**JOURNAL OF TROPICAL LIFE SCIENCE**
2023, Vol. 13, No. 1, 157 – 170
http://dx.doi.org/10.11594/jtls.13.01.16

**Research Article**

# Antioxidant Activity of Andrographolide from *Andrographis paniculata* leaf and Its Extraction Optimization by using Accelerated Solvent Extraction

Rabe'ah Adam [1], Faiqah Ramli [1], Mariani Abdul Hamid [1]*, Roswanira Abdul Wahab [2,3]

[1] Department of Bioprocess & Polymer Engineering, School of Chemical & Energy Engineering, Faculty of Engineering, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Malaysia
[2] Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Malaysia
[3] Enzyme Technology and Green Synthesis Group, Faculty of Science, Universiti Teknologi, 81310 UTM Johor Bahru, Malaysia

*Article history*:
Submission September 2021
Revised January 2022
Accepted December 2022

**Corresponding author:*
E-mail: mariani@utm.my

**ABSTRACT**

*Andrographis paniculata* is widely used as a medicinal plant in many countries and andrographolide is the major bioactive compound extracted from *A. paniculata* leaf. This study purposely to optimize statistical andrographolide extraction using the accelerated solvent extraction (ASE) technique. The Box Behnken design (BBD) was chosen to determine the optimum ASE conditions for the extraction temperature (ºC), cycle number, and extraction time (min) to achieve the highest yield of andrographolide. The optimum ASE conditions were identified as: extraction temperature of 60 ºC, using 3 cycles and 5 min extraction time, with maximum conversion yield as high as 335.2 ± 0.2 mg/g determined by High Performance Liquid Chromatography (HPLC) with the squared correlation coefficients ($R^{2)}$ of 0.97. The findings revealed the ASE method significantly enhanced andrographolide extraction and agreed closely with the predicted value at 337.5 mg/g. Andrographolide was isolated by preparative HPLC technique. *A. paniculata* leaf extract and extracted andrographolide displayed moderate radical scavenging activity in 2, 2−Diphenyl−1−picrylhydrazyl hydrate (DPPH) assay with $IC_{50}$ of 0.883 ± 1.597 mg/ml and 0.514 ± 0.285 mg/ml respectively as $IC_{50}$ for ascorbic acid was 0.048 ± 0.004 mg/ml. *A. paniculata* extract and andrographolide inhibited the tyrosinase enzyme with $IC_{50}$ of 0.749 ± 0.293 µg/ml and $IC_{50}$ of 2.441 ± 2.026 µg/ml indicated stronger tyrosinase inhibition abilities than kojic acid, $IC_{50}$ of 19.985 ± 0.557 µg/ml. These results suggest that *A. paniculata* leaf extract and andrographolide have greater potential as sources of biochemical compounds that can be used as skin depigmentation solutions.

*Keywords: Andrographis paniculata, Andrographolide, Aaccelerated solvent extraction, Box Behnken design, Extraction yield*

## Introduction

*Andrographis paniculata* or locally known as 'hempedu bumi' or "king of bitters" belongs to the family Acanthaceae that is widely distributed in Southeast Asia, China, India, and Taiwan [1]. The intensive investigations on diterpenoid lactones isolated from *A. paniculata* extracts and are reported to exhibit a wide spectrum of biological activities, involving antibacterial activities [2]. Extracts of the plant are used in complementary medicine and nutritional supplements to treat a wide spectrum of ailments such as fever, hepatitis, diabetes, bowel diseases, snake bites, and hypertension [3, 4, 5] following its inherent high composition of diterpenes, flavonoids and stigmasterols [6, 7]. The plant is also ethnobotanically used for treating snakebite, bug bite, diabetes, dysentery, fever, and malaria [8].

The compositions of phytoconstituents widely differ from one part to another and with the place, season, and time of harvest, comprising of more

*How to cite:*
Adam R, Ramli F et al. (2023) Antioxidant Activity of Andrographolide from *Andrographis paniculata* leaf and Its Extraction Optimization by using Accelerated Solvent Extraction. Journal of Tropical Life Science 13 (1): 157 – 170. doi: 10.11594/jtls.13.01.16.

than 55 *ent*−labdane diterpenoids, 30 flavonoids, 8 quinic acids, 4 xanthones, and 5 rare noriridoids [7]. The *A. paniculata* extract is also documented to be effective in reducing upper respiratory tract infections [9, 10]. The whole *A. paniculata* plant contains andrographolide diterpenoids and polyphenols, with leaf extracts and andrographolide showing variable hepatoprotective actions. Andrographolide also exhibits significant analgesic and anti−inflammatory activities with claims that aqueous decoction/extract of the plant could avert glucose−induced hyperglycemia, lower the blood glucose of diabetic animals, and restore the metabolic profile of obese−diabetic rats [10]. Ethanolic *A. paniculata* extract comparably reduced Glutathione (GSH) levels in diabetic rats as that of the control drug, metformin [12, 13].

Phytochemical investigations on the major andrographolide component (Figure 1) in *A. paniculata* revealed interesting pharmacological properties: anti-oxidant, anti−tyrosinase, anti-diabetic and anti−inflammatory [14, 15]. Andrographolide exhibited anticancer activities, appreciable antiproliferative effect, and increase tumor necrosis factor, that increased toxicity towards B16F0 and HT−29 [16, 17]. Andrographolide also suppresses the Akt/GSK3β/β−catenin signal pathway expression and inhibits melanin production [18]. However, the remarkable pharmacological properties of andrographolide in *A. paniculata* have been observed for extracts obtained by conventional−extraction techniques. While many studies used modern extraction methods to obtain andrographolide from *A. paniculata*, there are insufficient methods investigated and optimized for high yield and improved pharmacological properties.

Among the modern extraction techniques applied to the extraction of plants, materials include vacuum−assisted extraction, microwave−assisted extraction, and supercritical $CO_2$ extraction [19, 20, 21]. However, to the best of our knowledge, there is no report on the extraction of andrographolide from *A. paniculata* through accelerated solvent extraction (ASE). This alternative technique to solvent extraction offers the benefits of reduced extraction time and solvent consumption. The high temperature and pressure in ASE synergistically increase diffusion rates of Phyto−extractives, hence the effective sample extraction [22]. The method can simultaneously process multiple samples and effortlessly separate the solvent and solid matter, compared to cold maceration and microwave−assisted extraction [23, 24].

Noteworthily, the ASE benefits to extract the major andrographolide component from *A. paniculata* can be enhanced by the method of response surface methodology through the Box Behnken design (BBD). The technique has been extensively applied with success to analyze the relationships of experimental variables in improving the extraction efficiency of a new extraction method on different plants [25, 26, 27]. BBD establishes a quadratic response surface and independent quadratic design [28], and the methods do not combine maximum and minimum factors simultaneously [29].

