In Vitro Anti-Photoaging Properties of *Phylanthus urinaria* L. Herb Extract

Ines Septi Arsiningtyas, Desy Damayanti Simamora, Angela Merici Palimbongan, Fabiana Disa Widianingtyas, Sendy Junedi*

Faculty of Biotechnology, Universitas Atma Jaya Yogyakarta, Yogyakarta, Indonesia

Abstract

The overexposure to ultraviolet (UV) radiation from sunlight is related to photoaging of the skin and high risk of skin cancer. Sunscreen material and antioxidants are commonly used to protect skin from harmful UV. However, the application of synthetic compounds in sunscreen products is regulated to a limited amount because it can produce photosensitizers, inducing adverse reactions in the skin, and also harmful to the environment. Ethanol extract of *Phyllanthus urinaria* L. herb possesses a strong antioxidant activity similar to ascorbic acid, which makes it potential to substitute the synthetic compound in sunscreen products. Therefore, this research was conducted on the 70% ethanol extract of *P. urinaria* herb to analyze its *in vitro* anti-photoaging properties, *i.e.*, antioxidant, protein antiglycation and antiinflammation, also to determine the Sun Protection Factor (SPF) and the quantitative composition of the metabolites. The extract exhibited a strong antioxidant activity IC_{50} of 3.01 mg/L, a higher moderate antiglycation activity IC_{50} of 216.67 mg/L, but low anti-inflammatory activity IC_{50} of 86.61mg/L. The presence of saponin, phenolic compounds, flavonoids, tannins, and methyl linoleates is suggested to contribute to the anti-photoaging properties. According to the anti-inflammatory assay, the extract may not inhibit signaling pathways that follow cytokine expression but possibly inhibit those that precede cytokine expression due to its antioxidant activity. An SPF value of 5.95 at 50 mg/L meets the recommended range for the skin phototypes of Southeast Asian, dark-skinned Asian, and African people. These results indicate that *P. urinaria* extract has potential as an anti-photoaging material for sunscreen products.

Keywords: *Antiglycation, antiinflammation, antioxidation,* Phyllanthus urinaria *L., SPF.*

INTRODUCTION

Human exposure to UV radiation primarily comes from the sun. The ozone layer of the atmosphere blocks UVC (100-280 nm) and approximately 90% of UVB radiation (280-315 nm). As a result, UVA radiation (315-400 nm), along with a small amount of UVB radiation, is the dominant form of UV radiation that reaches the Earth's surface (World Health Organization, 2002). The UV radiation index in Indonesia, according to the Indonesia Meteorology,

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*Corresponding author: sendy.junedi@uajy.ac.id

Climatology, and Geophysical Agency, is moderate to very high risk to skin and immune system, ranging from 5 to 10 between 10 a.m. and 2 p.m. (World Health Organization, 2002).

Overexposure to UV radiation could elevate the production of Reactive Oxygen Species (ROS), and promote a variety of damaging effects, including gene mutation, which increases the risk of nonmelanoma skin cancer (D'Orazio, *et al*., 2013; Kim and He, 2014). Overproduction of ROS also initiates, leading to lipid peroxidation and glycoxidation reactions of cellular components (Moldogazieva, *et al*., 2019). These reactions produced advanced lipooxidation (ALE) and glycation end products (AGE), which disrupt cell signaling and function, leading to cell damage. Thus, excessive production of ALE and AGE is related to aging, especially photoaging of the skin (Masaki, *et al*., 1997; Moldogazieva, *et al*., 2019).

To prevent photoaging and skin cancer, sunscreen material and exogenous antioxidant has been commonly used (Shanbhag, *et al*., 2019). Antioxidants work by preventing the formation of UV-induced ROS (Shanbhag, *et al*., 2019). Meanwhile, sunscreen material protects the skin from harmful UV rays by absorbing or reflecting UV, which is expressed by SPF value (Shanbhag, *et al*., 2019; Paulina, *et al*., 2020). Synthetic compounds in sunscreen are regulated to a limited amount because they can produce photosensitizers, which can cause negative skin reactions (Heurung, *et al*., 2014). Ovobenzone and octyl methoxycinnamate are some examples of common synthetic compounds in sunscreen product that effectively absorb UV radiation but produce photoreactive compounds that cause adverse skin reactions (Abid, *et al*., 2017; Duale, *et al*., 2010). On the other hand, natural compounds such as flavonoids and polyphenols present in herbal extracts are typically part of a complex mixture that can have synergistic effects, reducing the overall photosensitizing potential (Radice, *et al*., 2016). Therefore, natural compounds in herbal extract offer a great potential to substitute the synthetic compounds in sunscreen products in order to reduce the quantity of synthetic compounds (Saewan & Jimtaisong, 2013). Besides the photosensitizing problem, synthetic compounds in sunscreen products are limited because many of them are harmful to the environment, causing pollution and affecting aquatic life when they wash off and enter water systems (Schneider and Lim, 2019). Many natural compounds have antioxidant, antiinflammation, and immunomodulatory activities to prevent the damaging effects of UV radiation (Saewan and Jimtaisong, 2013). Natural compounds such as polyphenols, alkaloids, and saponin have gained attention for their anti-aging ability, marking their potential to be material in sunscreen products (Afnan, *et al*., 2012; Saewan and Jimtaisong, 2013; Shanbhag, *et al*., 2019; Choi, *et al*., 2020).

