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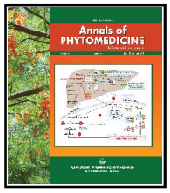

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Ethnobotanical activities of the *Erigeron bonariensis* (L.) Cronq. leaf extracts

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Abstract

An adhering population of sessile bacteria enclosed in an extracellular polymeric material (EPS) is called a biofilm. One important aspect of the pathogenicity of many bacteria that cause chronic illnesses is the production of biofilms. Many plants have been shown to possess antibacterial qualities and are utilized in the treatment of various infectious diseases. The purpose of this work is to examine the phytochemicals, antibacterial, and antibiofilm properties of the leaves of *Erigeron bonariensis* (L.) Cronq. methanolic extract. Standard biochemical procedures were used to conduct a preliminary phytochemical study. To determine which functional groups were present in the phytochemicals, FTIR analysis was performed. While MIC was ascertained by the Resazurin method, the well diffusion method was utilized to evaluate the antibacterial activity. DPPH and the hydrogen peroxide techniques were used to assess the antioxidant capabilities. An experiment for biofilm inhibition was run against the test organisms (*Staphylococcus aureus, Staphylococcus epidermidis,* and *Enterococcus faecalis*). There are secondary metabolites in the methanolic extract of *E. bonariensis* that are associated with different functional groups. Significant antibacterial, antibiofilm, and antioxidant properties were also demonstrated by *E. bonariensis* leaf extract. According to our research, *E. bonariensis* shows promise as a therapeutic candidate for the management of infectious diseases, especially those brought on by biofilm.

1. Introduction

Biofilms are an adherent population of sessile bacteria that stick to a live or inactive surface because they are enclosed in an extracellular polymeric substance (EPS) matrix (Lahiri *et al*., 2019). Numerous bacteria and an extracellular matrix consisting of proteins, lipids, nucleic acids, polysaccharides, and other biological or chemical materials make up its main components (Song *et al*., 2018). On a wide range of biotic and abiotic surfaces, these biofilms can form, including on medicinal supplies and foliage from plants. The complex process of biofilm formation requires the coordinated expression of multiple genes. A mechanism of cell-to-cell communication called quorum sensing controls, the change from planktonic to biofilm formation (Arnason and Ta, 2015). These extracellular polymeric substances (EPS) generate a protective covering on top of the bacterial cells, shielding them from exogenous stressors and antibiotics while also offering an escape route from the host's immune system. As a result, it presents numerous challenges for the different healthcare sectors (Alam *et al*., 2020). The formation of biofilms is a crucial aspect of pathogenicity for numerous bacteria that result in chronic diseases. Notably, interactions between the host and opportunistic bacteria are evident in biofilm infections, often within a heterogeneous microbiome (Koo *et al*., 2017).

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Medical researchers across a range of healthcare domains are becoming increasingly concerned about the antimicrobial resistance of biofilms. During the course of the treatment, hybrid antibiotics, combination therapy, and synthetic drugs were unable to achieve the anticipated results (Mishra *et al*., 2020). Biofilms are the cause of around 75 per cent of human microbial illnesses. The bulk of chronic infections and biofilm related diseases are brought on by the resilience of polymicrobial biofilm communities, which is why most antibiofilm drugs now in use, concentrate on just one type of bacteria (Miquel *et al*., 2016).

Mankind has been using medicinal plants in the treatment of various diseases since time unknown, and plant-originated remedies are still being used by 80% of the global population, and most such plants possess antibacterial qualities (Ayman *et al.,* 2023; Romha *et al*., 2018). As a result, the scientific community is interested in natural plant metabolites since they are thought to be a sustainable and ecologically benign source of new chemicals that have the potential to be highly effective biofilm inhibitors and effective against multidrug resistant pathogens (Srinivasan and Murali, 2022; Borges *et al*., 2015). Plants communicate with one another via these secondary metabolites. Numerous plant chemicals have demonstrated antibiofilm characteristics and have been shown to greatly limit the formation of biofilms (Song *et al*., 2018).

Native to and extensively spread throughout North America, *E. bonariensis* is a winter annual or biennial plant belonging to the Asteraceae family (Sung *et al*., 2014). It is generally known that Canadian horseweed frequently invades croplands and is a weed that is difficult to eliminate (Balicki *et al*., 2020). It features an upright stem with lateral branches and an inflorescence that is usually a terminal cluster with many small flower heads that are cream,

white, pink, or green in color (Benzarti *et al*., 2013). The flowering parts of this plant have been used in folk medicine to treat a wide range of ailments, such as cholecystitis, oedema, hematuria, and hepatitis (Sung *et al*., 2014). Conventional medicine frequently uses infusions derived from *E. bonariensis* as an astringent, diuretic, and antifungal agent (Balicki *et al*., 2020). In this study, we have investigated the phytochemicals, antibacterial, and antibiofilm activity of the methanolic extract of *E. bonariensis* leaves.