Compared to other RSM approaches, the use of BBD offers more benefits because of fewer points at the extreme of the cubic region [30, 31]. Having said that, this study aims to identify the best ASE parameters to obtain high yields of andrographolide from *A. paniculata*. Three parameters, namely extraction temperature ($X_1$), cycle number ($X_2$), and extraction time ($X_3$), were chosen in this study to establish a statistical model that could be applied for future scale-up andrographolide extraction. Under the optimal extraction conditions, the anti-oxidant of properties of andrographolide rich extract was assessed using DPPH assay, and the extracted component was identified by high−performance liquid chromatography.

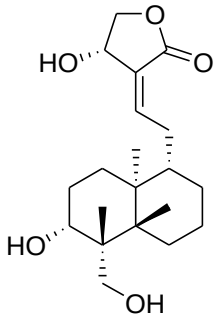


Figure 1. Structure of andrographolide.

## Material and Methods

### *Materials*

The dried leaves (200 g) of *A. paniculata* were purchased from Herba Bagus Sdn Bhd, Johor, and deposited at Forest Research Institute Malaysia, Kuala Lumpur, Malaysia (SBID 002/12). The *A. paniculata* plant was cultivated in a climate−controlled area and air−dried at room temperature of about 25°C. The dried leaves were ground into

powder form and passed through a 30 mesh sieve. The sample was preserved in a sealed plastic bag at −80ºC until further use. The chemical 2, 2−Diphenyl−1−picrylhydrazyl hydrate (95% purity) (DPPH) and the certified reference material (CRM) of andrographolide (95% purity) were purchased from Sigma–Aldrich (Madrid, Spain). Ethanol (95%) and HPLC−grade methanol were procured from J.T. Baker (Phillipsburg, NJ, USA).

### *Accelerated solvent extraction (ASE) of A. paniculata*

Each extraction was conducted using the ASE 100 Dionex System (Dionex Pty Ltd, Lane cove, 1595, NSW). Briefly, 3 g of the sample was mixed with diatomite to remove the remaining moisture before extraction. The obtained sample was then packed into a 10 ml stainless steel vessel extracted with ethanol at 1500 psi (10 MPa). The extraction was performed under variable extraction temperatures (60, 80, and 100 ºC), under three different cycle numbers (ranging from 1 to 3) at different extraction times (3 to 7 mins). Upon completion, the excess solvent was evaporated using a rotary evaporator (Heidolph MX07R−20, PolyScience, USA), and the extraction liquid sample was oven−dried to a constant weight. The extracts were then filtered by using a 0.22 µm microporous membrane for High Performance Liquid Chromatography (HPLC) analysis. All extractions were triplicated.

### *Experimental design and statistical analysis*

It is known that extraction temperature ($X_1$), cycle number ($X_2$), and extraction time ($X_3$) are among the significant factors that could affect the efficiency of ASE [32]. To access the best combination of extraction variables and their influence on the extraction yield of andrographolide, the extraction parameters were optimized with RSM by using a mathematical software package, Design-Expert version 13, DX13 (Stat−Ease, Inc., Minneapolis, USA). A total of 17 experiments comprised the three−variable−three−level Box Behnken design to determine the optimal Accelerated assisted extraction (ASE) conditions, with five replicates as the center point (Table 1). The selected variables were prescribed into 3 levels coded as −1, 0, and +1, and each experiment was randomized to avoid bias. Andrographolide content was quantitatively analyzed by HPLC analysis. In this experiment, regression analysis was performed on experimental data fitted to a second-order polynomial quadratic regression model Equation 1 as follows:

$$Y = \beta o + \sum \beta iXi + \sum \beta iixi^2 + \sum\sum \beta ijXiXj$$

(Equation 1)

Where Y = response parameters, $\beta_0$ = constant, $\beta_i$ = linear terms, $\beta_{ii}$ = quadratic terms, $\beta_{ij}$ = interaction terms.

The study performed statistical analysis via Analysis of Variance (ANOVA) to evaluate the statistical significance of the obtained model. Parameters used to gauge the model's quality involved observation of the regression coefficient, $R^2$, in which a high value indicates the model adequacy and is further supported by the F−test and lack of fit. The ANOVA also assessed the statistical significance ($p < 0.05$) of the dependent and independent variables while Fischer's test values (F-value) examine the adequacy of ASE models. Three-dimensional response surface plots were generated to show the interaction between variables and their effect on response parameters (concentration of andrographolide). In this study, an extra set of experiments were also conducted under the optimal conditions to validate the proposed BBD design model.

### *High-performance liquid chromatography (HPLC) analysis*

High performance Liquid Chromatography (HPLC) analysis was performed by using Zorbax® Eclipse plus HPLC column phase C18, 5 µm particle size, L × I.D. 150 mm × 4.6 mm, column oven temperature 25°C, mobile phase methanol, water (60:40) pumped at the flow-rate 0.7 ml/min detection at 223 nm injection volume 20 µl, using andrographolide as an internal standard for quantitation).

### *Calibration Curve and Andrographolide Quantification by Analytical HPLC*

A five−point calibration curve was used to quantify andrographolide (Supplementary 1). Andrographolide standards were prepared by diluting stock andrographolide in methanol, water (60:40) concentrations ranging from 200 to 1000 µg/ml.

For each concentration, 10.0 µl solution was injected into the analytical column at 25 °C. Accordingly andrographolide peaks were then eluted in mobile phase (60:40) solvent ratio at 1.5 ml/min flow rate.

For similar conditions, about 2 mg/ml *A. paniculata* leaf extract was dissolved in methanol, water solvent ratio (60:40), and injected into the same column. Andrographolide content in *A. paniculata* leaf extract was quantified based on reference to the andrographolide standard calibration curve. The calculation used the following equation 2:

$$\text{Concentration of andrographolide} = \frac{A - I}{M}$$

(Equation 2)

Where *A*: The peak area of andrographolide in sample test solution, *I*: the Y intercept in the calibration curve, *M*: The slope for calibration curve. Andrographolide percentage (w/w) in *A. paniculata* leaf extract was calculated as follows:

$$\text{Andrographolide percentage} = \frac{C \times DF \times V}{1000\ W}$$

(Equation 3)

Where, *C* = The concentration of analyte in the sample test solution (mg/L), *V* = The test solution (ml), *DF* = Dilution factor, *W*= weight of sample extract (g)

### *Purification of Andrographolide by Preparative HPLC*

*A. paniculata* leaf extract was dissolved at about 2 mg/ml in methanol, water ratio (60:40), and was then filtered using PVDF membrane (17 mm, porosity, 0.45 mm) for HPLC analysis. In HPLC analysis, the parameters of elution were developed and applied to preparative HPLC on Zorbax® Eclipse PrepHT (high throughput) C18 column (21.2 x 150 mm, 7µm). For sample injection, 400 µl and 15.0 ml/min flow rates were exerted per injection. The separation was achieved volume ratio (45:55) of 100% acetonitrile ($C_2H_3N$): 0.1% aqueous phosphoric acid ($H_3PO_4$) at 25 °C column temperature. The injections of samples were repeated with about 20 duplications for andrographolide purification. The effluent was observed using Ultraviolet-Visible spectroscopy (UV-Vis) at 223 nm and fraction peaks were collected based on the standard elution profile of andrographolide. The fraction of the solution was collected and stored at 4 °C before being used for spectral identification of the compound.

