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In situ gels loaded with naringin as ocular drug delivery carriers; development and preliminary characterization

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ABSTRACT: Ocular drug delivery of any molecule is quite complex and challenging due to the ophthalmic anatomy. The current ocular formulations such as drops, gels or ointments cannot deliver the active molecules efficiently, therefore newer dosage forms are being developed. *In situ* gels which are in the liquid state in room temperature or in certain pH but transform to gels when instilled onto the eye belongs to such innovative dosage forms. Herein, the fabrication of *in situ* gels for naringin ocular delivery is presented since there are very few studies examining the use of naringin as an active molecule for eye delivery. Naringin which is hydrolyzed in naringenin, is a flavonoid glucoside found on citrus fruits and has been studied as antioxidant and anti-inflammatory agent or potent antimicrobial agent. The naringin loaded *in situ* gels developed via cold method using Poloxamer 407, sodium alginate and hydroxypropyl methylcellulose E5. The temperature-responsive *in situ* gels were characterized for clarity, sol-gel transition temperature, gelling capacity, pH and viscosity. All the results were of desirable limits. Furthermore, in vitro drug release demonstrated that the *in situ* gels showed sustained pattern Antimicrobial studies indicated specific antimicrobial potency against Enterococcus faecalis. Future studies will involve in vivo studies and ocular irritation analysis.

KEYWORDS: Naringin; ocular; *in situ* gels; eye; antimicrobial; gelation

1. INTRODUCTION

The use of medicinal plants or phytopharmaceuticals has been widely exploited as potent molecules for various diseases as neurodegenerative, metabolic syndrome [1], autoimmune disorders [2], cancer [3] etc. Nonetheless, ocular drug delivery of natural plants based medicaments is not very common. Phytochemical compounds since they are abundant on fruits, vegetables and plants are cost-effective pharmaceutical agents and exert biological activities. Naringin, is a natural flavonoid found on citrus fruits peel especially on grapefruit. Naringin is responsible for the bitterness of the citrus fruits; despite its sour taste, naringin has been studied for its proliferation of human mesenchymal stem cells [4], anti-inflammatory action [5], angiogenesis [6], wound healing [7,8] and possible antinociceptive central mechanism [9]. Naringin, as drug has been classified in class II of Biopharmaceutical Classification System due to its low solubility and permeability. It is metabolized to naringenin an aglycone derivative and both molecules have similar structures besides the sugar moieties. Naringin, despite its numerous biological activities has not been clinically available in the market with main obstacle its low solubility and the fact that can be degraded when intravenously administered [10]. Nonetheless, these obstacles are not important for ocular delivery since the use of specific drug formulations as *in situ* gels can protect the molecule from degradation and

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improve its solubility. *In situ* gels are well documented formulations for topical application of drugs to the skin, vagina, mouth, eye etc [11–15].

In situ gels comprised from macromolecules which have the ability to transform from viscous liquids to gel nature via the differentiation of physiological conditions as pH, ionic strength, UV and temperature. *In situ* gels as ocular formulations are advantageous given that present enhanced corneal permeability due to the prolonged eye contact time. Various macromolecules either natural, semi-synthetic or synthetic have been used as gelling agents; i.e. poloxamers which are triblock copolymers consist of polyethylene oxide-polypropylene oxide-polyethylene oxide (PEO-PPO-PEO) blocks, sodium alginate (SA)- a derivative of alginic acid and hyrdroxypropylmethyl cellulose (HPMC)- a derivative of cellulose are some of the polymers used. Poloxamers, especially Poloxamer 407 (P407) and Poloxamer 188 (P188) can be changed to clear gel when contact water has been widely applied to ocular formulations [16]. However, poloxamers show limited mucoadhesion and therefore mucoadhesive hydrophilic molecules as cellulose and its derivatives [17] or sodium alginate [18] can be use in addition to poloxamers.

