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Evaluation of the Antibacterial Activity of Shilajit Aqueous Extract on Clinically Isolated Bacteria in Kalar District, Iraq

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Abstract:

Background: Multidrug-resistant (MDR) bacteria represent a significant danger to public health worldwide, complicating treatment strategies and contributing to high mortality rates. Among the prominent pathogens, *Staphylococcus aureus* and *Pseudomonas aeruginosa* exhibit robust resistance profiles against multiple antibiotics, necessitating the exploration of alternative therapeutic options. Shilajit, a traditional medicinal substance originating from the Himalayas and other mountainous regions, has garnered attention for its diverse biological activities, including antimicrobial properties.

Objectives: This study investigates the potential of Shilajit aqueous extract against clinically isolated MDR strains of *S. aureus* and *P. aeruginosa* from the Kalar District, Iraq.

Methodology: Antibacterial efficacy was evaluated using disk diffusion and broth micro-dilution methods. Statistical significance was determined using one-way ANOVA. All experiments were performed in triplicate, with $p < 0.05$ considered statistically significant.

Results: Results demonstrated significant inhibition of *S. aureus* at concentrations above 25 mg/mL, while no activity was observed against *P. aeruginosa*. These findings underscore the potential of Shilajit as a natural resource for developing alternative therapies amidst the growing concern of antibiotic resistance.

Conclusions: Shilajit's efficacy against *S. aureus* highlights its promise as a targeted treatment option, advocating for further exploration into its mechanisms and broader application in combating antibiotic-resistant infections.

Recommendations: Preventive programs focusing on encouraging the use of Shilajit and similar natural products should be considered. Further research is recommended to explore the mechanisms of Shilajit's antibacterial activity and to evaluate its efficacy in clinical settings.

Keywords: ***Shilajit, Minimal inhibitory concentration (MIC), Disk Diffusion Method, Antibacterial activity assay , Broth Micro-Dilution Method.***

Introduction: Infectious diseases cause serious challenges to human health in the world while the majority of these infections predominantly caused by pathogenic microorganisms especially bacteria, rickettsia, fungi, and viruses (1). According to reports, bacteria are the cause of up to 30% of all infections That accounting for millions of human deaths each year(2). In response to the threat crisis caused by microbial diseases, pharmaceutical markets have seen a significant development and introduction of synthetic antibiotics. However, The inappropriate and excessive use of synthetic antibiotics has over time led to the appear of drug-resistant microorganisms, creating a new worldwide therapeutic issue for the public health system known as antibiotic resistance(3). In recent years, research has focused on alternative antibiotics due to the increasing antibacterial resistance and adverse effects of antibacterial agents(4).

The search for safe and efficient antibacterial drugs is necessary in response to the problem of bacterial infection. Any approach to solve the problem focuses on developing new antibiotics that are effective against bacteria that resistant to existing antibiotics; However, opinions differ on which tactics can best make these discoveries(5). For decades natural products have been used as conceptual and material starting points in the search for new antibiotics. However, due to the inherent difficulties in making accurate chemical modifications of the structurally complex natural products (semisynthesis), the rate of novel drug discovery through this approach has significantly slowed(6).

Traditional medicine uses shilajit to treat and cure a wide range of illnesses. Therefore, a significant amount of conformational discover was designed to report the mechanism of action of herbo-medicinal extracts. Crude Shilajit could be used for a variety of purposes, including anti-ulcerogenic, anti-bacterial, anti-fungal, anxiolytic, anti-allergic, anti-inflammatory, analgesic, antioxidant, and antidiabetic effects(7-11).

The origin of shilajit is explained according to three main theories: geological, biological and biomineralogical. According to the biological theory, Shilajit is obtained from the decomposition of plant materials or animal wastes under specific physical and chemical conditions. According to geological theory, Shilajit was formed as a result of geological processes over a long period of time. According to the biomineral theory, shilajit originates from the mechanical contamination of alkaline precursors by combining them with mineral components(12). In addition, Moomiaii dissolves in water, with between 30 and 50 percent of its constituents moving into the liquid phase. The amount of sediments varies depending on sample purity(13)

Traditional herbal-medical treatment, such as shilajit, has gained widespread acceptance due to its effective antimicrobial activity with management of common ailments such as digestive issues, diabetes, chronic pain, anemia and osteoporosis. The current study we examined the antibacterial activity of shilajit by using in vitro models.

