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Optimization Of Extraction Conditions For Total Phenols, Flavonoids, And Antioxidant Activity Of Avocado Seeds (Persea Americana Mill.) Using Simplex Lattice Design

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ABSTRACT

Avocado pits contain a diverse array of bioactive substances, such as procyanidins, phenolic compounds, triterpenoids, acetogenins, fatty amino acids, and mild acids, all exhibiting antioxidant properties. Antioxidants function by preventing oxidation, reducing the formation of harmful compounds, and protecting against aging, inflammation, and cancer. This research centers on assessing the impact of solvent proportions, pinpointing the ideal solvent mixture, and measuring the overall phenolic and flavonoid levels, along with the antioxidant capacity, of the top-performing solvent applied for extracting compounds from avocado seeds. The research method used the SLD (Simplex Lattice Design) Design Expert method. The extract formula was prepared using sonication, with the results showing 17 formulas for three solvents: acetone, distilled water, and 70% ethanol. Test parameters included phytochemical screening tests, specific and non-specific parameter tests, total phenol tests, total flavonoid tests, and antioxidant activity tests using the DPPH method. Statistical data analysis was performed using Design Expert with ANOVA (Analysis of Variance) output according to the designed experimental design. The results obtained based on the research are the 12th run formula with a solvent ratio of acetone, distilled water, and 70% ethanol (100:0:0) has an extract yield of 3.25%, total flavonoids of 17.302 mgQE/g extract, total phenols of 46.274 mgGAE/g extract, and antioxidant activity of 81.68 ppm, the optimisation results show that the optimum solvent composition is 100% Acetone, without a mixture of 70% Ethanol or Distilled Water.

Keywords: Avocado seed, antioxidant, SLD, IC50, Design Expert.

Introduction

Free radicals can be defined as molecular entities or molecular fragments that are capable of standing alone ('free'). Free radicals have one or more unpaired electrons in the outer atomic or molecular orbitals ('radicals'). The electron's negative charge may be counteracted by the positron's positive nuclear charge, producing a neutral species or forming either a cationic or anionic radical (Martemucci et al., 2022).

Antioxidant compounds are capable of inhibiting oxidative reactions despite being present in minimal quantities, thus protecting cellular structures by diminishing or preventing the free radical-induced oxidation of lipids, proteins,

carbohydrates, and DNA. Their primary mechanism involves the donation of electrons. Chain-breaking antioxidants act as strong electron donors that neutralize free radicals before these radicals can damage vital biomolecules. As a result, the antioxidants themselves undergo oxidation and must subsequently be renewed or substituted. Clinical studies have demonstrated that the use of suitable natural antioxidants can help slow the progression of neurodegenerative diseases (Martemucci et al., 2022).

The demand for natural antioxidants has risen in recent years, as they have been found to decrease the reliance on synthetic antioxidants, which may pose risks of toxicity, carcinogenicity, or hepatotoxicity in the body (Martemucci et al., 2022). These natural antioxidants are frequently found in plants, such as vegetables, fruits, herbs, and culinary spices, all of which serve as abundant sources of vitamins, phenolic compounds, carotenoids, and trace element radicals. Plant products are inexpensive antioxidants that provide many benefits and deserve special attention, such as leaves and seeds in addition to fruits, which contain many antioxidants (Flieger et al., 2021).

Avocados are recognized as fruits with exceptionally high nutritional value. Originating from Mexico and Central America, these flowering plants are members of the Lauraceae family. Research has indicated that avocados exhibit notable antioxidant properties, primarily attributed to their abundance of secondary metabolites and diverse bioactive molecules such as flavonoids, phenolic compounds, tannins, tocopherols, fatty alcohols, carotenoids, and anthocyanins (Marra et al., 2024). The seeds, in particular, contain substantial amounts of polyphenolic substances and display stronger antioxidant capacity compared to the fruit pulp (Dabas et al., 2019). Among the antioxidant agents identified in avocado seed extracts, flavonoids represent the most dominant group (Kopon et al., 2020).

Munthe et al. (2023) investigated the antioxidant activity, total phenolic content, and total flavonoid content in avocado seed extracts prepared using 70% ethanol. The findings were evaluated according to extraction yield, revealing productivity levels of 43.07% and 39.58%, respectively. The antioxidant capacities of the 70% ethanol extracts prepared via maceration and reflux techniques were 77.298 µg/mL and 98.626 µg/mL, respectively, whereas vitamin C exhibited an IC_{50} value of 12.883 µg/mL. Furthermore, the total phenolic concentrations of the macerated and refluxed extracts were 276.96 mg Gallic Acid Equivalent/g and 294.96 mg Gallic Acid Equivalent/g, while their total flavonoid concentrations measured 1.73 mg Quercetin Equivalent/g and 12.70 mg Quercetin Equivalent/g, respectively.

According to Vo et al. (2019), avocado seeds consist of (7.14 ± 0.40) g lipids, (1.67 ± 0.03) g protein, and (54.0 ± 1.2) g carbohydrates in every 100 g sample, along with (62.0 ± 2.3) mg GAE for each gram of dry extract. Among the tested fractions, dichloromethane and ethyl acetate exhibited the strongest free radical scavenging effects, with IC_{50} values of (48.0 ± 3.4) µg/mL in the DPPH assay and (22.0 ± 1.8) µg/mL in the ABTS assay, respectively.

Optimisation of avocado seed extract has been conducted in previous studies on avocado seed extract optimisation. Drawing from the study's background on

optimization, it is essential to perform optimization of total phenol extraction, total flavonoid extraction, and antioxidant activity evaluation in avocado seed extract.

Methodology

Research Design

This study employed an experimental design, formula determination, and data processing using DE (Design Expert) software with the SLD. This research seeks to identify the conditions yielding the highest flavonoid, phenolic, and antioxidant contents, whilst placing no emphasis on extraction yield in the pursuit of optimal outcomes. Exactly 20 grams of the powdered material were used, whereas the solvent amount adhered to the results provided by the DE system in Table 1.

Materials

The material utilized in this study consisted of avocado seeds collected from several regions, including Sleman. The chemicals used included: 70% ethanol, acetone (Merck), distilled water, sodium acetate (Merck), aluminium (III) chloride (Merck), Dragendroff's reagent, sodium hydroxide (Merck), gelatin, iron (III) chloride (Merck), sulphuric acid (Merck), p.a ethanol (Merck), p.a methanol (Merck), ascorbic acid (Sigma), gallic acid (Sigma), quercetin (Sigma), Follin-Ciocalteu reagent (Merck), and 2,2-diphenyl-1-picrylhydrazil (DPPH), Sigma.

Plant identification

Plant identification was performed at the Setia Budi University Laboratory in Surakarta to ensure the precision and authenticity of the identified plant.

Powder production

Twenty grams (20 g) of avocado seed powder were measured and transferred into a container following the solvent ratio indicated in Table 2. The powder was then subjected to extraction in each trial using the sonication method with different solvents-acetone, distilled water, and 70% ethanol-to evaluate total phenolic content, total flavonoids, and antioxidant activity. The following is the solvent composition output from the Design Expert 13 system using the SLD method:

Table 1. Composition of extraction solvents

Composition	Acetone	Aquadest	Ethanol 70%
1	33,3333	33,3333	33,3333
2	33,3333	0	66,6667
3	16,6667	16,6667	66,6667
4	66,6667	0	33,3333
5	0	0	100
6	66,6667	33,3333	0
7	0	33,3333	66,6667
8	0	66,6667	33,3333
9	16,6667	66,6667	16,6667
10	100	0	0
11	0	0	100
12	100	0	0
13	0	100	0
14	0	100	0
15	33,3333	33,3333	33,3333
16	33,3333	66,6667	0
17	66,6667	16,6667	16,6667

Optimisation of avocado seed extraction using modified Ultrasound-assisted Extraction (UAE). Twenty grams of avocado seed powder in an Erlenmeyer flask was added to acetone, distilled water, and ethanol solvents at a ratio of avocado seed powder (b/v). The sample was subjected to extraction in a sonicator bath operating at a frequency of 40 kHz, with the temperature maintained between 40–50°C and an extraction duration of 40–50 minutes. The mixture obtained was subsequently filtered and evaporated under reduced pressure with a rotary evaporator to yield a viscous extract (Kunarto et al., 2019).

