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Original Article

Simple and Sensitive High Performance Liquid Chromatographic (HPLC) Method for the Determination of the Apigenin from Dried Powder of *Cosmos Bipinnatus, Apium Graveolens* and *Petroselinum Crispum*

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Abstract

Apigenin is best known as an irreversible inhibitor of monoamine oxidase (MAO), an intracellular enzyme associated with the outer membrane of mitochondria. The purpose of this study is to establish a reliable and quick method for the assignment of apigenin in *Cosmos bipinnatus, Apium graveolens,* and *Petroselinum crispum* by HPLC. A rapid and sensitive HPLC method has been developed for determination of apigenin. Mobile phase was composed water-acetonitrile (55:45 v/v) with a flow rate of 1 ml/min and the eluted peaks were detected by a UV detector set at wavelength of 340 nm. The method was validated in the range of apigenin concentrations from 0.01 to 500 µg/ml and the limits of detection (LOD) and quantitation (LOQ) of the method were 0.005 and 0.01 µg/ml, respectively. The average recovery throughout the linear concentration range was 97.28 percent and the averages for within-run and between-run accuracy values were 99.32 and 96.79 respectively. The method is quick, simple, sensitive, and precise for the screen, assignment, and evaluation of apigenin in plants by HPLC.

Keywords: Apigenin, HPLC, Cosmos bipinnatus, Apium graveolens and Petroselinum crispum

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1. Introduction

Natural plant compounds are used in treating various diseases [1-5]. Apigenin [figure 1] is a 4, 5, 7 trihydroxy flavone, belonging to a less toxic and non-mutagenic flavones subclass of flavonoids. It is derived from the *Cosmos bipinnatus* and many other plants. It has a variety of pharmacological activities, including antioxidant [6], anti-tumor [7], anti-inflammatory [8], ant-bacterial [9] anti-proliferative, [10] oxygenase inhibitor [11], and induced apoptosis[12]. The monomer apigenin is fit in to a pharmacophore model for ligands binding to the GABAA receptor benzodiazepine site [13]. It also act as inhibitors of IL-4synthesis and CD40 ligand expression by basophils[14]. It has protective effect on radiation-induced chromosomal damage in human lymphocytes [15-17]. Apigenin is one of the very important secondary metabolite produced by *C. bipinnatus*. *C. bipinnatus* has been used in a traditional herbal remedy for various diseases such as jaundice, intermittent fever, and anti-inflammatory activity [18, 19] reported antioxidative and antigenotoxic activity of extracts from *C. bipinnatus*. They suggested that it has significant antioxidant activity and protective effect against oxidative DNA damage [20]. The chemical composition of essential oils of *C. bipinnatus* and its antibacterial activity have been revealed [21]. Suggested that the hepatoprotective activity of apigenin is due to its antioxidant properties, acting as scavengers reported that a sesquiterpene lactone isolated from the roots of *C. bipinnatus* demonstrates an anti-inflammatory effect [22]. It exhibits anti-mutagenic, anti-inflammatory, antiviral, and purgative effects [22, 23] reporting that it can be a very useful anticancer apigenin candidate for chemotherapy and cancer prevention [24] [25]. In another study, Anti-proliferative effects of apigenin on human breast cancer cell lines have been demonstrated. It strongly inhibited tumor cell invasion in estrogen-insensitive breast tumor cell line [26], reporting that apigenin inhibited the growth of human cervical carcinoma HeLa cells through an apoptotic pathway. HPLC methods for the detection of apigenin from rat apigenin and mouse tissues have been reported in literature [27, 28]. Also, reverse phase HPLC method for the determination and quantitation of apigenin in human urine after oral administration of tablet of *Chrysanthemum moriflum* extract [29] and simultaneous determination of apigenin with other flavonoids from alcoholic extract of Pigeonpea [*Cajanuscajan*(L.) Millsp.] leaves are reported in literature [30]. The determination and quantitation of apigenin from dried root powder of *Gmelina arborea* L. has been

