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Development of novel antibacterial gel using clove and calendula extracts with colloidal silver nanoparticles

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This study focuses on obtaining an extract from clove and calendula plants with colloidal nanoparticles of silver in the presence of 40% propyleneglycol and preparation of hydrophilic gel based on the resulting extract. Also, the optimal composition of the gel and releasing of biologically active substances from gels based on chitosan, sodium carboxymethylcellulose, carbopol, used as gelling agents was evaluated. The results showed that the release of bioactive compounds from a chitosan-based gel is more complete and intensive. The antibacterial and antifungal properties of chitosan gel containing silver nanoparticles have been confirmed by microbiological studies.

KEYWORDS

Antimicrobial; antifungal; colloidal nanoparticles; chitosan.

Introduction

It is known that one of the urgent issues of pharmaceutical technology is the creation of natural antibacterial agents. At present, research is being conducted to develop new drugs with the complex antimicrobial and antiviral activity for the prevention and treatment of complex "somatic-infectious" diseases. These drugs affect the overall strengthening of the body as a whole without destroying the normal micro-flora. Because the silver preparations are very promising in this respect, the silver cluster preparations, as well as new colloidal forms of silver, micro-suspension (micro-emulsion) and silver ions have been obtained using new advances in nanotechnology. According to scientists, it is namely silver ions that have bactericidal activity, and as the concentration of these ions increases, the degree of activity of silver also increases. When the silver ion interacts with the nitrogenous compounds of deoxyribonucleic acids in bacteria, it leads to a violation of the DNA stability of these bacteria and their vital activity. In addition, the penetration of silver ions into the cytoplasmic membrane of the cell disrupts the function of the cell wall, blocking the growth of bacterial enzymes, which leads to the destruction of

microorganisms. Preparations of silver that produces products such as Collargol, Protargol, Argosulfan, Dermazin, Argonica, Argolit, Argovit, Argogel, Poviargol, Argocrem, ArqoVasnaSiren have been successfully used in gastrointestinal diseases, surgery, ophthalmology, urology, dentistry, dermatology, traumatology [4,5,6, and 10] for many years.

In many research laboratories around the world, including the Department of Pharmaceutical Technology and Management of the Azerbaijan Medical University, various products based on plant extracts and colloidal silver solution rich in biologically active components: lotions, shampoos, ointments and gels are created and produced. Currently, the development of an antibacterial gel with colloidal silver remains relevant. Taking into account the above-mentioned statements, we addressed the same issue in this research. The main purpose of the research was to develop a technology for obtaining antibacterial gel based on natural raw materials and evaluate its quality.

Experimental

General

Silver nanoparticles, Sodium hydroxide (NaOH), hydrochloric acid (HCl), phosphate-buffered saline (PBS) were purchased from Merck Sigma-Aldrich, Darmstadt, Germany. All the chemicals used were of analytic grade with high purity.

Calendula and clove flowers, colloidal silver solution, biopolymer-chitosan were used as the object of research [9]. The extraction method was maceration. The pH indicator of the prepared gel, organoleptic properties, assessment of thermo- and colloidal stability, statistical calculations were performed on the basis of methods specified in XIV SPh RF and relevant standards [2,11, and 12]. Determination of biologically active substances in the prepared phytoextract and gel by HPLC method was carried out in the Analytical Expertise Center of the Ministry of Health of the Republic of Azerbaijan. Devices and chromatography conditions: Analytical scales - KERN, AET-220-4nm; max 220g, d = 0.1mg; min 10mg, e=1 mg; Chromatograph - Agilent 1260 Series; Chromatographic column-ZORBAX CB-C18; 4.6x250 mm; Injection volume -20 mL; Detector -DAD, 360nm; Active phase: methanol: acetonitrile + 0.1% solution of H3PO4 (25:75, pH 3.5) Active phase speed -1 mL/min; Preparation of the active phase. The active phase consists of a solution of acetonitrile and 0.1% phosphoric acid in a ratio of 25:75. The decisive phase was prepared by adding 300 mL of 0.1% phosphoric acid solution and 245 mL of acetonitrile to the flask. The analysis was performed by high-efficiency liquid chromatography.

