Effect of solvent polarity levels on separation of xanthone and coumarin from *Calophyllum inophyllum* leaves extract

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Abstract. *Calophyllum inophyllum* has various benefits that can be utilized from root, stem, leaf, until seed. *C. inophyllum* leaves contain many bioactive compounds, such as xanthone and coumarin which are useful as antioxidant, and inhibitors of enzyme activity from HIV virus. The aim of this research was to investigate the effect of solvent polarity levels on the separation of xanthone and coumarin compounds contained in the crude extract of *C. inophyllum* leaves. Crude leaves extract was obtained by percolation method. Moreover, Liquid Liquid Extraction (LLE) was used for separating xanthone and coumarin compounds. It was performed by methanol (polar solvent) and hexane (non-polar solvent) with solvent ratio of 1. Methanol concentration in water used were 20%, 50%, 80%, and 100%. Each fraction obtained was tested qualitatively using Thin Layer Chromatography (TLC) and quantitatively using Gas Chromatography (GC) to analyze xanthone and coumarin. The best separation result was obtained by using 50% methanol. In this results, coumarin and xanthones were separated in methanol fraction (81.18% recovery) and in hexane fraction (81.91% recovery), respectively.

1. Introduction

Indonesia has many varieties of flora and fauna. The most common plant species in coastal areas in Indonesia are mangroves. According to FAO (2007), in 2005, mangrove forests in Indonesia reached 3.062.300 ha (19% of the world’s mangrove forest). The largest areas of mangroves in Southeast Asia are found in Indonesia (almost 60 percent of Southeast Asia total), Malaysia (11.7%), Myanmar (8.8%), Papua New Guinea (8.7%) and Thailand (5.0%) [1]. Mangroves contains a number of minerals, vitamins, amino acids that are essential for the growth of the world used for health [2].

One type of mangrove plant that has high economic value is *Calophyllum inophyllum* because almost all parts of this plant (stems, leaves, flowers, seeds, and sap) can produce various benefit products. *C. inophyllum* is a mangrove species from the family Clusiaceae. *C. inophyllum* is an evergreen tree with supports a dense canopy of glossy, elliptical leaves, fragrant white flowers and large round nuts. This habitat is primarily coastal and adjacent to lowland forests. This tree grows to height 8-20m (25-26 ft), sometimes reaching up to 35 m (115 ft) [3]. It has milky white sap. Leaves are opposite, deep glossy green, glabrous, simple, coriaceous with broadly elliptic or obovate-elliptic
lamina 10-20 cm long by 6-9 cm wide, rounded or emarginate apex, rounded or cuneate base, entire margin and distinct parallel lateral veins, perpendicular to mid rib [4].

Higher plants as sources of bioactive compounds play a dominant role in the maintenance of human health. The various parts of *C. inophyllum* plant contain many bioactive compounds, including among others: xanthones, coumarins, chromanones (flavonoids, biflavonoids), triterpenes and steroids [5]. The phytochemical compounds of *C. inophyllum* leaves had been studied previously [6]. Meanwhile, this plant contains phytochemical compounds that can be used as a cure of various diseases [7]. These compounds include inophynone, canophyllol, canophyllic acid, calophyllolide, inophyllolide, jacareubin, calanolide A, calophynone, and others. *C. inophyllum* contains chemopreventive cancer agents and coumarins have antimicrobial activity [8]. Coumarin is one of the secondary metabolites in plants. Coumarin compounds exhibit anti-HIV drugs belonging to the Non-nucleoside reverse transcriptase inhibitor (NNRTI) [9]. NNRTI is a group of compounds that inhibits the activity of the reverse transcriptase enzyme from HIV-1 [10]. Coumarin compounds and their derivatives have many biological activities such as anticoagulant blood, antibiotics, and inhibiting carcinogenic activity [11]. Some of xanthone compound such as caloxanthone A, inoxanthone, macluraxanthone, and caloxanthone B have antimicrobial activity [12]. In order to increase the utilization of *C. inophyllum* leaves in medical function, bioactive compounds must be extracted, concentrated, separated and purified.