1. Research Objectives:

1. To evaluate the antibacterial efficacy of Shilajit aqueous extract against clinically isolated multidrug-resistant (MDR) strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa* from the Kalar District, Iraq.

2. To investigate the potential of Shilajit as a natural therapeutic agent for the treatment of antibiotic-resistant infections, with a focus on its minimum inhibitory concentration (MIC) and comparative effectiveness relative to standard antibiotics.

Materials and methods

3.1. Bacterial isolates : Chra Medical Laboratory from Kalar District in the Kurdistan Region of Iraq provided two pathogenic bacterial isolates, one Gram-positive and one Gram-negative. These isolates, obtained from clinical specimens, were initially identified as Staphylococcus aureus and Pseudomonas aeruginosa . The Vitek-2 system was used for confirmatory diagnosis, which verified the identities of the isolates as S. aureus and P. aeruginosa and also determined their antibiotic susceptibility patterns. Then these two isolates had been used in the current study.

3.2. Antibiotics susceptibility test : The disk diffusion method that further recognized as the Kirby-Bauer method used for various classes of antibiotics for their effectiveness against the bacterial isolates , as outlined by Bauer et al. (14) Then, respectively following the guidelines of Clinical Laboratory Standard Institute (CLSI) (15). The sensitivity of the bacteria to each antibiotic was reported by using electronic caliper to measure the diameter of the inhibition zones around the antibiotic disks. Reference tables were then used to categorize the bacteria as resistant (R), susceptible, or intermediate (I) to the antibiotics based on these measurements.

3.3. Preparation of shilajit aqueous extract : Shilajit bought in the Kalar area of the Sulaymaniyah Governorate in the Kurdistan Region of Iraq from a local herbal-medicine store. First, shilajit had been washed, air-dried, before being pieces thinly as possible. After the pieces had dried fully in a food drier for 12 hours, they were crushed into a rather fine powder. Following that, aqueous shilajit extracts were made according tPerumal et al. (16).To prepare a stock solution with concentration of 50 mg/mL for antimicrobial testing, approximately 5 grams of Shilajit powder was weighed and added to 250 mL beaker containing 100 mL of double-distilled water .Then, the mixture was heated about 60°C for 15 minutes. After cooling it to 25°C (room temperature), the mixture was then filtered to inhibit any solid debris through Whatman No. 1 filter paper, causing in a clear solution. The filtrate was subsequently stored at 4°C for further analysis.

3.4. Disk Diffusion Method Antibacterial activity assay of shilajit aqueous extract : Analysis of shilajit aqueous extract of antibacterial activity against antibiotic-resistant S. aureus and P. aeruginosa was carried out (17, 18). As previously described, a crude aqueous extract of shilajit with a 50 mg/mL concentration stock solution prepared . To inoculate the solid media plates, 100 µL of bacterial inoculum that standardized (0.5 McFarland's standard that equals to1.5 × 108 CFU/mL) of each bacterial isolate were evenly spread using a sterile cotton swab. Blank disks with a diameter of 6mm were overloaded with different dilutions of the shilajit stock aqueous extract, resulting in concentrations ranged between 12.5, 25, 50, 100, and 200µg per disk. The discs were then located on agar plates after that incubated for 24-48 hours at 37°C.
The diameter of the zone of inhibition in millimetres was measured from the margin of the disc to the inner edge of the pathogen-free zone. A disk impregnated with D.W served as a negative

control. Positive controls included standards of colistin 25µg for P. aeruginosa and levofloxacin 5µg for S. aureus (Sigma Chemical Co., St. Louis, MO, USA) while moxifloxacin 5µg for both as an extra material, functioning as antibacterial controls, respectively. Three plates (replicates) have been used for each concentration to limit any errors that occurred throughout the experiment.

