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Research Article

An Approach for Developing a Simple and Quick Method for Separation of Asiatic Acid and Asiaticoside Rich Fraction From *Centella Asiatica* and Simultaneous Determination by Reversed-Phase High-Performance Liquid Chromatography

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Abstract: In Asian countries, *Centella asiatica is* exploited for abundant types of pharmacological activities due to the presence of opulent phytochemicals. Asiaticoside, madecassoside, and their sapogenin triterpene acids such as asiatic acid and madecassic acid are the most noticeable triterpenes present in *C. asiatica.* To date, numerous techniques/methods are used to extract and isolate the different kinds of phytoconstituents from *C. asiatica.* Still, most methods require some special requirements, and some procedures are monotonous and time-consuming. Meanwhile, previously reported methods used for the extraction and isolation were not validated for large-scale production, yield, and purity. The study's primary goal is to develop the methodology for extracting and isolating the Asiaticoside and asiatic acid from *C. asiatica* at the minimum time with the highest yield and purity. Asiaticoside and asiatic acid extraction and isolation involved the acid hydrolysis method and recovered in alcohol. The Reverse Phase-High Performace Liquid Chromatography (RP-HPLC) method was developed and validated as per ICH guidelines for quantifying both compounds. The obtained results indicate that the developed method produces asiaticoside and asiatic acid with good purity. As per the ICH guidelines, the RP-HPLC was developed and validated. The proposed method can be used to isolate the asiaticoside and asiatic acid from *C. asiatica.* Some modification in this method leads to the large-scale production of highly pure asiatic acid and asiaticoside for their versatile application in the area of cosmetics and phytopharmaceuticals.

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1. Introduction

Traditionally, in Asian countries, the *Centella asiatica* (Gotu kola) (Family-Apiaceae) is exploited, especially in Ayurveda and Unani, for memory enhancement, healing wounds, reducing

anxiety and stress, leprosy treatment, fever, syphilis, acne, allergies, eczema, psoriasis, etc. types of pharmacological activities. It also produces well-documented neuroprotective effects. Such types of activity/effects produce by *C. asiatica* due to the presence of opulent phytochemicals. To date, more than seventy compounds have been extracted and identified from *C. asiatica.* The majority of phytoconstituents belong to the triterpenes, flavonoids, and essential oils categories. Asiaticoside, madecassoside, and their sapogenin triterpene acids such as asiatic acid and madecassic acid are the most noticeable triterpenes present in *C. asiatica.* (Idris et al., 2021; Lu et al., 2021; Nouri et al., 2021; Ribeiro et al., 2021; Songvut et al., 2021; Sabaragamuwa et al., 2022) Polypharmacological properties of asiatic acid and asiaticoside sparked interest in the research community. Numerous researchers throughout the globe reported the different pharmacological properties of the active phytoconstituents such as anti-inflammatory, antioxidant, hepatoprotective properties, anti-diabetic properties, analgesic, antibacterial, cytotoxic, etc. (Lu et al., 2021; Pingyod et al., 2021; Ribeiro et al., 2021; Thong et al., 2021; Mohammed et al., 2022). These pharmacological activities associated with the asiatic acid and asiaticoside has been thoroughly studied and well documented.

Idris and Nadzir summarised the various techniques/methods (conventional and modern methods) used to extract and isolate the different kinds of phytoconstituents from *C. asiatica* to date. Techniques such as maceration, distillation, Soxhlet, ultrasound-assisted extraction, microwave-assisted extraction, vacuum microwave-assisted extraction, solvent-free microwave extraction, enzymatic pretreatment microwave extraction, and subcritical water extraction were enlisted for the extraction of constituents from *C. asiatica.* Most researchers used ethanol, methanol, water, and a mixture of water with ethanol or methanol to extract the desirable phytoconstituents. (Idris et al., 2021)

Ongoing through the literature on methods for extracting and isolating the phytoconstituents from *C. asiatica*, it was observed that most methods were targeted for extraction of asiaticoside, madecassoside, asiatic acid, and madecassic acid. The main reason behind such target-specific research is the diverse and effective pharmacological activities of these constituents from *C. asiatica.* Global demand for plant extracts with a high percentage of pure phytoconstituents has significantly increased but has not commensurate increased the supply. This urgency has attracted researchers from different communities to develop the extraction and isolation methodology for the essential constituents of *C. asiatica.*