Phyllanthus urinaria L. is a plant that has been widely used in traditional medicine across various cultures. This plant has been subjected to scientific research due to its potential bioactivities such as antioxidant, anticancer, antibacterial, antivirus, antihypertensive and hepatoprotective effects (Geethangili and Ding, 2018; Puspitarini, *et al*., 2022). The ethanolic extract of *P. urinaria* is reported to have strong antioxidant activity with an IC_{50} of 4.46 mg/L (Nurwijayanto, *et al*., 2020), similar to ascorbic acid activity as a potent and well-known antioxidant compound. It envisaged that the metabolites compounds in *P. urinaria* extract might prevent the oxidation stress and thus inhibit the damage caused by glycation of extracellular matrix proteins in the skin. Furthermore, flavonoids and alkaloids in the extract are expected to absorb UV rays due to the presence of chromophores in their structure.

This study builds on previous research to further examine the properties of the ethanol extract of *P. urinaria* herb as an anti-photoaging agent. The extract was analyzed for its protein glycation inhibitory, antioxidant, and anti-inflammatory activity, as well as its SPF value. To better understand the relationship between the metabolite

compounds in the extract and its bioactivity, the study also quantitatively determined the levels of secondary metabolites, including saponin, phenolic compound, flavonoid, tannin, alkaloid and fatty acids. Ultimately, the aim of this research is to demonstrate the effectiveness of the ethanolic extract of *P. urinaria* herb as an anti-photoaging material for use in future sunscreen products.

METHODS

Preparation of Extract

P. urinaria L. were collected from Institut Pertanian Bogor, and taxonomically identified by Unit Konservasi Budidaya Biofarmaka (UKKB), Pusat Studi Biofarmaka tropika LPPM IPB, Bogor. Voucher identification number was 006/IT3. L1.13/UKBB/I/2021. The herbs were dried at 48°C for 24 h then ground to obtain dry powder. Moisture content analysis was performed using a Moisture Analyzer (OHAUS MB120). Extraction was performed by adding 70% ethanol to 100 g of dried powder with a material-to-solvent ratio of 1:5 (v/v). The mixture was macerated at 120 rpm and 24°C for 48 h. The filtrate was concentrated using a vacuum rotary evaporator (IKA® RV 10 Basic) at 60 rpm and 50°C. The concentrated extract was dried in an oven (MMM Medcenter Venticell) at 45°C and weighed to calculate the yield (%). The extracts were stored at 4°C until next analysis.

Proximate Analysis: Water-Soluble and Ethanol-Soluble Extract

One hundred grams of *P. urinaria* herb powder were macerated with 500 mL of absolute ethanol and water-saturated chloroform separately at 120 rpm, 24°C for six hours, following with incubation for 18 h. The filtrate was concentrated with a vacuum rotary evaporator. The concentrated extract was dried in the oven and weighed to calculate %yield (Indonesian Ministry of Health, 2017).

Evaluation of DPPH Free Radical Scavenging Activity

DPPH 0.4 mM was prepared in ethanol, and one mL of the solution was added to 0.1 mL of ascorbic acid as a standard (final concentration of 1-3 mg/L), or *P. urinaria* herb extract (final concentration of 1-5 mg/L). The solutions were incubated for 30 minutes and the absorbance was measured at 517 nm. The scavenging activity was calculated as inhibition percentage using the equation:

$$\%\text{Inhibition} = \frac{\text{Absorbance of negative control} - \text{Absorbance of tested sample}}{\text{Absorbance of negative control}} \text{x} 100\%$$

Note: negative control = DPPH with solvent

The IC_{50} values were calculated by the regression equation from different concentrations of the samples. The analysis was performed in triplicate (Eshwarappa, *et al*., 2014).

