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Original Article

Antioxidant potential and xanthine oxidase inhibition of flavonol glycosides from *Phyllanthus emblica* L. leaves

[Potencial antioxidante e inhibición de la xantina oxidasa de los glucósidos de flavonol de las hojas de *Phyllanthus emblica* L.]

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Abstract

Context: Hyperuricemia is the cause of gout in the inflammatory joint condition. The xanthine oxidase enzyme is a therapeutic target for gout treatment because it plays a role in the generation of uric acid. Allopurinol is used to treat gout. It prevents the xanthine oxidase enzyme from producing as much uric acid. When selecting a medication, one must consider the various adverse effects of allopurinol. *Phyllanthus emblica* plants are among the medicinal plants that can be used as an alternative treatment for gout.

Aims: To evaluate isolated compounds from the *Phyllanthus emblica* as antihyperuricemia candidates.

Methods: The isolated compounds were characterized using High-Performance Liquid Chromatography Analysis and Thin Layer Chromatography-Densitometry. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) and cupric ion (CUPRAC) antioxidant capacity procedures were used to develop the antioxidant activity index. The ability to inhibit xanthine oxidase was determined using a spectrophotometer.

Results: Compound **1** was indicated as rutin having antioxidant capacity with an antioxidant activity index (AAI) DPPH value of 7.89 ± 0.03 and AAI CUPRAC value of 15.83 ± 0.04 stronger than compound **2** (quercitrin) with an AAI DPPH value of 3.72 ± 0.01 and AAI CUPRAC 3.24 ± 0.03. The IC_{50} for quercitrin's inhibition of xanthine oxidase is 23.85 ± 2.04, which was higher than rutin's IC_{50} value of 32.77 ± 4.49 µg/mL.

Conclusions: Flavonol glycosides present in the ethanol extract of *Phyllanthus emblica* leaves gave potent xanthine oxidase inhibitory activity stronger than the extract. Quercitrin gave stronger xanthine oxidase inhibitory activity, but this compound has weaker antioxidant capacity compared to rutin.

Keywords: antioxidants; hyperuricemia; *Phyllanthus emblica*; xanthine oxidase.

Resumen

Contexto: La hiperuricemia es la causa de la gota, una enfermedad inflamatoria de las articulaciones. La enzima xantina oxidasa es una diana terapéutica para el tratamiento de la gota porque interviene en la generación de ácido úrico. El alopurinol se utiliza para tratar la gota. Impide que la enzima xantina oxidasa produzca tanto ácido úrico. A la hora de elegir un medicamento, hay que tener en cuenta los diversos efectos adversos del alopurinol. Las plantas de *Phyllanthus emblica* se encuentran entre las plantas medicinales que pueden utilizarse como tratamiento alternativo para la gota.

Objetivos: Evaluar compuestos aislados de *Phyllanthus emblica* como candidatos antihiperuricemiantes.

Métodos: Los compuestos aislados se caracterizaron mediante análisis de cromatografía líquida de alto rendimiento y cromatografía de capa fina-densitometría. Se utilizaron los procedimientos de capacidad antioxidante 2,2-difenil-1-picrilhidrazilo (DPPH) e ion cúprico (CUPRAC) para desarrollar el índice de actividad antioxidante. La capacidad de inhibición de la xantina oxidasa se determinó con un espectrofotómetro.

Resultados: Se indicó que el compuesto **1** (rutina) tenía actividad antioxidante con un índice de actividad antioxidante (AAI) DPPH de 7,89 ± 0,03 y un valor AAI CUPRAC de 15,83 ± 0,04 más fuerte que el compuesto **2** (quercitrina) con un valor AAI DPPH de 3,72 ± 0,01 y un valor AAI CUPRAC de 3,24 ± 0,03. El IC_{50} para la inhibición de la xantina oxidasa por la quercitrina es de 23,85 ± 2,04, que fue superior al valor IC_{50} de la rutina de 32,77 ± 4,49 µg/mL.

Conclusiones: Los glucósidos de flavonol presentes en el extracto etanólico de las hojas de *Phyllanthus emblica* presentaron una potente actividad inhibidora de la xantina oxidasa, superior a la del extracto. La quercitrina presentó una actividad inhibidora de la xantina oxidasa más potente, pero este compuesto tiene una actividad antioxidante más débil en comparación con la rutina.

Palabras Clave: antioxidantes; hiperuricemia; *Phyllanthus emblica*; xantina oxidasa.

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INTRODUCTION

Hyperuricemia is the main etiological factor in gout. This condition will trigger the formation of urate crystal deposits in the joints that can cause inflammation. Hyperuricemia can also cause tissue inflammation, especially in the blood vessel walls. In recent decades, greater emphasis has been placed on the prevention of acute episodes in hyperuricemia therapeutic approaches. Hyperuricemia is typically treated by modulating the activity of enzymes embroiled in urate metabolism and excretion, such as xanthine oxidase (XO) and uric acid transporter (URAT). Xanthine oxidase inhibitors and uricosuric substances have been shown to effectively lower uric acid levels in both the bloodstream and peripheral tissues by mitigating the overproduction of uric acid. Conversely, the observed decrease in uric acid levels resulting from the inhibition of XO was expected to be associated with a concurrent attenuation of redox imbalance. It is recognized as a risk factor for the emergence of endothelial dysfunction. Therefore, this pathway might contribute to the pathophysiology of several diseases, including diabetes, hypertension, arteriosclerosis, and chronic heart failure. Hence, the implementation of scavenging mechanisms and the inhibition of oxygen radicals produced by XO emerges as a compelling novel therapeutic approach for mitigating tissue injury associated with gout (Gliozzi et al., 2016).

