การวิเคราะห์ด้วยโครมาโทกราฟีของเหลวสมรรถนะสูงและการสกัดด้วยตัวทำละลายของสารอิโมดินจากเถาวัลย์เหล็ก
HPLC Analysis and Solvent Extraction of Emodin from *Ventilago denticulata* Willd

นิพนธ์ต้นฉบับ

ปัณรส แช่มชมดาว[1,4], สริน ทัดทอง[2] และ วีระศักดิ์ สามี[3]*

[1] ขณะทำการศึกษาเป็นนักศึกษาระดับบัณฑิตศึกษา ชั้นปีที่ 4 คณะเภสัชศาสตร์
[2] สาขาวิชาเภสัชเวท คณะเภสัชศาสตร์
[3] สาขาวิชาเภสัชเภสัชเคมี คณะเภสัชศาสตร์
[4] กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข
[1-3] มหาวิทยาลัยศรีนครินทรวิโรฒ อ.องครักษ์ จ.นครนายก 26120
*** Corresponding author:** weerasak@g.swu.ac.th

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Original Article

Pannaros Chamchomdao[1,4], Sarin Tadtong[2] and Weerasak Samee[3]*

[1] 4th year graduate student, Faculty of Pharmacy
[2] Department of Pharmacognosy, Faculty of Pharmacy
[3] Department of Pharmaceutical Chemistry, Faculty of Pharmacy
[4] Department of Medical Sciences, Ministry of Public Health
[1-3] Srinakharinwirot University, Ongkharak, Nakhon-Nayok 26120, Thailand
*** Corresponding author:** weerasak@g.swu.ac.th

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บทคัดย่อ

วัตถุประสงค์: 1) เพื่อพัฒนาและตรวจสอบความถูกต้องของวิธีวิเคราะห์อิโมดินจากสารสกัดเถาวัลย์เหล็กด้วยเทคนิค HPLC และ 2) เพื่อประเมินตัวทำละลายที่เหมาะสมในการสกัดสารอิโมดินจากเถาวัลย์เหล็ก **วิธีการศึกษา:** พัฒนาวิธีการวิเคราะห์ปริมาณอิโมดินที่สกัดจากเถาวัลย์เหล็กด้วยตัวทำละลายชนิดต่าง ๆ ด้วยเทคนิค HPLC-PDA โดยใช้คอลัมน์ XSelect CSH ชนิด C18 ขนาดอนุภาค 5 ไมโครเมตร ขนาด 4.6x250 มิลลิเมตร อุณหภูมิคอลัมน์ 30 องศาเซลเซียส สารละลายตัวพาเป็นสารละลายกรดฟอสฟอริกความเข้มข้นร้อยละ 0.1 และ อะเซโทไนไทรล์ผสมกันแบบเกรเดียน อัตราการไหล 1.0 มล./นาที และตรวจวัดด้วยโฟโตไดโอดแอเรย์ที่ความยาวคลื่น 288 นาโนเมตร ศึกษาความเหมาะสมของค่าคงที่ไดอิเล็กทริกของตัวทำละลายในการสกัดอิโมดินจากเถาวัลย์เหล็ก ศึกษาโดยหมักผงเถาวัลย์เหล็กนาน 7 วัน ด้วยตัวทำละลาย ได้แก่ น้ำ, เมธานอลความเข้มข้นร้อยละ 20 - 100, เอธานอลความเข้มข้นร้อยละ 20 - 100, โพรพานอล และบิวธานอล **ผลการศึกษา:** อิโมดินให้พีคที่เวลาประมาณ 10.8 นาที โดยใช้เวลาวิเคราะห์ 20 นาทีต่อการฉีดแต่ละครั้ง พบว่าวิธีมีความจำเพาะกับอิโมดินสามารถแยกออกจากพีคข้างเคียงได้ พบความถูกต้องของวิธีด้วยค่าร้อยละการกลับคืนของอิโมดินที่ 101.99 ถึง 103.16 ค่าร้อยละของค่าเบี่ยงเบนมาตรฐานสัมพัทธ์เท่ากับ 0.09 ถึง 0.14 ปริมาณต่ำสุดของอิโมดินที่พบได้เป็น 0.008 มคก./มล. และปริมาณต่ำสุดที่ตรวจหาเชิงปริมาณได้เท่ากับ 0.02 มคก./มล. พบว่าตัวทำละลายที่สกัดได้อิโมดินปริมาณสูงที่ 500 ถึง 700 มก./กก. มีค่าคงที่ไดอิเล็กทริกอยู่ในช่วง 30 ถึง 50 โดยเอธานอลความเข้มข้นร้อยละ 60 ให้ปริมาณอิโมดินสูงที่สุดที่ 702.52 มก./กก. **สรุป:** วิธีวิเคราะห์ปริมาณอิโมดินมีความไว ความจำเพาะ ความแม่น และความเที่ยง ตามหลักเกณฑ์การตรวจสอบความถูกต้องของวิธีวิเคราะห์ของ ICH Guideline 2005 และ AOAC 2013 ตัวทำละลายที่เหมาะสมคือ เอธานอลความเข้มข้นร้อยละ 60 ซึ่งจะได้ปริมาณอิโมดินสูงที่สุดเพื่อนำไปแยกสารให้บริสุทธิ์และนำไปให้ประโยชน์ต่อไป

