Validation of Quantification Process for Total Polyphenol and Flavonoid in Propolis Hydrogel by UV Spectrophotometry

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Abstract

Propolis is a resin rich in polyphenols and flavonoids, providing strong antibacterial, antioxidant, and wound-healing properties. Therefore, propolis hydrogels are therefore promising wound dressing materials that accelerate the healing of minor injuries. This study aimed to validate a Ultraviolet spectrophotometric method for the quantification of total polyphenol and total flavonoid content in propolis hydrogels. The quantifications were validated for their selectivity, linearity, accuracy and precision. The results demonstrated excellent linearity for polyphenols, and flavonoids over a concentration range of (10-120) µg/mL at 754 nm and 427 nm, respectively. Furthermore, the method exhibited high specificity, accuracy (recovery rates between 95% and 102%), and precision (relative standard deviation < 2%) for both analytes. The TPC and TFC in the propolis hydrogel were (4.2392 ± 0.0495) mgGAE/g and (2.1733 ± 0.0817) mgQE/g respectively. These validation results are crucial for establishing robust quality control criteria in the manufacturing of propolis hydrogels.

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Keywords

Propolis hydrogel; polyphenols; flavonoids; method validation; UV spectrophotometry.

1 Introduction

A lesion is a damage or disruption of living tissue's cellular, anatomical, and functional integrity [1]. Wound healing is the body's natural response to restore the structure and function of the skin after injury. This process includes several complex steps such as hemostasis, anti-inflammation and tissue regeneration. Tissue repair time depends on many factors such as location, wound size, regeneration rate of damaged cells and the patient's health status. While minor wounds are naturally healed without medical interventions, large ones with a high risk of infection require external mediations to promote rapid recovery and ensure aesthetic.

Among numerous types of wound dressings, hydrogels are promising materials due to their rapid coagulation, antibacterial activity, biocompatibility and biodegradability [2]. In recent year, propolis has been loaded on hydrogels as wound dressing materials. Some polymers have been developed for the hydrogel film such as polyvinyl pyrrolidone (PVP), hydroxypropyl methylcellulose, polyacrylamide/methylcellulose, and chitosan/pectin [3-6]. The propolis hydrogels were developed to enhance wound healing, capitalizing on the 40% total polyphenol content (TPC) of propolis and the inherent swelling capability of hydrogels.

For determining TPC and the flavonoid content (TFC) of propolis hydrogel, UV spectrophotometry is a fast

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and efficient analytical technique [3, 5, 6]. However, no articles demonstrate validation of TPC and TFC methods uniquely within hydrogels.

The present study validated quantification process of TPC and TFC of propolis hydrogel using UV spectrophotometry to provide a reliable assessment of the antioxidant properties of propolis. By establishing a standardized quantification process, this study contributed to make quality control criteria for propolis hydrogel in industrial manufacturing.

The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guideline Q2(R2) on the validation of analytical procedures outlines several key elements for consideration, including specificity, working range, accuracy, and precision [7].

2 Materials and Methods

2.1 Materials and equipment

Propolis hydrogels were made in previous study [8]. Gallic acid (98% of purity), sodium carbonate, and aluminum chloride were purchased from Xilong, China. Quercetin (83.9% of purity) was purchased from National Institute of Drug Quality Control, Viet Nam. Folin-Ciocalteu reagent, and potassium acetate (≥ 99% of purity) were purchased from Merck (Merck Co., Darmstadt, Germany).

All equipment used in the study were located in the Faculty of Pharmacy at Nguyen Tat Thanh University, and routinely validated every 6 months.