Allopurinol is commonly prescribed as an initial treatment option for the management of gout. By inhibiting the enzyme xanthine oxidase, the intervention efficiently lowers plasma urate concentrations to a degree that is adequate for the dissolution of monosodium urate crystals. This reduction in uric acid levels inside the body has the potential to partially correct endothelial dysfunction in patients with comorbidities (Alem et al., 2018; Day et al., 2017). Although allopurinol side effects are rare, to certain people, they can be severe, such as hypersensitivity reactions, Drug responses involving eosinophilia and systemic symptoms, or severe cutaneous adverse drug reactions (SCARs), which are significant adverse events associated with the use of certain medications, agranulocytosis, myelosuppression, anemia, thrombocytopenia, and leukopenia, Stevens-Johnson syndrome, skin eruptions, fever and toxic epidermal necrolysis (TEN) is a medical condition that is distinguished by the necrosis and shedding of the epidermis, the outermost layer of the skin. This condition is often accompanied by symptoms such as a burning feeling and discomfort, and in severe cases, it can even result in mortality (Lee et al., 2021; Mari et al., 2011; Park et al., 2019; Saksit et al., 2017; Stamp and Chapman, 2020) Therefore, the development of alternative therapy that can reduce uric acid levels is very important.

Inflammation plays an important role in the pathogenesis of arthritis, and traditionally, the plants of the genus *Phyllanthus* have long been used as the main component in Ayurvedic formulations to treat it (Ongchai, 2019). Several studies report that the *Phyllanthus emblica* L. plant (*Phyllanthaceae*) has various pharmacological activities, including antioxidant, anticancer, immunomodulatory, anti-inflammatory, hepatoprotective, antidiabetic, dyslipidemia, obesity, cancer, liver disorders, arthritis, gingivitis, and wound healing. Apart from that, *Phyllanthus emblica* also has antiaging activities, such as antioxidants, anti-tyrosinase, and anti-melanogenesis, and can repair kidney damage (Chaikul et al., 2021; Kiran et al., 2021; Purena et al., 2018; Variya et al., 2016; Yadav et al., 2017). The fruit in question was subjected to toxicological investigations, which revealed the absence of adverse reactions at elevated dosages following oral ingestion. The presence of polyphenols, particularly flavonoids, in this botanical specimen renders it a potential therapeutic option for hyperuricemia, owing to its limited adverse effects. The plant's flavonoids have been found to exhibit xanthine oxidase inhibition, as indicated by IC_{50} values ranging from 0.44 µM to less than 50 µM. The plant under investigation exhibits the presence of phenolic compounds that have significant efficacy in scavenging radicals, displaying promising potential for chelating Fe^{2+} ions and exhibiting notable resistance against lipid peroxidation (Husnunnisa et al., 2022; Luo et al., 2011; Saini et al., 2022).

Based on ethnomedicine information or traditional use by people who use the *P. emblica* plant for the treatment of arthritis, as an anti-inflammatory, and as an antiaging and supported by literature studies where this plant contains phenolics and flavonoids which have xanthine oxidase inhibitory activity, active as an antioxidant and anti-inflammatory so we are interested in isolating compounds that have anti hyperuricemia activity from the *P. emblica* plant.

MATERIAL AND METHODS

Chemicals

Quercetin, gallic acid, xanthine, xanthine oxidase, 2,2-diphenyl-1-picrylhydrazyl (DPPH), cupric chloride, and neocuproine were obtained from Sigma-Aldrich Chemicals (St. Louise, MO, USA). The ascorbic acid was obtained from Merck (Darmstadt, Ger-

many). Allopurinol was purchased from TCI (Tokyo, Japan), and all other reagents were analytical grade.

Plant material

Phyllanthus emblica leaves to prepare extract were collected in August 2021 at the top of Mount Gadung, Cipongkor subdistrict, West Bandung, West Java, Indonesia, at GPS coordinates -6.951810,107.320000 and Herbarium Bandungense School of Life Bandung Institute of Science and Technology confirm the identification of the leaves and fruit of the plant with letter number 3519/IT1.C11.2/TA.00/2022 using the reference Bouman et al. (2022).

Preparation of plant extracts

Crude drug powder of *P. emblica* leaves (1900 g) was macerated thrice with 96% ethanol and then evaporated in vacuo at 50ºC to give a thick extract of 718.33 g.

Phenolic total content (TPC) of *P. emblica* extract

This test was performed according to Pourmorad et al. (2006). The total phenolic content was assessed using a Folin-Ciocalteu reagent. 0.5 mL of each extract or gallic acid as a standard phenolic compound was piped into 5 mL of 10% Folin-Ciocalteu reagent and 4 mL of 1 M sodium carbonate, which were then incubated for 15 minutes. A specific wavelength of 765 nm was used to test the absorbance (Orion Aquamate 8100 UV-Visible spectrophotometer, Thermoscientific, USA). Three replications of each extract's analysis were done. The results were expressed as gallic acid equivalents (GAE) per 100 g of extract (g GAE/100 g), with the reference being a gallic acid calibration curve ranging from 40 to 120 µg/mL (Pourmorad et al., 2006; Shahidi and Naczk, 2003).

Total flavonoid content (TFC) of *P. emblica* extract

The total flavonoid content was calculated using the Chang et al. (2002) method. Each plant extract or quercetin as a standard flavonoid compound 0.5 mL was mixed with 0.1 mL aluminum chloride 10%, 0.1 mL sodium acetate 1 M, 2.8 mL distilled water, and 1.5 mL ethanol, then incubated the mixture for 30 minutes. The absorbance was measured at a wavelength of 415 nm (Orion Aquamate 8100 UV-Visible spectrophotometer, Thermoscientific, USA). The samples were tested in triplicates. The values were given as g equivalents of quercetin (QE) per 100 g extract (g QE/100 g) according to the calibration curve of quercetin 40–120 µg/mL as a standard.

In vitro antioxidant activities by DPPH assay

Activation of the antioxidants was evaluated by utilizing the DPPH test adapted from the modification of the Nayaka et al. (2020) method. Each sample for some concentrations was combined in a 1:1 ratio with a 50 µg/mL DPPH solution, then incubated for 30 minutes, and a spectrophotometer UV-Vis (Thermo Scientific®) was used to measure the absorbance at 516 nm. The initial DPPH uptake was 0.579. As a control, a solution of methanol and DPPH was utilized. Ascorbic acid served as the standard. A triplicate was used to analyze the samples and standard. The equation was used to calculate the radical scavenging activity: I% = [(Abs0 – Abs1)/Abs0] x 100, where Abs0 is the absorbance of the control, and Abs1 is the samples at various concentrations. The calibration curve between concentration and percent inhibition determines the IC_{50} (concentration that inhibits 50% of DPPH). Antioxidant activity index (AAI) was calculated as AAI = [final DPPH concentration (µg/mL)]/ IC_{50} (µg/mL).

