# Application of machine learning models for green and simultaneous determination of asiaticoside and madecassoside in *Centella asiatica*

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## Abstract

The artificial neuron network (ANN), which is a subfield of machine learning, has been widely applied in analytical chemistry for classification/pattern recognition, prediction, and modeling. ANN combined with UV spectrometry can be used to tackle the problem of overlapping spectra of a complicated matrix of herbal medicine. In the present study, ANN has been used for simultaneous determination of asiaticoside and madecassoside in *Centella asiatica* collected from various provinces in Viet Nam based on UV spectra of standard reference and spiked samples. The absorption spectra of 108 *C. asiatica* samples were recorded in (190-250) nm of wavelength with an interval of 1 nm (61 variables) were used for data acquisition. An ANN model using traincgb function with 40 neural hidden layers was trained. The correlation coefficients were all higher than 0.9999. The concentration of asiaticoside and madecassoside in all tested samples revealed a good recovery, as compared to the referenced values. The ANN model can be considered as effective, time-saving and environment-friendly quantitative analysis tool for herbal medicine

## 1 Introduction

The use of ANNs is a new but expanding area in the field of pharmaceutical research. The potential applications of ANN methodology in the pharmaceutical sciences are broad, ranging from interpretation of analytical data (modeling the pharmaceutical analysis in quality control), drug design, and dosage form design to clinical pharmacy through bio-pharmacy [1, 2]. One of these applications, neural networks can recognize patterns even from noisy and complex data in which there is a considerable degree of variation and estimate non-linear relationships. Therefore, ANNs are useful in all fields of research where the recognition of peak-shaped signals in analytical data such as spectral data is important. ANNs are also beneficial in determining the composition of an unknown sample when the spectrum of the unknown is a superposition of known spectra. This technique uses the whole spectrum in the identification process instead of only the individual peaks [3, 4].

As a Vietnamese traditional medicine, *Centella asiatica* Linn (called *"Rau má"* in Vietnamese) has

been widely studied and used as a wound healing, memory enhancer, and anti-aging agent. The saponins, triterpenoids, including asiaticoside, madecassoside, centelloside, and flavonoids regarded as biomarker components have been isolated in this herbal drug [5, 6]. However, the pentacyclic triterpenoids components in *Centella* are known to depend on its growth location and the diverse environmental conditions it is subjected to [7, 8].



Asiaticoside ($C_{48}H_{78}O_{19}$)     Madecassoside ($C_{48}H_{78}O_{20}$)
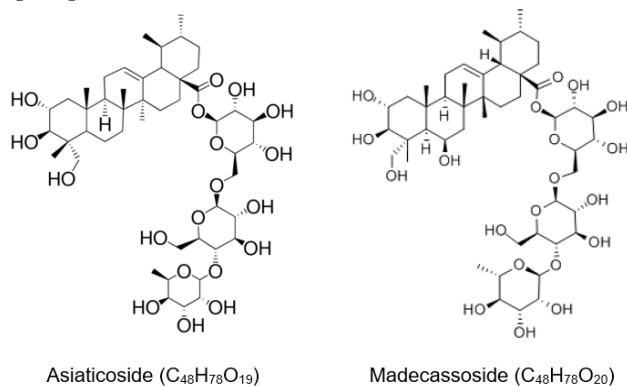
**Fig. 1** The chemical structures of asiaticoside and madecassoside

Quantification of triterpenes of *C. asiatica* has been successfully established by several researchers using HPLC-UV [9, 10] combined with PAD [11] or ELSD [12] detectors. In addition, LC/MS/MS has also been generally used for the determination of asiaticoside, asiatic acid, and madecassic acid [13]. However, HPLC and LC/MS/MS require pre-separation methods and prolonged time.

On the contrary, spectrophotometry is considered as the most convenient analytical technique because of its simplicity, low cost, and wide availability in most quality-control laboratories. As shown in the chemical structures of asiaticoside and madecassoside, the direct UV-Vis spectrophotometry essentially require chemometrics due to the complete overlapping of each component and other interferences in *C. asiatica matrix*.

