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Study the Effect of *Alchemilla vulgaris* on Biofilm Production by *Staphylococcus Aureus* That Isolated from Wounds and Respiratory Tract of Patients

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Abstract

Alchemilla vulgaris, an aggregate species commonly called lion's foot or Lady's mantle, is a perennial herbaceous plant from the Rosaceae family. Clinical specimens were cultured, including wound swabs, sputum, and throat swabs, and six isolates were identified as *Staphylococcus aureus* by colony morphology, Gram stain, cultivation on mannitol salt agar, coagulase test, and a set of biochemical tests, three isolates from wound swabs and three from the respiratory tract (sputum and throat swabs). *Alchemilla vulgaris*(L.) seeds ethanol extract was extracted by 70% ethanol, and aqueous extract by Soxhlet, then dried by lyophilizer. Phytochemical investigation was performed using petroleum ether, ethyl acetate, and 70% ethanol. Preliminary phytochemical examination showed the presence of alkaloids, flavonoids, phenols, glycosides, tannins, and saponins in both alcoholic and aqueous extracts of *Alchemilla vulgaris,* with more concentration in the alcoholic extract than in the aqueous extract. Antimicrobial sensitivity test was done by disk diffusion method using six antibiotics, and most isolates were multidrug resistant to oxacillin and gentamicin, while ciprofloxacin and levofloxacin showed high activity. Methanol extract shows more activity than aqueous extract, and *Staph. aureus* from wound sources were more susceptible than those isolated from the respiratory tract. Inhibition zone ranged from 20- 11 mm in diameter for methanol extract and 16- 8 mm in diameter for aqueous extract. MIC was 100 mg/ mL. Biofilm was detected by two methods, Congo red agar and microtiter plate assay reveal strong biofilm formation for all isolates. Antibiofilm activity was examined, and both ethanol and aqueous extracts decreased the bacterial biofilm formation.

Keywords: *Alchmilla vulgaris*, Biofilm, *Staphylococcus aureus,* Antimicrobial, Wound infection

Introduction

Recently, we have encountered a severe problem with bacterial resistance. The discovery of modern antibiotics and antibacterial chemicals cannot keep up with the growth of resistance. To address this issue, novel antibacterial chemicals, primarily from natural sources, need to be identified. Plants contain secondary metabolites with antibacterial properties [1]. Plants are a valuable source of antibacterial chemicals due to their ancient medicinal use.

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Combining plant extracts with commercial antibiotics can help prevent the spread of resistance [2]. Combining antibiotics can reduce the amount needed while increasing their potency at lower dosages. Genus *Alchemilla L* includes more than 300 species of herbaceous, clump-forming perennials that are found in mountainous areas of South America and Africa, as well as wet meadows, upland in Western Asia, Europe, and North America [3; 4]. Lady's mantle, or *Alchemilla vulgaris L*., is the best studied species in the genus. According to modern taxonomic interpretations, A. vulgaris is an assemblage of 12 apomictic microspecies with similar morphologies that frequently hybridize [5]. Around the world, lady's mantle is frequently employed in folk medicine. It has been stated that the upper portions of the plant can be used to treat wounds, rashes, inflammations, anemia, diabetes, ulcers, multiple sclerosis, hernias, gynecological diseases, abdominal diseases, and wounds [6;7]. *Alchemilla* species are used throughout Southeast Europe and the Balkans to treat a variety of inflammatory ailments, weight loss, skin disorders, lung infections, kidney and liver diseases, diabetes, diarrhea, and gynecological, menstrual, and menopausal complaints [8;9]. Additionally, this pharmaceutical plant has antibacterial, antiviral, and antifungal activities [10]. Regarding antibiotic resistance, plant medicines and extracts were regarded as powerful bactericidal agents that do not induce resistance to traditional antimicrobial agents [11]. An important human pathogen, *Staphylococcus aureus* can cause a variety of infections, including bacteremia, pneumonia, toxin-mediated illnesses, and infections of the skin and tissues. Simultaneously, 20% of people have S. aureus permanently colonizing their noses, and another 30% carry it sporadically [12]. One of the main risk factors for staphylococcal infections is colonization with S. aureus [13]. The significance of host factors is highlighted by the fact that 80% of nosocomial S. aureus bacteremia cases in carriers have an endogenous origin [14;15]. However, it has been challenging to provide a biological explanation for the variations observed in the disease-evoking potentials of S. aureus clones despite an abundance of evidence to support this claim [16;17]. S. aureus is generally considered to be one of the most common opportunistic human pathogens [18], capable of eluding immune system defenses and causing a wide range of infections from minor skin wounds to critically ill sepsis [19]. *S. aureus* is a well-known bacterium linked to wound infections among the many other types of infections; it typically colonizes the wound's outermost layer [20]. Specifically, wound infections caused by *S. aureus* may be considered a risk factor for MRSA concern [21], which has led to the development of untraditional antimicrobials that can replace conventional antibiotics. Furthermore, *S. aureus*, particularly MRSA, can stick to surfaces that are either living or inert by secreting an extracellular polymeric material called a biofilm that is made up of polysaccharides, proteins, nucleic acids, and water, the biofilm matrix serves as a barrier which hinders the drug's penetration into the bacterial colonies, aids in the microbe's resistance to conventional antibiotics, thereby reducing their impact [22].