CUPRAC test for *in vitro* antioxidant capacity

The CUPRAC AAI was calculated following the procedure of Apak et al. (2013). A sample or standard with various concentrations was added to the CUPRAC solution. CUPRAC reagent of 100 µg/mL (volume 1:1) and incubated for 30 min. The CUPRAC reagent was made by dissolving the mixture in ammonium acetate buffer pH 7 after combining $CuCl_2$ in distilled water and neocuproine in ethanol. At 450 nm, the absorbance value was determined using a spectrophotometer (Orion Aquamate 8100 UV-Visible spectrophotometer, Thermoscientific, USA). Ascorbic acid was used as a standard, and the analysis was repeated three times. The Cu (II) can be changed to Cu(I), and to make a chromophore with Cu (I), neocuproine is required., therefore the sample will display a yellow color if it functions as an antioxidant agent. The AAI of each extract was calculated by dividing the CUPRAC final concentration by the exhibitory concentration of 50% (EC_{50}), which can be found on the calibration curve.

Isolation of major constituents of *P. emblica*

The sample was leaves of *P. emblica*, which were sorted, dried, chopped, blended to obtain powder (1900 g), and macerated with 96% ethanol (3 × 1 day each). The combined ethanol extracts were filtered and evaporated *in vacuo* to give a solid residue (718.33 g). Using n-hexane, and ethyl acetate, 100 g of the extract was fractionated to obtain 3.75 g of the n-hexane fraction, 50.38 g of the ethyl acetate fraction, and 29.84 g of the water fraction.

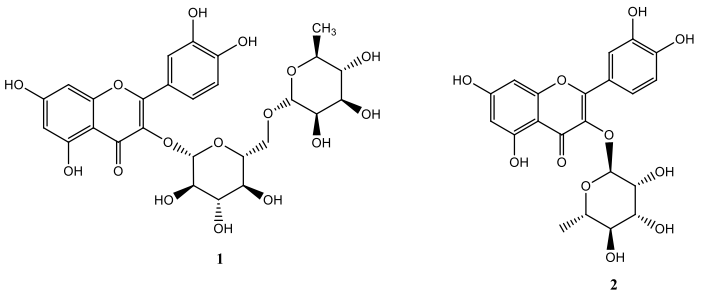

Figure 1. Structures of isolated compounds tested in the xanthine oxidase inhibitory and antioxidant capacity.

Vacuum liquid chromatography was used to separate a 30 g sample of ethyl acetate using silica gel 60 H and an eluted gradient of n-hexane, ethyl acetate, and methanol. The fractions that exhibit similar behavior to TLC are combined and evaporated. Subfractions 21, 22, 23, 24, 26, 27, 29, and 35 were tested for their inhibitory activity against xanthine oxidase. Subfraction 29 had the best inhibitory activity, and Classical column chromatography with silica gel 60 was performed to continue the separation, and n-hexane, ethyl acetate, and methanol served to elute the product in a gradient way to produce subfraction 29a-i. Subfraction 29a-i had tested for xanthine oxidase inhibitory activity. Subfraction 29a had the best inhibitory activity and was followed by TLC Silica gel 60 RP-18 F254S for preparative thin layer chromatography and water-acetonitrile-methanol (2:1:1) as mobile phases and compounds **1** and **2** were obtained (Fig. 1). This procedure refers to the Faleschini et al. (2023) and Ismed et al. (2012) methods with slight modifications.

Activity inhibitory of xanthine oxidase (XOI)

The method provided by Owen and Johns (1999) was modified slightly to assess the inhibitory action of xanthine oxidase. The extract was mixed with phosphate buffer before being dissolved in dimethyl sulfoxide (DMSO). The maximum DMSO concentration in the finished product was 0.5%. Allopurinol was used as a standard for comparison. In 96-well plates prepared by Corning®, UV-Transparent Clear Microplates, mix 50 µL of the sample with 88 µL of pH 7.5 phosphate buffer, 55 µL of xanthine substrate solution (0.15 mM in phosphate buffer pH 7.5), and then 7 µL of freshly prepared enzyme solution (phosphate buffer with pH 7.5, which contains 0.1 unit/mL xanthine oxidase). After the test solution 15 minutes of incubation at 25°C, the absorbance at 290 nm was measured using a microplate reader (Thermo Scientific Multiscan Skyhigh®). Allopurinol and each extract analysis were done in triplicate. A blank was made using the same process, but phosphate buffer pH 7.5 was used in place of the enzyme. I% = [(A - B)/A] × 100 was used to calculate the inhibitory activity of xanthine oxidase, A is the enzyme xanthine oxidase absorbance without the test extract—blank of A (absorbance without XO and test extract), and B is the test extract absorbance—blank of B (absorbance without XO). The concentration and percent inhibition calibration curve was used to get the IC_{50} value.

High-performance liquid chromatography analysis

Samples were analyzed utilizing a liquid chromatography system (LC-20AD) from Shimadzu, which is outfitted CTO-20A oven pump and SPD-20A UV/Vis detector (Japan). LiChrospher® 100 RP-18 (5 m) column, which has a diameter of 4 mm and a length of 125 mm, is a reversed-phase column. The mobile phase of the HPLC consists of water and methanol, and a linear gradient system of 40% methanol for 3 minutes, then a gradient of 40% to 60% methanol for 3 minutes, a gradient of 60% methanol for 3 minutes, followed by a gradient of 60% to 70% methanol for 3 minutes, and a gradient of methanol 70% to 15 minutes. The volume of injection was 20 µL, 1 mL/min of the flow rate, the UV wavelength at 360 nm, and the temperature of the column at 30 C. This procedure has been slightly modified from the Faleschini et al. (2023).

