Indian Journal of Natural Products and Resources
Vol. 12(1), March 2021, pp 128-135

Gallic acid-loaded microemulsion and microemulsion gel: Development, characterization, and evaluation of antioxidant effectiveness

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Received 25 May 2019; Revised 27 November 2020

In this study, it was aimed to develop, characterize, and improve the antioxidant activity of gallic acid (GA) by formulating it into microemulsion (ME) and microemulsion gel (MEg). Blank MEs were prepared using different proportions of oil/water/surfactant, which provide stable and transparent ME production. Their droplet sizes, zeta potentials and stabilities after holding, centrifugation, and freeze-thawing processes were determined. F2 and F5 coded MEs were selected among the blank MEs and GA was loaded into these formulations. Besides the characterization studies, pH and viscosity measurement, *in vitro* release, cytotoxicity test, cell permeation, and antioxidant activity studies were performed. *In vitro* released amount of GA was enhanced by formulating it into ME and MEg at the end of six hours and it showed a scavenging effect of $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals. In conclusion, increased efficacy, reduced toxicity, and prolonged antioxidant activity have been achieved with the use of new, non-toxic, and stable ME and MEg loaded with GA and it is thought that these formulations create the potential for topical application.

Key Words: Antioxidant effect, Cytotoxicity, Drug delivery system, Gallic acid, Microemulsion gel, Microemulsion.

IPC code; Int. cl. (2015.01)- A61K 9/00, A61K 36/00, A61P 39/00

Introduction

Microemulsions (MEs) are transparent or translucent thermodynamically stable systems with a droplet size of 20-200 nm, mainly containing oil, water, surfactant, and usually contains a co-surfactant. MEs are characterized by very low interfacial tension between oil and water phases and classified as water-in-oil (w/o), oil-in-water (o/w) or bicontinuous systems[1,2]. For the formation of low viscosity, transparent, and stable ME, the use of appropriate surfactant and co-surfactant in ideal proportions is important by causing an interface pressure that exceeds the interfacial tension at the oil-water interface[3]. Human skin, the external part of the body, prevents penetration of drugs as it acts as a barrier against different factors such as ultraviolet radiation, toxic substances, and microorganisms[4]. MEs are promising for topical and transdermal administration of both hydrophilic and lipophilic drugs as they improve the solubility, absorption, and permeation rate and hence the therapeutic effect of the drugs due to their unique formulation properties, thermodynamic stability, excellent biocompatibility, and hydrophilic & lipophilic fields[5-7]. MEs increase drug solubility by increasing the solubility of the drug, increasing the thermodynamic activity of drugs, the permeability enhancing effects of the formulation components and the hydration effect of MEs on the stratum corneum[8]. Microemulsion gels (MEgs) are prepared by the combination of MEs with bioadhesive gel such as Carbopol to increase contact time of the formulation with skin by increasing viscosity.

Gallic acid (3,4,5-trihydroxybenzoic acid) (GA) is a lipophilic polyhydroxyphenolic compound commonly found in many different plant families such as Anacardiaceae, Fabaceae, and Myrtaceaeas[9,10]. GA is known to have antibacterial, antiviral, antifungal, anti-inflammatory, antioxidant and prooxidative effects[11,12]. The chemical structure of GA is shown in Fig. 1.

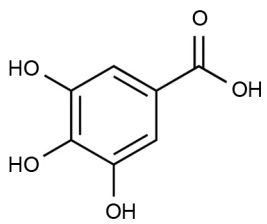

Fig. 1 — Chemical structure of gallic acid.

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Oil-in-water ME is preferred, as it is more suitable for delivery of lipophilic drugs[4]. Therefore, in the current study, it was aimed to develop and characterize the lipophilic antioxidant GA-loaded oil-in-water ME and MEg formulations, to evaluate cytotoxicity, transport properties through L929 cell line and antioxidant activity with using 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) and 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid ($ABTS^{\bullet+}$) radical scavenging activity assays.

