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Optimization of Flavonoid Compounds from Avocado Seeds Using Ethanol Solvent through *Response Surface Methodology* (RSM)

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A B S T R A C T

This study aimed to optimize the extraction of flavonoid compounds from avocado seeds (*Persea americana* Mill.) using ethanol solvent through Response Surface Methodology (RSM). The research investigated the effects of maceration time (1–5 days) and the ratio of avocado seed powder to solvent volume (1:10 to 5:10 w/v) on flavonoid yield. The results demonstrated that the highest flavonoid content (4.334%) was achieved at a maceration time of 5 days and a ratio of 5:10 (w/v). Optimization using RSM yielded an optimal flavonoid content of 3.61419% at 4.9 days and a 4.3:10 (w/v) ratio, with high model accuracy ($R^2 = 0.9909$). GC-MS analysis identified degraded flavonoid derivatives, such as avocadenofuran and avocadynofuran, indicating thermal degradation during analysis. The study concluded that maceration with food-grade ethanol is more suitable for alkaloid extraction, while flavonoid extraction may require alternative methods to preserve compound integrity.

Contribution to Sustainable Development Goals (SDGs):
SDG 3: Good Health and Well-being
SDG 9: Industry, Innovation, and Infrastructure
SDG 12: Responsible Consumption and Production
SDG 13: Climate Action

1. INTRODUCTION

1.1. Research Background

Avocado (Persea americana Mill.) is a plant native to Central America and is widely cultivated in tropical regions, including Tulungagung Regency, East Java Province, Indonesia. Nearly all parts of the plant—leaves, peel, stem, pulp, seeds, and roots—have known beneficial properties. Furthermore, avocado is frequently used as a raw material in pharmaceutical applications [1]. Although often underutilised, avocado seeds contain various secondary metabolites, including alkaloids, saponins, tannins, and triterpenoids, as well as other bioactive compounds such as flavonoids, glycosides, phenolics, and steroids [2]. Flavonoids are one of the most abundant classes of natural phenolic compounds found in green plants. These polyphenols exhibit strong antioxidant activity, free radical scavenging properties, enzyme inhibition, and anti-inflammatory effects [3].

Extraction is a crucial technique for isolating these bioactive compounds, with the choice of solvent being a critical factor that significantly influences extraction efficiency. Solvent polarity significantly affects the content and composition of the extracted compounds [4]. Selecting the appropriate solvent ensures the optimal extraction of the target compounds.Food-grade ethanol is widely used in the food and pharmaceutical industries due to its excellent solvating properties, low toxicity, and ease of evaporation. These characteristics make it suitable for extracting polar compounds such as flavonoids [5]. Therefore, in this study, food-grade ethanol was selected as the solvent to extract flavonoid compounds from avocado seeds

To achieve optimal results in a study, an optimization process is necessary, for which various methods can be employed. One commonly used approach is Response Surface Methodology (RSM). This method is applied to optimize and analyze the best conditions based on the collected data [6]. RSM has previously been used in a study involving the extraction of oil from avocado seeds using n-hexane as the solvent, contenting an optimized

predicted oil content of 12.518% and a free fatty acid content of 1.489% [7].

Based on the study conducted by [8], one of the strengths was the comparison of results obtained using different solvents. However, a limitation of the previous study was the absence of optimization regarding extraction time and the lack of results using food-grade ethanol as a solvent. Therefore, the present study aims to determine the optimal conditions for extracting flavonoid content from avocado seeds. The findings are expected to be applicable in both the food and pharmaceutical industries, particularly in the development of antioxidant-based products.

1.2. Literature Review

Avocado (*Persea americana* Mill.), a member of the Lauraceae family, is a plant that thrives in tropical regions, such as Tulungagung Regency, East Java Province, Indonesia. Often referred to as a "superfood," avocado is valued for its numerous health benefits and medicinal potential [9]. Avocado trees can grow up to 20 meters tall, with regular-patterned leaves and small, yellow-green flowers. The fruit is renowned for its distinctive texture and rich nutritional profile. In addition to the fruit, other parts of the avocado plant, including the seeds, peel, and leaves, also offer various health benefits [10].

Avocado seeds are one of the parts of the fruit that possess numerous health benefits due to their rich content of phytochemical compounds**.** These seeds contain various secondary metabolites**,** including flavonoids (0.1068%)**,** phenolics (0.0309%)**,** tannins (0.2044%)**,** and alkaloids (0.435%) [11]. In addition, avocado seeds contain two derivatives of 1,2,4-trihydroxy-nanodecane and six derivatives of 1,2,4-trihydroxy-decane, which have demonstrated potential as insecticidal agents. However, several factors can influence the phytochemical content of avocado seeds, including geographical location, temperature, climate, and soil fertility. Even within the same species, the chemical composition can vary significantly between different regions [10].

Table 1. Nutrient content and composition

No	Item	Composition
1	Karbohidrat	64.9 %
2	Protein	15.55 %
3	Kalsium	0.82 mg / 100 g
4	Kalium	4.16 mg / 100 g
5	Fosfor	0.09 mg / 100 g
6	Zinc	0.18 mg / 100 g
7	Sodium	1.42 mg / 100 g
8	Iron	0.31 mg / 100 g
9	Copper	0.98 mg / 100 g
10	Vitamin A	10 mg / 100 g
11	Thiamin	0.33 mg / 100 g
12	Ascorbic acid	97.8 mg / 100 g
13	Vitamin E	0.12 mg / 100 g
14	Stearic acid	5.06 µg/g
15	Oleic acid	5.32 µg/g

1.2.1. Flavanoid

Flavonoids are the largest group of phenolic compounds found in nature. They are a class of phenylpropanoids that function as water-soluble pigments located in the vacuoles of plant cells. The basic structure of flavonoids consists of a C6–C3–C6 carbon skeleton, comprising two six-carbon benzene rings (Ring A and Ring B) connected by a three-carbon heterocyclic ring (Ring C). Based on the degree of oxidation of the heterocyclic ring and the number and position of hydroxyl or methyl groups on the benzene rings, flavonoids can be classified into twelve subclasses: Chalcones, Stilbenes, Aurones, Flavanones, Flavones, Isoflavones, Phlobaphenes, Dihydroflavonols, Flavonols, Leucoanthocyanidins, and Anthocyanins [12].

1.2.2. Extraction Maceration

Extraction is a chemical separation technique used to isolate one or more components or compounds from a sample by employing a suitable solvent based on specific needs. There are various methods for performing extraction, each with its advantages, disadvantages, and applications. The choice of extraction method depends on the properties of the compounds and the solvent used.. Maceration is an extraction technique applied to materials that are sensitive to heat. This process involves soaking the material in a specific solvent for a designated period until a concentration equilibrium is reached between the solution and the solvent. The maceration process can be classified into two types: kinetic maceration and digestion. Kinetic maceration involves extraction through agitation, while digestion is a maceration technique carried out at temperatures higher than room temperature, typically between 40°C and 60°C [13].