Naringin ocular formulations are absent from literature; nonetheless, ocular administration of naringenin complexes with β-cyclodextrin [19] or eye drops for Choroidal neovascularization (CNV) management [20], naringenin-loaded sulfobutylether-β- cyclodextrin/chitosan nanoparticles against Age-related macular degeneration (AMD) [21] as well as Naringenin encapsulated polyvinylpyrrolidone (PVP) nanocomplexes for improved ocular bioavailability have been reported [22]. The aim of this study was to present an alternative use of naringin as ocular *in situ* gels for the management of microbial infections or inflammatory ocular diseases. Therefore, physicochemical characterization, rheological studies, stability, in vitro release, microbiological studies were performed.

2. RESULTS AND DISCUSSION

2.1. Preparation and physicochemical analysis of ocular *in situ* gels neat and loaded with naringin

In this study, *in situ* gels were successfully developed with loading naringin for ocular bacterial infections. *In situ* gels are both novel and versatile for ocular applications in addition, recently, naringin has drawn attention in pharmaceutic field. Thus, this study matter for an alternative ocular treatment based on naringing loaded *in situ* gels. Physicochemical characterization of *in situ* gels is the most important study when *in situ* gelling systems are prepared. This is because the permeation and release behavior of the formulations will be affected by these parameters. Table 1 summarizes the physicochemical properties of neat and naringin loaded *in situ* gels.

First of all, it should be noted that the *in situ* gelling systems were developed via cold preparation method involving the use of P407, SA and HPMC. Cold method was chosen due to the better effectiveness compared to hot process which may form polymeric lumps to the gel [23]. The developed formulations were loaded with 2% of naringin while their appearance was clear gels. According to the physicochemical characterization, besides F5 formulation, which was not displayed any gelation characteristics, the others were of desirable properties. More specifically, F1-4 both neat and naringin loaded were clear; clarity is very important when gels are developed as pharmaceutical products. Clarity was assessed via visually observed the *in situ* gels against a black and white background. Moreover, the pure *in situ* gels displayed gelation temperatures at about 28-31°C. F4 gelling temperature was decreased and according to some studies, SA inclusion may reversed the temperature [24]. On the other hand, gelation temperature of naringin *in situ* gel increased at about 32- 34 °C. This increment on the gelation temperature can be attributed to the inclusion of the drug. The aforementioned temperature is of desirable limits which is in the range of 32.9 and 36°C since they will easily be converted to gel when they installed the ocular surface [25]. It can be noted that the addition of SA and HPMC has impacted the viscosity of the gels which is normal due to their properties. In fact, viscosity of the *in situ* gels has been more than double by increasing the SA concentration [26].

In further, the gelling capacity of all temperature responsive formulations exhibited optimum gelling capacity and immediate gelation; this would be important since after the instillation of the formulations into the cul de sac of eye as a liquid drops, they would undergo a rapid sol-to-gel transition [12]. It can be said that gelling capacity was enhanced by enhancing SA concentration and the addition of HPMC. This fact has been already reported in the literature considering that viscosity enhancing agents as HPMC can positively impact gelling capacity [26,27].

Another important parameter which is always examined when ocular formulations are being prepared is pH; pH of eye has been measured at 7 to 7.4. Although, the eye can tolerate pH of lower or higher than 7-7.4 pH, it is better to keep the pH values of the ophthalmic formulations near the ocular range

of 6.6-7.8, as an acidic or alkaline pH value induces lacrimation, ocular pain, and discomfort [28]. Herein, the pH of the prepared formulations without naringin found between 6.493±0,2836 and 6.797±0,0850 while the pH of the prepared formulations with naringin ranged between 6.887±0.295 and 7.23±0.067. All the pH values are appropriate for ocular delivery since they were iso-hydric and non-irritancy on the ocular mucosa will be expected [12].