Materials and Methods

Materials

GA (G7384, Sigma, USA), Tween 20 (817072, Merck, Germany), Polietilen glikol 400 (PEG400, 25322-68-3, Loba Chemie, India), Olive oil (purchased from the market, Komili, Turkey) and Carbopol 980 (purchased from Abdi İbrahim Pharmaceutical, Industry and Trade Company, Turkey) were used. All other chemicals used were analytical grade. L929 cell line was provided from the American Type Culture Collection (ATCC® CCL-1™). Cell culture flasks and cell culture plates 6 well (3412, Transwell™) were purchased from Corning®. Cedex Smart Slides and Trypan Blue solution were purchased from Roche (Switzerland).

Quantification of GA

UV-spectrophotometer was used to measure drug content in ME and MEg. The Maximum spectrums of GA were measured from 200 to 550 nm with a spectrophotometer (Shimadzu 1800, Japan) and the maximum wavelength was found to be 284 nm[(ref 13)]. The calibration curve was linear from 40 to 2.5 µg/mL with a high correlation coefficient (r^2=0.997). A stock solution of GA was prepared in aqueous ethanol (1:3 v/v) at a concentration of 40 µg/mL and stored in the dark. Working solutions were prepared from stock solution with dilution down to a final concentration of 2.5 µg/mL for calibration curves. The standard curve was calculated by linear regression, according to the following formula: $y = ax + b$, where x is GA the concentration as µg/mL and y is the absorbance at the maximum spectrum (284 nm) of GA.

Preparation of MEs and Megs

The newly developed ME formulations were prepared by titration method[14]. Tween 20: PEG 400 mixture was added to the oil phase in a 2:1 ratio and a constant amount of water was added dropwise by stirring continuously in the magnetic stirrer. ME formulations were prepared by using different ratios of oil, surfactant: cosurfactant mixture and water were given in Table 1.

GA-loaded MEs were prepared using the oil: water: surfactant ratios given in Table 1 by adding the GA solution in ethanol to the oil phase. GA-loaded MEgs were also prepared by using Carbopol 980. Gels were first prepared, 1.0% (w/v) of Carbopol 980 was added to distilled water, adjusted to pH 5.5 with triethanolamine in 1:1 (v/v) ratio and allowed to swell overnight at room temperature, then, optimized MEs (F2 and F5) and gel were mixed in the ratio of 1:1 (v/v) of 1.0% (w/v) Carbopol 980 solution and GA-loaded ME.

Characterization of MEs

In the characterization studies, droplet size (DS), zeta potential (ZP), dilution tests, and viscosities of developed MEs were measured. In addition, after the centrifugation, retention, and freeze-thawing, stability of MEs was evaluated.

DS and ZP of MEs were measured using a Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, United Kingdom) (Table 2). The physical appearance of MEs was determined using a polarized microscope (Zeiss Primo Star, Germany). To determine whether the ME was monophasic or not, the MEs were diluted with distilled water to confirm the emulsion type and miscibility with the aqueous phase. The pH of GA loaded formulations was

Table 1 — Composition of microemulsion formulations

Formulation	Olive oil (%)	Tween 20:PEG 400 ratio	Tween 20:PEG 400 (%)	Water (%)
F1	5	2:1	50	45
F2	5	2:1	55	40
F3	7,5	2:1	45	47,5
F4	7,5	2:1	47,5	45
F5	10	2:1	45	45
F6	10	2:1	50	40

Table 2 — Droplet size and zeta potential of blank microemulsions

Formulation	Droplet size (µm±SD)	Zeta Potential (mV±SD)
F1	1.335±0.02	-20.1±0.2
F2	1.002±0.01	-33.6±0.3
F3	2.142±0.04	-13.5±0.2
F4	2.060±0.05	-21.3±0.4
F5	1.247±0.02	-35.7±0.4
F6	1.880±0.03	-21.3±0.3

Values are expressed as mean±standard deviation, n=3

measured using a Digital pH meter (Mettler Toledo Seven Compact, Switzerland). The viscosity was measured using spindle number 14 at 10, 20, 50, and 100 rpm at room temperature (Brookfield Viscometer LVDV-I Prime, USA). In addition, the formulations were centrifuged at 10.000 rpm for 30 minutes using a cold centrifuge (Thermo Scientific, USA), frozen at -80 ºC for two hours and dissolved at room temperature; and then the system was examined as monophasic or biphasic[15]. The stability of ME formulations was also investigated at 4 ºC for 30 days.