The past decade has seen an increase in the use of plant-based phytoconstituents to prevent or treat various diseases. The versatile use of both constituents states the importance of extraction and isolation in phytopharmaceuticals. All the developed methodologies in the literature are not suitable for large-scale production due to the limitation of each method. Such as, many researchers use column chromatography to isolate desirable constituents from *C. asiatica.* But the column chromatography has its limitations, such as being time-consuming, tedious, and laborious. The reproducibility for the column chromatography was significantly less. Therefore, column chromatography cannot be used for the large-scale production of asiatic acid or asiaticoside. Other methods where sophisticated instruments used for the separating such as supercritical fluid extraction, flash chromatography, etc led to an increase in the cost of the final product. The use of such kinds of appliances produces expensive products. Majorities of phytopharmaceutical industries need an economical method with higher yield and purity. The previously reported methods used for the extraction and isolation were not validated for large-scale production, yield, purity, and economical method. Therefore, there is a need for a simple and economical method for extracting and isolating both constituents from *C. asiatica.*

The availability of these constituents is challenging. We have strongly focused on addressing these obstacles related to the availability of these materials at low cost. This is the essential motivation behind this research. The main purpose of this study was to develop a generic, simple and accurate approach to the extraction and isolation of asiatic acid and asiaticoside from *C. asiatica.* Specifically, we aim to investigate the yield along with purity. To assist the purity, we perform the HPLC analysis of asiatic acid and asiaticosides. We developed the HPLC method and validate it as per International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines.

2. Material and Methods

2.1 Plant material

Dried powder of plant material was purchased from the local crude herbal drugs market, Mumbai. The voucher specimen (ICT/MNPRL/2021/CA-02) was deposited at the Medicinal Natural Products Research Laboratory, Institute of Chemical Technology, Mumbai.

2.2 Reference standards and chemicals

Laboratory reagent-grade solvents were used for extraction and isolation. All the solvents were obtained from Finar Limited. HPLC grade acetonitrile, *o*-phosphoric acid, and water were purchased from Finar Limited. Asiatic acid (Purity ≥ 97% by HPLC) was purchased from Sisco Research Laboratories Pvt Ltd, (SRL) Mumbai, India. Asiaticoside (Purity ≥ 95 % by HPLC) was procured from Sunpure Herbal extract Pvt Ltd, Delhi.

2.3 Method of isolation

In the Soxhlet apparatus, approximately 1000 g of dried powder of *Centella asiatica* was subjected to solvent extraction using petroleum ether (4.5 L) for 8 hr. The obtained extract was collected and stored for further studies. The powdered plant material was dried and further subjected to acid hydrolysis. The defatted material was macerated with 5 % hydro-alcoholic solution (70:30) of sulphuric acid for 6 hr. After hydrolysis, the material was separated using simple filtration. In the obtained filtrate, water was added to precipitate the product (10-12 g). The obtained precipitates were separated and washed with water till neutral for the litmus test. The obtained material was kept for drying under a vacuum. The dried product was further dissolved in alcohol, and activated charcoal was added to remove insoluble impurities. The charcoal containing alcoholic solution was filtered and water was added to the filtrate to precipitate the product. The obtained product was further recrystallized using methanol. The recrystallized product (7-8 g) was dried and used for further analysis. HPLC studies were performed to assess the sample's purity.

2.4 Instrument and chromatographic conditions

RP-HPLC analysis was performed on Thermo Vanquish HPLC System equipped with quaternary pump F, a variable wavelength UV-visible detector, Vanquish column compartment (Oven temperature range 5 ºC to 90 ºC), and an autosampler. The HPLC System is controlled by Chromeleon 7 software. The analysis takes place on the RP-C18 column (Zodiac C_{18}, 4.6 x 250 mm i.d, particle size 5 µm). The appropriate separation of compounds was achieved in the isocratic mode. Acetonitrile and 0.2 % orthophosphoric acid in water (85:15 v/v) with a flow rate of 0.6 mL/min was used as the mobile phase. The UV detector was set at 210 nm for the detection. The run time was 45 min for the analysis. Analysis was performed at room temperature.

