*Original Research Article*

*Biophytum reinwardtii* extracts as promising alternative agents against pathogenic bacteria

.

ABSTRACT

|  |
| --- |
| **Aims** With the growing resistance of pathogenic microbes to antibiotics, the search for novel approaches to address this challenge has increased. In this instance, plant-based medicine emerges as a promising alternative, having been utilized by humans for centuries. *Biophytum reinwardtii* (commonly known as Gas nidikumba), a small herb from the *Oxalidaceae* family, has a long history of use in traditional medicine. This study aimed to provide scientific evidence of the antibacterial potential of the whole plant *Biophytum reinwardtii* against organisms commonly isolated from diabetic chronic wounds (*Staphylococcus aureus, Pseudomonas aeruginosa,* and *Escherichia coli*), including their respective ATCC (American Type Culture Collection) strains.  **Study design** This research was conducted as a laboratory based *in vitro* experimental study.  **Place and duration of study** All the laboratory-based experiments were conducted at the Microbiology Laboratory, Faculty of Allied Health Sciences, General Sir John Kotelawala Defence University, Werahera, Sri Lanka. Between February 2023 and March 2024, cultures of test microorganisms were obtained from the clinical samples delivered for inspection to the microbiology laboratory at the National Hospital of Sri Lanka.  **Methodology**  The plant extracts were prepared using a Soxhlet apparatus with ethanol and methanol as solvents. Antibacterial activity was evaluated through disc diffusion and well diffusion assays, while the minimum inhibitory concentration (MIC) was determined using the macro dilution method. Qualitative phytochemical analysis was conducted to identify the phytochemical constituents.  **Results**  Both extracts exhibited antibacterial activity exclusively against *Pseudomonas aeruginosa* (ATCC 27853) at concentrations of 100 mg/mL and 50 mg/mL in both well diffusion and disc diffusion assays, with the unexpected occurrence of heteroresistance. The phytochemical analysis revealed the presence of flavonoids, glycosides, phenols, and alkaloids.  **Conclusion** *Biophytum reinwardtii* has a promising potential as an antibacterial agent and the phytochemicals present may contribute to the plant's antibacterial properties, highlighting the need for further research on this plant. |

*Keywords: Biophytum reinwardtii, heteroresistance, phytochemical, Minimum inhibitory concentration, antibacterial activity*

# 1. INTRODUCTION

The finding of antibiotics in 1928 enhanceda new dawn in modern medicine and revolutionized the treatment approaches. Apart from being utilized in eliminating pathogenic bacterial diseases, antibiotics have significantly contributed to the advancement of other areas of medicine. In the absence of antibiotics vital procedures such as surgeries, organ transplantation, or chemotherapy would be impracticable. Nevertheless, Sir Alexander Fleming forewarned that the use of antimicrobials could lead to the inevitable development of resistance in his Nobel prize address. Bacteria are simple yet adaptable microorganisms to unfavorable environmental conditions such as antibiotics, by modifying themselves (Caneschi *et al.*, 2023) and there have been reports of side effects associated with causing a significant challenge to the healthcare field (Mary and Raj, 2017). As a result, common infections that were once easily managed have become tougher to combat, leading to extended hospital stays, increased medical costs, and higher death rates (Muteeb *et al.*, 2023). Indeed, antibiotic resistance has been considered one of the three most important public health threats of the 21st century by the World Health Organization (Salam *et al.*, 2023). Therefore, identifying alternatives is crucial to address the limitations and challenges associated with the use of antibiotics. Hence, medicinal plants can serve as an alternative, which is more affordable and readily available.

Medicinal plants symbolize one of the most primordial modes of medication, extending back to thousands of years of traditional medicine in many countries around the world. Herbal medicine is thought to be more socially and culturally acceptable, less poisonous, and has fewer adverse effects. Other advantages include that the same plant can be used for various treatments and health benefits, has fewer side effects and can be used in a variety of ways (Sam, 2019). Yet, there are plenty of medicinal plants that have not been deeply investigated on their phytochemical makeup and possible health advantages, leaving a hopeful future for medicinal plants (Marrelli, 2021).

The chemical compounds generated by plants during their regular metabolic processes, known as "phytochemicals," have the potential to lead to the development of innovative treatments (Anand *et al*., 2019). According to Ibrahim and Fagbohun, bioactive compounds found in plants such as alkaloids, flavonoids, tannins, phenolic compounds, and glycosides are considered as important (Ojediran *et al.*, 2024). The general functions of most phytochemicals include antioxidant properties, antimicrobial properties, and many physiological activities (Nwozo *et al.*, 2023).

