TLC Densitometry of Isoquercitrin Content in Kenikir Leaves (*Cosmos caudatus* Kunth.) and Tyrosinase Inhibitory and Anti-*Propionibacterium acnes* Bioactivity Assays

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ABSTRACT

Isoquercitrin is a 3-O-glucoside of quercetin identified in the TLC profiles of kenikir (*Cosmos caudatus* Knuth.) leaves extract and fractions. Quantification of isoquercitrin was effected by TLC-densitometry analysis with ethyl acetate/water/formic acid (10:1:1) mobile phase, developing with citroborate reagent and detecting at wavelength 366 nm. The linearity equation, y = 23.404x + 402.16, with a correlation coefficient value of 0.9925, indicated isoquercitrin levels of 15.7 and 24.6 mg/g in the total extract and ethanol fraction, respectively. The minimum inhibition concentration (MIC) for *Propionibacterium acnes* was determined using microdilution with the addition of iodonitrotetrazolium as a chromogenic agent. The antibacterial activity of the ethanol fraction against *P. acnes* was twice that of chloramphenicol, with MIC 0.625 mg/ml. Tyrosinase inhibition was evaluated by IC_{50} spectrophotometrically. The ethanol fraction was more active in inhibiting tyrosinase enzyme than kojic acid, with IC_{50} 6.803 µg/ml. *C. caudatus* ethanol fraction containing the flavonoid isoquercitrin has good tyrosinase inhibitory and anti-*Propionibacterium acnes* activity.

Keywords: isoquercitrin; TLC-densitometry; *Cosmos caudatus* Kunth.; *Propionibacterium acnes;* Tyrosinase.

1. INTRODUCTION

Cosmos caudatus Kunth., an annual herbaceous plant with pink to purple flowers and pinnately compound leaves, is native to Latin America and has now been naturalized in Asia via the Philippines. Commonly known as kenikir or cosmos in Indonesia, in Malaysia it is called ulam raja, and, in Thailand, dauruang.[1] Kenikir has been used traditionally as an antihypertensive, antidiabetic, antioxidant, anti-osteoporosis, antifungal, and antibacterial agent.[2] Kenikir is often consumed raw as a fresh salad because of its unique and attractive aroma which adds variety and taste to the regional cuisine.[3] Kenikir leaf extract has been shown to reduce blood glucose levels and total cholesterol and to help regenerate pancreatic tissue in hypercholesterolemic mice.[4] It has a strong antioxidant activity based on DPPH and ABTS methods,[5,6] and its antibacterial activity against *Salmonella* spp., *Proteus mirabilis*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Vibrio cholerae* has been demonstrated.[5,7] n-Hexane, ethanol, and diethyl ether extracts inhibited the growth of *S. aureus* with minimum inhibitory concentration (MIC) values of 25, 6.25, and 6.25 mg/ml, respectively.[7]

C. caudatus contains bioactive compounds that support its pharmacological activities. The leaves contain several flavonoid constituents not found in the roots, including quercetin, kaempferol, myricetin, catechin, luteolin,

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apigenin, quercetin 3-O-ramnoside (quercitrin), quercetin 3-O-glucoside, quercetin 3-O-siloside, quercetin 3-O-arabinofuranoside, and rutin.[3] Of the total flavonoids (52.2 ± 4.1 mg/100 g of fresh leaves), the predominant flavonoid quercetin content is reported to be 51.3 ± 4.1 mg/100 g.[8] The contents of quercitrin, catechin, and rutin, are 36.9, 25, and 8.2 mg/g of *C. caudatus* extract, respectively.[9] While there is no quantitative data on isoquercitrin in the extracts or fractions of *C. caudatus*, isoquercetrin (Figure 1) can be used as a marker compound.[10,11] To determine the content of isoquercitrin and other pharmacological activities of the flavonoid group in *C. caudatus*, it is necessary to carry out defatting and dechlorophyllation processes.

