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# Antibacterial and antibiofilm activities of Tunisian mulberry (*Morus alba* L.) leaves aqueous extract against pathogenic bacteria

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**Abstract**
The misuse or excessive use of antibiotics in several fields, such as agriculture, food and pharmaceutical industries and medicine leads to the emergence of multi-resistant bacteria and the evolution of antimicrobial resistance genes with serious consequences on human health. Historically, mulberry has been effectively used as a traditional medicine in Asia for the treatment of various infectious and internal diseases. It is a rich source of bioactive compounds that can promote human healthy life. This study was undertaken with the aim to evaluate the antibacterial and antibiofilm activities of Tunisian cultivated mulberry (*Morus alba* L.) leaves aqueous extract (MLAE). The antibacterial activity of MLAE was evaluated by micro-dilution method and the anti-biofilm effect was assessed using a crystal violet test against two Gram-positive bacteria (*Staphylococcus aureus* and *Staphylococcus epidermidis*) and two Gram-negative strains (*Escherichia coli* and *Bacillus cereus*). The mulberry leaf extracts revealed significant antibacterial activity against all bacterial strains. The minimum inhibitory concentration (MIC) varied between 2.03 and 16.25 mg/mL and the minimum bactericidal concentration (MBC) ranged from 8.12 to 32.5 mg/mL. Also, the mulberry extracts exhibited a great ability of biofilm formation inhibition as well as the eradication of the pre-installed biofilm against all tested bacterial strains. The results showed that mulberry leaves extract has an effective potential as natural antibacterial and seemed to be useful in pharmaceutical, cosmetics, and food industries with beneficial properties to human health. Therefore, supplementing a balanced diet with mulberryleaves extract may have beneficial health effects.

**Keywords:** *Morus alba* L., leaves extract, pathogenic bacteria, antibacterial activity, antibiofilm effect

## 1. Introduction
Despite the rapid progresses in drug discovery, the bacteria being “smart” adopt many drug resistance strategies, such as drug molecule inactivation, mutant protein synthesis and biofilm production [1]. Bacteria within biofilms are more resistant to antibiotics and disinfectants than individual cells in suspension. Indeed, the majority of infections in humans are caused by microorganisms in a biofilm state [2]. Therefore, controlling bacteria biofilm formation’s still a challenging issue. Is requires discovery and analysis of effective and safe alternative antimicrobials that may be used to prevent antibiotic resistance and infection recurrence [3-6]. Consequently, natural bioactive molecules from aromatic and medicinal plants are the subject of several researches in order to discover and develop effective and safe alternative antibacterial agents, for the control of bacterial biofilms formation [2, 7-10]. The plant bioactive molecules, such as essential oils (EOs) and polyphenolic components, have been used for thousands of years as natural medicines to fight against a multitude of pathogens, such as bacteria, fungi and viruses [2,3,11].
Historically, mulberry has been effectively used as a traditional medicine in Asia for the treatment of various infectious and internal diseases. It is a rich source of bioactive compounds that can promote human healthy life [12-15]. Mulberry (*Morus alba* L.) of the Moraceae family is native to China. This plant is also widely cultivated in India, Japan, Korea, amongst other countries that have warm temperatures such as Mediterranean, sub-tropical and tropical environments including African and European countries [16, 17]. Mulberry leaves have been used for thousands of years in traditional chinese medicine, to treat a myriad of illnesses/diseases and can promote human healthy life [16]. Mulberry foliage is valued as the primary food for silkworms, supporting the silk industry for centuries.

In particular, the leaves of *M. Alba* are known to contain high concentrations of essential micronutrients, such as iron and vitamin C, alkaloids, flavonoids, polyphenols, and phenolic acids [18]. These compounds often exhibit wide range of biological activities that include antioxidant, anti-inflammatory, antihypertensive and antimicrobial properties [19-22]. However, to the best of our knowledge, no previous work has investigated the antibacterial and antibiofilm activities of Tunisian mulberry (*Morus alba* L.) leaves aqueous extract. In this context, this study was undertaken with the aim to evaluate the antibacterial and antibiofilm activities of Tunisian cultivated mulberry (*Morus alba* L.) leaves aqueous extract with a view to valorization of this plant growing in Tunisia.

