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EFFECT OF DIFFERENT SOLVENT ON PHYTOCHEMICAL CONTENT, TYROSINASE INHIBITION AND ANTIOXIDANT ACTIVITIES OF CAMPOLAY (POUTERIA CAMPECHIANA KUNTH. (BAEHNI.))

ABSTRACT

Pouteria campechiana leaves are reported to have phenol and flavonoid compounds. Phenolic and flavonoid compounds can act as tyrosinase inhibitor and antioxidant. The purpose of this study was to compare phytochemical content, tyrosinase inhibition, antioxidant activities and determine of marker compound from P. campechiana leaves extract with different polarities solvent. In addition, the content of marker compound from P. campechaina leaves extract was determined by HPLC. The highest total phenolic content 7.83 GAE/100 g extract, IC_{50} of tyrosinase 171.512 ± 1.352 and IC_{50} of DPPH 0.968 ± 0.008 was given by ethanolic extract (DE). Meanwhile ethyl acetate extract had the highest of total flavonoid content 2.544 ± 0.554 QEA/100 g extract. The total phenolic content correlated with tyrosinase inhibitory activity and antioxidant activity. Quercitrin was as marker compound from P.campechiana leaves extract, and quercitrin content in the ethyl acetate extract of P. campechiana leaves was 3.539%, while in the ethanol extract was 0.153%

Keywords: *P.campechaina, Tyrosinase, Antioxidant, Quercitrin*

INTRODUCTION

Exposure of UV light can produce Reactive Oxidative Singlet (ROS). If the mechanism action of ROS is not inhibited, it will increase the tyrosinase action [1] Tyrosinase is enzyme that play the role in biosynthesis of melanin in plant and mammalian cell, including human skin [2]. The mechanism action of tyrosinase enzyme divided into two, namely monophenolase activity using L-tyrosine substrate and diphenolase using L-DOPA substrate[2],[3]. Diphenolase activity will produce ortho-quinone, meanwhile melanin will be produced by non-enzymatic reaction. This process is called melanogenesis [4].

Antioxidants compounds can inhibit the reaction of free radicals. An example of a free radical is ROS which is found in the skin. When antioxidant can inhibit reaction of ROS which is caused by UV light exposure on the skin, it will reduce tyrosinase enzyme activity. Phenolic acid [5], flavonol [6] and stilbenoide [7] were antioxidant compounds as well as tyrosinase inhibitors.

Medicinal plants are a source of raw material which contain phenolic, flavonoids and polyphenols. Pouteria campechiana is one of the medicinal plants in Sapotaceae family. In Indonesia, P. campechiana was called as campolay. P. campechiana fruits extract contains gallic acid, myricitrin and cathecin which act as antioxidant compounds [8]. In addition, taxifolin, quercetin glycoside and myricetin glycoside were found in P. campechiana leaves which its toxicity had been tested [9]. The comparison of chemical content, tyrosinase inhibitory activity, antioxidant activity of P. campechiana leaves extract have not been reported with different solvent polarity levels.

In this study, the total chemical content was calculated, inhibitory of tyrosinase and antioxidant activities were tested, then marker compound from selected extract of P. campechiana was identified and determined.

EXPERIMENITAL

Material

P. campechiana leaves were collected from Bandung, West Java, Indonesia in December 2020.

Chemical material

DPPH (Sigma Aldrich), tyrosinase enzyme (T3824-25KU, Sigma Aldrich), L-DOPA (Sigma Aldrich), quercetin, kaemferol, apigenin, gallic acid, chlorogenic acid, luteolin 7-O glycoside, and rutin which were obtained from Sigma Aldrich, quercitrin (Markherb), methanol pro analysis, ethanol 96%, ethyl acetate, n-hexane, Folin-Ciocalteu reagent, aluminum chloride and other chemical materials which used in this study were analytical grade.