### *Spectral Identification of Compound*

The fraction sample solution was processed corresponding to the following procedure. About 100 µl of the liquid sample with 500 µl of ethyl acetate and 20 µl of IS solution were transferred into a 1.5 ml microcentrifuge tube and were vortex for about 5 minutes. The solution was then centrifuged for 10 minutes at 32000 x g. The supernatant solution was collected and blown by nitrogen. The solution was dissolved with methanol, and water solution (1:1) (100 µl). The sample was injected with about 10 µl and was analyzed by Liquid Chromatography with Tandem Mass Spectrometry (LC-MS/MS) analysis system.

### *DPPH (2, 2−Diphenyl−1−picrylhydrazyl hydrate) assay*

Anti-oxidant activity of *A. paniculata* leaf extracts and andrographolide were spectrophotochemically evaluated using DPPH assay as depicted by a recent study [33] with slight modifications. About each aliquot, 100 µl (100 µM) DPPH in methanol solution was transferred into 100 µl of sample solution. The mixture was then made into 7.5 - 500 µg/ml for the final concentration of the extract and andrographolide. Ascorbic acid was used as standard. The radical decay using blank solutions was used for the absorbance values correction. The absorbance of the samples was determined using Biobase−EL10A (Biobase Industry Co. Ltd, Shandong, China) at 515 nm after 30 min of incubation in a dark room. The calibration curve's linearity range of the prepared was between 0.00−10.00 mg/L (Supplementary 2) and the scavenging activity was estimated by the ascorbic acid standard curve, reported as mg/g of dried weight (DW) (Equation 2). All absorbances were measured in triplicate. The scavenging activity is calculated as the following:

$$Scavenging\ Activity\ (\%) = \frac{((Absorbance\ of\ control - Absorbance\ of\ sample) \times 100)}{Absorbance\ of\ sample}$$

(Equation 4)

Estimating the $IC_{50}$ values of the obtained *A. paniculata* leaf extract and andrographolide used the non−linear regression plot derived from the plotted data GraphPad Prism version 9.0.0 for Windows, GraphPad Software, San Diego, California USA (Supplementary 3). The concentration of each sample was required to give 50 % of the optical density shown by the control for the value of the $IC_{50}$ reference. All analyses of the samples were triplicated with mean and standard deviation determined in this study.

### *Mushroom Tyrosinase Inhibition Assay*

About 140 µl of *A. paniculata* leaf extract and andrographolide purified at proper concentration were made for in vitro mushroom tyrosinase inhibition assay. Sodium phosphate buffer (pH 6.8) at 10 mM was used in the sample preparation and was transferred into 96 well plates. At a concentration of 10 µg/ml of mushroom tyrosinase in 10 mM sodium phosphate buffer, 40 µl was added for each well. The 96 well then were incubated for about 10 minutes at room temperature. The sample was then mixed with 20 µl of 10 mM L-DOPA solution in 10 mM sodium phosphate buffer. The sample in 96 wells was incubated for 30 minutes. Absorbance reading was determined by using a microplate reader (PerkinElmer, USA) at 405 nm. The tyrosinase activity (%) was calculated by using the following equation 5:

$$\text{Mushroom Tyrosinase Activity (\%)} = \frac{C-A}{B} \times 100 \quad \text{(Equation 5)}$$

Where A = Absorbance value (sodium phosphate buffer supplemented with each concentration of andrographolide without mushroom tyrosinase), B = Absorbance value (Mushroom tyrosinase only treated sodium phosphate buffer), and C = Absorbance value (Sodium phosphate buffer supplemented with each concentration of andrographolide with mushroom tyrosinase).

The calibration curve's linearity range of kojic acid was prepared between 0.00−90.00 µg/ml (Supplementary 4). All absorbance values were measured at 405 nm. Estimating the IC50 values for mushroom tyrosinase inhibition (%) of *A. paniculata* leaf extract and andrographolide used the non−linear regression plot derived from the plotted data using GraphPad Prism version 9.0.0 for Windows, GraphPad Software, San Diego, California USA (Supplementary 5).

## Results and Discussion

### *Model fitting*

The analytical tool of Response Surface Methodology (RSM) is effective for measuring the interaction and correlation process variables and corresponding responses to optimize multiple variables for certain responses [29, 31]. In this study,

Table 1. Experimental design by Box-Behnken with independent variables.

| Run | Extraction Temp (°C) ($X_1$) | Cycle No ($X_2$) | Extraction time (min) ($X_3$) | Yield of Andrographolide (mg/g) | |
|---|---|---|---|---|---|
| | | | | Experiment values (mg/g) | Predicted values |
| 1 | 80 | 1 | 7 | 221.0 | 221.62 |
| 2 | 80 | 3 | 7 | 247.0 | 248.13 |
| 3 | 80 | 2 | 5 | 244.0 | 246.40 |
| 4 | 60 | 2 | 7 | 200.0 | 195.00 |
| 5 | 80 | 2 | 5 | 245.0 | 246.40 |
| 6 | 100 | 3 | 5 | 241.0 | 236.62 |
| 7 | 80 | 1 | 3 | 216.0 | 214.87 |
| 8 | 100 | 2 | 3 | 158.0 | 163.00 |
| 9 | 80 | 2 | 5 | 251.0 | 246.40 |
| 10 | 60 | 1 | 5 | 231.0 | 235.37 |
| 11 | 80 | 2 | 5 | 247.0 | 246.40 |
| 12 | 80 | 3 | 3 | 226.0 | 225.38 |
| 13 | 100 | 1 | 5 | 307.0 | 303.12 |
| 14 | 100 | 2 | 7 | 259.0 | 262.25 |
| 15 | 60 | 3 | 5 | 335.0 | 338.88 |
| 16 | 80 | 2 | 5 | 245.0 | 246.40 |
| 17 | 60 | 2 | 3 | 268.0 | 264.75 |

The codes are (− 1, 0, 1) and actual values of $X_1$ (60, 80, 100), $X_2$ (1, 2, 3) and $X_3$ (3, 5, 7).

the BBD's multiple regression analysis was done to investigate the relationship between the response of andrographolide yield and the experimental levels of independent variables in the ASE of the *A. paniculata* leaf extract. The experimental results for the extraction of andrographolide using ASE and the corresponding independent variables were tabulated in Table 1.

As shown in Table 1 the ASE significantly improved the extraction efficiency of andrographolide from *A. paniculata,* to yield andrographolide yield ranges from 158–335 mg/g. The highest andrographolide yield (335 mg/ml) was recorded at an extraction temperature of 60 ºC cycle number 3 for 5 min of extraction time. After the insignificant terms were removed, the empirical relationships between the response and the three independent variables were expressed by a second-order polynomial equation (Equation 4):

$$Y = 246.4 - 8.62X_1 + 9.25\,X_2 + 7.38X_3 - 42.5X_1X_2 + 42.24X_1X_3 + 4X_2X_3 + 12.92X_1^2 + 19.17X_2^2$$

(Equation 4)

Where Y = Yield of andrographolide, $X_1$ = extraction temperature, $X_2$ = cycle number, $X_3$ = extraction time.