*Biophytum reinwardtii* is a small annual herb from the *Oxalidaceae* family and is widely known for its medicinal properties. It is usually seen during the rainy season and distributed throughout the warmer regions of India, Indo-Malaysia, China, and Sri Lanka. It is reported that the leaves and the roots of this plant are used to treat common fever. The herb's leaves have diuretic and expectorant qualities and can be applied to wounds and bruises. In some regions, a decoction made from the leaves is given for hypertension, asthma, phthisis, and snake bite poisoning, while the root decoction is consumed to treat gonorrhea and bladder stones. The powdered seeds mixed with cow ghee can be applied topically to promote suppuration in abscesses (Nair and Sreeshma, 2021). However, there was no scientific evidence supporting its use in various instances.

The objective of this study was to explore the scientific proof behind the antibacterial properties of *Biophytum reinwardtii* and to identify the phytochemicals that are present in the plant. The results of this research may provide insights into the potential use of *Biophytum reinwardtii* in treating bacterial infections, and the detection of phytochemicals could aid in the development of new drugs. Further research is needed to determine the clinical significance of these findings and to explore the potential therapeutic applications of *Biophytum reinwardtii.*

# 2. MATERIAL AND METHODS

## 2.1 Sample collection and plant authentication

The mature and healthy whole plants of *Biophytum reinwardtii* were collected from a local coconut estate in the western province of Sri Lanka. (GPS) 7°01'24.2"N 79°56'04.1"E.

The collected plant preserved on a herbarium sheet was identified and authenticated by a botanist at Bandaranaike Memorial Ayurvedic Research Institute, Herbarium of Medicinal Plants, Nawinna, Maharagama, Sri Lanka.



**figure 1**:Preserved *Biophytum reinwardtii* plant

## 2.2 Preparation of dried plant powder

The whole plant was used for the extraction process. The plants were thoroughly cleaned to remove any impurities and defective plants were excluded. Plants were air dried under shade at 25 °C (Room Temperature) for about 30 days until a constant dry weight was achieved. The dried plants were ground into a coarse powder using a mechanical grinder. Care was taken to prevent overheating of the sample, by allowing cooling intervals during the grinding process. The resulting coarse powder was stored in an airtight, sterile container and away from sunlight at room temperature.

## 2.3 Soxhlet extraction of the plant extracts

The coarse plant powder was sieved through a 300-micron mesh to obtain a fine powder. One gram of the sieved powder was accurately weighed using an analytical balance, and its moisture content was measured using a moisture analyzer. Samples with a moisture content below 15% were selected for the extraction process. Ten grams of the sieved powder were weighed and placed into thimbles prepared from Whatman filter paper (No. 42). Three such thimbles were used in the procedure. The thimbles were sealed tightly to prevent the leakage of plant powder into the extract. Each thimble was extracted with 150 mL of 99% methanol using a Soxhlet apparatus at a temperature below 60°C (not exceeding the boiling point of methanol=64.7 °C) for 4 hours. A similar procedure was followed for the ethanolic extract of *Biophytum reinwardtii* using 99.8% ethanol. The extraction was carried out at a temperature not exceeding 75°C (boiling point of ethanol = 73.37°C) for 3 hours.

The methanolic and ethanolic extracts of *Biophytum reinwardtii* were separately concentrated using a rotary vacuum evaporator to ensure the complete removal of solvents. The concentrated extracts were then transferred into labeled sterile petri dishes and stored at −20°C for preservation until further analysis. A stock solution of the extracts was prepared in 5% DMSO (dimethyl sulfoxide) at a concentration of 100 mg/mL. Subsequently, serial dilutions were prepared from the stock solution to achieve concentrations of 50 mg/mL, 25 mg/mL, and 12.5 mg/mL.

## 2.4 Maceration technique as an alternative extraction procedure

In addition to Soxhlet extraction, the maceration technique was performed using methanol to obtain additional plant extract. Ninety grams of coarse plant powder was soaked in 900 mL of methanol at room temperature for 7 days with intermittent shaking. The mixture was then filtered through a clean muslin cloth, and the extract was transferred into labeled sterile petri dishes. It was evaporated at 60°C using a water bath. A stock solution with a concentration of 1600 mg/mL was prepared from the extract.

## 2.5 Maintenance of microbial cultures for antimicrobial assay

Cultures of test microorganisms, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli* were obtained from the clinical samples delivered for inspection to the microbiology laboratory at the National Hospital of Sri Lanka. Along with them the ATCC culture strains of the above-mentioned microorganisms (*Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923), *Escherichia Coli* (ATCC 25922)) were collected from the Medical Research Institute, Sri Lanka. The ATCC strains were used as control organisms. These microorganisms were then sub-cultured on Muller Hinton Agar and stored at 4 °C - 8 °C in the refrigerator for a maximum period of one week. Sub-culturing was done weekly until the end of the experimental procedure to maintain the viability of the organisms. Gram-staining was performed on each of the sub-cultured plates to confirm the continuity of the same microorganism.

