diosmin (**2**) in methanol, NaOH 5%, the *pH* of the reaction medium is preferably greater than 11, the reaction is refluxed for 15 minutes and diosmin can be subsequently isolated by adding HCl 0.5% to the reaction medium, so diosmin is precipitated and can be recovered by filtration to obtained diosmin (**3**), give the yield up to 95% and HPLC's purity 98.6%. According to Nguyen Thi My Duyen, diosmin (**3**) was synthesized from hesperidin through three steps. In the first step, some functional groups of hesperidin were protected, then it continuously suffered a process of halogenation on position C-3, and finally, it was dehalogenated to get diosmin which contents of 88.98% in HPLC [12]. Furthermore, Nguyen Van Thanh, diosmin was also synthesized *via* the oxidation of hesperidin with iodine in ion liquid including [BMIM]Br, [BMIM]BF4, [BMIM]OTf, [BPY]Br, [BPY]BF4 and [BPY]OTf. As a result, the process allows for obtaining diosmin with high yields of 79-85% [9].

4. Conclusion

In this study, diosmin (**3**) was synthesized through three steps with an efficiency per step of 90% and the overall yield of the process was 82.1%. Our synthesis is a suitable procedure for the semi-synthesis of diosmin with high yield at the laboratory scale. This is the initial result in the semi-synthesis of diosmin. It is necessary to study on process optimization to limit the use of some toxic solvents such as pyridine and MeOH, before scaling it up to the pilot scale.

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QUANTITATIVE DETERMINATION OF AGNUSIDE IN THE FRUIT OF *VITEX TRIFOLIA* L. COLLECTED IN VIETNAM

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Summary

Quantitative Determination of Agnuside in the Fruit of *Vitex trifolia* L. Collected in Vietnam

Agnuside is the main bioactive compound in *Vitex* species. A simple and sensitive high performance liquid chromatography (HPLC-UV) method was developed for the identification and quantification of agnuside in the fruit of *Vitex trifolia* L. The chromatography was achieved on a Cosmosil 5C18-AR-II (250 mm x 4.6 mm, 5 µm) column at 25°C, using acetonitrile and *O*-phosphoric acid - water (0.4%, v/v) as the mobile phases in the gradient elution mode. The flow rate of 1.4 ml/min and detection wavelength of 258 nm were selected. The developed method was validated as per the ICH guidelines for limit of detection, limit of quantification, linearity, accuracy, and precision for the quantitative analysis of agnuside in 22 fruit samples of *Vitex trifolia* L. The contents of agnuside ranged from 0.12 to 0.38%.

Keywords: *Vitex trifolia* L., *Agnuside, HPLC.*

1. Introduction

Medicinal plants have been documented to treat diverse human ailments for thousands of years and offer a rich resource of novel therapeutics. *Vitex trifolia* L. from the Verbenaceae family is a deciduous plant mainly found in coastal areas of Pacific - Asian regions, including countries such as China, Vietnam, Singapore, and India [1]. *Vitex trifolia* is traditionally used for various ailments. Dried ripe fruits of *Vitex trifolia* are documented in Traditional Medicine to treat inflammation of the eye, headaches, blurred vision, rhinitis, and the common cold [2],[3]. Flavonoids, iridoids, terpenes, and steroids are the major classes of compounds isolated from *V. trifolia*. The extracts of fruits of *Vitex trifolia* have many pharmacological actions like anti-arthritic, anti-inflammatory [4], pre-menstrual syndrome reduction [5], etc. Agnuside (AGN), an iridoid glycoside is reported as an important chemotaxonomic marker of the genus *Vitex*. It was isolated from *V. agnus-castus, V. negundo, V. triflolia,* etc [6],[7],[8],[9],[10].

Although, several HPLC methods for the analysis of agnuside in *Vitex* species have been reported. USP 44 used agnuside as a marker for controlling the quanlity of the dried ripened fruits of *Vitex agnus-castus* L. by HPLC with a total run time of 23 min using a column (12.5 cm x 3.1 mm; 5 µm packing L1), a gradient elution mode with acetonitrile and *O*-phosphoric acid-water (5.88 g/L), a flow rate of 1.3 ml/min, and detection wavelength of 258 nm [11]. Tushar Dhanani *et al.* reported a validated rapid and simple isocratic HPLC-PDA method for the determination of three bioactive compounds, *p*-hydroxybenzoic acid, negundoside, and agnuside in leaves and bark of *V. negundo* and *V. trifolia* using acetonitrile (15%) and 0.05% trifluoroacetic acid in water (85%) [13]. To the best of our knowledge, no HPLC method for identification and quantification of agnuside in the fruit of *Vitex trifolia* L. has been reported previously. In this paper, an HPLC-UV method was used with a few modifications for the quantitative evaluation of agnuside from the fruit of *V. trifolia* collected in Vietnam.