2. Materials and Methods

2.1 Sample collection

The sample was collected from the local market. To get rid of any debris, the leaves were thoroughly cleaned with running tap water and then deionized water. After that, it was shade-dried and ground into a fine powder with a machine grinder. For additional examination, the powdered leaf sample was kept in airtight receptacles.

2.2 Preparation of extract

Using the Soxhlet apparatus, 20 g of powdered leaf sample was extracted with 100 ml of methanol (Boggula and Peddapalli, 2017; Jyothiprabha and Venkatachalam, 2016). To get a sizable amount of extract, the extraction process was carried out multiple times. The remnants that were left behind were stored for use in further experiments once it was concentrated under low pressure.

2.3 Phytochemical screening

To ascertain whether secondary metabolites were present in the methanolic extract of *E. bonariensis*, a preliminary phytochemical screening was conducted (Boggula and Peddapalli, 2017; Jyothiprabha and Venkatachalam, 2016).

2.3.1 Tannins (Lead acetate test)

A few drops of a 1% lead acetate solution were combined with 2 ml of the test extract. The presence of tannins is indicated by the precipitate taking on a yellow hue.

2.3.2 Flavonoid (Alkaline reagent test)

2 mg of the test extract was mixed with a few drops of a 20% sodium hydroxide solution, which caused the extract to turn yellow. A successful outcome is shown by the color changing from yellow to colourless when diluted hydrochloric acid is added.

2.3.3 Saponin (Foam test)

After adding 6 ml of distilled water to 2 mm of test extract in a test tube and giving it a good shake, the creation of foam is a sign that the experiment was successful.

2.3.4 Steroids (Salkowski test)

10 ml of chloroform and 1 mg of the test extract were combined, and then an equal amount of concentrated H_2SO_4 was added. The presence of steroids is confirmed by the production of a red color in the upper layer and a yellowish-green sulphuric acid layer.

2.3.5 Terpenoids (Salkowski test)

3 ml of strong sulfuric acid were introduced to the test tube after 5 ml of test extract had been dissolved in 2 ml of the chloroform-containing test tube. The presence of terpenoids is indicated by the formation of the reddish-brown layer at the interface.

2.3.6 Glycosides (Keller Kiliani test)

The 5 ml of test extract was combined with 2 ml of glacial acetic acid and thoroughly mixed. 1 ml of concentrated sulfuric acid and a few drops of ferric chloride solution were added to the reaction mixture. The creation of a brown ring at the interface was considered a positive outcome.

2.3.7 Phenol (Lead acetate test)

3 ml of 10% lead acetate solution were added to 1 ml of test extract, and the appearance of a white precipitate signifies a successful outcome.

2.3.8 Alkaloids (Mayer's test)

After treating the extract (2 ml) with diluted hydrochloric acid, three drops of Meyer's reagent were added. The development of a creamy white precipitate was interpreted as a successful outcome.

2.3.9 Quinone

After treating 1 ml of the test extract with 1 ml of strong sulfuric acid, quinone was detected by the emergence of red color.

2.3.10 Proteins (Xanthoprotein test)

When 1 ml of strong nitric acid was added to the test extract, a precipitate with a white color appeared. It was then brought to a boil, let to cool, and then 20% ammonia or sodium hydroxide was added. The orange coloration is a telltale sign of aromatic amino acids.

2.4 FTIR analysis

To determine whether functional groups were present in the phytochemicals of the methanolic extract of *E. bonariensis* leaves, FTIR analysis was performed. The extract was put into the Shimadzu, Japan-based Fourier Transform Infrared Spectrometer, and it was scanned within the 400-4000 cm^{-1} infrared range.

2.5 Antioxidant activity

Hydrogen peroxide scavenging and the DPPH radical were used to assess the antioxidant activity of the methanolic extract of *E. bonariensis* leaves. The standard was ascorbic acid.

2.5.1 DPPH radical scavenging assay

Using the technique outlined by Formagio *et al*. (2014), the methanolic extract of *E. bonariensis* was tested for its ability to scavenge DPPH (1,1-Diphenyl-2-Picrylhydrazyl) radicals. 3 ml of 0.1 ìg/ml methanolic DPPH solution was combined with different aliquots of methanolic extract of *E. bonariensis* leaves (25, 50, 75, 100, 250, 500, 750, and 1000 µg/ml). After 30 min of incubation, the optical density of the reaction mixture was measured at 517 nm in comparison to the blank.

$$\text{Inhibition (\%)} = \frac{(\text{Absorbance of Control} - \text{Absorbance of Sample})}{(\text{Absorbance of Sample})} \times 100$$

