The extraction and purification of squalene from Nyamplung (Calophyllum Inophyllum L.) leaves

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Abstract. Squalene is a natural organic compound that is primarily found in shark liver oil. It can act as a skin moisturizing, anticancer, antivirus, and drug delivery agent. The advantages of people consuming healthy oil which contains squalene can lower the risk of heart disease. Due to the high market demand for squalene, it becomes a severe threat to shark existence. Therefore, investigation to find a new source of squalene is deemed necessary. Squalene is present in the leaves of nyamplung (Calophyllum inophyllum), a species of mangrove plant commonly found along Indonesia's coast. This study aims to extract and purify squalene from nyamplung leaves. First, nyamplung leaves were crushed and extracted with n-hexane. The extract was designated as a crude extract of nyamplung leaves. After that, squalene was purified from the crude extract by the multistage adsorption-desorption method at 3°C. The crude extract was dissolved in hexane, and silica gel was then added at 30 min. The mass ratios of silica gel to crude extract were varied at 1:1, 2:1 and 4:1 (g/g). N-hexane fraction was separated from silica gel by filtration paper. N-hexane fraction was designated as a non-polar fraction (NPF), while silica gel fraction was designated as a polar fraction (PF). The dark-colored of PF was employed for the desorption process using methanol until its color became colorless. Meanwhile, NPF was extracted with fresh silica gel until it reached the 10th stage of extraction. NPF and PF were then quantitatively and qualitatively analyzed using TLC and GC, respectively. The best result was obtained from silica gel to crude extract mass ratio of 1:1 (g/g) at the 5th stage with squalene purity of 35.96% (45.82% recovery).

1. Introduction
Squalene is a triterpene that has six isoprene units which the double bonds are nonconjugated [1]. It is an unsaturated hydrocarbon [2]. It has a pale yellow color. It is a type of lipid and is used as a biochemical precursor to nearly 200 different triterpenes [3]. Squalene is found approximately 13% on human skin surface [1]. It is often used as a natural skin moisturizer and as a cosmetic raw material. In the pharmaceutical industry, squalene is used as a drug, vaccine adjuvant, and nutritional supplement [4].

The advantages of people consuming healthy oil that contains a lot of squalene can result in a lower risk of heart disease. This is proven by the consumption of olive oil which is rich in squalene. It lowers the amount of cholesterol due to the content of monounsaturated and polyunsaturated fatty acids owned by squalene [5]. Utilization and demand for squalene can increase the danger of extinction of the shark as the main source. However, many countries have banned shark fishing since 2000. Thus, many researchers investigate other sources of squalene.
Ragas et al. [6] identified various triterpenes compounds in *Calophyllum inophyllum* leaves, including squalene. Nyamplung (*C. inophyllum*) is a versatile mangrove plant that is often found on the coast of Indonesia. *C. Inophyllum* functions as a coastal protector from abrasion, wind tidal waves, landslides, and a maintenance system of brackish water quality. Furthermore, this plant has a high economic value such that almost every part of it can be processed into high-value products. In India, all parts of the *C. inophyllum* can be used for the treatment of rheumatism and various skin diseases, although the active ingredients are still not clear. While the stems of *C. inophyllum* are being used to treat internal bleeding and as an astringent material for skincare products, the leaves are useful for treating eye irritation, vertigo, migraine, and heat stroke [7].

There is a considerable amount of *C. inophyllum* in Indonesia which has not been used properly. It has many benefits from various bioactive compounds that make it a new source of squalene. Squalene extraction has been carried out by using various methods on plants, yeast, and fungi. In plants, it has been detected in many edible crops, such as olive plant [8], amaranth [9], seeds of grape [10], walnuts, peanuts [11], and soybean [12].

Gunawan et al. [12] had succeeded in purifying squalene in soybean oil with high purity (95.90%) and recovery (93.09%) using the modified soxhlet extraction. Wejnerowska et al. [9] reported that the purity of squalene isolated from amaranth seed using the supercritical carbon dioxide was 60% (98.2% recovery). The extraction of squalene from camellia oil has been successfully carried out by Xiao et al. [13] using silver ion complexation and obtained purity of 37.8%, 108 times higher than the initial purity. However, there have been no studies on squalene extraction from *C. inophyllum* leaves. Therefore, this research aimed to extract and purify squalene from *C. inophyllum* leaves using maceration, followed by a multistage of adsorption-desorption methods.

2. Materials and method

2.1. Materials

*C. inophyllum* leaves were collected from Wonorejo Mangrove Education Ecotourism area in Surabaya, East Java, Indonesia. Hexane and methanol solvents were purchased from Chemical Indo Multi Sentosa (Surabaya, Indonesia). The filter paper was obtained from Nurra Gemilang (Malang, Indonesia). Silica Gel 60 (0.2–0.5 mm) was purchased from Merck KGaA (Darmstadt, Germany). Standards of squalene, coumarin, friedelin, xanthone were obtained from Sigma Aldrich (St. Louis, MO: USA).

