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Research Article

Validation of Rosmarinic Acid Quantification using High-Performance Liquid Chromatography in Various Plants

Andiri Niza Syarifah[1], Herman Suryadi[2,*], Abdul Mun'im[3]

ABSTRACT

Introduction: Rosmarinic acid has been utilized in traditional medicine as antioxidant, antiinflammation, anticancer and antibacterial. In order to control the herbal quality, validation of rosmarinic acid determination using high-performance liquid chromatography was developed. The objective of this report was to validate an HPLC technique for assessing rosmarinic acid levels. and application that method to determine rosmarinic acid in *Rosmarinus officinalis, Symphytum officinale, Mentha piperita, Orthosiphon stamineus* and *Salvia officinale*. **Methods:** The chromatographic separation was carried out on a reversed-phase C18 column with a mobile phase of 0,1% formic acid and acetonitrile and an isocratic elution at a flow rate of 0,5 mL/min. The wavelength for detection was set to 330 nm. The method has been validated for precision, accuracy, linearity, limit of detection, and limit of quantitation. **Result:** The concentration response of the detector was linear, with a coefficient of determination of 0.9933. The HPLC technique had an accuracy of 101,00 ± 6,43%. The precision was 6,36% when expressed as a coefficient of variation (CV). The highest level of rosmarinic acid was 214,86 ± 0,60 µg/mL in *Rosmarinus officinalis* extract. **Conclusion:** The HPLC method was valid to analyse rosmarinic acid level. The method can be applied in routine determination of rosmarinic acid of phytopharmaceutical products.

Key words: Rosmarinic acid, HPLC, *Laminaceae Borraginaceae*.

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INTRODUCTION

Some secondary metabolites of plants have numerous health benefits such as polyphenols. Polyphenols was found in variety plants and has therapeutic effects[1,2]. Previous study reported the phenolic compounds have antioxidant, antiinflammation and antibacterial activity [3–5]. Rosmarinic acid is one of the most typical phenolic compounds. As the caffeic acid ester of 3,4-dihydroxyphenyllactic acid, rosmarinic acid is a phenylpropanoid.[6,7].

Rosmarinic acid was reported occurs in Boraginaceae and Lamiaceae plant families [8,9]. One of Boraginaceae family was *Symphytum officinale*, commonly known as Comfrey. Rosmarinic acid level in *Symphytum officinale* was 7,557±0,005 mg/g in comfrey root sample [3,10]. *Rosmarinus officinalis L.* (Lamiaceae), also known as rosemary, is a herb that is utilized all over the world. Rosemary extract also contains a significant amount of phenolic chemicals, such as rosmarinic acid [11,12]. Peppermint (*Mentha piperita*) is a Lamiaceae herbaceous plant with a global distribution. Mentha species dried leaves have been used as a herbal tea and as a primary element in several pharmacological formulations with multiple health benefits in cooking as a herb for centuries[5]. *Orthosiphon stamineus*, the java tea plant, is widely grown throughout Southeast Asia, Australia, and southern China. It has been used as a tea in South Asia for thousands of years, and it's also a popular traditional Chinese medicinal herb for kidney stones[13]. There were substantial changes in the composition of phenolic extracts from different Salvia species, and the phenolic extracts of Salvia officinalis L. demonstrated considerable scavenging action of active oxygen, such as superoxide anion radicals, hydroxyl radicals, singlet oxygen, and lipid peroxides. The results show that the efficacy is due in part to the presence of phenolic acids, specifically rosmarinic acid, which has been found in large amounts in a number of therapeutic plants.[6].

On the previous study, rosmarinic acid was determined using gradient HPLC method, LC/MS/MS, spectrophotometry method and Fourier transform infrared[3,6,14]. HPLC is a simple and reliable methods for routine analysis and easily used for quantification of individual compounds. Most of research to determine rosmarinic acid used gradient HPLC. Although HPLC separation has been reported for calculation of rosmarinic acid in some plants, a fast, simple and valid analytical method is needed. Therefore, this study aims to develop and validate a novel isocratic HPLC method with photodiode array detector for the analysis of rosmarinic acid contents in Boraginaceae and Lamiaceae family plants.

