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Development and Evaluation of *Ginkgo biloba* L. Extract Loaded into Carboxymethyl Cellulose Sublingual Films

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Abstract: Oral bioavailability of flavonoids, including *G. biloba* extract, is limited due to their chemical complexity, which determines slow dissolution in vitro behavior of the extract. The overall research objective was to compare the effect of increasing freeze-dried *G. biloba* extract (GFD) concentrations in carboxymethyl cellulose (CMC) films on their mechanical properties, release profile of flavonoid glycosides, stability and disintegration time. Physicochemical evaluation of films was performed by SEM and FTIR. The mechanical properties and in vitro release profile of flavonoid glycosides from the prepared films were characterized in the study. The higher elongation at break and tensile strength values, quick release of flavonoids and good stability were observed in formulation, coded FRG—15 (the film contained 0.4 g of GFD, 0.3 g of glycerol and 2 g of 2% CMC), ($p < 0.05$). Dissolution rate tests showed that approximately 85% of loaded flavonoid glycosides had been released; the release profile of flavonoid glycosides from FRG-15 had levelled off after only 15 min. The results could lay the groundwork for further studies, concerning the development of sublingual films as *G. biloba* extract-based dosage forms, which might increase the multifunctional properties and pharmacological activity closer to the desired level.

Keywords: *Gingko biloba* L. freeze-dried extract; dissolution rate of flavonoids; mucoadhesive drug delivery system

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Citation: Rimkiene, L.; Baranauskaite, J.; Marksa, M.; Jarukas, L.; Ivanauskas, L. Development and Evaluation of *Ginkgo biloba* L. Extract Loaded into Carboxymethyl Cellulose Sublingual Films. *Appl. Sci.* **2021**, *11*, 270. https://doi.org/10.3390/app11010270

Received: 8 December 2020
Accepted: 28 December 2020
Published: 30 December 2020

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1. Introduction

Ginkgo biloba L. is a tree species with a very rich history of usage for medicinal purposes because of the various benefits to a human health it can provide [1]. After decades of studies, researchers are still interested in *G. biloba*, still studying its mode of action, still discovering new beneficial properties. Active components in *Ginkgo biloba* extract, such as terpenoids and flavonoids, possess antioxidant activity [2–4], antiasthmatic—contributing to bronchospasm relief activity [5], skin regenerating and wound-healing properties [6]. There are studies also showing the ability of *Ginkgo biloba* extract to improve blood circulation, prevent clot formation, support the walls of capillaries and their flexibility, and protect nerve cells from harm, which could be brought about when they are deprived of oxygen [7]. *G. biloba*, due to its noteworthy pharmacological properties and effects, is widely used for the treatment of different phases of Alzheimer 's disease, concentration difficulties or even concentration deficit disorder, dementia and memory impairment [8], cerebral insufficiency [9], intermittent claudication, which causes pain and cramps in legs induced by obstruction of the arteries [10], vertigo [11] and tinnitus, which usually causes hearing loss [12]. It should be mentioned that every single constituent of *Ginkgo biloba* extract has an individual therapeutic mechanism and all of these mechanisms taken together provide the unique pharmacological activity of the extract [13,14].

There are several routes of drug administration—oral or enteral, parenteral (injections, inhalation, transdermal, transmucosal) and topical. The most common is the oral route, but there are disadvantages, such as long onset time, lower bioavailability, dysphagia and even difficulties with precise dosing [15]. Sublingual films as a drug dosage form have been on the market for a while, yet it has not been tried as a *G. biloba* extract delivery system. Oromucosal films can be categorized into mucoadhesive buccal films (MBF's), orodispersible films (ODF's) and oromucosal patches (ORP's), which depends on their residence time in the mouth and disintegration [16]. Sublingual or orodispersible films are a dosage form that consists of a very thin oral strip, which should be placed on the tongue or beneath it and dissolves in less than a minute [17]. Such an administration method of *G. biloba* should bring many possible advantages—higher bioavailability, because of the possibility to prevent drug instability in the gastrointestinal tract, more precise dosing comparing to liquid forms, short onset time, fewer cases of mouth discomfort—especially in pediatric cases, lesser possibility of drug interactions, when multiple medications are used at the same time, because these drugs could be administered through separate routes [18–20]. There are several manufacturing methods of oral films, such as rolling, solid dispersion extrusion, hot melt extrusion, semisolid casting and solvent casting method [21]. The hot melt extrusion method and solvent casting method are the most commonly used methods of film manufacturing. The hot melt extrusion method is more often used to manufacture transdermal and transmucosal drug delivery systems, granules and sustained release tablets [22]. The solvent casting method was used in this study due to its simple preparation method and it is suitable for the hydrophobic active ingredient incorporation into the hydrophilic carriers.

The constituent polymers of sublingual films are chosen based on the desired physicochemical properties of the product [23]. Moreover, polymer employed should be non-toxic, non-irritant and devoid of leachable impurities. It also should have good wetting and spreadability properties [23]. Carboxymethyl cellulose (CMC) is a cellulose derivative produced by the reaction of cellulose with sodium monochloracetate. CMC is one of the commonly used polymers for preparation of sublingual films. It can produce films with excellent clarity and with the ability to carry a wide range of active ingredients, which has been proven to be useful in the preparation optimal polymeric matrices [24].