3.5. Broth Micro-Dilution Method: Activity of Shilajit extract against S. aureus and P. aeruginosa by minimal inhibitory concentration (MIC) measured by antimicrobial analysis using the broth micro-dilution method. To evaluate the MIC of shilajit aqueous extract, 5 dilutions at 50 mg mL-1 were made in double-distilled water. A quantity of 100 µL of each 5 dilutions and 100 µL of the microbial suspensions (0.5 McFarland's standard that equal to 1.5 × 108 CFU/mL) of each bacterial isolate were mixed then inoculated into the tubes of prepared Mueller-Hinton broth and incubated at 37°C for 48 hrs respectively. Controls included tubes with only substrate and either diluted extract (negative control) or microbial suspension (positive control). MIC was determined as the lowest concentration inhibiting visible microbial growth, To determine the presence or lack of growing turbidity, confirmed spectrophotometrically at 540 nm after 48 hours. .All experiments were conducted in triplicate to ensure accuracy and reproducibility of findings.

3.6. Statistical analysis: SPSS version 23.0 was used to analyze the collected data. To determine the statistical significance of differences between groups One-way analysis of variance (ANOVA) was performed. All experiments were performed in triplicate. and data are expressed as mean ± standard deviation. A value of $p < 0.05$ was considered statistically significant.

3.7. Ethics approval and consent to participate: Not applicable.

Results

4.1. Antibiotics susceptibility test : According to the results of antibiotic susceptibility testing using the Kirby–Bauer method, the two bacterial isolates exhibited multidrug resistance (MDR) patterns as shown in Table 1 .The Staphylococcus aureus isolate was sensitive to just two of the antibiotics tested: levofloxacin and moxifloxacin. On the other hand, the Pseudomonas aeruginosa isolate was only sensitive to colistin and resistant to all other antibiotics tested. This highlights the difficulty of treating infections caused by multidrug-resistant bacteria.

Table (1): - The antibiotic susceptibility test results of Pseudomonas aeruginosa and Staphylococcus aureus isolates.

Antibiotics	*lomonas Aeruginosa*	Antibiotics	*hylococcus Aureus*
Levofloxacin	R	Levofloxacin	S
Colistin	S	Ciprofloxacin	R
Ciprofloxacin	R	Vancomycin	R
Gentamicin	R	Oxacillin	R
Meropenem	R	Meropenem	R
Moxifloxacin	R	Moxifloxacin	S
Amikacin	R	Tetracycline	R

R, resistant; S, susceptible.

4.2. Antibacterial activity assay of shilajit aqueous extract : In this study results of the antimicrobial activity of of shilajit aqueous extract to inhibit the growth of pathogenic bacteria was performed by measuring the appearing of inhibition zones, as presented in Table 2. The raw aqueous shilajit extract presented considerable antibacterial activity versus Staphylococcus aureus at all concentrations meanwhile exhibited the best activity at 200 µg/disc, resulting in inhibition zones about 18±0.3 mm in diameter Fig.1. As the shilajit extract was diluted, its antibacterial activity steady decreased expectedly Fig.1, and the shilajit aqueous extract showed no activity against Pseudomonas aeruginosa at the specified shilajit aqueous concentrations used in the study Fig.2.Also the results that obtained from moxifloxacin showed resistance in Pseudomonas aeruginosa.

