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Green Chemistry in Medical Applications: Preliminary Assessment of Kuzu Starch Films with Plant-Based Antiseptics

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Abstract: The current state of the natural environment requires medical products, including dressings, to be manufactured in accordance with the principles of a sustainable economy. This assumption is perfectly met by dressings made of renewable materials and additionally filled with natural antiseptics. The use of such plant compounds is consistent with the principles of green chemistry. In this work, films based on Kuzu starch with rooibos extract and chili pepper oil extract were prepared and tested. Starch foil with silver nanoparticles and foil without additives were used as a comparative material. The chemical structures (ATR-FTIR) of the materials obtained, their thermal (DSC) and mechanical properties (tensile strength, hardness), density, swelling, water vapor permeability, water solubility, and effects on bacteria such as *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 were examined. The Kuzu rooibos film had the lowest antimicrobial activity. At the same time, it was the most flexible foil and was characterized by having the best water vapor permeability and water absorption capacity. The starch film with chili extract was the weakest mechanically speaking, but it significantly inhibited the growth of *Staphylococcus aureus* ATCC 25923 bacteria at a level similar to that of the film with silver nanoparticles. The preliminary tests carried out on the properties of Kuzu starch films with plant extracts from rooibos tea and chili peppers indicate that they may be suitable for further research on dressing materials.

Keywords: Kuzu starch material; wound dressings; sustainable materials; antibacterial additives

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1. Introduction

The global problems regarding oil depletion and the increasing pollution of the natural environment with plastic waste require a solution that will put a stop to both processes. Sustainable economy activities have led to the creation of new, safer, and more efficient production and processing technologies, as well as the effective recycling of traditional plastics and the use of environmentally friendly materials in place of traditional plastics. These activities are directly related to the twelve principles of green chemistry. In recent years, attention has been paid to the production of biodegradable materials from renewable polymers such as starch, collagen, albumin, cellulose, alginate, etc. [1–3]. There is also increasing interest in these materials in biomedical applications due to their excellent biocompatibility, among other things [4–7]. The source of substrates for obtaining such medical materials is renewable and often constitutes post-production waste. Moreover, the methods used to obtain these materials are energy-efficient and do not require the use of organic solvents and complex technologies.

For centuries, medicine has been looking for effective ways to treat wounds quickly and effectively. Wounds are characterized by the destruction of the structure of the skin (with or without tissue loss). Most wounds heal on their own. However, the long duration of this process and complications related to the risk of infection have forced people to look for ways to regenerate skin safely and quickly. The first therapeutic agents used were substances of natural origin, such as plant substances, butter, honey, and resin. Over the years and with the development of science, new methods and materials have emerged to support the reconstruction of damaged tissues. They have been designed to support the healing process in all four stages: hemostasis, inflammation, proliferation, and tissue remodeling [8–13]. The healing of wounds by preventing dehydration in a moist environment has been well-documented in the literature [14]. Wound dressings provide a moist wound environment, help absorb exudates, and accelerate tissue regeneration.

Contemporary investigations of dressings mainly focus on accelerating the wound healing process and replacing 'petrochemical polymers' with new materials from renewable sources.

Starch is one of the most common and inexpensive plant materials. Starch accumulates in the branches of trees near buds, in fruits, in seeds, in rhizomes, and in tubers. It plays an important role in energy storage. This biopolymer is composed of two different polymers: linear amylose and branched amylopectin [15].

The amylose/amylopectin ratio determines the film-forming properties of starch. The amylose content is mainly responsible for the film-forming capacity of starch. The concentration of amylose in starch is an essential characteristic of individual starch species. For example, the amylose content ranges from 20 to 24% for potato starch, from 20 to 27% for corn starch, and from about 20 to 30% for Kuzu starch, and the amylose content of arrow root starch is over 40% [16–19]. Films obtained from starch are biodegradable, colorless, nontoxic, odorless, and have good barrier properties against oxygen. The Japanese Kuzu root (*Pueraria lobata*) has been known in the East for more than 2000 years and, due to its large amounts of isoflavones and minerals, it is used in traditional Chinese medicine [20–22]. The Kuzu plant has a very large root, which is rich in starch with good film formation properties. Moreover, Kuzu starch has a relatively high amylose content and produces clear, colorless, and high-strength gels. Thus, this biopolymer can be an effective material when used as a wound dressing. It can absorb wound exudates and provide a moist wound environment, which accelerates wound healing [23,24].

However, starch membranes do not protect against infections caused by microorganisms. Therefore, citric acid, which can be used as an agent to cross-link starch and thus increase its strength, may influence the antibacterial effect. Citric acid is extremely beneficial in medical applications [25].

Currently, various substances of plant origin that have been used for centuries in traditional medicine are being introduced into natural and synthetic polymer matrices to give these polymers various desirable properties (e.g., biocompatibility, antimicrobial, or anti-inflammatory properties). The potential applications of such materials mainly pertain to, as aforementioned, the medical industry, but these materials could also have many other applications (for example, in molding plastic products).

Active substances with antibacterial activity can quite easily be introduced into the starch network [24]. Therefore, the inclusion of natural antimicrobial substances may also play an active role in wound healing processes [26]. Various starch-based polymers with incorporated antibacterial substances have been studied for wound healing applications [25,27–29]. Eskandarinia and others obtained films based on corn starch via the addition of propolis and hyaluronic acid [30]. The films exhibited antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli*. Hadisi et al. have developed a new bioactive gelatin-starch nanofibrous dressing containing *Lawsonia inermis* (henna) for the treatment of second-degree burn wounds. The gelatin–starch–henna material reduced the inflammatory response and the number of macrophages in the wound bed [31].

Two plant materials with known antimicrobial and anti-inflammatory properties were selected for this research study: rooibos tea leaves and chili peppers.

Rooibos (*Aspalathus linearisi*), a popular herbal tea, is a shrub legume indigenous to the mountains of South Africa [32]. In traditional medicine, rooibos leaf dressings have been used to treat difficult-to-heal wounds [33]. Its beneficial effect in supporting the treatment of surgical wounds was proven in the study by the authors of [34]. Rooibos contains many valuable ingredients, including polyphenols, which offer numerous health benefits [35]. Due to their immunomodulatory properties, polyphenols improve the phase of wound healing, accelerate the process, and lead to better healing effects. Hence, the antioxidant, antimicrobial, and analgesic effects of polyphenols are significant [36,37].

Chili peppers (*Capsicum annuum* L., also known as hot peppers, red hot peppers, or red peppers) are well known and frequently added to dishes and sauces to give them a sharp and burning taste [38]. Capsaicin, contained in these peppers, irritates and warms the skin and causes a feeling of warmth and redness but does not cause inflammation. Capsaicin expands the blood vessels of the skin and the organs beneath it; therefore, it is used externally as a medicine to warm joints and muscles (e.g., in ointments, warming dressings). It is present as a constituent of analgesics. Capsaicin extracted from chili peppers has been reported to have antibacterial and antifungal effects [39]. Chili peppers also contain polyphenols, the healing effects of which were mentioned above [38].