2.2 Quantification procedure of total polyphenol content in propolis hydrogel

TPC in the propolis hydrogel was determined using a modified Folin–Ciocalteu method [9]. Four mL of 10% Folin-Ciocalteu reagent were added to 1 mL of the tested solution. The mixture was shaken well and left in the dark in 3 minutes. Then, 8 mL of 7.5% sodium carbonate solution was added, mixed and incubated in the dark for 30 minutes. Absorbance was recorded at a wavelength from (615-760) nm using a UV-Vis spectrophotometer [10]. Each sample was analyzed in triplicate. TPC was calculated using Equation (Eq.) 1 and expressed as a gallic acid equivalent.

$$TPC\ (\frac{mgGAE}{g}) = \frac{C \times V \times K}{m} \qquad \text{(Eq.1)}$$

Whereas:

C: gallic acid concentration calculated (mg/mL)

V: test volume (mL)

K: dilution factor

m: propolis hydrogel weight (g)

2.3 Quantification procedure of flavonoid content in propolis hydrogel

TFC was determined using a modified aluminum chloride colorimetric method with slight modifications [11]. 1.5 mL of ethanol and 0.1 mL of 10% aluminum chloride solution were added to 0.5 mL of the sample solution. The mixture was stabilized for 6 minutes for proper reaction. Then, 0.1 mL of 1 M potassium acetate solution and 2.5 mL of distilled water were added. The mixture was shaken well and left at room temperature for 45 minutes. The absorbance was recorded at a wavelength of (410-430) nm [10], using a UV-Vis spectrophotometer. Each sample was analyzed in triplicate. TFC was calculated using Eq. 2 and expressed as quercetin equivalent.

$$TFC\ (\frac{mgQE}{g}) = \frac{C \times V \times K}{m} \qquad \text{(Eq. 2)}$$

Whereas:

C: quercetin concentration calculated (mg/mL)

V: test volume (mL)

K: dilution factor

m: propolis hydrogel weight (g)

2.4 Validation of quantification procedure

2.4.1 Maximum absorbance wavelengths determination

The standard solutions of gallic acid (40 µg/mL) and quercetin (20 µg/mL) were prepared in 70% ethanol. These were then mixed with other reagents, following the same procedure used for quantifying TPC and TFC. The resulting mixtures were then analyzed with a UV-Vis spectrophotometer, which scanned their absorption spectra between 400 nm and 800 nm for gallic acid and between 200 nm to 800 nm for quercetin. The maximum absorbance wavelengths (λ_{max}) for both gallic acid and quercetin were determined from these scans and were used for further experiment. Blank was 70% ethanol.

2.4.2 Selectivity

The hydrogels spiked with gallic acid or quercetin and blank hydrogel were dissolved in 80 mL of 70% ethanol and sonicated for 15 minutes. The mixtures were left to cool and diluted to 100 mL. After filtration through a 0.45 µm membrane, they were mixed with reagents similar to quantification procedure of TPC and TFC. Subsequently, the resulting solutions were scanned for their spectra between 400 nm and 800 nm for gallic acid and between 200 nm to 800 nm for quercetin using a UV-Vis spectrophotometer. The requirement is that the spectra of blank hydrogels must not show any peak absorbance at the λ_{max} for gallic acid or the λ_{max} for quercetin, while the spectra of spiked hydrogels demonstrate absorbance peaks at corresponding wavelengths of gallic acid and quercetin [7].

2.4.3 Linearity and working range

Gallic acid (standard) or quercetin (standard) were dissolved in 70% ethanol at concentrations of (10, 20, 40, 60, 80, 100, and 120) µg/mL. The amount of gallic acid or quercetin was evaluated according to the quantification procedure for TPC or TFC. The response of the measured quantity (peak area) at each concentration was determined by establishing a regression equation and plotting a graph that shows the correlation between the response signal (peak area) and the concentration. Linearity was then assessed using statistical methods based on the regression equation y = ax + b. The significance of the coefficients in the regression equation was checked using a *t*-test, while the overall adequacy of the regression equation was checked using an *F*-test.

2.4.4 Detection limit and quantification limit

The detection limit (DL) and quantification limit (QL) are the lowest concentrations of an analyte that can be reliably detected and quantified, respectively. These limits are calculated using Eq. 3 and Eq. 4 [7]:

$$DL = \frac{3.3 \times SD}{a} \quad \text{(Eq. 3)}$$

$$QL = \frac{10 \times SD}{a} \quad \text{(Eq. 4)}$$

Whereas:

a: the slope of the calibration curve

SD: the standard deviation of the response

2.4.5 Accuracy

Sample solution with a concentration of 40 µg/mL gallic acid or 20 µg/mL quercetin was prepared. The solution was spiked with gallic acid standard solution or quercetin standard solution at 3 concentrations of (80, 100 and 120)% of initial concentration. The gallic acid or quercetin amount was determined following the quantification procedure for TPC and TFC. The experiment was repeated 3 times to obtain the average value of each concentration. Accuracy was reported as the mean percent recovery by known added amount of gallic acid or quercetin and the accepted true value as Equation 5. The procedure is accurate if the percent recovery is between (95 and 102)% [12].

