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Specialty: Applied Biochemistry

Theme

Biogenic Synthesis of Metal Naroparticles with Antibacterial and Healing Properties for Burn Wound Management

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Dedication

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بسمه

Dedication

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إلى نفسي ...ثم إلى نفسي ...ثم إلى نفسي،

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يسرى

Dedication

إلى نفسي أولًا...

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آية

Dedication

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التي خاضت هذه الرحلة بكل صبر وإصرار، وتحملت التعب والضغوط والتحديات، ولم تفقد الأمل رغم الصعوبات. إلى نفسي التي آمنت بقدرتها على النجاح، وسعت بكل اجتهاد لتحقيق هذا الهدف حتى أصبح حقيقة أفتخر بها اليوم.

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شكرًا لرفقتكن الجميلة، ولدعمكن الصادق وكلماتكن المشجعة التي خففت عني مشقة الطريق وجعلت هذه الرحلة أكثر جمالاً.

كما أتقدم بخالص الشكر والتقدير إلى جميع أساتذتي الذين رافقوني خلال مسيرتي الدراسية، وأسهموا بعلمهم وتوجيهاتهم في وصولي إلى هذه المرحلة.

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إسلام

Abstract

In this study, a bimetallic hybrid nanosystem composed of silver (Ag) and zinc (Zn) nanoparticles was synthesized using Animal derived biological material during the preparation process. The synthesized nanoparticles were then incorporated into a suitable pharmaceutical base to obtain a topical therapeutic ointment intended for the treatment of skin burns. Physical characterization results revealed homogeneous nanoparticles with an average size of 19.98 nm, indicating the successful synthesis and good stability of the hybrid nanosystem, The prepared ointment was subjected to several physicochemical evaluations, which demonstrated good homogeneity, suitable stability, and high applicability for topical use. The biological activity of the ointment was also evaluated Brun wound healing using Albino Wistar rats with experimentally induced skin burns. The obtained results showed significant therapeutic efficacy, including accelerated burn wound healing, reduction of inflammation severity, and stimulation of skin tissue regeneration compared to untreated groups, The hybrid nanosystem significantly enhanced the biological performance of the formulation and improved the penetration of the active material into the damaged tissues, leading to higher therapeutic efficiency compared with conventional formulations. The obtained findings confirm that hybrid nanoparticles synthesized using an animal-derived biological material possess promising therapeutic properties, opening new perspectives for the development of modern and effective topical pharmaceutical formulations for burn treatment and skin care.

Key words: Animal-derived biological material, hybrid nanoparticles, therapeutic ointment, skin burns, wound healing, Brun wound healing.

Résumé

Au cours de cette étude, un nanosystème hybride bimétallique composé de nanoparticules d'argent (Ag) et de zinc (Zn) a été synthétisé en utilisant du Matériel biologique d'origine animale lors du procédé de fabrication. Les nanoparticules obtenues ont ensuite été incorporées dans une base pharmaceutique appropriée afin de formuler une pommade thérapeutique topique destinée au traitement des brûlures cutanées. Les résultats de la caractérisation physique ont montré l'obtention de nanoparticules homogènes avec une taille moyenne de 19,98 nm, confirmant ainsi la réussite de la synthèse et la bonne stabilité du nanosystème hybride, La pommade préparée a été soumise à plusieurs évaluations physicochimiques révélant une bonne homogénéité, une stabilité satisfaisante ainsi qu'une excellente aptitude à l'application topique. L'activité biologique de la pommade a également été évaluée Cicatrisation des plaies brulures sur des rats Albino Wistar présentant des brûlures cutanées expérimentales. Les résultats obtenus ont démontré une efficacité thérapeutique remarquable caractérisée par une accélération de la cicatrisation des brûlures, une diminution de l'inflammation et une stimulation de la régénération des tissus cutanés par rapport aux groupes non traités, Le nanosystème hybride a contribué de manière significative à l'amélioration des performances biologiques de la formulation ainsi qu'à une meilleure pénétration de la substance active dans les tissus lésés, conduisant ainsi à une efficacité thérapeutique supérieure aux formulations conventionnelles. Les résultats obtenus confirment que les nanoparticules hybrides synthétisées à partir d'un matériau biologique d'origine animale possèdent des propriétés thérapeutiques prometteuses, ouvrant la voie au développement de formulations pharmaceutiques topiques modernes et efficaces pour le traitement des brûlures et les soins cutanés.

Mots-clés : Matériel biologique d'origine animale, nanoparticules hybrides, pommade thérapeutique, brûlures cutanées, cicatrisation, Cicatrisation des plaies brun.

الملخص

قمنا خلال هذه الدراسة بتحضير نظام نانوي هجين ثنائي المعدن مكوّن من جسيمات الفضة والزنك النانوية باستعمال مادة حيوية ذات أصل حيواني في عملية التصنيع، ثم تم إدماج الجسيمات النانوية المحضّرة داخل قاعدة صيدلانية مناسبة للحصول على مرهم علاجي موضعي موجه لعلاج الحروق الجلدية .أظهرت نتائج التوصيف الفيزيائي الحصول على جسيمات نانوية متجانسة بمتوسط حجم بلغ 19.98 نانومتر، مما يدل على نجاح عملية التحضير والاستقرار الجيد للنظام النانوي الهجين، خضع المرهم المحضّر لعدة اختبارات فيزيائية وكيميائية أظهرت تجانساً جيداً وثباتاً مناسباً وقابلية عالية للاستعمال الموضعي .كما تم تقييم الفعالية البيولوجية للمرهم داخل الجسم الحي باستعمال فئران ألبينو ويستار المصابة بحروق جلدية تجريبية، حيث أظهرت النتائج فعالية علاجية معتبرة تمثلت في تسريع التئام الحروق، تقليل شدة الالتهاب، وتحفيز تجدد الأنسجة الجلدية مقارنة بالحالات غير المعالجة، وقد ساهم النظام النانوي الهجين بشكل واضح في تعزيز الأداء الحيوي للمستحضر وتحسين نفاذ المادة الفعالة داخل الأنسجة المصابة، مما أدى إلى رفع الكفاءة العلاجية للمرهم مقارنة بالتركيبات التقليدية .وتؤكد النتائج المتحصل عليها أن الجسيمات النانوية الهجينة المصنعة باستعمال مادة حيوية ذات أصل حيواني تمتلك خصائص علاجية واعدة، مما يفتح المجال أمام تطوير مستحضرات صيدلانية موضعية حديثة وفعالة في علاج الحروق والعناية الجلدية.