1.2.3. Organic Solvent

Organic solvents are compounds that typically contain carbon atoms and are widely used in industries as cleaning agents, solvents for fats, thinners, extraction media, and intermediates in chemical synthesis [14]. Examples include alcohols, ethers, esters, ethyl acetate, and ketones. Alcohols are organic compounds that contain one or more hydroxyl (-OH) groups attached to an alkyl chain, with the general formula R–OH. Alcohols are important in organic chemistry due to their ability to serve as precursors for various other compounds. Their reactions can lead to the formation of compounds with either R–O or O–H bonds. Ethanol (CH_3CH_2OH) is a simple alcohol that is polar and soluble in water due to its low molecular weight [15].

1.3. Research Objective

This study aims to determine the optimal conditions for extracting alkaloid and flavonoid compounds based on the variables of extraction time and the ratio of avocado seed powder weight to solvent volume. A maceration extraction using food-grade ethanol was conducted and optimised using Response Surface Methodology (RSM).

2. MATERIALS AND METHODS

2.1. Raw and Material

The raw materials used in the manufacture of avocado seed powder are avocado seeds obtained from Tulungagung, East Java. The chemicals used for pre-treatment avocado seed is aqudest . The chemicals used for the maceration extraction of avocado seed powder are 96% ethanol. For analysis used 10 % Pb Acetat.

The tools used for making avocado seed powder are grater, oven, cooper, and 100 mesh sieve. the tools used for maceration extraction are closed jar, and glass rod. The tools used to analyze of flavanoid are Test tube, Filter paper, Glass funnel, Dropping pipette and Analytical balance

2.2. Pre-treatment Method of Avocado Seeds

The first step in the treatment process for avocado seeds involves washing them with distilled water (Aquadest) until they are clean. The seeds are then reduced in size to facilitate the drying process. Following this, the seeds are dried in an oven at a temperature of 60°C for 3 to 4 hours. After drying, the seeds are further reduced in size until the desired dimensions are achieved. To standardize the size of the avocado seeds, a 100 mesh sieve can be used.

2.3. Maceration Extraction Process of Avocado Seed Powder and Flavonoid Content Analysis using Gravimetric Method

The extraction process begins with washing the avocado seeds using aquadest. Next, the seed size is reduced using a grater until the seeds are smaller, and then they are dried in an oven at 60 °C for 1 to 2 hours. To standardise the seed size, a 100-mesh sieve can be used. Following this, the maceration extraction process is carried out by soaking the avocado seed powder in 100 mL food grade ethanol. The beaker glass is securely covered to prevent evaporation of the ethanol. During this process, several variables are controlled, including the extraction time, which is set to 1 to 5 days, and the ratio of avocado seed powder weight to solvent volume, which is varied at 1:10, 2:10, 3:10, 4:10, and 5:10 (w/v). After the extraction process is complete, the resulting filtrate is separated from the residue. The filtrate undergoes gravimetric testing, where 1 mL of 10% lead acetate is added and shaken. A positive result is indicated by the formation of a yellow brown precipitate.

2.4. Optimization of Flavonoid Compounds Using Response Surface Methodology (RSM)

The optimisation process is conducted using the Response Surface Methodology (RSM) method, employing Design-Expert 13 software. The steps for using this method include defining the independent variables with their corresponding boundaries. The next step is to select the Central Composite Design (CCD) program and input the results of the Design of Experiments (DOE) analysis as determined by the software. Subsequently, a simulation analysis is performed, and the results are subjected to an Analysis of Variance (ANOVA). The analysis results should be compared with the requirements of the Response Surface Methodology (RSM). Suppose the results do not meet the required conditions. In that case, the boundary values of the independent variables must be adjusted, and the Design of Experiments (DOE) should be re-entered according to the initial steps. However, if the results comply with the RSM conditions, an optimal solution and a 3D surface graph will be displayed.

2.5. Flavonoid Compound Testing using Gas Chromatography – Mass Spectrometry (GC-MS)

The flavonoid content test is conducted using GC-MS (Gas Chromatography-Mass Spectrometry), commonly referred to as Gas Chromatography. This test aims to determine the alkaloid or flavonoid concentration in the filtrate obtained from the previous extraction. The working principle of Gas Chromatography-Mass Spectrometry (GC-MS) involves vaporising the sample under high vacuum and low pressure when heated, thereby determining the molecular weight and identity of the compounds in the sample. The GC-MS technique is a sample separation method performed through gas chromatography, followed by analysis using mass spectrometry. This method offers high sensitivity, enabling it to separate and analyse mixed compounds at very low concentrations.

3. RESULT AND DISCUSSION

3.1. The Influence of Extraction Time Variables and the Weight Ratio of Avocado Seed Powder on Solvent Volume and Flavonoid Compound Content

This research was also conducted to investigate the extent to which the variables of maceration extraction time and the ratio of avocado seed powder weight to solvent volume affect the resulting flavonoid content. The combination of these variables provides a more comprehensive overview of their interactive relationship with the flavonoid content. The research findings indicated that there were increases and decreases in the flavonoid content across the specified variable conditions. The observed flavonoid contents corresponding to these two variables are presented in Table 2.

Table 2. Flavonoid content analysis results.

Maceration Time (Day)	Ratio of avocado seed powder to solvent (% F/s)				
	10	**20**	**30**	**40**	**50**
1	0.915	1.054	0.807	0.740	1.130
2	0.924	1.139	1.011	1.422	1.638
3	1.130	1.358	1.418	1.678	2.583
4	2.139	2.258	2.247	2.430	2.591
5	3.067	3.076	3.458	3.7680	4.334

Table 2 illustrates that the content of flavonoid compounds increases with the duration of maceration across all ratios of avocado seed powder to solvent volume. It was observed that the lowest concentration of flavonoid compounds was generally found on the first day, while significant increases occurred on the fourth and fifth days. At a ratio of 5:10 (w/v), it was noted that the concentration of flavonoid compounds increased significantly, yielding the highest flavonoid concentration on the fifth day. This indicates that both the extended extraction time of maceration and the ratio of avocado seed powder to solvent volume have a significant impact on the increase in flavonoid content.[16]. It can be observed from the data that the content of flavonoid compounds tends to increase with the duration of maceration extraction and a higher ratio of avocado seed powder to solvent. The data presented in the table is not always linear, as fluctuations are evident in several combinations of the weight ratio of avocado seed powder to solvent volume and maceration time. Notably, on the second day of maceration, the content of flavonoid compounds actually decreased from a ratio of 2:10 (w/v) (1.1390) to a ratio of 3:10 (w/v) (1.0110), followed by a sharp increase to 1.4220 at a ratio of 4:10 (w/v). This indicates that the increase in flavonoid content did not meet expectations. Previous research [11] has shown that the flavonoid content from the maceration extraction of avocado seeds provides significant data. The results of this study align with those findings; however,

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in some variables, there were still significant decreases in flavonoid content, indicating the need for further research.