Preparation Extract

Extraction was conducted using Soxhlet with increasing polarity solvent, ranging from nonpolar (n-hexane), semi-polar (ethyl acetate), and polar solvent (ethanol 96%). 400 g of P. campechiana leaves powder was extracted with n-hexane, the filtrate and residue are separated. The residue was dried, then extracted with ethyl acetate. Finally, the residue was extracted using ethanol 96%. Each extract was thickened using rotary evaporator with a temperature of 50^0C to produce n-hexane extract (DN), ethyl acetate extract (DEA) and ethanol 96% extract (DE). Each extraction process with Soxhlet was carried out triplicate.

Total phenolic content

Determination of total phenolic content using modification of Pourmorad's method [10]. Each extract was tested triplicate. The test was measured at a wavelength of 765 nm. Total phenolic content was calculated by gallic acid calibration curve with concentration in the range of 10-250 µg/ml and expressed as gallic acid equivalent (GAE) per 100 g extract (g GAE/100 g extract).

Total flavonoid content

Determination of total flavonoid content using of Chang's method modified [11]. Each extract was tested triplicate. The test was investigated at a wavelength of 415 nm. Total flavonoid content was calculated by quercetin calibration curve with concentration ranged from 25 to 250 µg/ml and presented as quercetin equivalent (QE) per 100 g extract (g QE/100 g extract).

Tyrosinase Inhibitory Activity

Determination of tyrosinase inhibitory activity using as spectrophotometer in 96-well plates (Biologix®) following the procedure of Masuda's method [12] with slight modification. Tyrosinase inhibitory activity was determined by diphenolase mechanism, with L-Dopa as substrate. Standard of tyrosinase inhibitor (kojic acid) were dissolved in phosphate buffer (pH 6.8) which contained DMSO 5%. The enzyme concentration used was 300 unit/ml. Absorbance was investigated at λ 475 nm. Concentration of DN, DEA and DE 1000 µg/ml

were carried out for testing tyrosinase inhibitory activity. Then, 10 concentration series was prepared from the selected sample to find the IC_{50} value.

Antioxidant activity

Determination of antioxidant activity was used modified Blois's method[13]. The free radical DPPH with a concentration of 39,4 µg/ml in methanol. The standard was ascorbic acid. Absorbance measurement at λ 517 nm using a UV-Vis spectrophotometer (Beckman Coulter DU 720). Extracts and standard were performed in three replications. The final parameter used as antioxidant activity was antioxidant activity index (AAI), which applied the formula AAI = [final concentration of DPPH (µg/ml)]/IC_{50} (µg/ml) [14].

Statistical analysis

The results were presented with the mean ± SD value from three repetitions of the test, using MS excel software to evaluate the results of the IC_{50} value. Statistical analysis was also used to see the correlation between the total phenolic content and total flavonoid content to tyrosinase inhibitory and antioxidant activities with Pearson's method in Minitab-19 application.

Identification of marker compound from selected extract

Identification of marker compound was carried out qualitatively. The first method using TLC co-chromatography to compare the Retardation factor (Rf) values with various standard compounds. The second method used was HPLC (LC-20AD) by comparing with quercitrin as standard compound selected based on the TLC co-chromatography. The stationary phase in HPLC used LiChrospher® 100 RP-C18 5 µm (length 100 mm, diameter 4 mm, 20 mm in column (Merck)). The mobile phase used was 0.01 % H_3PO_4 (eluent A) dan methanol (eluent B). Eluent B, 2.5 min 40%, 2.51 to 7 min 60%, 7.01-12 min 70%, and 40% for linier gradient from 12 to 15 min. Flow rate used 1 ml/min, (CTO-20A pump, Shimadzu, Japan). Injection volume 20 µl. Detector used 366 nm (Detector UV/Vis SPD-20A, Shimadzu, Japan). The parameter used was retention time from standard compound (quercitrin) and sample. Sample and quercitrin were diluted in methanol with 50.000 µg/ml for DE, 10.000 µg/ml for DEA and 500 µg/ml for quercitrin.

Determination of marker content

Determination of marker content from selected extract used the one-point method. Calculation the content of marker was by comparing the area under curve (AUC) of marker in extract and AUC of standard compound.

