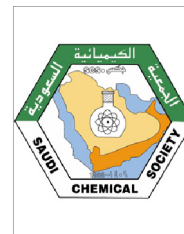




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ORIGINAL ARTICLE

Phenolic and flavonoid compounds extraction from *Calophyllum inophyllum* leaves



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Abstract *Calophyllum inophyllum* has been known as a part of the mangrove forest area. This species is distributed primarily in the coastal regions of Indonesia and Africa. It is rich in bioactive compounds and has been used as a traditional medication. This work employed a single replicate of the one-factor-at-a-time experiment method to investigate optimum conditions, which resulted in the highest TPC. The three factors studied were organic solvent type (acetone, ethanol, and methanol), organic solvent concentration in water (50–100%, v/v), and extraction temperature (30–60 °C). The extraction was conducted with the percolation method. The result shows that organic solvent type, organic solvent concentration in water, and extraction temperature significantly affect the TPC, TFC, and the yield of crude extract obtained. The highest TPC (289.12 mg GAE/g of the residue of *C. inophyllum* leaves) was achieved with 80% methanol in water at 30 °C for 48 h. Under this condition, TFC value of 410.4 mg QE/g of the residue of *C. inophyllum* leaves, the yield of 2.41%, and IC₅₀ value of 0.054 µg/mL were achieved. Moreover, bis (2-ethylhexyl) phthalate was firstly detected in the extract.

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1. Introduction

Natural antioxidants are the bioactive compounds from a plant that can inhibit the development of cancer, diabetes, and other various long-term diseases (Atanassova et al., 2011; Lai et al., 2012). Phenolics are the main category of antioxidants obtained from nature which become the most extensively researched. Phenolics can prevent various degenerative diseases and exhibit high antioxidant activity (Evans et al., 1997). There are many types of phenolic compounds from plants, including phenolic acids (hydroxycinnamic acids and hydroxybenzoic), coumarins, flavonoids, xanthenes, and polyphenols (lignins and tannins) (Do et al., 2014).

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Calophyllum inophyllum is one of the mangrove plants from the *Clusiaceae* family. The tree is commonly found with a minimum height of 8 m, a maximum height of 20 m, and widely distributed in various regions of Indonesia and Africa, especially in the coastal areas. Occasionally, the tree can reach a height of 35 m (Prabakaran and Britto, 2012). The leaves of *C. inophyllum* are hairless, smooth, and glossy. They have a dark green color, parallel veins that grow from the midrib, and elliptical edges around 6–9 cm wide and 10–20 cm long. The veins are aligned to each other and transversal to the midline of the leaf (Friday and Okano, 2006). Traditionally, they are used to treat various skin problems like skin rash, ulcers, and sores (Lim, 2012).

In Indonesia, the seed oil of *C. inophyllum* is used as a biodiesel feedstock, while the leaves are still underutilized. *C. inophyllum* leaves were reported to be potential sources for central nervous system depressant, antiinflammatory, antiulcer (Gopalakrishnan et al., 1980), antiviral (Patil et al., 1993), anticancer (Itoigawa et al., 2001), antimicrobial (Yimdo et al., 2004), and an inhibitor of Human Immunodeficiency Virus (HIV) (Laure et al., 2008). Several compounds have been found in *C. inophyllum* leaves, including coumarins (e.g., inophyllum C, inophyllum E, inophyllum A, inophyllum B, inophyllum P), chromanones (e.g., inophynone, isoinophynone), triterpenes (e.g., friedelin, canophyllol, canophyllal) (Su et al., 2008). Moreover, Susanto et al. (2017) found that alkaloids (11.51%), saponins (2.16%), triterpenoids (2.48%), flavonoids (2.37%), polyphenols (2.53%), and tannins (7.68%) of *C. inophyllum* leaves were soluble in methanol fraction. Because there are various bioactive contents in *C. inophyllum* leaves that can be utilized as herbal medicine ingredients, the research on these leaves has been widely developed.

Extraction, separation, and purification are involved in bioactive compound identification. Extraction is the crucial one to separate and recover phenolic compounds from plant matrixes. This process is affected by chemical properties, dimension of solid particles, solvent, extraction method, time (Nacz and Shahidi, 2004), and temperature (Kalt, 2005). Total phenolic content (TPC) and total flavonoid content (TFC) of *C. inophyllum* leaves in absolute methanolic extract had been reported in previous work (Dutta and Ray, 2014). Several types of phenolic or flavonoid compounds had also been isolated and identified (e.g., 3-oxofriedelin-28-oic acid, amentoflavone, calophyllic acid, canophyllic acid, and shikimic acid) (Prasad et al., 2012). However, the type of organic solvent, concentration of organic solvent in water, and temperature of extraction necessary for phenolic and flavonoid compounds extraction had not been previously studied. Thus, the purpose of this work was to investigate the effect of type of organic solvent, the concentration of organic solvent in water, and temperature of extraction toward the TPC, TFC, yield, and antioxidant activities against DPPH (2,2-diphenyl-1-picrylhydrazyl) free radicals of extract obtained from *C. inophyllum* leaves. Moreover, the type of compounds in the extract of *C. inophyllum* leaves was also investigated.

2. Material and methods

2.1. Materials

The dried leaves of *C. inophyllum* were obtained from the Jarak Lestari company in Central Java, Indonesia. Aluminum chloride anhydrous, Folin-Ciocalteu reagent, quercetin, sodium carbonate anhydrous, sodium hydroxide, sodium nitrite, 2,2-diphenyl-1-picrylhydrazyl, and gallic acid were purchased from Sigma Aldrich (Sternheim, Germany). All solvents were obtained from Chemical Indonesia Multi Sentosa (Surabaya, Indonesia).