The extraordinary properties of silver have been known for thousands of years. This chemical element occurs naturally in nature and belongs to the group of precious metals. In medicine, it is valued for its effective antibacterial properties. An ancient civilization used silver coins to disinfect and extend the life of drinking water [40]. The nonionic colloidal silver water dispersion solution has a proven antibacterial effect [41,42]. Nanoparticles of silver have the shape of flakes (plates) that give the maximum active surface. Colloidal silver can be found in the form of an aqueous solution, so it is easy to introduce it into the starch polymer matrix. However, there are studies that have showed the toxic effects of silver nanoparticles on living cells [43,44]. Despite this, they are often used in medical applications, including in wound dressings.

In this study, a new composite wound dressing with potential antimicrobial properties was developed. Kuzu starch-based composite films were prepared by adding rooibos infusion and chili pepper oil extract. To compare their properties (especially their antibacterial properties), a starch foil without additives (negative control film) and with the addition of a non-colloidal silver solution (positive control film) were also made. A commercial solution of silver nanoparticles with proven antimicrobial activity was used. The properties of the developed composite films were tested in terms of the requirements of biomedical applications; i.e., we tested their chemical structures (ATR-FTIR), physicochemical properties (thickness, density, swelling and solubility in water, permeability of water vapor), thermal (DSC) and mechanical properties (hardness, tensile strength, and elongation at break), and antibacterial activities (disk diffusion method).

These preliminary studies are part of a larger project, the aim of which is to analyze the impact of natural additives on the properties of starch films and to select those that are most suitable for further research on cells and living organisms, as well as for use in clinical trials, for their potential applications as dressing materials.

2. Materials and Methods

2.1. Materials

Kuzu starch (Terrasana, Leimuiden, The Netherlands), glycerol (Glycerol pure, Chempur, Piekary Śląskie, Poland), citric acid (Chempur, Piekary Śląskie, Poland), rooibos tea (William's Natural Products Sp. z o.o., Warsaw, Poland), chili pepper (fresh from a local store), and nonionic colloidal nanosilver solution (50 ppm, Vitacolloids, Rawa Mazowiecka, Poland) were used as received to prepare starch-based films. Standard *Staphylococcus aureus* ATCC 25923 (*S. aureus*) and *Escherichia coli* ATCC 25922 (*E. coli*) strains, obtained from Merck (MerckKGaA, Darmstadt, Germany) and Mueller–Hinton (M-H) agar (Bio-Rad, Poland Sp. zo.o., Warsaw, Poland), were used in our antimicrobial tests.

2.2. Preparation of Composite Films

Films were prepared by dispersing 5% (w/w) Kuzu in distilled water. The dispersions were stirred at 85 °C for 45 min to induce the gelatinization of the starch. Glycerol (2% w/w)

was added to the starch dispersion as a plasticizer, and citric acid (0.5% w/w) was added as a crosslinking agent. Citric acid carboxyl groups can form hydrogen bonds with starch hydroxyl groups to prevent recrystallization and retrogradation.

2.2.1. Film Prepared with Rooibos Extract (Kuzu Rooibos)

The extract was prepared from 5 g of rooibos tea (*Aspalathus linearis*) in 100 mL of boiling water for 5 min. The leaves were filtered off, the solution was cooled, and the mixture was stored under refrigeration conditions. The distilled water in the starch solution was replaced with an infusion of rooibos. Starch gelatinization was performed in a rooibos solution analogously to the procedure described above.

2.2.2. Films Prepared with Oil-based Chili Extract (Kuzu Chili)

Preparation of the Chili Pepper Extract: As capsaicin and its derivatives are soluble in alcohol and lipids and insoluble in water [45,46], a weighed amount of dry vegetable material (3 g of chili pepper, *Capsicum annuum Solanaceae*) was soaked in hemp oil (10 g) and left for 1 h in a dark place. Maceration was carried out at room temperature. The oily extract was mixed with 10 mL of distilled water using glycerol stearate (0.5%) as an emulsifier. After gelatinization, the water starch solution was chilled to a temperature in the range of 50 °C to 60 °C and the pepper extract was added.

2.2.3. Film Prepared with Nonionic Colloidal Silver (Kuzu Ag)

In this case, 50% of the distilled water in the Kuzu suspension was replaced by nonionic colloidal nanosilver solution.

The samples with silver nanoparticles (Kuzu Ag) and without additives (Kuzu 0) were used as the control samples.

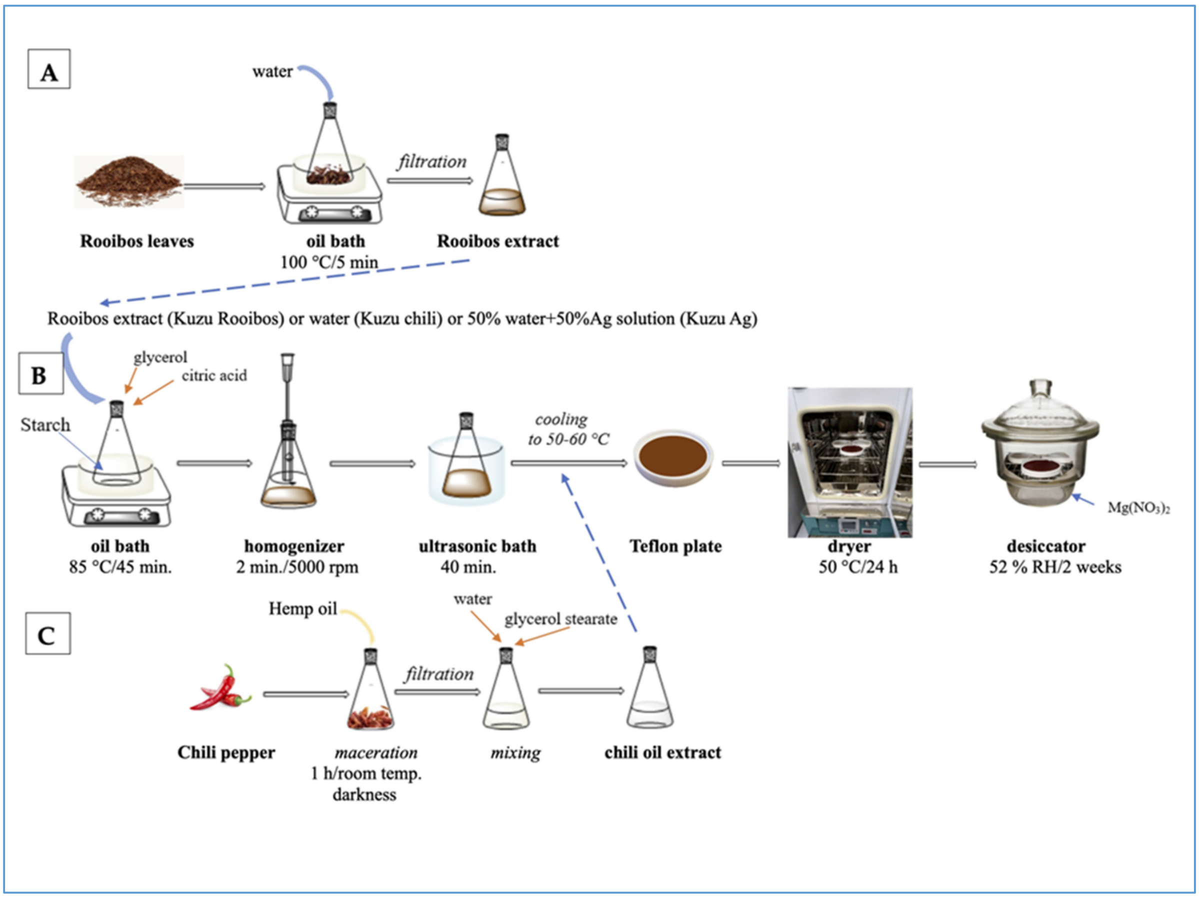
The quantification of additives was determined in such a way as to ensure the consistent maintenance of a starch concentration of 2% within each of the film-forming solutions. The compositions of the individual materials obtained based on Kuzu starch are presented in Table 1.

