nm were also recorded. To determine viable cells, a volume of 20 µl of (3-(4,5- dimethylthiazol- 2,5-diphenyltetrazolium bromide) (MTT) solution was added to each well of the 96-well microtiter plate and the plate was incubated for 120 minutes at 37°C. Optical density readings were recorded at 590 nm using a Tecan microplate reader. The results obtained were analyzed graphically.

Screening for antibiofilm effects

Effects on Static Biofilm Formation

The effects on the formation of *P. aeruginosa* biofilms were evaluated using tormentic acid, DCM: methanol, and ethanol: water extract. A static biofilm detachment assay was performed following a method by Chipenzi *et al.* with modifications [29]. An overnight culture of *P. aeruginosa* cells was centrifuged at 3500 rpm for 15 minutes and the supernatant was discarded. The remaining pellet was washed with 0.5 M phosphate buffered saline pH 7.2 and the supernatant was discarded. A volume of 10 mls media was added and the cells were standardized to 1.5×10^6 colony forming units. A volume of 2 ml of cells was added to each well and the cells were allowed to adhere to the well of a 24-well microplate by incubating the cells in a shaking incubator for 2 hours. A volume of 500 µl tormentic acid as well as each of the extracts were added at 500 µg/ml to the wells containing the cells to achieve a final concentration of 100 µg/ml. Ciprofloxacin was used as the positive control at different concentrations. Total volume for each well was 2500 µl ie 2000 µl of cells and 500 µl of test samples. The plate was further incubated for 72 hours at 37 °C in a humid non-shaking incubator (Labcon orbital incubator, Labotec Co., Cape Town, South Africa). After incubation, the contents of the plate were gently discarded using 1 ml pipette and the plate was gently washed three times with 0.5M PBS running 2 ml of buffer. After washing plates were inverted on a paper towel for 15 minutes in a biosafety cabinet (BioFlow-11,

Labotec Co., Cape Town, South Africa) to drain excess liquid. The plates were subsequently placed in an oven at 60 °C for 1 hour to fix the formed biofilm after which 0.1% crystal violet stain was added to each well and the plate incubated at room temperature for 20 minutes before the crystal violet was discarded. The plate was gently washed with sterile water and left to dry overnight. A volume of 2.5 ml 95% ethanol was added to each well and 200 µl of the contents were transferred to a 96-well plate. The optical density readings of the plate were taken at 590 nm.

Effects of tormentic acid and extracts on adhesion of *P. aeruginosa*.

An overnight culture of *P. aeruginosa* was grown at 37 °C. Cells were centrifuged at 3500 rpm for 15 minutes and the supernatant was discarded. The pellet remaining was washed with 0.5M phosphate buffered saline pH 7.2 and the supernatant was discarded. A volume of 10 ml media was added and the cells were standardized using 0.5 McFarland standard to culture 1.5×10^6 CFU/ml. An equal volume of cells and test compounds was added at once to the wells of a 24-well microplate and incubated for 72 hours in a non-shaking incubator (Labcon orbital incubator, Labotec Co., Cape Town, South Africa). After incubation, the biofilms were quantified, the contents of the plate were gently discarded using 1 ml pipette and the plate was gently washed three times with 0.5M PBS running 2 ml of the buffer. The method of biofilm quantification was done exactly as above.

Effects of ciprofloxacin, tormentic acid and extracts on mature biofilms

An overnight culture of *P. aeruginosa* was grown at 37 °C. The cells were centrifuged at 3500 rpm and the supernatant was discarded. The remaining pellet was washed with 0.5M PBS at pH 7.2. After washing, 10 mls of media was added and the cells were standardized using 0.5 McFarland to culture 1.5×10^6 CFU/ml. A volume of 2 ml of cells were allowed to adhere to the wells of a 24 well microplate by incubating the cells in a shaking incubator at 37°C for 2 hours (SI300 Incubated shaker, Jeiotech, Korea) in the absence of the test samples. After 2 hours of incubation the plate was then incubated in a nonshaking incubator for 72 hours to allow biofilm formation. Following the incubation, 200 µg/ml of each of the test samples was added to the plate. After the different concentration of test samples had been added to the preformed biofilm, the plate was then incubated for 24 hours at 37 °C without shaking in the Lab Doctor Mini Incubator (MID SCI, USA). The amount of biofilm that remained attached to the plate was determined by crystal violet staining as described before. Optical density (OD) of the stained adherent bacteria was measured with an absorbance microplate reader using ELx800 Tecan Genios-Pro microplate reader (Grödig, Austria) at wavelength of 590 nm and was proportional to the biofilm that had not been disrupted by the test samples.

