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Antioxidant and antibiofilm activity of *Ageratum conyzoides* L, and *Bidens pilosa* L against wound pathogens

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Biofilm infections represent a significant challenge in the medical field due to escalating antibiotic resistance. Plants offer a promising avenue for addressing this issue, as they harbor a diverse array of phytochemicals with various biological activities, including antioxidant properties crucial for preventing biofilm formation in wounds. This study aimed to explore the antioxidant and antibiofilm capabilities of *Ageratum conyzoides L. and Bidens pilosa L*. The plants underwent quantitative phytochemical screening, antioxidant activity assessment via the DPPH scavenging assay, and antibiofilm activity evaluation using the microtiter plate assay. All plants were found to contain tannins, flavonoids, polyphenols, and alkaloids, with alkaloids being particularly abundant. The antioxidant activity (IC50) of *A. conyzoides* was measured at 50.52 µg, while that of B. pilosa was 45.94 µg. Regarding the minimum concentration needed to inhibit 50% of *Pseudomonas aeruginosa* biofilms (MBIC50), it was 24.9 mg/ml for *B. pilosa* and 28.3 mg/ml for *A. conyzoides*, with the MBIC90 being 147.7 mg/ml for *A. conyzoides* and 139.5 mg/ml for *B. pilosa*. For Staphylococcus aureus biofilms, the MBIC50 was 11.4 mg/ml for B. pilosa and 18.6 mg/ml for *A. conyzoides*, while the MBIC90 was 69.3 mg/ml for *B. pilosa* and 97 mg/ml for *A. conyzoides.* In the biofilm removal assay for *P. aeruginosa*, the minimum concentration required to eliminate 50% of the formed biofilms (MBEC50) was 38.1 mg/ml for *A. conyzoides* and 200 mg/ml for *B. pilosa*. The MBEC90 was 151.7 mg/ml for *A. conyzoides* and 261 mg/ml for *B. pilosa. For S. aureus*, the MBEC50 was 96.93 mg/ml for *A. conyzoides* and 195 mg/ml for *B. pilosa*, with the MBEC90 being 334 mg/ml and 250 mg/ml for *A. conyzoides* and *B. pilosa*, respectively. The study findings confirm that plant extracts possess antioxidant activity and the potential to inhibit biofilm formation and disrupt mature biofilms. The antibiofilm activity observed in these plants underscores their potential as a solution to antibiotic resistance, warranting further research.

Key words: Antibiofilm activity, antioxidant activity, *A. conyzoides*, *B. pilosa*, *S. aureus, P. aeruginosa*.

INTRODUCTION

Wounds can pose significant health risks to patients if they become infected with biofilms, leading to prolonged

treatment and high costs. Biofilms, which are collections of microbes adhering to biotic and abiotic surfaces or clustered within extracellular polymeric substances (EPS), can compromise the host immune system and resist antibiotics, complicating treatment and increasing expenses (Percival et al., 2015). As biofilms mature, bacteria communicate through quorum sensing, affecting growth rate, structure, interactions, virulence, toxin and enzyme production, and antibiotic resistance (Gautam et al., 2013; Percival et al., 2015).

The primary concern with biofilms is their heightened resistance to antibiotics and host immune responses, being approximately 1000 times more resistant than planktonic microbes. This resistance contributes to increased patient harm, morbidity, and mortality (Song et al., 2018; Thi et al., 2020). Pathogenic biofilms account for approximately 80% of human tissue infections, according to the National Institutes of Health (Borges et al., 2015; Kou et al., 2020; Li and Zhao, 2020; Song et al., 2018). Annually in the United States, 17 million hospital cases and 550,000 deaths are attributed to biofilm infections, resulting in an expenditure of 94 billion US dollars on treatment and medical care (Kou et al., 2020). Consequently, biofilms have become a major focus of pharmaceutical research and development.

Pseudomonas aeruginosa and *Staphylococcus aureus* are among the common pathogens that form biofilms in wounds and exhibit resistance to antibiotics (Ciofu and Tolker-Nielsen, 2019). Additionally, *P. aeruginosa* has been identified by the World Health Organization (WHO) as one of the most deadly microbes, necessitating special attention in research (Thi et al., 2020). These two organisms often coexist and can lead to severe biofilm infections, particularly in chronic wounds (del Mar Cendra and Torrents, 2021). They also have the capability to alter susceptibility mechanisms against respective antibiotics in multispecies biofilms (Kranjec et al., 2021), underscoring the need for thorough consideration when developing new antimicrobials.

Aside from biofilms, oxidative stress is reported to impede wound healing. This stress arises from the accumulation of reactive oxygen species (ROS) generated during the inflammatory phase of wound healing, aimed at aiding the host immune system in combating bacteria. Oxidative stress disrupts antiprotease substances responsible for safeguarding tissue cells and the extracellular matrix, thereby delaying healing (Süntar et al., 2012). Consequently, antioxidants play a crucial role in wound management and treatment by mitigating oxidative stress through radical scavenging (Comino-Sanz et al., 2021). They are also instrumental in preventing biofilm formation (Ong et al., 2018).

