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FLAVONOID CONTENT DETERMINATION AND CYTOTOXICITY TEST OF CARRAGEENAN GEL MOUTHWASH

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ABSTRACT

Introduction: Mouthwash is an antibiotic-based product that fights bacteria, oral infections, cleans, deodorizes, refreshes the oral cavity, and is an antiseptic. Carrageenan is a class of galactan polysaccharides found as an intercellular matrix substance in red algae or algae from the Rhodophyta class. Carrageenan is also known to contain secondary metabolite compounds, namely flavonoids, alkaloids, triterpenoids, proteins, carbohydrates, and fats.
Aim: The aim of the study is to determine flavonoid levels and cytotoxicity assay of carrageenan gel mouthwash.
Methods: The samples of this study were carrageenan gel mouthwash and fibroblast cells. The samples used were divided into cell control groups (negative) and carrageenan gel mouthwash groups with concentrations of 0.5%, 1%, 2%, and 4%.
Results: Total flavonoid levels in carrageenan gel mouthwash preparations with 4 different concentrations have flavonoid levels with a 0.5% concentration of 45.42%, 1% concentration of 69.77%, 2% concentration of 65.09%, and 4% concentration of 38.97%. The cytotoxicity test showed cell viability of more than 60% at various concentrations. The highest cell viability in the carrageenan hydrogel mouthwash with a concentration of 2% at 80.57%.
Conclusion: The carrageenan hydrogel mouthwash that has the highest flavonoid content at a concentration 1% was 69.77% and the carrageenan gel mouthwash was non-toxic to fibroblast cells.

INTRODUCTION

Mouthwash is an antibiotic-based product that fights bacteria, oral infections, cleans, deodorizes, refreshes the oral cavity, and is an antiseptic. Gargling with mouthwash plays an important role in the oral hygiene of everyone, helps reduce the symptoms of gingivitis, gum disease and can be used to destroy bacteria.[1] According to the previous study, the use of mouthwash effectively reduces the number of bacteria that cause oral diseases, softens the mouth, can remove foreign objects from the mouth, and helps reduce oral inflammation.[2]

Hydrogels are polymers with a three-dimensional structure that can absorb water droplets. Hydrogels based on natural and synthetic polymers are attractive for cell layering and attractive as a

matrix for repairing and regenerating tissues in various parts.[3] Hydrogels can control drug release by changing the gel structure. In addition, Hydrogel can also incorporate many comonomers in the network that make it sensitive to different stimuli and can change its properties in response to the environment, including pH, temperature, electricity, heat, and pressure. One of the materials used as a gelling agent is carrageenan.[4]

Carrageenan is a galactan polysaccharide found in the intercellular matrix of red algae or Rhodophyta algae. In seaweed, carrageenan has a hydrophilic structure like agar that is flexible enough to withstand pressure and current movement in water. Due to its biological properties, carrageenan is used as a viscosity agent, stabilizer, thickener, and others.[5] Carrageenan can also be used in the production of toothpaste, light foam, shampoo gel, and cosmetic creams. Carrageenan is often used as an emulsifier, gel base, inhibitor, and viscosity enhancer in pharmaceutical products such as suspensions, emulsions, gels, creams, lotions, eye drops, suppositories, tablets, and capsules.[6] Carrageenan has a high anti-inflammatory content and has anti-cancer properties. In one study, carrageenan was associated with antitumor and cytotoxic activities.[7] Carrageenan is also known to contain secondary metabolite compounds namely flavonoids, alkaloids, triterpenoids, proteins, carbohydrates, and fats.[8]

The most common group of compounds in plants are flavonoids, which can be found in almost all plant parts. This family includes various types of flavones, flavanols, flavanones, flavanols, and isoflavones. Flavonoids have many health-promoting benefits, and are an important part of various medicines, and cosmetics. This is due to the various properties of flavonoids, namely antioxidants, anti-inflammatory, antimutagenic, and anticarcinogenic properties, as well as their ability to alter important cellular functions of enzymes.[3]

The basis of the cytotoxic assay is the ability of cells to survive in the presence of toxic compounds. Many effective cytotoxic from different plants are considered as biological compounds that can make anticancer drugs.[7] The exposure for 3 hours is sufficient to produce toxic effects in the cells.[6]

Based on the description above, carrageenan or seaweed is one type of plant that has many benefits related to oral health containing high anti-inflammatory and anticancer, where anti-inflammatory is a factor that affects the healing process of inflammation in the oral cavity. So, this research was carried out by making carrageenan gel mouthwash and determining the levels of flavonoids and cytotoxicity in the mouthwash so that the authors would determine the flavonoid levels and evaluate the toxicity of carrageenan hydrogel mouthwash.

