

Thermal stability and pH of antioxidant activity of ethanol extract of Ciplukan herbs (*Physalis angulata* Linn) along with determination of pKa

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ABSTRACT

In this study, thermal stability and pH for the antioxidant activity of the ethanol extract of ciplukan (*Physalis angulata* Linn) herbs had been studied. Among the antioxidant activity methods, the use of DPPH (2,2-diphenyl-1-picrylhydrazyl) reagent is simple and easy to use. The thermal effect was observed by warming the sample at 50, 70, and 100 °C in intervals of 30, 45, and 60 min. The results showed that the half-maximal Inhibitory Concentration (IC₅₀) of the extract was 185.53±1.35, which was classified as medium. Based on these results, a significant decrease was observed at 100 °C and pH=8. Thus, the pKa of the extract is recommended for maintaining stability at a pKa value of 3.83, which is proven to maintain a spot in the pH range of 4.

Keywords: Ciplukan (*Physalis angulata* Linn.); antioxidant activity; temperature

INTRODUCTION

Ciplukan (*Physalis angulata* Linn) herb as part of the Solanaceae family has empirical efficacy that has been tested preclinical, including antioxidant, hypoglycemic agent, microbicide, antiviral, immune system modulator, anti-fibrotic, anti-inflammatory, and antineoplastic [1–6].

Phytochemical screening of ciplukan from other studies revealed that the herb contains phenols (flavonoids, tannins, and phenylpropane), secosteroids (physalin, withanolid, etc.), and saponins [3-30]. Phyto-phenol, as a phytoconstituent, plays a major role in biological activities as an antioxidant and immunomodulator [29]. Antioxidant defenses have functions, including: suppress and scavenge ROS production, repair cell damage, gene damage protection, activate antioxidant proteins and enzyme expression [30]. Hence the antioxidant activity is universal and can represent the content of vitamin C and flavonoid compounds which are thermolabile and sensitive to changes in heating and pH, then the antioxidant activity is chosen as an indicator of the decrease in the ability of the extract [9-20]. Plant extracts have a complicated problem because all parts of the compound of the extract being regarded active substances, so

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all chemical constituents must be preserved. In the production process of herbal medicinal preparations, significant changes in pH and temperature are often encountered. At a certain pH, active substances from plants can change to their salt forms or other inactive forms. Meanwhile, at high temperatures, it can cause the decomposition of the active compound part due to not being able to withstand heat so that the inactive substance becomes and is impaired [22-24]. Among the antioxidant measurement methods, the inhibition DPPH method (2,2 diphenyl-1-picrylhydrazyl) was chosen because it has several advantages, it is easy, simple, and uses a few samples in a short time with good precision results [25]. Thus, it is necessary to investigate the antioxidant profile of the ethanol extract of Ciplukan herb on the effect of temperature and pH, because no one has studied it yet.

MATERIALS AND METHODS

Plant materials

All parts of Ciplukan herbs, except the roots, were gathered from December 2019 to February 2020 from several areas in West Java, Indonesia. The harvested specimens were identified at the Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran.

Chemical Materials

Acetic acid, boric acid, hydrochloric acid, acetone, 70 % ethanol, 95 % ethanol, ethyl acetate, potassium biphthalate, potassium dihydrogen phosphate, methanol, DPPH reagent (1,1- diphenyl-2-picrylhydrazyl) (Sigma Aldrich, St. Louis, MO, USA), silica plate F254, water-saturated toluene, sodium hydroxide, vitamin C (PT. Bratachem, Indonesia), distilled water. All additional compounds were purchased from local commercial vendors.

Preparation ethanol extract of Ciplukan herbs

The extraction method was the cold maceration process using a solvent that was stirred several times, carried out at room temperature. All parts of ciplukan herbs (except roots) were soaked with 70% ethanol for 3 × 24 hours. A rotary vacuum evaporator was used to thicken the filtrate.

Phytochemicals screening and Standardization of extract

Standardization was carried out to determine the quality such as organoleptic, ethanol-soluble extract content, water-soluble extract content, total ash content, acid-insoluble ash content, and drying shrinkage as Indonesian Herbal Pharmacopoeia (FHI) parameters [21].

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Evaluation of this parameter is intended to provide an initial information of the total amount of the chemical compounds in an herbal material.

Phytochemical screening conducted to determine the secondary metabolite content which has efficacy responsibility. The phytochemical screening included polyphenols (such as gallic acid), flavonoids (as like as quercetin), saponins, and secosteroids (such as physalin A and withanolid A).

Determination of the contents of physalin A and withanolid A was carried out using high-performance liquid chromatography (HPLC-UV) (Waters Alliance HPLC, Waters Corporation, USA), meanwhile the polyphenol, flavonoid, and saponin were using HPTLC system with different eluent system. The solvent of toluene: ethyl acetate: formic acid in ratio 5:4:1 for determination gallic acid, the same solvent system with ratio 1:9:1 for saponin and the same eluent system with ratio 10:10:1.5 for quercetin.

