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Methyl thiazolyl tetrazolium cytotoxicity analysis and evaluation of acyclovir-based organogel lipstick; an in-vitro and experimental study

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Abstract

Introduction: Acyclovir (ACV) is an antiviral medication primarily used to treat herpes simplex virus (HSV) infections. The methyl thiazolyl tetrazolium (MTT) assay is a reliable quantitative method for assessing cell viability.
Objectives: The study aimed to develop and evaluate the cytotoxic effects of an ACV-based organo-gel lipstick.
Materials and Methods: In this in-vitro and experimental study, formulations of an ACV-based organo-gel lipstick were created using varying amounts of 12-hydroxystearic acid (12HSA), beeswax, vitamin E, and oleic acid, resulting in a total of 34 formulations, of which 20 successfully gelled into lipsticks. To evaluate these formulations, a series of tests were conducted, including tabletop rheology to assess flow properties, pH measurement to ensure skin compatibility, spreadability testing to determine ease of application, and melting and breaking point analysis to evaluate thermal stability. Additionally, optical microscopy was employed to observe microstructural characteristics, in vitro release studies measured drug release profiles, skin irritation tests assessed biocompatibility using mice, permeation studies evaluated drug absorption through skin models, oscillatory rheology analyzed viscoelastic properties, and MTT cytotoxicity assays assessed cell viability in the presence of ACV.
Results: The results revealed diverse characteristics among the formulations, with gel-sol temperatures exceeding 34 °C and pH values ranging from 4 to 7, indicating their stability for topical applications. Spreadability testing showed poor performance for formulations F1 and F5, while other formulations demonstrated adequate spreadability. Notably, formulations F24 and F34 exhibited melting points above 50 °C, reflecting good thermal stability and the breaking point was highest in beeswax lipsticks across both solvents tested. Microscopic analysis indicated that oleic acid-based organogels formed spherulites, whereas vitamin E formulations displayed fibrillar networks. ACV release was significantly higher in beeswax formulations with oleic acid compared to those containing vitamin E in 12HSA organogels. Skin irritation tests on mice showed no signs of irritation, with formulation F34 demonstrating higher permeation through external lower and inner mucus bovine lips than formulation F21. Oscillatory rheology tests indicated that selected organogels exhibited strength, showing comparable storage modulus (G′) values, linear viscoelastic region (LVER), flow points, and frequency independence, except formulation F21. Additionally, the MTT assay results indicated that the F34 lipstick formulation significantly increased rhabdomyosarcoma cell viability against the HSV.
Conclusion: In conclusion, formulation F34, utilizing gelators in oleic acid, has shown significant promise as an effective delivery system for ACV. Its high melting point and excellent elastic properties further support its suitability for use in a lipstick-based organo-gel format. Additionally, the formulation's ability to enhance the inhibition of viral toxicity to rhabdomyosarcoma cells underscores its potential therapeutic efficacy against HSV, distinguishing it from other formulations that hinder ACV release.

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Introduction

Herpes simplex labialis, commonly known as oral herpes, is a prevalent viral infection primarily caused by the herpes simplex virus type 1 (HSV-1), although HSV type 2 can also infrequently contribute to this condition. This infection manifests as painful blisters or sores on the lips and surrounding areas, often referred to as cold sores, and typically follows an initial asymptomatic phase during childhood, with the virus remaining dormant in the trigeminal ganglion thereafter (1). After the initial infection with HSV-1, the virus replicates at the infection site before traveling along unmyelinated sensory fibers to the trigeminal ganglion, where it establishes a latent infection (2). These viruses are treated by acyclovir (ACV), an antiviral agent belonging to the Biopharmaceutical Classification System (BCS) class III (3,4). The major limitations associated with oral intake of this medication include low bioavailability (10%–20%) and a short half-life of approximately 3 hours, encouraging

Key point

The results of this study highlight several key points regarding the development and evaluation of acyclovir (ACV)-based organogel lipsticks. The formulations demonstrated stability for topical applications, with gel-sol temperatures exceeding 34 °C and pH values ranging from 4 to 7. While some formulations, specifically F1 and F5, exhibited poor spreadability, others performed adequately. Notably, formulations F24 and F34 showed high melting points above 50°C, indicating good thermal stability and the breaking point was highest in beeswax lipsticks. Microscopic analysis revealed distinct microstructural characteristics, with oleic acid-based organogels forming spherulites and vitamin E formulations displaying fibrillar networks. The release of ACV was significantly enhanced in beeswax formulations with oleic acid compared to those containing vitamin E in 12-hydroxystearic acid (12HSA). Importantly, skin irritation tests indicated no adverse effects and formulation F34 demonstrated superior permeation through biological membranes. The oscillatory rheology tests confirmed the strength of selected organogels, while the methyl thiazolyl tetrazolium (MTT) assay showed that F34 significantly increased rhabdomyosarcoma cell viability against the herpes simplex virus. These findings suggest that formulation F34 is a promising candidate for a topical ACV delivery system, offering enhanced drug release and antiviral efficacy while maintaining safety for potential clinical applications.

its frequent administration (5,6). Thus, many studies formulated ACV for topical applications; for instance, Kamal et al prepared ACV a topical cream formulated of propylene glycol, poloxamer, and sodium lauryl sulfate with mineral oils (7).

Organogels are semisolid bicontinuous systems formed by the combination of gelators and an apolar solvent, which becomes immobilized within the interstitial spaces of a three-dimensional network structure (8). These properties make organogels suitable for topical preparations, as they can effectively deliver active ingredients while ensuring stability and usability in various gel formulations (9).

Given the favorable characteristics of topical application for ACV, the primary objective of this research was to develop an ACV-based organogel lipstick formulation that offers multiple advantages, including targeted delivery to the site of action. The design of the lipstick facilitates self-application to the affected area, enhancing ease of use. To our knowledge, this study is the first to formulate ACV as a lipstick using organogel technology derived from beeswax and 12-hydroxystearic acid (12HSA) in various oily solvents, including vitamin E and oleic acid. Previous research has focused on ACV formulations such as niosomes made with cholesterol and surfactants like Span 60 and 80 (10), but none have explored its incorporation into an organogel-based lipstick formulation.