Xanthones are polyphenolic compounds that have molecular formula of C_{13}H_{18}O_{2}. Xanthones consist of a unique backbone with two benzene rings bridged across a carbonyl group and an oxygen. The most bioactive property of xanthones is their antioxidant ability. The derivatives of xanthones which isolated from *Garcinia mangostana* was found to act as the free radical scavenger and hence prevented the the oxidative damage of low density lipoprotein [13]. Coumarins are one of the member of the benzopyrone compounds. Coumarins have a benzene ring linked to a pyrone [14]. Coumarins are attractive to human for their physiological, bacteriostatic, and anti-tumour activity. 4-phenylcoumarins might be valuable as potential cancer chemopreventive agents (anti-tumour-promoters) [15].

Therefore, the objective of this work was to separate coumarin and xanthone contained in *C. inophyllum* leaves by liquid-liquid extraction method. This separation is based on the polarity index solvent. The separation technology of xanthone and coumarin is needed due to they have different benefits. The effect of solvent polarity levels on the separation of xanthone and coumarin compounds contained in the crude extract of *C. inophyllum* leaves were investigated.

2. Materials and methods

2.1. Materials

Dried *C. inophyllum* leaves were obtained from the “Koperasi Jarak Lestari”, Cilacap, Central Java, Indonesia. Thin-layer chromatograph (TLC) aluminum plates (20 x 20cm x 250 m) were purchased from Merck (Darmstadt, Germany), Advantec filter papers were obtained from Toyo Roshi Kaisha Ltd. (Tokyo, Japan). Standard of xanthone and coumarin were obtained from Sigma Aldrich (St. Louis, MO). Methanol, hexane, aquadest, ethyl acetate, acetic acid were obtained from commercial sources.

2.2. Preparation of the crude extract

*C. inophyllum* leaves were dried in the sun for 3 days to reduce their water content. They was chopped to a homogeneous size by a mill and soaked in 3 L of methanol for two times (72 h each time). Then, the solutions were filtered through filter paper. The filtrate were combined and then evaporated to dryness by distillation at 80 °C.
2.3. Separation of coumarin and xanthone from Calophyllum inophyllum leaves
In this study, Liquid-Liquid Extraction was used to separate the mixture. This method was carried out to remove impurities simultaneously, purify and separate the polar and nonpolar fraction in the crude extract. Crude extract (3 g, 12 g) was put into beaker glass to be mixed with methanol (150 g) with variable methanol concentration of 20%, 50%, 80%, 100%. Then, it was stirred for 30 minutes with magnetic stirrer. The mixture was added with hexane (150 g), then it was stirred again for 30 minutes. The whole mixture was separated in separation funnel, the mixture split into layers. Each layer’s content was analyzed.

2.4. Analysis by Thin Layer Chromatography (TLC)
TLC was employed to qualitatively analyze the sample, using authentic standards, as described research [16]. TLC plates that has been stained by the sample was immersed in a mobile phase of hexane : ethyl acetate : acetic acid at 90:10:1 (v/v/v). Then, TLC plates were analyzed by UV light at 254 nm.

2.5. Analysis by Gas Chromatography (GC)
The contents of bioactive compounds in each fraction were determined by GC [17] with some modification. External standard calibration curves were obtained by using 0.2-20 mg pure standard. Gas chromatographic analysis was performed on a Shimadzu GC-2010 (Kyoto, Japan) gas chromatography equipped with a Flame Ionization Detector. Separations were carried on a DB-5HT (5%-phenyl)-methylpolysiloxane non-polar column (15m x 0.32mm i.d.; Agilent Tech. Palo Alto, California). Temperatures of the injector and the detector were both set at 310°C. The temperature of the column was started at 80°C, increased to 300°C at rate of 15°C/min, and maintained at 300°C for 8 min. The split ratio was 1:50 using nitrogen as carrier gas with a linear velocity of 30 cm/s at 80°C. A twenty-milligram sample was dissolved in 1 mL ethyl acetate, and a 1 µL sample was taken and injected into the GC instrument.

