Development and validation of quantitative procedure of clotrimazole and eugenol in gel product using high-performance liquid chromatography

Mong To Tam Tran[1*], Thi Thanh Vy Dinh[1], Hoai Hieu Vo[1], Thi Hoang Yen Pham[1], Thi Loan Le[1], Dinh Nga Nguyen[2]

[1]Yersin University, 27 Ton That Tung Street, Ward 8, Da Lat City, Lam Dong Province, Vietnam
[2]University of Medicine and Pharmacy at Ho Chi Minh City, 217 Hong Bang Street, Ward 11, District 5, Ho Chi Minh City, Vietnam

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Abstract:

Vulvovaginitis is a prevalent gynaecological ailment worldwide, often attributed to bacteria, *Candida* fungi, or *Trichomonas*. The synergistic action of antifungal agents and essential oils can enhance the efficacy against pathogenic microorganisms. In this study, a simultaneous quantitative method for determining clotrimazole and eugenol in a laboratory-compounded gel product was developed using high-performance liquid chromatography with a photodiode array detector (HPLC-PDA). The HPLC-PDA system utilised in this study was the Shimadzu LC-20AD, with a HiQ Sil RP C18 HS column (250 x 4.6 mm, 5 µm), and a mobile phase consisting of methanol-water in an 80:20 (v/v) ratio, employing isocratic elution at a flow rate of 1.0 ml/min. The injection volume was set at 20 µl, and detection was performed at a wavelength of 229.0 nm. The analytical procedure was validated in accordance with ASEAN guidelines for the validation of analytical procedures, successfully meeting the criteria for specificity, accuracy, system suitability, repeatability, intermediate precision, and linearity within the concentration range of 2.25 to 36.00 ppm for eugenol and 12.00 to 200.00 ppm for clotrimazole. Subsequently, this validated procedure was applied to quantify the clotrimazole and eugenol content in bulk materials and three different lots of gel products. The results for clotrimazole and eugenol content in both the raw materials and the gel product lots fell within an acceptable range, with deviations of less than ±10% compared to the labelled content.

Keywords: **antibacterial activity, antifungal activity, clotrimazole, high-performance liquid chromatography, *Ocimum gratissimum* essential oil.**

Classification numbers: **2.2, 3.3**

1. Introduction

Vaginitis is a medical condition encompassing various conditions characterised by infection or inflammation of the vagina, with vulvovaginitis being the inflammation affecting both the vagina and vulva. In a healthy vaginal environment, *Lactobacillus* bacteria play a vital role by metabolising glucose into lactic acid, thereby maintaining a normal vaginal pH level ranging from 3.5 to 4.6. The acidic pH fosters the proliferation of *Lactobacillus* by providing advantageous conditions for their growth. *Lactobacillus* also produces hydroperoxide, which effectively combats bacteria and hampers the growth of pathogenic microorganisms. The presence of *Lactobacillus* in vaginal discharge typically amounts to approximately 10^6 colony-forming units per millilitre (CFU/ml) [1]. Pathogenic bacteria can reduce the concentration of *Lactobacillus* to below 10^3 CFU/ml. When vaginal pH levels rise, it creates a conducive environment for the proliferation of pathogenic bacteria, ultimately leading to vulvovaginitis [2].

Clotrimazole is a broad-spectrum antifungal compound, first discovered in the late 1960s, belonging to the azole group. This group is the largest class of antifungals used in clinical treatments, comprising two primary subgroups: imidazole and triazole, with clotrimazole falling under the imidazole subgroup [3]. Clotrimazole operates by inhibiting the methylation of 14-α-lanosterol through cytochrome P450, a critical step in the biosynthesis of ergosterol in fungi. This results in the replacement of ergosterol with 14-α-methyl sterol, an atypical sterol, causing disruptions in the flexibility and permeability of the fungal cell membrane. Clotrimazole not only impedes enzyme activity related to cell membrane synthesis but also enhances the leakage of cell contents through the membrane. Clotrimazole is considered an inhibitor at lower concentrations and a fungicidal agent at higher concentrations [4].

Furthermore, clotrimazole exhibits pharmacological effects such as inhibiting the activity of the Ca^{2+}-ATPase

Corresponding author: Email: totamtran910@gmail.com

channel, disrupting intracellular calcium storage, blocking calcium-dependent potassium channels, and voltage-dependent calcium channels. *In vitro* studies have shown that clotrimazole inhibits the growth of certain normal and cancer cell types. It is also reported to have neuroprotective effects, reducing the toxicity of metal cations to cells. Additionally, clotrimazole demonstrates activity against the malaria parasite *in vitro* due to its inhibitory action on the hemoperoxidase enzyme [4, 5].

