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Optimization of Broccoli (Brassica oleracea var. italica Plenck) and Carrot (Daucus carota L.) Extract Combination Concentration Using Control Composite Design on Antioxidant Activity and Total Phenolic Content

Arum Tri Handayani[1], Anna Fitriawati[2], Annie Rahmatillah[3]

[1,2,3]Duta Bangsa University
E-mail: arumtrihandayani0325@gmail.com, anna_fitriawati@udb.ac.id, annie_rahmatillah@udb.ac.id

Abstract

Oxidative stress caused by free radicals is a major contributor to premature aging and skin damage. Natural antioxidants from a combination of broccoli (Brassica oleracea L. var. italica) and carrot (Daucus carota L.) extracts have great potential to counteract these effects. This study aimed to optimize the concentration of the combined extract to achieve the highest antioxidant activity (lowest IC_{50}) and the highest total phenolic content. The method used for optimization was Central Composite Design (CCD) with the aid of the Design Expert software. Extracts were prepared through maceration with a 70% ethanol solvent. Antioxidant activity (IC_{50}) was measured using the DPPH method, total phenolic content was determined with the Folin-Ciocalteu method, and phytochemical screening was also conducted. The results showed that the optimal combination ratio was a 5%:5% broccoli and carrot extract, which yielded the highest antioxidant activity (IC_{50} of 25.23 µg/mL) and the highest total phenolic content (148.935 mg GAE/g). Increasing the concentration of both extracts significantly enhanced both responses, with the broccoli extract showing a more dominant effect. The high desirability value of 0.985 indicates that this solution is very close to the ideal outcome.

Keywords: Antioxidant, Phenolic, Broccoli, Carrot, Central Composite Design

INTRODUCTION

Research Phenomenon

The skin, as the body's outermost organ, is continuously exposed to ultraviolet (UV) radiation, which triggers the formation of reactive oxygen species (ROS) and accelerates oxidative stress, leading to premature aging and cellular damage. Although the stratum corneum naturally produces antioxidants, their levels decline with age and environmental stressors, making topical antioxidant supplementation increasingly important (Widgerow et al., 2023; Andarina & Djauhari, 2017). Vegetables such as broccoli (Brassica oleracea var. italica) and carrots (Daucus carota L.) are recognized for their rich antioxidant profiles, with broccoli containing glucoraphanin that converts to sulforaphane, and carrots being a source of beta-carotene, both contributing to free radical neutralization (Kim & You, 2023; Jung, 2024).

Recent studies highlight the synergistic potential of combining broccoli and carrot extracts, as their distinct antioxidant compounds—sulforaphane, flavonoids, vitamin C from broccoli, and carotenoids, polyphenols from carrots—may enhance overall antioxidant efficacy (Jiang et al., 2015; Maharani et al., 2025). The optimization of such combinations using Central Composite Design (CCD) and advanced analytical methods is crucial for developing effective topical formulations (Muttaqin, 2019; Dewantara et al., 2021).

Research Problem

Despite the known antioxidant benefits of broccoli and carrot extracts, the optimal concentration ratio for maximizing antioxidant activity and total phenolic content remains unclear. Previous research has primarily focused on single extracts, with limited exploration of their combined effects and the

mechanisms underlying their synergistic interactions (Handayani et al., 2022; Sami & Rahimah, 2015). The lack of standardized methods for evaluating and optimizing extract combinations further complicates the formulation of effective antioxidant gels (Pitaloka, 2024; Pandanwangi et al., 2018).

Moreover, the variability in extraction techniques, solvent selection, and phytochemical screening protocols can significantly influence the yield and efficacy of bioactive compounds (Saerang et al., 2023; Feninlambir et al., 2023). The challenge lies in systematically analyzing the impact of each extract's concentration on antioxidant activity (IC50) and total phenolic content, while ensuring reproducibility and statistical significance in laboratory settings (Firdayani et al., 2015; Dewi et al., 2024).

The absence of comprehensive optimization studies using robust experimental designs, such as CCD, limits the ability to identify the most effective extract ratios and validate their predicted outcomes through model verification and confirmation analysis (Pamungkas et al., 2016; Zaky et al., 2021). This gap underscores the need for research that integrates advanced statistical modeling with practical formulation strategies.

Research Purpose, Urgency, and Novelty

This study aims to optimize the concentration ratio of combined broccoli and carrot extracts to achieve the highest antioxidant activity (lowest IC50) and total phenolic content, utilizing Central Composite Design and validated analytical methods. The urgency of this research stems from the increasing demand for natural, effective topical antioxidants to combat oxidative stress and skin aging, as well as the need for standardized, reproducible formulation protocols (Aldila et al., 2023; Setyawati et al., 2024). The novelty lies in the systematic application of CCD for extract combination optimization, the integration of phytochemical screening, and the model verification process, which together provide a robust framework for developing high-efficacy antioxidant gels (Dewantara et al., 2021; Feninlambir et al., 2023).

RESEARCH METHODS

This study is an experimental laboratory research using the Central Composite Design (CCD) optimization method with the Design Expert version 13 software. This method was chosen to examine the effects of individual and combined concentrations of broccoli and carrot extracts on the observed responses of antioxidant activity and total phenolic content. The main objective is to find the optimal combination ratio that yields the highest antioxidant activity (lowest IC_{50}) and the highest total phenolic content.

Population, Sample, and Research Instruments

The population in this study consisted of all possible combinations of broccoli (Brassica oleracea L. var. italica) and carrot (Daucus carota L.) extracts that have the potential for antioxidant activity and total phenolic content. The sample was comprised of 9 experimental units, each representing a specific ratio of extract concentrations determined by the Design Expert software. The sampling technique used was the Central Composite Design (CCD), a method within the Design of Experiments (DoE), with randomization applied to the experimental units to ensure data validity. A wide range of laboratory instruments and materials were utilized, including a UV-Vis spectrophotometer for absorbance measurements, a rotary evaporator for the extraction process, and various common laboratory tools such as glassware and analytical scales, to prepare and test the samples.