2.2. Extraction of non-polar compounds

The leaves (2 kg) were ground and sieved to a size of 150 mesh. The non-polar compounds of *C. inophyllum* leaves were

removed using n-hexane as a solvent, and then the powder of *C. inophyllum* leaves (2 kg) was soaked in n-hexane (6 L) for 72 h. This mixture consisted of the solid and liquid phase, which was then separated through filter paper. After filtration, the residue (solid phase) was dried at ambient temperature for 24 h, and it was designated as RCILP (residue of *C. inophyllum* leaves powder). RCILP was refrigerated at 4 °C for further investigation. The flow diagram of non-polar compounds extraction is depicted in Fig. 1.

2.3. Extraction of polar compounds

RCILP was extracted with three different factors and each factor had three different levels: (i) polar solvents (acetone, methanol, ethanol), (ii) solvent concentration in water (50%, 80%, 100% (v/v)), and (iii) temperature (30 °C, 45 °C, 60 °C), to obtain phenolic compounds. At first, RCILP (20 g) was extracted with the various extracting solvents (100% acetone, 100% ethanol, and 100% methanol) at the volume of 200 mL. The extraction process was conducted by the percolation method at 30 °C for 48 h. The mixture was separated through filter paper to obtain its residue. This percolation method was repeated two times with the same condition. After filtration, the filtrate was combined, evaporated by distillation, and designated as a crude extract. Next, it was weighed to obtain the yield. TPC and TFC of these extracts were investigated to find the optimal solvent with the highest TPC value, as seen in Fig. 2.

After the optimal solvent had been determined, RCILP was extracted using the optimal solvent with various solvent concentrations in water (50, 80, 100 %, v/v). Other operation conditions remained the same. The flow diagram to determine optimal solvent concentration is shown in Fig. 3. The optimal solvent concentration was chosen based on the crude extract, which had the highest TPC value. After that, RCILP was extracted using solvent type and concentration which had the optimal result at 30 °C, 45 °C, and 60 °C with extraction time fixed at 48 h. The extraction process to obtain optimal temperature can be seen in Fig. 4.

2.4. Total phenolic content (TPC) analysis

The Folin-Ciocalteu method was used to estimate TPC in each extract as reported elsewhere (Dutta and Ray, 2014), with

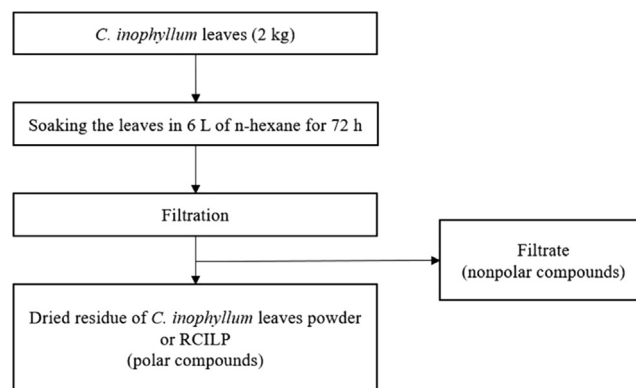


Fig. 1 Flow diagram of non-polar compounds extraction.

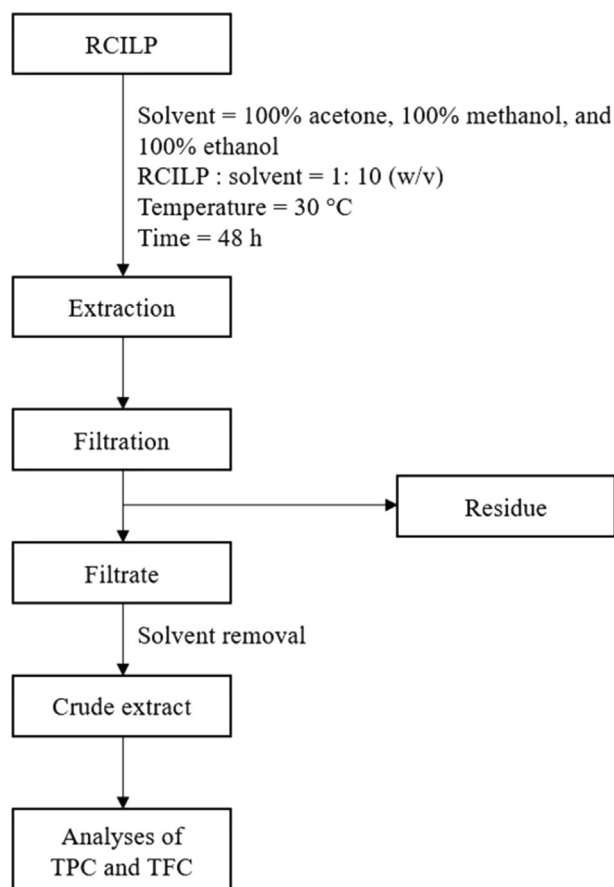


Fig. 2 Extraction of RCILP to determine the optimal solvent.