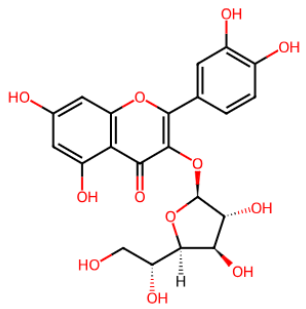

Figure 1. Structure of isoquercitrin.

According to the Global Burden of Disease study, it is estimated that acne affects around 10% of the global population, which places it as the eighth most common disease worldwide and the third most common dermatological condition.[12] Acne, or *acne vulgaris*, has a multi-factorial pathogenesis, starting from increased sebum production, changes in the quality of sebum lipids, hormonal dysregulation, and follicular hyper-keratinization. *Propionibacterium acnes*, the main factor causing inflammation,[13] is a gram-positive bacterium that attacks human skin and predominates in pilosebaceous skin follicles.[14] Inflammation caused by *P. acnes* results in hyperpigmentation after the healing process, usually called post inflammatory hyperpigmentation (PIH),[15] due to an increase in melanin production and a change in the density of activated melanocytes. Efforts to eliminate acne and inflammation can focus on inhibiting the activity of *P. acnes*, and inhibiting the activity of the tyrosinase enzyme can inhibit or prevent hyperpigmentation.[16]

Given the lack of quantitative data on the predominant flavonoid isoquerctirin, and the absence of studies on the antibacterial activity against *P. acnes* and on tyrosinase inhibition, we were interested to analyze isoquercitrin content in *C. caudatus* leaves extract and fractions, and to determine their activity.

2 MATERIALS AND METHODS

2.1 Materials

Fractionation of *C. caudatus* leaf ethanol extract was carried out using a solid-liquid method with n-hexane, ethyl acetate, and ethanol. TLC Silicagel 60 aluminum sheets (20 x 20 cm, Merck KGaA, Darmstadt, Germany, catalog no. 1.05554), were eluted with ethyl acetate/aquadest/formic acid (10:1:1).

Tyrosinase inhibitory activity was evaluated using tyrosinase from mushroom (Sigma Aldrich, T3824-25KU, CAS: 9002-10-2) with L-DOPA (3,4-dihydroxy-L-phenylalanine, Sigma Aldrich, D9628-5G, CAS: 59-92-7) as substrate and kojic acid (Sigma Aldrich, K3125-5G) as comparative active compound. Phosphate buffer was made using sodium dihydrogen phosphate monohydrate (Merck KGaA, Darmstadt Germany, catalog no. 1.06345) mixed with di-sodium hydrogen phosphate heptahydrate (Merck KGaA, catalog no. 1.06575).

Anti-bacterial activity was tested using the liquid microdilution method with chromogenic agent INT (iodonitrotetrazolium chloride) (Sigma Aldrich, 10406, CAS: 146-68-9) against *Propionibacterium acnes*.

2.2 Instrumentation

TLC densitometry was carried out with a TLC scanner 4 (CAMAG). Data acquisition and processing were recorded using the winCATS version 1.4.7 software [17]. Spectrophotometric evaluation of IC_{50} inhibition of

tyrosinase was carried out using a FlexA-200 microplate reader (Allsheng, China).

2.3 Fractionation and Chemical Profiling

C. caudatus leaves thick ethanol extract (5 g) was subjected to solid-liquid fractionation with n-hexane. After separating the n-hexane fraction, the residue was fractionated again using ethyl acetate. The residue was dissolved in ethanol. The total extract, n-hexane fraction, ethyl acetate fraction, and ethanol fraction were profiled by TLC using the eluent ethyl acetate/aquadest/formic acid (10:1:1) with rutin and isoquercitrin as reference compounds. Plate visualization was carried out under UV lamp 366 nm after spraying with citroborate reagent and heating at 100ºC for 5 minutes.