The effects of maceration extraction time and the ratio of avocado seed powder to solvent volume on the content of flavonoid compounds can be examined in more detail in the following graph.

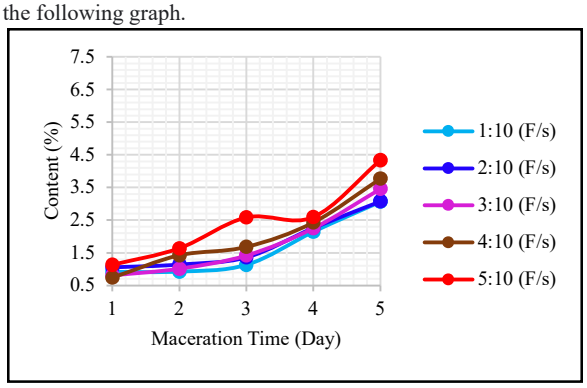

Fig 1. Graph of the effect of maceration extraction time (days) on flavonoid compound content.

Based on Fig. 1, the relationship between maceration extraction time and the content of flavonoid compounds shows a positive, non-linear correlation. The graph indicates that the content of flavonoids increases with longer maceration times, reaching a maximum on the fifth day at a 5:10 (F/s) ratio. This suggests that extended maceration time allows the solvent to extract flavonoid compounds more thoroughly, leading to optimal diffusion of active compounds into the solvent [17].

However, The graph also indicates fluctuations at specific maceration extraction times, such as on the first day of extraction with a ratio of 3:10 (w/v), where a decrease in flavonoid content was observed. This may be attributed to limitations of the solvent in dissolving active compounds, degradation of the compounds, or back diffusion.[18]. It is important to note that the data presented in the graph may not be entirely accurate due to specific experimental errors or uncertainties. Variations or deviations in the data resulting from these errors are illustrated in the following graph.

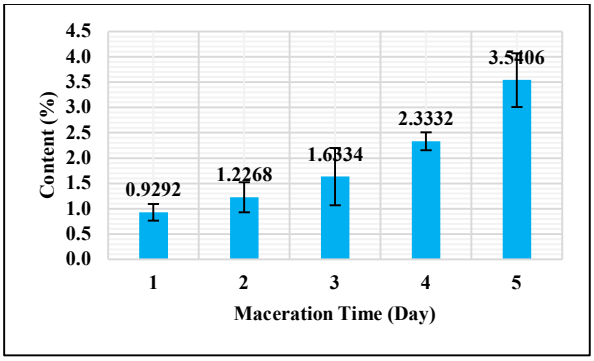

Fig 2. Standard deviation graph of flavonoid content at specific maceration extraction time (days)

Fig 2. illustrates the relationship between maceration extraction time and the average content of flavonoid compounds, along with their standard deviations. The average content of flavonoids tends to increase over the maceration extraction period, rising from 0.9292 on the first day to 3.9380 on the fifth day. This indicates that longer contact time between the material and the solvent results in a greater amount of compounds being successfully extracted, aligning with the fundamental principles of diffusion and solubility of active compounds during the maceration extraction process.

However, the increase in content is not always accompanied by consistent results across replicates, as evidenced by the fluctuations in standard deviation associated with each time point. Therefore, while the content of flavonoids increases with extended maceration time, the stability and consistency of the results must be considered, particularly by monitoring low standard deviation values to ensure the reproducibility of the findings [19].

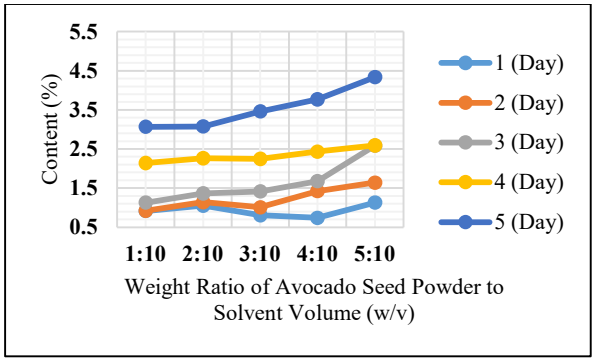

Fig 3. Graph of the effect of the ratio of avocado seed powder weight to solvent volume (% w/v) on flavonoid compound content.

Based on Fig. 3, a positive non-linear correlation exists between the ratio of avocado seed powder to solvent and maceration time, and the content of flavonoid compounds. On the fifth day, this relationship appears exponential, particularly with the increase in ratio from 4:10 to 5:10 w/v, resulting in a significant spike in flavonoid content. This indicates that increasing the ratio by adding more powder while keeping the solvent volume constant enhances the contact surface area between the solid and the solvent, thereby improving the solubility of the active compound [20]. The data presented in the graph indicate that from day 1 to day 4, the influence of the ratio on content appears to be weak and inconsistent. Increases in the ratio do not consistently lead to significant increases in content, suggesting a weak correlation between the variables. This implies that the content of alkaloid compounds is influenced by multivariate interactions, where other factors such as extraction time, compound degradation, and diffusion rate also play crucial roles in determining the effectiveness of the maceration process [21]. Furthermore, the data in the graph may not be entirely accurate due to experimental error or uncertainty at certain levels. The variation or deviation in the data resulting from these errors is illustrated in the following graph.

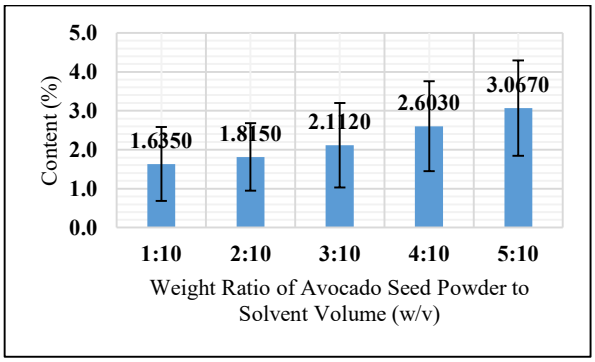

Fig 4. Standard deviation graph of flavonoid compound content at a specific ratio of avocado seed powder weight to solvent volume (% w/v).

