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INVESTIGATION OF ANTIMICROBIAL ACTION AND RHEOLOGICAL PROPERTIES OF CHITOSAN AND ALGINATE BASED HYDROGELS ENRICHED WITH VOLATILE OILS FOR BIOMEDICAL APPLICATIONS

SUMMARY OF DOCTORAL THESIS

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Introduction

In the context of significant advances in the field of biomedical materials, chitosan- and alginate-based hydrogels have garnered considerable interest due to their remarkable properties such as biocompatibility, biodegradability, and the ability to form three-dimensional hydrated networks. These characteristics are fundamental for various medical and biomedical applications, such as wound healing and controlled drug delivery. However, to fully harness the potential of these materials, it is essential to have a detailed understanding of their rheological properties and antimicrobial action.

The primary motivation of this study stems from the need to develop hydrogels with optimized performance through an in-depth exploration of their rheological behavior and antimicrobial capabilities. Specifically, understanding the rheological properties of these hydrogels is crucial for optimizing manufacturing processes and improving their clinical applicability. Rheology influences not only the administration and stability of hydrogels but also how they can be adapted to mimic biological tissues, thereby providing adequate structural and functional support. Regarding antimicrobial capabilities, chitosan is known for its activity against a variety of bacteria and fungi due to its cationic properties, which allow it to interact with microbial cell membranes. Although alginate is less active in terms of antimicrobial properties, it contributes through its film-forming ability and mucoadhesive properties, which can be enhanced through chemical modifications or by combining with other antimicrobial agents.

These properties are important for several reasons:

- Efficiency in biomedical applications: The rheological properties of hydrogels directly influence how they can be applied and manipulated in clinical settings, including wound dressings and drug delivery systems. Proper rheology can ensure uniform application and optimal adhesion to affected tissues.
- Safety and efficacy: Antimicrobial properties are essential for preventing and combating infections in wound healing applications. Hydrogels that can effectively inhibit the growth of pathogenic microorganisms can reduce the risk of infections and promote faster and more efficient healing.

- Development of innovative solutions: A detailed understanding of these aspects can lead to the development of new hydrogels with improved performance, contributing to innovations in the biomedical field and enhancing available treatments for patients.

This study aims to achieve the following main objectives, focusing on the rheological properties and antimicrobial action of chitosan- and alginate-based hydrogels:

- Characterization of rheological properties: The study will conduct a detailed analysis of the rheological behavior of chitosan- and alginate-based hydrogels. Rheology, the science that studies the deformation and flow of materials, provides essential information about the viscosity, elasticity, and fluidity of the studied materials. This analysis will examine how these parameters are influenced by the composition of the hydrogels and the formulation conditions. We will evaluate the hydrogels' behavior under various types of shear, their ability to recover their shape after deformation, and their structural stability over time. This rheological evaluation is crucial for understanding how hydrogels can be applied in various contexts, such as biomedicine or the food industry.

- Evaluation of antimicrobial activity: The second objective of the study involves testing the efficacy of hydrogels against a wide range of pathogenic microorganisms, including clinically relevant bacteria and fungi. Testing methods will include standardized techniques such as the disk diffusion method to determine the hydrogels' ability to inhibit bacterial and fungal growth. Antimicrobial activity is an essential attribute of hydrogels with biomedical applications where infection prevention is a priority.

- Optimization of composition: Another central objective of the research is to optimize the composition of the hydrogels by varying the concentration of key components: chitosan, alginate, and crosslinking agents. This investigation will focus on how these formulation variables influence the rheological properties and antimicrobial activity of the hydrogels. The ultimate goal is to identify optimal combinations that ensure an ideal balance between rheological performance and antimicrobial efficacy. This optimization is important for developing hydrogels with broad applicability and enhanced efficiency in various biomedical applications.

The proposed study aims to significantly advance the field of biomedical materials by providing valuable insights into the design and application of chitosan- and alginate-based

hydrogels. This research not only seeks to improve existing treatments but also to open new horizons for innovative applications in the biomedical field. The results obtained can form the basis for developing new, personalized, and effective therapeutic solutions tailored to modern clinical requirements.

The general- theoretical part

The first chapter provides a detailed analysis of the structure and functions of the skin, as well as the use of therapeutic hydrogels in healing skin lesions. The skin, the largest organ of the human body, plays an essential role in maintaining the integrity and health of the organism due to its multiple vital functions, including protection against mechanical, chemical, and biological agents from the external environment [1]. Additionally, the skin is crucial for homeostasis, body temperature regulation, sensory perception of temperature, touch, and pressure, mitigating the harmful effects of UV radiation, and strengthening the immune system to prevent infections. Moreover, the skin has a remarkable self-healing capacity and plays a significant role in the synthesis of vitamins [2,3].

Structurally, human skin is composed of three distinct layers: the epidermis, dermis, and hypodermis. The epidermis, the outer layer, is elastic and continuously regenerating. It is made up of various cell types (keratinocytes, corneocytes, melanocytes, Merkel cells, Langerhans cells), which fulfill the protective barrier function. Approximately 80% of the cells in the epidermis are keratinocytes, which are constantly generated through cell division and progressively migrate towards the skin surface. On the surface, these cells become flattened, dead, and shed through the natural exfoliation process. The outer keratinized and corneous layer of the epidermis is relatively impermeable.

Upon a skin injury, multiple organized reactions occur around the affected tissue, ultimately leading to tissue healing. This complex and dynamic physiological process consists of four stages (or three, according to some authors who include hemostasis in the inflammatory phase): hemostasis, the inflammatory phase, the proliferative phase (tissue growth), and the maturation and tissue remodeling phase [1,4]. Immediately after trauma, a series of well-coordinated events are triggered, mediated by receptors and cells involved in these processes, each playing an essential role in the progression of wound healing.

