Chemistry Research Journal, 2018, 3(2):33-41

Available online www.chemrj.org

Research Article

ISSN: 2455-8990
CODEN(USA): CRJHA5

Development of a Validated Reversed Phase HPLC-UV Method for a Simultaneous Determination of Hederacoside C and Thymol in Ivy-Thyme Cough Syrup

Ola M. Abdallah[a,b] , Amira Abdel Motaal[c,d] , Sara A.A. Elbahrawy[a,e*]

[a] Analytical Chemistry Department, Faculty of Pharmacy, Al-Azhar University (Girls), Cairo, Egypt.
[b] Pharmaceutical Chemistry Department, Faculty of Pharmacy, Badr University in Cairo (BUC), Badr City, Cairo, Egypt.
[c] Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Egypt.
[d] Pharmacognosy Department, Faculty of Pharmacy, King Khaled University, Abha, Kingdom of Saudi Arabia
[e] Pharmaceutical research and development department, Faculty of Pharmacy, Heliopolis University for sustainable development, Cairo, Egypt.

Abstract Thyme herb (*Thymus vulgaris, Lamiaceae*) and ivy leaves (*Hedera helix* L., Araliaceae) are evergreen plants which have culinary, medicinal, and ornamental uses. The extracts of thyme and ivy are commercially incorporated into a pharmaceutical dosage form (syrup) used as an expectorant to treat cough and bronchitis. The objective of the present study was to develop a simple, reliable, and validated analytical method for the quantification of hederacoside C "triterpene saponin" and thymol "phenol" simultaneously by using reversed phase high-performance liquid chromatography (RP-HPLC) with UV detection, a method that can be used efficiently for routine quality control and the analysis of the commercially available syrup containing ivy and thyme extracts. RP-HPLC gradient elution method was developed for quantitative determination of the hederacoside C and the thymol using acetonitrile and 0.2% ortho- phosphoric acid solution at wave length 205 and 274nm. A standard calibration curve, established for hederacoside C and thymol at a concentration range of 50-400 µg/ml and 30-90 µg/ml, respectively that showed good linearity with a correlation coefficient (R) of 0.999 for both standards. Furthermore, the mean recovery of the method for hederacoside C and thymol were 100.13% and 99.96%, respectively whereas the value of precision (relative standard deviation values <1%) was achieved. Finally, the limits of detection and quantification were 1 and 3.3 µg/ml for hederacoside C and 0.3 and 0.97 µg/ml respectively. A simultaneous RP-HPLC method was developed for the first time to achieve the separation of hederacoside C and thymol in the commercially available syrup. The developed method was found to be very simple, cheap, sensitive and fairly selective than other reported methods.

Keywords Ivy, thyme, hederacoside C, thymol, RP-HPLC, validation

1. Introduction

Ivy-thyme cough syrup (Thymotal plus) is a herbal medicine that combines two extracts: ivy leaves with thyme herb for the purpose of cough treatment.

As a medicinal plant, thyme (*Thymus vulgaris*) is an evergreen herb with culinary, medicinal, and ornamental uses, family (Lamiaceae) [1]. Thyme is used for the treatment of bronchitis, whooping cough, and upper respiratory inflammation [2] .

Oil of thyme was the important commercial product obtained by distillation of the fresh leaves and flowering tops of thyme. Its chief constituents are from 20-25% of the phenols, thymol and carvacrol (an isomeric phenol), rising in rare cases to 42 %. Phenols are the principal constituents of thyme oil, thymol being the most valuable for medicinal purposes [3].

Figure 1 illustrates the chemical structure for thymol.

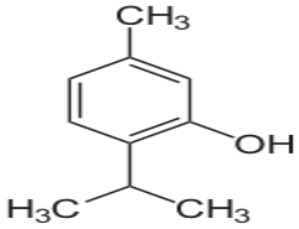

Figure 1: Chemical structure for thymol (5-Methyl-2-(propan-2-yl)phenol) [3]

Ivy *(Hedera helix L.*) is a rampant, clinging evergreen vine , family Araliaceae, native to most of Europe and western Asia [4]. Its efficacy against chronic inflammatory bronchial conditions and productive coughs due to its actions as an expectorant and its spasmolytic effect among children and adults [5, 6].