Considering the studies related to development of sublingual films as a carrier for freeze-dried *G. biloba* extract (GFD), the main purpose of this study was to exploit and optimize the amount of GFD in CMC-based sublingual films. Composition optimization of a drug carrier was performed in order to produce a positive impact on the desirable features of a drug formulation, such as more rapid drug release, higher release yields and improvement of drug stability. The sublingual region offers fast onset of action along with high bioavailability, which are desirable drug features for patients. Rapid onset of action and bioavailability of GFD sublingual films were also investigated for the first time. As was mentioned before, for this purpose, CMC was chosen as the film forming polymer and glycerol was selected as a plasticizer. To begin with, initial work focused on preparing and physicochemically characterizing formulations in terms of disintegration, tensile strength, Young's modulus and elongation at break. Finally, sublingual films and freeze-dried extract were compared to ascertain the merit of CMC as carriers for these poorly-aqueous soluble compounds.

2. Materials and Methods

2.1. Plant Material

The leaf samples were collected from the western climatic region of Lithuania (geographical coordinates: 55°39′40″ N; 21°10′30″ E) in 25th of September 2019 (voucher specimen: 1997/0304). Samples were collected during the stages of leaf aging. The samples were collected from branches located at a height of 1–3 m from the ground. During each sampling, about 40 leaves were collected from different parts of the crown of Ginkgo biloba.

To obtain a representative Ginkgo biloba leaf sample, leaves collected from different trees were mixed together into a single combined sample.

Ginkgo biloba leaves were dried at room temperature (25 ± 2 °C). The dried raw material was packed into paper bags and was stored in a sun-protected dry environment at room temperature and at 60 ± 5% relative humidity until the analysis. Prior to the analysis, the air-dried raw material was ground, and the wastage of Ginkgo biloba leaves was evaluated.

2.2. Reagents and Standards

Methanol (HPLC grade) was purchased by Roth GmbH (Lichtentanne, Germany). Ethanol (96.3% (v/v)) was purchased by Stumbras (Kaunas, Lithuania). Hydrochloric acid (≥37%) and ortho-phosphoric acid (≥85%) were purchased from Sigma-Aldrich GmbH (Saint Louis, MO, USA). The standards were used in the chromatographic analysis: kaempferol (≥99%) and isorhamnetin (≥99%), (Extrasynthese, Genay, France), quercetin dehydrate (≥98%) and CMC 200–400 cP were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Purified water was produced using a Millipore water purification system (Merck, Bedford, MA, USA). Polyethylene glycol (PEG—MW1500), glycerol and Arabic gum were supplied by Sigma-Aldrich (Saint Louis, MO, USA).

2.3. Preparation of Ginkgo biloba Leaves Ethanolic-Aqua Extract

During this stage, we applied the extraction technique proposed by Ding et al. [3]. Prior to the extract preparation, the dried *G. biloba* leaved were grounded in a cross-beater mill IKA A11 Basic Grinder (IKA Works, Guanghou, China) and sieved using vibratory sieve shaker AS 200 basic (Retch, UK) equipped with a 125 µm sieve. Powdered material (10 g) was then extracted with 100 mL of 70% (v/v) ethanol in a round bottom flask by heat-reflux extraction performed in a water bath Memmert WNB7 (Memmert GmbH & Co. KG, Schwabach, Germany) at 80 °C for 1 h. The prepared extract was filtered using a vacuum filter.

2.4. Preparation of Ginkgo biloba Leaves Freeze–Dried Extract

Ethanol was removed from *G. biloba* leaves ethanol–aqua extract using a rotary evaporator (Heidolph, Schwabach, Germany) for 10 min at 50 °C until dry residue was obtained. After evaporation, the required amount purified water, 1 g of PEG 1500 and 0.1 g of Arabic gum were added then the suspension was mixed for 15 min at 2000 rpm with a mechanic stirrer (Heidolph, Schwabach, Germany). The prepared dispersion was freeze-dried (LyoQuest Telstar, Germany). PEG 1500 and Arabic gum were used as a stabilizer to decrease deactivativation and destabilization of freeze-dried extract. After evaporation the purified water was added. The primary drying was carried out at 0.05 mbar for 12 h at a plate temperature of −50 °C. The secondary drying step was run for 12 h at 0 °C.