In recent years, researchers have focused on investigating reactive oxygen species (ROS), chemokines, and macrophage phenotypes as critical factors in the excessive

inflammation observed in skin or organ injuries. Polymers, whether natural or synthetic, undergo physical or chemical recombinations to achieve various functions and properties. Physically, they form hydrophobic associations, hydrogen bonds, and ionic interactions. Chemically, polymers are connected through a variety of covalent bonds, including disulfides, Schiff bases, and borate ester bonds. Cross-linking methods depend on the individual characteristics of the polymers [5].

Anti-inflammatory hydrogel dressings integrate specific drugs, small bioactive molecules, and novel biomimetic materials into a hydrogel matrix (Figure 5). These are capable of neutralizing excess free radicals, sequestering chemokines, and promoting M1-M2 macrophage polarization, thus having a beneficial effect on reducing excessive inflammation at the wound site and facilitating the healing process. These dressings work by stimulating angiogenesis (formation of new blood vessels to improve local vascularization), collagen deposition, epithelial cell migration, reducing fibrosis (pathological excessive development of connective tissues), and remodeling of the extracellular matrix [6].

Free radicals (ROS) include hydroxyl radicals (-OH), hydroxyl ions (OH^-), superoxide anions (O^{2-}), and peroxides (O_2^{2-}). Various studies have shown that a reduced concentration of reactive oxygen species (ROS) favors the tissue healing process. Hypoglycemia and infections are associated with an abnormal pro-inflammatory response, characterized by significant infiltration of inflammatory cells such as neutrophils and macrophages, leading to an increased concentration of ROS. This increase in ROS can have harmful effects, such as damage to DNA structures, lipids, and membrane proteins, cell injury, and apoptosis.

When ROS concentrations are low, they can attract lymphocytes from other regions to the injury site, promoting and modulating angiogenesis and tissue reperfusion processes in the affected area. ROS also play an important role in host defense against invading microorganisms. Clinical studies suggest that a concentration of 100 μM hydrogen peroxide (H_2O_2) can stimulate angiogenesis through vascular endothelial growth factor (VEGF) signaling.

It is well known that an excess of ROS can negatively affect the healing process. Consequently, researchers have sought innovative ways to control the excessive release of ROS. Recently, the use of compounds with antioxidant properties in hydrogels through various processes, such as combination, modification, and polymerization, has shown promising results in neutralizing excess ROS and promoting effective wound healing. Depending on their nature,

these compounds have been classified into five categories, as follows: 1) natural polyphenols, 2) polysaccharides, 3) amino acids, 4) synthetic polymers, 5) new metallic nanomaterials [7].

Phenolic hydroxyl groups in natural polyphenols have the ability to stabilize ROS through chemical modifications such as hydrogen exchange and electron transfer. Additionally, natural polyphenols can chelate transition metals and exert a protective and activating role on antioxidant enzymes, thus inhibiting oxidative enzymes resulting from oxidative stress. They are also involved in antimicrobial protection. Natural polyphenols, primarily from the flavonoid category (such as quercetin, catechin, catechol, and curcumin) and polyphenolic acid esters (such as ferulic acid, gallic acid, tannic acid, and ester derivatives), are essential compounds in this regard.

Curcumin, a major active ingredient in turmeric, demonstrates strong anti-infective, antioxidant, and anti-inflammatory properties, making it a promising agent for topical use in wound treatment [8]. In a study published by di Luca and colleagues [9], a multifunctional compound was developed by combining curcumin-loaded hydrogels and microparticle systems containing polyphenols with antimicrobial properties and quercetin. The final results showed that this system reduced H_2O_2-induced cellular oxidative stress and the proliferation of methicillin-resistant Staphylococcus aureus.

Another antioxidant bioactive compound is resveratrol (RSV), a polyphenol with excellent tissue regeneration capacity and the ability to modulate cytokine production and insulin sensitivity [10]. Gallic acid, a crucial polyphenolic substance, exhibits remarkable qualities, including anti-inflammatory, antimicrobial, antibacterial properties, and the ability to neutralize reactive oxygen species (ROS), thus significantly contributing to the acceleration of the wound healing process [11].

Ferulic acid, an organic phenolic compound derived from hydroxycinnamic acid, is present in the cell walls of most plants and is associated with molecules such as arabinoxylans [12]. Wei and his team [13] investigated the effects of ferulic acid by incorporating it into a hydrogel aimed at accelerating wound healing.

Another widely used natural polyphenol derived from plant species in bioengineering is tannic acid. Hydrogels containing this bioactive compound, due to its valuable properties, exhibit important characteristics such as adherence, antibacterial activity, and antioxidant effect. These hydrogels stimulate accelerated collagen deposition at the injury site and enhance

the expression of vascular endothelial growth factor (VEGF) while reducing tumor necrosis factor-alpha (TNF-α) levels.

Some amino acids and peptides can directly react with ROS through functional groups such as amino (-NH2), hydroxyl (-OH), carboxyl (-COOH), and sulfur. Particularly, a more pronounced antioxidant effect is observed in amino acids containing hydroxyl phenolic or sulfhydryl (-SH) groups. Among the amino acids incorporated into hydrogels that exert both antioxidant and antibacterial effects are arginine, silk fibroin peptides, and peptides derived from pearls.

All the aforementioned natural materials exhibit good biocompatibility. However, one of their main disadvantages is increased enzymatic degradation, as well as low physical and chemical stability. These aspects can limit their use in certain medical applications, as the durability and resilience of the material are critical factors for long-term therapeutic success [6]. However, these issues can be avoided or controlled through the use of synthetic materials, which will be discussed next.

Personal contributions

The personal contribution is detailed in **Chapter 2** and **Chapter 3**, which address both the rheological characteristics of chitosan and alginate-based hydrogels, as well as the preparation methodology and procedures used to test their antimicrobial action. This research reflects significant personal contribution in several aspects:

- Development and optimization of the preparation procedure for chitosan and alginate-based hydrogels enriched with volatile oils.
- Implementation and adaptation of rheological tests to evaluate the mechanical properties of the hydrogels.
- Conducting antimicrobial tests and analyzing data to determine the effectiveness of volatile oils in the hydrogel formulations.
- Interpreting results and formulating conclusions based on empirical data.

