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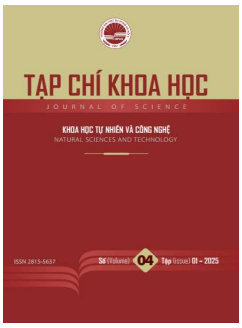

Article type: Research article

Optimization of extraction of flavonoid and polyphenol from *Camellia megasepala* leaves and their anticancer activity

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Abstract

Camellia megasepala Hung T. Chang & Trin Ninh, found in Na Hang district, Tuyen Quang province, contains a significant amount of flavonoids and polyphenols, which are important phenolic compounds known for their various biological activities beneficial to human health, including antibacterial and anti-inflammatory effects. This study applied the response surface methodology (RSM) to examine the influence of extraction factors on the polyphenol and flavonoid content in golden tea leaves. The optimal conditions for extracting polyphenols and flavonoids were temperature at 60°C, 70% ethanol concentration, extraction time of 80 minutes, and a material/solvent ratio of 1/20 (w/v). The experimental values were: flavonoid = 1.42 ± 0.0172 mgQE/g extract; polyphenol = 4.29 ± 0.0348 g/100g extract. The extract from golden tea leaves was evaluated for its cytotoxic activity against HepG2 liver cancer cells and A549 lung cancer cells. The inhibitory concentration (IC50) for the ethanol 70% extract was recorded as IC50 = 74.11 ± 1.37 µg/ml and IC50 = 67.11 ± 1.57 µg/ml for HepG2 and A549 cells, respectively. The results of this study suggest the potential application of golden tea leaf extract in inhibiting the growth of liver and lung cancer cells.

Keywords: Optimization, flavonoid, polyphenol, cancer, *Camellia*

1. Introduction

Yellow Camellia tea, derived from several species of the *Camellia* genus, particularly *C. tienii* and *C. sinensis*, is gaining attention for its unique properties and potential health benefits. This tea is characterized by its yellow flowers and leaves, which are rich in polyphenols and other beneficial compounds. *Camellia tienii* has been shown to contain over 158 polar metabolites, including flavonoids and catechins, which contribute to its medicinal properties [1]. Anti-Obesity Effects: Yellow leaf green tea (YLGT) derived from *C. sinensis* has demonstrated significant anti-obesity effects in studies,

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modulating metabolic pathways and gut microbiota in mice [2]. The Central Highlands of Vietnam are home to numerous yellow camellia species, with a high survival rate for cultivation, suggesting strong potential for commercial tea production [3]. The growing interest in yellow tea products, such as large-leaf yellow tea, highlights the economic viability of these plants within the food industry [4].

Optimization of extraction processes to obtain bioactive-rich compounds from plants is a topic of interest in the research and production of herbal products. Response Surface Methodology (RSM), coupled with data processing software, has become a valuable tool among modern experimental design methods. It enables researchers to conduct multi-factor optimization studies, thereby saving both time and costs [5]. The Box-Behnken design is a powerful statistical tool widely utilized in optimizing extraction processes from plant materials, enhancing yield and efficiency. This method allows researchers to systematically evaluate the effects of multiple variables on extraction outcomes, leading to improved extraction conditions for various bioactive compounds [6]–[9].

Numerous studies worldwide focused on optimizing the extraction processes and production of products from yellow camellia tea [10], [11]. However, in Vietnam, no research has yet applied Response Surface Methodology to optimize the extraction of polyphenols and flavonoids from yellow camellia tea leaves. Consequently, this study was conducted to provide foundational knowledge for applying yellow camellia tea leaves as a resource in healthcare.

2. Experimental section

2.1. Materials

Yellow camellia tea leaves were collected from Na Hang District, Tuyen Quang Province. The alcohol chemicals included ethanol (Vietnam), were provided by the Institute of Scientific and Applied Research, Hanoi Pedagogical University 2.