RESULTS AND DISCUSSION

Extraction of P. campechiana leaves

The yield obtained from n-hexane, ethyl acetate and ethanol extracts of P.campechiana leaves was showed in Table-1. The yield of the extracts indicated amount of the secondary metabolite which was extracted in each solvent with different polarity. DE had the largest yield compared to DN and DEA. This result showed that extraction of P. campechiana leaves by Soxhlet with ethanol 96% could attract more metabolite from P. campechiana leaves.

Tabel-1: The Yield of P.campechiana Leaves Extracts

Sample	*Yield of extract (g extract /100 g dried leaves)*
DN	*7.24*
DEA	*5.25*
DE	*23.00*

Note: DN (n-hexane extract), DEA (ethyl acetate extract), DE (ethanol extract)

Determination of phytochemical content

Determination of phytochemical content was conducted for total phenolic content (TPC) and total flavonoid content (TFC). This is based on phenolic and flavonoid play a role in tyrosinase inhibitory and antioxidant activities.

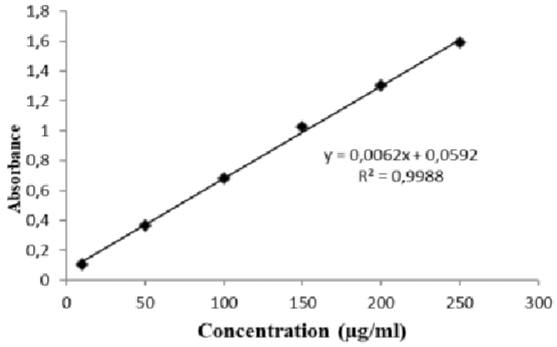

Fig.-1: Calibration curve of gallic acid

Total phenolic content was calculated using gallic acid curve with linier regression equation y=0.0061x + 0.0796; R² = 0.9943 (Fig.-1) and presented by g GAE/100 g extract. It has no report regarding TPC and TFC in P. campechiana leaves extract using the multilevel extraction method by Soxhlet. The results showed that the TPC of ethanol leaves extract was higher than n-hexane and ethyl acetate extracts. This mean, that compounds belonging to the phenolic groups which found in P. campechiana leaves were more suitable extracted with ethanol 96% as solvent. Phenolic compound is a group of secondary metabolites in plants. The phenolic group included polyphenols. Polyphenols included flavonoids, phenolic acids, stilbenoid and lignan [15]. The phenolic group which has more OH groups showed high polarity and will be more effective if extracted with a polar solvent, such as ethanol. The fewer OH groups in phenolic group, will be extracted with semipolar solvents such as ethyl acetate.

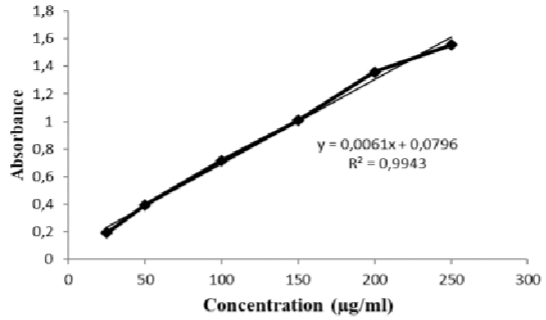

Fig.-2: Calibration curve of quercetin

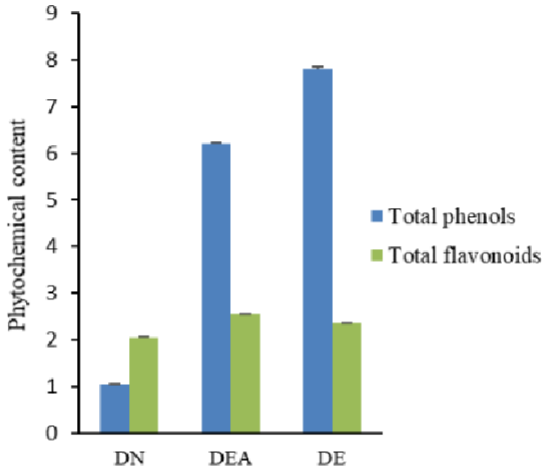

Fig.-3: Phytochemical content of P. campechiana leaves extract

The total flavonoid content was determined based on the quercetin standard curve, y = 0.0062x + 0.0592; R^2=0.9988 and exposed with g QE/100 g extract. The ethyl acetate extract had the highest total flavonoid content compared to ethanol and n-hexane extracts. This means, that ethyl acetate solvent was more effective for extracting flavonoids from P. campechiana leaves. Based on the structure, the flavonoid group can be divided into flavones group such as apigenin, flavonols group such as quercetin, quercitrin and myricitrin, flavan group, flavanol and anthocyanidin group [16].