Research Variables

1. Independent Variable
   The two independent variables being optimized are:
   a. Factor A: Broccoli Extract Concentration (1%−5%)
   b. Factor B: Carrot Extract Concentration (1%−5%)
2. Dependent Variables
   The two responses measured for each combination are:
   a. Response 1: Antioxidant Activity, expressed as the IC_{50} value.
   b. Response 2: Total Phenolic Content, expressed in mg GAE/g extract.
3. Control Variables
   All laboratory procedures were kept constant to ensure data validity. These variables include:
   a. Extraction Method: Maceration using a 70% ethanol solvent for 5x24 hours.

b. Antioxidant Activity Test Method: DPPH, 30-minute incubation, and absorbance measurement at 517 nm.
c. Total Phenolic Content Test Method: Folin-Ciocalteu, and absorbance measurement at 765 nm.

Materials and Instruments

A wide range of materials and instruments were utilized throughout the entire process, from preparation to quality testing. The materials used were broccoli (*Brassica oleracea L. var. italica*) and carrots (*Daucus carota L.*), 70% ethanol, distilled water (Aquadest), methanol p.a., carbopol, methyl paraben, propyl paraben, propylene glycol, *oleum rosae*, broccoli and carrot extracts, DPPH (1,1-Diphenyl-2-Picrylhydrazyl), quercetin, universal pH indicator, 1% HCl, magnesium, 1% $FeCl_3$, H_2SO_4, Mayer's Reagent, Dragendorff's Reagent, gallic acid, Folin-Ciocalteau Reagent, and sodium carbonate (Na_2CO_3).

The tools included a blender or grinder, digital scale, glass jars, Erlenmeyer flasks, sieves, funnels, filter paper, rotary evaporator, water bath, graduated cylinders, beaker glass, hot plate, magnetic stirrer, pH meter, Brookfield viscometer, spatulas, gel containers, vial bottles, UV-Vis Spectrophotometer, micropipettes, cuvettes, volumetric flasks, vortex mixer, glass slides and weights, stopwatch, thermometer, porcelain crucibles, crucible tongs, oven, porcelain dishes, aluminum foil, moisture balance, test tubes, furnace, and a desiccator.

Research Procedure

1. Sample Collection and Plant Determination

The plant samples were collected from Girikerto Village, Sine District, Ngawi Regency, East Java, Indonesia. Plant determination was conducted at the Biology Laboratory of the Faculty of Applied Science and Technology, Universitas Ahmad Dahlan, Yogyakarta, to confirm the identity of the samples as Brassica oleracea L. var. italica (broccoli) and Daucus carota L. (carrot).

2. Simplicia Preparation

a. Sample Preparation: Fresh broccoli and carrots were selected, washed with running water, and separated from unwanted leaves and roots.
b. Slicing: The washed materials were sliced into small pieces to facilitate the drying process.
c. Drying: The sliced samples were drained and then dried under the sun for 7 days, covered with a black cloth to protect them from external contamination.
d. Grinding and Sieving: The dried simplicia were ground into a fine powder using a blender and sieved with a 40-mesh sieve to obtain a homogeneous powder.

3. Extraction

Extraction was performed using the maceration method with 70% ethanol solvent. A total of 200 grams of simplicia powder was soaked in a maceration vessel with 2 liters of the solvent for 3x24 hours, with periodic stirring. The solvent was then filtered, and the residue was remacerated for another 2x24 hours. The combined filtrates were concentrated using a rotary vacuum evaporator at 50°C and 20 psi, followed by a water bath to obtain a thick, solvent-free extract.

4. Phytochemical Screening

A qualitative phytochemical screening was conducted on the extracts to identify secondary metabolites. The tests performed were:

a. Alkaloid Test: Using Mayer's, Dragendorff's, and Wagner's reagents.
b. Flavonoid Test: Using magnesium powder and concentrated HCl.
c. Saponin Test: By shaking a sample with hot water and adding HCl to check for stable foam formation.
d. Tannin and Phenolic Test: Using a 1% $FeCl_3$ solution.
e. Steroid and Triterpenoid Test: Using the Liebermann-Burchard reagent.

5. Evaluation of Extract Combination Responses

Nine variations of extract combinations were prepared according to the *Central Composite Design* matrix. Each combination was tested for two responses:

a. Antioxidant Activity Test

1) DPPH Solution Preparation

To prepare the DPPH solution, 4 mg (0.004 g) of DPPH was weighed and added to a 100 ml volumetric flask. Then, p.a. methanol was added little by little until it reached the

flask's fill line. The solution was homogenized by shaking until the DPPH was completely dissolved. To protect the solution from light exposure, which can cause degradation, the volumetric flask was tightly sealed with aluminum foil. DPPH solutions should be freshly prepared each time the test is conducted to ensure the accuracy of the test results (Julizan et al., 2019).

2) Preparation of the Test Solution Combination of Broccoli and Carrot Extracts

The stock solution was prepared by taking a certain amount of the broccoli and carrot extract combination according to the nine combinations recommended by the Design Expert software. From these nine extract combinations, the extract stock solution was made by dissolving each extract combination in 100 mL of p.a. methanol. Subsequently, intermediate dilution solutions with a concentration of 1000 ppm were prepared from each stock solution. These intermediate solutions were prepared at several concentration series (10, 20, 30, 40, and 50 ppm) by pipetting 0.1 mL, 0.2 mL, 0.3 mL, 0.4 mL, and 0.5 mL of the intermediate solution into a 10 mL vial bottle, respectively. The final volume of each solution was then adjusted using p.a. methanol until it reached the 10 mL mark.

3) Determination of Wavelength (λ max)

1 mL of 40 ppm DPPH solution was placed into a vial bottle that was tightly sealed with aluminum foil to prevent light exposure because DPPH is sensitive to light. After that, 3 mL of p.a. methanol was added and shaken until the solution was homogeneous. The solution was then incubated for 30 minutes at room temperature, and after incubation, its absorbance was measured at wavelengths of 400–800 nm. (Ainul Yahya et al., 2020; Zaky et al., 2021).