3. Results and discussion

3.1. Identification of xanthone and coumarin in C. inophyllum leaves crude extract
C. inophyllum leaves contain many bioactive compounds, such as xanthone and coumarin which are useful for medicinal value. Figure 1 shows the TLC of crude extract of C. inophyllum leaves, standard xanthone, and coumarin. It can be known that coumarin is more polar than xanthone. The coumarin spot (Rf = 0.28) was located below the xanthone spot in TLC plate (Rf 0.56). Component having a smaller Rf value, have more polar properties.

![Figure 1. TLC analysis of C. inophyllum leaves crude extract (a); standard coumarin (b) and standard xanthone (c).]
The aim of this research was to know effect of solvent polarity level on the separation of xanthone and coumarin compounds contained in the crude extract of *C. inophyllum* leaves. It was expected that the largest recovery of coumarin was in polar fraction, and the largest recovery of xanthone was in non-polar fraction.

3.2. Separation of xanthone and coumarin from *C. inophyllum* leaves crude extract

The separation process was done by Liquid - Liquid Extraction method were used polar solvent (methanol / water combination) and nonpolar solvent (hexane). Selection of solvent based on polarity index. Water is a polar solvent with a polar solvent with polarity index of 9, while methanol is semipolar solvent with a polarity index of 5.1 [18]. Hexane is a non-polar solvent with polarity index of 0. In this research, first, there were formed three layers. The lowest layer was insoluble fraction. The middle layer was the methanol fraction. The top layer was the hexane fraction. The insoluble fraction was separated by filter paper. Then, methanol and hexane fractions were inserted in separating funnel as shown in figure 2. Top layer (transparent yellow color) was hexane fraction which contained non-polar compounds, while the bottom layer (light brown color) was methanol fraction which contained polar compounds.

Table 1 shows the effect of methanol concentration to separation of coumarin and xanthone. The combination of methanol and water is expected to dissolve polar compounds contained in crude extract, while hexane is expected to dissolve non-polar compounds in crude extract. From Table 1, it appears that methanol 50% was excellent solvent for the separation of xanthones and coumarin. This is evident from the mass of coumarin in the methanol fraction is greater than the mass of coumarin in hexane fraction, while the mass of xanthones in the methanol fraction is smaller than the mass of xanthones in hexane fraction. Methanol 20% was not the good solvent for separation process of coumarin and xanthone, because the mass of coumarin was more on hexane fraction. No separation between xanthone and coumarin on the use of methanol 80% and 100%. This is because coumarin is more polar than xanthone (figure 1b). Water and its combination with organic solvent commonly used to extract bioactive components from plants. The largest extraction yield of flavonoid and phenolic content of the *Macademia tetrathylla* skin was obtained on the use of solvents with 50% methanol, 50% ethanol, 50% acetonitrile, and 50% acetone concentration (v/v) in water, respectively [19].

From table 1, it shows that the best separation of coumarin and xanthone was at methanol 50%, whereas %recovery coumarin in methanol fraction (3.35%) larger than in hexane fraction (0%). The different of solvent affects the recovery of bioactive compound such as total phenolic compound and total flavonoid compound, where the % recovery is best for bioactive compounds separation on the
combination of water and organic solvent (methanol, ethanol, acetonitrile) by 30-60% concentration [20]. This is caused by differences in the dielectric constant and polarity index of solvents. The dielectric constant of the solvent is the magnitude of the force acting between two payloads in the solvent. These constant determines the extent to which the level of ability of the solvent to dissolve components. Solvent that has a low dielectric constant is a good solvent for dissolve non-polar compounds and vice versa [21].