## 2.6 Preparation of antibiotic discs

Whatman filter papers (No. 4) were punched into 6 mm diameter discs and sterilized at 160°C for 2 hours in a hot air oven. Discs were prepared by loading 20 µL of plant extract at concentrations of 100 mg/mL, 50 mg/mL, 25 mg/mL, and 12.5 mg/mL. A disc loaded with 5% DMSO served as the negative control, while commercially available antibiotic discs, Erythromycin for *Staphylococcus aureus*, Cefepime for *Pseudomonas aeruginosa*, and Ciprofloxacin for *Escherichia coli* were used as positive controls following the CLSI guidelines (The Clinical and Laboratory Standards Institute (CLSI)- supplement M100, 2023).

## 2.7 Screening the antibacterial activity of the extracts using the Disc diffusion method

The antibacterial activity of *Biophytum reinwardtii* extracts (methanol and ethanol) was assessed using the Kirby-Bauer disc diffusion method (Hudzicki, 2009).

Saline suspensions of each test microorganism were prepared by suspending a single colony from the bacterial subculture into 1 mL of sterile, 0.9% w/v saline suspension (Sodium chloride infusion BP 0.9% w/v) and adjusted to equate the turbidity of 0.5 McFarland standard. MHA plates were inoculated by spreading lawn cultures for each microorganism, rotating the plate at an angle of 60° after each inoculation, using a sterile cotton swab. Two plates per organism (ATCC strains and clinical strains of the respective organism) were prepared: one with 100 mg/mL and 50 mg/mL concentrations, and the other with 25 mg/mL and 12.5 mg/mL concentrations of plant extracts. A 24 mm gap between discs was maintained to avoid overlapping inhibition zones. Positive and negative control discs were also included in each plate. After 15 minutes of pre-diffusion at room temperature, the plates were incubated at 35°C for 16-18 hours, and the inhibition zone diameters were measured. The procedure was conducted in triplicates.

## 2.8 Screening the antibacterial activity of the extracts using the Well-diffusion method

The well diffusion assay followed the same procedure as the disc diffusion assay for creating lawn cultures for each test microorganism on MHA plates. Wells with a 6mm diameter were punched into the agar using sterile micropipette tips. The bottom of the wells was sealed with10 μL of MHA to prevent leaking of the solutions throughout the plate and was left to solidify. The wells were filled with 100 μL of extracts at concentrations of 100, 50, 25, and 12.5 mg/mL and were allowed to diffuse at room temperature. Each plate had its negative control well with 100 μL of 5% DMSO and 100 μL of their respective standard positive control. Commercially obtained antibiotic vials were used as positive controls, Clindamycin (2µg) for *Staphylococcus aureus*, Ceftazidime (30 µg) for *Pseudomonas aeruginosa* and Ciprofloxacin (10 µg) for *Escherichia coli* (The Clinical and Laboratory Standards Institute (CLSI)- supplement M100, 2023). Plates were incubated at 35 °C for 16-18 hours, and the assay was performed in triplicates. Results were interpreted according to Natarajan *et al*., 2010.

## 2.9 Determination of Minimum inhibitory concentration (MIC) using the Macro dilution method

The standard broth dilution method, as per CLSI guidelines (M07), was used to evaluate the antimicrobial efficacy of *Biophytum reinwardtii* by assessing the visible microbial growth in the broth after overnight incubation.

A new stock concentration of 200 mg/ mL was prepared using each extract separately. A twofold serial dilution was prepared on a concentration ranging from 100 mg/ mL to 6.25 mg/ mL with adjusted bacterial concentration (108 CFU/ mL, 0.5 McFarland's standard) was used to determine MIC in Muller Hinton Broth (MHB). Seven sterilized test tubes were labeled and arranged according to concentration gradient along with negative control (only broth) and positive control/growth control (inoculated broth) tubes. One milliliter of MHB was added to each tube and then each dilution was mixed well and allowed to incubate at 35 °C for 16-18 hours. The visual turbidity of the tubes was noted, both before and after incubation to confirm the MIC value (CLSI guidelines, M07). Minimum inhibitory concentration values were determined as the lowest concentration of the compound that inhibited the growth of the test organism (Isaac and Thomas, 2013).

This procedure was repeated with the extract obtained from the maceration technique. The stock solution was prepared at a concentration of 1600 mg/ mL. Then a two-fold serial dilution was performed to obtain a concentration of 800 mg/ mL, 400 mg/ mL, 200 mg/ mL, 100 mg/ mL, 50 mg/ mL, 25 mg/ mL and 12.5mg/ mL.

## 2.10 Investigation of occurrence of heteroresistivity in *Pseudomonas aeruginosa* (ATCC 27853)

Colonies observed within the zone of inhibition on *Pseudomonas aeruginosa* (ATCC 27853) cultured plates at concentrations of 100 mg/mL and 50 mg/mL prompted further investigation to determine whether this occurrence was due to contamination or heteroresistance. Heteroresistance is a subpopulation of bacterial cells that shows a higher level of antibacterial resistance than the rest of the population in the same culture (Omar and Miguel, 2015).