2.5 Preparation of stock solutions for RP-HPLC

The standard stock solution of asiatic acid and asiaticoside was prepared by dissolving 10 mg of each in 10 mL methanol to give a 1 mg ml $^{-1}$ concentration. Further calibration levels (2-12 µg ml $^{-1}$) were prepared by diluting the standard stock solution of each standard with methanol to obtain appropriate concentrations. The mixture of both standards was also prepared as per above mention method. Standard solutions were stored at 4 °C. These prepared standards were used for the RP-HPLC analysis.

2.6 RP-HPLC analysis

The RP-HPLC method was `developed and validated as per ICH guidelines (Q2 (R1)). The method validation was performed for the linearity, accuracy, precision, specificity, selectivity, and sensitivity ((limit of detection (LOD) and limit of quantification (LOQ)).

2.6.1 Optimization of method

2.6.1.1 Selection of wavelength

Due to the different absorption maxima of both compounds, the selection of scanning wavelength was essential for detecting both compounds. The UV detection was carried out at a lower wavelength due to the absence of strong chromophores in both compounds. Therefore 210 nm was selected for the scanning because scanning at 210 nm gave the best sensitivity with minimum noise detected for both compounds.

2.6.1.2 Selection of stationary phase

C_{18} RP columns from different makers were tried during the method development. Zodiac C_{18} RP-HPLC column (4.6 * 250 mm, 5 µm) was selected for further studies.

2.6.2 Validation of method

The developed HPLC method was validated as per the ICH guidelines (Q2 (R1)).

2.6.2.1 Linearity

Linearity of both compounds was performed using six different concentrations ranging from 2 µg/mL to 12 µg/mL. All the measurements took triplicate and were plotted using linear regression of the mean peak area versus concentration. The obtained linear regression equation was utilized for further calculations.

2.6.2.2 Accuracy (recovery study)

Accuracy or recovery study of the method was performed using the standard addition method. The analysis was carried out in triplicates. In the recovery experiments, by spiking (Adding) a known amount of both standards at three different levels (spike level-1 (50 %), spike level-2 (100 %), and spike level-3 (150 %)) to a standard of known concentration. Calculate the percentage recovery for each spiked level.

2.6.2.3 Precision

To determine the method's precision, intraday and interday precision of standard solutions were performed. Intraday precision was measured in replicates ($n = 6$) of both standard solutions on the same day, while interday precision was achieved over six consecutive days ($n = 6$). The precision results were expressed in terms of % relative standard deviation (RSD) $\leq 2\%$. Values with % RSD $\leq 2\%$ for peak area responses were accepted.

2.6.2.4 Sensitivity (limit of detection and limit of quantification)

As per the ICH guidelines, the limits of detection and quantification of the developed method were calculated from the standard deviation of the response and slope of the calibration curve of markers using the following formulas:

Limit of detection = $3.3 \times \sigma / S$,

Limit of quantification = $10 \times \sigma / S$,

where σ is the standard deviation of the response and S is the slope.

2.6.2.5 Specificity and selectivity

The specificity of the developed method was estimated by studying the blank non-interference and comparing the retention time of target analyte peaks from the sample analyst with the reference standard. No difference was found in the peaks and spectra of reference standards and the sample analyzed. The peak purity tool was used to evaluate the peak purity of the samples. Hence the developed method demonstrates specificity and selectivity.

3. Results and Discussion

3.1. HPLC method development and validation

3.1.1 Optimization of chromatographic conditions

A novel RP-HPLC method was developed to quantify asiatic acid and asiaticoside in the extract and isolated fractions/compounds/material. Maintaining the optimum chromatographic condition throughout the experimentation is very important for the detection and quantification of asiatic acid and asiaticoside in the extract and isolated compounds/fractions. During the method development, various experimental trials were performed to obtain an accurate, rapid, precise, and sensitive RP-HPLC method with a high level of specificity. In the RP-HPLC method, various chromatographic conditions were considered, such as selecting the appropriate stationary phase, suitable type and mobile phase ratio, flow rate, and detection wavelength. Optimization of each chromatographic condition is described below. A standard solution of asiatic acid and asiaticoside was used for the method development.