Table 1. Compositions of gelatinized starch and its composites with antibacterial additives (in 100 mL of starch solution).

Sample	Kuzu Starch [g]	Glycerol [g]	Citric Acid [g]	Rooibos Extract [g]	Chili Extract [g]	Nanocolloid Ag [g]
Kuzu 0	5	2	0.5	-	-	-
Kuzu rooibos	5	2	0.5	92.5	-	-
Kuzu chili	5	2	0.5	-	20	-
Kuzu Ag	5	2	0.5	-	-	46.5

All solutions were homogenized using a homogenizer (MPV-302) at 5000 rpm for 2 min. An ultrasound bath was applied for 40 min to remove air bubbles from the solution. The gel was poured onto a Teflon casting plate (10 cm in diameter) with a levelled surface and dried at 50 °C for 24 h. After the drying process, the films were peeled from the Teflon plates and conditioned in a desiccator in 52% RH (saturated magnesium nitrate solution) for two weeks.

The preparation of the modifying solutions and the method of their introduction to the gelatinized starch are shown in Scheme 1.



Scheme 1. Scheme of how to prepare a rooibos tea solution (**A**) and a chili pepper solution (**C**) and obtain a starch film with modifying additives (**B**).

2.3. Tests and Characterization

Analyses of chemical structure, thickness, hardness, density, solubility in water, swelling index, permeability of water vapor, thermal properties, strength tests, and the influence on bacterial growth of the obtained starch films were performed.

2.3.1. Thickness

Film thicknesses were determined using a micrometer screw. An average of 10 measurements performed for each film was used for the calculation. The thicknesses were measured at different points in each starch sample film.

2.3.2. Hardness

Hardness was evaluated on Shore A according to PN-EN ISO 868:2005 [47]. The means of 9 hardness values were used for the calculation.

2.3.3. Density

The densities of the polymer samples were determined according to the PN-EN ISO 1183-1:2019-05 standard using an analytical scale equipped with a density determination kit (RADWAG AS 160.X2, Radom, Poland) [48]. The means of five density values were used for the calculation.

2.3.4. Swelling Index

The swelling behaviors of the films were measured using an electronic RADWAG AS 160.X2 scale with an accuracy of ±0.0001 mg. The films (2 cm × 2 cm) were initially weighed (W_1) and immersed in 50 mL of distilled water for 24 h at 23 °C. The film samples were removed from the water and wiped with a piece of filter paper to absorb the water on the surfaces of the films. The swollen films were weighed (W_2). The percentage of swelling (in triplicate) for each sample was calculated using the following equation:

$$Swelling\ \% = \frac{W_2 - W_1}{W_1} \times 100 \quad (1)$$

2.3.5. Solubility Index

The solubility in water was determined according to the Riar method [49]. Square pieces of film (2 cm × 2 cm) were dried (105 °C for 24 h) to determine the initial weight of dry matter (W_A). The films were immersed in 50 mL of distilled water for 24 h at 25 °C and then removed and dried at 105 °C for 24 h to determine the final weight of the dry matter (W_B). The weight of the solubilized dry matter (in triplicate) was calculated using the following equation:

$$Solubility\ \% = \frac{W_A - W_B}{W_A} \times 100 \quad (2)$$

2.3.6. Thermal Analyses

Thermal analyses of the starch films were performed on a SetaramLabsys TG-DTA/DSC Differential Scanning Calorimeter with an 800 °C rod. Characteristics of the thermal properties of the starch films, including the melting temperature (expressed in °C) and the melting enthalpy (expressed in J/g) were measured. The heating rate for the runs was 10 °C/min in a pure nitrogen atmosphere. Film samples weighing 20 mg were heated in aluminum cells at temperatures of 20 °C to 180 °C. An empty aluminum cell was used as a reference. Apparatus calibration was performed using an indium sample.

2.3.7. Water Vapor Permeability Test

The water vapor permeability test was performed using a moisture analyzer (RADWAG MAX 60/1/NH) equipped with a probe cell to determine permeability. The film samples (19.625 cm^2) were sealed with two rims and placed in a circular opening permeation cell containing a weighed water sample (~9 g). The whole assembly was weighed and placed in the chamber of the moisture analyzer. The temperature within the chamber was kept at 45 °C for 2 h. Changes in cell weight were recorded to 0.0001 g and plotted as a function of time. The water vapor permeability was calculated based on the following equation:

$$P = \frac{M_1 - M_2}{t \times P_p} \quad (3)$$

where:

P—permeability of water vapor [mg cm^{-2} h^{-1}]
M_1—mass of distilled water in the probe cell in the 1 h of the experiment,
M_2—mass of distilled water in the probe cell in the 2 h of the experiment.
t—time of the experiment (1 h),
P_p—surface of evaporation.

During the first hour of the experiment, the sample chamber is stabilized. The stability of permeability is assumed to occur after 1 h of heating of the tested films.

2.3.8. Tensile Tests

The MultiTest 1-xt Mecmesin tensile tester was used to determine tensile strength and elongation at break. The initial distance between the handles and the stretching speed were 70 mm and 10 mm/min, respectively. The tested foil samples were rectangular pieces that were 100 mm long and 10 mm wide.

2.3.9. Fourier-Transform Infrared Spectroscopy (ATR/FTIR)

The absorbance spectra of the starch-based films were recorded using a Thermo Scientific Nicolet 380 FT-IR Spectrometer with a high-performance diamond single-bounce ATR accessory. A resolution of 4 cm^{-1} and a scanning range from 600 to 4000 cm^{-1} were applied, and 48 scans were taken for each measurement.