Effects on Capsular Polysaccharide Content

The extraction and analysis of *P. aeruginosa* capsular polysaccharide was carried out with an aim of investigating the intervention of screened concentrations of ciprofloxacin, tormentic acid, DCM: methanol, and ethanol: water extracts of *C. Citrinus* on capsular polysaccharide production, using phase separation

by phenol [30]. An overnight culture of *P. aeruginosa* cells was standardised to 1×10^9 CFU/ml. The cells were incubated in 50 ml centrifuge tubes with the different test samples. A concentration (MIC) of 0.25 µg/ml was used for ciprofloxacin while tormentic acid and extracts were used at 100 µg/ml. Each tube had 5 mls of cells and 5 mls the test samples. Positive and negative controls were prepared to determine the effect of confounding variables and contaminants. The centrifuge tubes were incubated at 37°C for 4 hours without shaking. After 4 hours, cells were separated centrifugation at 4000 rpm for 15 minutes and washed three times with PBS. Cells were suspended in 450 µl of deionised water and an equal volume of saturated phenol was added. The mixture was incubated in 65°C water bath for 20 minutes. The volume of 300 µl of each tube was transferred to 1 ml microtubes in triplicate. After incubation, 150 µl chloroform was added and the mixtures were intensely vortexed. The cell suspension mixture was centrifuged and the supernatant was collected and prepared for the phenol-sulphuric assay. A volume of 50 µl of each supernatant was distributed in the respective wells on a microtiter plate. Mannose was used as a standard and a standard curve was prepared, and the samples were treated in the same way as capsular polysaccharide. Each well was added 5% phenol and the microtiter plate was placed in a static water bath of 90°C for 5 minutes. The plate was dried and absorbance was read at 492 nm on a stat fax 2 100 micro-plate reader (Awareness Technologies Inc, Westport, United States of America).

Statistical Analysis

Statistical analysis was performed the one-way analysis of variance (ANOVA) test. A further analysis of the results was carried out using Dunnett's multiple comparison post-test to compare all mean values with the mean value of a control column for each test. Values with p-values < 0.05 at 95% confidence intervals were statistically significant. Graphpad Prism 6® software (Version 6.0, Graphpad Software Inc, San Diego, United States of America) software was used for all graphical and statistical analyses.

Results

Antimicrobial effects of samples on *P. aeruginosa*.

The antibacterial activity of ciprofloxacin, tormentic acid, DCM: methanol and ethanol water extract against *P. aeruginosa* was evaluated. All test samples had an inhibitory effect on the growth of the bacteria (Fig. 1A, B, C, and D). The MIC of ciprofloxacin against *P. aeruginosa* was 0.125 µg/ml (Fig. 1A). Tormentic acid inhibited growth by 46% at 100 µg/ml (Fig. 1B), the ethanol: water extract caused 59% inhibition (Fig. 1C) and the DCM: methanol extract had 50% inhibition of growth on the bacteria (Fig. 1D).

Effects of test samples on *P. aeruginosa* biofilms

Effects of ciprofloxacin, extracts and tormentic acid on biofilm formation by *P. aeruginosa*

The ability of ciprofloxacin, tormentic acid, DCM: methanol and ethanol water extract to inhibit the formation of biofilms of *P. aeruginosa* was investigated. None of the test samples managed to completely inhibit the formation of biofilm by bacteria (Fig. 2A, 2B). However, ciprofloxacin had a

significant concentration dependent inhibition of biofilm formation by *P. aeruginosa* (Fig. 2A). DCM: methanol extract was the only extract that significantly reduced biofilm formation by 20% at 100 µg/ml. (Fig. 2B).

Effects of tormentic acid and extracts on adhesion of *P. aeruginosa*.

The ability of the test samples to inhibit the adhesion of *P. aeruginosa* cells was investigated by introducing the cells and tests compounds at the same time into the plate. If the sample manages to inhibit the adhesion of the cells, this would be evidenced by the absence of biofilm or the reduction of the biofilm formed relative to when the cells were first incubated to allow cell attachment prior to the addition of test samples. The results obtained indicate that ciprofloxacin inhibited the adhesion of *P. aeruginosa* and therefore, reduced the formation of biofilms by 88% (Fig. 3A). However, when comparing the biofilm formed (Fig. 3A) with that formed when cells were first incubated alone (Fig. 2A), the difference was insignificant. This would mean that ciprofloxacin has an insignificant effect to adhesion of *P. aeruginosa*. Tormentic acid was shown to have antiadhesion activity by reducing the amount of biofilms by 27%. Ethanol: water extracts have antiadhesion activity, resulting in a reduction in the biofilms formed by 58% and DCM: methanol extract had an inhibitory effect of biofilm formation of 43% (Fig. 3B). The amount of biofilm that was inhibited from forming when tormentic acid, and the two extracts were added at the same time with the cells was more than the biofilm inhibition when the cells were first allowed to adhere to the wells of the plate (Fig. 2B). This would mean that tormentic acid, DCM: methanol and ethanol water extracts significantly inhibit adhesion of *P. aeruginosa* cells.