คำสำคัญ: อิโมดิน, HPLC, เถาวัลย์เหล็ก, dielectric constant, การสกัดด้วยตัวทำละลาย

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Abstract

Objectives: 1) To develop and validate analytical method for determination of emodin content in *V. denticulata* extracts using HPLC, and 2) to evaluate a suitable solvent for emodin extraction from *V. denticulata*. **Methods:** HPLC-PDA method was developed for the determination of emodin from *Ventilago denticulata* Willd in various solvent extracts. Emodin was separated from an extract on a XSelect CSH-C18 (5 µm x 4.6 mm x 250 mm) column maintained at 30 °C, using 0.1% phosphoric acid solution and acetonitrile mixed by gradient system as a mobile phase, flow rate at 1.0 mL/min and photo diode array (PDA) detector at 288 nm. The relevance of dielectric constant of solvents and emodin content extracted from *V. denticulata* was studied. The dried herbs were macerated with water, 20 - 100% methanol, 20 - 100% ethanol, 1-propanol and 1-butanol for 7 days. **Results:** The chromatogram showed emodin peak at about 10.6 min. with a run time of 20 min. per injection. The specificity and selectivity parameters were proved by good separation of emodin from other compounds. The developed method was accurate and precise as shown by % recoveries of 101.99 - 103.16% and % relative standard deviation of 0.09 - 0.14%. The limit of detection (LOD) and limit of quantitation (LOQ) were 0.008 and 0.02 µg/mL, respectively. The correlation between emodin content and dielectric constant of extracted solvents was parabolic. The dielectric constant between 30 - 50 yielded the emodin content in the range of 500 - 700 mg/kg of dried weight. 60% ethanol provided the highest emodin content with 702.52 mg/kg dried weight. **Conclusion:** The developed HPLC analysis on emodin was sensitive, specific, accurate, and precise as indicated by the ICH 2005 guideline and AOAC 2013 guideline. 60% ethanol offered the highest amunt of emodin extracted from *V. denticulata* for furtther isolation and utilisation.

Keywords: emodin, HPLC, *Ventilago denticulata*, dielectric constant, solvent extraction

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Introduction

Ventilago denticulata Willd is in Rhamnaceae family known as “Tao wan lek” or “Rang dang” in Thai. The root is used for atonic dyspepsia, mild fever and debility. The vines and leaves are brewed as tea to use as diuretics, health tonic, and for the treatment of arthritis, high cholesterol and high blood sugar. The antioxidant and cytotoxic activities were found in its aqueous and ethanolic extracts.[1] Many kinds of substances in the anthraquinone group were isolated from vines of *V. denticulata* such as chrysophanol, physcion and emodin.[2]