Identification of Chemical Compounds

Determination of Alkaloids.

For each extract, 2 mL of diluted hydrochloric acid was used to dissolve the sample individually, followed by filtration. Subsequently, Dragendorff's test was conducted by introducing 3–4 drops of Dragendorff's reagent (potassium bismuth iodide solution) into the filtrate. The appearance of a reddish precipitate confirmed the presence of alkaloid compounds (Noorul et al., 2017).

Saponins Determination

A 0.5 g portion of the extract was combined with 2 mL of distilled water and agitated. The formation of a stable foam that lasted for 10 minutes signified the presence of saponins.

Tannins Determination

Gelatin Assay: Two milliliters of a 1% gelatin solution prepared in sodium chloride were mixed with the extract. The appearance of a white precipitate indicated the presence of tannins.

Determination of Phenolic Compounds

Ferric Chloride Test: The extract was combined with 3–4 drops of ferric chloride reagent, where the appearance of a blue-black hue signified the existence of phenolic substances.

Flavonoids Determination

In the Alkaline Reagent Test, the extract received three to four drops of sodium hydroxide solution. A vivid yellow hue emerging, which dissipated upon addition of diluted acid, indicated the presence of flavonoids.

Determination of Terpenoids

The combination of the extract with 2 mL sulfuric acid and 2 mL chloroform yielded a red coloration, indicating the presence of terpenoids.

Determination of Non-Specific Parameters

The evaluation of nonspecific parameters includes determining the moisture and drying loss levels, as well as measuring the total ash and acid insoluble ash contents.

Determination of Specific Parameters

The assessment of specific parameters includes the examination of identity and organoleptic characteristics, as well as the measurement of water-soluble and ethanol-soluble extract contents.

Determination of Total Phenols

Preparation of Sample Standard Solution

Weigh 40 mg of each avocado seed extract, add 3 ml of methanol to the mark, shake until dissolved, then place in a 5 ml volumetric flask and add p.a. methanol to make a concentration of 4000 µg/mL.

Preparation of Concentration Series

A series of gallic acid concentrations of 30, 45, 60, 75, and 90 ppm was prepared. The concentration series solution was prepared from a 400 ppm gallic acid stock solution. Pipette 0.75, 1.125, 1.5, 1.875, and 2.25 mL of the 400 ppm gallic acid stock solution, dissolve in p.a. methanol in a 10 mL volumetric flask, and homogenise.

Determination of Maximum Wavelength

Transfer 1 mL of the gallic acid solution to a vial and mix with 5 mL of 7.5% Folin–Ciocalteu reagent. Allow the mixture to stand for 8 minutes, followed by the addition of 4 mL of 1% NaOH solution. Incubate the mixture in darkness for 60 minutes. Measure the absorbance using a UV–Vis spectrophotometer within the wavelength range of 600 to 800 nm.

Determination of Operating Time (OT)

Transfer 1 mL of the gallic acid solution into a vial, followed by the addition of 5 mL of 7.5% Folin–Ciocalteu reagent. Let the mixture rest for 6 minutes, then incorporate 4 mL of 1% NaOH solution and store it in darkness for 60 minutes. Record the absorbance at the maximum wavelength with a UV–Vis spectrophotometer at various points from 0 to 60 minutes until the value stabilizes.

Preparation of the Gallic Acid Standard Curve

Gallic acid standard solutions were prepared in concentrations of 30, 45, 60, 75, and 90 ppm. A 1 mL portion from each concentration was pipetted into individual vials, then 5 mL of 7.5% Folin–Ciocalteu reagent was slowly introduced. Following an 8-minute reaction time, 4 mL of 1% sodium hydroxide was added, and the samples were incubated for 60 minutes. Absorbance was determined at λ_max using a UV–Vis spectrophotometer. Measurements were performed in triplicate for each concentration, with spectra collected between approximately 600 and 800 nm (Kemenkes RI, 2017).

Determination of Total Flavonoids

Preparation of Sample Standard Solution

Exactly 40 mg of each avocado seed extract was weighed out, after which 3 mL of absolute ethanol (p.a.) was added until the volume attained the calibration mark. The mixture was agitated thoroughly to ensure complete dissolution. Subsequently, the solution was transferred into a 5 mL volumetric flask, and ethanol (p.a.) was added up to the mark to yield a final concentration of 4000 µg/mL.

Preparation of Concentration Series

A quercetin concentration series of 25, 40, 55, 70, and 85 ppm was prepared from a 400 ppm quercetin stock solution. Aliquots of 0.6, 0.9, 1.2, 1.5, and 1.8 mL of the stock solution were pipetted into 5 mL volumetric flasks, diluted to volume with ethanol (p.a.), and homogenized.

Determination of Maximum Wavelength

A 0.5 mL portion of 50 ppm quercetin solution was transferred via pipette into a vial, after which 1.5 mL of ethanol (p.a.), 0.1 mL of 10% $AlCl_3$, 0.1 mL of 1 M sodium acetate, and 2.8 mL of distilled water were added sequentially. The absorbance was measured using a UV–Vis spectrophotometer across the wavelength range of 400–600 nm to determine the maximum absorbance peak.

Determination of Operating Time (OT)

The absorbance was measured with a UV–Vis spectrophotometer over the wavelength range of 400–600 nm to determine the maximum absorbance peak. In a vial, 0.5 mL of 50 ppm quercetin solution was mixed with 1.5 mL of ethanol (p.a.), 0.1 mL of 10% $AlCl_3$, 0.1 mL of 1 M sodium acetate, and 2.8 mL of distilled water. The absorbance of the resulting mixture was subsequently recorded at the established peak wavelength using a UV–Vis spectrophotometer from 0 to 30 minutes until the value stabilized.

Preparation of the Standard Curve for Quercetin

For every quercetin level (25, 40, 55, 65, and 80 ppm), 0.5 mL of the corresponding solution was transferred via pipette into a separate vial. To each vial, 1.5 mL of analytical-grade ethanol, 0.1 mL of 10% aluminum chloride solution, 0.1 mL of 1 M sodium acetate, and 2.8 mL of distilled water were added. The mixtures were vigorously agitated and then incubated at room temperature for 30 minutes. Absorbance readings were taken thereafter with a UV–Vis spectrophotometer at the predetermined λ_max. All experiments were conducted in three replicates (Kemenkes RI, 2017).

Determination of Antioxidant Activity

Preparation of DPPH solution

A 0.4 mM solution of DPPH was prepared by dissolving 15.77 mg of DPPH powder in methanol, followed by bringing the final volume to 100 mL (Alim et al., 2022).

Preparation of extract solution and standard series

To evaluate the antioxidant capacity of the avocado seed extract, 5 mg of the extract was dissolved in analytical-grade methanol and adjusted to a final volume of 10 mL, yielding a 500 ppm stock solution. From this stock, several aliquots were used to prepare working solutions with concentrations of 25, 50, 100, 150, and 200 ppm (Kunarto et al., 2019). Each working solution was subsequently mixed with 1 mL of 0.4 mM DPPH solution and additionally diluted using methanol to attain the ultimate volume. The resulting mixtures were incubated for 35 minutes, following which their absorbance readings were measured at 517 nm with a UV–Vis spectrophotometer (Septiawan, 2020).