## 2. Materials and Methods

### 2.1. Plant material collection

The plant material used consists of the leaves of white mulberry plants (*Morus alba* L.), which were harvested during the fruiting period (May 2021), in Gafsa and Sidi Bouzid regions (Table 1). After collection, the leaves were washed with tap water and were dried at room temperature for 10 days and afterwards dried in a forced-air drier at 35 ◦C for 48 h, until they reached a constant weight. The dried leaves were ground into a fine powder and stored in glass cans at 4 °C until use.

**Table 1:** Description of the collection sites and their eco-geographic characteristics

| Collection site | Bioclimaticstage | Rainfall (mm/year) | Geographical location | | |
|---|---|---|---|---|---|
| | | | Altitude (m) | Latitude (E) | Longitude (N) |
| MA. SB | Upper arid | 230.4 | 291 | 34°37'7.38’’ | 9°21'1.97’’ |
| MA. G | Lower arid | 222 | 298 | 9°16’1.2’’ | 34°28'1.2’’ |

**MA:** *Morus alba* L., **SB:** Sidi Bouzid, **G:** Gafsa

### 2.2. Preparation of mulberry leaves aqueous extract

The preparation of mulberry leaves aqueous extract (MLAE) was carried out by maceration according to the method reported by Eva *et al*. (2015) [23] with some modifications [24]. Briefly, 200 mg of grounded sample was extracted using 6 ml of distilled water for 24 hours at room temperature. Then, the mixture was filtered and centrifuged for 5 min at 4000 rpm. The supernatant of extract was dried in a forced-air drier at 37°C. Finally, the obtained residue was recovered by 5 ml of distilled water and was kept in vials at 4 °C until the corresponding analyses were conducted.

### 2.3. Bacterial strains and culture conditions

The used bacterial support was composed of four referenced pathogenic strains. Two gram-positive bacteria: S1: *Staphylococcus aureus* (ATCC 25923) and S3: *Staphylococcus epidermidis* (CIP 106510) and two gram-negative bacteria: S5:*Escherichia coli* (ATCC 35218) and S9: *Salmonella typhimurium* (ATCC 1408). All strains were provided by the Laboratory of Analysis, Treatment and Valorization of Environmental Pollutants and Products (Faculty of Pharmacy of Monastir). Bacterial strains were grown in Trypticase Soy Broth (TSB, Merck, Darmstadt, Germany) and incubated at 37°C. The bacterial suspensions were adjusted with sterile saline to a concentration of $10^6$ CFU/ml. To verify the absence of contamination and the validity of the inoculum, dilutions of the inoculum were cultured on solid medium.

### 2.4. Antibacterial activity

#### 2.4.1. Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of MLAE was determined by employing a broth microdilution assay in a 96-well microtiter plate [2]. 100 µL of TSB broth was added to each well of sterile 96-well microplates. Then, 100 µL of stock solution of MLAE (130 mg/ml), prepared in DMSO 1%, was placed in the first well of the 96-well microplate, followed by a twofold serial dilution to reach a final concentration ranging from 65 to 32.5,16.25, 8.12, 4.06 and 2.03 mg/mL. A 10 µL aliquot of each tested strain, at a final concentration of $10^6$ CFU/mL, was added to each well and incubated at 37 °C and three replicates were performed. Positive controls containing TSB medium with inoculum and negative control wells containing medium with and DMSO 1% (v/v) were used. Following incubation, 10 µL of TTC solution (2,3,5-triphenyl tetrazolium chloride, 5mg/mL) was added as a growth indicator, and the mixture was incubated for another 30 min at 37 °C in the dark. TTC is reduced to red formazan in the presence of bacteria, indicating cell activity and viability [25]. Therefore, the well with the lowest concentration of MLAE at which bacterial growth was prevented and no pink-red coloration was observed, was assigned as the MIC value of the studied MLAE.

