Physicochemical and phytochemical standardization, and antibacterial evaluation of Cassia alata leaves from different locations in Indonesia

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Received 21 October 2021 ♦ Accepted 11 November 2021 ♦ Published 15 December 2021

Citation: Angelina M, Mardhiyah A, Dewi RT, Fajriah S, Muthiah N, Ekapratiiwi Y, Dewijanti ID, Sukirno, Jamilah, Hartati S (2021) Physicochemical and phytochemical standardization, and antibacterial evaluation of Cassia alata leaves from different locations in Indonesia. Pharmacia 68(4): 947–956. https://doi.org/10.3897/pharmacia.68.e76835

Abstract

Physicochemical analysis for simplicia and extract, respectively: water content 2.12–4% for simplicia; drying losses 3.93–5.47% and 8.51–19.76%; total ash 5.14–9.41% and 6.22–17.07%; total ash acid-insoluble content 0.29–5.41% and 0.52–3.82%; total ash water-soluble content 1.26–7.14% and 1.43–8.54%; water-soluble content 19.60–39.41% and 58.45–77.51%; ethanol-soluble content 13.99–33.76% and 59.79–75.39%. The phytochemical analysis showed that the extracts contain alkaloids, flavonoids, saponins, tannins, and terpenoids. Total flavonoids and total phenolics content were 9.86–15.74% QE/g and 6.67–7.65% GAE/g, respectively. Based on LC-MS results, the extract contained emodin, kaempferol, kaempferol-3,7-diglucoside, and kaempferol-3-O-β-D-glucopyranoside. The extracts possessed antibacterial activity against bacteria tested.

Keywords

antibacterial activity, Cassia alata, herbal medicine, physicochemical, phytochemical, standardization

Introduction

Cassia alata (synonym: Senna alata (L.) Roxb.), is a herb plant from the Fabaceae family, found in intertropical areas. It is commonly known as ‘Ath thora’, ‘Eth thora’ (Sri Lanka), candle bush/tree (Malaysia), candle stick, Carriion Crow Bush, Winged Senna, Empress Candle plant, dadmardan (India), Roman Candle trees (Fiji), and ringworm shrub (Habtemariam 2019). In Indonesia, Cassia alata commonly known as ketepeng cina, but has several local names, such as ketepeng kebo (Java), ketepeng badak (Sunda), acon-aconan (Madura), sajamera (Halmahera), kupang-kupang (Ternate), tabankun (Tidore), daun kupang, daun kurapen, and gelinggang (Sumatra) (Asmah et al. 2020). As a traditional medicine, in many countries in the world C. alata leaves are used to treat digestive, dermatologic, anti-infectious, antidiabetic, and miscellaneous diseases (Hennebelle et al. 2009), including Indonesia where it is used for treating fungus on the skin which can cause hives by grinding or rubbing directly on the affected skin (Fatmawati et al. 2020).

Several studies have been reported the pharmacological activities of Cassia alata leaf extract, such as antibacterial (Ibrahim and Osman 1995), antifungal (Palanichamy...
Angelina M et al.: Physicochemical, phytochemical, and antibacterial study of Cassia alata

and Nagarajan 1990; Timothy 2012), antiviral (Angelina et al. 2017, 2020), antioxidant (Sagnia et al. 2014), cytotoxicity (Sugumar et al. 2019), antitumor (Pieme et al. 2008), anti-inflammatory (Moriyama et al. 2003), antidiabetic (Palanchamy et al. 1988), antihaptoxic and hepatoprotective effects (Mohammed et al. 2017). Some of the reported chemical constituents are phenolics (rhein, chrysophanol, kaempferol, aloe emodin, and glycosides), anthraquinones (alatitnine and alatatonal), fatty acids (oleic, palmitic, and linoleic acids), steroids, and terpenoids (sitosterol, stigmasterol, and campesterol) (Oladeji et al. 2020).

The chemical composition of medicinal plant not only varies because of varieties or species difference and genetic factors, but also depends on external variables, such as environmental conditions, i.e. type of soil, levels of precipitation, light intensity, humidity, etc., agricultural practices, and post-harvest handling, i.e. drying, storage, processing. Inconsistency and varying its biological effects encourage standardization of phytopharmaceuticals in order to obtain reproducible results in pharmacological, toxicological, and clinical studies (Nafiu et al. 2017).