Evaluation of Antiglycation Activity in BSA-Glucose Model

The extract was dissolved in 50% ethanol to obtain a concentration of 10,000 mg/L and diluted with deionized water to make a series of concentrations. The test solution was added to a solution containing BSA (20 mg/mL), D-fructose (235 mM), and D-glucose (235 mM) in potassium phosphate buffer (200 mM, pH 7.4). The mixture was incubated at 60°C for 40 h. The fluorescence intensity was measured by a spectrofluorometer (Shimadzu, RF-6000) at an excitation wavelength of 330 nm and an emission wavelength of 440 nm. Aminoguanidine hydrochloride was used as a standard inhibitor (Junedi, *et al*., 2023).

Evaluation of Anti-inflammatory Activity in Protein Denaturation Model

The stock solution of the extract and diclofenac sodium (positive control) was prepared by diluting in 50% ethanol and DMSO,

respectively. Various concentrations of those test solution was prepared by diluting with Tris buffer saline pH 6.3. Test solution was then mixed with 4.5 mL of BSA 0.2% w/v in Tris buffer saline pH 6.3. Each solution was incubated for 30 minutes at room temperature, then heated for 5 minutes at 80-82°C, and subsequently cooled down at room temperature for 25 minutes. Absorbance was measured at a wavelength of 660 nm, and the inhibition percentage of protein denaturation was calculated with the formula bellow:

$$\%\text{Inhibition} = \frac{\text{Absorbance of negative control} - \text{Absorbance of tested sample}}{\text{Absorbance of negative control}} \text{x}100\%$$

Inhibition percentage more than 20% indicated potential anti-inflammatory compound (Farida, *et al*., 2018).

Determination of Total Flavonoid Content

P. urinaria herb extract was prepared to get concentration of 5,000 mg/L, and quercetin as a standard was prepared with concentration of 250-1,000 mg/L. A volume of 0.5 mL of test solutions was added with 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate, 1.5 mL of absolute ethanol, and distilled water until 5 mL final volume. The solutions were incubated for 30 minutes at room temperature, and the absorbance was measured at 415 nm. The analysis was performed in triplicate, and the result was expressed as mg quercetin equivalent (QE)/gram extract (Indonesian Ministry of Health, 2017).

Determination of Total Phenolic Content

The extract was prepared to get concentration of 5,000 mg/L, and gallic acid as a standard was prepared at the concentration of 10-200 mg/L. A volume of 1 mL from test solution was added to 5 mL of 7.5% Folin-Ciocalteu, and then incubated for 8 minutes, followed by the addition of 4 mL of 1% NaOH and incubation for 1 h. The absorbance was measured at 730 nm. The analysis was performed in triplicate, and the results were expressed as mg gallic acid equivalent (GAE)/gram extract (Indonesian Ministry of Health, 2017).

Determination of Total Tannin Content

The extract was prepared to get concentration of 5,000 mg/L, and tannic acid as a standard was prepared at the concentration of 55-220 mg/L. A volume of 0.5 mL from the test solution was added to 2.5 mL of 10% Folin-Ciocalteu and 2.5 mL of 7.5% Na2CO3, followed by incubation for 1 h. The absorbance was measured at 775 nm. The analysis was performed in triplicate, and the results were expressed as mg tannic acid equivalent (TAE)/gram extract (Galvão, *et al*., 2018; Ahmed, *et al.*, 2019).

Determination of Total Alkaloid Content

The extract was prepared to get concentration of 5,000 mg/L with 2N hydrochloric acid, and atropine sulfate as a standard was prepared at the concentration of 20-100 mg/L. A volume of 1 mL from the test solutions was replaced to a separating tunnel, and then added with 5 mL of phosphate buffer pH 4.7, 5 mL of 10-4 M bromocrescol green solution and 5 mL chloroform. The mixture was shaken several times, and then the chloroform layer was collected. Absorbance was measured at 415 nm. The analysis was performed in triplicate (Patel, *et al*., 2015).

Determination of Total Saponin Content

The extract was diluted in 50% ethanol to get concentration of 5,000 mg/L. saponin as a standard (50-150 mg/L). A volume of 0.5 mL from test solutions was reacted with 0.5 mL of 8% vanillin solution and 4 mL of 72% sulphuric acid. The mixture was incubated at 60°C for 10 minutes, and the absorbance was measured at 544 nm. The analysis was performed in triplicate (Wu, *et al*., 2019).

Gas Chromatography-Mass Spectrometry (GC-MS)

The extract in 70% ethanol was injected into the GC-MS Shimadzu QP2010 SE (Japan). The column is Rtx-5MS (5% diphenyl/95% dimethyl polysiloxane), length 30 m, diameter 0.25 mm and thickness 0.25 µm, maximum column temperature 330˚C, helium carrier gas with flame ionization detector and detector M.S. Mass spectra data was compared with the chemical structure database in the Wiley library to determine the chemical compounds contained in essential oils.