#### 2.4.2. Determination of minimum bactericide concentration (MBC)

The MBC was determined by serial subculturing of the samples taken from each where bacterial growth was not detected. In order to evaluate MBC, 10 µL of MLAE sample, was plated in Tryptic Soy Agar medium. Plates were incubated at 37 ◦C for 24 h. The evaluation of MBC was defined as the lowest MLAE concentration able to reduce and kill more than 99.9% of the initial inoculum. MBC was carried out in triplicate.

### 2.5. Antibiofilm activity

The anti-biofilm activity of the studied MLAE was tested against the same strains previously mentioned. The inhibition and eradication of biofilms were assessed in 96-well microplates [26], with some modifications [2]. Biofilm biomass quantification was performed using an optical density (OD) assay with crystal violet (CV) staining test.

#### 2.5.1. Inhibition of initial cell attachment

The pathogenic bacterial strains were grown overnight in TSB broth at 37 °C and diluted (1:100) with fresh medium supplemented with 2% glucose to obtain a final OD600nm of 0.2. 100 µL of culture dilution was dispensed into each well. Then, 100 µL of MLAE was added to each well according to the MIC. Wells containing only TSB broth supplemented with glucose and MLAE served as negative controls. The wells contained TSB broth supplemented with glucose, and the tested bacteria served as positive controls. The plates were incubated for 24 h at 37 °C. After incubation, the wells were emptied by tapping the plates into a disposal vessel. Each well was washing three times with 200 µL of sterile phosphate-buffered saline (PBS, pH 7.2) to remove planktonic cells.

Then, the plates were dried at 60 °C for 1h. Each well was stained with 150 µL of crystal violet solution (1%) for 15 min at room temperature. Afterward, the wells were rinsed three times with sterile water to remove the excess of crystal violet. To each well 200 µl of glacial acetic acid 30% (v/v) was added, and the plates were incubated for 1h at room temperature. Finally, the optical density (OD) of each well was measured using a microplate reader (Multiscan FC, Thermo Fisher Scientific) at a wavelength of 570 nm. All tests were performed in triplicate. To prove the ability of the studied MLAE to inihibit biofilm formation, the percentage of adherent bacteria inhibited was calculated using the following equation:

$$\% \text{ Biofilm inhibition} = [(\text{OD growth control} - \text{OD sample})/\text{OD growth control}] \times 100$$

### 2.5.2. Effect on installed biofilm

The ability of MLAE to eradicate the pre-established biofilms was calculated according to the method previously described by Ellafi *et al*. (2023) [2] with slight modifications. The medium and non-attached bacteria were removed after biofilm formation for 24-48h, and the plates were washed three times with PBS. Then, 200 µL of MLAE were added to each well according to the MIC for each strain. The plates were further incubated at 37 °C for 24h. After incubation, the biofilms were stained with crystal violet as described previously. Each experiment was evaluated in triplicate. The control was a biofilm without MLAE. The percentage of biofilm eradication we calculated using the following formula:

$$\% \text{ Biofilm eradication} = [(\text{OD growth control} - \text{OD sample})/\text{OD growth control}] \times 100$$

## 3. Results and Discussion

### 3.1. Antibacterial activity

The results of the *in vitro* screening of mulberry leaves aqueous extract (MLAE) antibacterial activity against pathogenic microorganisms showed variation in the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) (Table 2). The evaluation of antibacterial activity shows that our extracts are capable of inhibiting/killing the tested bacterial strains at low concentrations with MICs and MBCs vary from 2.03 to 16.25 mg/ml. Based on these results; we can conclude that Gram+ bacteria are more sensitive to MLAE than Gram- one.In fact, Wang *et al*., (2012) [19], found MICs similar to our results against these same strains for extracts of the same variety of Chinese origin: 1.56 mg/ml; 6.25 mg/ml and 3.12 mg respectively *Staphylococcus aureus* and *Escherichia coli*. Except for *Staphylococcus epidermidis* which found MICs higher than those found by our extracts, which are of 25 mg/ml.