Standardization of herbal medicines is the process of prescribing a set of standards or inherent characteristics, constant parameters, definitive qualitative and quantitative values that carry an assurance of quality, efficacy, safety and reproducibility, which is used as a tool for quality control process (Kunle 2012). World Health Organization (1998) emphasized the need to ensure the quality of medicinal plant products, hence described a series of tests for assessing the quality of medicinal plant materials, i.e. macro and microscopic examination, determination of foreign organic matter, ash, extractable matter, moisture content, contaminants, chromatographic examination, qualitative and quantitative chemical examination, etc.

Several publications, United States Pharmacopoeia, British Herbal Compendium, British Herbal Pharmacopoeia, Chinese Pharmacopoeia, Physician's Desk Reference (PDR, Ayurvedic Pharmacopoeia of India carry monographs for herbal raw material. In Indonesia, based on the General Standard Parameter of Medicinal Plant Extract Handbook, released by the Ministry of Health of Indonesia (Depkes RI 2000), parameters which need to be standardized for herbal medicines are specific and non-specific parameters. Specific parameters are related to compounds found in plants, including organoleptic, water-soluble content, ethanol-soluble content, components and levels of chemical compounds. Meanwhile, non-specific parameters are not directly related to pharmacological activity but can affect the safety and stability aspects of the resulting extract or preparation, include ash content, acid-insoluble ash content, water-soluble ash content, water content, bacterial contaminants, mold and yeast contaminants and heavy metal contaminants. However, the information about C. alata standardization is inadequate. Previous studies reporting standardization of C. alata were limited to groups of compounds instead of marker compounds (Gritsanapan and Mangmeesi 2009). In the view of this background, the aims of our studies were to investigate and determine the physicochemical and phytochemical contents supplemented with data on marker compound and the antibacterial activity C. alata leaf obtained in four locations in Indonesia for further standardization of its usage as herbal medicine raw materials.

Materials and methods

Plant materials

The leaves of Cassia alata were collected from four different locations in Indonesia: Bogor Botanical Garden, West Java (6°35’51.0”S, 106°47’58.4”E), Bogor, West Java (6°23’03.3”S, 106°40’46.5”E), South Tangerang, Banten (6°21’13.1”S, 106°39’31.6”E) and Banjarmasin, South Kalimantan (3°19’38.7”S, 114°35’26.8”E). The plants were identified at Herbarium Bogoriense, Department of Botany, Indonesian Institute of Sciences, Research Center of Biology.

C. alata leaves were dried and ground using a blender to get a simplicia powder. The powder was macerated with 70% ethanol, with the ratio of 1:5 (ratio of simplicia powder and solvent) and stirred by a shaker at 110 rpm for 18 h. The extraction was repeated until the macerate color resembled the color of the solvent. Then, the extract was evaporated with a vacuum evaporator at 50 °C, yielding of the thick extract (Angelina et al. 2020).

Solvents, reagents, and standards

The chemical used in the study were 70% ethanol, ether, methanol, HCl, CHCl₃, H₂SO₄, HNO₃, FeCl₃, NaNO₂, AlCl₃, NaOH, H₂O₂, Na₂CO₃, NaCl, Mayer reagent, Dragendorff reagent, Bouchardat reagent, Folin-Ciocalteau, Quercetin, Gallic acid, purchased from Merck.

Microbial culture media used were Peptone Water, Potato Dextrose Agar (PDA), Selenite Cystine Broth (SCB), Luria Broth (LB), Mueller-Hinton Agar (MHA), and Brain Heart Infusion (BHI), purchased from Himedia.
Organoleptic tests
The organoleptic test was carried out to observe the shape and color of simplicia and extract according to (Depkes RI 2000).

Physicochemical analysis
Physicochemical parameters include loss on drying, water content, total ash content, acid-insoluble ash content, water-soluble ash content, water-soluble extract content, and ethanol-soluble content.

Determination of loss on drying (LOD)
LOD was determined by gravimetric determination. 2–5 g of sample was placed in a crucible porcelain, dried at 105 °C for 60 min, then moved into a desiccator. This process was repeated until the constant weight was achieved. LOD was expressed as gram per gram of air-dried (World Health Organization 1998).

Determination of water content
Water content was determined by gravimetric method. 1 g of sample was heated in the oven at 105⁰C for 5 h, and then weighed. The process was continued with 1 h intervals until the difference between 2 consecutive weighings is not more than 0.25% (Depkes RI 2000).

Determination of total ash content
1 g of sample was placed in a silicate crucible and weighed. Sample was spread in an even layer in the crucible, and the material ignited by gradually increasing the heat to 500–600 °C until free from carbon, cooled in a desiccator, and weighed. Repeatedly until a fixed weight is obtained (World Health Organization 1998).