2.5. Preparation of Sublingual Films

The films were prepared using the solvent casting method. CMC was used as a film-forming agent and glycerol was used as a plasticizer [22]. Aqueous solution 1 was prepared by dissolving 2 g of the CMC in 100 mL of distilled water and stirred for 4 h at 2000 rpm with a mechanic stirrer (Heidolph, Schwabach, Germany) to produce a clear solution, which was kept for 1 h to remove all air bubbles. Then, aqueous solution 2 was prepared by adding 0.3 g of plasticizer and 2 g of solution 1 and stirred for 1 h. Prepared solution 2 was kept in the refrigerator (15 ± 0.5 °C) for 24 h to remove all the air bubbles from the sample. The desired amount of GFD was added and mixed gently with solution 2 under magnetic stirring (Table 1). The solution was cast onto each glass petri dish cast (internal diameter = 9.03 cm) and then kept in the oven for drying at 60 ± 0.5 °C for 4 h, at relative humidity of 75%. The film was carefully removed from the petri dish and checked for any imperfection and cut according to the size required for testing a square film of

2 cm length, 2 cm width. The samples were stored in a glass container maintained at a temperature of 25 ± 0.5 °C and a relative humidity of 60 ± 5%, until further analysis.

Table 1. The formulation codes, composition, weight ratio of sublingual films based on carboxymethyl cellulose (CMC) (control sample) and sublingual films based on CMC and *G. biloba* ethanol extract.

Sample Name	CMC * (g)	GFD ** (g)	Glycerol (g)
CMC—0	2	0	0.3
FRG—12	2	0.3	0.3
FRG—15	2	0.4	0.3
FRG—18	2	0.5	0.3
FRG—21	2	0.6	0.3

* 2 % of CMC solution used for sublingual film preparation. ** freeze-dried *G. biloba* extract.

2.6. Sample Preparation for High-Performance Liquid Chromatography (HPLC) Flavonoid Analysis

Samples for Ginkgo flavonoid analysis were prepared according to the procedure described in the European Pharmacopoeia monograph [25]. The extracts for the determination of ginkgo flavonoids were produced by precise weighing the material (dried Ginkgo biloba leaves/freeze-dried G. biloba leave extract/film), they were then transferred to a 50.0-mL vial, rinsed with 20.0 mL of methanol, 15 mL of dilute hydrochloric acid, 5 mL of water and diluted with methanol to the 50.0 mL mark. The resulting solution (10 mL) was transferred to a 10-mL bottle with a pressure-resistant membrane and pressure-resistant stopper. The stopper of the bottle was closed in a nitrogen stream, and the well-sealed vial was heated in a boiling water bath (100 ± 2 °C) for 25 min. The solution was cooled to room temperature. Before the extracts were injected into the HPLC system, all samples were filtered through 0.45-µm nylon filter pens (Carl Roth Gmbh, Karlsruhe, Germany).

2.7. HPLC Conditions for Determination of Flavonoids

Flavonoids in Ginkgo biloba leaf samples were separated using a Waters 2695 chromatographer (Waters Corporation, Milford, CT, USA) equipped with a Waters 996 photo diode array detector (PDA) (Waters Corporation, Milford, CT, USA). An ACE C18 column (150 × 4.6 mm, 5 µm) (Advanced Chromatography Technologies, Aberdeen, Scotland, UK) was used for analysis. The column temperature was maintained at 25 °C. Gradient elution was performed with a mobile phase ortho-phosphoric acid (pH 2.0) (solvent A) and methanol (solvent B). A linear gradient profile was applied with the following proportions: 0 min to 1 min with 60% A and 40% B; 1 min to 20 min from 60% to 45% A and from 40% to 55% B; 20 min to 21 min from 45% to 0% A and from 55% to 100% B; 21 min to 25 min with an isocratic elution of 100% B; 25 min to 30 min from 0% to 60% A and from 100% to 40% B. The injection volume was 10 µL, and the elution flow rate was 1.0 mL/min.

Three selected flavonoids—quercetin, kaempferol and isorhamnetin were identified in G. biloba leaves. Flavonoids were identified by comparing the retention time and spectral characteristics (λ = 370 nm) of the eluting peaks with those of reference compounds.

The percentage content of flavonoid glycosides, was calculated using following formula:

$$X = 2 \times \frac{A_1 \times m_1 \times 2.514 \times p}{A_2 \times m_2} \quad (1)$$

where m_1—grams of quercetin dihydrate in the reference solution; m_2—grams of the extract to prepare the test solution; A_1—sum of the peak areas from quercetin to the isorhamnetin in the chromatogram obtained with the test solution; A_2—areas of the peak due to quercetin in the chromatogram obtained with the reference solution and p—percentage content of anhydrous quercetin in quercetin dihydrate.

2.8. FT-IR (Fourier Transform-Infrared) Imaging

The functional groups of CMCs, GFD and FRG—15 films were investigated using infrared an spectroscopy spectrum (IR tracer—100, Shimadzu, Kyoto, Japan). Transmission levels were measured for wave numbers of 4000–500 cm^{-1} with a resolution of 4 cm^{-1} for 20 scans and read as absorbance in triplicate before taking the averaged value.

2.9. Thickness Measurement

Screw gauge with an accuracy of 0.001 mm was used to measure the thickness of the film. The measurements were taken from different strategic locations i.e., the center and four corners of the film. The results are reported in mean ± SD.