Preparation of ascorbic acid reference solution

Ascorbic acid was employed as the reference antioxidant in this study. A stock solution at 1000 ppm concentration was prepared by dissolving 10 mg of ascorbic acid in analytical-grade methanol and bringing the total volume to 10 mL. From this stock, a series of working solutions containing 20, 40, 60, 80, and 100 ppm were prepared. Each prepared solution was combined with 1 mL of a 0.4 mM DPPH solution and then made up to volume. The resulting mixtures were gently agitated, covered to prevent light exposure, and left to stand for 30 minutes at ambient temperature. The

absorbance of every sample was determined at 517 nm with a UV–Visible spectrophotometer (Septyanani et al., 2023).

Determination of IC50 Value

The IC_{50} (50% Inhibitory Concentration) value denotes the sample concentration needed to achieve 50% reduction of DPPH free radicals (reflecting 50% antioxidant activity against oxidation). This IC_{50} was derived from the linear regression model y = bx + a, in which y represents the inhibition percentage and x denotes the sample concentration. Ascorbic acid solution was employed as the positive control (Agustin & Suroso, 2022).

Data Analysis

The investigation utilized a Box–Behnken design for experiments, assessing variables including extraction yield, total phenolic content, total flavonoid content, and antioxidant activity. Solvent optimization for extraction was achieved via a simplex lattice design (SLD) in Design Expert software version 13. Each experiment was replicated three times, with results reported as mean ± standard deviation. ANOVA ($p < 0.05$) was applied for statistical evaluation using Design Expert (DE) and Microsoft Excel 2021.

Result and Discussion

Powder production

Based on the drying results, 5000g of wet avocado seeds produced 1300g of dry weight with a yield of 26% w/w. The reduction in weight resulted from decreased water content and the evaporation of certain volatile substances, including essential oils, during drying at a steady temperature of 60°C. This value indicates that the yield of the crude drug produced has met the requirements for crude drug raw material yield, which is more than ≥ 10% (Kemenkes RI, 2017). The powdered avocado seed simplisia obtained after sieving using a 60-mesh sieve weighed 1100g, with 200g not passing through the sieve, resulting in a yield of 84.61% of the total dry weight of the simplisia.

Table 2. Organoleptic powder

Parameters	Observation Result
Smell	Characteristic of avocado seeds
Colour	Brown
Taste	Bitter
Texture	Fine Powder

Extract yield

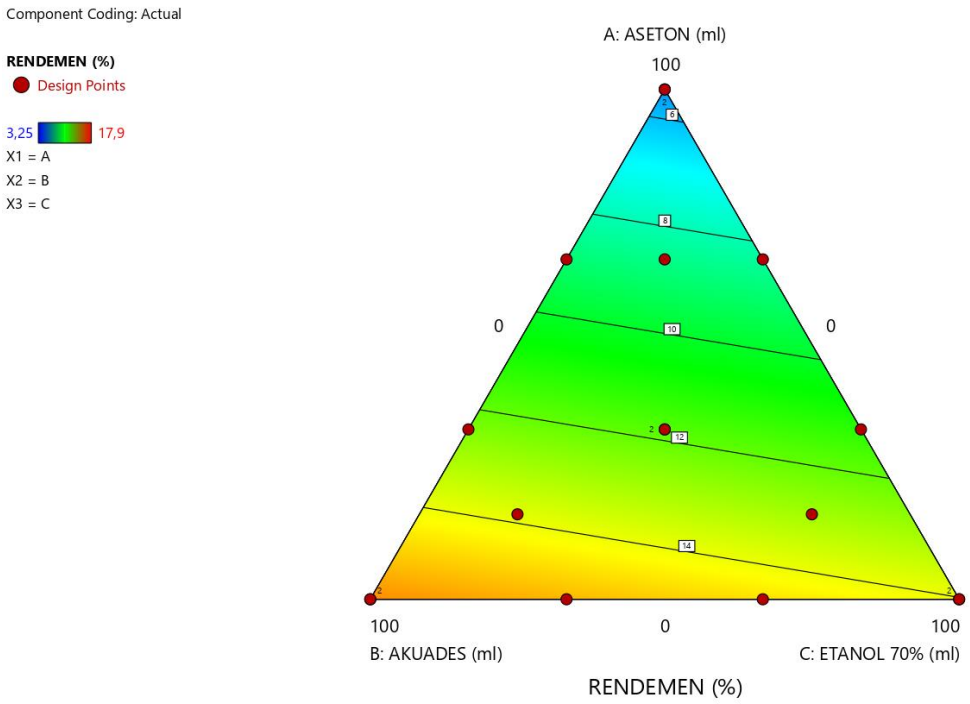
The research conducted using sonication extraction utilised high-frequency ultrasonic waves (>20 kHz) that caused acoustic cavitation. The experimental data showed variations in yield values for each run. extraction was carried out 4 times to obtain a high yield. The yield results are presented in Table 3 as follows:

Table 3. Extract yield

No	Run	Extract Weight (g)	Yield (%)
1	1	2,94	14,7
2	2	2,32	11,6
3	3	2,41	12,05
4	4	1,12	5,6
5	5	2,85	14,25
6	6	2,58	12,9
7	7	2,67	13,35
8	8	2,84	14,2
9	9	2,1	10,5
10	10	0,67	3,35
11	11	2,89	14,45
12	12	0,65	3,25
13	13	3,58	17,9
14	14	2,6	13
15	15	2,92	14,6
16	16	2,69	13,45
17	17	2,25	11,25

Based on the yield measurements from 17 experimental runs, the obtained yields ranged between 3.25% and 17.9%. The maximum yield (17.9%) was recorded in run 13, whereas the minimum yield (3.25%) appeared in runs 10 and 12. This variability could be attributed to several factors during the sonication process, including the duration of ultrasonic wave exposure, the intensity of the frequency, the temperature of the solution, and the uniformity of the simplicia particles. The application of polar solvents such as distilled water can dissolve not only polar compounds like flavonoids and tannins but also other substances such as proteins and carbohydrates (Azwan et al., 2020). The choice of solvent dramatically alters the range of extracted compounds, underscoring its pivotal influence on both the chemical makeup and bioactivity of extracts derived from avocado seeds (Satriawan & Wijaya, 2023). These polarity differences influence the solvent's capacity to disrupt bonds between bioactive compounds and the crude matrix, thereby directly affecting the resulting yield.

The results of statistical analysis using the SLD show that the quadratic model used to predict yield has good validity and feasibility. The model's R^2 of 0.6821 shows that the chosen predictors account for 68.21% of the yield's variability, with the leftover 31.79% stemming from unmodeled influences. The Adjusted R^2 value of 0.6367 shows that after being corrected for the number of predictors, the model still maintains its explanatory power well. The Predicted R^2 of 0.5400 confirms that this model is reliable enough to predict results outside the experimental data used in the model fitting process.



Picture 1. Countour plot

Based on the colour distribution, it can be seen that the use of aqua dest in high amounts (position near angle B) produces the highest yield (red-yellow), while acetone or 70% ethanol solvents tend to produce lower yields (blue-green). This is consistent with the nature of distilled water as a pure polar solvent that has a high ability to extract polar compounds such as flavonoid glycosides, phenolics, and tannins (Azwanida, 2015). The statistical evaluation via ANOVA demonstrates that the applied model is statistically meaningful, registering a p-value of 0.0003 (below 0.05) alongside an F-statistic of 15.02, which confirms the solvent's influence on output. The Lack of Fit assessment produced an insignificant outcome (p exceeding 0.05), implying strong alignment between observed measurements and forecasted figures.

