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# Antibacterial effect of plantago major extract on staphylococcus aureus biofilm

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**ABSTRACT**

The ability of microorganisms to attach to living and non-living surfaces and create a biofilm is the cause of numerous long-lasting illnesses, as well as their strong resistance to drugs. Bacterial biofilms consist of intricate assemblies of immobile bacteria. These are located in an extracellular matrix and adhere to various surfaces for a long period. The present study evaluated the antibacterial effectiveness of Plantago major extract against Staphylococcus aureus biofilm. The specimens analyzed in this investigation were skin infections of clinical origin. The current study was not previously studied, particularly in terms of S. aureus biofilm breakdown and inhibition. The disc diffusion method was used to test the antimicrobial activity of extracts on planktonic forms. Several antibiotic control tests were conducted utilizing several commercial disks, The sizes of the inhibitory zones were measured in millimeters and normalized. The anti-biofilm effects were evaluated using the microtiter plate technique. Out of 120 clinical samples, only 46 (38.3 %) had positive S. aureus isolates, whereas 74 (61.6%) were negative. The current study demonstrated that ethanolic P. major leaf extract has antibacterial effects on the development of S. aureus isolates with zones of inhibition ranging in size from 9 to 22 mm. Simultaneously, Antibiotic susceptibility tests showed that all isolates were resistant to erythromycin and clindamycin at an 80 % level. Cefoxitin and chloramphenicol resistance was found at 72 %, and 77 %, respectively. significance and impact of study: this research shown that Extracts from Plantago major can be employed as antibacterial agents against S. aureus, and also anti_staphyloccocus biofilm forms.

Keywords. Plantago major, staphylococcus aureus, Biofilm, Antimicrobial activities, Microtiter Plate.

## 1.INTRODUCTION

Antimicrobial resistance poses a substantial clinical concern. However, there have been significant advancements in the production of medications. The prevalence of microorganisms that are resistant to conventional antibiotics is increasing (Langdon et al., 2016). Staphylococcus aureus is a highly prevalent bacterial infection that causes a significant number of uncomplicated skin infections and potentially hundreds of thousands to millions of more severe, invasive diseases worldwide annually (Kuzina et al., 2001) (Mungole & Chaturvedi, 2011). Based on a 2012 assessment, the occurrence of S. aureus bacteremia ranges from 20 to 50 cases per 100,000 individuals per year. Of these cases, ten to thirty percent of patients succumb to this illness (Ferrazzano et al., 2015). Pharmaceutical researchers globally have recognized the therapeutic value of utilizing medicinal plants as a natural resource in the development of medicine, specifically in the treatment of germs that are resistant to several drugs (Fleer & Verspohl, 2007). Plant extracts are rich in bactericidal compounds, including flavonoids, alkaloids, terpenoids,

tannins, saponins, and phenols (Sharafzadeh & Alizadeh, 2012). In recent times, there has been a growing fascination among human societies with the utilization of natural herbal remedies. Medicinal herbs are renowned for their affordability and minimal likelihood of adverse consequences (Klevens et al., 2007) (Nazarizadeh et al., 2013). Plantago, a genus belonging to the Plantaginaceae family, is among countless plants that have been utilized for several purposes. P. major, a member of this genus, known as broadleaf plantain, has been used to cure several ailments including illnesses, skin conditions, infectious disorders, malignancies, high fever, pain, colds, hepatitis, and gastrointestinal and respiratory organ abnormalities (Rasigade et al., 2014). P. major is a globally distributed plant, including in Iran, and has historically been employed for the treatment of various ailments. The antibacterial and antioxidant activities of P. major on various bacteria, particularly Gram-positive bacteria, are now under investigation (van Hal et al., 2012). While there have been studies on the antibacterial properties of P. major against different microorganisms, there is limited evidence available regarding its impact on S. aureus. This study aimed to investigate the antibacterial and anti-biofilm properties of P. major against S. aureus. This work aims to elucidate the anti-staphylococcal and anti-biofilm properties of P. major.

