MJFAS MALAYSIAN JOURNAL OF FUNDAMENTAL AND APPLIED SCIENCES
PRINT ISSN: 2289-5981 | ONLINE ISSN: 2289-599X

RESEARCH ARTICLE

Separation of xanthone and vitamin E from *Calophyllum inophyllum* leaf

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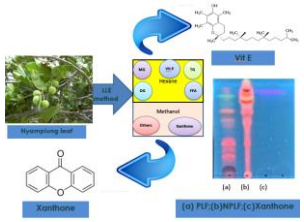
Article history
Received 21 November 2017
Revised 19 February 2018
Accepted 12 June 2018
Published Online 16 December 2018

Graphical abstract



Abstract

Calophyllum inophyllum (nyamplung) has many advantages start from stems, leaves, roots, to seeds. Indonesian society generally only know that seeds can produce biodiesel. Nyamplung leaves have bioactive compounds, such as xanthone that can be used for medical purposed. Recently, quantification of xanthone and vitamin E in the nyamplung leaf still doesn't exist. This research aims to isolate and quantify the content of xanthone and vitamin E from the crude nyamplung leaf. The separation is done through liquid-liquid extraction with methanol and petroleum ether (PE) to obtain two layers namely non-polar fraction (PE fraction) and polar fraction (methanol fraction). The mass ratio of solvent mixture-to-crude extract (10,30, 50 and 70) and the mass ratio of PE-to-methanol (1 and 3) were applied and analyzed by TLC, GC-MS, and UV-Vis. Xanthones (0.150 equilibrium constant, 15.16% purity and 94.43% recovery) were obtained in the methanol fraction based on the mass ratio of solvent mixture-to-crude extract of 70 and mass ratio of PE-to-methanol at 1.From GC-MS, Xanthones were detected in the methanol fraction with quality of 94% and Vitamin E was detected in the petroleum ether fraction with quality of 99%.

Keywords: *C. inophyllum* leaf, polarity, recovery, soxhlet solvent extraction, vitamin E

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INTRODUCTION

Indonesia is one country that has a diversity of tropical flora that can be used for traditional treatment for human health. Many species of plants that have reported benefits in the field of traditional medicine. One of them comes from the *Calophyllum genus* of *Clusiaceae* family. The *Calophyllum genus* is a tropical plant that consists of 180-200 different species of famous rich in a number of bioactive compounds (Su et al., 2008). Some species of the *Calophyllum genus* were reported useful as a topical medication for treating rheumatic diseases and inflammation of the eye (Heyne, 1987).

One species of the Calophyllum genus is *Calophyllum Inophyllum Lin* (nyamplung). Indonesian has many species of *C. Inophyllum* that spread over Sumatera, Java, Nusa Tenggara, Sulawesi and Bali (Heyne, 1987). C. *Inophyllum* grows well at 0-200 m above sea level, rainfall type of A and B with 1000-3000 mm/year, 4-5 months dry season and temperature average of 18-33°C. It begins to bear fruit at 7 years and fruits throughout the year. The fruits of *C. Inophyllum* are usually harvested in August-September. The tree of *C. Inophyllum* can still bear fruit until the age of 58. The number per kilogram of dry seed is 100-150 (Forestry Department, 2008).

Previous researches on *C. inophyllum* were the conversion of its oil into biodiesel and identification of its bioactive compounds. Venkanna and Reddy (2009) investigate the biodiesel production from C. Inophyllum oil via acid pre-treatment, transesterification, and post-treatment stages. A three stages transesterification process was also proposed to produce biodiesel from *C. Innopyllum* oil (Sahoo et al., 2007). Another researcher was carried out a two-step process to produce biodiesel from *C. Innopyllum* using a solid acid catalyst (Sathyaselvabala et al., 2011).