Determination of acid-insoluble ash content
Ash obtained from ash content testing was boiled with 25 mL HCl (~70 g/l) TS for 5 min. The ash is filtered with non-ash filter paper and washed with 5 mL hot water. The insoluble matter was transferred to the crucible, dried on a hot-plate and ignited to constant weight, and placed in a desiccator for 30 min, then weighed without delay. Content of acid-insoluble ash is calculated in mg per g of air-dried material (World Health Organization 1998).

Determination of water-soluble ash content
10 mg of extract was dissolved with 2 mL of 5% HCl and filtered. The filtrate was divided into 4 tubes. Drops of Mayer, Dragendorff, and Bouchardat reagents were added to each tube. The fourth filtrate serves as a positive control. The formation of a yellowish-white precipitate (Mayer), red–orange precipitate (Dragendorff), and brown precipitate (Bouchardat) indicates the presence of alkaloids (María et al. 2018).

Flavonoid screening
A few drops of concentrated HCl were added to a small amount of the extracts of the plant material. Immediate development of a red color was taken as an indication of the presence of flavonoids (Abdel-motaal et al. 2016).

Tannin screening
0.5 g of extract was boiled in 20 mL of water in a test tube and then filtered. A few drops of 0.1% FeCl₃ was added and observed for brownish green or a blue-black coloration (Edeoga et al. 2005).

Phenolic screening
0.2 g of thick extract was added with 2 mL of 5% FeCl₃ solution. A positive result is indicated by the formation of a bluish color (Adusei et al. 2019).

Saponin screening
0.5 g of extract was shaken with 10 mL of hot water. If foam produced persists for 5 min, it indicates the presence of saponins (Iqbal et al. 2015).

Terpenoid screening
Terpenoids screening was determined by Salkowski test 2 mL of sample was mixed in 2 mL of CHCl₃ and 2 mL concentrated H₂SO₄ was carefully added to form at a temperature of 450 °C until the weight remained. The reduction of the residue weight in mg is total ash weight. The water-soluble ash is calculated in mg per g of air-dried material (World Health Organization 1998).
a layer. A reddish brown color produced in the lower chloroform layer indicates the presence of terpenoid (Bargah 2015).

**Identification major compound using LC-MS**

Mass spectrometry was performed on a Xevo, G2-XS QTof (Waters MS Technologies) according to the method from (Angelina et al. 2020). Ionisation type is ESI. The scan range was from 100 to 1200 m/z. The capillary and cone voltage was set at 0.8 kV and 30 kV, respectively and was used positive electro spray mode. The desolvation gas was set to 1000 L/h at 500 °C and the cone gas was set to 50 L/h and the source temperature was 120 °C. The UPLC analysis was performed using a Waters Acquity Ultra Performance LC system. Chromatographic separation was carried out on an ACQUITY UPLC HSS T3 C18 column (100 mm x 2.1 mm, 1.7 μm) at a column temperature of 40 °C. The mobile phase consisted of solvent A (0.1% formic acid in water, v/v) and solvent B (0.1% formic acid in acetonitrile), with gradient polarity from 95:0.5 (A:B) to 0.5:95 (A:B). The flow rate was 0.3 mL/min. The column and auto sampler were maintained at 40 °C and 20 °C, respectively and the injection volume was 1 μL. The data acquisition and processing were performed using UNIFI. The parameter used was retention time (RT) in the range of 1–15 min. Determination of the dominant compound was carried out with semi quantitative determination based on the high intensity of the compound on the LC-MS/MS graph.

**Quantitative phytochemical analysis**

**Determination of total flavonoids content**

Total Flavonoid Content (TFC) was assessed by spectrophotometric method with minor modification (Dewi and Maryani 2015). For each sample, 500 μL of the samples (1000 µg/mL) were mixed with 2.2 mL of aquadest and 150 μL of 5% NaNO₂. After 5 min, 150 μL of 10% AlCl₃ was added. Then, 6 min later, 2 mL of 1M NaOH was added. The absorbance was read at 510 nm. Quercetin was taken as standard for the calibration curve. The total flavonoid content was expressed as mg quercetin equivalent/g dry extract.

**Determination of total phenolics content**

The total phenol content was determined by using the Folin-Ciocalteu method (Dewi and Maryani 2015) with few modifications. For each sample, 500 μL of the samples (1000 μg/mL) was added to 3.5 mL distilled water and 250 μL of 2N FC reagent. The mixture was incubated at room temperature for 8 min, and then 250 μL of 20% Na₂CO₃ was added to the mixture, and incubated again for 2 h. The absorbance was read at 765 nm. Gallic acid was taken as standard for the calibration curve. The total phenol content was expressed as mg gallic acid equivalent/g dry extract.