3.1.2 Selection of stationary phase

Numerous provisional runs were performed using C_{18} RP columns from different makers. Initially, we used the Agilent RP column (Agilent Zorbax Bonus-RP; 4.6 * 250 mm, 5 µm) to separate the asiatic acid and asiaticoside. Both compounds were not adequately resolved using this column, and the retention time (RT) of both compounds was found to be more than 10 min. We rejected the Agilent RP column because the carbon load has approx. 9.5 % and surface area is around approx. 180 m^2/g. Due to its specifications, it's not suitable for our analysis. The important objective of the method development was to reduce the cost and time of quantifying both compounds. To satisfy the purpose of the method development, we change the stationary phase. For further trials, we select, Cosmosil C_{18} (Cosmosil 5C_{18}-MS II; 4.6 * 250 mm, 5 µm) column. In this case, fairly resolved symmetrical peaks of both compounds were observed, along with RT of both compounds was found to be around 7-9 min. The results obtained from this stationary phase were also not satisfactory. We rejected the Cosmosil C_{18} column because the carbon load and surface area are around approx. 16 % and 300 m^2/g respectively. These specifications are also not suitable for the analysis of both compounds. Again, there was a need to select a suitable stationary phase that produces a good separation and peak symmetry and less than 10 min RT. To achieve the set goal, we performed the trials with Zodic C_{18} RP-HPLC column (4.6 * 250 mm, 5 µm). Both compounds separated properly, and the RT of both compounds was less than 8 min. The carbon load on Zodic C_{18} RP-HPLC column is approx. 24% and surface area approx. 440m^2/g. The column has a high carbon load and surface area as compared to the previous stationary phases as well as specifications of these columns were suitable for the analysis of both compounds. Therefore, for further study, this stationary phase was selected.

3.1.3 Influence of mobile phase and organic modifier

The reported literature suggested the use of methanol, acetonitrile, water, phosphoric acid, and acetic acid as a mobile phase for the chromatographic separation of both compounds. (Kaur et al., 2016; Monton et al., 2019) Initially, gradient elution of the mobile phase was tried for the separation. During the experimentation, the baseline was not stable as well as the RT of both compounds was very high. Therefore, the gradient elution method was rejected. To obtain the optimum separation, isocratic mixtures of several mobile phases were tried. The use of methanol instead of other organic solvents is the cost-effective approach for the quantification of several compounds. But in the case of asiatic acid and asiaticoside, methanol is not a suitable organic solvent for chromatographic separation. To ensure the proper chromatographic separation of both compounds, the organic content of the mobile phase was also considered. Substituting methanol with acetonitrile led to the improvement of chromatographic conditions. The effect of acetonitrile concentration in the acetonitrile: water mobile phase on the retention time of both compounds was studied. The water concentration in the mobile phase increased the retention time of both compounds (due to higher hydrophobic interaction between the stationary phase and both compounds). To improve the chromatographic conditions in water, *o*-phosphoric acid was added. The effect of *o*-phosphoric acid on the retention time of both compounds was investigated. Based on all performed trials, a combination of acetonitrile: water with 0.2 % *o*-phosphoric acid led to good chromatographic conditions. The expected results, i.e. optimum resolution with a short analysis

time, were achieved using this mobile phase combination. So, acetonitrile: water with 0.2 % *o*-phosphoric acid was selected as the mobile phase throughout the experiments.

3.1.4 Influence of flow rate

The flow rate of the mobile phase significantly affects the separation of the desirable constituents. To obtain a suitable flow rate for separating both compounds, we tried a range of different flow rates from 0.5 to 1.5 ml/ min of the mobile phase. An increase in the flow rate during the experiments led to faster elution of the compounds, but no proper separation of the desired product was observed. A sharp and symmetric peak was observed at the mobile phase's 0.6 ml/min flow.