Determination of SPF Value

P. urinaria extract was prepared at the concentration of 25 mg/L and 50 mg/L with 50% ethanol. Quercetin was used as a standard. The absorbance of samples were measured at UV-B wavelength (290-320 nm) with 5-nm increments and three determinations were made at each point (Junedi, *et al*., 2023). The SPF was calculated by applying equation:

$$\mathrm{SPF} = \mathrm{CF} \sum_{290}^{320} EE(\lambda).I(\lambda).Abs(\lambda)$$

Note: EE(λ), erythemogenic effect of wavelength radiation; I(λ), sun intensity at wavelength; λ, wavelength (Mansur, *et al*., 1986).

where CF (correction factor)=10 (constant); EE=erythemogenic effect; I=intensity of the sun and Abs=absorbance of sample. The constant EE and I were pre-defined according to Table 1.

Table 1. EE and I constants for the calculation of *in vitro* SPF.

λ (nm)	EE(λ) x I(λ)
290	0.0150
295	0.0817
300	0.2874
305	0.3278
310	0.1864
315	0.0839
320	0.0180

Statistical Analysis

The concentration of secondary metabolites and IC_{50} to evaluate antioxidation, antiglycation and antiinflammation activities were determined by linear regression or polynomial analysis using Microsoft Excel.

RESULTS

Proximate Analysis and Extraction

In order to maintain consistency in the composition of the extract, a proximate analysis of the simplisia was conducted prior to extraction. This analysis included parameters such as moisture content, water soluble extractive, alcohol soluble extractive, and extraction yield. It was observed that simplisia, which had been dried at 48°C for 24 h, contained 5.75% water (Table 2) below the minimum standard 10%. This suggests that microorganisms such as bacteria or yeast may find it difficult to thrive in simplisia, thereby preventing the degradation of metabolites (Manalu and Adinegoro, 2016). Metabolites that dissolve in water typically comprise phenolic compounds, sugars, organic acids, and certain proteins. Conversely, metabolites that dissolve in alcohol often include non-polar compounds like lipids, waxes, alkaloids, and some volatile compounds (Lefebvre, *et al*., 2021). The parameters for both types of water and alcohol extractives were relatively low, at 1.86% and 1.68% for water-soluble and alcohol-soluble extractives, respectively (Table 2). In order to extract a diverse array of metabolites ranging from polar to non-polar, a 70% ethanol solution was utilized to process the *P. urinaria* herb simplisia. This approach resulted in an extraction yield of 6.14% (Table 2),

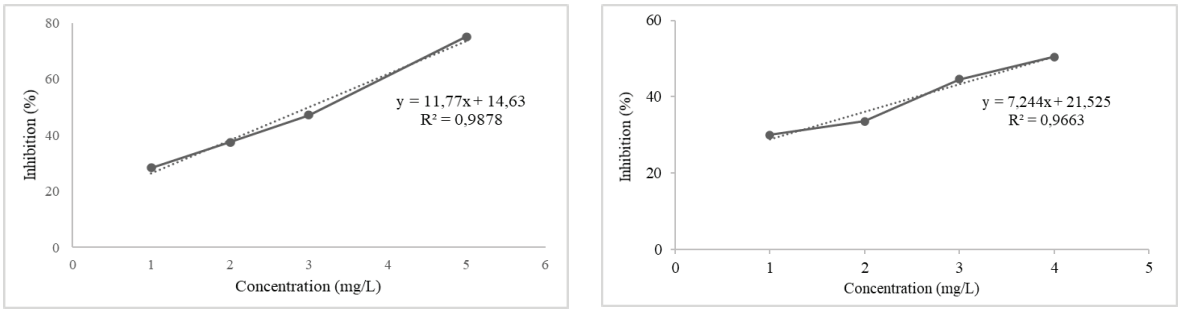
Table 2. Characteristics of *P. urinaria* herb simplisia.

Moisture content (%)	Water soluble extractive (%)	Alcohol soluble extractive (%)	Extraction yield (%)
5.75	1.86	1.68	6.14

surpassing the yields obtained using absolute ethanol (96%) or water. It should be noted that the proportion of extractives soluble in water or alcohol, as well as the overall extraction yield, may vary depending on factors such as plant species, plant part analyzed (*e.g*. leaves, roots, fruits), growing conditions, and choice of extraction method.