**Metal contamination evaluation**

The concentration of lead (Pb), cadmium (Cd), mercury (Hg), and arsenic (As) were determined by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). 1 g samples were digested with 9 mL HNO₃ and 3 mL H₂O₂. The mixture was heated at 180 °C for 25 min. After this destruction process, the sample was diluted with 0.5M HNO₃ until the volume reaches 50 mL and analyzed with Agilent 5110 ICP-OES.

**Microbial contamination evaluation**

Microbial contamination was investigated on Escherichia coli (LB), Salmonella sp & Shigella sp (SCB), Pseudomonas aeruginosa (Peptone Water), Total Plate Count (NA), Yeast & Mould Count (PDA). All mediums were dissolved with aquadest and sterilized 1mL of sample was placed at the petri dish (NA and PDA) and tubes (LB, SCB and Peptone Water), and mixed with 9 mL of each media tested. The plates and tubes were incubated at 27 °C for 24 h for bacteria and 3 days for molds/yeast.

**Antibacterial activity**

Antibacterial activity was assessed against Staphylococcus aureus ATCC 25923, Staphylococcus epidermidis ATCC 12228, Escherichia coli ATCC 25922, and Bacillus subtilis ATCC 6633. The minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined by the broth microdilution assay according to (Jorgensen and Ferraro 2009; Septama and Paniyachayupakaranant 2017) with few modifications. The bacteria were incubated with MHA at 37 °C for 24 h, and then transferred in the sterile 0.9% NaCl. The turbidity of the suspension was adjusted to the 0.5 McFarland standard solution, which was equivalent to 10⁶ CFU/mL. The MIC was performed using a 96-well microplate, consisted of 100 µL BHI for each well. For starting, 100 µL of 1000 µg/mL extract was added and diluted until final concentration on used was 500, 250, 125, 62.5 and 31.25 µg/mL. Afterwards, 100 µL of bacteria suspension was added. MIC was done at 37 °C after 24 h of incubation. MIC was defined as the lowest concentration of extracts at which the microorganism tested did not demonstrate visible growth. The MBC tests were performed by scratching suspension from the microplate at MIC, below and above MIC value. MBC was defined as the lowest concentration yielding negative subcultures or only one colony.

**Results and discussion**

**Organoleptic test**

The results of organoleptic observations from shape and color of simplicia and C. alata leaf can be seen in Fig. 2 and Table 1. The data obtained are used as a standard for good simplicia raw materials and extracts before being used for further testing. The simplicia of C. alata leaf from Bogor,
Table 1. Result of organoleptic test of simplicia and extracts C. alata leaf.

| Parameters          | Bogor Botanical Garden | South Tangerang | Kalimantan |
|---------------------|------------------------|-----------------|------------|
| Simplicia           |                        |                 |            |
| Shape               | Coarse powder          | Coarse powder   | Coarse powder |
| Color               | Brownish green         | Brownish green  | Brownish green |
| Extracts            |                        |                 |            |
| Shape               | Thick                  | Thick           | Thick      |
| Color               | Blackish brown         | Blackish brown  | Blackish brown |

Table 2. Results of physicochemical analysis from C. alata simplicia and extracts.

| Parameters          | Bogor Botanical Garden | South Tangerang | Kalimantan |
|---------------------|------------------------|-----------------|------------|
| Simplicia           |                        |                 |            |
| Loss on drying (%)  | 5.09                   | 5.15            | 5.47       | 3.93       |
| Water content (%)   | -                      | 3.44            | 2.12       | 4          |
| Total ash content (%)| 6.94                   | 9.41            | 7.10       | 5.14       |
| Acid-insoluble ash content (%)| 0.65 | 0.39 | 5.41 | 0.29 |
| Water soluble ash content (%)| 1.93 | 7.14 | 1.26 | 1.95 |
| Water soluble content (%)| 19.60 | 39.43 | 27.23 | 29.72 |
| Ethanol soluble content (%)| 22.91 | 13.99 | 24.18 | 33.76 |
| Extracts            |                        |                 |            |
| Loss on drying (%)  | 9.35                   | 19.76           | 17.38      | 8.51       |
| Water content (%)   | -                      | 17.07           | 6.22       | 6.94       |
| Total ash content (%)| 8.91                   | 17.07           | 6.22       | 6.94       |
| Acid-insoluble ash content (%)| 3.05 | 3.82 | 2.44 | 0.52 |
| Water soluble ash content (%)| 8.54 | 7.97 | 1.43 | 2.79 |
| Water soluble content (%)| 77.51 | 66.38 | 73.71 | 58.45 |
| Ethanol soluble content (%)| 59.79 | 69.53 | 75.39 | 75.20 |