In the pursuit of new antibiotic therapies to combat antibiotic resistance stemming from biofilms and other multidrug-resistant bacterial strains, plants have emerged as a focal point of research. Throughout history, plants have been utilized to treat various illnesses, including bacterial infections and wounds. They contain a plethora of phytochemicals with diverse biological functions, such as anti-inflammatory, antibacterial, anti-diabetic, anti-angiogenesis, antiproliferative, and wound healing properties. Plant phytochemicals also serve as valuable sources of antioxidants, a critical attribute in alleviating oxidative stress and promoting wound healing (Catherine et al., 2022).

A. conyzoides and B. pilosa are traditionally used in herbal medicine to address various ailments. Scientific research has demonstrated their effectiveness as antimicrobial, anti-inflammatory, antioxidant, antiulcer, and wound healing agents (Catherine et al., 2022). Their antibacterial activity against planktonic bacteria has been studied (Catherine et al., 2022), and their pharmacological properties have been extensively reviewed (Bartolome et al., 2013; Okunade, 2002; Singh et al., 2013). However, information regarding their activity against biofilms is limited.

Numerous studies have investigated various plant extracts and phytochemicals for their efficacy against biofilms, with several reviews on this topic (Mani and Mahalingam, 2017; Song et al., 2018). However, to the best of our knowledge, there is scarce research that directly compares the antibiofilm activity of A. conyzoides and B. pilosa against *P. aeruginosa and S. aureus* biofilms. Therefore, the aim of this study was to assess the antibiofilm activity of *A conyzoides and B. pilosa* against *P. aeruginosa and S. aureus* biofilms, along with their quantitative phytochemistry and antioxidant activity.

MATERIALS AND METHODS

Materials

Ethanol and ethyl acetate were procured from Loba Chemicals India. Folin-Ciocalteu, gallic acid, DPPH, and rutin were provided by the National Chemotherapeutic Research Laboratory in Uganda. Dimethyl sulfoxide (DMSO) (99.7%) was obtained from Acros Organics. Brain. heart infusion (BHI) and brain heart infusion supplemented (BHI-S) were sourced from Condalab, while Delbeco's phosphate buffer (PBS) was acquired from Lonza. All reagents were of analytical grade.

Collection of plants

The aerial parts of mature plants of *B. pilosa and A. conyzoides*

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were collected from Bbale, Kayunga district in Uganda between November 2019 and January 2020, during the hours of 9 am to 12 pm EAT. The collected plant parts, including leaves, flowers, and leaf stalks, were stored separately in netted bags to allow proper air circulation. Authentication of the specimens was conducted by Mr. Protase Rwaburindore, a botanist at Makerere University Herbarium, and assigned accession numbers; 51251 for *B. pilosa* and 51252 for *A. conyzoides*.

Plant preparation

The collected plant parts were cleaned, to remove dust and foreign matter then air dried overnight, after which they were placed in an oven at 27 to 30°C until they were crispy dry. The dried plants were pulverised into fine powder using an electric grinder and kept in airtight containers for further processing.

Plant extraction

The extracts used in this study were ethyl acetate extract of *A. conyzoides* extracted by soxhlet and ethanol extract of *B. pilosa* extracted by Soxhlet extraction. These plants extracts were chosen from preliminary tests done to ascertain their antibacterial activity (Catherine et al., 2022). These extracts showed better antibacterial activity than others thus, were chosen for further assessment.

Quantitative phytochemical analysis of plant material

Estimation of total tannins

The total tannins were determined using the Folin-Ciocalteu method (Gan et al., 2017) with minor modifications in the quantities used. 0.1 gram of the sample was extracted using 10 ml of distilled water. Then, 50 µl of the extracted sample was added to 7.5 ml of distilled water in a 10 ml volumetric flask containing 0.5 ml of phenol reagent, Folin-Ciocalteu, and 1 ml of 35% sodium carbonate solution. The mixture was diluted to 10 ml with distilled water, shaken well, and stored for 30 min at room temperature. A set of reference standard solutions of tannic acid was prepared (0, 10, 20, 40, 50 µg/ml). Absorbance for the standard solutions and test samples was measured at 725 nm using a UV/Visible spectrophotometer (U-2602, Labomed Inc, USA) against the blank (distilled water). The procedure was repeated three times, and the average tannin content was calculated and expressed in terms of mg/g of tannic acid equivalent (GAE) in the sample.