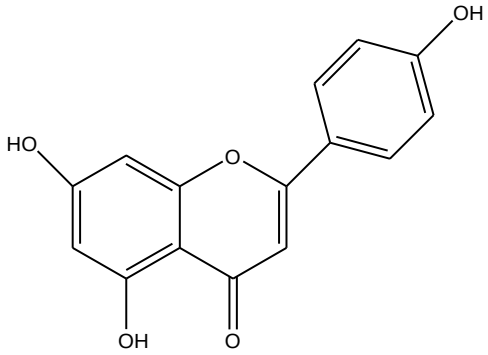


Figure 1. Chemical structure of apigenin.

reported. In the present study, we describe a simple, selective, and stable method using high performance liquid chromatography (HPLC) for the determination of apigenin in *Cosmos bipinnatus*, *Apium graveolens*, and *Petroselinum crispum*.

2. Materials and Methods

2.1. Materials

Apigenin standard (99.9% purity) was provided from Sigma- Aldrich (Aldrich Division; Steinheim, Germany) company. *Cosmos bipinnatus*, *Apium graveolens*, and *Petroselinum crispum* were collected from herbarium of Zanjan University of Medical Sciences.

2.2. Instrument and HPLC Method

The HPLC system to include of pump (WATER, USA), wavelength UV detector (WATER, model Breeze), USA) used at a wavelength of 340 nm with the outputs to record and analyze using with a software (Breeze, USA). The apigenin analization was performed using a C_8 analytical column (250 mm*4.6mm, particle size 5µm; Perfectsill, MZ-Analysentechnik, Germany) equipped by a guard column of the same packing. The mobile phase composed of water-acetonitrile (55:45 v/v) with a flow rate of 1 ml/min. Sample injection to system (20µl) was made by a loop injector (Rheodyne®7725i, Cotati, CA, USA).

2.3. Plant Material

Cosmos bipinnatus, Apium graveolens, and Petroselinum crispum were collected as wild plant, from Zanjan, Iran. A herbarium plants were prepared in duplicate and was authenticated from school of pharmacy, Zanjan University. The plant material was thoroughly washed to remove dust particles. The plant was separated and then air dried. Immediately after drying, plants were powdered using an electric mixer-grinder and sieved through a BSS mesh no. 85 sieve. The sieved powder was used for the present research work.

2.4. Preparation of Stock Solutions and Working Solutions

Stock solutions of apigenin was prepared in HPLC grade methanol at concentrations of 1mg/ml and were stored at 4 ˚C. Working solutions of apigenin were prepared daily in HPLC grade methanol by appropriate dilution at 0.01, 0.1, 5.0, 12.5, , 25.0, 50.0, 100, and 500µg/ml and an aliquot of 20 µl was injected into the HPLC system.

2.5. Preparation of sample solution

2.5.1. Extraction and Isolation

The 8.45 g of *Cosmos bipinnatus* were washed well to remove the dust, dried at room

temperature and were extracted with 1000 ml ethanol. The above procedures were repeated three times and the extracts obtained from extractions were combined. The extracts were filtered and allowed to stand for 72 h in Refrigerator and then concentrated under reduced pressure. The yield of apigenin was measured by HPLC [31]. The above procedures were repeated for extraction of apigenin from *Apium graveolens* and *Petroselinum crispum.*

2.6. Standard Curves

Proper volume of one of the above-mentioned working solutions to produce the standard curve point's equivalent to 0.01, 0.1, 5.0, 12.5, 25.0, 50.0, 100, and 500µg/ml of apigenin and each sample was processed as described. Finally, the nominal known concentrations were plotted against the corresponding peak areas to construct the standard curve.

