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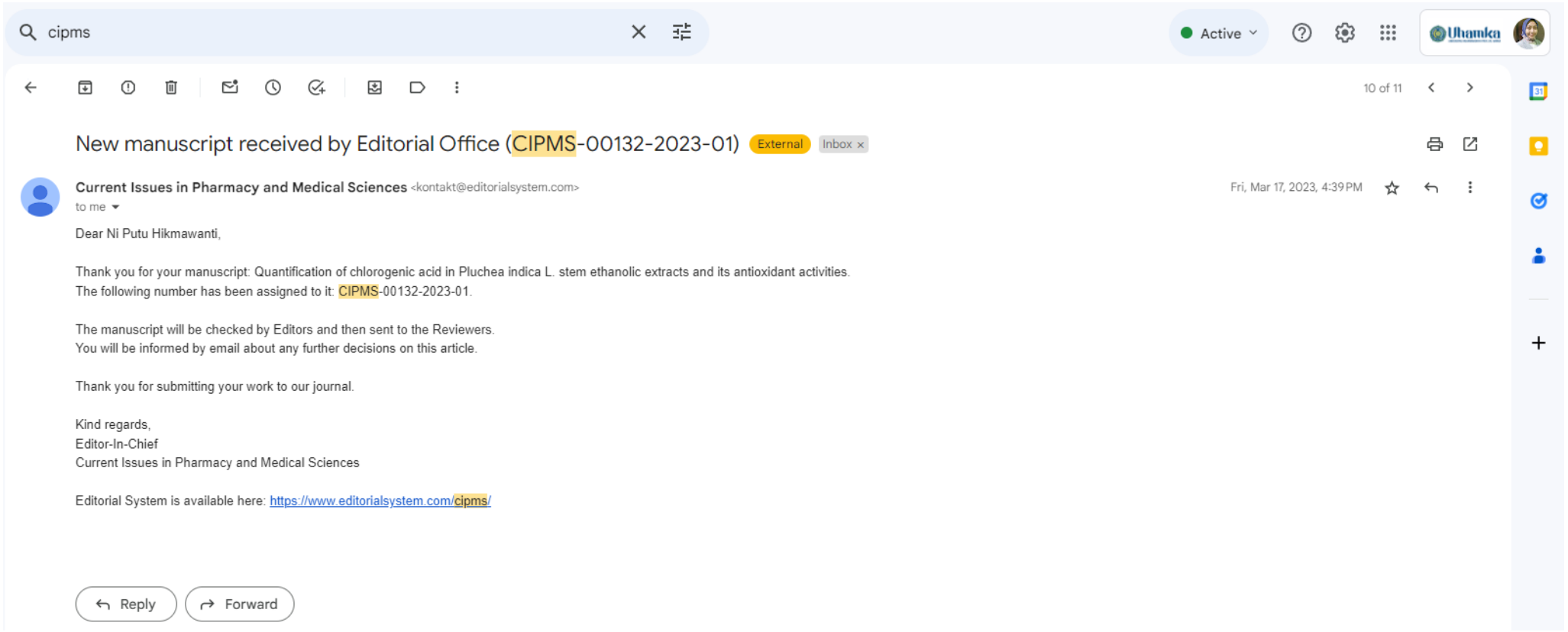
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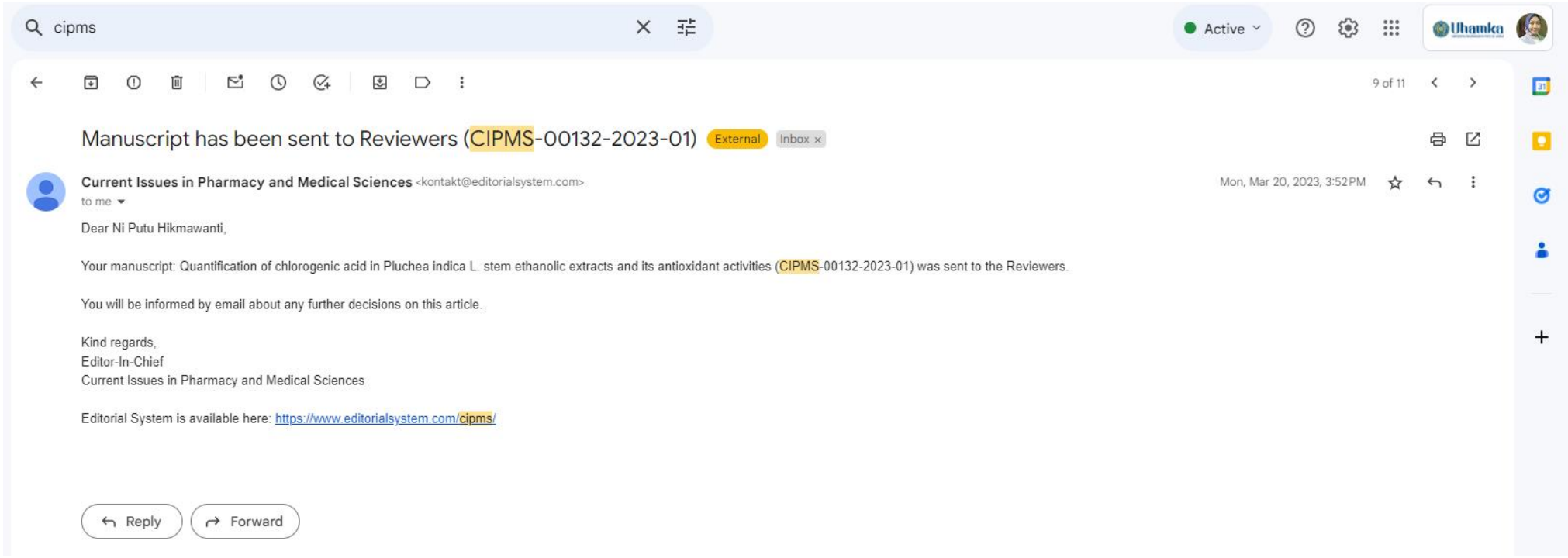
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Quantification of chlorogenic acid in Pluchea indica L. stem ethanolic extracts and its antioxidant activities

Keywords (in English)
Beluntas, Chlorogenic acid, DPPH, Ultrasonic-assisted extraction, Sequential extraction

Abstract (in English)
Chlorogenic acid (CA) is an important phenolic acid antioxidant. It is found in Pluchea indica L. (Asteraceae). It has been extensively studied in the leaves, while studies on the stems have not been reported. This study aimed to identify and measure the levels of CA in the stem extract of P. indica. The extract was also determined for its antioxidant activities. P. indica stems powder was extracted using the UAE technique using 50% ethanol as solvent directly and sequentially. The extract was then measured for total phenolic content (TPC) and CA content using RP-HPLC. Meanwhile, antioxidant activities were determined by the DPPH, ABTS, and reducing power (RP) methods. TPC in the sequential and the direct of P. indica stems ethanol extracts were 1.4694±0.0228 and 1.9314±0.0318 mgGAE/g DW, respectively. Nevertheless, the CA content of 50% ethanol extract of P. indica stems from sequential extraction (0.2045±0.0128 %, w/w) was higher than 50% ethanol extract from direct extraction (0.1984±0.0113 %, w/w). The two extracts demonstrated good antioxidant capacity, while the ethyl acetate and n-hexane extracts did not. Identifying of the other types of antioxidants phenolics using other methods of these extracts still needs to be studied further.

Quantification of chlorogenic acid in *Pluchea indica* L. stem ethanolic extracts and its antioxidant activities

ABSTRACT

Chlorogenic acid (CA) is an important phenolic acid antioxidant. It is found in *Pluchea indica* L. (Asteraceae). It has been extensively studied in the leaves, while studies on the stems have not been reported. This study aimed to identify and measure the levels of CA in the stem extract of *P. indica*. The extract was also determined for its antioxidant activities. *P. indica* stems powder was extracted using the UAE technique using 50% ethanol as solvent directly and sequentially. The extract was then measured for total phenolic content (TPC) and CA content using RP-HPLC. Meanwhile, antioxidant activities were determined by the DPPH, ABTS, and reducing power (RP) methods. TPC in the sequential and the direct of *P. indica* stems ethanol extracts were 1.4694±0.0228 and 1.9314±0.0318 mgGAE/g DW, respectively. Nevertheless, the CA content of 50% ethanol extract of *P. indica* stems from sequential extraction (0.2045±0.0128 %, w/w) was higher than 50% ethanol extract from direct extraction (0.1984±0.0113 %, w/w). The two extracts demonstrated good antioxidant capacity, while the ethyl acetate and *n*-hexane extracts did not. Identifying of the other types of antioxidants phenolics using other methods of these extracts still needs to be studied further.

Key-words: Beluntas; Chlorogenic acid; DPPH; Ultrasonic-assisted extraction; Sequential extraction

INTRODUCTION

CA is an important phenolic derivative of caffeoylquinic acid (CQA). CA is applicable as an antioxidant, anti-inflammatory, antidiabetic, anti-obesity, anti-hypertensive, antimicrobial, etc.(1). CA can be extracted from plant materials using ultrasound-assisted extraction (UAE)(2). CA can be analyzed qualitatively or quantitatively using *High-performance liquid chromatography* (HPLC) (3) with a C18 column with a UV or DAD detector (3).

CA is found in many plants(2). One source of CA from plants is *Pluchea indica* L. (Asteraceae), especially the leaves(4–7). The *P. indica* leaves were more explored than other plant parts. *P. indica* stems contained a relatively high total phenolic content after leaves in 50% ethanol extract compared to flowers and roots (8). The importance of CA compounds as a source of antioxidants that can reduce free radicals needs to be explored. The quantification of CA in 50%-ethanol extract of *P. indica* stems has never been reported. In the present study, quantification of the compound in *P. indica* stems was carried out on the ethanol extract (obtained directly and sequentially) using RP-HPLC analysis. In addition, the antioxidant activities of the extracts were also studied.

MATERIALS AND METHODS

1. Chemicals

CA and gallic acid as reference were purchased from MarkHerb, Institut Teknologi Bandung (ITB), Bandung, Indonesia. Ethanol, ethyl acetate, and *n*-hexane (PT. Brataco, Indonesia) were used as extraction solvents. Methanol, acetic acid, and water for HPLC grade (Merck, Germany) were used for HPLC analysis.

2. Plant material

P. indica stems were collected on November 2021 from Biopharmaceutical Cultivation Conservation Unit, Tropical Biopharmaca Research Center (TropBRC), Institut Pertanian Bogor (IPB), Bogor, Indonesia, with voucher number: BMK0188092016. The stems are cleaned and air-dried. The dried stems are then powdered and stored in a dry container protected from light.

3. Extraction

Extraction is divided into two ways, namely direct extraction and sequential extraction. Each five g of stem-dried powder was extracted with 50 mL of solvent. In summary, the extraction procedure is presented in **Fig. 1**. The extraction process was carried out using the UAE technique following the protocol in Kongkiatpaiboon et al., (2018)(5). Extraction was performed

in an ultrasonic bath (Branson 5510) 40 kHz at 40 °C for 15 min. The filtrate is separated from the residue by filtration. The residue was re-extracted using a new solvent 3 times. Each filtrate was collected and made up to 50 mL, after this referred to as liquid extract. Extraction of each sample was carried out for 5 replications.

4. Polyphenol Screening

Screening of phenolic compounds was carried out qualitatively using a 5% $FeCl_3$ reagent. The positive result of the reaction is indicated by the formation of a blue-black colour(9).

5. Total Phenolic Content Assay

Colorimetry was used to determine total phenolic content following the Hikmawanti et al., (2020)(10) procedure with slight modifications. Gallic acid was used as a standard. For calibration, the gallic acid solution was prepared with variation concentrations: 1.1×10^{-2}, 1.7×10^{-2}, 2.3×10^{-2}, 2.9×10^{-2}, and 3.5×10^{-2} mg/mL. Each extract was diluted 20 times using ethanol. Both extract and standard (0.3 mL) were mixed with 1.5 mL of the Folin-ciocalteu (1:10 in water) and left for 3 min. After that, the mixture was added with 1.2 mL of sodium carbonate solution (7.5%), homogenized, and then incubated at room temperature for 110 min. The absorbance was measured at 765.10 nm with a spectrophotometer UV-Vis (UV-1900i Series, Shimadzu, Kyoto, Japan). The total phenolic content was expressed as gallic acid equivalent (mg GAE/g of the dried sample). The test was carried out triplets (mean ± SD).

6. HPLC condition for analysis of CA

CA analysis was carried out following the protocol of Kongkiatpaiboon et al., (2018)(5) with slight modification at The Advanced Characterization Laboratories Cibinong–Integrated Laboratory of Bioproduct, National Research and Innovation Agency (BRIN), Indonesia. CA (as an external standard) was prepared at a concentration of 10 mg/mL in ethanol. The standard solution was then diluted to 1.0, 2.0, 6.0, and 7.5 mg/mL. Each solution was filtered using a 0.45 μm filter and injected into the HPLC system. The injection volume of the sample was 5 μL. The HPLC system (Shimadzu Prominence-i LC-2030C 3D Plus), which includes a

quaternary pump, a degasser, and an autosampler, is combined with a photodiode array (PDA) detector in the HPLC apparatus. Separation system with HPLC was performed using Shim Pack GIST C18 column (150 mmL. x 4.6 mm I.D., 4 μm) as the stationary phase at 25 °C. Acetic Acid 0,5% in Water (A) and methanol (B) was used as a mobile phase by gradient elution (as stated in Kongkiatpaiboon et al., (2018)(5)). The flow rate was 1.0 mL/min. Separation was monitored at 326 nm.

7. DPPH assay

A 0.1 mM DPPH solution was prepared in methanol. Quercetin (1×10^2 mg/mL) in methanol was used as a reference. Initially, each sample of both extract and quercetin (1 mL) was separately reacted with 0.1 mM DPPH (4 mL). After incubation for 30 min at room temperature in the dark, the reaction of the mixture was measured at 516 nm against an ethanol blank using a UV-Vis spectrophotometer (UV-1900 Series, Shimadzu, Kyoto, Japan)(11).