الكلمات المفتاحية :مادة حيوية ذات أصل حيواني، جسيمات نانوية هجينة، المرهم العلاجي، الحروق الجلدية، التئام الحروق.

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Abbreviation List

$AgNO_3$: Silver

DRX: X-ray Diffraction

DMSO: dimethyl sulfoxide

FCC: face-centered cubic

FNS: Full Blood Count

FTIR: Fourier Transform Infrared Spectroscopy

Hcl: Hydrochloric Acid

H_2O_2: hydrogen peroxide

IC50 : Inhibitory concentration

MDA: Malondialdehyde

NaOH: Sodium Hydroxide

NPs: nanoparticules

PBS: phosphate-buffered saline

Pla: Platelets

ROS: oxygen species

SPR: the surface plasmon resonance

TBA: Tris,Thiobarbituric Acide

TBARS: Thiobarbituric Acid Reactive Substances

TBS: Total Body Surface

Tris: hydroxymethyl

$Zn(CH_3CO_2)_2$:acetate Zinc

Content

Introduction

Living in a continuously hazardous environment, humans are constantly exposed to various threats that may compromise physiological integrity and vital functions. These threats include a wide range of physical, chemical, and biological factors such as thermal variations, toxic substances, and diverse microbial agents. To counteract these challenges, the human body is equipped with an integrated defense system composed of multiple natural barriers and protective mechanisms that work in coordination to maintain homeostasis and protect tissues and organs from damage. Among these natural barriers, the skin stands out as a fundamental first line of defense against external insults, playing a crucial role in preserving internal tissue integrity and ensuring overall bodily stability and function **(Hall, J. E., & Hall, M. E. 2021).**

The skin is the largest and one of the most complex organs of the human body. Its function extends beyond simple external coverage, as it constitutes a highly organized biological barrier involved in regulating body temperature, maintaining fluid and electrolyte balance, and participating in immune and sensory functions. Furthermore, the skin prevents the penetration of microorganisms and harmful agents, making it essential for survival. However, when this vital barrier is damaged or compromised, significant disruption occurs in its protective functions, rendering the body vulnerable to infections, fluid loss, and various physiological imbalances **(Tortora & Derrickson, 2021).**

Among the most severe skin injuries, burns are recognized as one of the most common and dangerous types of domestic and industrial trauma worldwide. They are associated with high mortality rates, long-term physical and psychological disabilities, and a significant economic and social burden on healthcare systems and societies.

Burns remain a major global public health problem, highlighting the urgent need for effective and safe therapeutic approaches. Therefore, the objective of this study was to develop an innovative burn-healing ointment based on chitosan and Ag/ZnO nanohybrid, According to reports from the World Health Organization (WHO), burns cause hundreds of thousands of deaths annually, while millions of individuals suffer from permanent complications and disfigurements requiring long-term treatment and continuous rehabilitation. The highest incidence rates are observed in developing countries due to multiple contributing factors, including overcrowding, inadequate safety measures, frequent household accidents, and limited access to specialized healthcare services **(World Health Organization, 2023).**

Burns are defined as acute tissue injuries resulting from exposure of the skin and underlying tissues to thermal, electrical, chemical, or radiation sources, leading to varying

degrees of tissue destruction. The severity of injury depends on exposure duration, depth of damage, and affected surface area. Burns are generally classified into superficial, partial-thickness, and full-thickness injuries. Beyond local tissue damage, burns can also induce complex systemic disturbances that may become life-threatening, including significant fluid and electrolyte loss, as well as severe inflammatory, immune, and metabolic responses **(Greenhalgh, 2019).**

Oxidative stress is one of the key factors involved in the exacerbation of burn injuries, as the acute inflammatory response leads to excessive production of reactive oxygen species **(ROS)**,

which attack cellular membranes, proteins, and lipids, resulting in extensive tissue damage. Malondialdehyde **(MDA)** is considered one of the most important biomarkers of lipid

peroxidation and tissue damage severity following burns. This oxidative imbalance also contributes to delayed wound healing, increased risk of bacterial infection, and the formation of scars and fibrotic tissue **(Akasaka *et al*., 2022)**.

Burned skin tissues respond through a complex and highly regulated series of biological processes known as wound healing, which includes three overlapping phases: inflammation, proliferation, and remodeling **(Gurtner *et al*., 2008)**.During these stages, multiple cells and biological factors are activated, including fibroblasts, keratinocytes, immune cells, growth factors, and collagen fibers, all working to restore and regenerate damaged tissue. However, the success of this process is influenced by several factors, most notably microbial infection, poor blood perfusion, and oxidative stress, which often result in delayed healing and chronic complications **(Rodrigues *et al*., 2019)**.

Historically, humans have relied on various traditional and home-based remedies for the treatment of burns and wounds, using natural resources and inherited folk practices. These remedies include honey, olive oil, aloe vera, and various plant-derived extracts known for their soothing and anti-inflammatory properties. Some animal-derived and natural substances have also been used to reduce pain, accelerate tissue healing, and prevent infection. Although many of these natural products have shown promising therapeutic effects due to their bioactive compounds, their clinical application remains limited due to insufficient scientific validation, lack of standardization, difficulties in dose control, and potential microbial contamination **(Vijayakumar & Malaikozhundan, 2023)**.

With the rapid advancement of medical sciences, modern therapeutic strategies for burn management have emerged, including advanced wound dressings, topical antibiotics, and therapeutic ointments such as silver sulfadiazine, as well as skin grafting and tissue engineering approaches in complex cases **(Negut *et al*., 2020)**.Modern technologies have also contributed to the development of smart dressings capable of maintaining wound moisture, preventing infection, and promoting tissue regeneration. However, these treatments still face several limitations, including cytotoxicity of some compounds, treatment-associated pain, high costs, and the increasing prevalence of multidrug-resistant **(MDR)** bacteria, which has reduced the effectiveness of many antibiotics against resistant strains such as Pseudomonas aeruginosa. This has created an urgent need for safer, more effective, and biologically compatible therapeutic strategies**(Church *et al*., 2006)**.