Generally, optimization and analysis of a fitted response surface often lead to misleading results. Then this study performed statistical analysis via Analysis of Variance (ANOVA) to identify the model's accuracy and predictive capability. The fitted quadratic polynomial model of extraction yield for andrographolide from *A. paniculata* were then determined from the ANOVA (Table 2).

The F- test (107.71) and very low probability values, $p < 0.0001$, suggested that the model was highly significant at a 95% confidence level. These data imply the significance of each experiment parameter towards the extraction rate of andrographolide. As tabulated in Table 2, all linear and quadratic terms significantly affected andrographolide with *a p*-value of < 0.05. Similarly, extraction temperature appeared to significantly interact with cycle number ($X_1X_2$) and extraction time ($X_1X_3$) with *a* p-value of < 0.0001. The relationship between the predicted and experimental conversions (mg/g) is shown in Figure 2a. For this study, andrographolide yield for ASE of *A. paniculata* leaf extract was within 152.00 - 333.13 mg/g.

Table 2. ANOVA results of the BBD model.

| Source | Sum of Squares | Degree of freedom | Mean Square | F−value | p−value |
|---|---|---|---|---|---|
| **Model** | 24090.17 | 9 | 2676.69 | 107.71 | < 0.0001 |
| ***Linear*** | | | | | |
| Extraction Temperature ($X_1$) | 595.12 | 1 | 595.12 | 23.95 | 0.0018 |
| Cycle No ($X_2$) | 684.50 | 1 | 684.50 | 27.55 | 0.0012 |
| Extraction Time ($X_3$) | 435.13 | 1 | 435.13 | 17.51 | 0.0041 |
| ***Interaction*** | | | | | |
| $X_1X_2$ | 7225.00 | 1 | 7225.00 | 290.74 | < 0.0001 |
| $X_1X_3$ | 7140.25 | 1 | 7140.25 | 287.33 | < 0.0001 |
| $X_2X_3$ | 64.00 | 1 | 64.00 | 2.58 | 0.1526 |
| ***Quadratic*** | | | | | |
| $X_1^2$ | 703.39 | 1 | 703.39 | 28.31 | 0.0011 |
| $X_2^2$ | 1548.13 | 1 | 1548.13 | 62.30 | < 0.0001 |
| $X_3^2$ | 6104.02 | 1 | 6104.02 | 245.63 | < 0.0001 |
| **Residual** | 173.95 | 7 | 24.85 | | |
| Lack of Fit | 142.75 | 3 | 47.58 | 6.10 | 0.0566 |
| Pure Error | 31.20 | 4 | 7.80 | | |
| **Correlation Total** | 24264.12 | 16 | | | |

The determination coefficient, $R^2$ value (0.99), is close to 1, implying the model could adequately represent the relationship of the chosen parameters. Moreover, the adjusted $R^2$ ($R_{adj}$: 0.98) and predicted $R^2$ (0.90) were in the satisfactory range with the determination coefficient, which further confirms the model's significance.

The "lack of fit" determines the model failure in data representation that is not included in the experimental domain. That said, the insignificant lack of fit value of the model (0.00566) signifies a good reproducibility of the experimental data and the reliability of the model to correlate the independent variables to the response [31, 34].

***Response surface analysis***

Response surface three-dimensional graphs represent the relationship between the response variable and the correspondence coefficients. Figure 3 demonstrates the mutual interactions of extraction temperature ($X_1$), cycle number ($X_2$), and extraction time ($X_3$) on the yield of andrographolide. A circular shape of contour plots indicates a negligible interaction between corresponding variables, while an elliptical shape of contour plots suggests the significance of the interactions between variables [35].

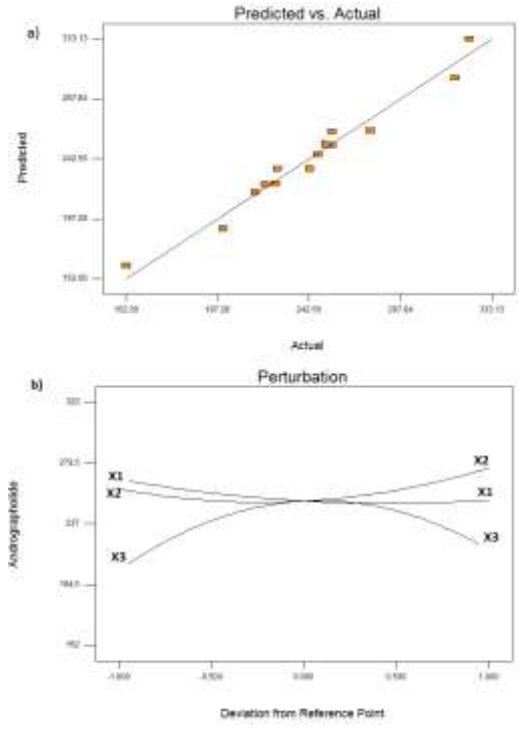


Figure 2. a) Comparison between the predicted and the actual values for andrographolide yield (mg/g) in extract. b) The deviation of the reference point for andrographolide yield for the effect of (X1), (X2) and (X3).

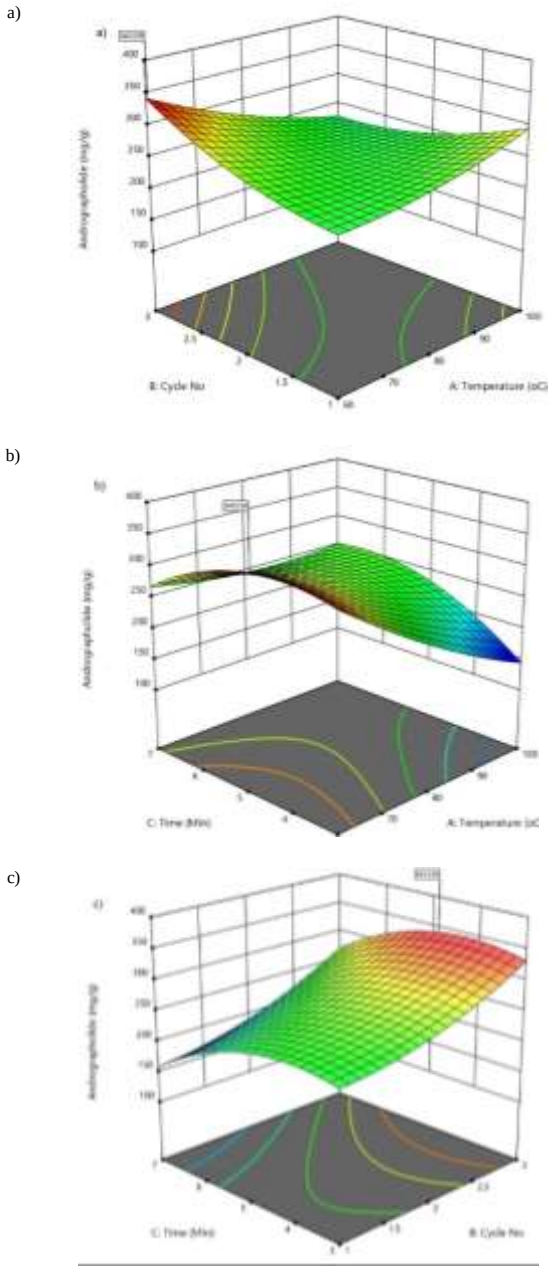


Figure 3. Response surface plots of experimental factors on andrographolide yield. a) Extraction temperature (60 − 80 °C) versus cycle number (1 − 3) at an extraction time of 5 minutes, b) Extraction temperature (60 − 80 °C) versus extraction time (3, 5 and 7 min) at a fixed extraction cycle (2), and c) cycle number (1 − 3) versus extraction time (3, 5 and 7 min) at a fixed extraction temperature of 80 °C.