Numerous studies have focused on quantifying clotrimazole in various sample types, including biological fluids. For instance, N. Tamilselvi, et al. (2016) [6] developed and validated a novel quantitative procedure for clotrimazole in human plasma using a reversed-phase C18 column and a mobile phase of 0.5% TEA in water-acetonitrile (20:80, v/v). M. Locatelli, et al. (2017) [7] established a robust method for the simultaneous determination of 12 azole compounds in body fluids (plasma and urine). This method employed HPLC-PDA combined with fabric phase sorptive extraction, a Luna C18 column, and a gradient elution mode. C. Campestre, et al. (2017) [8] analysed 12 azole drugs in biological samples using HPLC-PDA with micro-extraction by packed sorbent. The analytical procedure employed a C18 packing column and a gradient mode involving a mixture of phosphate buffer (pH 2.5) and acetonitrile.

Ocimum gratissimum essential oil is extracted from the aerial parts of *Ocimum gratissimum*, a member of the Lamiaceae family. Eugenol constitutes the predominant component, accounting for 45-75% of *Ocimum gratissimum* essential oil, followed by terpinolene and germacrene D. This essential oil effectively inhibits pathogenic bacterial strains, including *Staphylococcus aureus* and *Enterobacteriaceae* such as *Escherichia coli*, *Salmonella typhimurium*, *Salmonella typhi*, *Shigella flexineri*, *Salmonella enteritis*, and *Proteus mirabilis*, with a minimum inhibitory concentration (MIC) ranging from 3 to 12 mg/ml and a minimum bactericidal concentration (MBC) twice that of the MIC [9]. *Ocimum gratissimum* essential oil also demonstrates the ability to inhibit various pathogenic fungi, including *Botryosphaeria rhodia*, *Rhizoctonia* sp., *Alternaria* sp., *Cryptococcus neoformans*, and numerous skin fungi [10].

While clotrimazole is an effective antifungal agent, its activity against bacteria is limited, making it suitable primarily for treating vulvovaginitis resulting from fungal infections. In recent years, researchers have explored the combination of antifungal drugs and essential oils to broaden the spectrum of targeted microorganisms. Some studies have indicated that the coadministration of essential oils with antifungal drugs can significantly enhance their inhibitory activity against various microorganisms. However, limited research has been conducted on the combination of clotrimazole and essential oils. In a previous study, the combination of clotrimazole and *Ocimum gratissimum* essential oil exhibited potent activity against nine bacterial and fungal strains [11]. Building upon this finding, we formulated a laboratory-produced gel product. In this study, we have developed and validated a procedure for quantifying the content of clotrimazole and eugenol, the primary component of *Ocimum gratissimum* essential oil, in this gel product.

2. Materials and methods

2.1. Materials

Clotrimazole was supplied by the Medical Biomaterial and Pharmaceutical Joint Stock Company, Ho Chi Minh City, Vietnam, with a purity of 99%. *Ocimum gratissimum* essential oil, sourced from McWay Beauty Care company (Saroma Brand) in Ho Chi Minh City, Vietnam. Reference standards of clotrimazole were obtained from the Institute of Drug Quality Control Ho Chi Minh City, and eugenol from Sigma-Aldrich, with purities of 99.38 and 99.00%, respectively.

Gel product formulation: Clotrimazole 0.50 g; *Ocimum gratissimum* essential oil 0.15 g; ingredients (propylene glycol, hydroxypropyl methylcellulose, polyethylene glycol 4000, benzyl alcohol, stabiliser) until the total is 100.00 g.

2.2. Methods

Surveying quantitative procedure conditions: Initially, we investigated the composition and proportions of the mobile phase. Standard samples containing clotrimazole and eugenol references, as well as test samples with comparable concentrations, were prepared. The experiments were conducted under the suggested chromatographic conditions, which included:

- Column: HiQ Sil RP C18 HS (250 x 4.6 mm, 5 µm) or Supelco RP C18 (250 x 4.6 mm, 5 µm).
- Detection wavelength: 229.0 nm.
- Flow rate: 1.0 ml/min.
- Injection volume: 20.0 µl.
- Mobile phase: 1. Acetonitrile-water 90:10 (v/v) or 2. Methanol-water 80:20 (v/v) or 3. Methanol-water 90:10 (v/v).

Based on the chromatographic parameters for clotrimazole and eugenol peaks, we selected the most suitable mobile phase and column type. Subsequently, we explored two sample treatment methods:

Method 1: Weighed 1.0 g of gel, diluted it with methanol in a 10.0 ml volumetric flask, then further diluted this solution ten-fold with the same solvent. Afterwards, the solution was filtered through a 0.45 µm membrane filter to obtain the test sample.

Method 2: Weighed 1.0 g of gel, diluted it with dichloromethane, and completely evaporated the solvent. The resulting residue was then diluted with methanol in a 10.0 ml volumetric flask, followed by a ten-fold dilution with the same solvent. The solution was then filtered through a 0.45 µm membrane filter to obtain the test sample.

