biologically active lignans due to hydrophilic matrix. Dissolution profiles of GB were higher when increasing the ratio of PVP K30 in the formula, demonstrating a significant difference with a lower PVP K30 as this carrier could facilitate the diffusion of markers in medium dissolution. However, the formula containing 4 or 6 % of PVP exhibited a similar dissolution profile. Simultaneously, too much incorporated PVP K30 per unit can increase humidity and enhance average mass. Therefore, PVP K30 of 4% was selected to prepare the SD of lignans enriched from SS extract.

4. Conclusion

The study was successful in the formation of solid dispersion loaded lignans-enriched *Schisandra sphenanthera* extract with tween 80 and PVP K30 promising for improving the dissolution of poorly soluble lignans into aqueous medium.

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EVALUATION OF THE SKIN IRRITATION AND ANTI-INFLAMMATORY ACTIVITY OF A GEL CONTAINING *PERILLA FRUTESCENS* EXTRACT AND *GLYCYRRHIZA URALENSIS* EXTRACT

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Summary

Evaluation of the Skin Irritation and Anti-Inflammatory Activity of a Gel Containing *Perilla frutescens* Extract and *Glycyrrhiza uralensis* Extract

The study prepared the herbal gel with 0.75% of carbopol 940, 0.85% of *Perilla frutescens* (PF) extract, 0.7% of *Glycyrrhiza uralensis* Fisch (GU) extract. and other excipients. *In vitro* testing showed that the product was non-irritating and safe; the anti-inflammatory activity of the gel had an IC_{50} value of 303.33 µg/mL. The total polyphenol content and flavonoid content were 2.9 mg GAE/g and 1.82 mg QCE/g, respectively. The research prepared and evaluated the skin irritation of the gel by the HET-CAM (Hen's EggTest on the Chorioallantoic Membrane) method. At the same time, the study assessed the anti-inflammatory activity and quantified polyphenols and flavonoids in the finished gel.

Keywords: *Perilla frutescens, Glycyrrhiza uralensis, Gel, Anti-inflammatory, HET-CAM, Irritation.*

1. Introduction

As the quality of life improves. taking care of beauty has become essential. With the tendency to return to nature, the search for safe and effective treatment solutions has been researched and developed. *Perilla frutescens* (L.) Britt. (Lamiaceae) (PF) and *Glycyrrhiza uralensis* Fisch (Fabaceae) (GU) has been published on chemical composition and proven to have anti-inflammatory and antioxidant properties. Phenolic and flavonoids in PF leaf extract could inhibit inflammatory and allergic responses in mice [1]. Saponins and flavonoids in GU root extract could show a strong tyrosinase and melanin synthesis inhibitory activity [2] and reduce free radicals [3].

Nowadays, there are diverse kinds of skincare products available that contain natural ingredients. These products have had a tendency to combine extracts from many medicinal plants, enhancing beneficial bioactive substances and improving their effectiveness. The combination of the anti-inflammatory property of PF and the antioxidant and hyperpigmentation-fading effects of GU will increase the product's effectiveness.

This study aimed to prepare and evaluate the irritation of a gel containing PF extract and GU extract as an anti-inflammatory supportive therapy but not irritating the skin with acne. Due to being used through the skin. cosmetics must meet the requirements of texture, be stable in terms of physicochemical properties, and not cause any irritations or allergies to the skin even if used for a long time. Therefore, irritation is one of the factors that directly affect product quality and consumer choice. Currently, researchers evaluated skin irritation by methods such as rabbit skin irritation test, rat skin electrical resistance test. Episkin (skin corrosivity test), etc. [4], but these tests have disadvantages that are costly and involve ethical issues in research.