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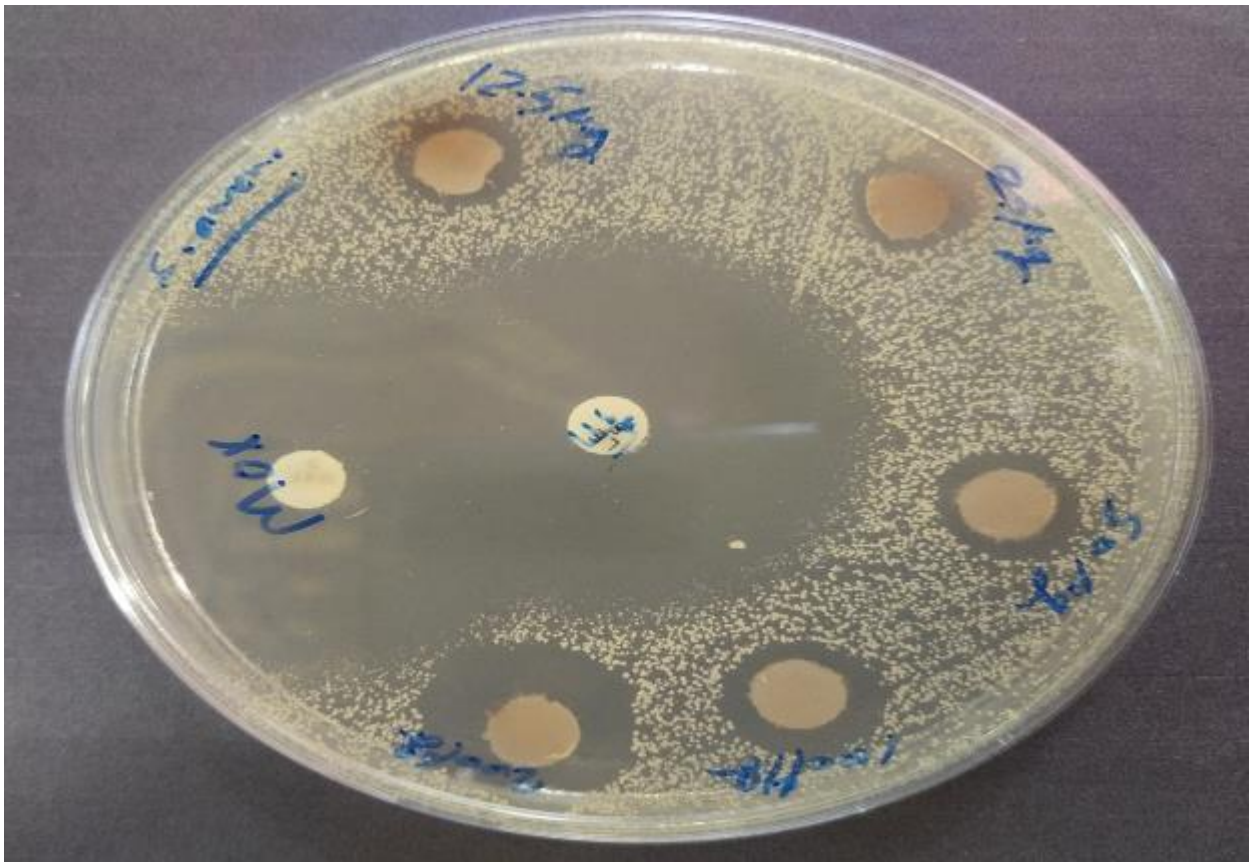

Figure (1). - Disk diffusion assay evaluating antibacterial activity against isolated Staphylococcus aureus.