In vitro release study of GA

The release study was performed using 12.000 dalton pore size dialysis membrane for six hours. Two mL GA-loaded ME and MEg were placed in the dialysis bag. The dialysis bags were suspended in 5 mL of distilled water and maintained at 37±0.5 °C with magnetic stirring at 400 rpm in a shaker (GFL 3032 Shaker, LABOTEC, Germany) during the experiment. At the end of six hours, the drug concentrations were analyzed by UV spectrophotometer and all experiments were performed in triplicate.

Cell culture studies

L929 cells were grown in a medium composed of EMEM containing 25 mM glucose, 5 mM glutamine supplemented with 10% horse serum, 1% gentamicin, and 7.5% sodium bicarbonate in an incubator at 37 ºC under 5% CO_2 atmosphere. The medium was changed with fresh EMEM every 48 hours. The presence of a confluent monolayer was controlled with a microscope.

Cytotoxicity assay

Cell viability on L929 cells was evaluated using the MTT test. In the test, L929 cells were seeded (10.000 cells/well) in 96-well culture plates[16]. Plates were kept at 37 ºC for 24 hours for cell adhering. Then, the cells were treated with different GA concentrations (62.5-1000 µg/mL), blank and GA loaded MEs and MEgs. After six hours, the medium was removed and 100 µL fresh medium and 13 µL MTT solution (5 mg/mL in phosphate-buffered saline) were added. After incubation for 4 hours at 37 °C, 100 µL of DMSO was added to each well to dissolve the formazan precipitate. The colour density was measured at 570 nm with a multi-well ELISA reader (Biotech Synergy HT, USA)[17]. The wells containing only the medium were regarded as a control group with the cell viability of 100% and the results were presented as the percentage using the control group values.

Transport experiments

The L929 cells were seeded on Transwell® inserts which consist of an upper (apical) chamber, a porous permeable membrane where cells attach and form a tight monolayer, and a lower (basolateral) chamber[18]. Transport experiments were performed from the apical to the basolateral compartment, with samples collected from the basolateral compartment and the amount of GA at the end of the six-hours.

Stability studies

Stability studies were carried out with pH, ZP, DS and viscosity analysis of the samples after 30 days storage at 4 °C. Also, after the centrifugation, retention and freeze-thawing, stability of MEs was evaluated[15] (Table 3).

$DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals scavenging activity

The ability of the formulations to scavenge $DPPH^{\bullet}$ was determined by the method of Gyamfi *et al.*[19]. A 50 µL aliquot of sample was mixed with 450 µL of Tris-HCl buffer (50 mM, pH 7.4) and 1.0 mL of 0.1 mM $DPPH^{\bullet}$ in MeOH. After 30 minutes of incubation in darkness and at ambient temperature, the resultant absorbance was recorded at 517 nm. The % inhibition was calculated using the following equation

$$\text{Inhibition \%} = [(Abs_{control} - Abs_{sample}) / Abs_{control}] \times 100$$

To further confirm the free radical scavenging activity of the formulations, an alternative synthetic radical $ABTS^{\bullet+}$ model was used, following the

Table 3 — Stabilization of microemulsions after retention, freeze-thawing and centrifugation

Formulation	Retention	Centrifugation	Freeze-thawing
F1	Not stable	Not stable	Not stable
F2	Stable, monophasic	Stable, monophasic	Stable, monophasic
F3	Not stable	Not stable	Not stable
F4	Not stable	Not stable	Not stable
F5	Stable, monophasic	Stable, monophasic	Stable, monophasic
F6	Not stable	Not stable	Not stable

method of Re *et al.*[20]. The $ABTS^{\bullet+}$ radical was generated by reacting a (7 mmol/L) $ABTS^{\bullet+}$ aqueous solution with $K_2S_2O_8$ (2.45 mmol/L, final concentration) in the dark for 12-16 hours, at ambient temperature, and adjusting the absorbance 734 nm to 0.700 with ethanol. After 990 µL $ABTS^{\bullet+}$ solution was added to 10 µL sample, the absorbance at 734 nm was recorded 1 minute after initial mixing and subsequently (for 30 minutes in total). The results are expressed as the Trolox equivalent antioxidant capacity (TEAC, mmol/L Trolox). Butylated hydroxyl anisole (BHA) was used as a positive control for both activities.