Moreover, several bioactive compounds, such as xanthone, coumarin, and benzodipiranons have been successfully identified from *C. inophyllum* plant. Xanthones were reported to show activity cytotoxic and anti-microbial (Noldin et al., 2006). They were found from seed (Noldin et al., 2006; Yimdjo et al., 2004; Linuma et al., 1994) and leaf (Patil et al., 1993). The second bioactive compound is coumarin that was showed inhibitory activity of the HIV virus (Patil et al., 1993), cytotoxic activity (Yimdjo et al., 2004), anti-inflammatory (Fylaktakidou et al., 2004), anticoagulants (Jung et al., 2001), and antitumor (Itoigawa et al., 2001). It was found from leaf and from seed (Itoigawa et al., 2001). The third bioactive is flavonoids that reported to have cytotoxic activity and anti-cancer (Ito et al., 1999; Kondo et al., 2009). It was successfully isolated from seed (Atabani et al., 2013; Berquin et al., 2008). Moreover, Triterpenoids and benzodiperanons were found in seed (Ajayi et al., 2008; Kumar et al., 1976) and leaf (Ali et al., 1999; Khan et al., 1996). Aparamarta et al (2018) identified twelve fatty acids in the C. inophyllum seed oil. The Phytochemical compounds were also identified from the *C. Inophyllum* leaf (Susanto et al., 2017). However, there is no information on the quantification of xanthones in the *C. Inophyllum* leaf.

Therefore, the focus of this research was to make an effective separation method and to quantify the content of xanthones from *C. inophyllum* leaves. In this research, crude leaf extract was separated into two fractions based on differences in polarity of the constituent components by liquid-liquid extraction. The effects of various factors, such as the mass ratio of solvent-to-the crude extract, and the mass ratio of PE-to-methanol on the separation performance, were investigated.

EXPERIMENTAL

Materials

C. inophyllum leaf powder was purchased from Klaten, Indonesia. Silica gel was obtained from Merck (New York, USA), xanthone, coumarin, triolein, tripalmitin and fatty acids Standards were purchased from Sigma Chemical Company (St. Louis, MO). All reagents and solvents were either analytical-grade or HPLC-grade and were purchased from commercial sources. Thin-layer chromatography (TLC) aluminium plates (20 cm x 20 cm x 250 µm) were obtained from Merck (Darmstadt, Germany).

Extraction of *C. inophyllum* leaf

A Soxhlet extractor was applied in this research. Fresh nyamplung leaves (5 kg) were dried and blended into the form of fine powder. The powder leaf (0.15 kg) was covered with filter paper and put in the extractor. Added 98% (v/v) Methanol (300 mL) in round-bottom flask and heated. The flask that contained crude extract of the leaf was obtained by extracting with the methanol for 5 h at 65°C as defined by Shiu *et al*. (2010). The crude extract was stored in a desiccator for 24 hours before it was weighed and used for followed procedures.

Isolation of Xanthone from C. *Innophyllum* leaf

Polar solvent (80% methanol) and non-polar (petroleum ether) solvents extraction were used in this study. A solvent mixture-to-crude extract mass ratio (10, 30, 50 and 70) and PE-to-methanol mass ratio (1 and 3) were used. The PE and methanol fractions were explored using UV Vis, GC-MS and TLC.

TLC analysis

TLC was used to get qualitatively result from the sample as defined by Gunawan *et al*. (2008). TLC paper that has been colored by the sample was immersed in a mobile phase of hexane: ethyl acetate: acetic acid at 90: 10: 1 (v/v/v).

GC-MS analysis

Analysis using GC-MS was used the Agilent 6890N GC and autosampler. Capillary column, HP5 5% fenilmetilsiloksan with length 30 m x 320 µm i.d. and layer thickness of stationary phase 0.25 µm, a detector using MS, Agilent 6971 inert mass selective detector (Agilent Tech Palo Alto, California, USA).The Injector temperature was set at 250°C. The temperature was raised 2°C/min until the temperature became 100°C. After that, it raised 5°C/min until temperature became 290°C. The temperature will be maintained at temperature 290°C for 10 minutes. Helium gas is used. Transfer line at 280°C, 150°C quadrupole MS and 230°C the MS source. Total volume injection was 1 µm, using 1:10 inlet model split and flow rate gas in the column was 1.3 ml/min