Identification of Chemical Compounds

Avocado seed extract phytochemistry tests reveal that solvent choice markedly alters the variety of extractable bioactive agents.

Table 4. Identification of Chemical Compounds

Test	Alkaloids	Saponins	Tannins	Phenols	Flavonoids	Terpenoids
1-17	+	+	+	+	+	+
Exp	Red sediment at the bottom of the tube	3 cm of foam for 10 minutes	White sediment in the tube	Blue-green colour	Clear colour change after adding dilute HCl	Faded red colour change

Determination of Extract Characterisation

Non-Specific Parameters

Water Content

The moisture content analysis revealed a value of 9.9%, which falls below the maximum threshold specified in the Indonesian Herbal Pharmacopoeia (FHI)-not exceeding 10% for dried simplicia and thick extracts (Kemenkes RI, 2017).

Drying shrinkage

The extract exhibited a drying shrinkage of 9.8%, aligning with the specifications of the Indonesian Herbal Pharmacopoeia (FHI), which stipulates a maximum limit of 10% (Kemenkes RI, 2017).

Quantity of overall residue and portion undissolved in acid

According to the Indonesian Herbal Pharmacopoeia, the ash quantity in the extract from avocado seeds amounted to 9.8% (Kemenkes RI, 2017), the permissible limit is 12%, representing the inorganic mineral fraction originating from the plant material itself as well as from possible external contaminants introduced during processing. The avocado seed extract exhibited non-acid-soluble ash levels from 0.37% to 0.55%, demonstrating exceptional purity and complete adherence to the 2017 Indonesian Herbal Pharmacopoeia requirement of under 2%.

Table 5. Result of Non-Specific Parameters

Run	Water content (%) ± SD	shrinkage (%) ± SD	Ash content (%) ± SD	Acid-insoluble ash content (%) ± SD
1	9,88±0,003	9,44±0,0016	9,83±0,16	0,49±0,0009
2	9,96±0,001	9,37±0,0009	9,14±0,12	0,37±0,0007
3	9,85±0,003	9,55±0,0011	9,89±0,18	0,48±0,0010
4	9,85±0,003	9,07±0,0010	9,88±0,06	0,43±0,0013
5	9,93±0,014	9,24±0,0015	9,84±0,13	0,43±0,0012
6	9,85±0,003	9,18±0,0015	9,23±0,18	0,40±0,0008
7	9,88±0,003	9,22±0,0012	9,76±0,09	0,51±0,0003
8	9,96±0,003	9,24±0,0006	9,82±0,03	0,49±0,0009
9	9,88±0,003	9,18±0,0017	9,68±0,26	0,41±0,0014
10	10,00±0,001	9,30±0,0006	9,77±0,18	0,44±0,0005
11	9,92±0,0019	9,27±0,0007	9,76±0,19	0,52±0,0003
12	9,92±0,0019	9,33±0,0006	9,64±0,46	0,43±0,0014
13	9,96±0,0019	9,23±0,0006	9,86±0,14	0,42±0,0004
14	9,92±0,0019	9,20±0,0004	9,93±0,03	0,47±0,0004
15	9,85±0,0019	9,28±0,0005	9,81±0,09	0,43±0,0009
16	9,96±0,0019	9,24±0,0004	9,67±0,16	0,51±0,0008
17	9,88±0,0019	9,22±0,0008	9,79±0,15	0,55±0,0003

Specific Parameters

Identity and Organoleptic Properties of Extracts

Table 6. Organoleptic of Extracts

Parameters	Observation Result
Smell	Characteristic of avocado seeds
Colour	Dark brown
Taste	Bitter
Texture	Thick extract

Dissolvable-in-water fraction amount

The analysis of water-soluble extract content indicated that the majority of water-soluble compounds in the avocado seed extract were within the range of approximately 75.67–76.73%. According to the Indonesian Herbal Pharmacopoeia (2017), this value satisfies the established quality standard, which requires that the water-soluble extract content be no less than 6.7% (Kemenkes RI, 2017).

Amount of extract that dissolves in ethanol

The test outcomes indicated that the portion of avocado seed extract dissolvable in ethanol showed an extremely tight variation, fluctuating solely from 89.2% to 89.9%. According to the 2017 Herbal Pharmacopoeia, which is often used as a quality standard for herbal simplisia and extracts, the minimum content is ≥10.3% (Kemenkes RI, 2017).

Table 7. Water-soluble and etanol extract content

Run	Amount of extract that dissolves in water (%) ± SD	Amount of extract that dissolves in ethanol (%) ± SD
1	76,27±1,81	89,3±0,29
2	75,67±1,16	89,9±0,61
3	76,73±1,10	89,5±0,26
4	76,03±1,17	89,5±0,32
5	76,20±1,64	89,5±0,40
6	76,63±0,68	89,5±0,35
7	76,13±0,84	89,4±0,36
8	76,50±1,97	89,2±0,26
9	76,57±1,16	89,5±0,45
10	76,20±1,21	89,4±0,31
11	76,37±1,72	89,5±0,42
12	76,00±0,95	89,6±0,44
13	76,37±1,58	89,4±0,40
14	76,57±1,16	89,5±0,36
15	76,23±1,59	89,3±0,30
16	76,23±1,18	89,6±0,40
17	76,30±1,61	89,4±0,29

Determination of Total Flavonoids

Measurement of Maximum Wavelength (λ)

The UV-Vis spectrophotometric analysis indicated absorbance values within the wavelength range of 400–500 nm. The maximum absorbance peak was observed at 441 nm, corresponding to an absorbance value of 0.2258.

Determination of Operating Time (OT)

The results showed that absorbance values began to stabilize from the 3rd minute and remained consistent up to the 33rd minute. After the 33rd minute, the absorbance stayed stable, suggesting that the complex remained intact and did not undergo significant degradation until the 40th minute.

Determination of Total Flavonoid Content

The flavonoid concentration in the avocado seed extract was determined based on a linear regression equation obtained from the calibration curve of quercetin

standards. The calibration curve was constructed using quercetin solutions at concentrations of 25, 40, 55, 70, and 80 ppm, resulting in the regression equation y = 0.0071x + 0.1546 and a correlation coefficient (R^2) of 0.9947, as analyzed using Excel 2021.