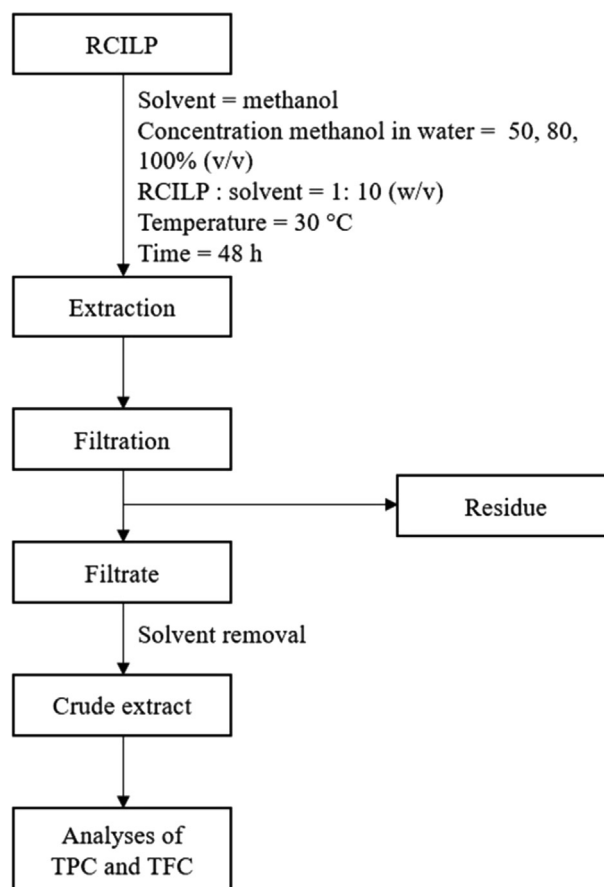


Fig. 3 Extraction of RCILP to determine the optimal solvent concentration.

some adjustments. The dried crude extract was added to distilled water, so the concentration of crude extract became 5 mg/L. Folin-Ciocalteu reagent was prepared in distilled water (5-fold dilution). Next, a Folin-Ciocalteu reagent (0.2 mL) was added to each diluted extract (1.6 mL) and vortexed for 3 min. Subsequently, the mixture was added by 0.2 mL of sodium carbonate (10%), and it was set aside for 30 min at room temperature. After that, UV-Vis spectrophotometer Genesys 10 (USA) was used to read the absorbance of each sample at 760 nm. TPC was calculated as milligram gallic acid equivalent per gram RCILP (mg GAE/g RCILP). Gallic acid with a concentration between 0 and 60 µg/mL was used to plot the calibration curve.

2.5. Total flavonoid content (TFC) analysis

The aluminum chloride colorimetric method was employed to estimate TFC in each extract as described by Dutta and Ray (2014), with some adjustments. Extract (1 mg) was liquefied in distilled water to a volume of 1 mL. Then, distilled water (1 mL) was added to it. After that, 3 mL of 5% sodium nitrite and 0.3 mL of 10% aluminum chloride were added to the mixture. Both sodium nitrite and aluminum chloride were prepared in distilled water. After 6 min, sodium hydroxide (1 M, 2 mL) was added to the mixture, continued by the addition of distilled water until the volume reached 10 mL. Then, the mixture was put in the darkroom, while the time was fixed

at 30 min. The absorbance of the mixture was recorded at 510 nm with a spectrophotometer. TFC was calculated as milligram quercetin equivalent per gram RCILP (mg QE/g RCILP).

2.6. Antioxidant activity

DPPH or 2,2-diphenyl-1-picrylhydrazyl was applied to evaluate the antioxidant activity of extract as previously described (Dutta and Ray, 2014), with some adjustments. DPPH is a compound famous for its free radical nature that becomes colorless when it accepts an electron or hydrogen radical. Fine powder DPPH was dissolved in methanol to yield a DPPH solution with a concentration of 0.02 mg/mL. Standard (gallic acid) and each extract were dissolved in methanol with different concentrations (0.01, 0.1, 1, and 10 µg/mL). Then, DPPH solution (2 mL) was reacted with 2 mL of each sample in the test tube. After that, they were put in the darkroom with the time fixed at 30 min. Each sample's absorbance was analyzed by using a spectrophotometer, and the wavelength was set at 517 nm.

2.7. Isolation and identification of bis (2-ethylhexyl) phthalate

A total of 3 g methanolic extract was extracted with n-hexane as a solvent. The ratio of methanolic extract and n-hexane was 1/50 (g/g). This solid-liquid extraction was processed until the

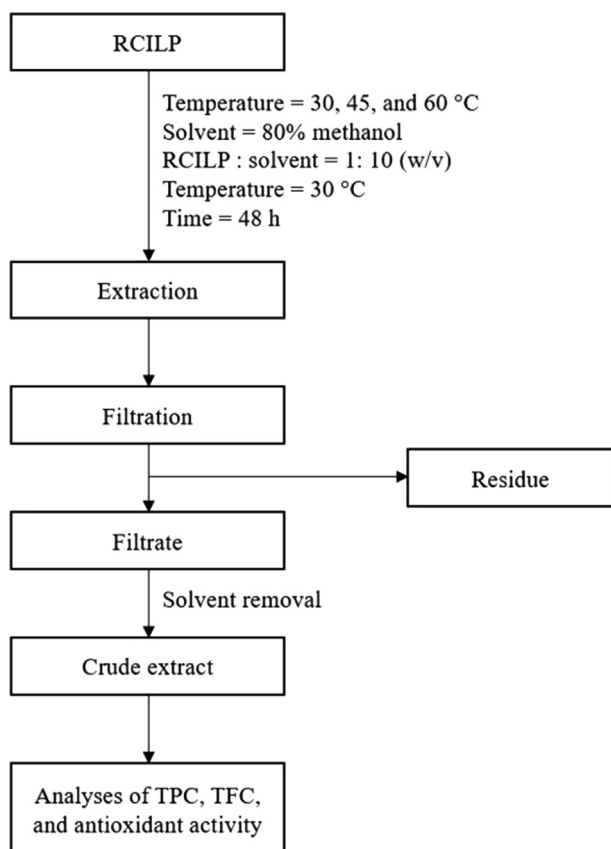


Fig. 4 Extraction of RCILP to determine the optimal temperature.