## 2.MATERIALS AND METHODS

### 2.1 Plant collection and preparation

Recently harvested P. major leaves were obtained from the Baghdad city marketplace in Iraq. The leaves were rinsed with tap water and air-dried in a shaded area for five days. The desiccated leaves were pulverized into a fine powder using both a mortar and pestle and a mechanical grinding apparatus. The resulting powder was then stored in opaque containers until it was extracted (Mahmood & Mahdi, 2022).

### 2.2 Maceration method

A total of 30 grams of powdered P. major leaves were soaked in a flask containing 300 milliliters of ethanol solvent in a ratio of 1:10. The maceration process lasted for 72 hours until the bioactive component was fully extracted, as indicated by previous research (Abd Razik et al., 2012) (Kassaw et al., 2018). After 72 hours, the extract was passed through Whitman No. 1 filter paper. The extract was stored in screw-cap bottles and refrigerated at a temperature of 4°C until it was used. The solvent used for extraction was evaporated, and the resulting extract was

concentrated either at ambient temperature or in an oven set at 40°C, resulting in the production of 4 grams of extracted plant material. A concentrated solution of the extract was created by dissolving 0.1 g in 100 mL of ethanol, resulting in a final concentration of 100 mg/mL. The concentrated solution was diluted with distilled water to achieve concentrations of 75, 50, 25, and 10 mg/mL.

**2.3 Bacterial isolation and identification**

Between September and December 2023, 46 S. aureus isolates were isolated from clinical samples in Baghdad, Iraq. Clinical samples were acquired from the Yarmouk Teaching Hospital, as well as several private clinics. Clinical isolates were skin infections. After being cultivated for 24 hours at 37°C on an enriched medium (blood agar) and a selective and differential media (mannitol salt agar), these isolates of bacteria were recognized as S. aureus. Under Bergey's guides of determinative bacteriology, use a mannitol fermenter and beta hemolysis test as identification for staphylococci (HOLTJ.G. et al., 1994) (ATLAS et al., 1995).

**2.4 Antibiotic Susceptibility Testing**

Commercial disks were used to conduct a comparative antibiotic control test concurrently (erythromycin, clindamycin, cefoxitin, tetracycline, vancomycin and chloramphenicol, ciprofloxacin, levofloxacin, doxycycline, and gentamycin), and the CLSI (Clinical and Laboratory Standard Institute) standardized the width of the inhibitory zones, which were then characterized as sensitive (S), intermediate (I), and resistant (R).

**2.5 Antibacterial activity**

The disk inhibition zone approach was used to evaluate P. major's antibacterial activity. The Kirby and Bauer method (Kose & Colak, 2021) was used, in which freshly produced bacterial cells were inoculated into the Muller-Hinton agar medium to produce growth. After the agar had solidified, several sterile disks with different quantities of extract (100, 75, 50, 25, and 10 mg/ml) were immersed and placed onto the plates. The antibacterial activity was evaluated by measuring the diameter of the inhibitory zone that developed around the disk after a 24-hour incubation period at 37 °C.

**2.6 Detection of biofilm formation Microtiter plate method**

In 96-well polystyrene tissue culture plates, S. aureus isolates were cultivated using Brain-Heart Infusion Broth which contained 1% glucose. The plates were thereafter placed in an environment with oxygen and incubated at a temperature of 37°C for 24 hours. Planktonic cells were rinsed three times with PBS following incubation. After that, 200 µl of pure methanol was used to fix the bacterial cells in each well for 20 minutes. The plates were dry and empty. Following the application of 200 µl of 0.1 percent crystal violet to the connected cells, which were treated for 20 minutes, the surplus stain was washed away. Following the process of air drying, the plates were thoroughly cleansed using PBS. 200 µl of 95% ethanol was used per well to dissolve the adhering cells' crystal violet dye. With a spectrophotometer set at 630 nm, the plates were analyzed. As the negative control, the absorbance of wells filled with sterile BHI was used (Christensen et al., 1985). And then classify as none/weakly, moderately, strong according to (table 1).