MATERIALS AND METHODS

Materials

Comfrey leaves, Rosemary leaves, Sage Leaves, Peppermint leaves and Java tea was obtained from local market in Yogyakarta Province, Indonesia. The solvent used in this research was pro analysis. Methanol p.a (Merck, Germany), Acetonitril p.a. (Merck. Germany), Aqua pro injection/ API (PT Ikapharmindo Putramas, Indonesia) and Formic acid (Merck, Germany). Rosmarinic acid standard was purchased from Phytolab, Germany.

Instrumentation

Analytical balance (VIBRA HT), High Performance Liquid Chromatography HPLC-0053 (Agilent,

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USA), Ultrasonic (Daihan, Korea), Zorbax Eclipse Plus C18 Analytical 4.6x150mm 5-micron (Agilent, USA) and micropipet 100-1000 μl (Thermo Scientific, USA).

Extraction

Each sample were diluted using methanol p.a. as a solvent in ratio 1:10. Extraction was carried using UAE methods in ultrasonic bath at 50^{0}C for 50 minutes. The extracts of sample were filtered, the supernatant was separated and evaporated using water bath until get the thick extract.

Standard solution preparation

Standard solution was prepared for rosmarinic acid. Rosmarinic acid solution was made into a stock solution with concentration of 1 mg/ml prepared in 30% acetonitrile. Series concentration of working solution were made by diluting the standard solution using 30% acetonitrile.

Chromatography conditions

Chromatography condition were supported with Zorbax Eclipse Plus C18 (4.6x150mm, 5μm) for stationary phase. The mobile phase used 30% acetonitrile and formic acid (0,1% v/v). The chromatography was set at isocratic mode and the flow rate 0,5 ml/min. Wavelength detection was 330 nm, injection volume 20 μl, temperature 30^{0}C and run time was set at 15 min.

System suitability test

System performance was check before the chromatographic runs. The tailing factor (T) and theoretical plates (N) are system suitability test variables.

Linearity Test

Linearity test was determined by using working standard solution of rosmarinic acid at series concentration (10-100 μg/ml). Each concentration was evaluated three times. Plotting the peak area against standard concentration produced the calibration curve. Data were evaluated for coefficients corelation (R^2) in linear regression method.

Accuracy

Percent recovery was the parameter of accuracy of the method. The accuracy was tested by adding a reference standard into sample. The total amount of 20 μl of solution was injected in the chromatography system triplicate.

Precision Test

Determination of precision of the method using addition of rosmarinic acid standard into sample. A total of 20 μl of solution was injected in the chromatography. The test was injected triplicate. Precision was evaluated as coefficient variance.

Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ under the proposed chromatography condition were evaluated by diluting the working standard solution to obtain the signal-to-noise (S/N) of analytes at 3:1 and 10:1 for LOD and LOQ, respectively.

Determination of rosmarinic acid level

Rosmarinic acid level was calculated for each sample. Sample were diluted in acetonitrile and 0,1% formic acid (30 % v/v). Sample was filtered using 0,45 μm membrane filter and homogenized before injected to chromatogram system.

RESULTS AND DISCUSSION

System suitability test

The specification of the method was assessed by peak purity using UV spectrum obtained from HPLC-DAD. The UV spectrum was recorded from 190 nm to 450 nm (Figure 1). At this study, rosmarinic acid detection used maximum wavelength at 330 nm.

Recently, rosmarinic acid was detected using several mobile phase systems of isocratic and gradient HPLC. The HPLC system used acetonitrile-water and methanol-water in combination with phosphoric acid showed the best separation of rosmarinic acid[6,14]. However, rosmarinic acid detection takes a lengthy period due to its long retention time. The retention time of rosmarinic acid was greater than 20 minutes on a gradient HPLC system. Meanwhile, rosmarinic acid was captured after 20 minutes using a mobile phase combination of methanol-water and formic acid[15]. On the other hand, mobile phase combination of acetonitrile-water revealed a fast retention time within 5 minutes but the recent study used 60% of acetonitrile in isocratic condition at flow rate of 1,5 ml/min[16]. The recent study needs some improvement such as efficiency of solvent use as mobile phase. At this study, chromatogram of rosmarinic acid showed the fast retention time within 6 minutes with 30% of acetonitrile-water in combination with formic acid at flow rate of 0,5 ml/min (Figure 2). It showed efficiency in using solvent of mobile phase.