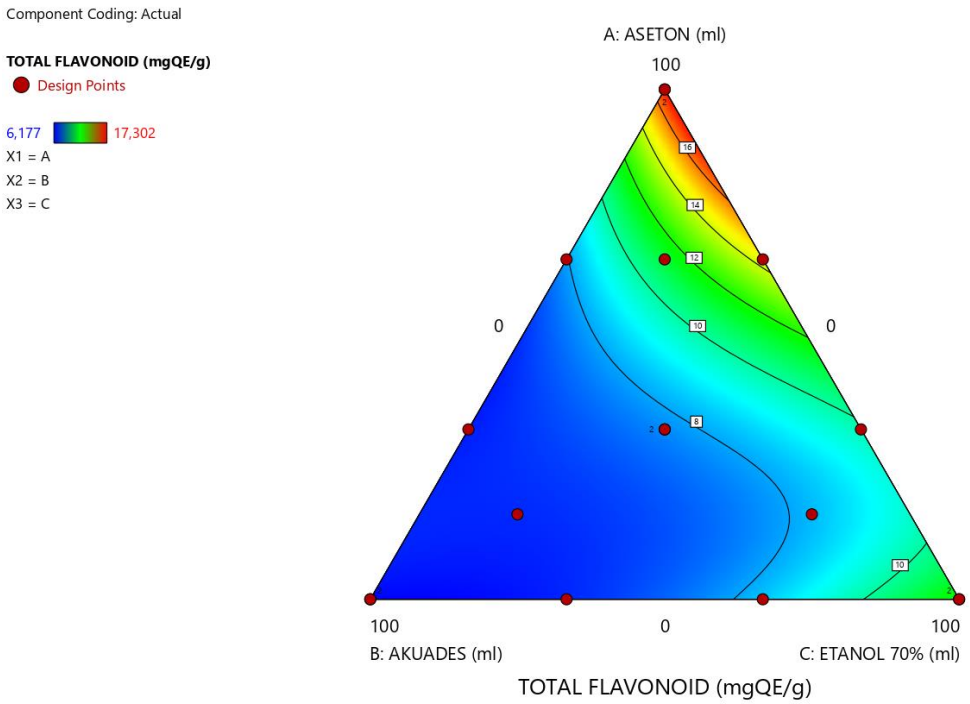
Table 8. Total Flavonoids Content

Run	Absorbance	Flavonoids Content (mgQE/g)±SD
1	0,307	7,79±0,14
2	0,410	9,55±0,44
3	0,351	8,52±0,32
4	0,727	14,63±0,07
5	0,560	12,14±0,43
6	0,323	8,07±0,11
7	0,336	8,19±1,15
8	0,254	6,69±0,08
9	0,234	6,54±0,56
10	0,812	16,80±0,05
11	0,559	12,00±0,83
12	0,874	17,30±0,17
13	0,216	6,47±0,43
14	0,213	6,17±0,12
15	0,305	7,65±0,10
16	0,226	6,46±0,16
17	0,501	10,86±0,08

The average absorbance values and average flavonoid content from 17 measurement runs showed that the measured absorbance values ranged from 0.213 to 0.874, with flavonoid content between 6.17-17.30 mgQE/g, indicating that the absorbance value and flavonoid content increased in line with the increase in the concentration of the analysed substance (Meija et al., 2016). Avocado seed extracts with 70% ethanol solvent in runs 5 and 11 produced absorbance values of ±0.560 and flavonoid content of approximately 12 mgQE/g. This indicates that 70% ethanol is quite effective in extracting flavonoids because this solvent has the appropriate polarity to dissolve flavonoid compounds (Azmir et al., 2013). Acetone solvent in runs 10 and 12 had higher absorbance values and flavonoid content above 0.8 and flavonoid content of more than 16 mgQE/g, indicating that acetone is also effective, especially for flavonoids that are slightly less polar. The distilled water solvent in runs 13 and 14 showed low absorbance of around 0.21–0.22 and flavonoid content of ±6 mgQE/g, indicating that distilled water is less effective in extracting flavonoids from the material because flavonoids are difficult to dissolve in distilled water (Azzahra et al., 2022).

The analysis results from the SLD indicate that the developed quadratic model effectively represents the relationship between solvent variables and flavonoid content. This model considers not only the individual effects of each solvent (A, B, C) but also their interactive effects (AB, AC, BC). The coefficient of determination (R^2 = 0.9977) demonstrates that the variation in flavonoid content is well explained by the model. Meanwhile, the adjusted R^2 value (0.9947) refines this measure by accounting for the number of predictors, and the predicted R^2 value (0.9673) confirms the

model's accuracy in forecasting flavonoid content for various solvent combinations. Furthermore, the Adeq Precision value of 53.18 (>4) indicates that the signal-to-noise ratio is excellent, confirming the model's reliability for optimisation purposes.



Picture 2. Countour plot

Based on the plot, increasing the proportion of acetone solvent (A) leads to a significant rise in extracted flavonoid content. The findings suggest that acetone is the most effective solvent for flavonoid extraction, as shown by the dominant red region alongside A (100%) in the ternary diagram. This agrees with previous studies reporting that acetone efficiently extracts flavonoids due to its strong ability to penetrate plant cell walls and dissolve phenolic compounds (Sasidharan et al., 2011). Similarly, acetone demonstrated a high capacity for flavonoid extraction, marked by the intense red colour in the diagram (Soledad et al., 2021).

The statistical evaluation using ANOVA demonstrated that the regression equation achieved significance, recording an F-statistic of 334.42 alongside a p-value of 0.0001 (below the 0.05 threshold). This confirms that variations in solvent composition have a significant effect on the total flavonoid content (mgQE/g). Additionally, the non-significant Lack of Fit result indicates that the regression model fits the experimental data well, allowing accurate prediction and optimisation of total flavonoid yield based on solvent combinations.

Determination of Total Phenols

Measurement of Maximum Wavelength (λ)

Based on the UV-Vis spectrum results obtained, the λmax value was 731 nm with an absorbance value of 0.3636. Measurements were taken in the wavelength range between 400-800 nm.

Determination of Operating Time (OT)

The stable absorbance value indicates that the ideal reaction time is around 53–58 minutes. This is in line with the FC method standard, which generally recommends incubation for 30 minutes before reading the absorbance. Molole et al., (2022) are consistent with research conducted on reaction times, which found that the ideal absorbance reading time is between 30–60 minutes (Sasidharan et al., 2011).

Determination of Total Phenols Content

The quantification of overall phenolic compounds in the extract from avocado seeds employed a calibration curve based on gallic acid standards prepared at concentrations of 25, 45, 65, 85, and 105 ppm, yielding an equation for linear regression. The analysis results showed a regression equation of y = 0.0049x + 0.2205 and R^2 = 0.9985.

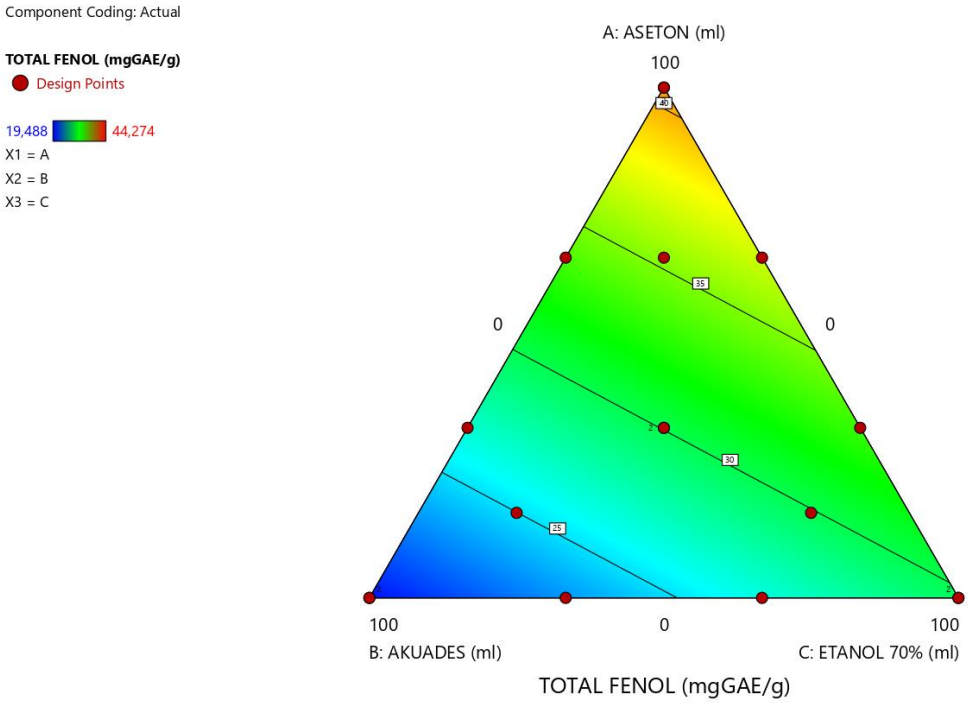
Table 9. Total Phenols Content

Run	Absorbance	Phenols content (mgGAE/g) ± SD
1	0,475	33,18±0,59
2	0,472	32,35±1,12
3	0,429	31,86±0,13
4	0,601	40,44±0,54
5	0,371	29,24±0,04
6	0,490	31,81±0,49
7	0,258	23,79±0,05
8	0,248	23,32±0,11
9	0,180	20,33±0,35
10	0,677	36,15±1,44
11	0,382	29,44±0,29
12	0,748	46,27±0,4
13	0,185	19,48±0,38
14	0,202	19,67±0,63
15	0,479	33,20±1,49
16	0,427	32,04±0,41
17	0,601	30,57±1,12