2.3.10. Antibacterial Tests

Standard *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 strains were used for the antimicrobial tests. Antibacterial activity against selected types of bacteria was determined via the disk diffusion method. This method is based on the principle that antimicrobial components at a certain concentration in the disk will diffuse into the medium and inhibit the growth of sensitive organisms, creating a cracking zone around the disk [50]. Mueller–Hinton agar, employed in bacterial cultivation, was poured into Petri dishes to achieve a uniform thickness of 4 mm. A suspension of 0.5 McFarland density in 0.85% sterile NaCl was prepared from 24 h bacterial cultures. A sterile cotton swab was immersed in the prepared suspension and then evenly spread over the agar. Ten-millimeter discs of starch films with natural rooibos extract, chili pepper extract, and silver colloid were placed on the medium inoculated with sterile tweezers, as was a disc of pure starch as a control. Each disc was gently pressed, ensuring even contact with the ground. The plates were incubated under aerobic conditions for 16–18 h at 35 °C [51]. After incubation, a caliper (Beta Poland Sp. zo.o., Skarbimierzyce, Poland) was used to determine the width of the microbial growth inhibition zone. The contact area was used to assess the growth inhibition under the film discs in direct contact with the target microorganisms on the agar. The entire area of the zone was calculated and then subtracted from the area of the film disk, and this difference area was reported as the inhibition zone (Equation (4)) [51]. The results are given as the zone of inhibition of bacterial growth in mm^2.

$$Inhibition\ zone = D_I - D_d \tag{4}$$

where:

D_I—Diameter of the entire inhibition zone [mm];
D_d—Diameter of the disc [mm].

2.3.11. Statistical Analysis

Measurement values are presented as the arithmetic mean ± standard deviation. To determine statistical significance, a one-way analysis of variance (ANOVA) was performed, followed by a Bonferroni post hoc analysis. A p value of less than 0.05 was considered indicative of a statistically significant difference.

3. Results and Discussion

The morphological characteristics of the Kuzu starch films with and without various additives are depicted in Figure 1. In particular, the incorporation of rooibos extract into the starch film resulted in increased gloss and a pronounced red coloration, as shown in Figure 1B. On the contrary, the addition of chili oil led to a reduction in the transparency of the film, resulting in opaque samples with small droplets of visible oil (Figure 1C). This effect can be attributed to the dispersion of oil droplets within the polymer matrix, which hinders light transmission [52]. Despite the inclusion of glycerol stearate as an emulsifier, the separation of hemp oil drops was still observed. Interestingly, the addition of nonionic colloidal silver did not significantly alter the appearance of the starch film, as illustrated in Figure 1D.

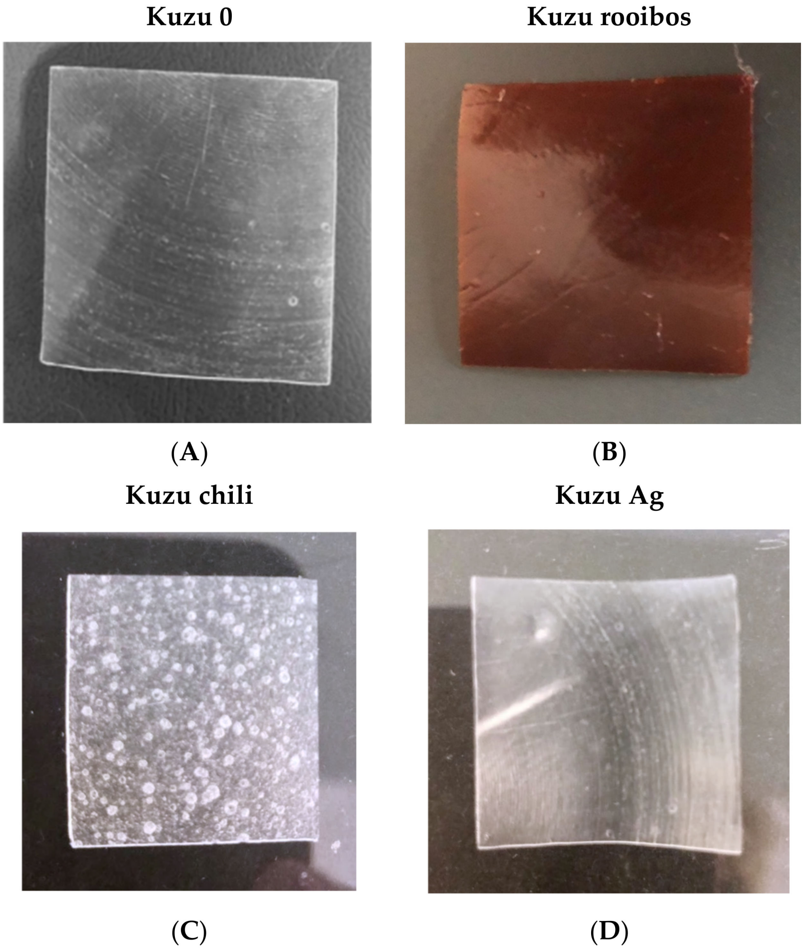


Figure 1. Photographs of the Kuzu starch films.

3.1. Thickness, Hardness, and Density

The physical properties of the wound dressing films, such as thickness (mm), hardness (Shore A), and density (g cm^{-3}), are presented in Table 2. The film thicknesses of the Kuzu 0, Kuzu rooibos, and Kuzu Ag samples were comparable and amounted to approximately 0.210–0.265 mm. However, the Kuzu chili films were almost twice as thick. However, this increased thickness of the Kuzu chili sample, compared to the Kuzu 0 sample, was expected [53]. This was due to the chemical components present in the chili oil extract, which could interact with starch to form agglomerates, thus imparting a distinct three-dimensional structure to the film. A similar effect was also observed for cellulose composite films prepared using essential oil [54]. Furthermore, the observed agglomeration of small droplets of hemp oil in the Kuzu chili foil could have resulted in an increase in the thickness of the prepared foil.

Table 2. Physical properties of the starch films.

Sample	Thickness [mm]	Hardness [Shore A]	Density [g cm^{-3}]
Kuzu 0	0.265 ± 0.0025	94.2 ± 8.07	1.3605 ± 0.03269
Kuzu rooibos	0.210 ± 0.0072 *	61.9 ± 4.39 *	1.3069 ± 0.02098 *
Kuzu chili	0.415 ± 0.0123 *	19.1 ± 4.99 *	1.2270 ± 0.01615 *
Kuzu Ag	0.250 ± 0.0011	93.9 ± 2.20	1.3609 ± 0.00850

Results are presented as the average ± standard deviation. Statistical analysis was performed by carrying out a one-way analysis of variance (ANOVA), followed by a Bonferroni post hoc test. Significant results ($p < 0.05$) are marked with *.

The films prepared using the rooibos and chili oil extract revealed lower densities than that of Kuzu 0. This effect may be caused by the incorporation of the low-molecular-weight substances contained in natural extracts (Kuzu rooibos and Kuzu chili) into the polymer matrix. The presence of these compounds in the structure of modified starch disturbed the interaction of the polymer chains, causing them to move apart. The formation of free interchain spaces probably hindered the formation of hydrogen bonds, which are an important factor, causing a highly dense assembly of polymer chains and thus increasing polymer density. The presence of the silver nanoparticles in the gelatinized starch network did not affect its density value.

The results of the density test are consistent with the results regarding the hardness values of the starch films. The samples with higher density values also had higher hardness values.