In the system suitability test, the standard solution of rosmarinic acid with a flow rate 0,5 ml/min was injected 20 μl at a concentration of 0,20 mg/ml. The test result showed the theoretical plates and tailing factor are in accordance with the literature specification[12]. Table 1 reports the research of the system suitability test.

Linearity test

The linearity test is occurred to explain the correlation of response and concentration. Linearity is showed by coefficient determination (R^2) that closes to 1. The linearity of method was met the parameter with R^2 of 0,987 (Figure 4). As a result, it is possible to conclude that the approach generates a linear response to rosmarinic acid concentrations.

Accuracy and Precision test

The accuracy and precision test are investigated by the standard enumeration method which is to add a reference standard into the sample. This method was chosen since it is unable to construct a sample matrix without the presence of analyte[17]. The method's calculated value's similarity to the nominal concentration of the analyte proved the accuracy of an analytical method (expressed in percentage). The

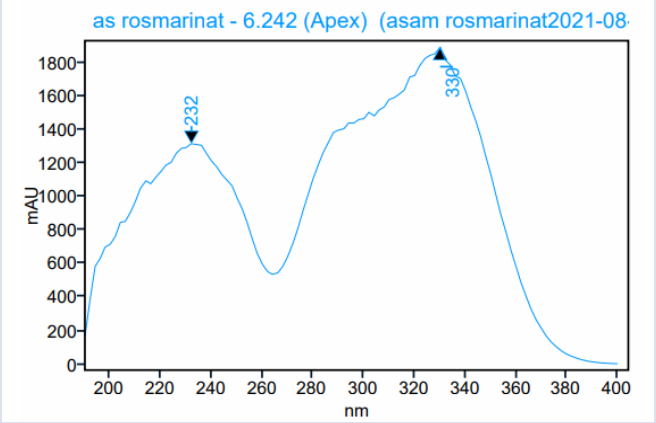

Figure 1: Rosmarinic acid UV spectrum.

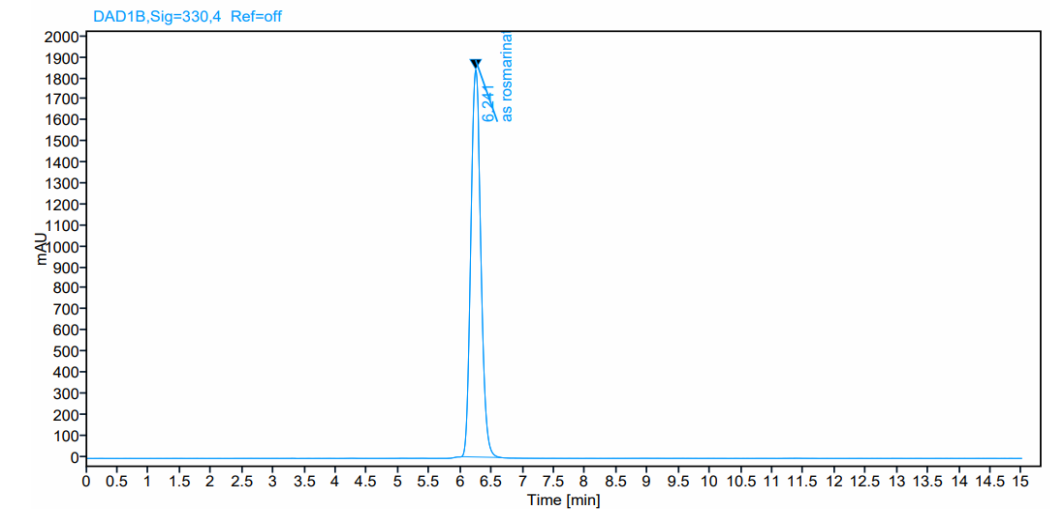

Figure 2: Chromatogram of rosmarinic acid.

Table 1: System suitability test of rosmarinic acid.

	Tailing Factor	Theoritical Plats	Repeatability (RSD, n=6)
Standard	1,22	9963,33	0,02
Literature specification	<2	>2000	<1%

Table 2: Accuracy test of rosmarinic acid.

Rosmarinic acid standard	Theorotical (µg/ml)	Found (µg/ml)	Recovery (%)
1	2,020	1,941	96,095
2	2,020	1,992	98,626
3	2,020	2,187	108,279

Table 3: Presicion test of Rosmarinic acid.