Tyrosinase inhibitory activity

The tyrosinase inhibitory activity of P. campechiana leaves extract was expressed in Table- 2. Determination of tyrosinase inhibitory activity using modification method of Masuda [12]. Kojic acid was used as tyrosinase inhibitor. Inhibitory of tyrosinase activity from ethanol, ethyl acetate and n-hexane extracts of P. campechiana leaves were the first time reported. The result of the study, showed that DE had the highest tyrosinase inhibitory activity compared to DEA and DN. The n-hexane extract had lowest of tyrosinase inhibitory activity which was 15.078 ± 1.785 at 1000 µg/ml. Therefore, DN was not continued to determine the IC_{50} tyrosinase inhibitory value. The difference in tyrosinase inhibitory activity in DE, DEA and

DN extract could be due to differences in phytochemical content that extracted in each extract. Extraction methods with different solvent polarity levels can extract different chemical constituents. Differences in the chemical structure of each compound will affect the strength of tyrosinase inhibitory and antioxidant activities [17]. Phenols and flavonoids can contribute to antioxidant and tyrosinase inhibitory activity [18]. The number of hydroxy groups can increase antioxidant activity and tyrosinase inhibitory activity [1]. According to the literature, Manilkara zapota belong to Sapotaceae family gave tyrosinase inhibitory activity. The chemical constituent of Manilkara zapota belonging to the triterpenoids, sterols, flavonoids and phenolic groups presented tyrosinase inhibitory activity with monophenolase and diphenolase. Triterpenoids showed strong inhibitory of tyrosinase activity with monophenolase mechanism. Myricitrin which was a flavonoids glycoside from Manilkara zapota leaves extract, showed weak of tyrosinase inhibitory activity [19].

Antioxidant activity

Antioxidant activity of P.campechiana leaves extract can be seen in Table- 3 and Fig.-4. The value of antioxidant activity was presented in IC_{50} and Antioxidant Activity Index (AAI). Ascorbic acid was used as standard. DPPH used as free radical. According to Scherer,[14] if AAI < 0.5 was weak antioxidant, 0.5 - 1 medium antioxidant, 1 - 2 strong antioxidant and more than 2 classified as very strong antioxidant. The highest AAI value was in DE, followed by DEA and finally by DN extract of P. campechiana leaves. However, if classification based on the AAI value, the ethanol extract, ethyl acetate extract and n-hexane extract of P. campechiana leaves were classified as very strong antioxidant. The AAI of DE, DEA, and DN was lower than the AAI of ascorbic acid. The phenolic group can contribute to antioxidant activity.[20] The position of the OH group on flavonoids can affect the antioxidant activity. The presence of OH groups in ring B, at C-3 and ortho-hydroxy groups in ring A and B can increase antioxidant activity. In addition to the OH group, the presence of a double bond at the C2-C3 position, and the presence of a carbonyl group at C4 can also increase antioxidant activity.[16]

Table-2: IC_{50} of DPPH and IC_{50} of Tyrosinase P. campechiana leaves extract

Extract	*Density*	*IC_{50} DPPH (µg/ml)*	*IC_{50} Tyrosinase (µg/ml)*
DN	*0.673 ± 0.094*	*8.602 ± 1.226*	-
DEA	*0.882 ± 0.015*	*1.040 ± 0.101*	*828.537 ± 0.962*
DE	*0.839 ± 0.099*	*0.968 ± 0.008*	*176.553 ± 4.613*
Ascorbic acid	-	*0.570 ± 0.002*	-
Kojic acid		-	*10.513 ± 0.707*