In this context, nanotechnology has emerged as one of the most advanced and influential scientific fields of the modern era, revolutionizing the understanding and application of materials across various scientific, industrial, and medical domains **(Bayda *et al*., 2020)**.

Nanotechnology refers to the design and manipulation of materials at the nanoscale, typically ranging from 1 to 100 nanometers. **(Chauhan *et al*., 2022)**.

The importance of nanomaterials lies in their high surface area-to-volume ratio, which enhances their chemical, physical, and biological reactivity, enabling stronger interactions

with biological systems. They also exhibit unique properties such as chemical stability, high thermal and electrical conductivity, and enhanced absorption and permeability, making them highly valuable in various scientific applications **(Khan *et al*., 2021)**.

Nanoparticles can be synthesized using physical, chemical, and biological methods. Physical methods include mechanical milling, evaporation, and physical vapor deposition, while chemical methods involve reduction reactions, precipitation, thermal decomposition, and sol-gel techniques. Biological methods rely on microorganisms, plant extracts, or natural biomolecules to produce nanoparticles in a more environmentally friendly and biocompatible manner **(Patra & Baek, 2017)** .

Due to growing environmental and health concerns associated with toxic chemical methods, the concept of biology synthesis has emerged as a promising and sustainable approach in nanotechnology.This method utilizes natural compounds found in plants or microorganisms to perform reduction and stabilization processes, resulting in stable and effective nanoparticles

while minimizing environmental impact and biological toxicity **(Saratale *et al*., 2023)**. Green synthesis has gained increasing attention due to its simplicity, low cost, sustainability, and high biocompatibility **(Hussain *et al*., 2024)**.

The applications of nanotechnology are not limited to a single field but extend across multiple sectors , Nanotechnology has become one of the most important and rapidly developing fields in modern science due to its wide range of applications in various sectors, particularly in medicine. In the medical field, nanotechnology has contributed significantly to drug delivery systems, targeted therapies, bioimaging, tissue engineering, biosensors, and the development of advanced therapeutic materials. Nanomaterials have enhanced drug efficacy by improving stability and enabling controlled and targeted release at specific tissues **(Hasan *et al*., 2024)**. Beyond medicine, nanotechnology has also expanded into several industrial and environmental domains, including food industries, water treatment, energy, agriculture, electronics, and smart materials. In food packaging, nanomaterials improve food preservation and safety, while in environmental applications they play an important role in pollutant removal and water purification. Furthermore, they are widely utilized in high-performance electronics, batteries, and solar cells due to their unique physicochemical properties **(Singh *et al*., 2023)**.

In regenerative medicine, nanotechnology has opened new horizons for the development of bioactive scaffolds that support cell growth and tissue regeneration. Nanostructures, nanofibers, and nanohydrogels mimic the extracellular matrix, promoting cell adhesion, proliferation, and tissue reconstruction. They also help regulate inflammatory responses and enhance regenerative processes, making them a central focus in modern biomedical research **(Augustine *et al*., 2024)**.

Among the most recent advancements are hybrid nanomaterials, which are designed by combining two or more components to achieve enhanced and multifunctional properties that introduction cannot be obtained by a single material alone. These materials exhibit combined functionalities such as antimicrobial activity, biological stability, high conductivity, and drug-loading capacity, making them highly effective in advanced applications **(Makvandi *et al*., 2020)**.

The concept of synergy is a key feature of hybrid nanomaterials, where the combination of multiple components within a single nanosystem results in enhanced functional and biological performance that exceeds the sum of individual effects. This synergistic interaction improves the physicochemical and therapeutic properties of hybrid materials, enabling the

development of intelligent multifunctional systems for medical, industrial, and biological applications **(Zare *et al*., 2024)**.

In light of these rapid developments in nanotechnology, nanomaterials have emerged as one of the most promising tools for addressing complex medical and environmental challenges **(Saleh, 2021)**. The integration of green synthesis strategies with hybrid nanomaterials offers significant potential for developing safer, more effective, and sustainable systems capable of meeting the growing demands of modern biomedical and regenerative medicine applications **(Gupta *et al*., 2024)**.

Although green nanoparticle synthesis has been widely explored using plant extracts, approaches involving other biological sources for the fabrication of hybrid nanomaterials remain relatively underexplored. These biological matrices, rich in proteins, peptides, lipids, vitamins, minerals, and other bioactive compounds, are of particular interest due to their ability to act as reducing and stabilizing agents while enhancing the biocompatibility, antioxidant potential, and tissue-regenerative properties of the resulting nanoparticles.

Despite these promising advantages, research on the synthesis of hybrid nanomaterials derived from biological systems for burn wound healing and regenerative medicine applications remains limited. Therefore, further investigations are required to evaluate their therapeutic potential, safety profile, and biological effectiveness in relevant physiological environments.

In the context of recent advances in biomedical nanotechnology, it has become essential to assess the performance of these materials in biological systems, particularly their ability to modulate tissue response and promote skin lesion repair. Moreover, the development of hybrid nanostructured systems opens new perspectives for obtaining materials with enhanced biological performance compared to conventional approaches.

In response to the limitations of conventional burn wound healing strategies, the development of innovative nanobiological systems represents a major advancement in regenerative medicine.

In this context, the present study highlights an original strategy based on the use of an animal-derived biological source for the synthesis of a hybrid nanocomposite with enhanced biological properties. This innovative approach exploits the natural richness in bioactive compounds to promote nanoparticle stabilization, optimize biocompatibility, and strengthen therapeutic potential.

This innovation opens new perspectives in the field of therapeutic biomaterials by offering an advanced alternative to conventional treatments, with strong potential applications in burn wound healing and regenerative medicine.

Applied Part

MATERIALS AND METHODES

Study location:

This study was conducted in the laboratory of the Faculty of Natural and Life Sciences at the University of Echahid Hamma Lakhdar, El-Oued, where nanoparticle synthesis was carried out. Their characterization of the nanoparticles was performed using, X-ray diffraction (XRD) Fourier Transform Infrared Spectroscopy (FTIR) . The anti-biofilm activities were evaluated at the private El Madjed laboratory. Blood analyses (FNS) on rat samples were performed at the medical-surgical emergency department laboratory of 8 May Hospital in El-Oued.