This personal contribution allows for obtaining relevant and innovative results that add value to the field of biomedical applications of hydrogels.

Simple Chitosan-Based Hydrogels with Antimicrobial Action

Formulating simple chitosan and alginate-based hydrogels involves a complex process that requires careful selection and rigorous preparation of the components involved. By optimizing each step, from dissolution to final cross-linking, hydrogels with improved properties for various biomedical applications can be obtained. This attention to detail ensures not only the quality and efficiency of the hydrogels but also their safety and efficacy in medical uses.

To evaluate the influence of chitosan concentration on the properties of the formed hydrogels, gels with concentrations of 2% (Formulation A), 3% (Formulation B), and 4% (Formulation C) enriched with volatile oils of turmeric and bay leaf were prepared. Additionally, the influence of alginate concentration on the properties of the formed hydrogels was evaluated using gels with the same concentrations of 2% (Formulation D), 3% (Formulation E), and 4% (Formulation F) enriched with volatile oils of copaiba and rosemary.

According to the experimental results obtained from the control tests (Table 1, Table 2), good stability was observed in all six formulations of hydrogels with volatile oils. However, in terms of consistency and pH, the best stability over time was presented by Formulations C (chitosan-based hydrogel) and F (alginate-based hydrogel). All formulations exhibited an acidic physiological pH, similar to the physiological value of human skin, ranging from 4.8 to 6.0.

Table 1. Characteristics of chitosan hydrogels

Characteristic	Formula A	Formula B	Formula C
Initial organoleptic examination	appearance: homogeneous, translucent; color: yellow-orange; smell: specific	appearance: homogeneous, translucent; color: yellow-orange; smell: specific	appearance: homogeneous, translucent; color: yellow-orange; smell: specific
Organoleptic examination after 30 days	characteristics initial constants	characteristics initial constants	characteristics initial constants

Organoleptic examination after 60 days	characteristics initial constants	characteristics initial constants	characteristics initial constants
pH initial	5.5-6	5.5-6	5.5-6
pH after 30 days	5.5-6	5.5-6	5.5-6
pH after 60 days	6-6.5	6-6.5	5.5-6
Viscosity	640 ± 0.66 mPas	855 ± 0.25 mPas	1488 ± 0.50 mPas
Viscosity after 30 days	636 ± 0.75 mPas	853 ± 0.66 mPas	1487 ± 0.33 mPas
Viscosity after 60 days	624 ± 0.36 mPas	850 ± 0.52 mPas	1476 ± 0.73 mPas

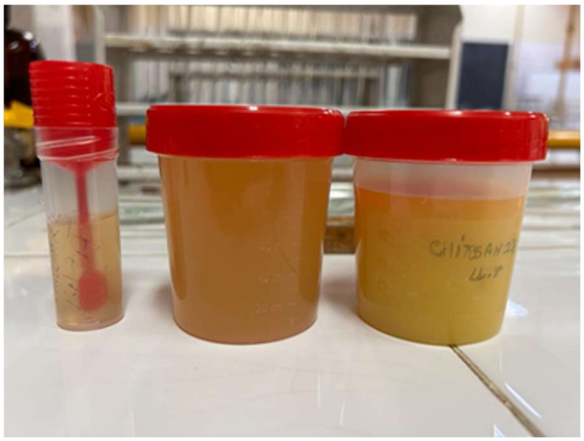

Figure 1. Hydrogels with chitosan: Formula A, B, C

Table 2. Characteristics alginate hydrogels

Characteristic	Formula D	Formula E	Formula F
Organoleptic evaluation – initial moment	appearance: homogeneous, translucent; color: white-yellowish; smell: specific	appearance: homogeneous, translucent; color: white-yellowish; smell: specific	appearance: homogeneous, translucent; color: white-yellowish; smell: specific

Organoleptic examination after 30 days	characteristics initial constants	characteristics initial constants	characteristics initial constants
Organoleptic examination after 60 days	characteristics initial constants	characteristics initial constants	characteristics initial constants
pH-initial	6-6.5	6-6.5	6-6.5
pH after 30 days	6-6.5	6-6.5	6-6.5
pH after 60 days	6.5-6.8	6.5-6.8	6-6.5
Viscosity-initial	682 ± 0.50 mPas	814 ± 0.33 mPas	1038 ± 0.46 mPas
Viscosity after 30 days	676 ± 0.66 mPas	807 ± 0.75 mPas	1036 ± 0.33 mPas
Viscosity after 60 days	664 ± 0.28 mPas	800 ± 0.22 mPas	1030 ± 0.64 mPas

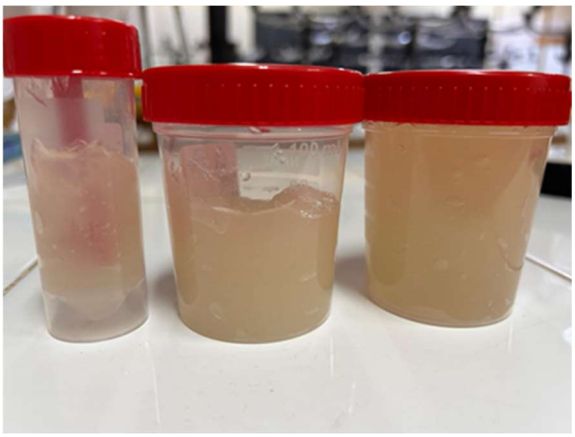

Figure 2. Hydrogels with alginate: Formula D, E, F

These results indicate that Formulations C and F are the most promising in terms of maintaining the stability of their essential characteristics, such as consistency and pH, over time. These properties are critical for the applicability of hydrogels in the medical field, especially in the context of interaction with human skin, where an appropriate pH can significantly influence the product's tolerability and effectiveness.