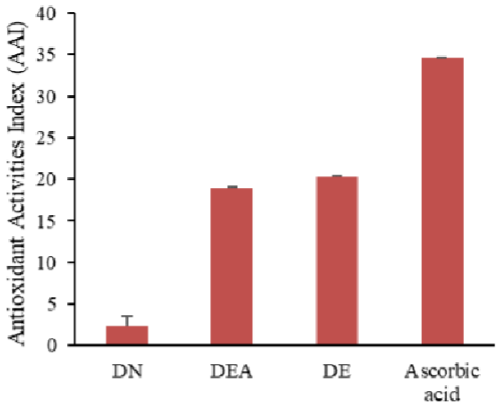

Fig.- 4: AAI of P.campechiana leaves extract

Correlation of total phenolic and flavonoid content with tyrosinase inhibitory and antioxidant activities

Total phenolic content (TPC) had a positive and significant correlation with AAI DPPH. The TPC also gave negative and significant correlation with IC_{50} tyrosinase (Table-3). This mean that when TPC increase, the AAI DPPH increase which showed high antioxidant activity. Increasing in TPC will correlate with decreasing in IC_{50} of tyrosinase, which presented high tyrosinase inhibitory activity. This correlation result was in line with the results of the study which showed that ethanolic extract had the higher TPC than the ethyl acetate and n-hexane extracts.

Table-3: Correlation of phytochemical content with tyrosinase inhibitory and antioxidant activities

	Pearson's correlation coefficient (r)	
Activity parameter	*TPC*	*TFC*
AAI	*0.981***	*-0.978***
IC_{50} tyrosinase	*-1.00***	*0.999***

Identification of marker compound

Marker compound was used to control the consistency of crude drug, extract or product of the extract. The results of the identification of compounds in the ethanol and ethyl acetate extracts by TLC co-chromatography (Fig.-5) showed that there were spots of DE (ethanol extract) and DEA (ethyl acetate extract) which had the same Rf value with quercetin (0.92), quercitrin (0.38) and gallic acid (0.48). The retention time of one peak in DE and DEA chromatograms coincided with the retention time on the quercitrin. Based on the HPLC chromatogram, quercitrin was dominant compound in DE and DEA. Then quercitrin was stated as marker compound in P.campechiana leaves extract. The HPLC chromatogram of DE and DEA of P.campechiana leaves and quercitrin standard was displayed in Fig.-6. It confirmed the identification of marker compound of DE and DEA by TLC.

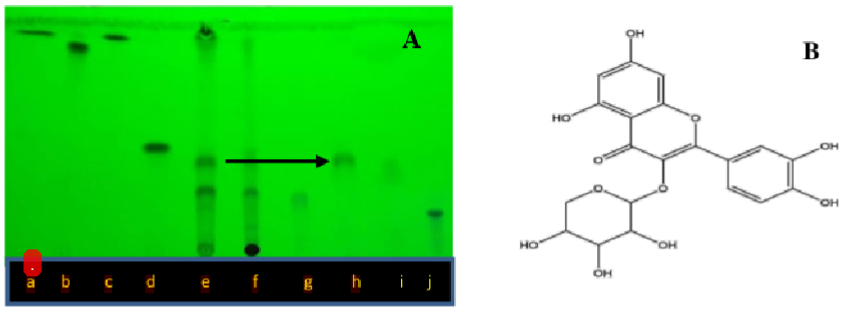

Fig.-5: A. TLC co-chromatography of DE and DEA with standard compounds: a. kaempferol, b. quercetin, c. apigenin, d. gallic acid, e. DEA, f. DE, g. chlorogenic acid, h. quercitrin, i. luteolin 7-O glycoside, j. rutin, monitoring under UV light λ 254 nm; B. quercitrin structure