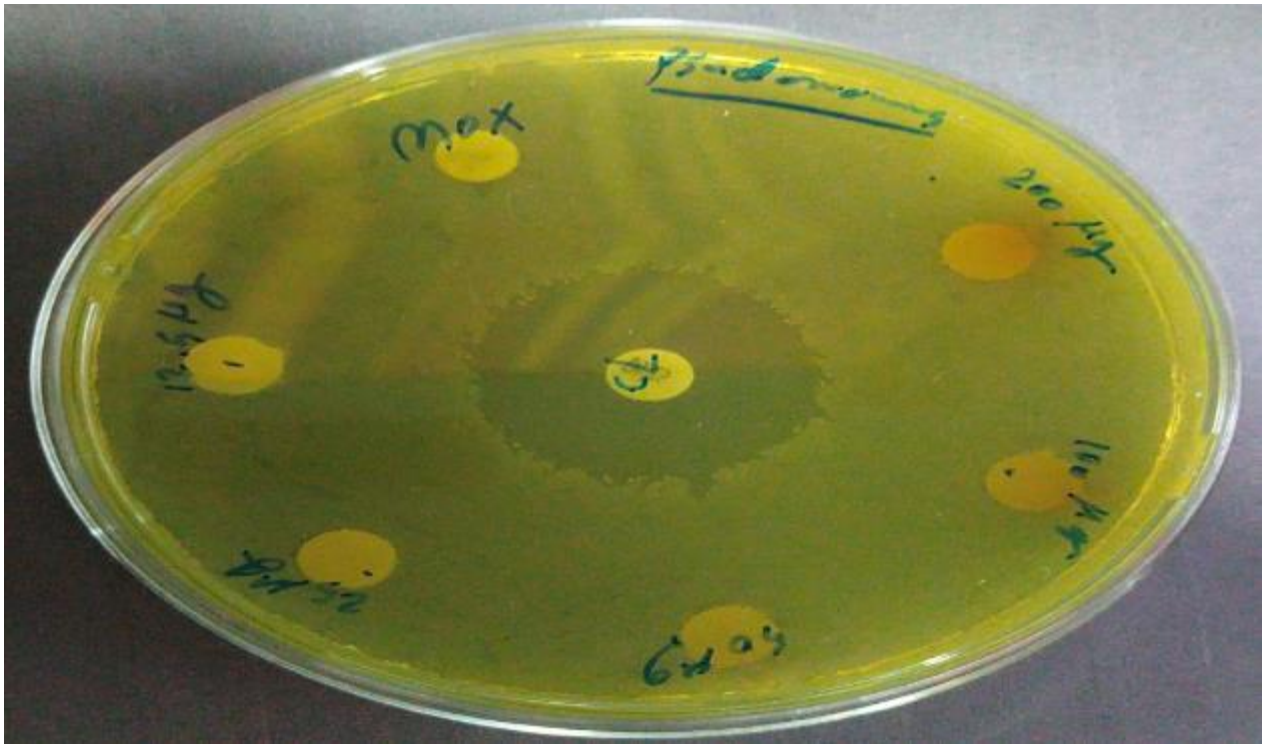

Figure (2). - Disk diffusion assay detecting antibacterial activity against isolated Pseudomonas aeruginosa

The minimum inhibitory concentration (MIC) was performed based on the assumption that the lower the MIC value, the more effective the herbo-medicinal extract in treating the infection. Many research have suggested that low dosages of herbo-medicinal extract required to generate a therapeutic response might result in lower toxicity and side effects (19). Table 3 shows the antibacterial activity of shilajit as MIC determined by the broth microdilution technique. The findings demonstrated inhibitory antibacterial activities against Staphylococcus aureus at concentrations greater than 25 mg/mL, but no action against Pseudomonas aeruginosa. The presence of active components such as fulvic, ferulic, humic,and gallic acids might account for the extract's growth inhibitory effect.

Table (2): - Antimicrobial activities of Shilajit aqueous extract, alongside standards Moxifloxacin, Colistin, and Levofloxacin against bacterial isolates, represented as mean ± SD of inhibition zone measurements (mm) in experimental groups (n = 3 per group).

	Mean Diameter of Inhibition Zone (Mm) ± SEM*					Colistin	Levofloxacin	Moxifloxacin
	200 (µg/Disc)	100 (µg/Disc)	50 µg/Disc)	25 (µg/Disc)	12.5 g/Disc)	25 µg/Disc)	5 (µg/Disc)	5 (µg/Disc)
Pseudomonas Aeruginosa	-	-	-	-	-	± 0.3	-	-
Staphylococcus Aureus	18± 0.3	13± 0.3	12± 0.3	11± 0.3	10± 0.3	-	37± 0.3	36 ± 0.2

Mean±SEM values in each column with different superscripts significantly differ(*P ≤ 0.05).(-):shows that no action was noticed at this Shilajit concentration or standard. *SEM: standard error of the mean.

Table (3): - The minimal inhibitory concentration (MIC) assay of the aqueous extract of Shilajit against bacterial isolates.

	MIC Of Shilajit (Mg / Ml)
Pseudomonas Aeruginosa	-
Staphylococcus Aureus	25.0

Mean ± standard error of the mean values with no shared superscript letters are significantly different (*P ≤ 0.05). (-):shows that no activity has been noticed at this Shilajit concentration or standard.

Discussion: The findings of the presented study indicated that the raw aqueous extract of shilajit exhibited an positive antimicrobial effect on Staphylococcus aureus at concentrations above 25 mg/mL, but had no effect on Pseudomonas aeruginosa. An earlier investigation found that the minimum inhibitory concentration (MIC) of Russian Shilajit is 1000 µg/mL for Bacillus subtilis, 125 µg/mL for Staphylococcus aureus, and 62.5 µg/mL for both Candida albicans and Escherichia coli. However, no MIC was detected for Pseudomonas aeruginosa within the range of diluted concentrations tested. (20).

the study by AlShubaily and Jambi underscores the therapeutic properties of Shilajit extract, identifying bioactive compounds like fulvic acid, gallic acid, and ferulic acid using LC-HRESIMS technology. Their research demonstrated significant antimicrobial activity against Staphylococcus aureus and Candida albicans, and cytotoxic effects on Hep G2 cell lines. These findings support the potential of Shilajit in antimicrobial applications, aligning with our exploration of its efficacy against bacteria in the Kalar District of Iraq(11).