Table 1. Physicochemical properties of neat and naringin loaded *in situ* gels**.**

Gel Code	Gelling temperature	Viscosity (cP)*	Viscosity (cP)**	pH (24°C)	Gelling capacity	Clarity
F1	28	146,6±11,5470	8633,3±404,1452	6.797±0,0850	++	+++
F2	30	140±20	19733,3±5604,7598	6.743±0,0666	+	+++
F3	31	180±0	26416,7±1376,8926	6.493±0,2836	++	+++
F4	28	453,3±11,5470	22333,3±144,3376	6.74±0,04	++	+++
F5	No gelation	466,7±11,5470	12333,3±144,3375	6.807±0,0551	-	+++
F1-N	32	446.667±9.428	1466.667±209.497	7.23±0.067	++	+++
F2-N	34	840±0.000	24250±1136.515	7.133±0.040	+	++
F3-N	34	1560±28.284	48416.67±1124.228	7.097±0.133	++	++
F4-N	32	1860±16.330	25343.33±846.023	6.887±0.295	+++	++

*At 4°C with using no 3 spindle with 30 rpm
**At 32°C with using no 4 spindle with 12 rpm

2.2. Stability and Drug content of the developed *in situ* gels

Stability is the most important issue for pharmaceutical products; instability can lead to major economical burden and therefore the stability studies were carried out at 4 ± 1 °C, for 90 days using the refrigerator. The developed formulations were visually observed periodically on every month. It was revealed that the *in situ* gels did not display any changes in visual appearance, clarity, and gelation time revealing their stability. After the 90 days study period, the pH values were not changed statistically. Moreover, drug content was found similar to before the initiation of the stability study. In fact, drug content was analyzed via UV - VIS spectrometry method. According to the results, the drug content before and after stability studies was high ranging from 90 to 100% (Table 2).

Table 2. Drug content of *in situ* gels before and after stability studies

Gel Code	% Drug Content before stability studies	% Drug Content after stability studies
F1	90.856 ±0.35427	90.505 ± 0.811734
F2	99.651 ±1.209277	92.498 ± 0.552296
F3	92.950 ±0.367701	90.908 ± 0.382715
F4	100.423 ±0.213179	99.247 ± 1.151659

2.3. *In vitro* drug release studies

In vitro release studies are crucial when formulations are first time being developed; in fact, the results from the analysis can possibly predict the behavior of the formulations in vivo. Herein, in vitro release studies were performed on Simulated Tear Fluid. Figure 1 depicts the the cumulative amount of naringin released over time from F1-F4. F5 formulation was excluded given that it has not gelling capacity. In general, it can be concluded that the drug released in a slow and sustain manner for a chronic period of 48 hours. F2, F3 and F4 released naringin in similar pattern for the first 12 hours compared to F1 formulation where drug was released quicker. After 24 hours, Naringin released slower from F1 and F4 in comparison to F2 and F3. The in vitro release results are similar to those reported in literature for *in situ* gels. More specifically, *in situ* gels are capable of controlling the drug release; according to Shen and Zhang, in situ gels containing Pemirolast Potassium, a drug for allergic conjunctivitis released the active molecule in a control and slow manner. Moreover, authors stated that as the polymer concentration increased, the drug release slowed down given that viscosity also increased. Consequently, the slower release at first for F2, F3, F4 *in situ* gels can be correlated with the increased SA content [29].