Material and Method

Isolation of bacteria:

Wound swabs, sputum, and bronchial wash were collected from patients in Baghdad medical city in 2024 and cultured on blood agar and chocolate agar for 24 hours of incubation.

Yielded bacteria were examined morphologically on blood agar and chocolate agar, stained by Gram stain, and examined microscopically and cultured on mannitol salt agar and human plasma. The identification was confirmed by the Vitek 2 system.

Preparation of *A. vulgaris* extracts

The extraction was prepared by mixing 500 grams of ready-to-use herbal powder with 1.5 L of water (for an aqueous extraction) or ethanol (for an alcoholic extraction) on a magnetic stirrer for 12 hours. Next, Whatman filter paper No. 1 was used to filter the

mixture. Supernatant convened. After the alcoholic extraction, the filtrates were dried at 55 °C, and the herbal material from the alcoholic extract was mixed with 1.5 milliliters of DMSO, and the mixture was then filtered through a Millipore filter with a 0.22 µm filter. The Stoke solution was then kept at 4 °C until it was needed. A powdered formulation was produced by lyophilizing the aqueous extraction in the interim.

Phytochemical evaluation

The active compounds were detected in the laboratories of the Biotechnology Research Center / Al-Nahrain University, using chemical reagents.

0.1mg of ethanol and methanol extract of Albizia leaves were weighed and dissolved in 10ml sterile distilled water (D.W), using chemical reagents.

Tanin detection

One milliliter of the extract is mixed with several drops of lead acetate (1%) to identify tannins. Tannins were present because a white, gelatinous precipitate developed.

Flavonoid investigation

Alkaline reagent test: a brilliant yellow color is produced by mixing a sodium hydroxide solution with a quantity of extraction solutions and then waiting, indicating the presence.

Alkaloid detection (Dragendorff test)

Alkaloids were detected using the Dragendorff detector. Two solutions were used to prepare the reagent:

Solution A: 0.2 ml of strong hydrochloric acid (HCl) was added to 60 mg of dissolved sub-nitrate bismuth.

One milliliter of distilled water is added to Solution B, which contains potassium iodide.

The extract is combined with the solution (A + B).

When alkaloids are present, the hue changes from o range to brown.

Detecting polysaccharides

One milliliter of the liquid extract and two milliliters of Benedict reagent are mixed, shaken thoroughly, put in a boiling bath for five minutes, then allowed to cool in order to detect if sugars are present. When there are sugars present, a crimson precipitate will form.

Identifying Saponins

The extract solution will be thoroughly shaken to complete the detection process. The presence of saponins will be shown by the creation of foam on top of the extract.

Polyphenolic Compounds Detection

Creation of a brown precipitate after adding several drops of ferric chloride 3% solution to the extraction solution.

Antibiotic resistance:

Utilizing the Kirby-Bauer technique, disk diffusion is carried out by inoculating agar plates with a standardized bacterial solution with turbidity about equal to McFarland 0.5, placing antibiotic disks on top, and then incubating at 37 °C. To determine whether the microorganism is susceptible or resistant, the inhibition zone around antibiotic disks was measured and then compared to the interpretation criteria.

Herbal extracts' antimicrobial properties:

Bactericidal activity has been investigated by the Mueller-Hinton agar disc diffusion method [23]. A sterile cork borer has been used for forming three wells on the Muller Hinton agar plate, which was covered by bacterial culture. Three wells have been used: one for an alcoholic extract, another for an aqueous extract, and the third, which served as a control, held only DMSO. After a 24-hour incubation period at 37°C, inhibitory zones on the plates were measured.

Plant extracts' minimum inhibitory concentration (MIC):

Sterile 96-well microtiter plates were used to determine the MIC in compliance with the Clinical and Laboratory Standards Institute (CLSI) Guidelines [24].