1. Materials and methods:

1.1 Materials:

1.1.1 Biological Materials:

1.1.1.1 The bacterial strains:

The bacterial strains used in this study include:

• Escherichia coli ATCC 25922

• Staphylococcus aureus ATCC 25932

• Bacillus subtilis ATCC 25973

• Pseudomonas aeruginosa ATCC 27853

1.1.1.2 Rats

In this *in vivo* study, Albino Wistar white rats weighing between 150 and 180 g were used to evaluate the efficacy of the prepared formulations to treat burns and eliminate scars . All rats were maintained under the same standard laboratory conditions and housed individually throughout the experimental period.

1.1.2 Laboratory equipment:

Beaker, Magnetic Stirrer Bar,Graduated Cylinder, SPATULA,Micro-pipette (1000 µL),Centrifuge Tubes, Pneumatic Trough, Thermometer.

1.1.3 Laboratory devices:

Magnetic Mixer and Heater, Analytic Balance, Centrifuge, pH Meter , Ultrasonic, UV-Visible Spectrophotometer, FTIR Spectrometer, Drying oven, DRX.

1.1.4 Products and reagents:

Ethanol (50%), Distilled Water , Silver ($AgNO_3$),acetate Zinc ($Zn(CH_3CO_2)_2$), Sodium Hydroxide(NaOH), Hydrochloric Acid (HCl) , Acetic acid, Chitosane,Goat milk fat, sodium Chloride (Nacl),Tris, Thiobarbituric Acide (TBA),chloroforme,(Tris) hydroxymethyl,vaseline.

2. Methodes:

2.1. Protocol for the Synthesis of Zinc Oxide Nanoparticles (ZnO NPs):

The following steps describe the synthesis of ZnO nanoparticles.

- Dissolve zinc acetate in distilled water, then add product A and stir.
-Add the NaOH solution.
-Centrifuge at 4,000 rpm for 20 minutes.
-Keep the solid precipitate (discard the supernatant).
-Perform several redispersions in deionized water for purification, followed by three centrifugation cycles at 3,000 rpm for 30 minutes, including one wash using 50% absolute ethanol.
-After the third washing step, centrifuge again and completely remove the supernatant.
-Carry out additional washing of the nanoparticle suspension to eliminate residual impurities.
-Dry in a hot-air oven at 45 °C for 72 hours.
-Collect the dry powder using a fine spatula and store it in an amber glass vial, protected from light.

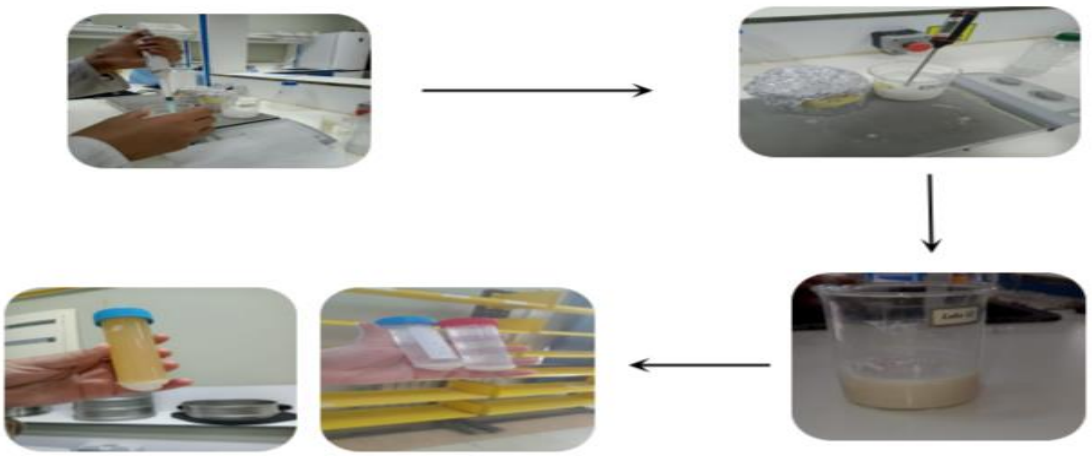

Figure 1: the synthesis procedure of ZnO NPs (Original photo, 2026).

2.2. Detailed Preparation Protocol for Silver Nanoparticles (AgNPs):

The following steps describe the synthesis of Ag nanoparticles:

-Dissolve silver nitrate ($AgNO_3$) in distilled water, then add product A under continuous stirring.

-Adjust the pH of the solution by adding NaOH.

-Subsequently increase the hotplate temperature to promote the reaction.

-Transfer the obtained colloidal solution into centrifuge tubes.

-Centrifuge at 4,000 rpm for 20 minutes.

-Discard the supernatant and collect the pellet.

-Perform several redispersions in deionized water to ensure purification, followed by three centrifugation cycles at 3,900 rpm for 15 minutes, including one washing step with absolute ethanol (50%).

-After the third washing step, centrifuge again and completely remove the supernatant.

-Carry out a final washing step of the nanoparticle suspension to eliminate any remaining residues.

-Spread the precipitate onto a clean surface covered with aluminum foil (appropriate tray).

-Dry in a hot-air oven at 45 °C for 24 hours.

-Store the obtained powder in an amber glass vial, tightly sealed, in a cool place and protected from light.