Statistical analysis

All data in this study were considered as means±SD. One-way ANOVA was used for statistical analysis. GraphPad InStat ver. 2 was used for the analysis program. Significant differences between means were determined by Tukey's pairwise comparison test.

Results

Analytical method and calibration

For the detection of GA, the optimal wavelength of 284 nm was found and the linearity plotting was ($y=0.0246x+0.0156$) ($r^2=0.997$) for working solutions.

Preparation of formulations

The MEs produced by the titration method given in Table 1 generally had a transparent and homogeneous appearance. MEg formulations were also homogeneous.

Characterization studies

For characterization of formulations, DSs and ZPs were measured and given in Table 2. The surface morphology of the F2 coded ME is shown in Fig. 2.

Determination of the stability of MEs after retention, freeze-thawing, and centrifugation

The stability of MEs was determined according to the remain of the monophasic structure after retention, freeze-thawing and centrifugation (Table 3).

Preparation and characterization of GA loaded MEs and MEgs

F2 and F5 coded MEs were selected as the ideal formulations considering stability, DS and ZP. GA-loaded F2 and F5 coded MEs were prepared by adding GA at a concentration determined from the cytotoxicity test. The DS, ZP and pH of GA-loaded F2 and F5 formulations are shown in Table 4.

The viscosities of F2 and F5 coded formulations were measured at different shear rate. The viscosity curves of the formulations are shown in Fig. 3.

***In vitro* drug release studies**

In vitro release studies of GA-loaded ME and MEg were performed as described in the method section. After 6 hours, the samples were measured at 284 nm by UV spectrophotometer and cumulative drug release was calculated. At the end of the sixth hour, only 43.2% of the GA was released from the solution, while the released amount of GA % was found to be

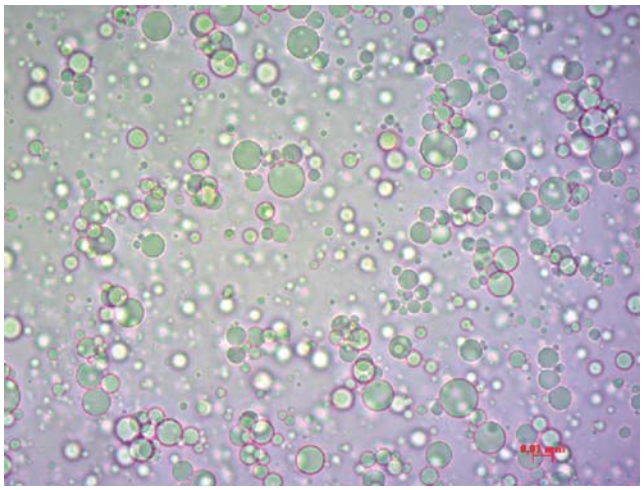

Fig. 2 — The physical appearance of F2 coded microemulsion.

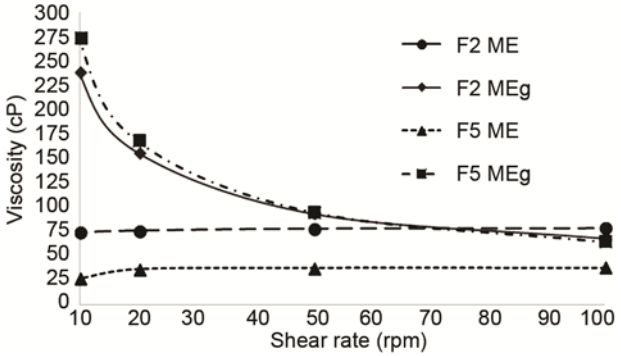

Fig. 3 — Rheology of gallic acid loaded microemulsions and microemulsion gels for F2 and F5 (n=3).