4) Determination of Operating Time

The operating time in this study was determined by modifying the previous study by Zaky et al. (2021). The first step is to take 50 µL (0.05 mL) of each previously prepared extract combination solution and place it into a volumetric flask that has been covered with aluminum foil. Then, 4 mL of DPPH solution was added to each sample. After that, the solutions were vortexed and their operating time was measured from minute 1 to 30 at the previously determined maximum wavelength. The time that produces the most stable absorbance will be set as the operating time.

5) Preparation of Blank

Solution 2 ml of DPPH solution was pipetted into a test tube, then 2 ml of p.a. methanol was added. The test tube was covered with aluminum foil and homogenized using a vortex. After that, the solution was incubated in a dark room for a predetermined operating time. Finally, after incubation, the absorbance of the solution was measured at the predetermined maximum wavelength based on previous measurements (Zaky et al., 2021).

6) Preparation of Vitamin C Stock

Solution To prepare the vitamin C stock solution, 10 mg of vitamin C was dissolved in 100 mL of p.a. methanol to obtain a stock solution with a concentration of 100 ppm. Subsequently, solutions with various lower vitamin C concentrations (1, 2, 3, 4, and 5 ppm) were prepared by pipetting 0.1 mL, 0.2 mL, 0.3 mL, 0.4 mL, and 0.5 mL, respectively, of the stock solution into 10 mL vials or volumetric flasks. The final volume of each solution was then adjusted with p.a. methanol to reach a limit of 10 mL. Next, 1 mL of each concentration of vitamin C solution was added to 2 mL of the prepared DPPH solution. This mixture of solutions was homogenized and then incubated for the operating time previously determined. The absorbance of each reacted solution was measured at the predetermined maximum wavelength. This process was repeated for three replicates (Zaky et al., 2021).

7) Antioxidant Activity Testing

A 2 mL sample of each concentration of the extract combination test solution was taken and mixed with 2 mL of DPPH solution. After homogenization, the solution was incubated in a dark room according to the predetermined operating time. The absorbance of each solution was measured using UV-Vis spectrophotometry at a maximum wavelength of 517 nm (Pandanwangi TW et al., 2018). The testing process for each solution was repeated three times.

b. Total Phenolic Content Test

1) Preparation of Stock Solution and Gallic Acid

Dilution 10 mg of gallic acid was weighed to make a 1000 ppm gallic acid stock solution. Then, it was dissolved in p.a. methanol until the volume reached 10 mL. Next, a 100 ppm intermediate solution was prepared from the 1000 ppm stock solution by taking 2.5 mL of the stock solution and adding methanol until the total volume was 25 mL. From this 100 ppm intermediate solution, several concentration series of 10, 20, 30, 40, and 50 ppm were prepared by pipetting the intermediate solution in amounts of 1, 2, 3, 4, and 5 mL, respectively, resulting in those concentrations. Then, by adding p.a. methanol, the volume was increased to 10 mL (Ahmad et al., 2015).

2) Preparation of Folin-Ciocalteu

Solution and Na_2CO_3 Solution Folin-Ciocalteu reagent and distilled water were mixed in a 1:10 (v/v) ratio to prepare the test Folin-Ciocalteu solution. Subsequently, the Na_2CO_3 solution was made by weighing 7.5 g of sodium carbonate, dissolving it in a 100 mL volumetric flask, and diluting it with distilled water to the mark (Feninlambir et al., 2023).

3) Determination of Operating Time (OT)

By modifying the method from Dewantara et al. (2021). A total of 1.5 mL of Folin-Ciocalteu reagent was mixed with 0.3 mL (300 µL) of gallic acid solution at a concentration of 30 ppm, shaken, and then allowed to stand for three minutes. After that, 1.2 mL of Na_2CO_3 solution was added, and the mixture was shaken until homogeneous. Then, the absorbance of the solution was measured periodically at a wavelength of 765 nm over a time range of 0 to 30 minutes.

4) Determination of Maximum Wavelength

A total of 1.5 mL of Folin-Ciocalteu reagent was mixed with 0.3 mL (300 µL) of gallic acid solution at a concentration of 30 ppm, shaken, and then allowed to stand for three minutes. Next, 1.2 mL of Na_2CO_3 solution was added, shaken until homogeneous, and allowed to stand at room temperature for the specified operating time. After that, the absorbance of the reacted solution was measured over a wavelength range of 400 to 800 nm (Dewantara et al., 2021).

5) Preparation of Extract Sample Solution

The stock solution of the sample was prepared by modifying the methodology from the study by Suryandari et al. (2025). According to the combination design from the Design Expert program, a number of extract concentration combinations from broccoli and carrots were used to create the solution, with a total of nine combinations. After that, the extract was dissolved in 100 mL of p.a. methanol, and each stock solution was then used to make intermediate dilution solutions. By pipetting 0.1 mL of the intermediate solution into a 10 mL vial, a 10 ppm concentration series was created from the 1000 ppm intermediate solution. After that, p.a. methanol was added until the 10 mL mark was reached.

6) Measurement and Calibration Curve

Construction of Gallic Acid Gallic acid solutions with concentrations of 10, 20, 30, 40, and 50 ppm were prepared to create a gallic acid calibration curve. A total of 1.5 mL of Folin-Ciocalteu reagent was added to 0.3 mL (300 µL) of each concentration series. After that, the mixture was stirred and allowed to stand for three minutes. Then, 1.2 mL of Na_2CO_3 solution was added, and everything was shaken until homogeneous. The solution was then incubated at room temperature for a predetermined operating time. The gallic acid calibration curve was created using the absorbance of the reacted solutions measured at the maximum wavelength of gallic acid (Dewantara et al., 2021). 7) Absorbance Measurement The test tubes were filled with 300 µL of each sample solution concentration. After that, 1.5 mL of Folin-Ciocalteu reagent (1:10) was added, mixed thoroughly using a vortex, and allowed to stand for three minutes. Then, 1.2 mL of Na_2CO_3 solution was added, mixed again with a vortex, and allowed to stand at room temperature for the specified operating time. Next, the absorbance of each solution was measured at its maximum wavelength (Feninlambir et al., 2023).

