AFSSAAE

Advances in Food Science, Sustainable Agriculture and Agroindustrial Engineering 2023, 6(4), 409-423
ISSN 2622-5921

ORIGINAL RESEARCH Open Access

Optimization of tobacco leaves extraction process with microwave assisted extraction (MAE) as an antibacterial agents

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KEYWORDS

Antibacterial activity
Extraction optimization
Inhibitory effects
Microwave-assisted extraction
Tobacco leaves

ABSTRACT

Tobacco (*Nicotiana tabaccum L.)* is an agricultural commodity in Indonesia, with production reaching 261.40 thousand tonnes in 2020. Tobacco leaves contain flavonoids and alkaloids, which are helpful as antibacterial, antifungal, and anti-insect agents. This study aimed to optimize the extraction process of tobacco leaves as an antibacterial agent. The factors used were material-to-solvent ratio (w/v) and extraction time. The microwave-assisted extraction (MAE) was used, with the response surface methodology (RSM) for data processing. This study showed that thematerial-to-solvent ratio and extraction time significantly affected the total flavonoid value, the extract inhibitory activity against *S. mutans* bacteria, and the extract yield. However, no significant effect was observed on the extract's pH. The highest total flavonoid value, inhibitory activity, and pH were obtained from the treatment with a ratio of 1:2172 (w/v) and 6 minutes extraction time, giving an average value of 524.67 mg EQ/g, 22.4 mm, and 5.6, respectively. The highest yield of 4.61% was obtained from the treatment with a ratio of 1:5 (w/v) and 6 minutes extraction time. The optimal solution for the tobacco leaves extraction process was obtained from material-to-solvent ratio of 1: 2.6 (w/v) and 7 minutes of extraction time, giving the total flavonoids, inhibitory activity, pH, and yield of 428.4 mg EQ/g, 20.7 mm, 5.8, and 3.1%, respectively.

Introduction

As one of the oldest Indonesian agricultural commodities, tobacco biomass had a total production of 261.40 thousand tons in 2020. The top producing provinces based on area, namely, East Java, Central Java, and West Nusa Tenggara, were around 136 thousand tons, 55.5 thousand tons, and 52.7 thousand tons (BPS, 2021). According to data from East Java Provincial Plantation Service (2011), Indonesia has been part of the top ten producers of tobacco, with a contribution of approximately 145 thousand tonnes (2.3%) for the world's tobacco needs. Furthermore, based on the data from Directorate General of Plantations (2014), the annual of tobacco exports in Indonesia for 2013 reached up to US$199,589,000. Nurhidayati et al. (2019) stated that around 10 million people's welfare could be supported by the farming and tobacco business, farmers, traders, factory employees, and people involved in transportation and advertising tobacco.

West (2017) stated that tobacco leaves are widely used as a raw material for cigarette production. After being harvested, the tobacco is separated between the leaves and the tobacco stalk. Tobacco leaves are dried for several days under the sun, then finely chopped and dried again until completely dry. In Indonesia, cigarette production is carried out by several large companies, e.g., PT Gudang Garam Tbk, PT Djarum, PT HM Sampoerna Tbk, etc. Cigarette production requires the best quality tobacco that has gone through several sorting processes. However, the tobacco's quality harvested by farmers is varied. Because smoking can cause serious health problems, it is necessary to develop non-smoking products that utilize tobacco leaves (Audrine, 2020).

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Received on 11 Desember 2023, Revised on 27 December 2023, Accepted on 29 December 2023

The chemical compounds contained in tobacco leaves include flavonoids, alkaloids, saponins, and polyphenols (Handayani et al., 2018). Nicotine, the main constituent of tobacco-derived alkaloid compounds, has antibacterial properties. The primary work starts by destructing the constituent components of peptidoglycan in bacterial cells. This causes the bacterial cell wall layer to lysis, followed by bacterial cell decease. The flavonoid compounds are composed of C6-C3-C6, the carbon skeleton in flavonoids consists of two clusters C_6 connected by C_3 aliphatic chains. Flavonoid compounds can act as antifungals by denaturing the proteins, damaging the permeability of bacterial cells, lysosomes, and microsomes (Zou et al., 2021). Putri et al. (2016) observed that the flavonoid compounds in tobacco leaves extract can inhibit the formation of pathogenic fungal spores and the growth of *Candida albicans*. In addition, Handayani et al. (2018) found that the flavonoid compounds in tobacco leaves extract could be used as insecticides on *Sitophilus* larvae, locust bugs, and brown planthoppers.

Extraction is a method of separating two or more components in the material by adding a solvent. The extraction process can be conducted either in hot or cold condition. Hot extraction includes both soxhlation and boiling extraction methods. The cold extraction consists of percolation, maceration, and sonification (Susanty et al., 2019). Putri et al. (2016) investigated the extraction of tobacco leaves with the maceration method. A 250 g of tobacco simplicia was added to 1.875 L of 96% ethanol, then stirred until homogeneous. The maceration process was carried out for 72 hours and stirred every 24 hours. The extract was then filtered using filter paper to separate the dregs and filtrate. Furthermore, evaporation was carried out at 50°C until a thick extract was obtained. Maceration extraction takes longer time. Moreover, extraction with the maceration method produce a yield lower than the MAE method (Oroian et al., 2020). Therefore, a method that can speed up the extraction is needed.

The extraction process can be carried out using microwave-assisted extraction (MAE), pressurized liquid extraction (PLE), ultrasonic-assisted extraction (UAE), and supercritical fluid extraction (SFE) methods (Zhang et al., 2018; Aini et al., 2019). These methods can shorten the extraction time and increase the yield. MAE extraction is an extraction method capable of speeding up time extraction, reducing the amount of solvent, and enhancing yield (Sari et al., 2020). MAE extraction is influenced by the ratio of ingredients, solvent, and extraction time. Zhang et al. (2018) researched extracting pectin from tobacco using two different methods, namely reflux and MAE. In the MAE extraction, the ratio of material: solvent (1:10, 1:15, and 1:20), extraction time factor (4, 5, and 6 minutes), and microwave power factor (470W, 550W, and 630W) were used. The optimization results obtained were in the treatment ratio of 1:20 b/v, 4 minutes, and 550W of power with a yield of 8.88 %. Meanwhile, Firdausiah et al. (2020) researched extracting tobacco using three different methods (i.e., maceration, soxhlet, and MAE) to determine the effect on tobacco extract yield and nicotine content. The result showed MAE extraction produce the highest yield and the lowest nicotine content which compared to the maceration and soxhlation methods. Therefore, this study aimed to optimize the extraction process of tobacco leaves as antibacterial agents.

Research and Methods

Materials

The materials used include tobacco leaves, ethanol 70%, ethanol pa, quercetin, distilled water, $AlCl_3$, sodium acetate, filter cloth, filter paper, NA medium, MHA medium, NB medium, antibiotic amoxicillin, and *S. mutans* bacterial isolation.

Material preparation

The tobacco leaves were obtained from farmers in Trantang Village, Kerek District, Tuban Regency, Indonesia. Tobacco leaves were sun-dried to make the simplicia. The dried tobacco leaves were chopped, re-dried in an oven at 60°C for one hour to reduce the moisture content optimally (Li et al, 2018). Then, the dried samples were ground using a blender and sieved through a 40-mesh sieve. The resulting powder was used for extraction trials.

Experimental set up

The experimental design in this study used the Response Surface Methodology (RSM) method with Central Composite Design (CCD). Two factors were employed, including material-to-solvent ratio (lower limit 1:3 w/v, midpoint 1:5 w/v, and upper limit 1:7 w/v) and extraction time (lower limit 4 minutes, midpoint 6 minutes, and an upper limit of 8 minutes). The response parameters composed of total flavonoids, inhibition, pH, and yield, as shown in Table 1.