To overcome the above limitation, the evaluation model on *in vitro* irritation has been researched. The HET-CAM (Hen's EggTest on the Chorioallantoic Membrane) method is routinely used to evaluate the potential eye irritation of raw materials but in some cases can be used to evaluate skin irritation. This test method was first proposed by Luenken (1985) and Lesepke and Kemper (1986) [5]. This is a novel method of irritation evaluation with high sensitivity, cost-effectiveness. easiness of implementation, and no use of test animals.

2. Materials and methods

2.1. Materials

Standard quercetin with a content of 95.8% was obtained from the Institute of Drug Quality Control Ho Chi Minh City. Standard gallic acid with a content of ≥ 98% was bought from Sigma Aldrich Company, in the USA. Diclofenac, ascorbic acid, nipagin, triethanolamine, sodium hydroxide, sodium carbonate, aluminum chloride, and potassium acetate were purchased from China. Carbopol 940, propylene glycol, and distilled water were made in Vietnam. Folin-Ciocalteu reagent, bovine serum albumin, and dimethyl sulfoxide were bought from the USA.

Fresh PF leaves and GU root (preliminarily processed) were purchased in Ninh Kieu district. Can Tho City in December 2022, and identified at the Department of Pharmacognosy -Traditional Pharmacy - Botany, Can Tho University of Medicine and Pharmacy.

Fresh PF leaves were preliminarily processed and dried at 70°C until a moisture content of < 7.5%. Then, they were finely ground to appropriate sizes and sieved through a sieve with a diameter of 0.2 cm. The powder was extracted with 60% of ethanol, a plant/solvent ratio of 1/35 w/v. and a pH of 3 at 50°C for 1 hour [6]. The extract was heated in a water bath at 50°C to obtain a concentrated PF extract (moisture content 8.0%). GU with a moisture content of < 12.0% were ground and sieved through a sieve with a diameter of 0.8 cm. GU powder was soaked in 80% of ethanol with a plant/solvent ratio of 1/15 w/v for 24 hours [7]. The extract was heated in water at 40°C to obtain a concentrated GU extract (moisture content 8.7%).

Chicken eggs in the HET-CAM test were eggs that were not older than 7 days and between 50 and 60 grams. Eggs were incubated for 9 days in conditions of a moisture content of 55-65% and temperature of 36-37°C. Eggs should be rotated at least 5 times per day for the first 8 days of incubation. Then, eggs were incubated for an additional 24 hours without rotation.

2.2. Methods

2.2.1. Investigation of *in vitro* anti-inflammatory and antioxidant effects of PF extract and GU extract:

Investigation of *in vitro* anti-inflammatory activity by inhibition of protein denaturation: reaction mixture consisting of 1 mL of the test sample was mixed with 1 mL of 5% bovine serum albumin solution, incubated at 27°C for 15 minutes. Protein was denatured at 60°C for 10 minutes. After cooling, the sample was measured at 660 nm. The reference drug was diclofenac [8].

Inhibition of protein denaturation of medicinal plant extracts was determined according to the following formula:

$$\% \text{ Inhibition} = (1 - Vt/Vc) \times 100 \qquad (2.1)$$

where Vt was the optical density of test samples of extracts or a standard drug; Vc was

the optical density of a sample of phosphate buffer (pH 3.2).

The antioxidant effect of extracts was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free-radical scavenging activity: The reaction mixture consisted of 40 µL of DPPH (1000 µg/mL) and 960 µL of the extract. The reaction mixture was incubated in the dark at 30°C for 30 minutes. After that, the absorbance of DPPH was measured at 517 nm. Vitamin C was used as a positive control. The percentage of the antioxidant property was determined according to the following formula:

Percentage of DPHH free-radical scavenging activity (%) = *(ODC-ODM) / ODC (2.2)*

where ODM and ODC were the optical density of the test sample and the negative control, respectively.

The regression equation was established from the percentage of protein denaturation inhibition and the percentage of DPPH free-radical scavenging activity, then the IC_{50} value was determined and played a role as a basis for comparing anti-inflammatory and antioxidant properties between samples. The lower the IC_{50} value was, the higher the activity was.