2.4 Quantification of Isoquercitrin

From a stock solution of isoquercitrin (10 mg) dissolved in ethanol (2 ml), standard solutions with concentrations 500, 250, 125, 62.5, and 31.25 µg/ml were prepared. An isoquercitrin calibration curve was established from the peak areas of the standard solution concentrations. Preparation of samples (extract and fractions) to 5000 µg/ml concentration was carried out by dissolving samples (10 mg) in ethanol (2 ml). A volume of 2 µl of standard and sample solutions was applied to the chromatographic plate and then developed at room temperature in flat bottom chamber using the eluent and plate visualization method described above.

2.5 Tyrosinase enzyme inhibition assay

The tyrosinase inhibition assay used in this study refers to the method developed by Momtez et al. (2008) with several modifications.[18] The total extract and each fraction were prepared to a final concentration of 250, 125, 62.5, 31.25, 15.625, 7.8125, and 3.9062 µg/ml. Kojic Acid was used with a concentration of 12.5, 6.25, 3.125, 1.563, 0.781, 0.391, and 0.196 µg/ml. A sample (50 µl) was added to phosphate buffer (80 µl) with pH 6.5, then incubated for 5 minutes, then tyrosinase (50 µl, 250 units/ml) and L-DOPA (20 µl, 5.07 mM) were added to each well and incubated for 30 minutes at room temperature. Absorbance was measured at a wavelength of 492 nm.

2.6 Anti-*Propionobacterium acnes* assay

The antibacterial assay employed a modified microdilution method with p-iodonitrophenyltetrazolium violet as an indicator of bacterial cell viability. Each sample was dissolved in DMSO (10% final volume) and diluted with Mueller-Hinton broth (Oxoid) medium. The total extract and all fractions were prepared to concentrations of 5, 2.5, 1.25, 0.625, 0.3125, 0.1562, and 0.0781 mg/ml. The comparison or positive control used was chloramphenicol prepared to concentrations of 1.5, 0.75, 0.375, 0.1875, 0.09375, 0.046875, and 0.00234375 mg/ml. Samples of each concentration (100 □l) were added to three microplate wells, and sterilization control (media + DMSO) and growth control (media + DMSO + bacteria) were carried out. Each microplate well was inoculated with bacterial suspension (5 µl, 106 cfu/ml) and was incubated at 37 ºC for 18 hours, followed by the addition of p-iodonitrotetrazolium (INT, 20 µl) in distilled water (0.5 mg/ml), and incubated again for 30 minutes. INT is a compound that is easily reduced by the presence of dehydrogenase enzymes in bacteria to form formazan, which gives a purple color. The change in color from yellow to purple indicates that there are still bacteria in the microplate wells.[19]

3 RESULTS AND DISCUSSION

The total extract of *C. caudatus* was fractionated with n-hexane and ethyl acetate to effect defatting and dechlorophyllization. Chlorophyll in *C. caudatus* was detected at 3.29 to 4.31 mg/ml.[20] Besides chlorophyll, *C. caudatus* is known to contain other non-flavonoid compounds such as phenolic compounds, benzoic acid derivatives, cyclohexene-1-carboxylic, chlorogenic, α-linolenic, and ascorbic acids, α-tocopherol, myo-inositol, α-D-glucopyranoside, 4,4′-bipyridine, diterpenoids, costunolide, stigmasterol, lycopene, and lutein.[3] These non-flavonoid compounds are less polar than the

flavonoids and can be separated into the n-hexane and ethyl acetate fractions.

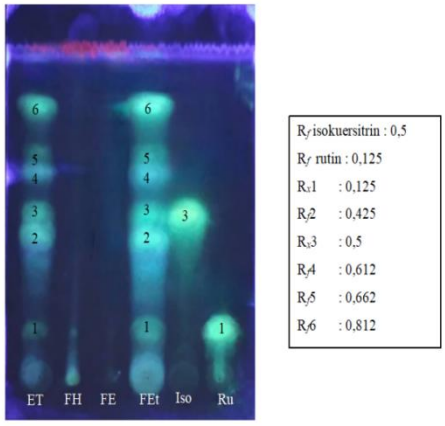

Figure 2. TLC profile of total extract and fractions (ET total extract, FH n-hexane fraction, FE ethyl acetate fraction, FEt ethanol fraction, Iso isoquersitrin, and Ru rutin) eluted with ethyl acetate/water/formic acid (10:1:1), developed with citroborate and visualized under UV light at 366 nm.