UV-Visible spectroscopy

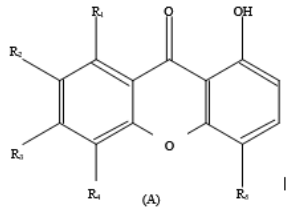
UV-VIS absorption spectra were recorded with a Thermo Scientific Genesys 10S UV Scanning (New York, USA). Absorbance measurements were obtained over the wavelength range of 190–900 nm at room temperature using quartz cuvettes (1 cm path length). The wavelength was selected by the largest absorbance. The maximum wavelength was used for xanthone at 332 nm. Peaks assignments were made by comparing the spectrum of analytes with xanthone standards. This standard solution used to find the wavelength of maximum absorbance at a concentration of 0.1 mg/mL and the calibration curve used in concentrations of 0.1; 0.08; 0.06; 0.03; 0.01; 0.005 mg/mL. The calibration curve was done by fitting the absorbance against concentration at the wavelength of maximum absorption. This procedure was repeated 5 times.

Statistical analysis

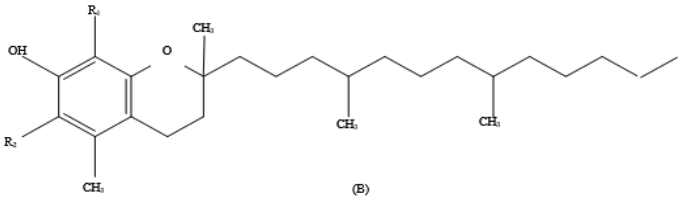
Reliability of the results was analyzed by a statistical software namely Design Expert 9.0.3 trial version. The differences in mean values were examined by analysis of variance (ANOVA). Moreover, the differences of this value, associated with $p<0.05$, were considered significant (Montgomery, 2005).

RESULTS AND DISCUSSION

Many bioactive compounds have been successfully identified from *C. inophyllum* such as xanthones (Yimdjo *et al*., 2004; Linuma *et al*., 1994), coumarine (Patil *et al*., 1993; Itoigawa *et al*., 2001), benzodipiranon (Ali *et al*., 1999; Khan *et al*., 1996), flavonoids (Linuma *et al*., 1994), triterpenoids (Yimdjo *et al*., 2004; Kumar *et al*., 1976) and steroid (Ali *et al*., 1999; Kumar *et al*., 1976). Most of them were found in the nyamplung seed (Atabani *et al*., 2013; Hathurusingha *et al*., 2011; Singh and Singh, 2010; Crane *et al*., 2005) and the leaf (Lim, 2012; Ali *et al*., 1999; Khan *et al*., 1996; Patil *et al*., 1993).



	R_1	R_2	R_3	R_4	R_5
1	H	OH	OH	isoprenyl	H
2	H	H	H	OH	CH_2=CHC(Me)$_2$
3	H	H	OH	OH	CH_2=CHC(Me)$_2$



Compound	R1	R2	Molecular Weight
α- tocopherol	CH_3	CH_3	430
β- tocopherol	CH_3	H	416
γ- tocopherol	H	CH_3	416
δ- tocopherol	H	H	402

Figure 1 Xanthone (A) and vitamin E (B) structure.

Xanthones are the most widely isolated compounds from *C. Inophyllum* (Su *et al*., 2008). The basic framework of two phenyl xanthone compounds connected by bridges and the carbonyl oxygen (ether). Biosynthesis of xanthone compounds is not known clearly but allegedly still closely related to the biosynthesis of flavonoids and stilbenoid. This can be seen from the type of oxygenation. It has two types, the first is aromatic rings that shows the characteristics derived from sikimat. The second is lines rings that show the characteristics derived from the acetate-malonate pathway. In addition, most of these compounds contain additional groups, especially groups isoprenoid that can be seen in Figure 1(A). Xanthone compounds that have been isolated from the bark of *C. Inophyllum* were caloxanton A (Yimdjo *et al*., 2004; Linuma *et al*., 1994), caloxanthone C (Linuma *et al*., 1994), and 3-hydroxyblancoxanthone (Linuma *et al*., 1994). The other kind of xanthones were isolated in the resin of *Garcinia morella and Garcinia hanburyi* namely gambogic acid (Palempalli *et al*., 2009).