IC_{50} with the higher value was selected to conduct the survey and calculate to select the active concentration as the minimum concentration. Active concentrations of PF and GU were converted into their percentages (%) in the finished gel formula according to the following formulas:

$$x = \frac{IC_{ht}^{tt} \times V\,(gel) \times 10^{-6}}{m\,(gel)}(\%) \qquad (2.3)$$

$$y = \frac{IC_{ht}^{ct} \times V\,(gel) \times 10^{-6}}{m\,(gel)}(\%) \qquad (2.4)$$

where, x and y (%) were the minimum percentages of PF and GU at the starting period of the survey, respectively;

IC_{ht}^{tt} and IC_{ht}^{ct} (µg/mL) were the active concentration of PF and GU, respectively;

m (g) was the weight of the finished gel (m = 100 g);

V (mL) was the volume of 100 g of finished gel;

10^{-6} was a conversion factor from µg into g.

2.2.2. Investigation of gel formulations:

Procedure: carbopol was soaked in 70 g water until fully expanded. It was adjusted with triethanolamine (TEA) until a pH of 6-7, then stirred to obtain the gel (1). Nipagin was dissolved in propylene glycol (2), then PF extract and GU extract were dissolved in (2), the remaining water was added and stirred well to obtain (3), (3) was slowly added to (1) and stirred to homogenize.

The gel formula was built based on the investigation of the ratio of carbopol 940 and plant extracts. The percentages of carbopol 940 used were 0.50%, 0.75%, 1.00%, 1.25% and 1.5%, respectively **(F1-F5)**. At the same time, the percentages of PF extract and GU extract (x and y, respectively) were fixed. After that, a survey was conducted on the percentages of PF extract and GU extract increasing as follows: x and y, 1.5x and 1.5y, 2x and 2y, 2.5x and 2.5y, and 3x and 3y **(F6-F10)**. The remaining ingredients included 0.2% of nipagin, 10% of propylene glycol, TEA (ad.), and distilled water (ad. 100%).

Table 1. The formula of the gel containing PF and GU

Fomula / Ingredients (%)	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
PF extract	x	x	x	x	x	x	1.5x	2.0x	2.5x	3.0x
GU extract	y	y	y	y	y	y	1.5y	2.0y	2.5y	3.0y
Carbopol 940	0.5	0.75	1.0	1.25	1.5	z	z	z	z	z
Triethanolamine	Adjust pH 6-7									
Nipagin	0.2									
Propylene glycol	10									
Distilled water	Fill up to 100									
z: result from F1-F5										

Criteria: appearance (light yellow and transparent gel), pH (from 6.5 to 7.5), viscosity (from 2000 to 4000 cP). and homogeneity (no particles detected).

In vitro skin irritation test: the HET- CAM (Hen's Egg Test on the Chorioallantoic Membrane) method was used [5] on 3 groups: 0.9% NaCl (negative control), 0.1 N NaOH (positive control), and test samples. Procedure: the eggshell along the air cell was removed; the white inner membrane was moistened with 0.9% (w/v) NaCl solution. The inner membrane was carefully removed immediately prior to use (not more than 20 minutes after removal of the eggshell). Exposure of the eggs to test substance for at least 5 minutes. The endpoint was measured by visual inspection:

- Hemorrhage: bleeding out of the blood vessels of the CAM with red blood dots around the vessels.
- Lysis: optical disappearance of small blood vessels in the CAM cave.

- Coagulation: thrombosis (intravascular dark spots), extravascular blood coagulation (dark spots), and denaturation of albumin.

The endpoint measurement for the skin irritation test is shown in Table 1. Each test sample was repeated three times and averaged.