Bioactivity Analysis (Antioxidation, Antiglycation and Antiinflammation)

The antioxidant potential of *P. urinaria* extract was evaluated using the DPPH method. DPPH contains a stable free radical nitrogen with a distinct dark purple hue in both solid and solution states. Upon encountering an antioxidant, DPPH undergoes electron transfer from the antioxidant, leading to a loss of color and transformation into a colorless form



A B

Figure 1. Free radical scavenging activity curve of *P. urinaria* extract (A) using the DPPH assay against ascorbic acid (B) as a positive control. Data was determined by linear regression analysis.

(Gangwar, *et al*., 2014). The IC_{50} value, which is the concentration that produces 50% scavenging of free radical DPPH, was found to be lower for *P. urinaria* extract (3.01 mg/L) as compared to the positive control, ascorbic acid (3.88 mg/L) (Figure 1 and Table 3). This suggests that the extract has antioxidant activity similar to that of ascorbic acid.

The reaction of fructose and glucose with BSA at high temperatures resulted in the creation of fluorescent AGE (Jing and Juxiu, 2017). However, when *P. urinaria* extract was added, the fluorescence intensity decreased, indicating that the extract hindered AGE formation. At a concentration of 500 mg/L, the *P. urinaria* extract inhibited 93.14±0.59% of AGE formation, with an IC_{50} of 216.67 mg/L (Figure 2 and Table 3). While the IC_{50} was higher than that of aminoguanidine as a positive control compound (which was 22.34 mg/L), the inhibition percentage of 400 mg/L extract was detected more

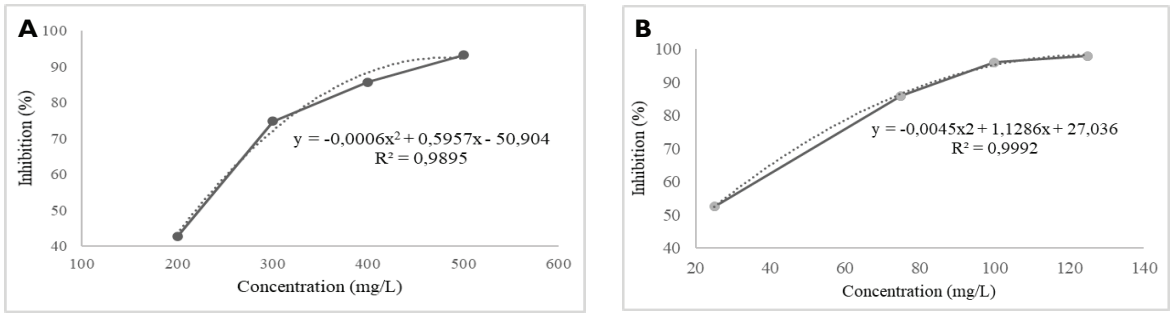
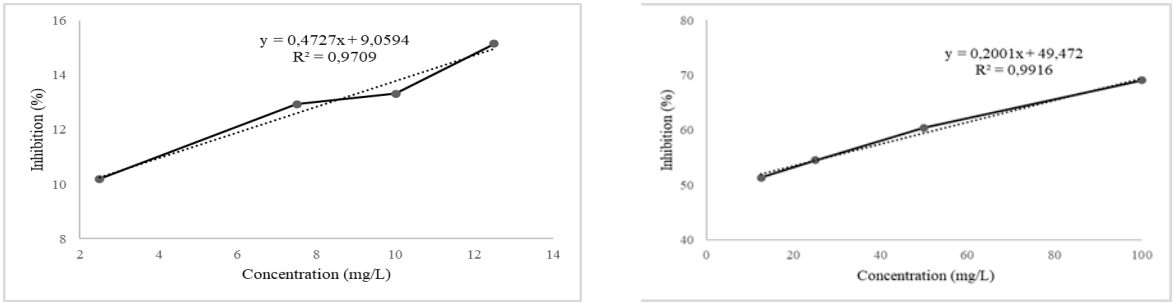


Figure 2. Antiglycation activity curve of *P. urinaria* extract (A) using the glycation inhibition assays against aminoguanidine (B) as a positive control. Data was determined by polynomial analysis.



A B

Figure 3. Anti-inflammatory activity curve of *P. urinaria* extract (A) using the BSA denaturation assay against diclofenac sodium (B) as a positive control. Data was determined by linear regression analysis.

than 80% (Figure 2). Based on the previous study, the concentration of extract 1000 mg/L will be categorized as moderate antiglycation activity if the inhibition percentage is more than 80% (Rungsang, *et al*., 2023). In this research, a concentration of extract below 1000 mg/L could inhibit more than 80% protein glycation. Therefore, the *P. urinaria* extract's antiglycation activity was categorized as higher than moderate level.