1. Filling of each microliter plate with 100 microliters using broth medium.

2. Filling of the first well with 0.1 ml of the extract at a concentration of 400 mg/ml.
3. In the container with the previous additions, move 100 microliters from the first well to the second well. 100 microliters should also be moved from the second well to the third. Lastly, remove 100 microliters from the final well and discard them.
4. After comparing it with McFarland, ten microns of activated bacteria were added to each well in the preceding row, except for the last two (one containing only broth with extract, and the other containing only broth as a control), and they were cultured for the entire night.
6. Generating a Resazurin dye concentration of 0.015%

The results will be shown by re-incubating the microtiter plate in the incubator for two hours after adding 0.015g in 100 D.W. and making sure that each well contains 20 Microtiter plate Solution [25].

Congo red agar: Congo red agar (CRA) is prepared from brain heart infusion (BHI) agar, sucrose, and Congo red. It is used for biofilm detection by cultivation of bacteria on CRA and incubation for 24 hours at 37 °C [26].

Microtiter plate assay: Microtiter plate assay (MPA) is used for biofilm detection by filling the well with 200 µl from diluted bacterial culture and incubation for 24 hours at 37 C then stained by crystal violet and reading the absorbance.

Results and Discussion

Isolation of bacteria: cultivation of wound swabs yielded three isolates of *Staphylococcus aureus;* similarly, cultivation of sputum and bronchial wash yielded three isolates.

Phytochemical evaluation

Phytochemical evaluation shows the presence of active compounds in distinct levels in both ethanol extract and aqueous extract of *Alchemilla vulgaris,* as shown in Table 1.

Compound	Ethanol extract	Aqueous extract
Tannins	+++	+++
Polysaccharides	++	++
Alkaloides	+++	+
Saponins	+++	++
Flavonoids	+++	+++
Polyphenolic compounds	-	+++

Polyphenol compounds in A. vulgaris extracts are responsible for many biological activities [27].

Antibiotic resistance

All isolates show antibiotic resistance patterns (Table 2) as six isolates were resistant to azithromycin and rifampin, with no sensitive isolate for each of them (0%), oxacillin and gentamicin with one sensitive isolate for each of them (16.67%) and sensitive to levofloxacin (50%) and ciprofloxacin (33.33%) as shown in figure (1).

Table (2) Antibiotic resistance

Isolate	OXA	AZM	CN	Rif	Cip	Lev
St-w1	R	I	I	R	I	I
St-w2	R	R	I	R	I	S
St-w3	S	I	I	R	S	S
St-r1	R	R	S	R	S	S
St-r2	R	I	I	R	R	R
St-r3	R	R	I	R	R	R

OXA: Oxacillin, AZM: Azithromycin, CN: Gentamicin, Rif: Rifampin, Cip: Ciprofloxacin, Lev: Levofloxacin, S: Sensitive, I: Intermediate, R: Resistant.

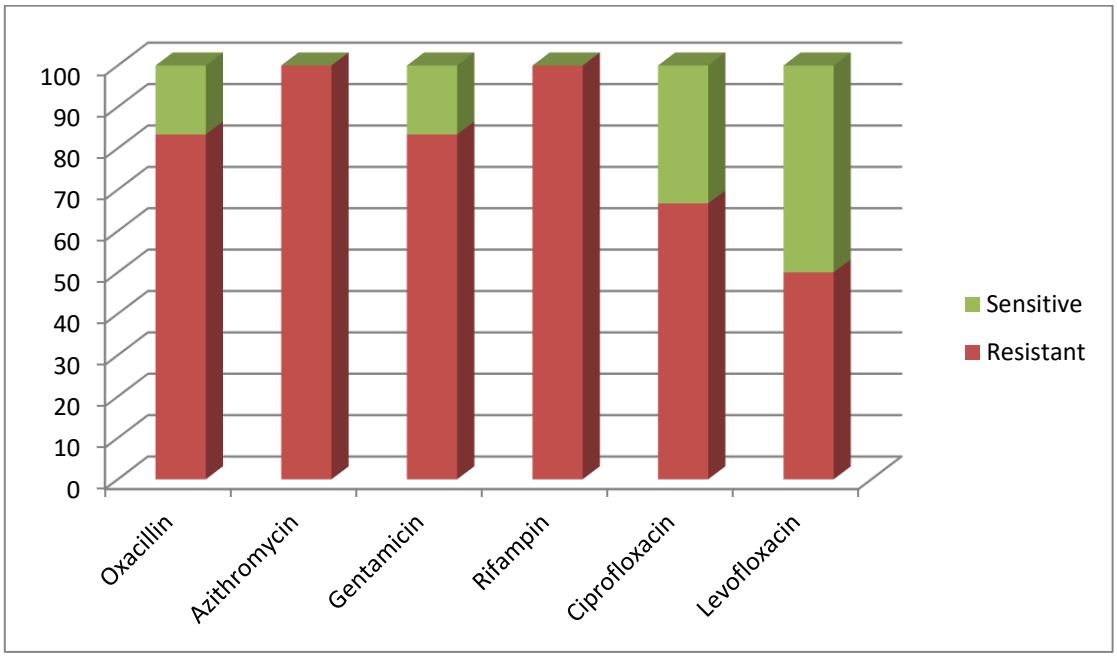

Figure 1: antibiotic resistance ratio.