MATERIALS AND METHOD

The research protocol has met the feasibility according to the Health Research Ethics Committee of the Faculty of Medicine, Baiturrahmah University (No.224/ETIK-FKUNBRAH/03/11/2023). The study was an experimental study which determined flavonoid levels and cytotoxicity. The study analyzes the total flavonoid compounds and cytotoxicity test of carrageenan gel mouthwash. The samples of this study were carrageenan hydrogel mouthwash and were divided into 4 test groups, they were: 1) carrageenan hydrogel mouthwash with a concentration of 0.5%; 2) carrageenan hydrogel mouthwash with a concentration of 1%; 3) carrageenan hydrogel mouthwash with a concentration of 2%; 4) carrageenan hydrogel mouthwash with a concentration of 4%.

The mouthwash preparation used the instruments such as: analytical balance (Mettler Toledo, USA), spatula, measuring cup (Glassco, India), beaker glass (Pyrex, Sigma Aldrich, USA), dropper pipette (Sigma Aldrich, USA), mortar, and stamper. The determination of flavonoid levels used some instruments involved UV-Vis spectrophotometry (Drawell, China), rotary evaporator (Henan Laphan, China), oven, , desiccator, porcelain cup, shaker, 70 mesh sieve, blender, container, spatula, test tube rack, cuvette, digital balance, funnel, 100 mL Erlenmeyer (Sigma Aldrich, USA), 250 mL volumetric flask, drip pipette (Sigma Aldrich, USA), stirring rod, volumetric flask, beaker glass, micro pipette, furnace and filter paper and cytotoxicity test: CO2 incubator, 96 well plate, aluminium foil, hemocytometer (Sigma Aldrich, USA), Pasteur pipette, microscope, and counter.

The materials used to prepare mouthwash were carrageenan, glycerin, ethanol, sodium benzoate, tween 80, aquadest ad. Determination of flavonoid content used some materials. They were methanol, aluminium chloride (AlCl3) 10%, NaNO2 5%, NaOH IN, quercetin powder, aluminium foil, distilled water, and tissue and cytocytosis test: fibroblast cell culture media, MTT solution, stopper reagent, PBS.

Preparation of Carrageenan Hydrogel Mouthwash

Carrageenan extract was put into the mortar and then crushed the glycerin until it dissolves. Then, put sorbitol into the mortar and grind until poured. Then, add distilled water to 100 milliliters. Finally, add peppermint oil and close the bottle.

Mouthwash Formulation

Table 1: Mouthwash Formulation

Materials	Concentration%				
	Control	**F1**	**F2**	**F3**	**F4**
Carrageenan	-	0,5	1	2	4
Glycerin	21	21	21	21	21
Ethanol	9,5	9,5	9,5	9,5	9,5
Natrium Benzoate	0,4	0,4	0,4	0,4	0,4
Tween 80	25	25	25	25	25
Aquadest ad	100ml	100ml	100ml	100ml	100ml

Determination of yield

Determination of yield is done by weighing the cleaned carrageenan (A) and the thick extract obtained (B) to calculate the yield. The yield value must at least meet the figures listed on each extract monograph. Weigh 5 grams of 5% NaNO2, then add 100 ml aquadest and stir until homogeneous. Weigh as much as 1gram of 10% AlCl3, then add 10 ml of distilled water and stir until homogeneous.

Preparation of mother liquor of quinine comparator

We weigh 50 mg of standard quinine, put it into a 50 ml volumetric flask and add the solvent. Then stir well to dissolve and dilute with solvent to the limit mark to obtain a solution with a concentration of 1000 ppm. Use a pipette to take 5 ml of 1000 ppm stock standard solution into a 50 ml bottle and shake well to mix until a solution with a concentration of 100 ppm is obtained. Use a pipette to take 12.5 ml of 100 ppm stock standard solution into a 50 ml volumetric flask. Dilute with solvent until the line mark and stir until homogeneous to obtain a solution with a concentration of 25 ppm.[10]

Determination of Maximum Wavelength

After putting the stock solution into a cuvette with a certain volume, check the wavelengths of 300, 310, 320, 330, 340, 350, 360, 380, and 400 nm. Then record the absorbance of each wavelength and draw a curve showing the relationship between wavelength and absorbance.[11]