Emodin (1,3,8-trihydroxy-6-methylanthraquinone) (Figure 1) belongs to the anthraquinones mostly found in root and bark of three plant families namely Fabaceae, Polygonaceae and Rhamnaceae (*Rhamnus* and *Ventilago* spp.).[3] It inhibits many types of microorganisms such as *Helicobacter pylori*, Methicillin-resistant *Staphylococcus aureus* (MRSA), *Mycobacterium tuberculosis*, and herpes simplex virus (HSV). Moreover, it can reduce inflammation and cancer cells particulary lung, liver, and breast cancer cells.[4] Emodin has gained considerable interest because of the increased use of herbal drugs as alternative medicine. *V. denticulata* is an emodin-containing plant that needs to be validated by standard analytical procedures. There are many studies on the extraction and analysis of emodin in other plants such as methanol extraction in *Rheum emodi*,[5] 95% methanol extration in *Semen Cassiae*,[6] and chloroform with sulfuric acid extraction in *Fagopyrum tataricum*.[7] These studies provided emodin amount in the range of 1 - 70 mg/Kg. In this work, we investigated effects of dielectric constant on emodin extraction. The reported HPLC methods for emodin struggled with long analysis time (25 min)[5] and unavailable selectivity profile of emodin in *V. denticulate*.[5-7] Moreover, we also developed a fast and sensitive HPLC method for emodin in *V. denticulate* by using gradient elution and optimization of the detection wavelength.

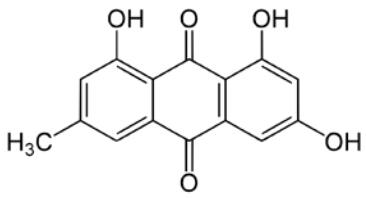

Figure 1 Chemical structure of emodin.

Methods

Materials

V. denticulata powder was purchased from Vejpong Pharmacy Co., Ltd. (Bangkok, Thailand). Emodin (Lot 029K1110V), 1-propanol, and 1-butanol were purchased from Sigma-Aldrich (St. Louis, USA). Methanol was purchased from Macron Fine Chemicals. Ethanol was purchased from Carlo Erba reagents S.r.l. Phosphoric acid was purchased from E. Merck KG. Acetonitrile (HPLC grade) was purchased from Honeywell Company. High purity water was obtained by Millipore Milli Q water purification system.

HPLC conditions

The HPLC system was carried out on a Waters Acquity UPLC H-Class System with a photodiode array detector. The quantification wavelength was set at 288 nm. The XSelect CSH, 5 µm C18 column, size 4.6x250 mm column was used for separation. 0.1% phosphoric acid solution and acetonitrile were mixed by gradient system as a mobile phase with the flow rate of 1 mL/min. The gradient program for elution are presented in Table 1. The injection volume was 20 µL. The column was maintained at 30 °C.

Table 1 Mobile phase gradient program.

Time (min)	0.1% Phosphoric acid (%)	Acetonitrile (%)
0	40	60
13	40	60
14	5	95
19	5	95
20	40	60

Validation of HPLC Method

The HPLC method was validated in terms of linearity, accuracy, and precision according to ICH guidelines[8] and AOAC guideline.[9] Linearity solutions were prepared and diluted to obtain the concentration range. The accuracy was evaluated with the percentage recoveries from three concentration levels of a standard in the sample which contained known amount of emodin. The system precision was performed by three standard solutions. The determination of limit of detection (LOD) and limit of quantification (LOQ) were defined based on the signal-to-noise ratio. LOD was defined as the signal-to-noise ration of 3:1 and LOQ as 10:1. To determine the robustness of the method, the final experimental conditions were purposely altered and the resolution, tailing factor and theoretical plate were examined. The flow rate, percentage of concentration of phosphoric acid and column temperature were varied by (±) 10%, (±) 10% and (±) 1 °C, respectively.

Extraction of sample

The powdered sample (5 g) and 50 mL of solvent were added into a 250-mL glass-stoppered flask and extracted by maceration for 7 days. The filtrate of 2 mL was transferred to a 10-mL volumetric flask, and diluted with methanol to volume. The solution was filtered through 0.22 µm filter before injection. The solvents were water, 20 - 100% methanol, 20 - 100% ethanol, 1-propanol, and 1-butanol.

Dielectric constant calculation of solvent mixtures

To estimate the dielectric constants of binary solvent mixtures, the weight percent of the solvent components (w) and dielectric constant of each solvent were used to calculation by the following equation.[10]

$$\varepsilon = \varepsilon_1(100-w_2)/100 + \varepsilon_2 w_2/100$$

where ε, ε_1 and ε_2 are the dielectric constant of the solvent mixture, pure solvent 1 and pure solvent 2, respectively, and w_2 is the weight percent of one of the mixture component.