13 stages of extraction. Afterward, n-hexane fraction from each stage was accumulated and followed by solvent removal. The dried n-hexane fraction was mixed with 100 mL of acetone and 100 mL of chloroform. Next, the mixture was put in the refrigerator (4 °C) for 48 h. The crystal and liquid fraction formed after 48 h were separated with filter paper. Then, methanolic extract, n-hexane fraction, crystal fraction, acetone, and chloroform fraction were analyzed using gas chromatography (GC) analysis. Meanwhile, the crystal fraction was further analyzed by gas chromatography-mass spectrometry (GC-MS) analysis. The flowchart is shown in Fig. 5.

2.8. Gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analysis

The methods of GC and GCMS analyses were described elsewhere (Susanto et al., 2019).

2.9. Statistical analysis

This study used the one-factor-at-a-time method with single replication. The software used to conduct statistical analysis was Minitab 18. Meanwhile, a one-way analysis of variance (ANOVA) with Tukey's test was used to check the significant difference of variables. The variable was considered significant when the p -value < 0.05.

3. Results and discussion

Phenolic compounds consist of aromatic rings with one or more hydroxyl groups attached directly to them. They are

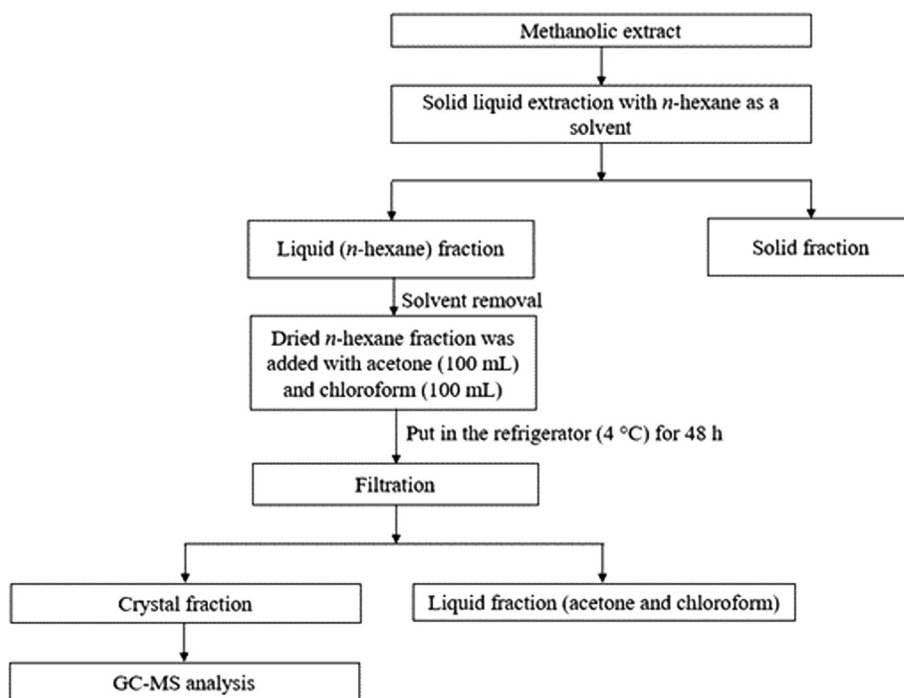


Fig. 5 Isolation and identification of bis (2-ethylhexyl) phthalate.

categorized as flavonoids and non-flavonoids. Flavonoids have 15 carbons (C6-C3-C6 carbon skeleton). Meanwhile, there are 8 (eight) subgroups, such as curcuminoids, coumarins, lignans, quinones, phenolic acids, stilbenes, and tannins which belong to the non-flavonoid group (Gan et al., 2019). By understanding the importance of phenolic compounds and their activities as antioxidants, the extraction of phenolic compounds from the leaf of *C. inophyllum* can lead to the development of mangrove areas.

Hydroxyl groups in phenolic compounds can be coupled with alkyl groups, acid, or sugar. Each one has a different polarity, making it difficult to extract all types of phenolic chemicals efficiently using a single approach. Moreover, phenolic compounds can be degraded at a high temperature of extraction (Kalt, 2005). Separating and isolating phenolic compounds from the leaf of *C. inophyllum* requires a thorough examination of the extraction condition.

Identifying a suitable solvent is critical in the herbal medicine development process because phenolic compounds are frequently found in small quantities. Method to obtain bioactive compounds from plant matrixes has been developed through years, such as maceration, microwave-assisted extraction, percolation, Soxhlet extraction, and ultrasound-assisted extraction. In this study, percolation was employed because it was the simplest method, and the temperature of extraction was adjustable. There were two steps for extraction. Firstly, non-polar compounds (terpenes and triterpenoids) of dried powder of *C. inophyllum* leaves were extracted by n-hexane. Secondly, the residue was extracted with three different factors, and each factor had three different levels: (i) polar solvents (acetone, methanol, ethanol), (ii) solvent concentrations in water (50%, 80%, 100%, v/v), and (iii) extraction temperatures (30 °C, 45 °C, 60 °C) to obtain phenolic compounds. The one-factor-at-a-time method was applied to select a set of baseline levels for each factor, then altering each factor over its range while keeping the other factors constant at the baseline level. The extracted compounds from *C. inophyllum* leaves were quantitatively measured and compared.