The biologically active compounds responsible for the medicinal use of ivy are triterpenoid saponins (2.5-6%),the bidesmosidic glycosides of hederagenin, oleanolic acid (hederacoside C,B,D,E,F,G,H,I) and the monodesmoside α- hederin [7]. Therefore, hederacoside C will be used in the present study to standardize the ivy extract.

Figure 2 illustrates the chemical structure of hederacoside C.

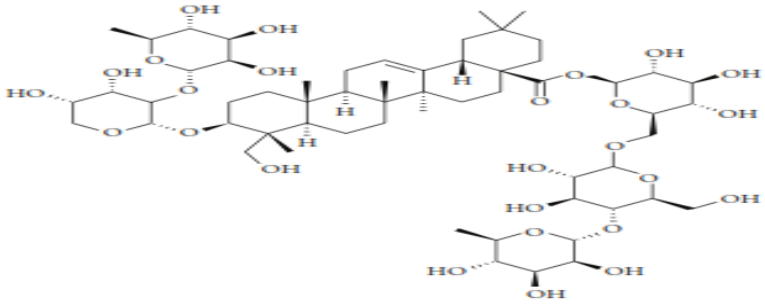

Figure 2: Chemical structure of hederacoside C (3-[[2-O-(-L-Rhamnopyranosyl)—L arabinopyranosyl]oxy]-23-hydroxyolean-12-en-28-oic acid 6-O-[4-O-(-L-rhamnopyranosyl)--D-glucopyranosyl]--D-glucopyranosyl ester) [8]

The objective of the present study was to develop a simple, reliable, and validated analytical method for the quantification of hederacoside C "triterpene saponin" and thymol "phenol" simultaneously by using reversed phase high-performance liquid chromatography (RP-HPLC) with UV detection, this method can be used efficiently for routine quality control and for the analysis of the commercially available syrup containing ivy and thyme extracts.

2. Experimental

2.1. Instrumentation

An Agilent technologies 1100 series HPLC was used, equipped with an Agilent 1260 series G1322A degasser, an Agilent 1260 series G1329B auto sampler, a G1311A Quaternary pump, a G1314A variable wavelength detector and an Agilent ChemStation software, Santa Clara, California, United States software.

2.2. Materials and Reagents

2.2.1. Pure samples

Hederacoside C standard was obtained from LGC standards, USA; with 99.6% purity.
Thymol standard was obtained from LOBA Chemie, India; with 99% purity.

2.2.2. Extracts and market samples

-Ivy powdered extract, manufactured by Finzelberg, Germany.
-Thyme liquid extract, manufactured by Atos Company, Egypt.
-Market sample: Thymotal plus syrup, batch no. L060315. Each 5 ml labeled to contain 35 mg ivy extract(which standardized to contain min 10% hederacoside C) and 2000 mg thyme extract (which standardized to contain min. 0.03% of phenolic compound calculated as thymol) , manufactured by Atos for production of medicinal herbs (Atos Pharma), Cairo, Egypt.

2.2.3. Chemicals

Chromatographic HPLC grade water and acetonitrile were purchased from Fischer scientific, UK and the analytical grade O-phosphoric acid was purchased from Honey well, Germany. The samples which will be introduced to the HPLC was filtered through 0.45 μm syringe filters, Agilent, USA, and the acidic aqueous solution used for HPLC analysis was filtered through 0.45 μm cellulose nitrate filter Agilent, USA.

Standard Solutions

Stock solutions of the standard drugs (1 mg ml^{-1}) were prepared by dissolving hederacoside C or thymol in methanol.

2.3. Chromatographic conditions

The chromatographic separation was performed at room temperature using a Zorbax RP HC-C18, 5 μm, 250 mm × 4.6 mm column (Agilent, USA) guarded by a 5 μm, 12.5 mm × 4.6 mm guard column, Agilent, USA, the mobile phase consisted of acetonitrile (solvent A) and 0.2% O-phosphoric acid in water (solvent B) with gradient elution (20-80% A and B) for 15 min then 50-50% till 30 min. The mobile phase was pumped at flow rate 1ml min -1 for at least 30 minutes before injecting the drug solutions to equilibrate the chromatographic column. The injected volume was 20 μl and the eluents were monitored at 205 nm for hederacoside C till 15 min and then at 274 nm for thymol.