Statistical analysis

The experiments were performed in triplicate and the result were expressed as means ± standard deviation. A one-way analysis of variance (ANOVA) was used to test the differences in emodin content by various extraction solvents. Statistical significance was set at a type I error of 5% or *P*-value < 0.05.

Results

Validation of HPLC Method

The previously reported HPLC methods for emodin utilized the detection wavelengths at 254 nm[5], 278 nm[6] and 436 nm.[7] However, our results showed that the detection at 288 nm (Figure 3) provided the optimal absorption and improved emodin sensitivity. Gradient elution shortened the analysis time of emodin from 25 min[5] to 10.6 min (Table 1).

Chromatographic system suitability

The resolution between emodin and another peak was 1.90. The tailing factor and theoretical plate of emodin peak were 1.00 and about 15000, respectively.

Specificity and selectivity

The identification of emodin in the sample was achieved by comparison of retention times and UV/VIS spectrum at 200-600 nm with the standard. The similarity of UV/VIS spectrum at 200 - 600 nm of emodin peak from standard solution and sample solution was shown in (Figure 2), the maximum absorption wavelength was 288 nm. The specificity was assessed by peak purity, using chromatogram from a diode array detector. The chromatogram of the emodin standard was presented at one peak at the retention time of 10.6 min (Figure 3). The chromatogram of the *V. Denticulata* sample extract showed one peak at 10.6 min similar to emodin standard. No interference peaks were observed in the region of the main peak of emodin peak (Figure 4).

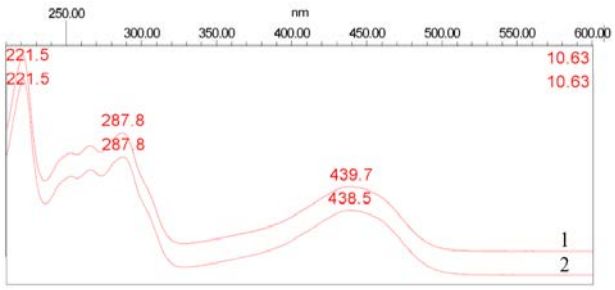

Figure 2 UV/VIS spectrum at 200-600 nm of emodin peak from 1: Standard solution, 2: Sample solution.

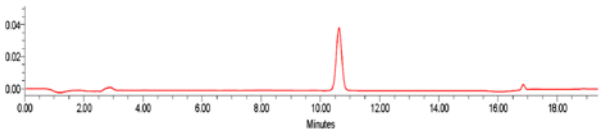

Figure 3 HPLC chromatogram of standard emodin.

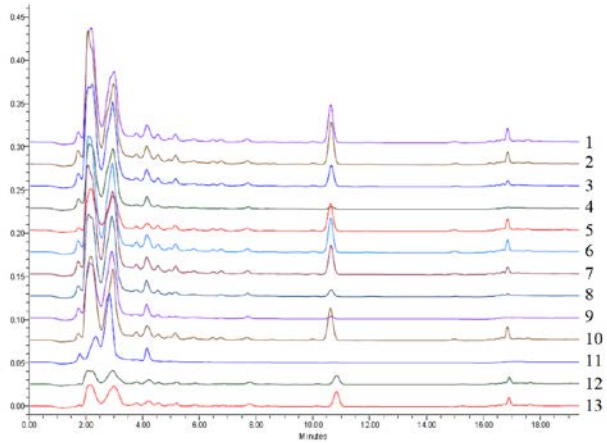

Figure 4 HPLC chromatogram of *V. denticulata* extracted by various solvents: 1 - 80% ethanol, 2 - 60% ethanol, 3 - 40% ethanol, 4 - 20% ethanol, 5 – ethanol, 6 - 80% methanol, 7 - 60% methanol, 8 - 40% methanol, 9 - 20% methanol, 10 – methanol, 11 – water, 12 - 1-butanol, and 13 - 1-propanol.

The peak purity results of emodin peak were proved by spectral contrast technique to compute the purity angle and purity threshold. This was done by comparing each spectrum within the peak against the peak apex spectrum by measuring the differences in vector direction. The purity angle was the weighted average of all spectral contrast angles and the purity threshold was the sum of purity noise angle and solvent angle.[11] The results indicated that the purity angle was less than the purity threshold. There was no spectroscopic evidence for coelution. The peak was spectrally homogeneous. The peak purity from HPLC chromatogram of emodin is shown in Figure 5. Emodin is a good marker for routine procedure in quality control of *V. Denticulata* sample.