2.5.2 Hydrogen peroxide scavenging assay

According to Ruch *et al*. (1989), the hydrogen peroxide (H_2O_2) scavenging ability of the methanolic extract of *E. bonariensis* leaves

was investigated. The leaves of *E. bonariensis* were metabolically extracted at different concentrations (25, 50, 75, 100, 250, 500, 750, and 1000 µg/ml) and combined with a 40 mM solution of hydrogen peroxide that was made using phosphate buffer (0.1 M, pH 7.4). Using spectrophotometry, the absorbance at 230 nm was measured during a 10 min incubation period at room temperature.

$$\text{Inhibition (\%)} = \frac{(\text{Absorbance of Control} - \text{Absorbance of Sample})}{(\text{Absorbance of Sample})} \times 100$$

2.6 Antibacterial activity

2.6.1 Test organisms

For the antibacterial activity assay, three distinct bacterial strains, including *E. faecalis*, *S. aureus*, and *S. epidermidis* were utilized. Every month, the test organisms were sub-cultured and kept at 4°C in Mueller-Hinton agar.

2.6.2 Well-diffusion method

The well diffusion method was used to assess the antibacterial activity of the methanolic extract of *E. bonariensis* against test organisms; namely, *S. aureus*, *S. epidermidis*, and *E. faecalis*. Test organisms were cultured overnight on Mueller-Hinton agar plates. Wells (6 mm) were created in the agar plates and different quantities of *E. bonariensis* methanolic extract (0.125 mg, 0.25 mg, 0.5 mg, and 1 mg) were added. The plates were then incubated at 37°C for a full day. Millimeters were used to estimate the diameter of the ensuing zone of inhibition (Boggula and Paddapalli, 2017).

2.6.3 MIC (minimum inhibitory concentration)

MIC of *E. bonariensis* methanolic extract against test organisms was ascertained by the use of the Resazurin method (Elshikh *et al*., 2016). The 96-well microplate with Muller-Hinton Broth (MHB) was filled with 50 il of an overnight culture of test organisms. The *E. bonariensis* methanolic extract was added to the wells at varying concentrations, and the mixture was then incubated for 24 h at 37°C. Following the incubation period, each well received 30 µl of resazurin (0.015%), and the wells were then incubated for a further 2-4 h to see if the color changed. A change in color from blue to pink indicated the presence of bacteria.

2.6.4 Biofilm inhibition assay

With a few minor adjustments, the biofilm inhibition assay was carried out in accordance with the Noumi *et al*. (2017) previously published protocol.

2.7 Cultivation of biofilm on microtiter plates

Following overnight culture, test strains of *E. faecalis*, *S. aureus*, and *S. epidermidis* were diluted 1:1000 in tryptone soy broth (TSB). The bacterial suspension was added to 1 X and 2 X MIC of methanolic extract of *E. bonariensis* leaves and distributed into wells of flat bottom 96-well microtiter plates (about 5×10^6 CFU/ml).

2.8 Biofilm inhibition assay

The test organisms that were cultivated without the extract served as the negative control, whereas TSB with 1% inoculation of the test organisms was used as the positive control. Crystal violet staining was used to quantify the biomass of biofilms after the microplates were incubated at 37°C for 24 h without movement. Three rounds of washing biofilms were conducted with phosphate buffer saline (PBS). The biofilms were then treated with 1% (w/v) crystal violet for 15 min and dried for an hour at 60°C. Acetic acid (33%), used to resolubilize biofilm-associated crystal violet, was used to measure the concentration by taking a microplate reader's optical density reading at 570.

$$\text{Inhibition (\%)} = \frac{(\text{Absorbance of Control} - \text{Absorbance of Sample})}{(\text{Absorbance of Sample})} \times 100$$

3. Results

3.1 Phytochemical screening

Table 1 and Figure 1 present the findings from the initial phytochemical screening of *E. bonariensis* methanolic extract. Protein, tannins, saponins, terpenoids, glycosides, phenol, and quinone were among the secondary metabolites that were revealed by the methanolic extract of *E. bonariensis*. In contrast, a negative outcome was noted for steroids, alkaloids, and flavonoids.

Table 1: Results of phytochemical screening of methanolic extract of *E. bonariensis*

S.No.	Phytochemical	Observation	Result
1.	Tannin	Formation of yellow precipitate	+
2.	Flavonoid	No colour change	-
3.	Saponin	Formation of foam	+
4.	Steroids	No formation of reddish-brown colour at the interface	-
5.	Terpenoids	The appearance of the reddish-brown layer at the interface	+
6.	Glycosides	Formation of the brown-coloured ring at the interface	+
7.	Phenol	Formation of white precipitate	+
8.	Alkaloids	The precipitate was not formed	-
9.	Quinone	The appearance of the red colour	+
10.	Protein	Occurrence of orange colour	+