Table 2. Endpoints for the skin irritation

Endpoints	Scores		
	0.5 minutes	2 minutes	5 minutes
Hemorrhage	7	5	3
Lysis	5	3	1
Coagulation	9	7	5

Classification of skin irritation was as follows: 0-0.9 scores: nonirritant; 1-4.9 points: mildly irritant; 5-8.9 points: moderately irritant; 9-21 points: severely irritant. Requirements: the product should not cause any irritations or mild irritations.

2.2.3. Evaluation of *in vitro* anti-inflammatory ability and quantification of polyphenol and flavonoid in gel:

The finished gel was evaluated for its anti-inflammatory and antioxidant properties as described above.

Polyphenol content was determined by the Folin-Ciocalteu method: A standard gallic acid solution was prepared at different concentrations of 0, 10, 20, 30, 40, 50, and 60 µg/mL. 250 µL of Folin-Ciocalteu reagent 10% was added to 100 µL of samples of gallic acid at different concentrations to obtain a solution (1). After 5 minutes, 200 µL of Na_2CO_3 2% solution was added to the solution (1) to obtain a solution (2). The solution (2) was incubated for 45 minutes at room temperature. The absorbance of the reaction solution was measured at 760 nm [9]. The experiment was repeated 3 times. Meanwhile, the absorbance values were recorded to draw a calibration line to determine the total polyphenol content in the gel samples.

Total flavonoid content was determined by the $AlCl_3$ colorimetric method: A standard quercetin solution was prepared at concentrations of 0, 10, 20, 40, 60, 80, and 100 µg/mL. 10 µL of $AlCl_3$ 10% was added to 50 µL of samples at different concentrations. After 6 minutes, 10 µL of CH_3COOK 1M and 280 µL of distilled water were added to this mixture, then shaken well. Next, it was stabilized at room temperature for 45 minutes. The absorbance of the reaction solution was measured at 510 nm [10]. The experiment was repeated 3 times. Meanwhile, the absorbance values were recorded to draw a calibration line to determine the flavonoid content in the gel samples.

3. Results

3.1. Investigation of in vitro anti-inflammatory and antioxidant effects of PF extract and GU extract

The results of the investigation of *in vitro* anti-inflammatory effects of PF extract and GU extract are presented in Table 3.

Table 3. Anti-inflammatory performance of PF extract and GU extract

C (µg/mL) / HS (%)	0	3.125	6.25	12.5	25	50	100	IC_{50}
PF extract								
HS1	0	4.160	7.132	11.293	20.059	35.067	64.042	75.726
HS2	0	4.094	7.749	11.842	19.883	38.012	62.573	75.771
HS3	0	4.329	6.494	9.957	24.820	37.085	62.338	75.585
The average IC_{50} = 75.70 µg/mL								
GU extract								
HS1	0	7.429	9.955	16.345	23.180	40.713	78.158	61.699
HS2	0	7.602	10.088	15.351	23.246	41.374	77.778	61.792
HS3	0	6.926	10.390	16.306	25.397	40.404	79.221	60.773
The average IC_{50} = 61.42 µg/mL								

HS1. HS2. and HS3 were the inhibition performance of test samples 1. 2. and 3. respectively.

Table 4. Results of the anti-inflammatory performance of diclofenac

C (µg/mL) / HS (%)	0	0.625	1.25	2.5	5	10	20	IC_{50}
HS1	0	5.847	10.495	15.142	27.136	51.424	91.604	10.336
HS2	0	4.341	11.008	14.264	26.667	50.853	89.922	10.546
HS3	0	4.863	10.790	13.678	24.164	49.544	89.818	10.701
The average IC_{50} = 10.528 µg/mL								

The results of the anti-inflammatory activity of PF extract and GU extract were 75.70 µg/mL and 61.42 µg/mL, respectively. This result shows that the anti-inflammatory effects of the two extracts were about 7 times lower than that of the standard diclofenac (IC_{50} = 10.528 µg/mL).

The results of *in vitro* antioxidant effects of PF extract and GU extract are presented in Table 5.