**Table 1: bacterial adherence classification using the tissue culture plate technique (Stepanović et al., 2007) (Atshan et al., 2012).**

| OD VALUES | ADHERENCE | BIOFILM FORMATION |
|---|---|---|
| < OD C | none | none |
| OD < OD ≤ 2*ODC | Weakly | Weakly |
| 2*ODC < ODT ≤ 2*ODC | Moderately | Moderately |
| 4ODC | Strong | Strong |

C= control, t = test, OD = Optical density at 630 nm

## 2.7 Inhibitory effect of P. major on S. aureus biofilm formation

Isolates were chosen based on their ability to create biofilms as well as the biofilm formation experiment described above. This strain was cultivated on a microtiter plate with serial doses of plant extract (100, 75, 50, 25, and 10 mg/ml) and then kept at 37 °C for 24 hours. The wells were stained with 200 µl (0.1 percent) crystal violet after the planktonic cells were removed. As a percentage of decreased biofilms compared to untreated control biofilms (positive control), the data is displayed. Following the incubation period, the wells were thoroughly rinsed and treated with a staining agent. The amount of light absorbed at a wavelength of 360 nm was measured using an ELISA reader. The formula for calculating inhibition-mediated reduction of biofilm development is as follows (Mathur et al., 2006).

$$\% \text{ of inhibition} = \frac{\text{OD in control} - \text{OD in treatment}}{\text{OD in control}} \times 100$$

## 3. RESULT

Out of 120 clinical samples, only 46 (38.3 percent) had positive S. aureus isolates, whereas 74 (61.6 percent) were negative. A modified Kirby-Bauer technique was employed to assess the susceptibility of S. aureus isolates to commonly used (commercial) antibiotics (Figure 1). Figure 1 shows that erythromycin and clindamycin resistance was 80% present in all samples. Resistance to cefoxitin and chloramphenicol was observed at 72% and 77%, respectively. By employing the diffusion technique for antimicrobial susceptibility testing, the antibacterial activity of different dosages of P. major plant ethanolic extracts against S. aureus was evaluated in this work (Table 2). Zones of inhibition ranging in width from 9 to 22 mm were observed in the current experiment to be associated with ethanolic P. major leaf extract's antibacterial activities on the development of S. aureus isolates (figure 2).