We conducted experiments using the chosen conditions with mobile phases 2 and 3. The most suitable sample treatment method was selected based on the chromatogram parameters. Lastly, we investigated the number of extractions with dichloromethane. The test sample was treated with Method 2, subjected to repeated extractions with dichloromethane, and chromatography was carried out until no clotrimazole and eugenol peaks were detected on the chromatograms.

Validation of the simultaneous quantitative procedure of clotrimazole and eugenol: The quantitative procedure was validated following the ASEAN guidelines for the validation of analytical procedures, which includes assessments for specificity, linearity and range, accuracy, and precision [12].

Determination of clotrimazole and eugenol contents in bulk materials and products: Standard samples of clotrimazole and eugenol were prepared with concentrations of 50.0 and 9.0 ppm, respectively. The clotrimazole sample was prepared by weighing 1.0 g of clotrimazole standard, dissolving it in methanol, and diluting it with the same solvent to obtain a 50.0 ppm solution. The *Ocimum gratissimum* essential oil sample was prepared by weighing 1.0 g of the oil, dissolving it in methanol, and diluting it with the same solvent to obtain a 9.0 ppm solution.

Both test samples and standard samples underwent the chromatography procedure, and the content of clotrimazole and eugenol in the materials was calculated. For the gel products, test samples and standard samples were prepared and subjected to the chromatography process according to the specified procedures. The contents of clotrimazole and eugenol in 100 g of the gel product were calculated using the following equation:

$$X = \frac{S_T}{S_S} \times \frac{a}{1000} \times C\% \times \frac{K_T}{K_S} \times \frac{100}{m}$$

where, X: the weight of eugenol (clotrimazole) in 100 g of gel product (g); S_T: the peak area of eugenol (clotrimazole) in the test sample; S_S: the peak area of eugenol (clotrimazole) in the standard sample; a: the weight of eugenol (clotrimazole) standard (g); $C\%$: the purity of eugenol (clotrimazole) standard; K_T, K_S: the dilution level of the test sample and standard sample; m: the weight of the product (g).

3. Results and discussion

3.1. Surveying quantitative procedure conditions

Until this point, there has been no prior research dedicated to the development of a simultaneous quantitative procedure for eugenol and clotrimazole in a product. This lack of research can be attributed to the disparate structural and physicochemical characteristics of these compounds. Eugenol is typically quantified using gas chromatography, whereas clotrimazole quantification methods often rely on HPLC. Considering this, and after consulting relevant literature, it was established that both eugenol and clotrimazole could be quantified individually using HPLC, which is a widely adopted and suitable technique for laboratory use. Consequently, we proceeded to develop a simultaneous quantitative procedure for eugenol and clotrimazole utilising HPLC-PDA.

Figure 1 illustrates that the Supelco column had the capability to separate the eugenol and clotrimazole peaks, but the associated parameters did not meet the desired criteria. Conversely, the HiQ Sil column effectively separated the eugenol and clotrimazole peaks while yielding superior parameters. The use of an acetonitrile-water solvent led to an asymmetrical peak and prolonged chromatographic duration. Hence, we opted for the HiQ Sil column in conjunction with a methanol-water mobile phase for the chromatographic procedure.

Most references in the field employed a reversed-phase C18 column for the quantification of eugenol and clotrimazole. We investigated two types of columns, Supelco and HiQ Sil, both of which employed octadecyl carbon chain bonded silica as the stationary phase. However, they differed in terms of stationary phase particles and silanisation content [13, 14]. The HiQ Sil column employed high-purity silica gel particles and a high carbon loading content, resulting in sharper and more symmetrical peaks. Our quantitative findings revealed that the eugenol and clotrimazole peaks obtained using the Supelco column exhibited tailing, whereas those achieved with the HiQ Sil column displayed

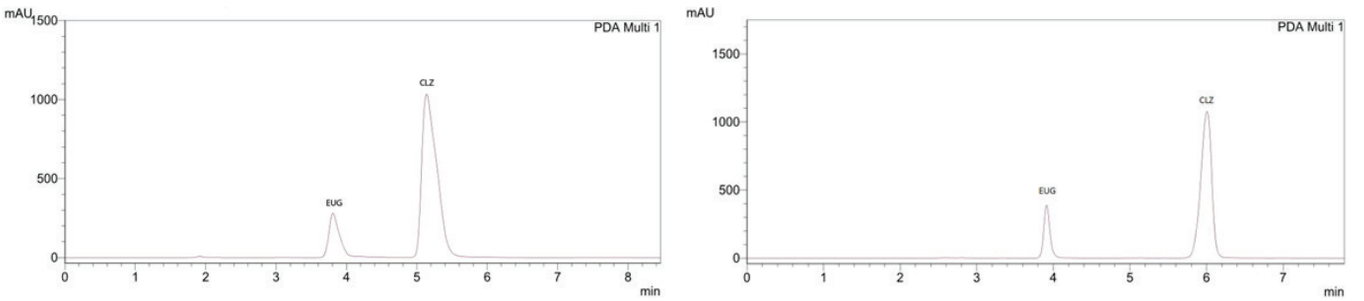

Fig. 1. Chromatograms of eugenol and clotrimazole with Supelco column (left) and HiQ Sil column (right).

satisfactory asymmetry factors. Consequently, we selected the HiQ Sil column for the simultaneous quantification of eugenol and clotrimazole.