Preparation of the standard solutions of Rutin

To prepare 0.2 g/l rutin solution, it was pre-dried at a temperature of 130^0 C for 3 hours to constant weight. 0.0200 g of rutin, 10 mL of 70% ethyl alcohol was added to a chemical beaker and was mixed. The mixture was placed in a 100 mL volumetric flask; the chemical beaker was washed 3 times with 10 mL of 70% ethyl alcohol, then the rutin solution was heated to 50–60 °C. After dissolution, the content of the flask was cooled to room temperature and made up to 100 m with 70% alcohol. The working solution of rutin was prepared in the sequence of dilution of the initial solution of 70% ethyl alcohol. The solution was stable for 1 month in a place protected from light. 0.0514 mL of clove extract was measured and placed in a 25 mL volumetric flask. The volume was raised to the required measure with the water. 10 mL was taken from it, placed in a 100 mL flask, dissolved in the mobile phase and chromatographed.

The amount of flavonoids in 10 mL of calendula extract was calculated by the following formula:

$$X_{cal.} = \frac{S_{sam.} \times m_{st.} \times 25 \times 100}{S_{st.} \times m_{sam.} \times 25} = \frac{764{,}154 \times 0{,}0514 \times 25 \times 100}{15452{,}3 \times 25} = 0{,}254 q/ml$$

The amount of flavonoids in 10 g of hydrophilic gel was calculated by the following formula:

$$X_{gel} = \frac{S_{sam.} \times m_{st.} \times 100}{S_{st.}} = \frac{76{,}373 \times 0{,}0514 \times 100}{15452{,}3} = 0{,}0254 q/ml$$

Ssam denotes the average index of peak areas calculated in the chromatogram of the studied sample solution; Sst. signifies the average index of peak areas calculated in the chromatogram of the Working Standard Sample solution; mst. shows weight taken for the preparation of the Working Standard Sample, in grams; msam. is the sample weight of the test solution, in grams; 25 and 100 - dilutions in mL.

Optimal chromatographic conditions were selected by quantifying phytoextracts containing flavonoids and hydrophilic gel by HPLC method. As a result of the analysis, it was determined that the peak area of the rutin was 764, 154 in 2,533 minutes in calendula extract; clove extract 1351.72302 in 2,415 minutes; 76,373 at 2,396 minutes in hydrophilic gel; The standard rutin was 15,452.3 relative unit at 2,479 minutes.

The flavonoids in clove extract prepared during the calculations were 0.450 g/mL, 0.254 g/mL in calendula extract and 0.0254 g/mL in gel. Scientific substantiation of the ingredients included into the composition of the antibacterial gel. One of the ingredients in the antibacterial gel is a colloidal silver solution. Water-soluble bactericidal drugs of protargol and collargol, consisting of colloidal compositions containing silver, stabilized, protective high-molecular protein nature, unknown chemical structure, are known, where, protargol contains 7.5-8.0% of colloidal silver oxide, and collargol contains 70% of colloidal metallic silver. Colloidal silver consists of metallic Ag particles ranging in size from 1nm to several micrometers. It is known that colloidal silver solution is widely used in surgery: In postoperative purulent-sepsis complications and infectious wounds; in bone and bone-joint panaricias; in phlegmon and abscesses; in diabetic and trophic wounds; paraproctitis and hemorrhoids; in osteomyelitis; in carbuncles and furuncle; in traumatology: post-traumatic purulent-sepsis complications, incisions at the site of trauma, bruises, edema, inflammatory foci and tumors; in combustology: in the prevention and treatment of purulent-inflammatory processes after complex burns of various etiologies; in dermatology: herpetic rashes, furunculosis, microbial and true eczema, drug toxoderma, dermatosis and psoriasis of various etiologies, aggravated secondary infections, scaly and zonal scabs, dermatomycosis, skin integrity disorders (cracks) [6, 7, 8, 10].