Thermal and pH treatments

Heat Treatment on Ciplukan herbs Extract
A stock solution of extract was made with a concentration of 5000 ppm using 96 % ethanol as solvent. The extract solution was

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then put into several brown vials and then heated at 50, 70 and 10⁰ C, respectively, and at interval storage of 30, 45 and 60 min. Then tested for antioxidant activity.

pH Treatment on Ciplukan herbs Extract

A stock solution of extract was made with a concentration of 5000 ppm using 96 % ethanol as solvent. The extract solution was then put into several brown vials and a buffer solution of pH 1, 4 and 8 was added in a ratio of 1:1. Then stirred using a magnetic stirrer for 30 min and tested for antioxidant activity.

Thin-Layer Chromatography (TLC) profile of treatment results

Prepared a solution of temperature and pH test results along with controls, then smeared on the Silica gel F254 TLC plate and eluted using toluene-acetone-acetic acid (6:6:0,05).

The test solution used was made by making an extract solution with a concentration of 15 % in 70 % ethanol and as a comparison made a quercetin solution with a concentration of 0.3 % in 96 % ethanol.

The TLC plate was removed and air dried. Then the TLC plate was observed under UV light at 254 and 366 nm. If necessary, the spots can be sprayed with a spot viewer using LP citronella detection and then

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heated at 100 °C for 5-10 min and checked again at 366 nm UV light and then the Rf value was calculated.

Antioxidant activity test

The relatively stable DPPH radical had been used widely to test the ability of compounds to act as free radical scavengers or hydrogen donors. This capability was used to evaluate antioxidant activity. DPPH reagent was weighed as much as 5 mg and dissolved in 50 mL of pure ethanol. Various extract solutions were made with concentrations of 1000 ppm, 1400 ppm, 1800 ppm, 2200 ppm, 2600 ppm and 3000 ppm. Each concentration of samples and positive control (ascorbic acid) as much 100 µL was added to a 96-well microplate. Each well was added 100 µL of DPPH and absorbance control was made, then the sample was incubated for 30 min. Samples were measured with a microplate reader and then observed the absorbance value then antioxidant values were calculated by the formula.

DPPH scavenging equals

$$\frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100\%$$

Determination of pKa Ciplukan herbs extract

Estimation of pKa values of Ciplukan herbs extract was performed by spectrophotometric measurement in buffers with a pH range of 1.2–10. Preparation of buffers at pH between 1.2–4 was conducted using HCl, NaCl, and KHP with different compositions. At pH 5, KHP and NaOH were used, and at pH 6 and 7, KH₂PO₄ and NaOH were used. Meanwhile, at pH 8 and 9, H₃BO₃, KCl, and NaOH were used following the Indonesian Pharmacopeia. Samples (10 mg) were dissolved in a methanol and buffer solution with a ratio of 4:6 up to 100 mL, and the pH result was checked using a Mettler Toledo S20 pH meter. The sample solution was filtered through Millipore 0.45- μ m filter paper and suitably diluted before measurement by UV spectroscopy at 300 nm.

RESULTS

Phytochemical Screening and Standardization of Ciplukan herbs extract

The phytochemical content according to the standardization of *Physalis minima* leaves in the Indonesian herbal pharmacopeia (FHI) in the form of total flavonoids as quercetin in the extract was carried out to identify the presence of standardized phytochemicals and

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standardization of other parameters of extract [21,26–27]. The results following Table 1 obtained that all meet the standard with a value above the FHI standard.

Standardization of antioxidant activity of extract

Antioxidants will react with DPPH through an electron donor mechanism, which is stabilized by decreasing the intensity of the purple color of DPPH and slowly turning yellow due to the formation of DPPH-H. DPPH acts as a hydrogen donor for antioxidant compounds of several hydroxyls (-OH) and methyl (-CH₃) substitutions in phenolic compounds in the phytochemical content of the extract [8–19]. This color-reducing activity can be observed by visible spectrophotometry at 500–600 nm, in this study the maximum wavelength found at 517 nm. As the results in Table 2 with vitamin C as a strong antioxidant standard, obtained according to the calculation of the antioxidant activity of the extract having moderate activity (medium) with a result of 185.53 ± 1.35 μ g/mL.

Effect of Thermal and pH on antioxidant activity of extracts

Regarding the effect of thermal and pH on the antioxidant activity of Ciplukan herbs extract, it was carried out to determine the

extent of the influence of these factors during the processing that was able to change its quality [22-24].