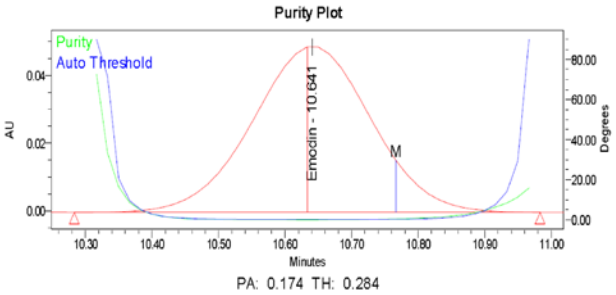

Figure 5 Peak purity from HPLC chromatogram of emodin extracted by 60% ethanol.

Linearity

Linearity was obtained at six concentration levels. The association between concentration and area under the curve was linear over the range of 1 to 10 µg of emodin per mL. The standard curve in correspondence with a linear regression of y = 88160.1*x + 2068.7 with a correlation coefficient of 0.9999 (*r*) was evident (Figure 6).

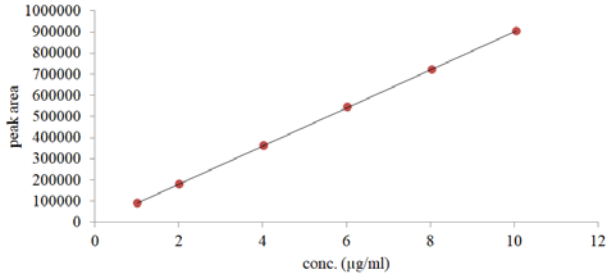

Figure 6 The relationship between standard emodin concentrations (µg/mL) and peak area.

Accuracy

The accuracy of the method was determined by spiking standards in samples solution extracted by ethanol. percent recoveries were in the range of AOAC acceptable limit (95 - 105%) (Table 2).

Table 2 Recovery study of emodin.

Quantity added (µg/mL)	Quantity found (µg/mL) (n = 3)	Recovery (%) (n = 3)	Relative standard deviation (%) (n = 3)
1.838	1.874	101.99	0.05
3.675	3.765	102.44	0.34
5.513	5.687	103.16	0.94

Precision

The system precision was performed by six injections of standard solutions in the different day gave % reltive standard deviation (%RSD) of 0.14, 0.09 and 0.12. The precision of an analytical method was within the AOAC acceptable limit at 1000 ppm (3.7%).

Sensitivity

The LOD and LOQ were 0.008 and 0.02 µg/mL, respectively.

Robustness

With all the deliberately varied chromatographic conditions, the chromatogram of sample solution showed satisfactory resolution (Rs), tailing factor (T), theoretical plates number (N) and peak area (A). To evaluate the effect of each parameter, the average and % RSD of peak area corresponding to altered conditions was observed. The %RSD of peak area was within the acceptable AOAC limit of 3.7% (Table 3). The highest variation in peak area was 2.48%, when the flow rate of mobile phase was altered.

Table 3 Effects of the analytical parameters on resolution, tailing factor, theoretical plates number and peak area.

Effect	Rs*	T*	N*	A*	Mean of A	%RSD of A
Temperature (29 °C)	1.68	0.98	14295	519388		
Temperature (30 °C)	1.65	0.98	14886	518643	518703	0.13
Temperature (31 °C)	1.51	0.98	15010	518079		
Flow rate (0.95 mL/min)	1.66	0.98	15275	522261		
Flow rate (1.00 mL/min)	1.65	0.98	14886	518643	513184	2.48
Flow rate (1.05 mL/min)	1.61	0.98	14485	498650		
Phosphoric acid (0.09%)	1.62	0.98	14956	522544		
Phosphoric acid (0.10%)	1.65	0.98	14886	518643	521669	0.52
Phosphoric acid (0.11%)	1.62	0.98	14931	523822		

Note: Rs = resolution, T = tailing factor, N = theoretical plates number, A = peak area, and RSD = relative standard deviation.

* Average of the triplicate values.