2.2. Development of an optimized extraction protocol flavonoid and polyphenol from Camellia megasepala leaves

The research process was carried out sequentially through the following steps: (1) Conducting single-variable analysis of factors affecting the extraction process to select the survey limits and central points of these factors (independent variables). (2) Designing and implementing an optimization experimental model. (3) Analyzing experimental data. (4) Extracting yellow camellia tea leaves under optimal conditions and comparing the results of the model with the actual experiment. (5) Determining the polyphenol and flavonoid content in yellow camellia tea leaf extract under optimal extraction conditions [5], [12].

Flavonoid determined using the color method $AlCl_3$ by Sultana et al. (2007) with correction [13].

Polyphenol determined according to the method of Singleton et al. (1999) with correction [14].

Analysis of individual factors affecting the extraction process

Sequentially investigating the influence of ethanol concentration, extraction temperature (°C), and extraction time (minutes) on the polyphenol and flavonoid content in the extract of yellow camellia tea leaves. At each experimental step, the value of the factor under investigation varies while the other parameters are kept constant; polyphenol and flavonoid content are then determined. This helps in selecting the appropriate factor to use in subsequent experiments. The ethanol concentrations tested are 50%, 60%, 70%, 80%, and 90%. The temperature levels examined are 40°C, 50°C, 60°C, 70°C, and 80°C. The extraction times studied are 20, 40, 60, 80, and 100 minutes.

2.3. Methodology for in vitro cell culture

HepG2 liver cancer cells and A549 lung cancer cells were used and cultured in DMEM or RPMI media, respectively, supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic solution (penicillin 100 units/mL, 100 µg/mL streptomycin sulfate). The cells were cultured in an incubator at 37°C, 5% CO_2, and the culture medium was changed regularly every 2–5 days depending on cell growth.

2.4. Cytotoxicity evaluation using the mtt assay

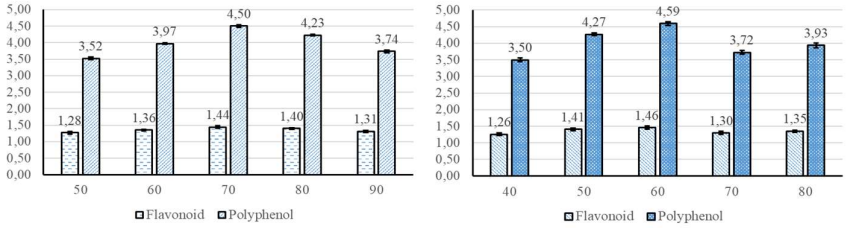
The MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was used to test in vitro cytotoxic activity on HepG2 liver cancer cells and A549 lung cancer cells. HepG2 and A549 cells were seeded in 96-well plates at 10,000 cells/well density. The cells were incubated overnight to stabilize the plates. Then, the compound under investigation was added in triplicate at different concentrations, from low to high. Dimethyl sulfoxide (DMSO) was used as a negative control under the same conditions. The samples were incubated for 24 hours. Afterward, 10% of the well volume of MTT ((4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) at a concentration of 5 mg/mL (Sigma-Aldrich) was added to the wells, and they were incubated for 4 hours at 37°C. The media was carefully removed, and the cells were washed twice with phosphate buffer. Cell viability was measured using the MTT assay. Formazan crystals were dissolved in DMSO, and absorbance was measured at 562 nm. Data were analyzed and compared to the control group. Cytotoxicity was determined using the formula: Cytotoxicity percentage (%) = (control - sample) x 100 / control. Cells surviving after treatment were observed under a microscope at 40x magnification [15].

Minitab statistical software and all experiments were repeated 3 times with 95% confidence.