2. Experimental

2.1. Materials

Fruits of *V. trifolia* were collected from Ninh Binh, Thanh Hoa, and Nghe An in 3/2021, 12/2021 and authenticated by MSc Phan Van Truong and Dao Minh Hieu at the Center of Medicinal Material Resources, NIMM. Fruits of *V. trifolia* were dried in the oven at 50°C, crushed into fine powder, and stored in the refrigerator at the Department of Phytochemistry, NIMM.

2.2. Chemicals

HPLC grade solvents (methanol, acetonitrile, and ethanol) were purchased from Fisher (Korea) and phosphoric acid was purchased from Merck (India). Deionized water was prepared using a WaterPro PS Polishing system (18.2 megohm-cm). Standard agnuside (purity ≥ 98%) was purchased from Chengdu Pufei De Biotech Co., Ltd (China). All other solvents were of analytical grade.

2.3. Instrumentation

The HPLC system for chromatographic analysis consisted of a separation module (Shimadzu LC-20AD), a quaternary pump, a degasser, an auto sample, an injector, and a detector (Shimadzu SPD-20A). The chromatographic separation was carried out on a C18 column (Cosmosil, Nacalai Tesque-Japan, 250 mm x 4.6 mm, 5 µm).

2.4. Methods

2.4.1. Sample preparation:

The sample preparation method was selected to achieve the highest agnuside yield. Sample preparation factors were investigated including extraction methods (ultrasonic, reflux, soxhlet, and maceration extraction methods), extraction solvents (methanol, ethanol, the mixture ethanol-water with different ratios), and particle size of the sample.

2.4.2. Standard solutions:

The standard stock solution of agnuside (460 ppm) was prepared by dissolving 4.6 mg of standard agnuside in 10 ml methanol. The working solutions of lower concentration were prepared by appropriate dilution of stock solutions. Five concentrations in the range of 14.7 - 460.0 ppm were used for the construction of the calibration curve. These solutions were stored at 4°C and brought to room temperature before use.

2.4.3. Chromatographic condition:

The chromatographic condition was investigated to separate the agnuside peak from other peaks on the chromatogram and achieve chromatographic purity.

2.4.4. Method validation:

The analytical method was validated for system suitability, specificity, calibration curve, accuracy, precision, limit of detection (LOD), and limit of quantitation (LOQ) according to the current ICH guideline [15]. The LOD and LOQ were determined based on the signal-to-noise

ratio. The accuracy of the analytical method was assessed by spike recovery experiments at different levels.

2.4.5. Calculation:

Use the validated method to quantify agnuside content in real samples from different regions. The percentages of agnuside were calculated from the sample taken:

$$X\,(\%) = \frac{C \times V \times 50}{m \times (100 - a)}$$

C: the concentration of agnuside in the sample solution (ppm). V: volume of the sample solution (ml). m: weight of the test sample taken to prepare the sample solution (mg). a: the moisture of powder (%).

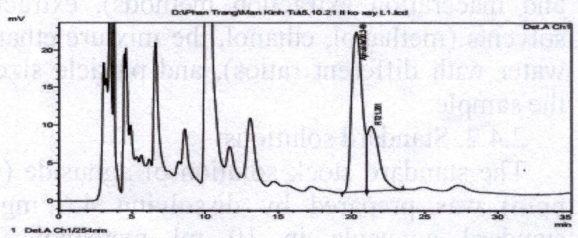
3. Results and discussion

3.1. Optimization of the chromatographic conditions

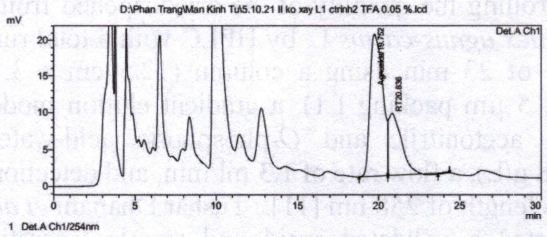
A reversed-phase C18 column was used and several different elution systems were tried. The mobile phase condition and chromatogram examined are presented in Table 1 and Fig. 1, respectively. The results showed that the resolution of peaks of agnuside was unsatisfactory when either acetonitrile: water or methanol: water was used as a mobile phase. Several modified acids, such as *O*-phosphoric acid, and acetic acid were used for optimization of chromatographic separation. Acetonitrile provided better resolution than methanol in the organic phase. Different percentages of acid in water and percentages of acetonitrile in the mobile phase were also optimized. The best results were obtained as a mobile phase consisting of a mixture of acetonitrile and *O*-phosphoric acid (0.4%) in gradient elution mode with a flow rate of 1.4 ml/min (Condition VI).