$$Percent\ recovery = \frac{Accepted\ true\ value\ of\ added\ amount\ of\ analyte}{Known\ added\ amount\ of\ analyte} \times 100 \quad \text{(Eq.5)}$$

2.4.6 Precision

The precision test was performed by evaluating six determinations of a 40 µg/mL gallic acid solution or a 20 µg/mL quercetin solution. From the results, the relative standard deviation (RSD) was calculated. The procedure was considered precise if the RSD was less than or equal to 2% [7].

2.5 Determination of TPC and TFC in propolis hydrogels

The propolis hydrogel (approximate 1 g) was dissolved in 80 mL of 70% ethanol and sonicated for 15 minutes. The mixtures were left to cool down and diluted to 100 mL. After filtration through a 0.45 µm membrane, they were mixed with reagents similar to quantification procedure of TPC and TFC. The resulting solutions were measured the absorbance at λ_{max} of gallic acid and quercetin. The experiment was repeated 3 times. The data were then used to calculate the total polyphenol content and total flavonoid content.

3 Results and Discussion

3.1 Maximum absorbance wavelengths of gallic acid and quercetin

The absorbance peaks for gallic acid and quercetin obtained in standard solutions from the scan spectrum are shown in Figure 1. The result exhibited that the λ_{max} for gallic acid and quercetin were 754 nm (Figure 1A) and 427 nm (Figure 1B), respectively (Figure 1A and Figure 1B)

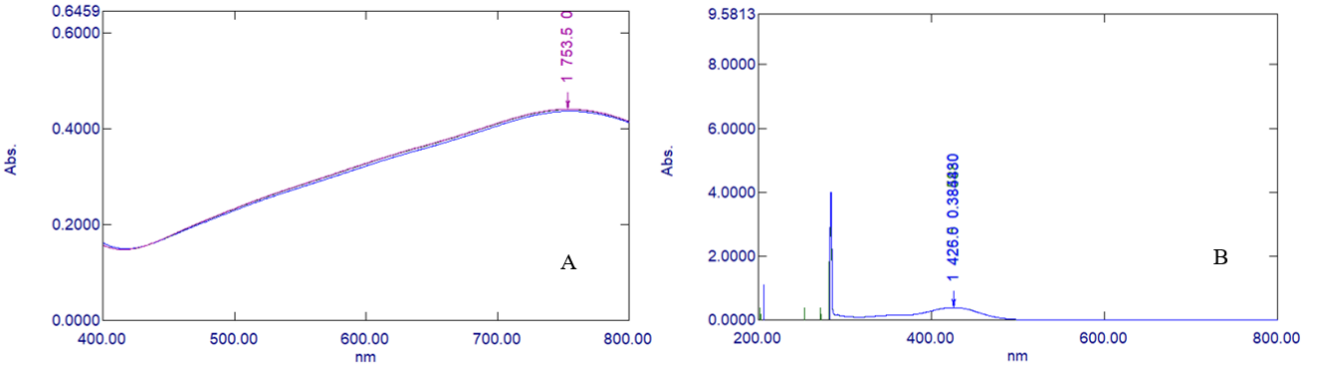

Figure 1 λ_{max} for gallic acid (A) and quercetin (B)

3.2 Selectivity

Figure 2 and Figure 3 showed the scan spectra of the blank hydrogel and the spiked hydrogel with gallic acid and quercetin, respectively. There are no absorption peaks at 754 nm and 427 nm on the scan spectra of the blank hydrogel (Figure 2A and Figure 3A, respectively). Meanwhile, the spiked hydrogels' spectra display absorption bands maximum at 754 nm for gallic acid (Figure 2B) and 427 nm for quercetin (Figure 3B). This performance exhibited that the excipients contained in hydrogels did not interfere with the absorbance of gallic acid and quercetin.