Objectives

The objective of this study is to develop and evaluate an ACV-based organogel lipstick, focusing on its cytotoxic effects as assessed by the MTT assay. Specifically, the study aimed to formulate various organogel combinations using 12HSA, beeswax, vitamin E, and oleic acid to optimize the delivery system for ACV, enhancing its bioavailability and therapeutic efficacy against HSV infections. Through a series of physicochemical evaluations and biological assays, the research sought to establish the organogel's stability, release profile, skin compatibility, and antiviral effectiveness, ultimately contributing to improved topical treatment options for HSV.

Materials and Methods

Formulation of organogels

Every organogel formulation underwent two manufacturing steps. The first step was the organogel preparation, followed by pouring the liquid organogel preparation into the lipstick's mold, representing the second step in organogel formulation. The beeswax and 12HSA in isolation or both in combination were melted in these concentrations (5, 8, 10, 12, and 15) w/w % in 2 solvents—the vitamin E and oleic acid. The oily solvents were added to bring the volume to one gram in vials to be subjected to 70 °C while being in the water bath. After the organogelator's melting; 50 mg of ACV was physically distributed throughout the oily mixture while being magnetically stirred at 100 rpm for ten minutes (11,12). The medicated organogel step preparation was followed by pouring the preparations into the lipstick mold. The indication of organogel formulation was after cooling for a specific period at room temperature as all vials were flipped, monitoring for the solidifying of the content, signing organogel when there was no flow of content, and the opposite indicated no organogel formulation (Table 1).

Tabletop rheology (transitions temperature)

Samples were immersed in a water bath heated from 34 °C to 70 °C, with a 15-minute hold at each 2 °C increment, to determine the sol-gel and gel-sol phase transition temperatures. At each predetermined temperature, samples were removed from the water bath and tilted for 30 seconds to assess whether the formulations exhibited "flowing" or "not flowing" behavior. This evaluation was conducted for both organogels containing ACV and those without (13).

PH determination

The pH of selected ACV organogels was measured using a pH meter, which probed the melted organogel to ensure accurate readings. The measurements were conducted while monitoring the pH levels until equilibrium was established, allowing for a thorough assessment of the formulations' acidity or alkalinity (14).

Spreadability test

At an angle of 45°, applying the lipstick organogel to a clear glass slide at room temperature (25.0 ± 3.0 °C) by hand as observations visually against a dark background to assess the consistency of the produced layer on the slide and determine whether the organogel stick broke, fragmented,

Table 1. The formulations number and their concentration

Formulation number	12HSA (G)	BW (G)	Vit E (G)	OA (G)
F1	0.05		0.9	
F2	0.08		0.87	
F3	0.10		0.85	
F4	0.12		0.83	
F5	0.15		0.8	
F6	0.05			0.9
F7	0.08			0.87
F8	0.10			0.85
F9	0.12			0.83
F10	0.15			0.8
F11		0.05	0.9	
F12		0.08	0.87	
F13		0.10	0.85	
F14		0.12	0.83	
F15		0.15	0.8	
F16		0.05		0.9
F17		0.08		0.87
F18		0.10		0.85
F19		0.12		0.83
F20		0.15		0.8
F21		0.3	0.65	
F22		0.4	0.55	
F23		0.4		0.55
F24		0.8		0.15
F25	0.05	0.05	0.85	
F26	0.08	0.08	0.79	
F27	0.10	0.10	0.75	
F28	0.12	0.12	0.71	
F29	0.15	0.15	0.65	
F30	0.05	0.05		0.85
F31	0.08	0.08		0.79
F32	0.10	0.10		0.75
F33	0.12	0.12		0.71
F34	0.15	0.15		0.65

BW, Beeswax; 12HSA, 12-hydroxystearic acid; Vit E, Vitamin E; OA, Oleic acid.

or distorted during application. Accordingly, the features below categorized the lipstick products into three groups:

- G – Good: Consistent, did not leave shards; flawless application, preventing the lipstick from deforming.
- I – Intermediate: Consistent, left few shards, applied correctly, and the lipstick deformed very little.
- B – Bad: Severe deformation of the lipstick, improper, incorrect application, left many pieces, and was not uniform (15).

Melting point test

The melting point of the prepared lipstick was evaluated using the capillary tube method, wherein a capillary tube was filled with the sample and placed in a capillary apparatus for observation. This setup allowed for continuous monitoring of the melting process, providing precise data on the temperature at which the lipstick transitioned from solid to liquid (16).

Breaking point test

The lipstick bar was pushed out one inch from its house and held horizontally by the holder and stand. Then, 5 grams of two magnetic stirrers were put over the pushed organogels. The weight was increased by a specified amount (10 g) over 30 seconds, and the breaking weight was deemed the breaking point (17).

Optical microscopy

An optical microscope was used to investigate the morphology of organogels by preparing slides and transferring a drop of melted organogel to a 25×75×1 mm glass slide. This was followed by covering the hot melted organogels with coverslips. After cooling at room temperature, the slides were investigated on the microscope stage to take pictures using the digital microscope camera MC500, utilizing ×40 objectives and the program MicroCapture assisting in image capturing (18).

In vitro drug release studies

A modified Franz diffusion cell was employed, utilizing a glass cup with a 2 cm^2 cross-sectional area, and 1 g of the ACV organogels were added. The cellulose membrane then covered the cup to seal it with a rubber band around the cellulose membrane. After cup inversion, the membrane was leveled by 0.5 mm underneath the surface of 50 mL of pH 7.4 with (1% w/w Tween 80) phosphate buffer as the temperature was set to 34 °C (19, 20). A 3 mL sample was taken out at predetermined intervals of 1, 2, 3, 4, 5, 6, 7, and 8 hours, and they were replaced with the same amount (21). A control was run parallel to the tested organogels using 50 mg of ACV that had been separately made in oleic acid and vitamin E. ultraviolet-visible spectrophotometry at a selected wavelength of 253 nm was used to measure the concentration of ACV in the samples against a properly established calibration curve equation ($y = 0.0255x+0.0283$) and R^2 equals 0.9945 (20). In addition, the marketed 5% w/w ACV cream was subjected to the *in vitro* release study for comparison purposes. The release study was conducted in a triplicate.

Skin irritation test

One female Laca mouse per group (15–20 g) was placed into eight groups. An animal hair clipper measuring 0.1 mm was used to remove the animal's dorsal hair. A cotton swab soaked in saline was used to wipe the skin three or four times. Groups 1, 2, 3, 4, 5, and 6 were given selected organogels, whereas group 7 received treatment with formalin alone, and group 8 was used as a control. Every animal had its cage and was kept at a constant temperature (22-24 °C) and light-controlled environment on an alternate 12:12 hours light-dark cycle, with full access to food and water. After a week of application, the formulation kept on the skin was carefully removed and swabbed with cotton soaked in saline. The mice's shaved

skin was observed for redness, edema, and erythema following the topical application of the formulation.