2.10. Mechanical Properties

Mechanical properties of the prepared films were measured using a Universal Testing Instrument (Model H5KS, Tinius Olsen, Horsham, PA, USA), with a 10-N load cell. The samples from each film formulation were cut in the direction of machine flow into rectangles (150 mm long × 10 mm wide) and the test was performed according to the American Society for Testing and Materials (ASTM) International Test Method for Thin Plastic Sheeting (D 882—02). The film formulations were placed between two clamps. Each film was pulled by the upper clamp at a rate of 50 mm/min. The force and elongation at the point of break were measured. Three mechanical properties were calculated for the evaluation of the prepared films: tensile strength, elastic modulus and elongation at break. Measurement was done in triplicate and the average value was taken. Values of the modulus of elasticity (the E-modulus (E)) were calculated from the linear part of the stress–strain curve [26]. Elongation as a function of the applied load was recorded at the moment of rupture. Elongation is the increase in length produced in the gage length of the sample by a tensile load. It is usually measured at the moment of film rupture (present elongation at rupture) [27].

2.11. Disintegration Time

Disintegration was studied with the USP disintegration testing apparatus (Sotax, Aesch, Switzerland). Distilled water was used as medium. The films were cut into 2 cm^2 pieces and each film was placed in the tubes and disintegration time was recorded (n = 6) [27].

2.12. SEM Analysis

The microstructures of the films were evaluated by scanning electron microscopy (SEM) using an FEI Quanta 200 FEG (FEI, OR, USA), which is a high resolution field emission scanning electron microscope with a Schottky-type electron gun where the samples can be investigated under controlled pressure water steam atmosphere. The SEM was operated at 10 kV with magnification of 5000–10,000 times.

2.13. In Vitro Release Studies

The FRG—15 sample was cut into rectangles (150 mm long × 10 mm wide). Dissolution profiles of active ingredients (flavonoids glycosides) in prepared FRG—15 film and GFD (extract/PEG/Arabic gum mixture) samples were determined using a SOTAX brand AT7 smart model semi-automated dissolution tester (Aesch, Switzerland). The basket method was applied using a simulated saliva at pH 6.8, (37 ± 0.5 °C), 50 rpm, and 500 mL. Aliquots (5 mL) were manually extracted from parallel dissolution vessels at 1, 3, 5, 7, 10, 15, 20, 25 min time points, filtered through a nitrocellulose membrane (0.45 µm) and quantified by HPLC. The dissolution media in each vessel was topped off with fresh dissolution fluid (5 mL) to maintain constant volume. The evaluation of dissolution profiles was carried out in triplicate.

2.14. Evaluation of Stability

Four ounce amber glass containers, each containing the FRG—15 sample (the size of the film was 2 × 2 cm and one film mass was 0.95 g) were kept according to The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH Q1A (R2)) guidelines for the long-term storage conditions in standard atmosphere (temperature: 25 ± 2 °C, and relative humidity: 60 ± 5%) and were also subjected to accelerated storage conditions at 40 ± 2 °C and with a relative humidity of 75 ± 5% [28].

The required containers were withdrawn after 0, 3, and 6 months in triplicate for analysis. The main active ingredients in the prepared sublingual films were flavonoid glycosides. The identification and quantified tests (by HPLC) were performed to evaluate the storage influence on the stability of the film.

2.15. Statistical Analysis

Data are expressed as a mean ± SD. The software package Prism v. 5.04 (GraphPad Software Inc., La Jolla, CA, USA) was used to perform the analysis of variance (ANOVA) to detect the differences among the mean values of responses and for the curve fitting. A value of $p < 0.05$ was taken as the level of significance.

3. Results and Discussion

3.1. Mechanical Properties of the Prepared Sublingual Films

The mechanical properties of the films determine the packaging, transport and storage conditions along with application. The mechanical features of designed films were examined by three different parameters: tensile strength, elongation at break and Young's modulus (Table 2). For sublingual drug administration, flexible and strong films are preferable. They should be characterized by low value of Young's modulus and by high value tensile strength, also high elongation at break [29]. The film samples contained different amount (0.3, 0.4, 0.5, 0.6 g) of GFD, 0.3 g of glycerol and 2 g of 2% CMC (the code explanation is shown in Table 1). The results showed that increasing concentration of GFD in films induced a significant reduction in tensile strength and Young's modulus compared with control (CMC—0) sample. Moreover, the elongation parameter was significantly increased in FRG—12, FRG—15, FRG—18 and FRG—21 formulations in comparison with the CMC—0 sample (additional information about sample composition is presented in Table 1). Furthermore, the increasing amount of GFD in film also increased interactions between macromolecules, which may have caused the reduction in intermolecular forces between a freeze-dried extract and polymer, consequently reducing the mechanical resistance of the polymer [30]. Moreover, the glycerol which was used as a plasticizer which may have influenced the Young's modulus and tensile strength results. Glycerol increased the free volume between polymer chains by reducing interactions between the chains, thereby making the polymer more flexible, and making the polymer chains easily slide past one another to yield lower values of tensile strength with enhanced ductility [31].

Table 2. Mechanical properties of sublingual films based on CMC with freeze-dried *G. biloba* extract (GFD).