The chemometrics coupling with UV-(Vis), near-infrared measurements were also reported as the effective methods for simultaneous determination of several components in water [14], pharmaceutical formulation [15], food [16 ], oil [17], herbal medicine [18 ]. Multiple linear regression (MLR) and ANN that constructed a non-linear regression model [11] have been developed and tested according to the standard solutions and external validation prediction sets and successfully applied for simultaneous determination of several substances in the mixtures containing known components.

To use ANN-assisted UV spectrophotometry in traditional medicine analysis, one of the major problems is the limitation of the unrecognizable effect of the unknown components. Therefore, it is necessary to apply the advanced machine learning algorithms combined with training sets based on standard reference samples in UV measurements.

In this research, the ANN algorithm was applied to the UV spectra data of the *C. asiatica* samples to simultaneously determine the asiaticoside and madecassoside. First, the contents of 2 analytes in real samples were determined by reference analytical methods (HPLC and LC/MS/MS) to gain the concentration matrices then UV spectra data matrices of the real samples were recorded. The ANN model will be used to reflect the relationships between concentration and absorbance.

## 2 Experimental design

### 2.1 Reagents and chemicals

For the extraction of crude samples, methanol 80% and double distilled water were used. Asiaticoside and madecassoside primary standard were purchased from Sigma Aldrich (Singapore) with a purity of $\geq 95$ %. Asiaticoside and madecassoside stock standard solutions were prepared by dissolving the primary standards in 80 % methanol to obtain concentrations of 688 µg/mL and 205 µg/mL, respectively. Stock standard solutions were kept in the dark at a temperature between (3-5) °C. The working standard solutions were prepared in HPLC mobile phase solution and in distilled water with UV measurement.

For the ANN method, a concentration set of 108 *C. asiatica* referenced samples containing asiaticoside and madecassoside in their non-linear concentration ranges of [(0-100) and (0-556)] µg/mL , respectively, were obtained from the spiked analyte solutions of samples.

### 2.2 Sampling and sample preparation

Twenty-one crude samples of *C. asiatica* were collected from various provinces in Viet Nam. The whole plant, including the roots, had been manually harvested,stored in small plastic bags and kept inside a locker in an air-conditioned room. For sample preparation, weigh 0.5 g (to the nearest 0.1 mg) of the powdered sample, place in a 100-mL round-bottom

flask, and add 20 mL of methanol (80 %). Reflux the mixture for 30 min and cool down to room temperature. Transfer the solution to a 50-mL centrifuge tube then centrifuge at about 3.000 g for 5 min, filter through a 0.45-µm PTFE membrane to 25 mL volumetric flask and fill up to the mark by distilled water to obtain solution A, which was used to determine the concentration of asiaticoside by HPLC method. The remaining solutions were kept for continuous analysis of madecassoside by LC/MS/MS, followed by 10 times dilution with methanol.

For ANN-assisted UV spectrophotometry, 0.2 mL of solution A were diluted to 25.00 mL and then a portion of 0.25 mL was mixed with standard solutions containing 2 compounds at different concentrations, diluted with distilled water to 5.00 mL before measuring the absorbance.

2.3 Analytical procedures

*Determination of asiaticoside by HPLC:* The analytical procedure was carried out following the Centellae Herba monograph in Hong Kong Chinese Materia Medica Standards. Chromatographic measurements were performed on a HPLC-DAD (Shimadzu). The liquid chromatograph was equipped with a DAD (205 nm) and C18 column (250 mm × 4.6 mm, 5 µm). The column temperature was maintained at 20 ℃ during the separation. The flow rate was 1.0 mL/min, and the injection volume was 10 µL. The mobile phase was set at 0.15 % phosphoric acid (channel A) and acetonitrile (channel B) following the isocratic at (0-15) min (21 % channel B), and linear gradient in (15-32) min (21-36) % channel B), (32-50) min (36-40) % channel B), (50-60) min (40-80) % channel B). The standard curve of asiaticoside was linear over a range of (6.87-687) µg/mL with a correlation coefficient of 0.9991. The limit of determination and the limit of quantification was assessed to be 0.15 µg/mL and 0.45 µg/mL, respectively.