Statistical analysis

The IC_{50} and EC_{50} values were evaluated using MS Excel Software. The data were shown as means ± standard error of the mean (SEM). The ANOVA (One-Way) was used to calculate statistical significance - post hoc Tukey by SPSS 25, with $p<0.05$ considered significant. Antioxidant and xanthine oxidase activities of the total phenolic and flavonoid contents were correlated with Pearson's technique.

RESULTS

The chemical constituents of *P. emblica* were extracted using the maceration method using 96% ethanol as the solvent because of its non-toxic properties and its capacity to successfully extract a variety of

Table 1. Yield, extract density, total phenolics and flavonoids content of the extract (n = 3).

Sample	Yield (%)	Extract density 1% (g/mL)	Phenolic content (g GAE/100 g)	Flavonoid content (g QE/100 g)
Ethanol extract from *P. emblica* leaves	37.81	0.916	44.31 ± 0.133	8.17 ± 0.280

polar, semi-polar, and non-polar compounds. The quantity obtained from this particular method was 718.33 g. The density of the extract was assessed to ascertain its viscosity level. The density of a 1% w/v extract was determined, yielding a value of 0.916 g/mL. Table 1 presents the data about the yields and density extract.

Content of phenolic and flavonoids of *P. emblica* extract

The total phenol content of this extract was 44.31 ± 0.133 g GAE/100 g extract (Table 1). The equation y = 0.0075x - 0.0213; R^2 = 0.997 was used to calculate the gallic acid equivalent and calculate the total phenolic content (TPC) of the extract. This extract's total flavonoid content (TFC) was determined by quantifying quercetin using the equation of the calibration curve, which was y = 0.0069x - 0.0892; R^2 = 0.990. This extract had a flavonoid content of 8.17 ± 0.280 g QE/100 g extract. The total phenol and flavonoid content results are shown in Table 1.

Identification of isolates in the *P. emblica*

High-performance liquid chromatography was employed to confirm the presence of rutin **(1)** and quercitrin **(2)** in isolates **1** and **2**, where the retention time of isolate **1** was 8.016 minutes (Fig. 2), and the presence of isolate **2** with retention time was 8.947 min (Fig. 3). TLC-densitometry analysis showed that isolate **1** has a UV spectrum similar to rutin, while isolate **2** has a UV spectrum similar to quercitrin as illustrated in Fig. 4. The chromatograms were obtained after scanning at 254 nm wavelength. By comparing the Rf values of the isolates to the reference standard apigenin-7-O-glycosides, kaempferol, quercitrin, luteolin, rutin, and quercetin we were able to identify each compound peak. Isolate **1** was identified as rutin with an Rf of 0.54 compared to standard rutin (Rf **=** 0.55), while compound **2** as quercitrin compared to standard quercitrin had the same Rf **=** 0.44. The UV spectra of flavonoids exhibit two prominent absorption peaks, where band II of the A-ring benzoyl system has maximum absorption at a wavelength of 240-285 nm, while band I of the B-ring cinnamoyl system exhibits peak absorption within the wavelength region of 300-400 nm. The characteristic feature of flavonols is that they have a maximum absorption band I in the 352-385 nm range. Substitution, hydroxylation, methylation, and glycosylation of the structure of flavonoids will affect the absorption shifts of both hypsochromic and bathochromic (Markham and Mabry 1975). The maximum absorption in the UV spectrum given by isolate **1** in band I of the B-ring cinnamoyl system was at a wavelength of 364 nm, similar to the maximum absorption given by rutin, which was 365 nm, while the maximum absorption of isolate **2** in band I of the B-ring cinnamoyl system was at a wavelength of 354 nm similar to the maximum absorption given by quercitrin which was 353 nm.

Antioxidant capacity

DPPH, which was soluble in methanol, was used to evaluate antioxidant capacity. It has a wavelength of 516 nm at its optimum. *P. emblica* extract had antioxidant capacity with an AAI DPPH value of 8.39 ± 0.042, close to the positive control ascorbic acid AAI DPPH value of 10.42 ± 0.032. Testing the antioxidant capacity using CUPRAC extract of *P. emblica* had an AAI value of 13.19 ± 0.059; ascorbic acid had an AAI value of 10.53 ± 0.031. Among the fractions of n-hexane fraction, ethyl acetate, and water, the ethyl acetate fraction possessed the highest antioxidant capacity with AAI DPPH values of 12.61 ± 0.033 and AAI CUPRAC 11.42 ± 0.053.

The antioxidant capacity of isolate **1** (rutin) was confirmed to have better antioxidant capacity than isolate **2** (quercitrin) with AAI DPPH values of 7.90 ± 0.027; 3.72 ± 0.003 respectively and AAI CUPRAC 7.91 ± 0.021; 1.62 ± 0.011 respectively. The antioxidant capacity is represented in Table 2 by the antioxidant activity index (AAI). Table 3 illustrates the correlation between the two antioxidant testing methodologies, DPPH and CUPRAC. There exists a statistically significant positive connection at a $p<0.01$ significance level between the AAI DPPH and AAI CUPRAC n-hexane fraction, ethyl acetate fraction, isolate **2**, and ascorbic acid, with correlation coefficients of r = 1.000; 0.915; 0.866; and 0.849, respectively. At p=0.05, a statistically significant positive correlation between AAI DPPH and AAI CUPRAC ethanol extract and isolate **1**, each with a value of r = 0.724; and 0.655. While the positive correlation that was not significant between AAI DPPH and AAI CUPRAC water fraction was with a value of r = 0.500.