Ex vivo ACV permeation study

A Franz diffusion cell that was precisely the same size as the one used in the *in vitro* release investigation was utilized to assess *ex vivo* penetration on both the bovine lip membrane extended to the inner mucus membrane as one piece (INLIP) and the external lower part of lips (EXLIP) as these tissues were obtained from local slaughter. Since all existing tissues were immersed in buffer pH 7.4 and then incubated in a water bath at a temperature of 37 ºC to be subjected periodically to read at 253 nm absorbance in ultraviolet spectrophotometry until zero absorbance was reached. The receptor compartment was filled with phosphate buffer solution pH 7.4 (1% w/w Tween 80), and the temperature was maintained at 34 ± 0.5 ºC. The excised tissues were placed over the recipient compartment (2 cm^2) as the dermis was in contact with the receptor medium. A 3 mL fresh phosphate buffer solution was added to the receptor medium as a 3 mL was removed at predetermined intervals of 1, 2, 4, 6, 8, 12, and 24 hours to maintain the sink state.

Oscillatory rheology studies

A plate-plate (PP 25 SN 61895) related to Anton Paar MCR-302 rheometer was used to study the oscillatory rheology on a selection of ACV organogels. Every measurement was done twice at 34 °C, and the data extraction process was done using Rheoplus software. A sufficient scoop of the chosen organogel was placed between the two plates for the amplitude and frequency sweep experiments (PP 25/ SN 61895).

Amplitude sweep

The angular frequency was constant at 10 rad s^{-1} while the oscillatory strain range was adjusted from 0% to 100%.

Frequency sweep

The second oscillatory test was the frequency sweep; based on the LVER data derived from the amplitude sweep test for each formulation, the strain applied was between 0.01% and 0.075%, and, at the same time, the angular frequency range was 0.1 to 100 rad s^{-1}.

MTT cytotoxicity test

Maintenance of cell cultures

Rhabdomyosarcoma cells (RDs) were large, spindle-shaped, multinucleated and utilized for this test maintained in RPMI-164, 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin. Then, the cells in Trypsin-EDTA were reseeded at 80% confluence twice a week and incubated at 37 °C.

Cytotoxicity assays

To determine the cytotoxic effect of HSV, the MTT test application aids in identifying the MTT reduction to the purple formazan product and its proportion related to the cell viability by the impact of mitochondrial dehydrogenase in 96-well plates (22).

Cells were seeded at 1×10^4 cells/well, and after 48 hours, the confluent monolayer was achieved in a 96-well plate to be treated with HSV at a specific multiplicity of infection M.O.I (cell: virus) 1: 2, and 1:10 in the presence of 50 µg/mL of ACV organogel and control samples (blank formulation without ACV addition). Cell viability was measured after 24 hours of virus treatment by removing the medium, adding 100 µL of 2 mg/mL of MTT, and incubating the cells for 2.5 hours at 37 °C. After observing the appearance of the purple formazan product, the MTT solution was removed. Then, the crystals remaining in the wells were solubilized by adding 130 µL of DMSO followed by incubation at 37 °C with shaking for 15 minutes (23,24). The absorbance of solubilized formazan was determined on a microplate reader at 492 nm. This assay was performed in triplicate. Cell growth inhibition rate (the percentage of cytotoxicity) was calculated using the following equation (25).

$$Inhibition\ rate = A - \frac{B}{A} * 100$$

Where A is the optical density of the control, and B is the optical density of the samples (25).

Statistical analysis

The obtained data were statically analyzed using an unpaired T-test with GraphPad Prism 6. The values were presented as triplicate measurements' mean ± standard deviation (SD).

Results

The organogel formulation was developed according to solidifying the formulation content and was assigned as inverted vials. Any contents that showed fluidity were not assigned as organogels.

The formulations (F1 to F5) containing vitamin E formed gels, while F6 and F7 with oleic acid did not create organogels; therefore, the 8% w/w concentration represented the lowest amount of 12HSA capable of holding oleic acid. Interestingly, the formulations from F11 to F20 that represent the beeswax in both solvents vitamin E and oleic acid were entirely not gelled in all prepared concentrations. This necessitated increasing the concentration of beeswax to 30% and 40% w/w in vitamin E, and to 40% and 80% w/w in oleic acid, resulting in the gelation of all four organogels. Additionally, it was found the combination of beeswax and 12HSA in equal proportions of the 5, 8, 10, 12, and 15% w/w in both solvents vitamin E and oleic acid showed the same gelation results as 12HSA alone since the combined 5% w/w of beeswax and 12HSA in oleic acid did not gel. Moreover, the gelation time, as presented in Table 2, was

in the opposite relationship with the gelator concentration, and it was noted that the gelator combination shortened the gelation time. Moreover, Table 2 signifies that all successful formulations, such as organogels, effectively formed lipstick based on organogels. For the next steps in our study, the selected organogel formulations - F1, F5, F7, F10, F21, F22, F23, F24, F25, F29, F31, and F34- represent the lowest and the highest concentrations for each 12HSA and beeswax in both solvents in addition to the combined gelators (Table 2).

Each formulation exhibited distinct gel-sol and sol-gel transition temperatures when tested with and without ACV, indicating their stability and performance in different conditions. Additionally, the melting points varied among the formulations, reflecting their structural integrity and suitability for topical applications. The breaking points also differed significantly, showcasing the mechanical strength of each formulation. The evaluation of the selected organogel formulations demonstrated varying thermal properties and mechanical strengths across different conditions. For the gel-sol transition temperatures with ACV, formulations F1, F5, F7, F10, F21, F22, F23, F24, F25, F29, F31, and F34 exhibited temperatures ranging from 38 °C to 54 °C. In contrast, the gel-sol transition temperatures without ACV were similar for most formulations, with values between 38 °C and 54 °C. The sol-gel transition temperatures with ACV varied from 27 °C to 43 °C, while those without ACV ranged from 26 °C to 41 °C. The melting points of the formulations were also notable, with values spanning from 51 °C to 64 °C. Additionally, the breaking points varied significantly among the formulations, with some exhibiting strengths as low as 10 grams (F1, F5, F7, F10) and others reaching up to 60 g (F24) (Table 3).

