

Optimization of meniran (*Phyllanthus niruri* L.) leaf extract using natural deep eutectic solvent basis choline chloride-glycol on antioxidant

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ABSTRACT: *Phyllanthus niruri* L. has many benefits because it is known that meniran leaves contain secondary metabolites in the form of alkaloids, flavonoids, tannins, saponins and phenolics. The content of phenol compounds which are constituent compounds for antioxidant activity. This research was conducted to determine the combination of Natural Deep Eutectic Solvents (NADES) which can produce extracts with strong antioxidant activity. The preliminary stage determines the best combination of NADES with choline chloride as the Hydrogen Bond Acceptor and the type of sugar as the Hydrogen Bond Donor. The NADES screening obtained the best combination of choline chloride: glycerol, then the optimization stage was carried out using the RSM method with a Central Composite Design. Three variables used were temperature (25°, 40°, 60°C), time (10, 30, 60 minutes) and the ratio of NADES usage (50%, 70% and 90%). Antioxidant activity was determined with the value of IC_{50} in Meniran extract with positive control of vitamin C. The best NADES combination was choline chloride-glycerol (1:1) with an IC_{50} value of 18.039 ppm. Optimization with variations in temperature time and ratio variables obtained optimal conditions at temperature of 60°C with an extraction time of 44 minutes and ratio of 90% NADES obtained IC_{50} values of 8.040 ppm and positive control of vitamin C obtained IC_{50} values of 4.307 ppm. The best results for antioxidant activity are at a temperature of 60°C gives an IC_{50} value of 8.050 ppm was obtained and the average IC_{50} is 8.075 ppm.

KEYWORDS: Antioxidant; IC_{50} meniran; NADES; RSM.

INTRODUCTION

Meniran (*Phyllanthus niruri* L.) is known as a plant that contains chemicals including alkaloids, flavonoids, tannins, saponins and phenolics [1]. This meniran is known to have antioxidant activity, antioxidants are compounds that can inhibit free radicals by donating one electron [2]. Free radicals are molecules that have one or more electrons that do not have a partner in their outer orbit so they are relatively unstable [3].

In research by Risma, et al (2019) [1] examining the antioxidant activity of the meniran herb using 70% ethanol solvent extracted using the maceration method, the average IC_{50} value was 17.59 ppm. Based on the two studies that have been carried out, it can be interpreted that meniran has strong antioxidant activity. The use of NADES as a new solvent to replace organic solvents is rapidly being researched. Using Natural Deep Eutectic Solvent (NADES) and ethanol with different extraction methods, the results obtained were a coumarin concentration from cinnamon with NADES UAE extraction of 11.6 ± 0.11 mg/g, with ethanol solvent using the soxhletation method of 4.25 ± 0.05 mg/g, the reflux method was 3.94 ± 0.03 mg/g, and the maceration method was 4.36 ± 0.05 mg/g. This research shows that NADES with UAE extraction provides higher coumarin content compared to other methods.

This research was conducted to determine the antioxidant activity of meniran leaves extracted using the Ultrasound Assisted Extraction (UAE) method using Natural Deep Eutectic Solvent (NADES) as a solvent with the Hydrogen Bond Acceptor (HBA) component choline chloride and the Hydrogen Bond Donor (HBD) component of several types of sugar, and determined the optimum conditions for the water ratio factor (%), temperature (°C), and extraction time (minutes), then data processing was carried out using the Response Surface Methodology (RSM) method using the Central Composite Design (CCD) design.

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MATERIALS AND METHODS

Materials

The materials used in this research were meniran leaf powder (*Phyllanthus niruri* L.), distilled water, 70% ethanol (Merck®, USA), methanol (Merck®, USA), Bouchardat, Mayer, and Wagner reagents, magnesium powder (Mg), acid sulfate 2 N, hydrochloric acid, gelatin, NaCl-gelatin, iron (III) chloride (FeCl₃), DPPH (Himedia®, India), vitamin C (Merck®, USA), Choline Chloride (Xi'an Rongsheng®, China), glycerin, sorbitol, glucose, maltose, fructose, propylene glycol, and sucrose.

Organoleptic test of simplicia

This test was carried out by observing the smell, taste, shape and color of meniran leaf simplicia powder using the five senses.