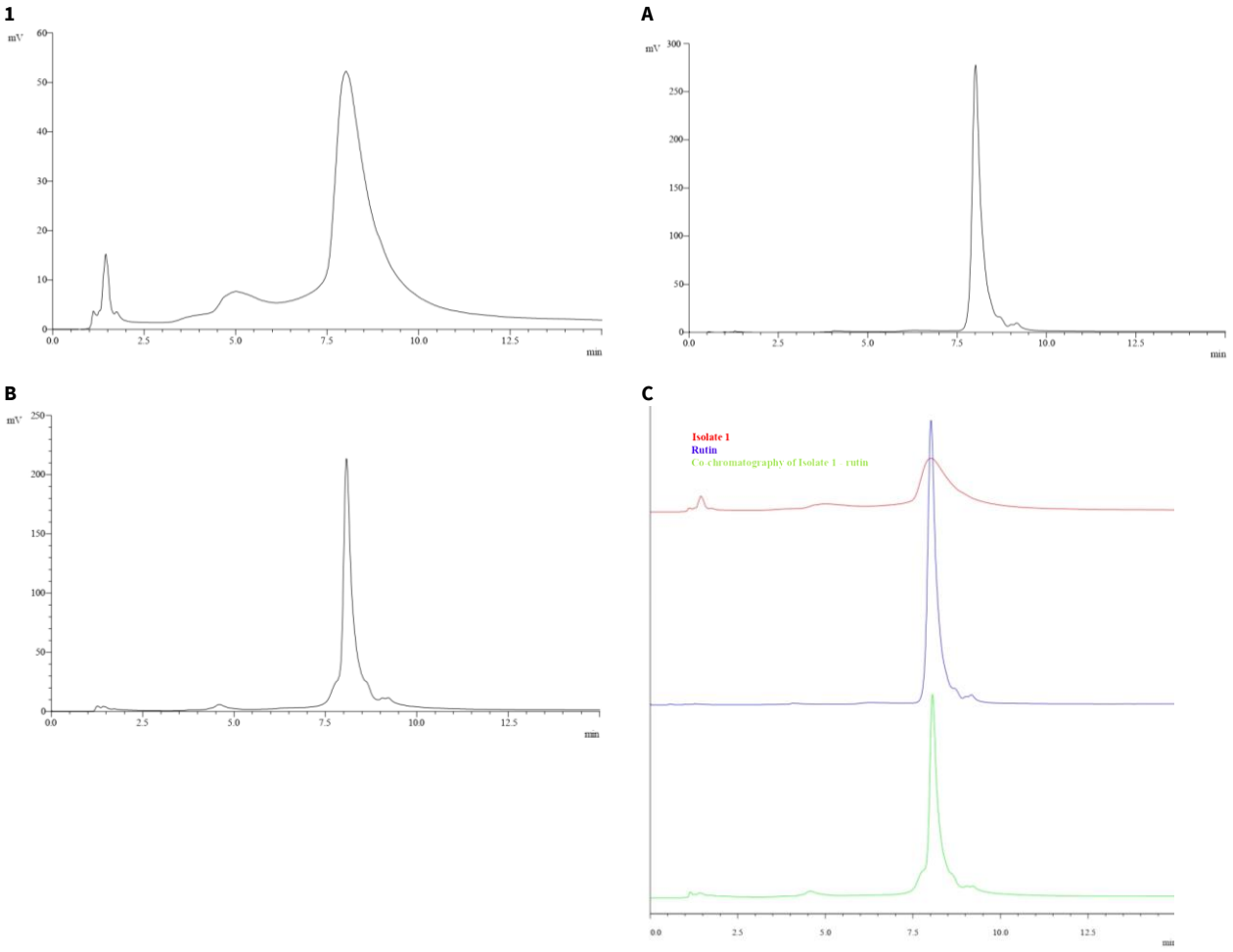

Figure 2. HPLC chromatogram from **1** (isolate **1**).

(A) Rutin as the reference standard; **(B)** Co-chromatography of Isolate **1** and reference standard (rutin); **(C)** Overlay of isolate **1**, rutin (reference standard), and co-chromatography of isolate **1** and reference standard (rutin).

The positive correlation between AAI, TPC, and TFC indicated a relationship between phenolic and flavonoid content and antioxidant capacity. The amount of phenolic and flavonoid compounds in the extract and its antioxidant capacity were positively correlated. With r = 0.917 and r = 0.939, respectively, and a $p<0.01$, there was a significant association between TPC, AAI DPPH, and AAI CUPRAC. While there was a substantial association between TFC and AAI DPPH (r = 0.683; $p<0.05$), the correlation between TFC and AAI CUPRAC (r = 0.998; $p<0.01$) was much stronger (Table 4).

Inhibitory activity of xanthine oxidase (XOI)

The test's results for inhibiting xanthine oxidase activity of isolate **2** (quercitrin) IC_{50} value of 23.85 ± 2.04 µg/mL was higher than that of isolate **1** (rutin) with an IC_{50} value of 32.77 ± 4.49 µg/mL, this could be due to the higher sugar content in rutin than quercitrin so that activity of rutin had reduced. The results of xanthine oxidase inhibitory activity tests of extracts, fractions, and isolates are seen in Table 5.

DISCUSSION

In the human body, unpaired electrons are produced through the food eaten and metabolic processes, which have chemical activity and high reactivity and are known as ROS (reactive oxygen species). ROS attack proteins, nucleic acids, and other biological macromolecules at production sites and other places far from production sites through free radical chain reactions, then cause structural and functional abnormalities in cells, resulting in various diseases such as arteriosclerosis, vasospasm, cancer, asthma, arthritis, retinal damage, hepatitis, and liver damage. Antioxidants can trap free radicals, so by increasing antioxidant activity, you can prevent various diseases. Several studies show that intake of antioxidants from outside the body can reduce excess ROS *in vivo* and have additional therapeutic effects on many diseases