Fig 4 above illustrates the relationship between the weight ratio of avocado seed powder to solvent volume (% w/v) and the average content of flavonoid compounds produced, along with its standard deviation. The data indicate a tendency for the content of flavonoid compounds to increase with the rising ratio, with the highest content of 2.5910 achieved at a ratio of 50% w/v. However, this increase does not follow a linear pattern, as the content of flavonoids at the 20% and 30% w/v ratios decreased compared to the 10% w/v ratio. Subsequently, the content of flavonoids significantly increased again at the 40% w/v ratio, peaking at the 50% w/v ratio. Therefore, while the content of flavonoids increases with prolonged maceration time, the stability or consistency of the results must be considered, particularly by monitoring low standard deviation values to ensure the reproducibility of the results [22].

3.2. Optimization of Flavonoid Compound Content Using Response Surface Methodology (RSM).

To optimize the content of alkaloid compounds, the Central Composite Design (CCD) experimental design can be utilized within the framework of Response Surface Methodology (RSM). The CCD experimental design is chosen for its ability to explore the response area and identify optimal conditions thoroughly. This design enables the assessment of the relationship between the variables of maceration time (A) and the weight ratio of avocado seed powder to solvent volume (% w/v) (B), as well as the content of flavonoid compounds (Y). The resulting experimental design matrix is presented in Table 3.

Table 3 above presents data in the form of a design matrix from the application of Response Surface Methodology (RSM) for the optimisation process of flavonoid content, concerning the variables of maceration extraction time (in days) and the weight ratio of avocado seed powder to solvent volume (% w/v). The results of the design matrix indicate that the highest flavonoid content was obtained in experiment number 25, which involved a maceration extraction time of 5 days and a weight ratio of avocado seed powder to solvent of 5:10 (w/v). Conversely, the lowest flavonoid content was found in experiment number 4, with a maceration extraction time of 1 day and a weight ratio of avocado seed powder to solvent of 4:10 (w/v). It can be observed that in the experiment with a maceration extraction time of 1 day, there were fluctuations in the data compared to other maceration extraction times, which showed a significant increase in flavonoid content using the same weight ratio of avocado seed powder to solvent as on other days. To determine the significance of each factor, an analysis of variance (ANOVA) is necessary. Table 4 presents the results of the ANOVA based on the content of flavonoid compounds obtained under the conditions specified in the experimental design matrix.

Table 3. Experimental design matrix for response surface methodology (RSM).

Run	Factor 1 A: Time	Factor 2 B: Ratio	Response 1 *Contents*
1	1	10	0.9150
2	1	20	1.0540
3	1	30	0.8070
4	1	40	0.7400
5	1	50	1.1300
6	2	10	0.9240
7	2	20	1.1390
8	2	30	1.0110
9	2	40	1.4220
10	2	50	1.6380
11	3	10	1.1300
12	3	20	1.3580
13	3	30	1.4180
14	3	40	1.6780
15	3	50	2.5830
16	4	10	2.1390
17	4	20	2.2580
18	4	30	2.2470
19	4	40	2.4310
20	4	50	2.5910
21	5	10	3.0670
22	5	20	3.0760
23	5	30	3.4580
24	5	40	3.7680
25	5	50	4.3340

Table 4. Analysis of variance (ANOVA) for the quadratic model of flavonoid compounds (25 data points)

Source	*Sum of Squares*	df	*Mean Square*	F - value	P – value	
Model	25,79	5	5,16	63,98	< 0,0001	*Significant*
A - Waktu	22,69	1	22,69	281,41	< 0,0001	
B – Rasio	1,07	1	1,07	13,30	0,0017	
AB	0,9320	1	0,9320	11,56	0,0030	
A^2	1,04	1	1,04	12,89	0,0019	
B^2	0,0607	1	0,0607	0,7534	0,3962	
Residual	1,53	19	0,0806			
Cor Total	27,32	24				

Through the analysis of variance (ANOVA), various significant insights were obtained that can aid in interpreting the

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experimental results. The ANOVA table indicates the significance of the factors and interactions affecting the flavonoid content. The model used showed an F value of 63.98 with a p-value < 0.0001, indicating that the model as a whole is highly significant. This suggests that the quadratic model applied can effectively describe the relationship between the independent variables (macération extraction time and the weight ratio of avocado seed powder to solvent) and the flavonoid content. Further analysis revealed that factor A (time) has the most significant influence on flavonoid content, with an F value of 281.41 and a p-value < 0.0001. This indicates that variations in maceration extraction time have a strong impact on the flavonoid content obtained. Factor B (ratio) also showed a significant effect with an F value of 13.30 and a p-value of 0.0017, which is well below the significance threshold of 0.05.

The interaction between maceration extraction time and the weight ratio of avocado seed powder to solvent (AB) showed an F value of 11.56 with a p-value of 0.0030, indicating that this interaction has a significant effect on flavonoid content, as it is also below the significance level of 0.05 (Rohmawati and Izzati, 2021). The quadratic interaction A^2 showed significance regarding flavonoid content with an F value of 12.89 and a p-value of 0.0019, while the quadratic interaction B^2 had an F value of 0.7534 with a p-value of 0.3962, indicating a lack of interaction between this factor and flavonoid content. The data suggests a non-linear relationship between these factors and flavonoid content, as changes in variable values do not always yield linear results in flavonoid content.

The residual value of 1.53 with 19 degrees of freedom indicates the extent of data variation that cannot be explained by the model. Meanwhile, the total correlation value of 27.32 represents the total variation in the data. In the Fit Statistics analysis, this model has an R^2 value of 0.9439, an adjusted R^2 of 0.9292, and a predicted R^2 of 0.8740. These values indicate that the model has low accuracy in predicting experimental outcomes, as the R^2, adjusted R^2, and predicted R^2 values do not approach the predictive value of 1.

After analyzing variance (ANOVA), equations in both coded and actual forms can be derived to predict the response of flavonoid content based on each factor, namely maceration extraction time (days) and the weight ratio of avocado seed powder to solvent (w/v). Below are the equations derived from the coded and actual calculations for each factor:

$$content\ (Flavanoid) = 1{,}59 + 1{,}35A + 0{,}2929B + 0{,}3862AB + 0{,}4874A^2 + 0{,}1178B^2 \quad \text{.....(1)}$$

$$content\ (Flavanoid) = 1{,}35990 - 0{,}347163A - 0{,}031992B + 0{,}009654AB + 0{,}121857A^2 + 0{,}000295B^2 \quad \text{.....(2)}$$

Statistically, the above equations can be explained in Table 5, which presents the coefficient of determination derived from the optimization of 25 data points.

Based on Table 5, the optimisation contained an R^2 value of 0,9439, an adjusted R^2 value of 0,9292, and a predicted R^2 value of 0,8740. This indicates that some of the 25 data points show a significant difference among them, as the resulting R^2 value does not approach the predictive value of 1. An R^2 value closer to 1 indicates higher accuracy of the model in predicting the dependent variable based on the independent variables. Conversely, an R^2 value approaching 0 suggests that the model is poor and has low accuracy in predicting the response or dependent variable [23] Therefore, further optimization was conducted using 13 content data points of flavonoid compounds that exhibited a lower degree of variation compared to the overall research data. This step aims to enhance the model's ability to predict response values. Below are the 13 content data points of flavonoid compounds used in the subsequent optimization process.