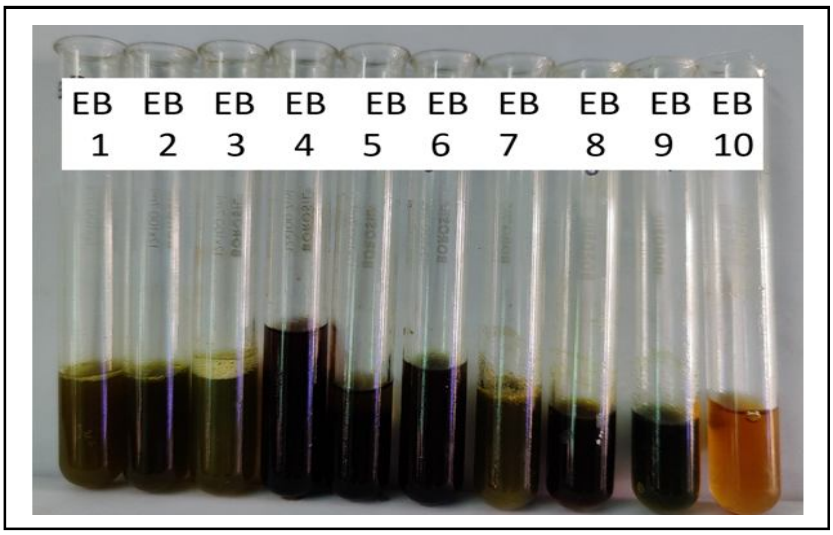

Figure 1: Phytochemical screening of methanolic extract of *E. bonariensis* leaves.

3.2 FTIR analysis

Figure 2 and Table 2 display the FTIR spectral peak values obtained for the *E. bonariensis* methanolic extract. The spectral peaks at 416.62, 470.63, 601.79, 671.23, 1195.87, 1635.64, and 3263.56 were identified by the FTIR analysis. Functional groups such as carboxylic acid, amine, conjugated alkene, alkyl, and halogen compounds were all proven to exist.

Table 2: FTIR results of methanolic extract of *E. bonariensis* leaves

Peak value	Functional group	Structure
416.62	Alkyl	Ca”C
470.63	Alkyl halides	C-Br
601.79	Halo compound	C-Br
671.23	Halo compound	C-Br
1195.87	Tertiary alcohol; sulfonate	C-O; S=O
1635.64	Amine; Conjugated alkene	N-H; C=C
3263.56	Carboxylic acid	O-H

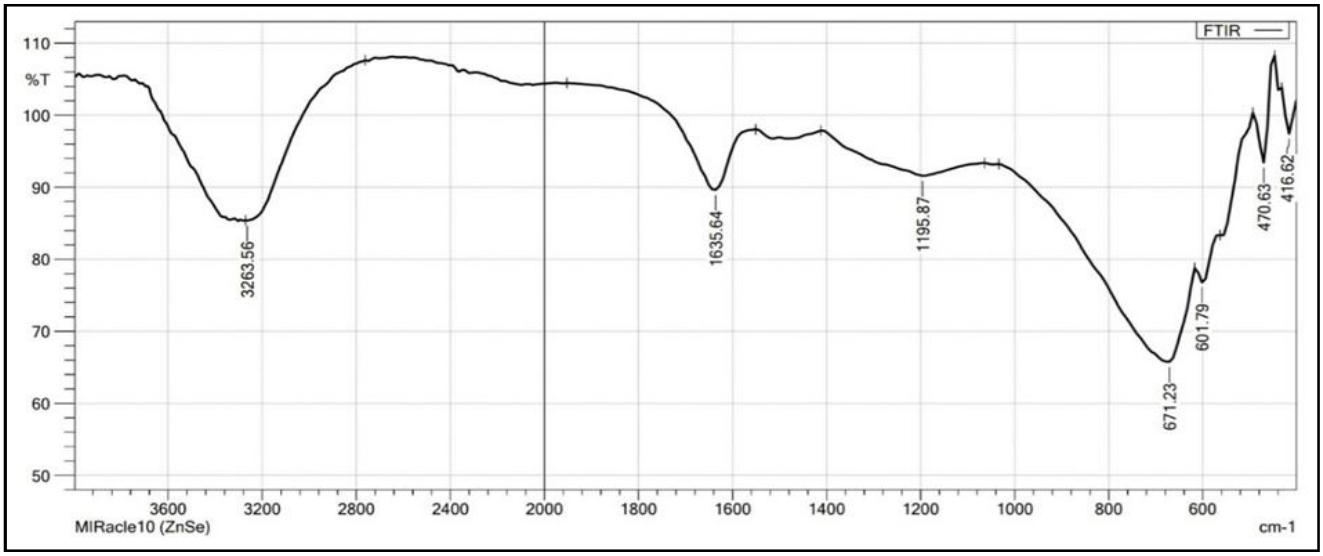

Figure 2: FTIR spectrum of *E. bonariensis* leaves.