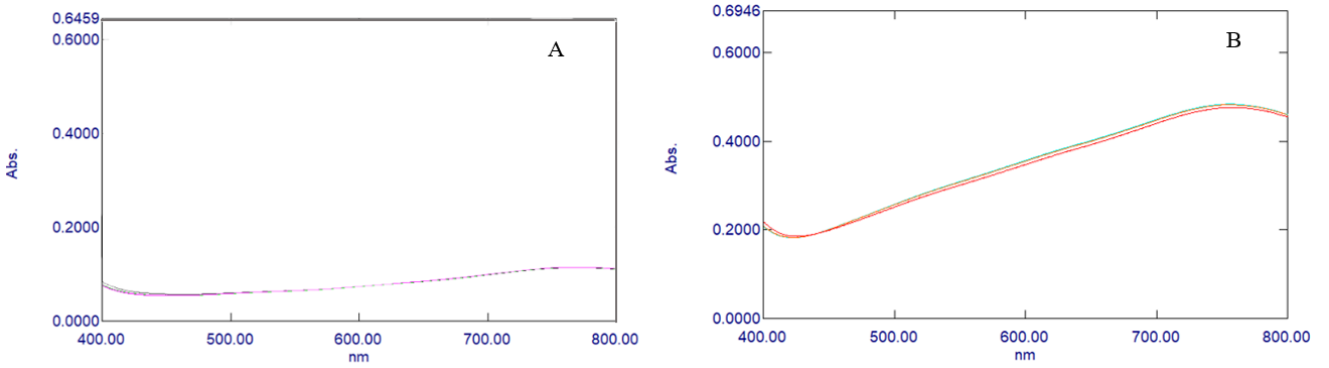

Figure 2 Scan spectrum of blank hydrogel (A) and spiked hydrogel with gallic acid (B)

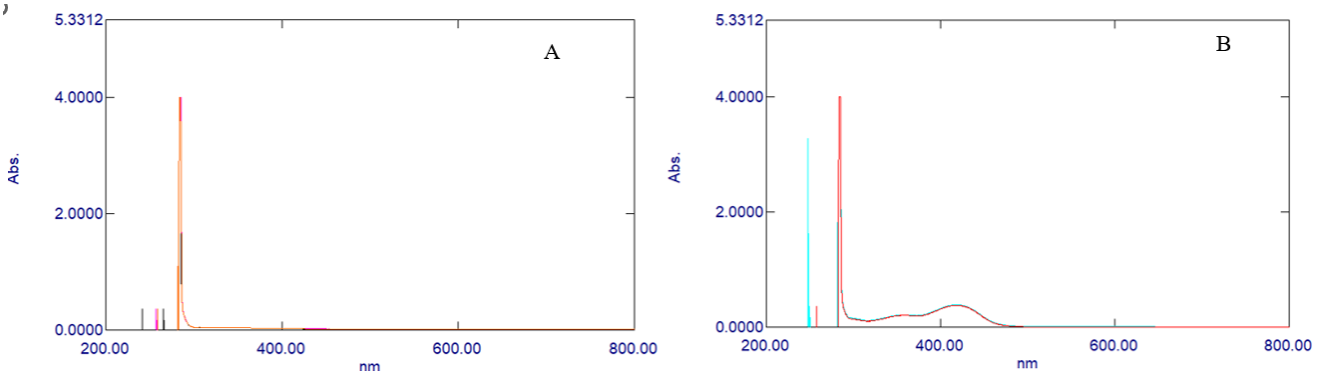

Figure 3 Scan spectra of blank hydrogel (A) and spiked hydrogel with quercetin (B)

The TPC and TFC quantification procedure in propolis hydrogel using UV spectrophotometry was selective.

3.3 Linearity and working range

Table 1 shows the absorbance of standard solutions of gallic acid and quercetin at concentrations from (10-120) µg/mL. The measurements were taken at 754 nm and 427 nm, respectively.