3.1.5 Influence of detection wavelength

The absorption maxima for both compounds were different. Selecting an appropriate scanning wavelength was crucial for the experiments to detect both compounds. Several scanning wavelengths were tried to obtain good sensitivity and minimum noise during method development. Initially, we selected a few scanning wavelengths mentioned in the literature, such as 200 nm, 205 nm, and 206 nm. The strong chromophores are absent in both compounds therefore UV detection is carried out at lower wavelengths (200–210 nm). During the analysis of the experimental trials, it was observed that there was a single unwanted peak observed between the asiaticoside and asiatic acid at scanning wavelengths of 200 nm, 205 nm, and 206 nm. To find out the unknown peak, several experiments were repeated (trials with a single compound analysis and a mixture of both compounds). Finally, a blank run of Methanol (without any compound) was performed with the developed chromatographic conditions. The obtained results suggested that the methanol produces the peak at selected scanning wavelengths. Therefore, the unwanted peak observed during the analysis was confirmed as methanol because the samples were dissolved in the methanol. So, to minimize the area of the methanol from the chromatograph, we tried slightly higher scanning wavelengths for detection. Scanning at 210 nm gave the best sensitivity with minimum noise detected for both compounds, and the unwanted peak of methanol was also reduced (shows the minimum area in the chromatograph).

Finally, the effect of the stationary phase, mobile phase, organic modifier, flow rate, and detection wavelength was investigated to develop the RP-HPLC method for quantification of the asiaticoside and asiatic acid. The Zodic C_{18} RP-HPLC column was selected as a stationary phase for the chromatographic analysis. Acetonitrile in combination with 0.2 % OPA in water (ratio of 85: 15 (v/v) respectively) was selected as the mobile phase with 0.6 ml/min flow rate. At 210 nm, the samples were analyzed. The RT for Asiaticoside and Asiatic acid were found to be at 4.1 min and 5.9 min respectively.

The developed RP-HPLC method was validated as per the ICH guidelines. The observations of Calibration curves, Repeatability, Recovery Studies, and Sensitivity of Asiaticoside and Asiatic acid are reported in Table 1, Table 2, Table 3, and Table 4, respectively.

Table 1. Parameters of calibration curves for two quantified compounds (Asiaticoside and Asiatic acid)

Compound	Regression Equation	R^2	Linear Range (µg ml $^{-1}$)
Asiaticoside	y = 0.4721x - 0.3732	0.9997	2-12
Asiatic acid	y = 1.0697x - 0.5505	0.9995	2-12

Table 2. Repeatability of Asiaticoside and Asiatic acid

Compound	Repeatability RSD (%) (n = 6)
Asiaticoside	0.2301
Asiatic acid	0.3695

Table 3. Recovery Studies of Asiaticoside and Asiatic acid

Compound	Level (%)	Initial Amount (µg ml $^{-1}$) (n =3)	Added (µg/ml) (n =3)	Found (µg ml $^{-1}$) (n =3) ± SD	Recovery (%) (n =3)	RSD (%)
Asiaticoside	50	4	2	5.98 ± 0.065	99.66	1.098
	100	4	4	8.00 ± 0.017	100.08	0.216
	150	4	6	10.04 ± 0.019	100.40	0.191
Asiatic acid	50	4	2	5.99 ± 0.007	99.92	0.129
	100	4	4	8.02 ± 0.047	100.28	0.596
	150	4	6	9.99 ± 0.086	99.90	0.870

Table 4. Sensitivity Studies of Asiaticoside and Asiatic acid

Compound	LOD (µg/ml)	LOQ (µg/ml)
Asiaticoside	0.5049	1.5302
Asiatic acid	0.6931	2.1003

3.2. Extraction method development

Various methods were tried to optimize the isolation process to extract the Asiatic acid and asiaticoside from the *Centella asiatica*. During the development process, methods already mentioned in the literature were also attempted. Acid hydrolysis and basic hydrolysis methods were utilized for the procedure (Table 5). Based on various observations, the acid hydrolysis method was selected during the process development, and the yield obtained from this method was high compared to the other methods.

3.2.1 Basic hydrolysis method trials

Firstly, the plant material was subjected to the basic hydrolysis method. The aqueous solution of 2% potassium hydroxide was used for basic hydrolysis. The mixture of the plant material and the basic solution was kept for 5 hr. for hydrolysis. After completing the basic hydrolysis, the material was separated, and in the filtrate (dark green solution) addition of the dilute hydrochloric acid (5 %) took place. Adding hydrochloric acid leads to the formation of the precipitate of the crude triterpenoids. The obtained precipitated was washed till neutral to litmus paper. After the washing, the residue was kept for drying and used further for recrystallization. For the recrystallization process, crude triterpenoids were dissolved in the ethanol; some water was added in the ratio of 1:5 and kept in a cooling condition. After a few hours, there was the formation of the buff white color precipitate takes place. The precipitate was separated, identified, and analyzed for purity.