Table 1. Trial design

No	Factor		Factors		Responses			
	X1	X2	Material-to-solvent ratio (g/mL)	Extraction time (min)	Total flavonoid (mg EQ /L)	Inhibition power (mm)	pH	Yield (%)
1	-1	-1	1:3	4	Y1	Y2	Y3	Y4
2	+1	-1	1:7	4	Y1	Y2	Y3	Y4
3	-1	+1	1:3	8	Y1	Y2	Y3	Y4
4	+1	+1	1:7	8	Y1	Y2	Y3	Y4
5	-1.414	0	1:2.172	6	Y1	Y2	Y3	Y4
6	+1.414	0	1:7.828	6	Y1	Y2	Y3	Y4
7	0	-1.414	1:5	3.172	Y1	Y2	Y3	Y4
8	0	+1.414	1:5	8.828	Y1	Y2	Y3	Y4
9	0	0	1:5	6	Y1	Y2	Y3	Y4
10	0	0	1:5	6	Y1	Y2	Y3	Y4
11	0	0	1:5	6	Y1	Y2	Y3	Y4
12	0	0	1:5	6	Y1	Y2	Y3	Y4
13	0	0	1:5	6	Y1	Y2	Y3	Y4

Extraction of tobacco leaves

A 100 g of tobacco simplicia powder was weighed, then 70% ethanol was added at a specified ratio (i.e., 1:3, 1:5, and 1:7 w/v), and stirred until homogeneous using a stirrer. The solution was put into the MAE tool and set for different extraction time (i.e., 4, 6, and 8 minutes) at 100W power. After that, the extracted solution was filtered with a filter cloth. The resulting filtrate was evaporated at 50°C, 100 mbar pressure, and 65 rpm for 90 minutes to obtain a concentrated extract (Liu et al., 2021). The evaporation separates the solvent and extract (Firdausiah et al., 2020).

Parameter analysis

a. Total flavonoid test

A standard curve of 100 ppm quercetin was prepared by dissolving 2.5 mg of quercetin in 25 mL of ethanol. Followed by preparing a concentration series of 20, 30, 40, 50, and 60 ppm. Furthermore, 0.5 mL of quercetin standard solution was added with 0.1 mL of $AlCl_3$ 10%, 2.8 mL of distilled water, and 0.1 mL of 1 M sodium acetate. One of the standard solution concentrations was taken, and the absorbance was measured with a UV-Vis spectrophotometer at a wavelength of 437 nm. The next step is to make a linear regression equation based on the absorbance value obtained from each standard solution concentration. This equation was used to calculate the total value of flavonoids from tobacco leaves extract (Haeria et al., 2016).

The total flavonoid test was carried out by taking a sample of 0.05 mL, dissolved in 10 mL of ethanol, and then, incubated for 30 minutes in dark conditions. A 0.5 mL of sample mixture was taken and added with 0.1 mL $AlCl_3$ 10%, 0.1 mL sodium acetate 1 M, and 2.8 mL distilled water. The solutions were then incubated for 10 minutes and the absorbance was measured with a UV-Vis spectrophotometer at a wavelength of 437 nm. The total value of flavonoids was calculated based on the linear regression equation made based on the quercetin standard curve. The total flavonoids expressed as a number of milligrams quercetin equivalent per gram of extract, as shown in the equation below (Manurung et al., 2017):

$$Total\ flavonoid\ \left(\frac{mgQE}{g}\right) = \left(\frac{sample\ adsorbance - b}{a}\right) X\ fp\ X\ \frac{v}{m} \quad ..(1)$$

Where:

c is the flavonoid concentration from the standard curve (mg/mL)

v is the volume of extract (mL)

m is the extract weight (g)

fp is the dilution factor

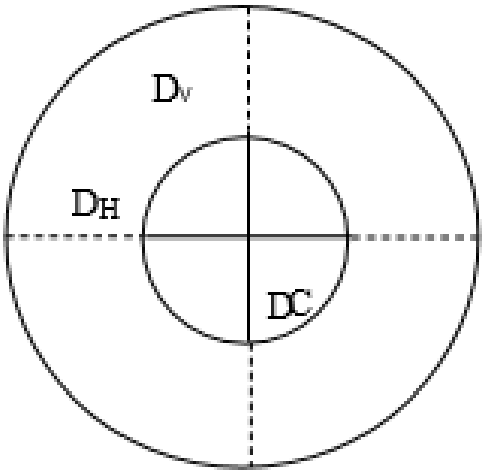

Figure 1. Extract inhibition zone measurement (Bempa et al., 2016)

b. Inhibition test

Nutrient Broth (NB) media was prepared by dissolving 1.3 g of powdered NB media in 100 mL of distilled water. Then, the mixture was placed in a 5-mL reaction tube and sterilized for 15 minutes by autoclaving at 121°C and 1 atm pressure (Oroh et al., 2015).

Media Mueller Hinton Agar (MHA) was prepared by dissolving 19 g of powdered MHA media in 500 mL of distilled water, then sterilized using an autoclave at 121°C with a pressure of 1.5 atm for 15 minutes (Hudaya et al., 2014).

The nutrient agar (NA) (NA) medium was made by dissolving 6 g of NA media powder in 300 mL of distilled water, and heating it until completely dissolved. A 5 mL mixture solution was placed in a test tube and sterilized by autoclave at 121°C and 1 atm pressure for 15 minutes. Then, the sample was cooled and let to solidify at 15 °C. Pure cultures of *S. mutans* were inoculated aseptically in the NA media and incubated for 24 hours at 37°C (Oroh et al., 2015).

The bacterial suspension was prepared by taking one ose of *S. mutans* in NA medium aseptically, placing in 5 mL of nutrient broth (NB) media, and mixing it using a vortex. The bacterial suspension was incubated for 24 hours at 37°C. Then, turbidity values were measured by comparing with the turbidity standard solution of MCfarland 0.5 ($1.5x10^8$ CFU/mL). The accuracy of theMcFarland standard solution can be measured using a spectrophotometer with a wavelength of 625 nm and an absorbance of 0.08-1 (Idroes et al., 2019). *Amoxicillin* (antibiotic) was used as a positive control, as it acts as an antibiotic to treat toothache caused by bacterial growth. The negative control used was distilled water.

A bacterial inhibition test was carried out by culturing *S. mutans* bacteria, which was taken from NB media and then aseptically distributed into sterile Petri dishes containing MHA media. Paper discs with a diameter of 8 mm were prepared as a medium to evaluate the inhibition of the extract against bacteria. Paper discs were inserted and soaked in tobacco leaves extract, *Amoxicillin* antibiotic solution (as a positive control), and distilled water (as a negative control). Soaked disc paper, *tetracycline* stock solution, and distilled water were placed aseptically on the surface of the NA medium surface NA, which previously inoculated with *S. mutans* (Haerussana et al., 2022). The prepared samples were then incubated at 37˚ for 24 hours. The clear zone formed around the disc paper was observed, and its diameter was measured. Inhibition zone measurements were carried out, as shown in Figure 1.

The extract inhibition zone for bacteria was calculated using the formula below (Bempa et al., 2016):

$$\text{Inhibition} = \frac{(Dv-Dc)+(Dh-Dc)}{2} \quad \text{..... (2)}$$

Where:
Dv is the vertical diameter (mm)
Dh is the horizontal diameter (mm)
Dc is the disk diameter (mm)

c. pH

The pH of the samples was measured using a standard method by a conventional pH electrode, as described in Sánchez-Clemente et al. (2018). Prior to the test, the pH electrode was cleaned using distilled water and calibrated following the range of pH measurement (i.e., pH 4, 7, or 9). Between each sample measurement, the pH electrode was washed with distilled waste to avoid contamination or error measurement.