According to the experimental results obtained from the control tests, all six formulations of hydrogels with volatile oils showed good stability. However, in terms of consistency and pH, the best stability over time was demonstrated by Formulations C (chitosan-based hydrogel) and F (alginate-based hydrogel). All formulations exhibited an acidic physiological pH, similar to the physiological range of human skin, from 4.8 to 6.0.

Analysis of the rheograms presented in Figure 3 shows a non-Newtonian behavior for Formulations B, C, D, and E.

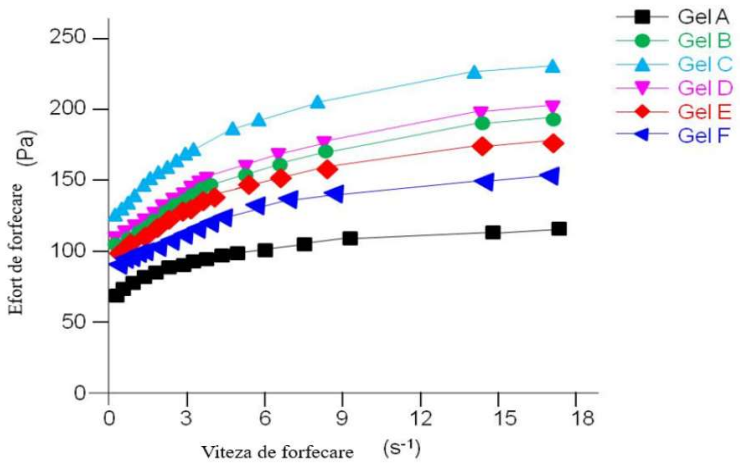

Figure 3. Rheograms of analyzed hydrogels

The identification of non-Newtonian behavior in the studied formulations enhances our understanding of the rheology of hydrogels, aligning with findings established in the literature. Due to their complex polymer network structures, hydrogels often exhibit intricate rheological responses that deviate from Newtonian fluid behavior. This phenomenon has been documented in the literature, where various hydrogel formulations have demonstrated shear-thinning, shear-thickening, or viscoelastic behavior, depending on factors such as polymer composition, concentration, and cross-linking density [14].

The antimicrobial activity of hydrogel Formulations B, C, and D—those with the most favorable rheological characteristics—was tested. Additionally, the antimicrobial action of the volatile oil mixtures in the chitosan-based hydrogel formulations was assessed, including Mixture U1 (equal parts turmeric oil and bay leaf oil, 1/1 g/g). The antimicrobial activity of the

volatile oil mixture in the alginate-based hydrogel formulations was also tested, including Mixture U2 (equal parts rosemary oil and copaiba oil, 1/1 g/g).

The purpose of these tests was to determine the potential efficacy of the formulations in combating pathogenic microorganisms.

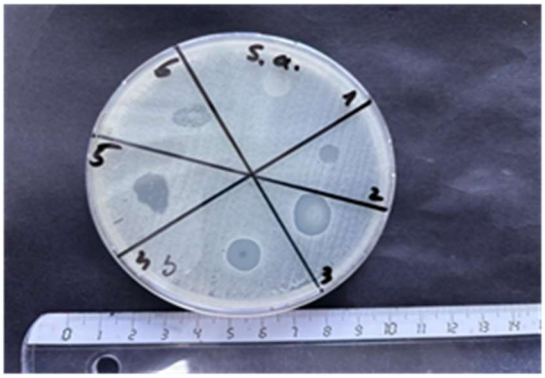

Figure 4. a. Zones of inhibition on *Staphylococcus aureus strains* after 24h

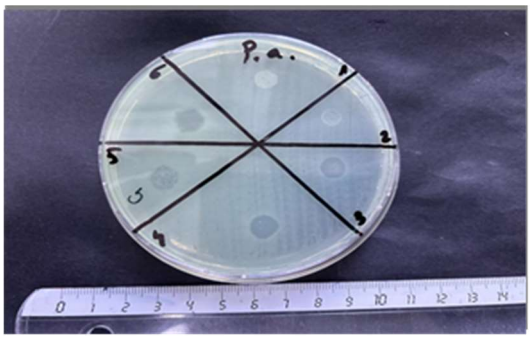

Figure 4.b. Zones of inhibition on *Pseudomonas aeruginosa strains* after 24h

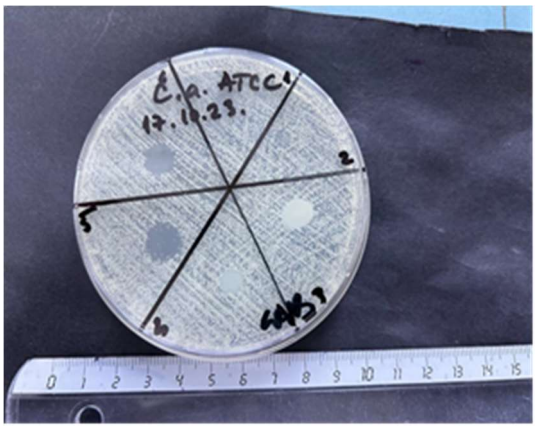

Figure 4.c. Zones of inhibition on *Candida albicans strains* after 24h

The formation of inhibition zones indicates antimicrobial activity, with larger inhibition zones typically suggesting stronger antimicrobial activity, demonstrating the formulations' ability to hinder microbial proliferation.

Using an adapted diffusion method on agar plates, both chitosan and alginate gels exhibited moderate antimicrobial activity against the tested bacterial strains. The diameters of the growth inhibition zones for *Staphylococcus aureus* and *Pseudomonas aeruginosa* ranged from 7 to 11 mm.