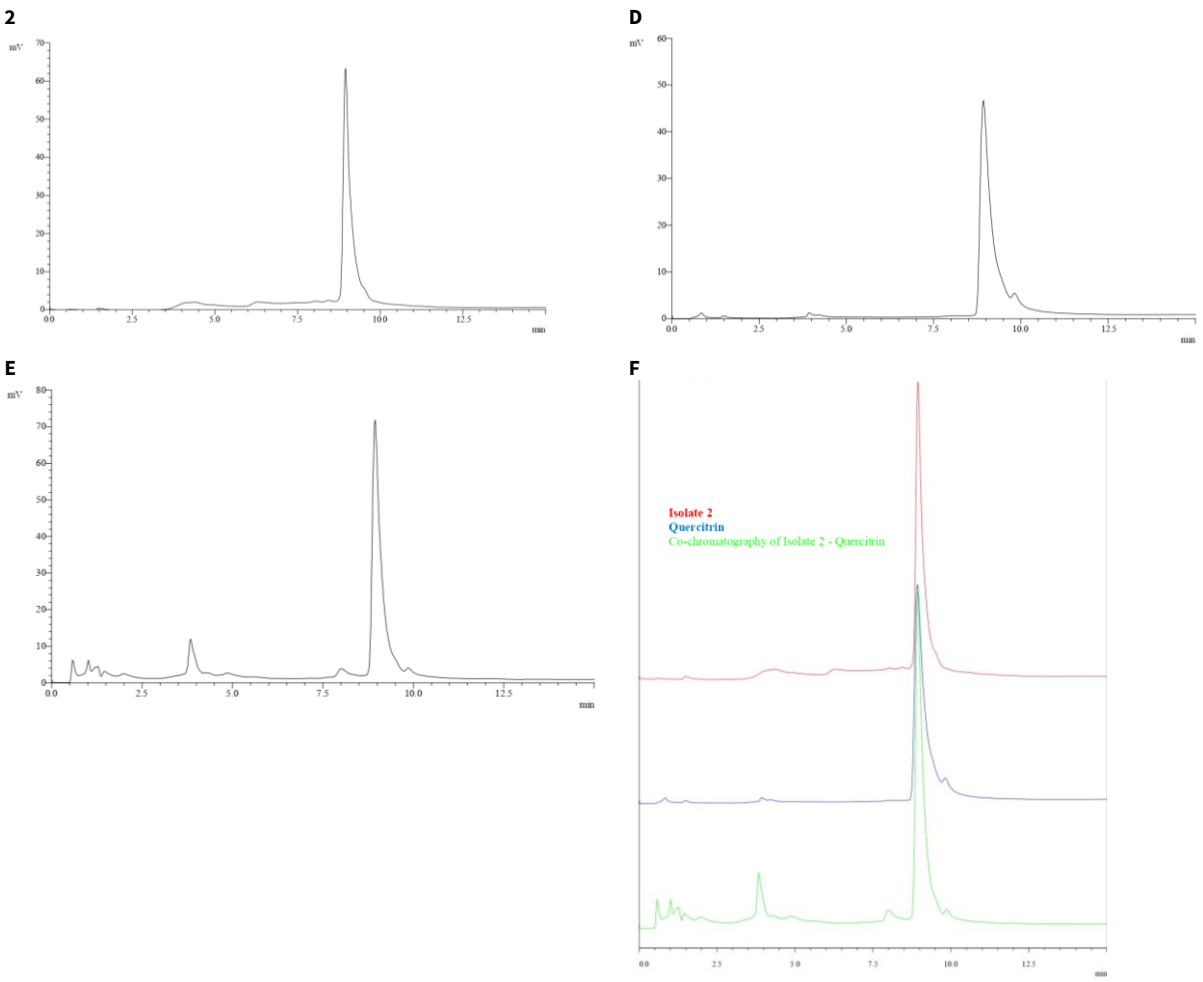

Figure 3. HPLC chromatogram from **2** (isolate **2**).

(D) Quercitrin as the reference standard; **(E)** Co-chromatography of Isolate **2** and reference standard (quercitrin); **(F)** Overlay of isolate **2**, quercitrin (reference standard), and co-chromatography of isolate **2** and reference standard (quercitrin).

(Hu et al., 2021; 2023). The antioxidant capacity test results of *P. emblica* extract showed strong activity, namely with the AAI DPPH value of *P. emblica* extract 8.39 ± 0.04, AAI CUPRAC *P. emblica* extract 13.19 ± 0.06, close to the control AAI value, namely ascorbic acid where the AAI DPPH value of ascorbic acid was 10.42 ± 0.03 and AAI CUPRAC was 10.53 ± 0.03.

The antioxidant capacity provided by rutin in this study with an AAI value of 7.90 ± 0.03 was higher than the AAI value of rutin reported by Rusmana et al. (2017), 5.47 ± 0.05. The aglycone compound of rutin and quercetin, namely quercetin, showed a stronger antioxidant capacity than rutin and quercetin (AAI quercetin = 55.2 ± 0.00). This is estimated to be the influence of the presence of the OH group at position 3 in quercetin, which can increase antioxidant capacity. Meanwhile, in rutin and quercitrin, the presence of sugar, which takes the position of the H atom attached to O at the C3 position, reduces the antioxidant activity (Rusmana et al., 2017). The effect of antioxidants on preventing DPPH radicals was thought to be due to their ability to donate hydrogen. From the results of our research and the research reported by Rusmana et al. (2017), it is a challenge to carry out further research related to testing the antioxidant activity of quercetin, rutin, and quercitrin so that information can be obtained on the influence of added sugar as well as the amount of sugar contained in the aglycone compound on antioxidant activity.

The inhibitory capacity of flavonoids against free radicals is influenced by their chemical structure. The type of substitution in the flavonoid's structure influenced the radical scavenging and chelating activity. The methoxy group exerts an unfavorable steric effect and increases lipophilicity and membrane partitioning. Double bonds and carbonyl groups in heterocyclic or polymerization of nuclear structures can enhance more stable radical scavenging activity through electron conjugation and delocalization (Heim et al., 2002). Antioxidant activity is enhanced in flavonoids with ortho dihydroxy groups on C3' and C4', OH on C3, oxo groups on C4, and two covalent bonds on C2 and C3. Flavonoids with orthodihydroxy groups gave the best antioxidant activity at C-3'-C-4' (Sukrasno et

al., 2017). Various antioxidant capacity evaluations will yield distinct outcomes. Two methods with different mechanisms of antioxidants may or may not produce linear results. Pearson correlation can determine whether or not the AAI of each method produces linear results. DPPH is composed of both hydrogen and electron transfer. CUPRAC, meanwhile, only transfers electrons. When the two methods exhibit a positive and linear correlation, they will produce linear outcomes (Dirgantara et al., 2022).