Table 1 Absorbance of gallic acid concentrations at 754 nm and of quercetin concentrations at 427 nm

Gallic acid concentration	Absorbance at 754 nm				
	No.1	**No.2**	**No.3**	**Average**	**SD**
10	0.0614	0.0590	0.0838	0.0681	0.0137
20	0.1492	0.1423	0.1456	0.1457	0.0035
40	0.3034	0.2984	0.3022	0.3013	0.0026
60	0.4509	0.4371	0.4469	0.4450	0.0071
80	0.6018	0.5857	0.5993	0.5956	0.0086
100	0.7604	0.7481	0.7488	0.7524	0.0069
120	0.9026	0.8854	0.9065	0.8982	0.0112
Quercetin concentration (µg/mL)	**Absorbance at 427 nm**				
	No.1	**No.2**	**No.3**	**Average**	**SD**
10	0.0757	0.0829	0.0809	0.0798	0.0037
20	0.1554	0.1495	0.1587	0.1545	0.0047
40	0.3556	0.3434	0.3383	0.3458	0.0089
60	0.5180	0.5074	0.5035	0.5096	0.0075
80	0.7017	0.6797	0.6860	0.6891	0.0113
100	0.8524	0.8626	0.8758	0.8636	0.0118
120	1.0417	1.0126	1.0553	1.0365	0.0218

As shown in Table 2, a statistical regression analysis revealed a high linear correlation between gallic acid concentration and its absorbance at 754 nm (R^2 = 0.9999), as well as between quercetin concentration and its absorbance at 427 nm (R^2 = 0.9998). The *p*-values for the coefficients in both cases are less than 0.05, indicating that the coefficients in the regression equations are significant. The *F*-statistic for the significance of the gallic acid equation's y-intercept (b) is greater than 0.05, indicating it is not statistically significant. In contrast, the *F*-statistic for the quercetin equation's y-intercept is less than 0.05, meaning it is significant.

Table 2 Results of regression analysis

Value	TPC	TFC
Standard substance	Gallic acid	Quercetin
Regression equation y = ax + b	y = 0.0075x – 0.0052	y = 0.0087x – 0.0115
R square (R^2)	0.9999	0.9998
p-value of a	3.0099E-11	2.3066E-10
Significance *F*	0.0796 > 0.05: b is not significant	0.0394 < 0.05: b is significant
Standard error	0.0033	0.0058
Working range (µg/mL)	10-120	10-120
Linear equation	y = 0.0075x	y = 0.0087x – 0.0115

As a result, the procedure meets the linearity requirements, with a working range of (10-120) µg/mL for both experiments. The standard curves for TPC are y = 0.0075x (R^2 = 0.9999) (Figure 4A), and for TFC it is y = 0.0087x – 0.0115 (R^2 = 0.9998) (Figure 4B).

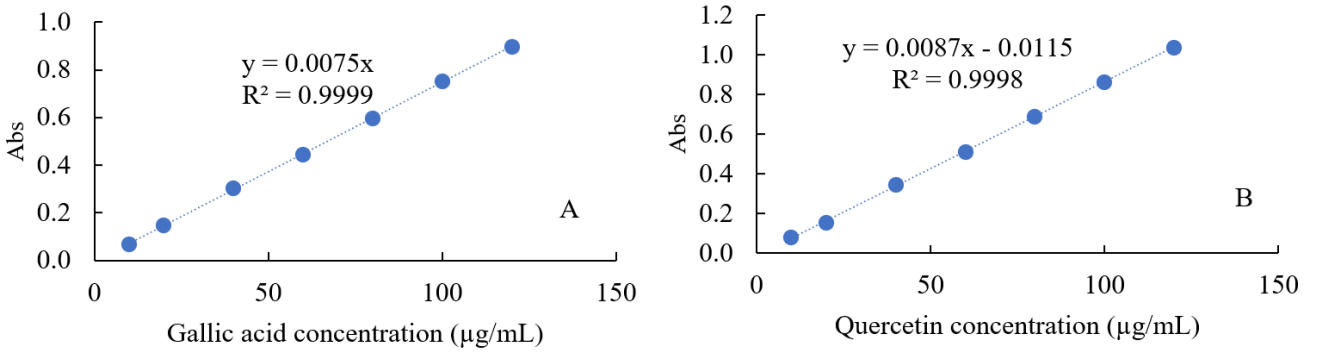

Figure 4 Standard curves of gallic acid (A) and quercetin (B)

3.4 Detection limit and quantification limit

The value of Standard Error and the slope of the calibration curve (a) shown in Table 2 was used to calculate the DL and QL. The results can be observed in Table 3.