### 2.10.1 Evaluating the purity of the pre-inoculum and post-inoculum of *Pseudomonas aeruginosa* (ATCC 27853)

From the 0.5 MacFarland standard bacterial suspension used for assessing the antibacterial activity of *Pseudomonas aeruginosa* (ATCC 27853), a loopful was streaked onto a blood agar plate on the same day. The plate was incubated at 35 °C for 16–18 hours and observed the following day.

In order to check for the purity of the post-inoculum, 1–2 colonies from the zone of inhibition and 1–2 colonies from the lawn of the cultured MHA plate used in the antibacterial assay were separately streaked onto two equal areas of a divided blood agar plate, referred to as the post-inoculum plate. The plate was incubated at 35 °C for 16–18 hours and observed the following day.

### 2.10.2 Investigation of the progression of Heteroresistance in *Pseudomonas aeruginosa* (ATCC 27853)

The continuity of heteroresistivity in *Pseudomonas aeruginosa* (ATCC 27853) across generations was assessed using the following procedure. Colonies from the inhibition zone of an MHA plate suspected of heteroresistivity were used to prepare a 0.5 McFarland standard. The spreading of the plate was done as described in Hudzicki, 2009. Impregnated discs containing 100 mg/mL and 50 mg/mL of methanol plant extract were placed alongside a Cefepime disc (positive control) and a 5% DMSO disc (negative control). Then the plates were allowed to incubate at 35 °C for 16 to 18 hours. Zone diameters were measured the following day.

The same procedure was repeated for colonies from the lawn outside the inhibition zone and continued for two consecutive generations, labeled as first, second, and third generations. Gram staining was performed on colonies from the initial plate and the third generation.

### 2.10.3 Confirmation of the organism as *Pseudomonas aeruginosa* using BD PHOENIX 100 system

Organisms isolated within the inhibition zone and the lawn, outside the zone of inhibition were individually examined to their species level with the use of a BD PHOENIX 100 system.

## 2.11 Qualitative phytochemical screening of *Biophytum reinwardtii* extracts

Phytochemical analysis was carried out as follows.

### 2.11.1Tests for Alkaloids

One milliliter of extract was mixed with 10 mL of 1% Hydrochloric acid (Sigma Aldrich, Germany). The solution was then warmed and filtered accordingly.

**Wagner’s test:** A few drops of Wagner's reagent were added to 2 mL of filtrate along the sides of the test tube. A positive result indicates a reddish-brown precipitate (Banu and Cathrine, 2015).

### 2.11.2 Tests for Phenolic compounds

Two milliliters of 2% FeCl3 (Sigma Aldrich, Germany) were mixed with the plant extract. A blue-green or black color change indicates the presence of phenols (Mashini *et al.,* 2023).

### 2.11.3 Test for Tannins

**Gelatin Test**: 1% gelatin solution (BDH Laboratory Chemicals Division, England) containing 10% sodium chloride (Sigma Aldrich, Germany) was added to 1 mL of extract. The formation of a white precipitate indicates the presence of tannins (Banu and Cathrine, 2015).

### 2.11.4 Test for flavonoids

**Lead acetate Test**: An extract of 0.5 mL was treated with a few drops of 10% lead acetate solution (Thermo Fisher Scientific, Poland). The appearance of pale-yellow color precipitation indicates the presence of flavonoids (Banu and Cathrine, 2015).

### 2.11.5 Test for glycosides

Concentrated HCL was used to hydrolyze 0.5 mL of the extract by placing it in a water bath for 2 hours and then subjected to the following test for glycosides.

**Bontrager’s Test:** Chloroform (Sigma Aldrich, Germany) (3 mL) was added to 2 mL of filtered hydrolysate and shaken. The separated Chloroform layer was treated with a 10% ammonia solution (Sigma Aldrich, Germany). Pink coloration indicates the presence of glycosides (Banu and Cathrine, 2015).