As shown in Figure 3a, the mutual interaction between extraction temperature and cycle number, $X_1X_2$, yielded the highest andrographolide yield at

60 ºC and cycle number 3. Based on the highest andrographolide content obtained in experiment 2, it could be inferred the cycle number is a dominant factor in andrographolide extraction. This was

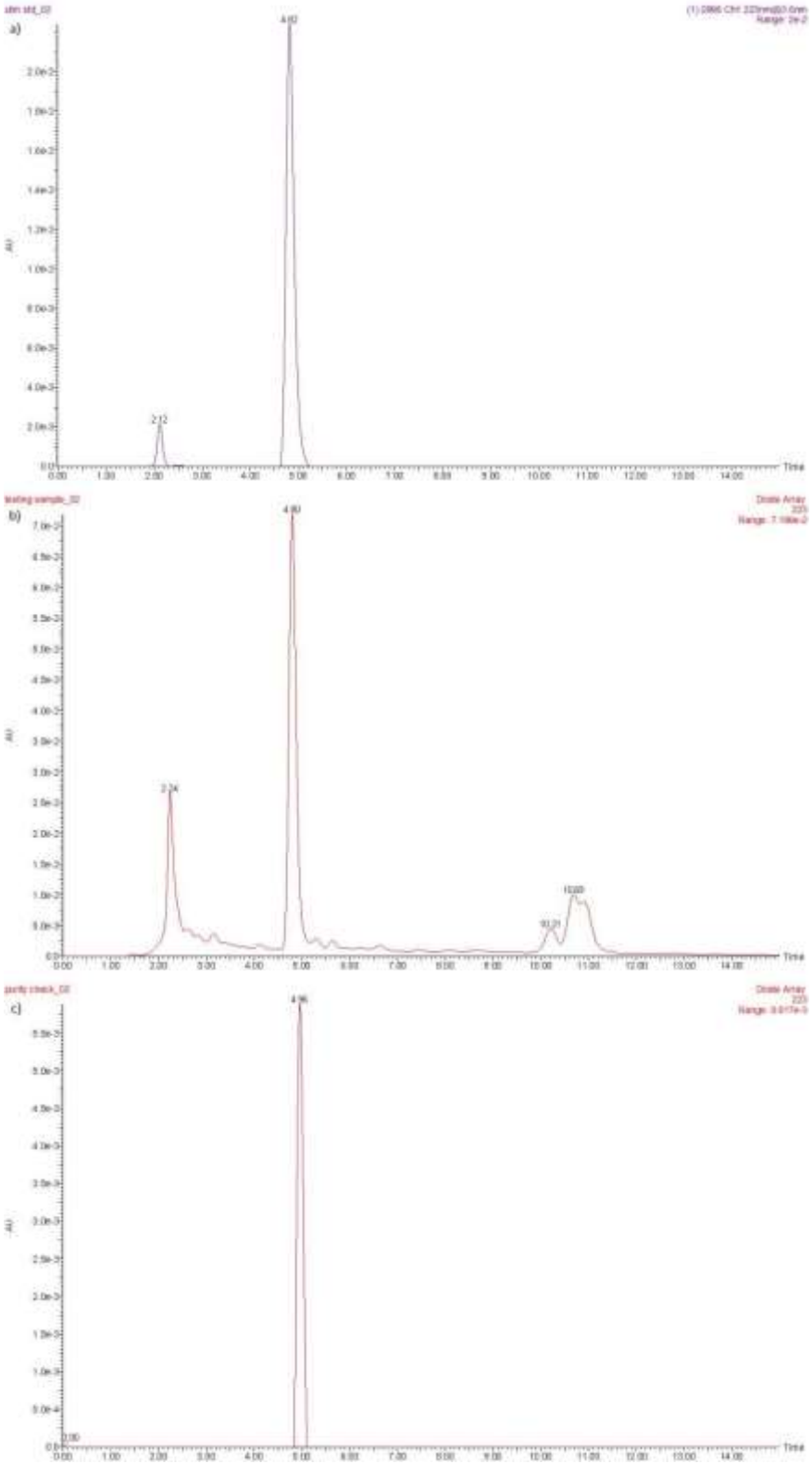


Figure 4. HPLC chromatograms read at 223 nm for a) andrographolide standard, b) *A. paniculata* leaf extract, and c) isolated andrographolide from *A. paniculata* leaf extract.

shown by the larger F value (27.55) over the extraction temperature (23.75) (Equation 3). Furthermore, the temperature (23.75) (Equation 3). Furthermore, the negative interaction term (−42.5 $X_1X_2$) (Equation 3) signifies an antagonist effect between extraction temperature and cycle number, which meant that high andrographolide content is obtainable at low cycle number and high temperature, and vice versa. Furthermore, a recent study reported that andrographolide is a thermally labile compound, and thus, it is highly susceptible to degradation at high extraction temperatures [21].

Hence, higher cycle numbers would only further degrade the andrographolide and result in lower content of the substance in the *A. paniculata* leaf crude extract. Extraction of andrographolide from *A. paniculata* leaf extract slightly decreased as the extraction temperature exceeded 50 ºC. While other studies that reported a high extraction temperature could increase the kinetic removal of the substances from plant cells, the condition could backfire and cause the leaves structure to be swollen.

Despite that, this above-said phenomenon likely contributed to the reduced andrographolide content at temperatures beyond 60°, as seen in this study, because of the mass transfer limitation of the substance from the plant matrix into the surrounding solvent. The trend seen here corroborates an observation of a previous work that found 65 ºC being the highest yield of diterpenoids extracted from *A. paniculata* leaf using vacuum-assisted extraction [36].

Figure 3b depicts the effect of extraction temperature and time of extraction, $X_1X_3$, towards the concentration of andrographolide in *A. paniculata* leaf extract. It is apparent that the extraction temperature of (F value 23.75) is a larger controlling factor than that of the extraction time of (F value 17.51) (Table 2) to maximize andrographolide recovery for this mutual interaction, with the maximum andrographolide yield achieved at ~60 ºC.

The general trend of the *A. paniculata* ASE revealed the andrographolide content increased monotonically until the mid-point of the extraction time, to reach a maximum of 340.5 mg/g after 5.3 min (Figure 2b). This agreed with the regression equation's positive value for the two interacting factors (+42.24$X_1X_3$) (Equation 3).