During the trials with the basic hydrolysis method, various concentrations of potassium hydroxide (aqueous as well as alcoholic) such as from 2% to 10 %, and for hydrochloric acid, the range of 2% to 10 % were also tried. The crude triterpenoids obtained from the basic hydrolysis method were sticky and challenging the processing to obtain the desired product. For the recrystallization process, ethanol, methanol, and ethyl acetate were used. But, ethanol gives better yield and purity than the other solvents. The results did not demonstrate a direct correlation between purity and the yield of the desired product.

After the basic hydrolysis, the solution was very difficult for separation/filtration. The plant material absorbed a large amount of solution/solvent, and laboratory-scale filtration takes time for the separation of absorbed solvent/solution. Therefore, we used the mechanical method to separate the basic solution from the plant material. The developed method performs the worst in terms of the filtration process, nature of the material (sticky material obtained), purity, and yield.

We are left with the conclusion that this approach was limited in terms of purity and yield of the desired product. A low performance was observed with this approach; therefore, the method was rejected.

3.2.2 Acid hydrolysis

For the acid hydrolysis method development, hydrochloric acid and sulphuric acid were used. At initial trials, various concentrations of hydrochloric acid were used, but the yield and purity of the product were obtained with the sulphuric acid trials.

3.2.2.1 With hydrochloric acid

Initially, the plant material was kept for acid hydrolysis with 10 % hydro-alcoholic solution (70:30) of hydrochloric acid for 12 hr. After the hydrolysis, the plant material was separated by filtration, and the filtrate was processed for further experimentation. The filtrate's volume was reduced to 30 % to 50 % from its initial concentration (to remove the alcohol). The concentrated filtrate was kept aside for a while to sediment some residue of crude triterpenoids. The formed precipitate was separated, dried, and processed for recrystallization with the ratio of methanol and water. Diethyl ether, methanol, ethanol, and ethyl acetate were used as the solvent for recrystallization. The product was obtained in the portion of methanol and water. The obtained product was further processed for the HPLC analysis. Based on HPLC analysis, it was observed that the method needs modifications concerning the yield and purity of the final product.

To enhance the yield and purity of the product, the following modifications were made in the previous acid hydrolysis methodology such as there a range of 2 to 10 % solution of hydrochloric acid was tried as well as alcohol and methanol were used as a solvent along with water at various concentrations from a range of 50 % to 90 %. During the method development of the acid hydrolysis with hydrochloric acid, it was observed that the yield was significantly less for processing the sample for further studies. In the final product, the green color was observed, which may be due to various impurities. The purity of the obtained product was also less compared with the desired standards.

3.2.2.2 With sulphuric acid

The same methodology was utilized with sulphuric acid instead of hydrochloric acid. Various trials with modifications were performed to obtain more yield and purity of the product. Change in the concentration of acid, change in the solvent (methanol and alcohol), change in the proportion of the water concerning solvent, variation time of the hydrolysis, etc. modification was tried with the sulphuric acid hydrolysis method. The material was recrystallized using methanol, alcohol, diethyl ether, and ethyl acetate. The yield of crude triterpenoids was more as compared to hydrochloric acid hydrolysis. The obtained results show that the product's purity and yield are satisfactory compared to the previous acid hydrolysis and basic hydrolysis methods. The results conclude that no apparent advantage exists in utilizing our method for the industrialization of the laboratory to a large scale.

Table 5. Few experimental trials for the extraction of Asiaticoside and Asiatic acid