Upon reviewing prior research, we ascertained that standard mobile phases such as acetonitrile-water and methanol-water were typically used, with buffer solutions designed specifically for clotrimazole quantification. S.S. Santos, et al. (2012) [15] employed methanol-water (90:10, v/v) with isocratic elution at a flow rate of 1.0 ml/min to determine clotrimazole content. S.J. Liu, et al. (2010) [16] utilised methanol-water (65:35, v/v) in an isocratic elution mode at a flow rate of 1.0 ml/min for eugenol quantification. Although acetonitrile was also tested as a mobile phase on both columns, the resulting peaks did not exhibit satisfactory asymmetry factors and necessitated extended analysis times. As a result, acetonitrile was excluded from the quantification process. Following experimentation with various methanol-water ratios, we concluded that the ratio of 80:20 (v/v) was optimal for the simultaneous quantification of eugenol and clotrimazole.

Results of the test sample treatment method survey are depicted in Fig. 2. Method 1 was straightforward and easily implemented, but the ensuing chromatograms displayed multiple impurity peaks that remained unresolved from the primary analytical peaks. Conversely, Method 2, although more intricate and comprising multiple stages, effectively eliminated a substantial portion of impurities and separated the primary peaks. The peak obtained using methanol-water 80:20 (v/v) achieved the desired level of purity and met all other requisite parameters, while the peak obtained using methanol-water 90:10 (v/v) exhibited unsatisfactory purity.

In terms of the number of extraction times, the chromatogram obtained after the 4th extraction did not

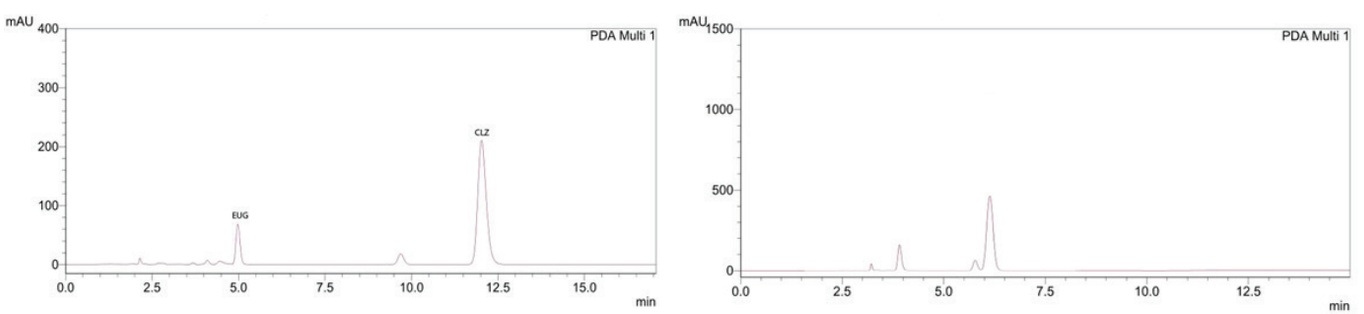

Fig. 2. Chromatograms of test samples treated by Method 2 using methanol-water 80:20 (v/v) (left) and methanol-water 90:10 (v/v) (right).

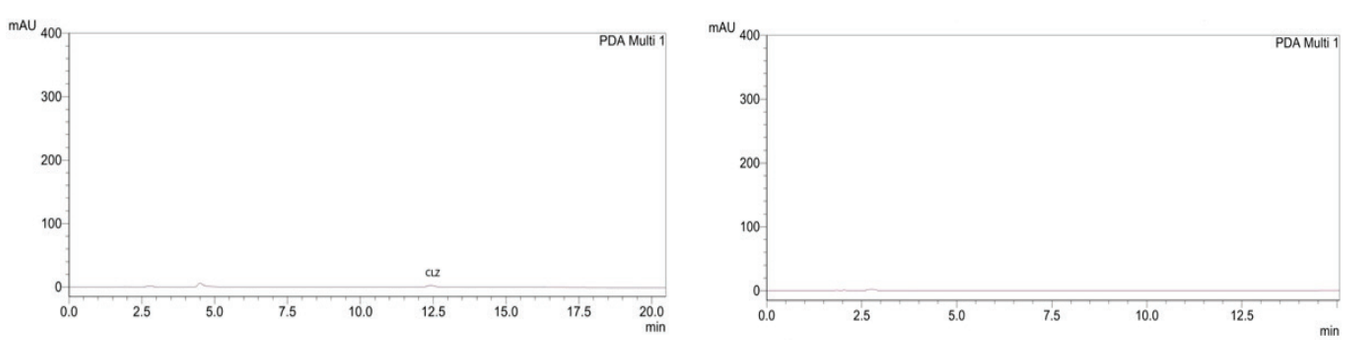

Fig. 3. Chromatograms of eugenol and clotrimazole of the 3rd (left) and 4th (right) extraction.