2.4. Construction of Calibration Curve

Aliquots of stock solutions of the standard drug (1 mg ml^{-1}) containing 500-4000 μg ml^{-1} of hederacoside C were introduced into a series of 10 ml volumetric flasks.
Into another set of 10 ml volumetric flasks, aliquots of stock solutions of the standard drug (1 mg ml^{-1}) containing 300-900 μg ml^{-1} of thymol were introduced. The volume of both sets was adjusted to 10 ml using methanol. Triplicate of 20 μL injections were made for each concentration and chromatographed under the specified chromatographic conditions described previously. The Peak area of each concentration was plotted against the corresponding concentration to obtain the calibration graph of hederacoside C and thymol.

2.5. Assay of Laboratory Prepared Mixture

Different aliquots of hederacoside C (1 mg ml^{-1}) and thymol (1 mg ml^{-1}) were introduced into a series of 10 ml volumetric flasks, and then adjusted to the volume using methanol. 20μL of each obtained solution was injected into the HPLC column and the corresponding chromatograms were monitored at 205 nm for hederacoside C and at 274 nm for thymol using the same previous chromatographic conditions. The concentration of each drug in the prepared mixtures was calculated from the regression parameters.

2.6. Application to Pharmaceutical Dosage Form

Each 15 ml of Thymotal plus syrup labeled to contain 10.5 mg hederacoside C and 1.8 mg thymol. 15 ml of syrup was transferred into a 50 ml volumetric flask, extracted with methanol, mixed well, then shaked for 15 min using an

ultrasonic and adjusted to the volume using methanol, and finally filtered via 0.45 µM syringe filter PTFE and used for injection. The drug concentrations were calculated from the appropriate regression parameters.

3. Results and Discussion

3.1. Method Development and Optimization

The aim of the present study was the development of RP-HPLC analytical method for the simultaneous determination of hederacoside C and thymol.

The development of this method is considered a challenge which is attributed to the presence of hederacoside C and thymol in a complex combination of the cough syrup. Therefore, the initial efforts to develop an isocratic reversed-phase HPLC method were unsuccessful.

Because of the different polarity of the active compounds, so it was necessary to develop a gradient method.

In addition, hederacoside C, like almost all other saponins, lacks a chromophore; it absorbs UV-light at wavelengths below 210 nm [9, 10], which makes its analysis by the use of gradient elution not an easy task [11].

Also, the presence of other ingredients of the cough syrup may interfere with the chromatographic peak of both compounds.

Actually, the developed and validated reversed phase high-performance liquid chromatographic (RP-HPLC) method is considered to be a novel one for simultaneous determination of the hederacoside C and thymol (figure 3&4), in addition to its application on the analysis of the finished product (Thymotal plus syrup) which contains the two extracts (figure 5).

From literatures, ivy extract was analyzed alone by RP-HPLC [12] while thyme extract was analyzed separately in another work by spectrophotometer, GC or RP-HPLC methods [12-14]. Unlike previous methods of analysis for the compounds of interest, those methods consume time and chemicals, while the

RP-HPLC method, hederacoside C and thymol were determined simultaneously by a simple, rapid, low cost, and fairly selective procedure. It also had the advantage of being accurate (Table1, 2).

Optimization of this method was required several trials using different mobile phases. Acetonitrile - 0.2% O-phosphoric acid, acetonitrile - 0.05 M O-phosphoric acid, acetonitrile – water and 0.1% glacial acetic acid in water - 0.1% glacial acetic acid in acetonitrile in different ratios with isocratic and gradient elution were tried. The best peak shape and adequate separation of the two drugs was obtained by using acetonitrile(solvent A) - 0.2% O-phosphoric acid(solvent B) with gradient elution (20-80% A and B) for 15 min then 50-50% till 30 min.

Different flow rates (0.8-1.5 ml min^{-1}) were tested; good resolution was obtained using 1 ml min^{-1}

Five wave lengths were tried (205, 260, 274, 300 and 370 nm), much sensitive detector response was obtained at 205 nm for hederacoside C and 274 nm for thymol.

Under the described HPLC parameters. The achieved peaks were well defined and resolved free from tailing with retention times of 9.6 minutes for hederacoside C and 25.2 minutes for thymol; Figure (5).

3.2. Method Validation

The developed method was validated according to the guidelines of the International Conference on Harmonization (ICH) for validation of analytical procedures [15]**.** Typical validation characteristics which should be considered are: Linearity, Range, Detection Limit, Quantitation Limit, Accuracy, Precision (Repeatability &Intermediate Precision), Selectivity and Robustness.