Table 1. Effect of methanol concentration and solvent to crude extract ratio (mL/g) on separation of xanthone and coumarin.

| Methanol concentration | Compound     | Solvent / crude extract = 50 mL/g | Solvent / crude extract = 12.5 mL/g |
|------------------------|--------------|----------------------------------|------------------------------------|
|                        |              | Methanol Fraction | Hexane Fraction | Insoluble Fraction | Methanol Fraction | Hexane Fraction | Insoluble Fraction |
| 20%                    | Coumarin     | 0.011a                      | 0.04                        | 0.06                  | 0.05                  | 0.10                  | 0.09                  |
|                        |              | (0.96)b                       | (3.13)                      | (95.90)               | (1.04)               | (10.05)               | (88.90)               |
|                        | Xanthone     | 0.001                        | 0.13                        | 0.19                  | 0.04                  | 0.28                  | 0.29                  |
|                        |              | (0.04)                        | (3.70)                      | (96.26)               | (0.26)               | (9.65)               | (90.09)               |
| 50%                    | Coumarin     | 0.026                        | 0.29                        | 0.04                  | 0.03                  | 0                      | 0.08                  |
|                        |              | (34.56)                       | (4.30)                      | (61.13)               | (3.35)               | 0                      | (96.65)               |
|                        | Xanthone     | 0.13                         | 0.69                        | 0.13                  | 0.02                  | 12.12                 | 0.25                  |
|                        |              | (7.55)                        | (27.97)                     | (64.48)               | (1.02)               | (0.22)                | (92.5)                |
| 80%                    | Coumarin     | 0.06                         | 0.09                        | 0.03                  | 0.05                  | 0.12                  | 0.09                  |
|                        |              | (50.74)                       | (5.46)                      | (43.79)               | (15.69)              | (8.74)                | (75.56)               |
|                        | Xanthone     | 0.08                         | 0.27±0.14                  | 0.10                  | 0.02                  | 0.22                  | 0.25                  |
|                        |              | (24.38)                       | (23.96)                     | (51.66)               | (1.74)               | (5.72)                | (92.5)                |
| 100%                   | Coumarin     | 0.04                         | 0.05                        | NA                    | 0.09                  | 0.09                  | NA                    |
|                        |              | (51)                         | (48.99)                     | NA                    | (68.52)              | (31.48)               | NA                    |
|                        | Xanthone     | 0.07                         | 0.05                        | NA                    | 0.11                  | 0.45                  | NA                    |
|                        |              | (63.29)                       | (36.71)                     | (36.20)               | (63.79)              |                       |                       |

a Content (%wt)

b Recovery (%)

NA : Not available

In this research, besides there methanol fraction and hexane fraction, there are also insoluble solid fraction. From table 1, % recovery xanthone and coumarin in solid fraction is still very large, so we need to get back extraction of solid fraction to get greater of % recovery xanthone and coumarin in methanol and hexane fraction. The insoluble fraction from variable 50 (mL/g) solvent / crude extract ratio was chosen to be extracted again. The best separation between xanthone and coumarin occurs in the solvent / crude extract variable ratio of 50 mL/g. This can be seen from the differences of %recovery xanthone and coumarin far enough between in methanol fraction and hexane fraction. from table 1, it appears that for the variable methanol 50%, %recovery of coumarin in insoluble fraction of variable solvent / crude extract ratio 50 mL/g (61.13%) smaller than variable solvent / crude extract ratio 12.5 mL/g (96.65%), and %recovery of xanthone in insoluble fraction of variable solvent / crude extract ratio 50 mL/g (64.48%) smaller than variable solvent / crude extract ratio 12.5 mL/g (92.5%). The insoluble solid fraction of variable solvent / crude extract ratio 50 mL/g was extracted with the same method and solvent (methanol 50% and hexane) repeatedly. The insoluble fraction of variable solvent / crude extract ratio 50 mL/g (2.424 g) extracted with methanol 50% as polar solvent and hexane as non-polar solvent with the same amount with first LLE. Extraction was done in a glass beaker with stirring with magnetic stirrer for 30 minutes. Once extraction was complete will be formed of three layers, namely the lower layer insoluble hereinafter referred to solid fraction, the middle layer is methanol fraction, and the top layer is hexane fraction. But, extraction is done with new solvent
repeatedly until the insoluble fraction depleted. Repeated extraction of this insoluble fraction has done as much as 6 stages (table 2).