6. Data Analysis

a. Antioxidant Activity

Percentage of inhibition of antioxidant activity was measured using IC_{50} (Inhibition Concentration 50%), which is the concentration of the sample capable of eliminating 50% of DPPH radicals. The IC_{50} value was obtained from the percentage of DPPH radical inhibition at each concentration of the sample solution (Ghozaly & Safitri, 2016). The following formula is used to calculate the percentage of inhibition:

% Inhibition = (Blank Absorbance - Sample Absorbance) / (Blank Absorbance) × 100%

b. Antioxidant Activity IC_{50}

The IC_{50} value is a parameter used to measure the antioxidant activity of a compound. This value can be calculated using the following formula:

y = bx + a
50 = bx + a
IC50 = (50 - a) / b

The IC_{50} value is calculated using the standard curve equation created from the relationship between solution concentration (x) and percentage inhibition (y). The IC_{50} (Inhibition Concentration 50%) value is an important parameter for indicating the effectiveness of a compound's antioxidant properties. This value is calculated using linear regression, which describes the relationship between the concentration of the test solution (x-axis) and the percentage of inhibition (y-axis). After obtaining the regression equation, the value of y is substituted with the number 50 (representing 50% inhibition), and the resulting value of x from the calculation is the IC_{50} value (Feninlambir et al., 2023).

c. Total Phenolic Content

Gallic acid absorbance data were analyzed using linear regression with the equation y = ax+b, where absorbance values are represented as y and the concentration of the standard solution as x. The following formula is used to calculate the total phenolic content of the extract:

Total Phenolic Content = (C x V x fp) / g

Explanation:
C: Phenolic content or x value (µg/mL)
V: Volume of extract sample (mL)
Fp: Dilution factor of the solution
g: Sample weight (g)

RESULTS AND DISCUSSION

Plant Determination

Plant determination aims to identify the plants that will be used in the research. The determination process was carried out at the Biology Laboratory, Faculty of Science and Applied Technology, Ahmad Dahlan University, located at Jl. Ahmad Yani (South Ringroad) Tamanan, Banguntapan, Bantul, Yogyakarta. The samples determined in this study were Broccoli (Brassica oleracea var. italica Plenck) and Carrots (Daucus carota L.) obtained from Ngrambe Village, Ngrambe District, Ngawi Regency, East Java. Based on letter number 478/Lab.Bio/B/VII/2025, the results of the determination show that the plants are indeed Broccoli (Brassica oleracea var. italica Plenck) and Carrots (Daucus carota L.).

Preparation of Sample and Extract

A total of 5 kg of broccoli and 5 kg of carrots were wet-sorted and washed thoroughly with running water, then chopped to facilitate the drying process. Next, the chopped broccoli and carrots were dried in the sun for 7 days. To aid in the drying process and protect them from dust and insects, the pieces are covered with black cloth. This drying process serves to reduce the moisture content of the material, which can help prevent the growth of fungi and mold, and stop enzymatic processes that could potentially damage the physical and chemical properties of the raw material. This ensures its quality remains stable during storage. (Priamsari et al., 2019).

The sample preparation process was successful, yielding results that met the required standards. A total of 5 kg each of fresh broccoli and carrots were processed into simplicia powder. After drying, the broccoli simplicia powder weighed 504 grams, with a yield of 10.08%. Meanwhile, the carrot simplicia powder weighed 507 grams, with a yield of 10.14%. Both of these yields, being more than 10%, indicate that the drying process was efficient and met the established standards (BPOM, 2013).

After the stage of making the crude drug, the next process is extraction to obtain bioactive compounds. In this study, the maceration method was used for extraction for 3 days, followed by re-maceration with 2 ethanol 70% as the solvent. The maceration method was chosen because it is a simple, relatively inexpensive extraction technique suitable for heat-sensitive materials, as the process is carried out at room temperature, which can minimize the degradation of active compounds. The selection of 70% ethanol as a solvent is based on its optimal polarity characteristics for extracting a wide range of secondary metabolite compounds, particularly phenolic compounds known for their antioxidant activity.

The extraction process was conducted using 200 grams of simplicia powder for each material. The maceration method using 70% ethanol was effective, yielding 97.72 grams of thick broccoli extract and 90.38 grams of thick carrot extract. The extract yield for broccoli was 46.86% and for carrots it was 45.19%. These high yield values, which also exceeded the 10% standard, indicate that the extraction method was very effective in drawing out the bioactive compounds from the simplicia (Saerang et al., 2023).

Standardization of Simplicia

The standardization results of the broccoli and carrot simplicia showed that both have good quality and meet the requirements set for traditional medicine raw materials. Organoleptically, broccoli simplicia is characterized by a dry powder, brownish-green color, and a characteristic broccoli aroma, while carrot simplicia is also a dry powder, reddish-orange in color, with a characteristic carrot aroma.

Table 1.
Results of Determining the Moisture Content of Herbal Medicine

Simplicia	Weight Before Analysis (gr)	Weight After Analysis (Drying) (gr)	*Water Content (Water Loss)*
Broccoli	2,021	2,009	1,28%
Carrot	2,059	1,981	3,79%

Based on the measurement data obtained, it was found that both crude drugs had low moisture content, namely 1.28% for the carrot crude drug and 3.79% for the broccoli crude drug, making them more resistant to fungal growth. According to Materia Medika Indonesia, the requirement for crude drug moisture content is <10% (Hartati et al., 2023), thus it can be said that the moisture content of the test crude drugs (carrots and broccoli) has met the applicable requirements.