This procedure could not detect the presence of madecassoside due to its signals not being separated from interferences by this procedure.

*Determination of madecassoside by LC-ESI/MS/MS:* The HPLC Triple Quadrupole Mass Spectrometer LCMS-8045 (Shimadzu, Japan) was managed with C18 column (150 mm × 4.6 mm; 5 µm) and positive ESI mode, molecular ion $[M^+Na]^+$ (m/z 997.6 at Q1 and m/z 493.15 at Q3); Dwell time: 100; CE = 35. The HPLC system used mobile phase acetonitrile – acid

formic 0.1 % (80:20, v/v); flow rate 0.5 mL/min; column temperature 25 ℃; injection volume 1 µL. The standard curve of madecassoside was linear over a range of (20-400) ng/mL with a correlation coefficient of 0.9999. The limit of determination and the limit of quantification were 4 ng/mL and 12 ng/mL, respectively.

*Simultaneous determination of asiaticoside and madecassoside using ANN-UV spectrophotometry:* The spectrophotometric measurements were performed on a UV-1601PC (Shimadzu, Kyoto, Japan) connected with a computer loaded with UV-Win PC software. All absorption spectra [from (190-250) nm with 1 nm interval] were saved and subsequently exported to Microsoft Excel for statistical manipulation. In addition, Matlab 2019a version software using neural network toolbox was employed to determine the concentrations of combinations for calibration and validation sets.

# 3 Results and discussion

## 3.1 Design of mixture

The extensively overlapped absorption spectra of asiaticoside, madecassoside, and other components in *C. asiatica* sample (Figure 2) indicated that simultaneous determination of two selected components by direct UV-Vis spectrophotometry or MLR could not produce reliable results for quantitative analysis. The non-linear multivariate calibration (like ANN) should be applied and require suitable experimental design based on many known real samples.

The training input and test models were constructed from 18 extracted real samples, which had known concentrations (determined by HPLC/DAD and LC/MS/MS), and known different amounts of asiaticoside and madecassosid. The concentration matrix (108 × 2) was a symbol as Y matrix in the machine learning models. The matrix X (108 × 61) was obtained by recording absorption spectra of 108 solutions in the range of (190-250) nm with an interval of 1 nm. The relationship between X and Y would be set up by using ANN calibration model.
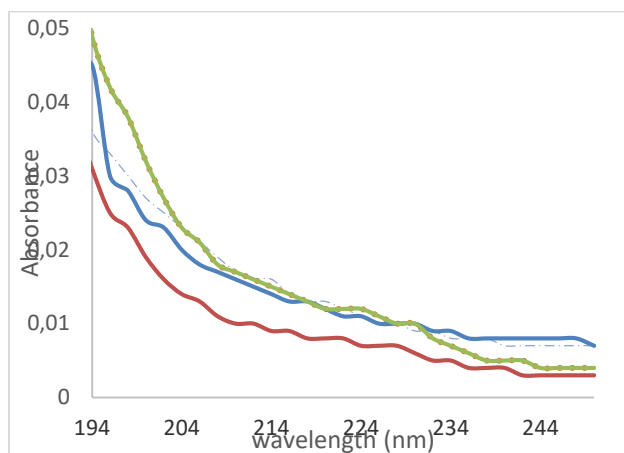
**Fig. 2** Overlay absorption spectra of madecassoside (0.0513 μg/mL), asiaticoside (0.0688 μg/mL), their mixture and *C. asiatica* samples (cuvette length 1 cm).