Reference standard	Intra-day precision	Inter-day precision
Rosmarinic acid	6,37 %	3,46 %

Table 4: Validation method parameter.

Parameter	Rosmarinic acid
Regression equation	y = 83,82 x + 977,62
Coefficient corelation (R^2)	0,9866
Linearity range	1-100 µg/ml
LOD	14,19 µg/ml
LOQ	43,00 µg/ml

Table 5: Determination of rosmarinic acid in various plant extracts.

Extracts sample	Rosmarinic acid level (µg/g)			Mean	SD
	1	2	3		
Symphytum officinale	0,169	0,167	0,170	0,169	0,068
Rosmarinus officinalis	8,599	8,568	8,616	8,594	0,598
Mentha piperita	0,189	0,189	0,190	0,190	0,023
Orthosiphon stamineus	0,454	0,353	0,242	0,350	5,295
Salvia officinale	0,052	0,080	0,051	0,061	0,413

result showed in Table 2 that the recovery at the addition of 100% rosmarinic acid were 101,00 ± 6,43 % (n = 3).

The analytical method's precision indicates the consistency of repeated individual analyte determinations. The coefficient of variation is used to express precision (CV). The result showed that the CV values in intraday at the addition of 100% rosmarinic acid was 6,37% (n = 3) and the inter-day was 3,46% (n=3). The precision test showed in Table 3. According to the International Conference on Harmonization, this suggested technique meets the accuracy and precision requirements[18].

Limit of detection (LOD) and limit of quantification (LOQ)

The results of rosmarinic acid validation parameter describe in Table 4. The LOD and the LOQ were less than 14,19 µg/mL and 43,00 µg/