Table 2.
Results of the Determination of Drying Loss of Herbal Raw Materials

Simplicia	Replication	Empty Crucible Weight (gr)	Crucible + Simplicia Weight (Before Heating) (gr)	Crucible + Simplicia Weight (After Heating) (gr)	Loss on Drying Simplicia Broccoli	Average
Broccoli	1	49,195	51,205	50,867	6.87%	6,51%
	2	44,749	46,746	46,619	6.36%	
	3	43,767	45,768	45,642	6.30%	
Carrot	1	46,547	48,549	48,379	8.49%	8,50%
	2	44,448	46,460	46,287	8.60%	
	3	44,959	46,960	46,792	8.40%	

Based on Table 2, the drying loss of broccoli herbal medicine was found to be 6.51% and carrot herbal medicine 8.50%. According to BPOM (2013), good drying loss for herbal medicine is <10%, so these test results can be said to have met the requirements. This result indicates a substantial reduction in the weight of the crude drug during the drying process. This weight reduction indicates that good quality and stability were achieved in the production of herbal medicine (Dewi et al., 2024).

Table 3.
Results of Simplisia Ash Content Determination

Simplicia	Replication	Empty Crucible Weight (g)	Crucible and Sample Weight (Before Incineration) (g)	Crucible and Sample Weight (After Incineration) (g)	Broccoli Extract Ash Content (%)	Average
Broccoli	1	45,105	47,108	45,184	3.94%	4,00%
	2	45,469	47,528	45,551	3.98%	
	3	45,325	47,408	45,410	4.08%	
Carrot	1	45,749	47,697	45,826	3.95%	4,12%
	3	44,915	46,924	45,003	4.38%	
	2	45,037	47,019	45,117	4.04%	

According to Samodra (2019), the total ash content value obtained must be below the limit required by Materia Medica Indonesia IV, which is <14%. Based on the results of the ash content test for broccoli and carrot Simplicia, only 4.00% and 4.12% of the Simplicia mass is composed of minerals or inorganic materials. Therefore, this crude drug can be said to have relatively high purity with minimal mineral contribution to its total weight.

Standardization of Extract

The standardization results of the broccoli and carrot extracts showed good quality and met the established standards. This indicates that the extraction method used was very effective in drawing out the bioactive compounds from the simplicia. Organoleptically, the broccoli extract was a thick, brownish-green liquid with a distinct broccoli smell. The carrot extract was a thick, brownish-orange liquid with a characteristic carrot aroma.

Table 4.
Hasil Penetapan Kadar Air Ekstrak

Extract	Weight Before Analysis (g)	Weight After Analysis (Drying) (g)	Water Content (Water Loss) (%)
Broccoli	2,000	1,913	4,35%
Carrot	2,003	1,912	4,54%

The moisture content of broccoli and carrot extracts is 4.35% and 4.54%, respectively. These relatively low figures indicate good extract stability, as high moisture content risks triggering the growth of microorganisms, hydrolysis reactions, and other detrimental chemical reactions. According to Dewi et al. (2024), low water content in extracts tends to extend their shelf life and protect them from microbial contamination. According to the Indonesian Herbal Pharmacopeia (FHI) 2000, the moisture content requirement for extracts is <10% (Najib et al., 2017). So, it can be concluded that the water content in

the broccoli and carrot extracts has met the quality standards.

Table 5.
Results of Drying Loss Determination for Extract

Extract	Replication	Empty Crucible Weight (gr)	Crucible + Simplicia Weight (Before Heating) (gr)	Crucible + Simplicia Weight (After Heating) (gr)	Loss on Drying Result	Average
Broccoli	1	44,123	46,124	45,953	8.55%	8,22%
	2	44,557	46,568	46,407	8.01%	
	3	45,013	47,012	46,850	8.10%	
Carrot	1	44,234	46,235	46,039	9.79%	9,63%
	2	44,678	46,681	46,482	9.93%	
	3	45,122	47,136	46,951	9.19%	

From the test results, the average drying shrinkage value for broccoli extract was 8.22% and for carrot extract, it was 9.63%. These values indicate the components that evaporated from the extracts due to heating, such as essential oils, water, or residues from the ethanol solvent. The drying loss values of both extracts were below the requirement limit of <10%, so they were considered to meet quality standards. When the drying loss value exceeds this limit, it can potentially disrupt the stability of the extract (Ramdhini, 2023).

Table 6.
Results of Extract Ash Content Determination

Extract	Replication	Empty Crucible Weight (g)	Crucible and Sample Weight (Before Incineration) (g)	Crucible and Sample Weight (After Incineration) (g)	Broccoli Extract Ash Content (%)	Average
Broccoli	1	45,345	47,387	45,412	3.28%	3,32%
	2	45,718	47,810	45,795	3.68%	
	3	46,062	48,194	46,126	3.01%	
Carrot	1	45,892	47,965	45,987	4.58%	4,66%
	3	46,215	48,278	46,311	4.65%	
	2	46,754	48,813	46,852	4.76%	

Based on the test results, the ash content values obtained for broccoli extract are 3.32% and for carrot extract are 4.66%. Based on these data, it can be said that the ash content of both extracts is below the requirement limit, so they are considered to have met the extract quality standards. This also indicates that during the extraction process, some internal and external minerals, such as sand and soil, were not carried along with the extract.

Phytochemical Screening

The phytochemical screening conducted on the broccoli and carrot extracts showed the presence of various secondary metabolite compounds with antioxidant potential.

Table 7.
Phytochemical Screening Results

Test	Reagent	Observation of Broccoli Phytochemical Screening	Conclusion	Observation of Carrot Phytochemical Screening	Conclusion
Alkaloid	*Mayer*	Formed a yellowish-white precipitate	+	White or yellow precipitate did not form	-
	Dragendorff	Formed an orange precipitate	+	Reddish-orange precipitate did not form	-
	Wagner	Formed a reddish-	+	Brown precipitate did	-

		brown precipitate		not form	
Flavonoid	Mg + HCl Conc + Amyl Alcohol	The color changed to yellow	+	The color changed to reddish-yellow	+
Phenolic	$FeCl_3$ 5%	The color changed to bluish-green	+	The color changed to green	+
Tannin	$FeCl_3$ 1%	The color changed to brownish-black	+	The color changed to greenish-black	+
Saponin	Distilled water	Formed stable foam	+	Formed stable foam	+
Steroid/Triterpenoid	$(CH_3CO)_2O$ + conc. H_2SO_4	The color changed to bluish-green	+	Formed a red color	+

For the broccoli extract, the tests yielded positive results for almost all compound groups, including alkaloids, flavonoids, phenols, tannins, saponins, steroids, and triterpenoids. Meanwhile, the carrot extract also showed positive results for most compounds, specifically flavonoids, phenols, tannins, saponins, steroids, and triterpenoids. However, the alkaloid test on the carrot extract yielded a negative result, indicating that this compound was either not detected or its content was very low. The presence of these compounds provides a strong scientific basis for explaining the antioxidant activity and total phenolic content measured in this study.