8. ABTS assay

Antioxidant activity assay against ABTS radicals was carried out by microtiter assay. As much as 180 µl of ABTS radical ($ABTS^{\bullet+}$), prepared 12-16 h beforehand, was reacted with 20 µl of the extract test solution in a 96-well flat-bottom microplate. After 5 min of incubation, the absorbance was measured at 750 nm using an iMark microplate reader (BioRad, Shanghai, China) (12). Trolox was used as a reference (0.1-0.8 mmol/L). The results are expressed as μmol Trolox equivalent per g dry weight sample (μmol TE/g DW).

9. RP assay

The ability of antioxidants to reduce iron was determined by reducing power assay following the protocol of Khatua et al. (2017) (12). The tests were carried out in 96-well flat-bottom microplates. A total of 10 µl of the extract test solution was reacted with 25 µl of 0.2 M phosphate buffer (pH 6.6), and 25 µl of potassium ferricyanide (1%, w/v). After 20 min incubated (at room temperature), 25 μl of TCA 10% (w/v) was added. Then, the mixture was added with 85 μl of distilled water and 8.5 μl of Iron (III) chloride. The mixture was shaken for

10 s at medium speed, then incubated at room temperature for 15 min. The absorbance was measured at 750 nm using the iMark microplate reader (BioRad, Shanghai, China) (12). Trolox was used as a reference (0.1-0.8 mmol/L). The results are expressed as μmol Trolox equivalent per g dry weight sample (μmol TE/g DW).

RESULTS

Based on the results, the 50%-ethanol extracts of *P. indica* stems, both obtained from direct and sequential extraction, were detected to contain phenolics based on the test with a 5% $FeCl_3$ reagent (**Fig. 2**). The two extracts were then analysed for the total phenolics content using a UV-Visible spectrophotometer. Total phenolics content in the sequential and the direct *P. indica* stems ethanol extracts were 1.4694±0.0228 and 1.9314±0.0318 mgGAE/g dried powder, respectively.

Analysis of CA in ethanol extracts of *P. indica* stems was performed using RP-HPLC at 326 nm as the maximum wavelength (5). **Fig. 3** shows the HPLC chromatograms of the standard CA (A, retention time = 23.042 min), the sequential-ethanol extract (B), and the direct-ethanol extract (C) of *P. indica* stems. The chromatogram showed the presence of CA in the sequential-ethanol extract and the direct-ethanol extract of *P. indica* stems at retention times of 22.702 dan 22.946 min, respectively. The equation of the line obtained from the CA calibration curve is y=1628x–3E+06 (R^2 = 0.9991). The quantification of CA in the sequential-ethanol extract and the direct-ethanol extract of *P. indica* stems were 0.2045±0.0128 and 0.1984±0.0113% (w/w), respectively.

The antioxidant activity of the stem extract of *P. indica* was tested using the DPPH, ABTS, and RP methods. **Tab. 1** shows the summary of the antioxidant activity of the extracts. The two ethanol extracts (direct and sequential) provide an overview of the activity of reducing radicals (both DPPH• and ABTS•+) and metal ions (through iron reduction). Meanwhile, the ethyl acetate and *n*-hexane extracts did not provide an overview of antioxidant capacity.

DISCUSSION

Extraction is the initial step in separating metabolites from the plant matrix for identification (13). In this study, phenolic extraction was performed using 50% ethanol solvent, directly and sequentially, by the UAE technique. Direct extraction allows metabolites with a polarity corresponding to 50% ethanol to be extracted in it (crude), so the concentration of the target compound in this extract is lower. Meanwhile, through sequential extraction, it is expected that other compounds extracted sequentially in *n*-hexane and ethyl acetate are not found in the 50% ethanol extract. With the elimination of these compounds (such as components of fat, chlorophyll, etc.), it is hoped that phenolics (especially CA) can be extracted well in 50% ethanol solvent (14).

According to Kongkiatpaiboon et al. (2018), to ensure the quality of *P. indica* extract and its nutritional components, the HPLC technique might be used for routine analysis(5). This method is proven to be fast, precise, and accurate. In the study, it was reported that CA, as one of the phenolics of *P. indica* leaves, can be extracted well using 50% ethanol solvent with UAE. Meanwhile, in this study, CA was detected and quantified in 50% ethanol extract (both obtained from direct and sequential extraction). Compared with the levels of CA in *P. indica* leaves in the study of Kongkiatpaiboon et al. (2018)(5), the content of this compound in the ethanol extract of *P. indica* stems was lower. The levels of CA in *P. indica* leaves were in the range of 2.67% (w/w), while in stem extracts were in the range of 0.19-0.2% (w/w). CA is a compound biosynthesized through the shikimate pathway in plants from various families, including Asteraceae (15). It is accumulated in leaves, stems, roots, fruits, and other organs at different levels (16).

In this study, the high phenolic content in the extract was linear with its antiradical DPPH activity. The direct 50% ethanol extract had high phenolic content and the DPPH radical scavenging activity better than other extracts. The extract had a lower CA content than the sequential 50% ethanol extract. Thus, it is suspected that CA is not the only phenolic in *P. indica* stems that plays a role in scavenging DPPH radicals. The presence of other phenolic

compounds, or other natural compounds that are synergistic in the 50% ethanol extract, is suspected of playing a role in providing better antiradical activity than the graded 50% ethanol extract. Different things happened in the ABTS and RP tests. In both tests, 50%-ethanol (sequential) gave better antioxidant activity.

There are several chemical approaches for measuring plant antioxidant capacity. The antioxidant chemical-based assay's mode of action, namely electron transfer, hydrogen transfer, and metal reduction (17). The DPPH and ABTS methods are included in reactions based on electron and hydrogen transfer. The DPPH technique is an in vitro approach that is simple, inexpensive, and frequently used to assess the antioxidant activity of natural materials (18). This method describes the antiradical activity of the sample more than as an antioxidant activity. Antiradical activity refers to a compound's capacity to react with a free radical, whereas antioxidant activity refers to a compound's ability to block the oxidation process (19). Meanwhile, ABTS is capable of measuring the capacity of both lipophilic and hydrophilic antioxidants. ABTS radicals are more reactive than DPPH radicals. The chemical interaction between antioxidants and ABTS radicals is relatively quick (20). Another method, RP, is based on an increase in absorbance from the reaction between the antioxidant and a mixture of potassium ferricyanide, trichloro acetic acid, and ferric chloride reagents, which demonstrating the strength of antioxidants in reducing metals (18). However, measuring antioxidant activity in vitro has limitations since DPPH and ABTS are not naturally occurring oxidants in the body. The radical quenching reactions that occur in these assays are often quick, unlike in the occurrence of a slower reaction in the body. In addition, whereas antioxidants have significant antioxidant effects in chemical tests, they can function as pro-oxidants in dietary and biological systems (17).

The variety of phenolic types and the complexity of the composition of these chemicals in plants make the separation of antioxidants phenolic inefficient and expensive (14). Other compounds that can also act as antioxidants are phytosterols (21), flavonoids, minerals, organosulfur, vitamins, carotenoids, etc.(22). The crude often provides more significant

antioxidant benefits than the individual forms. In addition, the measurement of antioxidant activity with specific methods was only carried out under adjusted conditions. Determining the overall antioxidant capacity of the sample should also be done using additional techniques, exceptionally those capable of evaluating the efficiency of antioxidants against specific ROS/RNS (14). Studying the types of other phenolic compounds in *P. indica* stems is still possible. Thus, the use of *P. indica* stems as a source of antioxidants can continue to be developed, for example, in the therapy of metabolic syndrome, as a preservative, and as natural food supplements.

CONCLUSIONS

Based on this research, CA in *P. indica* stems can be extracted well using 50% ethanol solvent either by direct or sequential extraction. The sequential-ethanol extract contains more CA than the ethanol extract obtained from direct extraction. Meanwhile, more phenolic content was found in extracts produced from direct extraction. The extracts also provided better antioxidant activity. It is still important to search for phenolic substances besides CA, which functions as an antioxidant in *P. indica* stems.

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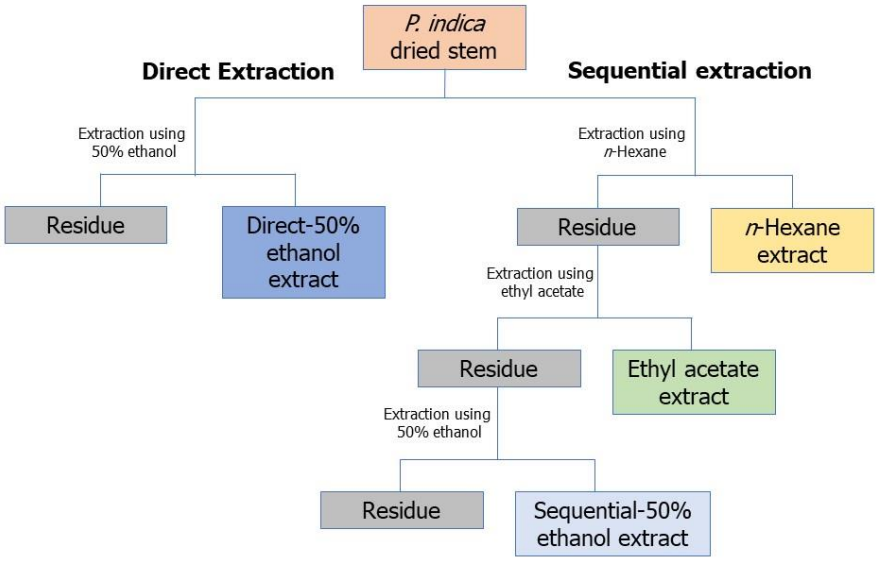
Tab. 1. Antioxidant activity from *P. indica* stem extracts.

Samples	Antioxidant activities		
	DPPH (mg QE/100 g DW)	ABTS (μmol TE/g DW)	RP (μmol TE/g DW)
50%-ethanol extract (direct)	189.210±0.01	17.624±0.345	40.069±0.214
50%-ethanol extract (sequential)	143.198±0.02	34.837±0.126	42.854±0.909
Ethyl acetate extract (sequential)	1.209±0.05	nd	nd
n-hexane extract (sequential)	0.597±0.03	nd	nd

Note: DW = dry weight; nd = not detected; QE = quercetin equivalent; TE = Trolox equivalent

277 ## Figure Legends



278 **Fig. 1**. Extraction procedures of *P. indica* dried stem

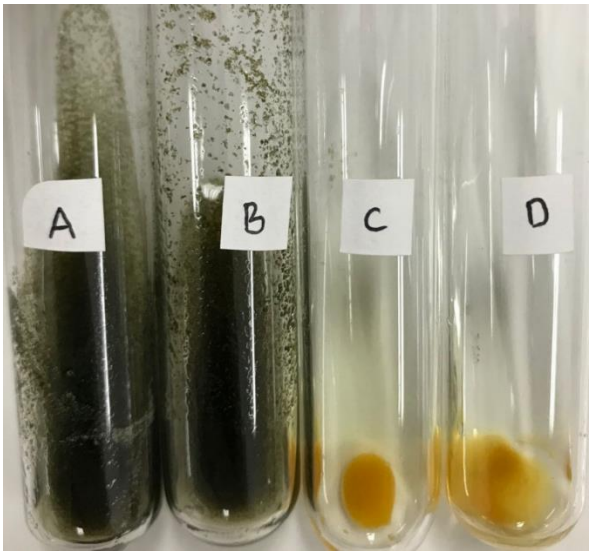


Fig. 2. The results of phenolic compounds screening in *P. indica* stem extracts with 5% $FeCl_3$ reagent. A = 50%-ethanol extract (direct); B = 50%-ethanol extract (sequential); C = ethyl acetate extract (sequential); D = *n*-hexane extract (sequential)

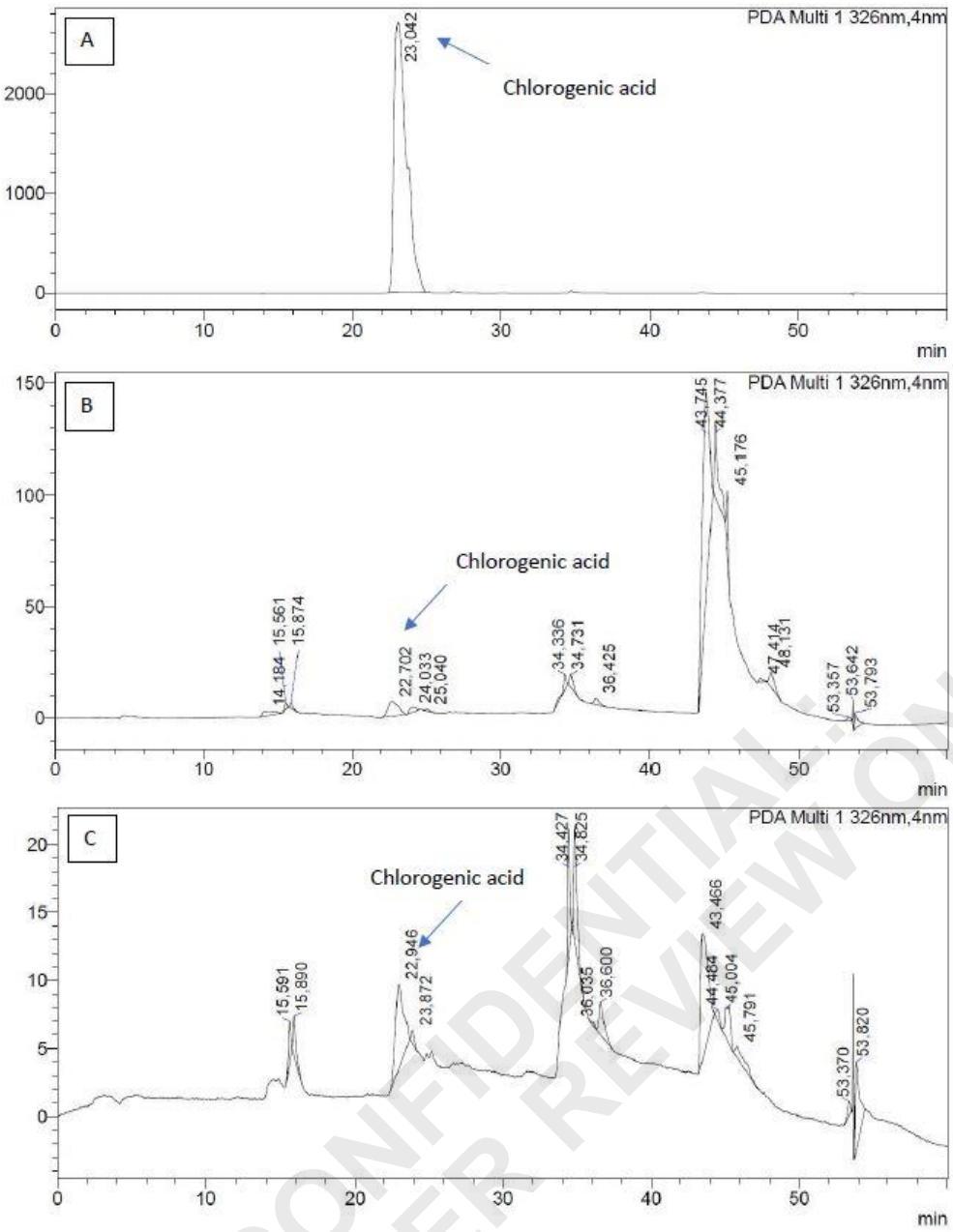


Fig. 3. RP-HPLC Chromatogram of chlorogenic acid (as a standard) and ethanolic extracts of *P. indica* stem. A = chlorogenic acid; B = 50%-ethanol extract (sequential); C = 50%-ethanol extract (direct)