Estimation of total flavonoids

Total flavonoids were estimated using Aluminum chloride colorimetry method (Gan et al., 2017) with some modification (in amounts and different model of instruments used). 1 gram of the sample was extracted in 10 ml of 80% methanol. To 0.1 ml of sample, 0.5 ml of 2% $AlCl_3$ in ethanol solution was added and shaken. A yellow color indicated the presence of flavonoids. A set of reference standard solutions of rutin (0, 10, 20, 40, and 80 µg/ml) was also prepared. After one hour, the absorbance at 420 nm and at room temperature using a UV–visible spectrophotometer. The experiment was repeated thrice to get a mean value. Total flavonoid contents were calculated as rutin (mg/g) using the equation based on the standard calibration curve. Total flavonoid content of the extract samples was expressed as rutin equivalent (RE) milligrams per gram of dry extract.

Estimation of total polyphenolics

Total phenolic contents were determined using Folin–Ciocalteau method (Gan et al., 2017) with some modification (in quantities used). 0.1 gram of the sample was extracted in 10 ml of distilled water. Folin–Ciocalteau reagent (0.5 ml) was added to the extracted solution (0.1 ml) followed by distilled water to make 8.5 ml. The contents were shaken thoroughly and kept at room temperature. After 10 min 20% sodium carbonate (1.5 ml) was added and the contents incubated in a water bath for 20 min at 40ºC. Distilled water was used as the blank. Reference standard solutions (0, 10, 20, 40, and 80 µg/ml) of gallic acid were prepared. Absorbance for the test and standard solutions was measured at 755 nm with a UV/Visible spectrophotometer against at 755 nm. A standard calibration curve was prepared using gallic acid to quantify the total phenols in the extract. The estimation of the total polyphenol content was carried out thrice. Total phenolic content of the extract samples was expressed as gallic acid equivalent (GAE) milligrams per gram of the dry extract.

Estimation of total alkaloids

The procedure was done as described by Ezeonu and Ejikeme (2016) but with slight modification in amounts used. 5 grams of the sample was weighed into a 250 ml beaker followed by 200 ml of 10% acetic acid in ethanol. The mixture was covered and kept at room temperature for 4 hours. The extract was concentrated in a water bath to a quarter of the original volume after filtration. $Al(OH)_3$ was added dropwise to the extract until precipitation was complete. The solution was left to stand for 10 min, and the precipitate was collected, washed with dilute ammonium hydroxide, and filtered. The residue is alkaloid. It was dried and weighed. Procedure was done in triplicate.

Determination of antioxidant activity by DPPH scavenging

The antioxidant activity was determined based on radical scavenging activity using the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) according to the procedure described by Mensor et al. (2001) with modifications in the amounts used. Initially, 0.1 gram of the sample was extracted with methanol (10 ml) overnight, filtered, and adjusted to a final volume of 10 ml. Then, 3.94 mg of DPPH was dissolved in methanol (100 ml), of which 3 ml were added to predetermined volumes of the extract (ranging from 50 to 100 µl), followed by the addition of methanol (2 ml). The mixtures were thoroughly mixed and left to stand at room temperature.

For the positive control, ascorbic acid (0.01 g) was dissolved in methanol (10 ml) to make a 1 mg/ml stock solution, which was used as the standard. From this, 1 ml was pipetted and made up to 10 ml with methanol to yield a solution of 1µg/10µl. Next, 3 ml of DPPH was added to different volumes of the ascorbic acid stock solution (ranging from 50 to 200 µl), followed by 2 ml of methanol, and thoroughly mixed. The mixtures were then incubated in the dark at 37°C for 30 min.

The decrease in absorbance of each solution was read at 517 nm using a UV/VIS spectrophotometer. DPPH in methanol was taken as the blank and used for background correction. The percentage of radical scavenging activity was calculated using the equation: % Free radical scavenging activity = (Ac - As) / Ac x 100, where Ac represents the absorbance of the control (blank) and As represents the absorbance of the extract or ascorbic acid. The concentration of the sample required to scavenge 50% of the DPPH free radical (IC50) was calculated by linear regression of the plots, with the x-axis representing the various concentrations of the extracts, and the y-axis representing the % inhibition (free radical scavenging activity).

Inhibition of Biofilm formation

Microtiter plate (MTP) assay

This is a simple high-throughput method used to monitor microbial attachment to an abiotic surface, adapted from Merritt et al. (2011) and Kırmusaoğlu (2019), where the assay is performed in a 96-well microtiter plate. Standard isolates of *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923 were obtained from the Microbiology laboratory at the School of Biomedical Sciences, College of Health Sciences, Makerere University. The plant extract was standardized by dissolving 0.5 g in 1ml of DMSO.

In a sterile 96-well microtiter polystyrene plate, 100 µl of Mueller Hinton broth supplemented with 1% glucose was dispensed in each well. Subsequently, 100 µl of standardized extracts were added carefully to the first well and then diluted using a 2-fold serial dilution technique by transferring 100 µl of the resultant mixture from the first well to the subsequent wells until the last well, with 100 µl discarded.