Another study demonstrated that the crude aqueous extract of shilajit with concentrations greater than 50 mg/mL had an antimicrobial effect on Staphylococcus aureus NCIMB 6571 and on Candida albicans NCPF 3255 with an MIC greater than 12.5 mg/mL and a minimum fungicidal concentration (MFC) greater than 50 mg/mL. Additionally, the methanolic extract of shilajit showed 95% spore inhibitory activity against Alternaria cajani at a concentration of 5000 µg/mL (21) .The variability in antimicrobial impact of fulvic acid and concentration contributes to the antimicrobial effects observed in the studied shilajit sample(21).

Muratova and Shakirov confirmed the positive antimicrobial activities of shilajit against various common pyogenic microbial strains such as coliform bacteria, streptococci, enterococci, staphylococci, and Proteus (22). Some investigations reported a correlation between the antimicrobial activities and the constituents of shilajit extract, including fulvic and benzoic acids. Variations in herbs, regional species, and climate conditions lead to differences in their antimicrobial properties. Therefore, based on the primary constituents as reported by several authors, the different physiological activities of shilajit samples may be related to their native regions around the world (11, 23).

The broad antimicrobial spectrum of the major shilajit component, fulvic acid, has been confirmed against various microbial strains, including A. actinomycetemcomitans, P. gingivalis, E. faecalis, F. nucleatum, S. mutans and S. mitis. Additionally, fulvic acid possesses anticancer cytotoxic effects in vivo (24),

Conclusions:

Current study evaluated the antibacterial activity of shilajit aqueous extract on clinically isolated bacteria in the Kalar district of Iraq, specifically focusing on Staphylococcus aureus and Pseudomonas aeruginosa. The results demonstrated that the raw shilajit extract exhibited significant antibacterial activity against clinically isolated Staphylococcus aureus at concentrations above 25 mg/mL, while no effect was observed on Pseudomonas aeruginosa. This aligns with previous studies that have shown shilajit to be effective against various bacterial strains, with varying minimum inhibitory concentrations (MICs) for different organisms.

The variability in antimicrobial activity observed in different studies can be associate with the concentration and impact of active components within shilajit such as fulvic acid. Additionally, the differences in the antimicrobial properties of shilajit samples from various regions are likely due to the variations in their major organic constituents and environmental factors.

Purification of the shilajit chemical constituents may increase its ability in inhibiting microorganisms, eventually leading to the discovery of a new broad-spectrum antimicrobial in the future with herbal composition.

Recommendations:

1. **Promote Shilajit Awareness:** Increase awareness of Shilajit and similar natural products as potential treatments for multidrug-resistant bacterial infections.
2. **Advance Research:** Conduct further studies to understand Shilajit's antibacterial mechanisms and active components.
3. **Conduct Clinical Trials:** Initiate clinical trials to evaluate Shilajit's safety, dosage, and efficacy in treating antibiotic-resistant infections.
4. **Compare with Standard Treatments:** Compare Shilajit's effectiveness with standard antibiotics to determine its clinical utility.
5. **Inform Policy:** Influence healthcare policies to incorporate findings supporting the use of natural antimicrobial agents.

References:

1. Shameem N, Kamili AN, Ahmad M, Masoodi F, Parray JA. Antimicrobial activity of crude fractions and morel compounds from wild edible mushrooms of North western Himalaya. Microbial pathogenesis. 2017;105:356-60.
2. Ganesan P, Reegan AD, David RHA, Gandhi MR, Paulraj MG, Al-Dhabi NA, et al. Antimicrobial activity of some actinomycetes from Western Ghats of Tamil Nadu, India. Alexandria journal of medicine. 2017;53(2):101-10.
3. Chanda S, Rakholiya K, Parekh J. Indian medicinal herb: Antimicrobial efficacy of Mesua ferrea L. seed extracted in different solvents against infection causing pathogenic strains. Journal of acute disease. 2013;2(4):277-81.
4. Sathiyaraj S, Suriyakala G, Gandhi AD, Babujanarthanam R, Almaary KS, Chen T-W, et al. Biosynthesis, characterization, and antibacterial activity of gold nanoparticles. Journal of Infection and Public Health. 2021;14(12):1842-7.
5. Shore CK, Coukell A. Roadmap for antibiotic discovery. Nature microbiology. 2016;1(6):1-2.
6. Wright PM, Seiple IB, Myers AG. The evolving role of chemical synthesis in antibacterial drug discovery. Angewandte Chemie International Edition. 2014;53(34):8840-69.
7. Agarwal SP, Khanna R, Karmarkar R, Anwer MK, Khar RK. Shilajit: a review. Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives. 2007;21(5):401-5.

8. Bhattacharya SK. Shilajit attenuates streptozotocin induced diabetes mellitus and decrease in pancreatic islet superoxide dismutase activity in rats. Phytotherapy Research. 1995;9(1):41-4.
9. Ghosal S. Chemistry of shilajit, an immunomodulatory Ayurvedic rasayan. Pure and Applied Chemistry. 1990;62(7):1285-8.
10. Acharya S, Frotan M, Goel R, Tripathi S, Das P. Pharmacological actions of Shilajit. Indian journal of experimental biology. 1988;26(10):775-7.
11. AlShubaily F, Jambi E. LC/MS Profiling of Shilajit Extract for Antimicrobial & Antifungal and Cytotoxic Activities. Int Trans J Eng Manag Appl Sci Technol. 2022;13:1-13.
12. Frolova L, Kiseleva T. Chemical composition of mumijo and methods for determining its authenticity and quality (a review). Pharmaceutical Chemistry Journal. 1996;30:543-7.
13. Schepetkin I, Khlebnikov A, Kwon BS. Medical drugs from humus matter: Focus on mumie. Drug development research. 2002;57(3):140-59.
14. Bauer A, Kirby W, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. American journal of clinical pathology. 1966;45(4_ts):493-6.
15. Wayne P. CLSI Performance Standards for Antimicrobial Susceptibility Testing. CLSI supplements M. 2020;100.
16. Perumal P, Sathakkathulla NA, Kumaran K, Ravikumar R, Selvaraj JJ, Nagendran V, et al. Green synthesis of zinc oxide nanoparticles using aqueous extract of shilajit and their anticancer activity against HeLa cells. Scientific Reports. 2024;14(1):2204.
17. Balouiri M, Sadiki M, Ibnsouda SK. Methods for in vitro evaluating antimicrobial activity: A review. Journal of pharmaceutical analysis. 2016;6(2):71-9.
18. Ferraro MJ. Performance standards for antimicrobial disk susceptibility tests2000.
19. Garima Srivastava GS, Rohit Jain RJ, Nitya Vyas NV, Archana Mehta AM, Sumita Kachhwaha SK, Kothari S. Antimicrobial activity of the methanolic extract, fractions and isolated compounds from Citrullus colocynthis (L.) Schrad. 2013.
20. El-Sayed M-IK, Amin H-K, Al-Kaf A-G. Anti-microbial, anti-oxidant and anti-ulcerogenic effects of shilajit on gastric ulcer in rats. 2012.
21. Shalini SR, Srivastav R. Antifungal activity screening and HPLC analysis of crude extract from Tectona grandis, Shilajit, Valeriana wallachi. The International Journal of Alternative Medicine. 2008;5(2):1540-2584.
22. Muratova Kh N, Shakirov D. [Clinical treatment of suppurative wounds with mumie]. Khirurgiia (Mosk). 1968;44(9):122-4.
23. Ghosal S, Lal J, Singh SK. The core structure of Shilajit humus. Soil Biology and Biochemistry. 1991;23(7):673-80.
24. Sherry L, Millhouse E, Lappin DF, Murray C, Culshaw S, Nile CJ, et al. Investigating the biological properties of carbohydrate derived fulvic acid (CHD-FA) as a potential novel therapy for the management of oral biofilm infections. BMC Oral Health. 2013;13:1-10.