Table 2. Optimization results with RSM

No.	Variable code		Factor		Responses			
	X1	X2	material-to-solvent ratio (w/v)	Extraction time (min)	Total flavonoid (mgEQ/g)	Inhibition (mm)	pH	Yield (%)
1	-1	-1	1:3	4	240.21	14.6	6.1	2.64
2	+1	-1	1:7	4	92.16	10.55	5.8	3.01
3	-1	+1	1:3	8	361.75	18.8	6	3.45
4	+1	+1	1:7	8	217.16	14.4	5.9	2.13
5	-1.414	0	1:2.172	6	524.67	22.4	5.6	2.17
6	+1.414	0	1:7.828	6	310.08	17.05	6.6	2.56
7	0	-1.414	1:5	3.172	62.75	10.2	5.8	4.13
8	0	+1.414	1:5	8.828	105.00	10.8	6.1	3.55
9	0	0	1:5	6	157.88	12.4	5.9	4.52
10	0	0	1:5	6	185.93	13.2	5.8	4.53
11	0	0	1:5	6	162.15	12.55	5.8	4.59
12	0	0	1:5	6	125.63	12.8	5.8	4.61
13	0	0	1:5	6	176.44	12.6	5.9	4.33

d. Yield

The extraction yield was calculated using the following formula (Ngamkhae et al., 2022):

$$Initial\ mass = mass\ of\ tobacco\ leaves\ powder + mass\ of\ ethanol\ 70\% \quad ..(3)$$

$$Final\ extract\ mass = extract\ volume\ X\ extract\ density \quad ..(4)$$

$$Yield\ (\%) = \frac{Initial\ mass}{Final\ extract\ mass}\ X\ 100\% \quad ..(5)$$

Data analysis

Data processing was conducted using design expert software (i.e., Stat-Ease Design-Expert v12.0.3.0). A two-way analysis of variance (ANOVA) and response surface methodology was carried out to evaluate effect of independent variables factor on the parameters responses and the model obtained ($P \leq 0.05$). The statistical software used was OpenStat 30.0.

Results and Discussion

Optimization results

The total flavonoid value of tobacco leaves extract ranged from 62.75 – 524.67 mgEQ/g (Table 2). The highest total flavonoid value was obtained from the ratio of 1:2.172 w/v treatment and 6 minutes extraction time, with a of 524.67 mg EQ/g. The lowest total flavonoid value was obtained from the ratio of 1:5 w/v treatment and 3.172 minutes extraction time, with a value of 62.84 mg EQ/g. Table 2 shows that both factors affect the total flavonoids value. Increasing the material-to-solvent ratio was parallel to decreasing the total flavonoid value of. In contrast, the results indicate that the prolonged extraction time increased the total flavonoid value of tobacco leaves extract, which aligns with the findings reported by Koesnadi et al. (2021).

The highest inhibition value was obtained from the ratio of 1:2.172 w/v treatment and 6 minutes extraction time, which can inhibit the growth *S. mutants* bacteria of 22.4 mm. The lowest inhibition power of 10.2 mm was obtained from treatment with a ratio of 1:5 (w/v) and 3.172 minutes extraction time. The higher inhibition values indicate that the extract is better at inhibiting the growth of *Streptococcus mutans* bacteria. The total flavonoids can release transducing energy from the membrane cytoplasm of bacteria and inhibit bacterial motility (Manik et al., 2014). The hydroxyl groups in the structure of flavonoids can change organic components and transport nutrients that cause toxic effects on bacterial growth.

The pH of the extract ranged from 5.6 to 6.6 (Table 2), which indicates that the material-to-solvent ratio and extraction time did not significantly affect the pH value. No significant pH changes were observed in all 13 treatments.

Hidayanto et al. (2017) explained that the pH standard for a mouthwash product ranged from 5 to 7. Therefore, the resulting tobacco leaves extract could potentially be used as a raw material for making herbal mouthwash.

The highest extract yield resulted from the treatment with a ratio of 1:5 (w/v) and an extraction time of 6 minutes, with a value of 4.61%. Meanwhile, the lowest yield was produced at a ratio of 1:7 (w/v) and 8 minutes extraction time, with a value of 2.13%. The results demonstrate that increasing the material-to-solvent ratio up to 1:5 (w/v) or prolonging the extraction time to 6 minutes increased the yield. However, when a much longer extraction time (i.e., 8 minutes) was applied, the yield tends to decrease due to potential evaporation occurrence. A previous study reported that a high volume of solvent used during extraction is parallel to an increase in the yield (Yudharini et al., 2016). Similarly, the longer the extraction time, the more extended contact between the solvent and the material occurs, causing a yield increase (Handayani et al., 2018). Furthermore, Wahyuni and Widjanarko (2015) explained that the length of extraction time would allow the solvent to penetrate the cell walls of the material and pull out the compounds present in the raw material, hence increasing the resulting yield.

ANOVA results and model analysis

a. Total flavonoid

The results of ANOVA are presented in Table 3, indicating that the selected model was quadratic with a *p-value* of <0.0001. Since the p-value was much lower than 5%, the quadratic model was a better fit to explained the effect on the total flavonoids, as previously described by Kurniawan et al. (2018). The lack of fit test value was 0.1336 or higher than 0.05, indicating no data deviation in the model.

The R^2 value for the total flavonoid response was 0.9610, indicating that the material-to-solvent ratio and extraction time influenced the total flavonoid value of the extract by 96.10%. As the R^2 value was close to 1 (or 100%), both independent variables significantly affect the total flavonoids, as explained by Ratnaningsih et al. (2018). Furthermore, the adjusted R^2 value from the model was 0.9332 and the predicted R^2 value was 0.7838, giving the difference of 0.1494. This result was interpreted reasonable since the difference value was lower than 0.2. While, the value of adequate precision of the selected model was 22.117, which categorized as very good because of higher than 4 (Prabudi et al., 2018).

The optimal condition for total flavonoid response is depicted in polynomial equation order 1 (Y1) and 2 (Y2), as follows:

$$Y1 = 161.61 - 74.51\ A + 38.29\ B + 0.8650\ AB + 112.18\ A^2 - 44.57\ B^2 \quad (6)$$

$$Y1 = 602.06539 - 344.01288\ X1 + 151.76216\ X2 + 0.216250\ X1X2 + 30.5481\ X1^2 - 11.14169\ X2^2 \quad (7)$$

Table 3. ANOVA results on total flavonoid

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.859E+05	5	37181.53	34.54	< 0.0001	Significant
A-Material-to-solvent ratio	44419.30	1	44419.30	41.26	0.0004	
B-Extraction time	11726.74	1	11726.74	10.89	0.0131	
AB	2.99	1	2.99	0.0028	< 0.0001	
A^2	1.039E+05	1	1.039E+05	96.46	0.0089	
B^2	13817.01	1	13817.01	12.83		
Residual	7536.26	7	1076.61		0.1336	Not Significant
Lack of Fit	5416.10	3	1805.37	3.41		
Pure Error	2120.16	4	530.04			
Cor Total	1.934E+05	12				
Std. Dev	32.81		**R^2**		0.9610	
Mean	209.37		**Adjusted R^2**		0.9332	
C.V. %	15.67		**Predicted R^2**		0.7838	
			Adeq Precision		22.1175	

Based on the equation above, it is known that the regression coefficient at the central point was 928.02097. The values of X_1 and X_2 were linear coefficients, while the value of X_1X_2 was the interaction coefficient of the two factors. The values of X_1^2 and X_2^2 are quadratic coefficients. The resulting equation also showed positive and negative coefficients. A positive coefficient means that the response value increases if the value is increased, and vice versa for a negative coefficient. In the polynomial equation of order 2, it can be seen that increasing the material-to-solvent ratio may decrease the total flavonoid value. However, the total flavonoid value increases if the extraction time increases.

The normal plot graph describes the normal distribution of the experimental data (or actual value). The actual value is depicted with a box symbol and the normal line is depicted with a red linear line. Figure 2a shows that the data is normally distributed and can proceed to the next test (Breig and Luti, 2021).

Contour plot graphs are widely used to evaluate the influence of the combination of independent variables and the responses displayed in different colors. On the contour graph, lines connect points with the same response value (Breig and Luti, 2021). Figure 2b shows a similar trend that the combination of material-to-solvent ratio and extraction time was mutually influence the response of total flavonoids. The different colors was observed in the graph, indicates that the total flavonoid content obtained differed. The lowest total flavonoid value of 62.75 mgEQ/g was shown in dark blue, while the highest value of 524.667 mgEQ/g was in red .