3.3 Antioxidant activity

3.3.1 DPPH radical scavenging assay

The methanolic extract of *E. bonariensis* exhibited concentration-dependent DPPH radical scavenging action. The highest percentage of inhibition observed was 52.37% at a concentration of 1000 ìg/ml, while the lowest percentage of inhibition was 7.78% at a concentration of 25 µg/ml. The resultant IC_{50} was 8.01 µg/ml. Figure 3 and Table 3 display the percentage of inhibition that each tested concentration revealed.

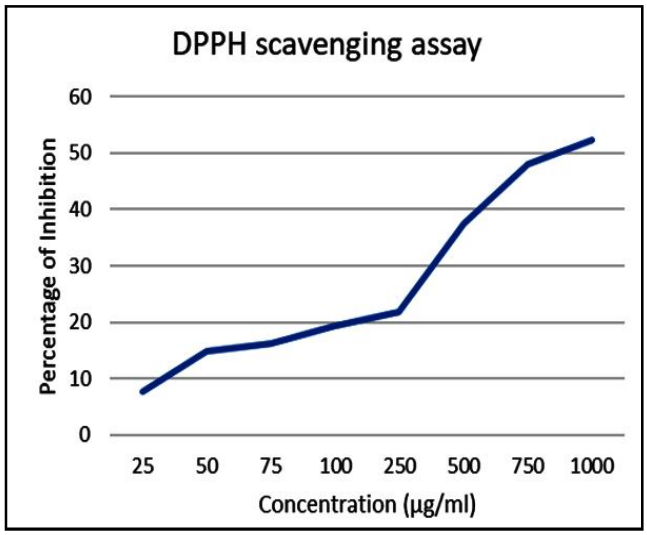

Figure 3: DPPH scavenging assay.

Table 3: DPPH scavenging activity of methanolic extract of *E. bonariensis* leaves

Concentration (µg/ml)	% of inhibition
25	7.78
50	14.91
75	16.21
100	19.36
250	21.85
500	37.43
750	48.12
1000	52.37
IC_{50}	8.01

3.3.2 Hydrogen peroxide scavenging assay

Significant hydrogen peroxide scavenging activity was shown by the methanolic extract of *E. bonariensis* at all tested doses. At 1000 µg/ml (48.75%), the highest inhibitory percentage was achieved, while at 25 µg/ml, the lowest inhibition was 0.75% (Figure 4 and Table 4). For this experiment, the IC_{50} value was 9.28 µg/ml.

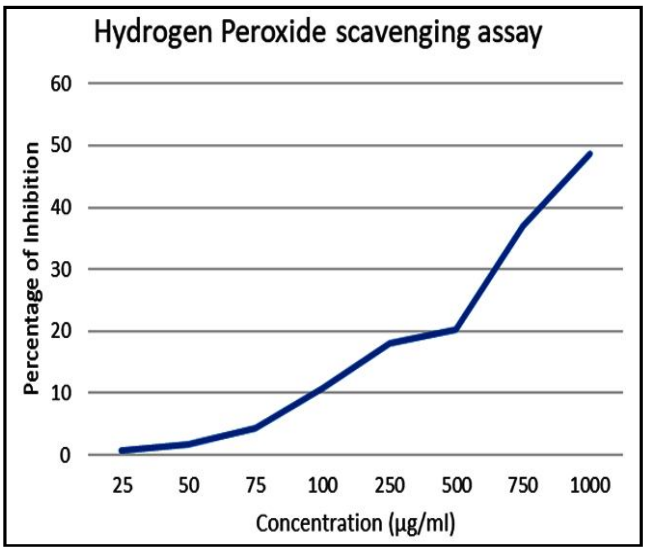

Figure 4: Hydrogen peroxide scavenging activity.

Table 4: Hydrogen peroxide scavenging activity of methanolic extract of *E. bonariensis* leaves

Concentration (µg/ml)	% of inhibition
25	0.75
50	1.80
75	4.38
100	10.84
250	18.12
500	20.25
750	37.01
1000	48.75
IC_{50}	9.28

3.4 Antibacterial activity

The growth of test organisms, *S. aureus*, was decreased by the methanolic extract of *E. bonariensis* leaves at all tested concentrations (0.125 mg, 0.25 mg, 0.5 mg, and 1 mg). *E. faecalis* and *S. epidermidis).* At a dosage of 1 mg/ml, the highest zone of inhibition was found for *S. epidermidis* (26 mm), *E. faecalis* (24 mm), and *S. aureus* (23 mm). At the lowest dose (0.125 mg/ml), the zone of inhibition for *E. faecalis, S. aureus,* and *S. epidermidis* was measured to be 11 mm, 14 mm, and 17 mm, respectively (Figure 5 and 6, and Table 5).