Effects of ciprofloxacin, tormentic acid and extracts on biofilm disruption

Effect of the test samples on disrupting mature biofilms of *P. aeruginosa* was determined by adding the test cells to a preformed mature biofilm and incubating for 24 hours. Tormentic acid was shown to have 23% disruptive activity against mature *P. aeruginosa* biofilms. The dichloromethane: methanol extract was shown to have 25% disruptive activity against mature biofilms (Fig. 4B), the amount of biofilm decreased indicating a disruption of the biofilms of *P. aeruginosa* by the extract. The ethanol: water extract had 5% activity against mature biofilms. Ciprofloxacin was shown to have no activity against mature *P. aeruginosa* biofilms as the drug concentration increased (Fig. 4A).

The effect of ciprofloxacin, tormentic acid, and extracts on the polysaccharide content of biofilms.

The amount of capsular polysaccharides in biofilms of *P. aeruginosa* formed in the presence of 0.25 µg/ml (MIC), ciprofloxacin and 100 µg/ml of tormentic acid as well as the extracts was evaluated. The results for capsular polysaccharide analysis show an overall significant decrease in the amount of polysaccharides after growing *P. aeruginosa* against 100 µg/ml tormentic acid, ethanol: water and DCM: methanol extracts. (Fig. 5). However, there was no significant reduction in polysaccharide content after growing *P. aeruginosa* against ciprofloxacin at 0.25 µg/ml

Discussion

The objective of this study was to investigate the effects of tormentic acid and *C. citrinus* extracts on *P. aeruginosa* biofilms, as bacterial resistance to antibiotics and the survival of *P. aeruginosa* cells are associated with its ability to form biofilms [31]. Planktonic bacterial cells are 1000 times less resistant as compared to bacterial biofilms [32]. The solutions that could be used to inhibit *P. aeruginosa* biofilms should at least focus on the critical stages in the biofilm formation cycle. These stages include 2 hours after adhesion, before cell adhesion, and after matured biofilms. Bioactive compounds can be used to inhibit biofilms that dwell inside medical devices such as catheters [33]. This study involved use of tormentic acid, ciprofloxacin and two extracts from *C. citrinus*, against different stages of *P. aeruginosa* biofilms. Tormentic acid was used because it was studied and shown to have antibacterial potential, therefore it could also have antibiofilm properties [29]. Crude extraction using DCM: methanol solvent results in extracts that have a range of less polar and nonpolar phytochemicals, including torment acid. Ethanol: water extracts from *C. citrinus* have a range of polar to more polar phytochemicals [41].

Ciprofloxacin served as a positive control. Ciprofloxacin is one of the drugs currently being used as a treatment for *P. aeruginosa* infections, although there has been increased resistance from *P. aeruginosa*. Antibacterial growth tests are used to determine whether bacterial cells are susceptible to antibacterial agents. The clinical break point is used to determine the efficiency of the antibiotic against bacterial cells. If the MIC value is close to or above the clinical break-point-value, that means that the efficiency of the drug is reduced. The MIC for ciprofloxacin was found to be 0.25 µg/ml, which is less than the clinical breakpoint of ciprofloxacin which is ≤ 0.5 against *P. aeruginosa*. The MIC of ciprofloxacin is getting closer to the clinical break point which means that *P. aeruginosa* is gradually getting resistant against ciprofloxacin. Antibiotic resistance from *P. aeruginosa* is due to its ability to form biofilms. The pentacyclic terpenes are known to have a wide range of uses including the potential to have antimicrobial properties. Tormentic acid MIC was studied and observed to be 125 µg/ml for *S. aureus* NBIMNCC 3359 [34]. The activity of tormentic acid against growth of bacteria studies have shown to be high for gram positive as compared to gram negative. According to this study tormentic inhibited the growth of *P. aeruginosa* cells by 46% growth inhibition at highest concentration (100 µg/ml). The ethanol: water extract had the highest percentage inhibition, which was 59% and DCM: methanol had 50% inhibition at 100 µg/ml. This shows that tormentic acid and crude extracts have potential as antibacterial agents. The antibacterial activity of the plant extracts is due to the presence of plant secondary metabolites.

Biofilm formation in *P. aeruginosa* was studied during all the stages of the biofilm formation cycle, after 2 hrs of cell adhesion, before cell adhesion and after 72 hrs to allow biofilms to mature. Ciprofloxacin was able to inhibit biofilm formation in the early stages of the cycle. Ciprofloxacin inhibited the continuation of cell adhesion and inhibited the adhesion process from the beginning. The anti-adhesion activity of ciprofloxacin results in a significant reduction in biofilm formation by *P. aeruginosa*. Tormentic acid and hydroethanolic extract did not significantly reduce biofilm formation by *P. aeruginosa* after 2 hours of initial cell adhesion. DCM: methanol extract reduced biofilm formation by 20%, by inhibiting the continuation of the adhesion process, which is an important stage for biofilm formation process to

proceed. Tormentic acid and the 2 extracts had significant antiadhesion activity, which results in a reduced amount of biofilms formed.. The plant phytochemicals which are present in the plant extracts are responsible for the anti-adhesion activity.