The other component included in the composition is the Calendula flowers (*Flores Calendulae*). Calendula preparations have local anti-inflammatory, antimicrobial and reparative effects. The mechanism of action is related with the active ingredients in its content : calendulozides A, E, F, H (2-10%) from triterpene saponins, flavonoids (astragalin, hyperoside, quercetin, isocversetin and rutin), sesquiterpenes (karyophylline) and triterpenes (α- və β-amyrins, lupeol, lupenone) and essential oils.

The component of flavonoids and essential oils has an antimicrobial effect to the gram-positive bacteria of the calendula (Staphylococcus aureus, Bacillus subtilis, Sarcina lutea), as well as the gram-negative bacteria (Escherichia coli, Klebsiella spp., Pseudomonas aeruginosa), also the pathogenic fungi (Candida albicans, Candida monbicans, Candida monobicans). Calendula flavonoids have an antioxidant effect, restore free radicals (hydroxyl, peroxide, superoxide), block the enzyme xanthine oxidase in tissues,

and have the ability to generate new superoxidase molecules. They participate in the synthesis of collagen in the vascular wall (enter the transverse sutures in collagen and desmosin molecules), regulate the colloidal state of intercellular substances (inhibits the activity of hyaluronidase), and regulate the permeability of the capillary wall. The flavonoids component of the calendula has a capillary strengthening effect. Calendula drugs accelerate the process of tissue accumulation in the peripheral areas of the wound, regulate the formation of granulation and epithelialization of the wound defect, improve the perfusion of tissue microvasculature, and reduce chronic venous insufficiency, such as the oedema, pain, cramps, trophic disorders, varicose eczema and ulcers. Immediately after use of the drug, it has a weak irritating effect in the form of a warm sensibility on the affected area. This is directly related to the activation of triterpenes in it by activating thermoreceptors in tissues. After stimulation of the receptors, the impulses are transmitted to the brain through sensitive nerve endings. Sympathetic impulses stimulate blood circulation in soft tissues and skin, accelerate metabolism and regeneration processes in the skin. Calendula drugs create an anti-inflammatory effect in the deep layers of soft tissue. The local anti-inflammatory effect reduces the release and synthesis of inflammatory mediators due to the triterpene components in calendula, which are the result of the acceleration of metabolism, inhibition of cyclooxygenase and lipoxygenase activity. The propylene glycol liquid extract of calendula contains the mixture of biologically active substances in medicinal plant raw materials with the help of propylene glycol, which also contain active substances soluble in water and oil, antimicrobial, disinfectant, healing, anti-inflammatory, regenerating, soothing, moisturizing, soothing, and increase the strength of capillaries, and restore the hydrolipid balance in the skin [9, 11]. The next component is clove flowers (Flores Caryophylli), which is also rich in essential oils. It also contains vaccinations items, phenolic compounds, alcohols, flavonoids and sterols. Clove extract is used to disinfect the oral cavity and throat. Due to the chemical composition of eugenol, this plant has a bactericidal, strong antiseptic, and anti-inflammatory effect. Eugenol has the ability to reduce pain levels by stimulating the appropriate receptors. One of the components of antibacterial gel is chitosan. Chitosan - has gel-forming properties. It is compatible with human tissues, stimulates regeneration and metabolism, has a wound-healing effect, has the ability to penetrate immediately into the deeper layers of the skin, provides transport of drugs and nutrients, and has bactericidal, antifungal and antiviral properties. Chitosan has the following properties: It is compatible with the tissues of the human body, has immunocorrectivity, has the ability to biodegrade and is completely eliminated from the body. It forms a thin protective coating on the wound surface, which provides gas permeability, has a healing effect on the wound by stimulating regeneration and exchange processes, has the ability to immediately penetrate into the deeper layers of the skin, and provides transportation of drugs and nutrients. It has bactericidal, antifungal and antiviral properties [12,16]. Another component included in the antibacterial gel is propyleneglycol. Propyleneglycol has a neutral pH level, does not contain alkaline, does not accumulate in the body, is hypoallergenic and completely harmLess for all ages [16-20]. At the same time, this substance helps the active components of cosmetics to penetrate into the deeper layers of the skin, significantly increases their effectiveness, as well as softens and moisturizes the skin and hair, and prevents loss of moisture and nutrients. In addition, propylene glycol is a component of antiperspirants and aerosols, where it has been found to have a bactericidal effect. The effect of PG is associated with its penetration