Microscopic test

Meniran leaf fragments were identified in order to conduct this test. The simplicia sample was placed on a preparation glass, two drops of chloral hydrate were added, and the sample was covered with a glass object before the observations were made under a microscope with the appropriate magnification.

Water content test

In order to determine the water content, two grams of meniran leaf simplicia powder were weighed and then placed into a steam cup that had previously been weighed. The steam cup was then placed in the oven for fifteen minutes, at 105°C, and the simplicia powder was added. This was followed by an hour-long drying process. The technique was repeated until a consistent weight was achieved. The gravimetric approach is employed.

Ash content test

Weighing two grams of meniran leaf simplicia powder, which was subsequently mixed with a silicate powder that had been tared and dried at 600°C, allowed us to determine the ash content. The sample-containing pot is lit and kept that way until the charcoal runs out. Following cooling, the rate is weighed as a set weight. Determine the amount of ash in materials that have been air-dried.

Preparation of Natural Deep Eutectic Solvent (NADES)

NADES is made with at least 2 components, namely HBA and HBD with a predetermined molar ratio. Table 1 lists the molar ratios and HBD and HBA combinations that were employed. After the preparation and mixing of the HBA and HBD components, the mixture is heated to 40°C for 30 minutes, during which time it is agitated with a magnetic stirrer until it melts. Subsequently, 30% distilled water was included [4].

Table 1. Combination of HBA and HBD.

No	Combination HBA and HBD	Molar Ratio
1	ChCl : Glycerol	1 : 1
2	ChCl : Glucose	1 : 1
3	ChCl : Fructose	1 : 1
4	ChCl : Sorbitol	1 : 2
5	ChCl : Sucrose	1 : 2
6	ChCl : Maltose	1 : 2
7	ChCl : Propylene Glycol	1 : 2

Screening Natural Deep Eutectic Solvent (NADES)

One gram of powdered meniran leaf simplicia was placed in a vial with 10 milliliters of NADES, and the sample was extracted using the *Ultrasound Assisted Extraction* (UAE) method for thirty minutes at 40°C. Following the extraction procedure, UV-Vis spectrophotometry was used to determine the antioxidant activity of each extract. The *Ultrasound Assisted Extraction* (UAE) method's extraction optimization process will make use of the best NADES combination findings that were obtained.

Preparation of 1 mM DPPH reagent

To create a DPPH solution with a concentration of 1 mM, 0.0394 grams of DPPH in total were weighed, dissolved in methanol p.a. to the 100 mL mark, and agitated until homogenous.

Preparation of Vitamin C standard solution

Ten milligrams of vitamin C in total were weighed, added to a 100 mL measuring flask, dissolved in distilled water to the fullest extent possible, and shaken until completely mixed. A 100 ppm concentration of standard liquor was produced.

Preparation of Vitamin C standard series solutions

Pipetted from the 100 ppm stock solution, a total of 0.2 mL, 0.4 mL, 0.6 mL, 0.8 mL, and 1 mL were added to a 10 mL volumetric flask and diluted with distilled water until the mark was homogenous. The standard series of vitamin C was created with changes in concentrations of 2 ppm, 4 ppm, 6 ppm, 8 ppm, and 10 ppm. The UV-Vis spectrophotometry was used to measure the absorbance at different concentrations [5].

Determination of DPPH maximum wavelength

Four mL of a 1 mM DPPH solution were pipetted, added to a vial along with 2 mL of methanol:water (1:1), and then left in a dark location for thirty minutes. Between 400 and 800 nm in wavelength, UV-Vis spectrophotometry was used to conduct the measurements [5].

Preparation of blank solution

The vial was filled with a total of 2 mL of methanol:water (1:1), 2 mL of 1 mM stock DPPH, and UV-vis spectrophotometry was used to quantify the absorbance and the maximum wavelength that was achieved [6].

Preparation of meniran leaf extract test solution

The concentration of the generated NADES-UAE and Main Ethanol-Maceration samples was 100,000 ppm. Subsequently, the stock solution was diluted by pipetting 0.1 mL of the stock solution into PA methanol until it reached the 10 mL volumetric flask threshold. Thus, a 1000 ppm concentration is attained. The solution with a concentration of 1000 ppm was then pipetted into volumes of 0.1 mL, 0.2 mL, 0.3 mL, 0.4 mL, and 0.5 mL, with concentration fluctuations of 10, 20, 30, 40, and 50 ppm. Next, transfer it into a vial, add 1 mL of 1 mM DPPH solution, and dissolve it with Pa methanol until the volumetric flask reads 10 mL. Finally, store it in a dark location for 30 minutes. Next, at a maximum wavelength of 520 nm, UV-Vis spectrophotometry was used to quantify the test solution's absorbance. If the absorbance value is excessively high, dilution is used [6].