The principle behind examination of biofilm detachment is to investigate the effect of a detergent on biofilm mass exposed to an antibacterial agent. Biochemical substances account for the mechanical stability of biofilms by mediating the cohesive and adhesive forces [36]. Bioactive compounds have the capacity to weaken the mechanical stability of biofilms [33]. Weakened biofilms structures are easily detached by detergents. The data obtained showed that ciprofloxacin was not able to detach the mature biofilms formed by *P. aeruginosa*, which demonstrates its resistance to antibiotics. Tormentic acid and DCM: methanol had disruptive activity on matured biofilms. Tormentic acid being largely non-polar might have been sorbed by the abundant polysaccharides. Ethanol: water extract had higher activity, this might be because the phytochemicals extracted ranged from polar to more polar as compared to DCM: methanol extract which had less polar and nonpolar extracts. The detachment activity of the test samples could be due to increased disruption of the EPS as the bacteria prepares to detach and revert to planktonic form at the dispersal stage, which is the last stage of biofilm formation cycle. After biofilm detachment they can be easily wiped off by detergents. As the biofilm formation process continues after establishment of biofilms, it might be more important to study the inhibition of dispersal processes that allow cells to continue the biofilm formation cycle.

The formation of extracellular proteins, DNA and polysaccharides have been identified as the most important virulence factors of bacteria that form biofilms. Mashezha *et al*/ found out that tormentic acid completely inhibited the production of extracellular proteases which is one of the virulence mechanisms used by *S. aureus* [35]. Ciprofloxacin was not able to reduce the amount of extracellular polysaccharides formed by *P. aeruginosa* during the biofilm formation process. These results can be supported by reports of ciprofloxacin that it targets DNA gyrase in bacteria, therefore, inhibiting bacterial DNA replication and transcription [36]. The mechanism of action used by *P. aeruginosa* results in low amounts of eDNA and is more affective in planktonic bacteria compared to mature biofilms. A study by Whitchurch and colleagues involving DNase in growth medium, lead to the biofilms failure to grow. This shows that eDNA is a necessary component in biofilm development but it can be irreversibly to altered in mature biofilms [40]. Exopolysaccharides, protein, nucleic acids and lipids are the biochemical components found in biofilms primarily [37]. Reduced extracellular compounds have a significant role in the formation and detachment of biofilms by *P. aeruginosa*. The role of the capsular polysaccharide is to mediate biofilm formation by allowing cells to adhere to surface and cell-cell adhesion. Hindering the production should indirectly inhibit biofilm formation. Highest concentrations (100 µg/ml) of tormentic acid ethanol: water extract, DCM: methanol extract and ciprofloxacin (0.25 µg/ml) were used to analyse the intervention of CPS production. Psl is the predominant polysaccharide that is synthesized by *P. aeruginosa* cells. The psl polymer is made up of pentameric repeat units made up of D-mannose, D-glucose and D-rhamnose [38, 39]. Psl is the first polysaccharide to be synthesized by bacteria during the biofilm formation. Psl is involved during attachment and recruitment of bacteria cells by acting as an adhesive. Mannose standards estimated the relative amount of capsular polysaccharide effectively. The test samples were

able to significantly reduce the amount extracellular polysaccharides formed. These results means that tormentic acid and the extracts use reducing extracellular polysaccharides as their mechanism of action in reducing cell adhesion process during biofilm formation process. Wojnicz *et al.* studied the effect of individual pentacyclic triterpenes, ursolic acid and asiatic acid, on biofilm eradication [26]. The analogues of tormentic acid were shown to have the same negligible effect on biofilm eradication, which might have been due to sorption by the EPS.

Conclusion

Tormentic acid and the extracts (DCM: methanol and ethanol water extract) from *C. citrinus* have an inhibitory effect on the growth of *P. aeruginosa* cells. Tormentic acid and the two extracts were not able to detach the irreversible cell attachment in static biofilm formation. Tormentic acid and extracts significantly reduced adhesion of *P. aeruginosa* cells, leading to indirect reduction of biofilm formed. Tormentic acid and DCM: methanol extract significantly enhanced biofilm detachment. The extracellular polysaccharide content was reduced by tormentic acid, hydroethanolic, and DCM: methanol extracts. Proposed mechanism of antiadhesion and disruptive activity of the test samples can be concluded from their ability to reduce the amount of extracellular polysaccharide content required for cell attachment, an important stage of biofilm formation cycle.

Abbreviations

DCM Dichloromethane

MIC Minimum Inhibitory Concentration

MBIC Minimum Biofilm Inhibitory Concentration

VAP Ventilator Associated Pneumonia

CF Cystic Fibrosis

DMSO Dimethyl sulfoxide

EPS Extracellular Polysaccharide Substance

Declarations

Ethics approval and consent to participate

The present study was approved by the Ethical Committee of the Department of Biochemistry Board, University of Zimbabwe (RD/R159502H of 2019).