Figure 2. (A–D) The organoleptic profiles of C. alata simplicia. A: Bogor; B: Bogor Botanical Garden; C: South Tangerang; D: Kalimantan. It had rough shape with a brownish green color. (E, F) The organoleptic profiles of C. alata ethanol extract. E: Bogor; F: Bogor Botanical Garden; G: South Tangerang; H: Kalimantan. It was thick and brownish black in color.

Physicochemical analysis

Table 2 shows the result of physicochemical analysis of C. alata simplicia and extracts. The loss of drying (LOD) determines the maximum limit range of the compound lost during the drying process. During the drying process, the air and compounds with a lower boiling point than air will evaporate (Depkes RI 2000). According to the results, losses of drying from simplicia from the lowest were C. alata from Kalimantan 3.93%, Bogor 5.09%, Bogor Botanical Garden 5.15%, and South Tangerang 5.47%. While losses on drying of extract from the lowest were Kalimantan 8.51%, Bogor 9.35%, South Tangerang 17.38%, and Bogor Botanical Garden 19.76%, respectively. The LOD value of the extract we examined was higher than the previous study (Gritsananp and Mangmeersri 2009) whose LOD value was no more than 6%. One of the causes is due to the difference in solvents in the extraction process. In this study we used ethanol 70% while in the previous study used ethanol 80%. The high water content in the solvents we use is one of the things that cause the extract’s LOD value to be higher.

Determination of total ash content was carried out to measure the total amount of external and internal mineral in simplicia or extract from the initial process to the end of manufacture. The high value of the total ash content indicates that the sample contains minerals. Total ash content in simplicia was 5.4%–9.41% and in extract was 6.94%–17.07%. In this test there are two parameters to determine type of nutrient absorbed by plants, acid-insoluble ash content and water-soluble ash content. Water-soluble ash content was tested by dissolving the ash into water, and determined the amount of mineral soluble in water, while the acid-insoluble ash content indicates the amount of mineral insoluble in acid. The high value of acid-insoluble ash content indicates that the sample has a silicate component associated with soil or sand, silver, lead or mercury (Salim 2016). Acid-insoluble ash content in simplicia was 0.29%–5.41% and in extract was 0.52%–3.82%. Water-soluble ash content in simplicia was 1.26%–7.14% and in extract was 1.43%–8.54%. Based on the results, the value of water-soluble ash content is higher in simplicia and extract indicates that the sample does not contain much silica component or other impurities.

The soluble content parameter determines the amount of chemical components in simplicia and extracts in certain solvents. The chemical compounds in the sample which can be dissolved are related to reproducibility in the pharmacodynamic activity of the crude drugs. The ability of each plant to dissolve in water or other organic solvents is different. Water solvent used to dissolve polar compounds and ethanol to dissolve the less polar compounds (Wirnawati et al. 2020). Water-soluble content in simplicia was between 19.60%–39.43%, and in extract was 58.45%–77.51%. The ethanol-soluble content in simplicia was 13.99%–33.76% and in extract was 59.79%–75.39%. Based on the results (Table 2) water-soluble content value...
in simplicia and extract is higher than the levels of ethanol-soluble content, which indicates that the sample of simplicia and extract contain more polar substances which make it easier to dissolve in water than ethanol.

**Phytochemical analysis**

Phytochemical screening was carried out to identify the phytoconstituents present in the *C. alata* leaf extract. Based on the screening (Table 3), the compounds contained in the ethanol extract of *C. alata* leaves from Bogor, Bogor Botanical Garden, South Tangerang, and Kalimantan are alkaloid, flavonoid, terpenoid, saponin, and tannin. The results obtained are in accordance with the research conducted by (Kumavat et al. 2011) which showed that the phytochemical components contained in the ethanol extract of *C. alata* leaves were alkaloids, tannins, steroids, glycosides, sterols, terpenoids, flavonoids. As well as research conducted by (Raji et al. 2015; Nagarajan 2018) which showed that the content of phytochemical compounds in the ethanol extract of *C. alata* leaves were phenols, steroids, flavonoids, terpenoids and alkaloids.