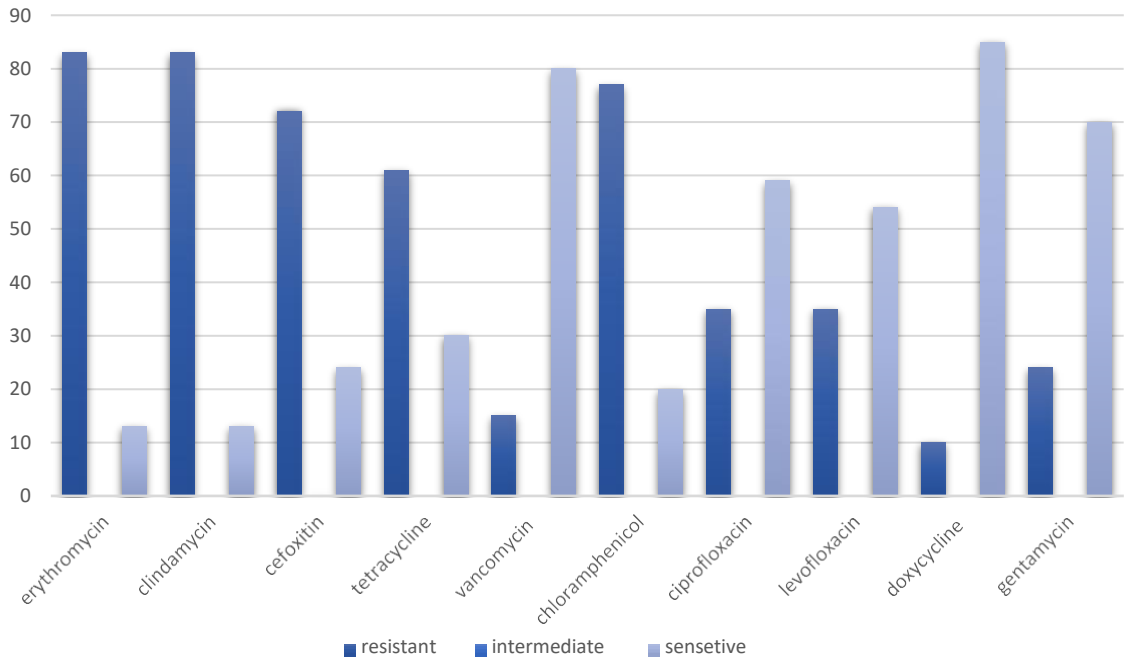


**Figure-1: Resistance of S. aureus isolates to selected antibiotics [n = 46].**

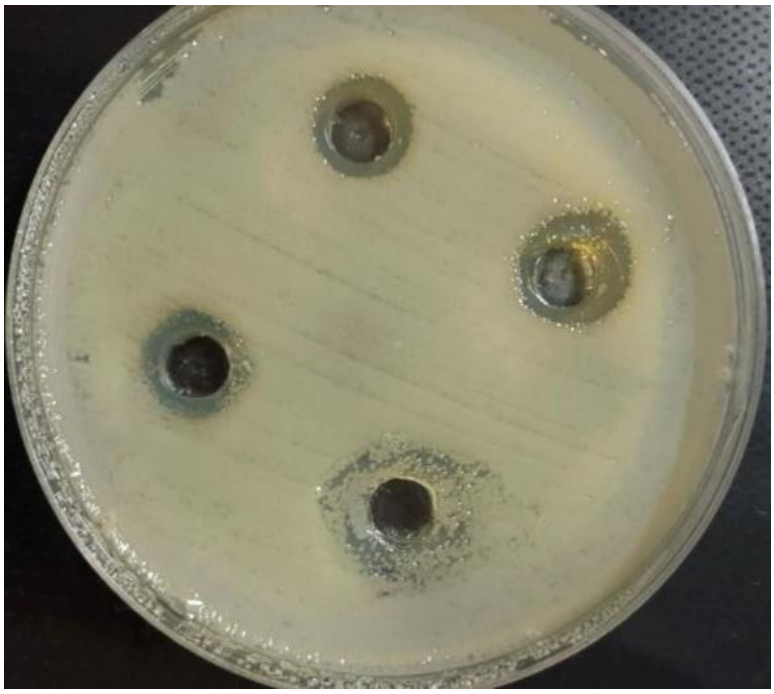

**Figure 2: antibacterial activity of P.major on staph. Aureus.**

**Table 2: P. major ethanolic extract exhibited dose-dependent inhibitory effects on S. aureus isolates.**

| Extracts' concentration % | The inhibitory zone's average diameter (mm) |
|---|---|
| 100 | 27 |
| 75 | 21 |
| 50 | 17 |
| 25 | 12 |
| 10 | 9 |

### 3.1 Biofilm formation

S. aureus isolates formed biofilms, as shown in (table 3). The isolates were recognized as high biofilm producers because their 4ODC, among the forty-six (100 percent) S. aureus biofilm result by Micro-titer plate test reveal 11 (23.9 percent) separates as strong, 17 (36.9 percent) separates as moderate, and 18 (39.1 percent) separates as weak/non-biofilm producers (figure 3). The ability to form biofilms differed widely between isolates due to a variety of factors such as S. aureus' physical qualities, the physical interface between machines, the type of external surface to which the biofilms adhere, temperature, pH, etc. The differences in biofilm thickness

were caused by a variety of factors and variations in each isolate's capacity to create biofilm, maybe the principal quantity of adherent cells, and differences in the production of auto-inducers (signaling molecules for quorum sensing) in terms of both quality and quantity (Brady et al., 2008) (Beenken et al., 2010).