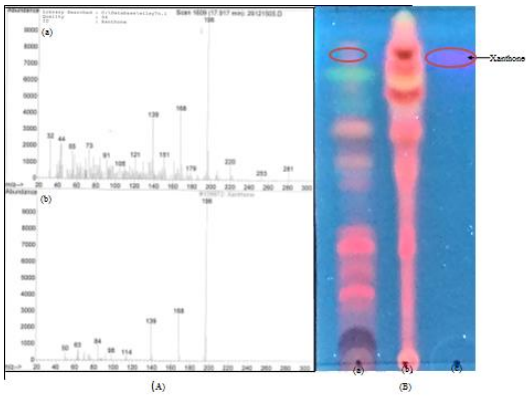


Figure 2 Result of GC-MS (A) of sample (a) and standard solution of xanthones (b) and typical TLC analysis (B) developed in mobile phase = Hexane: Ethyl Acetate : Acetic Acid = 60:40:1 (v/v) of methanol fraction (a) and PE fraction (b) developed in mobile phase.

In this study, xanthones and vitamin E were isolated from *C. inophyllum* leaf extract by liquid-liquid extraction. Two immiscible phases solvents, namely petroleum ether (PE) and methanol were used. The election of the solvents is based on their polarity indices. PE is a non-polar solvent with a polarity index of 0, while the methanol is more polar solvent with a polarity index of 5.1 Sadek, 2002).

Liquid-liquid extraction was applied to separate crude *C.Inophyllum* leaf into two fractions namely non-polar fraction (PE fraction) and polar fraction (methanol fraction) based on polarity indices different of compounds as described by Hakun *et al*. (2016). It was found that PE phases are in the top layer. It is because the density of PE is less than the density of methanol. The density of PE and methanol are 653 and 792 kg/m³, respectively.

A distribution of the component between the immiscible phases occurs. The theory of chemical equilibrium to describe the reversible distribution as:

$$X_A <=> X_B \quad (1)$$

And the equilibrium constant was referred to Nernst distribution law:

$$K_D = \frac{[X]_B}{[X]_A} \quad (2)$$

where the bracket denotes of X was identified as the content of xanthones at a constant temperature in the methanol (denote as B) and PE (denote as A). The larger value of K_D is preferred, a high degree of extraction from PE to methanol. If K_D is small, fewer xanthones are transferred from PE into methanol.

Recovery was used in this cases because no chemical reaction is taking place. Recovery and purity of xanthones were used as the indicator of the separation performance. It is calculated as follows:

$$\text{Recovery} = \frac{\text{Mass of xanthone recovered (g)}}{\text{Mass of crude extract of leaf (g)}} \times 100$$

Purity was calculated as follows:

$$\text{Purity} = \frac{\text{Mass of xanthone in methanol fraction (g)}}{\text{Mass of methanol fraction (g)}} \times 100$$

The experimental design matrix development was evaluated with Design Expert 9.0.3 trial version. Triple replicates of the factorial design were applied. Those were an experimental design with two factors at different levels, such as (A) mass ratio between solvent mixture-to-crude extract at levels of 10, 30, 50 and 70 and (B) mass ratio of PE-to-methanol at levels of 1 and 3. A total of 27 runs were implement in random order. The responses were equilibrium constant (K_D), recovery and purity of xanthones in the polar fraction of *C.Inophyllum* leaf as confirmed by TLC analysis, GC-MS (Figure 2) and UV Vis spectrophotometry.