Tests were conducted according to relevant microbiological standards, and the results were analyzed to assess the ability of the hydrogels and their volatile oil mixtures to inhibit the growth and development of bacteria and fungi, including standardized strains used in antimicrobial studies.

Dimethyl sulfoxide (DMSO) was used as the solvent for dissolving the essential oils and their mixtures, which generally has no antimicrobial activity. DMSO and the gel bases (chitosan and alginate) were used as controls for all tested strains.

This approach ensures that the observed effects in the antimicrobial tests are specifically attributed to the active compounds (essential oils and their mixtures), eliminating the possibility that the solvent or gel bases influenced the results.

The use of DMSO and gel bases as controls is essential for the accurate interpretation of the antimicrobial activity of the tested compounds compared to standardized laboratory conditions.

All determinations were performed in triplicate, and results were expressed as mean ± standard deviation (SD). Statistical evaluation of the clinical results was performed using the Student’s t-test and analysis of variance (ANOVA).

This method provides robust validation of the data by repeating measurements three times for each set of experiments. Calculating the mean and standard deviation allows for a precise description of the results, highlighting variability between measurements and the degree of accuracy of each determination.

The U1 combination (turmeric and bay leaf essential oils) generally showed a slightly higher inhibitory potential than the other analyzed volatile oil mixtures against the three tested strains (Figure 5).

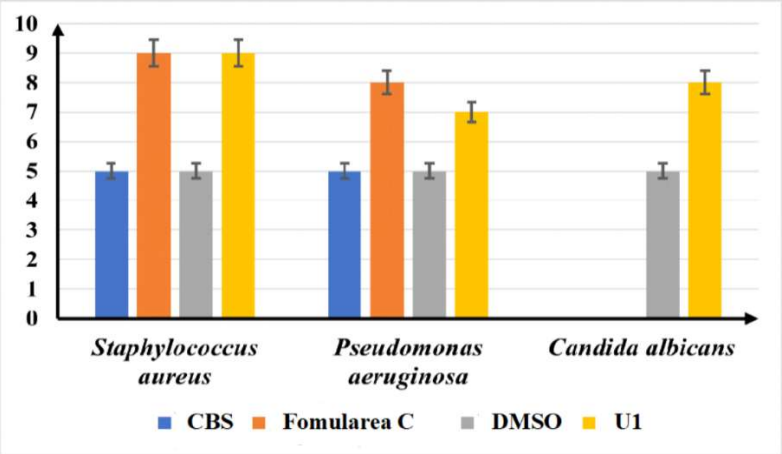

Figure 5. Diameters of the zone of growth inhibition (GZID) for tested formulations of chitosan- based hydrogels (CBS), Formulation C, DMSO and the mixture of volatile oils (U1).

Overall, these findings highlight the potential of chitosan and alginate-based hydrogels (particularly formulation C), as well as the volatile oil mixtures (especially U1), as antimicrobial agents. These results were anticipated, as essential oils of turmeric and bay leaf have demonstrated remarkable antimicrobial activity according to literature data.

Antimicrobial activity and the rheological properties of hydrogel formulations play a significant role in practical applications, particularly in wound healing and tissue engineering. The stretchability of hydrogels, as observed in our study, reflects their mechanical integrity and ability to conform to various wound geometries [15][16]. Our results show that all hydrogel formulations maintain robust stretchability over time, with minimal variation, even after extended storage. This suggests the stability of the hydrogel matrix and their potential for long-term biomedical applications.

Comparison with literature data highlights the complex interaction between antimicrobial efficacy, rheological properties, and hydrogel composition. While previous studies have reported antimicrobial activity of chitosan and alginate-based hydrogels, few have investigated the combined effects of volatile oils on antimicrobial activity and rheological

behavior [17][18]. Our study addresses this gap by demonstrating the synergistic impact of volatile oil-enriched hydrogel formulations, alongside their favorable rheological properties.

Mixed Hydrogel Formulation with Antimicrobial Activity

Hydrogels composed of alginate and chitosan represent a significant innovation in the field of biomedical materials due to the unique combination of properties of these two natural polysaccharides. These materials offer remarkable advantages in various applications, especially in biomedicine and tissue engineering, due to their physical, chemical, and biological characteristics.

Combining alginate with chitosan significantly enhances the mechanical properties of hydrogels. Alginate, known for its ability to form stable gels, provides the hydrogel with a robust structure, maintaining its integrity even under varying environmental conditions. At the same time, chitosan adds flexibility and mechanical strength, making the hydrogels more elastic and adaptable to mechanical stresses. This combination of properties allows these hydrogels to be used in applications requiring durable and versatile materials.

Combining chitosan and alginate in hydrogel formulations leverages the advantages of each polymer, resulting in materials with improved properties. Hydrogels obtained from these two biopolymers exhibit excellent biocompatibility, adjustable mechanical properties, and enhanced antimicrobial capabilities. These hydrogels can release antimicrobial agents in a controlled manner and form protective barriers against pathogenic microorganisms.

The primary goal of this research was to develop complex hydrogels with mixed alginate-chitosan bases at alginate concentrations of 2% and chitosan of 1.5% (Formula A), as well as 1.5% alginate (Formula B), enriched with volatile oils (turmeric, bay leaf, and rosemary), and compare them to a 3% chitosan-based hydrogel (Formula C) to identify the advantages of mixed bases concerning stability, rheological properties, and antimicrobial activity.

The development of the alginate and chitosan-based hydrogel complex for topical application involved careful selection and preparation of the ingredients according to specific requirements. This process included rigorous selection of alginate and chitosan, adjusting their

proportions to ensure effective interaction and uniform distribution in the hydrogel, as well as using appropriate crosslinking technology to provide optimal properties for skin application.

Through this meticulous approach, a topical hydrogel complex was achieved with the capability to release active substances in a controlled manner and suitable biological compatibility for interaction with the skin. By establishing concentrations of 1.5% and 2%, we aimed to obtain colloidal dispersions with optimal rheological and gelling properties to ensure the hydrogel's suitability for topical application.