Table 2. Formulation number, organogel formulation with lipstick, and gelation duration

Formula number	Organogel formulation	Lipstick	Gelation time (min)
F1	Yes	Yes	12
F2	Yes	Yes	9
F3	Yes	Yes	5
F4	Yes	Yes	5
F5	Yes	Yes	5
F6	No	No	No gel
F7	Yes	Yes	15
F8	Yes	Yes	12
F9	Yes	Yes	6
F10	Yes	Yes	4
F11	No	No	No gel
F12	No	No	No gel
F13	No	No	No gel
F14	No	No	No gel
F15	No	No	No gel
F16	No	No	No gel
F17	No	No	No gel
F18	No	No	No gel
F19	No	No	No gel
F20	No	No	No gel
F21	Yes	Yes	6
F22	Yes	Yes	3
F23	Yes	Yes	3
F24	Yes	Yes	3
F25	Yes	Yes	7
F26	Yes	Yes	5
F27	Yes	Yes	5
F28	Yes	Yes	5
F29	Yes	Yes	2
F30	No	No	No gel
F31	Yes	Yes	8
F32	Yes	Yes	5
F33	Yes	Yes	3
F34	Yes	Yes	3

Optical microscopy

Optical microscopy aimed to investigate the cross-sectional morphology of the organogel scaffold. Results illustrated that each organogel had an aggregate pattern. The images of F1, F5, F21, F22, F23, F24, F25, and F29 formulations showed a fibrillar network, whilst F7, F10, F31, and F34 images showed a spherulite scaffold. It was noted that most organogels prepared in oleic acid were spherulites, while the ones in vitamin E showed fibrillar networks, except F23 and F24 were spherulites in their images, as we think the oleic acid was in low amounts (Figure 1).

In vitro drug release studies

This study was carried out for 8 hours in phosphate buffer with a pH of 7.4 to assess the depot feature of organogels and the amount of ACV released from the generated organogels. In addition to the organogel's release study, a control experiment was carried out, as shown in Figure 2. All prepared organogels were compared with the controls in both solvents, and the ACV release was slowed. Moreover, it was apparent that the ACV/vitamin E control release was prolonged and gradual compared with ACV/oleic acid control, which had a very rapid release, liberating the 50 mg of ACV in one hour. This study categorized the organogels into three groups. The first group as shown in Figure 2A included organogels made from 12HSA, vitamin E, and oleic acid, which slowed ACV release. Specifically, formulations F1 and F5 exhibited a gradual release profile. In contrast, the control formula containing ACV in oleic acid showed rapid release. Similarly, organogels made from 12HSA and oleic acid (F7 and F10) also demonstrated a slower ACV release. It was noted that the impact of vitamin E on ACV slowing release was higher than that of the 12HSA gelator. Surprisingly, the low concentration of 12HSA/vitamin E organogels slowed the ACV release than the higher concentration organogels of 12HSA/oleic acid. The second group of beeswax gelators in both solvents, as represented in Figure 2B, showed opposite outcomes to the first as F21, F22 (beeswax/vitamin E) released 40 mg within 8 hours, and F23, F24 (beeswax/oleic acid) released

Table 3. Temperatures at which the phase transitions from gel to sol and vice versa, melting point and breaking points of the selected formulas

Formula number	(Gel-Sol)°C with ACV	(Gel-Sol)°C without ACV	(Sol-Gel)°C with ACV	(Sol-Gel)°C without ACV	Melting Point °C	Breaking Point (G)
F1	38	38	30	29	51	10
F5	45	45	41	41	54	20
F7	39	38	30	27	56	10
F10	44	44	32	33	60	10
F21	39	40	31	32	61	40
F22	40	40	35	32	60	50
F23	38	38	32	29	51	40
F24	44	46	43	41	60	60
F25	44	48	29	26	55	20
F29	46	50	35	40	57	30
F31	39	40	27	26	60	20
F34	41	40	32	28	64	30

around 38 mg within the 8 hours of the release study. The third group represented the organogels of mixed gelators, as shown in Figure 2C. It was found the F31 and F34 (12HSA+beeswax/oleic acid) released around 46 mg and 48 mg of ACV respectively within 8 hours, while the mixed gelators in vitamin E prolonged the release as F29 liberated 34 mg of ACV within 8 hours, and no ACV was released within 8 hours from F25. This difference could be justified because F25 was composed of mixed gelators, but the vitamin E amount was still higher than F29. Figure 2D showed that the marketed 5% ACV cream release was close to F34, representing the mixed gelators in oleic acid.

Skin irritation test

The skin irritation test was conducted to evaluate the safety of repeated topical applications of ACV organogels, focusing on potential skin irritation and assessing the formulation's dermal tolerability. Selected organogels (F1, F10, F21, F23, F29, and F34) based on the chosen organogel of each group depended on the included ingredients as they were subjected to skin irritation test; the outcomes were compared visually to formalin, the irritant in this study. Erythema and edema were monitored for skin irritation after seven days of application, and these changes were not found in all mice in this study. Conversely, formalin resulted in significant edema and erythema, improving that the selected organogel formulations are non-irritant.

Ex vivo ACV permeation study

The selected formulations (F7, F10, F21, F31, and F34), the organogels lipstick, showed the best ACV release, guaranteeing the earliest ACV reaching the site of action. In ex vivo permeation, the study examined the penetration of the selected formulations of ACV organogels through