Consent for publication

Not applicable

Availability of data and materials

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

Competing interests

The authors declare no competing interests.

Funding

Support from the Swedish International Development Agency (SIDA) through the International Science Programmes (ISP) (ISP IPICS: ZIM01, Uppsala University, Uppsala, Sweden). ISP IPICS: ZIM01 supported the research under the title “Biomolecular Interactions Analyses”. Support from the Alliance for Global Health and Science (University of California, Berkeley, USA) is acknowledged.

Authors' contributions

RD conducted all the experimental studies. WM assisted in the data analyses and writing of the manuscript. SM conceptualized, designed and directed the study. All authors read and approved the final manuscript.

Acknowledgments

The authors acknowledge the assistance of Mr. Simbarashe Sithole with technical assistance.

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Figures

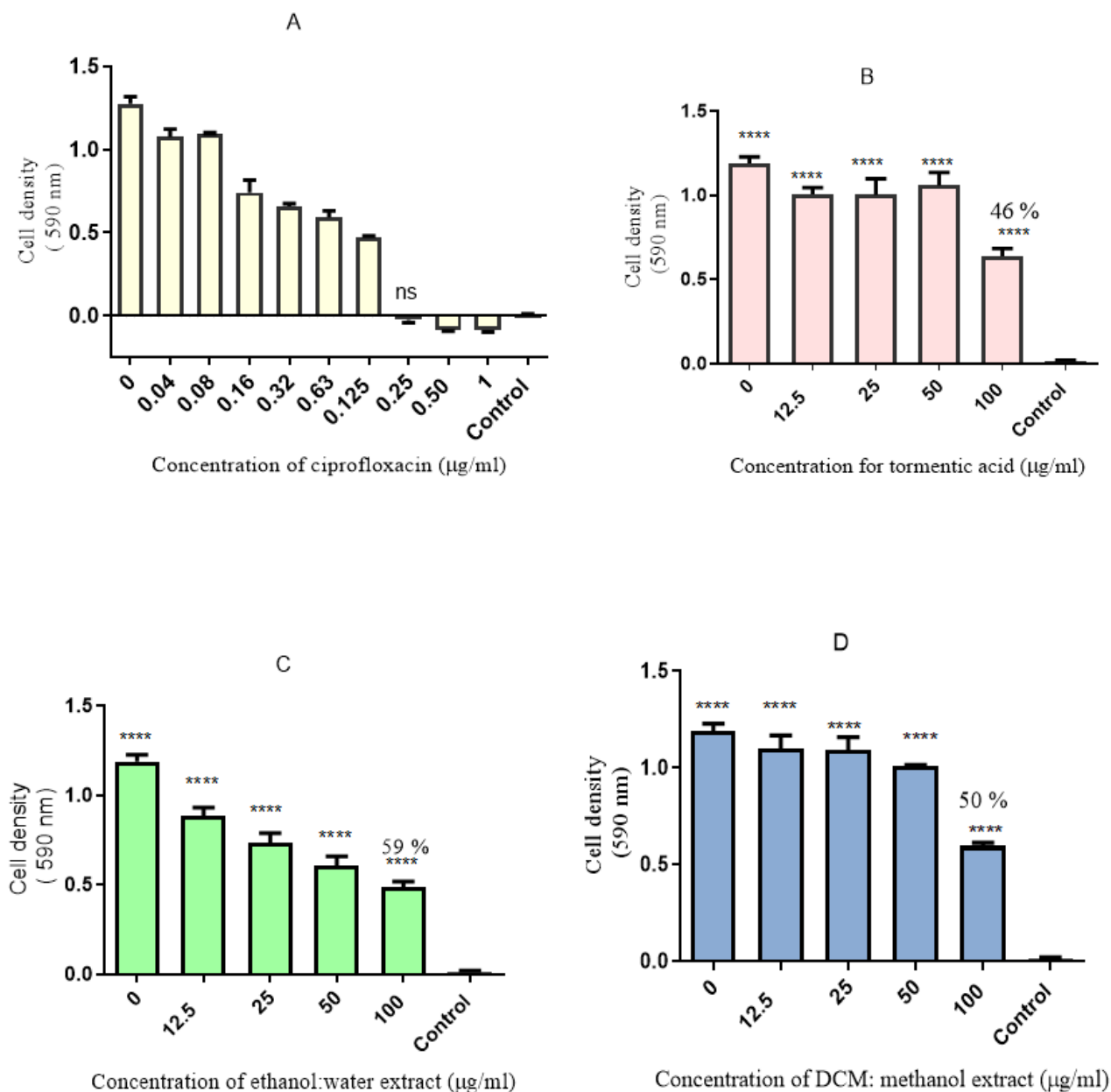


Figure 1

The effects of ciprofloxacin, extracts, and tormentic acid on *P. aeruginosa* growth. The test compounds were incubated with 2×10^6 CFU/ml of *P. aeruginosa* for 24 hours at 37 °C. The optical density was read at 590 nm using a spectrophotometer. The error bars indicate standard the deviation from the mean of the readings taken (n=6). One way ANOVA was used to analyze the results, with Dunnett's multiple comparison post-test to compare the significant difference at 95 % confidence interval. The asterisks (***) indicate statistical differences from the control (media only) where = $p < 0.0001$.