Control cultures were resuscitated using brain heart infusion broth and streaked on Mannitol salt agar and King's medium for *S. aureus and P. aeruginosa*, respectively. Bacterial suspensions prepared from 24-h-old cultures in brain heart infusion broth supplemented with 1% glucose were adjusted to a 0.5% McFarland standard (approximately 1x10^8 CFU/ml) and further diluted by a 20-fold factor to a final concentration of 5x10^6 CFU/ml.

Subsequently, 20 µl of this cell suspension was dispensed into each well, except those containing only DMSO (negative control). For each plate, one column of wells served as a blank, another contained the bacterial suspension without extract, and another contained a positive control (0.5 mg/ml Ciprofloxacin) with the bacterial suspension. The plates were then incubated at 37ºC for 16-24 h to allow for biofilm formation.

After incubation, the components of the microtiter plates were poured out; leaving the biofilms adhered to the plate wells. The plates were washed twice with distilled water. The formed biofilms were fixed by incubating the plates at 60°C for 1 h, and then stained with 150 µl of 0.1% Crystal Violet and left to stand for 15 min.

Subsequently, the Crystal Violet was removed, and the plates were rinsed thrice with Dulbecco's phosphate-buffered saline and air-dried. Then, 95% Ethanol (150 µl) was added, and the absorbance of the contents in the plates was measured at 620 nm using a microplate reader (Thermo Scientific MULTISKAN FC).

The percentage reduction of biofilm formation of the isolates was calculated as follows (Kırmusaoğlu, 2019);

$$\mathbf{PR} = \frac{(\mathbf{ODC}-\mathbf{ODB}) - (\mathbf{ODT}-\mathbf{ODB})}{(\mathbf{ODC}-\mathbf{ODB})} \mathbf{x\ 100}$$

ODC: Optical density of positive control wells (wells inoculated with test organisms and Mueller Hinton broth but with no agents), ODB: Optical density of negative control wells (blank wells-wells with no organisms and agents), ODT: Optical density of wells treated with the extracts.

The minimum biofilm inhibition concentration (MBIC) was determined by plotting the curve of the percent reduction against concentration. MBIC50 and MBIC90 which are the minimum concentrations of agents that inhibits formation of 50 and 90% of the biofilm respectively (Kırmusaoğlu, 2019) were calculated from the equations obtained through extrapolation of the curve.

Biofilm removal assay

Biofilm removal was conducted following the procedure adapted from Kırmusaoğlu (2019). Initially, 180 µl of sterile Mueller Hinton broth was dispensed into wells of sterile polystyrene microtiter plates. Subsequently, 20 µl of the standardized bacterial suspensions (5x10^6 CFU/ml) was added to each well, and the plates were incubated at 37°C for 72 h to facilitate biofilm formation. After incubation, the contents of the plates were poured out, leaving the formed biofilm rings adhered to the walls of the wells.

Then, 200 µl of each extract, following a fold dilution, was added to each well of the microplate containing the formed biofilm. Ciprofloxacin was utilized as a positive control. The plates were further incubated at 37°C for 24 h. Subsequently, the contents were discarded, and the plates were washed with distilled water and ethanol before staining with 0.1% Crystal Violet.

The absorbance was measured as previously described, and a curve for percent reduction against concentration was plotted. The minimum concentration of the extract required eradicating mature biofilm, or Minimum Biofilm Eradication Concentration (MBEC), was determined. MBEC50 and MBEC90, indicating the minimum concentrations of agents eradicating 50 and 90% of mature biofilm formed, respectively, were calculated from the equations obtained through extrapolation of the curve (Kırmusaoğlu, 2019).

Statistical analysis

The means and standard deviation were analysed in Microsoft Excel 2018. Where necessary, the difference in means was analysed in Minitab 19 software using one way analysis of variance at significance level of 0.05 (P-value).

RESULTS AND DISCUSSION

Quantitative phytochemistry and antioxidant activity

Secondary metabolites such as tannins, flavonoids, phenols, and alkaloids are responsible for the bioactivity of plants. Figure 1 displays the amounts of total tannins, total flavonoids, total polyphenols, and total alkaloids present in each of the plants used in this study. *A. conyzoides* exhibited a significantly higher amount (p-value = 0.000) of total tannins (23.57 ± 1.1 GAE mg/g) compared to *B. pilosa* (6.987 ± 0.25 GAE mg/g), while *B. pilosa* showed significantly higher amounts (p-value = 0.000) of total flavonoids (8.24 ± 0.16 RE mg/g) than *A. conyzoides* (4.59 ± 0.45 mg/g RE). For total polyphenols, *A. conyzoides* exhibited a significantly higher amount (p-value = 0.000) of 37.34 ± 1.72 compared to *B. pilosa* (15.5 ± 1). Similarly, *A. conyzoides* demonstrated a significantly higher amount (p-value = 0.000) of alkaloids (51.3 ± 3.73) compared to *B. pilosa* (31.69 ± 1.25).