**Table 3: Number of biofilm-forming S. aureus isolates.**

| Biofilm formation (n = 46) | Microtiter plate (MTP) |
|---|---|
| Strong | 11 (23.9%) |
| Moderate | 17 (36.9%) |
| Weak/none | 18 (39.1%) |

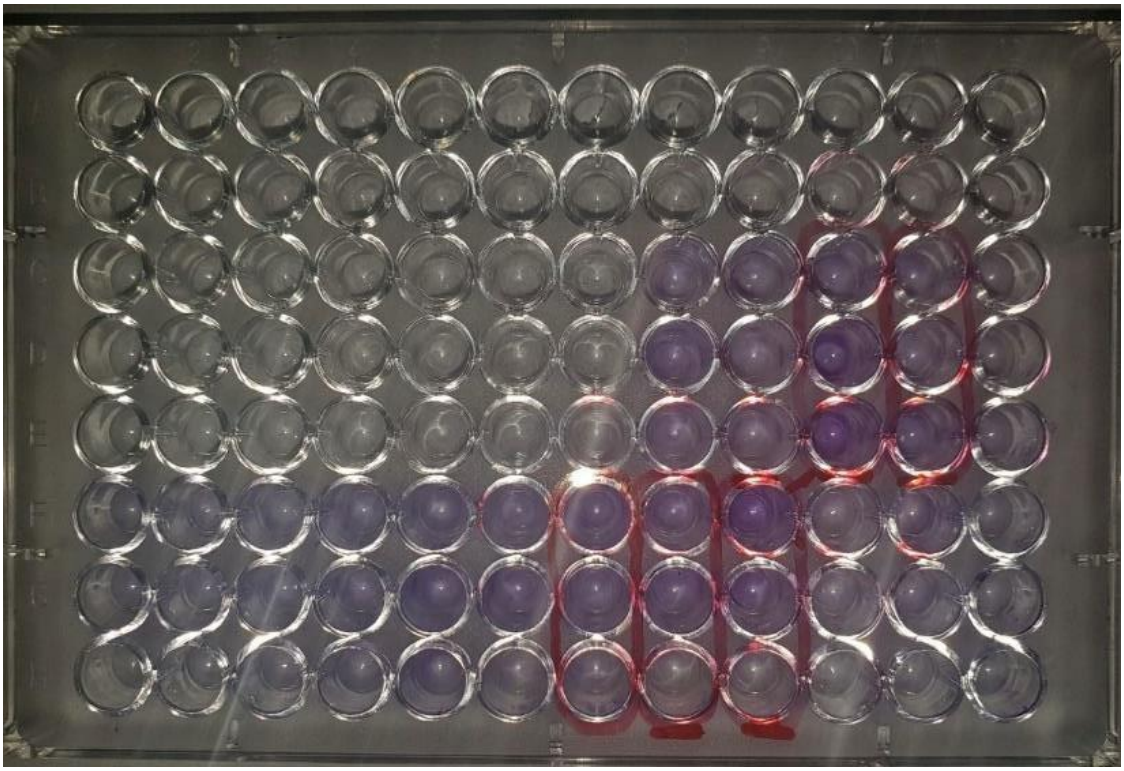

**Figure- 3: shown the biofilm formation of staph. Aureus.**

### 3.2 Plant extract's impact on the production of biofilms

Plant extracts were used to conduct biofilm inhibition investigations. At all investigated concentrations, S. aureus biofilm development was successfully suppressed. All plant extracts examined suppressed biofilm in a dose-dependent manner. P. major extracts at 10, 25, 50, and 75 µg/ml inhibited biofilms by 22.0, 34.5, 47.5, and 59.0 percent, respectively. At the same time, 100 µg/ml of P. major resulted in 65% inhibition (figure 4).