3.2.1. Linearity and Range

Linearity was determined by injecting five different concentrations of hederacoside C standard solution (50-400 µg ml^{-1}) and thymol standard solution (30-90 µg ml^{-1}).

Linear regression analysis of hederacoside C and thymol was performed by plotting the mean peak area versus concentration. The correlation coefficient (R^2) of the standard calibration curve was 0.999 for both standards over the range of 50-400 µg ml^{-1} of hederacoside C at 205 nm and 30-90 µg ml^{-1} of thymol at 274 nm, Table (1).

3.2.2. LOD and LOQ

Limit of detection (LOD) and limit of quantification (LOQ) were determined based on the standard deviation of the response (σ) and the slope of the calibration curve (S) according to the ICH guidelines, as follows:

LOD = 3.3 (σ/S), LOQ = 10 (σ/S).

The LOD and LOQ for hederacoside C and thymol were 1 and 3.3 μg ml^{-1} and 0.3 and 0.97 μg ml^{-1}, respectively, Table (1).

3.2.3. Accuracy and Precision

The accuracy and precision of the procedure were determined using three different concentrations of pure samples of the drug covering the specified range, each in triplicate, within one day for intraday analysis and different three days for interday analysis.

To determine the intra- and inter-day precision of the method, hederacoside C and thymol were assayed at three different concentrations (100, 200 and 300 μg ml^{-1}) and (60, 70 and 80 μg ml^{-1}), respectively. The experiment was performed using nine replicates (n=9) for intra-day and on 3 separate days in triplicates (n=9) for inter-day. RSD % was taken as a measure of precision while the accuracy of the method was expressed as (R%); the results were summarized in Table (2).

3.2.4. Selectivity

Method selectivity was assured by applying it to laboratory prepared mixtures of studied drugs at different concentrations within the linearity range.

Analysis of the laboratory prepared mixtures indicated high selectivity of this method for simultaneous determination of hederacoside C and thymol in binary mixtures; Table (3).

3.2.5. Robustness

The robustness was checked by studying the effect of different sources of acetonitrile: Fischer and Sigma-Aldrich, and the effect of changing the percentage of O-phosphoric acid.

It was found that, these deliberate variations did not affect the system suitability parameters; capacity factor (k`), number of theoretical plates (N), resolution (R), selectivity (α) and tailing factor (T) confirming robustness of the method and the RSD found to be < 2% which was also in favor of the developed RP-HPLC method; Table (4).

3.2.6. Stability of Standard Solutions

The stability of thymol and hederacoside C solutions was evaluated by analyzing two different solutions; one of them was kept at room temperature while the other was kept in the refrigerator at 4^0C against freshly prepared standards. The results showed that thymol solution was stable for one week either kept at room temperature or in refrigerator, while hederacoside C was stable for five hours at room temperature and one week being kept in the refrigerator.

3. 3. Application to Pharmaceutical Dosage Form

The HPLC method was applied for the simultaneous determination of both hederacoside C and thymol in the pharmaceutical dosage form. Three replicate determinations were made and the obtained results were in good agreement with the label claim, where no interference from excipients and additives was observed as shown in Table (5).

The recovery of the procedure was assessed by applying standard addition technique, results are shown in Table (5). The results obtained from the HPLC analysis of Thymotal plus syrup were statistically compared with those obtained by the reported methods, [15] for determination of hederacoside C and [16] for determination of thymol. As revealed in Table (6), the calculated t- and F- values are less than the tabulated ones indicating no significance difference between the proposed and reported methods, confirming accuracy and precision at 95% confidence limit.

However, the developed method is more sensitive and more selective than the reported ones which could not achieve simultaneous determination of both components.

