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Optimisation of the Extraction Process of a Flavonoid-rich Extract from the Leaves of *Abutilon indicum* with Anti-inflammatory Activity and Cytotoxicity against Selected Cancer Cell lines

Do Huynh Anh[1], Do Le Anh Thu[1], Tran Chi Linh[2], Nguyen Ba Hoang[1,2], Do Van Mai[1]*

[1]*Faculty of Pharmacy, University Nam Can Tho*
[2]*Faculty of Medicine, University Nam Can Tho*

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ABSTRACT

In recent years, increasing attention has been directed toward the study of flavonoids isolated from medicinal plants, due to their broad pharmacological properties and lower toxicity than synthetic agents. This study aimed to optimise the extraction process for a flavonoid-rich extract from *Abutilon indicum* (L.) leaves and to evaluate its sweet taste, anti-inflammatory, and cytotoxic activities. A Box–Behnken Design under Response Surface Methodology (RSM) was employed to investigate the effects of ethanol concentration, extraction temperature, ultrasound time, and solvent-to-material ratio. Total flavonoid content was determined using the aluminium chloride ($AlCl_3$) colourimetric method. The optimised extract was assessed for *in vitro* anti-inflammatory activity by measuring nitric oxide (NO) inhibition in LPS-induced RAW264.7 macrophages and for cytotoxicity against three human cancer cell lines—HepG2 (liver), SK-LU-1 (lung), and MKN7 (gastric)—using the Sulforhodamine B (SRB) assay. Under the optimal extraction conditions (68.5 % ethanol, 50 °C, 20 minutes ultrasound), the extract yielded a total flavonoid content of 306.55 ± 4.62 mg QE/g. The extract exhibited anti-inflammatory activity with a cell protection rate of 54.92 ± 2.29 %, and demonstrated promising cytotoxicity against cancer cell lines with IC_{50} values of 148.29 ± 4.55 µg/mL (SK-LU-1), 164.39 ± 6.94 µg/mL (HepG2), and 164.76 ± 7.73 µg/mL (MKN7). These findings suggest that *Abutilon indicum* leaves are a promising natural source of bioactive flavonoids with potential anti-inflammatory and anticancer effects.

Keywords: *Abutilon indicum*, flavonoids, extraction optimisation, Box–Behnken Design, anti-inflammatory, cytotoxicity, cancer.

Introduction

Cancer and inflammation remain two major disease categories that continue to threaten global public health due to their increasing incidence and mortality rates. Among them, inflammation plays a pivotal role in both physiological and pathological processes, promoting the initiation and progression of several cancers.[1] Natural products with anti-inflammatory potential have demonstrated promising effects *in vivo*, supporting their therapeutic value in managing inflammation.[2] Current treatment strategies mainly depend on chemotherapy, radiotherapy, and synthetic anti-inflammatory drugs; however, these are often limited by severe side effects, high costs, and the risk of resistance.[3] Consequently, there is growing scientific interest in identifying naturally derived compounds that combine anti-inflammatory and anticancer properties, particularly from medicinal plants.[4] *Abutilon indicum* (L.) Sweet, belonging to the family Malvaceae, is a common tropical species traditionally used to manage dysuria, urinary tract inflammation, joint pain, and cough.[5] Modern pharmacological research has revealed that its leaves and stems contain flavonoids, sterols, organic acids, and polysaccharides.[6]

*Corresponding author. Email: dvmai@nctu.edu.vn
Tel.: +62 822 2221 3115

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Among these, flavonoids are recognised as the most biologically active components owing to their antioxidant, anti-inflammatory, and cytotoxic activities.[7] Recent studies also reported the antioxidant and anti-inflammatory potential of related medicinal plants, emphasising the pharmacological relevance of flavonoid-rich extracts.[8] The biological efficacy of plant-derived extracts depends heavily on the extraction process. Conventional extraction methods often yield poor results and require excessive solvent use.[9] Advanced statistical designs, such as the Box–Behnken Design (BBD) under Response Surface Methodology (RSM), have been widely applied to optimise factors influencing extraction efficiency, including solvent concentration, temperature, and solid-to-solvent ratio.[10] Evaluating the anti-inflammatory and cytotoxic activities of optimised extracts can therefore provide a scientific basis for the development of potential phytopharmaceutical formulations.