3.2 Training and optimization of ANN models

ANN is a simulation of a real neuron system containing a collection of neuron units communicating via axon connections. Each neuron contains input, weights associated with each input, transfer function, and output [20]. To optimize ANN calibration model, different neuron sizes and various networks such as multilayer perceptron, convolutional neural net, and recurrent neural net were tested. In optimized recurrent neural network optimization, the *log-sigmoid* activation function was used for hidden and pureline function used in output layers as the linear correlation between absorbance and concentrations.

For training the data set, the *traincgb* function, which is a network training function that updates weight and bias values according to the conjugate gradient backpropagation with Powell-Beale restarts[ 21] was used to train a network because it minimized the mean squared error (MSE) between the network output and actual values. The MSE is a quality measure for the estimator by dividing Residual Sum of Squares (RSS) by total observed data points.

$$\text{MSE} = \frac{1}{N}\text{RSS} = \frac{1}{N}\sum(f_i - y_i)^2$$

Where $f_i$ and $y_i$ are actual and targeted values of the ith output nodes, N is the number of training examples.

Any training sets aim to reach the smallest MSE value in the shortest possible time while avoiding the overtraining problem.

In *traincgb* function that updates weight and bias values according to the conjugate gradient back-propagation with Powell-Beale restarts, the calibration

set was randomly divided into two subsets, one used for training (including 80 % of the calibration samples), and the other samples used for testing. The test set was held out during training, which avoids the overlap between training data and test data, yielding a more accurate estimate for the generalization performance of the algorithm.

To perform supervised training and prediction, we need a method to evaluate the ANN output. During gradient-descent back-propagation of the ANN algorithm, the relationship between the MSE of the network and epochs for the training set was established. From the matrix (108 × 61) spectra data for 108 samples, the effect of 11, 13, 17, and 40 hidden layers to MSE was destroyed and depicted in Fig. 3. The results showed that the increase of hidden neuron layer leads to increasing trueness of the models due to the decrease of MSE with fewer epochs. The best number of neurons (40) was selected in each layer based on the lowest mean square error (MSE). The calculated MSE reached a value of $3.98 \times 10^{-7}$ after 8 epochs as shown in the performance plot of the optimum network in Fig. 3.

The correlation coefficients between target and output concentrations of all calibration sets also increased from 0.99 (with 11 hidden layers) to 0.9999 (if 40 hidden layers were used) (Fig.4). This indicated that the training model is suitable for simultaneous determination of asiaticoside and madecassoside in *C. asiatica*. Finally, a training network of 61 neurons in the input layer, 40 neurons in hidden layers, and 2 outputs were found optimal for performing calibration and prediction steps and, consequently, found to be suitable for the construction of an ANN calibrator for the simultaneous quantitative prediction of the unknown *C. asiatica* samples.