Dilution of Quinone Solution

Weigh 50 mg of quinine and dissolve 50 ml of methanol as a stock solution, then make dilutions of quinine with concentrations of 30, 35, 40, 45 and 50 ug/ml as a comparative quinine solution.[11]

Quinones Maximum Wavelength Measurement

Take each concentration of 30, 35, 40, 45, and 50 µg/ml as quercetine comparison solution. Dilute the quercetine comparison solution with methanol then take 0.5 ml of each concentration and add 0.1 ml of AIC1; 10%. 0.1 ml NaNO, 5%, 2 ml NaOH IN and 2.3 ml distilled water. After incubation for

30 minutes, the absorbance of the comparison solution was measured using visible light UV-Vis spectrophotometry at the maximum wavelength of quinine.[10]

Measurement of Total Flavonoid Level in Carrageenan Hydrogel Mouthwash

The tested samples for each formula were dissolved in methanol at a concentration of 1000 ug/ml for preparation. The sample weighed: 50 ml diluted with 50 ml methanol, 0.5 ml test sample plus 2 ml distilled water. NaNO, 5% 0.1 ml, AICI, 10% 0.1 ml, add 2 ml of methanol. After incubating for 30 minutes. The absorbance of the reference solution was measured by ug/ml spectrophotometer. Visible light is at the maximum wavelength of quinine. Determination of flavonoid content can be calculated using the formula.[12]

$$F = \frac{c\ x\ v}{m} x100$$

Notes:

F = Flavonoid content (mg/100 grams)

C = Quercetin equivalence (mg/L)

V = Volume (L)

m = Sample weight (gram)

Cytotoxicity Test with Microtetraziolium Method (MTT) Harvest Fibroblast Cells

Fibroblast cells were human dermal fibroblasts-adult (HDFa) cell culture (Gibco C-013-5C, USA) harvested after the cells reached 80% confluence. Fibroblast cells were taken from the CO Incubator, media was removed using a micropipette in the dish and cells were washed with 500 ul of sterile PBS. To detach the cells at the bottom of the petri dish, add 0.25% trypsin evenly to 100 µl. For three minutes, incubate in a 5% CO2 incubator at 37°C. To inactivate trypsin, culture medium was added in 2 ml. After the cells were resuspended with a pipette until all the cells were detached individually (not clustered) and put into a new conical tube containing 2-3 ml DMEM, the cells were resuspended again. The cell harvest was taken in 10 µl and pipetted into a hemacytometer for counting with a counter.

Calculation and Preparation of Fibroblast Cell Concentration to be Used for Samples

A hemocytometer is a device used to count the number of cells that consists of four counting chambers, each consisting of sixteen squares. The four chambers of the hemocytometer are used to count the number of fibroblast cells. Only dark (dead) fibroblast cells and cells on the left and bottom borders are counted; cells on the top and right borders are not. The formula for calculating the number of cells/ml. The number of cells obtained was transferred as required into a conical pipe, and complete medium was added according to the desired cell concentration.

Cytotoxicity Assay (MTT Assay)

1. Fibroblast cells were prepared in cell suspension at 2 x 10' cells/180 ul culture medium. To allow fibroblast cells to adapt and adhere to the wells, 180 ml of fibroblast cell suspension was added to each 96-well tube. Then, incubator co for 24 hours at 37°C.
2. Discard the culture medium and wash the cells with 100 l pbs into each well with 100 l culture medium.
3. Add mouthwash formulation with predetermined concentration.
4. Incubate for 24 hours in a CO incubator at 37 degrees Celsius.
5. At the end of incubation, the culture medium was discarded.
6. Each well was added 100 al of MTT solution. Next, the cells were incubated again for 4 hours in a CO incubator at 37 degrees Celsius.
7. Stopper reagent 100 µl wrapped in aluminium foil was used to stop the MTT reaction. Overnight, incubate in a dark place (room temperature).
8. Next, the absorbance was measured with an Elisa reuder at 595 nm. The result is shown in optical thickness (absorbance). Make an absorbance graph and calculate the percentage of viability using the cell viability percentage formula.

Cell viability was calculated using the formula:

$$\% \text{ viability cell} = \frac{\text{Mean optical density eksperimen}}{\text{Mean optical density kontrol}} \; x \; 100\%$$

The measurement results obtained are interpreted according to the table below:

Table 2. Classification of Toxicity Based on Cell Viability

Cell Viability %	Toxicity
>90%	Biocompatible
60 – 90%	Mildly Toxic
30 – 59 %	Moderately Toxic
<30%	Highly Toxic

The data analysis technique used is descriptive analysis. Data obtained from the results of testing total flavonoid levels and cytotoxicity tests are made in the form of tables and diagrams which are then described.