2.6.1 Preparation of Quality Control Samples

Quality control samples were prepared daily by spiking different samples with proper volume of the corresponding standard solution to produce a final concentration equivalent to low level (0.01µg/ml), middle level(25.0 µg/ml), and high level (500.0 µg/ml) of apigenin. The following procedures were the same as described above.

2.7. Method Validation

The method was validated for selectivity, linearity, accuracy, precision, recovery, stability, detection limit, and quantitation limit according to the principles of the FDA industry guidance [31-33]**.**

2.7.1. Assay Specificity

To evaluate the matrix effect on the ionization of analytes, five different concentration levels of apigenin (0.01,0.1, 5.0, 12.5, , 25.0, 50.0, 100, and 500µg/ml) were prepared as five sample series using five different lots of the apigenin and the samples were processed, as described, and injected to HPLC. The same concentrations were prepared in mobile phase and analyzed for apigenin concentration using the same procedure. A comparison of the matrix effects of the two variants was made as an indicator of the method specificity.

2.7.2. Linearity

Standard curves of ten concentrations of apigenin ranged 0.01–500.0 µg/ml were assayed. Blank samples were analyzed to ensure the lack of interferences but not used to construct the calibration function. The limit of detection (LOD) was estimated from the signal-to- noise ratio. This parameter was defined as the lowest concentration level resulting in a peak area of three times the baseline noise. The limit of quantification (LOQ) was defined as the lowest concentration level that provided a peak area with a signal-to- noise ratio higher than 5, with precision (% CV) within ± 20% and accuracy (% recovery) between 80% and 120%.

2.7.3. Precision and Accuracy

2.7.3.1. Within-Run Variations

In one run, three samples with concentrations of 0.01, 25, and 500µg/ml (from high, middle, and low regions of the standard curve) were prepared in triplicate and analyzed by developed HPLC method. Then, the coefficient of variations (CV %) of the corresponding determined concentrations were calculated in each case.

2.7.3.2. Between-Run Variations

On three different runs, samples from upper, intermediate, and lower concentration regions used for construction of standard curve (the same as within-run variations test) were prepared and analyzed by HPLC method. Then, the corresponding CV% values were calculated.

2.7.3.3. Repeatability Test

To test the method repeatability, six independent samples with an apigenin concentration of 25 µg/ml were prepared as described. A single injection of each preparation was made to HPLC and the %RSD between the results was determined as the repeatability of the method.

2.7.4. Extraction Recovery

Three samples with concentrations of 0.01, 25, and 500µg/ml (from high, middle, and low regions of the standard curve) were prepared in triplicate and analyzed by developed HPLC method. Then, the ratio of the recorded peak heights to the peak heights resulted from the direct injection of the aqueous solutions of apigenin with the same concentrations were determined as percentage in each case.

2.7.5. Intermediate Precision

On a different day to that of the repeatability study, a second analyst executed analysis of a further six samples prepared as described in repeatability test procedure. The analysis was carried out using fresh reagents and a different HPLC column. The % RSD between six measurements was determined along with the % RSD between the total of 12 measurements from the repeatability and intermediate precision tests.

2.7.6. Reproducibility

Mean results for the same sample analysis between our laboratory and two different test facilities were obtained and the % difference between content measurements was calculated using the equation:

[(highest value − lowest value) /mean value]× 100.

2.7.7. Stability

2.7.7.1. Freeze and Thaw Stability

Three concentration levels of QC samples were stored at the storage temperature (−20 ◦C) for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 24 h under the same conditions. The freeze-thaw cycle were repeated twice, then the samples were tested after three freeze (−20 ◦C)-thaw (room temperature).

2.7.7.2. Short-term temperature stability

Three concentration levels of QC apigenin samples were kept at room temperature for a period that exceeded the routine preparation time of samples (around 6 h).

2.7.7.3. Long-Term Stability

Three concentration levels of QC samples kept at low temperature (−20 ◦C) were studied for a period of 4 weeks.