3.3 External validation of ANN model using real samples

To assess the applicability of the proposed method to the analysis of real samples, the selected ANN model was applied to the determination of madecassoside and asiaticoside in 18 *C. asiatica* samples. Three replicate measurements were carried out. First, absorption spectra of the tested samples were prepared similar to the details described in the experimental section. Data extracted from the absorption spectra was then fed into the established network. The predicted concentration and recoveries obtained from ANN optimal model are shown in Table 1.
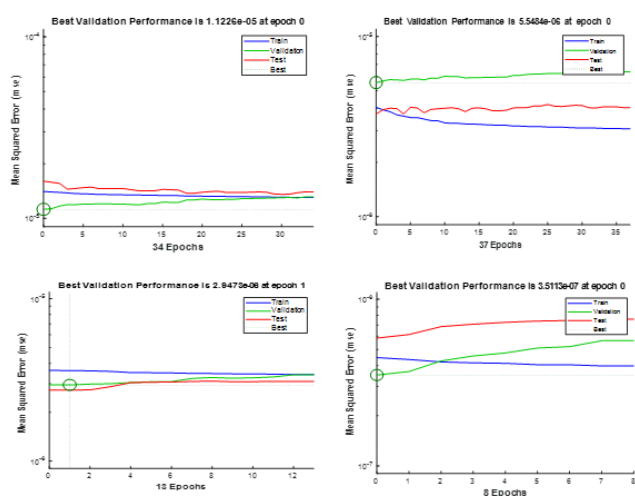
**Fig. 3** MSE values (for 2 analytes) versus the number of neurons for *traincgb* function with (a) 11 layers, (b) 13 layers, (c) 17 layers and (d) 40 layers.
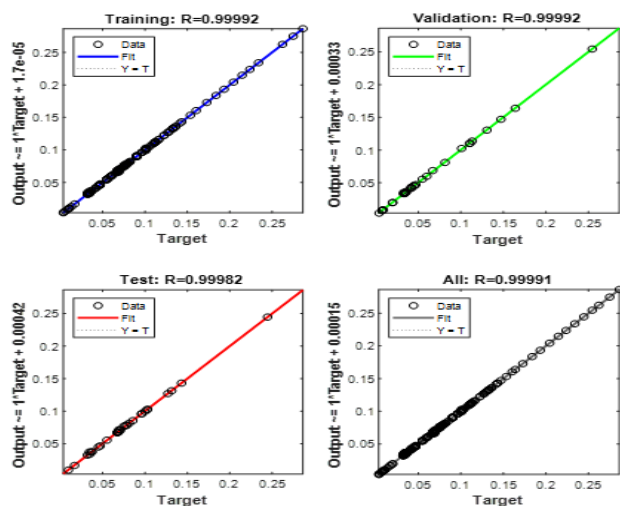


**Fig. 4** Training, validation and test diagrams of the *traincgb* function related to 2 analytes in the optimum ANN model (40 hidden layers).

**Table 1** Obtained recovery by application of traincgb function in 18 real samples

| | Asiaticoside | | | | Madecassoside | | |
|---|---|---|---|---|---|---|---|
| No. | Actual content (µg/mL) (HPLC results) | Predicted content (µg/mL) | Recovery (%) | No. | Actual content (µg/mL) (LC/MS/MS results) | Predicted content (µg/mL) | Recovery (%) |
| 1 | 0.090 | 0.092 | 101.3 | 1 | 0.105 | 0.105 | 100.0 |
| 2 | 0.067 | 0.067 | 99.9 | 2 | 0.096 | 0.096 | 99.9 |
| 3 | 0.070 | 0.071 | 100.7 | 3 | 0.127 | 0.127 | 99.9 |
| 4 | 0.045 | 0.045 | 100.4 | 4 | 0.112 | 0.112 | 99.9 |
| 5 | 0.042 | 0.043 | 104.0 | 5 | 0.110 | 0.110 | 99.9 |
| 6 | 0.035 | 0.037 | 106.3 | 6 | 0.080 | 0.080 | 100.1 |
| 7 | 0.047 | 0.047 | 100.0 | 7 | 0.113 | 0.114 | 100.4 |
| 8 | 0.077 | 0.077 | 100.2 | 8 | 0.133 | 0.133 | 100.1 |
| 9 | 0.055 | 0.054 | 99.1 | 9 | 0.061 | 0.061 | 100.0 |
| 10 | 0.034 | 0.034 | 98.7 | 10 | 0.090 | 0.090 | 100.1 |
| 11 | 0.033 | 0.034 | 101.9 | 11 | 0.010 | 0.010 | 100.0 |
| 12 | 0.069 | 0.068 | 99.1 | 12 | 0.017 | 0.017 | 99.0 |
| 13 | 0.037 | 0.038 | 101.4 | 13 | 0.086 | 0.085 | 99.9 |
| 14 | 0.078 | 0.078 | 100.0 | 14 | 0.154 | 0.153 | 99.9 |
| 15 | 0.067 | 0.069 | 102.4 | 15 | 0.204 | 0.204 | 100.0 |
| 16 | 0.101 | 0.099 | 98.2 | 16 | 0.234 | 0.234 | 100.0 |
| 17 | 0.032 | 0.032 | 99.2 | 17 | 0.070 | 0.071 | 100.4 |
| 18 | 0.075 | 0.075 | 99.8 | 18 | 0.109 | 0.109 | 100.0 |
| | **RMSE (root MSE)** | | 0.0010 | | **RMSE (root MSE)** | | 0.0002 |
| | **Mean recovery (%)** | | 100.7 | | **Mean recovery (%)** | | 100.0 |