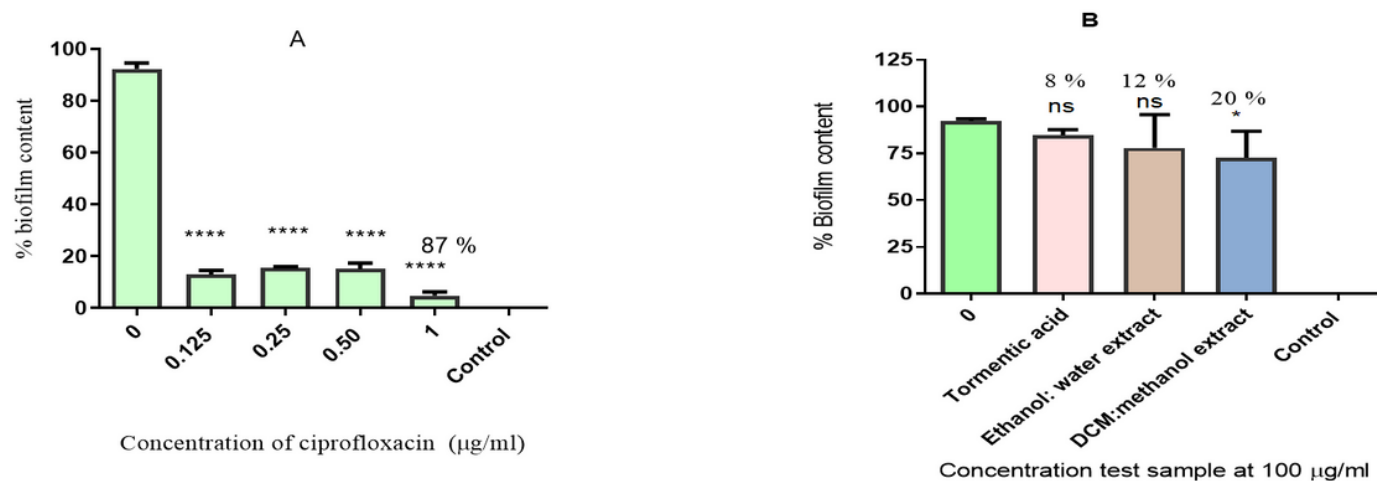


Figure 2

The effects of ciprofloxacin (2A), tormentic acid and extracts (2B) on static biofilm formation in *P. aeruginosa*. Cells were incubated with the drug for 72 hours in a non-shaking incubator. At the end of the incubation period, the biofilms were quantified using 0.1 % crystal violet solution. One way ANOVA was used to analyze the results, with Dunnett's multiple comparison post-test to compare the significant difference at 95% confidence interval. The asterisks (***) indicate statistical differences to the control (cells only) where * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. **** = $p < 0.0001$.