Effective separation is demonstrated by the TLC profiles in Figure 2.[11] The ethanol fraction contains significant flavonoids. Spots 1 and 3 have the same retention factor values as the reference compounds rutin and isoquercitrin. Spots 2, 4, 5, and 6 also gave positive reactions with the citroborate reagent. The color change reaction in flavonoids is caused by H_3BO_3, a main component of citroborate that can form a chelate complex with the ortho-dihydroxy and ortho-hydroxy carbonyl groups in flavonoids.

TLC densitometry was carried out on the total extract and ethanol fraction that positively contained isoquercitrin. There have been no reports of isoquercitrin quantification of the extract or fractions of C. caudatus. The isoquercitrin Rf value of 0.53 was detected at a wavelength 366 nm. Based on the correlation between the concentration of isoquercitrin standard (x) and peak area (y), the equation y = 23.404x + 402.16 (r = 0.9925) was obtained. The highest isoquercitrin content of 23.2 mg/g was detected in the ethanol fraction, equivalent to 3.7 mg/g dried leaves of C. caudatus, while the total extract contained 15.7 mg/g equivalent to 2.6 mg/g dried leaves (Table 1). There is no visible trace of isoquercitrin in the n-hexane or ethyl acetate fractions, indicating a highly effective fractionation process. Isoquercitrin levels are greater in the fraction than the extract because defatting and dechlorophyllation concentrate the flavonoids in the fraction. It can be concluded that isoquercitrin as a marker compound in C. caudatus is a minor component.

Table 1. Calculation of isoquercitrin concentration in extract and dried leaves of C. caudatus.

No.	Sample	Part of 5 g extract (g)	Isoquercitrin concentration	
			per 1 g extract (mg)	per 1 g dried leaves (mg)
1	n-hexane fraction	0.196	0	0
2	ethyl acetate fraction	0.237	0	0
3	ethanol fraction	4.567	23.2	3.7
4	total extract		15.7	2.6

The anti-Propionibacterium acnes activity of C. caudatus leaves extract and fractions was tested using the liquid microdilution method to determine the minimum inhibitory concentration (MIC) using the chromogenic agent INT (iodonitrotetrazolium),[19] a method which requires only small quantities of sample and reagents, has high sensitivity, and provides quantitative results.[21,22] The MIC is the smallest concentration of an antimicrobial compound that can inhibit the growth of the test microbe.[23] Observations to determine MIC can be made visually by

adding the p-iodonitrotetrazolium (INT) reagent. The MIC is determined in the well with the lowest antimicrobial concentration that does not give a purple color after adding INT. A change in color from yellow to purple indicates that there are still bacteria in the well. p-Iodonitrotetrazolium (INT) is reduced by the dehydrogenase enzymes in bacteria to form purple-colored formazan.[24]

The total ethanol extract, n-hexane fraction, ethyl acetate fraction, and active ethanol fraction inhibited *P. acnes* with MIC values of 1.25, 2.5, 5, and 0.625 mg/ml, respectively (Figure 3). The MIC of the ethanol fraction was half that of chloramphenicol, suggesting that the inhibitory activity of the ethanol fraction toward *P. acnes* bacteria is 2 times higher than that of chloramphenicol. The flavonoid content in the ethanol fraction provides high anti-*P. acnes* activity.