into the intercellular lipid layers (areas of liquid crystal structure), which results in their swelling. During this process, water, along with propylene glycol, enters the lipid layers, further enhancing the penetration process. According to the Cosmetics Ingredient Review (CIR) assessments, up to 50% of propylene glycol is safe to use in cosmetics. According to the Food Drug Administration (FDA), PG is safe to use as a dietary supplement. According to the standards (CIR), pure propylene glycol can be used in doses of 1.6-3.3%, and glycolic extracts in doses of 5-10%. The last component included is the essential oil of tea tree (Melaleuca alternifolia), which contains about ten useful natural elements. The main essential oils are: terpinene-4-ol -30-48%, gamma-terpinene-10-28%, alpha-terpinene-5-13%, sineol-5%. They also have high antimicrobial activity. Tea tree oil is effective in treating skin diseases such as herpes, eczema, furunculus or dermatitis. Due to the antiseptic and antifungal effect of the oil, the skin is restored and rejuvenated. With regular use, the skin acquires a gentle whitening effect, and acne disappears. Even tea tree essential oil stimulates metabolic processes in the deeper layers of the skin and helps cells to regenerate, perfectly tones them, and restores firmness and elasticity. At the same time, it is included in the composition we have chosen for this purpose, as it contains a number of antibacterial gels.

Results and discussion

The actual problem of pharmacy is the creation and standardization of drugs for the treatment of wound injuries of various etiologies. Currently, the search continues searching for optimal methods to stimulate the healing of cancer, burns, trophic ulcers. Local conservative treatment using ointments, gels, bandages, liniment plays an important role in the complex therapy of skin lesions and shortens the healing time of wounds. One of the promising areas for the creation of new drugs, including those with a wound healing effect, is the use of nanomaterials in the composition of dosage forms. It was found that metal nanoparticles have physicochemical properties that differ both from the properties of massive metal objects and from the properties of individual atoms [21-22]. When nano-sized particles of metals enter living organisms, they cause a biological response that differs from the action of the traditional ionic form of elements. Nanoparticles easily penetrate into all organs and tissues and have a prolonged action. First of all, to determine the nature of the extractant, cloves and calendula are crushed separately in a shredder machine and passed through a 2 mm sieve. 5 g of each plant raw material is weighed on an electronic scale and placed in a clean flask with a volume of 250 mL, extracted at room temperature with constant mixing of 130 mL with 70% alcohol in a ratio of 1:10 for 72 hours. At the end of this period, the extract is filtered, the plant residue is squeezed and combined with the previous extract. Finally, the extract is filtered through a paper filter and centrifuged. It is exposed to the analysis. Propyleneglycol solution has been used as a penetrating substance in cosmetology to accelerate the extraction process, to achieve the maximum separation of extractives from plant raw materials, as well as to ensure the penetration of biological active substances (BAS) into the deeper layers of the skin; extraction time - 24 hours, raw extractant ratio - 1:10, extractant - 40% propyleneglycol solution.

The operations continued in the above-mentioned sequence. The extraction process was also repeated with purified water. In order to obtain comparable results for aqueous-alcoholic, aqueous-propylene glycol and aqueous extracts, the diffusion method was applied (Figure 1).

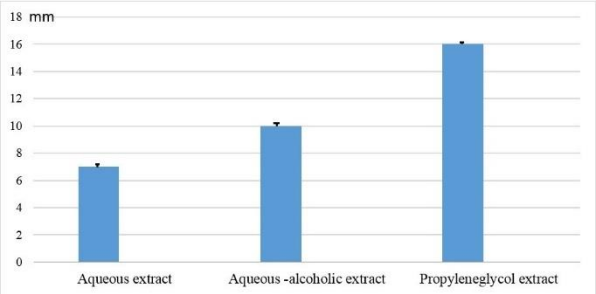

FIGURE 1 Effect of solvent type on release of biological active substances

Based on the results, it was determined that the result obtained with propylene glycol extractant is more satisfactory. With this in mind, propylene glycol extracts were analyzed by the HPLC method. The analyses were performed on Agilent 1260 chromatographs at 360 nm and 254 nm wavelengths. The effect of propylene glycol extract of clove and calendula flowers on microorganisms was also studied (Figure 2).