Table 4 — Droplet size, zeta potential and pH of gallic acid-loaded F2 and F5 formulations

Formulation	Droplet size (µm±SD)	Zeta potential (mV±SD)	pH±SD
F2 ME	1.04±0.02	-30.3±0.2	5.34±0.04
F2 MEg	2.73±0.05	-26.8±0.4	5.04±0.03
F5 ME	1.05±0.03	-37.2±0.2	5.32±0.01
F5 MEg	3.29±0.07	-33.0±0.3	5.06±0.02

Values are expressed as mean±standard deviation, n=3

72.0 and 66.8% from F2 and F5 coded MEs, respectively. On the other hand, in F2 and F5 MEg, the released amount of GA was determined as 57.2 and 43.0%, respectively.

Cell viability

The effects of GA solutions and ME formulation components were determined on the L929 cell viability for 6 hours using the MTT method. Cell viability of different concentrations of GA solutions and GA-loaded formulations is given in Fig. 4.

Permeation studies

The amount of transported GA form the L929 cell line was up to 41 and 27% for F2 and F5 ME, respectively. However, MEg formulations have limited the cell permeation of GA and it was found to be only 15 and 11% for F2 and F5 MEg, respectively.

Stability studies

The pH of F2 ME, which was kept at 4 ºC for 30 days, was 5.22±0.03, while ZP was -26.7±0.2 mV, and DS was 1.60±0.03 µm (n=3). The viscosity curve of the formulations after stability studies is shown in Fig. 5.

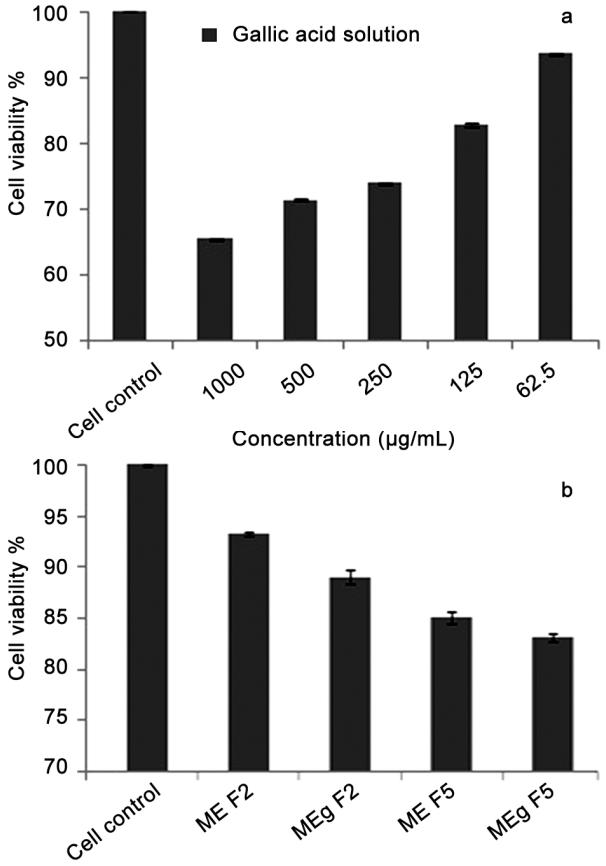

Fig. 4 — Gallic acid solution with different concentrations, a) Gallic acid loaded microemulsion formulations, b) on L929 cell viability (n=8).

Antioxidant activity assay

The released GA succeeded in scavenging DPPH$^{\bullet}$ radicals at physiological pH values. As shown in Table 5, it was found that the ability of the released GA to scavenge DPPH$^{\bullet}$ was determined and the percentage inhibition was found 52.79±1.29%.

As can be seen in Fig. 6, the released GA manage to inhibit ABTS$^{\bullet+}$ radical in a time-dependent manner. For released GA, TEAC values were found to be 2.55±0.00 mmol/L/Trolox.

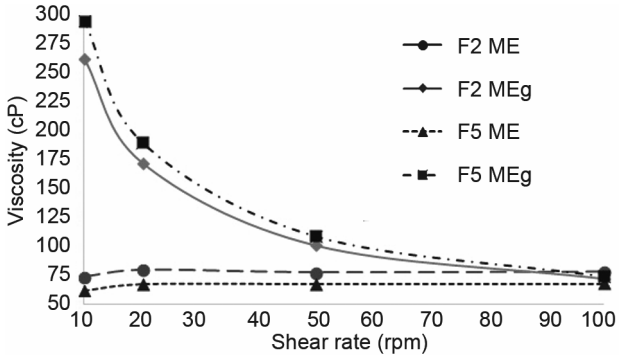

Fig. 5 — Rheology of formulations after stability studies (n=3).