TPC of extracts was estimated from the Folin-Ciocalteu method by plotting gallic acid concentration (x) versus absorbance (y). TPC is stated as milligram gallic acid equivalent per gram residue of *C. inophyllum* leaves powder (mg GAE/g RCILP). Calibration curve obtained ($R^2 = 0.9443$) is:

$$y = 0.0274x + 0.1739, \quad (1)$$

Meanwhile, the colorimetric method was required to estimate the TFC of extracts by plotting quercetin concentration (x) versus absorbance (y). TFC is stated as milligram quercetin equivalent per gram residue of *C. inophyllum* leaves powder (mg QE/g RCILP). TFC calibration curve obtained ($R^2 = 0.9769$) is:

$$y = 0.0005x + 0.0124 \quad (2)$$

3.1. Effect of solvent type

In this study, three polar solvents were employed to figure out the optimal solvent regarding phenolic compounds extraction from *C. inophyllum* leaves. They were acetone, methanol, and ethanol. The difference in solvent polarity influenced extraction yield and the solubility of chemical components pre-

sent in a mixture. There was no study found about the extraction yield of *C. inophyllum* leaves. Table 1 shows the effect of organic solvent type on the extraction yield, TPC, and TFC. The maximum extraction yield (2.58%) was achieved using methanol, followed by ethanol (2.44%) and acetone (2.27%). It has shown that the extraction yields of *C. inophyllum* leaves are significantly affected by solvent polarities because acetone, ethanol, and methanol are polar solvents with relatively equal polarity index of 5.4, 5.2, and 6.6, respectively (Snyder, 1974). Moreover, several factors affect the solubility of phenolic compounds, such as intramolecular interaction, polymerization degree, and insoluble complexes compound formation (Naczak and Shahidi, 2004).

The determination of a suitable solvent is necessary to optimize the recovery of phenolic and flavonoid compounds from the plant (Zhao et al., 2006). In this research, the highest TPC and TFC value belong to the methanolic extract, 143.14 mg GAE/g RCILP and 804.30 mg QE/g RCILP, respectively. These results agree with previous work that the optimal solvent for extracting bioactive components from *Severinia buxifolia* branches was methanol, compared to distilled water, ethanol, acetone, chloroform, and dichloromethane (Truong et al., 2019). It shows that strongly polar solvent has the highest extraction performance and solubility for phenolic compounds. Pure acetone has the lowest number of phenolic compounds. It could be attributed to polyphenols' limited solubility in acetone, caused by interactions between hydrogen in polyphenols and hydrogen in proteins structure (Sripad et al., 1982). Tan et al. (2013) reported that methanol is the optimal solvent to extract a higher phenolic acid and catechin yield. The suitable solvent to extract flavonoid and catechol is ethanol. Acetone provides a better yield to extract tannins. Another study by Dai and Mumper (2010) mentioned that methanol is excellent in lower molecular weight polyphenols extraction (e.g., flavonoids). Thus, methanol is the optimal solvent for extraction due to its high yield, TPC, and TFC value. The finding in this work shows that the TPC value is lower than the TFC value. TPC value is expressed as gallic acid equivalent. Meanwhile, the TFC value is expressed as quercetin equivalent.

3.2. Effect of solvent concentration

Various bioactive compounds have different solubility in a solvent (Turkmen et al., 2006). A combination of water and the organic solvent was frequently employed for recovering polyphenols from plant matrixes. Water content in organic solvent improves extraction performance for compounds that are highly soluble in organic solvents and/or water (Boskov et al.,

Table 1 The effect of organic solvent type on the extraction yield, TPC, and TFC from RCILP.^a

Solvent type	Yield (%)	TPC (mg GAE/g RCILP)	TFC (mg QE/g RCILP)
Acetone	2.28	65.76	585.70
Methanol	2.58	143.14	804.30
Ethanol	3.33	124.89	479.60

^a Operation conditions: Extraction temperature of 30 °C and 100% organic solvent.

2021). Moreover, using pure water as an extracting solvent result in a significant quantity of contaminants (organic acids, sugars, soluble proteins) in the extract, which may obstruct phenolic identification and quantification (Chirinos et al., 2007). Therefore, solvent concentrations in water at 50%, 80%, and 100% were investigated in this study.

Methanol concentrations ranging from 50 to 100% (v/v) were employed to obtain phenolic compounds from *C. inophyllum* leaves. Solvent concentrations significantly affected the yield of extract, TPC, and TFC value (p -value < 0.05), as shown in Table 2. Extraction by absolute methanol produced the highest yield of extract. However, 80% methanol was the best concentration of methanol to extract phenolic compounds from *C. inophyllum* leaves (289.12 mg GAE/g RCILP).

From some studies, combining water with organic solvents (ethanol, methanol, and acetone) can increase the polarity index of pure organic solvents, thereby resulting in a higher polarity medium that decreases the solubility of phenolic compounds (Spigno et al., 2007). On the contrary, the enhancement of water concentration in the mixture of organic solvent decreases the extraction yield of *C. inophyllum* leaves. It may be because the nature of phenolic compounds in *C. inophyllum* leaves is less soluble in highly polar mediums, such as 50% (v/v) aqueous methanol. For extracting phenolic chemicals from plants, each solvent has varying extraction efficiency. Phytochemical screening of *Kirkia wilmsii* shows that combining organic solvents (acetone, ethanol, or methanol) and water produced good extraction yield extracts (Chigayo et al., 2016). Pure water was ineffective in extracting phenolic compounds since they were generally more soluble in organic solvents with a lower polarity index than water (Kim and Lee, 2002). The result of this work is suitable with other research that 75% aqueous methanol extract from *Limnophila aromatica* provides the highest TPC, compared to 50% aqueous methanol and 100% methanol extract (Do et al., 2014). Moreover, 80% methanol extracts more polyphenols and exhibits higher antioxidant activity from *Moringa oleifera* leaf (Siddhuraju and Becker, 2003), *M. oleifera* root, *Ficus religiosa* fruit, and *Aloe barbadensis* leaf (Sultana et al., 2009). However, several works are in contrast with our result. As reported by Al-Farsi and Lee (2008), the most effective solvent for phenolic extraction from date seeds was 50% aqueous acetone. Also, Kallithraka et al. (1995) reported that 70% aqueous acetone shows good performance for extracting grape seed's phenolic compound (proanthocyanidins). Metrouh-Amir et al. (2015) found that the highest yield of *Matricaria pubescens* extract was achieved by 50% aqueous acetone, while the highest phenolic content was achieved by 50% aqueous methanol. In addition, Gonzalez-Montelongo et al. (2010) revealed that 50% aqueous acetone is efficient in extracting banana peels'

phenolic compounds. These results occurred because aqueous acetone was reported as an excellent solvent to extract higher molecular weight flavanols (Dai and Mumper, 2010).