Previous studies recorded increased antibiotic resistance in *Staphylococcus aureus,* and this resistance was mediated by varied mechanisms like β-lactamase production and biofilm formation. Methicillin-resistant *Staphylococcus aureus* (MRSA) is the most prevalent virulence pattern

Biofilm formation

All isolates were biofilm producer and their ability to produce biofilm ranged between moderate and strong, as shown in Table 3.

The biofilm is a frequent virulence factor in *Staphylococcus aureus,* which enables bacteria to attach to different surfaces for long durations.

Table (3) Biofilm production

Wound isolates		**Respiratory tract isolates**	
Isolate	**Biofilm production**	**Isolate**	**Biofilm production**
St-w1	Moderate	St-r1	Strong
St-w2	Strong	St-r2	Strong
St-w3	Strong	St-r3	Strong

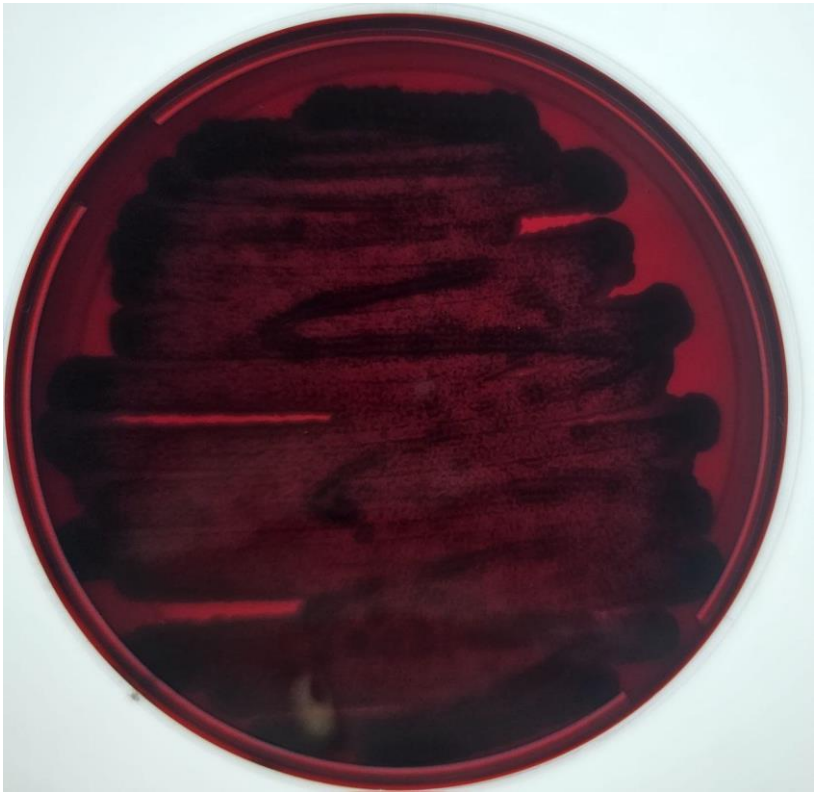

Figure (2) Biofilm producer *Staphylococcus aureus* on Congo red agar, colonies appear in black color

Herbal extract activity by disc diffusion

Isolates show an inhibition zone ranging in diameter, and alcoholic extract show larger bacterial inhibition zone than aqueous extract, as listed in tables (4) and (5) below.

Table (4) Aqueous extract inhibition zone for different concentrations (mg/ mL)

Isolate	100	50	25	12.5
St-w1	14 mm	12 mm	11 mm	9 mm
St-w2	16 mm	12 mm	16 mm	18 mm
St-w3	12 mm	11 mm	10 mm	12 mm
St-r1	-	-	12 mm	14 mm
St-r2	13 mm	11 mm	11 mm	12 mm
St-r3	10 mm	9 mm	9 mm	8 mm

Table (5) Alcoholic extract inhibition zone for different concentrations (mg/ mL)

Isolate	100	50	25	12.5
St-w1	8 mm	8 mm	12 mm	14 mm
St-w2	20 mm	17 mm	18 mm	15 mm
St-w3	14 mm	12 mm	13 mm	12 mm
St-r1	-	-	-	-
St-r2	14 mm	13 mm	12 mm	11 mm
St-r3	15 mm	13 mm	14 mm	15 mm

These results are consistent with [27] that *A. vulgaris* extracts have an antibacterial activity against a spectrum of gram-positive and negative bacteria.