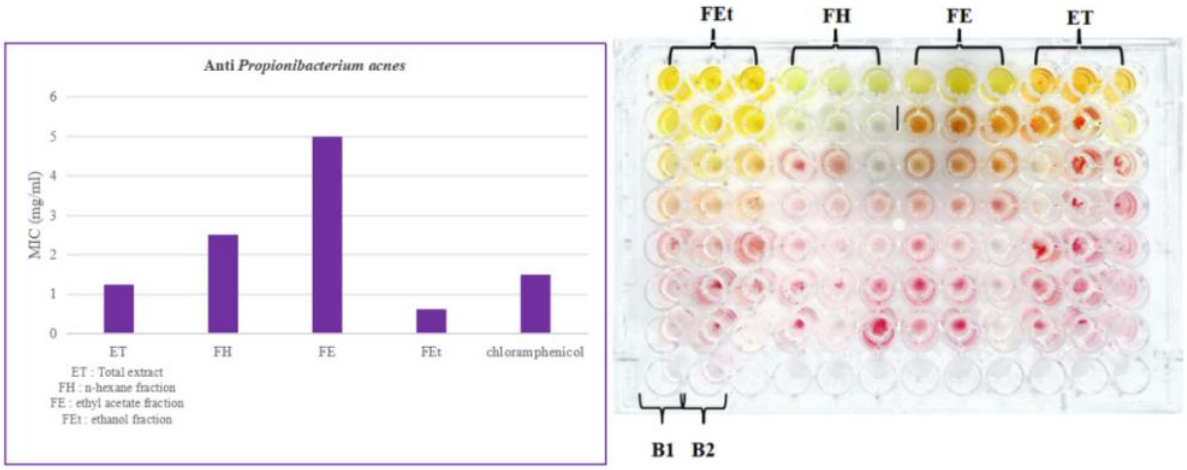

Figure 3. Anti-*P. acnes* activity of total extract (ET), n-hexane fraction (FH), ethyl acetate fraction (FE), ethanol fraction (FEt), and chloramphenicol.

Tyrosinase inhibitory activity testing was carried out using the method developed by Momtez, et. al, with various modifications.[18] Tyrosinase is an enzyme that plays a role in the process of melanogenesis, or melanin biosynthesis. The melanogenesis process begins with hydroxylation of phenylalanine to L-tyrosine, or directly from L-tyrosine, which is then hydroxylated to L-dihydroxyphenylalanine (L-DOPA). L-DOPA is then oxidized to L-DOPAquinone (DQ). Both reactions are catalyzed by the enzyme tyrosinase.

The total extract, n-hexane fraction, ethyl acetate fraction, and ethanol fraction of *C. caudatus* leaves actively inhibited the tyrosinase enzyme with IC_{50} values of 7.17, 15.659, 8.571, 6.803, and 7.327 µg/ml, respectively (Figure 4 and Table 2). The ethanol fraction has better activity than the reference comparison compound kojic acid. Flavonoids are phenolic compounds whose production is quinoid, which absorbs in a different spectral range to dopachrome.[25] When this phenolic function shows strong affinity for the enzyme, the formation of dopachrome can be prevented.[26]

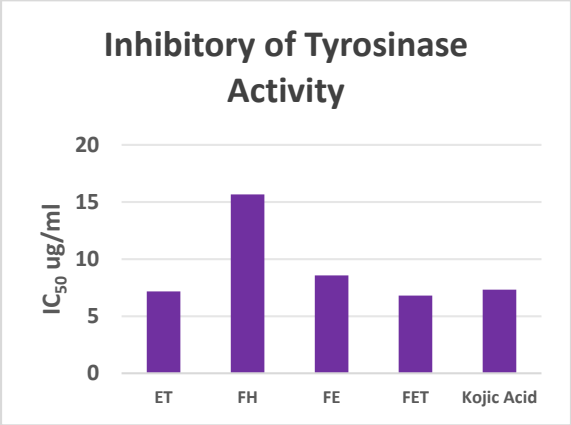

Figure 4. Tyrosinase inhibitory activity of total extract (ET), n-hexane fraction (FH), ethyl acetate fraction (FE), ethanol fraction (FEt) and kojic acid.