3. Results and discussion

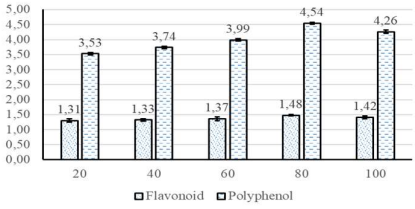
Results of univariate survey of factors affecting the extraction process

The results of a univariate survey of factors affecting the extraction process are shown in Figure 1.



a. Ethanol concentration (%)

b. Extraction temperature (ºC)



c. Extraction time (minutes)

Figure 1. Effects of ethanol concentration, extraction temperature, and extraction time on polyphenols and flavonoid content

In the solvent concentration survey, increasing the ethanol concentration from 50% to 90% resulted in a significant increase in both polyphenol and flavonoid content. At a concentration of 70%, the content of polyphenols and flavonoids reached the highest level, and the difference was statistically significant. At concentrations of 80% and 90%, the content of polyphenols and flavonoids decreased. Based on these findings, the ethanol concentration ranged from 60-80%, and the central point of 70% were selected for investigation.

An appropriate increase in temperature can increase extraction efficiency by reducing viscosity, increasing solvent penetration into cells, and increasing the solubility and diffusion coefficient of extracted compounds [16]. However, compounds belonging to polyphenols and flavonoids are also easily oxidized or decomposed at inappropriate extraction temperatures [16]. In the temperature survey, when the temperature increased from 40-80 ^{o}C, the content of polyphenols and flavonoids gradually increased, reaching the highest at 60 ^{o}C and then gradually decreasing as the temperature increased. These differences were all statistically significant for the samples, remaining concentration. From this result, it can be seen that high-temperaturetemperatures will reduce TPC and TFC [17], [18]. Therefore, we chose the survey range of 50-70 ^{o}C; The temperature of 60 ^{o}C is the center point.

In the survey of extraction times ranging from 20-100 minutes, the content of polyphenols and flavonoids obtained reached the highest at about 80 minutes. When the extraction time increased beyond 100 minutes, the content of polyphenols and flavonoids decreased, and the differences in parameters were statistically significant. Similarly, polyphenols and flavonoids tend to decrease with increasing ultrasound time. It can be seen that compounds belonging to the polyphenols and flavonoids group are released quickly within a short time of ultrasound. In addition, compounds belonging to the group of polyphenols and flavonoids can be decomposed and emulsified when the ultrasound time is prolonged [19], [20]. Therefore, the range from 60-100 minutes and the central point of 80 minutes were chosen as the survey domain.

Results in optimization of the extraction process

Table 2. Experimental Design Based on Box-Behnken and Results for Polyphenol and Flavonoid Content

STT	Time	Temp	Ethanol	Flavonoid	Polyphenol
1	80	60	80	1,46	4,6
2	100	70	80	1,39	4,28
3	80	60	70	1,47	4,61
4	100	70	60	1,4	4,03
5	80	60	70	1,46	4,6
6	80	60	70	1,46	4,6
7	60	50	80	1,34	3,8
8	100	50	60	1,44	4,43
9	80	60	70	1,44	4,56
10	60	50	60	1,31	3,7
11	100	50	80	1,43	4,22
12	80	60	70	1,44	4,57
13	80	60	70	1,46	4,6
14	60	60	70	1,38	3,93
15	60	70	60	1,29	3,6
16	80	70	70	1,46	4,6
17	100	60	70	1,42	4,13
18	60	70	80	1,32	3,71
19	80	50	70	1,47	4,57
20	80	60	60	1,46	4,6

The Box-Behnken experimental design model is implemented on Design Expert 13 software. The model includes 20 experimental units. When conducting experiments on extracting yellow flower tea

leaves under 20 designed experimental conditions, the obtained polyphenols and flavonoid values are shown in Table 2.

The suitability and significance of the model were evaluated through ANOVA (analysis of variance) and correlation indices (Table 3). The significance of the regression coefficients was tested using Fisher's F-test, with p-values < 0.05 indicating that the regression coefficients are statistically significant.