Several studies based on antibacterial and antiviral activity have proven that *Morus alba* L. leaf extracts are effective in inhibiting the growth of bacteria, particularly against *Staphylococcus aureus*; *Staphylococcus epidermidis*; *Bacillus cereus* and *Salmonella typhimurium*. Moreover, the lack of antiviral drugs has allowed *M. alba* to outstand as an effective alternative to prevent and control viral diseases [27]. Actually, large numbers of studies have confirmed that mulberry extracts are often used as antibacterial agents because of their antibacterial activity against both gram-positive and gram-negative bacteria [28-30].

**Table 2:** Minimal inhibition concentration (MIC) and minimal bactericidal concentration (MBC) (mg/mL), of MLAE against the tested strains.

| | S1 | | S3 | | S5 | | S9 | |
|---|---|---|---|---|---|---|---|---|
| | **MIC** | **MBC** | **MIC** | **MBC** | **MIC** | **MBC** | **MIC** | **MBC** |
| **SB** | 2.03 | 16.25 | 8.12 | - | 16.25 | - | 16,25 | - |
| **G** | 2.03 | 8.12 | 2.03 | 16.25 | 8.12 | - | 16,25 | 32,5 |

**SB:** Sidi Bouzid**, G:** Gafsa**, S1***: Staphylococcus aureus*, **S3**: *Staphylococcus epidermidis*, **S5**: *Escherichia coli*, **S9**: *Salmonella typhimurium*, (-): not determined.

### 3.2. Antibiofilm Activity

Based on the results of biofilm inhibition, it is noted that MLAE of Sidi Bouzid (SB) and Gafsa (G) have a significant inhibition capacity against the tested germs. For the SB extract, the percentage of biofilm inhibition is significant, especially against *Staphylococcus aureus* (S1) and *Staphylococcus epidermidis* (S3), of 60.33% and 63.54% respectively. On the other hand, the capacity to inhibit the formation of *Salmonella typhimurium* (S9) biofilms for the same extract is low; the percentage of inhibition does not exceed 30% (Figure 1). Likewise for MLAE of G, which showed an inhibition capacity for the formation of biofilms against all strains tested. Indeed, the extract of G shows a very significant inhibition capacity against *Staphylococcus aureus* (S1) with a percentage of around 76%. Similarly for *Staphylococcus epidermidis* (S3) and *Salmonella typhimurium* (S9) the percentage of inhibition was around 51.57% and 45.73%, respectively (Figure 1).

Regarding the eradication of biofilms, both extracts show positive activity against all the pathogens studied. However, the SB extract shows a significant capacity to eradicate *Staphylococcus aureus* (S1) and *Staphylococcus epidermidis* (S3) biofilms with 70.73% and 78.37% respectively, but it is weak against *Salmonella typhimurium* (S9) with only 18.28%. Similarly, the G extract showed a very high efficiency in eradicating biofilms with percentages of 80.02% and 63.40% for *Staphylococcus aureus* (S1) and *Staphylococcus epidermidis* (S3) respectively, but a low capacity to eradicate *Salmonella typhimurium* (S9) biofilms (20.41%) (Figure 2).

Both extracts showed a significant ability to inhibit the formation of biofilms of different strains as well as their eradication. The MLAE therefore represent very powerful antibacterials by crossing the barriers of bacterial resistance. Several recent studies have shown that plant extracts rich in phenolic compounds having antimicrobial and antibiofilm properties [2, 7, 10, 30, 31-33]. Indeed, the topic of effects of white mulberry extracts on the formation and/or eradication of bacterial biofilms is not well studied, which explains the lack of information.