Table 3. Qualitative and quantitative phytochemical determination of extract *C. alata* leaf extract.

| Parameters | Bogor | Bogor Botanical Garden | South Tangerang | Kalimantan |
|------------|-------|------------------------|-----------------|------------|
| Alkaloid   | -     | -                      | -               | -          |
| Meyer      | -     | -                      | -               | -          |
| Bouchardat | +     | +                      | +               | +          |
| Flavonoid  | +     | +                      | +               | +          |
| Terpenoid  | +     | +                      | +               | +          |
| Saponin    | ++    | ++                     | ++              | ++         |
| Tanin      | +     | +                      | +               | +          |
| Total flavonoids content (%QE) | 14.13 | 10.15 | 9.86 | 15.74 |
| Total phenolics content (%GAE) | 6.75 | 6.67 | 7.65 | - |

Alkaloids are semi polar, have a nitrogen atom that has a lone pair of electrons that will bond covalently to the iodine ion in the reagent and have substituents such as phenol, amine, amide and methoxy (Kurmukov 2013). Alkaloids qualitative tests were carried out using Dragendorf, Meyer and Bouchardat reagents. The results showed the presence of alkaloids with the formation of orange sediment in Dragendorf’s reagent and brown sediment in Bouchardat’s reagents, while in Meyer’s reagents there was no sediment. Terpenoids consist of several compounds including volatile monoterpenes, sesquiterpenes, volatile diterpenes, non-volatile triterpenes and sterols. It is characterized by the formation of a red, pink or violet color. The results showed that the extract of *C. alata* contained terpenoids. Saponin is a secondary metabolite belonging to surface compounds and found in many plants. Saponins are characterized by the presence of foam after shaking in aqueous solution. The results showed that the extract of *C. alata* leaf contains saponins. Tannins are compounds that are commonly found in vascular plants, have a phenol group and astringent taste (Kurmukov 2013). Based on the results, the extract of *C. alata* leaf contained tannin compounds. This was indicated by a change in the color of the solution to blackish green.

The measurement data using LC-MS is seen at Table 4 and the chromatograms are shown in Fig. 3. The chemical constituents detected were emodin, kaempferol, kaempferol-3,7-diglucoside, and kaempferol-3-O-β-D-glucopyranoside (Fig. 3). It can be seen that the % area detected from each compound from four sources in Indonesia was different, hence it can be concluded that the phytochemicals of extracts from different locations were different.

From our previous study (Angelina et al. 2020), the ethanolic extract of *C. alata* leaf contained 5,7,2,5'-tetrahydrox-flavone, daturametelin H, and kaempferol-3,7-diglucoside. Metabolic profiles of medicinal plants are mainly controlled by genetic factors, but their production is also influenced by environmental factors (Muraina et al. 2008; Mohammadi Bazargani et al. 2021). In another studies, it has been reported that chemical constituents of *C. alata* leaf extract were aloe emodin, emodin, ω-hydroxyemodin, lunatin, phycion, zиганин, apigenin, 7,4'-dihydroxy-5-methoxyflavone, diosmetin, kaempferol, luteolin, trans-dihydrokaempferol, trans-resveratrol (Promgool et al. 2014). From numerous studies, flavonol compounds which have been identified were kaempferol, kaempferol-3-O-β-D-glucopyranoside, 3,5,7,4'-tetrahydroxy flavone, 2,5,7,4'-tetrahydroxy isoflavones, antirrhoine and kaempferol 3-O-gentiobioside (Fatmawati et al. 2020). Alkaloid compounds from *C. alata* leaves have also been identified, such as adenine, chrysoeriol, quercetin, 5,7,40-trihydrallavanone, kaempferol-3-O-beta-D-glucopyranosyl-(1→6)-beta-D-glucopyranoside, n-dotriacontanol, n-triacontanol, stearic acid, palmitic acid, diostem, luteolin (Joel et al. 2017), and 1,3,5-trihydroxy-7-methylanthracene-9,10-dione (Promgool et al. 2014; Joel et al. 2017; Fatmawati et al. 2020).