This study's response exhibited a downward trend when the extraction time exceeded 6 min, agreeing with other ASE experiments. Previous ASE trends consistently showed an extraction time longer than 5 min was impractical and does not substantially impact the process efficiency to maximize andrographolide recovery [37, 38]. The prolonged extraction time plausibly exacerbated the disintegration of the *A. paniculata* andrographolide, a well-reported occurrence for diterpenoids extracted by extreme physical processes [22].

### *Model validation*

In this study, BBD was used to evaluate the optimum ASE parameters to maximize the andrographolide yield from *A. paniculata*. Using the proposed values from the Design-Expert version 13, DX13, the experiment established that the highest andrographolide yield (337 mg/g) at lower extraction temperature, higher number cycles, and a definite contact time between solvent and plant matrix at 60 ºC, 2.9 cycles and 4.72 minutes, respectively. The validation experiment was repeated using a set of experiments with modified conditions (3 cycles and 5 min of static time, extraction temperature remains unchanged) in order to verify the predicted data was not intolerant of the experimental value.

HPLC method was carried out in the quantification of andrographolide in *A. paniculata* leaf extract. As a reference, a standard solution of andrographolide was injected and retention time was monitored at 4.82 minutes (Figure 4a). A calibration curve was prepared through the peak area and concentration of the standard solutions. The linear regression with correlation coefficient ($R^2$ = 0.9847).

The solution of *A. paniculata* extract (2 mg/ml) was injected retaining the reference standard for identical chromatographic conditions. Andrographolide from the extract was determined at 4.80 minutes of the retention time (Figure 4b).

The quantity of andrographolide was approximated at 335.2 ± 0.2 mg/g (Figure 4b), which amounts to only a 0.534 % difference between the two values. The comparable values for the predicted and experimental andrographolide yields affirmed the BBD model obtained by this study is reliable and could adequately predict the optimized ASE conditions.

### *Purification of Andrographolide from A. paniculata leaf extract using Preparative HPLC*

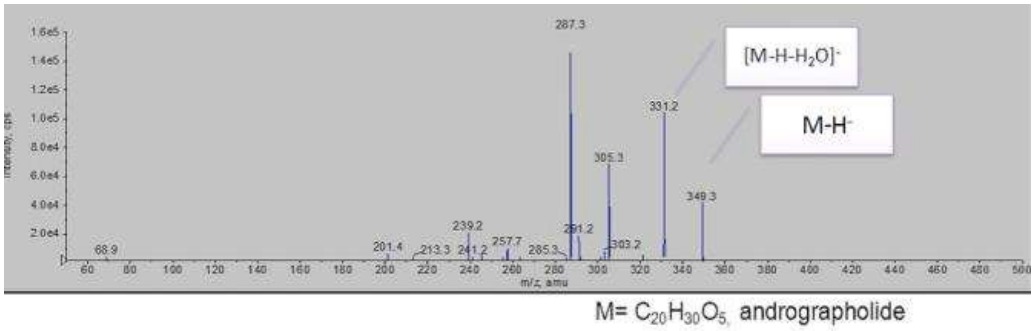


Figure 5. LC-MS/MS spectra of isolated andrographolide.

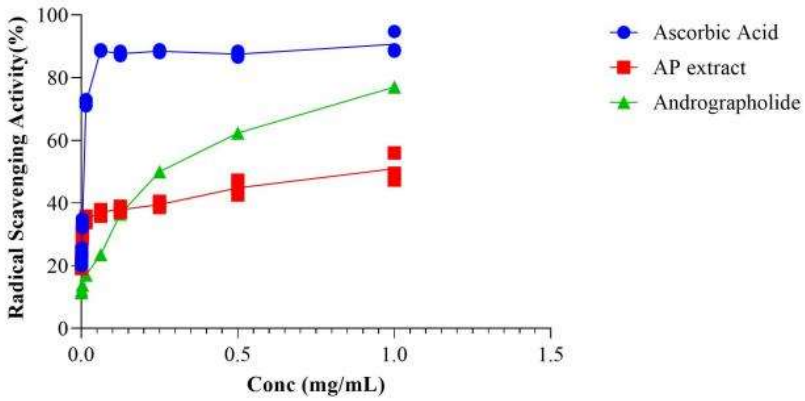


Figure 6. DPPH radical scavenging activity of Ascorbic acid, *A. paniculata* leaf extract and andrographolide.

Preparative HPLC was used for the purification of andrographolide which was obtained from *A. paniculata* leaf extract in HPLC analysis. A major peak was shown in the chromatogram of andrographolide significantly at 4.80 minutes of retention time (Fig. 4a). An identical andrographolide peak at 4.80 minutes of retention time was determined in *A. paniculata* extract as shown in Fig. 4b. Duration time between 4.70 minutes until 5.80 minutes as time trigger mode was used in the fraction collector set up. For each injection, about 20 ml was collected from a single fraction and 22.0 ml/min was used as the volume flow rate. The collective fraction was verified by using HPLC analysis and the structure of andrographolide was confirmed by LCMS/MS. Andrographolide concentration was determined at about 26.3170 mg/l in preparative HPLC (Fig. 4c). Concentrated extracts in acetonitrile were indicated to be 97% of purity recovery.

### *Spectral analysis*

The presence of andrographolide in the fraction collected was further confirmed through LCMS/MS analysis. LC-MS/MS method was used to determine the molecular weight of the compound and the value shown at 349.3 [M-H] (Figure 5), similar as reported in the previous finding [38, 39].

### *Anti-oxidant activity of A. paniculata extract and extracted andrographolide*

Investigation of the antioxidant activity of *A. paniculata* leaf extract and extracted andrographolide was carried out using a DPPH assay. In this assay, radical scavenging activities were perceived as an antioxidant's ability to donate hydrogen and consequently changed the purple DPPH radical to yellow−colored diphenylpicryhydrazine [39]. The extracted andrographolide exhibited the highest mean anti-oxidant activity of an $IC_{50}$ of 0.514 ± 0.285 mg/ml while the *A. paniculata* leaf extract displayed moderate radical scavenging activity with an $IC_{50}$ of 0.883 ± 1.597 mg/ml (Fig.6). In this study, ascorbic acid was used as the positive control showed a good anti-oxidant activity with $IC_{50}$ value of 0.048 ± 0.004 mg/ml. This tyrosinase inhibition (%) displays stronger tyrosinase inhibitory abilities than kojic acid tyrosinase inhibition $IC_{50}$ of 19.985 ± 0.557 µg/ml. The data indicates that the ex-

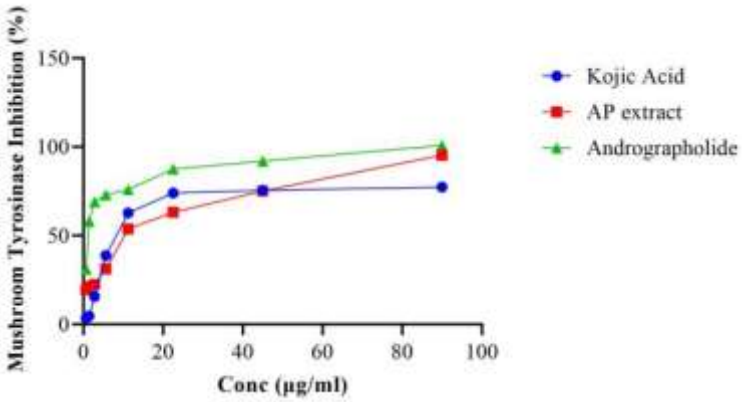


Figure 7. Mushroom tyrosinase activities of kojic acid, *A. paniculata* leaf extract, and isolated andrographolide.

tracted andrographolide showed better anti-oxidant activity over the *A. paniculata* leaf extract and ascorbic acid.