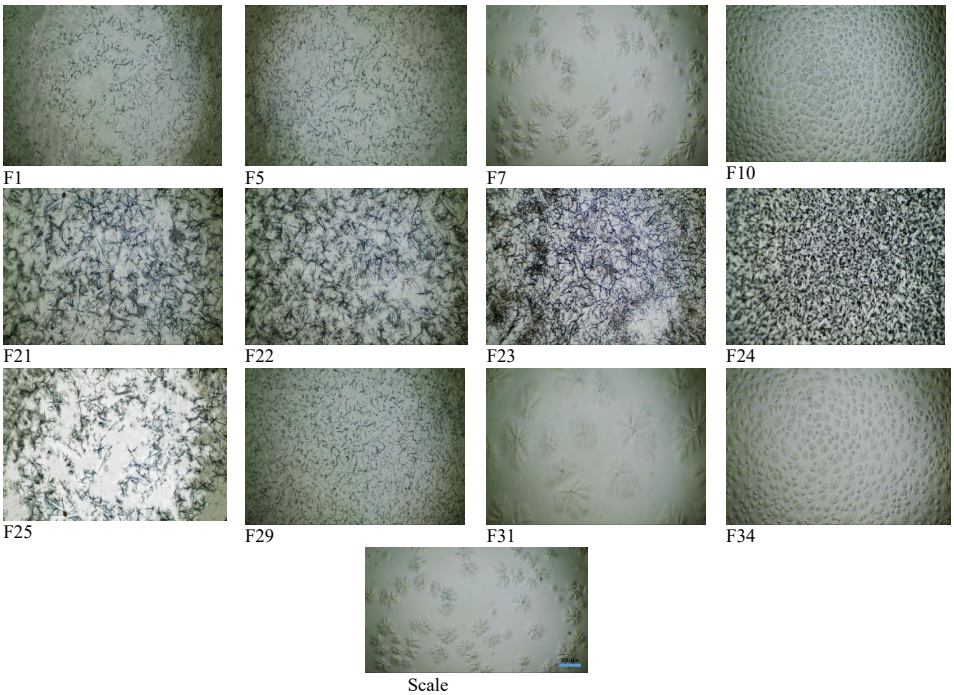

Figure 1. Optical microscopy image of ACV organogel at ×40 magnification and scaled against 200 µm.

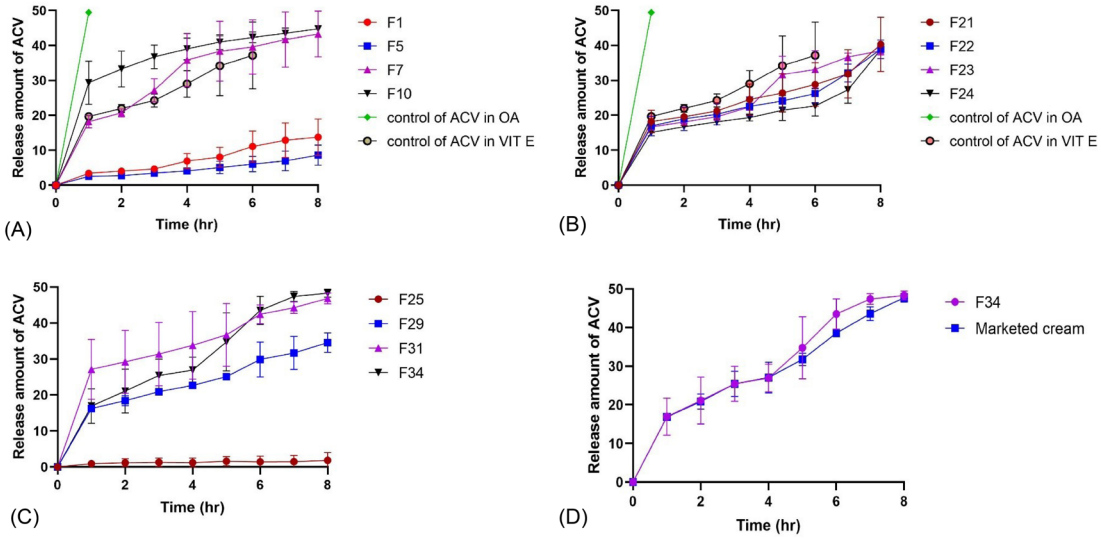

Figure 2. In-vitro ACV organogel release in pH 7.4 phosphate buffer solution (containing 1% w/w Tween 80) at 34 °C as each release curve is an average of triplicate ± standard deviation.

INLIP and EXLIP, as presented in Figure 3A and 3B, respectively. It was generally noticed that the highest penetration amount of ACV organogel was from F34, while the lowest amount was from F21. The F34 contained both gelators, which led to a higher ACV penetration amount, assumed to be the potent mixture that enhanced the permeation. This permeation could be attributed to the presence of oleic acid, the organic solvent in F34, F31, F7, and F10 that acted as a penetration enhancer (Figure 3).

Amplitude sweep

In amplitude study, this test featured robustness and was applied to the selected organogels (F7, F10, F21, F31, and F34) by identifying the G' (storage modulus), G" (Loose modulus), LVER (linear viscoelastic region) and the flow point. The strength of organogel characterizes the elasticity, and the solid scaffold content was represented by G'. Whilst the fluidity and broken organogel were indicated by G". Also, the elasticity can be further assessed by the LVER that gives an idea about the confrontation of the organogel to the increased applied strain by showing almost constant values of G' that after specific strain value, these G' values approach a continuous decrease as well in the same time an increased in the G" values until they meet at the same strain value representing the flow point and the whole destruction of the organogel's scaffold. The scooped organogel was subjected to increasing strain, from 0% to 100%.

Figure 4 demonstrates that all selected organogels showed parallel G' and G" curves, and G' values in LVER were one order greater than G". This feature demonstrates organogel formation, Regarding flow point, F21 had the highest value compared to the other organogels, whereas F31 had the lowest; however, this high value of flow point in F21 did not relate to the strength or elasticity as the G' curve was declining, indicating a decreasing in G' values as this formulation showed the lowest G' values compared with other selected organogels. To clarify the flow point, it represents where the organogel lost its elasticity and

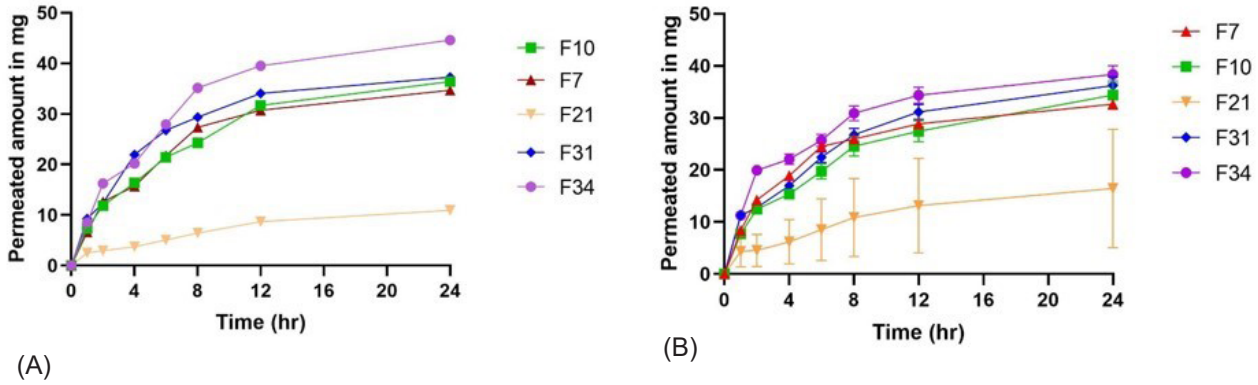

Figure 3. The permeation of ACV organogels of F7, F10, F21, F31, and F34 through (A) EXLIP and (B) INLIP is an average of triplicate ± standard deviation.