Sr No	Experimental Conditions	Observation	Conclusion
	Basic Hydrolysis Methods		
1.	Plant material --» Defatting with Petroleum ether (Pet ether) --» Defatted plant material kept for basic hydrolysis with 2 % aqueous KOH (5 hr) --» Dark green basic solution obtained --» Addition of 5 % aqueous HCl solution --» Formation of Precipitae of Crude triterpenoids (CTT) --» Wash PPT till neutral to litmus --» Crude triterpenoids subjected to Recrystallization using various solvents. Recrystallization using Methanol Recrystallization using Alcohol Recrystallization using Ethyl acetate	➢ The alcohol recrystallized product was sticky. The product was difficult for the separation. The obtained product was dark green. ➢ Methanol recrystallized product was not sticky. The obtained product was light gray. Methanol recrystallization product yields more yield as compared to alcohol recrystallized product. ➢ The ethyl acetate recrystallization process does not yield any product.	HPLC results are not favorable to the process. Need to change the process or recrystallization process. Because there is green coloration was observed in the product.
2.	Plant material --» Kept for basic hydrolysis (2 % Aq. KOH) (5 hr) --» Dark green basic solution obtained --» Addition of 10 % aqueous HCl solution --» Formation of CTT --» Wash PPT till neutral to litmus --» Crude triterpenoids subjected to Recrystallization using methanol-water combination (1:5 ratio) at cooling conditions	The obtained crude triterpenoids were sticky. No product was obtained from the recrystallization method.	The method was rejected because no product was obtained during the recrystallization. As well as the obtained crude Triterpenoids are sticky.
3.	Plant material --» Defatting with Pet ether --» Defatted plant material kept for extraction with methanol --» Methanolic extract --» Addition of water --» Formation of CTT --» Rejected due to the sticky nature of the product	The obtained crude triterpenoids were sticky.	The obtained material was sticky therefore the method was rejected.

Table 5. Few experimental trials for the extraction of Asiaticoside and Asiatic acid (continue)

Sr No	Experimental Conditions	Observation	Conclusion
	Acid Hydrolysis Methods		
4.	Plant material --» Kept for Acid hydrolysis (HCl) (15 hr.) (70:30 Alcoholic Solution) 10 % of acid --» Addition water --» Kept aside for formation of CTT --» Crude Triterpenoids --» Subjected to Recrystallization Using Ethyl acetate at the cooling condition	Greenish PPT obtained.	Need to change the method for the better results.
5.	Plant material --» Kept for Acid hydrolysis (H_2SO_4) (15 hr.) (70:30 Alcoholic Solution) 10 % of acid --» Addition water --» Kept aside for formation of CTT --» Crude Triterpenoids --» Subjected to Recrystallization Using Ethyl acetate at the cooling condition	The H_2SO_4 method produces the cruder triterpenoids as compared to the HCl method. Therefore for further studies, we select H_2SO_4.	Need to change the method for the better results.
6.	Defatted plant material --» Kept for Acid hydrolysis (H_2SO_4) (70:30 Alcoholic Solution) 10 % of acid --» Addition water --» Kept aside for formation of CTT --» Crude Triterpenoids --» Subjected to Recrystallization Using Ethyl acetate at the cooling condition	No Compound was observed in the recrystallization process.	Need to change the recrystallization process.
	Acid-Base Titration Methods		
7.	Defatted plant material --» Kept for basic hydrolysis with 2 % Aqueous KOH --» Filter --» In the filtrate addition of 5 % Dil. HCl --» Formation of CTT --» Wash the PPT till neutral to litmus --» Dry the PPT & Dissolved in Methanol --» Addition of water in the Methanolic solution of PPT --» Formation of precipitae --» Subjected to Recrystallization	No Compound was observed in the recrystallization process.	Need to change the recrystallization process.
	Final Developed Method		
8.	Defatted plant material --» Kept for Acid hydrolysis (H_2SO_4) (70:30 Alcoholic Solution) 10 % of acid --» Filter --» Addition water --» Kept aside for formation of CTT --» Crude Triterpenoids --» Separate the obtained PPT and dried under vacuum --» Dried product dissolved in methanol/alcohol --» addition of activated charcoal and filter --» In filtrate addition of water --» Formation of PPT --» Separated and dried --» Used for further analysis.	The obtained material was light brown with a good yield.	HPLC analysis confirms the presence of asiatic acid and asiaticoside.