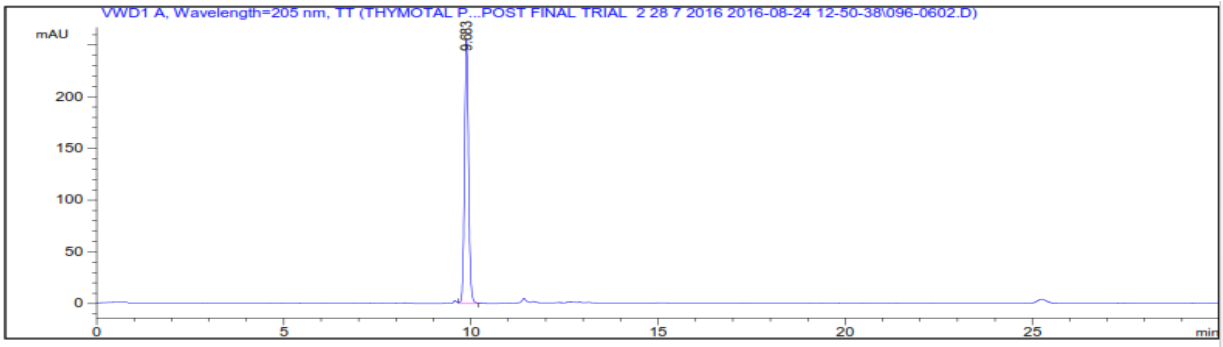

Figure 3: HPLC chromatogram of hederacoside C standard at 205 nm

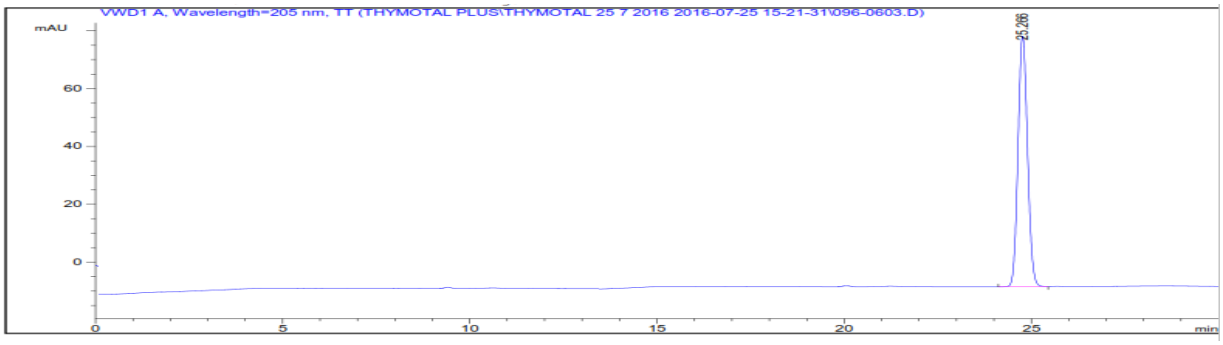

Figure 4: HPLC chromatogram of thymol standard at 274 nm

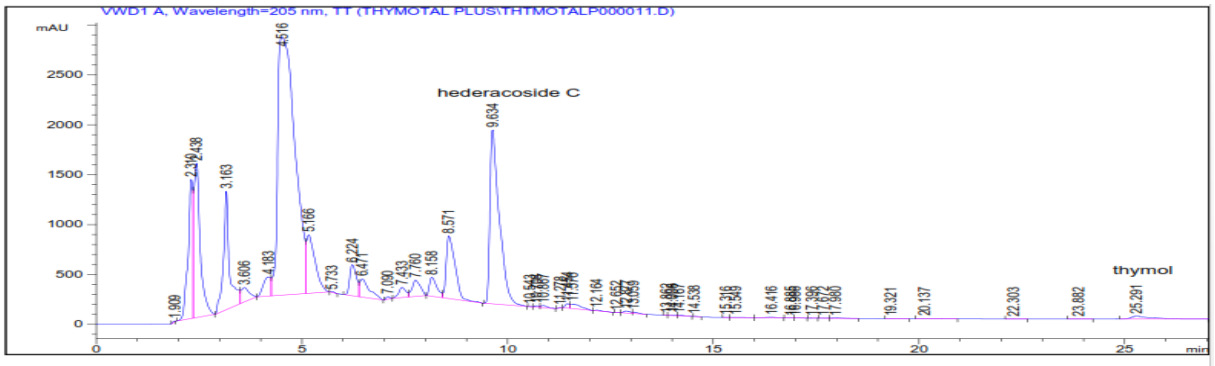

Figure 5: HPLC chromatogram of the Thymotal plus syrup

* Rt for hederacoside C and thymol were 9.6 and 25.2, respectively.