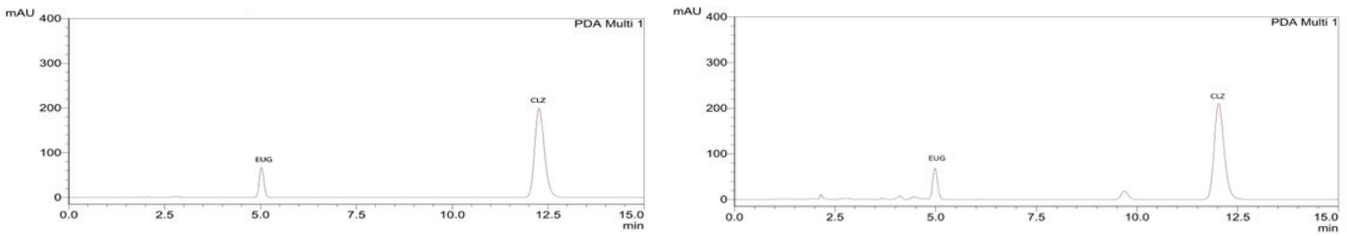

Fig. 4. Chromatograms of eugenol and clotrimazole in the standard sample (left) and test sample (right) under the optimal conditions.

exhibit any eugenol and clotrimazole peaks (Fig. 3). This implies that conducting 3 extraction cycles was adequate to fully extract eugenol and clotrimazole from the test sample.

The effectiveness of the simultaneous quantitative procedure for eugenol and clotrimazole relied on the treatment method employed to eliminate impurity peaks from the chromatograms, which can impact the quantification results. To extract eugenol and clotrimazole from the gel product and eliminate impurity peaks, we utilised dichloromethane as a solvent, followed by dissolving the extracted components in methanol. This process successfully removed numerous impurity peaks, completely separated the primary peaks, and yielded favourable chromatographic parameters. Incomplete extraction of the active components would have introduced quantification errors. Consequently, we also investigated the number of extraction cycles with dichloromethane, ultimately determining that a three-step extraction process was optimal for sample treatment.

The optimal chromatography conditions were chosen:
- Instrument: HPLC-PDA Shimadzu LC-20AD System.
- Column: HiQ Sil RP C18 HS (250 x 4.6 mm, 5 µm).
- Mobile phase: Methanol-water 80:20 (v/v).
- Elution mode: Isocratic.
- Flow rate: 1.0 ml/min.
- Injection volume: 20.0 µl.
- Detection wavelength: 229.0 nm.

With these selected chromatography conditions, the chromatograms exhibited well-resolved eugenol and clotrimazole peaks with satisfactory peak parameters (Fig. 4). The retention time of eugenol and clotrimazole in the test sample matched that in the standard sample.

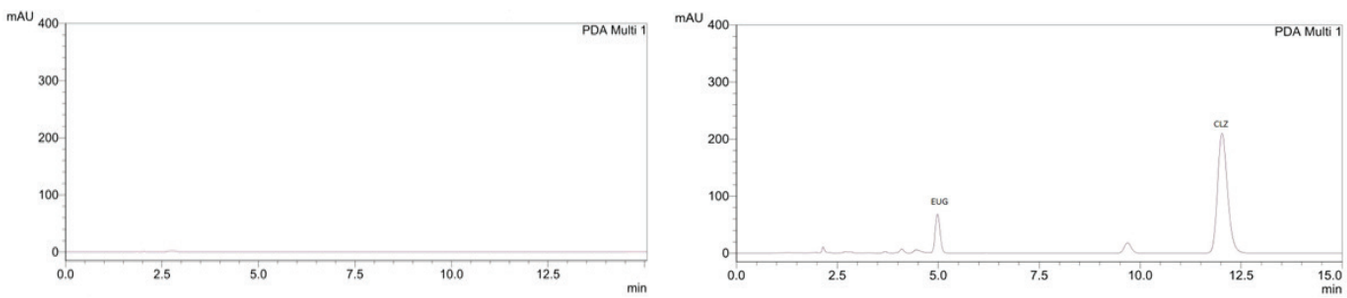

Fig. 5. Chromatograms of the blank sample (left) and test sample (right).