3.2. Swelling Index, Solubility in Water, Permeability of Water Vapor

In 1937, Frederick Carson suggested that the mechanism of moisture transpiration through various materials involves the diffusion of gases and water molecules, the movement of moisture as a function of the hygroscopic moisture content, and the migration of adsorbed water along internal surfaces [55]. This clearly indicates a direct dependence of the ability to transmit water on the physicochemical properties of the material, including its density, the degree of "throughput" of internal spaces, and hydrophilicity. For this reason, the study of new materials suitable for use as wound dressings also included the determination of their swelling, solubility, and water vapor permeability. The permeability of the water vapor in the wound dressing prevents the accumulation of secretion in heavily exuding wounds. Moisture vapor transmission thus has important implications for the ability of the dressing to cope with exudate production. The swelling index and permeability of the starch dressings were determined to assess the capacity of the dressings to handle exudates. High fluid absorption will prevent the accumulation of exudates in wound beds [56].

The water swelling index (water absorption capacity) of the Kuzu starch films was determined to assess their ability to transfer hydrophilic body fluids. This was carried out to ensure that the exudates from the wound could be absorbed by the dressings, preventing the maceration of the wound and accelerating the healing process [57]. Moreover, the permeability of products such as dressings is an important parameter that ensures safety and, on the other hand, comfort of use. Too much water permeability could cause the wound tissue to become dehydrated, while too little may increase the risk of bacterial infection. It should be noted that the test used to determine the water vapor permeability of wound dressings has not been standardized, so there is basically no reference point. However, reports in the literature indicate that the value of the water vapor transmission of the wound dressing should usually be 2000–2500 g $m^{-2}day^{-1}$, which gives 8.3–10.4 mg $cm^{-2}h^{-1}$ [24]. Considering this, the high permeability of the tested films could lead to excessive wound drying. However, in some cases, for example, to prevent granulating wounds, it is necessary to use dressings with increased water vapor permeability. For example, for first-degree burns, dressings with a permeability of 3000 g $m^{-2}day^{-1}$ (12.5 mg $cm^{-2}h^{-1}$) are suggested, while severe burns require dressings with a permeability of 5000 g $m^{-2}day^{-1}$ (20.8 mg $cm^{-2}h^{-1}$) [25]. According reports in the literature, the water lost from normal skin amounts to about ~250 g $m^{-2}day^{-1}$ at 35 °C (after unit conversion: 1.04 mg $cm^{-2}h^{-1}$) [24], while for damaged skin, it amounts to 1.16–21.41 mg $cm^{-2}h^{-1}$ [58]. Altaf et al. noted that 178 g m^{-2} h^{-1} (17.8 mg $cm^{-2}h^{-1}$) is the permeability for a second-degree burn wound [57]. Taking into account the aforementioned values, the determined water vapor permeabilities of the starch materials featured in this study are suitable, especially in the context of their potential uses as dressings for wounds such as burns (Figure 2C).

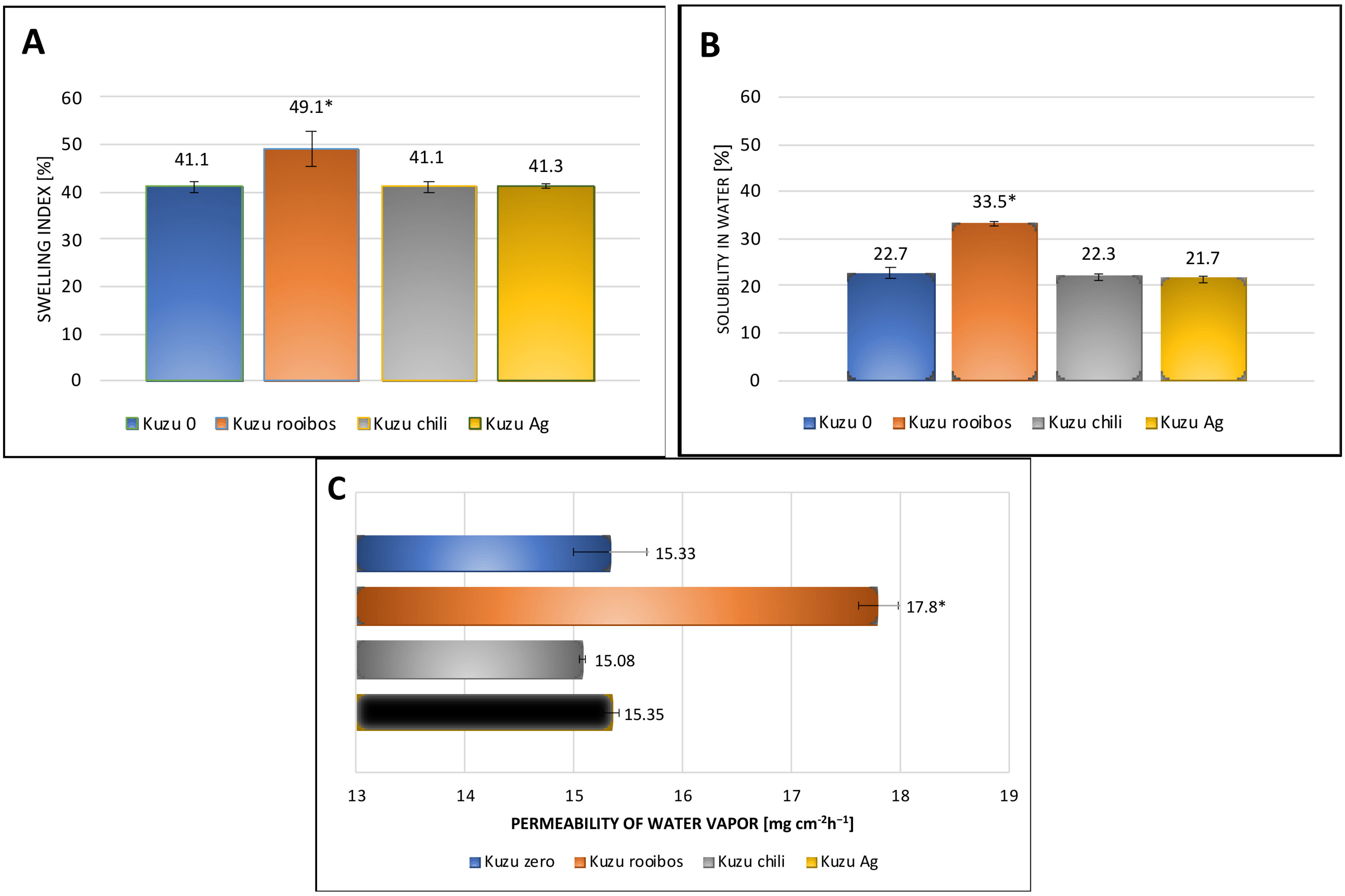


Figure 2. Swelling (**A**), solubility (**B**), and water vapor permeability (**C**) of the starch films. Results are presented as average ± standard deviation. Statistical analysis was performed by carrying out a one-way analysis of variance (ANOVA), followed by a Bonferroni post hoc test. Significant results ($p < 0.05$) are marked with *.

The degrees of the swelling of the starch composite films in distilled water are presented in Figure 2A. The degree of swelling of the Kuzu rooibos film (49.1 ± 3.71%) was higher compared to the other films. This was confirmed by the permeability results (Figure 2C). This suggests that the material effectively absorbs water molecules and also facilitates their evaporation, thereby aiding in the removal of excess moisture from the wound. However, the very high water vapor permeability value of the Kuzu rooibos foil poses a risk of excessive amounts of water being drained from some wounds. Therefore, it seems that the Kuzu rooibos film is not suitable for use in dressings for every type of wound.