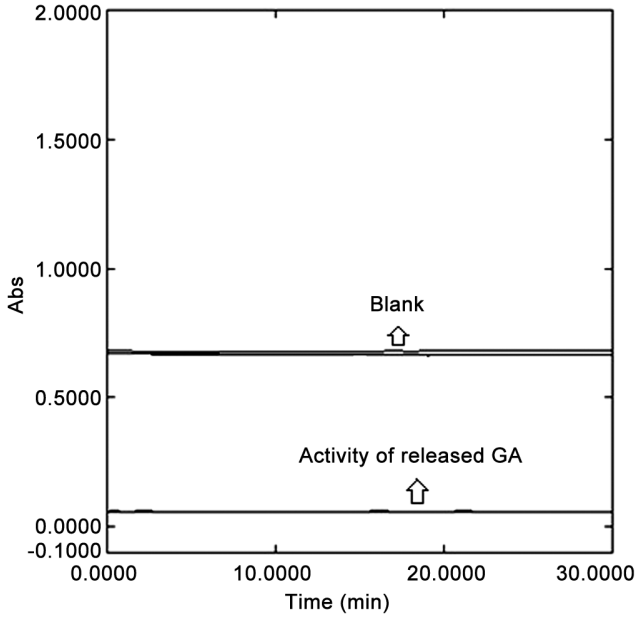

Fig. 6 — ABTS$^{\bullet+}$ radical scavenging activity of the release GA for 30 min.

Table 5 — Results of antioxidant activity of released gallic acid

TEAC (mmol/L/Trolox)	DPPH (% İnhibition)
2.55±0.00	52.79±1.29

Values are expressed as mean±standard deviation, n=3

Discussion

Thermodynamically stable MEs and MEgs are promising formulations for topical treatment that significantly increases drug absorption compared to solutions[21]. In the current study, stable ME and MEg formulations containing strong antioxidant GA were successfully developed and their characterizations and antioxidant activities were evaluated.

Blank ME formulations were prepared using different ratios of oil:water:surfactant. Blank MEs were found to have DS between 1.0-2.1 µm and the ZP values between (-13.5) - (-35.7) mV (Table 2). The formulation with the smallest droplet size was the F2 which had the lowest fat and the highest surfactant ratio.

The increase in ZP results in lower agglomeration of the droplets. Colloidal systems with a ZP value of greater than 30 mV and less than -30 mV are considered to be stable[22]. F2 and F5 formulations had the smallest DSs (1 and 1.2 µm) and appropriate ZPs (-33.6 mV and -35.7 mV) indicating that they were stable (Table 2). F2 and F5 coded MEs with low DS and ZP values close to -30 mV, were also found to maintain their homogenous, monophasic structures after retention, freeze-thawing and centrifugation (Table 3). Oil-in-water MEs which were miscible with water also maintained their homogeneous and monophasic phase structures after dilution with distilled water. F2 and F5 formulations have been identified as ideal formulations because they were stable with the smaller DS and appropriate ZP. Therefore, ME and MEg formulations containing GA have been prepared with F2 and F5 coded MEs.

As can be seen in Table 4, the ZP values of F2 and F5 coded ME and MEg formulations were close to -30 mV. While the DS of MEs was approximately 1 µm, the DS of F2 and F5 MEg increased to 2.73 and 3.29 1 µm, respectively (Table 4). The increase in the size of the droplets in the gels results from the higher viscosity of the gels. When the viscosities of the formulations were evaluated, there was no significant change in viscosity in F2 and F5 MEs and F2 and F5 MEg (P >0.05). MEs behaved like a newtonian fluid, did not cause a change in the viscosity of MEs with the change of the shear rate. On the other hand, the MEg formulations behaved like a pseudoplastic fluid, displayed a decreasing viscosity with an increasing shear rate (Fig. 3). The pseudoplastic behaviour of MEg resulted from the pseudoplastic characterization of carbopol[23].