Table 5: Antibacterial activity of methanolic extract of *E. bonariensis* leaves

Concentration (mg/ml)	Zone of inhibition (mm)			
0.125	0.25	0.5	1	
S. aureus	14	18	21	23
S. epidermidis	17	21	23	26
E. faecalis	11	17	22	24

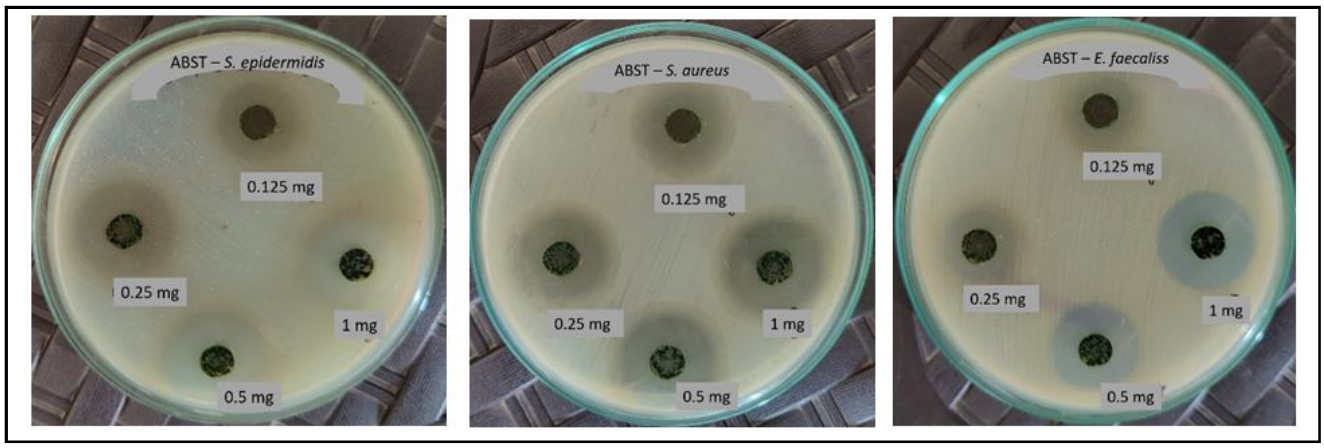

Figure 5: Antibacterial activity.

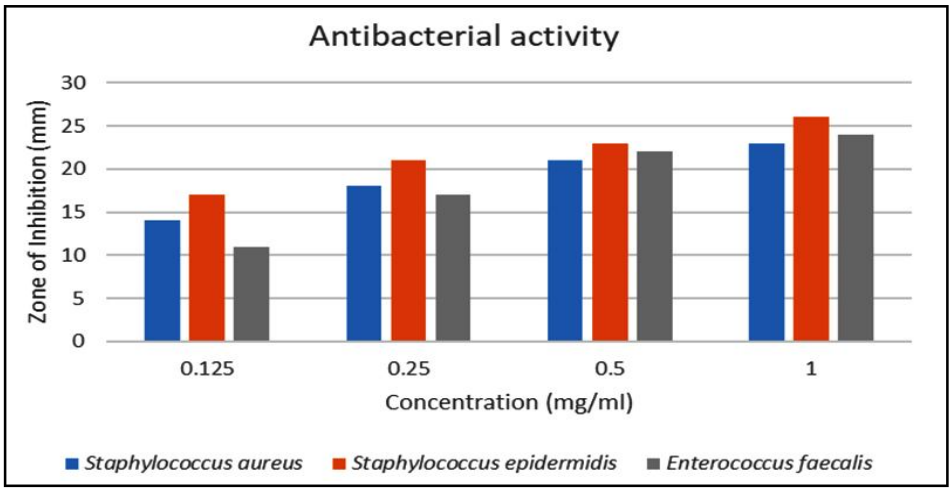

Figure 6: Antibacterial activity - comparative analysis.

3.5 MIC (minimum inhibitory concentration)

The Figure 7 and the Table 6 display the minimum inhibitory concentration of the *E. bonariensis* methanolic extract as determined by the Resazurin technique. *S. epidermidis* had the highest MIC value (12.5 ± 0.00 µg/ml), indicating resistance to the methanolic extract of *E. bonariensis* leaves. The most susceptible organism was determined to be *E. faecalis*, with a MIC value of 2.34 ± 0.78 µg/ml.