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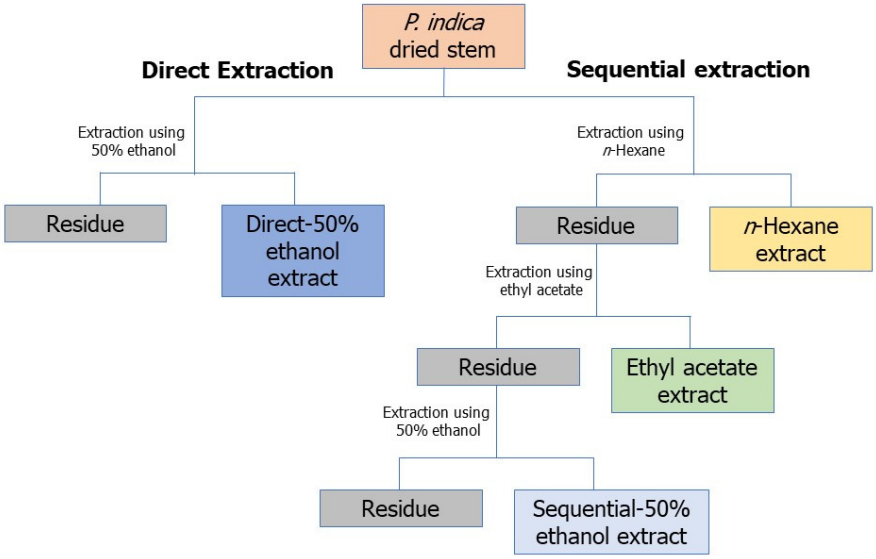
n-hexane extract (sequential)	0.597±0.03	nd	nd

Note: DW = dry weight; nd = not detected; QE = quercetin equivalent; TE = Trolox equivalent



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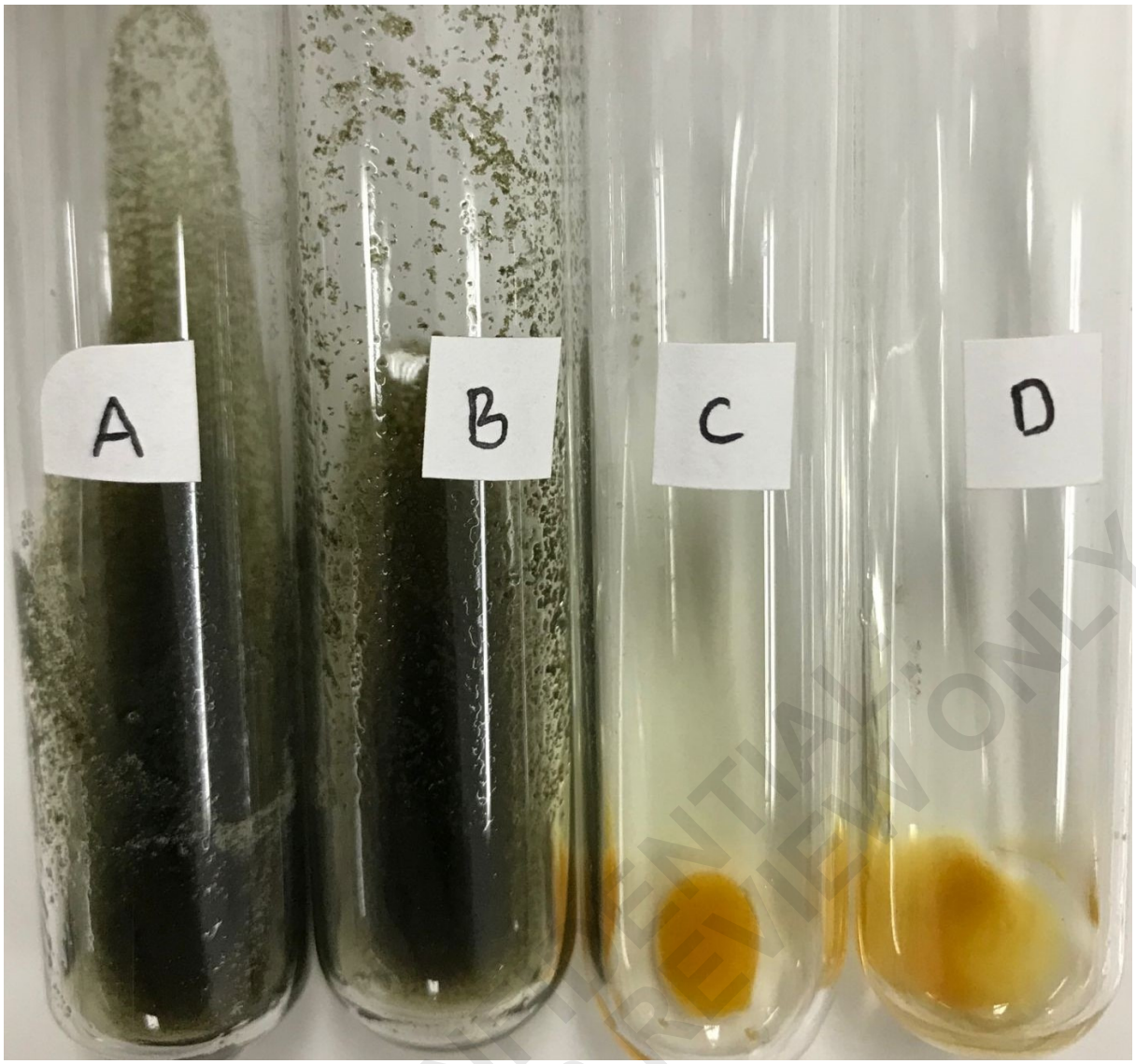

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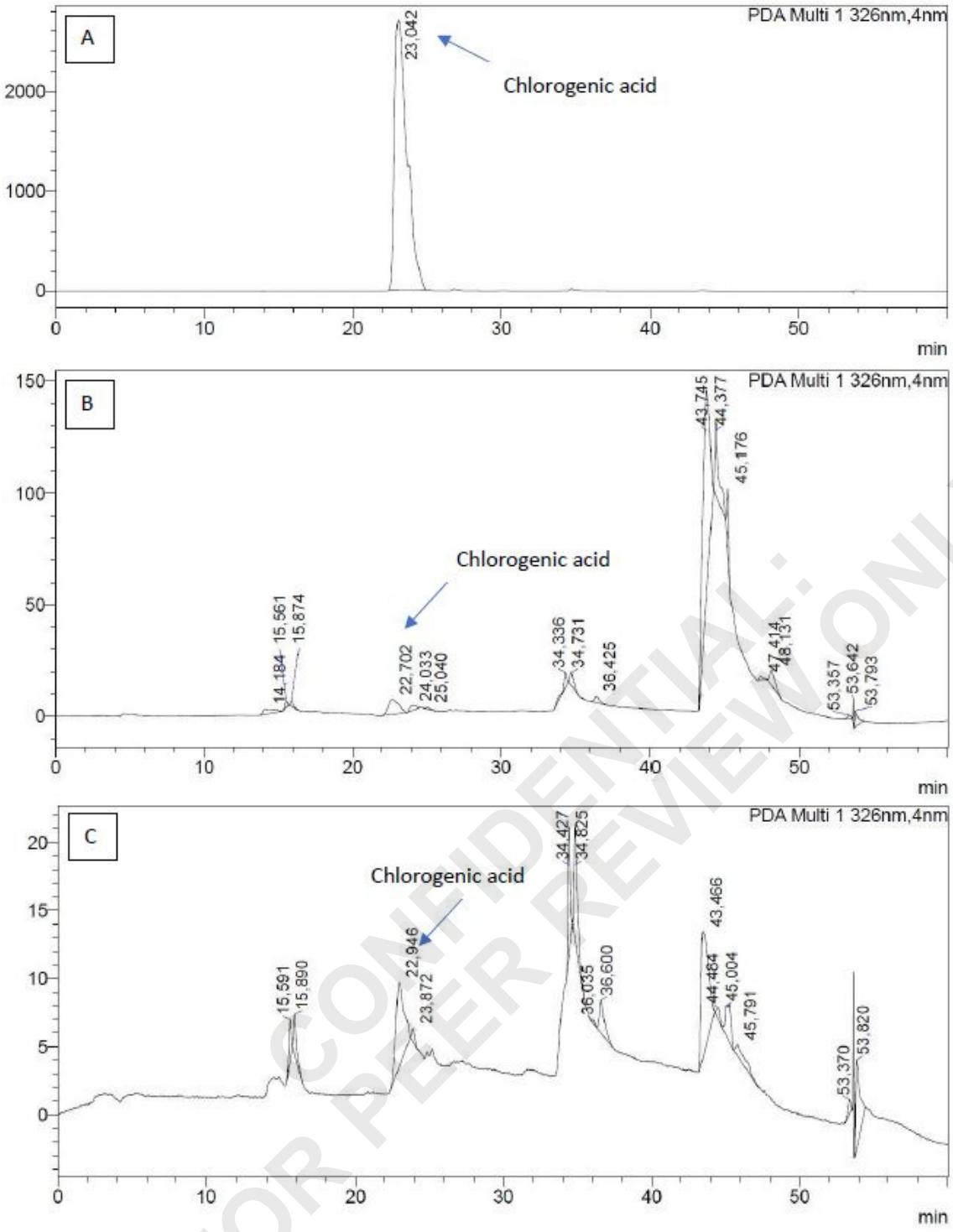

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Fig. 1. Extraction procedures of P. indica dried stem

Figure 2 - Download source file (2.19 MB)

Fig. 2. The results of phenolic compounds screening in P. indica stem extracts with 5% FeCl3 reagent. A = 50%-ethanol extract (direct); B = 50%-ethanol extract (sequential); C = ethyl acetate extract (sequential); D = n-hexane extract (sequential)

Figure 3 - Download source file (220.76 kB)

Fig. 3. RP-HPLC Chromatogram of chlorogenic acid (as a standard) and ethanolic extracts of P. indica stem. A = chlorogenic acid; B = 50%-ethanol extract (sequential); C = 50%-ethanol extract (direct)

2. Bukti hasil *review* dan file komentar *reviewer* (28 Agustus 2023)

Decision: **accept after changes suggested by reviewer**

August 28, 2023
CIPMS-00132-2023-01
Quantification of chlorogenic acid in Pluchea indica L. stem ethanolic extracts and its antioxidant activities

Dear Ni Putu Hikmawanti,

I am pleased to inform you that your manuscript, entitled: Quantification of chlorogenic acid in Pluchea indica L. stem ethanolic extracts and its antioxidant activities, might be accepted for publication in our journal, pending some minor changes suggested by reviewers (see below).

Please revise your paper strictly according to the attached Reviewers' comments. Your manuscript won't be taken into consideration without the revisions made according to the recommendations.

Authors are requested to prepare a revised version of their manuscript as soon as possible. This may ensure fast publication if an article is finally accepted.

Thank you for submitting your work to us.