Antioxidant activity testing with the DPPH method

Utilizing UV-Vis spectrophotometry with a preset maximum wavelength, the absorbance of the test solution, a standard vitamin C solution, is measured to assess antioxidant activity.

Optimization of extraction experimental design response surface methodology (RSM) method

The CCD (*Central Composite Design*) design will be used in experiments to optimize the extraction of the antioxidant activity of meniran leaves, this method makes it easier to determine optimum conditions.

Optimization of response surface methodology (RSM) design extraction procedure methods

Based on the NADES screening results, a vial containing a gram sample of meniran leaf *simplicia* powder was filled with 10 mL of the best NADES. In order to maximize the extraction of meniran leaves' antioxidant activity, the *Central Composite Design* (CCD) design will be employed in the experiments. This design facilitates the identification of ideal circumstances. Three factors, temperature (°C), extraction time (minutes), and NADES ratio: water (%), are employed to optimize the extraction process. Table 3 shows the time and temperature that have been set for extracting the sample. The *Ultrasound Assisted Extraction* (UAE) technique was used to perform the extraction. After that, the resultant solution is placed in a vial after being filtered via filter paper. After that, UV-Vis spectrophotometry was used to assess the antioxidant activity. We will apply *Response Surface Methodology* (RSM) to process the resultant data. The goal of RSM data analysis is to identify the ideal UAE extraction circumstances.

Table 2. Extraction process optimization design.

Factor	Unit	Lower value	Top value
Temperature	°C	25	60
Extraction Time	Minute	10	60
Ratio NADES : Water	%	50	90

Table 3. Optimization of extraction of CCD design results.

Std	Run	Temperature(°C)	Time (Minute)	Water ratio (%)
8	1	60	60	90
20	2	40	30	70
19	3	40	30	70
1	4	25	10	50
11	5	40	10	70
5	6	25	10	90
6	7	60	10	90
10	8	60	30	70
14	9	40	30	90
12	10	40	60	70
2	11	60	10	50
4	12	60	60	50
18	13	40	30	70
17	14	40	30	70
13	15	40	30	50
15	16	40	30	70
3	17	25	60	50
7	18	25	60	90
16	19	40	30	70
9	20	25	30	70

Verify optimization results

The most ideal NADES mixture and the UAE method which obtained the ideal water, temperature, and time ratio based on the CCD RSM method, were used to extract Meniran leaf simplicia powder. The antioxidant activity of the isolated samples was then assessed six times using UV-Vis spectrophotometry.

RESULTS

Macroscopic test

Organoleptic testing, which includes color, smell, and taste, was done on simplicia meniran powder under a microscope. Table 4 displays the results of the macroscopic tests.

Table 4. Macroscopic test results of simplicia meniran powder.

Parameter	Result	Comparison (Depkes RI. 2017)
Colour	Yellowish green	Yellowish green
Smell	Aromatic specific	Specific
Flavor	Bitter and chelate	Bitter

Water content

Weight measurements serve as the foundation for the gravimetric method, which is used to test for water content. The findings of evaluating the water content of meniran powder were 7.701% (Table 5), but the requirements that fulfill the standards are less than 10%, according to the Indonesian Ministry of Health.

Table 5. Results of simplicia meniran powder water content.

Parameter	Result (%)	\bar{X}
Water content	7.5958	7.701 ± 0.105
	7.8061	

Microscopic Test

Microscopic testing of meniran powder simplicia was carried out using a microscope.