No significant changes in swelling index and the solubility and permeability of the films with chili extract and Ag colloid were observed compared to the unmodified films (Figure 2A–C). This was particularly surprising in the case of the starch film modified with a hydrophobic chili oil extract, as the hydrophobic chili oil extract should reduce the affinity of the Kuzu chili for water [53,57]. However, Cerqueira et al. observed that adding a small amount of corn oil to a galacannan film increased its solubility, but a further increase in the amount of oil reduced the ability of the film to dissolve in water [59]. Song et al. observed a slight decrease in the permeability of lemon oil-modified starch films [54]. Furthermore, Chen et al. [60] observed that the addition of essential mustard oil did not show a change in the permeability of the cellulose film. It is hypothesized that a more extensive dispersion of the oil phase in the Kuzu chili layer would have had significant effects. Specifically, if the diameter of the oil droplets were significantly smaller, the material's interaction with water would have been markedly enhanced.

A decrease in the swelling of the Kuzu Ag samples was also expected for the reasons indicated by Nadtoka et al. [61]. They believe that the reduced swelling of samples containing Ag nanoparticles is due to the additional cross-links between the nanoparticles and the electron-rich O present in the polymer (in this case starch) chains. These should limit the ability of these types of samples to absorb water molecules and reduce their overall swelling behavior. However, as indicated by the lack of the changes in mechanical properties (Figure 3) and by the peak shift values in the FTIR spectra (Figure 4), the number of hydrogen bonds formed between the starch chains and Ag nanoparticles in Kuzu Ag is negligible; therefore, it was deemed to only have a minor impact on the affinity for water.

Differential scanning calorimetry measurements were performed, and the thermal properties of the tested Kuzu films are shown in Table 3. No changes related to the transition to the glassy state were observed in the DSC thermograms. According to Chiumarelli et al., the glass transition of carbohydrate films with a plasticizer is difficult to determine using DSC analysis because the change in heat capacity in this process is very small [62]. The films tested exhibited an endothermic peak at approximately 90 °C. This peak is associated with the melting of starch crystallites [63]. The thermogram of the Kuzu film prepared using rooibos extract indicated a slight shift in the peak to lower temperatures (85 °C).

Table 3. Thermal properties of the starch films.

Sample	T_m [°C]	ΔH [J/g]
Kuzu 0	88.5	359.5
Kuzu rooibos	85.0	325.3
Kuzu chili	88.9	190.8
Kuzu Ag	89.0	360.6

where: T_m—melting temperature; ΔH—melting enthalpy.

The natural extracts of rooibos contained, among other things, polyphenols. Many authors have reported that antioxidants such as polyphenols decrease the number of molecular interactions among starch polymer chains. These phenomena could be a cause of the increase in the mobility of starch chains. These results indicate that a reduced amount of thermal energy (lower melting temperature) is needed during melting to break the interactions between starch polymer chains in the structures of films with natural extracts [64,65]. The slight reduction in the melting enthalpy of the Kuzu rooibos film (325.3 J/g) suggests that some crystalline regions may have been damaged by the reduction in the number of hydrogen bonds. The natural rooibos extract, which contains polyphenols, modified the structure of starch raw materials in a similar way to plasticizing substances such as glycerol, sorbitol, urea, and polyethylene glycol [66]. The reduced crystallinity of the Kuzu rooibos film increased its affinity for water.

However, despite the significantly reduced crystallinity of the Kuzu chili film, these samples swelled and dissolved in water the least and were the least permeable to water vapor molecules among all the starch-based materials (Figure 2). The reason for this is, as mentioned above, the increased hydrophobicity of Kuzu chili. The high decrease in the melting enthalpy of the film containing the chili oil extract was probably associated with the destruction of the crystalline regions in the polymer network. This decrease in the crystallinity of the starch films after oil modification was confirmed by data from the literature [67]. The authors concluded that the addition of natural extracts to the edible film has a similar effect to that of a plasticizer; it decreases the melting temperature (T_m) because it facilitates chain mobility. This also explains why, among all the starch-based materials tested, this composite had the lowest hardness and density values (Table 2).

4. Tensile Tests

Characteristics such as tensile strength and elongation at break are important in the context of wound dressing materials, and they may contribute to the prediction of wound dressings' mechanical properties as medical materials [54]. The tensile strength of human skin can range from 1 MPa to 32 MPa, while elongation at break ranges from 17% to 207% [68]. Therefore, the mechanical parameters of dressing materials should also have similar values. As data on the properties of selected commercially available dressings indicate, their maximum strength is within the range of 0.17–4.60 MPa [69].

As shown in Figure 3, it can be said that the starch-based materials tested in this study meet this condition; only the addition of chili oil extract to the starch significantly reduced the tensile strength of this composite. For the Kuzu chili, the tensile strength decreased to 0.39 MPa, and the elongation at break slightly increased to 29.44%, respectively, compared to the control film (Kuzu 0). Researchers studying films containing oils usually note this relationship [53]. The decrease in tensile strength may be attributed to two main factors. Firstly, it could be due to the introduction of hydrophobic substances. These substances usually reduce tensile strength because they create a heterogeneous film structure with discontinuities [54]. Secondly, the decrease might be due to the partial replacement of starch chain interactions with weaker starch–oil interactions in the film network [70].The clear separation of the oil phase from the polymer matrix (Figure 1) causes a significant disturbance in the formation of hydrogen bonds between the starch chains, which has a huge impact on the strength of this material. The significant reduction in the strength of the Kuzu chili composite is also directly related to it having the lowest crystallinity among the materials tested (Table 3). It is understood that an increase in the crystalline phase of the polymer enhances its breaking strength and simultaneously decreases its elongation.

Meanwhile, the addition of rooibos extract to the Kuzu film matrix practically did not affect the value of the sample's tensile strength. However, an observed increase in the elongation of the Kuzu rooibos film from 26.7% to 46.3% was noted. It is assumed that the presence of compounds contained in the rooibos tea extract, such as polyphenols with OH groups, resulted in the destruction of the hydrogen interactions between the gelatinized starch chains. As a consequence, the chains became more mobile and the elasticity of the material increased. This is consistent with the DSC results, which show that the crystallinity of Kuzu rooibos is lower than that of Kuzu 0 (Table 3), which increases the elongation at break of the polymer sample (Figure 3).