The 3D surface graph was used to show the ideal conditions of each response for each parameter that affects the response (Prasetiyo et al., 2021). The results indicate that the optimal conditions for producing the highest total flavonoid response were at a ratio of 1:2.172 (w/v) and 6 minutes extraction time (marked in red), as shown in Figure 2c. When the ratio was increased to 1:5 (w/v), a decrease in total flavonoid value was observed. However, an improvement tendency was projected if the ratio was increased above 1:5 (w/v). Figure 2c also indicated that prolonged extraction time increased the total flavonoid values. However, a reduction in total flavonoid values were evident when applying more than 6 minutes of extraction time. This study's results align with Koesnadi et al. (2021) that longer extraction time may cause more flavonoid compounds to be released due to the intensive contact occurred on the active compounds.

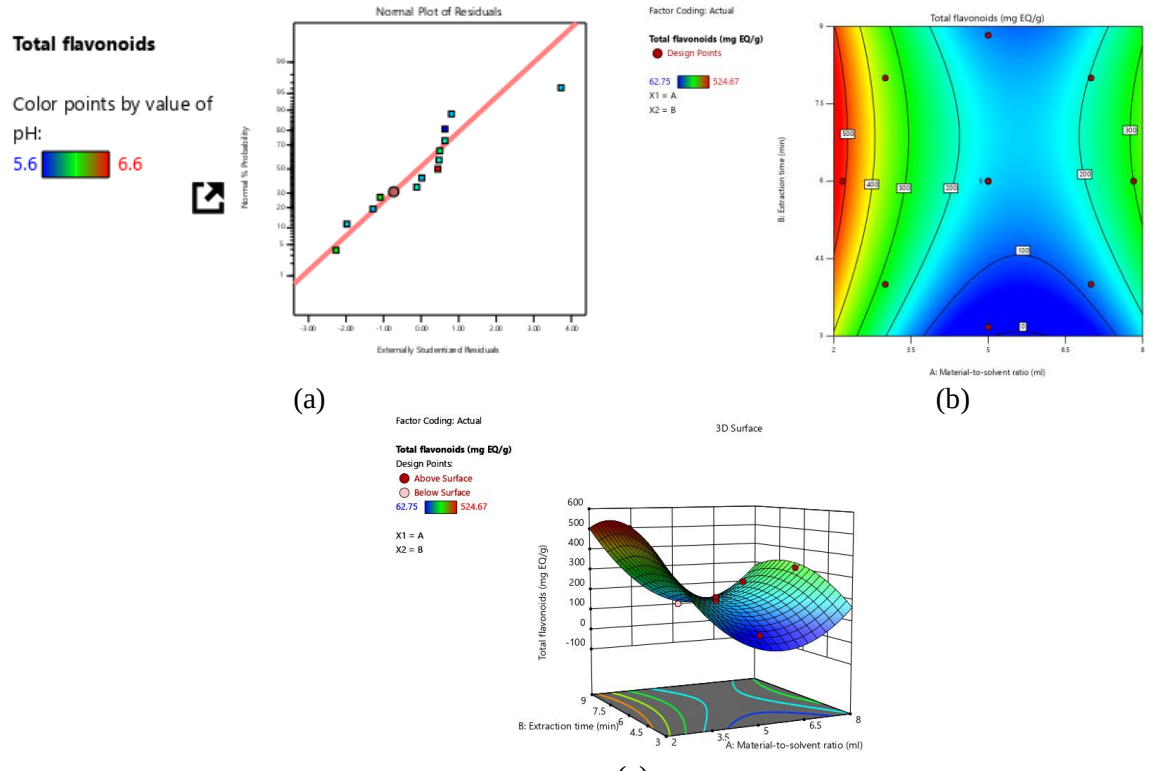

Figure 2. Response surface of total flavonoid: (a) Normal plot, (b) Contour plot, and (c) 3D surface

Table 4. ANOVA results on inhibitory activity

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	141.18	5	28.24	26.31	0.0002	Significant
A-Material-to-solvent ratio	32.06	1	32.06	29.87	0.0009	
B-Extraction time	9.90	1	9.90	9.22	0.0189	
AB	0.0306	1	0.0306	0.0285	0.8706	
A^2	79.30	1	79.30	73.88	< 0.0001	
B^2	10.63	1	10.63	9.91	0.0162	
Residual	7.51	7	1.07			
Lack of Fit	7.13	3	2.38	24.89	0.0048	Significant
Pure Error	0.3820	4	0.0955			
Cor Total	148.70	12				
Std. Dev	1.04		**R^2**		0.9495	
Mean	14.03		**Adjusted R^2**		0.9134	
C.V. %	7.39		**Predicted R^2**		0.6549	
			Adeq Precision		19.3640	

b. Inhibitory activity

Similarly, the quadratic model was selected to better explain the effect of both factors on the inhibitory activity of tobacco leaves extract, with a p-value of 0.0002 (0.02%), as shown in Table 4. The results also show that R^2 value was 0.9495, indicating significant effects on inhibitory activity, as previously explained by Sylvia et al. (2017).

The polynomial equations order 1 and order 2 can depicted to calculate the optimal results, as follows:

$$Y2 = 12.71 - 2A + 1.11\ B - 0.0875\ AB + 3.38\ A^2 - 1.24\ B^2 \quad (8)$$

$$Y2 = 23.69713 - 9.31038\ X1 + 4.37428\ X2 - 0.021875\ X1X2 + 0.844063\ X1^2 - 0.309063\ X2^2 \quad (9)$$

Using the equations order 2, the regression coefficient at the central point was 23.69713. The result demonstrates that increasing the material-to-solvent ratio was parallel to a decrease in the inhibitory activity, and vice versa. In contrast, the inhibitory activity increases when increasing the extraction time .

Figure 3a shows several colors on the normal contour plot, ranging from dark blue, light blue, green, yellow, orange, and red. The dark blue color represents the lowest inhibition response, while red represents the highest response. Figure 3b shows the contour plot of the relationship between the material-to-solvent ratio and extraction time on the inhibitory activity, which formed a half-ellipse shape expanding towards the extraction time. This was also confirmed by a red color on the Y axis (i.e., extraction time), denoting its significant effects on the inhibitory activity. This finding aligns with a previous study by Octaviani et al. (2017), which showed that applying longer extraction time with lower material-to-solvent ratio increased the inhibitory activity of the extract against bacteria (. This could allow longer contact between the material and the solvent, releasing more chemical compounds, such as flavonoid. Flavonoid compounds have antibacterial properties, so a higher flavonoid content corresponds to a higher inhibitory activity of the extract (Zou et al., 2021).

Figure 3c shows that the optimum condition to produce the highest inhibitory activity was treatment with a ratio of 1:2 (w/v) and 6 minutes (depicted in red color). While, the lowest inhibitory activity was observed from treatment with a ratio of 1:5 (w/v). Therefore, the findings confirmed that increasing material-to-solvent ratio reduces the inhibitory activity. . However, at some point, when increasing the ratio from 1:6 to 1:8 (w/v), an increase in the inhibitory activity against *S. mutans* was observed.

c. pH responds

The ANOVA results indicate that the linear model was suitable for describing the effects of all factors on pH. This model demonstrates that no significant effect was found on pH of the extract as a p-value was 0.3010 (or higher than p-value <0.05), as seen in Table 5. In addition, the R^2 value of the model

was 0.2135, much lower than 1. The adjusted R^2 and predicted R^2 values were 0.0562 and -0.6584, respectively. Therefore, it can be identified that the material-to-solvent ratio and extraction time did not significantly affect the extract pH, similar to a previous finding by Ratnaningsih et al. (2018).

The polynomial equations order 1 and order 2 were obtained to calculate the optimal results, as follows:

$$Y3 = 5.93 + 0.1268\ A + 0.0530\ B \quad \text{.....(10)}$$

$$Y3 = 5.45473 + 0.063388\ X1 + 0.026517\ X2 \text{ ..(11)}$$

From equation 11, the coefficient value of the material-to-solvent ratio was 0.063388, while extraction time was 0.026517. This means that increasing the ratio and extraction time contribute to an increase in pH value.