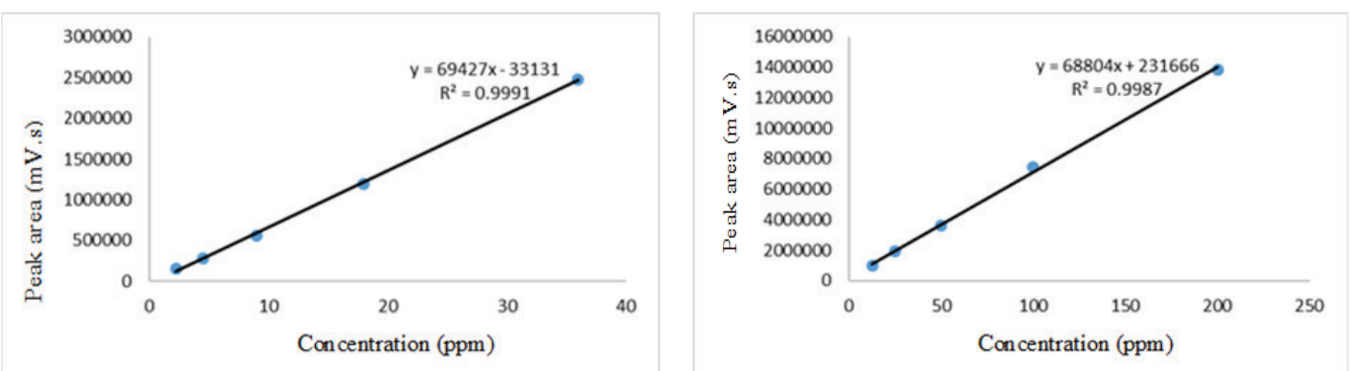

Fig. 6. Regression equations of eugenol (left) and clotrimazole (right) peaks.

3.2. Validating the quantitative procedure

Specificity: Fig. 5 confirms that the chromatograms of the blank sample did not display any peaks with the same retention time as the eugenol and clotrimazole standards. In the chromatogram of the test sample, eugenol and clotrimazole peaks with similar retention times to those of the standards were observed. In the chromatograms of the standard samples, the eugenol and clotrimazole peaks were entirely separated from impurity peaks. The eugenol and clotrimazole peaks in the chromatograms of both the test and standard samples exhibited good purity (peak impurity of approximately 100%). The spectral overlay coefficient of the eugenol and clotrimazole peaks in the chromatograms of the test and standard samples was approximately 1.0.

Linearity and range: Fig. 6 illustrates the linearity of the quantitative procedure for eugenol and clotrimazole. The suitability of the regression equation and the significance of the coefficients "a" and "b" were verified using the T-test, F-test, and ANOVA analysis with Microsoft Excel software. The regression equation for eugenol was $\hat{y}$=69,427x (R^2=0.9991), and for clotrimazole, it was $\hat{y}$=68,804x (R^2=0.9987). The linear range for eugenol and clotrimazole was 2.25-36.00 ppm and 12.50-200.00 ppm, respectively, with R^2 values exceeding 0.99.

Accuracy: Table 1 illustrates the accuracy of the quantitative procedure for eugenol and clotrimazole.

The quantitative procedure exhibited a recovery rate of 95.0-105.0% for eugenol and 97.0-103.0% for clotrimazole, with RSD%≤2.0% (Table 1).

Table 1. The accuracy of the eugenol and clotrimazole quantitative procedure.

Concentration	Eugenol			Clotrimazole		
	Amount of added standard	*Amount of found standard*	*Recovery rate (%)*	*Amount of added standard*	*Amount of found standard*	*Recovery rate (%)*
80%	7.20	6.83	94.84	40.00	40.61	101.53
	7.20	6.83	94.87	40.00	39.76	99.40
	7.20	6.98	96.93	40.00	41.07	102.67
	Average		*95.55*	*Average*		*101.20*
	RSD%		*1.25*	*RSD%*		*1.64*
100%	9.00	8.70	96.65	50.00	50.24	100.48
	9.00	8.77	97.48	50.00	51.56	103.12
	9.00	8.52	94.66	50.00	49.91	99.83
	Average		*96.26*	*Average*		*101.14*
	RSD%		*1.51*	*RSD%*		*1.73*
120%	10.80	10.47	96.98	60.00	60.29	100.49
	10.80	10.41	96.43	60.00	60.11	100.18
	10.80	10.54	97.61	60.00	60.74	101.24
	Average		*97.01*	*Average*		*100.64*
	RSD%		*0.61*	*RSD%*		*0.54*

Table 2. The system suitability of the eugenol and clotrimazole quantitative procedure.