In general, the amount of alkaloids present in all plants was significantly higher than the tannins (p-value = 0.000), flavonoids (p-value = 0.000), and polyphenols (p-value = 0.001). In vivo and clinical studies have provided evidence that alkaloids possess antibacterial, anticancer, anti-inflammatory, and antiviral properties (Yan et al., 2021). Tannins and flavonoids, both being polyphenols, contribute to the higher overall amount of polyphenols present in all plants. Flavonoids are recognized for their anti-allergic, anticancer, antifungal, and anti-inflammatory properties (Górniak et al., 2019); while tannins are renowned for their antibacterial activity and play a crucial role in plant defense mechanisms (Samrot et al., 2021).

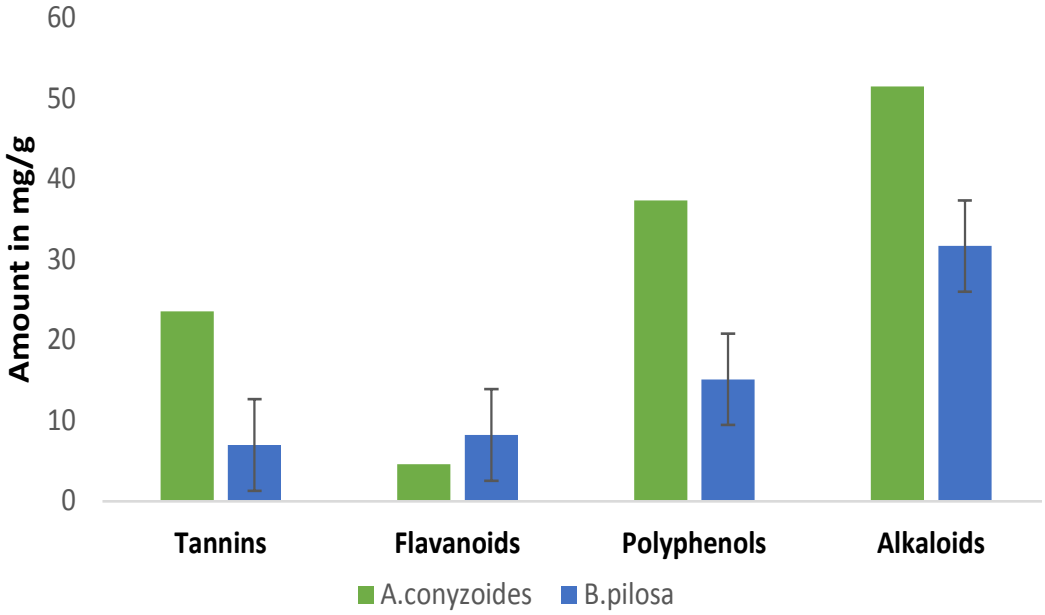

Figure 1. Amount of total tannins, total flavonoids, total polyphenols and total alkaloids present in A. conyzoides (AG), B. pilosa (BP).

Alkaloids, flavonoids, and polyphenols are essential antioxidants in plants, and their antioxidant activities have been extensively investigated in various studies (Gan et al., 2017). The antioxidant activity of the two plants was evaluated against ascorbic acid, a well-known antioxidant, using the DPPH scavenging method, which is widely employed in research to assess the free radical scavenging activity of plant extracts. This method utilizes DPPH, a stable radical, which undergoes a color change from purple to yellow upon reaction with an antioxidant agent through hydrogen transfer. A lower IC50 value indicates higher antioxidant activity (Safari and Ahmady-Asbchin, 2019). The concentration of sample required to scavenge 50% of the DPPH free radical (IC50) was 45.92 mg/ml for *B. pilosa* and 50.52 mg/ml for *A. conyzoides*, while ascorbic acid exhibited the lowest IC50 value of 11.85 µg.

Flavonoids are renowned for their antioxidant activity, so it could be presumed that their higher content in *B. pilosa* led to significantly higher antioxidant activity (p-value = 0.000) compared to *A. conyzoides*. It is also reported that a higher phenolic content leads to better DPPH scavenging activity, hence better antioxidant activity (Safari and Ahmady-Asbchin, 2019). However, *A. conyzoides* exhibited less antioxidant activity despite having higher polyphenolic content than *B. pilosa*. Some studies have suggested that antioxidant activity does not solely depend on the phenolic content because plants consist of a mixture of different compounds responsible for their bioactivity (Kaur and Mondal, 2014).

Therefore, it can be stated that antioxidant activity is attributed to the complex structural chemistry of a particular plant.