Table 1. Investigated mobile phase condition

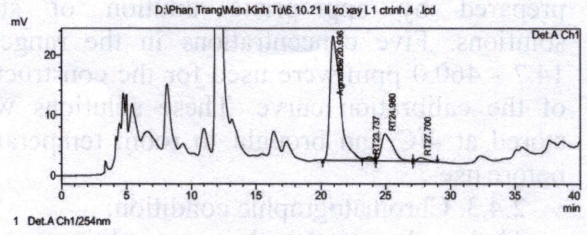
Condition	Elution program
I	Acetonitrile - 0.5% *ortho* phosphoric acid (15:85), flow rate: 1 ml/min
II	Acetonitrile - 0.05% TFA (15:85), flow rate: 1 ml/min
III	Methanol - 0.1% acetic acid (gradient), flow rate: 0.8 ml/min
IV	Methanol - 0.1% acetic acid (gradient), flow rate: 0.7 ml/min
V	Acetonitrile - 0.4% *ortho* phosphoric acid (gradient), flow rate: 1.3 ml/min (according to the USP 44)
VI	Acetonitrile - 0.4% *ortho* phosphoric acid (gradient), flow rate: 1.4 ml/min (according to the USP 44)



Condition I



Condition II



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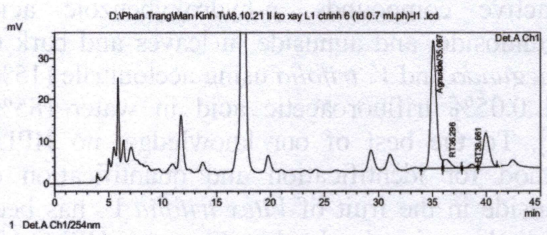
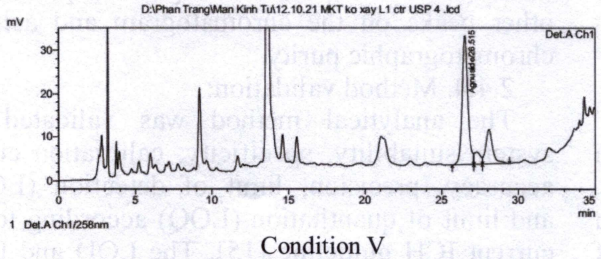
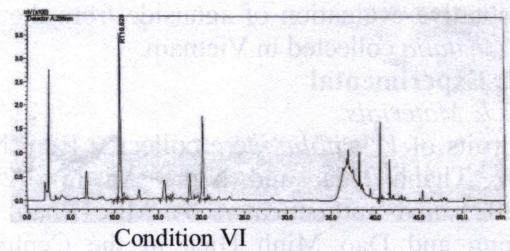


Condition IV



Condition V



Condition VI

Fig. 1. HPLC chromatograms of *V. trifolia* extract with different chromatography conditions

Under the optimized conditions, agnuside standards were well resolved with relatively high sensitivity at a mean retention time of 20.683 min when absorption was measured at 258 nm. At this wavelength, the best resolution between peaks as well as a baseline was achieved, and no interfering peaks were observed in the blank. The spectral purity of the peaks of standard agnuside and samples was verified in terms of the purity angle and purity threshold values. The purity angle was found to be lower than the purity threshold value, depicting the homogeneity of the peak.

3.2. Optimization of extraction conditions:

Various extraction methods, including particle size of sample, and solvents were evaluated to obtain maximum extraction efficiency. The results are presented in Table 1. From the investigation results, the final extraction condition was selected as follows: Powdered samples (2.0 g) were extracted with ethanol 70% (20 ml) for 2 hours at reflux temperature. The ethanol extracts were transferred to a 50 ml volumetric flask. The extraction was repeated two times with 70% ethanol (16 ml x 2) for 1 hour. All supernatants were transferred to the volumetric flask and made up to the mark with 70% ethanol. The sample solution was filtered through a 0.45 µm PTFE filter.