No.	Eugenol				Clotrimazole			
	RT	*Peak area*	*Peak height*	*Asymmetry*	*RT*	*Peak area*	*Peak height*	*Asymmetry*
1	5.026	537,646	59,206	1.17	12.355	3,435,165	188,728	1.18
2	5.028	513,952	56,550	1.17	12.329	3,266,724	179,641	1.18
3	5.033	521,251	57,303	1.17	12.330	3,319,747	182,651	1.18
4	5.031	525,383	57,758	1.16	12.347	3,325,773	183,014	1.24
5	5.034	525,366	57,847	1.17	12.353	3,345,920	183,582	1.18
6	5.029	532,316	58,980	1.17	12.344	3,362,791	180,214	1.19
Average	5.030	525,986	57,941		12.343	3,342,687	182,972	
SD	0.003	8,294.70	1,006.38		0.011	55,766.49	3,234.52	
RSD%	0.06	1.58	1.74		0.09	1.67	1.77	

Precision: The system suitability: The RSD% values for the retention time and peak area of eugenol and clotrimazole were ≤2.0%. All other parameters of the two active ingredients met the standard criteria for liquid chromatography methods (Table 2).

Repeatability: The RSD% values for the eugenol and clotrimazole quantitative procedure in test samples were ≤2.0%. Other parameters of the two active components met the standard criteria for the liquid chromatography method (Table 3).

Table 3. The repeatability of the eugenol and clotrimazole quantitative procedure.

	Active ingredient content (%)	
	Eugenol	*Clotrimazole*
1	0.088	0.496
2	0.088	0.501
3	0.089	0.507
4	0.089	0.510
5	0.089	0.511
6	0.088	0.496
Average	0.088	0.503
SD	0.0004	0.0068
RSD%	0.49	1.36

Intermediate precision: In test samples, the RSD% value for the eugenol and clotrimazole quantitative procedure was ≤2.0%, and the difference in active ingredient content between the two analysts was ≤2.0% (eugenol 1.67% and clotrimazole 1.54%) (Table 4). In summary, the method was validated according to ASEAN guidelines for the validation of analytical procedures, encompassing specificity, linearity and range, accuracy, system suitability, repeatability, and intermediate precision.

Table 4. The intermediate precision of the eugenol and clotrimazole quantitative procedure.

	Eugenol		Clotrimazole	
	Analyst 1	*Analyst 2*	*Analyst 1*	*Analyst 2*
1	0.088	0.090	0.496	0.515
2	0.088	0.089	0.501	0.492
3	0.089	0.092	0.507	0.517
4	0.089	0.091	0.510	0.503
5	0.089	0.088	0.511	0.502
6	0.088	0.091	0.496	0.505
Average	0.088	0.090	0.503	0.506
SD	0.0004	0.0015	0.0068	0.0092
RSD%	0.49	1.70	1.36	1.81

The procedure was developed to quantify eugenol and clotrimazole in a combination gel product containing 0.5% clotrimazole and 0.15% *Ocimum gratissimum* essential oil (equivalent to 0.09% eugenol). The peaks of eugenol and clotrimazole met all specified parameters. The procedure was validated according to the ASEAN Guidelines for the validation of analytical procedures, covering specificity, linearity and range, accuracy, system suitability, repeatability, and intermediate precision. Validation results for all criteria were excellent, with linear ranges of 2.25-36.00 ppm for eugenol and 12.50-200.00 ppm for clotrimazole.

3.3. Determination of the eugenol and clotrimazole content in material and gel products

The content of clotrimazole and eugenol in the material and three lots of gel products has been documented in Tables 5 and 6.

Table 5. Content of eugenol and clotrimazole in the material.

Bulk sample	Eugenol content (%)	Clotrimazole content (%)
Ocimum gratissimum essential oil	60.71	
Clotrimazole		98.46

Table 6. Content of eugenol and clotrimazole in three lots of gel product.

Gel sample	Content of active ingredients in 100 g of gel product	
	Eugenol (g)	*Clotrimazole (g)*
Lot 1	0.087	0.483
Lot 2	0.092	0.498
Lot 3	0.084	0.527

The procedure was employed to ascertain the eugenol and clotrimazole content in the bulk and three lots of gel products, which were compounded in the laboratory. The clotrimazole content in the bulk and eugenol in the *Ocimum Gratissimum* essential oil was found to be consistent with the specifications provided in the bulk certificate of analysis. These materials were deemed pure and of high quality, suitable for use in the formulation of pharmaceutical products. In accordance with the Vietnamese Pharmacopoeia 5th Edition, the allowable variation for active ingredient content in topical products was within ±10% of the label contents [17]. Analysis of the eugenol and clotrimazole contents in the three lots of gel products revealed that they fell within the permissible limits.

4. Conclusions

This study has successfully developed and validated a simultaneous quantitative procedure for determining the content of clotrimazole and eugenol. The method was effectively applied to assess the content of these components in the raw materials and in three batches of the final product. The procedure exhibited robust performance in terms of specificity, linearity, accuracy, system suitability, repeatability, and intermediate precision. The results obtained from the analysis of the bulk materials and three batches of the gel product demonstrated that the eugenol and clotrimazole content adhered to the standards set by the Vietnamese Pharmacopoeia 5th Edition.