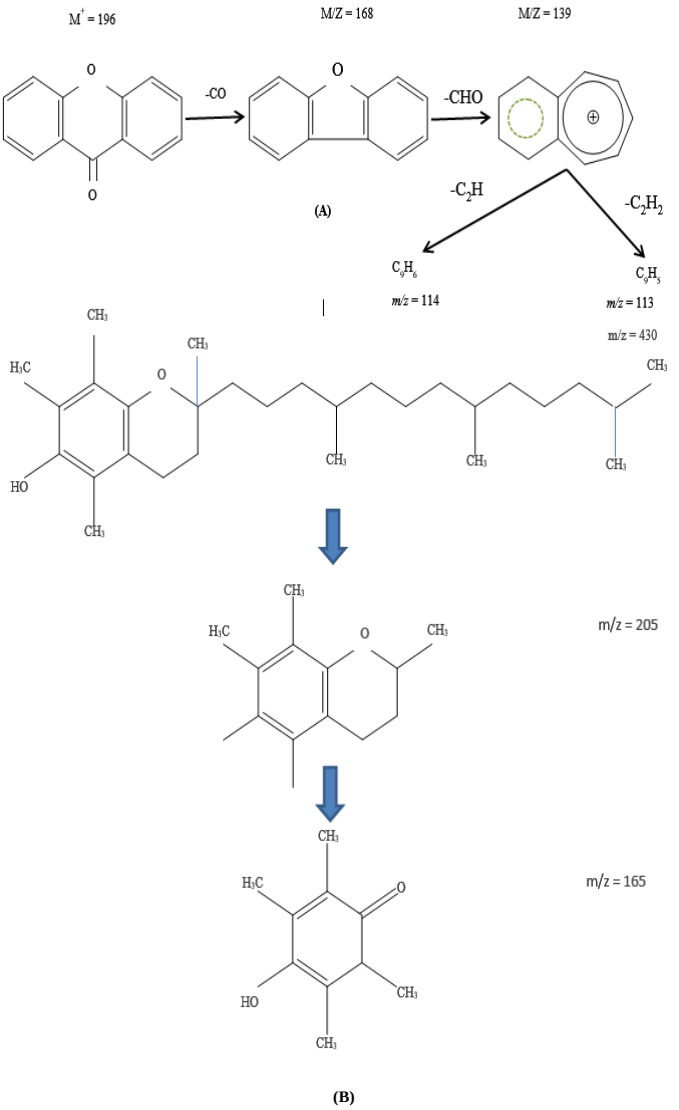


Figure 3 The typical GC-MS result and fragmentation of xanthones (A) and vitamin E (B).

Besides the TLC analysis, this work was applied GC-MS analysis. Best of our knowledge and literature survey there is no report of gas chromatography and mass spectrum analysis to identify the chemical compounds from the plant species of *C. Inophyllum*. From methanol fraction, The xanthones compound can be determined from the base peak of mass spectrometric ions. Xanthones compound was detected at retention time 17.95 minutes. The ion at m/z at196, 168.139, 114, 113, 98, 84, 63, and 50 were the base ion peak of xanthones, respectively. The ion at m/z 196 was the best peak of xanthones. Typical GC-MS fragmentation of xanthones is shown in Figure 3(A) Their GC-MS fragmentation was confirmed by M^+, (M^+-CO), (M^+-CHO), (M^+-C_2H) and (M^+-C_2H_2).

From petroleum extract, vitamin E can be determined too. This result was the same with the previous research as documented by Kumar *et al*. (2014). They found that vitamin E appears in the *Adiantum capillus-veneris* with GC-MS analysis. Vitamin E became of importance for human health because it contains a lot of advantages as an antioxidant (Kumar *et al*., 2014), protects and prevents lipids for the oxidation of polyunsaturated fatty acids (Whitney and Sharon, 2011) as can be seen on Figure 1(B). Vitamin E compound was detected at retention time 29.58 minutes. The ion at m/z at 430, 205.187, 165, and 43 were the base ion peak of , respectively. The ion at m/z 165 was the best peak of vitamin E. Typical GC-MS fragmentation of vitamin E is shown in Figure 3(B). Their GC-MS fragmentation was confirmed by M^+, (M^+-2,6,10 trimethyl $C_{13}H_{24}$), and (M^+-C_3H_4).