Descriptive analysis is one type of statistics whose level of work is collecting, organizing, and processing data so that it can be presented and provide a clear picture of the situation or certain events where the data is collected. Descriptive analysis usually presents data in the form of tables, graphs, or diagrams.

Column row tables, contingency tables and frequency distribution tables are the three most used types of tables. In addition, graphs depict the ebb and flow of a situation using lines or pictures. In real life, making graphs or diagrams does not only depend on lines that lie on cartesian coordinates.

Results and Discussions

Flavonoid content determination of carrageenan hydrogel mouthwash was carried out using UV-Vis spectrophotometric method with concentrations of 0.5%, 1%, 2%, and 4%. The following are the results of the determination of flavonoid levels with spectrophotometric tools. The results of measuring the absorbance of quercetin standard solution as a comparison with concentrations of 30 ppm, 35 ppm, 40 ppm, 45 ppm, and 50 ppm using UV-Vis spectrophotometry at 373 nm in the table below:

Table 3. Absorbance Measurement Results of Quinones Standard Solution

No	Quercetin Concentration	Absorbance (Y)
1.	30	0,325
2.	35	0,387
3.	40	0,454
4.	45	0,517
5.	50	0,580
Total (∑)	200	2,263

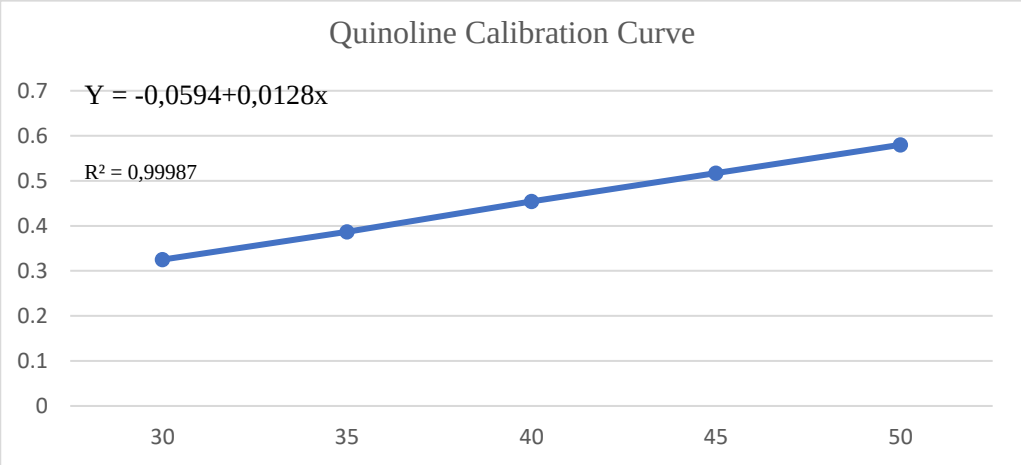

Figure 1. Curve of Quercetin Concentration with Absorbance

The absorbance value (y) and flavonoid content of the sample were calculated with the line equation y = -0.0594 + 0.0128x. The correlation coefficient value obtained was R2 = 0.99987. The above curve shows that the absorbance value is positively correlated with the concentration of quinine. Calculation of absorbance measurements of samples in carrageenan hydrogel mouthwash preparations using a wavelength of 373 nm. Flavonoid levels can be determined by matching the absorbance to the concentration curve of quinine solutions. The following is a table of absorbance result data on each sample formula:

Table 4. Sample Absorbance Result Data on Mouthwash Preparations

No.	Replication	Absorbance			
		Sample 0,5%	**Sample 0,5%**	**Sample 2%**	**Sample 4%**
1.	Replication I	0,231	0,828	1,609	1,930
2.	Replication II	0,229	0,833	1.606	1.937
3.	Replication III	0,234	0,840	1,606	1,942
	Mean	0, 2313	0,8337	1,6070	1,9363

Based on table 4 the results of absorbance measurements using concentration samples of 0.5%, 1%, 2%, and 4%. The average absorbance value in each sample preparation whose absorbance is higher in the 4% concentration sample of 1.9363 with stable results, the 2% concentration sample of 1.6070, the 1% concentration sample of 0.8337 and the smaller one in the sample with a concentration of 0.5% of 0.2313. The results of the calculation of Flavonoid Level Determination in this study can be seen in the table below:

Table 5. Result Data of Flavonoid Level of Carrageenan Hydrogel Mouthwash

No.	Sample	Flavonoid Content%
1.	Concentration 0,5%	45,42%
2.	Concentration 1%	69,77%
3.	Concentration 2%	65,09%
4.	Concentration 4%	38,97%

Based on the table and graph above, the results obtained in carrageenan hydrogel mouthwash at a concentration of 0.5% were 45.42%, 1% concentration was 69.77%, 2% concentration was 65.09%, and 4% concentration was 38.97%. The highest level is found in the 1% concentration sample, it can be concluded that each mouthwash preparation has different flavonoid levels in each concentration.

In this study, the cytotoxicity test of carrageenan hydrogel mouthwash was determined to determine that the mouthwash preparation is non-toxic. This test was carried out with 4 repetitions using the microtetazoliun (MTT) method on fibroblast cells. The following are the results of the cytoctosis test:

Table 6. Cytotoxicity Test Result of Carrageenan Hydogel Mouthwash

No	Sample	Viability (%)
1.	Control Cell	100%
2.	Concentration 0,5%	77,56%
3.	Concentration 1%	71,86%
4.	Concentration 2%	80,57%
5.	Concentration 4%	78,56%

Based on the table and graph above, the results obtained for cell viability in cell control are 100% and in carrageenan hydrogel mouthwash with a concentration of 0.5% of 77.56%, 1% concentration of 71.86%, 2% concentration of 80.57%, and 4% concentration of 78.56. Judging from the results of cell viability, the higher the concentration of each carrageenan hydrogel mouthwash, the relative

increase in biocompatibility. It can be concluded that the carrageenan hydrogel mouthwash is non-toxic to fibroblast cells.

DISCUSSION

Carrageenan hydrogel mouthwash is a solution or liquid that has properties as an antiseptic in the form of a gel preparation containing flavonoid compounds with raw materials in the form of carrageenan derived from seaweed extraction. Carrageenan has demonstrated potential bioactive qualities, including antiviral, anti-inflammatory, antibacterial, antihyperlipidemic, anticoagulant, antioxidant, antitumor, and immunomodulatory properties.[13] Three types of carrageenan, called κ-, ι- and λ-carrageenan, are of commercial importance. These carrageenan's are of interest due to their rheological properties as stabilizers and thickening or gelling agents. Both κ- and ι-carrageenan can form gels upon cooling or in the presence of K+ or Ca2+ opponent ions, whereas λ-carrageenan is difficult to form gels, or requires high concentrations of K+ to produce weak gels. Therefore, it is used as a thickening agent. They can be used in food, pharmaceutical, cosmetic, printing and textile industries. In addition, its use as a renewable, ecological, and non-toxic mobility control agent is reported. Besides its commercial importance, carrageenan is also known to have biological properties as well as low toxicity.[14]

The initial process of this research was carried out by making carrageenan hydrogel mouthwash preparations. Making mouthwash preparations made 4 formulations with different concentrations of each active substance. Formula I with a concentration of 0.5%, formula II with a concentration of 1%, formula III with a concentration of 2%, and formulation IV with a concentration of 4%. In making mouthwash preparations, additional ingredients are needed, consisting of glycerin, ethanol, sodium benzoate, tween 80, and distilled water. The use of glycerin as a humectant has a function to keep the active substance from evaporating and improve the stability of an ingredient in the long term[9]. Sodium benzoate as a preservative has a function to prevent product damage, preventing the growth of microorganisms in mouthwash. Tween 80 which functions as an emulgator in solution will cause a decrease in the surface tension of the solution.[15]

Analysis of total flavonoid levels in carrageenan hydrogel mouthwash preparations was carried out later using the UV-Vis spectrophotometric method. This method was chosen because it is very easy to do, requires a small sample, produces more valid data with a high level of accuracy. The purpose of determining the overall flavonoid compound content is to determine the number of flavonoid compounds that depend on the mouthwash preparation that has been made. The maximum wavelength was measured with a standard solution of quinine and compared with the sample solution. using quinine as a standard comparator to measure the number of flavonoids. Furthermore, the optimal

wavelength was carried out to determine the maximum wavelength that would be used to measure on UV-Vis spectrophotometry. The maximum wavelength found was 373 nm.[10]