The hydrogel formulation process involves several steps:

1. Preparation: Alginate and chitosan are dispersed in aqueous solutions, forming separate colloidal dispersions.
2. Incorporation: Volatile oils are incorporated using methods such as emulsification or direct diffusion to ensure uniform dispersion within the polymer matrix.
3. Combination: Alginate and chitosan solutions are combined, reacting to form a stable hydrogel.
4. Optimization: Parameters such as pH, temperature, and component concentrations are optimized to achieve the desired hydrogel characteristics, including texture, porosity, and release capacity of volatile oils.

Two complex hydrogels based on alginate and chitosan with volatile oils and a chitosan-based hydrogel enriched with volatile oils were formulated.

Quality Control of Hydrogels [19-21]:

- Appearance Examination: Samples of hydrogels were spread in a thin layer on a microscope slide and examined with a magnifying glass (4.5X).
- Color Examination: Hydrogel samples were spread in a thin layer on a watch glass and observed against a white background. The color should remain stable over time.
- Odor Examination: The odor of hydrogel samples was assessed from a distance of 4-6 cm.
- pH Examination: A 1g sample of each hydrogel was dispersed in 5g of distilled water, filtered, and the pH of the aqueous phase was determined potentiometrically.

- Stretchability Examination: Conducted using the Ojeda-Arbussa method. A 1g sample was placed on a glass plate, covered with a second plate, and weights (50g, 100g, 150g, 250g) were applied at equal time intervals (1 minute). The diameters of the circles formed were measured both after applying the top plate and after each added weight, and the circle areas were calculated.
- Viscosity Examination: Conducted using a Brookfield rotational viscometer, model RTV (T.F. cylinder, 5 rpm, Helipath Stand). Results are expressed as mean ± standard deviation (SD).
- Rheogram Examination: The rheological behavior of the gels was studied using the Brookfield rotational viscometer, model RTV, with shear rates ranging from 0.08 to 18.8 s−1 at room temperature.

The quality control results of the hydrogel samples are presented in Table 3.

Table 3. Characteristics of mixed chitosan and alginate hydrogels

Characteristics	**Formulation A**	**Formulation B**	**Formulation C**
Organoleptic evaluation – initial moment	appearance: homogeneous, translucent; color: white-yellowish; smell: specific	appearance: homogeneous, translucent; color: yellowish white; smell: specific	appearance: homogeneous, translucent; color: white-yellowish; smell: specific
Organoleptic evaluation - after 30 days	characteristics initial constants	characteristics initial constants	Characteristics initial constants
Organoleptic evaluation - after 60 days	characteristics initial constants	characteristics initial constants	characteristics initial constants
pH –initial	5.5	5.5	5.5
pH – after 30 days	5.5 - 5.8	5.5	5.5 – 5.7
pH – after 60 days	5.5 - 6.0	5.5 – 5.7	5.5 – 5.8

Characteristics	Formulation A	Formulation B	Formulation C
Viscosity- initial	1158 ± 0.33 mPa·s	1575 ± 0.25 mPa·s	895 ± 0.35 mPa·s
Viscosity – after 30 days	1150 ± 0.45 mPa·s	1571 ± 0.66 mPa·s	887 ± 0.65 mPa·s
Viscosity – after 60 days	1142 ± 0.25 mPa·s	1568 ± 0.50 mPa·s	872 ± 0.53 mPa·s

Formulations A, B, and C exhibited similar organoleptic characteristics at the initial moment, all presenting a homogeneous and translucent appearance, though with minor color differences: formulation A was off-white to yellowish, while formulations B and C had an orange-yellow tint. The odor was specific and consistent across all formulations.

Over the 60-days period, the initial organoleptic characteristics remained stable for all formulations, indicating good stability under the tested conditions with no visible or olfactory changes suggesting degradation or unwanted chemical reactions.

pH measurements showed relative stability over time for all formulations. Initially, all formulations had a pH of 5.5. After 30 days, the pH of formulation A varied slightly between 5.5 and 5.8, formulation B remained constant at 5.5, and formulation C varied between 5.5 and 5.7. After 60 days, the pH of formulation A ranged between 5.5 and 6.0, formulation B between 5.5 and 5.7, and formulation C between 5.5 and 5.8. These minor variations are acceptable and do not indicate major instability.

Viscosity measurements showed a slight decrease over time for all formulations, but these decreases were small and consistent, indicating good hydrogel structure stability. Initially, the viscosity of formulation A was 1158 ± 0.33 mPa·s, formulation B was 1575 ± 0.25 mPa·s, and formulation C was 895 ± 0.35 mPa·s. After 30 days, the viscosity slightly decreased to 1150 ± 0.45 mPa·s for formulation A, 1571 ± 0.66 mPa·s for formulation B, and 887 ± 0.65 mPa·s for formulation C. After 60 days, the viscosity was 1142 ± 0.25 mPa·s for formulation A, 1568 ± 0.50 mPa·s for formulation B, and 872 ± 0.53 mPa·s for formulation C.

In conclusion, the data from Table 3 suggest good stability of the three developed hydrogel formulations, with formulation B showing the best maintenance of constant

characteristics over time, followed by formulation C. It can be appreciated that the mixed complex gels have additional stability.

Antimicrobial Efficacy Evaluation

The antimicrobial efficacy was assessed against standard strains, including *Staphylococcus aureus* ATCC 25923 and *Candida albicans* ATCC 10231, as well as clinical isolates such as *Staphylococcus aureus* 1(50), *Staphylococcus aureus* 2(45), *Candida albicans* 5529, and *Candida albicans* 7262 (from the Microbiology Laboratory collection, Faculty of Biology, University of Bucharest).