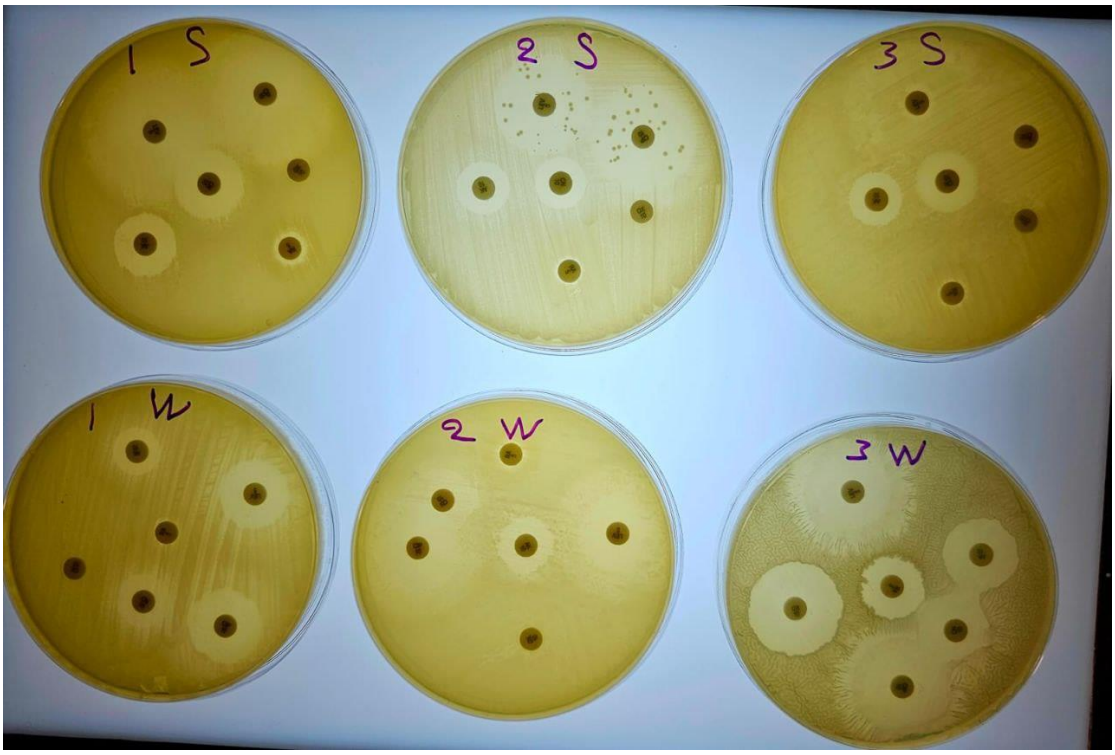

Figure 3: MIC of extracts for *Staphylococcus aureus* by the disk diffusion method

Minimal inhibitory concentration

The minimal inhibitory concentration (MIC) for *Alchemilla vulgaris* extracts was examined by microplate assay using Resazurin. MIC regarding the examined *Staphylococcus aureus* isolates was 100 mg/ mL for both ethanol extract and aqueous extract. The results are compatible with [28] that *Staphylococcus aureus* is more weakly susceptible to *A. vulgaris* extracts than other bacterial species.