# 3. RESULTS AND DISCUSSION

Result

## 3.1 Evaluation of antibacterial activity of *Biophytum reinwardtii*

The effectiveness of antibacterial activity of ethanol and methanol extracts of *Biophytum reinwardtii* compared to standard antibiotics, using the disc diffusion method and well diffusion method, was observed only in *Pseudomonas aeruginosa* (ATCC 27853). Methanol extract (Table 1) showed inhibition zones (mm) of 40.66 ± 0.66 at 100 mg/ mL and 23.33 ± 1.33 at 50 mg/ mL in disc diffusion for *Pseudomonas aeruginosa* (ATCC 27853).  In well diffusion using methanol extract (Table 1), *Pseudomonas aeruginosa* (ATCC 27853) showed 27.66 ± 14.25 at 100 mg/ mL and 18.00 ± 4.00 at 50 mg/ mL. Well diffusion performed using ethanol extract (Table 2) showed 24.33 ± 10.03 at 100 mg/ mL and 17.00 ± 11.93 at 50 mg/ mL for *Pseudomonas aeruginosa* (ATCC 27853). In disc diffusion for ethanol extract, (Table 2) *Pseudomonas aeruginosa* (ATCC 27853) showed 33.66 ± 5.89 at 100 mg/ mL and 26.00 ± 2.64 at 50 mg/ mL. Other organisms including the ATCC strains of *Staphylococcus aureus (ATCC 25923), Escherichia Coli (ATCC 25922*), and the clinical strains of *Staphylococcus aureus, Escherichia Coli* and *Pseudomonas aeruginosa* showed no zone of inhibition at any of the selected concentrations of the plant extract but showed inhibition zones for their respective positive controls.

**Table 1: Zones of Inhibition (mean ± standard error of mean) of disc diffusion and well diffusion of *Pseudomonas aeruginosa* (ATCC 27853) on the methanol extract**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Strain type** | **method** | **100 mg/ mL** | **50 mg/ mL** | **Positive control** |
| ATCC *Pseudomonas aeruginosa*  (ATCC 27853)  ATCC  *Escherichia coli*  (ATCC 25922)  ATCC *Staphylococcus aureus*  *(ATCC 25923)*  **CLINICAL STRAINS**  *Pseudomonas aeruginosa*  *Escherichia coli*  *Staphylococcus aureus* | Disc diffusion  Well diffusion  Disc diffusion  Well diffusion  Disc diffusion  Well diffusion  Disc diffusion  Well diffusion  Disc diffusion  Well diffusion  Disc diffusion  Well diffusion | 40.66 ± 0.66  27.66 ± 14.25  0  0  0  0  0  0  0  0  0  0 | 23.33 ± 1.33  18.00 ± 4.00  0  0  0  0  0  0  0  0  0  0 | 29.66 ± 0.57  33.66 ± 0.88  26.66 ± 0.33  33.33 ± 0.88  21.33 ± 0.66  23.00 ± 1.00  29.66 ± 0.40  34.66 ± 0.33  27.00 ± 0.57  31.33 ± 0.66  21.66 ± 0.33  23.66 ± 0.88 |

*Note- The zones of inhibition were measured by calculating the average of the triplicates, and the standard error of mean was obtained using Microsoft Excel*

**Table 2: Zones of Inhibition (mean ± standard error of mean) of disc diffusion and well diffusion of *Pseudomonas aeruginosa* (ATCC 27853) on the ethanol extract**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Strain type** | **method** | **100 mg/ mL** | **50 mg/ mL** | **Positive control** |
| ATCC *Pseudomonas aeruginosa*  (ATCC 27853)  ATCC  *Escherichia coli*  (ATCC 25922)  ATCC *Staphylococcus aureus*  *(ATCC 25923)*  **CLINICAL STRAINS**  *Pseudomonas aeruginosa*  *Escherichia coli*  *Staphylococcus aureus* | Disc diffusion  Well diffusion  Disc diffusion  Well diffusion  Disc diffusion  Well diffusion  Disc diffusion  Well diffusion  Disc diffusion  Well diffusion  Disc diffusion  Well diffusion | 33.66 ± 5.89  24.33 ± 10.03  0  0  0  0  0  0  0  0  0  0 | 26.00 ± 2.64  17.00 ± 11.93  0  0  0  0  0  0  0  0  0  0 | 29.66 ± 0.33  33.66 ± 0.88  34.33 ± 2.33  35.00 ± 0.57  27.66 ± 0.88  29.33 ± 0.33  29.66 ± 0.40  32.00 ± 1.15  31.00 ± 2.51  31.00 ± 1.00  28.66 ± 0.88  29.00 ± 0.57 |

*Note- The zones of inhibition were measured by calculating the average of the triplicates, and the standard error of mean was obtained using Microsoft Excel*

## 3.2 Evaluation of the occurrence of heteroresistivity in *Pseudomonas aeruginosa (ATCC 27853)*

*Pseudomonas aeruginosa* (ATCC 27853) showed heteroresistance for both ethanol and methanol extracts. The blood agar plates prepared to check for the purity of the cultures showed no contamination for pre and post-inoculums. The continuation of heteroresistance was observed throughout the three consecutive generations, assessed on the organisms picked from the lawn, outside the zone of inhibition. While the organisms picked from the zone of inhibition, showed complete resistance in all three generations. BD Phoenix 100 system confirmed that the two organisms picked each from the lawn, outside the zone of inhibition and within the zone of inhibition were none other than *Pseudomonas aeruginosa* (ATCC 27853). The oxidase test was also performed on both organisms and they showed positivity for the test.