Materials and Methods

Collection and preparation of plant material

The leaves of *Abutilon indicum* (L.) Sweet were collected from Nguyen Van Cu extension area, An Binh Ward, Can Tho City, Vietnam (GPS coordinates: latitude 10°00′53.3″ N, longitude 105°44′04.7″ E) in June 2025. Fresh leaves of *A. indicum* were washed thoroughly with water to remove impurities such as dust and debris, and any leaves showing signs of pest infestation or physical damage were discarded. The plant material was authenticated based on morphological characteristics described in the Dictionary of Vietnamese Medicinal Plants by Vo Van Chi (2021).[11] The morphology of *A. indicum* is illustrated in Figure 1.

Sample Preparation

After cleaning, the plant material was shade-dried, then further dried to brittleness at 60 °C. The dried material was ground into a fine powder

and passed through a sieve to obtain particles of 60 mesh size. The moisture content of the powdered sample was 11.37 ± 0.65%, not exceeding the limit of 13.0%, as determined according to the procedures described in the Vietnamese Pharmacopoeia V.[12]

Figure 1: Morphology of *Abutilon indicum* (L.) Sweet

Chemicals and reagents

The following reagents and chemicals were used: dimethyl sulfoxide (DMSO; Sigma-Aldrich, Switzerland), Griess reagent (FUJIFILM Wako, Japan), sodium nitroprusside (Sigma-Aldrich, Switzerland), sodium nitrite (Xilong, China), lipopolysaccharide (FUJIFILM Wako, Japan), Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, Switzerland), fetal bovine serum (FBS; Gibco, UK), penicillin–streptomycin (Sigma-Aldrich, Switzerland), Cell Counting Kit-8 (CCK-8 kit, Dojindo, Japan), N-nitro-L-arginine methyl ester (L-NAME; Sigma-Aldrich, Switzerland), curcumin (Sigma-Aldrich, Switzerland), and phosphate-buffered saline (PBS; pH 7.4; Biosharp, China).

Equipment

The study utilised the following instruments: refrigerated centrifuge (Mikro 12-24, Hettich, Germany), analytical balance (AB104-S, Mettler Toledo, Switzerland), drying oven (BE 200, Memmert, Germany), incubator (Memmert, Germany), vortex mixer (ZX3, Velp, Italy), micropipette (Thermol Labsystems), microplate spectrophotometer (Multiskan GO, Thermo Scientific, Finland), moisture analyser (MA37, Sartorius, Germany), rotary evaporator (Buchi, Switzerland), induction cooker (SHD 6800, Shunhouse, China), and biosafety cabinet (Thien Truong Scientific, Vietnam).

Investigation of the Effect of Single Factors on the Extraction Process of Flavonoid-Rich Extracts

The single factors investigated included ethanol concentration, extraction temperature, extraction time, and solid-to-solvent ratio.

Temperature

In this study, the extractions were conducted at 30 °C, 40 °C, 50 °C, 60 °C, 70 °C, 80 °C, and 90 °C, with 99.5% ethanol (concentration), ultrasonic extraction time (10 min), and solid-to-solvent ratio (1:10, w/v) kept constant. Each determination was performed in triplicate, and flavonoid content was measured.

Time

Ultrasonic extraction was performed for 5, 10, 15, 20, 25, 30, and 35 minutes, with the temperature fixed at 30 °C, ethanol concentration at 99.5%, and solid-to-solvent ratio at 1:10 (w/v). Each test was conducted in triplicate, and the flavonoid yield was determined for each condition.

Ethanol concentration

In this determination, extractions were carried out with ethanol concentrations of 40%, 50%, 60%, 70%, 80%, 90%, and 99.5%, while maintaining a temperature of 30 °C, an ultrasonic extraction time of 10 minutes, and a solid-to-solvent ratio of 1:10 (w/v). Each condition was performed in triplicate. Flavonoid content in the extracts was used as the primary evaluation metric.

Solid-to-solvent ratio study

In this study, ratios of 1:10, 1:15, 1:20, 1:25, 1:30, 1:35, and 1:40 (w/v) were tested, while temperature was maintained at 30 °C, ethanol concentration at 99.5%, and ultrasonic extraction time at 10 minutes. Each extraction was performed in triplicate, and the flavonoid content was assessed.