From the results of 17 experiments, the solvent makeup clearly plays a major role in determining the overall phenol content obtained. The value obtained in the 12th run, where 100% acetone was used, showed the highest phenol content of 46.27 GAE/g, indicating that acetone is a very effective solvent for extracting phenolic compounds from alpukat seed extract. In runs 13 and 14, distilled water was used as the sole solvent (100%), and the phenol content obtained was the lowest, namely 19.48 GAE/g and 19.67 GAE/g, indicating that distilled water is not effective enough as a solvent for phenolic compounds because it can only dissolve highly polar phenolic compounds. Ethanol 70% was also used both alone and in mixtures. In runs 5 and 11, the phenol content produced was 29.24 mg GAE/g and 29.44 mg GAE/g, respectively. Seventy percent ethanol is polar, but not as strong as water, so its ability to dissolve phenolic compounds is between that of water and acetone.

The findings from applying the Simplex Lattice Design (SLD) approach indicate that the formulated quadratic equation accurately captures the connection

between solvent factors and overall phenolic levels. Statistical evaluation shows an R^2 value of 0.8049, indicating that 80.49% of the variation in total phenolic content is explained by the regression model. This indicates that the framework effectively captures the connection linking the input factor (solvent makeup) to the output measure (phenol level). The adjusted R^2 figure at 0.7770 alongside the predicted R^2 figure at 0.7206 additionally verify the framework's strong capability for interpreting and forecasting values outside the dataset employed in its development. The Adequate Precision value of 14.9730 (>4) signifies a high signal-to-noise ratio, implying that most of the data variation is due to factors accounted for by the model rather than random error or noise.



Picture 3. Countour plot

The effect of aquadest on the lowest phenol content (blue colour) is located at the bottom left of the contour, which is the area of the plot with aqua dest variation approaching 100%. This indicates that aqua dest is less effective in extracting phenolic compounds, especially when compared to organic solvents such as acetone and ethanol. Water tends to only dissolve highly polar compounds (Dai & Mumper, 2010). Solvents using 70% ethanol showed fairly good results in increasing phenol levels, although not as high as pure acetone. The area on the lower right side of the contour (the area dominated by 70% ethanol) shows a green-yellow colour, which means that the phenol levels are moderate to fairly high.

Based on the profile examination, one may conclude that acetone functions as the optimal medium for isolating overall phenolic compounds from avocado seed material. A mixture of acetone and 70% ethanol also yields relatively high phenolic levels, though still lower than those obtained with pure acetone. Statistical analysis via ANOVA confirms the model's validity, showing a highly significant effect ($p = 0.0001$, below 0.05 threshold; $F = 334.42$), which proves that changes in the predictor strongly affect the outcome (total phenolics). The insignificant lack-of-fit term

indicates excellent alignment between the model and observed data, ensuring accurate depiction of the process.

Determination of Antioxidant Activity

Maximum Wavelength (λ) Measurement

The maximum wavelength (λmax) was identified to determine the peak absorption (maximum absorbance) of the DPPH solution within the wavelength range of 515–517 nm. From the λmax determination results using a 40 ppm DPPH solution, the obtained wavelength was 516 nm.

Determination of Operating Time (OT)

The measurement results indicate that the standard operating time value for ascorbic acid ranges from 41 to 47 minutes, while the optimal extract shows an operating time of 54 to 58 minutes for the best-performing sample, run 12.

Antioxidant Activity of Avocado Seed Extract

According to the results presented in the table above, the IC_{50} values for runs 1 through 17 range from 81.68 to 178.65 µg/mL. According to the system outlined in Jin et al. (2023), extracts exhibit exceptionally potent antioxidative effects from 1 to 50 µg/mL, robust effects between 50 and 100 µg/mL, fair effects in the 100–150 µg/mL range, and feeble effects above 150 µg/mL. The avocado seed extract contains several bioactive compounds with notable antioxidant potential, with β-sitosterol and avocadenofuran identified as the key contributors to this activity.

Table 10. Antioxidant Activity

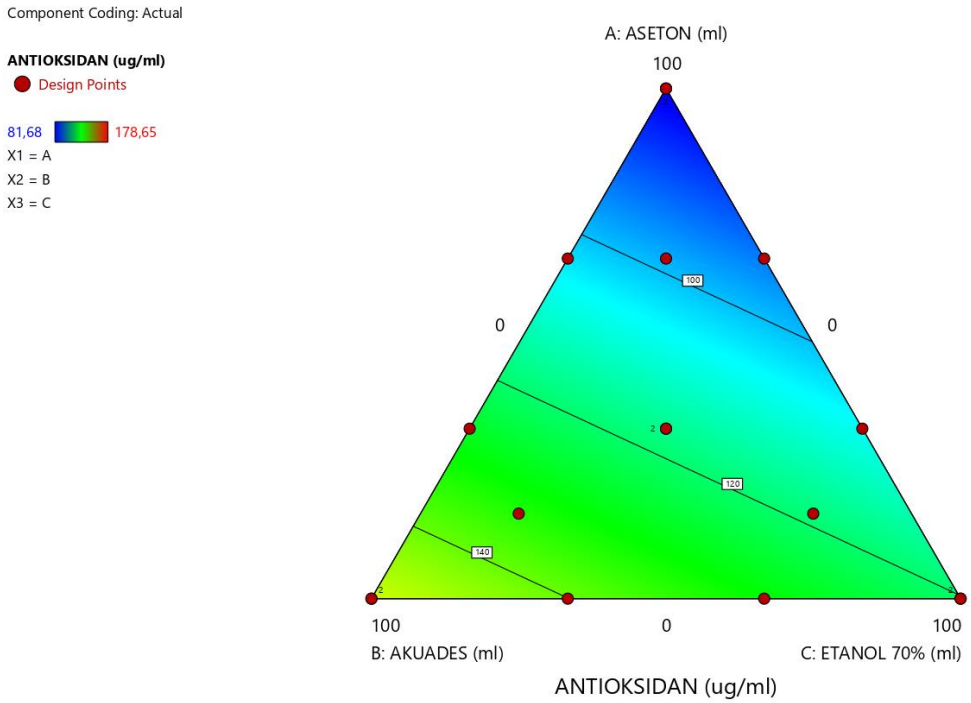
Run	DPPH Absorbance	Average			IC50± SD	Antioxidant Category
		a	b	r		
Ascorbic acid	0,811	2,4043	2,9565	0,9696	16,09±0,12	Veri strong
1	0,784	1,4200	0,4374	0,9596	111,05±0,59	Moderate
2	0,811	11,33	0,3602	0,9071	107,33±0,51	Moderate
3	0,784	1,3137	0,4058	0,9483	120,05±3,12	Moderate
4	0,784	5,6675	0,4747	0,9733	93,42±2,68	Strong
5	0,784	0,2806	0,4030	0,9905	123,37±1,63	Moderate
6	0,784	5,4294	0,4445	0,8876	100,26±0,18	Moderate
7	0,811	4,9075	0,3637	0,9790	124,01±2,58	Moderate
8	0,811	3,1894	0,3436	0,9773	136,23±0,26	Moderate
9	0,784	4,0136	0,338	0,9638	136,10±2,32	Moderate
10	0,784	6,9515	0,4985	0,9394	86,35±0,14	Strong
11	0,811	4,1636	0,3676	0,9882	124,68±0,73	Moderate
12	0,784	26,522	0,2874	0,9848	81,68±0,55	Strong
13	0,784	1,2032	0,2731	0,9365	178,65±1,59	Weak
14	0,784	7,8359	0,3084	0,9904	136,72±2,81	Moderate
15	0,784	2,1343	0,4332	0,9290	110,49±0,73	Moderate
16	0,784	4,0178	0,4021	0,9882	114,32±2,39	Moderate
17	0,784	7,2534	0,4281	0,9111	99,84±0,25	Strong