Antimicrobial activity was determined using the spot diffusion method [22][23]. Bacterial cell suspensions with a density of 1.5 × 10^8 CFU/mL (equivalent to the 0.5 McFarland standard) and fungal suspensions with a density of 3 × 10^8 CFU/mL (equivalent to the 1 McFarland standard) were prepared from cultures grown on Nutrient Agar (for bacterial species) and Sabouraud Agar (for fungal species).

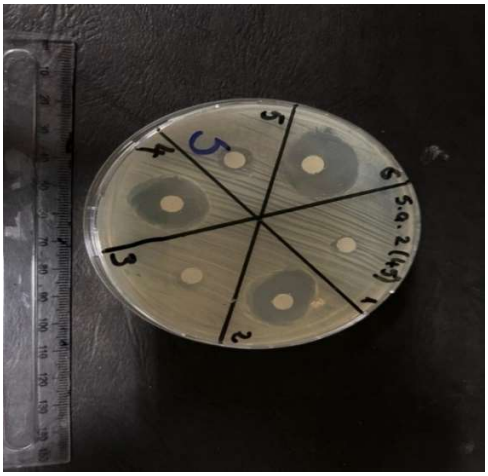

Figure 6.a. Zone of inhibition *S. aureus* ATCC 25923 Figure 6.b. Zone of inhibition *S. aureus* 2(45)

Figure 6.c. Zone of inhibition *S. aureus* 1(50)

Figure 6.d. Zone of inhibition *C. albicans* ATCC 10231

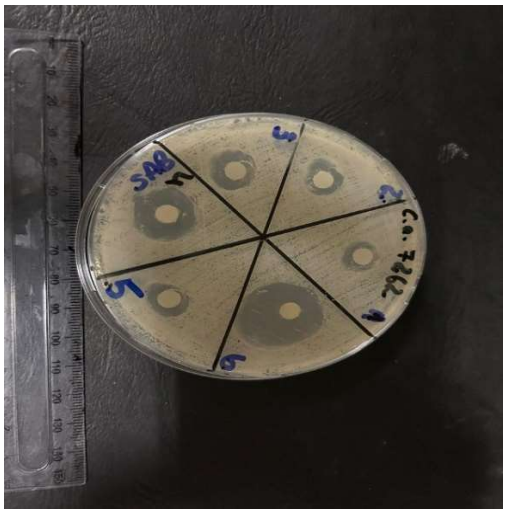

Figure 6.e. Zone of inhibition *C. albicans* 7262

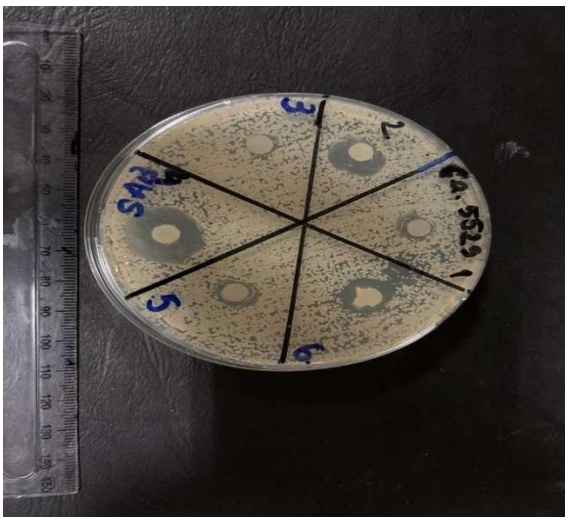

Figure 6.f. Zone of inhibition *C. albicans* 5529

Legend: 1- chitosan gel base 3%, 2 - Formulation C, 3 - alginate complex gel base 2% and chitosan 1.5%, 4 - Formulation B, 5 - alginate complex gel base 1.5% and chitosan 2%, 6 - Formulation A

All determinations were performed in triplicate, and using the diffusion method, significant antimicrobial and antifungal activity of the alginate and chitosan-based hydrogel complex, as well as of the chitosan gel on the culture plate, was observed. These results indicate that both formulations have the capacity to inhibit the growth and development of bacteria and fungi, suggesting a promising potential in combating microbial infections. It is important to note that this antimicrobial activity could be particularly useful in various medical and pharmaceutical applications, including the development of wound materials and skin care products.

Wathoni N. and colleagues emphasized that chitosan and alginate-based hydrogels have great potential in treating bacterial infections at the skin level [16]. Cruz Sánchez E. and collaborators experimentally demonstrated the inhibitory effect of lavender essential oil on the growth of bacterial species such as Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, and the fungus Candida albicans by incorporating the oil into chitosan, alginate, and chitosan-alginate membranes [24].

The diameters of the inhibition zones for Staphylococcus aureus ATCC 25923 varied between 6 and 18 mm. Notably, the pharmaceutical form F2 achieved the highest values among the gel samples analyzed, while the pharmaceutical forms B1, B3, and F3 recorded lower values. For Staphylococcus aureus 1(50), all gel formulations showed approximately the same inhibition zone diameters, with the pharmaceutical form F2 having an inhibition zone diameter twice as large as that of pharmaceutical form F3. For Staphylococcus aureus 2(45) strains, pharmaceutical form F2 showed the highest values. These findings suggest that these formulations could represent promising options for the development of antimicrobial treatments.

For Candida albicans ATCC 10231 strains, pharmaceutical form F2 showed slightly higher values compared to pharmaceutical forms F1 and F3. This trend was also evident for other Candida albicans strains, such as Candida albicans 7262 and Candida albicans 5529. These results indicate that pharmaceutical form F2 might have a greater potential in inhibiting the growth and development of Candida albicans strains compared to the other tested formulations.

While many studies focus on adding external antimicrobial agents to improve the effectiveness of hydrogel formulations, this research suggests that the hydrogel base itself can play a significant role in controlling infections. To better understand these mechanisms and

fully exploit the potential of hydrogel formulations in combating fungal and microbial infections, further research is needed. This research could focus on elucidating how the chemical and physical properties of the hydrogel affect its antimicrobial capacity and developing strategies to enhance this intrinsic antimicrobial activity.