Table 1: Regression data for the determination of hederacoside C and thymol by HPLC method

Parameter	Hederacoside C	Thymol
λ_{max} (nm)	205	274
Linearity range (µg ml^{-1})	50-400	30-90
Regression Parameters		
Slope ±SD (S_x)	4.40 ± 0.017	20.141 ± 0.177
Intercept ±SD (S_y)	66.06 ± 4.265	- 187.806 ± 11.771
Correlation coefficient (r^2)	0.999	0.999
LOD	1.09	0.322
LOQ	3.305	0.975

Table 2: Intra- and Inter-day accuracy and precision results for the determination of hederacoside C and thymol by HPLC method

Analyte	Taken conc. (µg mL^{-1})	Intra-day			Inter-day		
		Found conc. ±SD (µg mL^{-1})	(R%)	(RSD%)	Found conc. ±SD (µg mL^{-1})	(R%)	(RSD%)
Hederacoside C	100	99.89±0.17	99.06	0.17	99.90±0.25	99.47	0.25
	200	200.45±0.12	100.85	0.12	200.91±0.16	101.02	0.16
	300	300.27±0.14	101.10	0.13	300.99±0.14	101.33	0.14
Thymol	60	59.95±0.09	99.93	0.09	60.05±0.18	100.08	0.18
	70	70.13±0.22	100.19	0.22	70.18±0.12	100.25	0.12
	80	79.83±0.13	99.79	0.13	79.9±0.09	99.88	0.09

Table 3: Determination of hederacoside C and thymol in laboratory prepared mixtures by HPLC method

Sample No.	Hedercoside C to thymol Ratio (µg mL^{-1})	Hedercoside C R %	Thymol R %
1	360/60	100.0149	99.6462
2	60/90	100.4454	100.2926
3	90/60	99.97312	99.77952
4	240/120	100.0079	100.1576
5	120/240	99.73697	99.95503
Mean ±SD%		100.036±0.26	99.97±0.26

Table 4: Robustness results for the determination of hedercoside C and thymol by HPLC method

Parameters		K`		N		R		α		T	
		HC*	TH**	HC	TH	HC	TH	HC	TH	HC	TH
1-Fischer scientific		1.55	1.06	43107	46557	76.26		2.68	7.5	0.96	0.91
2-Sigma aldrich		1.61	1.12	43570	46930	76.01		3.05	8.02	1.09	1.14
Changing	(0.19%)	1.51	1.01	44082	46940	76.21		2.57	7.69	1.08	0.93
O-phosphoric acid%	(0.2%)	1.55	1.06	43107	46557	76.26		2.68	7.5	0.96	0.91
in the mobile phase:	(0.21%)	1.69	1.15	43298	47035	75.96		3.21	8.01	1.04	1.15

*HC=Hederacoside C, **TH=Thymol

K` is capacity factor, N is number of theoretical plates, R is resolution factor, α is selectivity factor.

Table 5: Application of standard addition technique for the determination of hederacoside C and thymol by HPLC method in Thymotal plus syrup

Preparation	Hederacoside C				Thymol			
	Mean± SD%	Claimed taken (µg mL^{-1})	Pure added (µg mL^{-1})	R % of added	Mean± SD%	Claimed taken (µg mL^{-1})	Pure added (µg mL^{-1})	R % of added
Thymotal		175	50	100.15		30	20	99.95
plus	99.96±	175	175	99.85	100.01±	30	30	100.02
syrup	0.33	175	225	100.08	0.12	30	50	99.99
Mean ±SD%				100.03±0.16				99.99±0.02

Table 6: Statistical analysis of the results obtained by the proposed and reported methods [15] and [16] for the determination of hederacoside C and thymol, respectively in Thymotal plus syrup.

Parameters	Proposed method		Reported methods	
	For hederacoside C	**For thymol**	**For hederacoside C [15]**	**For thymol [16]**
Linearity range (µg ml^{-1})	(50-400)	(30-90)	(200-400)	(50-90)
N	5	5	5	5
Mean %	99.96%	100.01%	99.78%	99.86%
SD	0.33	0.12	0.46	0.25
Variance	0.109	0.014	0.212	0.063
t-	0.71 (1.86)	1.20 (1.86)	-	-
F-	1.94 (5.19)	4.46 (5.19)	-	-

-Ref [15]: involved determination of hederacoside C by HPLC with gradient elution on C18 column (125×4mm i.d.) 5 µm particle size using mobile phase of acetonitrile: water (140:880,v/v) and adjust to PH 2.0 with phosphoric acid[solvent A],phosphoric acid: acetonitrile (2:998,v/v) [solvent B], at flow rate 1.5 ml min^{-1},UV detection at 205 nm and injection volume :20µl.