Apart from treating other illnesses, the plants used in this study are traditionally used for wound healing (Catherine et al., 2022). Hence, the antioxidant activity of these plants justifies their use in treating wounds, where antioxidant activity is crucial for healing. Antioxidants mitigate tissue damage caused by oxidative stress and protect the multiplication of fibroblasts and keratinocytes at the wound site, facilitating the healing process (Oso et al., 2019). Antioxidants also play a crucial role in preventing biofilm formation. Planktonic bacteria easily transform into biofilms due to oxidative stress, as oxidative stress facilitates redox defense mechanisms, production of the extracellular polymeric substance (EPS), and heterogeneity during biofilm formation (Ong et al., 2018).

Biofilm inhibition

Staphylococcus aureus and Pseudomonas aeruginosa are the leading causes of several infections and are the most common pathogens isolated from chronic wounds (Serra et al., 2015). *S. aureus* is typically found on the upper surface of the wound, while *P. aeruginosa* tends to be confined to the deeper tissue of the wound site, significantly impeding the healing process (Serra et al., 2015).

The results of biofilm inhibition of the plant extracts against *P. aeruginosa and S. aureus* are illustrated in Figure 2. The positive control, ciprofloxacin, inhibited 100% of the biofilm of both *S. aureus and P. aeruginosa*

at the lowest concentration of 0.0098 mg/ml. *P. aeruginosa*, belonging to the Pseudomononaceae family, is the most common gram-negative bacterium capable of surviving in various environments (Pang et al., 2019). This organism exhibits high resistance to antibiotics compared to other gram-negative bacteria due to its impermeable cell wall. *P. aeruginosa* forms dense mucoid biofilms protected by a hard-to-penetrate extracellular polymeric substance (EPS), comprising polysaccharides such as Pel, psl, and alginates, as well as DNA (Thi et al., 2020).

The equations derived from the curves for inhibition against *P. aeruginosa* biofilms were Y = 22.19ln(x) - 4.054 and Y = 24.27ln(x) - 21.038 for *B. pilosa and A. conyzoides*, respectively. Both plants inhibited more than 50% of the formed biofilm of *P. aeruginosa* at MBIC50 concentrations of 24.91 mg/ml for *B. pilosa* and 28.3 mg/ml for *A. conyzoides*. The MBIC90 was 147.7 mg/ml for *A. conyzoides* and 139.5 mg/ml for *B. pilosa*.

While *S. aureus* typically does not form strong biofilms like *P. aeruginosa*, during coinfection with the latter, they create a multispecies biofilm that sustains chronic infection and increases antimicrobial resistance, thus prolonging healing (Serra et al., 2015). For *S. aureus* biofilms, the equations obtained from the curves were Y = 22.19ln(x) - 4.054 and Y = 22.19ln(x) - 4.054 for *B. pilosa and A. conyzoides*, respectively. *B. pilosa* inhibited more than 50% of the biofilms with MBIC50 of 11.43 mg/ml, while *A. conyzoides* showed 18.67 mg/ml. The MBIC90 was 69.31 mg/ml for *B. pilosa* and 97.03 mg/ml for *A. conyzoides*.

The ability of the plant extracts to inhibit biofilm formation in both organisms may be attributed to their interference with the attachment and maturation phases of biofilm formation. The life cycle of biofilms involves initial attachment, where bacteria adhere to human cells and abiotic surfaces, followed by maturation, which includes further adhesion and multiplication to form a protective extracellular polymeric substance (EPS) impermeable to antibiotics. During this stage, the microbes exhibit different gene expressions governed by quorum sensing (Borges et al., 2015). Inhibiting quorum sensing interferes with the bacteria's ability to cause infection without exerting significant effort; thus, quorum sensing inhibitors can potentially replace conventional antibiotics in treating resistant strains (Li and Zhao, 2020). For instance, in one study, a hydro-methanolic extract of *A. conyzoides* was found to reduce quorum sensing virulence factors and biofilm formation in P. aeruginosa (Compaore et al., 2022). Therefore, it is presumed that the plant extracts could disrupt the mechanisms of attachment and EPS formation or interfere with the maturation stage of biofilm formation, as well as quorum sensing, thereby inhibiting biofilm formation.

The antibiofilm activity of the plant extracts can be attributed to the high content of flavonoids, alkaloids, tannins, and polyphenols revealed in the quantitative phytochemical analysis. These bioactive compounds have been reported to interfere with biofilm formation through various mechanisms. Flavonoids, for example, prevent the synthesis of the extracellular matrix by deactivating or inhibiting quorum sensing enzymes in the extracellular structure, or they bind to the lipid bilayer of the ECM (Górniak et al., 2019). They also inhibit the production of virulence factors by interfering with N-acyl homoserine lactones (AHLs) and their receptors (Górniak et al., 2019). Tannins can chelate ferric iron from the bacterial surroundings, thereby reducing its availability and inhibiting biofilm growth (Farha et al., 2020). Alkaloids inhibit the production of virulence factors and efflux pumps, interfere with adhesins through non-sortase-mediated mechanisms, and act as quorum sensing inhibitors (Cushnie et al., 2014).