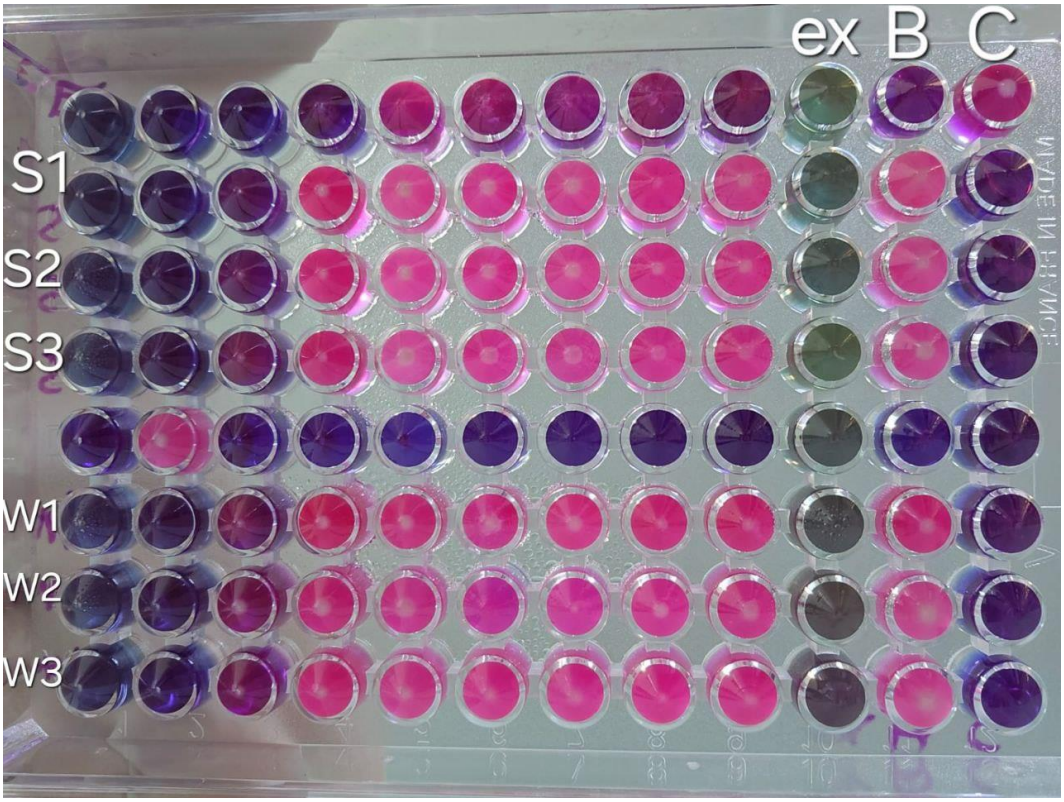

Figure 4: MIC of ethanol extract for *Staphylococcus aureus* by microtiter plate method using resazurin

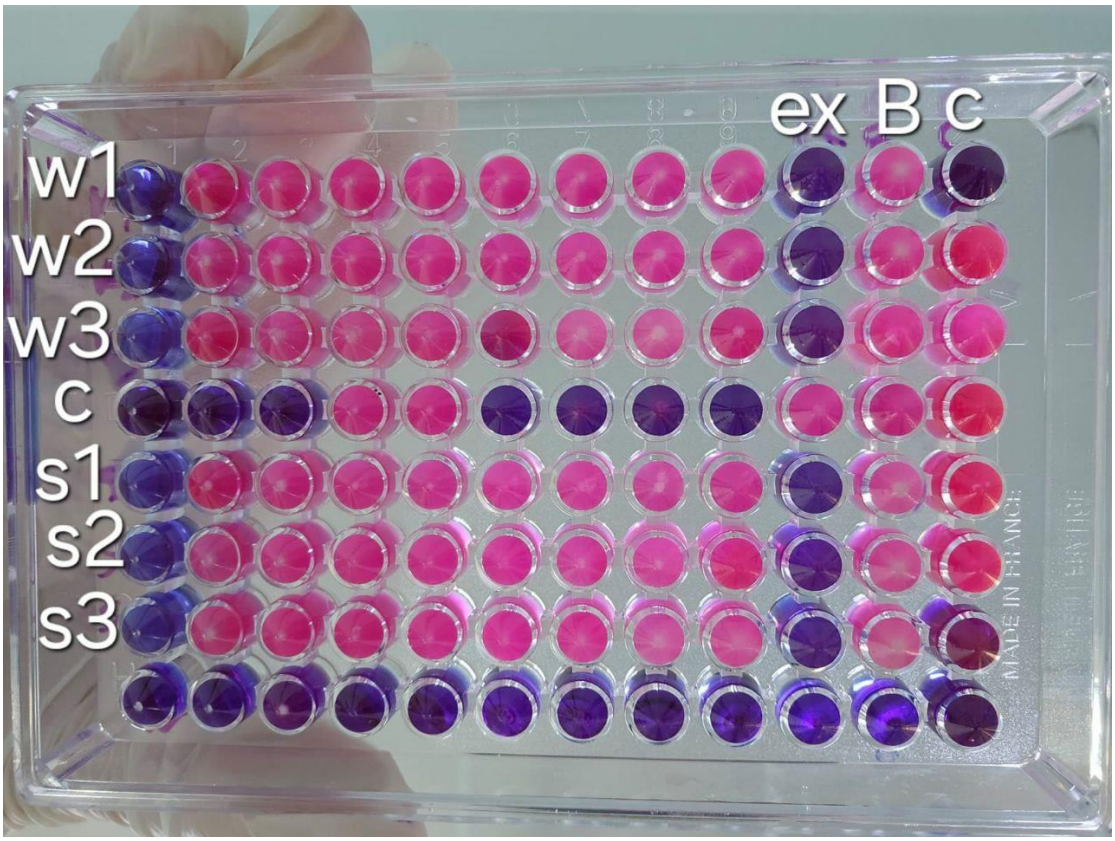

Figure 5: MIC of aqueous extract for *Staphylococcus aureus* by microtiter plate method using resazurin