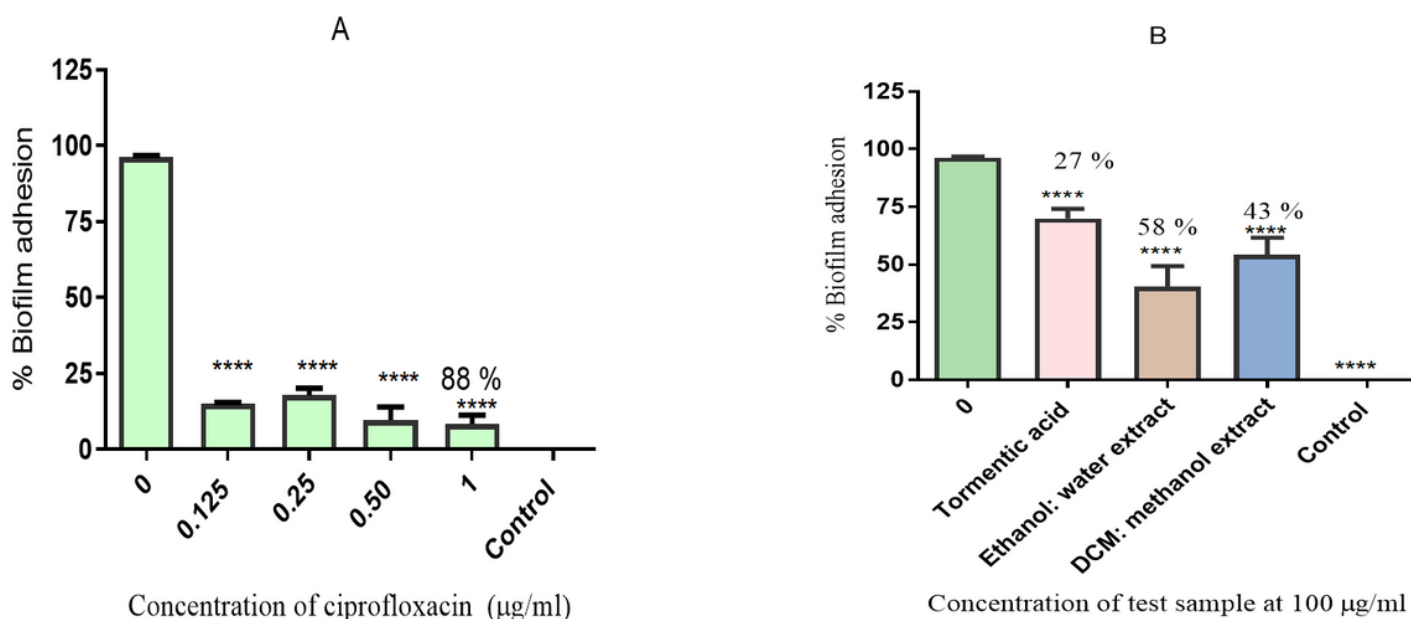


Figure 3

The effects of ciprofloxacin (3A), tormentic acid, and extracts (3B) on the adhesion of *P. aeruginosa*. A concentration of 2×10^6 CFU/ml of *P. aeruginosa* was incubated at 37°C with the test samples in a ratio

of 4:1 (v/v) for 72 hours. At the end of the incubation period the late was gently washed and the amount of biofilm present in the wells was determined by crystal violet staining. The optical density was read at 590 nm. One way ANOVA was used to analyze the results, with Dunnett's multiple comparison post-test compared the significant difference at 95% confidence interval. The asterisks (***) indicate statistical differences to the control (cells only) where * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. **** = $p < 0.0001$.

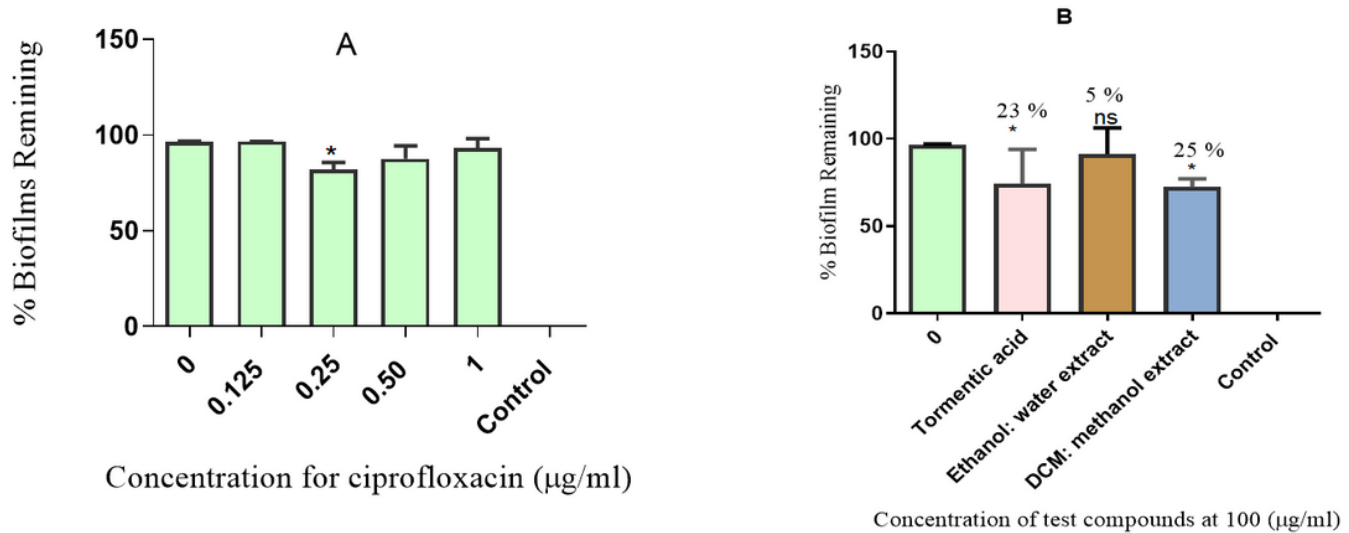


Figure 4

The effects of ciprofloxacin (4A), tormentic acid and extracts (4B) on biofilm disruption in *P. aeruginosa*.