The measurement and analysis results revealed that the 4th run achieved an antioxidant activity value of 93.42±2.68 µg/mL, the 10th run 86.35±0.14 µg/mL, and the 12th run 81.68±0.55 µg/mL, all of which were classified as having strong activity. Most of the remaining 17 runs fell into the moderate activity category, whereas the 13th run showed a value of 178.65±1.59 µg/mL, categorised as weak. The observed variation in IC_{50} values among runs is likely due to differences in the composition and

interactions of the bioactive compounds present in each sample. Polyphenols such as anthocyanins, catechins, and proanthocyanidins function vitally in deactivating reactive oxygen species through the transfer of protons or charged particles, thus rendering the species inert and halting propagation of damage cascades (Esati et al., 2022).

According to the findings of Loizou et al. (2010), avocadenofuran isolated from avocado seed extracts prepared with semipolar solvents such as acetone exhibited radical scavenging activity comparable to that of synthetic antioxidants like BHT (butylated hydroxytoluene). The furan ring structure, with its conjugated electron system, enables these compounds to stabilise free radicals through resonance after donating hydrogen atoms or electrons. This mechanism halts the propagation of oxidative chain reactions in lipids and other biomolecules. Moreover, the polar functional groups in avocadenofuran facilitate interactions with transition metal ions such as Fe^{2+} or Cu^{2+}, thereby inhibiting the generation of hydroxyl radicals via the Fenton reaction.

Analysis using the Simplex Lattice Design (SLD) approach demonstrated a correlation between the solvent type employed and the observed antioxidant activity. The model achieved an R^2 of 0.8401, meaning it accounts for 84.01% of the variation in antioxidant activity. An Adjusted R^2 of 0.8172 offered a refined estimate after penalizing for additional predictors, and a Predicted R^2 of 0.7088 demonstrated solid performance on unseen observations. Furthermore, an Adeq Precision value of 16.8375 (>4) suggested an adequate signal-to-noise ratio, confirming that the model was reliable for interpretation and optimisation purposes.



Picture 4. Countour plot

The colours in the diagram indicate the level of antioxidant activity produced by each solvent combination. The blue colour depicts low antioxidant activity (around 81.68 µg/mL), while the green to red colour shows an increase in antioxidant activity, with the highest value recorded at 178.65 µg/mL. Based on the colour distribution in the diagram, it can be seen that the dominant solvent combination of Acetone and

70% Ethanol resulted in higher antioxidant activity. This is in line with the literature which states that semipolar organic solvents such as Acetone and Ethanol effectively extract phenolic compounds and flavonoids that act as antioxidants (Rozemeijer et al., 2019). Polar distilled water solvents tend to be less effective in extracting these active compounds from certain natural ingredients, which causes low antioxidant activity in formulations with high distilled water content (Asra et al., 2019).

ANOVA test results showed a p value = 0.0001 (<0.05) that the model is statistically significant. The solvent variation as independent variable significantly influenced the antioxidant activity as response variable. The Lack of Fit test which resulted in an insignificant value indicates that the quadratic model has a good fit with the experimental data.

Optimisation of Optimum Formula

Optimisation of solvent composition on yield value, total flavonoid content, as well as antioxidant activity was conducted through comparative analysis of the most effective solvent formulation. The procedure entailed evaluating four key variables—yield, total flavonoid concentration (expressed as mg QE per g of extract), total phenolic concentration (expressed as mg GAE per g of extract), and antioxidant potency (measured as IC_{50} in ppm)—via the Simplex Lattice Design approach incorporating three components across four degrees, analyzed using Design Expert software release 13.

Table 11. Determination of the optimum formula

Solvent Parameters		Limits	
Solvents	Low composition	High composition	Target
Acetone	0	100%	In Range
Aquadest	0	100%	In Range
Etanol 70%	0	100%	In Range
Parameters test	Upper limit	Lower limits	Target
Yield	17,9 %	3,25 %	Maximize
Flavonoid Content	17,30 mgQE/g	6,17 mgQE/g	Maximize
Phenol content	46,27 mgGAE/g	19,48 mgGAE/g	Maximize
Antioxidants	81,68 µg/mL	178,65 µg/mL	Maximize

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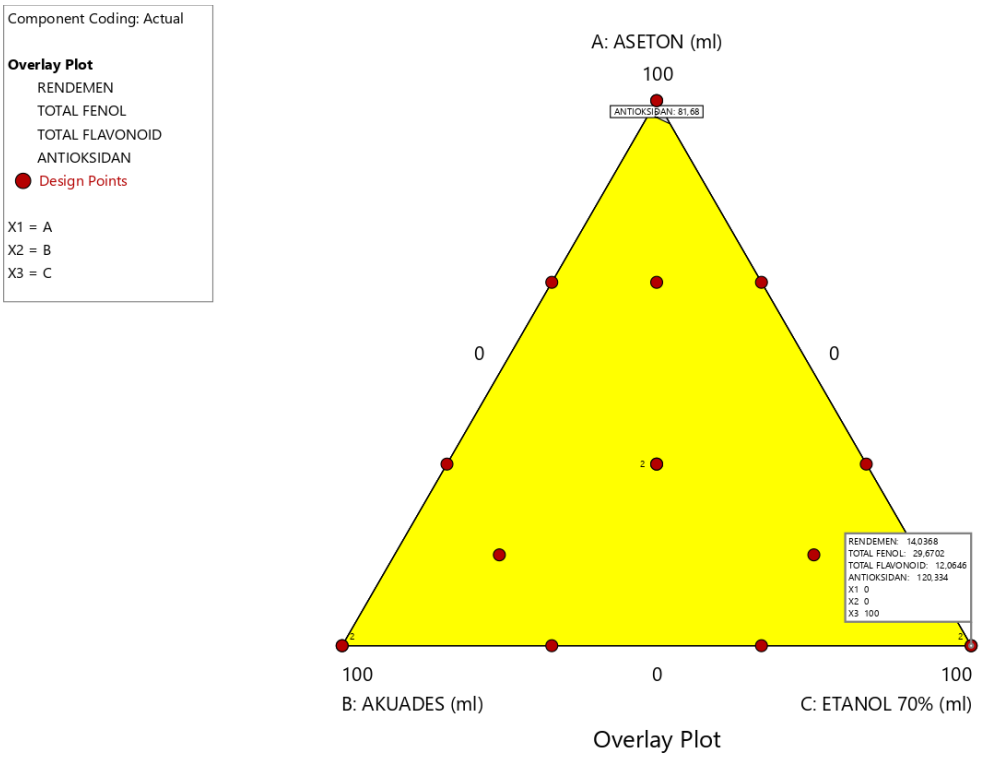