Sample Name	Tensile Strength (MPa)	Elongation at Break (%)	Young's Modulus (MPa)
CMC—0	51 ± 3	3.6 ± 0.2	3200 ± 11
FRG—12	46 ± 5 [a]	4.2 ± 0.3 [d]	2800 ± 14 [g]
FRG—15	40 ± 2 [a,b]	5.0 ± 0.4 [d,e]	2200 ± 13 [g,h,k]
FRG—18	36 ± 4 [a,b,c]	5.3 ± 0.5 [d,e]	1800 ± 15 [g,h,k,j]
FRG—21	30 ± 2 [a,b,c]	5.9 ± 0.2 [d,e,f]	1300 ± 16 [g,h,k,j]

Mean values in the same column with different letters indicate statistically significant differences (ANOVA/Tukey's test, $p < 0.05$). [a] $p \leq 0.05$ vs. CMC—0; [b] $p \leq 0.05$ vs. FRG—12; [c] $p \leq 0.05$ vs. FRG—15; [d] $p \leq 0.05$ vs. CMC—0; [e] $p \leq 0.05$ vs. FRG—12; [f] $p \leq 0.05$ vs. FRG—15; [g] $p \leq 0.05$ vs. CMC—0; [h] $p \leq 0.05$ vs. FRG—12; [k] $p \leq 0.05$ vs. FRG—15; [j] $p \leq 0.05$ vs. FRG—15.

3.2. Physical Properties

Thickness is one of the most important features characterizing the quality of sublingual films, because it can affect their barrier properties (by prolonging disintegration time) as well as mechanical properties. In this study, prepared films were very easily removed from containers after drying. Appropriate thickness for films is considered to be below 0.25 mm [32], the average thickness values of films were between 0.06 and 0.12 mm. As Table 3 shows, incorporation of GFD to sublingual CMC films had a significant effect on their thickness values ($p < 0.05$): increasing GFD concentration raised thickness values. The low standard deviations of results suggest homogeneousness of films. Our findings correspond with results of Dashipour et al.; it was reported in their manuscript that increasing concentration of CMC expanded film thickness, which could be happening due to entrapment of oil particles in film matrix pores [33]. Moreover, Mirzaei-Mohkam [34] reported that addition of vitamin E to CMC films increased film thickness.

Table 3. Physical properties of sublingual films based on CMC with *G. biloba* ethanol extract (GFD).

Sample Name	Thickness, (mm)	Disintegration Time, (s)
CMC—0	0.056 ± 0.002	100 ± 1
FRG—12	0.065 ± 0.005 [a]	134 ± 3 [b,d,]
FRG—15	0.078 ± 0.001 [a]	150 ± 4 [b,c]
FRG—18	0.086 ± 0.004 [a]	189 ± 6 [b,c,d]
FRG—21	0.095 ± 0.003 [a]	197 ± 7 [b,c,d]

Mean values in the same column with different letters indicate statistically significant differences (ANOVA/Tukey's test, $p < 0.05$). [a] $p \leq 0.05$ vs. CMC—0; [b] $p \leq 0.05$ vs. FRG—12; FRG—15; FRG—18; FRG—21; [c] $p \leq 0.05$ vs. FRG—15; FRG—18; FRG—21; [d] $p \leq 0.05$ vs. FRG—12; FRG—18; FRG—21.

Disintegration is significantly related to how quickly the drug is exposed to the environment and to drug dissolution rate. This process is slow for hydrophobic drugs [35]. The dashed line at a disintegration time of 180 s represents the maximum time limit, required by the Ph. Eur. for disintegration of orodispersible tablets [25]. Moreover, a higher disintegration rate is an important parameter for sublingual CMC films containing GFD, because the main active ingredients in the films (flavonoid glycosides) are hydrophobic. As presented in Table 3, the higher amount of GFD in sublingual films statistically increased disintegration time in comparison with the control CMC sample. Only the FRG—18 and FRG—21 samples did not fulfil disintegration time requirements of Ph. Eur [25] for orodispersible tablets ($t \leq 180$ s). These effects can be explained also by CMC swelling on the film surface and irregular diffusion through the film layer of small volume disintegration medium, which results in prolonged disintegration times [36,37]. Furthermore, it was observed that higher GFD concentrations also significantly increased the thickness of the film. Thicker films resulted in them being more resistant to water penetration than films with reduced thickness, and hence longer disintegration times were observed for the films containing

a higher concentration of GFD (Tables 2 and 3). Similar results were reported by Esim and coauthors [15], suggesting that increasing the hydrophobic drug (dihydroergotamine mesylate) amount in maltodextrin–pullulan sublingual films significantly increased the disintegration time.

SEM images of CMC-0 and FRG—15 are shown in Figure 1. SEM analysis was done only for the sample coded FRG—15, since it showed higher elongation and tensile strength, and a faster disintegration time, which are important physicochemical properties for production. To understand the morphology of freeze-dried extract after incorporation as well their distribution into the film, SEM images of film formulations were obtained. Blank film (CMC—0) shows uniform structure. Moreover, the SEM images of prepared sublingual film show the uniform surface morphology of the FRG—15 sample with homogenous distribution of GFD and with no crystalline structures visible on SEM images.