The appearance of flavonoid changes (quercetin as a reference) from the extract

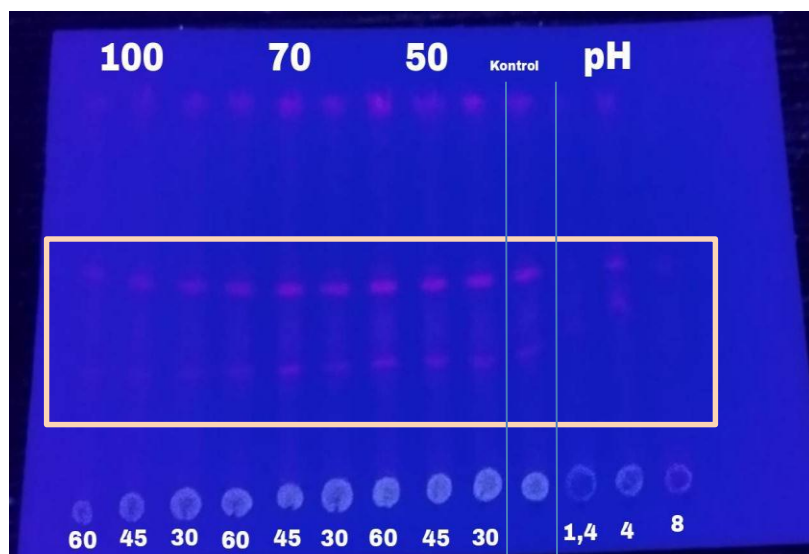
due to treatment was seen through Thin-Layer Chromatography (TLC) as shown in Figure 1.

Table 1. Parameter standardization extract of Ciplukan herbs (*Physalis angulata* Linn.) Vs *Physalis minima* (from FHI)

Quality parameter of Extract	<i>Physalis angulata</i> Linn herbs	<i>Physalis minima</i> leaves (from FHI)
Specific parameters		
Organoleptic	Dark greenish brown viscous extract, very thick, smells of extract	Extract thick, dark brown, and bitter taste.
Water soluble extract content	27.70 %	> 8.10 %
Ethanol soluble extract content	7.21 %	> 2.80 %
Total flavonoid content (with quercetin as standard)	0.82 %	≥ 0.58 %
Physalin A	0.968 %	-
Withanolid A	0.312 %	-
Gallic acid	0.264 %	-
Saponin	2.584 %	-
Non Specific parameters		
Density of ethanol extract	1.02	-
Water content	12.00 %	< 10 %
Loss on drying	12.53 %	< 10 %
Total ash content	17.14 %	-
Acid insoluble ash content	4.83 %	-
Extract rendement	13.70 %	> 9.6 %

Table 2. IC₅₀ values of ascorbic acid and extracted Ciplukan herbs

Samples	Linear regression	IC ₅₀ (µg/mL)	Average IC ₅₀ (µg/mL)	IC ₅₀ in DPPH means
Vitamin C (standard)	$y = 21.226x + 12.272$	1.777	1.775 ± 0.0015	Very active < 50 µg/mL Active 50 – 100 µg/mL Medium 101 – 250 µg/mL Weak 250 – 500 µg/mL Inactive > 500 µg/mL
	$y = 21.203x + 12.384$	1.774		
	$y = 20.987x + 12.712$	1.776		
<i>P. Angulata</i> Extract	$y = 0.175x + 17.491$	185.766	185.538 ± 1.3544	Very active < 50 µg/mL Active 50 – 100 µg/mL Medium 101 – 250 µg/mL Weak 250 – 500 µg/mL Inactive > 500 µg/mL
	$y = 0.174x + 17.503$	186.764		
	$y = 0.178x + 17.233$	184.084		

**Figure 1.** Thin Layer Chromatography (TLC) appearance spot of extract ethanol of Ciplukan herbs after treatments and quercetin (as control) in a UV lamp at 366 nm.

The TLC system uses a solvent or mobile phase which is the result of optimization, namely Toluene-Acetone-Acetic Acid (6:6:0.05) with the stationary phase used is silica gel 60 F254. The total flavonoid spot obtained from the extract and the spot on the sample has the same Rf value as quercetin 0.467. As shown in Figure 1, the stains gradually disappeared towards the highest temperature and storage time. Whereas in the pH treatment looked, the spot were still visible at pH 4.

To proof of the stability of the extract at pH variations, the pKa determination was made by dissolving the extract in various solutions with pH variations 1–10 [28]. The results as shown in Figure 2 were obtained from the equation of the line that intersects the absorbance logarithm of 3.83.

Thermal treatments were performed at temperatures of 50⁰, 70⁰, and 100⁰C with a storage period of 30, 45, and 60 min, while pH treatments were conducted at pH 1,4, 4, and 8. Considerations for choosing temperature and storage time were coming

into the most drying treatments of simplicia and extracts, while the pH was based on the condition of the digestive segment and the ionic ability limit of the extract when served with other additives supplemented.