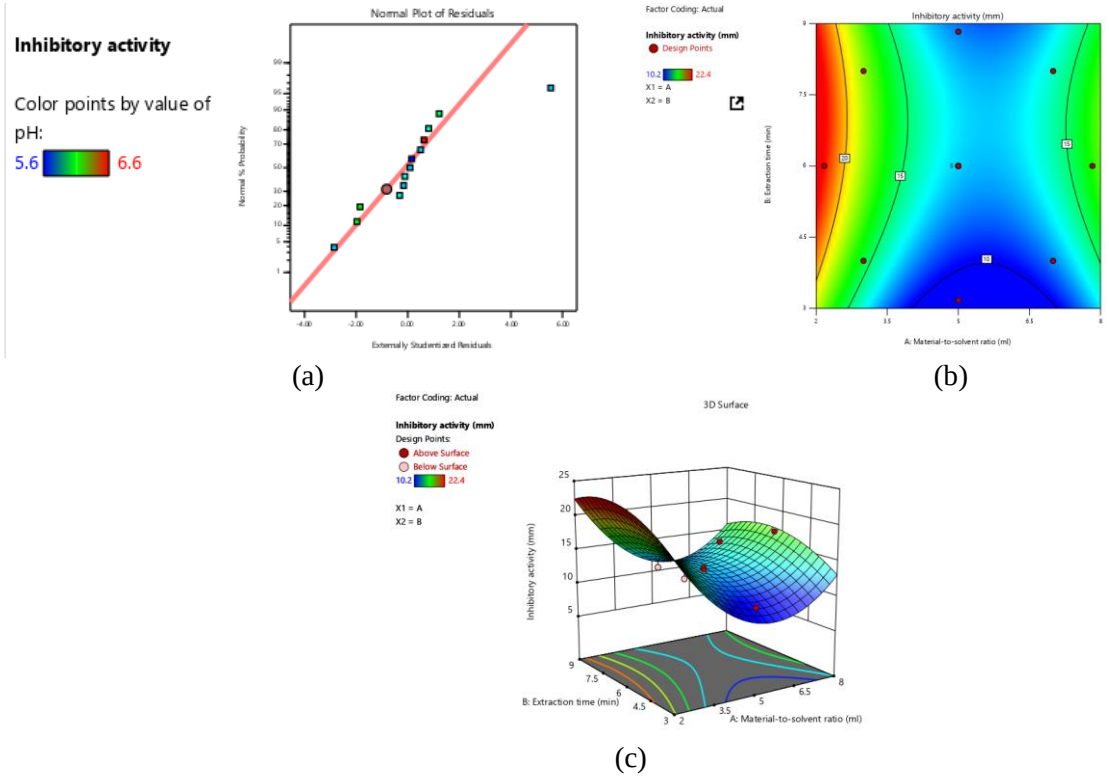

Figure 3. Response surface of inhibition: (a) Normal plot, (b) Contour plot, and (c) 3D surface

Table 5. ANOVA results on pH

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.1511	2	0.0755	1.36	0.3010	Not Significant
A-Material-to-solvent ratio	0.1286	1	0.1286	2.31	0.1595	
B-Extraction time	0.0225	1	0.0225	0.4042	0.5392	
Residual	0.5566	10	0.0557			
Lack of Fit	0.5446	6	0.0908	30.26	0.0027	Significant
Pure Error	0.0120	4	0.0030			
Cor Total	0.7077	12				
Std. Dev	0.2359		**R^2**		0.2135	
Mean	5.93		**Adjusted R^2**		0.0562	
C.V. %	3.98		**Predicted R^2**		-0.6584	
			Adeq Precision		3.1731	

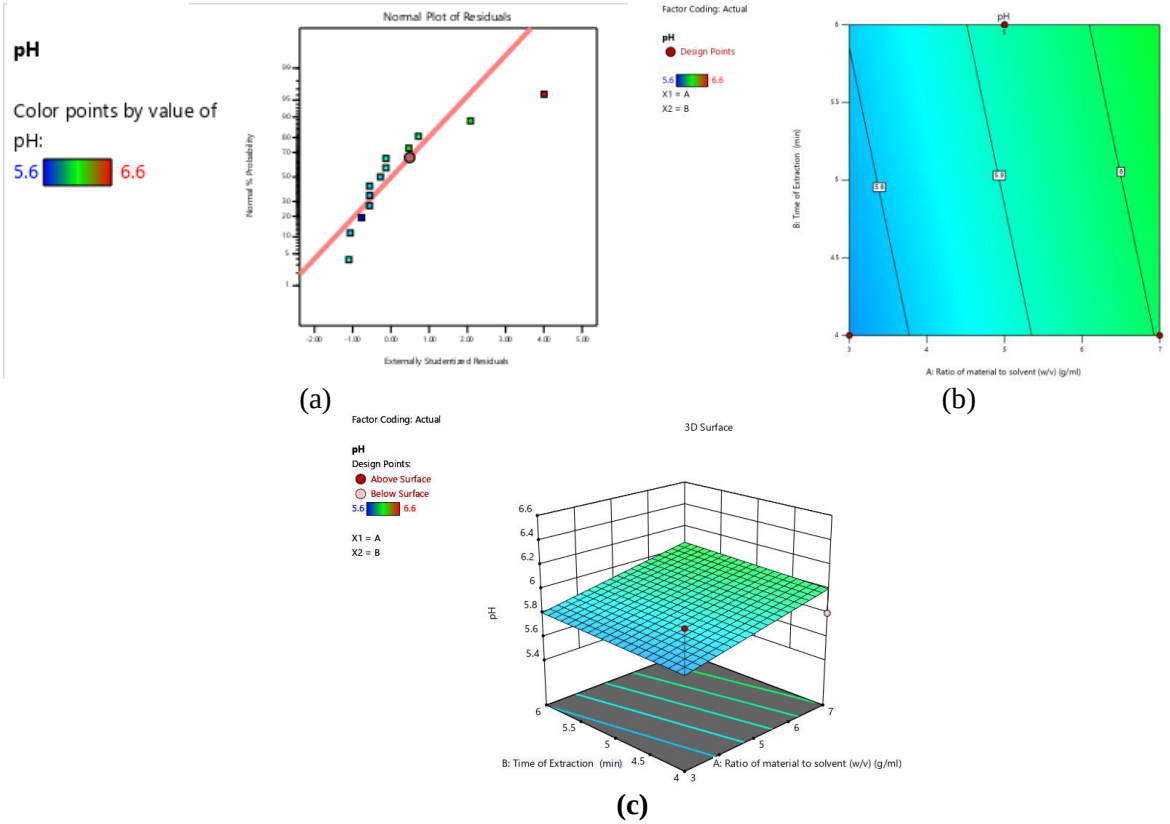

Figure 4. Response surface of pH: (a) Normal plot, (b) Contour plot, and (c) 3D surface

Figure 4a shows that the data spreads linearly (or in normal distribution data). The plot graph illustrates that the combination of the material-to-solvent ratio and extraction time was mutually influence the pH of tobacco leaves extract. Figure 4c indicates color gradations from dark blue to green, showing changes in the pH response. Limited studies have been reported on the influence of the material-to-solvent ratio and extraction time on the extract pH. A previous study found that the pH value of the tobacco leaves extract can be related to the ability of absorption nicotine. Where, an acidic pH may lower the absorption capability of nicotine in the body, and vice versa for an alkaline pH. This is because in acidic conditions, nicotine will be ironized (Alegantina, 2018). Figure 4c also shows that the higher the material-to-solvent ratio and the prolonged the extraction time increases the pH of the extract.

d. Yield

Table 6 shows that the quadratic model was better suited to explain the effect of the material-to-solvent ratio and extraction time on the extract yield response, with a p-value of <0.0002. The p-value < 0.05 indicates that all factors significantly affect the yield response (Hidayat et al., 2020). The R^2 value was 0.9481, meaning both factors affect the extract yield by 94.81%.

Based on the ANOVA results, the first-order and second-order quadratic model equations were obtained as follows:

$$Y4 = 4.52 - 0.0498\ A - 0.1113\ B - 0.4225\ AB - 1.15\ A^2 - 0.4118\ B^2 \quad (12)$$

$$Y4 = -9.08295 + 3.48197\ X1 + 1.70773\ X2 - 0.105625\ X1X2 - 0.287313\ X_1^2 - 0.102938\ X_2^2 \quad (13)$$

Based on equation 13, the coefficient of the material-to-solvent ratio and extraction time were positive. This indicates that increasing both factors increases the extract yield by 3.48197 (for the ratio) and 1.70773 (for extraction).