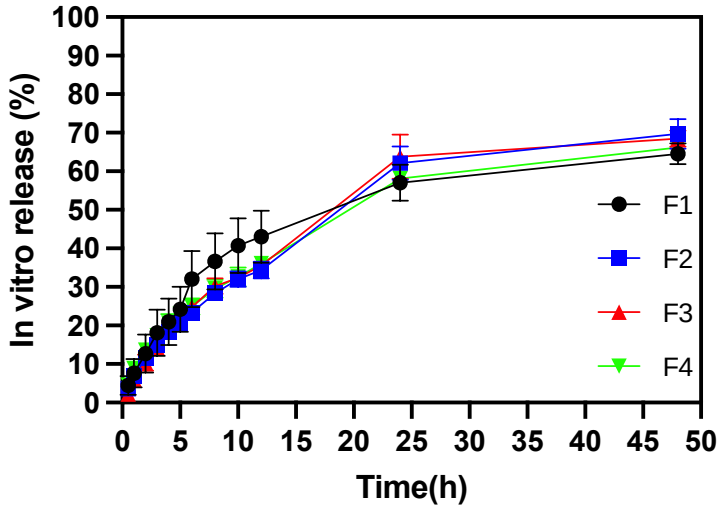

Figure 1. In vitro release studies of naringin from *in situ* gels

2.4. Antimicrobial studies of *in situ* gels

The antimicrobial activity of *in situ* gels loaded with naringin was determined against Candida *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 8739) and *Enterococcus faecalis* (ATCC 29212) by agar well diffusion method as recommended by CLSI. Neither naringin solution nor the formulations were found to be effective on the test microorganisms except *Enterococcus faecalis*. The assay was completed in triplicate and the mean zone diameter was detected. The zone diameters are given in table 3. Microbiological studies demonstrated that the formulations showed increased zone inhibition on *Enterococcus faecalis*, a gram positive bacterium which is known to cause severe endophthalmitis [30]. It is also known that *Enterococci* have high rates of resistance commonly used antibiotics such as clindamycin, cephalosporins and aminoglycoside [31]. Therefore, the obtained results would be promising for ocular infections developed by *Enterococcus faecalis.*

Table 3. Inhibition zone diameters in mm

	S. aureus	*P. aeruginosa*	*E. coli*	*E. faecalis*
F1	-	-	-	21.3 (20, 23, 21)
F1 blank	-	-	-	17.1 (18.5, 16, 17)
F2	-	-	-	23.1 (23.3, 23, 23.1)
F2 blank	-	-	-	19.3 (22, 18, 18)
F3	-	-	-	19 (22.1, 17, 18)
F3 blank	-	-	-	17.1 (20.5, 16, 15)
F4	-	-	-	21.4 (20.3, 22, 22)
F4 blank	-	-	-	20.3 (19.9, 20, 21)
Naringin 0.4 %	-	-	-	-

3. CONCLUSION

In the present preliminary work, an *in situ* gel based on Poloxamer, Sodium Alginate and Hydroxypropylmethyl cellulose was developed and analyzed as possible ocular carrier of Naringin. According to the results, the formulations which differ on the concentration of Sodium Alginate, present optimum stability, rheological properties and viscosity. Moreover, in vitro drug release studies depicted sustained release with slow drug release; as the concentration of sodium alginate increased, accordingly slower was the release behavior. Finally, antimicrobial studies depicted specific antimicrobial potency against Enterococcus faecalis. It can be concluded that the developed *in situ* gels could present an alternative use of naringin for ocular infections due to or inflammatory diseases.

4. MATERIALS AND METHODS

4.1. Materials

SA was provided from SAFC. Poloxamer 407 was obtained from Sigma Aldrich. HPMC E5 was kindly gifted from Colorcon. $CaCl_2$ was provided from Tekkim, Turkey. NaCl and $NaHCO_3$ were obtained from Yasin Teknik, Turkey.

4.2. Preparation method

So as to prepare *in situ* gels, naringin was dissolved in 2 ml of ethanol and 8 ml of water was added afterwards. Subsequently, in various concentrations of sodium alginate and HPMC were added to each sample. When the clear solution was obtained, they were cooled to 5°C and Poloxamer 407 was added and mixed for 2 hours. In order to complete swelling the formulations were kept in refrigerator overnight as it is known as cold method [32]. The composition of the gels was summarized in Table 4.

Table 4. The composition of the *in situ* gels

Formulation Code	Poloxamer 407 (a/h %)	Sodium Alginate (a/h %)	HPMC (a/h %)	Naringin (a/h %)
F1	20	1	0.5	0.4
F2	20	1.5	0.5	0.4
F3	20	2	0.5	0.4
F4	20	2.5	0.5	0.4
F5	20	3	0.5	0.4

4.3. Characterization methods

4.3.1. Gelation temperature

The gelation temperature was detected with using magnetic stirrer. The formulations were mixed with 300 rpm and heated with 2°C/min rate. The gelling temperature was determined as the magnetic bar stopped. The test was carried out in triplicate [13].