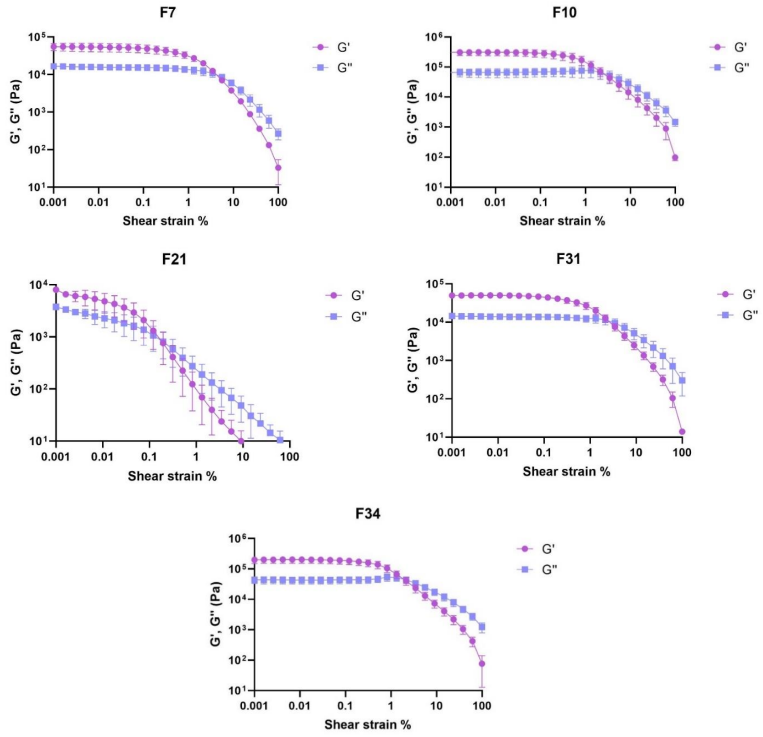

Figure 4. The amplitude sweep test for organogels of F7, F10, F21, F31, and F34 as the strain was set from 0 to 100%, with a temperature of 37°C with an angular frequency of 10 rad s^{-1}.

shifted to the liquid phase, possibly due to the complete dissociation of hydrogen bonds between fibers that build the three-dimensional scaffold. Overall, the selected organogels demonstrated sufficient strength regarding G', G", LVER, and flow points except F21 (Figure 4).

Frequency sweep

The frequency sweep study examined how organogels retained their solid shape in a 3-dimensional scaffold when motion was at different speeds and parallel curves of G' and G" did not intersect. Figure 5 illustrates that the G' curves were higher and parallel to the G" curves of all organogels, without crossing at any frequency rate except for F21; the beeswax-based organogels, the G' and G" curves were crossed, indicating very weak organogel. The selected organogels were frequency independent except the F21, as this harmonized with the amplitude sweep outcomes. Beeswax formulations (F21) showed a higher breaking point than other selected formulations. At the same time, in oscillatory rheology, its attitude was not elastic as this could be attributed to the type of motion since the breaking point study applied direct stress without any motion, opposite to the oscillatory studies of frequency and amplitude sweeps (Figure 5).

The analysis was conducted under controlled conditions with parameters ranging from 0% to 100%, utilizing an angular frequency of 10 rad/s at 37 °C. The formulations examined include F7, F10, F21, F31, and F34, each exhibiting distinct characteristics in terms of their mechanical properties and flow behavior. For instance, F7 demonstrates a relatively moderate storage modulus and flow point, while F10 shows a significantly higher storage modulus compared to the others. Conversely, F21 presents the lowest values among the formulations for both storage modulus and loss volume energy ratio. F31 and F34 also display varying degrees of mechanical strength and flow points, indicating a diverse range of properties across the organogel samples analyzed (Table 4).

MTT cytotoxicity test

This test was carried out on the proliferation of RD (rhabdomyosarcoma) cells to investigate the ACV lipstick's (F 34 was selected) antiviral activity. The bars D and G represent the ACV organogel impact on RD cells and showed a highly significant ($P \leq 0.001$) inhibition to the virus cytotoxicity in RD cells in comparison with blank organogels the negative (-ve) control (bars C and F) as well as the positive (+ve) control (bars B and E). Also, the impact of the high virus proportion on the cell is clear, as seen in the purple bars, which show a decrease in the inhibition rate of the virus cytotoxicity (Figure 6).

RD cell viability was observed using an optical microscope, as shown in Figure 7A; however, the white batches were found Figure 7B, showing the virus cytotoxicity as this highly decreased in Figure 7D, clarifying the inhibition effect of ACV to the virus cytotoxicity (Figure 7).

Discussion

For thermosensitive organogels, tabletop rheology is a process that explains the phase transitions from sol to gel