**Table 2. Result of each stage of repeated extraction of insoluble fraction.**

| Stage | Compound | Methanol Fraction | Hexane Fraction | Solid Fraction |
|-------|----------|------------------|----------------|----------------|
| 1     | Coumarin | 0.24<sup>a</sup> | 0.03%          | 0.05%          |
|       |          | (39.75)<sup>b</sup> | (3.60%)       | (61.13%)       |
|       | Xanthone | 0.13             | 0.70%          | 0.16%          |
|       |          | (7.55)           | (27.97%)       | (64.48%)       |
| 2     | Coumarin | 0.04             | 0.01%          | 0.06%          |
|       |          | (5.37)           | (0.91%)        | (54.85%)       |
|       | Xanthone | 0.03             | 0.62%          | 0.17%          |
|       |          | (1.20)           | (22.34%)       | (58.15%)       |
| 3     | Coumarin | 0.04             | 0.01%          | 0.09%          |
|       |          | (4.91)           | (3.26%)        | (46.68%)       |
|       | Xanthone | 0.03             | 0.15%          | 0.22%          |
|       |          | (1.06)           | (16.81%)       | (40.28%)       |
| 4     | Coumarin | 0.04             | 0.09%          | 0.1%           |
|       |          | (4.53)           | (7.48%)        | (34.66%)       |
|       | Xanthone | 0.08             | 0.29%          | 0.24%          |
|       |          | (2.72)           | (8.58%)        | (28.98%)       |
| 5     | Coumarin | 0.02             | 0.02%          | 0.18%          |
|       |          | (1.47)           | (2.50%)        | (30.69%)       |
|       | Xanthone | 0.06             | 0.22%          | 0.33%          |
|       |          | (1.55)           | (7.83%)        | (19.60%)       |
| 6     | Coumarin | 0.001            | 0.25%          | NA             |
|       |          | (0.24)           | (10.50%)       |               |
|       | Xanthone | 0.03             | 0.46%          | NA             |
|       |          | (0.48)           | (19.12%)       |               |

<sup>a</sup> Content (%wt)  
<sup>b</sup> Recovery (%)  
NA : not available

Based on table 2, it can be seen that the recovery of xanthone and coumarin on the solid fraction will decrease. So, enrichment coumarin in methanol fraction and xanthone in hexane fraction can be done. table 3 shows the total distribution of xanthone and coumarin in the methanol and hexane fraction.

**Table 3. Total xanthone and coumarin in methanol and hexane fraction.**

| Compound | Methanol Fraction | Hexane Fraction |
|----------|------------------|----------------|
| Coumarin | 0.089<sup>a</sup> | 0.057          |
|          | (81.18)<sup>b</sup> | (18.82)        |
| Xanthone | 0.067            | 0.351          |
|          | (18.09)          | (81.91)        |

<sup>a</sup> Content (%wt)  
<sup>b</sup> Recovery (%)  

Based on table 3, it appears that there is a separation between xanthones and coumarin between hexane fraction and methanol fraction, where the % total recovery coumarin in the methanol fraction is equal to 81.18%, while the % total recovery xanthone in hexane fraction is equal to 81.91%. So, it
can be indicated that this multiple extraction method with methanol 50% and hexane solvent suitable for separation of xanthones and coumarin from methanolic C. inophyllum leaves extract.

4. Conclusion
Repeated Liquid – liquid Extraction (LLE) with methanol and hexane can be used for separating xanthone and coumarin from C. inophyllum leaves crude extract. Solvent polarity levels have effect for separating xanthone and coumarin. Methanol 50% gives the best performance for separating xanthone and coumarin from C. inophyllum leaves crude extract. Coumarin was succeeded separately in the methanol fraction with recovery of 81.18%, while xanthone was succeeded separately in the hexane fraction with recovery of 81.91%.

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