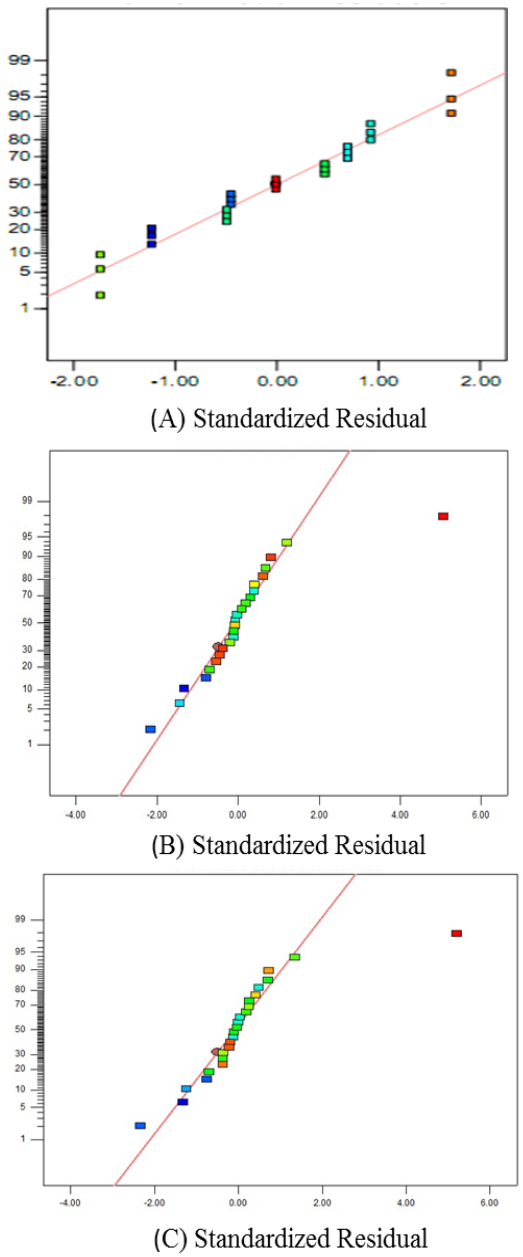


Figure 4 Normal probability plot of residuals for KD (a), Recovery of xanthone (b) and purity of xanthone (c) in methanol fraction.

Moreover, the goodness of model fit for purity, recovery, and K_D of xanthone was analyzed by using normal probability plot as shown in Figure 4. It can be seen that both plots were straight lines, which are associated with a normal distribution.

Table 1 Analysis of variance for equilibrium constant (K_D).

Source	Sum of Squares	df	Mean Square	F Value	the Prob > F
Model	0.0190	8	0.0023	1284.69	< 0.0001
A[a]	0.0150	2	0.0073	4020.88	< 0.0001
B[b]	0.0024	2	0.0018	654.51	< 0.0001
A*B	0.0017	4	0.0004	231.69	< 0.0001
Pure Error	< 0.0001	18	< 0.0001		
Cor Total	0.0190	26			
S =	0.0014		Rsq =	99.83%	

[a] Mass ratio of the solvent mixture to crude extract
[b] Mass ratio of PE to methanol

Table 2 Analysis of variance for purity of xanthone.

Source of variations	Sum of squares	Degrees of freedom	Mean square	F-value	P-value
A*)	0.0079	2	0.0040	183.93	< 0.0001
B**)	0,0015	2	0.0007	33.59	< 0.0001
A*B	0.0009	4	0.0002	10.05	< 0.0001
Error	0.0004	18	< 0.0001		
Total	0.0107	26			
S =	0.0047	R-Sq=	96,35%		

[a] Mass ratio of the solvent mixture to crude extract
[b] Mass ratio of PE to methanol

Table 3 Analysis of variance for recovery of xanthone.

Source	Sum of squares	Degrees of freedom	Mean square	F-value	P-value
A[a]	1791.5	2	895.75	158.27	< 0.0001
B[b]	1373.12	2	686.56	121.31	< 0.0001
A*B	113.22	4	28.31	5	0.007
Error	101.87	18	5.66		
Total	3379.7	26			
S=	2.37898	R-Sq=		96.99%	

[a] Mass ratio of the solvent mixture to crude extract
[b] Mass ratio of PE to methanol

The ANOVA for K_D, purity and recovery xanthone in methanol fraction are summarized in Tables 1 to 3. P value was used to determine the significance of the effect of factors. A P value less than 0.05 indicates that the factor is significant (Montgomery, 2005).

From Tables 1 to 3, It can be seen that the effect of solvent mixture-to-crude extract mass ratio (A) and PE-to-methanol mass ratio (B) were significant ($p<0.05$). It was also found that the interaction of solvent mixture-to-crude extract mass ratio and PE-to methanol mass ratio (AB) is significant. The P value below than 0.05 was indicated that this interaction was significant factor to get higher K_D , recovery and purity of xanthones in methanol fraction.