Kind regards,
Editor-In-Chief
Current Issues in Pharmacy and Medical Sciences

Review 1:

Good

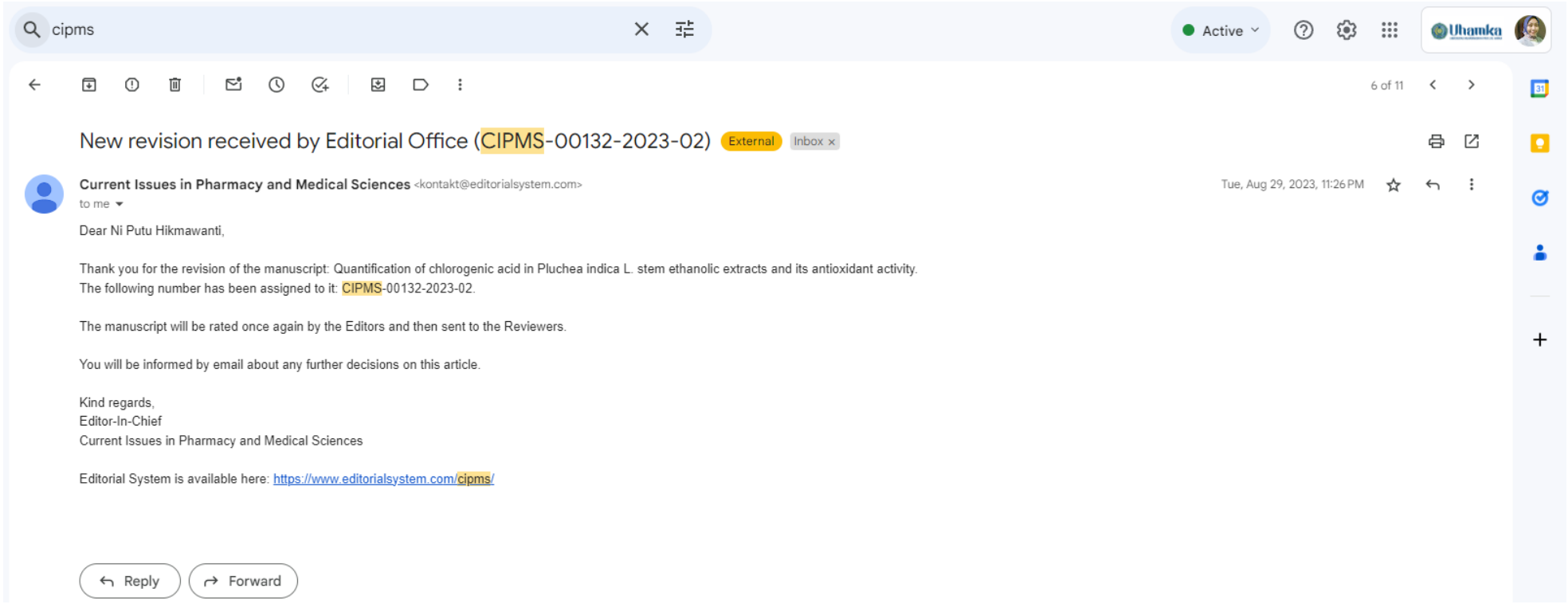
Review 2:

1. Title: How many antioxidant activities are in CA? if one, pls change it to “antioxidant activity”.
2. Abstract: provide the full form of UAE.
3. The introduction part is not compact enough. The authors have to include the chemical structure of CA and elaborate on the functional groups responsible for its activity. The introduction will become even more informative and engaging, preparing readers for the detailed research and findings presented in the subsequent sections.
4. In the discussion part: The authors have to provide an explanation based on chemistry about why the 50% ethanol extract has the highest concentration of CA than the others.

5. In section 7. DPPH assay: Please indicate on what basis was the ethanol selected as a blank while the DPPH was initially prepared in methanol ??.
6. HPLC method needs more explanation such as :
-At what ratio of the mobile phase you have got the best HPLC chromatogram? Provide such details.

-Pls specify at what concentration the chromatogram of the CA standard (Fig. 3A) is displayed.

3. Bukti hasil revisi dan artikel yang direvisi (29 Agustus 2023)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New revision received by Editorial Office (CIPMS-00132-2023-02)
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Tue, Aug 29, 2023, 11:26 PM
Dear Ni Putu Hikmawanti,
Thank you for the revision of the manuscript: Quantification of chlorogenic acid in Pluchea indica L. stem ethanolic extracts and its antioxidant activity.
The following number has been assigned to it: CIPMS-00132-2023-02.
The manuscript will be rated once again by the Editors and then sent to the Reviewers.
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Comments for reviewers

Title: Quantification of chlorogenic acid in *Pluchea indica* L. stem ethanolic extracts and its antioxidant activity

Comments of Reviewers	***Comments of Author***
1. Title: How many antioxidant activities are in CA? if one, pls change it to "antioxidant activity".	has been revised.
2. Abstract: provide the full form of UAE.	has been revised.
3. The introduction part is not compact enough. The authors have to include the chemical structure of CA and elaborate on the functional groups responsible for its activity. The introduction will become even more informative and engaging, preparing readers for the detailed research and findings presented in the subsequent sections.	has been added to the introduction. Chlorogenic acid (CA) is an important phenolic derivative of caffeoylquinic acid (CQA). In chemical structure, CA (or 5-CQA) consists of a quinic acid core which is acylated with one moiety of caffeic acid at the C-5 (1) (**Fig. 1**). The caffeic acid group in CA plays critical role in its activity as an antioxidant. According to Kritsi et al. (2022), CA has antioxidant activity through the interaction of hydrogen bonds on the receptor binding sites of NADPH oxidase, cytochrome P450, and myeloperoxidase (2).
4. In the discussion part: The authors have to provide an explanation based on chemistry about why the 50% ethanol extract has the highest concentration of CA than the others.	has been added In general, CA is soluble in low concentrations of alcohol or alcohol-water mixtures. This compound is insoluble in non-polar solvents, such as benzene, chloroform, or ether. The high solubility in an alcohol-water mixture is related to the large number of free hydroxyl groups (18).
5. In section 7. DPPH assay: Please indicate on what basis was the ethanol selected as a blank while the DPPH was initially prepared in methanol ??.	**Clarification:** Methanol was used as a blank because the solvent used was methanol. This was done to reduce interference during absorbance readings.
6. HPLC method needs more explanation such as : -At what ratio of the mobile phase you have got the best HPLC chromatogram? Provide such details. -Pls specify at what concentration the chromatogram of the CA standard (Fig. 3A) is displayed.	 has been added. (10% B to 50% B in A for 40 min and 100% B for 10 min) has been added.

Quantification of chlorogenic acid in *Pluchea indica* L. stem ethanolic extracts and its antioxidant activity

ABSTRACT

Chlorogenic acid (CA) is an important phenolic acid antioxidant. It is found in *Pluchea indica* L. (Asteraceae). It has been extensively studied in the leaves, while studies on the stems have not been reported. This study aimed to identify and measure the levels of CA in the stem extract of *P. indica*. The extract was also determined for its antioxidant activities. *P. indica* stems powder was extracted using the ultrasonic-assisted extraction (UAE) technique using 50% ethanol as solvent directly and sequentially. The extract was then measured for total phenolic content (TPC) and CA content using RP-HPLC. Meanwhile, antioxidant activities were determined by the DPPH, ABTS, and reducing power (RP) methods. TPC in the sequential and the direct of *P. indica* stems ethanol extracts were 1.4694±0.0228 and 1.9314±0.0318 mgGAE/g DW, respectively. Nevertheless, the CA content of 50% ethanol extract of *P. indica* stems from sequential extraction (0.2045±0.0128 %, w/w) was higher than 50% ethanol extract from direct extraction (0.1984±0.0113 %, w/w). The two extracts demonstrated good antioxidant capacity, while the ethyl acetate and *n*-hexane extracts did not. Identifying of the other types of antioxidants phenolics using other methods of these extracts still needs to be studied further.

Key-words: Beluntas; Chlorogenic acid; DPPH; Ultrasonic-assisted extraction; Sequential extraction

INTRODUCTION

Chlorogenic acid (CA) is an important phenolic derivative of caffeoylquinic acid (CQA). In chemical structure, CA (or 5-CQA) consists of a quinic acid core which is acylated with one moiety of caffeic acid at the C-5 (1) (**Fig. 1**). CA is applicable as an antioxidant, anti-inflammatory, antidiabetic, anti-obesity, anti-hypertensive, antimicrobial, etc.(1). The caffeic

acid group in CA plays critical role in its activity as an antioxidant. According to Kritsi et al. (2022), CA has antioxidant activity through the interaction of hydrogen bonds on the receptor binding sites of NADPH oxidase, cytochrome P450, and myeloperoxidase (2). CA can be extracted from plant materials using ultrasound-assisted extraction (UAE)(3). CA can be analyzed qualitatively or quantitatively using *High-performance liquid chromatography* (HPLC) (4) with a C18 column with a UV or DAD detector (4).

CA is found in many plants(3). One source of CA from plants is *Pluchea indica* L. (Asteraceae), especially the leaves(5–8). The *P. indica* leaves were more explored than other plant parts. *P. indica* stems contained a relatively high total phenolic content after leaves in 50% ethanol extract compared to flowers and roots (9). The importance of CA compounds as a source of antioxidants that can reduce free radicals needs to be explored. The quantification of CA in 50%-ethanol extract of *P. indica* stems has never been reported. In the present study, quantification of the compound in *P. indica* stems was carried out on the ethanol extract (obtained directly and sequentially) using RP-HPLC analysis. In addition, the antioxidant activities of the extracts were also studied.

MATERIALS AND METHODS

1. Chemicals

CA and gallic acid as reference were purchased from MarkHerb, Institut Teknologi Bandung (ITB), Bandung, Indonesia. Ethanol, ethyl acetate, and *n*-hexane (PT. Brataco, Indonesia) were used as extraction solvents. Methanol, acetic acid, and water for HPLC grade (Merck, Germany) were used for HPLC analysis.

2. Plant material

P. indica stems were collected on November 2021 from Biopharmaceutical Cultivation Conservation Unit, Tropical Biopharmaca Research Center (TropBRC), Institut Pertanian Bogor (IPB), Bogor, Indonesia, with voucher number: BMK0188092016. The stems are

cleaned and air-dried. The dried stems are then powdered and stored in a dry container protected from light.

3. Extraction

Extraction is divided into two ways, namely direct extraction and sequential extraction. Each five g of stem-dried powder was extracted with 50 mL of solvent. In summary, the extraction procedure is presented in **Fig. 2**. The extraction process was carried out using the UAE technique following the protocol in Kongkiatpaiboon et al., (2018)(6). Extraction was performed in an ultrasonic bath (Branson 5510) 40 kHz at 40 °C for 15 min. The filtrate is separated from the residue by filtration. The residue was re-extracted using a new solvent 3 times. Each filtrate was collected and made up to 50 mL, after this referred to as liquid extract. Extraction of each sample was carried out for 5 replications.

4. Polyphenol Screening

Screening of phenolic compounds was carried out qualitatively using a 5% $FeCl_3$ reagent. The positive result of the reaction is indicated by the formation of a blue-black colour(10).

5. Total Phenolic Content Assay

Colorimetry was used to determine total phenolic content following the Hikmawanti et al., (2020)(11) procedure with slight modifications. Gallic acid was used as a standard. For calibration, the gallic acid solution was prepared with variation concentrations: 1.1×10^{-2}, 1.7×10^{-2}, 2.3×10^{-2}, 2.9×10^{-2}, and 3.5×10^{-2} mg/mL. Each extract was diluted 20 times using ethanol. Both extract and standard (0.3 mL) were mixed with 1.5 mL of the Folin-ciocalteu (1:10 in water) and left for 3 min. After that, the mixture was added with 1.2 mL of sodium carbonate solution (7.5%), homogenized, and then incubated at room temperature for 110 min. The absorbance was measured at 765.10 nm with a spectrophotometer UV-Vis (UV-1900i Series, Shimadzu, Kyoto, Japan). The total phenolic content was expressed as gallic acid equivalent (mg GAE/g of the dried sample). The test was carried out triplets (mean ± SD).

6. HPLC condition for analysis of CA

CA analysis was carried out following the protocol of Kongkiatpaiboon et al., (2018)(6) with slight modification at The Advanced Characterization Laboratories Cibinong–Integrated Laboratory of Bioproduct, National Research and Innovation Agency (BRIN), Indonesia. CA (as an external standard) was prepared at a concentration of 10 mg/mL in ethanol. The standard solution was then diluted to 1.0, 2.0, 6.0, and 7.5 mg/mL. Each solution was filtered using a 0.45 μm filter and injected into the HPLC system. The injection volume of the sample was 5 μL. The HPLC system (Shimadzu Prominence-i LC-2030C 3D Plus), which includes a quaternary pump, a degasser, and an autosampler, is combined with a photodiode array (PDA) detector in the HPLC apparatus. Separation system with HPLC was performed using Shim Pack GIST C18 column (150 mmL. x 4.6 mm I.D., 4 μm) as the stationary phase at 25 °C. Acetic acid 0,5% in water (A) and methanol (B) was used as a mobile phase by gradient elution (10% B to 50% B in A for 40 min and 100% B for 10 min) as stated in Kongkiatpaiboon et al., (2018)(6). The flow rate was 1.0 mL/min. Separation was monitored at 326 nm.

7. DPPH assay

A 0.1 mM DPPH solution was prepared in methanol. Quercetin (1×10^2 mg/mL) in methanol was used as a reference. Initially, each sample of both extract and quercetin (1 mL) was separately reacted with 0.1 mM DPPH (4 mL). After incubation for 30 min at room temperature in the dark, the reaction of the mixture was measured at 516 nm against a methanol blank using a UV-Vis spectrophotometer (UV-1900 Series, Shimadzu, Kyoto, Japan)(12).

8. ABTS assay

Antioxidant activity assay against ABTS radicals was carried out by microtiter assay. As much as 180 μl of ABTS radical ($ABTS^{\bullet+}$), prepared 12-16 h beforehand, was reacted with 20 μl of the extract test solution in a 96-well flat-bottom microplate. After 5 min of incubation, the absorbance was measured at 750 nm using an iMark microplate reader (BioRad, Shanghai, China) (13). Trolox was used as a reference (0.1-0.8 mmol/L). The results are expressed as μmol Trolox equivalent per g dry weight sample (μmol TE/g DW).

9. RP assay

The ability of antioxidants to reduce iron was determined by reducing power assay following the protocol of Khatua et al. (2017) (13). The tests were carried out in 96-well flat-bottom microplates. A total of 10 µl of the extract test solution was reacted with 25 µl of 0.2 M phosphate buffer (pH 6.6), and 25 µl of potassium ferricyanide (1%, w/v). After 20 min incubated (at room temperature), 25 µl of TCA 10% (w/v) was added. Then, the mixture was added with 85 µl of distilled water and 8.5 µl of Iron (III) chloride. The mixture was shaken for 10 s at medium speed, then incubated at room temperature for 15 min. The absorbance was measured at 750 nm using the iMark microplate reader (BioRad, Shanghai, China) (13). Trolox was used as a reference (0.1-0.8 mmol/L). The results are expressed as µmol Trolox equivalent per g dry weight sample (µmol TE/g DW).

RESULTS

Based on the results, the 50%-ethanol extracts of *P. indica* stems, both obtained from direct and sequential extraction, were detected to contain phenolics based on the test with a 5% $FeCl_3$ reagent (**Fig. 3**). The two extracts were then analysed for the total phenolics content using a UV-Visible spectrophotometer. Total phenolics content in the sequential and the direct *P. indica* stems ethanol extracts were 1.4694±0.0228 and 1.9314±0.0318 mgGAE/g dried powder, respectively.