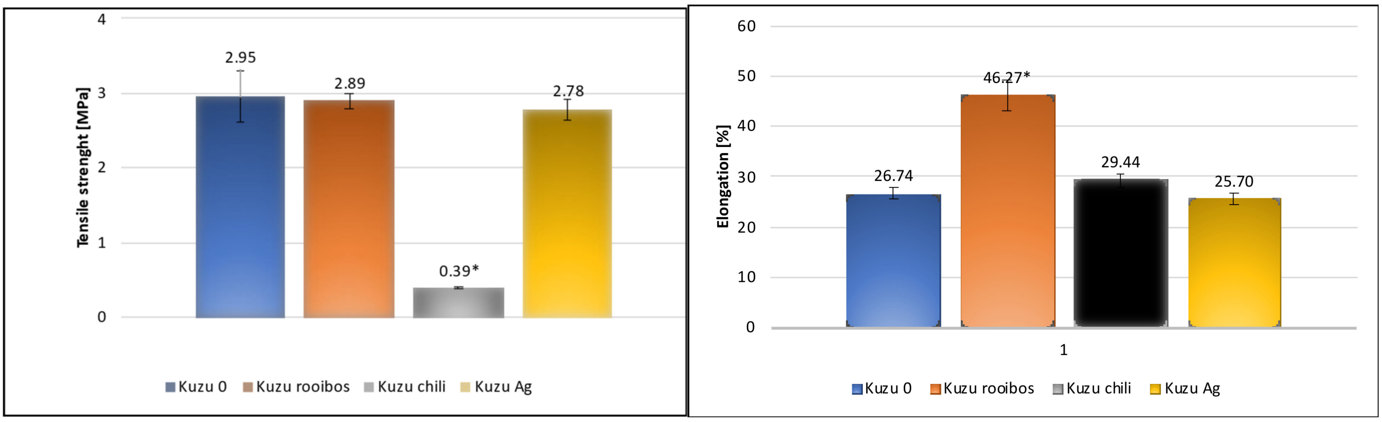


Figure 3. Tensile strength and elongation at break values of the starch films. Results are presented as the average ± standard deviation. Statistical analysis was performed by carrying out a one-way analysis of variance (ANOVA), followed by a Bonferroni post hoc test. Significant results ($p < 0.05$) are marked with *.

5. Chemical Structure

The absorbance spectra of the starch- and modified starch-based films were recorded on a FT-IR spectrometer with an ATR attachment and are shown in Figure 4. Bands corresponding to the main chemical groups of starch molecules were found in the spectra of each starch material. The wide band in the range of 3700–3000 cm^{-1} is due to the stretching vibrations of OH bonds (Figure 4). The bands closest to this peak (about 2900 cm^{-1})

are related to the symmetric and asymmetric deformation vibrations of the CH_2 groups. Infrared spectral bands characteristic of starch can be detected at 950–1200 cm^{-1}. These bands mainly correspond to the stretching vibrations of CO in the ring and the binding of the COC and COH groups [71]. In each tested sample, the stretching vibration bands of the acetyl groups from the citric acid are visible at 1713–1719 cm^{-1}, while the band at 1637–1646 cm^{-1} corresponds to H-O bending vibrations [72]. This last band is also identified with the presence of water molecules bound by hydrogen bonds to polymer chains. The presence of a peak at 1150 cm^{-1}, attributed to the stretching of the C-O bonds of the aliphatic esters, also confirms the chemical reaction between the acid and the hydroxyl groups of starch.

The addition of antibacterial additives to the starch network had a slight effect on the spectra of the tested materials. Slight shifts in some bands of the composites relative to those of the starch film indicate the influence of these additives on the hydrogen interactions between the gelatinized starch chains. The destruction of hydrogen bonds including the participation of OH starch groups in the composites is associated with this band shifting toward higher wavenumbers [73]. This is especially visible for the band for the Kuzu rooibos foil. The maximum shift of this band from 3257 cm^{-1} to 3273 cm^{-1} the in FTIR spectra is related to the destruction of the hydrogen bonds between the carbonyl and hydroxyl groups of gelatinised starch by the components of the rooibos extract, such as its polyphenols. At the same time, the intensity of the band at 1644 cm^{-1} on the Kuzu rooibos and Kuzu chili spectra increased significantly due to the stronger binding of water to the starch matrix network through the formation of additional hydrogen bonds with the molecules of the natural extracts used. The increase in elongation (Figure 3) and decrease in crystallization (Table 3) of both of these composites suggest that the hydrogen bonds between the carbonyl groups of citric acid and the hydroxyl groups of starch were replaced by citric acid—hydroxyl group interactions regarding the components of the rooibos and chili extracts (polyphenols and capsaicin). This imparted greater mobility to the chains and thus increased the flexibility of the film. This also affected the affinity of these materials for water, especially in the case of the Kuzu rooibos (Figure 2).

The smallest changes in the chemical structure of starch were found for Kuzu Ag. The slight shift in the bands in the FTIR spectra within the hydroxyl groups of the bound water (1646 $cm^{-1} \rightarrow$ 1638 cm^{-1}) and carbonyl groups (1716 $cm^{-1} \rightarrow$ 1713 cm^{-1}) towards lower wave number values indicates an increase in the number of hydrogen bonds. This implies a physical interaction between the silver nanoparticles and polymer matrix and water molecules, without chemical bond formation. However, as mentioned above, the number of interactions between the starch chains and Ag nanoparticles is small. Senevirathna et al. found that a silver vein graphite composite could act as a filler but that it did not chemically bind to starch chains [74].