### *Mushroom tyrosinase inhibition activity*

A mushroom tyrosinase inhibition assay is performed on the *A. paniculata* leaf extract and extracted andrographolide in order to observe tyrosinase activity inhibition. Kojic acid was used in this assay as standard as its prominently used tyrosinase inhibitor. The data indicate that the *A. paniculata* extract and andrographolide inhibited the tyrosinase enzyme with $IC_{50}$ of 0.749 ± 0.293 µg/ml and $IC_{50}$ of 2.441 ± 2.026 µg/ml (Fig. 7).

This shows that *A. paniculata* leaf extract and extracted andrographolide can inhibit melanin synthesis and tyrosinase activity. Since *A. paniculata* leaf extract and andrographolide showed greater tyrosinase inhibition activity, both have the potential to simulate tyrosine of amino acid and binding enzyme tyrosinase catalytically. As well as the formation of pigment is blocked by the deep cells on the skin and mitigates the melanogenesis process [40].

## Conclusion

The effects of ASE conditions on the yield of andrographolide from *A. paniculata* were evaluated using BBD and RSM. Notably, the study found the response yield depended on the quadratic terms of cycle number and extraction time, and interactions between extraction time and cycle number, and extraction time. Based on the polynomial regression model, the optimum conditions for extraction of andrographolide yield were extraction temperature of 60 ºC, 3 extraction cycles, and 5 min of extraction time to afford the highest yield of andrographolide of 335.2 ± 0.2 mg/g. Quantitative analysis of andrographolide in the crude extract was achieved by HPLC analysis and andrographolide purification by preparative HPLC. Hence, the BBD concluded that the temperature was the main factor for the total yield of extraction. It was clear that a low extraction temperature played an important role in maximizing andrographolide extraction from *A. paniculata*. From the food and drug industry point of view, this study's result could provide a vast green and economic prospect applicable to extracting andrographolide using ASE at an industrial scale.

## Acknowledgment

This work was supported by the NKEA Research Grant Scheme from Ministry of Agriculture and Agro-Based Industry, Malaysia (MOA) and also Ministry of Higher Education (MOHE), Malaysia (R.J130000.7909.4H020).

## References

1. Mishra SK, Sangwan NS, Sangwan RS (2007) *Andrographis paniculata* (Kalmegh): A Review. Pharmacognosy Reviews.
2. Zhang L, Bao M, Liu B et al. (2020) Effect of Andrographolide and Its Analogs on Bacterial Infection: A Review. Pharmacology 105 (3-4): 1-12. doi: 10.1159/000503410.
3. Ajaya KR, Sridevi K, Vijaya KN, Nanduri S, Rajagopal S (2004) Anticancer and immunostimulatory compounds from *Andrographis paniculata*. Journal of Ethnopharmacology 92 (2-3): 291-295. doi: 10.1016/j.jep.2004.03.004.
4. Rahman H, Kim M, Leung G, Green JA, Katz S (2017) Drug-Herb Interactions in the Elderly Patient with IBD: a Growing Concern. Current Treatment Options in Gastroenterology 15 (2): 618–636. doi:10.1007/s11938-017-0154-y.
5. Holleran G, Scaldaferri F, Gasbarrini A, Currò D (2020) Herbal medicinal products for inflammatory bowel dis-