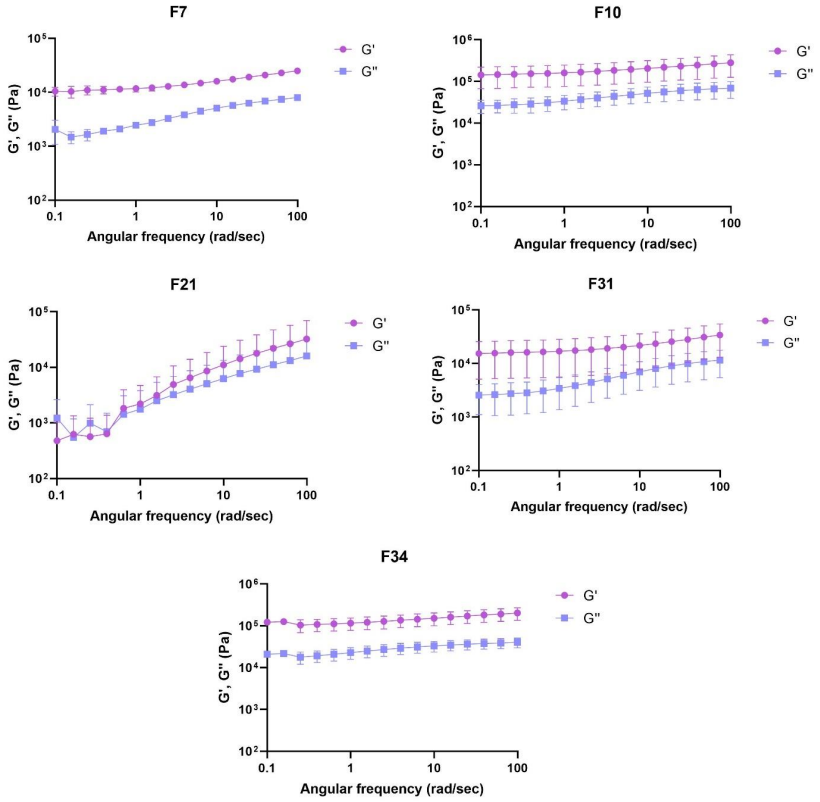

Figure 5. Frequency sweep test represents organogel of F7, F10, F21, F31, and F34. The angular frequency was set from 0 to 100 rad s^{-1}, and the temperature was 37 °C

(T sol-gel) and from gel to sol (T gel-sol). This test was applied to these formulations (F1, F5, F7, F10, F21, F22, F23, F24, F25, F29, F31, and F34) that showed gelation and to the organogels that were prepared without ACV. This was to investigate the impact of ACV addition on the thermal stability of the prepared successful organogels. Table 3 illustrates that the gel-sol transition temperatures were higher than the sol-gel temperatures; the results were similar to the previous study utilizing 12HSA with triacetin as solvent (26). In addition, all gel-sol temperatures were notably higher than 34 °C, ensuring its solidity and coherence at the application site. Furthermore, it was noticed that the high organogel concentration generally showed higher transition temperatures; however, the combined gelator organogel's transition temperatures gel-sol were almost the same as the 12HSA gelator alone. As seen in the earlier study that used diosgenin-based organogel, as the concentration of organogel's gelator increased from 1% to 6% w/w, the transition temperature also increased (27). Finally, adding ACV to the organogels did not remarkably change the thermal transitions.

The pH measurements were done since the lipstick-based organogel was applied topically to the skin, lip tissues, or surfaces, and an improper pH could irritate them (28). The results demonstrated that pH values of ACV organogels ranged from 4 to 7, the same range in previous lipstick and lip cream studies, indicating no danger of irritation (29,30).

The spreadability test showed the dispersion or spreading of lipstick preparation topically after application, which adds to the therapeutic efficacy. Furthermore, this is a crucial factor in the lipstick consistency (31). The results indicated that F1 and F5 had bad spreadability results, while F7, F10, F25, F29, and F31 had intermediate spreadability. However, F21, F22, F23, F24, and F34 showed good spreadability results, this result is in line with a study

Table 4. Amplitude sweep parameters (G', G'', LVER, and flow points) for organogels, with an average and their standard deviation

Formula number	G' (pa) ±SD	G'' (pa) ±SD	LVER(%) ±SD	Flow point (%) ±SD
F7	46963	15006	0.053 ± 0.061	5.135 ± 0.997
F10	261920	54145	0.071 ± 0.017	2.955 ± 0.827
F21	8513.7	3439.8	0.0021 ± 0.021	5.595 ± 2.128
F31	53297	14603	0.072 ± 0.041	4.705 ± 0.417
F34	243490	53294	0.070 ± 0.034	1.935 ± 0.643

The study's parameters ranged from 0% to 100%, angular frequency of 10 rad.s^{-1}, and temperature 37°C.

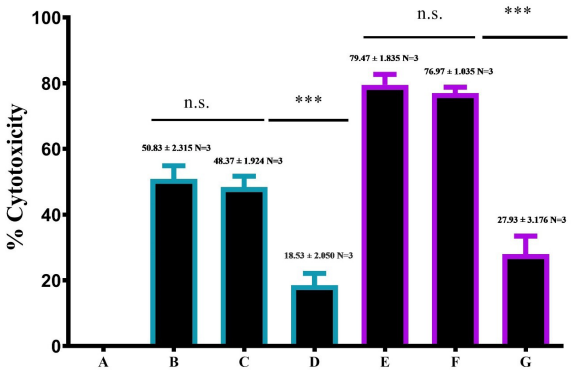

Figure 6. Cytotoxicity effect of HS virus in RD cells. A, uninfected RD cells. B, RD cells infected with HSV (M.O.I. 1:2). C, RD cells infected with HSV (M.O.I. 1:2) + (-ve Control). D, RD cells infected with HSV (M.O.I. 1:2) + (ACV organogel). E, RD cells infected with HSV (M.O.I. 1:10). F, RD cells infected with HSV (M.O.I. 1:10) + (-ve Control). G, RD cells infected with HSV (M.O.I. 1:10) + (ACV organogel). Data are represented as mean ±SD. n.s (non-significant). *** $P \leq 0.001$.

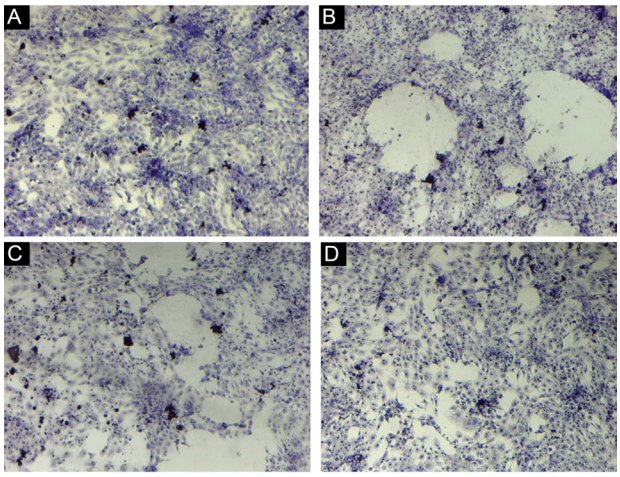

Figure 7. Microscopic images in which A represents uninfected RD cells, B represents RD cells after being infected with HSV, whilst C shows the RD cells after being infected with HSV with blank formulation, which is the negative control. The last image, D shows RD cells after being infected with HSV and treated with the ACV organogel

by Prajapati *et al* that prepared ketoconazole lipstick that showed good spreadability composed of beeswax and carnauba wax (32).