Optimisation of Flavonoid Extraction Process

The results of the single-factor experiments were used to identify the most influential parameters — ethanol concentration, extraction temperature, and ultrasonic duration — for optimisation. The experimental design and statistical evaluation were performed using Design-Expert software version 12.0 within the framework of Response Surface Methodology (RSM) to determine the optimal extraction conditions for maximising flavonoid yield.

Anti-Inflammatory Activity Assay

The anti-inflammatory activity of the extracts was assessed through their ability to inhibit nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. Cells were incubated with the extract, with or without LPS, for 18 hours. After incubation, 100 µL of the culture supernatant was mixed with an equal volume of Griess reagent and incubated at room temperature for 10 minutes. The absorbance of the resulting solution was measured at 540 nm using a microplate reader. N-nitro-L-arginine methyl ester (L-NAME; 100 µM, Sigma-Aldrich, Switzerland) served as the positive control.[13,14]

Cytotoxicity Assay on Cancer Cell Lines

The cytotoxic effect of the extract was determined using the *in vitro* screening protocol standardised by the U.S. National Cancer Institute (NCI) and initially established by Monks *et al.*[15], with minor modifications. This assay quantifies total cellular protein content by measuring optical density (OD) after staining with the sulforhodamine B (SRB) dye. Briefly, trypsinised cells were seeded into 96-well microplates and allowed to adhere overnight. The cells were then exposed to varying concentrations of the extract (10, 40, 80, 120, 160, and 200 µg/mL) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 48 hours. Cells in the control wells (untreated) were fixed with trichloroacetic acid (TCA) after 1 hour, while the remaining treated cells were fixed after the 48-hour incubation period. Following fixation, the cells were stained with SRB for 30 minutes, rinsed with 1% acetic acid to remove unbound dye, and air-dried. The bound SRB was then dissolved in 10 mM unbuffered Tris base solution, and the absorbance was read at 515 nm using an ELISA plate reader (Bio-Rad, USA). Ellipticine served as the positive control, and 10% DMSO as the negative control. The percentage inhibition of cell growth was calculated using Equation (1).

$$\text{Inhibition efficiency (\%)} = \frac{1 - \text{OD}_{\text{test sample}}}{\text{OD}_{\text{negative control sample}}} \times 100 \quad (1)$$

Data Processing and Analysis

Experimental data were processed in Microsoft Excel 2016, and statistical analysis was performed in Minitab 22.0 using Tukey's test. Results were presented as mean ± standard error (Mean ± SE). The optimisation experiments for factors affecting the extraction of *Abutilon indicum* leaf extract were designed using the Box–Behnken model and analysed with Design-Expert software version 11.0. Illustrative graphs were generated using Microsoft Excel 2016.

Results and Discussion

Extraction at different temperatures ranging from 30 °C to 90 °C resulted in the lowest flavonoid content of 176.74 ± 5.38 mg QE/g extract at 90 °C and the highest content of 258.19 ± 2.85 mg QE/g extract at 50 °C. Flavonoid yield increased from 30 °C to reach a peak at 50 °C, but decreased sharply when the temperature was further raised to 90 °C. This indicates that extraction temperature significantly influences flavonoid recovery, with 50 °C being the optimal temperature for obtaining the highest flavonoid yield.

When ultrasound-assisted extraction was performed for different durations ranging from 5 to 35 min, the lowest flavonoid content (176.50 ± 4.24 mg QE/g extract) was observed at 35 min. In comparison, the highest content (239.65 ± 4.93 mg QE/g extract) was