Experimental Design of Extract Combination

The optimization design in this study was conducted using the Central Composite Design (CCD) method with Design-Expert 13 software. This method was chosen for its ability to optimize two independent variables (factors) to achieve a desired response. The independent variables used were the concentrations of broccoli extract (Factor A) and carrot extract (Factor B), with a concentration range of 1% to 5%. The dependent variables, or responses, that were measured for each combination were antioxidant activity (IC_{50}) and total phenolic content. This design resulted in 9 experimental runs with varying concentration ratios. The purpose of this design was to systematically analyze the effect of these factors on the responses to find the optimal combination that yields the lowest IC_{50} and the highest total phenolic content.

Table 8.
Results of the Experimental Design for Formulation and Evaluation of the Combined Extract Response of Broccoli and Carrots

Std.	*Run*	Factor 1. A: Broccoli Extract Concentration (%)	Factor 2. B: Carrot Extract Concentration (%)	Response 1. Antioxidant Activity (IC_{50}) (ppm)	Response 2. Total Phenolic Content (mg GAE/g)
6	1	5.8	3	27.16	139.84
2	2	5	1	31.74	103.12
1	3	1	1	43.27	26.16
5	4	0.2	3	41.54	31.71
9	5	3	3	34.44	86.65
4	6	5	5	24.9	143.13
7	7	3	0.2	39.07	52.67
8	8	3	5.8	29.65	119.72
3	9	1	5	36.77	69.26

Assay of Antioxidant Activity

The antioxidant activity test in this study was conducted using the DPPH method, which is known for its simplicity, speed, and sensitivity. The procedure involved several stages

1. Results of Maximum DPPH Wavelength Measurement

   To obtain optimal absorbance, the maximum wavelength of DPPH was determined by measuring the absorbance of the DPPH compound over the range of 400–800 nm. This maximum

wavelength is identified from the peak of the curve. At that point, the compound showed the highest absorbance and sensitivity values (Mulangsari et al., 2017). The highest absorbance, which is 0.605, was achieved at a wavelength of 517.0 nm. This point was chosen because the solution showed the highest sensitivity. Therefore, a wavelength of 517 nm will be used for all DPPH antioxidant activity tests to ensure data accuracy and consistency.

2. Results of Operating Time (OT)

Determination To determine stable measurement times, operating time (OT) was established. This process involved measuring the relationship between time and the absorbance of the solution (Sania et al., 2024). Operating time (OT) was measured by reacting the DPPH solution with the extract combination sample, measuring absorbance every 5 minutes over a time range of 0 to 30 minutes at a wavelength of 517 nm. The results show a decreasing trend in absorbance over time, indicating that the reaction between the DPPH free radical and the sample's antioxidant compounds continued to occur. The absorbance value appears to stabilize starting at the 25th minute, with an absorbance value of 0.615, and remains constant until the 30th minute. This stable time indicates that the reaction has reached equilibrium. Therefore, 26 minutes was chosen as the optimal reaction time (operating time) for all DPPH antioxidant activity test measurements to ensure that the results obtained were accurate and consistent.

3. Results of Vitamin C

Antioxidant Activity Measurement The Vitamin C antioxidant activity test, used as a positive control, was conducted to validate the DPPH method employed. Ascorbic acid (Vitamin C) is a naturally occurring antioxidant that is readily available and commonly used as a standard (Karim et al., 2015).

The results of the vitamin C standard antioxidant activity test showed a response relationship. A significant increase in vitamin C concentration significantly increases the percentage of free radical inhibition. The data show that at a concentration of 1 ppm, the inhibition is only 36.56%, while at a concentration of 5 ppm, the inhibition increases to 62.11%. This strong correlation is also reflected in the correlation coefficient (R^2) value in the regression equation y = 6.4385x + 31.203, which is 0.9849. This R2 value, which is close to 1, confirms that the regression model has an excellent fit and the relationship between concentration and percentage of inhibition is linear and reliable.

Based on the linear regression equation, the IC_{50} value was calculated and resulted in a value of 2.91 ppm. This relatively low IC_{50} value indicates that vitamin C has very strong antioxidant activity. An antioxidant is classified as very strong if its IC_{50} value is <50 ppm (Masruroh & Tukiran, 2017). This supports the statement made by Karim et al. (2015) that the smaller the IC_{50} value, the higher the antioxidant activity

4. Result of Antioxidant Activity

Based on the results presented in Table 8, the antioxidant activity test of each combination of Broccoli (Brassica oleracea var. italica Plenck) and Carrot (Daucus carota L.) extract concentrations showed very good results. The IC_{50} (Inhibitory Concentration 50%) values obtained from all experimental runs ranged from 22.36 ppm to 43.28 ppm. A lower IC_{50} value indicates stronger antioxidant activity (Firdayani et al., 2015). Therefore, these results confirm that the two extracts, when combined, have the potential to be very strong and effective antioxidant agents, as all IC_{50} values were below 50 ppm, which is the criterion for antioxidants categorized as very strong (Indriani, 2021).

The lowest IC_{50} value, which was 22.36 ppm, was found in Run 6, indicating the strongest antioxidant activity, while Run 3 had the highest IC_{50} value of 43.28 ppm. Nevertheless, both values are still within the very strong antioxidant classification. This indicates that variations in concentration within this combination affect its antioxidant effectiveness, even though its overall potential is very high.