The melting point test showed that the limit of safe storage is crucial, and a flawless lipstick must be solid enough to resist pressure when applied and have standard melting point values between 50-70 °C (33, 34). In the present study, all lipstick organogels were within the range, as presented in Table 3, over 50 °C, and F24 and F34 showed the highest melting points, as the gelator amount could justify this. The tabletop rheology test and melting point test showed the same concept; the increase in the concentration of organogel led to the rise in the melting point and tabletop rheology transition temperature value. The breaking point test determines the strength and hardness of prepared lipstick, as presented in Table 3, and the highest values were interestingly found in the lipsticks prepared from the beeswax in both solvents. Nareswari et al stated that there are no definite values or range for a breaking point, and their coconut sunscreen lipstick's breaking point was 64, close to our formulation based on beeswax, the F24 (34).

In this study, the release was slow because beeswax gelators concentration increased, and the vitamin organogel release of ACV was higher than the ones with the oleic acid; however, there was no vast difference in ACV release amongst this group of organogels. In a study, beeswax was formulated in capryol 90 oil as a hollow suppository for vaginal delivery and presented the same trend, as higher concentration preparation retarded the lawsone release (35).

The results were found the F31 and F34 (12HSA+beeswax/oleic acid) released around 46 mg and 48 mg of ACV respectively within 8 hours, while the mixed gelators in vitamin E prolonged the release as F29 liberated 34 mg of ACV within 8 hours, and no ACV was released within 8 hours from F25. This difference could be justified because F25 was composed of mixed gelators, but the vitamin E amount was still higher than F29. Furthermore, results showed that the marketed 5% ACV cream release was close to F34, representing the mixed gelators in oleic acid. The existence of vitamin E within the gel, which might function as a diffusion barrier, is probably the cause of the longer release durations for medicine. The path molecules travel to diffuse from inside the gel to the fluid reservoir was longer due to this barrier (36).

It was generally noticed that the highest penetration amount of ACV organogel was from F34, while the lowest amount was from F21. The F34 contained both gelators, which led to a higher ACV penetration amount, assumed to be the potent mixture that enhanced the permeation. This permeation could be attributed to the presence of oleic acid, the organic solvent in F34, F31, F7, and F10 that acted as a penetration enhancer. Oleic acid diminishes the skin barrier's function, creates a transient and reversible disturbance of the corneum layer, and promotes the fluidization of the intercellular lipid medium (37). A previous study that used oleic acid with other surfactants and cosurfactants showed oleic acid was beneficial to employ as an oily phase because it increased skin permeability (38). Th F21 exhibited the most minor penetration level, and we think the presence of vitamin E acts as a barrier to penetration.

Rheology is fundamentally dependent on both amplitude and frequency sweep testing, which are essential techniques for characterizing the viscoelastic properties of materials (39). These two tests assist in the judgment of the organogel as their motion nature, the oscillatory, gives continuous and gradual destruction and deformation process showing and mimicking the human body tissues as well dosage administration. In amplitude study, this test featured robustness and was applied to the selected organogels (F7, F10, F21, F31, and F34) by identifying the

G' (storage modulus), G" (loose modulus), LVER and the flow point. The strength of organogel characterizes the elasticity, and the solid scaffold content was represented by G' (27). Whilst the fluidity and broken organogel were indicated by G". Also, the elasticity can be further assessed by the LVER that gives an idea about the confrontation of the organogel to the increased applied strain by showing almost constant values of G' that after specific strain value, these G' values approach a continuous decrease as well in the same time an increased in the G" values until they meet at the same strain value representing the flow point and the whole destruction of the organogel's scaffold. The scooped organogel was subjected to increasing strain, from 0% to 100% (40).

The results demonstrated that all selected organogels showed parallel G' and G" curves, and G' values in LVER were one order greater than G". This feature demonstrates organogel formation, as suggested by Yan et al (41). This was also identical to the monoglyceride organogels, which revealed the same association between the increase in concentration and G' with G" (42). G' values varied from 10^3 to 10^6, as did the produced 12HSA organogels in canola oil (43). The LVER percentages were highest in F7 and lowest in F21. The varying LVER values of chosen organogels can be attributed to the expanding gaps in the 3-dimensional scaffold caused by the strain effect.

Conclusion

The results of this study demonstrate that the formulations exhibit a range of characteristics suitable for topical applications, with gel-sol temperatures exceeding 34 °C and pH values between 4 and 7 indicating stability. While formulations F1 and F5 showed poor spreadability, others performed adequately, and formulations F24 and F34 displayed excellent thermal stability with melting points above 50 °C. Microscopic analysis revealed distinct structural features, with oleic acid-based organogels forming spherulites and Vitamin E formulations exhibiting fibrillar networks, which influenced ACV release rates—higher in beeswax formulations with oleic acid compared to those with Vitamin E. Skin irritation tests confirmed safety, particularly for formulation F34, which also demonstrated superior permeation through bovine lip tissues. Additionally, rheological tests indicated that the selected organogels maintained strength and stability, while the MTT assay highlighted formulation F34's significant increase in RD cell viability against the HSV, suggesting its potential as a therapeutic agent.

Limitations of the study

The following limitations should be addressed in future research to improve assessment:

(a) Lack of human *in vivo* testing: the formulations were not tested on human subjects. Hence, its effectiveness and safety in real-world use remain uncertain.

(b) Long-term stability of the formulation was not evaluated.

(c) The skin diffusion data was limited because the study used bovine lip membranes, which may not perfectly mimic human skin ACV diffusion.

Authors' contribution

Conceptualization: Zahraa Hassan Abed and Masar Basim Mohsin Mohamed.
Data curation: Masar Basim Mohsin Mohamed.
Formal analysis: Zahraa Hassan Abed.
Investigation: Zahraa Hassan Abed.
Methodology: Masar Basim Mohsin Mohamed.
Project administration: Masar Basim Mohsin Mohamed.
Resources: All authors.
Software: Masar Basim Mohsin Mohamed.
Supervision: Zahraa Hassan Abed.
Validation: Masar Basim Mohsin Mohamed.
Visualization: Zahraa Hassan Abed.
Writing–original draft: All authors.
Writing–review & editing: All authors.

Conflicts of interest

The authors declare no conflict of interest.

Ethical issues

The research and the protocol of this study followed the guidelines of animal studies and were approved by the Ethics Committee of the Mustansiriyah University College of Pharmacy (Ethical Code #131). We adhered to the guidelines related to animal experiments, approved by the United States National Institutes of Health (NIH, 1978). Besides, the authors have ultimately observed ethical issues (including plagiarism, data fabrication, and double publication).

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