Table 2. Results of the sample preparation method

Extraction methods	
Condition	**Agnuside (%)**
Sonication, m = 2.0 g, MeOH, solvent volume: 10 ml x 5 times, extraction time: 30 min x 5 times	0.170 ± 0.006
Reflux, m = 2.0 g, MeOH, solvent volume: 20, 16, and 16 ml, extraction time: 120, 90, and 60 min	***0.232 ± 0.001***
Soxhlet, m = 2.0 g, MeOH, solvent volume: 50 ml, extraction time: 30 hours	0.243 ± 0.006
Maceration, m = 2.0 g, MeOH, solvent volume: 50 ml, extraction time: 24 hours	0.141 ± 0.005
The particle size of the samples Reflux, m = 2.0 g, MeOH, solvent volume: 20, 16, and 16 ml, extraction time: 120, 90, and 60 min	
Condition	**Agnuside (%)**
Fruits	0.081 ± 0.018
Fine powder	***0.231 ± 0.002***
Extraction solvents Reflux, fine powder, m = 2 g, MeOH, solvent volume: 20, 16, and 16 ml, extraction time: 120, 90, and 60 min	
Condition	**Agnuside (%)**
MeOH	0.231 ± 0.002
EtOH 96%	0.215 ± 0.006
EtOH 70%	***0.243 ± 0.009***
EtOH 50%	0.232 ± 0.006
EtOH 30%	0.228 ± 0.007

3.3. Method validation

3.3.1. Specificity: The experiments were performed with a blank sample, an agnuside standard, and a sample solution according to the selected chromatographic condition. The obtained chromatograms of the specification test are shown in Fig. 2. The results showed that the peak signal of agnuside is sharp and well separated. The chromatogram of the test sample had a peak with a retention time corresponding to the agnuside pic in the standard solution and there was no pic in the chromatogram of the bank sample at the retention time corresponding to the agnuside pic in the standard solution.

3.3.2. System suitability: The results are presented in Table 2. Retention time and peak area of six replicates have RSD% ≤ 2%. The tailing factor met the requirement.

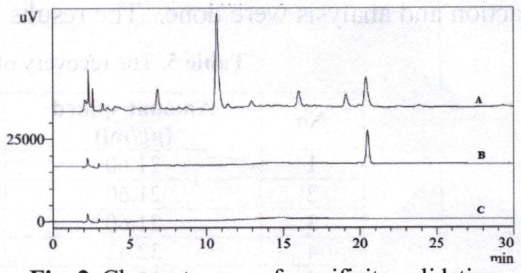


Fig. 2. Chromatogram of specificity validation
(A: The fruit of *Vitex trifolia* L., B: Agnuside, C: Blank sample)

Table 2. System suitability parameters

No	t_R (min)	Area (uV.s)	T
1	20.377	6106315	1.316
2	20.597	6243773	1.322
3	20.732	6252881	1.321
4	20.729	6266368	1.323
5	20.783	6276611	1.322
6	20.881	6244810	1.316
Average	20.683	6362246.836	
SD	0.176	62779.739	
RSD (%)	0.850	1.007	

3.3.3. Limit of detection (LOD) and limit of quantification (LOQ): The LOD and LOQ were determined by diluting the standard solution to the concentration that gave the minimum signal to noise (S/N) of 3 to 10, respectively. The result showed that the LOD is 1.176 µg/ml and the LOQ is 3.881 µg/ml.

3.3.4. Linear range and calibration curve: Calibration curves were constructed as a function of the concentrations of agnuside standard analytes (X) and their peak areas (Y). The calibration curves were linear over the concentration range (14.7 µg/ml - 367.5 µg/ml) (Fig. 3). The calibration curve equation was Y = 21367X + 3917.2, and the linear correlation coefficient (R^2) value was 0.9998.

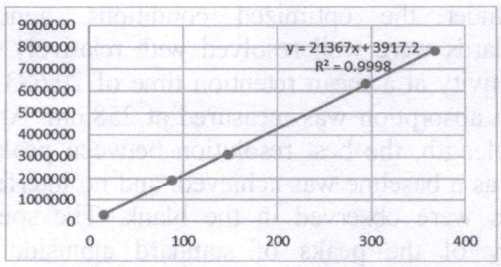


Fig. 3. Calibration curve of agnuside

3.3.5. Precision: The results of the intra-day and inter-day precision experiments are shown in Table 4. The developed method was found to be precise as the RSD values for repeatability of intra-day and inter-day precision studies were below 2.0%, which is under the limit as per the recommendations of the ICH guidelines.