Analysis of CA in ethanol extracts of *P. indica* stems was performed using RP-HPLC at 326 nm as the maximum wavelength (6). **Fig. 4** shows the HPLC chromatograms of the standard CA (A, retention time = 23.042 min), the sequential-ethanol extract (B), and the direct-ethanol extract (C) of *P. indica* stems. The chromatogram showed the presence of CA in the sequential-ethanol extract and the direct-ethanol extract of *P. indica* stems at retention times of 22.702 dan 22.946 min, respectively. The equation of the line obtained from the CA calibration curve is y=1628x–3E+06 (R^2 = 0.9991). The quantification of CA in the sequential-

ethanol extract and the direct-ethanol extract of *P. indica* stems were 0.2045±0.0128 and 0.1984±0.0113% (w/w), respectively.

The antioxidant activity of the stem extract of *P. indica* was tested using the DPPH, ABTS, and RP methods. **Tab. 1** shows the summary of the antioxidant activity of the extracts. The two ethanol extracts (direct and sequential) provide an overview of the activity of reducing radicals (both DPPH• and ABTS•+) and metal ions (through iron reduction). Meanwhile, the ethyl acetate and *n*-hexane extracts did not provide an overview of antioxidant capacity.

DISCUSSION

CA is a compound biosynthesized through the shikimate pathway in plants from various families, including Asteraceae (14). It is accumulated in leaves, stems, roots, fruits, and other organs at different levels (15). Extraction is the initial step in separating metabolites from the plant matrix for identification (16). To measure the quantity of CA in *P. indica* extract, the HPLC technique might be used for routine analysis (6). This method is proven to be fast, precise, and accurate. In previous study, it was reported that CA, as one of the phenolics of *P. indica* leaves, can be extracted well using 50% ethanol solvent with UAE (6). In the present study, phenolic extraction was performed using 50% ethanol solvent, directly and sequentially, by the UAE technique. CA was detected and quantified in 50% ethanol extract (both obtained from direct and sequential extraction). Compared with the levels of CA in *P. indica* leaves in the study of Kongkiatpaiboon et al. (2018)(6), the content of this compound in the ethanol extract of *P. indica* stems was lower. The levels of CA in *P. indica* leaves were in the range of 2.67% (w/w) (6), while in stem extracts (in present study) were in the range of 0.19-0.2% (w/w). Direct extraction allows metabolites with a polarity corresponding to 50% ethanol to be extracted in it (crude), so the concentration of the target compound in this extract is lower. Meanwhile, through sequential extraction, it is expected that other compounds extracted sequentially in *n*-hexane and ethyl acetate are not found in the 50% ethanol extract. With the elimination of

these compounds (such as components of fat, chlorophyll, etc.), it is hoped that phenolics (especially CA) can be extracted well in 50% ethanol solvent (17). In general, CA is soluble in low concentrations of alcohol or alcohol-water mixtures. This compound is insoluble in non-polar solvents, such as benzene, chloroform, or ether. The high solubility in an alcohol-water mixture is related to the large number of free hydroxyl groups (18).

In this study, the high phenolic content in the extract was linear with its antiradical DPPH activity. The direct 50% ethanol extract had high phenolic content and the DPPH radical scavenging activity better than other extracts. The extract had a lower CA content than the sequential 50% ethanol extract. Thus, it is suspected that CA is not the only phenolic in *P. indica* stems that plays a role in scavenging DPPH radicals. The presence of other phenolic compounds, or other natural compounds that are synergistic in the 50% ethanol extract, is suspected of playing a role in providing better antiradical activity than the graded 50% ethanol extract. Different things happened in the ABTS and RP tests. In both tests, 50%-ethanol (sequential) gave better antioxidant activity.

There are several chemical approaches for measuring plant antioxidant capacity. The antioxidant chemical-based assay's mode of action, namely electron transfer, hydrogen transfer, and metal reduction (19). The DPPH and ABTS methods are included in reactions based on electron and hydrogen transfer. The DPPH technique is an in vitro approach that is simple, inexpensive, and frequently used to assess the antioxidant activity of natural materials (20). This method describes the antiradical activity of the sample more than as an antioxidant activity. Antiradical activity refers to a compound's capacity to react with a free radical, whereas antioxidant activity refers to a compound's ability to block the oxidation process (21). Meanwhile, ABTS is capable of measuring the capacity of both lipophilic and hydrophilic antioxidants. ABTS radicals are more reactive than DPPH radicals. The chemical interaction between antioxidants and ABTS radicals is relatively quick (22). Another method, RP, is based on an increase in absorbance from the reaction between the antioxidant and a mixture of potassium ferricyanide, trichloro acetic acid, and ferric chloride reagents, which demonstrating

the strength of antioxidants in reducing metals (20). However, measuring antioxidant activity in vitro has limitations since DPPH and ABTS are not naturally occurring oxidants in the body. The radical quenching reactions that occur in these assays are often quick, unlike in the occurrence of a slower reaction in the body. In addition, whereas antioxidants have significant antioxidant effects in chemical tests, they can function as pro-oxidants in dietary and biological systems (19).

The variety of phenolic types and the complexity of the composition of these chemicals in plants make the separation of antioxidants phenolic inefficient and expensive (17). Other compounds that can also act as antioxidants are phytosterols (23), flavonoids, minerals, organosulfur, vitamins, carotenoids, etc.(24). The crude often provides more significant antioxidant benefits than the individual forms. In addition, the measurement of antioxidant activity with specific methods was only carried out under adjusted conditions. Determining the overall antioxidant capacity of the sample should also be done using additional techniques, exceptionally those capable of evaluating the efficiency of antioxidants against specific ROS/RNS (17). Studying the types of other phenolic compounds in *P. indica* stems is still possible. Thus, the use of *P. indica* stems as a source of antioxidants can continue to be developed, for example, in the therapy of metabolic syndrome, as a preservative, and as natural food supplements.

CONCLUSIONS

Based on this research, CA in *P. indica* stems can be extracted well using 50% ethanol solvent either by direct or sequential extraction. The sequential-ethanol extract contains more CA than the ethanol extract obtained from direct extraction. Meanwhile, more phenolic content was found in extracts produced from direct extraction. The extracts also provided better antioxidant activity. It is still important to search for phenolic substances besides CA, which functions as an antioxidant in *P. indica* stems.

ACKNOWLEDGEMENT

The authors thank Research and Development Institute or Lembaga Penelitian dan Pengembangan (Lemlitbang), Universitas Muhammadiyah Prof. DR. HAMKA for funding this research and publication with grant number: 713/F.03.07/2021. The authors also thank the facilities, scientific and technical support from Advanced Characterization Laboratories Cibinong–Integrated Laboratory of Bioproduct, National Research and Innovation Agency through E-Layanan Sains (ELSA), Badan Riset dan Inovasi Nasional (BRIN), Indonesia.

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Tab. 1. Antioxidant activity from *P. indica* stem extracts.

Samples	Antioxidant activities		
	DPPH (mg QE/100 g DW)	**ABTS (μmol TE/g DW)**	**RP (μmol TE/g DW)**
50%-ethanol extract (direct)	189.210±0.01	17.624±0.345	40.069±0.214
50%-ethanol extract (sequential)	143.198±0.02	34.837±0.126	42.854±0.909
Ethyl acetate extract (sequential)	1.209±0.05	nd	nd
n-hexane extract (sequential)	0.597±0.03	nd	nd

Note: DW = dry weight; nd = not detected; QE = quercetin equivalent; TE = Trolox equivalent

Figure Legends

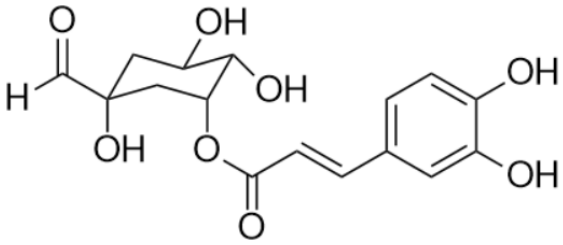

Fig 1. Chlorogenic acid (5-CQA)

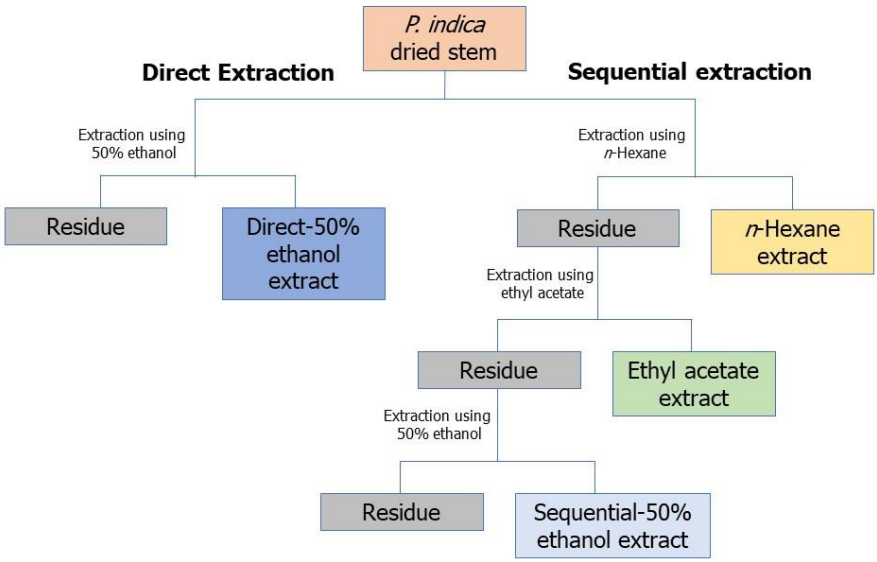

Fig. 2. Extraction procedures of *P. indica* dried stem

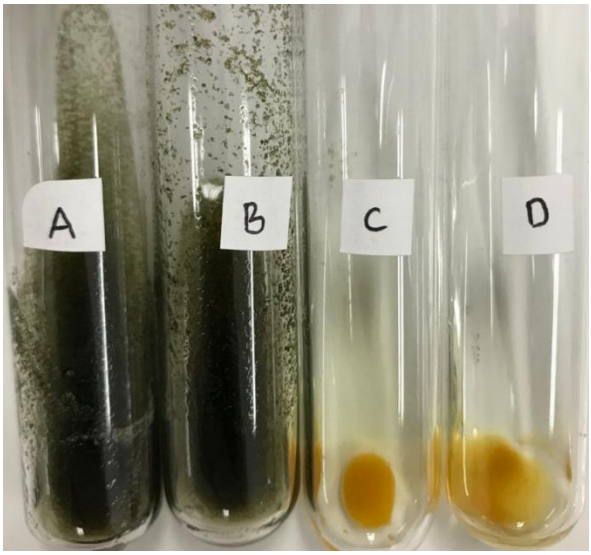

Fig. 3. The results of phenolic compounds screening in *P. indica* stem extracts with 5% $FeCl_3$ reagent. A = 50%-ethanol extract (direct); B = 50%-ethanol extract (sequential); C = ethyl acetate extract (sequential); D = *n*-hexane extract (sequential)

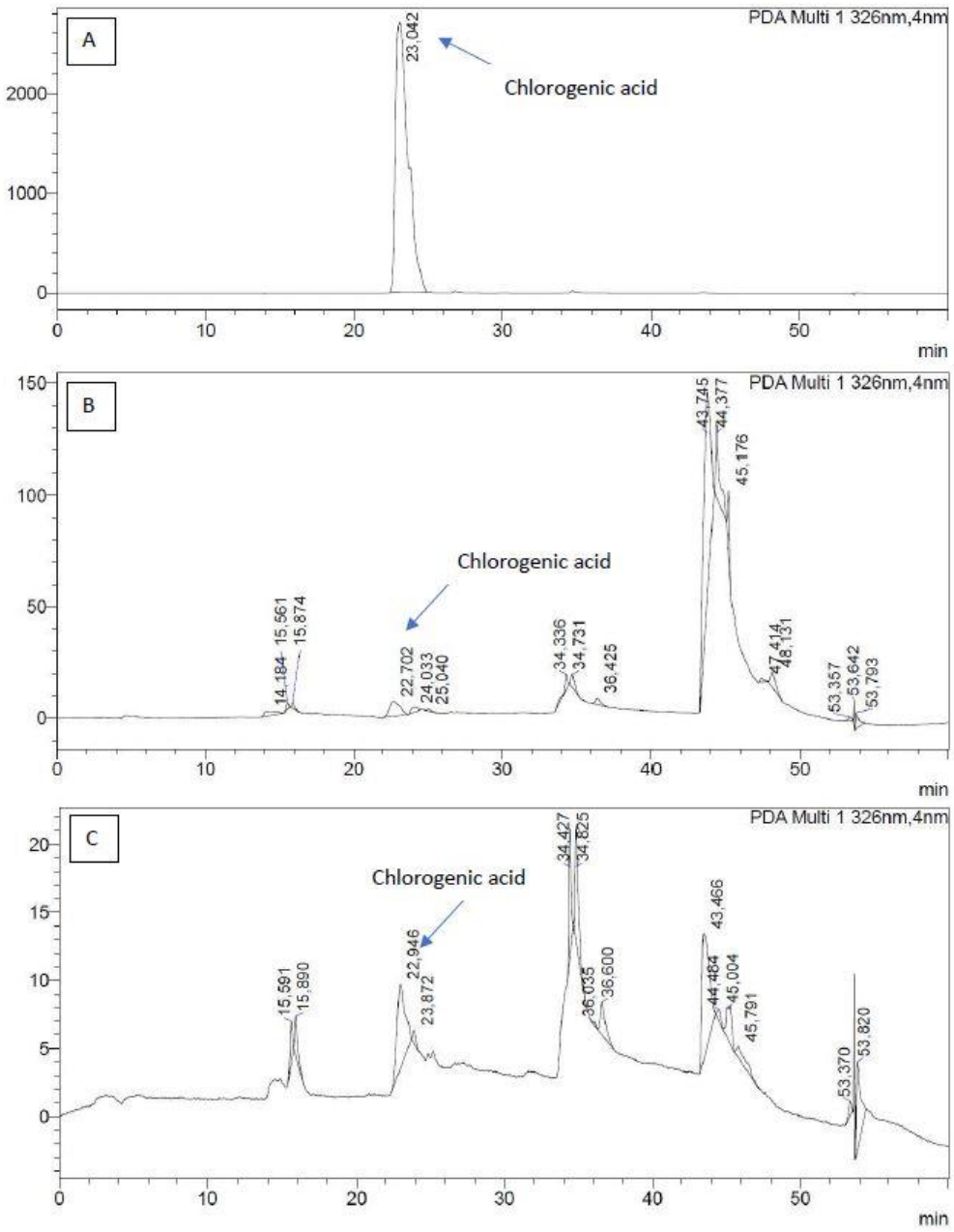

Fig. 4. RP-HPLC Chromatogram of chlorogenic acid (CA) as a standard (at 2.0 mg/mL) and ethanolic extracts of *P. indica* stem. A = chlorogenic acid; B = 50%-ethanol extract (sequential); C = 50%-ethanol extract (direct)

4. Bukti artikel diterima (7 September 2023)

Decision: **initial accept**

Dear Ni Putu Hikmawanti,

I am pleased to inform you that your manuscript, entitled: Quantification of chlorogenic acid in Pluchea indica L. stem ethanolic extracts and its antioxidant activity (CIPMS-00132-2023-02), has been initially accepted for publication in our journal.