Similarly, Figure 5a also shows that the experimental data were normally distributed. The highest yield response was found in the treatment with a ratio of 1: 5 (w/v) and 6 minutes of extraction time, as depicted in red color (Figure 5b). Figure 5c shows that the curve of the extract yield response was in a half-parabola shape, with colors of blue, green, yellow, and red. The findings confirmed that increasing the material-to-solvent ratio and extraction time increases the extract yield and vice versa. Increasing the solvent volume leads to a more optimal extraction for releasing the target compound from the material to the solvent.

Table 6. ANOVA results on yield

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	10.51	5	2.10	25.26	0.0002	Significant
A-Material-to-solvent ratio	0.0198	1	0.0198	0.2415	0.6382	
B-Extraction time	0.0991	1	0.0991	1.21	0.3086	
AB	0.7140	1	0.7140	8.69	0.0215	
A^2	9.19	1	9.19	111.78	< 0.0001	
B^2	1.18	1	1.18	14.35	0.0068	
Residual	0.5754	7	0.0822			
Lack of Fit	0.5262	3	0.1754	14.28	0.0133	Significant
Pure Error	0.0491	4	0.0123			
Cor Total	11.08	12				
Std. Dev	0.2867		**R^2**		0.9481	
Mean	3.56		**Adjusted R^2**		0.9110	
C.V. %	8.06		**Predicted R^2**		0.6554	
			Adeq Precision		12.1627	

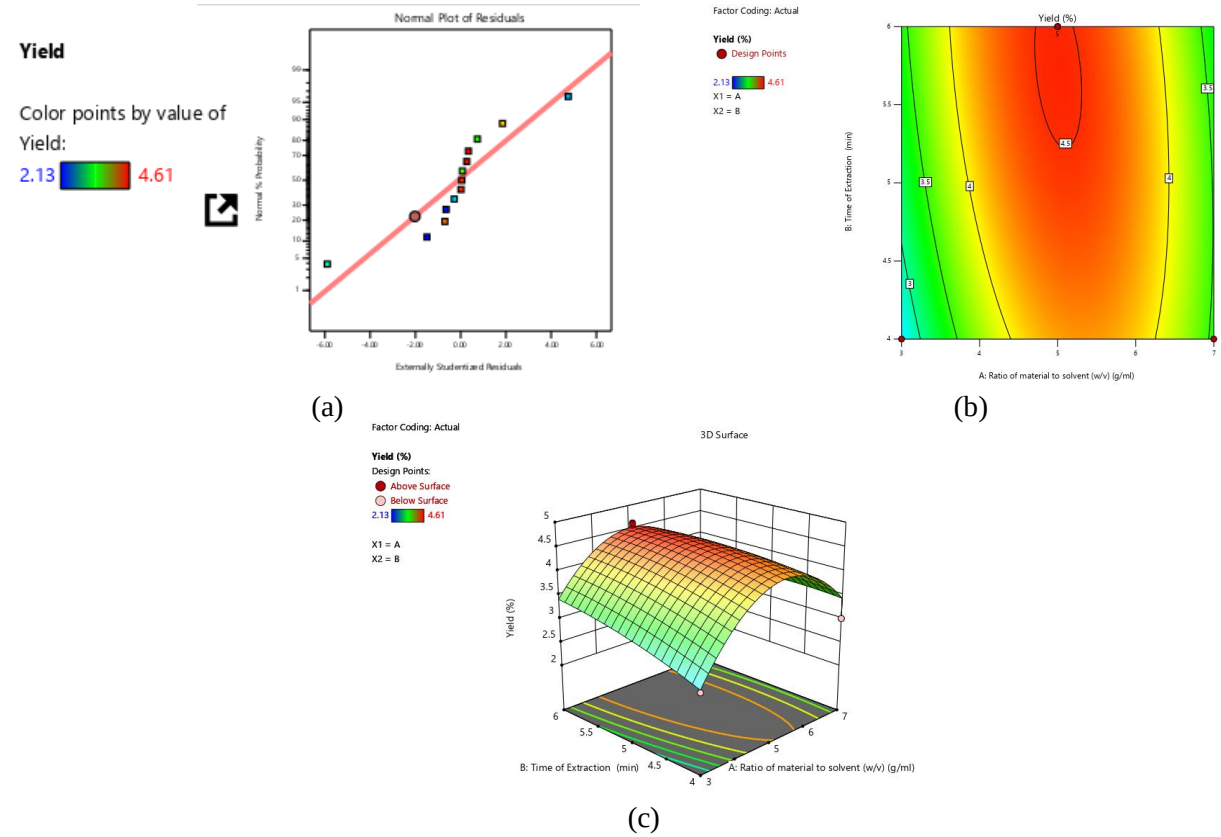

Figure 5. Response surface of yield: (a) Normal plot, (b) Contour plot, and (c) 3D surface

However, at a certain ratio and extraction time, the extract yield starts to decrease and experience saturation. This could potentially due to a prolonged extraction process may cause active compound deterioration or evaporation (Rifai et al., 2018). The results show that the optimal condition to produce the highest yield was at the material-to-solvent ratio of 1:5 (w/v) and extraction time of 6 minutes. But, after passing this optimal point, the extract yield decreases due to various factors, as previously explained..

Optimization solution, validation, and verification

The optimal solutions from the extraction of tobacco leaves are shown in Table 7. This was predicted using the material-to-solvent ratio ranged from 1:2 to 1:7.8 and extraction time from 3 to 9 minted.

Table 7. Results of optimization solution in RSM

Number	Material-to-solvent ratio	Extraction time	Total flavonoid	Inhibitory activity	pH	Yield	Desirability
1	2.665	6.920	422.80	19.94	5.80	3.097	**0.645**
2	7.280	5.895	233.27	14.75	6.07	2.996	0.365

Table 8. Verification results from the selected optimal solution

Solution	95% PI low	Predicted Mean	95% PI high	Std Dev	Data Mean	Difference	Accurancy
Total flavonoid	351.41	426.509	501.607	32.8117	428.4	0.44%	99.56%
Inhibitory activity	17.674	20.0416	22.4129	1.03603	20.7	3.28%	96.72%
pH	5.34202	5.80428	6.26654	0.235927	5.8	0.07%	99.93%
Yield	2.41517	3.07134	3.72752	0.286695	3.1	0.93%	99.07%

The estimation was done to obtain the maximum point of three responses (i.e., the total flavonoid, inhibitory activity, and yield) and the minimum point of pH response. The selected optimal solution was treatment with desirability value closer to 1, as explained by Ramadhany et al. (2017). The optimal treatment was a material-to-solvent ratio of 1:266 (w/v) and an extraction time of 7 minutes, with desirability value of 0.645. The predicted values of total flavonoid, inhibitory activity, pH, and yields responses were 422.802 mg EQ/g, 19.945 mm, 5.8, and 3.097%, respectively.

The validation and verification were carried out to evaluate the accuracy of the program in predicting the existing results. This was done by repeating the experimental research based on the selected optimal solution. Table 8 shows the actual results for total flavonoid (428.4 mg EQ/g), inhibitory activity (20.042 mm), pH (5.804), and yield (3.10%), with accuracy of 99.56%, 96.72%, 99.93%, and 99.07%, respectively. The results indicate high accuracy as the deviation value was < 5% and the accuracy level was > 95%, aligns with the statement of Hiovenaguna and Widjarnarko (2017). Thus, the selected model was appropriate for the optimization of extraction and the deviation did not have a significant difference. The total flavonoid and inhibitory activity of the resulting tobacco extract were much higher than that of 8.8 – 14.3 mg EQ/g (Docheva et al., 2014) and 7.34 mm (Putri et al., 2014). However, the extract yield was still much lower than the value of 26.77% reported by Firdausiah et al. (2020). Therefore, in-depth investigation is critical to further enhance the bioactive compound extraction in tobacco leaves for antibacterial agents.