2.7.7.4. Post-Preparative Stability

The auto sampler stability was conducted reanalyzing extracted QC samples kept under the auto sampler conditions (4 ◦C) for 12 h.

2.7.8. Robustness

The robustness of the method was determined to assess the effect of small but deliberate variation of the chromatographic conditions on the determination of apigenin. Robustness was determined by changing the mobile phase flow rate to 0.75 and 1.25 mL min^{-1}.

3. Results and Discussion

3.1. Method Development

Considering the complex biological matrix of the samples to be analyzed and the nature of the method to be used for apigenin assay, the method development efforts were made in two different areas of sample preparation and analyte separation which are discussed in detail in the following sections: Typical chromatograms produced from the developed method are shown in figure 2. The retention time of apigenin was 2.18 min. per sample, no interferences of the analyte were observed.

3.2. Method Validation

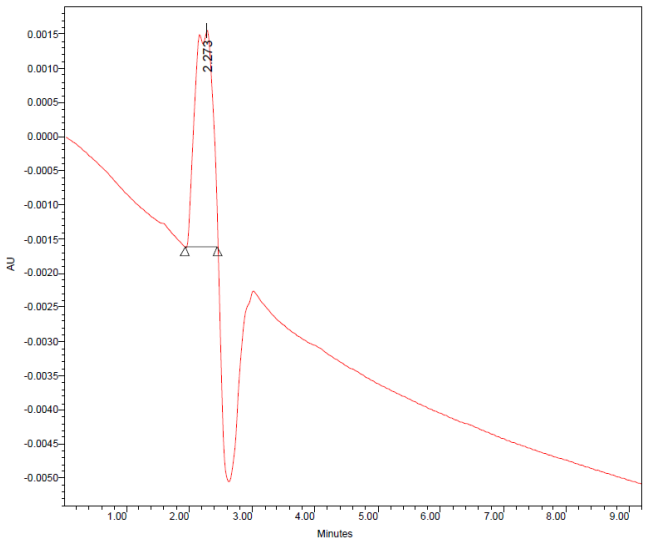
3.2.1. Assay Specificity

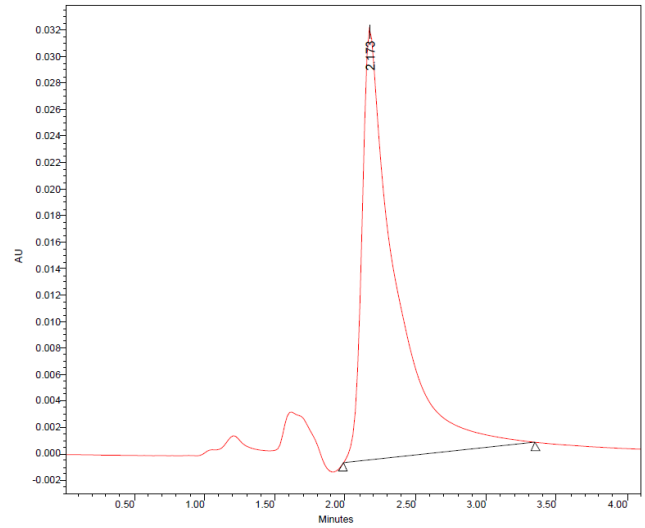
As it is clearly evident from the typical chromatograms of the developed method shown in figure 2, there are no discernible interferences between the matrix factors and the analyte. This, in turn, ensures obtaining