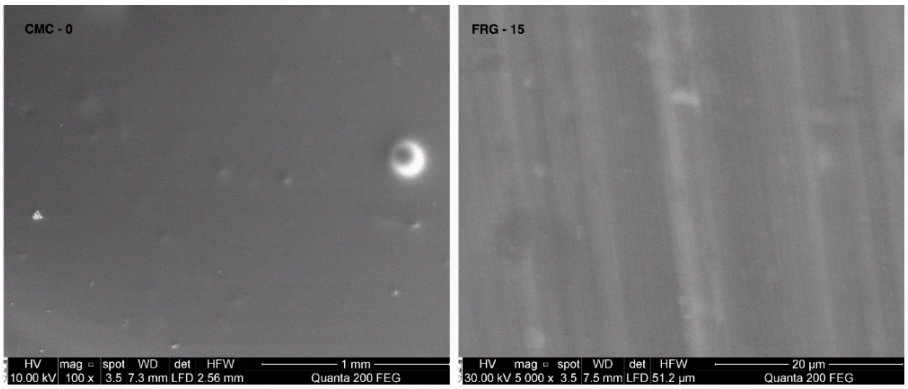

Figure 1. Scanning electron micrographs of films loaded with GFD (FRG—15 sample containing 0.4 g of GFD, 0.3 g of glycerol and 2 g of 2% CMC and without GFD CMC—0—the film sample containing 0.3 g of glycerol and 2 g of 2% CMC.

3.3. Dissolution Studies

The dissolution profile of the FRG—15 sample and GFD are presented in Figure 2. Release assay was performed only for the sample coded FRG—15 (the size of the film was 2 × 2 cm and the mass of one film was 0.95 g), since it showed higher elongation and tensile strength, and faster disintegration time, which are important physicochemical properties for production and marketing of this dosage form (Figure 2).

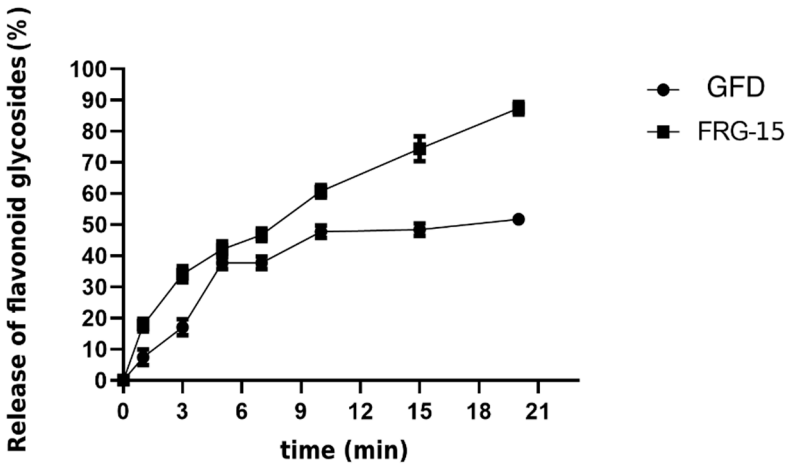

Figure 2. In Vitro release profile of flavonoid glycosides from film based on CMC with incorporated *G. biloba* ethanol extract of FRG—15 formulation and *G. biloba* freeze-dried extract (GFD).

Three selected flavonoids—quercetin, kaempferol and isorhamnetin—were identified in freeze-dried *Ginkgo biloba* extract. Flavonoids were identified by comparing the retention

times and spectral characteristics (λ = 370 nm) of eluting peaks with those of reference compounds. The percentage content of flavonoid glycosides was calculated using following formula, which is described in HPLC conditions for determination of flavonoids. An example of a chromatogram is presented in Figure 3. The obtained results showed that flavonoid glycosides, presented in FRG—15, demonstrated 1.3–2.0-fold higher dispersion in solvent than GFD. Moreover, the film exhibited maximal release of flavonoid glycosides in 10 min—it was more than 87 % in sample coded FRG—15. Consequently, by comparing two different preparation methods, the freeze-dried and film casting techniques, it was observed that the FRG—15 sample had a significantly better in vitro dissolution profile than the freeze-dried extract. Moreover, high dissolution data might influence the choice of polymer as a drug carrier, which plays an important role in drug delivery systems [27]. CMC has the ability to increase viscosity, high pigment binding capacity, bioadhesion and biocompatibility, which enhance the bioavailability of active compounds, in particular those with low aqueous solubility [26,38–40]. Moreover, the fast dissolution rate of the films has been attributed to a number of properties, which include a hydrophilic nature of the polymer and flavonoid glycosides, a high surface area of the film structure. Tedesco [41] and coauthors reported that according to the in vitro release profile, 80% of phenolic compounds were released in 5 min from hydroxypropyl methylcellulose films with incorporated peanut skin extract.