Biofilm removal

The results of biofilm removal of the extracts are depicted in Figure 2c. In this study, the plants demonstrated the ability to remove 50 and 90% of *P. aeruginosa* biofilms. Ciprofloxacin removed 50% of biofilms at concentrations above 0.0098 mg/ml. The equations obtained from the curves (c) for biofilm removal of *P. aeruginosa* were y = 8E-06x^3 - 0.0022x^2 + 0.3879x - 3.9377 and y = 28.966ln(x) - 55.46 for *B. pilosa and A. conyzoides* respectively. The MBEC50 was 200.76 mg/ml for *B. Pilosa* and 38.12 mg/ml for *A. conyzoides*, while the MBEC90 was 261.37 mg/ml for *B. pilosa* and 151.67 mg/ml for *A. conyzoides*. The presence of narrow porins in the outer membrane of *P. aeruginosa* hinders access of hydrophobic compounds, and the expression of efflux pumps prevents penetration of antibiotic compounds through the cell, hindering them from reaching the target (Cooper et al., 2014; Lambert, 2002). This makes the removal of biofilm difficult, explaining the high concentration values of the extracts compared to inhibition.

The biofilm activity of the plant extracts against *S. aureus* biofilms is depicted in Figure 2d. *A. conyzoides* exhibited an MBEC50 of 96.96 mg/ml, while *B. pilosa* showed an MBEC50 of 195 mg/ml. The MBEC90 values were 334.42875 mg/ml for *A. conyzoides* and 250.16 mg/ml for *B. pilosa. S. aureus* expresses MSCRAMMs (microbial surface components recognizing adhesive matrix molecules), allowing it to adhere strongly to plastic or abiotic surfaces, contributing to its prevalence in catheters and implantable devices (Otto, 2018). This strong adherence makes biofilm removal challenging, hence the observed high concentration values required for eradication.

The ability of the plant extracts to demonstrate potential in biofilm removal could also be attributed to the presence of plant phytochemicals identified in this study.