The calculation of total flavonoid content and absorbance of the average sample in the results shown in table 4. These results were entered into the linear regression equation of quercetin with y = -0.0594 + 0.0128x, with a correlation coefficient of R2 = 0.99987. After calculation, the total flavonoid content of carrageenan hydrogel mouthwash preparation at 0.5% concentration was 45.42%, 1% concentration was 69.77%, 2% concentration was 65.09%, and 4% concentration was 38.97%. The highest levels were found in the 1% concentration sample. The difference in flavonoid levels at each concentration can be caused by a less-than-optimal extraction process, in this study, the secondary metabolite compounds contained were not fully extracted. The temperature used when drying and evaporating using an evaporator can also have an effect; temperatures that are too high can damage secondary metabolite compounds.[16] Environmental factors growing on plants can also affect the levels of plant secondary metabolites, as in research conducted by Setyo Utomo, Elizabeth and Anggara 2020, the results showed that levels of secondary metabolites, including flavonoids, can be influenced by environmental factors, including the temperature of the place of growth, pH of the place of growth, light intensity, and the height of the growing location.

The next research is the cytocytosis test of carrageenan hydrogel mouthwash. In addition, the biocompatibility of the mouthwash preparation to fibroblast cells was also tested. The number of living cells and their growth is indicated by the percentage of cell viability. This study used the MTT method, which has advantages such as being easy to perform, requires a relatively short time, is very accurate, and can be used to measure very large samples. The principle of the MTT assay to measure cell viability is to measure mitochondrial function by measuring the activity of mitochondrial enzymes, such as the enzyme succinate dehydrogenase. MTT assay results are read with an ELISA reader.[17]

Each carrageenan hydrogel mouthwash preparation in fibroblast cells is non-toxic, because after calculation of the percentage of the number of living cells, all percentages are more than 60%. According to research on dental materials, the materials used in dentistry should be non-toxic, non-irritating, and biocompatible, in addition, the materials made should not affect the cytemic or local biological habitat. The MTT toxicity parameter is based on CD50, which means that the material is considered toxic if the percentage of live cells exposed to it is less than 60%.[18,19,20]

The calculation of the number of living cells in the treatment of carrageenan hydrogel mouthwash has a value above 60% in each group, namely in the mouthwash preparation of 0.5% concentration of 77.56%, 1% concentration of 71.86%, 2% concentration of 80.57%, 4% concentration of 78.56%. This means that carrageenan hydrogel mouthwash is not only safe but also has the potential to increase fibroblast cell proliferation. In another study, tested purified native and degraded carrageenan and its

disaccharides, obtained from extracts of potential cytotoxic and antitumor *compounds Hypnea musciformis, Iridaea undulosa and Euchema spinosumas*. The results showed that kappa-carrageenan and iota-carrageenan as well as carrageenan oligosaccharides had cytotoxic effects on LM2 tumour cells. Some oligosaccharides were also more cytotoxic than their parent compounds, indicating that lower molecular weight is one of the factors that enhance their cellular ability. These results suggest the potential of using disaccharide units, such as carrabiosis in combination with antineoplasics, to enhance their cytotoxicity and antimetastatic properties, and the use of iota-carrageenan as an adjuvant or carrier in anticancer treatment.

This study uses fibroblast cells derived from human skin called Human Dermal Fibroblast adult (HDFA) cells. HDFA cells are primary cultures of fibroblasts taken from adult skin specimens. Fibroblasts are known as differentiated mesenchymal cells forming connective tissue. Fibrocytes are also called dormant mature fibroblasts. These cells are spindle-shaped, smaller and smaller than fibroblasts, have acidophilic cytoplasm and little rough endoplasmic reticulum. During the wound healing process, fibrocytes can be stimulated and fibroblasts can resume synthetic activity. [20,21] Two to three days after wounding, fibroblast cells appear in the injured tissue and peak at seven to fourteen days after wounding.

Carrageenan hydrogel mouthwash promotes fibroblast proliferation, which helps the wound healing process, as the percentage of live fibroblast cells is high. In the field of dentistry, carrageenan hydrogel mouthwash can be considered for use as a wound medication and is safe when used in the oral cavity.

CONCLUSION

The results of total flavonoid content in carrageenan hydrogel mouthwash preparations with 4 different concentrations had the highest flavonoid content at a concentration of 1% at 69.77%. Carrageenan hydrogel mouthwash is non-toxic to fibroblast cells, the results showed cell viability of more than 60% at various concentrations. There was the highest cell viability in carrageenan hydrogel mouthwash with a concentration of 2% at 80.57%.

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