Table 5. Coefficient of determination for the regression model obtained from optimization of 25 data points

Std. Dev.	0.2839
Mean	1.89
C.V. (%)	15.01
R^2	0.9439
Adjusted R^2	0.9292
Predicted R^2	0.8740
Adequate precision	25.2229

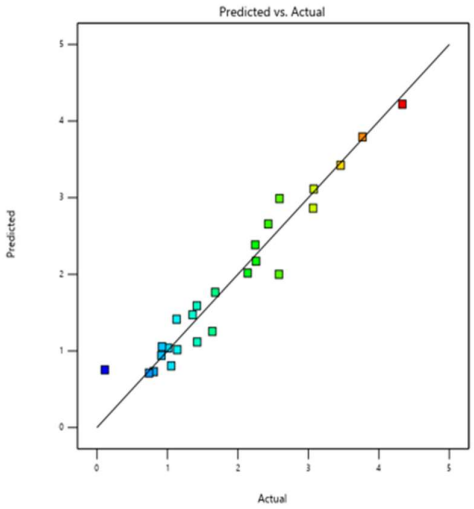

Fig 5. Correlation between Predicted and Experimental Data for Flavonoid Compounds across 25 Trials.

Based on Figure 5, the plot of 25 predicted versus actual data points reveals a lack of alignment between the model equation and the actual process. This is indicated by several data points scattered far from the linear regression line (y=x), suggesting that the model's accuracy in representing the process is limited.

To address this, further optimization was conducted using 13 flavonoid content data points that exhibited smaller deviations compared to the full dataset. These 13 data points were selected to cover the full range of flavonoid contents (low, medium, and high) while avoiding clustered points and prioritising those closest to the regression line (y = x). This approach aimed to enhance the model's predictive capability. Table IV.13 presents the 13 flavonoid content data points used in the subsequent optimization process**.**

Table 6. Experimental design matrix for response surface methodology (RSM).

Run	Factor 1	Factor 2	Response 1
	A: Time	B: Ratio	*Contents*
1	1	10	0,9150
2	1	20	1,0540
3	1	30	0,8070
4	1	40	0,7400
5	2	10	0,9240
6	2	20	1,1390
7	3	20	1,3580
8	3	30	1,4180
9	3	40	1,6780
10	4	20	2,2580
11	4	30	2,2470
12	5	20	3,0760
13	5	30	3,4580

Table 6 above presents data in the form of a design matrix from the application of Response Surface Methodology (RSM), which was used to further optimise the content of flavonoid compounds, reducing the dataset from 25 to 13 data points. The results of the design matrix indicate that the highest content of alkaloid compounds was achieved in experiment number 13, corresponding to a maceration extraction time of 5 days and a weight ratio of avocado seed powder to solvent volume of 3:10 w/v. Conversely, the lowest content of alkaloid compounds was observed in experiment number 4, with a maceration extraction time of 1 day and a weight ratio of avocado seed powder to solvent volume of 4:10 w/v. It is noted that in the experiment with a 1-day maceration extraction time, data fluctuation was observed compared to other extraction times, which showed a significant increase in alkaloid content using the same weight ratio of avocado seed powder to solvent volume as on the other days. This indicates the presence of complex interactions among the variables, degradation of compounds, or the influence of process conditions. To assess the significance of each factor, an analysis of variance (ANOVA) is necessary. The following presents the results of the ANOVA based on the content of alkaloid compounds obtained under the conditions specified in the experimental design matrix.

Table 7. Analysis of variance (ANOVA) for the quadratic model of flavonoid compounds (13 data points).

Source	*Sum of Squares*	df	*Mean Square*	F - value	P – value	
Model	9,29	5	1,86	153,28	< 0,0001	*Significant*
A - Waktu	6,35	1	6,35	523,23	< 0,0001	
B – Rasio	0,0324	1	0,0324	2,67	0,1462	
AB	0,1211	1	0,1211	9,99	0,0159	
A^2	0,8676	1	0,8676	71,54	< 0,0001	
B^2	0,0076	1	0,0076	0,6247	0,4552	
Residual	0,0849	7	0,0121			
Cor Total	9,38	12				

Based on Table 7, an analysis of variance (ANOVA) was conducted using a quadratic model. This model shows an F-value of 153.28 with a p-value < 0.0001, indicating that the model as a whole is highly significant. This suggests that the model effectively explains the relationship between the independent variables —namely, maceration extraction time (in days) and the weight ratio of avocado seed powder to solvent volume (% w/v) —in a representative manner, with respect to the content of flavonoid compounds. Further analysis reveals that factor A (maceration extraction time) has a very significant influence on increasing the content of flavonoid compounds, with an F-value of 523.23 and a p-value < 0.0001. In contrast, factor B (the weight ratio of avocado seed powder to solvent volume) shows a less significant effect on increasing the content of flavonoids, with an F-value of 2.67 and a p-value < 0.1462.

The table indicates that the interaction between maceration extraction time (days) and the weight ratio of avocado seed powder to solvent volume (% w/v) (AB) has an F-value of 9.99 with a p-value of 0.0159. This suggests a strong interaction between these two factors regarding the content of flavonoid compounds, as the p-value for each interaction among these factors is below 0.05. Additionally, the quadratic interaction A^2 shows significance concerning the content of flavonoid compounds, with an F-value of 71.54 and a p-value < 0.0001. In contrast, the quadratic interaction B^2 has an F-value of 0.6247 with a p-value of 0.4552, indicating a lack of interaction between this factor and the content of flavonoids. This suggests that changes in variable values do not always content linear results concerning the content of alkaloid compounds.

In Table 7, a residual value of 0.0849 with 7 degrees of freedom indicates the extent of data variation that cannot be explained by the model. Meanwhile, the total correlation value of 9.38 represents the total variation in the data. From the Fit Statistics analysis, this model has an R^2 value of 0.9909, an adjusted R^2 of 0.9845, and a predicted R^2 of 0.9734. These values indicate that the model has high accuracy in predicting experimental outcomes, as the R^2, adjusted R^2, and predicted R^2 values are close to the predictive value of 1. After conducting the analysis of variance (ANOVA), equations in both coded and actual forms can be derived to predict the response of flavonoid content based on each factor, namely maceration extraction time (days) and the weight ratio of avocado seed powder to solvent (w/v). Below are the equations derived from the coded and actual calculations for each factor:

$$Yield\ (Flavanoid) = 1{,}54 + 1{,}27A + 0{,}1728B + 0{,}3814AB + 0{,}6135A^2 - 0{,}1192B^2 \quad \text{.....(1)}$$

$$Yield\ (Flavanoid) = 1{,}33717 - 0{,}568851A - 0{,}002091B + 0{,}009536AB + 0{,}153367A^2 - 0{,}000298B^2 \quad \text{.....(2)}$$

Statistically, the equations above can be explained in Table 8, which presents the coefficient of determination derived from the optimization of the 13 data points.