In this study, extract of 50% methanol possessed much higher flavonoid content than that in the 80% and 100% methanol extract, whereas its TPC and yield value were the lowest. When the concentration of water increases, the polarity of solvent also increases. It causes flavonoid glycosides, which have a higher total polarity surface than flavonoid aglycones (Chuang et al., 2017), to become easily soluble and vice versa. A similar result was also reported from extract *M. pubescens*. The aqueous methanol (50%) extract of *M. pubescens* contains higher flavonoid content than that of aqueous acetone (50%) and pure methanol extract (Metrouh-Amir et al., 2015).

3.3. Effect of extraction temperature

Heating can contribute to thermal degradation, oxidation, and leaching of phenolic and flavonoid compounds from fresh vegetables (Kalt, 2005). It also affects the composition of antioxidant compounds in vegetables (Makris and Rossiter, 2001). By applying the best solvent and concentration (80% methanol), the extraction yield and TPC recovery of *C. inophyllum* leaves have been significantly affected by the differences in extraction temperatures (p -value < 0.05). Extraction yield and TPC decreased when the temperature raised from 30 °C to 60 °C, as demonstrated in Table 3. The best extraction temperature was observed at 30 °C, resulting in high a yield (2.41%) and TPC value (289.12 mg GAE/g RCILP). Therefore, 30 °C was selected as the optimal temperature to extract phenolic compounds from *C. inophyllum* leaves.

The maximum temperature employed in this extraction process was 60 °C because a higher extraction temperature decreased the stability of the phenolic and flavonoid compounds (Dorta et al., 2012). The increasing temperature can improve the extraction efficiency due to the breakdown of leaves' cell membranes and the breakage of phenol-protein complexes. As can be seen in Table 3, TFC significantly increased from 30 °C to 60 °C because high temperature enhances diffusivity and solubility of bioactive compounds and extraction yield. Increasing temperature also diminishes the viscosity of solvent and surface tension (Dorta et al., 2012). In the previous work, it was revealed that the increasing temperature of extraction (up to 120 °C) is responsible for the increasing flavonoid level in six varieties of the onion because quercetin is resistant to heat destruction (Sharma et al., 2015). The unbound fraction of phenolic acids, phenolic compounds, and antioxidant activity of peel citrus also increased by the heat treatment (Xu et al., 2007). However, applying higher temperatures (45 °C and 60 °C) in this study reduced the yield

Table 2 The effect of solvent concentration in water on the extraction yield, TPC, and TFC from RCILP.^a

Solvent Concentration	Yield (%)	TPC (mg GAE/g RCILP)	TFC (mg QE/g RCILP)
50 %	2.18	78.18	1289.00
80 %	2.41	289.12	410.40
100 %	2.58	143.14	574.90

^a Operation conditions: Extraction temperature of 30 °C and methanol as an organic solvent.

Table 3 The effect of extraction temperature on the extraction yield, TPC, and TFC from RCILP.^a

Temperature	Yield (%)	TPC (mg GAE/g RCILP)	TFC (mg QE/g RCILP)
30 °C	2.41	289.12	410.4
45 °C	2.12	146.79	522.9
60 °C	1.39	80.37	594.4

^a Operation condition: 80% methanol as an organic solvent.

Table 4 Comparison of extraction conditions and solvent used for TPC and TFC extraction.

No.	Author	Plant	Solvent studied	Best extraction conditions	Result
1	This study	<i>C. inophyllum</i> leaves	Acetone, ethanol, methanol.	Time = 48 h; Temperature = 30 °C; Solid : solvent = 1 : 10 (w/v); Solvent = 80% methanol.	The highest TPC and TFC value are 289.12 mg GAE/g and 410.4 mg QE/g, respectively.
2	Truong et al. (2019)	<i>Severinia buxifolia</i> branches	Distilled water, methanol, ethanol, chloroform, dichloromethane.	Time = 24 h; Temperature = 60 °C; Solid : solvent ratio = 1 : 20 (w/v); Solvent = 100% methanol.	The highest TPC and TFC value are 13.36 mg GAE/g DW and 1.92 mg QE/g dry weight (DW), respectively.
3	Do et al. (2014)	<i>Linnophila aromatica</i>	Distilled water, 100% methanol, 75% methanol, 50% methanol, 100% ethanol, 75 %ethanol, 50% ethanol, 100% acetone, 75% acetone, 50% acetone.	Time = 5 min; Solid : solvent ratio = 30 mg/1.6 mL; Solvent = 100% ethanol.	100% ethanol gives the highest TPC value (40.50 ± 0.88 mg GAE/g) and TFC value (31.11 ± 0.43 mg QE/g).
4	Tan et al. (2013)	Henna (<i>Lawsonia inermis</i>) stem	Distilled water, boiling water, acetone, ethanol, methanol.	Time = 270 min; Temperature = 55 °C Solid : solvent ratio = 1 : 10 (w/v); Solvent = 40% acetone.	40% acetone has the highest TPC value (5554.15 ± 73.04 mg GAE/100 g DW).
5	Turkmen et al. (2006)	Black tea and mate tea	Distilled water, acetone, N,N-dimethylformamide (DMF), ethanol and methanol.	Time = 1 h; Solid : solvent = 0.2 g/20 mL.	In black tea, 50% DMF extract gives the highest polyphenol content of 99.8 mg GAE/g. In mate tea, 50% acetone gives the highest polyphenol content of 120.4 mg GAE/g.
6	Metrouh-Amir et al. (2015)	<i>Matricaria pubescens</i>	Distilled water, aqueous acetone (50%), aqueous methanol (50%), aqueous ethanol (50%), acetone (100%), methanol (100%), and ethanol (100%).	Solid : solvent = 1 : 125 (g/mL); Time = 3 h; Room temperature; Solvent = 50% methanol.	Highest TPC (2.64 g GAE/100 g) was obtained with aqueous methanol (50%) and aqueous ethanol (50%). Highest TFC (0.93 g QE/100 g) was obtained with aqueous ethanol (50%).
7	Chigayo et al. (2016)	<i>Kirkia wilmsii</i> tubers	Distilled water, ethanol, methanol, methanol/chloroform/water (MCW), 80% methanol, 60% methanol, dichloromethane, chloroform, acetone, hexane, diethyl ether, ethyl acetate.	Solid : solvent = 2 g/25 mL; Time = 60 min; Solvent = 100% methanol.	100% methanol has the highest TPC value (122.84 ± 0.31 mg GAE/g) and TFC value (917.02 ± 0.10 mg QE/g).
8	Boskov et al. (2021)	Black locus (<i>Robinia pseudoacacia</i>) flowers	Distilled water, ethanol, methanol, and alcoholic mixtures.	Ultrasound-assisted extraction at 40 kHz; Temperature = 60 ± 1 °C; Power = 150 W; Solvent = 50% methanol.	50% methanol has the highest TPC value (3.78 g GAE/100 g DW).