4.3.2. In vitro gelling capacity

In vitro gelling capacity was determined in accordance with Gugleva et al. study [32]. One drop of each formulation was stowed into the test tube at 35 ± 1°C including 2 ml simulated tear fluid which was prepared by dissolving 0.67 g sodium chloride, 0.2 g sodium bicarbonate and 0.008 g calcium chloride dihydrate in 100 ml of distilled water with pH equal to 7.4. The results were evaluated in terms of gel formation time and dissolution and indicated as follows: + : gel formation in a few minute and fast dissolution, ++ : gel formation instantly and remaining for a few hours, +++ : gel formation instantly and remaining for longer periods. The experiment was carried out in triplicate.

4.3.3. *Determination of viscosity*

The viscosity of the formulations was detected with using rotational viscometer at 4 ± 1°C and 32 ± 1°C. The test was conducted in triplicate [33,34].

4.3.4. Physical appearance, clarity, and pH of the gels

The physical appearance and clarity of the formulations were assessed by visually. The physical appearance was evaluated in terms of color. The clarity was scored as follows: +++: Very clear, ++: Clear, +: Turbid. The pH values were determined with digital pHmeter. The studies were carried out in triplicate.

4.3.5. Drug content uniformity

0.25 g of the developed formulations was dissolved in 50 ml ethanol and drug concentration was analyzed by UV - VIS spectrometry [35].

4.3.6. UV – VIS spectrometer analysis

The amount of naringin was determined with a UV-VIS spectrometer (INESA L7, China). Samples were analyzed at 290 nm which is the maximum wavelength of naringin at room temperature. Samples were dispersed in ethanol and ethanol was used as a blank. The method was validated for linearity, limit of

detection (LOD) and limit of quantitation (LOQ), precision, accuracy and specificity, selectivity, and stability. The linearity between peak area and concentration was analyzed using calibration curve obtained from standard solutions of naringin (1–50 µg/ml). 5 µg/ml solution was injected ten times in order to evaluate method precision, standard deviation (SD) and coefficient of variation.

4.4. *In vitro* release studies

In vitro release studies were conducted in simulated tear fluid (STF) (as described at 4.3.2) 100 ml to be harmony with ocular environment. So as to ensure sink condition, STF was mixed with ethanol in ratio of 20:80 EtOH:STF. 5 g of formulations were put into dialysis membrane (Spectra/Por Regenerated Cellulose, Molecular weight cut off 12–14 kDa) and tied knot. Dialysis membranes were placed into 100 ml simulated tear fluid and stirred at 50 rpm (32±0.1°C). 2 ml of sample was taken at a predetermined times from 30 min to 48 h and the same volume of fresh medium was replaced. The samples were analyzed with UV for determination of the drug content [36].

4.5. Microbiology studies

The antibacterial activity of *in situ* gels loaded with naringin was determined against *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli (ATCC 8739)* and *Enterococcus faecalis* (ATCC 29212) by agar well diffusion method as recommended by CLSI (Clinical and Laboratory Standards Institute) (CLSI, 2015).

Bacterial strains were incubated on Nutrient Agar at 37 ± 1°C for 18h. Active cultures were aseptically suspended in 0.85% saline solution and were arranged to give an inoculum with an equivalent 0.5 McFarland cell density. 100 µl of prepared suspensions were transferred onto Mueller-Hinton II Agar (Sigma-Aldrich 70191) and spreaded evenly with a drigalski spatula under aseptic conditions and allowed to dry. Then, 9 mm wells were opened in aseptic conditions on the media (in Class II laminar air cabinets), and gels containing naringin and and blank gels were pipetted into each well. As positive control naringin solution (dissolved in 2 ml of ethanol and 8 ml of water) and as negative control sterile 0.85% saline solution containing 20% ethanol was also pipetted to the wells. The petri dishes were incubated at 37° C for 24 hours and the inhibition zone (IZs, in millimeters) diameters were measured by a digital ruler. The assay was completed in triplicate and the mean zone diameter was detected [37,38].

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