## 3.3 Determination of the minimum inhibitory concentration (MIC)

After 24 h of incubation under aerobic conditions at 37 °C, visual turbidity was noticed in the test tubes starting from 100 mg/ mL to 6.25 mg/ mL for ethanol extract and methanol extract of *Biophytum reinwardtii* indicating the growth of bacteria. However, *Pseudomonas aeruginosa* (ATCC 27853) had shown a slight reduction in turbidity at 100 mg/ mL and 50 mg/ mL in both the ethanol and methanol plant extracts obtained from Soxhlet extraction. MIC performed on the extract obtained from the maceration method showed no results even at the highest concentration of 800 mg/ mL.

## 3.4 Phytochemical analysis of *Biophytum reinwardtii*

The results of the qualitative analysis of phytochemicals in *Biophytum reinwardtii* showed the presence of flavonoids, glycoside, phenols, alkaloids and the absence of tannins.

**Table 3 Results of the qualitative phytochemical analysis**

|  |  |  |  |
| --- | --- | --- | --- |
| **phytochemical** | **Method** | **Water extract** | **Ethanol extract** |
| Flavonoid | lead acetate test | Present | Present |
| Glycoside | Bontrager’s test | Present | Absent |
| Phenol | Fecl3 test | Present | Present |
| Tannin | Gelatin test | Absent | Absent |
| Alkaloid | Wagner’s test | Present | Not done |

*Note- the presence or absence of each extract tested for the phytochemicals was marked based on the observation*

# 4. Discussion

*Biophytum reinwardtii* has been used in traditional medicine (Nair and Sreeshma, 2021), but its antibacterial activity has not been scientifically validated. Even though the antibacterial properties of ethanol and methanol extracts of *Biophytum reinwardtii* have not been previously explored, extensive studies on *Biophytum sensitivum*, a related species in the *Oxalidaceae* family, provided a basis for comparison with our findings.

The evaluation of the antibacterial activity of *Biophytum reinwardtii* using agar disc and well diffusion methods revealed that both methanol and ethanol extracts exhibited antibacterial activity against *Pseudomonas aeruginosa* (ATCC 27853) at concentrations of 100 mg/mL and 50 mg/mL (Tables No 1 and 2). However, no activity was observed at lower concentrations (25 mg/mL and 12.5 mg/mL), indicating that effective inhibition occurs only at higher concentrations. In contrast, no antibacterial activity was noted for other tested ATCC strains and clinically isolated organisms at any concentration.

Comparable studies on *Biophytum sensitivum* demonstrated antibacterial activity with acetone, ethanol, and petroleum ether extracts against *Staphylococcus aureus* and *Serratia marcescens* (Thomas and Isaac, 2013). Additionally, sequential Soxhlet extraction of *Biophytum sensitivum* root extracts showed ethanol extraction to be effective against multiple organisms, including *Staphylococcus aureus, Pseudomonas aeruginosa, Proteus vulgaris, Salmonella typhi,* *Escherichia coli* (Raj and Mary, 2017). In India, Natarajan *et al*., 2009 demonstrated that the leaf extracts of *Biophytum sensitivum* (methanol, chloroform, acetone, and petroleum ether) exhibited antimicrobial activity against several human pathogenic bacterial strains using the agar well diffusion method. These findings further support the potential of species within the *Biophytum* genus as a source of antibacterial agents.

In contrast, the Sri Lankan *Biophytum reinwardtii* in the present study demonstrated limited antimicrobial activity, which may be influenced by the type of solvent used for extraction and the geographical variation of phytoconstituents.

According to a study conducted on the phytochemical analysis of *Biophytum sensitivum*, it was discovered that this plant contains alkaloids, carbohydrates, tannins, glycosides, saponin, phytosterols, and phenols (Sreedevi and Retna, 2019). In the present study, similar phytochemicals were found. Phytochemical screening was carried out on both aqueous and ethanol extracts of *Biophytum reinwardtii* plant. Aqueous extract revealed the presence of flavonoids and phenols. While the ethanol extract revealed the presence of flavonoids, glycosides, phenols, and alkaloids (Table no 3). These phytochemicals are known for their antibacterial properties. Alkaloids exhibit antimicrobial activity by interfering with DNA, cell division, bacterial membranes, virulence genes, and enzyme inhibition (Yan *et al.,* 2021). Phenols act as enzyme inhibitors (Rahman *et al*., 2021), while flavonoids inhibit DNA gyrase, disrupt membrane functions, and interfere with cellular metabolism (Balouiri *et al*., 2016).

Therefore, it can be postulated that the antibacterial activity of the plant *Biophytum* *reinwardtii* is due to the phytochemicals present in it. This is further supported by previous studies reporting the presence of terpenoids, steroids, tannins, flavonoids, and saponins in *Biophytum reinwardtii* extracted using 50% alcohol (Oraon and Sinha, 2012).