Extraction of sample

The content of emodin extracted from *V. denticulata* by 7-days maceration with different solvents was varied, with the highest content by 60% ethanol (702.52 mg/kg dried weight) and the lowest by water (10.32 mg/kg dried weight) (Table 4). It was foud that the differences in emodin content by various extraction solvents were statistically significant (*P*-value < 0.05).

Table 4 Emodin contents and dielectric constants from different solvent extracts by 7-days maceration.

Solvent	Emodin content (mg/kg dried weight)	Dielectric constant (ε)
80% ethanol	618.13	37.70
60% ethanol	702.52	49.81
40% ethanol	343.48	60.80
20% ethanol	41.52	70.82
80% methanol	548.50	44.35
60% methanol	421.26	54.54
40% methanol	108.89	63.79
20% methanol	36.60	72.25
Water	10.32	80.00
Methanol	527.87	33.10
Ethanol	460.96	24.30
1-Propanol	264.90	20.10
1-Butanol	161.18	17.80

Note: Differences in emodin contents between extraction solvents were statistically significant (*P*-value < 0.05) by ANOVA.

Discussions and Conclusion

According to its chemical properties, emodin can be dissolved in alcohol. [12,13] Among four alcoholic solvents (methanol, ethanol, 1-propanol, and 1-butanol), the highest emodin content was extracted by methanol because methanol had the highest dielectric constant (Figure 7).

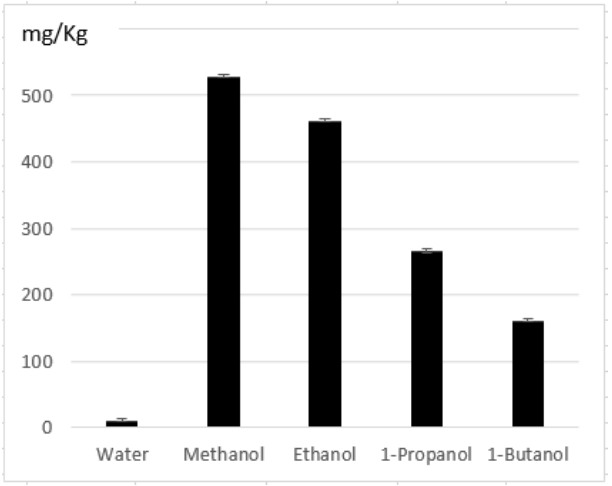

Figure 7 Emodin content from different solvent extracts: water, methanol, ethanol, 1-propanol and 1-butanol.

It could be assumed that solvent with higher dielectric constant could offer a higher yield. To prove the assumption, water was added to adjust the dielectric constant of mix solvents. The emodin content and dielectric constant of solvents was found to be in parabolic correlation (Figure 8). Water, the most polar solvent, showed the lowest emodin content while 1-butanol, the most non-polar solvent used in this study, showed lower emodin content than other alcohols. The optimal dielectric constant of extractive solvent resulting in acceptable emodin content (500 - 700 mg/kg of dried weight) was in the range of 30 - 50.

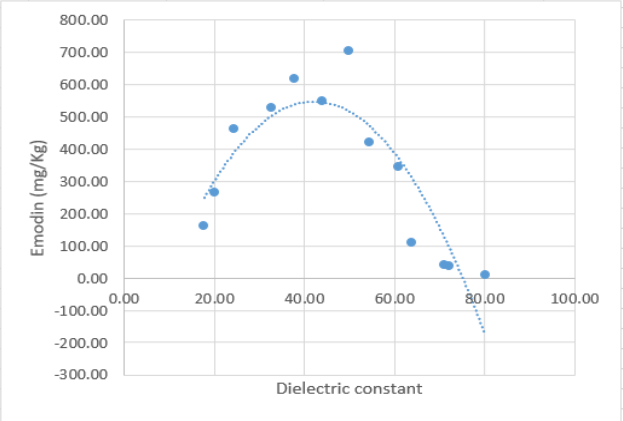

Figure 8 Relationship between emodin content and dielectric constant of solvents.