To receive a full acceptance and to have the article published it is required from you to:

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Quantification of chlorogenic acid in *Pluchea indica* L. stem ethanolic extracts and its antioxidant activity

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ABSTRACT

Chlorogenic acid (CA) is an important phenolic acid antioxidant. It is found in *Pluchea indica* L. (Asteraceae). However, it has only been extensively studied in the leaves, while studies on the stems have not been reported. This study aimed to identify and measure the levels of CA in the stem extract of *P. indica*. The extract was also determined for its antioxidant activities. In the course of the work, *P. indica* stems powder was extracted using the ultrasonic-assisted extraction (UAE) technique employing 50% ethanol as solvent directly and sequentially. The extract was then measured for total phenolic content (TPC) and CA content using RP-HPLC. Meanwhile, antioxidant activities were determined by the DPPH, ABTS, and reducing power (RP) methods. TPC in the sequential and the direct of *P. indica* stems ethanol extracts were 1.4694±0.0228 and 1.9314±0.0318 mgGAE/g DW, respectively. We found that the CA content of 50% ethanol extract of *P. indica* stems from sequential extraction (0.2045±0.0128 %, w/w) was higher than 50% ethanol extract from direct extraction (0.1984±0.0113%, w/w). The two extracts demonstrated good antioxidant capacity, while the ethyl acetate and n-hexane extracts did not. Identifying of other antioxidants phenolics using other extracting methods still needs further study.

INTRODUCTION

Chlorogenic acid (CA) is an important phenolic derivative of caffeoylquinic acid (CQA). CA (or 5-CQA) consists of a quinic acid core which is acylated with one moiety of caffeic acid at the C-5 (Figure 1) [1]. CA is applicable as an antioxidant, anti-inflammatory, antidiabetic, anti-obesity, anti-hypertensive, antimicrobial, etc.[1]. The caffeic acid group in CA plays a critical role in its activity as an antioxidant. According to Kritsi *et al*., CA has antioxidant activity through the interaction of hydrogen bonds on the receptor binding sites of NADPH oxidase, cytochrome P450, and myeloperoxidase [2]. CA can be extracted from plant materials using ultrasound-assisted extraction (UAE) [3]. CA can be analyzed qualitatively or quantitatively using High-performance liquid chromatography (HPLC) [4] with a C18 column with a UV or DAD detector [4].

CA is found in many plants [3]. One source of CA from plants is *Pluchea indica* L. (Asteraceae), especially the leaves [5-8]. The *P. indica* leaves were more explored than other plant parts. *P. indica* stems also contained a relatively high total phenolic content in 50% ethanol extract compared to flowers and roots [9]. The importance of CA compounds as a source of antioxidants that can reduce free radicals needs to be explored. Indeed, the quantification of CA in 50%-ethanol extract of *P. indica* stems has never been reported. In the present study, quantification of the compound in *P. indica* stems was carried out as an ethanol extract (obtained directly and sequentially), using RP-HPLC analysis. Subsequently, the antioxidant activities of the extracts were studied.

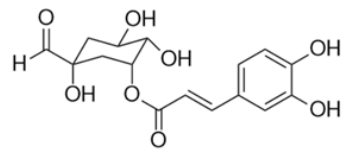

Figure 1. Chlorogenic acid (5-CQA)

MATERIALS AND METHODS

Chemicals

CA and gallic acid as reference were purchased from MarkHerb, Institut Teknologi Bandung (ITB), Bandung, Indonesia. Ethanol, ethyl acetate, and n-hexane (PT. Brataco, Indonesia) were used as extraction solvents. Methanol,

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acetic acid, and water for HPLC grade (Merck, Germany) were used for HPLC analysis.

Plant material

P. indica stems were collected on November 2021 from the Biopharmaceutical Cultivation Conservation Unit, Tropical Biopharmaca Research Center (TropBRC), Institut Pertanian Bogor (IPB), Bogor, Indonesia, with voucher number: BMK0188092016. The stems were cleaned and air-dried. The dried stems were then powdered and stored in a dry container protected from light.

Extraction

Extraction is divided into two ways, namely direct extraction and sequential extraction. In our work, each five g of stem-dried powder was extracted with 50 mL of solvent. In summary, the extraction procedure is presented in Figure 2. The extraction process was carried out using the UAE technique following the protocol in Kongkiatpaiboon *et al*., [6], and was performed in an ultrasonic bath (Branson 5510) 40 kHz at 40°C for 15 min, with the filtrate being separated from the residue by filtration. The residue was then re-extracted using new solvent 3 times. Each filtrate was subsequently collected and made up to 50 mL (here-to-after, referred to as 'liquid extract'). Extraction of each sample was carried out for 5 replications.

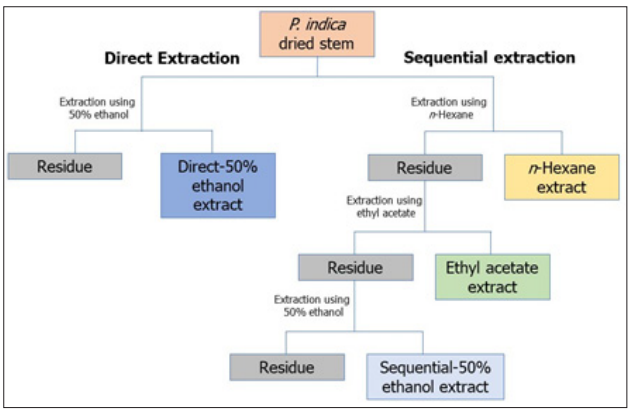


Figure 2. Extraction procedures of *P. indica* dried stem material

Polyphenol Screening

Screening of phenolic compounds was carried out qualitatively using a 5% $FeCl_3$ reagent. The positive result of the reaction is indicated by the formation of a blue-black colour [10].

Total Phenolic Content Assay

Colorimetry was used to determine total phenolic content following the Hikmawanti *et al*., [11] procedure with slight modifications. Gallic acid was used as a standard. For calibration, the gallic acid solution was prepared with variation concentrations: 1.1×10^{-2}, 1.7×10^{-2}, 2.3×10^{-2}, 2.9×10^{-2}, and 3.5×10^{-2} mg/mL. Each extract was diluted 20 times using ethanol. Both extract and standard (0.3 mL) were mixed with 1.5 mL of the Folin-ciocalteu (1:10 in water) and left for 3 min. Afterwards, 1.2 mL of sodium carbonate solution (7.5%) was added to the mixture, homogenized, and then incubated at room temperature for 110 min. The absorbance was measured at 765.10 nm with a spectrophotometer UV-Vis (UV-1900i Series, Shimadzu, Kyoto, Japan). The total phenolic content was expressed as gallic acid equivalent (mg GAE/g of the dried sample). The test was carried out in triplet (mean ± SD).

HPLC condition for analysis of CA

CA analysis followed the protocol of Kongkiatpaiboon *et al*., (2018)(6) with slight modification, and was undertaken at The Advanced Characterization Laboratories Cibinong-Integrated Laboratory of Bioproduct, National Research and Innovation Agency (BRIN), Indonesia. CA (as an external standard) was prepared at a concentration of 10 mg/mL in ethanol. The standard solution was then diluted to 1.0, 2.0, 6.0, and 7.5 mg/mL. Each solution was filtered using a 0.45 µm filter and injected into the HPLC system. The injection volume of the sample was 5 µL. The HPLC system (Shimadzu Prominence-i LC-2030C 3D Plus), which includes a quaternary pump, a degasser and an autosampler, is combined with a photodiode array (PDA) detector in the HPLC apparatus. HPLC separation was performed using a Shim Pack GIST C18 column (150 mmL. × 4.6 mm I.D., 4 µm) as the stationary phase at 25°C. Acetic acid 0,5% in water (A) and methanol (B) was used as the mobile phase via gradient elution (10% B to 50% B in A for 40 min and 100% B for 10 min), as stated in Kongkiatpaiboon *et al*., [6]. The flow rate was 1.0 mL/min. Separation was monitored at 326 nm.

DPPH assay

A 0.1 mM DPPH solution was prepared in methanol. Quercetin ($1x10^2$ mg/mL) in methanol was used as a reference. Initially, each sample of both extract and quercetin (1 mL) was separately reacted with 0.1 mM DPPH (4 mL). After incubation for 30 min at room temperature in the dark, the reaction of the mixture was measured at 516 nm against a methanol blank, using a UV-Vis spectrophotometer (UV-1900 Series, Shimadzu, Kyoto, Japan) [12].

ABTS assay

Antioxidant activity assay against ABTS radicals was carried out by microtiter assay. Accordingly, 180 µl of ABTS radical ($ABTS^{\bullet+}$), prepared 12-16 h beforehand, was reacted with 20 µl of the extract test solution in a 96-well flat-bottom microplate. After 5 min of incubation, the absorbance was measured at 750 nm, using an iMark microplate reader (BioRad, Shanghai, China) [13]. Trolox was employed as a reference (0.1-0.8 mmol/L). The results are expressed as µmol Trolox equivalent per g dry weight sample (µmol TE/g DW).

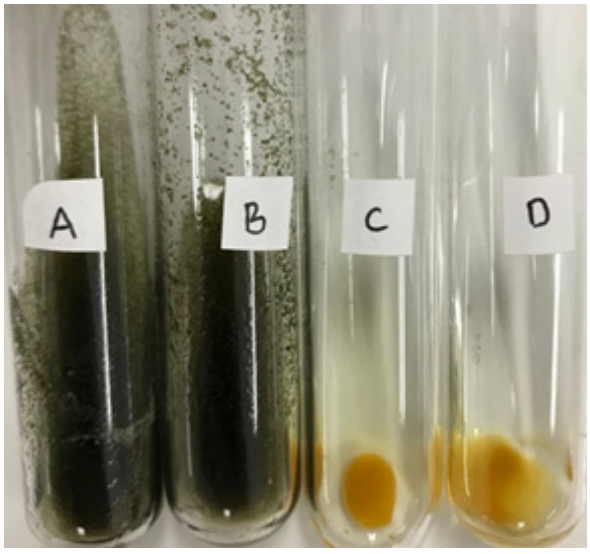
RP assay

The ability of antioxidants to reduce iron was determined by reducing power assay following the protocol of Khatua *et al*. [13]. The tests were carried out in 96-well flat-bottom microplates. A total of 10 µl of the extract test solution was reacted with 25 µl of 0.2 M phosphate buffer (pH 6.6), and 25 µl of potassium ferricyanide (1%, w/v). After 20 min incubated (at room temperature), 25 µl of TCA 10% (w/v) was added. Subsequently, 5 µl of distilled water and 8.5 µl of

Iron (III) chloride was added in. The mixture was shaken for 10 s at medium speed, then incubated at room temperature for 15 min. The absorbance was measured at 750 nm using a iMark microplate reader (BioRad, Shanghai, China) [13]. Trolox was employed as a reference (0.1-0.8 mmol/L). The results are expressed as µmol Trolox equivalent per g dry weight sample (µmol TE/g DW).

RESULTS

The 50%-ethanol extracts of *P. indica* stems, both obtained from direct and sequential extraction, were detected to contain phenolics based on the test with a 5% $FeCl_3$ reagent (Figure 3). The two extracts were then analysed for the total phenolics content via a UV-Visible spectrophotometer. Total phenolics content in the sequential and the direct *P. indica* stems ethanol extracts were 1.4694±0.0228 and 1.9314±0.0318 mgGAE/g dried powder, respectively.

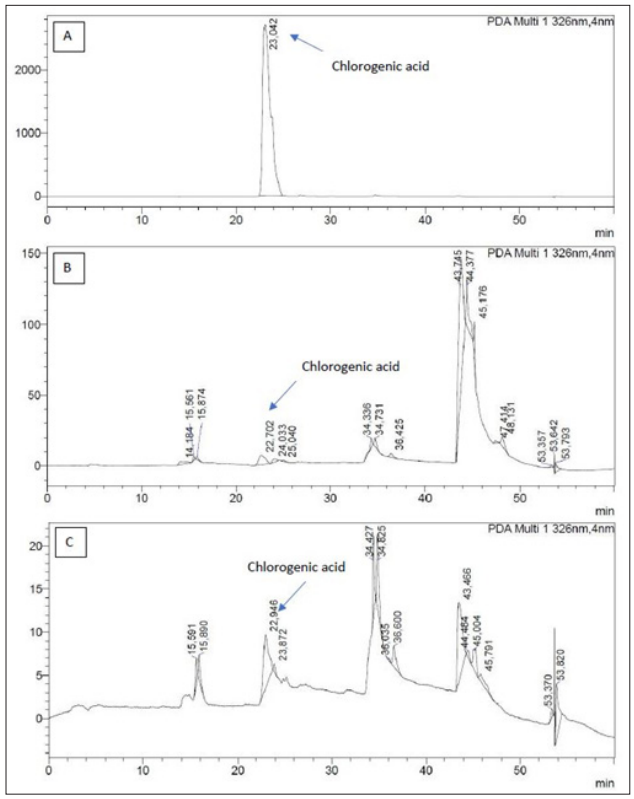


A – 50%-ethanol extract (direct); B – 50%-ethanol extract (sequential); C – ethyl acetate extract (sequential); D – n-hexane extract (sequential)

Figure 3. The results of phenolic compounds screening in *P. indica* stem extracts with 5% FeCl3 reagent

Analysis of CA in ethanol extracts of *P. indica* stems was performed using RP-HPLC at 326 nm as the maximum wavelength [6]. Figure 4 shows the HPLC chromatograms of the standard CA (A, retention time = 23.042 min), the sequential-ethanol extract (B), and the direct-ethanol extract (C) of *P. indica* stems. The chromatogram showed the presence of CA in the sequential-ethanol extract and the direct-ethanol extract of *P. indica* stems at retention times of 22.702 dan 22.946 min, respectively. The equation of the line obtained from the CA calibration curve is y=1628x–3E+06 (R^2=0.9991). The quantification of CA in the sequential-ethanol extract and the direct-ethanol extract of *P. indica* stems were 0.2045±0.0128 and 0.1984±0.0113% (w/w), respectively.