Previous studies on *Biophytum sensitivum* in India utilized extraction processes lasting over 24 hours, whereas the current study on *Biophytum reinwardtii* was limited to three to four hours of Soxhlet extraction. The shorter extraction duration may have influenced the antibacterial activity, as extended extraction times generally result in higher yields and potentially greater antibacterial efficacy.

The minimum inhibitory concentration (MIC) results from the Macro broth dilution method in the present study showed no antibacterial activity for methanol and ethanol extracts of *Biophytum reinwardtii* against any bacterial strains at concentrations ranging from 100 mg/mL to 6.25 mg/mL, except for a slight color reduction atconcentrations of 100 mg/mL and 50 mg/mL for *Pseudomonas aeruginosa* (ATCC 27853). In contrast, a similar study by Raj *et al*., 2013 on *Biophytum sensitivum* reported MIC values for methanolic extracts ranging from 7.81 mg/L to 31.25 mg/L against *Streptococcus pyogene*s, *Staphylococcus aureus*, *Bacillus cereus*, and *Bacillus coagulans*. This discrepancy may reflect differences in extraction methods, phytochemical composition, or bacterial susceptibility.

Our study was also carried out utilizing the maceration technique with methanol solvent due to insufficient yield from Soxhlet extraction. Although maceration produced a higher yield, no antibacterial activity was observed even at higher concentrations, such as 800 mg/mL, in the macro broth dilution method. Despite soaking the plant material for seven days, the residue obtained was not effective, indicating that maceration is not a suitable extraction method for *Biophytum reinwardtii.* Therefore, conducting the extraction using the Soxhlet method for longer durations would be an ideal approach. The observation of colonies within the inhibition zone for *Pseudomonas aeruginosa* (ATCC 27853) at 100 mg/mL and 50 mg/mL suggested the occurrence of heteroresistance in this strain. Heteroresistance is an intermediate phase where bacteria transition from susceptibility to complete resistance under unfavorable conditions, such as antibiotic pressure (Lin *et al*., 2022; Xu *et al*., 2019).

To investigate heteroresistance progression, colonies from the inhibition zone and the lawn were isolated and analyzed using the disc diffusion method across three generations for reproducibility (Xu *et al*., 2019). Colonies from the lawn, outside the inhibition zone consistently reproduced heteroresistance, while colonies within the inhibition zone demonstrated persistent resistance, suggesting adaptation under antibiotic pressure. These isolates were identified at the species level using the BD PHOENIX 100 system.

Although no definitive mechanism for heteroresistance in *Pseudomonas aeruginosa* has been established, studies have implicated the downregulation of oprD (antibiotic binding site) and upregulation of efflux pump genes (mexE and mexY) as potential contributors to imipenem heteroresistance (Xu *et al*., 2019). These studies utilized PCR, real-time PCR, biofilm assays, and population analysis profiles (PAP). Due to limited resources, further investigation into the heteroresistance mechanisms observed in this study could not be pursued.

# 5. CONCLUSION

This study suggests that *Biophytum reinwardtii* possesses potential antibacterial activity against microbial pathogens. The ATCC strain of *Pseudomonas aeruginosa* (ATCC 27853) exhibited susceptibility at concentrations of 100mg/mL and 50 mg/ML for both ethanol and methanol extracts. The observation of heteroresistance in *Pseudomonas aeruginosa* (ATCC 27853) highlights the need for further investigation to better understand this phenomenon and its possible contributing factors. Phytochemical analysis of aqueous and ethanol extracts revealed the presence of flavonoids, glycosides, phenols, and alkaloids, which may be responsible for the antibacterial activity observed. Additionally, optimizing extraction conditions and extending the duration of extraction in future studies may enhance the antibacterial potential of *Biophytum reinwardtii* and provide deeper insights into its bioactive properties.

# ETHICAL APPROVAL

The ethical approval for obtaining the clinical isolates of test microorganisms was obtained from the Ethics Review Committee of the National Hospital of Sri Lanka. (REF: AAJ/ETH/COM/2023/AUG)

Disclaimer (Artificial intelligence)