Table 4. The precision of the proposed procedure

No	Agnuside content (%)		
	Day 1	Day 2	
1	0.231	0.230	Average: 0.231 (%) RSD: 0.971%
2	0.229	0.234	
3	0.233	0.228	
4	0.233	0.227	
5	0.233	0.231	
6	0.232	0.229	
Average	0.232	0.230	
SD	0.002	0.002	
RSD (%)	0.862	1.080	

3.3.6. Accuracy: The accuracy was evaluated by adding a known amount of agnuside standard at three different levels to the known sample and then extraction and analysis were done. The results are presented in Table 5. The overall recovery percentages were in the range of 98.75-100.52%. These results demonstrated that the developed method is reproducible with good accuracy.

Table 5. The recovery of agnuside by the proposed procedure

No	Amount spiked (µg/ml)	Amount found (µg/ml)	Recovery (%)
1	21.60	21.35	98.82
2	21.60	21.33	98.75
3	21.60	21.39	99.05
4	32.42	32.09	98.98
5	32.42	31.89	98.37
6	32.42	32.36	99.80
7	41.25	41.46	100.52
8	41.25	40.86	99.06
9	41.25	40.76	98.83
10	92.80	91.65	98.76
11	92.80	91.10	98.17
12	92.80	92.07	99.21

3.4. Sample analysis

The developed method was applied for the determination of agnuside content in some samples of fruits of *Vitex trifolia* L. Each sample was analyzed in triplicate to determine the mean content (%). Analytical results are shown in Table 6.

Table 6. The content of agnuside in the fruits of *Vitex trifolia* L.

No	Sample name	Collected place of sample	Collected time of sample	Agnuside content Avg. ± SD (%)
1	MKT 1 NB	Dinh Hoa, Kim Son, Ninh Binh	12/2021	0.321 ± 0.003
2	MKT 2 NB	Hung Tien, Kim Son, Ninh Binh	3/2021	0.353 ± 0.004
3	MKT 2 TH	Nga Son, Thanh Hoa	12/2021	0.124 ± 0.006
4	MKT 3 NB	Xuan Chinh, Kim Son, Ninh Binh	3/2021	0.333 ± 0.004
5	MKT 3 TH	Trieu Son, Thanh Hoa	12/2021	0.131 ± 0.002
6	MKT4 NB	Van Hai, Kim Son, Ninh Binh	3/2021	0.337 ± 0.008
7	MKT 4 TH	Quamg Xuong, Thanh Hoa	12/2021	0.233 ± 0.002
8	MKT 5 NB	Dong Huong, Kim Son, Ninh Binh	3/2021	0.282 ± 0.005
9	MKT 5 TH	Hau Loc, Thanh Hoa	12/2021	0.386 ± 0.003
10	MKT 6 TH	Nghi Son, Thanh Hoa	12/2021	0.338 ± 0.002
11	MKT 6 NA	Hoang Mai, Nghe An	3/2021	0.175 ± 0.006
12	MKT 7 NA	Quynh Lien, Hoang Mai, Nghe An	12/2021	0.182 ± 0.004
13	MKT 7	Hoang Mai, Nghe An	12/2021	0.169 ± 0.007
14	MKT 8	Quynh Lien, Hoang Mai, Nghe An	3/2021	0.309 ± 0.001
15	MKT 9	Hoang Mai, Nghe An	3/2021	0.317 ± 0.002
16	MKT 10	Quynh Giang, Quynh Luu, Nghe An	3/2021	0.309 ± 0.007
17	MKT 11	Dien Hung, Dien Chau, Nghe An	3/2021	0.297 ± 0.006
18	MKT 12	Dien Hai, Quynh Chau, Nghe An	3/2021	0.376 ± 0.003
19	MKT 13	Nghi Tan, Cua Lo, Nghe An	3/2021	0.122 ± 0.002
20	MKT 14	Nghi Huong, Cua Lo, Nghe An	3/2021	0.296 ± 0.005
21	MKT 15	Hoang Mai, Nghe An	3/2021	0.170 ± 0.004
22	MKT 16	Quynh Thien, Hoang Mai, Nghe An	3/2021	0.185 ± 0.002

This is the first report of a validated HPLC method for rapid identification and quantification of agnuside in the fruits of *Vitex trifolia* L. The sample preparation is simple and does not require the use of a solid phase extraction cartridge, thus using this method for quality and quantitative control of fruits and extract as well as commercial samples of *Vitex trifolia* L. can be carried out. The current Pharmacopoeia of Vietnamese has been recognized as a monograph on *Fructus Viticis trifoliae* but it does not currently have a method for quantitative analysis. In this study, the agnuside was detected in 22 samples and ranged from 0.122 ± 0.002% to 0.386 ± 0.003%. Therefore, a proper method for quantitative determination of agnuside in fruits of *Vitex trifolia* L. is required.