Conclusions

The findings concluded that material-to-solvent ratio and extraction time significantly affect total flavonoids, inhibition power, and yield at various degrees. However, no significant effect on pH was found. Increasing the material-to-solvent ratio and lowering the extraction time decreases the total flavonoid. Meanwhile, lowering the material-to-solvent ratio and prolonging the extraction time increase the inhibitory activity of the extract against *S. mutans* bacteria. In contrast, increasing the material-to-solvent ratio and extraction time increase the extract yield. However, at a certain point, the yields tend to decrease due to potential evaporation and saturation during a prolonged extraction. The suggested model for the total flavonoids, inhibitory activity, and yield was quadratic model, while . the pH response was a linear model. The treatment with a ratio of 1:2.172 (w/v) and an extraction time of 6 minutes produced tobacco extract with the highest total flavonoid value (524.67 mgEQ/g) and inhibitory activity (22.4 mm). The highest pH value (6.6) was found in the treatment of 1:7.8 (w/v) and 6 minutes of extraction time. The highest yield (4.61%) was obtained from the 1:5 (w/v) treatment and 6 minutes of extraction time. The optimal solution was the treatment with a ratio of 1:2.66 (w/v) and an extraction time of 7 minutes, producing the tobacco extract with total flavonoid (422.802 mg EQ/g), inhibitory activity (19.945 mm), pH (5.8), and yields (3.097%).

Declarations

Conflict of interests The authors declare no competing interests.

References

Aini, N., Zubaidah, N., and Wulandari Mirzayanti, Y. (2019) 'Ekstraksi minyak atsiri tembakau (*Nicotiana tabacum L*) dengan menggunakan metode microwave-asisted extraction (MAE) (Extraction of tobacco essential oil (*Nicotiana tabacum L*) using the microwave-assisted extraction (MAE) method)', *Seminar Nasional Kimia Dan Pembelajarannya (SNKP)*, pp. 236–242 [In Indonesian]

Alegantina, S. (2018) 'Penetapan kadar nikotin dan karakteristik ekstrak daun tembakau (*Nicotiana tabacum L.*) (Determination of nicotine content and characteristics of tobacco leaf extract (*Nicotiana tabacum L.*))*', Jurnal Penelitian Dan Pengembangan Pelayanan Kesehatan*, 1(2), pp. 113-119 [In Indonesian]

Audrine, P. (2020). *A Policy Perspective on Tobacco Farming and Public Health in Indonesia*. Center for Indonesian Policy Studies

Bempa, S. L. P., Fatimawali., and Parengkuan, W. G., (2016) 'Uji daya hambat ekstrak daun sukun (*Artocarpus altilis*) terhadap pertumbuhan bakteri *Streptococcus mutans* (Test the inhibitory power of breadfruit (*Artocarpus altilis*) leaf extract on the growth of *Streptococcus mutans* bacteria)', *Pharmacon*, 5(4), pp. 2302-2493 [In Indonesian]

BPS. (2021) Produksi Tanaman Perkebunan Tahun 2018-2020 (*Plantation Crop Production 2018-2020*. Badan Pusat Statistik [In Indonesian]

Breig, S, J, M., and Luti, K, J, K. (2021) 'Response surface methodology: A review on its applications and challenges in microbial culture', *Materials Today: Procedings*, 42, pp. 2277-2284

Firdausiah, S., Firdaus, Hamdayanty, H., Hidayat, T., and Alfliadhi, M. (2020) 'The comparison of three different methods on extraction of cigarette butt as natural insecticide', *Jurnal Akta Kimia Indonesia (Indonesia Chimica Acta)*, 13, pp. 55-59

Haeria, Hermawati, and Pine, A. T. U. D. (2016) 'Penentuan kadar flavonoid total dan aktivitas antioksidan ekstrak etanol daun bidara (*Ziziphus spina-christi L*) (Determination of total flavonoid content and antioxidant activity of ethanol extract of bidara leaves (*Ziziphus spina-christi L*))', *Journal of Pharmaceutical and Medicinal Sciences*, 1(2), pp. 57-61 [In Indonesian]

Haerussana, A, N, E, M., Angreni, A., Siti, F, Y., Hana, A, B., and Widyastiwi. (2022) 'Taro (*Colosia esculenta*) leaves extract inhibits streptococcus mutans ATCC 31987', *Borneo Journal of Pharmacy*, 5(3), pp 268 - 278

Handayani, S., Prastowo, D., Boesri, H., Oktsariyanti, A., and Joharina, A. (2018) 'Efektivitas ekstrak daun tembakau (*Nicotiana tabacum L*) dari Semarang, Temanggung, dan Kendal sebagai larvasida *Aedes aegypti L* (Effectiveness of tobacco leaf extract (*Nicotiana tabacum L*) from Semarang, Temanggung, and Kendal as a larvicide for *Aedes aegypti L*)', *Balaba: Jurnal Litbang Pengendalian Penyakit Bersumber Binatang Banjarnegara*, 2, pp. 23-30 [In Indonesian]

Hidayanto, A., Manikam, A. S., Pertiwi, W. S., and Harismah, K. (2017) 'Formulasi obat kumur ekstrak daun kemangi (*Ocimum basilicum l*) dengan pemanis alami stevia (*Stevia rebaudiana bertoni*) (Formulation of basil leaf extract mouthwash (*Ocimum basilicum l*) with natural sweetener stevia (*Stevia rebaudiana bertoni*))', *Proceeding The 6th University Research Colloquium URECOL*, pp. 189–194.

Hidayat, Y. M., Munizar., Harsono, A. B., Winarno, G. N. A., Hasanuddin., and Salima, S. (2020) 'Chemokine ligand 5 to predict optimal cytoreduction in ovarian cancer', *International Journal of General Medicine*, 13, pp. 1201-1206

Hudaya, A., Radiastuti, N., Sukandar, D., and Djajanegara, I. (2014) 'Uji aktivitas antibakteri ekstrak air bunga kecombrang terhadap bakteri *e. coli* dan *s. aureus* sebagai bahan pangan fungsional (Test the antibacterial activity of kecombrang flower water extract against bacteria *e. coli* and *s. aureus* as a functional food ingredient)', *Al-Kauniyah Jurnal Biologi*, 7(1), pp. 9-15 [In Indonesian]

Idroes, R., and Fakri, F. (2016) 'Skrining Aktivitas Tumbuhan yang Berpotensi sebagai Bahan Antimikroba di Kawasan IE SEU-UM (Outflow Geothermal Zone) Aceh Besar (Screening of Plant Activity that has Potential as an Antimicrobial Agent in the Aceh Besar IE SEU-UM (Outflow Geothermal Zone) Area. Undergraduate Thesis. Universitas Syiah Kuala, Aceh. [In Indonesian]

Koesnadi, E. A., Putra, I. N. K., and Wiadnyani, A. A. I. S. (2021) 'Pengaruh waktu ektraksi terhadap aktivitas antioksidan ekstrak daun rambusa (*Passiflora foetida L.*) menggunakan metode microwave assisted extraction (MAE) (Effect of extraction time on the antioxidant activity of rambusa leaf extract (*Passiflora foetida L.*) using the microwave assisted extraction (MAE) method)', *Jurnal Ilmu dan Teknologi*

Pangan (ITEPA), 3, pp. 357-366 [In Indonesian]

Kurniawan, A. W., Tahir, B., Pasda, S., and Kurniawan Widhi, A. (2018) 'The Influence of market orientation, Innovation, and Entrepreneurial competence on competitiveness and Performance of Small And medium Enterprises of Silk weaving Industry', *IOSR Journal of Business and Management*, 20, pp. 1–9

Li, B., Wenkui, Z., Pengfei, W., Duanfeng, L., Le, W., and Bing, W. (2018) 'Fast drying of cut tobacco in drop tube reactor and its effect on petroleum ether tobacco extract', *Drying Technology an International Journal*, 36(11), pp 1304 – 1312

Liu R., Guo, C., Feng, M., and Liu, C. Z. (2021) 'Efficient microwave-assisted extraction of salidroside from *Rhodiola crenulata*', *SN Appiled Sciences Journal*, 3(93), pp. 1-9