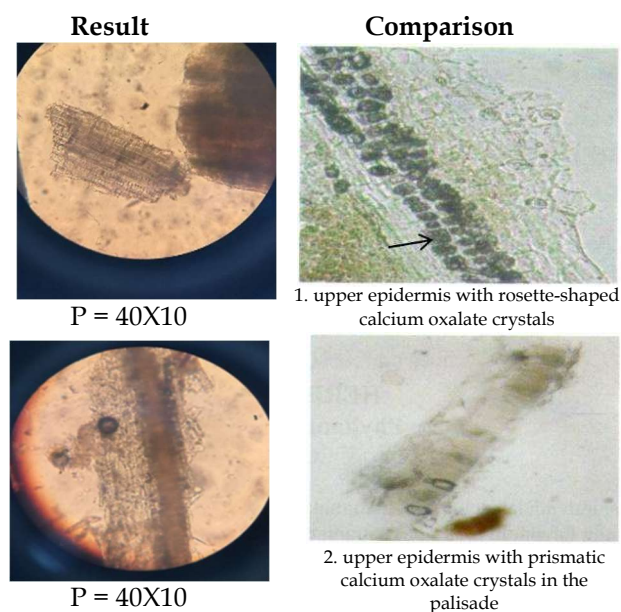


Figure 1. Microscopic test result of simplicia meniran powder.

Ash content

Meniran simplicia's ash content was tested, and the results showed 4.102%. An overview of the sample's mineral content is intended to be provided by the ash content test

Table 6. Results of simplicia meniran powder ash content.

Parameter	Result (%)	\bar{X}
Ash content	4.164	4.102 ± 0.062
	4.040	

Antioxidant analysis results

In this work, the 2,2-Dhyphenil-1-Picrylhidrazil (DPPH) technique was used for antioxidant analysis. A shift in the purple color's intensity serves as the basis for the DPPH technique. In order to complete the missing electron and create a stable antioxidant radical, the antioxidant will contribute its hydrogen atom to the DPPH radical, which functions as a free radical. The purple solution in the DPPH method changes to a yellow solution to indicate the color change.

Wavelength determination results

To find the highest absorption point, the maximum wavelength had to be determined. The purple-colored DPPH solution was employed. 520 nm is the maximum wavelength that was attained. This is consistent with theory, which states that the wavelength at which the absorption point of a purple solution sample is examined is between 500 and 560 nm [7].

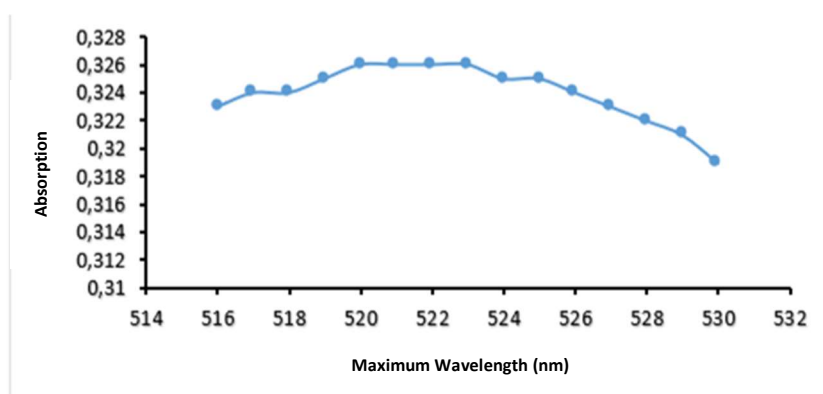


Figure 2. Results of determining maximum wavelength.

Determination of incubation time

The stable measurement time, or the amount of time the antioxidant sample will neutralize free radicals, is ascertained by measuring the incubation period. The resultant incubation period was thirty minutes.

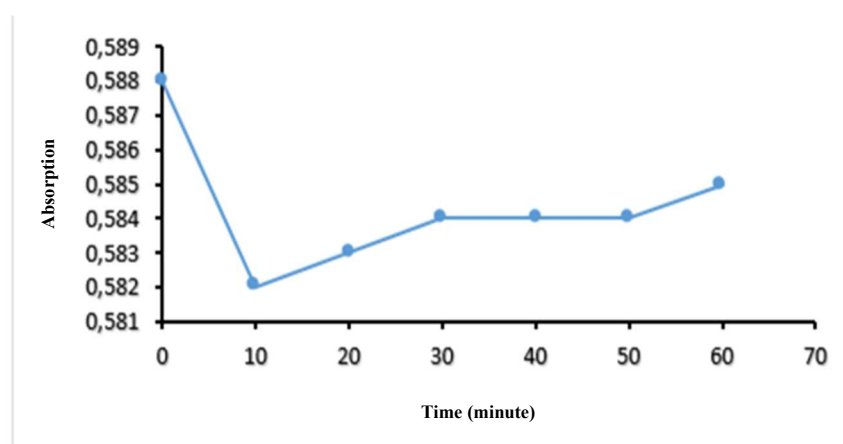


Figure 3. Results of determining incubation time.