Authors hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

# REFERENCES

1. Caneschi, A, Bardhi, A, Barbarossa, A and Zaghini, A, (2023): The use of antibiotics and antimicrobial resistance in veterinary medicine, a complex phenomenon: A narrative review. *Antibiotics*, *12*(3), p.487.
2. Muteeb, G, Rehman, M T, Shahwan, M and Aatif, M, (2023): Origin of antibiotics and antibiotic resistance, and their impacts on drug development: A narrative review. *Pharmaceuticals*, *16*(11), p.1615.
3. Salam, M.A., Al-Amin, M.Y., Salam, M.T., Pawar, J.S, Akhter, N, Rabaan, A A and Alqumber, M A, (2023): Antimicrobial resistance: a growing serious threat for global public health. In *Healthcare* (Vol. 11, No. 13, p. 1946). Multidisciplinary Digital Publishing Institute.
4. Mary, L and Raj, T (2017): Antibacterial activity of root extract of Biophytum sensitivum (L.) DC, *International Journal of Biology Research* 2 (3), pp 16-19.
5. Sam, S (2019): Importance and effectiveness of herbal medicines, *Journal of Pharmacognosy and Phytochemistry* 8(2), pp 354-357.
6. Marrelli, M (2021): Medicinal plants, *Plants* *10* (7), p 1355.
7. Anand, U, Jacobo-Herrera, N, Altemimi, A, and Lakhssassi, N (2019): A comprehensive review on medicinal plants as antimicrobial therapeutics: potential avenues of biocompatible drug discovery, *Metabolites*  9 (11), p 258.
8. Shahbaz, M., Imran, M., Alsagaby, S.A., Naeem, H., Al Abdulmonem, W., Hussain, M., Abdelgawad, M.A., El-Ghorab, A.H., Ghoneim, M.M., El-Sherbiny, M. and Atoki, A.V., 2023. Anticancer, antioxidant, ameliorative, and therapeutic properties of kaempferol. *International Journal of Food Properties*, *26*(1), pp.1140-1166.
9. Ojediran, T K, Alagbe, O J, Victor, D and Adewale, E, (2024): Analysis of *Kigelia africana* (Lam.) Benth. Fruit powder's antioxidant and phytochemical properties. *Brazilian Journal of Science*, *3*(7), pp.38-49.
10. Yan, Y, Li, X, Zhang, C, Lv, L, Gao, B and Li, M, (2021): Research progress on antibacterial activities and mechanisms of natural alkaloids: A review. *Antibiotics*, *10*(3), p.318.
11. Nair, B R, (2021): A simple protocol for the isolation of amentoflavone from two species of Biophytum DC.(Oxalidaceae) and evaluation of its antiproliferative potential. *Industrial crops and products*, *160*, p.113099.
12. Banu, K S and Cathrine, L (2015): General techniques involved in phytochemical analysis, International journal of Advanced Research in Chemical Science 2 ( 4), pp 25 32.
13. BD Phoenix Automated Microbiology System User's Manual (2020): USA Becton, Dickinson and Company.
14. CLSI supplement M100 (2023): Performance Standards for Antimicrobial Susceptibility Testing. Available at: <http://em100.edaptivedocs.net/dashboard.aspx>
15. Hudzicki, J (2009): Kirby-Bauer disk diffusion susceptibility test protocol, American Society for Microbiology, 15, pp 55-63.Available: <https://www.asm.org/Protocols/Kirby-Bauer-Disk-Diffusion-Susceptibility-Test-Pro>
16. Isaac, J and Thomas, T (2013) Antibacterial evaluation of Biophytum sesitivum (L) DC. var. candolleanum [wigh] Edgew. and Hook. F, Bioprospecting of Plant Genetic Resources, pp 53-55.
17. Natarajan, D, Shivakumar, M S and Srinivasan, R (2010): Antibacterial activity of leaf extracts of Biophytum sensitivum (L.) DC, Journal of Pharmaceutical Sciences and Research 2 (11), pp 710-720.
18. Oraon, A and Sinha, B N (2012): Subchronic toxicity potential of the alcoholic extract of Biophytum reinwardtii whole plant, Journal of Natural Sciences Research 2 (6), pp 94-99.
19. Sreedevi, A and Retna, A M (2019): Evaluation of phytochemical standardization and antioxidant activity of leaf part of Biophytum sensitivum, International Journal of Advanced Scientific Research and Management (4).
20. Rahman, M M, Rahaman, M S, Islam, M R, Rahman, F, Mithi, F M, Alqahtani, T, Almikhlafi, M A, Alghamdi, S Q, Alruwaili, A S, Hossain, M S and Ahmed, M, (2021): Role of phenolic compounds in human disease: Current knowledge and future prospects. *Molecules*, *27*(1), p.233.
21. Mashini, P, Chihomvu, P, Pillay, M and Takaidza, S, (2023): Phytochemical analysis and anti-mycobacterium activity of Bidens pilosa crude extracts. *Journal of Biotech Research*, *2023*(15), pp.116-137.
22. CLSI M07 (2022) Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. 12th ed. Wayne, PA: Clinical and Laboratory Standards Institute
23. Xu, Y, Zheng, X, Zeng, W, Chen, T, Liao, W, Qian, J, Lin, J, Zhou, C, Tian, X, Cao, J and Zhou, T (2019): Mechanisms of heteroresistance and resistance to imipenem in Pseudomonas aeruginosa, Infection and drug resistance, pp 1419-1428
24. Lu, Y, Liu, Y, Zhou, C, Liu, Y, Long, Y, Lin, D, Xiong, R, Xiao, Q, Huang, B and Chen, C (2022): Quorum sensing regulates heteroresistance in Pseudomonas aeruginosa, *Frontiers in microbiology 13*.