Table 3. Results of ANOVA analysis with flavonoid content

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0,0598	6	0,0100	33,54	< 0.0001	significant
A-Time	0,0194	1	0,0194	65,12	< 0.0001	
B-Tempe	0,0017	1	0,0017	5,68	0,0330	
C-Ethanol	0,0002	1	0,0002	0,5382	0,4762	
AC	0,0008	1	0,0008	2,69	0,1249	
A^2	0,0186	1	0,0186	62,58	< 0.0001	
C^2	0,0008	1	0,0008	2,84	0,1157	
Residual	0,0039	13	0,0003			
Lack of Fit	0,0031	8	0,0004	2,60	0,1541	not significant
Pure Error	0,0007	5	0,0001			
Cor Total	0,0637	19				

R^2 = 0,9393, Adjusted R^2 = 0,9113, Predicted R^2 = 0,8385. Adjusted R^2 helps adjust the model to fit the theory Predicted R^2

The final regression equation representing the relationship between flavonoid content (output parameter) and the variables of the quadratic model in the Box-Behnken response surface methodology for the extraction process is described by the following equation: Flavonoid = +1,46 + 0,0440A - 0,0130B + 0,0040C - 0,0100 AC - 0,0763A^2 - 0,0162 C^2.

Table 4. Results of ANOVA analysis with Polyphenol content

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	2,54	9	0,2827	232,93	< 0.0001	significant
A-Time	0,0200	1	0,0200	16,48	0,0023	
B-Tempe	0,0004	1	0,0004	0,3708	0,5562	
C-Ethanol	0,0063	1	0,0063	5,15	0,0466	
AC	0,0036	1	0,0036	2,98	0,1152	
BC	0,0276	1	0,0276	22,75	0,0008	
A^2	1,85	1	1,85	1522,82	< 0.0001	
ABC	0,0253	1	0,0253	20,85	0,0010	
A^2B	0,0106	1	0,0106	8,70	0,0145	
AB^2	0,0456	1	0,0456	37,54	0,0001	
Residual	0,0121	10	0,0012			
Lack of Fit	0,0101	5	0,0020	5,07	0,0497	significant
Pure Error	0,0020	5	0,0004			
Cor Total	2,56	19				

R^2= 0,9953, Adjusted R^2 = 0,9910, Predicted R^2 = 0,9332.

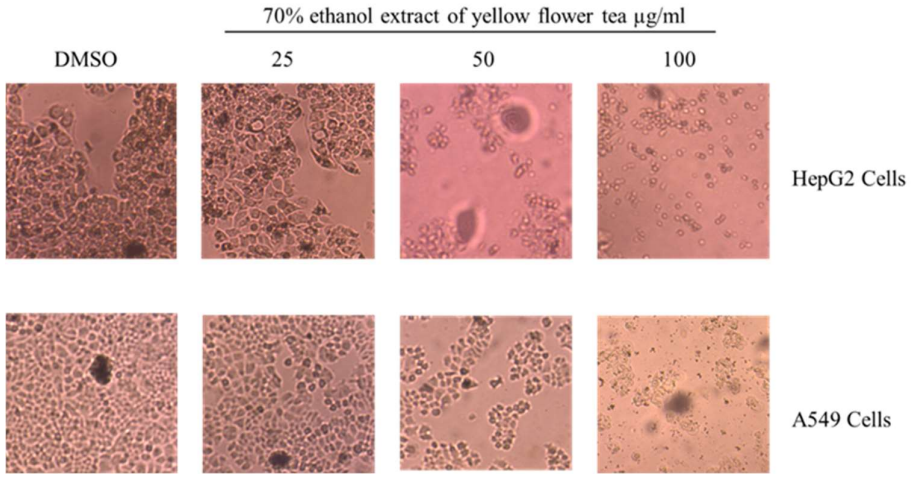
The final regression equation represents the relationship between the Polyphenol content (output parameter) and the variables of the second-order Box-Behnken response surface model for the extraction process, as described in the equation. The equation in terms of coded factors can be used to predict the response for given levels of each factor. By default, the high levels of the factors are coded as +1, and

the low levels are coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.