The data on the percentage of inhibition (% inhibition) for each run also show a consistent relationship. As the extract concentration increases (10-50 ppm), the resulting percentage of

inhibition also increases. This proves a positive relationship, where an increase in the number of bioactive compounds from the extract is directly proportional to its increased ability to capture and neutralize free radicals

This strong antioxidant activity is likely due to two factors. First, the abundant phenolic compound content in broccoli and carrots. Second, the presence of a synergistic effect between the bioactive compounds from both extracts. This synergy occurs when the combined effect of both is greater than the effect of each extract individually, thus optimizing their antioxidant capabilities (Pitaloka, 2024).

Compared to previous studies that tested single broccoli or carrot extracts (D. E. Handayani et al., 2022; Sami & Rahimah, 2015). The IC_{50} value of the combination of the two extracts in this study showed better performance. The IC_{50} value in Run 6 (22.36 ppm) was significantly lower, indicating that combining the two extracts is an effective approach to enhancing antioxidant activity.

Assay of Total Phenolic Content

The total phenolic content (TPC) was determined using the Folin-Ciocalteu method. The procedure involved several stages

1. Results of Maximum Wavelength Absorption Measurement

   Determining the maximum wavelength aims to ensure the most accurate absorbance and sensitivity results (Primadiamanti et al., 2020). In this study, the maximum wavelength was determined by finding the highest absorbance value within the range of 400 - 800 nm. Based on the results obtained, the maximum wavelength was found to be 765 nm with an absorbance value of 0.829 nm. This predetermined maximum wavelength will then be used for testing so that a calibration curve can be created. This curve is important because it's from this that the regression equation used to calculate the total phenolic compound content in the sample is derived.
2. Results of Operating Time

   Determination After determining the maximum wavelength of gallic acid, the operational time was then determined. This time is sought to ensure the reaction reaches optimal stability (Guntarti et al., 2021). Based on the results, the optimal operating time was obtained at the 20th minute, with the maximum absorption wavelength at 765 nm. The results of the operating time and maximum absorption wavelength were then used to measure the absorbance by creating a gallic acid calibration curve.
3. Results of Gallic Acid Calibration Curve

   Preparation Gallic acid was chosen as the standard because it is a phenolic compound with a simple structure, is stable, and is available in pure form. To create a calibration curve, gallic acid was prepared in various concentration series including 10, 20, 30, 40, and 50 ppm. The absorbance measurement of gallic acid was performed using a maximum wavelength of 765 nm and an operating time of 22 minutes. The linear regression equation obtained from the calibration curve was then used to measure the total phenolic content in the combination of broccoli and carrot extracts (Dewantara et al., 2021).

   The linear regression equation $y = 0.0475x + 0.2718$ was obtained, with an R^2 value of 0.9957. According to Pamungkas et al. (2016), mathematically this equation shows the correlation between gallic acid concentration and its absorbance. The R^2 value itself indicates the strength of the relationship between concentration and absorbance value. Because the R2 value is close to 1, this regression equation is considered linear and is subsequently used to calculate the total phenolic content in the extract combination samples.
4. Results of Total Phenolic Content

   Determination of Samples The total phenolic content was determined using the regression

equation from the gallic acid calibration curve. In this process, the absorbance value of the sample served as the y variable to obtain the x value, which was then interpreted as the concentration of gallic acid in mg/L. Subsequently, this gallic acid concentration in mg/L was used to calculate the total phenolic content by comparing it to the sample concentration (g/L) (Pamungkas et al., 2016).

Based on the results presented in Table 8, there is a significant variation in the total phenolic content values for each extract combination tested. This measurement was conducted because phenolic compounds are widely known as the main bioactive components that play a role in antioxidant activity. Thus, this data becomes one of the important indicators for evaluating which extract combination is most effective and optimal.

Overall, the average total phenolic content varied from the lowest in Run 3 (26.39 mg GAE/g extract) to the highest in Run 6 (143.58 mg GAE/g extract) and Run 1 (139.62 mg GAE/g extract). This striking difference confirms that the concentration of the broccoli and carrot extract combination has a direct impact on the amount of phenolics. The higher the total phenolic content, the greater the antioxidant potential. This is supported by previous research by Indriyah et al. (2023), who successfully demonstrated a correlation between antioxidant activity and total phenolic content, stating that total phenolic content is directly proportional to the antioxidant activity of the sample.

Data Analysis

All data obtained from the response tests were entered into the *Design-Expert* software for analysis. The analysis included:

1. **Statistical Analysis and Model Evaluation**

The data analysis performed with the Design-Expert software revealed that the built regression model had excellent quality and statistical significance for predicting the responses of antioxidant activity (IC_{50}) and total phenolic content. The model was highly significant, as evidenced by a p-value much smaller than 0.0001 from the ANOVA for both responses. The model's quality was further confirmed by high and consistent values for R2, Adjusted R2, and Predicted R2, all above 0.99, which demonstrates a strong predictive capability. Based on the model's coefficients, both broccoli (A) and carrot (B) extracts had a very significant influence on both responses, with the analysis showing that broccoli extract had a more dominant effect.

2. **Optimization**

The numerical optimization analysis successfully found that the optimal combination ratio of extracts is 5%:5%. At this optimal ratio, the model predicted an antioxidant activity (IC_{50}) of 25.436 µg/mL and a total phenolic content of 146.311 mg GAE/g. The success of this optimization was supported by a very high desirability value of 0.985, which is close to 1 and indicates that the solution is very close to the ideal conditions.

The chosen optimization approach is numerical optimization, considering that the factors to be optimized consist of two extract concentrations. The process of determining the optimal formula begins by setting the desired goal values for each observed response. In this context, the concentration of broccoli extract and the concentration of carrot extract serve as independent variables, while antioxidant activity (IC_{50} value) and total phenolic content are the dependent variables. After obtaining the test results for IC_{50} values and total phenolic content, the collected data was then analyzed using Design-Expert software. This analysis is based on specific criteria set for each physical response, such as minimizing, maximizing, being within a certain range, reaching a target, or being equal to a specific value.