Table 2. Inhibitor concentration 50% of total extract, n-hexane fraction, ethyl acetate fraction, and ethanol fraction of *C. caudatus*

Samples	IC_{50} (µg/ml)
total extract	7.172
n-hexane fraction	15.659
ethyl acetate fraction	8.571
ethanol fraction	6.803
kojic acid	7.327

CONCLUSION

TLC profiling of *C. caudatus* ethanol fraction showed significant levels of flavonoids and isoquercitrin. The fractionation process was successfully demonstrated by the absence of isoquercitrin in the n-hexane and ethyl acetate fractions, and higher isoquercitrin content in the ethanol fraction after defatting and dechlorophyllation. Isoquercitrin as a marker compound in *C. caudatus* is only a minor component. The inhibitory activity of the ethanol fraction against *P. acnes* bacteria is twice that of chloramphenicol. The ethanol fraction also has better activity than kojic acid as a tyrosinase inhibitory agent. Overall, the *C. caudatus* ethanol fraction has a high flavonoid content and good activity as a tyrosinase inhibitor and anti-*Propionibacterium acnes* agent.

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قياس كثافة TLCلمحتوى الإيزوكيرسيترين في أوراق الكينيكر (Cosmos caudatus Kunth) واختبار النشاط الحيوي كمثبط للتيروزيناز ومضاد للبروبيونِية العدية (Propionibacterium acnes)

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ملخص

إيزوكيرسيترين هو 3–أو–جلوكوزيد من الكيرسيتين الموجود في ملف تعريف تفريق لوني على طَبقة رقيقة (TLC) لمستخلص وأجزاء من أوراق كينيكير (Cosmos caudatus Kunth). الهدف من هذه الدراسة هو تحديد مستوى الإيزوكيرسيترين كمركب علامة في خلاصة مذنب ج (C. caudatus). تم تحديد مستوى الإيزوكيرسيترين باستخدام تحليل قياس الكثافة– تفريق لوني على طَبقة رقيقة (TLC-densitometry) مع طور متحرك من أسيتات الإيثيل: ماء مقطر: حمض الفورميك (10:1:1) مع إضافة محلول سيتروبورات. تم الكشف عن الإيزوكيرسيترين عند طول موجة 366 نانومتر بعد رشه بمحلول سيتروبورات. معادلة الخطية، واي=402.1623.404 + x، مع معامل ارتباط 0.9925، ومستوى الإيزوكيرسيترين 15.7 و 24.6 مج/ج في الخلاصة الكلية وجزء الإيثانول. يعرف الإيزوكيركترين كمركب علامة بتركيز أدنى. الحد الأدنى لتركيز التثبيط (MIC) لمضاد للبروبيونِية العدية (Propionibacterium acnes) يستخدم ميكرودلوسي مع إضافة أيودونيتروترازوليوم كعامل كروموجينيك. كان نشاط مضاد للبروبيونِية العدية (Propionibacterium acnes) لجزء الإيثانول أكثر فعالية بمرتين من الكلورامفينيكول مع الحد الأدنى لتركيز التثبيط 0.625 (MIC) مج/مل. تم تقييم تثبيط التيروزيناز بواسطة آى سي50 (50IC) طيفياً. كان جزء الإيثانول أكثر فعالية في تثبيط إنزيم التيروزيناز من حمض الكوجيك مع آى سي50 6.803 (50IC) ميكروغرام/مل. يتمتع جزء الإيثانول بنشاط جيد كمثبط للتيروزيناز ومضاد للبروبيونِية العدية (Propionibacterium acnes) مع وجود الإيزوكيرسيترين كأحد مكونات الفلافونويد مع الإيزوكيرسيترين كمركب علامة.

الكلمات الدالة: إيزوكيرسيتين؛ قياس الكثافة بكروماتوغرافيا الطبقة الرقيقة (TLC)؛ كوسموس كاوداتوس كونث؛ بكتيريا البروبيونية العدية (أو البكتيريا المسببة لحب الشباب)؛ تيروسيناز.

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