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obtained at 20 min. Flavonoid yield increased steadily from 5 minutes to 20 minutes, followed by a marked decline with prolonged sonication. At 35 min, the flavonoid content was 1.4-fold lower than at 20 min. This demonstrates that ultrasound duration has a pronounced impact on flavonoid recovery, with 20 minutes being the optimal extraction time. Varying ethanol concentration from 40% to 99.5% showed that the lowest flavonoid content (203.97 ± 6.69 mg QE/g extract) occurred at 99.5% ethanol, whereas the highest (261.24 ± 5.47 mg QE/g extract) was obtained at 70% ethanol. Flavonoid yield increased gradually from 40% to 60% ethanol, peaked sharply at 70%, and then decreased significantly at higher concentrations. At 99.5% ethanol, the yield was 1.3-fold lower than at 70%. This suggests that flavonoids, which generally have moderate polarity, dissolve most effectively in ethanol at intermediate concentrations. Thus, 70% ethanol was identified as the optimal solvent concentration. Similarly, the material-to-solvent ratios ranging from 1:10 to 1:40 (w/v) resulted in a slight increase in flavonoid content from 209.84 ± 4.79 to 220.63 ± 9.93 mg QE/g extract. The changes were minimal, indicating that this factor has little influence on flavonoid yield. Increasing the amount of plant material beyond a certain level may even risk solvent saturation, without improving extraction efficiency. Overall, the experimental results indicate that among the single factors studied, extraction temperature, ethanol concentration, and ultrasound time exerted the most significant influence on the flavonoid content of *Abutilon indicum* leaf extracts. Furthermore, following the experimental investigation, three individual factors—extraction temperature, ethanol concentration, and ultrasonic extraction time—were identified as significantly influencing the flavonoid content in *Abutilon indicum* leaves. Table 1 presents the effects of these three factors based on the experimental results and the corresponding predictions generated by the Box–Behnken design model.

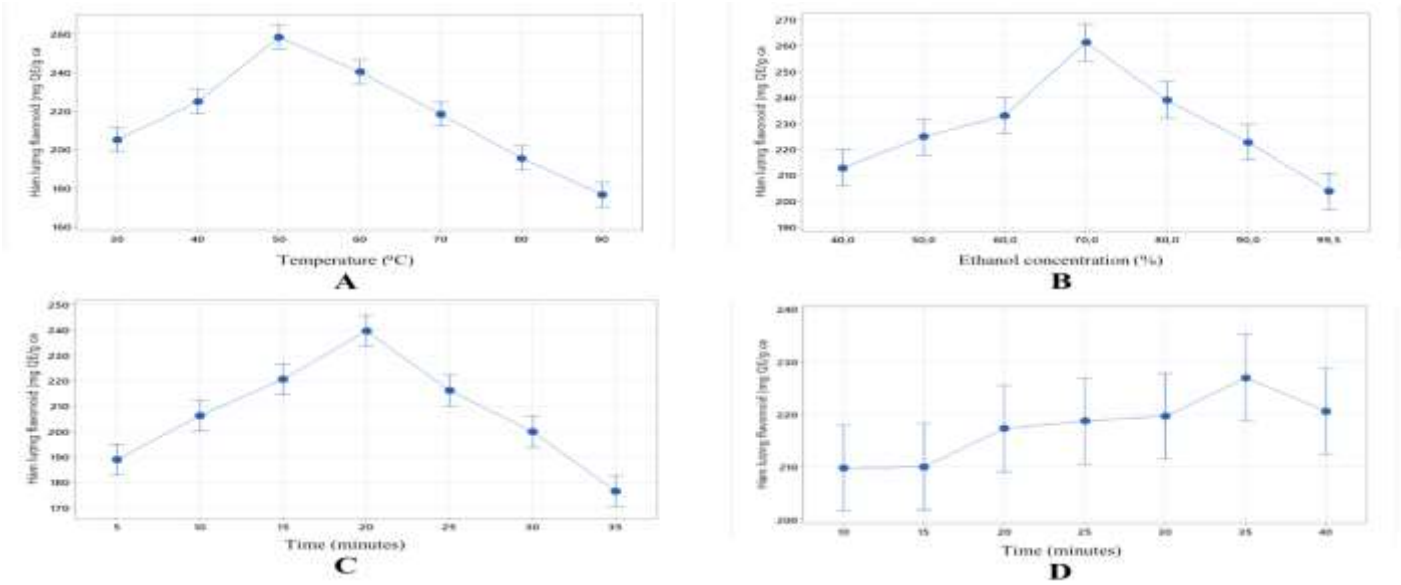

Figure 2: Flavonoid content in *Abutilon indicum* leaf extract and the effects of individual factors. Note: (A) Effect of extraction temperature on flavonoid content; (B) Effect of ethanol concentration on flavonoid content; (C) Effect of ultrasound time on flavonoid content; (D) Effect of material-to-solvent ratio on flavonoid content.