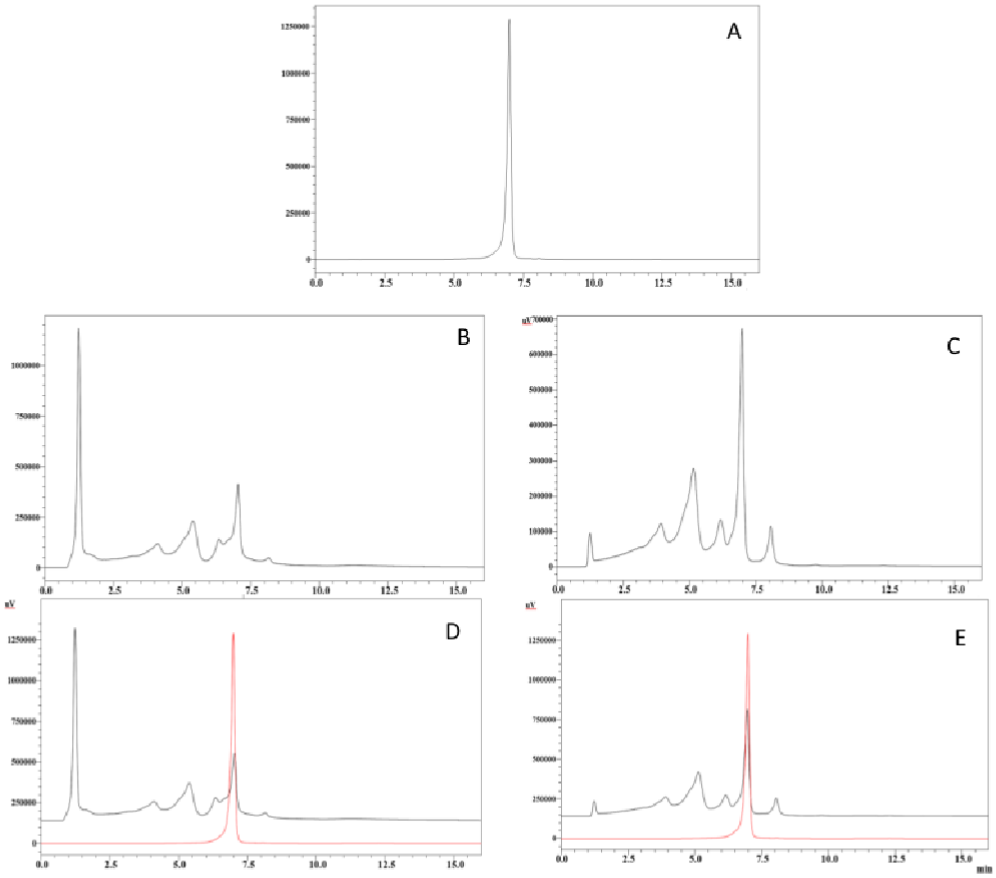

Fig.-6: A. Chromatogram HPLC of quercitrin; B. DE; C. DEA; D. overlay quercitrin with DE; E. overlay quercitrin with DEA

Marker compound content in P. campechiana leaves extract

Determination of marker compound content in DE and DEA were used one point method and the result showed in Table-5. The content of quercitrin in DEA was higher than in DE. Quercitrin is a compound that belongs to the flavonoid glycoside group. The benefit of quercitrin include being able to act as an antioxidant, antibacterial [21] and can protect the skin from exposure to UV B rays [22].

Table-5: Quercitrin content in ethanol and ethyl acetate extract of P.campechiana leaves

Sample	*Concentration*	*Retention time (min)*	*AUC*	*Quercitrin content*
Quercitrin	*500 µg/ml*	*6.998*	*15032928*	-
DE	*50000 µg/ml*	*6.930*	*2295223*	*0.153%*
DEA	*10000 µg/ml*	*6.972*	*10639291*	*3.539%*

CONCLUSION

Pouteria campechiana leaves extract had a potential as an antioxidant and tyrosinase inhibitor. Ethanol extract of P.campechiana leaves had the highest potential for tyrosinase inhibitory and antioxidant activities. The highest total phenolic content was in the ethanol extract and the highest total flavonoid content was in the ethyl acetate extract of P.campechiana leaves. Total phenolic content have a correlation with tyrosinase inhibitory and antioxidant activities. Quercitrin was chosen as a marker compound from P.campechiana leaves extract and the quercitrin content in the ethyl acetate extract was higher than in ethanol extract. Subsequent research will isolate compounds that can contribute to tyrosinase inhibitory and antioxidant activities.

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