The obtained recoveries range from 98.2 % to 106.3 % and the root mean squared errors (RMSE) values for asiaticoside and madecassoside show 0.0010 and 0.0002, respectively. These results showed that the

proposed method is suitable for the simultaneous determination of two chemical components in *C. asiatica*.

## 4 Conclusion

ANN using UV spectra data was successfully set up and applied to simultaneously determine the presence of asiaticoside and madecassoside in *C. asiatica*. The training data set from 108 *C. asiatica* reference samples indicated a good accuracy in recoveries obtained by analyzing 18 real samples. The test set has proved that using reference samples helped reduce the effect of interference in the complicated matrix of traditional herbal medicine. ANN could be useful when the signal of analytes in the samples was not linear and influenced by a complicated matrix. This research proposed a time-saving and environmental friendly method as a replacement for chromatographic methods.

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# Ứng dụng mô hình học máy trong phân tích asiaticoside và madecassoside trong Rau má (*Centella asiatica*)

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Tóm tắt: Mạng nơ ron nhân tạo (ANN), một lĩnh vực của học máy, hiện đã được ứng dụng rộng rãi trong hóa học phân tích với nhiều mục đích khác nhau như phân loại/nhận dạng mẫu, dự đoán và mô hình hóa. Nghiên cứu này cho thấy ANN đóng vai trò quan trọng như một phương pháp hồi quy phi tuyến, kết hợp với phương pháp quang phổ UV để giải quyết vấn đề chồng phổ sử dụng ma trận chứa nền mẫu phức tạp của thuốc thảo dược. Mô hình ANN đã được sử dụng thành công để xác định đồng thời asiaticoside và madecassoside trong mẫu rau Má (*Centella asiatica*) thu từ nhiều tỉnh khác nhau ở Việt Nam dựa trên phổ UV của mẫu chuẩn đối chiếu và mẫu thêm chuẩn. Phổ hấp thụ của 108 mẫu *C. asiatica* (có nồng độ asiaticoside được xác định bằng phương pháp HPLC và nồng độ madecassoside được phân tích bằng phương pháp LC/MS/MS) ở vùng phổ từ (190-250) nm với khoảng cách đo độ hấp thụ quang 1 nm (61 biến/cột) được sử dụng để thu thập dữ liệu đầu vào. Một mô hình ANN sử dụng hàm traincgb với 40 lớp ẩn đã được huấn luyện. Các hệ số tương quan thể hiện mối quan hệ giữa giá trị thực tế (trong các mẫu tham chiếu) và giá trị dự đoán của bộ mẫu hiệu chuẩn và bộ mẫu kiểm tra đều cao hơn 0,9999. Nồng độ asiaticoside và madecassoside trong 18 mẫu *C. asiatica* được xác định bằng mô hình ANN tối ưu cho thấy độ thu hồi tốt (lần lượt là 100,7 % và 100 %) so với giá trị tham chiếu thu được bằng HPLC và LC/MS/MS. Mô hình ANN đã chứng tỏ là công cụ định lượng tốt để phân tích đồng thời hai chất trong nền mẫu thảo dược với quy trình chuẩn bị mẫu đơn giản và quang phổ UV. Phương pháp phân tích đề xuất cho thấy có thể tiết kiệm thời gian, dễ dàng xác định nồng độ và thân thiện với môi trường.

**Từ khóa** mạng nơron nhân tạo (ANN), xác định đồng thời, asiaticoside, madecassoside, *Centella asiatica*, quang phổ UV. học máy