Based on Table 8, the R^2 value obtained is 0.9909, the adjusted R^2 is 0.9845, and the predicted R^2 is 0.9734. This indicates that among the 13 data points, there is a small difference between the data, as the R^2 value is very close to 1. The R^2 value obtained meets one of the criteria for a regression model in explaining the variation in the observed data, as a good R^2 value is considered to be at least 0.8, or approximately 80%. A good regression model is also indicated when the difference between

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the adjusted R^2 and predicted R^2 values is a maximum of 20%. The selection of these 13 data points was also based on the graph showing the relationship between the predicted data and the actual data.

Table 8. Coefficient of Determination for the Regression Model Obtained from Optimization of 25 Data Points

Std. Dev.	0,1101
Mean	1.62
C.V. (%)	6.79
R^2	0.9909
Adjusted R^2	0.9845
Predicted R^2	0.9734
Adeq precision	35.8724

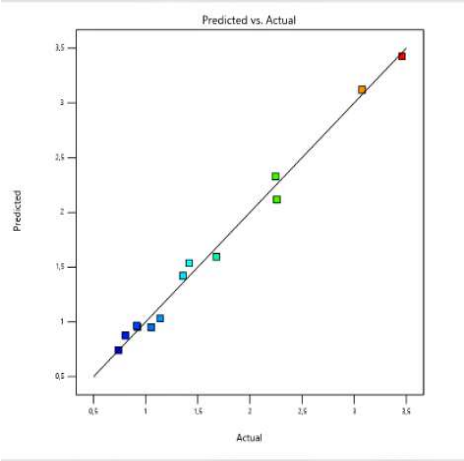


Fig 6. Correlation between predicted and experimental data for flavonoid compounds across 13 trials.

Based on Figure 6, the plot of the 13 predicted data points and actual data points shows the model's equation alignment with the process. This is indicated by the data points being close to the linear regression line. This also serves as the basis for selecting the optimization using the 13 content data points of flavonoid compounds, as the plot for the 25 content data points showed a distribution that was far from the regression line (y = x), indicating lower accuracy. In selecting the plot of the 13 predicted and actual data points, consideration was also given to the lowest, medium, and highest contents of flavonoid compounds, ensuring that they are not too close together so that the data does not overly converge on one another. With this approach, parameters can be obtained to optimise the content of flavonoid compounds in relation to the independent variables. Table 9 presents the optimisation results, which lead to the determination of the optimum point.

Table 9. Optimal solution based on response surface methodology (RSM) from 13 experimental data points.

Maceration Time (Days)	4.99414
Ratio (% w/v)	39.6367
Content	3.65815
Desirability	1.000

Based on Table 9, the results of the optimisation calculations using response surface methodology (RSM) indicate that the optimal conditions for achieving the best results occur at a maceration extraction time of 4.99414 days and a weight ratio of avocado seed powder to solvent volume of 39.6367% (w/v). With this combination, the content of alkaloid compounds reaches 3.65815, demonstrating a relatively high process efficiency. The data also reveal the desirability value, which is used to measure how well the optimal solution achieves the desired objectives. In this experiment, a desirability value of 1.000 was obtained, indicating that the optimum point found is very good as it reaches the maximum value. This shows that the combination of variables used can provide results that meet expectations.

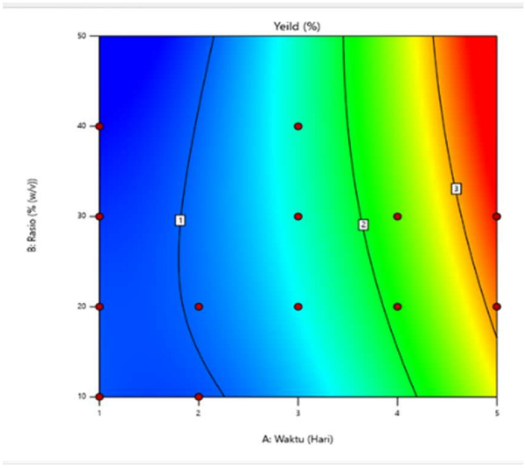


Fig 7. Contour plot illustrating the influence of maceration extraction time and avocado seed powder-to-solvent ratio on flavonoid compound content.

Figure 7 above is a contour plot, a two-dimensional representation of the optimisation process using response surface methodology (RSM). The graph illustrates the relationship between maceration extraction time and the weight ratio of avocado seed powder to solvent volume, showing the content of flavonoid compounds produced during the process. From the graph, it is evident that the optimum content of flavonoid compounds is achieved at a maceration extraction time of 4.90821 days and a weight ratio of avocado seed powder to solvent volume of 4.31341:10 w/v. The graph indicates that as the maceration extraction time and the weight ratio of avocado seed powder to solvent increase, they significantly influence the content of flavonoid compounds produced. The dominant blue color in this graph suggests that under these conditions, the process is less supportive of achieving maximum content of flavonoid compounds. This implies that extraction times and weight ratios of avocado seed powder to solvent volume outside the optimal range may lead to a decrease in the content of flavonoid compounds.

Figure 8 is a surface plot, a three-dimensional representation of the optimisation process using response surface methodology (RSM). This graph illustrates that maceration extraction time and the weight ratio of avocado seed powder to solvent volume play a crucial role in determining the content of alkaloid compounds. The graph identifies minimum, maximum, and saddle points within the process. In the graph, the minimum point is indicated at a maceration extraction time of 1 day with a weight ratio of avocado seed powder to solvent volume of 4:10 (w/v). The maximum point is shown at a maceration extraction time of 5 days with a weight ratio of avocado seed powder to solvent volume of 3:10 (w/v), while the saddle point is located at a

maceration extraction time of 3 days with a weight ratio of avocado seed powder to solvent volume of 3:10 w/v.