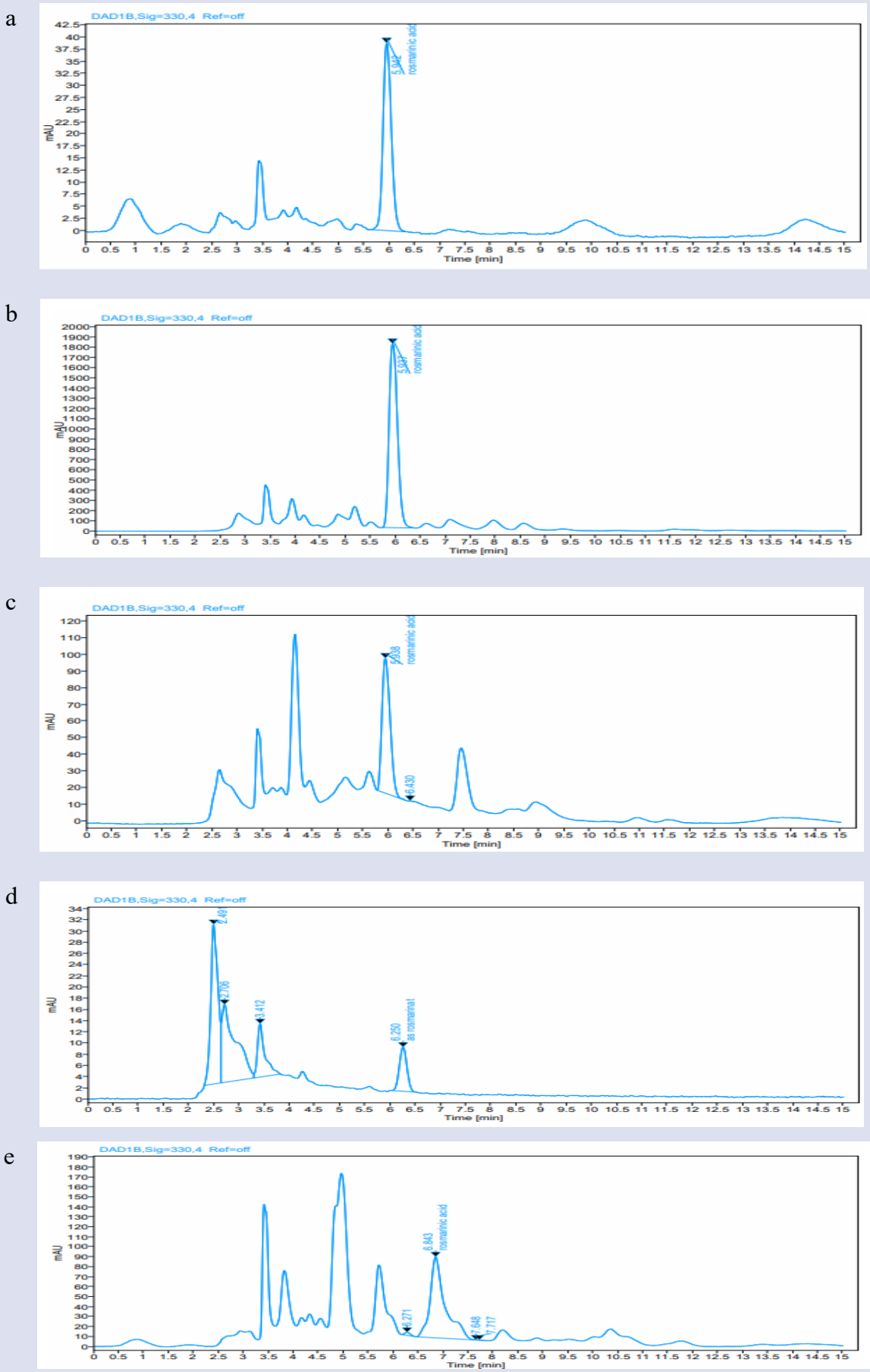

Figure 3: Chromatogram of, *Symphytum officinale* (a), *Rosmarinus officinalis* (b), *Mentha piperita* (c), *Orthosiphon stamineus* (d) and *Salvia officinale* (e).

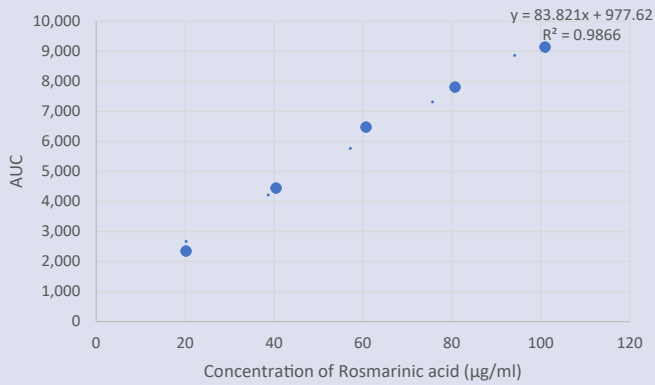

Figure 4: The linearity test of rosmarinic acid.

mL rosmarinic acid, respectively. The limitations are low enough to determine rosmarinic acid, as would be seen from these results.

Quantification of rosmarinic acid in various plants

The ratio of peak area values was used to quantify rosmarinic acid in every case. The yield of the extract and the weight of the plants were used to estimate the amount of rosmarinic acid in the extract. The test was performed three times. Table 5 showed the rosmarinic acid level in *Rosmarinus officinalis, Symphytum officinale, Mentha piperita, Orthosiphon stamineus* and *Salvia officinale.* The analyses were run under the validated condition that have describe above. *Rosmarinus officinalis* has the highest level of rosmarinic acid. Rosmarinic acid level in *Rosmarinus officinalis* was 8,599 *µ*g/gram dry weight. *Ultrasound assisted extraction* (UAE) help to extract rosmarinic acid in plants. Methanol extract rosmarinic acid higher than water. It proved the HPLC method above suitable to quantified rosmarinic acid[6]. *Rosmarinus officinalis* was known as rosemary. It has been used as flavouring herbs and folk medicine[19]. The previous study and this study have similarity of rosmarinic acid level[5,20–22]. Harvesting time, climate and regional condition contribute to difference amount of rosmarinic acid[23].

On the previous study, rosmarinic acid level of *Symphytum officinale* root was 7,557mg/gram and it has been extracted using reflux[3]. The result of this study was different with the previous because this study used the leaves part of *Symphytum officinale.* Beside that, the amount of rosmarinic acid content is affected by the extraction procedure. On *Mentha piperita, Salvia officinale* and *Orthosiphon stamineus* has been reported contain rosmarinic acid 28,3 mg/g, 39,3 mg/g and 9,10 mg/g respectively[24,25]. Solvent, extraction method and pretreament of sample effect the capacity of rosmarinic acid extraction[25,26].