Table 5. Results of DPPH free-radical scavenging performance of PF extract and GU extract

HS (%) \ C (μg/mL)	0	100	500	1000	1500	2000	2500	IC_{50}
PF extract								
HS1	0	7.076	17.318	35.940	51.676	67.598	87.430	1434.873
HS2	0	5.777	18.939	36.269	51.610	68.845	87.784	1424.208
HS3	0	7.728	19.779	35.787	53.358	69.181	86.937	1411.263
The average IC_{50} = 1423.448 μg/mL								
GU extract								
HS1	0	4.376	14.711	27.840	43.389	57.263	70.484	1755.815
HS2	0	4.451	12.216	27.841	43.277	60.417	71.023	1729.500
HS3	0	4.140	16.835	27.415	42.778	60.166	70.285	1733.431
The average IC_{50} = 1739.582 μg/mL								

Table 6. Results of DPPH free-radical scavenging performance of vitamin C

HS (%) \ C (μg/mL)	0	1	2	4	6	8	10	IC_{50}
HS1	0	10.598	18.750	34.511	51.268	65.036	77.627	6.129
HS2	0	09.415	16.254	31.715	50.248	63.132	76.809	6.322
HS3	0	11.271	19.116	35.618	51.578	64.382	75.834	6.170
The average IC_{50} = 6.207 μg/mL								

The IC_{50} values for the antioxidant activities of PF extract and GU extract were 1423.448 μg/mL and 1739.582 μg/mL, respectively. This result shows that the antioxidant effects of the two extracts were lower than that of the standard vitamin C (IC_{50} = 6.207 μg/mL).

3.2. Investigation of gel formulations

Table 7. Survey formulations

Formula	Appearance	pH	Viscosity (cP)	Homogeneity	Irritation
F1	Gel was yellow and transparent	7.30	1926	Pass	Non irritant
F2		7.35	3535	Pass	Non irritant
F3		7.97	4519	Pass	Non irritant
F4		7.80	5608	Pass	Non irritant
F5		8.23	6777	Pass	Non irritant
F6		6.93	3511	Pass	Non irritant
F7		7.55	3300	Pass	Non irritant
F8		7.78	3523	Pass	Non irritant
F9		7.38	3479	Pass	Non irritant
F10	Gel was yellow and cloudy	7.14	3516	Pass	Non irritant

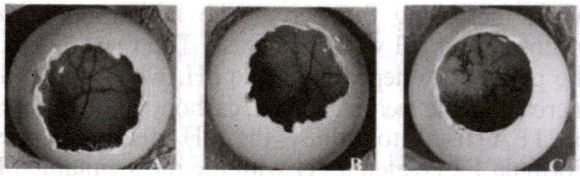

Fig. 1. Skin irritation test
(A: test sample (F9); B: negative control NaCl 0.9%; C: positive control NaOH 0.1N)

F2 with 0.75% of carbopol was consistent with the criteria, F2 was selected for the next test, F6-F9 met the requirements, F9 was the selected formula with 0.87% of PF extract and 0.70% of GU extract because of the highest percentage of medicinal plants.

3.3. Evaluation of in vitro anti-inflammatory ability and quantification of polyphenol content and flavonoid content

Table 8. Survey results of the gel's anti-inflammatory performance

HS (%) \ C(μg/mL)	0	12.5	25	50	100	200	400	IC_{50}
HS1	0	0.15	0.60	2.99	3.08	20.26	80.86	310
HS2	0	2.32	2.70	6.49	7.31	23.47	89.63	300
HS3	0	3.27	4.87	7.04	6.98	35.13	89.56	300
The average IC_{50} = 303.33 μg/mL								