Table 9.
Optimization Constraints and Objectives Table

Name	*Goal*	*Lower Limit*	*Upper Limit*	*Lower Weight*	*Upper Weight*	*Importance*
A: Broccoli Extract Concentration	*is in range*	1	5	1	1	3
B: Carrot Extract Concentration	*is in range*	1	5	1	1	3

Antioxidant Activity (IC_{50})	*minimize*	24.9	43.27	1	1	3
Total Phenolic Content	*maximize*	26.39	143.58	1	1	3

Based on Table 9, this analysis shows the setting of criteria for the optimization process where four variables are considered. The concentration factors for broccoli extract and carrot extract were set within the range of 1% to 5%. Meanwhile, for the response variables, the optimization goal was to minimize the IC_{50} value (antioxidant activity) within the range of 24.9 to 43.27 µg/mL and maximize the total phenolic content within the range of 26.39 to 143.58 mg GAE/g. Since the importance value for all variables was set at level 3, all these criteria were considered to have equal weight. Thus, the Design-Expert software will search for the best solution, which is a compromise point between minimizing the IC_{50} and maximizing the total phenolic content within the established concentration range.

Table 10.
Optimal Combination Results and Their Predicted Values (Solutions Table)

No.	Broccoli Extract Concentration (%)	Carrot Extract Concentration (%)	Antioxidant Activity (IC_{50})	Total Phenolic Content	*Desirability*	
1	**5**	**5**	**25.436**	**146.328**	**0.985**	***Selected***
2	5	4,787	25.793	143.941	0.975	
3	5	4,632	25.053	142.201	0.962	

Based on Table 10, the optimization results show three potential extract combinations that meet the established criteria. The best selected solution is combination number 1, which represents the optimal point for minimizing IC_{50} and maximizing total phenolic content. This combination consists of 5% broccoli extract and 5% carrot extract. In this combination, the model predicted an antioxidant activity value (IC_{50}) of 25.436 µg/mL and a total phenolic content of 146.328 mg GAE/g. The success rate of the optimization is indicated by a high desirability value of 0.985. This result is close to 1, which indicates that the solution is very close to the ideal condition. These findings are consistent with previous graphical analyses showing that the optimal conditions for both responses are at the highest extract concentration.

3. Model Verification

The confirmation analysis stage is performed to validate the optimization model by comparing the model's predicted values with the actual results from the experiment. Based on Table 11, confirmation was performed on the optimal combination recommended by the model (5% broccoli extract concentration and 5% carrot extract concentration). The purpose was to validate the optimization model by comparing the model's predicted values with the actual results of the experiment.

Table 11.
Verification Test Results (Confirmation Table)

Solution 1 of 3 Response	*Predicted Mean*	*Predicted Median*	*Std Dev*	*n*	*SE Pred*	*95% PI low*	*Data Mean*	*95% PI high*
Antioxidant Activity (IC_{50})	254.363	254.363	0.435365	1	0.508396	24.1923	25.23	26.6803
Total Phenolic Content	146.328	146.328	193.369	1	225806	140.803	148.935	151.854

Based on Table 33, confirmation was performed on the optimal combination recommended by the model, which is a 5% broccoli extract concentration and a 5% carrot extract concentration. The results show excellent agreement between the predicted and actual (observed) values. For the antioxidant activity response (IC_{50}), the predicted value (25.4363) is very close to the actual value (25.23), and this actual value falls within the 95% prediction interval (24.9123 to 26.6803). Similarly, for the Total Phenolic Content response, the predicted value (146.328) is also very close to the actual value (148.935), which falls within the 95% prediction interval (140.803 to 151.854). This high degree of fit proves that the model built is accurate, reliable, and has strong predictive capabilities,

thus validating the recommended optimal formulation.

4. Post Analysis Design Expert

After the optimization process, further analysis was conducted to evaluate the built model. This analysis focuses on the model coefficients for each response, presented in Table 12. These coefficients provide a quantitative overview of the influence of each factor (broccoli and carrot extract concentrations) on antioxidant activity (IC_{50}) and total phenolic content, as well as their statistical significance.

Table 12.
Coefficient Model Post Analysis Optimal Extract Combination

	Intercept	**A**	**B**
Antioxidant Activity (IC_{50})	34,2822	-5.49646	-3.34949
p-values		< 0.0001	< 0.0001
Total Phenolic Content	85,8467	38.0742	22.4076
p-values		< 0.0001	< 0.0001

After the optimization process, further analysis was conducted to evaluate the built model. This analysis focuses on the model coefficients for each response, which are presented in Table 12. This coefficient provides a quantitative overview of the influence of each factor (broccoli and carrot extract concentration) on antioxidant activity (IC_{50}) and total phenolic content, as well as their statistical significance. Based on Table 12, it can be concluded that both factors (broccoli and carrot extract concentration) have a highly significant influence on both responses tested. For the antioxidant activity response (IC_{50}), the intercept coefficient value of 34.2822 represents the average IC_{50} value when both factors are at their midpoint. The negative coefficients for broccoli extract (-5.49646) and carrot extract (-3.34949) indicate that increasing the concentration of both extracts will decrease the IC_{50} value, which means increasing antioxidant activity. The extremely small p-value (<0.0001) for both of these factors confirms that their influence is highly significant. Additionally, the absolute value of the broccoli extract coefficient is greater, giving it a more dominant influence.

For the Total Phenolic Content response, the intercept coefficient is 85.8467. The positive coefficients for broccoli extract (38.0742) and carrot extract (22.4076) indicate that increasing the concentration of both extracts will increase the total phenolic content. Just like the IC_{50}, the p-value for both factors is also highly significant (<0.0001). The effect of broccoli extract is also more dominant because its coefficient value is greater.

CONCLUSION

The optimal ratio of the combination of broccoli (*Brassica oleracea* var. *italica* Plenck) and carrot (*Daucus carota L.*) extracts to produce the highest antioxidant activity (lowest IC_{50}) and highest total phenolic content is a concentration ratio of 5%:5%. An increase in the concentration of both broccoli and carrot extracts significantly lowers the IC_{50} value and increases the total phenolic content, with a p-value <0.0001. The broccoli extract, with coefficient values of -5.50 for antioxidant activity and 38.07 for total phenolic content, was found to have a more dominant effect on both responses compared to the carrot extract, which had coefficients of -3.35 and 22.41 respectively.

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