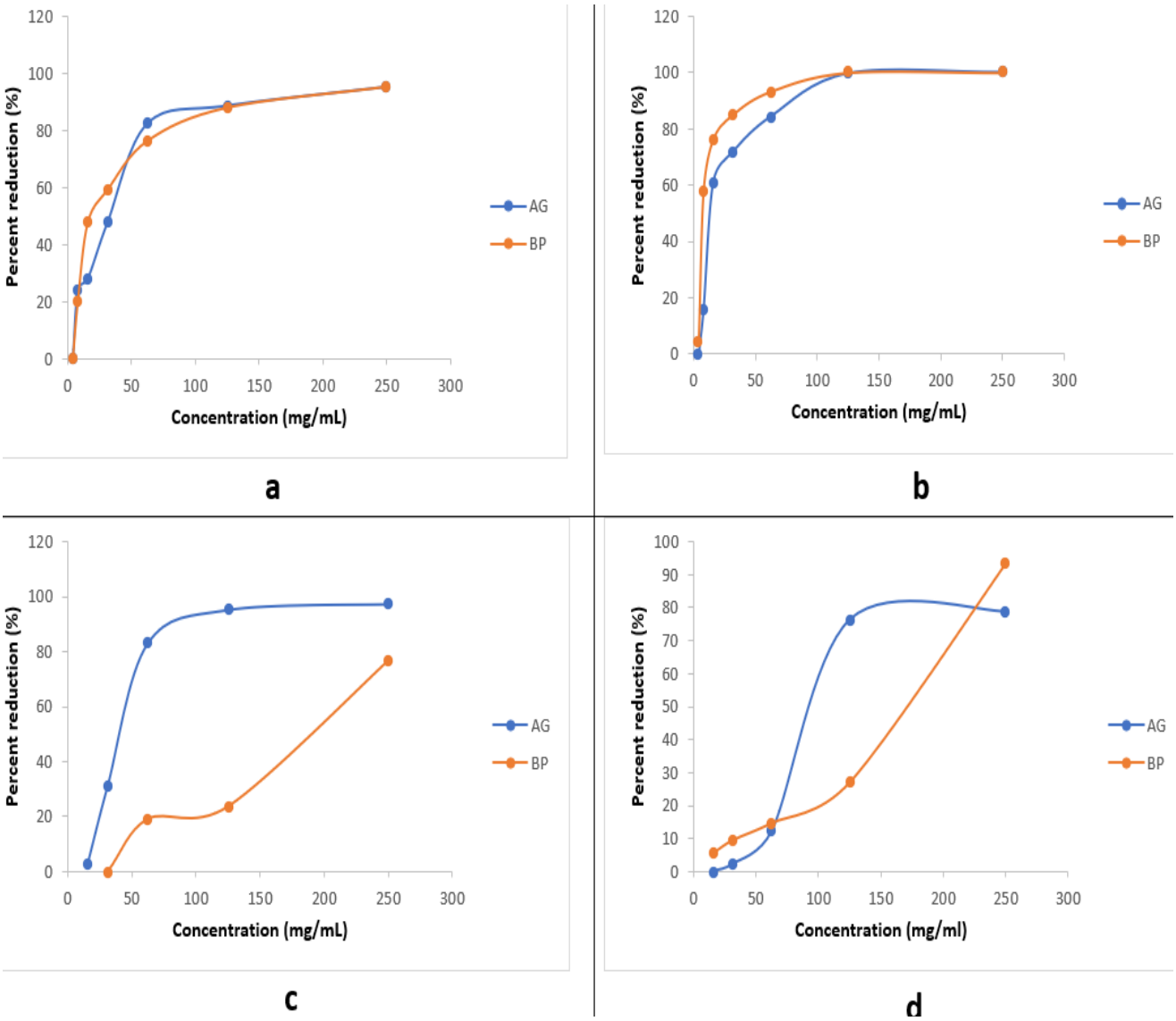

Figure 2. Upper graphs; biofilm inhibition of A. conyzoides (AG) and B. pilosa (BP) against P. aeruginosa (a) and S. aureus (b). Lower graphs; biofilm removal of B. pilosa and A. conyzoides against P. aeruginosa (c) and S. aureus (d).

These compounds might have interfered with the biochemistry of the extracellular polymeric substance (EPS) and penetrated it, affecting the integrity of the biofilm. Flavonoids, for instance, are reported to inhibit nucleic acid synthesis and porins on the bacterial cell membrane, as well as alter cell membrane permeability (Samrot et al., 2021). Phenols deactivate energy production by enzyme inhibition (Simoes et al., 2009).

Alkaloids, the most abundant compounds in these plants, affect DNA topoisomerase and respiration, interfere with membrane permeability, inhibit nucleic acids, and protein synthesis, ultimately leading to cell membrane destruction (Yan et al., 2021). Tannins inhibit the synthesis of the bacterial cell wall by directly binding to it or deactivating enzymes involved in membrane synthesis (Farha et al., 2020).

Mature biofilms consist of a range of cells with various functional and physiological variations, suggesting that multiple inhibitory antimicrobial interventions could be more effective than a single antibiotic (Cooper et al., 2014). Therefore, a combination of these phytochemicals would be important in antibiotic development to combat biofilm infections. The effectiveness of these metabolites varies depending on the type of plant and bacteria. This could explain why different plants showed different levels of antibiofilm activity. *B. pilosa* exhibited better biofilm inhibition, but biofilm removal was more challenging. Its biofilm activity has been investigated elsewhere, where the aqueous extract of the plant inhibited the biofilm of some bacterial pathogens but did not show inhibition of

P. aeruginosa (Brandelli et al., 2015). Our study, however, demonstrates that the ethanol extract of the plant inhibited *P. aeruginosa* (MBIC50 at 16 mg/ml). The difference could be due to variations in the solvents used for extraction, extraction methods, as well as environmental and geographical conditions.

A. conyzoides also inhibited biofilm formation of both organisms and eradicated some of the biofilm. Its effect on *P. aeruginosa* was notably better than that of *B. pilosa*. The activity of these plants justifies their use in traditional medicine for the treatment of various diseases and infections. Generally, plants showed more activity in inhibiting biofilm formation rather than removing the biofilm, indicating that the prevention of biofilm formation or early treatment of bacterial infections in clinical practice is crucial.

Conclusion

A. conyzoides and B. pilosa were found to contain a variety of phytochemicals responsible for their antioxidant and antibiofilm activities. These plants exhibited antioxidant properties, crucial for preventing biofilm formation and alleviating oxidative stress in wounds. Moreover, they demonstrated the ability to inhibit biofilm formation and eradicate pre-existing biofilms, supporting their traditional use in wound treatment and infection management. The capacity of these plant extracts to combat biofilms formed by resilient pathogens like *P. aeruginosa and S. aureus* marks a significant step in addressing antibiotic resistance. Importantly, the phytochemicals present in these plants operate through mechanisms distinct from those of antibiotics. Therefore, combining isolated plant compounds with conventional antimicrobials, each with different modes of action, may offer synergistic effects for effectively targeting biofilm cells and reducing the emergence of resistance. Nevertheless, extensive basic and clinical research is necessary to isolate active compounds, elucidate their mechanisms of action both in vitro and in vivo, determine appropriate doses, study their pharmacokinetics and pharmacodynamics, and develop suitable extraction technologies.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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