CRediT author statement

Mong To Tam Tran: Methodology, Investigation, Writing; Thi Thanh Vy Dinh: Formal analysis, Validation; Hoai Hieu Vo: Validation; Thi Hoang Yen Pham: Writing; Thi Loan Le: Editing; Dinh Nga Nguyen: Conceptualisation, Supervision.

COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

REFERENCES

[1] K. Itriyeva (2020), "Evaluation of vulvovaginitis in the adolescent patient", *Current Problems in Pediatric and Adolescent Health Care*, **50(7)**, DOI: 10.1016/j.cppeds.2020.100836.

[2] Department of Obstetrics and Gynecology (2007), *Obstetrics and Gynecology*, Medical Publishing House, **2**, Ho Chi Minh City, pp.752-755 (in Vietnamese).

[3] The Ministry of Health (2007), *Medicinal Chemistry*, Medical Publishing House, **2**, Hanoi, pp.190-191 (in Vietnamese).

[4] P.D. Crowley, H.C. Gallagher (2014), "Clotrimazole as a pharmaceutical: Past, present and future", *Journal of Applied Microbiology*, **117(3)**, pp.611-617, DOI: 10.1111/jam.12554.

[5] E.J. Ernst, P.D. Rogers (2005), *Antifungal Agents - Methods and Protocols*, Publisher: Humana Press, New Jersey, 220pp.

[6] N.Tamilselvi, H. Sinha, D. Visakh, et al. (2016), "Bio-analytical method development and validation for the estimation of Clotrimazole in human plasma by RP-HPLC method", *Research J. of Pharm. and Tech*, **9(6)**, pp.671-676, DOI: 10.5958/0974-360X.2016.00126.8.

[7] M. Locatelli, A. Kabir, D. Innosa, et al. (2017), "A fabric phase sorptive extraction-High performance liquid chromatography-Photo diode array detection method for the determination of twelve azole antimicrobial drug residues in human plasma and urine", *Journal of Chromatography B*, **1040**, pp.192-198, DOI: 10.1016/j.jchromb.2016.10.045.

[8] C. Campestre, M. Locatelli, P. Guglielmi, et al. (2017), "Analysis of imidazoles and triazoles in biological samples after MicroExtraction by packed sorbent", *Journal of Enzyme Inhibition and Medicinal Chemistry*, **32(1)**, pp.1053-1063, DOI: 10.1080/14756366.2017.1354858.

[9] A.S. Adesegun, F.O. Samuel, O.B. Anthony, et al. (2013), "Antioxidant and inhibitory properties of essential oil of *Ocimum gratissimum* against extracellular protease of *Escherichia coli*", *IOSR Journal of Pharmacy*, **3(1)**, pp.50-55, DOI: 10.9790/3013-31305055.

[10] R.K. Joshi (2013), "Chemical composition, *in vitro* antimicrobial and antioxidant activities of the essential oils of *Ocimum gratissimum*, *O. sanctum* and their major constituents", *Indian Journal of Pharmaceutical Science*, **75(4)**, pp.457-462, DOI: 10.4103/0250-474X.119834.

[11] T.M.T. Tam (2022), "Evaluating the antifungal and antibacterial activities of clotrimazole in combination with essential oils", *Yersin Journal of Science*, **13**, pp.86-97 (in Vietnamese).

[12] Association of South East Asian Nations (2012), *ASEAN Guidelines for Validation of Analytical Procedures*, https://asean.org/wp-content/uploads/2012/10/Asean-Analytical-Validation-gl.pdf, accessed 16 February 2023.

[13] KYA Technologies Corporation (2013), *HiQ Sil™ HPLC Column*, pp.2-5, https://gimitec.com/file/HiQsil.pdf, accessed 16 February 2023.

[14] Sigma-Aldrich (2014), *HPLC and UHPLC Column Selection Guide*, pp.35-36, https://www.sigmaaldrich.com/deepweb/assets/sigmaaldrich/product/documents/340/058/hplc-columns-guide-br7614en-mk.pdf, accessed 16 February 2023.

[15] S.S. Santos, L.M. Ferreira, S.R. Schaffazick, et al. (2012), "A simple RP-HPLC method for the determination of clotrimazole from acrylic nanocapsule suspensions", *Latin American Journal of Pharmacy*, **31(3)**, pp.482-486.

[16] S.J. Liu, Z.T. Lin, H. Wang, et al. (2010), "Quantification of eugenol and bancroftione in *Caryophylli* Fructus using high-performance liquid chromatography", *Journal of Chinese Pharmaceutical Sciences*, **19**, pp.459-463, DOI: 10.5246/jcps.2010.06.061.

[17] The Vietnam Ministry of Health (2017), *Vietnamese Pharmacopoeia*, 5th Edition, Medical Publishing House, **1-2**, Hanoi, 2120pp (in Vietnamese).