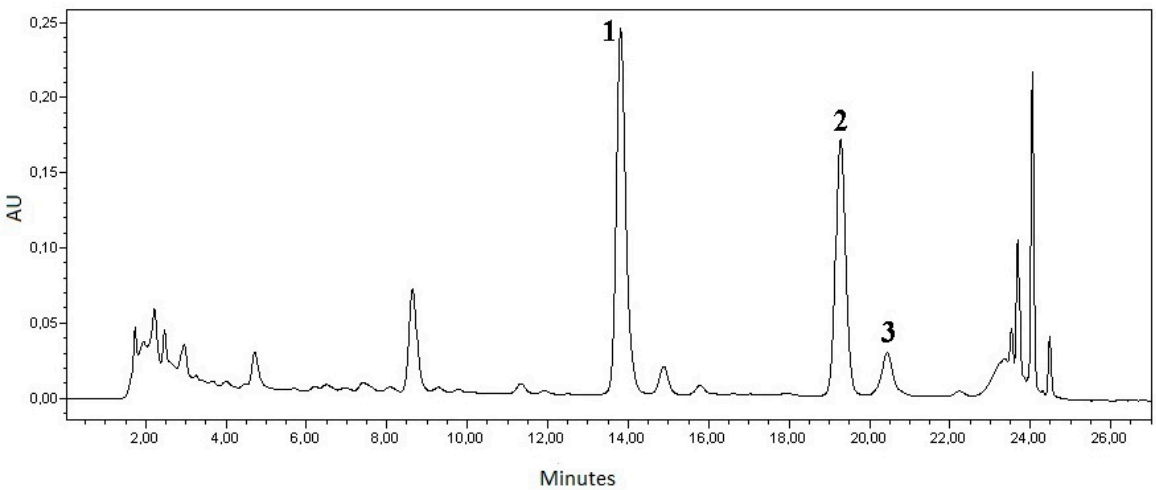

Figure 3. HPLC—PDA chromatogram of identified flavonoids in freeze-dried *Ginkgo biloba* extracts (1-quercetin, 2-kaempferol, and 3-isorhamnetin).

3.4. FTIR Analysis

FTIR spectra of (A) freeze-dried *G. biloba* leave extract, (B) CMC and (C) film based on CMC and freeze-dried *G. biloba* leave extract are shown in Figure 4. FTIR spectra shows the existence of specific functional groups in the samples. Figure 4A depicts the characteristic bands of *G. biloba* leaf extract, which appear at 1694, 1646 and 1068 cm^{-1}. The absorption at 1068 cm^{-1} was attributed to C–OH stretching vibration in alcohols and carboxylic acids [42]. The absorption at 1647 cm^{-1} was attributed due to the C=C and C=N stretching vibration and the absorption at 1694 cm^{-1} was assigned to C=O [43]. In Figure 4C, the broad absorption peak at 3364 cm^{-1} was due to the stretching vibrations of the hydroxyl group (OH) for FTIR spectra of CMC. The peak located at 2931 cm^{-1} was attributed to the stretching vibration of C-H [44,45]. A comparison Figure 4A–C for fast dissolving films based on CMC and *G. biloba* ethanol extract signified that the hydroxyl group (OH) and C-H group appeared in both figures at 3364 cm^{-1} and 2931 cm^{-1}, respectively. According to reported data, the peaks at wavelength of 1068, 1647 and 1694 cm^{-1} represented in freeze-dried *G. biloba* leaf extract were also determined in sample of fast dissolving film based on CMC and freeze-dried *G. biloba* leaf extract.