and TPC value. An increase in temperature above a certain value contributes to the risk of non-flavonoid compounds degradation. As a result, the TPC value decreased at 45 °C and 60 °C. Moreover, a higher temperature may cause solvent loss during extraction by vaporization because extraction was conducted at a temperature close to the boiling point of the solvents (Chan et al., 2009). From the standpoint of industrialization, high temperature is the cause of cost escalation in the extraction process (Mokrani and Madani, 2016). The comparison of extraction conditions in this study and others can be seen in Table 4. It shows that the most studied factors are time, temperature, solid to solvent mass ratio, solvent type, and organic solvent concentration in water.

The optimum condition for extracting phenolic and flavonoid compounds from *C. inophyllum* leaves is employing

80% methanol as extracting solvent at 30 °C for 48 h. This condition is quite beneficial because methanol is cheaper than other extracting solvents, such as ethanol, acetone, dichloromethane, dimethylformamide (DMF), and chloroform. The temperature also contributes to minimizing the cost of heating. In addition, *C. inophyllum* leaves are rarely utilized by Indonesian people, which can contribute to the low material cost.

3.4. DPPH radical scavenging activity

TPC is an essential factor because it significantly contributes to antioxidant activity (Chavan et al., 2013). The extract with higher phenolic content is very beneficial for humans because it can suppress free radical damage or primary oxidizing agents. Determination of scavenging stable DPPH free radical

Table 5 IC₅₀ of methanol extract of *C. inophyllum* leaves obtained at different temperature.^a

Extracts	TPC (mg GAE/g RCILP)	IC ₅₀ (μg/mL)
Standard gallic acid	—	0.002
Methanol 30 °C	289.12	0.054
Methanol 45 °C	146.79	0.070
Methanol 60 °C	80.37	0.075

^a Operation condition: 80% methanol as an organic solvent.

is the simplest method to assess the antioxidant activity of *C. inophyllum* leaves extracts. It is quick, accurate, and requires a low concentration of samples or reagents. This method is associated with the capacity of DPPH to deal with hydrogen donor species found in extract materials, such as phenolic compounds (Shabir et al., 2011). The antiradical capacity of antioxidant compounds can be analyzed by assessing the reduction in DPPH absorbance at 517 nm. In a DPPH molecule, the odd electrons on the nitrogen atom can delocalize across the molecule, giving the DPPH solution its deep violet hue. The absorbance diminished, and the hue shifted from deep violet to yellow when the DPPH radical received hydrogen donation creating a stable DPPH-H molecule.

The optimal solvent type and solvent concentration were employed for DPPH radical scavenging activity assay at concentrations of 0.01, 0.1, 1, 10, and 100 μg/mL. Gallic acid was employed as the positive control because it is well-known for its high antioxidant activity. These extracts and gallic acid were indexed in IC₅₀ value. The IC₅₀ of a compound is calculated using interpolation from logarithmic regression analysis and is represented as the quantity of antioxidant demand to reduce DPPH concentration by 50%. A compound with a lower IC₅₀ value has higher antioxidant capacity because it can lessen DPPH concentration by 50% with a small amount of concentration. The IC₅₀ values are shown in Table 5.

The extract obtained from 80% methanol at 30 °C possessed a high TPC value (289.12 mg GAE/g RCILP) and DPPH radical scavenging activity (IC₅₀ = 0.054 μg/mL). Meanwhile, the extract obtained from 80% methanol at 60 °C had the lowest TPC value (80.37 mg GAE/g RCILP) and DPPH radical scavenging activity (IC₅₀ = 0.075 μg/mL). Higher temperature results in lower TPC and higher IC₅₀ values. Therefore, it indicates that TPC is proportional to antioxidant activity in the polar fraction of *C. inophyllum* leaves. These results are similar to the previous work, which reported that brewer's spent grains extracts obtained from 80% methanol had the highest antioxidant activity according to DPPH assay (Meneses et al., 2013).