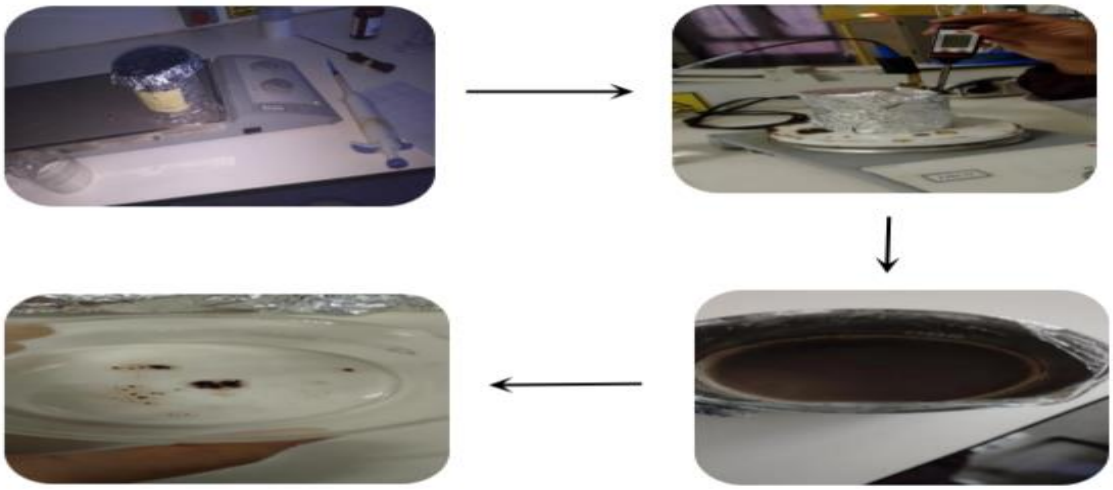

Figure 2: the synthesis procedure of Ag NPs (Original photo, 2026).

2.3. Synthesis of Ag/ZnO Nanoparticls:

The following steps describe the synthesis of Zn/Ag nanoparticles:

-Dissolve zinc acetate and silver nitrate ($AgNO_3$) in distilled water under vigorous stirring.

-Add product A to the metal solution under continuous stirring.

-Adjust the pH of the solution to the desired value.

-After complete addition, increase the temperature while maintaining stirring to promote the reaction.

-Transfer the obtained colloid into centrifuge tubes previously wrapped with aluminum foil.

-Centrifuge at 3,900 rpm for 20 minutes.

-Discard the supernatant and collect the dark brown pellet corresponding to the precipitate.

-Perform several redisperse steps in deionized water for purification, followed by three centrifugation cycles at 3,900 rpm for 15 minutes, including one wash with absolute ethanol (50%).

-After the third wash, perform an additional centrifugation and completely remove the supernatant.

-Carry out a final washing step of the nanoparticle suspension to remove residual impurities.

-Spread the precipitate on a clean surface covered with aluminum foil (suitable tray).

-Dry in a hot-air oven at 45 °C for 24 hours.

-Store the obtained powder in an amber glass vial, tightly sealed, in a cool place and protected from light.

Figure 3: The synthesis procedure of Ag NPs (Original photo, 2026).

Figure 4: Ag/Zno NPs(Original photo, 2026).

3. Extraction of Chitosan from Agaricus bisporus:

Chitosan was prepared in the research laboratory of the Faculty of Natural and Life Sciences under the supervision and assistance of **Dr. Ilham Ben Amor**. An appropriate laboratory protocol was followed for the extraction and preparation of chitosan from the fungal raw material, while respecting the different experimental conditions required to obtain a product with suitable quality and physicochemical properties for biochemical applications. Her scientific guidance and continuous support throughout the different stages of the work greatly contributed to the accurate and well-organized completion of this practical part of the study.

3.1 Mushroom Collection:

Agaricus bisporus mushrooms were obtained from the local market of El-Oued and used as the raw material for chitin extraction and its subsequent conversion into chitosan.

3.2 Preparation:

Initially, the mushroom samples were thoroughly washed with water to remove adhering impurities and contaminants, then cut into small pieces in order to increase the contact surface area and improve the efficiency of the extraction process.

3.2.1 Chitosan Extraction Procedure:

3.2.1.1 Demineralization:

The demineralization step was carried out in order to remove inorganic salts and mineral components present in the fungal biomass. Briefly, the mushroom samples were treatedhydrochloric acid (HCl) under continuous stirring . After treatment, the mixture was filtered and the resulting residue was washed several times with distilled water until complete removal of residual acid. traces.

3.2.1.2 Deproteinization:

To eliminate proteins associated with the chitinous matrix, a 10% sodium hydroxide (NaOH) solution was prepared . The sample was then treated with this alkaline solution under continuous stirring. Subsequently, the mixture was filtered and washed several times with distilled water until a neutral pH was achieved, resulting in a purified chitin fraction.

3.2.1.3 Decolourization:

The decolourization process was performed to remove pigments and colored compounds associated with the extracted chitin. The sample was treated with hydrogen peroxide (H_2O_2) solution. under continuous stirring. The resulting product was then thoroughly washed with

distilled water until a neutral pH was reached, yielding decolourized chitin with improved purity.

3.2.1.4 Deacetylation and Chitosan Preparation:

Chitosan was obtained deacetylation of chitin using a concentrated sodium hydroxide solution. The chitin sample was immersed in the prepared solution and treated under continuous stirring. After completion of the reaction, the sample was filtered and thoroughly washed with distilled water to remove alkali residues. Finally, the resulting product was dried in an oven at 50°C for 24 h to obtain dry chitosan powder.

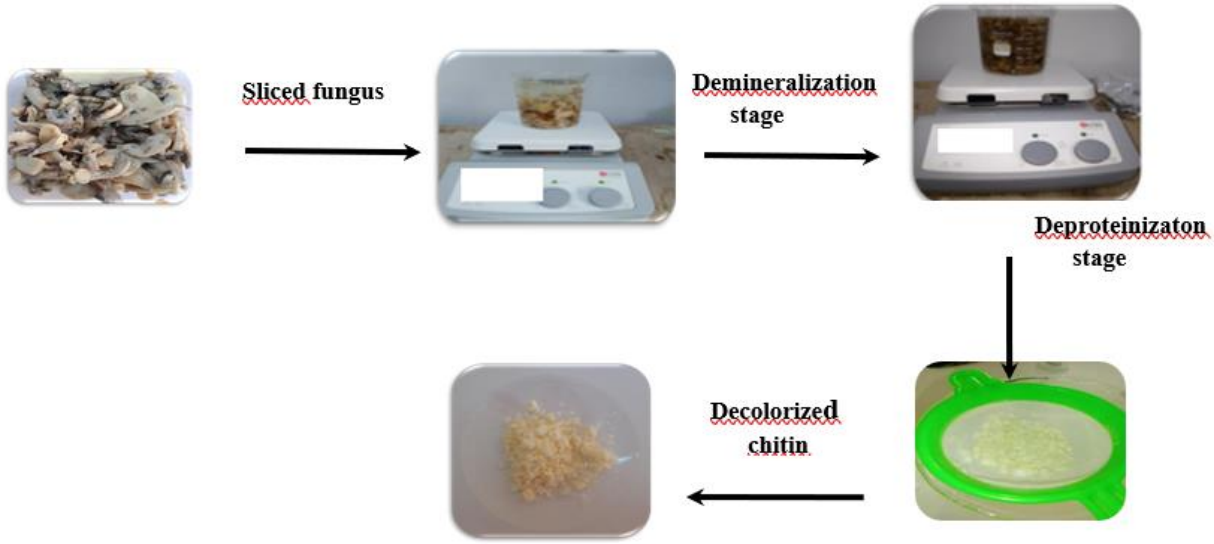

Figure 5:Preparation of Chitosan(Original photo, 2026).