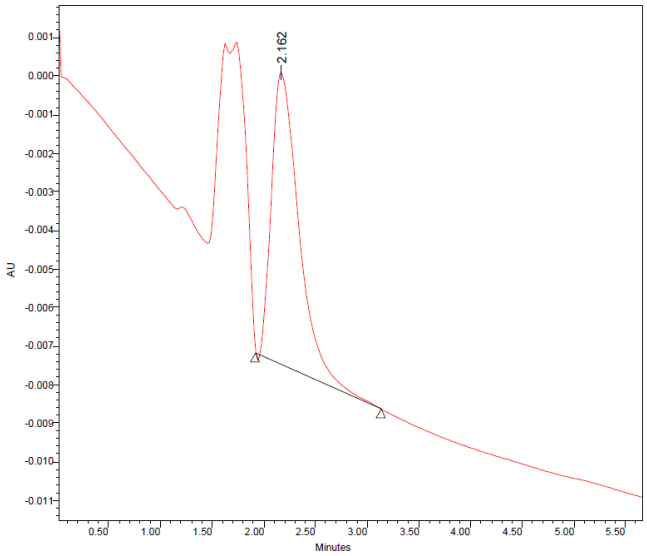
Table 1. Within–run variations and accuracy of the HPLC method for quantitation of apigenin (n=3).

Nominal Added Concentration (µg/ml)	**Sample Number**	**Measured Concentration (µg/ml)**	**Mean (SD)**	**CV%**	**Accuracy**	**Mean (SD)**
0.01	1	0.011	0.0103	5.9	95.23	95.54
	2	0.0099	(0.0006)		94.36	(1.37)
	3	0.010			97.05	
25	1	25.8	25.63	2.24	99.24	97.12
	2	26.1	(0.57)		95.89	(1.84)
	3	24.99			96.23	
500	1	499.46	500.33	0.18	100.02	99.23
	2	501.32	(0.93)		99.02	(0.70)
	3	500.23			98.65	

reliable results from the method for determination of concentrations of apigenin.







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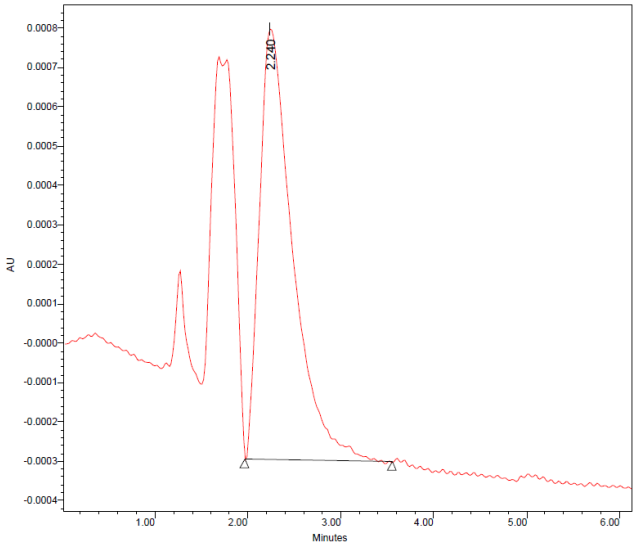

Figure 2. Typical chromatograms of the HPLC method developed for apigenin assay in plants: Fig. 2 A) chromatogram of standard apigenin with concentration 0.01 µg/ml (LOQ) of the method; Fig. 2.B chromatogram of apeginin extracted from Cosmos bipinnatus, Fig. 2.C chromatogram of apeginin extracted from Apium graveolens Fig. 2.D chromatogram of apeginin extracted from Petroselinum crispum

3.2.2. Linearity and LOQ

The method produced linear responses throughout the apigenin concentration range of 0.01-500µg/ml, which is suitable for intended purposes. A typical linear regression equation of the method was: y = 36689x +49747, with x and y representing apigenin concentration (in µg/ml) and peak area (in arbitrary units), respectively, and the regression coefficient (r) of 0.9975.The lower limit of quantification for apigenin was proved to be 0.01µg/ml and the limit of detection was 0.005µg/ml. figure. 2. A shows the chromatogram of an extracted sample that contained 0.01µg/ml (LOQ) of apigenin.