Vitamin C antioxidant analysis results

Utilizing the DPPH technique, antioxidant activity is measured. To produce more accurate results, a calibration curve technique was used during the testing process. Samples of vitamin C containing 2, 4, 6, 8, and 10 ppm were prepared. The linearity equation is found by varying the concentration. By fitting the result data into a curve where X represents the percentage inhibition and Y represents the sample concentration, the linearity equation can be found. The IC_{50} value is calculated using this equation. The findings of the vitamin C IC_{50} test were 4.307 ppm, and the results of the blank measurement were 0.927. The Strong antioxidant includes the IC_{50} value results. Using the linear regression equation, which is as follows: $R^2 = 0.9993$ and $y = 7.5891x + 17.314$.

Sample antioxidant analysis results

Meniran (*Phyllanthus niruri* L.) extract samples were employed in this antioxidant analysis utilizing the *Ultrasound Assisted Extraction* (UAE) extraction method and the *Natural Deep Eutectic Solvent* (NADES) solvent. Extracts were also used using the 70% Ethanol maceration extraction solvent. The purpose of the experiment, which used a 70% ethanol extract and the maceration procedure, was to see whether the antioxidant activity of the solvent could be increased. The first step in the testing of antioxidant activity on samples is the NADES screening procedure.

NADES preliminary screening results

The results of antioxidant activity utilizing NADES as a solvent and extraction using the *Ultrasound Assisted Extraction* (UAE) method had a greater IC_{50} value in the first screening of *Natural Deep Eutectic Solvent*

(NADES) than the results of maceration extraction with 70% ethanol solvent. The screening procedure starts with the NADES preparation step, which uses seven distinct NADES combinations, including sugar as the hydrogen bond donor and choline chloride as the hydrogen bond acceptor. Additionally, a mixture of NADES that produces crystals is used in this method; specifically, the mixture of NADES choline chloride - maltose (NADES 6-UAE) and sorbitol (NADES 5-UAE) forms crystals because sorbitol and maltose are used in a ratio of two parts. Choline chloride has an impact on the properties of NADES, and the density of NADES can be influenced by the HBD employed in NADES.

Table 7. NADES screening IC_{50} test results.

Sample	IC_{50} (ppm)	
	Without Control	With Control
NADES 1-UAE (Choline Chloride - Glycerol)	18.039	18.345
NADES 2-UAE (Choline Chloride- Glucose)	46.349	46.577
NADES 3-UAE (Choline Chloride- Fructose)	44.896	45.296
NADES 4-UAE (Choline Chloride- Sorbitol)	52.000	51.130
NADES 7-UAE (Choline Chloride- Propylenglycol)	41.075	40.898

An *Independent T test* was performed to ascertain the difference in influence between the two groups after the IC_{50} values were obtained for each NADES combination and from groups utilizing control and without control. A significant value of 0.998, which is more than 0.05, is displayed in the test results. As a result, data without control is used for additional testing since it indicates that there is no discernible difference in effect between groups with and without control. Table 8 displays the findings of the IC_{50} value with and without the use of controls.

Table 8. Final results of the NADES screening IC_{50} test.

Sample	IC_{50} (ppm)
NADES 1-UAE (Choline Chloride - Glycerol)	18.039 ^a
NADES 2-UAE (Choline Chloride - Glucose)	46.349 ^b
NADES 3-UAE (Choline Chloride - Fructose)	44.896 ^b
NADES 4-UAE (Choline Chloride - Sorbitol)	52.000 ^b
NADES 7-UAE (Choline Chloride - Propylene glycol)	41.075 ^b

Based on the NADES screening results, the combination of Choline Chloride - Glycerol (NADES-1 UAE) has the best effect, the use of NADES based on Choline Chloride - Glycerol has a polarity close to methanol so it is suitable and suitable for extracting phenolic compounds [8].