Table 3 Detection limit (DL) and Quantification limit (QL)

Limit value	Unit	Total polyphenol content	Flavonoid content
Detection limit (DL)	µg/mL	1.4469	2.1744
Quantification limit (QL)	µg/mL	4.3845	6.5891

3.5 Accuracy

Table 4 Recovery rate of gallic acid in accuracy experiment

Sample	No.	Absorbance	Gallic acid concentration (µg/mL)	Average concentration (µg/mL)	SD	Found standard (µg/mL)	Added standard (µg/mL)	Percent recovery (%)
Sample (40 µg/mL)	1	0.3189	42.2923	38.9893	3.0212			
	2	0.2742	36.3656					
	3	0.2889	38.3100					
Sample + 80 % standard	1	0.5678	75.2927	70.5443	7.0784	31.5550	31.1914	101.2
	2	0.4706	62.4088					
	3	0.5575	73.9314					
Sample + 100 % standard	1	0.5816	77.1249	78.5884	3.1374	39.5992	38.9893	101.6
	2	0.6198	82.1901					
	3	0.5765	76.4503					
Sample + 120 % standard	1	0.6400	84.8590	86.0652	1.2090	47.0759	46.7871	100.6
	2	0.6582	87.2770					
	3	0.6490	86.0595					

Table 5 Recovery rate of quercetin in accuracy experiment

Sample	No.	Absorbance	Quercetin concentration (µg/mL)	Average concentration (µg/mL)	SD	Found standard (µg/mL)	Added standard (µg/mL)	Percent recovery (%)
Sample (20 µg/mL)	1	0.1787	21.7504	20.2348	1.6958			
	2	0.1682	20.5507					
	3	0.1494	18.4032					
Sample + 80 % standard	1	0.3129	37.1045	36.6657	0.6688	16.4310	16.1878	101.5
	2	0.3120	36.9967					
	3	0.30236	35.8960					

Sample + 100 % standard	1	0.3232	38.2767	40.5655	2.0512	20.3308	20.2348	100.5
	2	0.3486	41.1823					
	3	0.3578	42.2376					
Sample + 120 % standard	1	0.386	45.4951	44.4352	0.9470	24.2005	24.2817	99.7
	2	0.3703	43.6723					
	3	0.3744	44.1384					

To verify the accuracy of TPC and TFC quantification method, the concentration of the test sample and the samples spiked with (80, 100, and 120)% of the standard was determined, as shown in Table 4 and Table 5. The results for the gallic acid percent recovery at the (80, 100, and 120)% levels were (101.2, 100.6, and 100.6)%, respectively, which is within the required range of (95-102)%. Similarly, the results for the quercetin percent recovery at the (80, 100, and 120)% levels were (101.5, 100.5, and 99.7)%, respectively, also falling within the required range of (95-102)%. Therefore, the total polyphenol and flavonoid content quantification procedure in propolis hydrogel using UV spectrophotometry was accurate.

3.6 Precision

Table 6 Absorbance of analyte concentrations in precision experiment

No.	Gallic acid		Quercetin	
	Absorbance	Concentration (µg/mL)	Absorbance	Concentration (µg/mL)
1	0.3034	40.2270	0.1801	21.9128
2	0.2984	39.5633	0.1819	22.1175
3	0.2989	39.6394	0.1863	22.6220
4	0.3022	40.0782	0.1754	21.3747
5	0.3107	41.2047	0.1805	21.9677
6	0.3122	41.4042	0.1787	21.7473
Average		40.3528	-	21.9570
SD		0.7816		0.4130
RSD		1.9370		1.8812

The absorbance and concentrations of 6 determinations for gallic acid and quercetin were exhibited in Table 6. The average concentration for gallic acid and quercetin was (40.3528 ± 0.7816) µg/mL and (21.9570 ± 0.4130) µg/mL, respectively. The RSD of 1.9370% and 1.8812% was less than 2%, meeting the requirements of the repeatability validation.

The TPC and TFC quantification method using UV spectrophotometry achieved repeatability.