Antibiofilm activity

The extracts had an antibiofilm activity and decreased biofilm formation at sub-MIC doses (50, 25, and 12.5) mg/ mL of both ethanol extract and aqueous extract; this is similar to previous studies that some plant extracts have an antibiofilm effect for *Staphylococcus aureus* [29].

Figure 6: Antibiofilm activity for *Staphylococcus aureus* by microtiter plate method

Conflict of interest: **NIL**

Funding: **NIL**

References

1. Abdallah, E. M., Alhatlani, B. Y., de Paula Menezes, R., & Martins, C. H. G. (2023). Back to nature: Medicinal plants as promising sources for antibacterial drugs in the post-antibiotic era. Plants, 12(17), 3077.
2. Almeida, G.C.M.; dos Santos, M.M.; Lima, N.G.M.; Cidral, T.A.; Melo, M.C.N.; Lima, K.C. Prevalence and factors associated with wound colonization by Staphylococcus spp. and Staphylococcus aureus in hospitalized patients in inland northeastern Brazil: A cross-sectional study. BMC Infect. 2014, 14, 328.
3. Atef, N.M.; Shanab, S.M.; Negm, S.I.; Abbas, Y.A. Evaluation of Antimicrobial Activity of Some Plant Extracts Against Antibiotic Susceptible and Resistant Bacterial Strains Causing Wound Infection. Bull. Natl. Res. Cent. 2019, 43, 144.
4. Bradley, P. British Herbal Compendium: A Handbook of Scientific Information on Widely Used Plant Drugs; British Herbal Medicine Association: Bournemouth, UK, 2006; Volume 2, ISBN 9780903032124.
5. Cheesman, M. J., Ilanko, A., Blonk, B., & Cock, I. E. (2017). Developing new antimicrobial therapies: are synergistic combinations of plant extracts/compounds with conventional antibiotics the solution?. Pharmacognosy reviews, 11(22), 57.
6. CLCI (2011) Performance standards for antimicrobial susceptibility testing. Wyne. PA: CLC
7. Ergene, B.; Acikara, Ö.B.; Bakar, F.; Saltan, G.; Nebioglu, S. Antioxidant Activity and Phytochemical Analysis of ˇ Alchemilla persica Rothm. Ank. Univ. Eczaci. Fak. Derg. 2010, 39, 145–154.
8. Filippova, E.I. Antiviral Activity of Lady's Mantle (Alchemilla vulgaris L.) Extracts against Orthopoxviruses. Bull. Exp. Biol. Med. 2017, 163, 374–377.
9. Gehrke, B.; Bräuchler, C.; Romoleroux, K.; Lundberg, M.; Heubl, G.; Eriksson, T. Molecular Phylogenetics of Alchemilla, Aphanes and Lachemilla (Rosaceae) Inferred from Plastid and Nuclear Intron and Spacer DNA Sequences, with Comments on Generic Classification. Mol. Phylogenetics Evol. 2008, 47, 1030–1044.
10. Ghedira, K.; Goetz, P.; Le Jeune, R. Alchemilla vulgaris L.: Alchémille (Rosaceae). Phytothérapie 2012, 10, 263–266.
11. Guo, Y., Song, G., Sun, M., Wang, J and Wang, Y. (2020). Prevalence and Therapies of Antibiotic-Resistance in *Staphylococcus aureus*. Front. Cell. Infect. Microbiol, 10.
12. Harika K, Shenoy VP, Narasimhaswamy N, Chawla K. Detection of Biofilm Production and Its Impact on Antibiotic Resistance Profile of Bacterial Isolates from Chronic Wound Infections. J Glob Infect Dis. 2020 Aug 29;12(3):129-134.
13. Idrees, M.; Sawant, S.; Karodia, N.; Rahman, A.; Health, P. Staphylococcus aureus biofilm: Morphology, genetics, pathogenesis and treatment strategies. Int. J. Environ. Res. 2021, 18, 7602.
14. Ke, Y.; Ye, L.; Zhu, P.; Zhu, Z.J.F.i.M. The clinical characteristics and microbiological investigation of pediatric burn patients with wound infections in a tertiary hospital in Ningbo, China: A ten-year retrospective study. Front. Microbiol. 2022, 13, 5238.
15. Lindsay, J. A., C. E. Moore, N. P. Day, S. J. Peacock, A. A. Witney, R. A. Stabler, S. E. Husain, P. D. Butcher, and J. Hinds. 2006. Microarrays reveal that each of the ten dominant lineages of Staphylococcus aureus has a unique combination of surface-associated and regulatory genes. J. Bacteriol. 188: 669–676.