The antioxidant activity of the stem extract of *P. indica* was tested by way of application of the DPPH, ABTS and RP methods. Table 1 shows the summary of the antioxidant activity of the extracts. The two ethanol extracts (direct and sequential) provide an overview of the activity of reducing radicals (both $DPPH^{\bullet}$ and $ABTS^{\bullet+}$) and metal ions (through iron reduction). The ethyl acetate and n-hexane extracts did not provide an overview of antioxidant capacity.



A - chlorogenic acid; B - 50%-ethanol extract (sequential); C - 50%-ethanol extract (direct)

Figure 4. RP-HPLC Chromatogram of chlorogenic acid (CA) as a standard (at 2.0 mg/mL) and ethanolic extracts of *P. indica* stem

Table 1. Antioxidant activity from *P. indica* stem extracts

Samples	Antioxidant activities		
	DPPH (mg QE/100 g DW)	ABTS (µ mol TE/g DW)	RP (µ mol TE/g DW)
50%-ethanol extract (direct)	189.210±0.01	17.624±0.345	40.069±0.214
50%-ethanol extract (sequential)	143.198±0.02	34.837±0.126	42.854±0.909
Ethyl acetate extract (sequential)	1.209±0.05	nd	nd
n-hexane extract (sequential)	0.597±0.03	nd	nd

Note: DW – dry weight; nd – not detected; QE – quercetin equivalent; TE – Trolox equivalent

DISCUSSION

CA is a compound biosynthesized through the shikimate pathway in plants from various families, including Asteraceae [14]. It is accumulated in leaves, stems, roots, fruits and other organs at different levels [15]. Extraction is the initial step in separating metabolites from the plant matrix for identification [16]. To measure the quantity of CA in *P. indica* extract, the HPLC technique might be used for routine analysis [6]. This method is proven to be fast, precise and accurate. In previous study, it was reported that CA, as one of the phenolics of *P. indica* leaves, can be efficently extracted using 50% ethanol solvent with UAE [6]. In the present study, phenolic extraction was performed using 50% ethanol solvent, directly and sequentially, by way of use of the UAE technique. CA was detected and quantified in 50% ethanol extract (both obtained from direct and sequential extraction). Compared with the levels of CA in *P. indica*

leaves in the study of Kongkiatpaiboon *et al*. [6], the content of this compound in the ethanol extract of *P. indica* stems was lower. The levels of CA in *P. indica* leaves were in the range of 2.67% (w/w) [6], while that in stem extracts (in the present study) were in the range of 0.19-0.2% (w/w). Direct extraction allows metabolites with a polarity corresponding to 50% ethanol to be extracted in it (crude), thus the concentration of the target compound in this extract is lower. Through sequential extraction, it is expected that other compounds extracted sequentially in n-hexane and ethyl acetate are not found in the 50% ethanol extract. With the elimination of these compounds (such as components of fat, chlorophyll, etc.), it is hoped that phenolics (especially CA) can be effectively extracted in 50% ethanol solvent [17]. In general, CA is soluble in low concentrations of alcohol or alcohol-water mixtures. This compound is insoluble in non-polar solvents, such as benzene, chloroform, or ether. The high solubility in an alcohol-water mixture is related to the large number of free hydroxyl groups [18].

In this study, the high phenolic content in the extract was linear with its antiradical DPPH activity. The direct 50% ethanol extract had high phenolic content and the DPPH radical scavenging activity was better than that with other extracts. The extract had, however, a lower CA content than the sequential 50% ethanol extract. Thus, it is suspected that CA is not the only phenolic in *P. indica* stems that plays a role in scavenging DPPH radicals. The presence of other phenolic compounds, or other natural compounds that are synergistic in the 50% ethanol extract, is suspected of playing a role in providing better antiradical activity than the graded 50% ethanol extract. In the ABTS and RP tests, 50%-ethanol (sequential) gave better antioxidant activity.

There are several chemical approaches for measuring plant antioxidant capacity. The antioxidant chemical-based assay's mode of action is that of electron transfer, hydrogen transfer and metal reduction [19]. The DPPH and ABTS methods are included in reactions based on electron and hydrogen transfer. The DPPH technique is an in vitro approach that is simple, inexpensive and frequently used to assess the antioxidant activity of natural materials [20]. This method describes the antiradical activity of the sample more than as an antioxidant activity. Antiradical activity refers to a compound's capacity to react with a free radical, whereas antioxidant activity refers to a compound's ability to block the oxidation process [21]. Meanwhile, ABTS is capable of measuring the capacity of both lipophilic and hydrophilic antioxidants. ABTS radicals are more reactive than DPPH radicals. The chemical interaction between antioxidants and ABTS radicals is relatively quick [22]. Another method, RP, is based on an increase in absorbance from the reaction between the antioxidant and a mixture of potassium ferricyanide, trichloro acetic acid and ferric chloride reagents, which demonstrates the strength of antioxidants in reducing metals [20]. However, measuring antioxidant activity in vitro has limitations, since DPPH and ABTS are not naturally occurring oxidants in the body. The radical quenching reactions that occur in these assays are often quick, unlike in the occurrence of a slower reaction in the body. In addition, whereas antioxidants have significant antioxidant effects in chemical tests, they can function as pro-oxidants in dietary and biological systems [19].

The variety of phenolic types and the complexity of the composition of these chemicals in plants make the separation of antioxidants phenolic inefficient and expensive [17]. Other compounds that can also act as antioxidants are phytosterols [23], flavonoids, minerals, organosulfur, vitamins, carotenoids, etc. [24]. Moreover, the crude often provides more significant antioxidant benefits than the individual forms. In addition, the measurement of antioxidant activity with specific methods is usually only carried out under adjusted conditions. Determining the overall antioxidant capacity of the sample should, therefore, be also done using additional techniques, exceptionally those capable of evaluating the efficiency of antioxidants against specific ROS/RNS [17]. Studying the types of other phenolic compounds in *P. indica* stems is still possible. Thus, the use of *P. indica* stems as a source of antioxidants can continue to be developed, for example, in the therapy of metabolic syndrome, as a preservative, and as natural food supplements.

CONCLUSIONS

Based on this research, CA in *P. indica* stems can be efficently extracted using 50% ethanol solvent either by direct or sequential extraction. The sequential-ethanol extract contains more CA than the ethanol extract obtained from direct extraction. ~~Meanwhile, more phenolic content was found in extracts produced from direct extraction.~~ The extracts also provided better antioxidant activity. It is important, therefore, to search for phenolic substances besides CA that function as antioxidants in *P. indica* stems.

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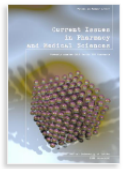
Ni Putu Ermi Hikmawanti, Agustin Yumita and Jihan Esa Siregar | Apr 11, 2024



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sequential extraction.

ABSTRACT

Chlorogenic acid (CA) is an important phenolic acid antioxidant. It is found in *Pluchea indica* L. (Asteraceae). However, it has only been extensively studied in the leaves, while studies on the stems have not been reported. This study aimed to identify and measure the levels of CA in the stem extract of *P. indica*. The extract was also determined for its antioxidant activities. In the course of the work, *P. indica* stems powder was extracted using the ultrasonic-assisted extraction (UAE) technique employing 50%-ethanol as solvent directly and sequentially. The extract was then measured for total phenolic content (TPC) and CA content using RP-HPLC. Meanwhile, antioxidant activities were determined by the DPPH, ABTS, and reducing power (RP) methods. TPC in the sequential and the direct of *P. indica* stems ethanol extracts were 1.4694±0.0228 and 1.9314±0.0318 mgGAE/g DW, respectively. We found that the CA content of 50%-ethanol extract of *P. indica* stems from sequential extraction (0.2045±0.0128%, w/w) was higher than 50%-ethanol extract from direct extraction (0.1984±0.0113%, w/w). The two extracts demonstrated good antioxidant capacity, while the ethyl acetate and *n*-hexane extracts did not. Identifying of other antioxidants phenolics using other extracting methods still needs further study.

INTRODUCTION

Chlorogenic acid (CA) is an important phenolic derivative of caffeoylquinic acid (CQA). CA (or 5-CQA) consists of a quinic acid core which is acylated with one moiety of caffeic acid at the C-5 (Figure 1) [1]. CA is applicable as an antioxidant, anti-inflammatory, antidiabetic, anti-obesity, anti-hypertensive, antimicrobial, etc.[1]. The caffeic acid group in CA plays a critical role in its activity as an antioxidant. According to Kritsi *et al*. [2], CA has antioxidant activity through the interaction of hydrogen bonds on the receptor binding sites of NADPH oxidase, cytochrome P450, and myeloperoxidase [2]. CA can be extracted from plant materials using ultrasound-assisted extraction (UAE) [3]. CA can be analyzed qualitatively or quantitatively using High-performance liquid chromatography (HPLC) [4] with a C18 column with a UV or DAD detector [4].

CA is found in many plants [3]. One source of CA from plants is *Pluchea indica* L. (Asteraceae), especially the leaves [5-8]. The *P. indica* leaves were more explored than other plant parts. *P. indica* stems also contained a relatively high total phenolic content in 50%-ethanol extract compared to flowers and roots [9]. The importance of CA compounds as a source of antioxidants that can reduce free radicals needs to be explored. Indeed, the quantification of CA in 50%-ethanol extract of *P. indica* stems has never been reported. In the present study, quantification of the compound in *P. indica* stems was carried out as an ethanol extract (obtained directly and sequentially), using RP-HPLC analysis. Subsequently, the antioxidant activities of the extracts were studied.

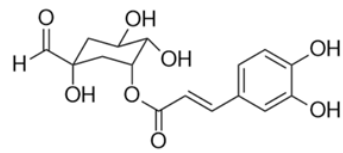

Figure 1. Chlorogenic acid (5-CQA)

MATERIALS AND METHODS

Chemicals

CA and gallic acid as reference were purchased from MarkHerb, Institut Teknologi Bandung (ITB), Bandung, Indonesia. Ethanol, ethyl acetate, and *n*-hexane (PT. Brataco, Indonesia) were used as extraction solvents. Methanol, acetic acid, and water for HPLC grade (Merck, Germany) were used for HPLC analysis.

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Plant material

P. indica stems were collected on November 2021 from the Biopharmaceutical Cultivation Conservation Unit, Tropical Biopharmaca Research Center (TropBRC), Institut Pertanian Bogor (IPB), Bogor, Indonesia, with voucher number: BMK0188092016. The stems were cleaned and air-dried. The dried stems were then powdered and stored in a dry container protected from light.

Extraction

Extraction is divided into two ways, namely direct extraction and sequential extraction. In our work, each five g of stem-dried powder was extracted with 50 mL of solvent. In summary, the extraction procedure is presented in Figure 2. The extraction process was carried out using the UAE technique following the protocol in Kongkiatpaiboon *et al*. [6], and was performed in an ultrasonic bath (Branson 5510) 40 kHz at 40°C for 15 min, with the filtrate being separated from the residue by filtration. The residue was then re-extracted using new solvent 3 times. Each filtrate was subsequently collected and made up to 50 mL (here-to-after, referred to as 'liquid extract'). Extraction of each sample was carried out for 5 replications.

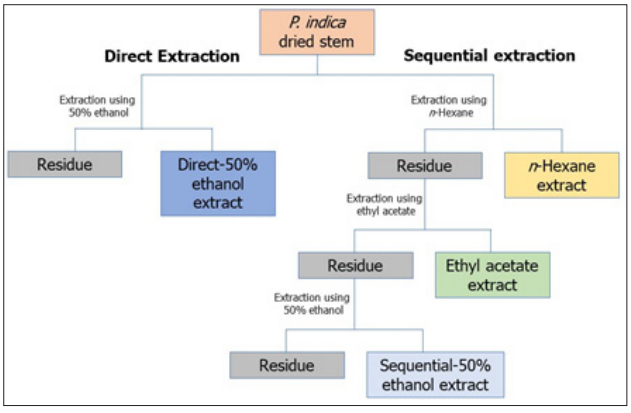


Figure 2. Extraction procedures of *P. indica* dried stem material

Polyphenol Screening

Screening of phenolic compounds was carried out qualitatively using a 5% $FeCl_3$ reagent. The positive result of the reaction is indicated by the formation of a blue-black colour [10].

Total Phenolic Content Assay

Colorimetry was used to determine total phenolic content following the Hikmawanti *et al*. [11] procedure with slight modifications. Gallic acid was used as a standard. For calibration, the gallic acid solution was prepared with variation concentrations: 1.1×10^{-2}, 1.7×10^{-2}, 2.3×10^{-2}, 2.9×10^{-2}, and 3.5×10^{-2} mg/mL. Each extract was diluted 20 times using ethanol. Both extract and standard (0.3 mL) were mixed with 1.5 mL of the Folin-ciocalteu (1:10 in water) and left for 3 min. Afterwards, 1.2 mL of sodium carbonate solution (7.5%) was added to the mixture, homogenized, and then incubated at room temperature for 110 min. The absorbance was measured at 765.10 nm with a spectrophotometer UV-Vis (UV-1900i Series, Shimadzu, Kyoto, Japan). The total phenolic content was expressed as gallic acid equivalent (mg GAE/g of the dried sample). The test was carried out in triplet (mean ± SD).