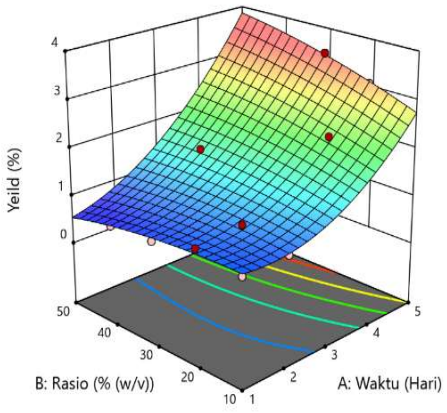


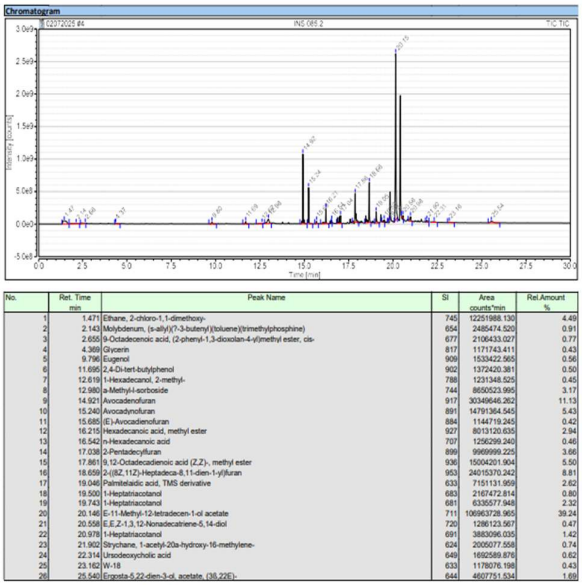
Fig 8. Surface plot illustrating the combined effect of maceration extraction time and avocado seed powder-to-solvent ratio on flavonoid content.

It can be concluded that by using response surface methodology (RSM) for optimization, the optimum operating conditions were obtained at a maceration extraction time of 4.90821 days and a weight ratio of avocado seed powder to solvent of 4.31341:10 (w/v), resulting in a flavonoid content of 3.61419.

3.3. Analysis of Alkaloid and Flavonoid Content Utilizing Gas Chromatography–Mass Spectrometry (GC-MS) Techniques.

The analysis of alkaloid and flavonoid content in this study was conducted using Gas Chromatography–Mass Spectrometry (GC-MS), which combines gas chromatography for compound separation and mass spectrometry for identification based on the mass-to-charge ratio (m/z). The process begins with the injection of the sample into the GC system, where the compounds are vaporized and carried through a capillary column by an inert carrier gas such as helium or nitrogen. The compounds are separated based on their polarity and interactions with the stationary phase, and then directed to the mass spectrometry (MS) component for ionization, resulting in molecular ions and fragments with characteristic patterns. These ions are sorted based on their m/z values and detected to produce a unique mass spectrum for each compound. This spectrum, represented as a histogram of relative intensity against m/z, serves as a molecular fingerprint. The high-vacuum system in the MS reduces background interference and enhances ion detection sensitivity. The resulting spectra are compared with reference libraries such as NIST, EPA, and NIH for accurate compound identification [24].

The analysis of flavonoid content was conducted using Gas Chromatography–Mass Spectrometry (GC-MS) on samples derived from the maceration extraction filtrate. The selected variable was day 4.99414 (5), with a weight-to-solvent volume ratio of 39.6367% w/v (40% w/v) for avocado seed powder. This sample selection was based on optimization results for alkaloid and flavonoid contents using Response Surface Methodology (RSM).



No.	Ret. Time min	Peak Name	SI	Area counts*min	Rel.Amount %
1	1.471	Ethane, 2-chloro-1,1-dimethoxy-	745	12251988.130	4.49
2	2.143	Molybdenum, (s-allyl)(7-3-butenyl)(toluene)(trimethylphosphine)	654	2485474.520	0.91
3	2.655	9-Octadecenoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester, cis-	677	2106433.027	0.77
4	4.369	Glycerin	817	1171743.411	0.43
5	9.796	Eugenol	909	1533422.565	0.56
6	11.695	2,4-Di-tert-butylphenol	902	1372420.381	0.50
7	12.619	1-Hexadecanol, 2-methyl-	788	1231348.525	0.45
8	12.980	a-Methyl-l-sorboside	744	8650523.995	3.17
9	14.921	Avocadenofuran	917	30349646.262	11.13
10	15.240	Avocadynofuran	891	14791364.545	5.43
11	15.685	(E)-Avocadienofuran	884	1144719.245	0.42
12	16.215	Hexadecanoic acid, methyl ester	927	8013120.635	2.94
13	16.542	n-Hexadecanoic acid	707	1256299.240	0.46
14	17.038	2-Pentadecylfuran	899	9969999.225	3.66
15	17.861	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	936	15004201.904	5.50
16	18.659	2-((8Z,11Z)-Heptadeca-8,11-dien-1-yl)furan	953	24015370.242	8.81
17	19.046	Palmitelaidic acid, TMS derivative	633	7151131.959	2.62
18	19.500	1-Heptatriacontanol	683	2167472.814	0.80
19	19.743	1-Heptatriacontanol	681	6335577.948	2.32
20	20.146	E-11-Methyl-12-tetradecen-1-ol acetate	711	106963728.965	39.24
21	20.558	E,E,Z-1,3,12-Nonadecatriene-5,14-diol	720	1286123.567	0.47
22	20.978	1-Heptatriacontanol	691	3883096.035	1.42
23	21.902	Strychane, 1-acetyl-20a-hydroxy-16-methylene-	624	2005077.558	0.74
24	22.314	Ursodeoxycholic acid	649	1692589.876	0.62
25	23.162	W-18	633	1178076.198	0.43
26	25.540	Ergosta-5,22-dien-3-ol, acetate, (3ß,22E)-	644	4607751.534	1.69

Fig 9. Sample chromatogram.

Based on Fig 9, it can be observed that the sample produced 26 peaks with varying retention times and area percentages. The chromatogram indicates a dominant presence of compounds within the retention time range of 14 to 22 minutes, with the highest peak occurring around the 20-minute mark. A larger percentage of the area and an increasing peak height suggest that the detected compounds are predominant in the sample [25]. In this analysis, a total of 26 compounds were identified, and a classification was performed to determine the flavonoid compounds, as shown in Table 10 below.

Table 10. Classification of flavonoid compounds.

Flavonoid Compounds	Time
Avocadenofuran*	(57938615.379) & (21055644.668)
Avocadynofuran*	(37632253.431)
E-Avocadienofuran*	(4317657.603)
2-((8Z,11Z)-Heptadeca-8,11-dien-1-yl) furan*	(28056138.826)

The analysis indicates the presence of flavonoid compounds in their degraded forms within the samples. This is evidenced by the identification of compounds such as Avocadenofuran, Avocadynofuran, E-Avocadienofuran, 2-((8Z,11Z)-Heptadeca-8,11-dien-1-yl) furan, and 2-Pentadecylfuran. The characterization of these compounds is substantiated by the mass spectrometry (MS) results presented in Figures IV.23 through IV.27 below. These findings suggest that the flavonoid content has undergone degradation, which may impact their biological activity and potential applications

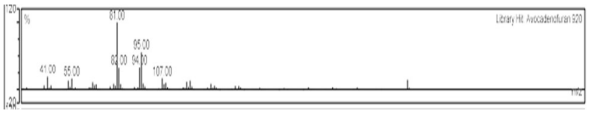


Fig 10. Mass Spectrometry (MS) results of avocadenofuran compounds.