The BSA solution undergoes denaturation under certain conditions, including exposure to heat or chemical denaturants. This process alters the protein's secondary and tertiary structures, causing it to lose its natural conformation. The ability of extract to interact and inhibit BSA denaturation demonstrates its effectiveness in protecting the protein structure from inflammatory agents (Mendez-Encinas, *et al*., 2023). The activity of *P. urinaria* extract to inhibit BSA denaturation was below 20% and much lower than that of diclofenac sodium as a positive control (Figure 3). IC_{50} of *P. urinaria* extract was 86.61 mg/L, which is significantly higher than that of diclofenac sodium (2.63 mg/L) (Tabel 3). The compound that inhibits BSA denaturation by less than 20% indicates having low anti-inflammatory activity (Farida, *et al*., 2018). Therefore, it can be suggested that the *P. urinaria* extract has low anti-inflammatory activity.

Determination of SPF Value

The technique commonly used to determine the SPF value for screening sun protecting materials involves measuring the spectral transmittance at UVB wavelengths ranging from 290 nm to 320 nm (Ebrahimzadeh, *et al*., 2014). The SPF value of *P. urinaria* extract at concentration 50 mg/L was determined to be 5.95, which is half the value of the positive control compound, quercetin, at 12.37 (Figure 4). The ratio

Table 3. IC_{50} of antioxidation, antiglycation and anti-inflammatory activities of *P. urinaria* extract and the positive control compounds.

Sample/positive control compound	IC_{50} (mg/L)		
	Antioxidation	Antiglycation	Antiinflammation
***P. urinaria* extract**	3.01	216.67	86.61
Ascorbic acid (positive control of antioxidation assay)	3.93	N.A	N.A
Aminoguanidine (positive control of antiglycation assay)	N.A	22.34	N.A
Diclofenac sodium (positive control of antiinflammation assay)	N.A	N.A	2.63

Note : N.A = not applicable

of SPF between *P. urinaria* extract and quercetin remained constant at both low (25 mg/L) and high (50 mg/L) concentrations. Furthermore, there was a clear correlation between the SPF value of *P. urinaria* extract and the concentration used.

Phytochemical Content

Through quantitative analysis using spectrophotometry UV-Vis, we examined five secondary metabolites present in *P. urinaria* extract: phenolic compound, flavonoid, tannin, alkaloid, and saponin. These metabolites are characterized by a benzene ring chromophore that can absorb UV rays. Prior research on other plant extracts has indicated that these metabolites possess antioxidant properties. Our investigation revealed that saponin had the greatest quantity among the examined secondary metabolites, with phenolic compounds, flavonoid, tannin, and alkaloid following in descending order (Table 4).

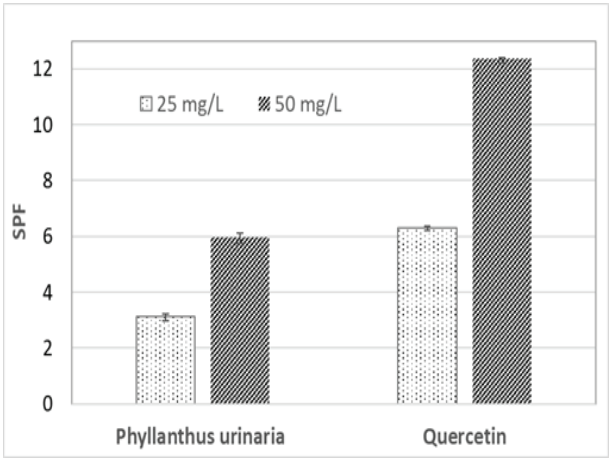


Figure 4. The SPF value of *P. urinaria* extract and quercetin (positive control) at the concentration of 25 and 50 mg/L. Data given as mean±standard deviation of triplicate test.

According to GC-MS analysis, three significant fatty acids were identified in *P. urinaria* extract, which are ethyl palmitate, methyl linolelaidate, and methyl linoleate. Methyl linoleate was the most abundant constituting 40% of all detected metabolites in GC-MS analysis (Table 5). It is classified as a polyunsaturated fatty acid, which has the ability to scavenge free radicals (Richard, *et al*., 2008).

Table 4. Phytochemical contents of *P. urinaria* extract.