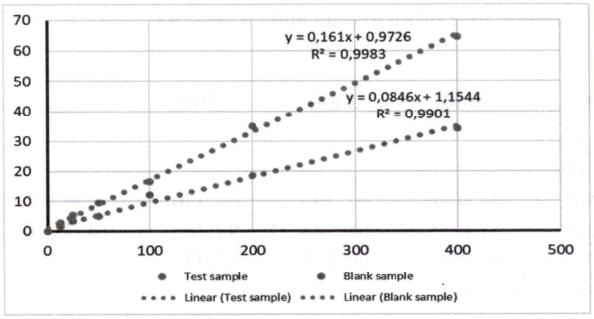

Fig. 2. Linear regression equation of gel's inhibition performance of protein denaturation

The inhibition performance of protein denaturation was calculated based on the percentage of reduction in the spectral absorbance of BSA on the test sample and the blank sample (without PF and GU). The result of the anti-inflammatory activity of gel containing PF extract and GU extract was IC_{50} of 303.33 µg/mL. At the concentration of 400 µg /mL, the maximum inhibition percentage of protein denaturation (%) of the finished gel was 89.56%.

Table 9. Results of antioxidant performance of the finished gel

C(µg/mL) / HS (%)	0	1000	2000	4000	6000	8000	10000	IC_{50}
HS1	0	19.39	21.14	35.13	44.61	58.86	92.12	7040
HS2	0	16.78	22.41	37.27	43.01	57.92	90.73	7142
HS3	0	15.01	20.66	31.96	42.27	57.47	91.81	6761
The average IC_{50} = 6981 µg/mL								

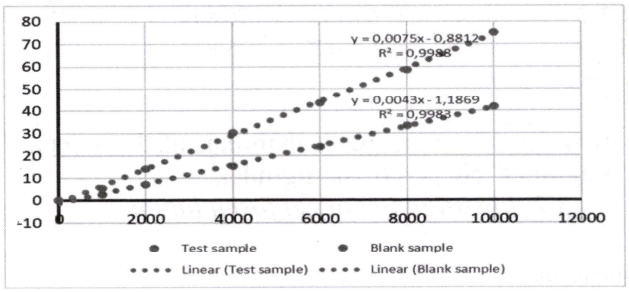

Fig. 3. Linear regression equation of the free-radical scavenging performance (%) of the finished gel

The IC_{50} value for the antioxidant performance of the finished gel containing PF extract and GU extract were 6981 µg/mL. At a concentration of 10000 µg/mL, the maximum free-radical scavenging performance (%) of the finished gel was 92.12%.

The total polyphenol content (mg/g) was converted from the equation of the standard gallic acid curve: $y = 0.019 x + 0.0004$ ($R^2 = 0.9997$). The total flavonoid content was converted from the equation of the standard quercetin curve: $y = 0.0067 x + 0.0059$ ($R^2 = 0.9988$). The average quantified results of the polyphenol content and the flavonoid content were 2.9 mg and 1.82 mg, respectively.

Table 10. Total contents of polyphenol and flavonoid in gel

No.	Total polyphenol content (mg GAE/g sample)	Total flavonoid content (mg QCE/g sample)
1	2.94	1.85
2	2.77	1.79
3	2.98	1.83
Average	**2.90**	**1.82**

4. Discussion

The study selected a concentration with antioxidant activity for further tests to optimize the product's activity. Previous studies demonstrated that certain shortcomings of the IC_{50} value in the DPPH experiment could affect the accuracy of the results. Therefore. JuDong Yeo et al (2019) [11] proposed a new value, IC_{100} which would be not affected by other components co-existing in the medicinal sample. Studies on cosmetics from medicinal plants also used the IC_{100} value to evaluate the survey formulas as the study by Arif Budiman et al (2022) [12]. So IC_{100} was chosen as the initial concentration for investigation.