CONCLUSION

This method is simple, sensitive and valid enough to analyse rosmarinic acid in *Rosmarinus officinalis, Symphytum officinale, Mentha piperita, Orthosiphon stamineus* and *Salvia officinale.* The approach is regarded to be appropriate for quick routine analysis.

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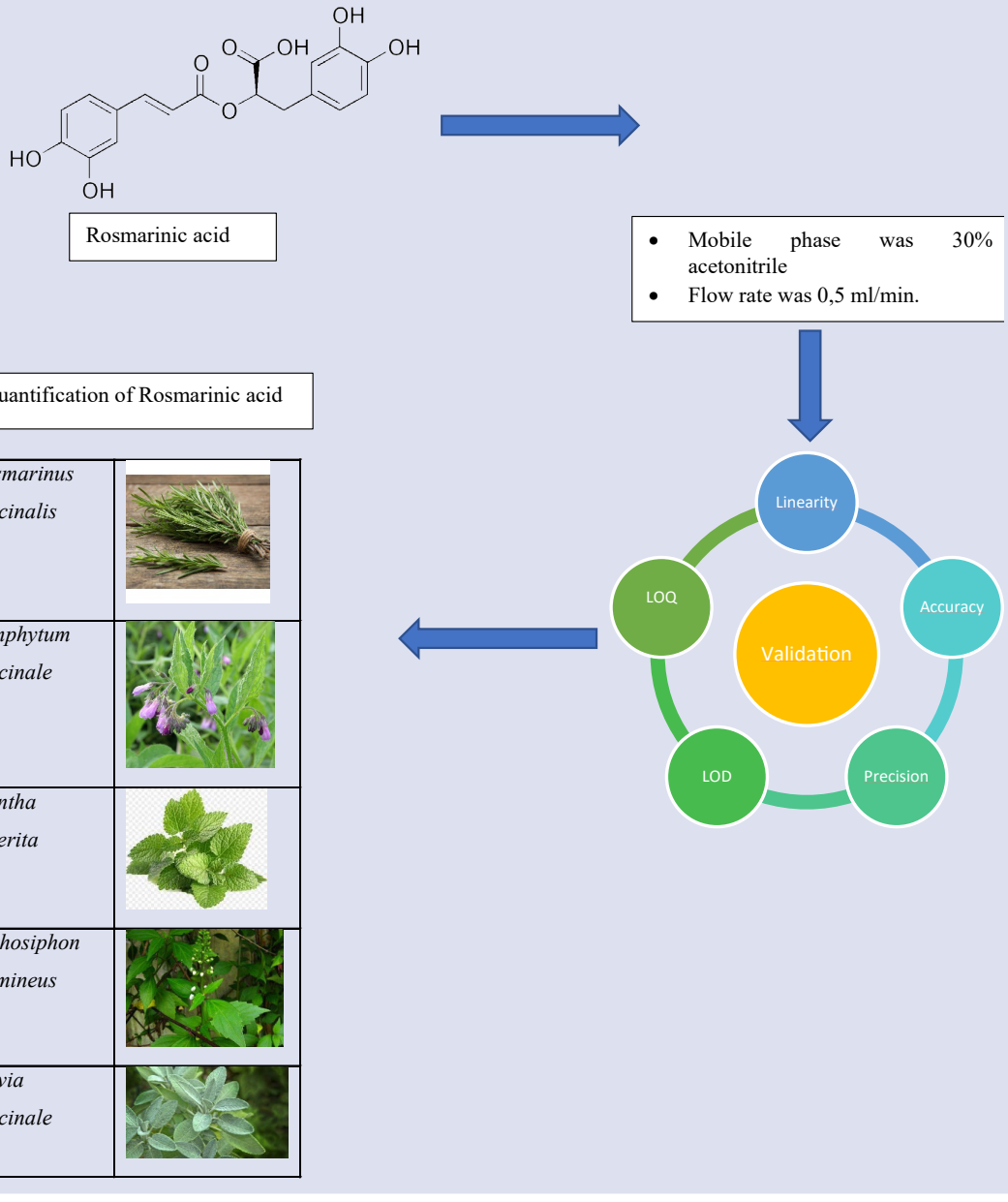
GRAPHICAL ABSTRACT



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