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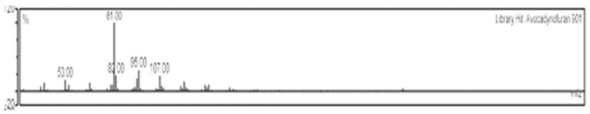

Fig 11. Mass Spectrometry (MS) results of avocadynofuran compounds.

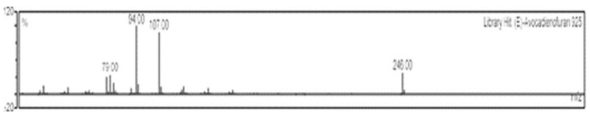

Fig 12. Mass Spectrometry (MS) results of e-avocadienofuran compunds.

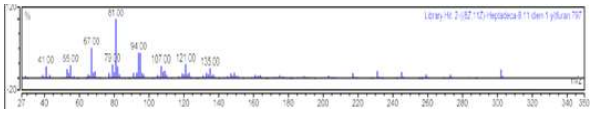

Fig 13. Mass Spectrometry (MS) results of 2-((8Z,11Z)-Heptadeca-8,11-dien-1-yl) furan compunds.

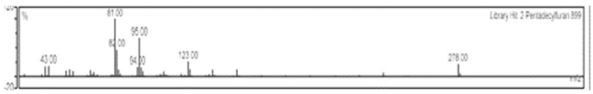

Fig 14. Mass Spectrometry (MS) results of 2-Pentadecylfuran compounds.

Fig. 10-14 present the mass spectrometry (MS) results for each compound, indicating the presence of flavonoid compounds in their degraded forms. The retention times, similarity indices, and relative area percentages for each compound are summarized in Table 11 below. This data provides a comprehensive overview of the flavonoid content and its degradation status within the samples.

Table 11. Retention Time Data, Similarity Index, and Relative Area Percentage of Compounds Indicating the Presence of Flavonoid Compounds

Compound	Time ret (Menit)	*Similarity Index*	Relative Area Percentage (%)
Avocadenofuran	14.929	920	9.87
Avocadynofuran	15.248	901	6.41
E-Avocadienofuran	15.685	925	0.74
2-((8Z,11Z)-Heptadeca-8,11-dien-1-yl) furan	18.659	947	4.78
2-Pentadecylfuran	17.038	899	3.66

Table 11 indicates that the compounds serving as indicators of flavonoid presence in their degraded forms exhibit relatively high area percentages. This suggests that these flavonoid indicators were detected in significant quantities. In terms of chemical structure, the compounds Avocadenofuran, Avocadynofuran, E-Avocadienofuran, 2-((8Z,11Z)-Heptadeca-8,11-dien-1-yl) furan, and 2-Pentadecylfuran can be utilized as indicators of flavonoid presence. The chemical structures of these compounds are illustrated in Figures 10 through 15, providing further insight into their structural characteristics.

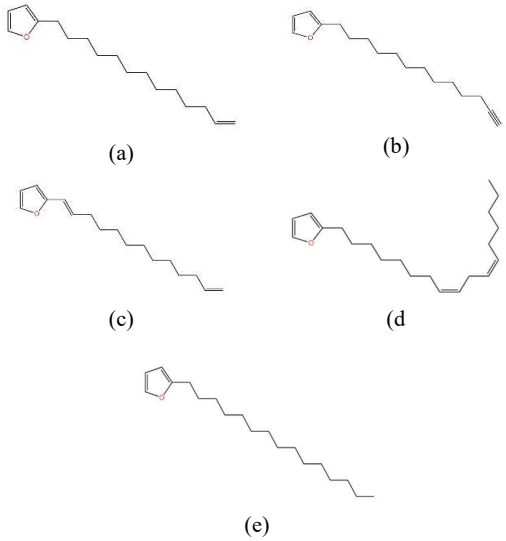

Fig 15. Chemical Structure of (a). Avocadenofuran, (b) Avocadynofuran, (c) E-Avocadienofuran, (d) 2-((8Z,11Z)-Heptadeca-8,11-dien-1-yl) furan, (e) 2-Pentadecylfuran/

Fig. 15 illustrate the chemical structures of the compounds that serve as indicators of flavonoid presence. The presence of a furan ring in the detected compounds evidences this. The formation of the furan ring is attributed to thermal degradation of flavonoid compounds during the analysis using Gas Chromatography–Mass Spectrometry (GC-MS). Flavonoids are particularly susceptible to high temperatures, which can lead to the breakdown of heterocyclic structures and glycosides, resulting in the formation of furan derivatives. Consequently, the results obtained from the GC-MS analysis do not reflect the original flavonoid compounds but rather the fragmentation products of furan.

Thus, this study demonstrates that the maceration method using "food grade" ethanol is more suitable for the extraction of alkaloid compounds than for flavonoids from avocado seeds. Alkaloids such as W-18 and Strychne were successfully identified through GC-MS due to their structural stability during the extraction process. In contrast, flavonoid compounds like Avocadenofuran and its derivatives underwent degradation into furan compounds, as evidenced by the GC-MS results and supported by literature from [25]. This degradation occurs because flavonoids are more vulnerable to heat and extraction conditions. Therefore, while the maceration-ethanol method is effective for alkaloids, the extraction of flavonoids may require alternative methods such as Soxhlet extraction or ultrasonic extraction with more suitable solvents to maintain compound stability.

4. CONCLUSION

Based on the research conducted, several conclusions can be drawn as follows: (1) The optimal flavonoid content obtained was 3.61419% at a maceration extraction time of 4.9021 days (approximately 5 days) and a weight ratio of 4.31341:10 (w/v). The optimization model derived from the analysis of 13 data points on flavonoid content using Response Surface Methodology (RSM) resulted in the following equations :

$$Coded: 1{,}54 + 1{,}27A + 0{,}1728B + 0{,}3814AB + 0{,}6135A^2 - 0{,}1192B^2$$

$$Actual: 1{,}33717 - 0{,}568851A - 0{,}002091B + 0{,}009536AB + 0{,}153367A^2 - 0{,}000298B^2$$

(2) GC-MS analysis confirmed that the optimized samples (samples 1 and 2) contained alkaloid compounds, specifically W-18 and Strychane, 1-acetyl-20a-hydroxy-16-methylene, as well as indicative flavonoid compounds such as Avocadenofuran, Avocadynofuran, E-Avocadienofuran, 2-((8Z,11Z)-Heptadeca-8,11-dien-1-yl) furan, and 2-Pentadecylfuran.

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