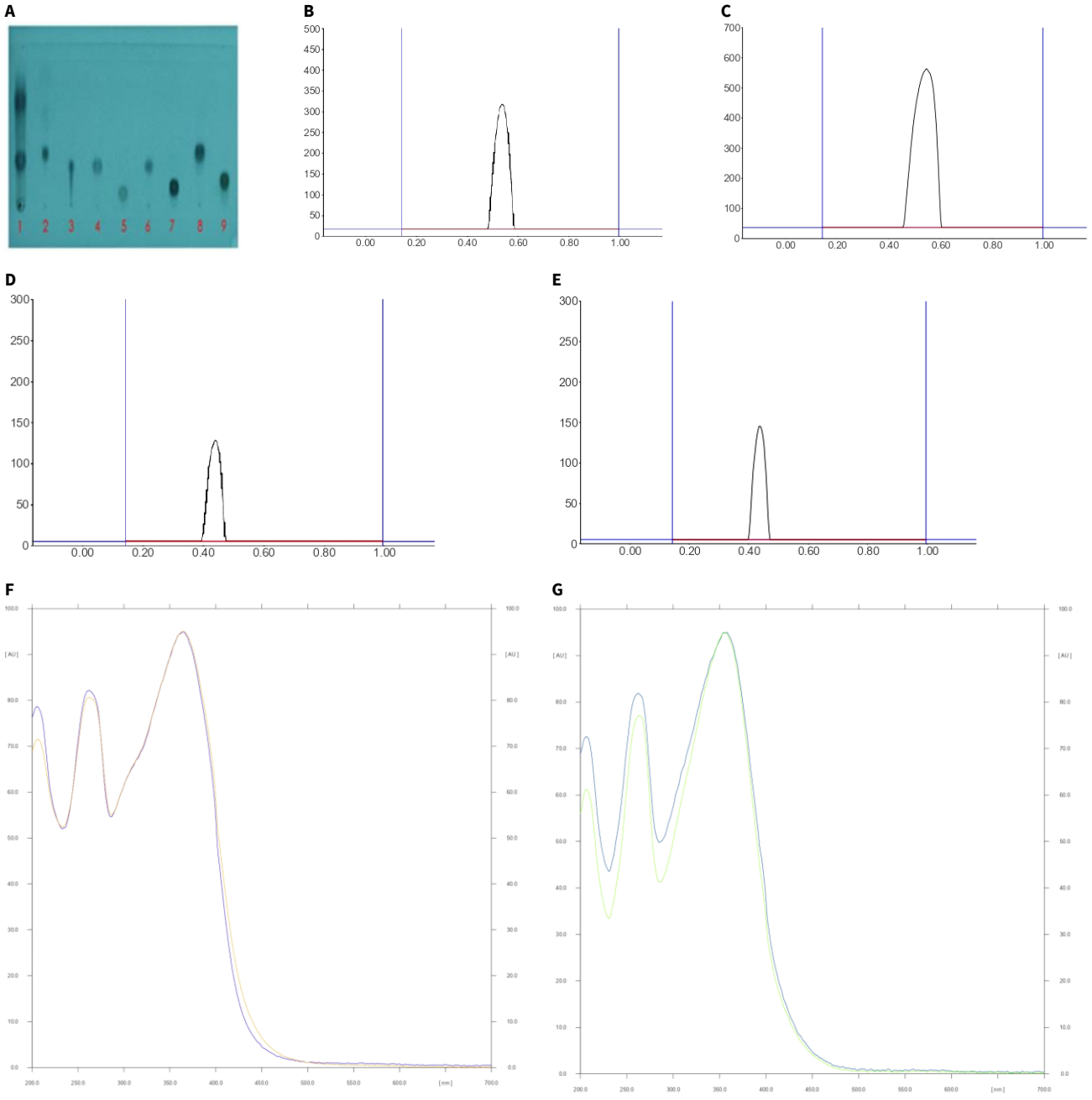

Figure 4. TLC profile of *P. emblica* extracts with 1: extract; 2: isolate **(1)**; 3: isolate **(2)**; 4: apigenin-7-O-glucoside; 5: kaempferol; 6: quercitrin; 7: luteolin; 8: rutin; and 9: quercetin.

(A) UV light 254 nm; **(B)** Densitogram isolate **1**; **(C)** Densitogram rutin; **(D)** Densitogram isolate **2**; **(E)** Densitogram quercitrin; **(F)** UV spectra of isolate **1** and rutin; navy: isolate **1** and orange: rutin; **(G)** UV spectra of isolate **2** and quercitrin; blue: isolate **2** and green: quercitrin.

Table 2. Antioxidant capacity of extract, fractions, and isolates of *P. emblica* leaves.

Sample	IC_{50} DPPH (µg/mL)	AAI DPPH	EC_{50} CUPRAC (µg/mL)	AAI CUPRAC
Ethanol extract *P. emblica*	5.96 ± 0.030^{c}	8.39 ± 0.042^{c}	7.58 ± 0.032^{c}	13.19 ± 0.059^{g}
n-hexane fraction	23.22 ± 0.080^{g}	2.15 ± 0.007^{e}	28.24 ± 0.076^{f}	1.77 ± 0.006^{b}
Ethyl acetate fraction	3.96 ± 0.010^{a}	12.61 ± 0.033^{f}	4.38 ± 0.021^{a}	11.42 ± 0.053^{f}
Water fractions	13.59 ± 0.076^{f}	3.68 ± 0.020^{a}	13.32 ± 0.026^{e}	3.75 ± 0.007^{c}
Compound 1 (rutin)	6.33 ± 0.022^{d}	7.90 ± 0.027^{b}	6.32 ± 0.017^{b}	7.91 ± 0.021^{d}
Compound 2 (quercitrin)	13.46 ± 0.010^{e}	3.72 ± 0.003^{a}	30.81 ± 0.214^{g}	1.62 ± 0.011^{a}
Ascorbic acid	4.80 ± 0.015^{b}	10.42 ± 0.032^{d}	9.5 ± 0.026^{d}	10.53 ± 0.031^{e}

Values are reported as mean ± SD (n = 3). The same letter in a column indicates no significant differences at p<0.05 with ANOVA and *post hoc* Tukey. AAI: Antioxidant activity index.