Response surface methodology (RSM) optimization results

The most ideal extraction conditions, as determined by the NADES 1-UAE optimization results, were 60°C, 44 minutes, and 90% NADES usage ratio. Under these conditions, an extremely potent antioxidant with an IC_{50} value of 8.050 ppm was produced. Additionally, the ANOVA analysis was done using the *Design Expert* application and the RSM method, with a quadratic model, after the IC_{50} value for each optimization condition was determined. Table 9 displays the ANOVA test results, whereas Table 10 displays the IC_{50} optimization value results.

Table 9. Optimization ANOVA test results.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1889.91	9	209.99	738.30	< 0.0001	<i>significant</i>
A-Temperature	1632.55	1	1632.55	5739.87	< 0.0001	
B-Time	18.39	1	18.39	64.67	< 0.0001	
C-Ratio	71.90	1	71.90	252.80	< 0.0001	
Lack of Fit	2.07	5	0.4134	2.66	0.1535	<i>not significant</i>
Pure Error	0.7775	5	0.1555			
Cor Total	1892.75	19				

Table 10. IC₅₀ value from optimization results.

RUN	Temperature(°C)	Time (Minute)	Ratio NADES:Water (%)	IC ₅₀ (ppm)
1	60	60	90	8.050
2	40	30	70	19.450
3	40	30	70	19.754
4	25	10	50	41.947
5	40	10	70	23.022
6	25	10	90	36.030
7	60	10	90	11.601
8	60	30	70	11.064
9	40	30	90	17.438
10	40	60	70	20.501
11	60	10	50	18.974
12	60	60	50	13.917
13	40	30	70	19.423
14	40	30	70	19.200
15	40	30	50	21.623
16	40	30	70	20.171
17	25	60	50	39.655
18	25	60	90	36.102
19	40	30	70	19.093
20	25	30	70	37.461

Significant findings from the ANOVA analysis are displayed, and there is also a lack of fit in the table. The model fits well, as evidenced by the P-Value value of 0.1535, which is greater than 0.05 and renders the lack of fit results non-significant. response is obtained, allowing the analysis to forecast that the analysis data is regarded as legitimate. Factor analysis is then performed, and the studied results are shown as graphs. There are two lines on the graph: the dotted line represents the 95% confidence interval, and the straight line represents the process's results. In Figure 4, the interaction graph is displayed.

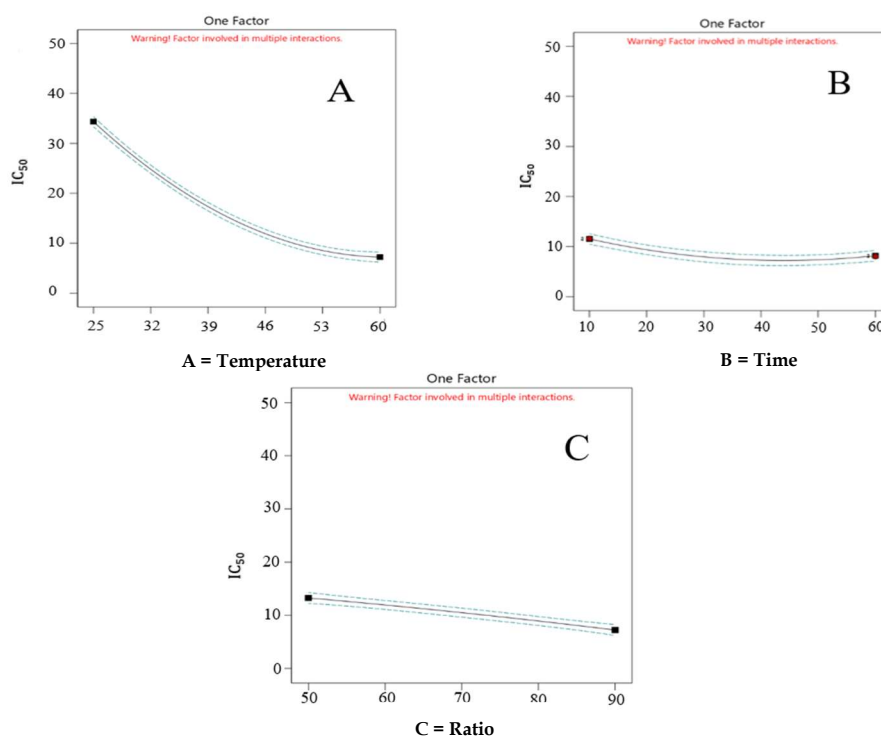


Figure 4. Optimization results graph. (A) temperature to IC₅₀ (B) Extraction time to IC₅₀ (C) NADES ratio to IC₅₀.