The total flavonoids content (Table 3) ranged from 9.86% to 15.74% QE. The highest concentration was found in *C. alata* from Kalimantan (15.74%) followed by Bogor (14.13%), Bogor Botanical Garden (10.5%) and South Tangerang (9.86%). Quantitative analysis of total

Table 4. LC-MS analysis of *C. alata* leaf extract.

| Compounds          | Molecular weight | Molecular formula | RT % Area | RT % Area | RT % Area | RT % Area |
|--------------------|------------------|-------------------|-----------|-----------|-----------|-----------|
| Emodin             | 270.03282        | C13H14O3          | 6.66 & 7.58 | 2.64 | 6.58 & 7.5 | 3.99 | 6.58 & 7.5 | 3.03 | 6.60 & 7.5 | 2.24 |
| Kaempferol         | 286.04774        | C13H13O3          | 6.73      | 8.04 | 6.65 & 12.86 | 9.66 | 6.67 | 12.06 |
| Kaempferol-3,7-diglucoside | 587.15338 | C13H13O3 | 4.48 | 9.77 | 4.48 | 14.59 | 4.48 | 18.41 | 4.48 | 37.70 |
| Kaempferol-3-O-β-D-glucopyranoside | 425.10056 | C13H14O5 | 4.99 | 3.04 | 4.98 | 4.17 | 4.98 | 4.18 | 4.98 | 3.31 |
flavonoid compounds was carried out with measurements based on the formation of complexes between AlCl₃ with ketone groups on C-4 atoms and hydroxyl groups on C-3 or C-5 atoms adjacent to flavones and flavonols (Nurlinda et al. 2021). The standard solution in this study was quercetin, a flavonol that is widespread in plants, and made up to 60–75% of flavonoids (Nurlinda et al. 2021).

The concentration of total phenolic content is given in terms of GAE/g of the extract (Table 3) and ranges from 6.67 to 7.65% GAE. The highest amount of phenolic content was observed in *C. alata* extract from South Tangerang (7.65%), followed by Bogor (6.75%), and botanical garden (6.67%).

Phenolic compounds are widely distributed in a variety of plants. Natural phenolic can range from simple molecules, such as phenolic acid, to highly polymerised compounds, such as tannins (Debebe et al. 2016). The total phenolic content of the extract *C. alata* leaf was determined using Folin-Ciocalteu’s reagent. Phenols reacted with the Folin-Ciocalteu reagent in the presence of sodium carbonate to form a blue-coloured complex. The intensity of the blue color is proportional to the amount of reactive phenolic compounds in the sample (Kupina et al. 2019). It is also mentioned in (Debebe et al. 2016), that the presence of compounds in plants is largely influenced by genetic factors and environmental conditions, other factors such as germination, degree of ripeness, variety, processing and storage. Based on the results obtained, it shows that the area where *C. alata* grows affects the total amount of flavonoids and phenolics.

**Contamination evaluation**

Heavy metal contamination testing aims to determine the levels of metal content, i.e. Pb, Cd, Hg and As contained in extract, which is dangerous and toxic to the body. The results obtained on the levels of Pb, Cd, Hg and As shows the absence of heavy metals in *C. alata* leaf extract (Table 5), hence the samples meet the standards, where the maximum limit for Pb is ≤ 10 mg/kg, Cd ≤ 0.3 mg/kg, Hg ≤ 0.5 mg/kg and As ≤ 5 mg/kg in herbal capsule containing dry extract (Vinet and Zhedanov 2011).

Microbial contamination reveals the impurity in medicinal plants, which come from the preparation or final products. Based on microbial contamination test, the total plate number shows the absence of bacteria in the extract of *C. alata* leaf (Table 5), hence the samples meet the standard of (BPOM 2014) which shows that the maximum limit range for the total plate number test is ≤ 10⁵ CFU/g.

The results of total yeast and mold number test extract of *C. alata* leaf from Bogor, South Tangerang and Kalimantan did not contain yeast and mold, while extract of *C. alata* leaf from Bogor Botanical Garden contained yeast and mold of 1×10² CFU/g. The result was still below the limit, where the limit of mold and yeast contamination that may be present in capsule preparations containing dry extract is ≤ 10³ CFU/g (BPOM 2014).

The identification test of pathogenic bacteria is carried out to detect the presence of pathogenic bacteria including *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella* sp, and *Shigella* sp. Based on the test results, in all samples there were no pathogenic bacteria tested. This met the standard of (BPOM 2014) which shows that the

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**Table 5. Metal and microorganism contamination of extract C. alata leaf.**

| Parameters               | Bogor | Bogor Botanical Garden | South Tangerang | Kalimantan |
|--------------------------|-------|------------------------|-----------------|------------|
| Pb                       | -     | -                      | -               | -          |
| Cd                       | -     | -                      | -               | -          |
| Hg                       | -     | -                      | -               | -          |
| As                       | -     | -                      | -               | -          |
| Total plate number       | 0     | 0                      | 0               | 0          |
| Total yeast and mold number | 0   | 1×10⁰                  | 0               | 0          |
| Pathogen bacteria        |       |                        |                 |            |
| *Escherichia coli*       | -     | -                      | -               | -          |
| *Pseudomonas aeruginosa* | -     | -                      | -               | -          |
| *Salmonella* sp          | -     | -                      | -               | -          |
| *Shigella* sp            | -     | -                      | -               | -          |
maximum limit of *E. coli* contamination is ≤ 10 CFU/g, for *Salmonella* and *Shigella* negative/g.