4. Syntheses of final product :

Preparation of Zn/Ag hybrid nanoparticles coated with chitosan and Product G (Zn/Ag NPs/Chitosan/Product G) and their incorporation into a petrolatum-based ointment.

4.1 Materials:

- Zn/Ag hybrid nanoparticles.

- Chitosan (powder).

- Acetic acid.

- Distilled/deionized water.

- Proudct G, solid at room temperature.

- Petrolatum (Vaseline, USP grade).

- Centrifuge and centrifuge tubes.

- Magnetic stirrer (or overhead stirrer).

- Water bath or hot plate (40–45 °C).

- Ethanol 70% (for cleaning purposes only).

- Chitosan was selected due to its extensive use in wound biomaterials and its documented synergistic antimicrobial activity when combined with silver nanoparticles in infected-wound management systems **(Alven , Aderibigbe , 2024).**

4.2 Step A — Preparation of Chitosan Solution (0.5% w/v):

Chitosan was dissolved in mildly acidic aqueous media, a standard approach to obtain homogeneous polymer solutions suitable for coating and film formation **(Rinaudo, 2006)** . A 1% (v/v) acetic acid solution was first prepared in distilled water. Chitosan was then dissolved in the acetic acid solution to obtain a final concentration of 0.5% (w/v), The solution was stirred at room temperature until complete dissolution was achieved (several hours to overnight).

4.3 Step B — Coating of Zn/Ag Nanoparticles with Chitosan:

Chitosan coating of nanoparticles is widely employed to enhance colloidal stability, biocompatibility, and surface functionality for biomedical applications, and is typically

followed by a purification step to remove excess unbound polymer **(Rinaudo, 2006)**.The Zn/Ag hybrid nanoparticles were dispersed in distilled water under gentle magnetic stirring; brief bath sonication was applied only if necessary, while avoiding any temperature increase. The chitosan solution then slowly added to the nanoparticle dispersion under continuous stirring. The mixture was stirred at room temperature to allow the adsorption and association

of chitosan onto the nanoparticle surface, leading to the formation of Zn/Ag NPs@Chitosan with a moderate coating density.

The nanoparticle pellet coated with chitosan, after removal of excess water, was directly added to the molten Product G and gently but thoroughly mixed to ensure homogeneous dispersion.

The mixture was then allowed to cool to room temperature, resulting in the solidification of the lipid phase and the formation of Zn/Ag NPs@Chitosan@Product G. In this configuration, the chitosan layer acts as a polymeric interface, while the molten lipid encapsulates the coated nanoparticles and solidifies upon cooling, in accordance with established mechanisms of lipid-based nanocarrier formation **(Khairnar *et al*., 2022; Musielak *et al*., 2023).**

The final composite was incorporated into petrolatum base to obtain an ointment formulation. The formulation was then cooled to room temperature and stored in a clean container.

5. Safety Considerations:

Systemic silver exposure has been reported in burn patients treated with silver sulfadiazine-based formulations, with elevated serum Ag^{+} levels observed, highlighting the need for a cautious low-dose approach in the development of silver-based topical systems (Tredget *et al*., 2019).Further evaluation of skin irritation, cytotoxicity, and antimicrobial efficacy is recommended prior to any *in vivo* application **(Tredget *et al*, 2019)**.

6. Caracterisation of nanoparticules :

6.1 X-ray diffraction (XRD):

To evaluate the crystallinity and crystallite size of the green-synthesized nanoparticles, powder X-ray diffraction (XRD) analysis was performed using Cu Kα radiation on a MiniFlex 600 Rigaku X-ray diffractometer (λ = 1.5406 Å) over a 2θ scanning range of 25°–90°. The crystallite size was estimated from the most intense diffraction peak using the Scherrer equation:

$$D = \frac{K.\lambda}{\beta.\theta}$$

In the Scherrer equation, D represents the crystallite size, k is the shape factor (0.9), λ is the X-ray wavelength (0.154281 nm, Cu Kα), β corresponds to the full width at half maximum (FWHM) of the diffraction peak, and θ is the Bragg diffraction angle.

6.2. FTIR Analysis :

To identify the functional groups and chemical interactions present in the Product A synthesized nanoparticles, Fourier Transform Infrared Spectroscopy (FTIR) analysis was performed using an FTIR spectrometer over the spectral range of 4000–400 cm^{-1}. This technique is based on measuring the absorption of infrared radiation by the various chemical bonds present in the sample, generating an absorption spectrum that correlates wavenumber (cm^{-1}) with absorbance or transmittance intensity.

The characteristic peaks observed in the FTIR spectra were used to identify the main functional groups involved in the reduction and stabilization of the synthesized nanoparticles. The positions and intensities of the absorption bands were interpreted according to established reference values for molecular vibrations, where each absorption band corresponds to a specific vibrational mode of a chemical bond. Furthermore, comparison of the spectra obtained from the precursor materials and the synthesized nanoparticles enabled the

identification of structural changes and chemical interactions associated with the green synthesis process.