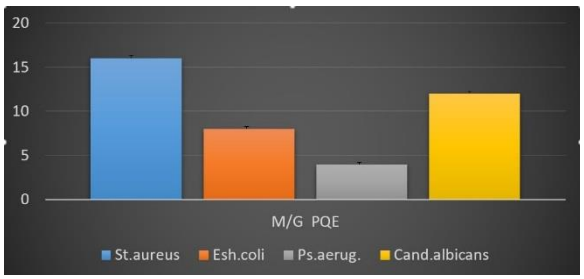

FIGURE 2 The effect of extract of clove and calendula flowers based on propyleneglycol on microorganisms

As can be seen from the diagram, the aqueous-propylene glycol extract of clove and calendula has the following effect on microorganisms: Staphylococcus aureus - 16 mm, Esherichia coli - 8 mm, Pseudomonas aeruginoza - 4 mm, Candida albicans - 12 mm. After that, we conducted research on the selection of the optimal basis. For the prevention and treatment of wounds it is advisable to use gels based on various polymers: polysiloxanes, carbomers, cellulose esters, as well as biopolymers.

As can be seen in Figure 2, the effect on microorganisms in the gel containing the 1st component, i.e. colloidal silver, is Staphylococcus aureus - 21 mm, Esherichia coli - 12 mm, Pseudomonas aeruginoza - 6 mm, Candida albicans - 12 mm. In the second composition, ie in the gel without colloidal silver ions, the effect on microorganisms was as follows: Staphylococcus aureus – 14 mm, Esherichia coli - 6mm, Pseudomonas aeruginoza – 4 mm, Candida albicans – 11 mm. Subsequently, we also evaluated some quality indicators of the antibacterial gel that we developed. It should be noted that gels, unlike creams and ointments based on hydrophilic bases, do not have a dehydrating effect and hyperosmolar properties. As a rule, they moisturize the site of inflammation, and protect the wound on the upper layer of the skin or mucous membranes from drying out. The mechanism of action of hydrogels is explained by their ability to form a transparent protective coating on the wound surface, protect it from mechanical damage, improve elasticity and prevent the development of the wound. Taking this into account, we developed 3 ingredients consisting of sodium carboxymethylcellulose, carbopol and chitosan gel (Table 1).

TABLE 1 Ingredients for choosing the optimal gel base

Components	Compositions		
	I	II	III
Aqueous-propylene glycol extract of clove-calendula, mL	5	5	5
Sodium carboxymethylcellulose, gr	-	7	-
Chitosan, gr	5	-	-
Citric acid, gr	2	-	-
Carbopol, gr	-	-	1
Sodium hydroxide, gr	0, 42	-	0, 42
Glycerin, mL	-	10	5
Purified water, mL	100	100	100

Next, we determined the relative bioavailability of the gels we developed in order to determine which gel-forming substance was optimal. The main active ingredient of the gel we studied was aqueous-alcohol-propyleneglycol extract of clove-calendula. A comparison in vitro study of suitable gels was made. The study was performed by diffusion of the active substance into gelatin with the addition of 3% aluminum chloride solution as an indicator. The degree of release of BAS from the drug form was assessed according to the diameter of the stained area (yellow). For this purpose, 4 Petri dishes were taken. The prepared gelatin-indicator solution was placed in Petri dishes. After the formation of gelatin, 3 tests were taken from the prepared gel with the help of a glass rod and placed in 3 different places in a dish with 0.1 g so that the gel was in good contact with the gelatin. The dishes were then placed in an adjustable thermostat at 37 °C. This process was performed in the same manner for each gel base. The BAS contained in the gel reacted with aluminum chloride reagent to form colored zones. After 1,4,8,12 hours, the diameter of the painted areas was measured with a ruler. Since large and small diameter ellipses were formed, the colored zone was defined as the arithmetic mean. The results are shown in Table 2.