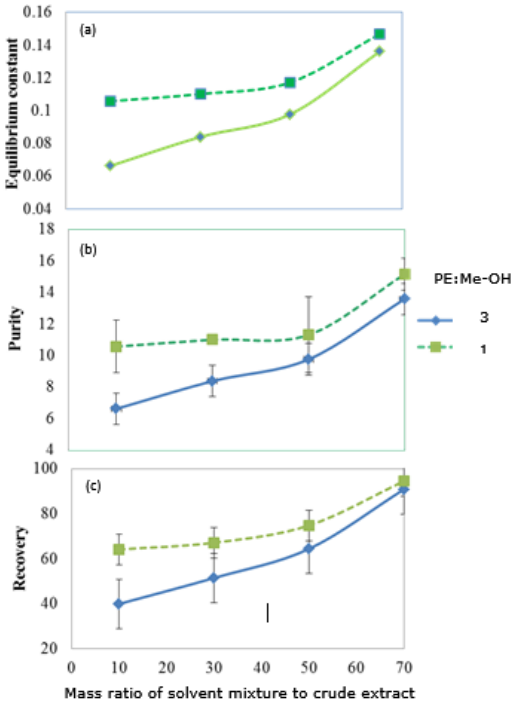


Figure 5 The graphic of (a) equilibrium constant (kd), (b) purity, and (c) recovery of xanthone in mass ratio of solvent mixture-to-crude extract.

The equilibrium constant (K_D), purity and recovery of xanthone at solvent mixture-to-crude extract mass ratio (10, 30, 50 and 70) and PE-to-methanol mass ratio (1 and 3) could be seen in Figure 5a, 5b, and 5c. The increasing content of mass ratio solvent mixture-to-crude extract results in the higher of equilibrium constant (K_D), recovery, and purity of xanthone in methanol fraction. This effect because the higher ratio of solvent can increase the chance of bioactive compound to contact with a solvent (Wong *et al*., 2013). This result agrees with another literature that organic solvent can significantly increase the recovery yields of the phenolic compound from plant materials (Ye *et al*., 2015). This study recommends that higher mass ratio solvent to crude should be used for xanthones isolation in the *C.Inophyllum* leaf

Moreover, it can be seen that mass ratio of PE-to-methanol of 1 has the better result than that of 3**.** This result was comparable with the previous research as documented by Goli *et al*. (2004). They found that the extraction of bioactive compounds is highly depending on the polarity of the solvent. Polar compounds were easily extracted using polar solvent. Xanthone was polar compound so can soluble in the polar solvent like ethanol (Yoswathana, 2013). Syukriah *et al*. (2014) reported that bioactive compound in *Quercus infectoria* (Manjakani) is easier to extract with solvents that are more polar. From these work, the mass ratio of PE to methanol of 1 was considered to be adequate for the process.

Optimization of this system was applied with the target to maximize K_D, recovery, and purity of xanthones in methanol fraction. It was found that the variables can be used to produce the optimum isolation of xanthones. This condition happens at the variable of solvent mixture-to-crude extract mass ratio of 70 and PE-to-methanol mass ratio of 1. The optimum isolation of xanthones in methanol fraction is 0.150 for K_D, 94.43% for recovery, and 15.16% for purity.

CONCLUSION

The mass ratio of solvent mixture-to-crude extract and the mass ratio of PE-to-methanol play on important role in xanthones isolation. Xanthones were successfully isolated from crude of *C. Inophyllum* leaf by liquid-liquid extraction. At mass ratio of the solvent mixture to a crude extract of 70 and mass ratio PE to methanol of 1 were the optimum condition for Xanthone isolation. With GC-MS analysis, xanthones were detected with the quality at 94% and % area at 0.056.

ACKNOWLEDGEMENT

This research was supported by a grant (897/PKS/ITS/2017) provided by the Institute of Research and Public Services (LPPM) - ITS, Indonesia. The authors thank to Mr Tholib and Mrs. Dewi for worth technical support.

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