Picture 5. Countour plot

The overlay plot presented above illustrates the outcomes derived from the simplex lattice grid design. The optimisation analysis clearly identifies the pure ethanol extract as the most effective formulation. The yellow region delineates the feasible formulation space that satisfies the minimum acceptable criteria across all measured parameters. The design points, denoted by red markers and distributed throughout the triangular plot, represent the experimental runs that were actually conducted. At the vertices of the triangle—corresponding to pure acetone (A), distilled water (B), and 70% ethanol (C)—the expert design software has determined, post-optimisation, that the optimum composition is a ternary ratio of 0:0:100 (acetone : distilled water : 70% ethanol). This optimal formula yields an extraction yield of 14.0368%, a total flavonoid content of 12.0646 mg QE/g extract, a total phenolic content of 29.6702 mg GAE/g extract, and an antioxidant activity expressed as an IC_{50} value of 120.334 ppm.

The limit values for each parameter were obtained from the results of the study Yield ranged from 3.25% to 17.90%, Total flavonoids between 19.48 to 46.27 mgQE/g, Total phenols between 6.177 to 17.302 mg GAE/g, and IC50 antioxidant activity between 81.68 to 178.65 µg/mL. Although the extraction yield itself was comparatively modest, this particular combination was chosen as the optimal one owing to its delivery of the highest concentrations of flavonoids, total phenolics, and antioxidant activity across all the conditions evaluated. In phytopharmaceutical studies, it is a well-established practice to favour the enrichment of bioactive compounds over maximising crude yield, since the therapeutic value and biological effectiveness of plant extracts depend chiefly on the quality and potency of their secondary metabolites rather than on the sheer amount of raw material obtained (Azmir et al., 2013).

Acetone proved to be the most effective solvent in extracting phenolic compounds and enhancing antioxidant activity, the 12th run produced the best IC_{50} value and the highest total phenol content, despite the relatively small yield. The use of pure acetone solvent (100% (100:0:0)) in the 12th run proved to give optimum results for antioxidant activity and highest phenolic compound content suggesting that acetone is the most effective solvent to extract bioactive compounds from specific plant materials in the context of this study. Despite having a low yield, the quality of the extract is much higher than other solvents.

The software identifies pure (100%) ethanol as the best operating condition because, when the ternary contour is examined, the point closest to the ethanol vertex (approximately 70% on the plot, but the model extrapolates toward absolute purity) delivers the highest simultaneous performance across critical outputs, namely total phenolic content (reaching 29.07 mg GAE/g) and antioxidant capacity (120.334%). This superiority stems from the regression-based multi-objective optimization that the program applied. Being a strongly polar solvent, ethanol excels at extracting polar bioactive molecules like flavonoids and other phenolic antioxidants, which explains why extracts obtained in that zone exhibit the strongest biological properties particularly when the optimization goal prioritizes extraction yield over aspects like solvent price or safety profile. Compared to formulations containing acetone or water, the predicted results with neat ethanol are markedly superior.

The primary reason the desirable (yellow) zone extends so widely toward pure ethanol in the Design Expert output is usually an inconsistency in how the desirability function was configured. For instance, placing excessively permissive upper bounds on antioxidant activity or phenolic content allows the “sweet spot” to shift and enlarge toward 100% ethanol, ignoring practical considerations such as long-term extract stability, operational cost, or regulatory restrictions on solvent residues. Another common mistake is relying on an ordinary quadratic model without proper validation (lack of lack-of-fit testing or external validation), which tends to inflate performance predictions near the ethanol corner. Additionally, omitting real-world constraints like mixture viscosity, boiling-point differences, or evaporation rate further distorts the feasible region, making it appear unrealistically large for industrial application. Consequently, the software may suggest absolute ethanol even when a blended system would be far more realistic. This artifact can be fixed by assigning appropriate importance weights or priorities to secondary factors (e.g., incorporating some acetone to improve drying properties or reduce costs) and by tightening the optimization constraints to reflect actual production requirements (Do et al., 2014).

Acetone’s superior performance stems primarily from its intermediate polarity (Reichardt polarity index ~0.355 or dielectric constant value 20.7), placing it in an optimal “sweet spot” for solubilizing semi-polar secondary metabolites. Flavonoids and other phenolics typically possess both hydrophilic hydroxyl moieties and hydrophobic aromatic scaffolds, making them poorly extracted by either highly polar solvents (water, aqueous ethanol) or highly non-polar ones (hexane). Highly polar solvents co-extract large amounts of polysaccharides, sugars, and organic acids, inflating crude extract mass while diluting the target bioactives; conversely, non-polar solvents largely exclude phenolics altogether (Do et al., 2014). Acetone, by

contrast, effectively partitions the desired semi-polar compounds into solution while leaving most polar impurities and non-polar lipids behind, achieving a markedly higher concentration of bioactive principles per unit of dry extract (Boeing et al., 2014). Regarding selectivity, acetone stands out for its highly targeted action: it efficiently solubilizes key flavonoids and phenolic compounds while leaving behind most chlorophyll, waxes, lipids, and other non-phenolic contaminants that are readily co-extracted by highly polar solvents such as water or aqueous ethanol. Consequently, acetone-derived extracts exhibit a markedly higher concentration of bioactive constituents per gram of dry material and deliver significantly greater antioxidant potency on a weight-for-weight basis (Ilbay et al., 2014).

Pure acetone typically delivers a markedly lower crude extract yield (often just 3–8% w/w) compared to distilled water (15–35% w/w) or 70% aqueous ethanol (12–25% w/w). Far from being a disadvantage, this modest yield signals exceptional selectivity for medium-polarity phenolics and flavonoids. Both water and 70% ethanol readily solubilize abundant hydrophilic matrix components sugars, polysaccharides, organic acids, and soluble proteins producing bulky extracts in which the target bioactives are heavily diluted. Acetone, with its intermediate dielectric constant of ≈20.7, largely excludes these highly polar impurities while efficiently capturing aglycone flavonoids, phenolic acids, and related semi-polar antioxidants. Consequently, although the recovered dry mass is small, the resulting acetone fraction routinely exhibits 2- to 4-fold higher total phenolic content (TPC), 3- to 6-fold greater total flavonoid content (TFC), and antioxidant capacity (whether measured by DPPH IC_{50} or FRAP) up to five times more potent on a per-milligram basis than extracts obtained with water or 70% ethanol (Ilbay et al., 2014). Thus, the reduced crude yield is not a limitation but clear evidence of superior enrichment and purity of the biologically active principles, rendering acetone the solvent of choice whenever maximum specific activity rather than sheer extract quantity is the primary objective.

Conclusion

The evaluation of various solvents significantly influenced the overall phenolic levels, flavonoid quantities, and antioxidative capacity. Using the SLD (simplex lattice design) method, the optimal formulation was identified in the 12th experimental run, which employed a solvent ratio of acetone, distilled water, and 70% ethanol (100:0:0). This optimized solvent composition produced a yield of 3.25%, contained 17.30 mgQE/g extract of total flavonoids, 46.27 mgGAE/g extract of total phenolics, and demonstrated antioxidant activity with an IC_{50} value of 81.68 µg/mL.

Statement on Conflicts of Interest

The author states that there were no business ties or monetary exchanges that could be seen as a possible conflict of interest during the execution of this study.

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