3.2.3. Precision and Accuracy

3.2.3.1. Within-Run Variations And Accuracy

The within-run variations of the developed HPLC method as well as the corresponding absolute recoveries are shown in Table 1.These data clearly show that the developed method has an acceptable degree of repeatability and accuracy within an analytical run.

3.2.3.2. Between-Run Variations and Accuracy

The between-run variations of the developed HPLC method as well as the corresponding absolute recoveries are shown in Table 2. As stated for the previous test, these data clearly show that the developed method has an acceptable degree of reproducibility and accuracy between different analytical runs.

3.2.3.3. Repeatability Test

The repeatability of the method is shown in Table 3. As shown, the method has a remarkable repeatability for the apigenin assay.

Table 2. Between–run variations and accuracy of the HPLC method for quantitation of apigenin (n=3).

Nominal Added Concentration (µg/ml)	Run Number	Measured Concentration (µg/ml)	Mean (SD)	CV%	Accuracy	Mean (SD)
0.01	1	0.0098	0.01006	3.76	97.90	99.23
	2	0.0105	(0.0003)		98.78	(1.62)
	3	0.0099			101.02	
25	1	24.98	25.58	2.24	96.55	98.79
	2	25.65	(0.58)		101.23	(2.34)
	3	26.12			98.59	
500	1	499.08	499.72	0.11	101.02	99.63
	2	499.97	(0.56)		99.32	(1.25)
	3	500.12			98.56	

Table 3. Repeatability of the test results for spiked apigenin containing 25 µg mL-1 apigenin.

Sample	Peak area	Mean(SD)	CV%	Retention time (min)	Mean (SD)	CV %
1	5099782	5100465(1042)	0.026	2.18	2.18(0.014)	0.64
2	5099687			2.17		
3	5099932			2.19		
4	5102312			2.16		
5	5101110			2.20		
6	5099967			2.18		

Table 4. Relative recovery of apigenin by the HPLC method (N=3).

Nominal Added Concentration (µg/ml)	Sample Number	Recovery (%)	Mean (SD)
0.01	1	95.21	98.77
	2	102.01	(3.41)
	3	99.11	
25	1	96.18	96.62
	2	101.14	(4.30)
	3	92.56	
500	1	101.25	99.21
	2	102.04	(4.22)
	3	94.36	

3.2.4. Relative Recovery (Matrix Effect)

The extraction recovery determined for apigenin was shown to be consistent, precise and reproducible. Data were shown below in Table 4.These data indicate that there is no significant matrix effect on the outputs of the assay method.

3.2.5. Intermediate Precision

The results of the intermediate precision test are shown in Table 5. As indicated, the developed method shows an acceptable intermediate precision for apigenin assay.

3.2.6. Reproducibility

The highest test result of the spiked apigenin with 25µg mL-1 apigenin was5099876 and the lowest value was5099021with the mean value of 5099296. Therefore, the % difference was 0.016% which means a high reproducibility for the method.

3.2.7. Stability

Table 6 summarizes the freeze and thaw stability, short term stability, long-term stability and post-preparative stability data of apigenin. All the results showed the stability behavior during these tests and there were no stability related problems during the samples routine analysis for the pharmacokinetic, bioavailability or bioequivalence studies. The stability of working solutions was tested at room temperature for 6 h. based on the results obtained; these working solutions were stable within 6 h.

3.2.8. Robustness

There was no significant change in the

Table 5. Intermediate precision of the test results for spiked apigenin containing 25 µg mL-1 apigenin

Sample	**Peak area**	**Mean(SD)**	**CV%**	**Retention time (min)**	**Mean (SD)**	**CV %**
1	5099563	5099296(417)	0.0088	2.16	2.18(0.016)	0.747
2	5098675			2.20		
3	5099321			2.18		
4	5099021			2.17		
5	5099321			2.20		
6	5099876			2.19		

Table 6. Data showing stability of apigenin in human apigenin at different QC levels (n=5)

Stability	**0.01(µg/ml)**	**25 (µg/ml)**	**500(µg/ml)**
Short-term stability	87.86	87.98	86.39
Freeze and thaw stability	98.65	98.78	97.89
Long-term stability	83.65	89. 21	84.56
Post-preparative stability	96.54	96.32	94.78

retention time of apigenin when the flow rate of the mobile phase was changed. The low values of the RSD, shown in Table 7, indicated the robustness of the method.