The scavenging activity of the Ciplukan herb extracts during treatment was reduced, along with spot fading from the TLC profile due to the influence of thermal and pH variations. It can be observed from Tables 3 and 4. The most significant reduction in antioxidant activity was observed at 100⁰C, while the least effect was observed at 50⁰C. Similarly, for the antioxidant activity at the pH of the treatment, where it appeared at pH 4, there was no significant change as the spot only appeared in the TLC profile. This was shown for the extract stability at the drying or processing temperature and pH conditions that need to be maintained.

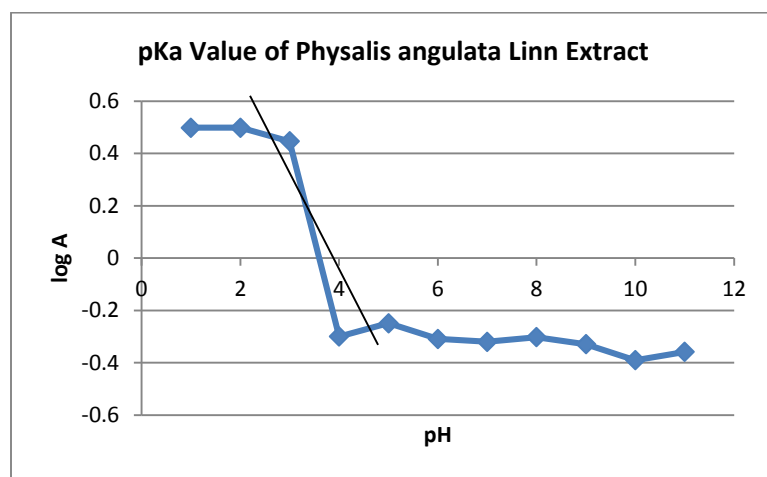
Table 3. The IC₅₀ value and the antioxidant activity reduction of extract ethanol Ciplukan herbs after thermal treatments

Temperature	60 minutes		45 minutes		30 minutes	
	IC ₅₀ % (ppm)	% reduction	IC ₅₀ % (ppm)	% reduction	IC ₅₀ % (ppm)	% reduction
100 °C	265.616± 0.91	43.159	253.093±0. 9	36.410	244.618±1. 8	31.842
70 °C	199.787± 1.40	7.679	195.076±1. 7	5.141	193.507±1. 3	4.295
50 °C	197.056± 1.12	6.207	192.870±2. 3	3.952	188.085±1. 6	1.372
Control	185.538 ± 1.3544					

*The data was presented as mean ± standard deviation of IC₅₀, The most active DPPH scavenging activity was ascorbic acid with the lowest value of IC₅₀

Table 4. The IC₅₀ value and the antioxidant activity reduction of extract Ciplukan Herbs after pH variation treatments

pH conditions	IC ₅₀ (ppm)	% Reduction
pH 1.4	238.473±0.85	28.530
pH 4	184.779±0.55	-0.409
pH 8	2502.768±3.6	1248.924
Control	185.538 ± 1.3544	



Line Equation	R ²	pKa Value
$y = -0.2988x + 1.1445$	0.80	3.83

Figure 2. The pKa value determination of *Physalis. angulata* Linn extract

DISCUSSION

The range of parameters is influenced by various factors, including the age of the plant, harvest time, climate, and geographical location. All results already represent necessary information on the origin of plant materials and common phytochemistry content, which should be checked as specific and non-specific parameters before use to ensure their quality. As shown in Table 1. The parameters are appropriate for FHI standards.

The activity of antioxidant of this extract shows the similarity of results with various studies of antioxidants in previous

Ciplukan herbs extracts which are in the range of 130-820 $\mu\text{m}/\text{mL}$ [2-3,5-8]. There is much published literature related to the measurement of antioxidant activity using the DPPH method on ciplukan herb extracts, but the changes in antioxidant ability due to other factors have not yet been studied.

CONCLUSION

The phytochemical examination results and standardization of the ethanol extract of Ciplukan herbs were conformed to the standard parameters of the Indonesian Herbal Pharmacopoeia and the parameters others ethanol extract of Ciplukan herbs

had been published previously. The antioxidant activity of the ethanol extract of Ciplukan herbs belongs to the intermediate classification, possibly because the amount of polyphenols and flavonoids, has lower than the content of secosteroids (physalin and withanolide), the antioxidant activity is obtained at $185.53 \pm 1.35 \mu\text{g/mL}$. Application of temperature and pH showed a decrease in antioxidant ability, seen past temperatures above 70°C for 60 min to 100°C there was a significant decrease in activity. Likewise with the pH treatment where the ability of the ethanol extract of *Physalis angulata* Linn was sufficient to survive at pH 4 conditions compared to alkaline conditions. This looks synergistically with the determination of the pKa of the extract at a pKa 3.83.

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