Table 2. Dynamics of release rate of biological active substances from carbopol, Na-CMC and chitosan-based gel

Bases	1hour	4hour	8hour	12hour
Carbopol	4,7±0,23mm	7,8±0,12mm	12,3±0,11mm	17,9±0,8mm
Na-CMC	2,5±0,4 mm	4,6±0,34mm	7,2±0,12mm	9,5±0,7mm
Chitosan	6,8±0,2 mm	11,6±0,04mm	19,7±0,03mm	22,8±0,03mm

As can be seen from Table 2, based on the results of the determination of the degree of release of the BFM complex from the gel, it was found that the diameter of the painted zone for 1 hour was 4.7±0.2 mm in carbopol, 2.5 ± 0.4 mm in Na-KMS, 6.8 ± 0.2 mm in chitosan. After 4 hours, the diameter of the painted zone in carbopol - 7.8 ± 0.12 mm, in Na-KMS - 4.6 ± 0.34 mm; in chitosan - was 11.6 ± 0.04 mm. After 8 hours, the diameter of the painted zone in carbopol - 12.3 ± 0.11 mm, in Na-KMS - 7.2 ± 0.12 mm; in chitosan - was 19.7 ± 0.03 mm; after 12 hours, the diameter of the painted zone in the appropriate gels in carbopol was - 17.9 ± 0.8 mm; in Na-KMS - 9.5 ± 0.7 mm; in chitosan - 22.8 ± 0.03 mm. The results showed that the release of BAS in the form of a chitosan-based gel drug was more complete and intensive. This shows that chitosan-based hydrogel is superior. Next, we developed the gel composition according to the colloidal solution of silver (Table 3). 94 mL of purified water was added to a 150 mL chemical beaker and heated to 80 °C and 2 g of citric acid, stirring constantly. 4.0 g of chitosan powder was added to the obtained solution and mixed until a homogeneous mass was obtained, and respectively 5.0-10.0 g of calendula and clove propylene glycol extract, 0.5-1.0 g of silver colloidal solution, 0,1-0.3 g of tea tree essential oil, was added and mixed. Prepared I-containing gel has the pleasant odor, light brown color, based soft consistency. The gel of the second composition has the pleasant odor, light brown color, based solid consistency. In order to find out which of the gel ingredients that we developed had a higher antibacterial effect, we presented them for microbiological research. The results obtained are shown in Figure 3.

TABLE 3 Ingredients developed according to the colloidal silver solution

Examples	Clove - aqueous-propylene glycol extract of calendula, g	Colloidal solution of silver, g	Essential oil of tea tree,q	Chitosan gel, q
1	5,0	0,5	0,3	Up to 100 grams
2	5,0	-	0,3	Up to 100 grams