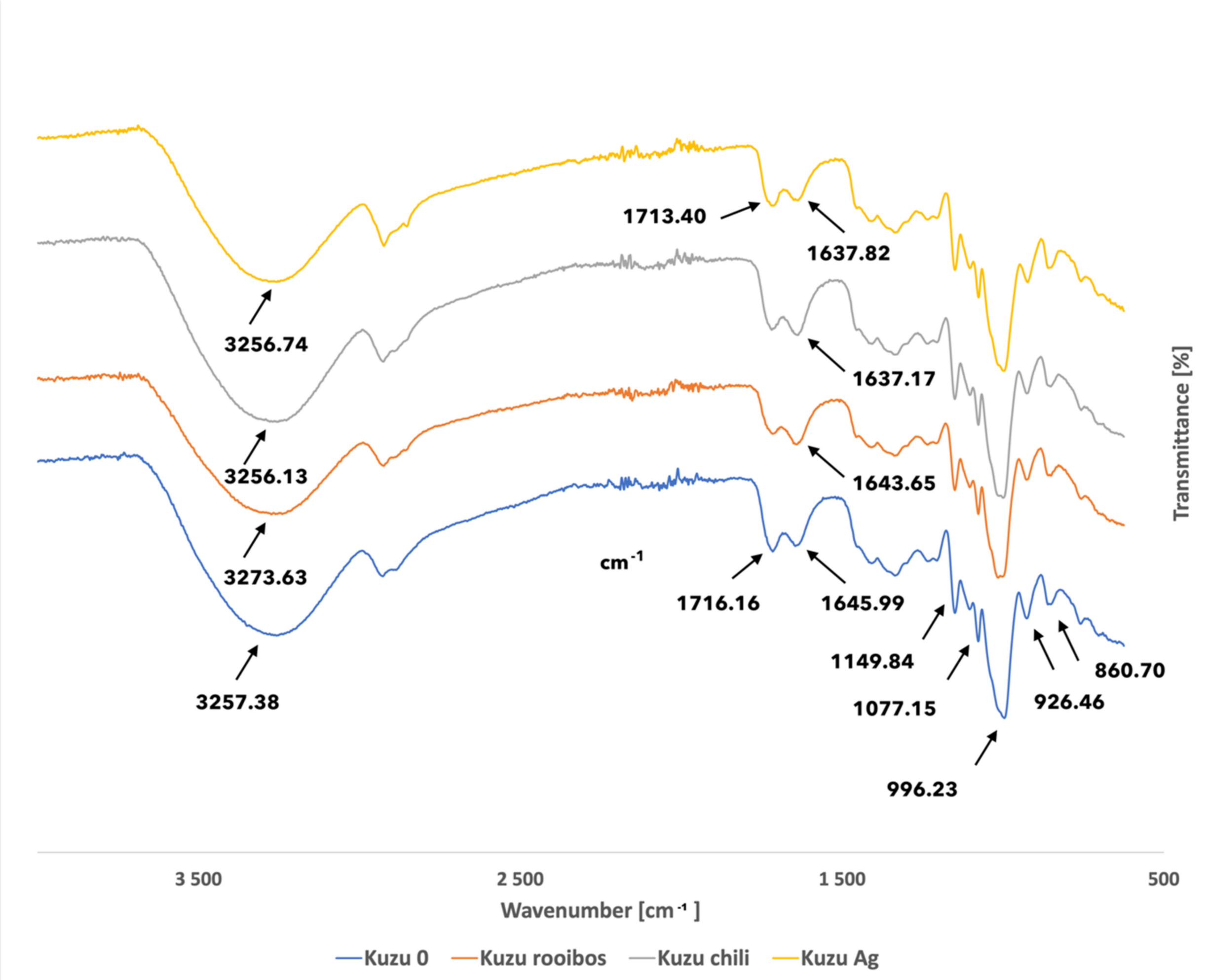


Figure 4. Spectra of Kuzu films.

6. Antibacterial Tests

The antibacterial substance contained in the disk diffuses radially, creating zones with a concentration gradient. Its highest concentration occurs at the edges of the disc and decreases with distance from it. The size of the inhibition area of bacterial growth is directly proportional to the degree of its sensitivity to the substance; the larger the inhibition area, the more sensitive the microorganism [75].

Some of the most frequently isolated microorganisms from wounds are *Staphylococcus aureus* and *Escherichia coli*. Therefore, Table 4 presents the results of the test conducted to evaluate the inhibition of bacterial growth around starchy materials containing natural extracts of rooibos and chili pepper, as well as silver nanoparticles. Kuzu without additives (negative control film) was not effective against any of the microorganisms used in the study. The same results have been reported by Ariaii [51]. It was expected that the presence of citric acid might cause some inhibition of bacterial growth. However, the small amount of acid and its incorporation into the starch network as a crosslinker resulted in the bacteria being unaffected.

The additives used in the starch discs in this study showed an antibacterial effect against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923; however, this effect varies depending on the type of additive. The results showed that *S. aureus* ATCC 25923 was more susceptible to starch discs containing silver nanoparticles (positive control film), followed by discs containing chili pepper extract and, finally, rooibos extract. The same direction of changes in the sensitivity of *E. coli* ATCC 25922 to the tested films with antimicrobial agents was observed, but with a smaller area of inhibition than in the case of *S. aureus* ATCC 25923 (Table 4).

Table 4. Antibacterial activity of starch films containing different additives against selected bacteria.

Sample	*S. aureus* ATCC 25923 Gram (+)	*E. coli* ATCC 25922 Gram (−)
	Inhibition Area [mm^2] *	
Kuzu 0	0.00 ± 0.00	
Kuzu rooibos	5.3 ± 2.04 *	22.0 ± 1.55 *
Kuzu chili	87.1 ± 3.59 *	29.4 ± 2.59 *
Kuzu Ag	94.7 ± 1.07 *	87.6 ± 3.59 *

* Means ± standard deviation (n = 3). Statistical analysis was performed by carrying out a one-way analysis of variance (ANOVA), followed by a Bonferroni post hoc test. Significant results ($p < 0.05$) are marked with *.

The use of chili pepper extract caused an inhibition area that was almost three times higher for *S. aureus* ATCC 25923 than for *E. coli* ATCC 25922 (Table 4). The antibacterial properties of chili pepper extract may result from the presence of capsaicin in chili fruit and cannabinoids in hemp oil [38,76]. Previous studies have reported that flavonoids and other active components such as capsaicin have potential antibacterial activity [45]. Some researchers have reported that capsaicin only slows *E. coli* growth [77], but it has an antibacterial effect on *S. aureus* [78]. The obtained value of the inhibition area of *S. aureus* bacteria is particularly satisfactory and is close to that of the control foil (Kuzu Ag) with silver nanoparticles and strong antibacterial properties.

The results showed that the Kuzu starch films containing the natural extract of rooibos were effective against both bacteria (Table 4). Rooibos has been shown to have anti-inflammatory (due to the dihydrochalcones aspalatin and nothofagin), antimutagenic (due to the flavonoids luteolin and chrysoeriol), and antimicrobial (due to the inhibitory effects of rooibos against certain microorganisms, such as the properties of *E. coli*, *S. aureus*, *Bacillus cereus*, *Listeria monocytogenes*, *Streptococcus mutans*, and *Candida albicans*) properties [32].

However, the highest antibacterial effect was observed for the starch film containing silver colloid. This is consistent with data from the literature indicating that silver is highly effective against both Gram-positive and Gram-negative bacteria cells [79], and these data were the reason we chose this additive to obtain a positive control foil. It is known that silver nanoparticles have the ability to bind to bacterial biomolecules, penetrate cells, produce reactive oxygen species and free radicals, and act as modulators during signal transduction by microorganisms [80]. The sensitivity of both bacteria to silver colloid was almost identical. Kim et al. also showed similar silver colloid antibacterial activity against *S. aureus* and *E. coli* [81]. The reason for this is the different structures of the bacterial cells. Gram-negative bacteria have an outer impermeable membrane that protects the peptidoglycans of the cell wall; hence, they have greater resistance to silver nanoparticles compared to Gram-positive bacteria such as *S. aureus* [76].

7. Conclusions

In recent years, active biodegradable films have proven to be very beneficial because they can be used to replace commercial, synthetic polymers. Moreover, the use of natural polymers from renewable sources is beneficial for the environment and in line with the principles of a sustainable economy and the principles of green chemistry. The results of this study indicate that films obtained based on Kuzu starch and natural antibacterial agents such as rooibos and chili oil extract may be suitable for further research focusing on obtaining active wound dressings. The lowest antibacterial activity, though significant compared to the negative control sample (Kuzu 0), was demonstrated by the Kuzu rooibos film; however, this film was the most flexible and had the best water vapor permeability and moisture absorption capacity of all the samples tested. The separation of the oil phase of the chili extract from the aqueous phase of starch, visible in the form of oil droplets dispersed in the polymer matrix, resulted in a significant decrease in the mechanical properties of Kuzu chili. At the same time, this material was characterized by good antibacterial properties against *Staphylococcus aureus* ATCC 25923, similar to the properties of starch foil with silver nanoparticles. Therefore, a better procedure for obtaining Kuzu chili films should be developed to reduce the diameter of the dispersed oil droplets and, at the same, time increase the compatibility of both phases. This would allow us to obtain a material with much better mechanical properties than the material used in this study. Further research is necessary to assess the biodegradability, biocompatibility, and effectiveness of these films as new active dressing materials in practice.

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