**Antibacterial activity**

Antibacterial activity of *C. alata* leaf can be seen in Table 6. From the MIC value, the extract from all areas showed similar antibacterial strength against all bacteria tested whether Gram positive bacteria (*S. aureus*, *E. coli*, and *S. subtilis*) or Gram-negative bacteria (*E. coli*). The MIC of all samples against *S. aureus* was 250 µg/mL, meanwhile for the rest of bacteria tested the MIC was 125 µg/mL. From the MBC value, the lowest concentration is from extract from South Tangerang and Kalimantan which was 1000 µg/mL against *S. aureus* and 250 µg/mL against other bacteria, whilst the highest MBC was from Bogor which was 1000 µg/mL against *S. aureus* and 500 µg/mL against other bacteria. It can be seen that the geographical factor from the plants affects its antibacterial activity.

The antibacterial activity from this study confirms previous study (Khan et al. 2001; Somchit et al. 2003) where it reported *C. alata* leaves extract antibacterial activity. The MIC and MBC values against *E. coli* bacteria obtained in this study were similar to the values of MIC and MBC in previous studies (El Mahmood AM 2008). The antibacterial effect of the *C. alata* leaves extracts may be due to presence of flavonoids, phenols, alkaloids, saponins, tannins, and terpenoids detected (Wikaningtyas and Sukandar 2016). Several researches have investigated the isolated compound of *C. alata* which are responsible for antibacterial activity. Bioactive compounds such as kaempferol, luteolin, and aloemodin isolated from the methanolic leaves extract showed antibacterial activity against MDR bacteria strains, which could be linked to the anthraquinone and flavonoids compounds detected (Joel et al. 2017). Compounds detected in this study also were reported have antibacterial activity. Emodin isolated from *C. alata* showed strong antibacterial activity against MRSA with MIC value of 4 µg/mL and moderate antibacterial activity against *B. cereus* and *S. aureus* MIC value of 16 µg/mL, but showed weak antibacterial activity against Gram-negative bacteria (MICs 128 µg/mL) (Promgool et al. 2014). Kaempferol 3-O-β-glucopyranoside exhibited MIC values of 13.0 ± 1.5 µg/mL against MRSA (Hazni et al. 2008). Kaempferol and kaempferol-3,7-diglucoside also have been investigated to have antibacterial activity (Saito et al. 2012; Mahmood 2014). Other studies have also reported that *C. alata* has activity against microorganisms that cause skin diseases in human (Ibrahim and Osman 1995).

**Table 6. Antibacterial activity of extract *C. alata* leaf (µg/mL).**

| Bacteria              | Bogor Botanical Garden | South Tangerang | Kalimantan |
|-----------------------|------------------------|-----------------|------------|
|                       | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC |
| *Escherichia coli*    | 125 | 500 | 125 | 250 | 125 | 125 | 250 | 250 |
| *Staphylococcus aureus*| 250 | 1000| 250 | 1000| 250 | 1000| 250 | 1000|
| *Staphylococcus epidermidis* | 125 | 500 | 125 | 500 | 125 | 250 | 125 | 250 |
| *Bacillus subtilis*   | 125 | 500 | 125 | 250 | 125 | 250 | 125 | 250 |

In summary, findings from this investigation suggest that the physicochemical and phytochemical properties of *C. alata* from several locations in Indonesia fulfilled the requirements as raw material for herbal drugs and showed similar antibacterial activity. Future experiments aimed at separation and isolation of individual compounds, as well as other *C. alata* pharmacological effects and its mechanisms which are still unexplored sufficiently.

**Funding**

This work was supported by LPDP Covid-19 Mandatory Program BRIN for Research Grant 2020/2021.

**Conflicts of interest**

The authors declare no conflicts of interest.

**Acknowledgements**

The authors would like to thank the LPDP/BRIN Covid 19 Funding Programme, The Deputy of Engineering Sciences, Research Center for Chemistry, National Research and Innovation Agency Republic of Indonesia (BRIN) who has provided facilities for conducting research. The first author who is also the correspondent author is the main contribution to this work.

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