The gel dosage form has various advantages that it is not greasy, quickly absorbed, and easy to wash off. Moreover, it does not interfere with the normal physiological activity of the skin, creating a more comfortable feeling for users. The survey showed when increasing the percentage of carbopol. the viscosity and pH would increase. The reason is that the gel texture depends on the pH, when gradually increasing the percentage of carbopol, the amount of TEA used to adjust the pH also increases. Formula F2 created a gel with the most suitable pH and texture without causing any irritations when used. The results of the survey on herbal plant extracts showed that when gradually increasing the percentages of extracts in the formula, there was a change in the transparency and color of the gel. As for formula F10 (1.05% of PF extract and 0.84% of GU extract), the gel was cloudy.

In the study, the HET-CAM test was used to evaluate the irritant effects of formulas and finished gels. All gel formulas were non-irritating. This can be explained by the fact that the research used safe excipients within the allowable limits prescribed by

the Ministry of Health. At a concentration of 400 μg/mL, the gel inhibited 89.56% of protein denaturation. This result shows a higher anti-inflammatory ability compared to the study by Jamadar et al (2017) on a gel preparation extracted from *Clerodendrum serratum* at a concentration of 500 μg/mL inhibiting only 69.7% of protein denaturation [5]. Another research by Sekar et al (2019) studied gel with embelin isolated from *Embelia ribes* at a concentration of 1000 μg/mL inhibiting 31.08% of protein denaturation [13]. From the above results. it can be seen that the gel from PF extract and GU extract had superior anti-inflammatory activity compared to other preparations with the same dosage form.

Results of DPPH antioxidant activity of gels containing PF extract and GU extract with IC_{50} of 6981 μg/mL. A study by Aziz et al. evaluated the antioxidant activity of gel containing *Polygonum amplexicaule* with a concentration of medicinal plant extract of 5% and IC_{50} of 446 μg/mL [15]. At a concentration of 1000 μg/mL, the finished gel containing GU extract and PF extract had a free-radical scavenging performance of 19.39%. Compared with the research by Sekar et al. on a gel with a concentration of embelin of 5% isolated from *Embelia ribes*, the DPPH free radical scavenging performance was 50.12% at a concentration of 1000 μg/mL [13]. It can be seen that the antioxidant activity of the finished gel in this study was lower because a concentration of extracts of 1.55% was much lower than that of Aziz's study (5% of extracts) [14]. Based on IC_{50} of anti-inflammatory and antioxidant properties of the two types of medicinal plant extracts, antioxidant activity was chosen to conduct the survey. The IC_{100} value of antioxidant activity (IC_{100} is defined as the amount of DPPH radical required to oxidize all the antioxidants in the reaction medium) was selected as the minimum active concentration to conduct the test. The results of conversion from the active concentrations of GU extract and PF extract into their percentages (%) in the finished gel formula were x of 0.34% and y of 0.28%. These were the minimum percentages to start investigating the amount of medicinal plant extracts in the gel formula.

According to the results of quantification. total polyphenol content was much higher than that of the study by Yousif et al. (2011) who prepared two herbal gels containing tannin extracts obtained from *Acacia nilotica* Del. fruits (Qarad) and *Quercus infectoria* Oliv. galls (Oak gall) with total polyphenol content of 0.1253 mg GAE/g and 0.064 mg GAE/g, respectively [15]. The gel in the study combined two types of medicinal herbs with ingredients rich in flavonoids and phenolic acids. Besides, the extraction process of the study was based on the previous optimized one. For the above reasons, the finished gel had higher polyphenol content and flavonoid content than that of the other studies on the herbal gel preparation.

5. Conclusions

The study evaluated the irritant effect and anti-inflammatory activity of *Perilla frutescens* extract and *Glycyrrhiza uralensis* extract through *in vitro* tests. The gel was formulated with 0.75% of carbopol 940, 0.85% of PF extract, 0.7% of GU extract, and other excipients, containing 2.90 mg GAE/g polyphenol and 1.82 mg QCE/g flavonoid. The gel was assessed to have good anti-inflammatory activity. The product has a lot of potential applications to develop into a specialized cosmetic line for acne skin. especially sensitive skin.

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