HPLC condition for analysis of CA

CA analysis followed the protocol of Kongkiatpaiboon *et al*. [6] with slight modification, and was undertaken at The Advanced Characterization Laboratories Cibinong-Integrated Laboratory of Bioproduct, National Research and Innovation Agency (BRIN), Indonesia. CA (as an external standard) was prepared at a concentration of 10 mg/mL in ethanol. The standard solution was then diluted to 1.0, 2.0, 6.0, and 7.5 mg/mL. Each solution was filtered using a 0.45 µm filter and injected into the HPLC system. The injection volume of the sample was 5 µL. The HPLC system (Shimadzu Prominence-i LC-2030C 3D Plus), which includes a quaternary pump, a degasser and an autosampler, is combined with a photodiode array (PDA) detector in the HPLC apparatus. HPLC separation was performed using a Shim Pack GIST C18 column (150 mmL. × 4.6 mm I.D., 4 µm) as the stationary phase at 25°C. Acetic acid 0,5% in water (A) and methanol (B) was used as the mobile phase via gradient elution (10% B to 50% B in A for 40 min and 100% B for 10 min), as stated in Kongkiatpaiboon *et al*. [6]. The flow rate was 1.0 mL/min. Separation was monitored at 326 nm.

DPPH assay

A 0.1 mM 2,2-Diphenyl-1-picrylhydrazyl (DPPH) solution was prepared in methanol. Quercetin (1×10^2 mg/mL) in methanol was used as a reference. Initially, each sample of both extract and quercetin (1 mL) was separately reacted with 0.1 mM DPPH (4 mL). After incubation for 30 min at room temperature in the dark, the reaction of the mixture was measured at 516 nm against a methanol blank, using a UV-Vis spectrophotometer (UV-1900 Series, Shimadzu, Kyoto, Japan) [12].

ABTS assay

Antioxidant activity assay against 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals was carried out by microtiter assay. Accordingly, 180 µL of ABTS radical ($ABTS^{\bullet+}$), prepared 12-16 h beforehand, was reacted with 20 µL of the extract test solution in a 96-well flat-bottom microplate. After 5 min of incubation, the absorbance was measured at 750 nm, using an iMark microplate reader (BioRad, Shanghai, China) [13]. Trolox was employed as a reference (0.1-0.8 mmol/L). The results are expressed as µmol Trolox equivalent per g dry weight sample (µmol TE/g DW).

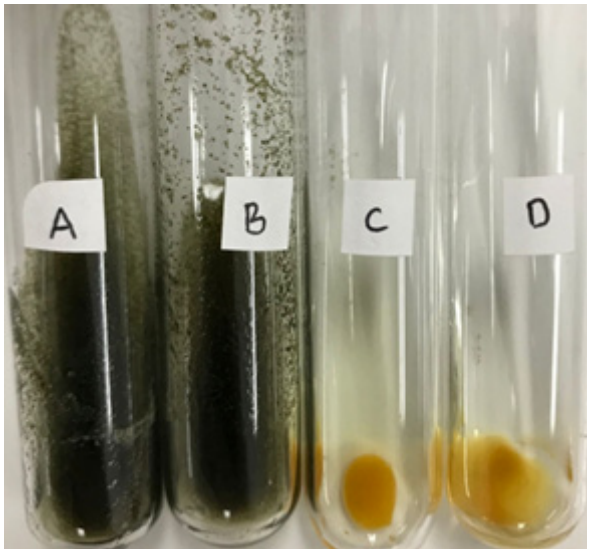
RP assay

The ability of antioxidants to reduce iron was determined by reducing power (RP) assay following the protocol of Khatua *et al*. [13]. The tests were carried out in 96-well flat-bottom microplates. A total of 10 µL of the extract test solution was reacted with 25 µL of 0.2 M phosphate buffer (pH 6.6), and 25 µL of potassium ferricyanide (1%, w/v). After 20 min incubated (at room temperature), 25 µL of Trichloroacetic Acid (TCA) 10% (w/v) was added.

Subsequently, 5 µL of distilled water and 8.5 µL of Iron (III) chloride was added in. The mixture was shaken for 10 s at medium speed, then incubated at room temperature for 15 min. The absorbance was measured at 750 nm using a iMark microplate reader (BioRad, Shanghai, China) [13]. Trolox was employed as a reference (0.1-0.8 mmol/L). The results are expressed as µmol Trolox equivalent per g dry weight sample (µmol TE/g DW).

RESULTS

The 50%-ethanol extracts of *P. indica* stems, both obtained from direct and sequential extraction, were detected to contain phenolics based on the test with a 5% $FeCl_3$ reagent (Figure 3). The two extracts were then analysed for the total phenolics content via a UV-Visible spectrophotometer. Total phenolics content in the 50%-ethanol extract of *P. indica* stems from sequential and direct extraction were 1.4694±0.0228 and 1.9314±0.0318 mgGAE/g dried powder, respectively.

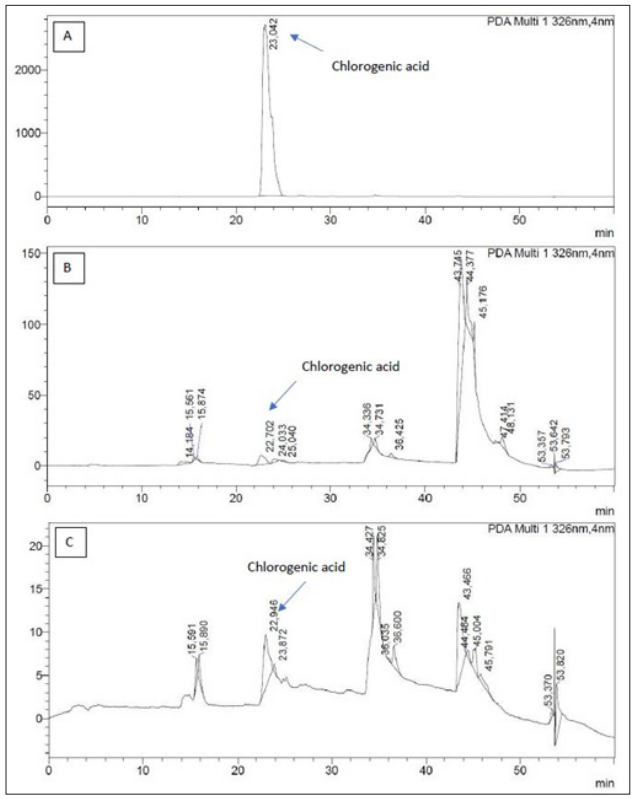


A – 50%-ethanol extract (direct); B – 50%-ethanol extract (sequential); C – ethyl acetate extract (sequential); D – *n*-hexane extract (sequential)

Figure 3. The results of phenolic compounds screening in *P. indica* stem extracts with 5% $FeCl_3$ reagent

Analysis of CA in ethanol extracts of *P. indica* stems was performed using RP-HPLC at 326 nm as the maximum wavelength [6]. Figure 4 shows the HPLC chromatograms of the standard CA (A, retention time = 23.042 min), the 50%-ethanol extract (sequential) (B), and the 50%-ethanol extract (direct) (C) of *P. indica* stems. The chromatogram showed the presence of CA in the 50%-ethanol extract (sequential) and the 50%-ethanol extract (direct) of *P. indica* stems at retention times of 22.702 dan 22.946 min, respectively. The equation of the line obtained from the CA calibration curve is y=1628x–3E+06 (R^2=0.9991). The quantification of CA in the 50%-ethanol extract (sequential) and the 50%-ethanol extract (direct) of *P. indica* stems were 0.2045±0.0128 and 0.1984±0.0113% (w/w), respectively.

The antioxidant activity of the stem extract of *P. indica* was tested by way of application of the DPPH, ABTS and RP methods. Table 1 shows the summary of the antioxidant activity of the extracts. The two ethanol extracts (direct and



A - chlorogenic acid; B - 50%-ethanol extract (sequential); C - 50%-ethanol extract (direct)

Figure 4. RP-HPLC Chromatogram of chlorogenic acid (CA) as a standard (at 2.0 mg/mL) and ethanolic extracts of *P. indica* stem

sequential) provide an overview of the activity of reducing radicals (both $DPPH^{\bullet}$ and $ABTS^{\bullet+}$) and metal ions (through iron reduction). The ethyl acetate and *n*-hexane extracts did not provide an overview of antioxidant capacity.

Table 1. Antioxidant activity from *P. indica* stem extracts

Samples	Antioxidant activities		
	DPPH (mg QE/100 g DW)	ABTS (µmol TE/g DW)	RP (µmol TE/g DW)
50%-ethanol extract (direct)	189.210±0.01	17.624±0.345	40.069±0.214
50%-ethanol extract (sequential)	143.198±0.02	34.837±0.126	42.854±0.909
Ethyl acetate extract (sequential)	1.209±0.05	nd	nd
n-hexane extract (sequential)	0.597±0.03	nd	nd

Note: DW – dry weight; nd – not detected; QE – quercetin equivalent; TE – Trolox equivalent

DISCUSSION

CA is a compound biosynthesized through the shikimate pathway in plants from various families, including Asteraceae [14]. It is accumulated in leaves, stems, roots, fruits and other organs at different levels [15]. Extraction is the initial step in separating metabolites from the plant matrix for identification [16]. To measure the quantity of CA in *P. indica* extract, the HPLC technique might be used for routine analysis [6]. This method is proven to be fast, precise and accurate. In previous study, it was reported that CA, as one of the phenolics of *P. indica* leaves, can be efficently extracted using 50%-ethanol solvent with UAE [6]. In the present study, phenolic extraction was performed using 50%-ethanol solvent, directly and sequentially, by way of use of the UAE technique. CA was detected and quantified

in 50%-ethanol extract (both obtained from direct and sequential extraction). Compared with the levels of CA in *P. indica* leaves in the study of Kongkiatpaiboon *et al*. [6], the content of this compound in the ethanol extract of *P. indica* stems was lower. The levels of CA in *P. indica* leaves were in the range of 2.67% (w/w) [6], while that in stem extracts (in the present study) were in the range of 0.19-0.2% (w/w). Direct extraction allows metabolites with a polarity corresponding to 50%-ethanol to be extracted in it (crude), thus the concentration of the target compound in this extract is lower. Through sequential extraction, it is expected that other compounds extracted sequentially in *n*-hexane and ethyl acetate are not found in the 50%-ethanol extract. With the elimination of these compounds (such as components of fat, chlorophyll, etc.), it is hoped that phenolics (especially CA) can be effectively extracted in 50%-ethanol solvent [17]. In general, CA is soluble in low concentrations of alcohol or alcohol-water mixtures. This compound is insoluble in non-polar solvents, such as benzene, chloroform, or ether. The high solubility in an alcohol-water mixture is related to the large number of free hydroxyl groups [18].

In this study, the high phenolic content in the extract was linear with its antiradical DPPH activity. The 50%-ethanol extract (direct) had high phenolic content and the DPPH radical scavenging activity was better than that with other extracts. The extract had, however, a lower CA content than the 50%-ethanol extract (sequential). Thus, it is suspected that CA is not the only phenolic in *P. indica* stems that plays a role in scavenging DPPH radicals. The presence of other phenolic compounds, or other natural compounds that are synergistic in the 50%-ethanol extract, is suspected of playing a role in providing better antiradical activity than the 50%-ethanol extract (sequential). In the ABTS and RP tests, 50%-ethanol extract (sequential) gave better antioxidant activity.

There are several chemical approaches for measuring plant antioxidant capacity. The antioxidant chemical-based assay's mode of action is that of electron transfer, hydrogen transfer and metal reduction [19]. The DPPH and ABTS methods are included in reactions based on electron and hydrogen transfer. The DPPH technique is an in vitro approach that is simple, inexpensive and frequently used to assess the antioxidant activity of natural materials [20]. This method describes the antiradical activity of the sample more than as an antioxidant activity. Antiradical activity refers to a compound's capacity to react with a free radical, whereas antioxidant activity refers to a compound's ability to block the oxidation process [21]. Meanwhile, ABTS is capable of measuring the capacity of both lipophilic and hydrophilic antioxidants. ABTS radicals are more reactive than DPPH radicals. The chemical interaction between antioxidants and ABTS radicals is relatively quick [22]. Another method, RP, is based on an increase in absorbance from the reaction between the antioxidant and a mixture of potassium ferricyanide, trichloro acetic acid and ferric chloride reagents, which demonstrates the strength of antioxidants in reducing metals [20]. However, measuring antioxidant activity in vitro has limitations, since DPPH and ABTS are not naturally occurring oxidants in the body. The radical quenching reactions that occur in these assays are often quick, unlike in the occurrence of a slower reaction in the body. In addition, whereas antioxidants have significant antioxidant effects in chemical tests, they can function as pro-oxidants in dietary and biological systems [19].

The variety of phenolic types and the complexity of the composition of these chemicals in plants make the separation of antioxidants phenolic inefficient and expensive [17]. Other compounds that can also act as antioxidants are phytosterols [23], flavonoids, minerals, organosulfur, vitamins, carotenoids, etc. [24]. Moreover, the crude often provides more significant antioxidant benefits than the individual forms. In addition, the measurement of antioxidant activity with specific methods is usually only carried out under adjusted conditions. Determining the overall antioxidant capacity of the sample should, therefore, be also done using additional techniques, exceptionally those capable of evaluating the efficiency of antioxidants against specific ROS/RNS [17]. Studying the types of other phenolic compounds in *P. indica* stems is still possible. Thus, the use of *P. indica* stems as a source of antioxidants can continue to be developed, for example, in the therapy of metabolic syndrome, as a preservative, and as natural food supplements.

CONCLUSIONS

Based on this research, the total phenolic content in the 50%-ethanol extract of *P. indica* stems from direct extraction is more than the 50%-ethanol extract obtained from sequential extraction. Meanwhile, the 50%-ethanol extract from sequential extraction contains more CA than the 50%-ethanol extract obtained from direct extraction. The extracts also provided better antioxidant activity. It is important, therefore, to search for phenolic substances besides CA that function as antioxidants in *P. indica* stems.

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