3.5. Isolation and identification of bis (2-Ethylhexyl) phthalate from methanolic extract of *C. inophyllum* leaves

As depicted in Fig. 6, there are four highest peaks in methanolic extract. Peak 1 and 2 were *trans*-2-[2-(trifluoromethyl)phenyl]-10b,10c-dimethyl-10b, 10c-dihydropyrene, and anti-4-az

a-B-homo-5.alpha-cholestane-3-one, respectively (Susanto et al., 2019). Methanolic extract and n-hexane fraction show the absence of bis (2-ethylhexyl) phthalate's peak due to the low concentration of bis (2-ethylhexyl) phthalate in methanolic extract. After the n-hexane fraction had been mixed with acetone and chloroform at 4 °C for 48 h, the crystal fraction was formed. Based on gas chromatography (GC) analysis, there were two peaks in the crystal fraction, and these two peaks were not present in the acetone and chloroform fractions. The highest peak in crystal fraction was identified as bis (2-Ethylhexyl) phthalate by gas chromatography-mass spectrometry (GCMS) analysis at a retention time of 16.161 min. The yield and purity of bis (2-Ethylhexyl) phthalate isolated from methanolic extract of *C. inophyllum* leaves was 1.25% and 79.51%, respectively. Acetone is a type of ketonic solvent acknowledged for its ability to crystallize ester compounds, such as bis (2-Ethylhexyl) phthalate (Gunawan et al., 2006).

The ion characterization *m/z* (relative intensity) of bis (2-ethylhexyl) phthalate is as follows: 29 (7.84%), 43 (17.65%), 57 (29.41%), 71 (15.69%), 104 (6.86%), 149 (100%), 167 (25.49%), 279 (7.84%). The base peak at *m/z* 149 (as shown in Fig. 7) is possibly formed when the ester bonds are broken, followed by the shifting of two hydrogen atoms and one more hydrogen atom, ended by water elimination (Sutrisno, 2018). Bis (2-ethylhexyl) phthalate is mainly used to increase plastic flexibility in cosmetics, furniture, toys, food, and beverages packaging products. It effectively inhibits the human erythroleukemic K562 cell line, as reported by Priya and Jayachandran (2012). Nevertheless, this compound is also reported to cause several serious illnesses, such as neurotoxicity, hepatotoxicity, cardiotoxicity, renal toxicity, and endometriosis (Rowdhwal and Chen, 2018).

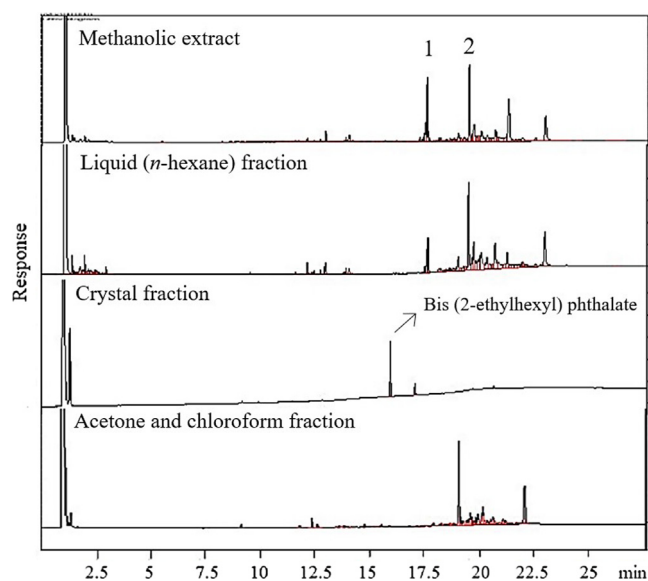


Fig. 6 Chromatogram of methanolic extract from *C. inophyllum* leaves by GC analysis.

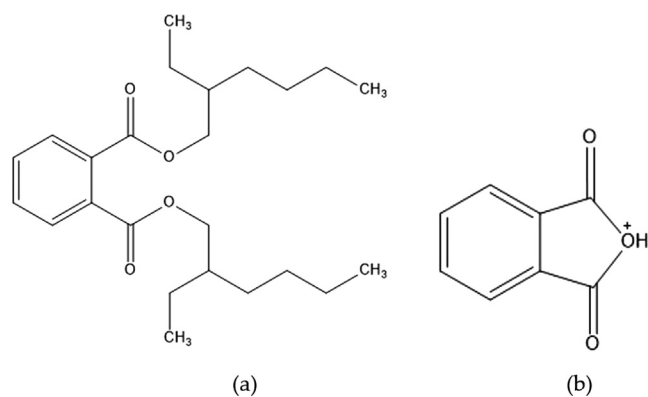


Fig. 7 The structure of bis (2-Ethylhexyl) phthalate (a) and the ion structure at m/z 149 (b).

4. Conclusion

The optimization of total phenolic compounds extraction from *C. inophyllum* leaves was evaluated using a single factor experiments approach in this work, which investigated certain factors (the effect of solvent type, concentration, and temperature). There was no data regarding extraction parameters that influence phenolic and flavonoid compounds from *C. inophyllum*. In general, the solvent type had a minor effect on the yield of extraction. Meanwhile, the TPC value of extracts was notably affected by solvent types, the concentration of solvent in water, and temperature. The most effective solvent to extract phenolic compounds from *C. inophyllum* leaves was 80% methanol. The optimal conditions of extraction were 80% methanol at 30 °C for 48 h. It contributed to the extraction yield of 2.58%, 289.12 mg GAE/g RCILP for TPC, and 410.4 mg QE/g RCILP for TFC. Extraction of *C. inophyllum* leaves with optimal conditions shows that the leaves exhibit antioxidant activity with an IC_{50} value of 0.054 μ g/mL. Moreover, bis (2-ethylhexyl) phthalate was identified in the methanolic extract. This study will be based on future investigations to separate and isolate phenolic compounds from *C. inophyllum* leaves.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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