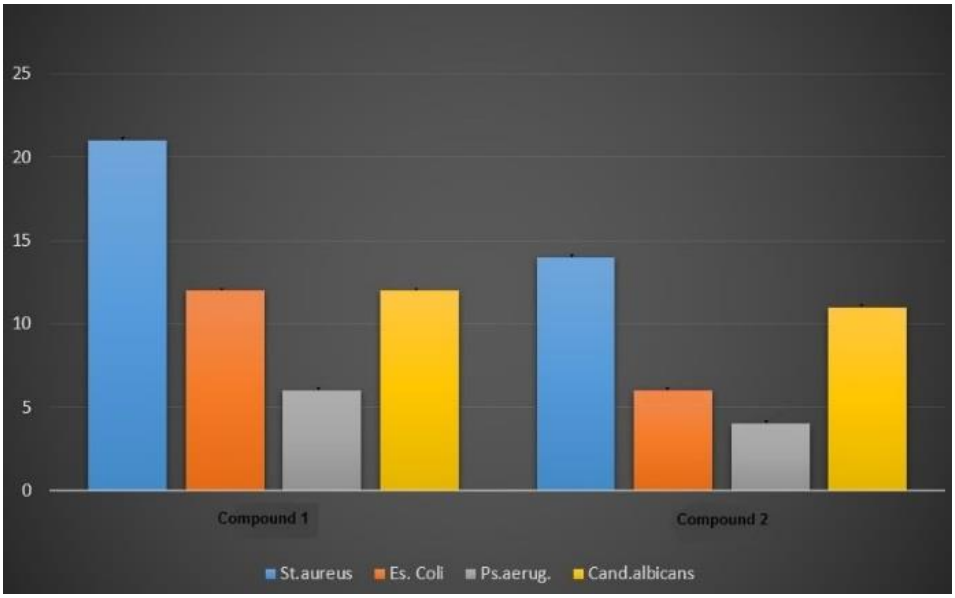

FIGURE 3 The effect of the developed ingredients on microorganisms

The results are given in Table 4. After that, we determined the stability of the antibacterial gel. One of the important features of the gel is that it can retain its properties during the storage process, ie it must not react with carriers, retain its chemical properties, remain microbiologically clean and not react with the packaging material. 2 series 30g antibacterial gel was stored in a well-sealed orange bottle at a temperature of 15-20 °C. The analyses were performed for 4 to 12 months using pre-developed identification and quantification methods. The results are given in Table 5.

TABLE 4 Evaluation of some quality indicators of antibacterial gel

Name of indicators	Methods	Norms and features
Description	Visual, organoleptic	Homogeneous, soft-textured consistency, without impurities, light brown specific odor
Flavonoids	1. Take 1 mL of the alcohol solution, place it in a test tube and add 1 mL of 10% ammonia solution. 2. Take 1 mL of the alcohol solution and place it in a test tube, add FeCl3 solution. 3. Cyanide test (Synod test). 1 mL of the filtrate was removed, placed on a watch glass, 20-30 mg of Zn granules and 5-6	1. Yellow color is observed 2. dirty-green staining is observed 3. red-brown coloring

	drops of HCl were added, and placed in a water bath.	
Hydrogen indicator pH	Potentiometric	5,5±0,4
Thermostability	Termostate	Stable
Colloid stability	Centrifugation	Stable
Microbiological purity	XI SP	2A category
Quantity appointment: Flavonoids	HPLC	2,54±0,02 %

TABLE 5 Stability testing of antibacterial gel

Criteria	Shelf life					
	4 months		8 months		12 months	
	1example	2example	1example	2example	1example	2example
Description Homogeneous, soft base with no consistency, specific odor of light brown color	corresponds		corresponds		Corresponds	
pH, 10% aqueous solution	5,5	5,5	5,3	5,4	5,4	5,6
Identity test	corresponds		corresponds		Corresponds	
The amount of flavonoids, in%	2,53	2,55	2,54	2,55	2,54	2,53
Microbiological purity	corresponds		corresponds		Corresponds	

For the first time, a composition consisting of cloves and calendula plants was developed. Optimal parameters for maximum extraction of extractives in this composition included: Extractant-40% propylene glycol solution, raw material-extractant ratio-1:10, raw material crushing rate-2 mm, extraction method-maceration, extraction conditions-20 °C, extraction time: 24 hours , the number of extractions :1 time). The amount of flavonoids (according to rutin) from biologically active substances in the studied extracts was determined by high-efficiency liquid chromatography. Hydrophilic gel composition was developed on the basis of extract from clove and calendula plants and colloidal silver ions (colloidal solution of silver - 0.5 mL, propylene glycol extract of clove and calendula - 5 mL, essential oil of tea tree - 0.3 mL, chitosan gel-residue). The quality indicators of the prepared hydrophilic gel were studied: light brown, homogeneous mass; smell - specific smell; pH 5.5 ± 0.4; shelf life - 12 months; has thermo- and colloidal stability.

Microbiological studies have shown that the gel prepared in the laboratory has antibacterial activity. The gel prepared during the examinations of patients at the Department of Therapeutic Dentistry of the Azerbaijan Medical University was found to be effective in the treatment of dental diseases such as stomatitis, gingivitis, and periodontitis.

Conclusion

Bioactive compounds of chitosan-based gel are more complete and intensive and are suggested to be used as an antibacterial and antifungal medication for various oral diseases.

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