3.7 Determination of total polyphenol and flavonoid content in propolis hydrogels

Table 7 Total polyphenol and flavonoid content in propolis hydrogels

Propolis hydrogel	Weight (g)	Moisture content (%)	Abs at 754 nm	Gallic acid concentration (µg/mL)	Total polyphenol content (mgGAE/g)	Abs at 427 nm	Quercetin concentration (µg/mL)	Flavonoid content (mgGAE/g)
1	1.0708	10.7300	0.3097	41.0681	4.2963	0.1646	20.1411	2.1070
2	1.0225	10.7300	0.2900	38.4606	4.2135	0.1692	20.6705	2.2646
3	1.0188	10.7300	0.2886	38.2684	4.2077	0.1593	19.5379	2.1482
Average				39.2657	4.2392	-	20.1165	2.1733
SD				1.5639	0.0495	-	0.5667	0.0817

The validated procedures for determining TPC and TFC in propolis hydrogels were applied to evaluate the total polyphenol and flavonoid content in the propolis hydrogels that were previously prepared [8]. The results are shown in Table 7. Accordingly, the TPC and TFC are (4.2392 ± 0.0495) mgGAE/g and (2.1733 ± 0.0817) mgQE/g, respectively.

4 Conclusion

The quantification procedure of total polyphenol and flavonoid content in propolis hydrogels using UV spectrophotometry was validated and achieved selectivity, linearity, accuracy, and precision. Accordingly, for total polyphenol, at 754 nm, the procedure demonstrated a linear equation y = 0.0075x (R^2 = 0.9999) with a detection limit of 1.4469 µg/mL and a quantification limit of 4.3845 µg/mL. For flavonoid, at 427 nm, the procedure demonstrated a linear equation y = 0.0087x – 0.0115 (R^2 = 0.9998) with a detection limit of 2.1744 µg/mL and a quantification limit of 6.5891 µg/mL. Both procedures verified a working range of (10-120) µg/mL, accuracy within (95-102)%, and precision with an RSD < 2%. The TPC and TFC are (4.2392 ± 0.0495) mgGAE/g and (2.1733 ± 0.0817) mgQE/g, respectively. This research is essential for establishing quality control criteria for propolis hydrogel in industrial manufacturing. Compared with more complex techniques such as HPLC, this method offers a faster, cost-effective and robust analytical approach that aligns with ICH Q2(R2) requirements.

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Thẩm định quy trình định lượng polyphenol tổng và flavonoid trong hydrogel keo ong bằng phương pháp quang phổ tử ngoại

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Tóm tắt Keo ong là một hỗn hợp nhựa giàu polyphenol và flavonoid, mang lại các đặc tính kháng khuẩn, chống oxy hóa và làm lành vết thương. Do đó, hydrogel keo ong là vật liệu băng vết thương đầy hứa hẹn, giúp đẩy nhanh quá trình làm lành các vết thương vừa và nhỏ. Nghiên cứu này nhằm mục đích thẩm định phương pháp định lượng polyphenol tổng (TPC) và định lượng flavonoid tổng (TFC) trong hydrogel keo ong. Các phép định lượng đã được thẩm định về tính đặc hiệu, độ tuyến tính, độ chính xác và độ đúng. Kết quả cho thấy độ tuyến tính cao đối với polyphenol và flavonoid trong khoảng nồng độ (10-120) µg/mL lần lượt tại bước sóng 754 nm và 427 nm. Hơn nữa, phương pháp thể hiện tính đặc hiệu cao, độ chính xác (tỷ lệ thu hồi nằm trong khoảng 95 % đến 102 %) và độ lặp lại cao (độ lệch chuẩn tương đối dưới 2 %) cho cả hai chất phân tích. Hàm lượng TPC và TFC trong mẫu hydrogel keo ong lần lượt là (4,2392 ± 0,0495) mgGAE/g và (2,1733 ± 0,0817) mgQE/g. Việc thẩm định này cần thiết trong xây dựng chỉ tiêu định lượng trong sản xuất hydrogel keo ong.

Từ khóa hydrogel keo ong, polyphenol, flavonoid, thẩm định phương pháp, quang phổ UV