Table 1: Experimental and predicted flavonoid content of *Abutilon indicum* leaves according to the Box–Behnken model

Formula	Independent variables			Flavonoid content (mg QE/g extract)	Predicted Flavonoid content
	Temperature (°C)	Ethanol concentration (%)	Time (minute)		
1	40	60	20	157.25^{f}±3.66	160.86
2	60	60	20	179.55^{e}±3.61	177.24
3	40	80	20	109.37^{gh}±4.62	111.68
4	60	80	20	90.59^{i}±2.85	86.98
5	40	70	15	221.10^{b}±4.13	217.46
6	60	70	15	194.58^{d}±3.07	196.87
7	40	70	25	212.18^{b}±3.52	209.90
8	60	70	25	218.52^{b}±2.82	222.16
9	50	60	15	177.68^{e}±1.86	177.71
10	50	80	15	98.57^{hi}±4.69	99.89
11	50	60	25	179.79^{e}±6.02	178.47
12	50	80	25	116.878^{g}±1.626	116.85
13	50	70	20	291.29^{b}±4.30	300.45
14	50	70	20	303.97^{a}±4.69	300.45
15	50	70	20	302.56^{ab}±1.77	300.45
16	50	70	20	300.45^{ab}±2.47	300.45
17	50	70	20	303.97^{a}±4.69	300.45

Note: Means followed by different letters are significantly different at the 5% level ($p < 0.05$).

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The results revealed pronounced variations in flavonoid content across the three evaluated factors. Among the 17 experimental runs, Runs 13, 14, 15, 16, and 17 (all conducted under identical conditions: temperature 50 °C, ethanol concentration 70%, and ultrasonic time 20 min) yielded the highest flavonoid content. In particular, Runs 14 and 17 reached a peak value of 303.97 ± 4.69 mg QE/g extract, while Run 16 produced 300.45 ± 2.47 mg QE/g extract, which was nearly identical to the model's predicted value of 300.45 mg QE/g extract. This consistency underscores the predictive model's stability and reliability under optimal conditions. Conversely, Run 4 (temperature 60 °C, ethanol concentration 80%, ultrasonic time 20 min) produced the lowest flavonoid content (90.59 ± 2.85 mg QE/g extract), indicating that excessively high temperature and ethanol concentration can significantly reduce the extraction efficiency of flavonoid compounds.

Also, the second-order polynomial regression model was established as follows: $Y_{Flavonoid} = -7154.98239 + 52.63791 \times A + 164.26643 \times B + 50.85094 \times C - 0.102700 \times A \times B + 0.164319 \times A \times C + 0.080986 \times B \times C - 0.489437 \times A^2 - 1.17312 \times B^2 - 1.59624 \times C^2$.

Where Y is the predicted flavonoid content extracted from *Abutilon indicum* leaf powder, A is the extraction temperature (°C), B is the ethanol concentration (%), and C is the ultrasonic extraction time (min).

The analysis of variance (ANOVA) results presented in Table 2 indicate that the regression model developed to predict the flavonoid content in *Abutilon indicum* leaf powder is highly significant, with a confidence level of 99.99% ($p < 0.0001$), confirming that the model is appropriate for explaining the relationship between the experimental factors and flavonoid content.

Table 2: Analysis of correlation coefficients of the factors affecting the flavonoid content from *Abutilon indicum* leaf powder

Source	Flavonoid ANOVA analysis				
	Sum of Squares	**Df**	**Mean Squares**	**F-value**	**P-value**
Model	91671.72	9	10185.75	374.64	< 0.0001
A– Temperature	34.72	1	34.72	1.28	0.2957
B– Ethanol concentration	9721.29	1	9721.29	357.56	< 0.0001
C– Time	157.05	1	157.05	5.78	0.0472
AB	421.89	1	421.89	15.52	0.0056
AC	270.01	1	270.01	9.93	0.0161
BC	65.59	1	65.59	2.41	0.1643
A^2	10086.24	1	10086.24	370.98	< 0.0001
B^2	57945.91	1	57945.91	2131.32	< 0.0001
C^2	6705.25	1	6705.25	246.63	< 0.0001
Residual	190.31	7	27.19		
Lack of Fit	77.24	3	25.75	0.9108	0.5110
Pure Error	113.07	4	28.27		
Cor Total	91862.04	16	R^2= 0.9979	R^2_{Adj}=0.9953	R^2_{Pre}=0.9846