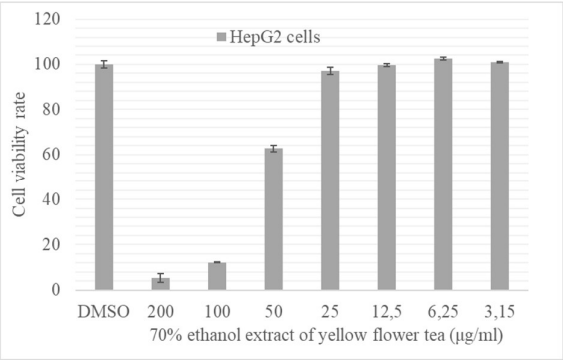
Polyphenol = 4,591+ 0,1A + 0,015B + 0,025 C - 0,02125AC + 0,05875BC - 0,608A^2 + 0,05625ABC -0 ,081249999999999A^2B + 0,16875AB^2

The 70% Ethanol Extract of Yellow Camellia Tea Evaluation for Cytotoxic Activity

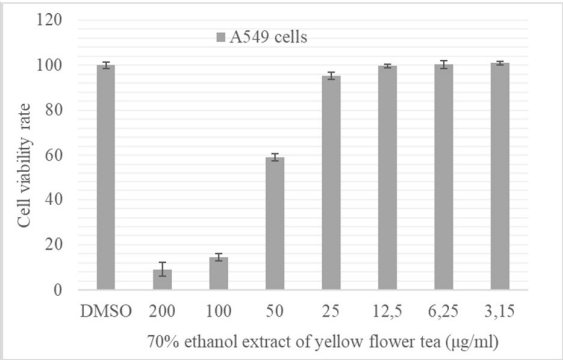
The 70% ethanol extract of yellow camellia tea was evaluated for its cytotoxic activity on HepG2 liver cancer cells and A549 lung cancer cells. The results, presented in Figure 1, show a significant decrease in the density of both HepG2 and A549 cells upon treatment with the 70% ethanol extract. The level of cytotoxicity was also concentration-dependent. The growth inhibition concentration (IC50) of the 70% ethanol extract was recorded as IC50 = 74.11 ± 1.37 µg/mL for HepG2 cells and IC50 = 67.11 ± 1.57 µg/mL for A549 cells. Other studies have reported varying IC50 values for different tea extracts; for instance, a functional tea extract showed an IC50 of 143 µg/mL against Hep3B cells (Chen et al., 2024) [21]. Yellow camellia extracts demonstrated moderate cytotoxicity against HepG2 and A549 cells, with IC50 values ranging from 34.73 to 80.58 µg/mL (An et al., 2023) [22].



a. Cell density and morphological changes of HepG2, A549 cells treated



b. Viability rate of HepG2 cells following treatment



c. Viability rate of A549 cells following treatment

Figure 2. Cytotoxic Activity of the 70% Ethanol Extract of Yellow Camellia Tea on HepG2 Liver Cancer Cells and A549 Lung Cancer Cells.

4. Conclusion

The results of this study mark the first application of the response surface methodology to optimize the polyphenol and flavonoid content in yellow flower tea leaves. The optimal extraction conditions proposed in this study are as follows: 70% ethanol solvent concentration, extraction temperature of 60°C, and extraction time of 80 minutes. Using these conditions, the yellow flower tea leaves extraction yielded polyphenol and flavonoid contents of 4.29 ± 0.348 mg/100g and 1.42 ± 0.0172 mgQE/g, respectively. The extract demonstrated in vitro antioxidant activity with an IC50 value of 42.07 µg/mL. Furthermore, the yellow flower tea extract demonstrated growth inhibitory effects on HepG2 and A549 cancer cells, with IC50 values of 74.11 ± 1.37 µg/mL and 67.11 ± 1.57 µg/mL, respectively.

Acknowledgments

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