7. Effect of Ag/Zn NPs Anti_biofilm formation:

To assess the effect of ZnO/Ag nanoparticle on biofilm formation, four reference bacterial strains were used, including Escherichia coli ATCC 25922 and Pseudomonas aeruginosa

ATCC 27853 as Gram-negative bacteria, and Staphylococcus aureus ATCC 25932 and Bacillus subtilis ATCC 25973 as Gram-positive bacteria. Overnight cultures of each strain were prepared in fresh growth medium and then diluted to obtain standardized inocula. Aliquots of the bacterial suspensions were dispensed into sterile 96-well flat-bottom microplates, and the Ag/Zno NPs, previously dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 2.5% (v/v), was added at sub-inhibitory concentrations of 80, 40, 20, 10, and 5% (v/v). Wells containing the same concentration of DMSO without Ag/Zno NPs served as the untreated control. The plates were incubated statically at 37 °C for 48 h to allow biofilm formation. After

incubation, the culture medium was gently removed, and the wells were washed twice with sterile phosphate-buffered saline (PBS) to eliminate non-adherent

cells. The remaining attached biofilms were air-dried and stained with 200 µL of 0.1% (w/v) crystal violet for 30 min at room temperature.

Excess stain was removed by thorough washing with distilled water, and the plates were left to dry. Bound crystal violet was then solubilized by adding DMSO, and the absorbance was measured at 570 nm using a microplate reader to quantify biofilm biomass. All experiments were performed in triplicate, and the results were expressed relative to the untreated control to determine the inhibitory effect of Ag/Zn NPs on biofilm formation **(Fan *et al.*, 2018)**.

The percentage of biofilm inhibition was calculated using the following equation:

Inibition %=(Abs(co positive)_Abs (sample))/Abs(co positive). the whole experment was replicated three times independently.

the entire experiment was performed independently in triplicate to ensure reproducibility and reliability of the results.

8. Experimental protocol for inducing burns in rats and evaluation of treatment:

Before the experiment, the rats were allowed to undergo a one-week adaptation period under standard laboratory conditions. After acclimatization, the lower dorsal region of each rat was shaved, and third-degree thermal burns were induced. The rats were then randomly divided into four experimental groups, with three rats in each group.

After burn induction, the different treatments were applied daily according to the protocol assigned to each experimental group. The treatment and follow-up period lasted for one month, during which the progression of burn healing was regularly monitored. Morphological changes, including reduction of the burn area, formation of new tissue, and gradual decrease of inflammatory signs, were recorded throughout the study..

Group 1 (G1): Burn + Vaseline.

Group 2 (G2): Burn + nanoparticle formulation mixed with Vaseline.

Group 3 (G3): Burn + prepared final product.

Group 4 (G4): Burn + 1% Silver Sulfadiazine ointment.

9. Collection of Biological Samples:

At the end of the experiment, the rats were sacrificed on March 15, 2026, using a sterile surgical blade. Blood samples were collected immediately after sacrifice and distributed into different tubes according to the required biological analyses.

whereas tubes containing EDTA were used for complete blood count analysis (CBC).

Skin samples were also collected from the burn area after removal of the affected tissue. The collected tissues were preserved for subsequent biochemical analyses, particularly the determination of malondialdehyde (MDA), an important marker of oxidative stress and lipid peroxidation.

9.1 Determination of Malondialdehyde (MDA):

The level of malondialdehyde (MDA) was determined using the thiobarbituric acid reactive substances (TBARS) assay in order to evaluate oxidative stress in skin tissues.

9.2 Preparation of Tissue Homogenate:

One gram of skin tissue collected from the burn area was homogenized with Tris Buffered Saline (TBS) using a mortar to obtain a homogeneous tissue extract.

The homogenates were then centrifuged at 3900 rpm for 20 min in order to separate cellular debris and membrane residues from the supernatant. Only the supernatant was collected for biochemical analyses, as it contains enzymes, oxidative products such as MDA, and measurable proteins.

9.3. Preparation of TBARS Reagent:

The acidic TBARS reagent was prepared using:

1% phosphoric acid (H_3PO_4)

0.67% thiobarbituric acid (TBA)

9.4 Assay Procedure:

A volume of tissue supernatant was mixed with the prepared TBARS reagent. The samples were then incubated at 95°C for 1 h.

After incubation, the samples were cooled using an ice bath and centrifuged to remove precipitates. Finally, the absorbance of the resulting supernatant was measured at 532 nm using a spectrophotometer.

9.5 Calculation of MDA Concentration:

The concentration of malondialdehyde (MDA) was calculated according to the Beer–Lambert law using the following equation:

$$C = \frac{A}{E \times L}$$

C: concentration of MDA (mol/L).

A: absorbance measured at 532 nm.

L: optical path length of the cuvette (1 cm).

E: molar extinction coefficient of the MDA–TBA complex (1.56×10^5 $L \cdot mol^{-1} \cdot cm^{-1}$).

10. statistical analysis:

Control vaseline, Treated Ag/Zn NPs, Treated Ag/Zn NPs @chitosan-product G, and Silver sulfadiazine 1%. Results are presented as Mean ± SE.

Group interpretation: Control vaseline = lesion control group treated with vaseline alone; Treated Ag/Zn NPs = nanoparticle treatment; Treated Ag/Zn NPs @chitosan-product G = hybrid treatment; Silver sulfadiazine 1% = reference treatment.

Methodological note: no value was added, repeated, or generated. Clearly identified outliers were excluded before statistical calculation in order to limit their excessive influence on Mean ± SE, ANOVA, and values expressed as Mean ± SE. Statistical comparison was

performed using One-way ANOVA followed by Dunnett post hoc test versus Control vaseline. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS: not significant..

Results and discussion

Conclusion

Bibliographic References

Bibliographic References

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Annexes

Figure 6:Final prepared extract (Original photo, 2026).