The temperature factor in graph (A) indicates that when the extraction temperature is raised, the IC₅₀ value decreases. At 60°C, there is a reduction in the graph, which represents the drop in the IC₅₀ value. Additionally, when the extraction time increases in graph (B), the IC₅₀ value decreases. At 44 minutes, the graph shows a reduction that corresponds to the IC₅₀ value. As the NADES utilization ratio rises, the IC₅₀ value also decreases in graph (C). Antioxidant activity was investigated in this study, and the more active the antioxidant activity, as indicated by the graph's tendency to decline, the smaller the IC₅₀.

After the ideal conditions have been reached in this manner, a verification procedure will be conducted. The optimum NADES combination, choline chloride - glycerol (1:1) with ideal conditions for optimization outcomes is used in the verification phase. Table 11 displays the optimization formula's findings.

Table 11. Optimization results formula.

No	Temperature	Time	Ratio	IC ₅₀	Desirability	
1	60.000	44.000	90.000	7.226	1.000	Selected
2	60.000	44.000	89.823	7.257	0.999	
3	59.836	44.000	90.000	7.233	0.999	
4	60.000	43.739	90.000	7.226	0.998	
5	59.699	44.000	90.000	7.240	0.998	

The optimization formula yielded a desirability value of 1,000, indicating that the optimal extraction results will yield a response of 100% when temperature, time, and NADES utilization ratio are used [9]. The optimal value is displayed when the desirability value is near to 1, as this indicates the precision of the optimization process [10].

Verification results

Using the extraction conditions that come from the optimization, retesting is done in the verification stage to validate the anticipated optimum circumstances. If the test is conducted in accordance with the parameters of the optimization results, the Design Expert's projected value will yield an IC₅₀ value of 7.226 ppm. The verification values for samples of meniran leaf extract (*Phyllanthus niruri* L.) at 60°C, 44 minutes of extraction duration, and 90% NADES utilization ratio are shown in Table 12.

Table 12. IC₅₀ value verification results.

Verification	IC ₅₀	\bar{x}	Information
1	8.089		
2	8.074		
3	8.065	8.075 ± 0.013	very active
4	8.053		
5	8.091		
6	8.079		

Table 13. Predicted results and actual results of IC₅₀ value.

IC ₅₀	Predicted Mean	Predicted Median	Std Dev	SE Mean	95% CI low for Mean	95% CI high for Mean
	7.22621	7.22621	0.533313	0.457149	6.20762	8.2448

Meniran leaf extract (*Phyllanthus niruri* L.) has an IC₅₀ value that falls between 95% CI (Confidence Interval) low and 95% CI (Confidence Interval) high, according to the verification results. The IC₅₀ value is not far from the projected value because the results of the six IC₅₀ value runs fall within the 95% confidence interval.

DISCUSSION

The antioxidant activity test showed that the combination of choline chloride - glycerol (NADES-1) had a significantly different effect on choline chloride - glucose (NADES-2), choline chloride - fructose (NADES-3),

choline chloride - sorbitol (NADES-4) and choline chloride - propylene glycol (NADES-7). The combination of Choline Chloride - Glycerol (NADES-1 UAE) has the best effect. The aforementioned findings demonstrate that variables related to temperature, time, and NADES ratio can affect antioxidant activity. It is simpler for the solvent to diffuse into the pores and dissolve the compounds in the sample properly when the extraction temperature is raised since this will cause the solvent molecules to move swiftly and randomly. In order for extraction to proceed as best it can, extending the extraction time also lengthens the duration of contact between the solvent and the sample. Additionally, the longer the extraction time, the longer the sample is exposed to ultrasonic waves, which breaks down the cell walls of the sample and allows the solute to dissolve in the solvent.

CONCLUSION

With an IC₅₀ value of 18.04 ppm, the combination of NADES-1 Choline chloride and Glycerol produced the greatest results for antioxidant activity. At 60°C, 44 minutes of extraction time, and a 90% NADES (Natural Deep Eutectic Solvent) usage ratio, the ideal conditions for UAE (Ultrasound Assisted Extraction) extraction yield the best results for antioxidant activity. An IC₅₀ value of 8.050 ppm is obtained, and the verification results show that the average IC₅₀ is 8.075 ppm.

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