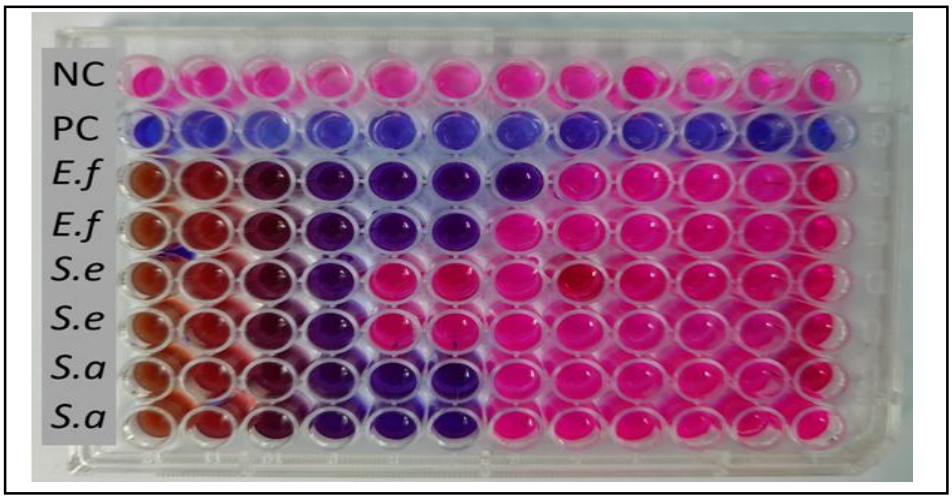

Figure 7: MIC (minimum inhibitory concentration).

NC-negative control, PC-Positive control, E.f - *E. faecalis*, S.e - *S. epidermidis*, S.a - *S. aureus*

Table 6: MIC of methanolic extract of *E. bonariensis*

Test bacteria	MIC (µg/ml)
S. aureus	3.125 ± 0.00
S. epidermidis	12.5 ± 0.00
E. faecalis	2.34 ± 0.78

3.6 Biofilm inhibition assay

E. faecalis demonstrated the highest biofilm inhibition at both the 1 X (90.41%) and 2 X (94.52%) MIC doses (Figure 8, Table 7). *S. aureus* (84.89%) showed the lowest percentage of inhibition at 1 X MIC concentration, while *S. epidermidis* (90.00%) showed the lowest percentage of inhibition at 2 X MIC concentration.

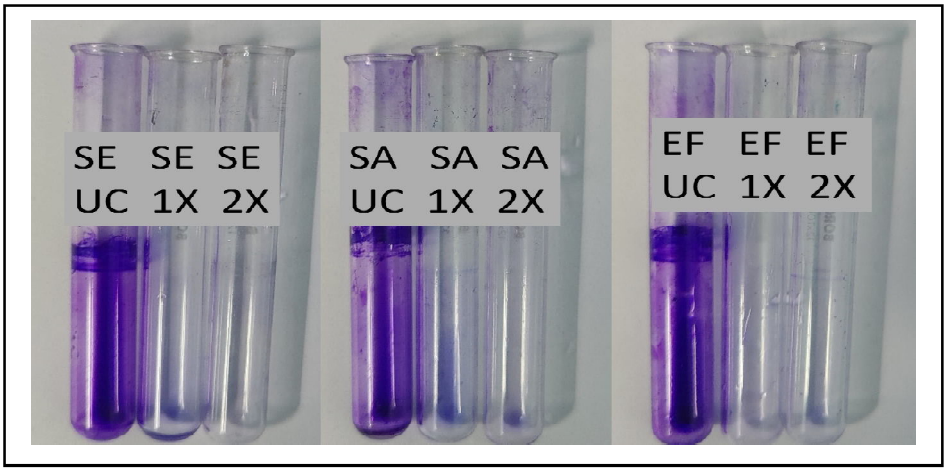

Figure 8: Biofilm inhibition activity.

Table 7: Biofilm inhibition activity of methanolic extract of *E. bonariensis*

Test bacteria	Control	% of inhibition	
		1 X MIC	2 X MIC
S. aureus	1.92	84.89	91.66
St. epidermidis	1.80	89.44	90.00
E. faecalis	1.46	90.41	94.52

4. Discussion

Numerous academic studies have emphasized the ability of phytochemicals found in a variety of plants and plant extracts to both prevent the growth of new biofilm and break down preexisting biofilm (Song *et al*., 2018). Many species of *Erigeron* yield essential oils that are rich in terpenoids and physiologically active polyacetylene compounds. These oils have been demonstrated to possess a range of biological characteristics, such as genotoxic, fungicidal, and bactericidal activity (Awen *et al*., 2010). Sesquiterpenes, monoterpenes, diterpenoids, polyacetylene chemicals, and bioactive pyrone derivatives are the main components of *Erigeron* species (Mabrouk *et al*., 2011).