Table 7. Results from testing of the robustness of the method by changing the mobile phase flow rate. (The concentration of the solution analysed was 25 μg mL−1)

Flow rate (ml/min)	Mean area (SD) N=3	Mean t_R(SD) N=3	CV %
0.75	5098675 (417)	2.19 (0.015)	0.0082
1	5099563 (564)	2.18 (0.016)	0.011
1.25	5097865 (467)	2.20 (0.017)	0.0092

Table 8. The extraction recovery determined for apigenin from plants

Plants	Weight of plants(mg)	Weight of apigenin(mg)	Recovery (%)
C. bipinnatus,	8450	30.49	0.36
Apium graveolens	8450	4.37	0.051
Petroselinum crispum	4650	2.855	0.061

3.3 Effects of extraction solvent

For finding an effective solvent for the extraction of apigenin various solvents were tested. The use of ethanol produced the highest yield of extract apigenin 30.49 mg from 8.45 g, 4.37 mg from 8.45 g, and 2.855 mg from 4.65 g of dry *C. bipinnatus*, *Apium graveolens*, and *Petroselinum crispum* plants respectively. While yields of extract from acetone, methanol, contain apigenin 11mg, 2.2 mg and 0.7mg, respectively from *C. bipinnatus*, *Apium graveolens* and *Petroselinum crispum* plants respectively. Figure 2.B chromatogram shows apeginin extracted from Cosmos bipinnatus and also figure 2.C shows chromatogram of apeginin extracted from *Apium graveolens* figure 2.D chromatogram of apeginin extracted from *Petroselinum crispum*. These results revealed as the first report. In our experiments, ethanol was used due to high yield and the lower toxicity of ethanol compared to the other solvents tested in this study. The extraction recovery determined for apigenin from plants is shown in Table 8.

3.3.1. Effects of extraction temperature and time

The extractions were carried out at various temperatures under the conditions described in Section 2.2.4. The recovery yield of extract and the apigenin content generally increased with an increase in extraction temperature up to 80°C and these results are probably due to a higher solubility and diffusivity of solute in liquidate higher temperature. Since the increase in the extraction yield was very large from 70–90°C, extraction temperatures. So, 80°C was considered optimal temperature for achieving a high recovery yield of extract containing apigenin. Using the optimal extraction conditions selected in sections

extractions were conducted for various extraction time lengths. The extract recovery yield appeared to increase by increasing extraction time up to 6h, but the further extending of extraction time did not result in an increase of extract recovery yield.

4. Conclusion

This study demonstrates to an effective process for separating and fractionating of apigenin. Optimal conditions for the recovery high content of apigenin were determined to be extraction with ethanol at 80 C for 6 h, followed by fraction at ion with ethanol. Apigenin was obtained in crystal form with over purity through relatively low cost sequential processes only requiring water, ethanol. This economically feasible process can be readily applied in those industries demanding high purity apigenin, with high value due to its various biological and physiological activities. It was concluded that a simple and sensitive HPLC methods has developed and validated by using C_8 column. Statistical analysis of the results shows that all the proposed procedures have good precision and accuracy shown in table. The procedure will successfully apply to the determination of the studied compound in pharmaceutical formulation without any interference from the additives and endogenous substances. This method offers several advantages such as a rapid and simple extraction scheme and a short chromatographic run time the method suitable for applicable to pharmacokinetic and pharmacodynamics studies and for routine applications in the quality control laboratories because of the simplicity, economic, accuracy, sensitivity and reproducibility.

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