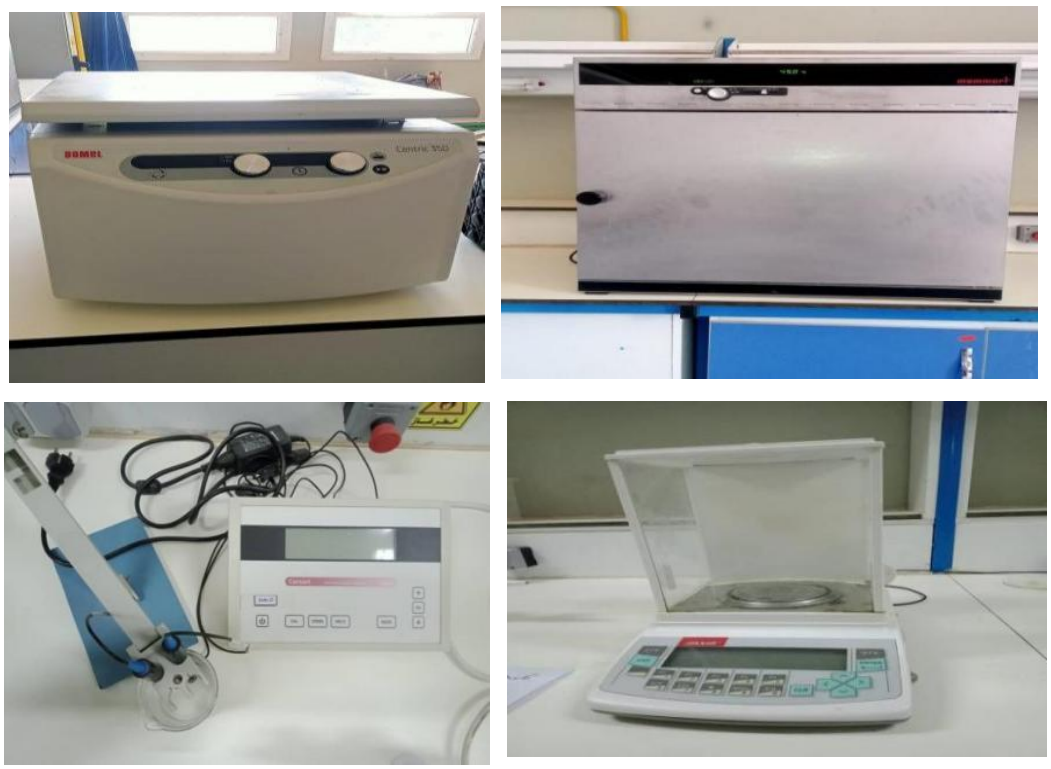

Figure 7:Steps and tools used whenPreparation for Nanoparticles (Original photo, 2026).

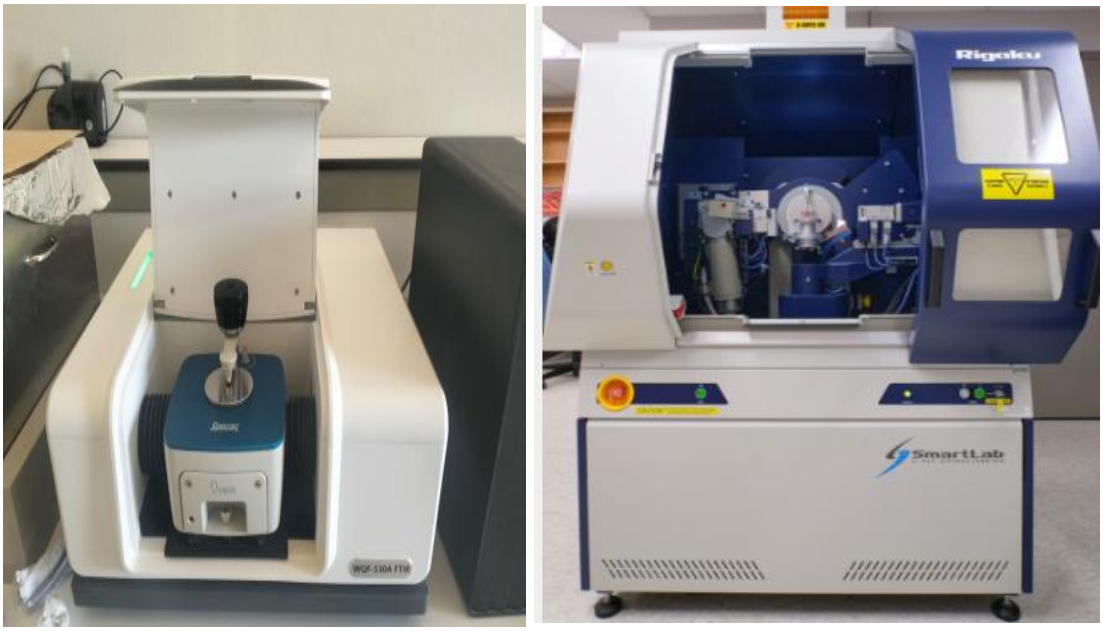

Figure 8:Laboratory Equipment Used for the Evaluation of the Prepared Samples' Efficacy (Original photo, 2026).

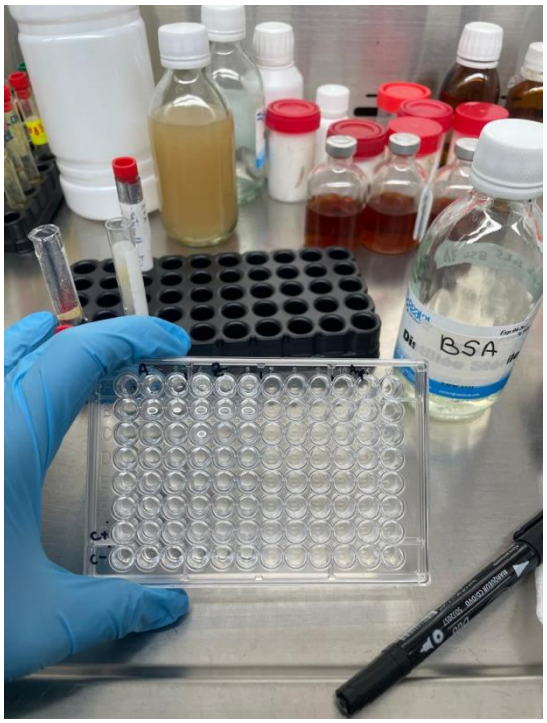

Figure 9:Protein Denaturation Inhibition Assay Using Bovine Serum Albumin.

Figure 10:Rat cages (Original photo, 2026).

Figure 11:Preparation of the rat by shaving the lower back area (Original photo, 2026).

Figure 12:Experimental induction of cutaneous burn in the rat (Original photo, 2026).

Figure 13:Sacrifice, blood drawing (Original photo, 2026).

Figure 14:Preparation of skin homogenates for MDA assay (Original photo, 2026).

Figure 15:Samples used for the MDA test (Original photo, 2026).