We considered the emodin contents and dielectric constants from ethanolic and methanolic extracts (Figure 9). It was found that the lines of the two solvents did not cross, even at the same dielectric constant. More content of emodin was more likely to be extracted by ethanol than methanol (Figure 9). This indicated that there are more other factors effect on the solubility of emodin. Dielectric constant cannot explain all about polarity and solubility of a compound in a solvent. Ethanol possesses more solubility parameter related to Van der Waals force. Vice versa, methanol possesses more solubility parameter related to H-bonding. Although dilute these solvents with water to obtain the same dielectric constant, but other properties still be not the same and affect the solubility.

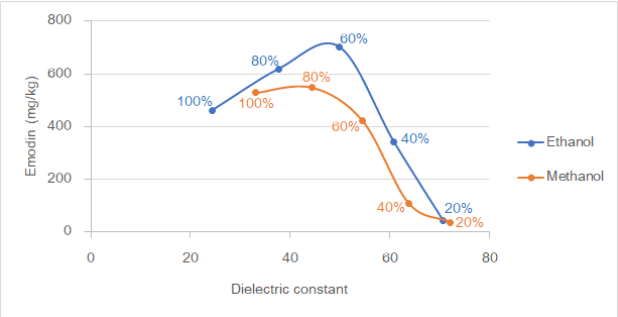

Figure 9 Relationships between emodin content and dielectric constant of 20-100% methanol and 20-100% ethanol.

In conclusion, emodin was considered to be an analytical marker for quality control of *V. denticulata* extracts. The HPLC method for the determination of emodin from *V. denticulata* was validated. The validation parameters proved that the method was specific, reliable, valid and robust. The results of different solvents showed that 60% ethanol as an extractive solvent provided the highest emodin content. The optimal dielectric constant of extractive solvent should be in the range of 30 - 50.

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References

1. Pongjanta A, Pangjit K, Srichairatanakool S. Antioxidant activity and cytotoxic effect of *Ventilago denticulata* Willd leaves extracts. *J Med Assoc Thai* 2016;99(suppl 1):S51-S57.
2. Molee W, Phanumartwiwath A, Kesornpun C, et al. Naphthalene derivatives and quinones from *Ventilago denticulata* and their nitric oxide radical scavenging, antioxidant, cytotoxic, antibacterial, and phosphodiesterase inhibitory activities. *Chem Biodivers* 2018;15(3): e1700537.
3. Rao BK, Hanumaiah T, Rao CP, Rao GSR, Rao KVJ, Thomson RH. Anthraquinones in Ventilago species. *Phytochemistry* 1983;22(11):2583-2585.
4. Izhaki I. Emodin - A secondary metabolite with multiple ecological functions in higher plants. *New Phytologist* 2002;155:205-217.
5. Tabin S, Gupta RC, Bansal G, Kamili A. Comparative HPLC analysis of emodin, aloe emodin and rhein in Rheum emodi of wild and in vitro raised plants. *J Pharmacog Phytochem* 2016;5(2):121-130.
6. Xu L, Chan CO, Lau CC, Yu Z, Mok DK, Chen S. Simultaneous determination of eight anthraquinones in *Semen Cassiae* by HPLC-DAD. *Phytochem Anal* 2012;23(2):110-116. (doi:10.1002/pca.1331)
7. Peng LX, Wang JB, Hu LX, et al. Rapid and simple method for the determination of emodin in tartary buckwheat (*Fagopyrum tataricum*) by high-performance liquid chromatography coupled to a diode array detector. *J Agric Food Chem* 2013;61(4):854-857. (doi:10.1021/jf304804c)
8. ICH. ICH Harmonised Tripartite Guideline, validation of analytical procedures: text and methodology Q2 (R1). USA. ICH Sterring Committee, 2005.
9. AOAC International. Appendix K: Guidelines for dietary supplements and botanicals. *J AOAC Int* 2013.
10. Pdfhall. Dielectric constants of common materials. 2020. (Accessed on Jan. 15, 2020, at https://pdfhall.com/dielectric-constants_5b3988f5097c47616e8b4578. html)
11. Water Corporation. PDA software user's guide. USA. Waters Corporation, 1994.
12. NCBI. PubChem Database. Emodin, CID=3220. (Accessed on Jan. 15, 2020, at https://pubchem.ncbi.nlm.nih.gov/compound/Emodin)
13. Budavari S. The Merck index: an encyclopedia of chemicals, drugs, and biologicals. Rahway, N.J., U.S.A. Merck, 1989.