3.2.3 Final developed method

The plant material was defatted with the pet ether to remove the wax, lipids, and phytosterols. These phytoconstituents create interfere with the product yield as well as purity. The defatted plant material was kept for acid hydrolysis with 10 % hydro-alcoholic solution (70:30) of sulphuric acid. After the hydrolysis, the plant material was separated by a simple filtration method. Now, in the filtrate, the addition of water takes place to precipitate the product. (Change of pH from 1 to 3 – 4) The obtained precipitate was separated by simple filtration and dried under a vacuum. The dried product was the other process for recrystallization with alcohol and methanol. Before the recrystallization, the material was dissolved in alcohol or methanol, and activated charcoal was added to absorb the impurities. Filter the solution and in the filtrate addition of water takes place. After the addition of water, there was the formation of a precipitate takes place. The formed precipitate was separated and dried for further analysis.

After the analysis, it was observed that the purity of the obtained product concerning the desired product was around 65-70 % only. To enhance the purity, some modifications in the previously developed methodology take place. Up to the addition of the charcoal, the process was repeated, and the precipitate was formed by the addition of water into the filtrate. The formed precipitate was dissolved in methanol and concentrated in the methanolic solution. The concentrated solution was kept aside for some time to form the desired product. After some time, a buff white color product was obtained (7 - 8 gm). The practical yield of the method was 0.7 - 0.8 % with respect to the purity of the product. The obtained material was subjected to analysis. The RP-HPLC analysis of the obtained material shows the presence of asiatic acid around 12-15 % and asiaticoside 65-68 % (Figure 1). The obtained material is the rich fraction of both compounds. The results surpass the earlier work in this area regarding purity and yield (Table 5).

This method produces more crude triterpenoids, asiatic acid yield, and purity was also high compared to the previously developed methodologies. This method has demonstrated a marked improvement in the quality of desirable compounds. In all of our experiments, we found that the proposed solution was better than the previous methods presented in the literature.

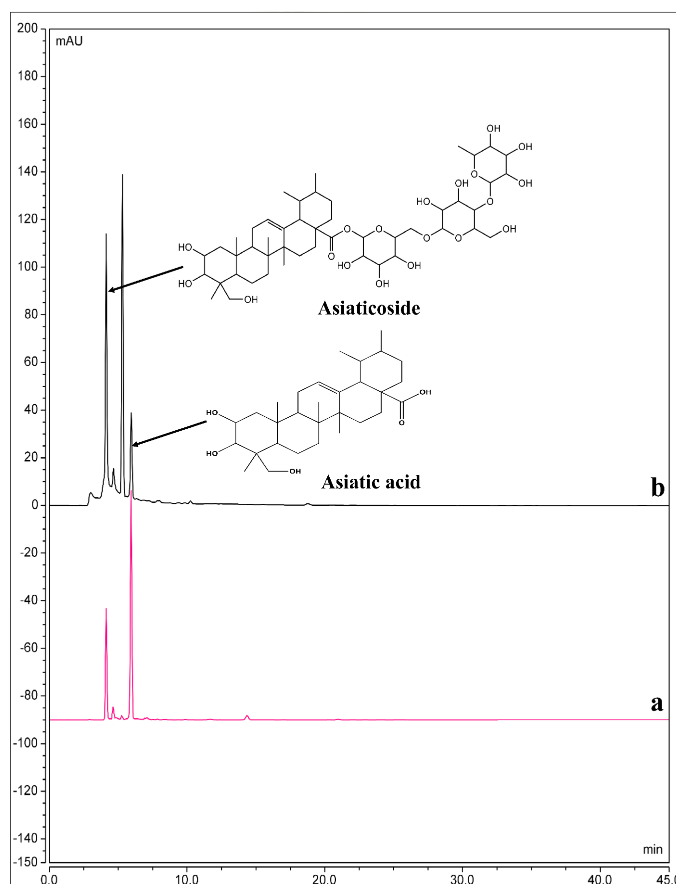

Figure 1. Overlaid HPLC Chromatograph of a) Standard of Asiaticoside and Asiatic acid b) extracted rich fraction of Asiaticoside and Asiatic acid.

Conclusion

The outcome of various experiments led to the conclusion that the developed method is simple enough to be applied for the extraction of a rich fraction of the asiatic acid and asiaticoside. It is also producing a good quality product with significant yield and purity as compared to the previous methodologies. This has been regarded as a useful method for the large-scale production of both compounds. The developed method presents some practical advantages, especially in the case of large-scale production because the materials (Chemicals) which are we used during the extraction method development are accepted by the industries for the pilot-plant setups. Overall, the methodology produces good results for both compounds and can be applied to industries.

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