Among the factors, ethanol concentration (B) and the quadratic terms (A^2, B^2, C^2) exhibited highly significant effects ($p < 0.0001$), indicating that including non-linear components in the model is essential to capture variation in flavonoid content accurately. In addition, ultrasonic extraction time (C) significantly influenced flavonoid content (p = 0.0472, < 0.05). Although extraction temperature (A) did not show a significant individual effect (P = 0.2957), its interactions with ethanol concentration (AB, p = 0.0056) and ultrasonic time (AC, p = 0.0161), as well as its quadratic term (A^2, p < 0.0001), were statistically significant. This finding indicates that temperature cannot be disregarded, as it plays a crucial role when combined with other factors, consistent with previous reports that flavonoid extraction efficiency is often governed by the interplay of conditions rather than single-factor effects.[16] In contrast, the interaction between ethanol concentration and ultrasonic time (BC) was not statistically significant (p = 0.1643).

The model's F-value (374.64) was substantially higher than the residuals', confirming its strong explanatory power for the observed data variability. Notably, the Lack-of-Fit value was not statistically significant (p = 0.5110), indicating no substantial deviation between the predicted and experimental values, thereby reflecting the model's high degree of fit. This is further supported by the coefficient of determination (R^2 = 0.9979), suggesting that 99.79% of the variation in flavonoid content could be explained by the variables included in the model.[17]

Based on this analysis, three-dimensional (3D) response surface plots illustrating the interactions among the factors and their effects on flavonoid content are shown in Figure 3. The optimal conditions for maximising flavonoid-rich extract yield from *Abutilon indicum* leaves are presented in Figure 4. The results indicated that the predicted maximum flavonoid content was 303.128 mg QE/g extract when extraction was performed at 49.98 °C, with 68.53% ethanol, for 20.24 minutes. Experimental validation was conducted at 50 °C, 68.5% ethanol, and 20 minutes of ultrasonic extraction. The flavonoid content obtained was 306.55 ± 4.62 mg QE/g extract, which did not differ significantly from the predicted value (p > 0.005), confirming the high accuracy and reliability of the predictive model.

Furthermore, the anti-inflammatory activity of the extracts was evaluated by their ability to scavenge free radicals, specifically nitric oxide ($NO^{\bullet}$) radicals.

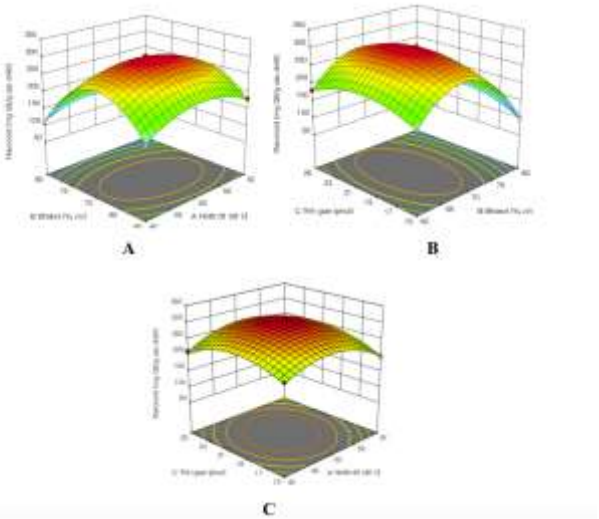

Figure 3: Response surface plots of the flavonoid content in *Abutilon indicum* leaf powder extract. Note: A – Interaction model between temperature and ethanol concentration; B – Interaction model between ethanol concentration and extraction time; C – Interaction model between temperature and extraction time.

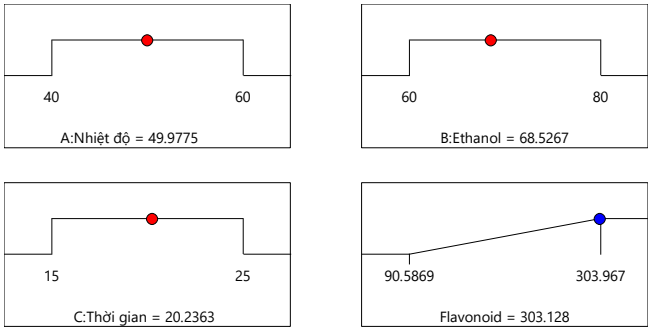

Figure 4: Predicted response and optimal extraction conditions for maximising flavonoid content in *Abutilon indicum* leaf extract.