Total phenolic content (mg GAE/g extract)	Total flavonoid content (mg QE/g extract)	Total tannin content (mg TAE/g extract)	Total alkaloid content (mg AE/g extract)	Total saponin content (mg SE/g extract)
145.01±4.98	51.19±1.64	39.37±0.47	1.1±0.37	412.02±1.39

DISCUSSION

A previous study about *P. urinaria* herb by Nurwijayanto, *et al.* (2020) found that a 96% ethanol extract of *P. urinaria* herb demonstrated strong scavenging activity against DPPH free radicals, with an IC_{50} of 4.46 mg/L- only two times higher than the IC_{50} of ascorbic acid, which was 2.06 mg/L. In our study, we used the same antioxidant method and found that a 70% ethanol extract of *P. urinaria* exhibited even more potent antioxidant activity than ascorbic acid, with an IC_{50}

Table 5. Three most prominent compounds in *P. urinaria* extract detected by GC-MS.

Compound name	Retention time (min)	Area (%)
Methyl linoleate	16.738	40.30
Ethyl palmitate	14.990	22.89
Methyl linolelaidate	16.650	11.49

lower than that of ascorbic acid. These results suggest that 70% ethanol may be more effective at extracting antioxidant compounds from the *P. urinaria* herb than 96% ethanol. Compared to ethanol 96%, ethanol 70% has chemical properties that could extract a wider range of polyphenolic compounds from semipolar to polar, while ethanol 96% extracted more semipolar than polar metabolites. The higher concentration of polyphenolic compounds in ethanol 70% than that in ethanol 96% was correlated well with the antioxidant activity of the extract (Lohvina, *et al*., 2022). UV-Vis spectrophotometry has identified the presence of secondary metabolites, such as saponin, phenolic compound, flavonoid, and tannin, which are responsible for the observed antioxidant activity of *P. urinaria* extract. The effectiveness of the extracts in scavenging DPPH free radicals is due to the presence of hydroxyl groups. These groups serve as proton donors and are abundant in phenolic compounds, saponins, certain flavonoids, and tannins. It's also noteworthy that other plant extracts that contain these compounds have shown antioxidant activity in previous studies (Antasionasti, *et al*., 2021; Xing, *et al*., 2024; Zeng, *et al*., 2024). According to the GC-MS findings, the *P. urinaria* extract is found to contain methyl linoleate a stable form of linoleic acid that belongs to the Omega-6 fatty acids group. Methyl linoleate has a conjugated double bond structure that helps it stabilize free radicals, thereby enhancing the antioxidant properties of the extract. Previous research has shown that flavonoids, in particular, can work via both direct and indirect pathways to protect against oxidative stress. The direct pathway involves transferring electrons to free radicals and chelating metal catalysts, while the indirect pathway involves activating antioxidant enzymes and inhibiting enzyme oxidase within biological systems (Saewan and Jimtaisong, 2013). In this study, the DPPH method was used to determine the direct pathway of these metabolites to transfer electrons to free radicals.

Exposure to UV from sunlight can cause skin damage, such as wrinkles and pigmentation. One reason for this damage is the formation of UV-induced AGEs. To prevent the production of AGEs, it's essential to inhibit nonenzymatic glycation. The BSA-glucose assay for antiglycation is a two-step process that involves a spontaneous non-enzymatic glycation reaction. In the first step, glucose and fructose are oxidized, creating ketoaldehyde and radical superoxide. The ketoaldehyde then reacts with BSA's amino groups, producing amadori products. In the second step, the amadori products undergo autooxidation, leading to the creation of active dicarbonyl compounds, AGEs, and radical superoxide. This glycation reaction causes the carboxylation of BSA's cysteine residues, reducing the reduced thiol content (Rombouts, *et al*., 2015). In this study aminoguanidine was used as a synthetic positive control compound that can prevent the formation of AGEs by reacting with amadori-derived products in the second step of the glycation process (Abbas, *et al*., 2016).

Various research studies have highlighted that plant extracts containing compounds such as phenolic, flavonoid, tannin, and

saponin exhibit antiglycation activity (Kaewnarin, *et al*., 2014; Sekowski, *et al*., 2023; Silva, *et al*., 2022). These compounds can perform different roles in preventing the formation of AGEs. In the first step, they can inhibit the rearrangement of Schiff bases and reduce the formation of amadori products. In the second step, they can block the oxidation or hydrolysis of amadori products. According to several research studies, the mechanism behind the inhibitory effect of polyphenols on glycation is due to their antioxidant properties, which can impede the auto-oxidation of Amadori products in second step (Crascì, *et al*., 2018). The antiglycation activity of *P. urinaria* extract was categorized as higher than moderate activity because the extract exhibited more than 80% antiglycation inhibition at a concentration of 400 mg/L (below 1000 mg/L). This activity may be caused by the antioxidant activity of the phenolic compound, saponin, flavonoid, and tannin in the extract, which inhibits auto-oxidation in the second step of AGE formation. The higher than moderate level of antiglycation activity of *P. urinaria* extract indicates that the extract has the potency to inhibit the glycation of proteins in the photoaging mechanism.