4. Conclusion

A simple and efficient reversed-phase HPLC method was developed for the identification and quantification of agnuside in the fruit of *Vitex trifolia* L. The method is simple, precise, and accurate. Further, it was also validated as per ICH guidelines. The obtained results provide useful information for the quality control of the fruit of *Vitex trifolia* L. and for improving the monograph *"Fructus Viticis Trifoliae"* in Vietnamese Pharmacopoeia in the future.

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IN VITRO ANTI-INFLAMMATORY ACTIVITIES OF FISSISPALLIN B ISOLATED FROM *FISSISTIGMA PALLENS*

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Summary

In vitro* Anti-Inflammatory Activities of Fissispallin B Isolated from *Fissistigma pallens

Inflammation refers to the process from the body against stimulation of infections, injuries, toxins, etc. Acute inflammation could become chronic with a category of other serious symptoms and diseases. Being a rich source of potential bioactivities, plants and their chemical components present a promising reservoir for developing new, safer anti-inflammatory drugs. *Fissistigma pallens* (Finet & Gagnep.) Merr. (Annonaceae), growing in the Northern regions of Vietnam has been used in traditional medicine to treat several diseases. In this study, the anti-inflammatory activity of fissispallin B (**FPB**) isolated from *F. pallens* was evaluated. Its nitric oxide (NO), tumor necrosis factor (TNFα), and interleukin 6 (IL-6) inhibitory activity and IL-10 stimulatory activity using LPS-induced RAW264.7 macrophage inflammatory model. The compound illustrated its promising NO inhibitory effects with IC_{50} values of 2.24 ± 0.17 μM. Besides, fissispallin B significantly expressed the inhibiting capacity on pro-inflammatory cytokines (IL-6, TNF-α) while it had not much effect on stimulation of anti-inflammatory IL-10 cytokine. Treatment of the fissispallin B at 2.5 μM and 5 μM in RAW 264.7 cells remarkably decreased NF-kB protein expression to 36.1% and 51.2% compared to that of the control, respectively ($P<0.05$) by using Western-blot analysis. On the other hand, the high percentage of cell viability indicated this compound had a weak cytotoxic effect in this experiment. In conclusion, fissispallin B showed potential *in vitro* anti-inflammatory bioactivity.

Keywords: *Fissistigma pallens, RAW264.7, Anti-inflammation, Nitric oxide, Fissispallin B (FPB).*

1. Introduction

Inflammation, which is composed of temporary (acute) or permanent (chronic) state, is a mechanism that protects the body from infections or injury [1]. Chronic inflammation is reported to contribute to many diseases such as heart attack, diabetes, etc. [2]. During the inflammatory response, tumor necrosis factor-α (TNF-α) induced vasodilation recruits more immune cells, such as monocytes and neutrophils, to the inflammation site [3]. The overproduction of TNF-α could result in disseminated intravascular coagulation, which leads to the failure of organs such as the liver, kidneys, heart, and lungs [4]. Interleukin 6 (IL-6) enhanced macrophage development and helped them and other leukocytes infiltrate the inflammatory site [4]. TNF-α and IL-6 were pro-inflammatory cytokines that could cause diseases such as asthma, other auto-immune diseases, and even cancer with an excess amount. Hence, the inhibition of these cytokines could control the inflammation. The primary biological function of IL-10 is limited to inflammatory responses. Additionally, IL-10 prevented the infiltration of macrophages into the inflammation site. Thus, IL-10 was a robust controller of inflammation [5]. Moreover, and as reported, Nuclear factor-κB (NF-κB) represents a family of inducible transcription factors, which regulates a category of genes involved in regulation of the inflammatory responses [6].

Many patients with inflammation were advised to consume non-steroidal anti-inflammatory drugs (NSAID), but they still have several adverse effects such as gastrointestinal