Overall, these findings highlight the potential of the alginate and chitosan-based hydrogel complex, as well as the chitosan hydrogel, as promising antimicrobial agents. Identifying formulation F2 as the most promising underscores the importance of optimizing composition to achieve desired antimicrobial results. Its superior ability to inhibit microorganism growth, along with its mechanical flexibility, reinforces formulation F2's position as a particularly promising candidate for further development. However, continued research is essential to better understand the underlying mechanisms behind the observed antimicrobial effects and to adapt formulations according to specific biomedical applications. In conclusion, our study's results offer significant insights into the design and development of enhanced antimicrobial hydrogels with favorable rheological properties. By leveraging the synergistic potential of chitosan and alginate, along with volatile oils, new avenues are opened for addressing microbial infections and improving biomedical interventions.

General conclusions

Hydrogels with a mixed alginate-chitosan base offer several advantages due to the combination of properties from these two natural polysaccharides. These materials are biocompatible, biodegradable, have enhanced mechanical and antimicrobial properties, and allow controlled drug release. The combination of alginate with chitosan improves the mechanical properties of the hydrogels, providing them with a robust and flexible structure. Alginate ensures structural stability, while chitosan imparts elasticity and mechanical strength. Additionally, the presence of chitosan adds antimicrobial properties, helping to prevent infections—an essential aspect for biomedical applications such as wound dressings and implant materials.

Furthermore, mixed alginate-chitosan hydrogels have improved rheological properties due to interactions between the two polysaccharides and optimization of synthesis and formulation parameters. Our study highlighted the importance of optimizing composition and manufacturing processes to maximize the effectiveness of alginate and chitosan-based hydrogels. Critical variables, such as the alginate-chitosan ratio, antimicrobial agent concentration, and reaction conditions, significantly impact the final performance of the hydrogel. Optimizing these parameters can enhance the consistency of antimicrobial activity and ensure controlled and efficient release of incorporated drugs or active substances.

Based on the observations and results obtained, it is evident that alginate and chitosan-based hydrogel formulations, enriched with volatile oils, represent a promising direction in the development of effective antimicrobial products with therapeutic potential. Identifying formulation F2, a hydrogel complex of 2% alginate and 1.5% chitosan with volatile oils (turmeric, bay leaf, rosemary), as the most effective in this study underscores the importance of optimizing composition and manufacturing processes to achieve desired results. The enhanced efficacy of formulation F2 in inhibiting microorganism growth, as highlighted by experimental results, along with its favorable rheological properties, suggests that this formulation could be a particularly promising candidate for future biomedical applications.

For simple gels, six formulations of chitosan and alginate-based hydrogels and volatile oil mixtures were developed and evaluated for their rheological characteristics and antimicrobial activity. Among the tested formulations, formulations C (4% chitosan hydrogel enriched with volatile oils of turmeric and bay leaf) and D (2% alginate hydrogel enriched with

volatile oils of copaiba and rosemary) exhibited the most favorable rheological properties, justifying further investigation. All three hydrogel formulations demonstrated significant antimicrobial activity against selected bacterial strains. Notably, formulation D showed the strongest antimicrobial activity against S. aureus and P. aeruginosa compared to the other formulations. Additionally, formulation D includes a combination of volatile oils with strong antimicrobial properties, including essential oils of turmeric and bay leaf. This highlights the synergistic effect of combining alginate-based hydrogels with antimicrobial volatile oils and the potential of these formulations for biomedical applications.

This formulation is a promising candidate for further optimization and possible clinical translation, offering a dual benefit of favorable rheological properties and potent antimicrobial activity. The findings of this study emphasize the importance of considering both rheological characteristics and antimicrobial activity in the development of hydrogel-based pharmaceutical preparations. Further research is needed to elucidate the underlying mechanisms and optimize the formulation to enhance efficacy and expand applicability in wound healing and antimicrobial treatments.

The development process of hydrogel formulations involved optimizing the composition, ratios of alginate and chitosan, and integrating volatile oils with antimicrobial properties. Chitosan, due to its recognized antimicrobial capacity, was a natural choice to be integrated into the alginate matrix, thereby enhancing the activity of the formulations against pathogenic microorganisms. Formulation D stood out for demonstrating the strongest antimicrobial activity compared to the other tested formulations. This result suggests that incorporating volatile oils, such as those from turmeric and bay leaf, into the alginate and chitosan-based hydrogel matrix can create a strong synergistic effect, amplifying the antimicrobial properties of the hydrogel.

The clinical applicability of these formulations is crucial for advancing biomedical treatments. The favorable rheological properties of formulation D suggest that it could be effectively used in wound dressings, where appropriate consistency and drug release control are critical for healing. Enhanced antimicrobial activity could also offer innovative solutions in the treatment of antibiotic-resistant bacterial infections. However, continued research is necessary to better understand the underlying mechanisms driving the antimicrobial activity of these formulations and to adapt them appropriately to specific biomedical application needs. These efforts could include more detailed investigations of the interactions between hydrogel

components and microorganisms, as well as optimization of manufacturing parameters to achieve maximum efficacy.

In conclusion, our study makes significant contributions to the field of antimicrobial product development, highlighting the potential of alginate and chitosan-based hydrogel formulations in combating microbial infections and advancing biomedical interventions. These results open new research directions and provide the necessary foundation for the development of innovative and effective therapies in the future.

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1. Joița F.A, Mititelu M., Musuc A.M., Oprea E., Marinescu F., Lupuliasa D., Hîncu L., Nicolescu T.O., Pop A.L., Popescu I.O., Investigation of antimicrobial activity and rheological properties of chitosan- and alginate-based hydrogels enriched with volatile oils for biomedical applications, *Farmacia*, **2024,** Vol. 72, 3, doi: 10.31925/farmacia.2024.3.7 .

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