Manik, D. F., Hertiani, T., and Anshory, H. (2014) 'Analisis korelasi antara kadar flavonoid dengan aktivitas antibakteri ekstrak etanol dan fraksi-fraksi daun kersen (*Muntingia calabura l.*) terhadap *Staphylococcus aureus* (Correlation analysis between flavonoid levels and the antibacterial activity of ethanol extract and cherry leaf fractions (*Muntingia calabura l.*) against *Staphylococcus aureus*)', *Khazanah*, 6(2), pp. 1-11 [In Indonesian]

Manurung, H., Wawan, K., Kusuma, I. W., and Marjenah. (2017) 'Total flavonoid content and antioxidant activity of tabat barito (*Ficus deltoidea Jack*) on different plan organs and ages. *Journal of Medicinal Plants Studies*, 5, 6, pp 120-125 [In Indonesian]

Ngamkhae, N. Monthakantirat, O., Chulikhit, Y., Boonyarat, C., Maneenet, J., Khamphukdee, C., Kwankhao, P., Pitiporn, S., and Daodee, S. (2022) 'Optimization of extraction method for Kleeb Bua Daeng formula and comparison between ultrasound-assisted and microwave-assisted extraction', *Journal of Applied Research on Medicinal and Aromatic Plants*, 28, pp. 1-11

Nurhidayati, T., Purnobasuki, H., and Hariyanto, S. (2019) *Tanaman tembakau pada cekaman genangan* (1 ed.). Yogyakarta: Deepublish.

Nururrahmah. (2011) 'Pengaruh rokok terhadap kesehatan manusia (The effect of smoking on human health)', *Jurnal Dinamika*, 2(2), pp. 45-51 [In Indonesian]

Octaviani, M. A., Dewi, D. R. S., and Asrini, L. J. (2017) 'Optimasi faktor yang berpengaruh pada kualitas lilin di UD.X dengan metode response surface (Optimization of factors that influence wax quality in UD.X using the response surface method)', *Jurnal Ilmiah Widya Teknik*, 16(1), pp. 29-38 [In Indonesian]

Oroh, S. B., Kandou, F. E. F., Pelealu, J., and Pandiangan, D. (2015) 'Uji daya hambat ekstrak metanol *Selaginella delicatula* dan *Diplazium dilatatum* terhadap bakteri *staphylococcus aureus* dan *escherichia coli* (Test of the inhibitory power of the methanol extract of *Selamatella delicatula* and *Diplazium dilatatum* against the bacteria *Staphylococcus aureus* and *Escherichia coli*.. *Jurnal Ilmiah Sains*, 15(1), pp. 52-58 [In Indonesian]

Oroian, M., Florina, D., and Florin, U. (2020) 'Comparative evaluation of maceration, microwave and ultrasonic assisted extraction of phenolic compound from propolis*', Journal Food Science Technology*, 27(1), pp 70-78

Prabudi, M., Nurtama, B., and Purnomo, E. H. (2018) 'Aplikasi response surface methodology (RSM) dengan historical data pada optimasi proses produksi burger (Application of response surface methodology (RSM) with historical data in optimizing the burger production process)', *Jurnal Mutu Pangan: Indonesian Journal of Food Quality*, 5(2), pp. 109-115 [In Indonesian]

Prasetiyo, A. B., Sekarjati, K. A., and Tontow, A. E. (2021) 'Application of RSM method in bio-composite materials (Polymethyl-Methacrylate/Hydroxyapatite) tension strength optimization by 3D printing machine process', *Journal of Energy, Mechanical, Material, and Manufacturing Engineering*, 6(2), pp. 119–126

Putri, R. H., Barid, I., and Kusumawardani, B. (2016) 'Daya hambat ekstrak daun tembakau terhadap pertumbuhan mikroba rongga mulut (Inhibitory effect of tobacco leaf extract on oral microbial growth)', *Stomatognatic-Jurnal Kedokteran Gigi*, 11(2), pp. 27-31 [In Indonesian]

Ratnaningsih, Richana, N., Suzuki, S., and Fujii, Y. (2018) 'Effect of soaking treatment on anthocyanin, flavonoid, phenolic content and antioxidant activities of *Dioscorea alata* flour', *Indonesian Journal of Chemistry*, 18(4), pp. 656-663

Rifai, G., Rai Widarta, I. W., and Ayu Nocianitri, K. (2018) 'Pengaruh jenis pelarut dan rasio bahan dengan pelarut terhadap kandungan senyawa fenolik dan aktivitas antioksidan ekstrak biji alpukat (*Persea americana mill.*) (Effect of solvent type and ratio of ingredients to solvent on phenolic compound content and antioxidant activity of avocado seed extract (*Persea americana mill.*)', *Jurnal Ilmu dan Teknologi Pangan*, 7(2), pp. 22-32 [In Indonesian]

Sari, B. L., Triastinurmiatiningsih, T., and Haryani, T. S. (2020) 'Optimasi metode microwave-assisted extraction (MAE) untuk menentukan kadar flavonoid total alga coklat *Padina australis* (Optimization of the microwave-assisted extraction (MAE) method to determine the total flavonoid content of the brown alga *Padina australis*)', *ALCHEMY*

Jurnal Penelitian Kimia, 16(1), pp. 38-49 [In Indonesian]

Susanty, S., Yudistirani, S. A., and Islam, M. B. (2019) 'Metode ekstraksi untuk perolehan kandungan flavonoid tertinggi dari ekstrak daun kelor (*Moringa oleifera Lam*) (Extraction method to obtain the highest flavonoid content from (*Moringa oleifera Lam)* leaf extract)', *Konversi*, 8(2), 31-36 [In Indonesian]

Sylvia, N., Meriatna, M., Hasfita, F., and Hakim, L. (2017) 'Optimasi adsorpsi ion Mg2+ pada fixed bed column dengan menggunakan response surface methodology (Optimization of Mg2+ ion adsorption on a fixed bed column using response surface methodology)', *Reaktor*, 17(3), pp. 126-131 [In Indonesian]

Wahyuni, D. T., and Widjanarko, S. B. (2015) 'Pengaruh jenis pelarut dan lama ekstraksi terhadap ekstrak karotenoid labu kuning dengan metode gelombang ultrasonic (The effect of solvent type and extraction time on pumpkin carotenoid extract using the ultrasonic wave method)', *Jurnal Pangan dan Agroindustri*, 3(2), pp. 390-401 [In Indonesian]

West, R. (2017) 'Tobacco smoking: Health impact, prevalence, correlates and interventions', *Psychology & Health*, 32(8), pp. 1018 – 1036

Yudharini, G. A. K. F., Suryawan, A. A. P. A. W., and Wartini, N. M. (2016) 'Pengaruh perbandingan bahan dengan pelarut dan lama ekstraksi terhadap rendemen dan karakteristik ekstrak pewarna dari buah pandan (*Pandanus tectorius*) (The effect of the ratio of ingredients to solvent and extraction time on the yield and characteristics of coloring extract from pandan fruit (*Pandanus tectorius*)', *Jurnal Rekayasa Dan Manajemen Agroindustri*, (3), pp.36–46 [In Indonesian]

Zhang, M., Zeng, G., Pan, Y., and Qi, N. (2018) 'Difference research of pectins extracted from tobacco waste by heat reflux extraction and microwave-assisted extraction', *Biocatalysis and Agricultural Biotechnology*, 15, pp. 359-363

Zou, X., BK, A., Rauf, A., Saeed, M., Al-Awthan, Y. S., Al-Duais, M. A., Bahattab, O., Khan, M. K., and Suleria, H. A. R. (2021) 'Screening of polyphenols in tobacco (*Nicotiana tabacum*) and determination of their antioxidant activity in different tobacco varieties', *ACS Omega*, 6(39), pp. 25361-25371

Zou, X., BK, A., Abu-Izneid, T., Aziz, A., Devnath, P., Rauf, A., Mitra, S., Emran, T., Mujawah, A. A. H., Lorenzo, J. M,, Mubarak, M. S., Wilairatana, P., and Suleria, H. A. R. (2021) 'Current advances of functional phytochemicals in nicotiana plant and related potential value of tobacco processing waste: A review', *Biomedicine and Pharmacotherapy*, 143, pp. 1-13