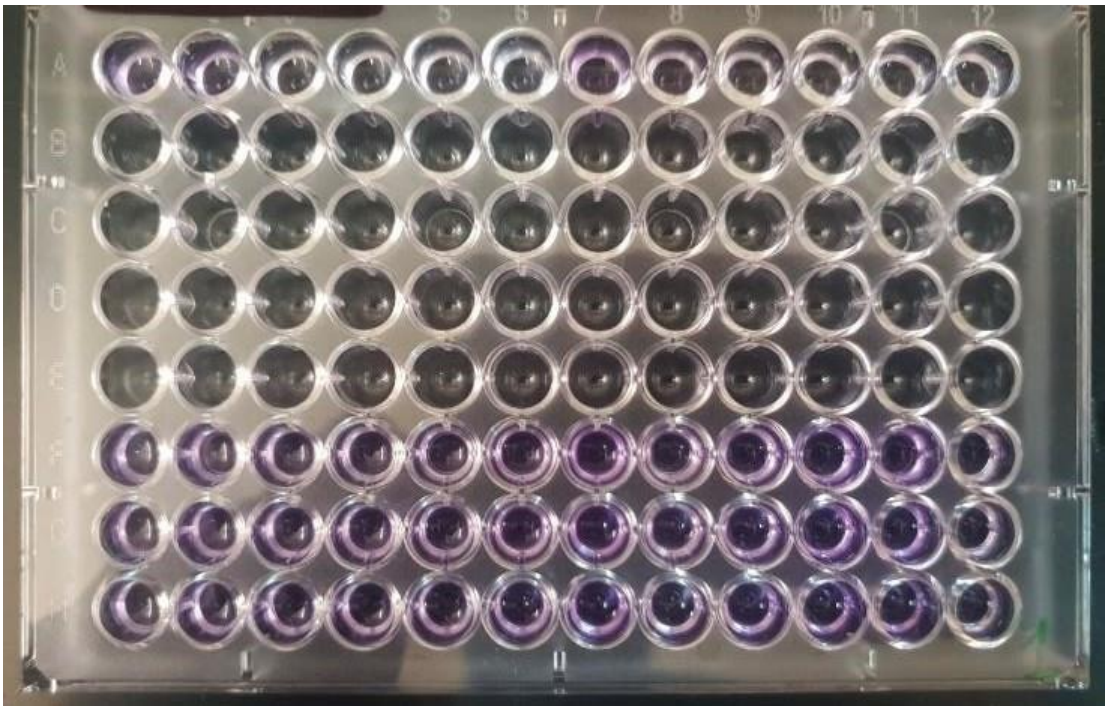

**Figure 4: ant-biofilm activity of P.major on s. aureus**

(Table 4) shows that there was a significant difference in bacterial inhibition by using a crude extract of P. major leaves for all treatment strains compared to the control, indicating that the extracts had antibacterial and anti-biofilm effects, inhibiting bacterial growth as well as biofilm formation against S. aureus (Carneiro et al., 2011) (Kaiser et al., 2013).

**Table 4 Anti-biofilm activity of P. major extract leaves on S. aureus.**

| S.No. | Biofilm inhibition (%) | | | | |
|---|---|---|---|---|---|
| | **10** | **25** | **50** | **75** | **100** |
| **1** | 22.0 | 34.5 | 47.5 | 59.0 | 65.0 |

| | | | | | |
|---|---|---|---|---|---|
| **2** | 12.0 | 32.0 | 43.0 | 51.0 | 65.0 |
| **3** | 12.0 | 21.0 | 30.0 | 37.6 | 41.9 |
| **4** | 10 | 16.2 | 23.4 | 29.0 | 33.0 |

## 4. DISCUSSION

biofilm-related etiology of S. aureus infections is complicated and involves host variables, patients are at risk and treatment might be challenging (Samuelsen, 2000). The current investigation found that the P. major extract significantly reduced S. aureus growth while also having a substantial inhibitory effect on biofilm formation. These findings are consistent with past data (Zubair et al., 2012). People have sought healing from nature since antiquity. Plants have been employed because they are effective, low-cost, and easily accessible. Medicinal herbs are an important natural resource because of their antibacterial properties (Tamilvanan et al., 2008). Compared to synthetic medications, their side effects are usually milder. In rural and isolated areas where people rely on herbal treatments and folklore, they are vital to their healthcare systems. P. major is a plant whose medicinal properties have been used for years (Holetz et al., 2002) (Hassawi & Abeer, 2006).

## 5. CONCLUSION

1. When compared to the overall growth of S. aureus, P. major extract showed antibacterial action.
2. Strong suppression of S. aureus biofilm development was shown by P. major extract.

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