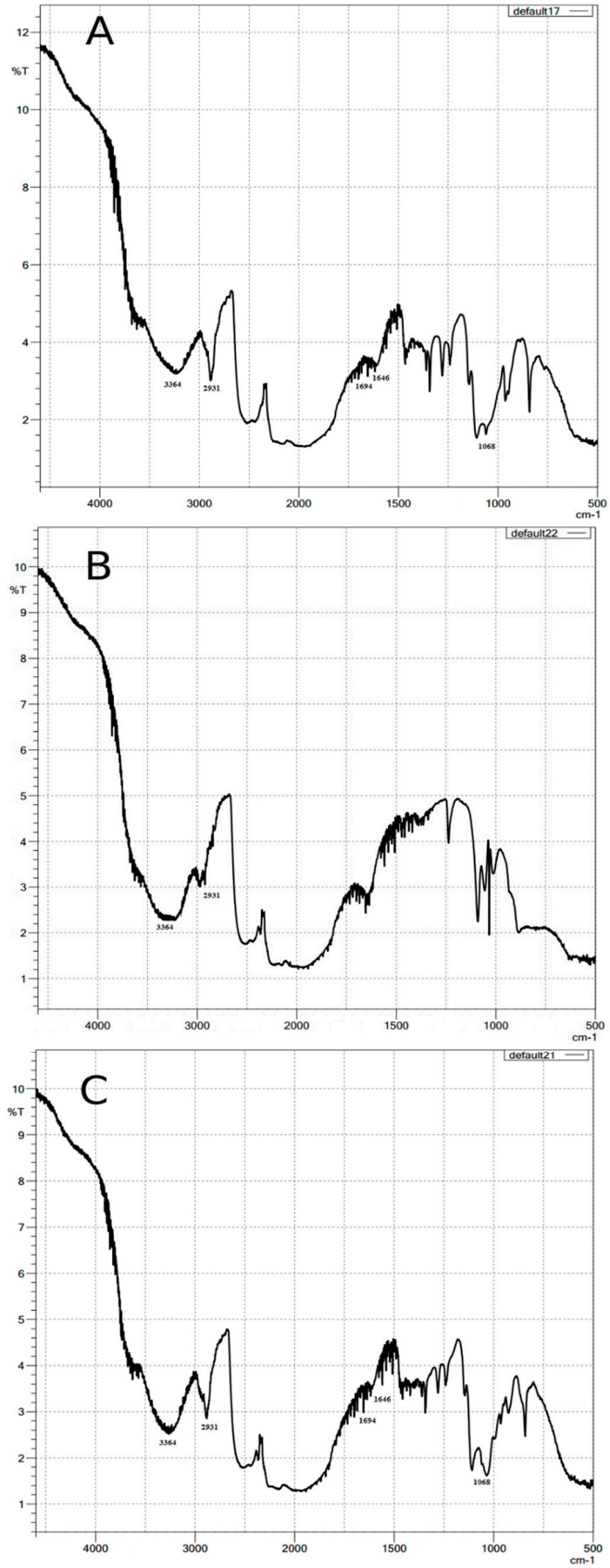

Figure 4. FTIR spectra of a GFD (**A**), CMC powders (**B**) and sublingual film containing FRG (FRG—15 formulation) (**C**).

3.5. Stability Studies of the FRG—15 Sample

The freeze-dried extract was very hygroscopic after 28 days; therefore, the stability test could not be continued. As was mentioned before, the moisture content in the sample increased 1.6 times ($p < 0.05$) in comparison to the fresh one. The humidity test was highly positive for freeze-dried material. For this reason, only the FRG—15 sample stability studies were evaluated by average weight content of film and flavonoid glycosides' stability in the sample under the accelerated stress conditions and long-term conditions for 6 months (Table 4).

Table 4. The FRG—15 formulation's (the film formulation contained 0.4 g of GFD, 0.3 g of glycerol and 2 g of 2% CMC) active compound stability studies were performed during 6 months storage under accelerated and long-term conditions, ($n = 3$).

Results			
Samples	**The Percentage Content of Flavonoid Glycosides, (%)**		
	Initial	**3 Months**	**6 Months**
	Accelerated Conditions [1]		
FRG—15	0.52 ± 0.05	0.51 ± 0.07	0.49 ± 0.08
	Long-Term Conditions [2]		
FRG—15	0.52 ± 0.04	0.50 ± 0.03	0.49 ± 0.02

[1] temperature: 40 ± 2 °C and relative humidity: 75 ± 5%; [2] temperature: 25 ± 2 °C and relative humidity: 60 ± 5%.

Moreover, the results revealed there was no significant changes ($p > 0.05$) of flavonoid glycoside content after aging under accelerated stress conditions (40 ± 2 °C, relative humidity 75 ± 5%) and long-term conditions (25 ± 2 °C, relative humidity 60 ± 5%). The results mentioned above also show that there are no interactions between flavonoid glycosides and polymer CMC molecular structures, because CMC is an ionic polymer and due to this reason, there was no flavonoid–polymer hydrogen bonding forces in this formulation [22,46].

4. Conclusions

In this study, the sublingual films were manufactured, containing GFD, using CMC as a film forming agent and glycerol as a plasticizer. The prepared films were transparent and easily removable from the Petri dishes. In terms of the physicomechanical properties (the elongation at break, tensile strength, disintegration time) the FRG—15 sample (the film formulation containing 0.4 g of GFD, 0.3 g of glycerol and 2 g of 2% CMC) was chosen as an optimal formulation. According to FTIR data, no interaction was observed between CMC and GFD. Moreover, the in vitro release profile, FRG incorporation into CMC sublingual film dramatically improved dispersion/dissolution traits of flavonoid glycosides compared to the freeze-dried control extract GFD. Furthermore, FRG—15 was remarkably stable over the 6-month storage period even under conditions of substantial humidity. These findings indicate that a CMC film containing the freeze-dried *G. biloba* extract can improve the dispersibility and dissolution rate of flavonoid glycosides, thus implying a potentially promising alternative technology for the mucosal application of such therapeutics.

Author Contributions: Conceptualization, L.I. and J.B.; methodology, L.R.; software, L.R., J.B.; validation, L.J., M.M., L.I.; formal analysis, L.I.; investigation L.R. and J.B.; resources, J.B.; data curation, L.R.; writing—original draft preparation, L.R., J.B.; writing—review and editing, J.B., L.I.; visualization, M.M.; supervision, L.I.; project administration. L.J. All authors have read and agreed to the published version of the manuscript.

Funding: The financial support is provided by Science Foundation of Lithuanian University of Health Sciences.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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