16. Melles, D. C., R. F. J. Gorkink, H. A. M. Boelens, S. V. Snijders, J. K. Peeters, M. J. Moorhouse, P. J. van der Spek, W. B. van Leeuwen, G. Simons, H. A. Verbrugh, and A. van Belkum. 2004. Natural population dynamics and expansion of pathogenic clones of Staphylococcus aureus. J. Clin. Investig. 114:1732–1740.
17. Nouwen, J., M. Fieren, S. Snijders, H. Verbrugh, and A. van Belkum. 2005. Persistent (not intermittent) nasal carriage of Staphylococcus aureus is the determinant of CPD-related infections. Kidney Int. 67:1084–1092.
18. Pollitt, E.J.; Szkuta, P.T.; Burns, N.; Foster, S.J. Staphylococcus aureus infection dynamics. PLoS Pathog. 2018, 14, e1007112.
19. Sarker SD, Nahar L, Kumarasamy Y. Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals. Methods (San Diego, Calif.). 2007; 42: 321-4
20. Šavikin, K.; Zduni´c, G.; Menkovi´c, N.; Živkovi´c, J.; Cuji´c, N.; Tereš˘cenko, M.; Bigovi´c, D. Ethnobotanical Study on Traditional Use ´ of Medicinal Plants in South-Western Serbia, Zlatibor District. J. Ethnopharmacol. 2013, 146, 803–810.
21. Sepp, S.; Bobrova, V.K.; Troitsky, A.K.; Glazunova, K.P. Genetic Polymorphism Detected with RAPD Analysis and Morphological Variability in Some Microspecies of Apomictic Alchemilla. Ann. Bot. Fenn. 2000, 37, 105–123.
22. Serra, R.; Grande, R.; Butrico, L.; Rossi, A.; Settimio, U.F.; Caroleo, B.; Amato, B.; Gallelli, L.; De Franciscis, S. Chronic wound infections: The role of Pseudomonas aeruginosa and Staphylococcus aureus. Expert Rev. Anti-Infect. Ther. 2015, 13, 605–613.
23. Tadi´c, V.; Krgovi´c, N.; Žugi´c, A. Lady's Mantle (Alchemilla vulgaris L., Rosaceae): A Review of Traditional Uses, Phytochemical Profile, and Biological Properties. Lek. Sirovine 2020, 40, 66–74.
24. Tadic, V., Krgovic, N and Žugic, A. (2020). Lady's mantle (Alchemilla vulgaris L., Rosaceae): A review of traditional uses, phytochemical profile, and biological properties. Natural Medicinal Materials, 40: 66-74.
25. von Eiff, C., K. Becker, K. Machka, H. Stammer, and G. Peters. 2001. Nasal carriage as a source of Staphylococcus aureus bacteremia. N. Engl. J. Med. 344:11–16.
26. Wertheim, H. F., D. C. Melles, M. C. Vos, W. van Leeuwen, A. van Belkum, H. A. Verbrugh, and J. L. Nouwen. 2005. The role of nasal carriage in Staphylococcus aureus infections. Lancet Infect. Dis. 5:751–762.
27. Wertheim, H. F., M. C. Vos, A. Ott, A. van Belkum, A. Voss, J. A. Kluytmans, P. H. van Keulen, C. M. Vandenbroucke-Grauls, M. H. Meester, and H. A. Verbrugh. 2004. Risk and outcome of nosocomial Staphylococcus aureus bacteraemia in nasal carriers versus non-carriers. Lancet 364:703–705.
28. Winn, Jr., W., et al. 2006. Konemann's color atlas and diagnostic text of microbiology, 6th ed., p. 945–1021. Lippencott Williams & Wilkins Publishers, Philadelphia, PA.
29. Wu, X., Wang, H., Xiong, J., Yang, G.X., Hu, J.F., Zhu, Q and Chen, Z. (2024). *Staphylococcus aureus* biofilm: Formulation, regulatory, and emerging natural products-derived therapeutics. Biofilms, 7, 100175.