A concentration of 2×10^6 CFU/ml of *P. aeruginosa* was incubated in a non-shaking incubator at 37 °C for 72 hours. At the end of the incubation period, biofilms were quantified using 0.1 % crystal violet solution to determine the amount of biofilm that remained attached to the plate. The amount of biofilms was quantified at 590 nm. One way ANOVA was used to analyze the results, with Dunnett's multiple comparison post-test to compare the significant difference at 95 % confidence interval. The asterisks (***) indicate statistical differences to the control (cells only) where * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. **** = $p < 0.0001$.

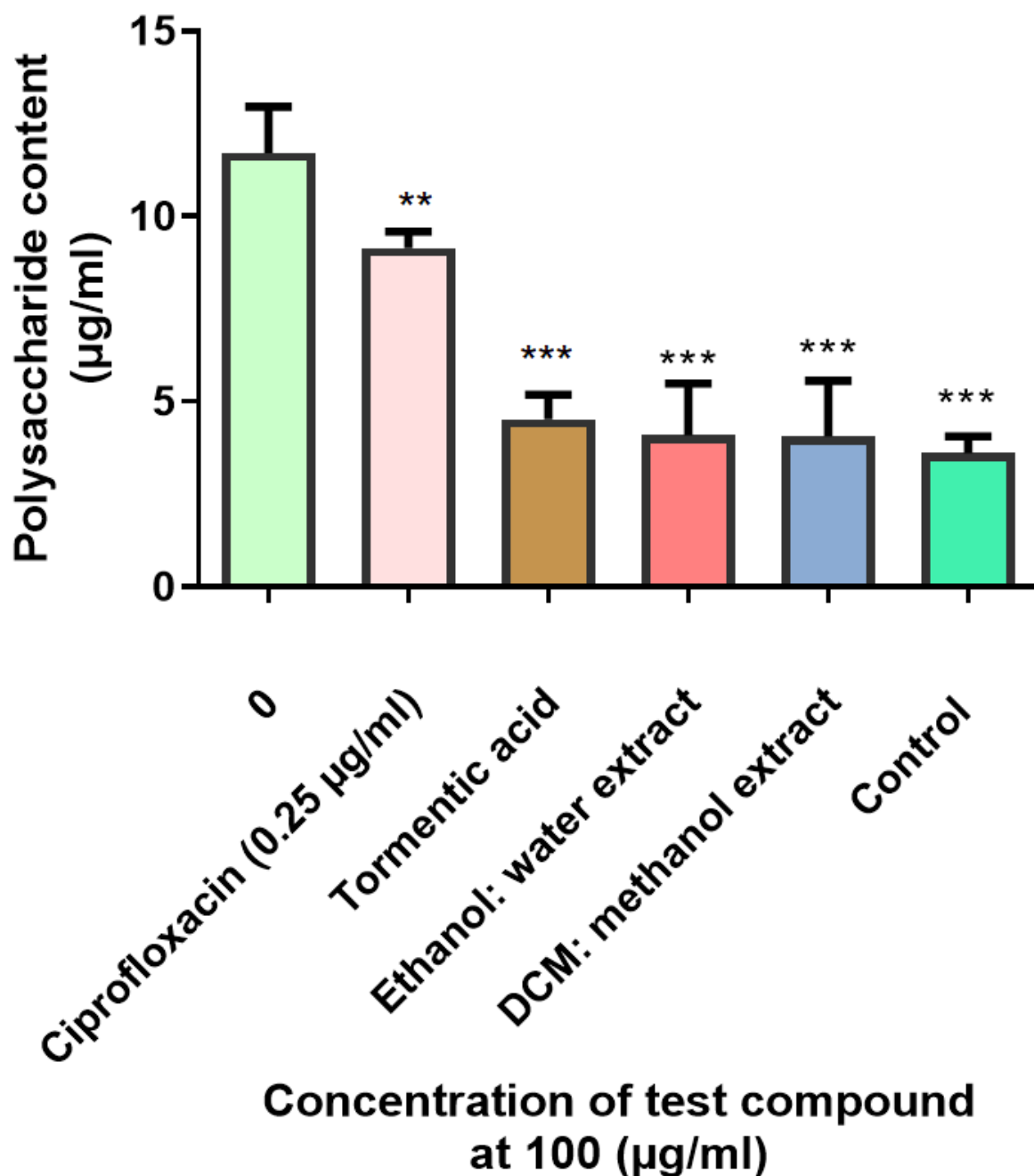


Figure 5

The effects of ciprofloxacin, tormentic acid, and extracts on the polysaccharide content of biofilms *in P. aeruginosa*. The amount of capsular polysaccharides in each tube was determined by extracting and assaying using the phenol-sulphuric assay after further growth for 4 hours. The quantity of mannose was determined at 492 nm using a spectrophotometer. Error bars indicate the standard deviation of the mean of the readings taken (n=4). One way ANOVA was used to analyze the results, with Dunnett's multiple comparison post-test compared the significant difference at 95% confidence interval. The asterisks (***) indicate statistical differences from the control (media only) where * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. **** = $p < 0.0001$.