-Ref [16]: involved determination of thymol by HPLC with gradient elution on column packed with Inertsil ODS-3v, Japan (250×4.6mm i.d.) 5 µm particle size using mobile phase of 0.05% orthophosphoric acid in water [solvent A],acetonitrile [solvent B],flow rate 1.5 ml min^{-1},diode array detection(DAD), injection volume :20µl.

-Figures in parenthesis are the theoretical t- and F- values at p= 0.05.

4. Conclusion

A simple and reliable reversed phase high-performance liquid chromatographic method with UV detection has been developed and validated for the evaluation of ivy extract (which standardized by hederacoside C) and thyme extract (which standardized by thymol) in their binary mixture in cough syrup. The proposed HPLC method could be applied successfully for the routine quality control analysis, necessary for providing herbal medicines with high safety and efficacy.

Acknowledgments

The authors are thankful to the pharmaceutical research and development lab, Faculty of Pharmacy, Heliopolis University for sustainable development as this study was partially funded by them.

Reference

1. Thyme *In Wikipedia, The Free Encyclopedia. Retrieved 15:10, December 21, 2016, from https://en.wikipedia.org/w/index.php?title=Thyme&oldid=756023786*. 2016.
2. ESCOP, *Thymi herba Monographs on the Medicinal Uses of Plant Drugs, European Scientific Cooperative on Phytotherapy, Exeter, UK, .* 1997.
3. The United State Pharmacopoeia 32, N., *Asian Ed. Rand Mc Nally, USA.* 2010.
4. Helix., H., *In Wikipedia, The Free Encyclopedia. Retrieved 13:43, December 19, 2016, from https://en.wikipedia.org/w/index.php?title=Hedera_helix&oldid=755668683.* 2016.
5. Demirci, B., et al., *HPLC profiling and quantification of active principles in leaves of Hedera helix L, Die Pharmazie, vol. 59, no. 10, pp. 770-774.* 2004.
6. Blumenthal, M., et al., *The Complete German Commission E Monographs-Therapeutic Guide to Herbal Medicines. Austin: American Botanical Council; Boston: Integrative Medicine Communications.* 1998.
7. Yulia, L., et al., *Hedera helix as medicinal plant. Herba Polinica;56(1).* 2012.
8. Khdair, A., et al., *A Validated RP HPLC-PAD Method for the Determination of Hederacoside C in Ivy-Thyme Cough Syrup.* International Journal of Analytical Chemistry, 2010. 2010.

9. Oleszek, W. and Z. Bialy, *Chromatographic determination of plant saponins--an update (2002-2005).* J Chromatogr A, 2006. 1112(1-2): p. 78-91.
10. Bruneton, J., *Pharmcognosy, Phytochemistry, Medicinal Plants, Technique & Documentation, Paris, France, 2nd edition.* 1999.
11. Crespin, F., et al., *High-performance liquid chromatographic determination of saponins from Hedera helix L. using a light-scattering detector, Chromatographia, vol. 38, no. 3-4, pp. 183-186,* . 1994.
12. Khdair, A., et al., *A Validated RP HPLC-PAD Method for the Determination of Hederacoside C in Ivy-Thyme Cough Syrup.* Int J Anal Chem, 2010. 2010.
13. Fadhil, G., *Spectrophotometric Determination of Thymol in Pharmaceutical Preparations Via Oxidative Coupling Reaction with 2,4- dinitrophenylhydrazine in the Presence of Potassium Periodate.* Iraqi Journal of Science, 2014. Vol 55, No.1: p. pp:27-34.
14. Hajimehdipoor, H., et al., *A validated high performance liquid chromatography method for the analysis of thymol and carvacrol in Thymus vulgaris L. volatile oil.* Pharmacognosy Magazine, 2010. 6(23): p. 154-158.
15. (EDQM), T.E.D.f.t.Q.o.M., *Ivy leaf*, in *European pharmacopoeia 7.0*. 2011. p. 1157-1158.
16. Ghada M. Hadad, et al., *HPLC – DAD Determination of Seven Antioxidants and Caffeine in Different Phytopharmaceuticals.* Journal of Chromatographic Science 2013: p. 617–623.