As shown in Table 3, the *in vitro* anti-inflammatory activity of the flavonoid-rich extract obtained under optimal extraction conditions (50 °C, 68.5% ethanol, 20-minutes ultrasonic extraction) was promising. Under LPS-stimulated conditions (inflammation-mimicking environment), both extracts showed strong cytoprotective effects, with the optimised extract achieving 83.06 ± 1.66% protection—higher than that of the non-optimised extract (78.71 ± 2.40%). However, when the extract concentration increased from 50 to 200 µg/mL, cell viability gradually decreased in both samples, indicating a dose-dependent effect, possibly related to cytotoxicity at higher concentrations. The anti-inflammatory potential was indirectly assessed by inhibiting NO production and protecting against inflammation-induced cellular damage. Higher cell viability indicated stronger anti-inflammatory efficacy of the extract.

Table 3: Inhibitory activity against NO• radicals of different extracts.

Sample concentration (µg/mL)	**Cell viability (%)**	
	Crude extract	**Optimal extract**
0	17.91±0.22	19.07±1.45
LPS	78.71±2.40	83.06±1.66
L-NAME	48.28±1.90	45.74±2.17
50	71.25±3.33	54.92±2.29
100	61.39±2.17	45.60±2.02
200	51.75±1.20	40.88±1.63

Table 4: IC_{50} values of *Abutilon indicum* leaf extracts against various cancer cell lines.

Cell lines	**IC_{50} value (µg/mL)**		
	Crude extract	Optimal extract	Ellipticine
SK-LU-1 lung cancer cells	177.44±1.65	148.29±4.55	0.67 ± 0.03
HepG2 liver cancer cells	201.04±8.39	164.39±6.94	0.74 ± 0.04
MKN7 gastric cancer cells	273.08±12.65	164.76±7.73	0.72 ± 0.02

The cytotoxic activity screening of *Abutilon indicum* leaf extracts against selected cancer cell lines, determined by the ability to inhibit 50% of the biological activity or cancer cell proliferation (IC_{50}), is presented in Table 4. The optimised extract exhibited a lower IC_{50} against SK-LU-1 lung cancer cells (148.29 ± 4.55 µg/mL) compared to the non-optimised extract (177.44 ± 1.65 µg/mL), indicating superior cytotoxic potential. However, its potency remained considerably lower than that of ellipticine (IC_{50} = 0.67 ± 0.03 µg/mL). Similarly, the optimised extract showed enhanced cytotoxicity against HepG2 liver cancer cells (IC_{50} = 164.39 ± 6.94 µg/mL) compared with the non-optimised extract (IC_{50} = 201.04 ± 8.39 µg/mL). Although both extracts demonstrated much weaker activity than ellipticine (IC_{50} = 0.74 ± 0.04 µg/mL), these findings highlight the improvement in biological activity achieved through the optimised extraction process. For MKN7 gastric cancer cells, the optimised extract yielded a markedly lower IC_{50} (164.76 ± 7.73 µg/mL) than the non-optimised extract (273.08 ± 12.65 µg/mL), confirming the superiority of the optimised protocol. Despite the substantially lower potency compared to ellipticine (IC_{50} = 0.72 ± 0.02 µg/mL), the results still support the potential of *A. indicum* leaf extracts for anticancer research.

Conclusion

This study successfully established an optimised extraction protocol for obtaining a flavonoid-rich extract from *Abutilon indicum* leaves using reflux extraction, Box–Behnken design, and Minitab software for process optimisation. Four extraction parameters were investigated: ethanol concentration, extraction temperature, solvent-to-material ratio, and extraction time. The optimal conditions were determined as ethanol concentration of 68.5%, extraction temperature of 50 °C, and extraction time of 20 minutes. Under these conditions, the total flavonoid content reached 306.55 ± 4.62 mg QE/g extract, which was significantly higher than under non-optimised conditions. The optimised extract exhibited notable anti-inflammatory activity by scavenging NO• radicals and demonstrated cytotoxicity against SK-LU-1 lung cancer cells, HepG2 liver cancer cells and MKN7 gastric cancer cells, suggesting potential applications in inflammation and cancer management. These findings indicate that the flavonoid-rich extract from *A. indicum* leaves is a promising candidate for the development of phytopharmaceutical products.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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