Table 3. Pearson correlation of the DPPH and CUPRAC approaches in antioxidant activity index.

Parameter	CUPRAC of *P. emblica* ethanol extract	CUPRAC of n-hexane fraction	CUPRAC of ethyl acetate fraction	CUPRAC of water fraction	CUPRAC of isolate 1	CUPRAC of isolate 2	CUPRAC of ascorbic acid
DPPH of *P. emblica* ethanol extract	0.724*						
DPPH of n-hexane fraction		1.000**					
DPPH of ethyl acetate fraction			0.915**				
DPPH of water fraction				0.500^{ns}			
DPPH of isolate 1					0.655*		
DPPH of isolate 2						0.866**	
DPPH of ascorbic acid							0.849**

*p<0.05 and **p<0.01 indicate statistical significance.

Table 4. Correlation between total phenolic (TPC) and flavonoid (TFC) contents, AAI DPPH, and AAI CUPRAC by Pearson's test.

Parameter	TPC g GAE/100g	TFC g QE/100g
AAI DPPH	0.917**	0.683*
AAI CUPRAC	0.939**	0.998**

*p<0.05; and **p<0.01 indicate statistical significance. AAI: Antioxidant activity index.

Table 5. Xanthine oxidase inhibitory activity of extract, fractions, and isolates.

Sample	IC_{50} value (µg/mL)
Ethanol extract *P. emblica*	72.55 ± 1.22^{e}
n-hexane fraction	185.70 ± 11.55^{f}
Ethyl acetate fraction	42.13 ± 1.16^{cd}
Water fractions	48.98 ± 1.19^{d}
Isolate 1	32.77 ± 4.49^{bc}
Isolate 2	23.85 ± 2.04^{b}
Allopurinol	1.49 ± 0.05^{a}

Values are reported as mean ± SD (n = 3). The same letter in a column indicates no significant differences at p<0.05 with ANOVA and *post hoc* Tukey.

In this research, information was obtained that SF29A2, known as a quercitrin compound, has stronger activity than SF29A1 (rutin). It could be because there are more sugar groups in the rutin compound (glucose and rhamnose) than in quercitrin (rhamnose), so the activity of the rutin compound is weaker than quercitrin. In previous studies, information was also obtained that the aglycone of the compound rutin and quercetin, namely quercetin, has better xanthine oxidase inhibitory activity compared to rutin and quercitrin. The presence of a glycoside group substitution at C-3 of quercetin weakens the flavonoid glycosidic bond with the enzyme. The inhibitory activity of XO by flavonoids is determined largely based on their structural properties. Pharmacologically, carbonyl compounds usually act through direct interactions with enzymes. The reactivity of carbonyl and hydroxyl at position 7 of flavones is also based on structural characteristics such as planarity which play a role in the interaction between flavonoids and enzymes. The hydrogen atoms of the OH group at positions 3 and 5 of flavones can form intramolecular hydrogen bonds with the oxygen atom of the carbonyl, supporting molecular planarity and π electron delocalization (Ponce et al., 2000). Substitution of different groups at various positions in the flavonoid structure greatly affects the binding of compounds with the active site XO, resulting in differences in inhibitory activity. The existence of groups of hydroxyls at locations C5, C-7, and C4', along with double bonds at C2-C3, has been found to enhance the inhibitory activity of XO. In contrast, hydroxylation at positions C3, C6, and C5' and glycosylation at C3 and C7 can reduce the inhibition activity of XO. Still, hydroxyl groups at positions C3 and C3' can increase the activity of reducing superoxide. The classification of flavonoids based on XO inhibitory activity and superoxide scavenging is as follows: scavenger superoxide without activity inhibiting XO; inhibition of xanthine oxidase blockers without activity to scavenge superoxide; XO inhibitor with superoxide scavenger activity; XO inhibitors with pro-oxidant activity; minor XO inhibition with pro-oxidant activity; and flavonoids that are devoid of XO inhibition and superoxide scavenging activity (Atmani et al., 2009; Lin et al., 2015; Mehmood et al., 2019; Nagao et al., 1999; Zhao et al., 2020).

In other studies, information has been obtained that there are rutin and quercitrin in *P. emblica* fruit (Ahmad et al., 2021; Huang et al., 2023) but no one has reported the presence of quercitrin, which is active in inhibiting xanthine oxidase found in *P. emblica* leaf extract, so the novelty in our research is the presence of flavonol glycosides namely rutin and quercitrin found in *P. emblica* leaves which are active in inhibiting xanthine oxidase.

CONCLUSION

The ethanol extract of *Phyllanthus emblica* leaves contains flavonol glycosides that inhibit xanthine oxidase with an IC_{50} value < 35 µg/mL stronger activity than the extract with an IC_{50} value of 72.55 ± 1.22 µg/mL. They were of rutin and quercitrin. Quercitrin has stronger xanthine oxidase inhibitory activity than rutin but has less antioxidant capacity. Rutin has strong antioxidant capacity with an AAI DPPH value of 7.89 ± 0.03 and AAI CUPRAC 15.83 ± 0.04, while quercitrin has antioxidant capacity with an AAI DPPH value of 3.72 ± 0.01 and AAI CUPRAC 3, 24 ± 0.03. These findings may serve as scientific evidence that flavonol glycosides have the potential to be used in the development of anti-hyperuricemia drugs with potent antioxidants.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	Husnunnisa	Hartati R	Mauludin R	Insanu M
Concepts or ideas	x	x	x	x
Design	x	x	x	x
Definition of intellectual content	x	x	x	x
Literature search	x	x	x	x
Experimental studies	x			
Data acquisition	x	x	x	x
Data analysis	x	x	x	x
Statistical analysis	x	x	x	x
Manuscript preparation	x	x	x	x
Manuscript editing	x	x	x	x
Manuscript review	x	x	x	x

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