ease: A focus on those assessed in double-blind randomised controlled trials. Phytotherapy Research 34 (1): 77-93. doi: 10.1002/ptr.6517.
6. Jarukamjorn K, Nemoto N (2008) Pharmacological aspects of *Andrographis paniculata* on health and its major diterpenoid constituent andrographolide. Journal of Health Science 54 (4): 370-381. doi: 10.1248/jhs.54.370.
7. Hossain MS, Urbi Z, Sule A, Rahman KMH (2014) *Andrographis paniculata* (Burm. f.) Wall. ex Nees: A review of ethnobotany, phytochemistry, and pharmacology. Scientific World Journal 2014: 1-28. doi: 10.1155/2014/274905.
8. Burkill IH, Birthwistle W, Foxworthy F, Scrivenor J, Watson J (1966) A Dictionary of the Economic Products of the Malay Peninsula. Kuala Lumpur, Ministry of Agriculture and Co-operatives.
9. Coon JT, Ernst E (2004) *Andrographis paniculata* in the treatment of upper respiratory tract infections: A systematic review of safety and efficacy. Planta Medica 70 (4): 293-298. doi: 10.1055/s-2004-818938.
10. Banerjee S, Kar A, Mukherjee PK et al. (2020) Immunoprotective potential of Ayurvedic herb Kalmegh (*Andrographis paniculata*) against respiratory viral infections – LC–MS/MS and network pharmacology analysis. Phytochemical Analysis 32 (4): 629-639. doi: 10.1002/pca.3011.
11. Akbar S (2020) *Andrographis paniculata* (Burm. f.) Nees. (Acanthaceae). in Handbook of 200 Medicinal Plants (2020): 267–283. doi: 10.1007/978-3-030-16807-0_26.
12. Zhang XF, Tan BKH (2000) Antihyperglycaemic and anti-oxidant properties of *Andrographis paniculata* in normal and diabetic rats. Clinical and Experimental Pharmacology and Physiology 27 (5-6): 358-363. doi: 10.1046/j.1440-1681.2000.03253.x.
13. Gaur P, Sharma S, Pandey S et al. (2018) Pharmacological and Clinical Effects of *Andrographis paniculata*. International Journal of Life-Sciences Scientific Research 4 (4): 1889-1896. doi: 10.21276/ijlssr.2018.4.4.6.
14. Koteswara RY, Vimalamma G, Venkata RC, Tzeng YM (2004) Flavonoids and andrographolides from *Andrographis paniculata*. Phytochemistry 65 (16): 2317-21. doi: 10.1016/j.phytochem.2004.05.008.
15. Kumar G, Singh D, Tali JA et al. (2020) Andrographolide: Chemical modification and its effect on biological activities. Bioorganic Chemistry 95: 103511. doi: 10.1016/j.bioorg.2019.103511.
16. Rajagopal S, Kumar RA, Deevi DS, Satyanarayana C, Rajagopalan R (2003) Andrographolide, a potential cancer therapeutic agent isolated from *Andrographis paniculata*. Journal of Experimental Therapeutics and Oncology 3 (3): 147-158. doi: 10.1046/j.1359-4117.2003.01090.x.
17. Khan I, Khan F, Farooqui A, Ansari IA (2018) Andrographolide Exhibits Anticancer Potential Against Human Colon Cancer Cells by Inducing Cell Cycle Arrest and Programmed Cell Death via Augmentation of Intracellular Reactive Oxygen Species Level. Nutrition and Cancer 70 (5): 787-803. doi: 10.1080/01635581.2018.1470649.
18. Zhu PY, Yin WH, Wang MR et al. (2015) Andrographolide suppresses melanin synthesis through Akt/GSK3β/β-catenin signal pathway. Journal of Dermatological Science79 (1): 74-83. doi: 10.1016/j.jdermsci.2015.03.013.
19. Bhan MK, Dhar AK, Khan S et al. (2006) Screening and optimization of *Andrographis paniculata* (Burm.f.) Nees for total andrographolide content, yield and its components. Scientia Horticulturae. 107: 386–391.
20. Mohan M, Khanam S, Shivananda BG (2013) Optimization of Microwave Assisted Extraction of Andrographolide from *Andrographis paniculata* and its Comparison with Refluxation Extraction Method. Journal of Pharmacognosy and Phytochemistry 2 (1): 342-248.
21. Kumoro AC, Hasan M, Singh H (2019) Extraction of Andrographolide from *Andrographis paniculata* Dried Leaves Using Supercritical CO2 and Ethanol Mixture. Industrial and Engineering Chemistry Research 58 (2): 742–751. doi: 10.1021/acs.iecr.8b02243.
22. Ji Y, Li X, Wang Z et al. (2020) Extraction optimization of accelerated solvent extraction for eight active compounds from Yaobitong capsule using response surface methodology: Comparison with ultrasonic and reflux extraction. Journal of Chromatography A 1620: 460984. doi: 10.1016/j.chroma.2020.460984.
23. Herrero M, Martín-Álvarez PJ, Señoráns FJ, Cifuentes A, Ibáñez E (2005) Optimization of accelerated solvent extraction of antioxidants from *Spirulina platensi*s microalga. Food Chemistry 93 (3): 417-423. doi: 10.1016/j.foodchem.2004.09.037.
24. Kataoka H (2019) Pharmaceutical analysis | sample preparation. in Encyclopedia of Analytical Science. doi: 10.1016/B978-0-12-409547-2.14358-6.
25. Sixt M, Gudi G, Schulz H, Strube J (2018) In-line Raman spectroscopy and advanced process control for the extraction of anethole and fenchone from fennel (*Foeniculum vulgare* L. MILL.). Comptes Rendus Chimie 21 (2): 97-103. doi: 10.1016/j.crci.2017.12.004.
26. Cunha SC, Fernandes JO (2018) Extraction techniques with deep eutectic solvents. TrAC - Trends in Analytical Chemistry 105: 225-239. doi: 10.1016/j.trac.2018.05.001.
27. Armenta S, Garrigues S, Esteve-Turrillas FA, de la Guardia, M (2019) Green extraction techniques in green analytical chemistry. TrAC - Trends in Analytical Chemistry 116: 248-253. doi: 10.1016/j.trac.2019.03.016.
28. Jibril S, Basar N, Sirat HM et al. (2019) Application of Box–Behnken design for ultrasound-assisted extraction and recycling preparative HPLC for isolation of anthraquinones from *Cassia singueana*. Phytochemical Analysis 30 (1): 101-109. doi: 10.1002/pca.2795.
29. Romes NB, Hamid MA, Hashim SE, Wahab RA (2019) Statistical modelling of ultrasonic-aided extraction of *Elaeis guineensis* leaves for better-quality yield and total phenolic content. Indonesian Journal of Chemistry 19 (3): 811 - 826. doi: 10.22146/ijc.41603.
30. Teng K, Shen P, Sun J, Zhang HF (2020) Optimization of Ultrasonic-assisted Extraction of Antioxidant Compounds from Asari Radix et Rhizoma Using Response Surface Methodology. Chinese Pharmaceutical Journal 11 (43): 463–469. doi: 10.4103/0973-1296.160451.
31. Yahya NA, Wahab RA, Hamid MA et al. (2020) Statistical optimization and characterization of acoustically extracted *Ananas comosus* peel powder with enhanced antioxidant capacity. Jurnal Teknologi 82 (4): 1-10. doi: 10.11113/jt.v82.14486.
32. Rodríguez-Solana R, Salgado JM, Domínguez JM, Cortés-Diéguez S (2014) Characterization of fennel extracts

and quantification of estragole: Optimization and comparison of accelerated solvent extraction and Soxhlet techniques. Industrial Crops and Products. 52: 528–536.
33. Prabakaran G, Manivarman S, Bharanidharan M (2021) Catalytic synthesis, ADMET, QSAR and molecular modeling studies of novel chalcone derivatives as highly potent antioxidant agents. Xi'an Dianzi Keji Daxue Xuebao/Journal of Xidian University 14 (38): 85-106. doi: 10.1016/j.matpr.2020.11.166.
34. Chen M, Zhao Y, Yu S (2015) Optimisation of ultrasonic-assisted extraction of phenolic compounds, antioxidants, and anthocyanins from sugar beet molasses. Food Chemistry 172: 543–550. doi: 10.1016/j.foodchem.2014.09.110.
35. Ramli F, Hamid MA, Wahab RA et al. (2020) Ultrasonic-Assisted Extraction of Phalerin from *Phaleria macrocarpa*: Response Surface Methodology and Artificial Neural Network Modelling. Arabian Journal for Science and Engineering. doi: 10.1007/s13369-020-04639-8.
36. Wang YQ, Wu ZF, Ke G, Yang M (2015) An effective vacuum assisted extraction method for the optimization of labdane diterpenoids from *Andrographis paniculata* by response surface methodology. Molecules 20 (1): 430-445. doi: 10.3390/molecules20010430.
37. Kang JH, Kim S, Moon B (2016) Optimization by response surface methodology of lutein recovery from paprika leaves using accelerated solvent extraction. Food Chemistry 205: 140-145. doi: 10.1016/j.foodchem.2016.03.013.
38. Richter BE, Jones BA, Ezzell JL et al. (1996) Accelerated solvent extraction: A technique for sample preparation. Analytical Chemistry 68 (6): 1033-1039. doi: 10.1021/ac9508199.
39. Neagu E, Radu GL, Albu C, Paun G (2018) Antioxidant activity, acetylcholinesterase and tyrosinase inhibitory potential of *Pulmonaria officinalis* and *Centarium umbellatum* extracts. Saudi Journal of Biological Sciences 25 (3): 578-585. doi: 10.1016/j.sjbs.2016.02.016.
40. Lim J, Nam S, Jeong JH, Kim MJ, Yang Y, Lee MS, Lee HG, Ryu JH, Lim JS (2019) Kazinol U inhibits melanogenesis through the inhibition of tyrosinase-related proteins via AMP kinase activation. British Journal of Pharmacology 176 (5): 737-750. doi: 10.1111/bph.14560.

This page is intentionally left blank.