Skin pH is generally assumed to be between 5-6, however, there are also studies showing that the pH of the skin is below 5[(ref 24)]. In our study, the pH of the formulations was in the range of 5-5.3, indicating that the formulations were also suitable for applying to the skin (Table 4). The F2 coded ME had an appropriate pH (5.3) for applying to the skin has been chosen as the ideal formulation.

In the literature, ME based herbal formulations were prepared by using herbal medicines such as furocoumarin psoralen, curcumin, and triptolide for enhanced activity and reduced toxicity[25-27]. Although, no GA-loaded ME formulation was found in the literature. It is known that drug release can be altered by changing formulation parameters from MEs developed with different drugs[28,29]. In our study, it was also aimed to improve *in vitro* GA release by preparing ME formulation of GA. As expected, *in vitro* GA release was increased by loading GA into F2 and F5 coded MEs and F2 coded MEg (P <0.05). *In vitro* GA release of F5 MEg and GA solution was similar (P >0.05). At the end of 6 hours, GA release was found to be 43.2% from GA solution and the amount of GA released increased to 72% in F2 ME and up to 66.8% in F5 ME. On the other hand, the *in vitro* GA release was determined as 57.2 and 43.0% in F2 and F5 MEg, respectively. In MEs, *in vitro* GA release was greater. Due to the increase in viscosities and DSs of the MEg formulations led to decreased *in vitro* GA release according to ME (Fig. 2, Table 4). The reduction in the DS results in an increase in surface area and hence drug release.

For cytotoxicity studies, we used the MTT test, which is the most commonly used. The effects of different concentrations of GA solution and MEs on L929 cell viability were investigated for 6 hours. According to the MTT test results, GA caused no cellular toxicity with the used dose in preparing MEs. Additionally, F2 and F5 coded MEs and MEgs were also not found to be toxic to cells, cell viability was over 80%. These high viability rates have shown that MEs can be used safely.

Permeation studies were carried out for F2 and F5 MEs and MEgs through L929 cells. MEg formulations have limited the cell permeation of GA because of high viscosity and it was found to be only 15 and 11% for F2 and F5 MEg, respectively. The amounts of GA were lower at permeation study than release study. The reason for this is that it has been

interpreted as tight junction regions in the cell layer and in particular, a high viscosity in MEg formulations. In the literature, to the authors' knowledge, there is no data to compare our results, so this is the first study of MEs containing GA as a bioactive molecule.

pH, ZP, DS and viscosity of F2 ME did not change at 4 °C for 30 days. After stability studies, the pH of F2 ME was 5.22 and still between 5-5.3. ZP value decreased to -26.7 from -30.3 mV. The fact that the ZP was still close to -30 mV after stability studies proved that the F2 coded ME maintained its stability at 4 ºC for 30 days. Although DS of F2 ME increased to 1.6 from 1.04 µm after 30 days at 4 °C, it had an acceptable DS. As can be seen in viscosity results, viscosity changed in all formulations except F2 ME after stability studies. The viscosity at 50 rpm of F2 and F5 MEg was increased to 102 and 109 cP from 94 and 95 cP, respectively. While viscosity of F5 ME was increased to 77 from 38 cP (P <0.05), the increase in F2 ME was only to 84 from 78 cP at 50 rpm. The minimum change in the viscosity, which was not statistically significant, was observed in F2 ME (P >0.05). So all the stability studies showed that F2 coded ME was stable at 4 °C for 30 days.

Removal of harmful and pathogenic radicals from the body is very important. For this purpose, in the measurement of the antiradical effects of the samples, nitrogen-based stable radical, $DPPH^{\bullet}$ radical and a blue/green coloured $ABTS^{+\bullet}$ radical cation were used. As a result of studies conducted with the release sample, the cleansing effect of GA on the physiological pH of DPPH radicals was higher than 50% (Table 5). The TEAC analysis is based on the inhibition of ABTS radical cation absorbance by antioxidants. As shown in Fig. 6, the released GA exhibited strong antioxidant activity by almost bleaching the entire radical. It was concluded that the prolonged potent antiradical activity could be sustained for 6 hours.

Conclusion

As a result, it has been observed that novel MEs were successfully developed and the antioxidant effect of GA-loaded MEs has been found to be prolonged for 6 hours. Also, these formulations were proposed for the field of cosmetics and topical application based on the strong antioxidant properties of GA.

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