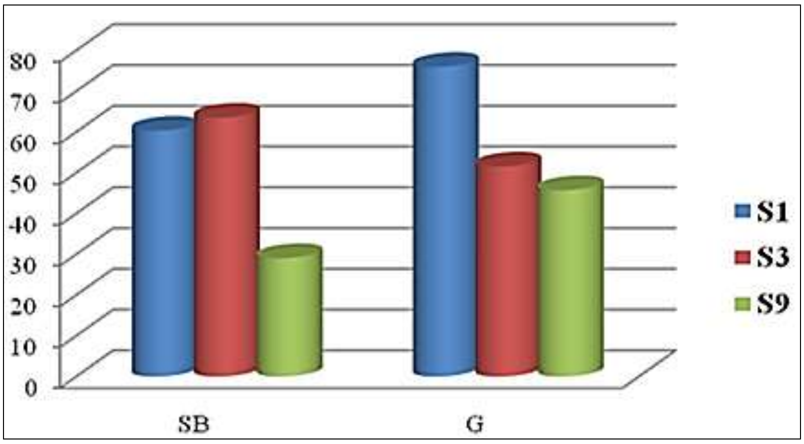


**Fig 1:** Effect of the studied MLAE on the inhibition of pathogenic bacteria biofilm formation (%). Code: SB: Sidi Bouzid, G: Gafsa, S1: *Staphylococcus aureus* (ATCC 25923), S3:*Staphylococcus epidermidis* (CIP 106510), S9: *Salmonella typhimurium* (ATCC 1408).

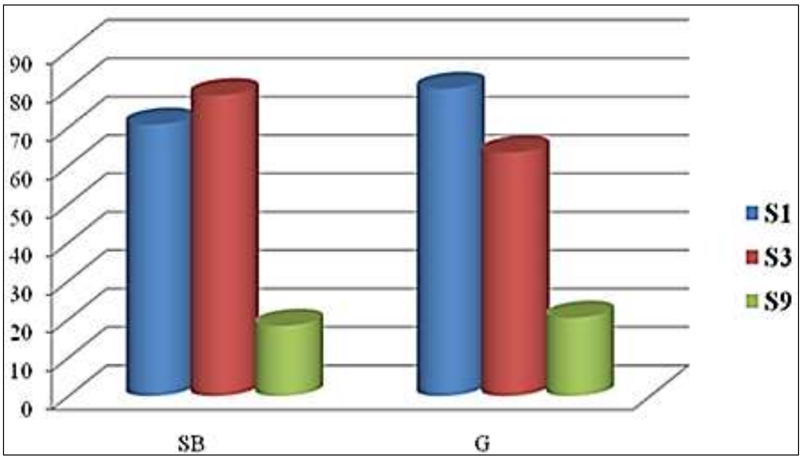


**Fig 2:** Effect of the studied MLAE on the eradication of established biofilm. Code: SB: Sidi Bouzid, G: Gafsa. S1: *Staphylococcus aureus* (ATCC 25923), S3:*Staphylococcus epidermidis* (CIP 106510), S9: *Salmonella typhimurium* (ATCC 1408).

## 4. Conclusion

The antibacterial activity results of mulberry leaves aqueous extract (MLAE) showed that the different extracts exhibited important inhibitory and bactericidal effects. Furthermore, the anti-biofilm activity showed that the studied MLAE has a potential anti-biofilm activity. Similarly, in the eradication activity, the majority of the tested MLAE was able to eradicate the bacterial preinstalled biofilms. The present study provides additional data in support of mulberry leaves extract as natural antimicrobial and antioxidant agents. These considerations warrant the introduction of mulberry bioactive molecules into complementary medicine as well as in the food and pharmaceutical industries. Future *in vivo* and clinical research is needed to explore the pharmacological applications and mechanisms of MLAE action.

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