In this work, we assessed the methanolic extract of *E. bonariensis* for its phytochemical profile as well as its antibacterial and antibiofilm properties. The methanolic extract of *E. bonariensis* was subjected to phytochemical screening, which revealed the presence of proteins, phenol, quinones, saponins, terpenoids, and tannins. Tannin, glycoside, and phenol were also discovered in the methanol extract of *E. bonariensis* leaves. The methanol extract of *E. bonariensis* leaves only revealed phenol and tannin. However, the aqueous extract included flavonoids (Gajanan *et al.,* 2022*). E. bonariensis* essential oil has been shown to contain both monoterpenes and diterpenes *(Elgamal et al., 2021).*

Functional groups such as carboxylic acid (3263.56 cm^{-1}), amine (1635.64 cm^{-1}), conjugated alkene (1635.64 cm^{-1}), alkyl (416.62 cm^{-1}), and halogen compounds (470.63 cm^{-1}, 601 cm^{-1}, 671.23 cm^{-1}) were found in the current study. The absorption peaks of *Senna auriculata* leaves were observed at 3390 cm^{-1} (-OH), 2929cm^{-1} (C-H), 1627 cm^{-1} (C=C), and 1055 cm^{-1} (C-O) in the methanolic extract. According to Jain *et al*. (2016), *Mentha spicata's* FTIR spectra showed an absorption band at 599.76 cm^{-1}, 1054.89 cm^{-1}, 1384.66 cm^{-1}, 1633.44 cm^{-1}, and 3349.81 cm^{-1}. These absorption bands suggested functional groups such as the aromatic ring, C-O, C-H, C=C, Ca"C, and –OH.

The DPPH and hydrogen peroxide scavenging assays in the current study demonstrated concentration-related antioxidant capability for the methanolic extract of *E. bonariensis* leaves (IC_{50} = 8.01 ìg/ml and IC_{50} = 9.28 µg/ml, respectively). The results of Thabit *et al*. (2018) are noteworthy since they demonstrated the strong antioxidant activity of *Conyza bonariensis* extracts by the use of the DPPH radical and the â-carotene scavenging assay. In the DPPH test, the hexane, ethyl acetate, and methanol extract of *Conyza sumatrensis* aerial parts verified their capacity to scavenge free radicals (Aiyelaagbe *et al*., 2016). Sesquiterpenes, another component of *E. bonariensis* essential oil, have the potential to be a potent antioxidant (Elgamal *et al*., 2021). *Conyza bonariensis*'s ethyl acetate, methanol, butanol, and chloroform extracts also demonstrated strong antioxidant capacity (Shahwar *et al*., 2012).

Salmonella typhi, Bacillus subtilis, P. aeruginosa, E. coli, S. aureus, and *K. pneumonia* were all suppressed in growth by the methanol extract of *Conyza sumatrensis* (aerial portions) (Aiyelaagbe *et al*., 2016). Similarly, our results showed that test strains of *S. aureus*, *S. epidermidis*, and *E. faecalis* were inhibited in growth by the methanolic extract of *E. bonariensis* leaves, with MIC values of 3.125 ± 0.00, 12.5 ± 0.00, and 2.34 ± 0.78 µg/ml, respectively. According to Ayaz *et al*. (2017), the essential oil derived from aerial portions had the

greatest antibacterial action against *Escherichia coli* RSKK 234 (MIC: 0.039 g/ml). The agar diffusion method was used to test the bactericidal qualities of oil derived from *E. canadensis* against *E. faecalis* (ATCC29212), *Streptococcus pyogenes* (HNCMB80002), and *S. aureus* (ATCC25923) (Curini *et al*., 2003).

In this study, the methanolic extract of *E. bonariensis* demonstrated significant antibiofilm action against *Enterococcus faecalis*, *S. aureus*, and *S. epidermidis*. *S. aureus*'s biofilm was suppressed by the ethanol fraction of *Arctium lappa* leaves (Asteraceae) (Tang *et al*., 2014). At every tested dosage, the extracted substance from *Vernonia adoensis* (Asteraceae) significantly eradicated formed *P. aeruginosa* biofilms. According to Mozirandi *et al*. (2019), *P. aeruginosa* biofilms had to be broken up by a chondrillasterol concentration of 1.6 g/ml (MIC). Three new polyacetylene glycosides that were extracted from the aerial portion of *Launaea capitata* (Asteraceae) were evaluated for their ability to inhibit the formation of biofilms by *S. aureus* (Emad *et al*., 2020).

5. Conclusion

The methanolic extract of *E. bonariensis* in this study contained secondary metabolites, as shown by the phytochemical screening. Different absorption peaks were visible in the FTIR spectrum, indicating the presence of phytochemicals from different functional groups. There was notable antibacterial, antibiofilm, and antioxidant activity in the leaf extract of *E. bonariensis*. Therefore, the leaf extract from *E. bonariensis* may present a promising avenue for therapeutic development, particularly in the context of treating biofilm-related infections. Therefore, more investigation is needed to identify the compounds in the extracts that are pertinent to the effects that have been described and to understand how they work.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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