Exposure to UV rays can cause the production of free radicals, which not only triggering glycation but also releasing cytokines like TNF-α and IL-1. These cytokines bind to receptors on the cell membrane and induce the breakdown of IκB through the ubiquitination-proteasome pathway (Tanveer, *et al*., 2023). IκB is a crucial inhibitor protein of the NF-κB transcription factor. With the degradation of IκB, NF-κB can enter the cell nucleus and activate inflammatory genes (Shambharkar, *et al*., 2007). Moreover, TNF-α can also break down E-cadherin, which can compromise the integrity of the epithelial barrier and facilitate the movement of inflammatory cells (Alotaibi, *et al*., 2023). The principle of anti-inflammatory *in vitro* testing using the BSA degradation method in this study is based on the degradation of inflammation-promoting proteins. Materials that can inhibit BSA degradation, triggered by heating as a model of proinflammatory agents, show their ability to prevent inflammatory reactions (Bastos, *et al*., 2022).

The ability of *P. urinaria* extract to interact with protein and inhibit the degradation of BSA is 33 times lower than that of diclofenac sodium (a positive control), as shown by the IC_{50} of *P. urinaria* extract in the anti-inflammatory test. The anti-inflammatory mechanism of diclofenac sodium has been well known by interacting with cyclooxygenase (COX) enzymes and inhibiting COX activity (Gan, 2010). The interaction between diclofenac sodium and BSA protein in this assay might be used as a model of the interaction of diclofenac sodium with COX as a protein. In this assay, metabolites in *P. urinaria* extract has much lower activity in interacting with BSA than diclofenac sodium. Possibly, in inflammatory reaction the extract did not inhibit signaling pathways following cytokine expression, but rather those preceding cytokine expression, possibly due to its high antioxidant activity. Cytokine expression can be inhibited by oxygen or nitrogen free radicals scavenging materials (antioxidant materials) by inactivating various intracellular signaling pathways, including the mitogen-activated protein kinase (MAPK) pathways (such as ERK, JNK, and p38 MAPK) and the phosphoinositide 3-kinase (PI3K)/Akt pathway, which lead to the restraint of production and release of cytokines (Kim, *et al*., 2020).

The skin type determines the minimum SPF value needed to prevent erythema caused by UVB exposure. In 1988, Thomas B. Fitzpatrick classified skin into 6 types based on its reaction to sunlight exposure, sunburn risk, and tanning ability. Skin types range from type I (very fair skin that burns easily) to type VI (very dark skin that rarely burns). *P. urinaria* herb extract can be used to

protect skin types V and VI, as it has an SPF value of 5.95 at a concentration of 50 mg/L. Skin type VI, which is prevalent among individuals of African origin, Aborigines, and dark-skinned Asians such as Tami, need an SPF value minimum 4. Skin type V, on the other hand, is common among individuals of South East Asians, and some Indians, Pakistanis and Latin, and need an SPF value ranging from 5 to 10 (Del Bino, *et al*., 2018). Raising the concentration of *P. urinaria* extract can enhance photoprotection because the SPF value is linked with concentration of constituent. The SPF values of some medical plant extracts were proven to correlate with the amount of phenolic compound (Ebrahimzadeh, *et al*., 2014). Therefore, the phenolic compound in *P. urinaria* extract may significantly contribute to the SPF value. However, other secondary metabolites in *P. urinaria* extract such as saponin, flavonoid, tannin, and methyl linoleate also have the potential to contribute to the SPF value due to their various chromophore structures.

The evaluation of the 70% ethanol extract of *P. urinaria* herb through antioxidant and antiglycation assays, along with SPF value determination, yielded positive results. The extract demonstrated potent antioxidant properties, which have the potency to inhibit glycation reactions. Moreover, the presence of phenolic compound and other secondary metabolites with high chromophore structures in the extract resulted in a suitable SPF value for skin types V and VI, making it an effective photoprotector.

CONCLUSION

In conclusion, the 70% ethanol extract of *P. urinaria* herb has the potential for future research and development as a sunscreen material that can prevent photoaging. This is due to its ability to provide both physical and chemical protection. The extract offers physical protection to skin types V and VI through its SPF value, and chemical protection through its function as an antioxidant and antiglycation agent. These bioactivities were possibly due to saponin, phenolic compounds, flavonoids, tannins and methyl linoleates. Further investigation into its anti-inflammatory capabilities during cytokine formation is necessary to comprehensively understand its anti-inflammatory effects, given that inflammation is a common reaction to photoaging.

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