2.2. Preparation of *C. inophyllum* leaves crude extract

The fresh *C. Inophyllum* leaves were air-dried for 14 days. After that, they were milled into coarse leaves powder using a chopping machine. Coarse leaves powder (1 kg) was macerated using 3 L of hexane, and then the mixture was stirred for 5 min every 24 hr for three days. The filtrate and residue were then separated. The residue was macerated twice with the same method. All filtrates were mixed, and the solvent was then evaporated by distillation at 72°C until the filtrate became semi-solid. It was designated as crude extract and stored at 4°C.

2.3. Multistage of adsorption-desorption

About 1 gr of crude extract was added to 150 mL of hexane, stirred for 15 minutes at a speed of 300 rpm. Then it was added with silica gel (1, 2, and 4 g) and stirred again for 30 min. The mixture was filtered using fine filter paper to separate the filtrate and residue. The filtrate was designated as a non-polar fraction (NPF) which was added with silica gel in the same ratio up to the 10th stage. Meanwhile, the residue was added with methanol and stirred for 15 min. This process was repeated until the adsorbent and solution were colorless. The filtrate was evaporated to remove the methanol and then, it was designated as a polar fraction (PF).

2.4. Gas Chromatography (GC) analysis

Based on research conducted by David et al.[14], a Shimadzu GC-2010 gas chromatography system with a flame ionization detector and a DB-5HT (5 percent phenyl)-methylpolysiloxane non-polar
column (15 m 0.32 mm id; Agilent Tech. Palo Alto, California, USA) was used to investigate C. Inophyllum leaves extractions.

2.5. Statistical analysis
The credibility of the results was checked by a statistical analysis Design of Experiment (DOE) with factorial design software Minitab 19.1, trial version. Analysis of variance was used to assess the variations in mean values (ANOVA). Differences with a p-value of less than 0.05 were judged significant.

3. Result and discussion

3.1. Extraction of C.Inophyllum leaves
C.inophyllum leaves crude extract has a blackish green color, dry, and sticky. It contains 0.009% coumarin, 0.1188% xanthones, 3.9388% friedelin, 11.0262% squalene and 84.9064% other components. Based on the GC chromatogram, friedelin and squalene are the main compounds of this crude extract, where both of them are triterpenes. This is in line with research conducted by Ragasa et al. [6] that succeeded in isolating various triterpenes compounds, such as friedelin, squalene, canophylllic acid, a mixture of canophyllalic acid, and canophyllol. Filho et al. [15] also said that plants of the genus Calophyllum mainly contain coumarins, xanthones, flavonoids, steroids, triterpenes, some of which have involved biological activities. However, in this study, coumarin and xanthone compounds were found in C.inophyllum plants, but they were not the main compounds of this species because of their low concentrations.

In addition, a high squalene content was obtained in the crude extract due to the choice of hexane solvent in the extraction process. Hexane is a non-polar solvent, so it tends to bond with non-polar compounds, such as squalene. These results agree with observations by Conforti et al. [16] on the extraction of Amaranthus caudatus seeds using methanol, ethyl acetate, and hexane as solvents which said that the highest squalene content was obtained in the hexane extract. Besides, hexane is also safer as a non-polar solvent than others because it can be recycled, has low energy costs, has a high selectivity against oil, is extraction efficient and effective [17]. Due to the high squalene content in C.inophyllum leaves crude extract, compared to other compounds, a further purification process was carried out.

3.2. Multistage adsorption-desorption
Selection of the adsorbent is an essential process in the adsorption-desorption. In that, the adsorbent can affect the polarity of the target compound [18]. Silica gel is a polar adsorbent, so squalene is not easily soluble. This purification process is considered successful if there is only squalene in the NPF, while the other polar components are adsorbed by silica gel. Because the adsorption-desorption process is generally exothermic therefore the temperature has an important role. This is because the process of decreasing the adsorption-desorption temperature results in better separation of polar and non-polar components [12,19]. The process is carried out at a temperature of 3°C. It cannot be lower due to equipment limitations.

Figure 1 shows the illustration of the NPF of each stage of several mass ratios of silica gel to crude extract. It can be seen that the higher the silica gel ratio, the faster the color of the crude extract will fade. It can be seen in the mass ratio of silica gel to crude extract of 4, and the NPF color changes to colorless after stage 4. Meanwhile, in the mass ratio of silica gel to crude extract of 2 and 1, the NPF color begins to turn colorless at stage 9 and stage 10, respectively. The resulting appearance of NPF at each stage was the overall color of green-colored concentrated toward the pale yellow color to colorless. This colorless process indicates that many polar compounds are entrained into silica gel. The pale yellow colored NPF indicates that the squalene content is very high because of the color of squalene.
Figure 1. The appearance of NPF at each stage with a mass ratio of silica gel to crude extract of (a) 1:1, (b) 2:1, and (c) 4:1 (g/g).

In table 1, the mass ratio of silica gel to crude extract of 1 has a more significantly increased in % purity of squalene. The highest purity of 35.96% (recovery of 45.82%) was obtained at stage 5. It was the highest % purity of squalene compared to the other ratios. This is in line with the findings of Fabian et al. [19], who found that in a stirred batch adsorption procedure, a lower silica gel utilization ratio is preferable for separating non-polar chemicals in soybean oil since it takes less adsorbent and results in a greater non-polar value. In the process of separating chemicals, however, a tiny amount of adsorbent is less effective.

The effect of the mass ratio of silica gel to crude extract on the NPF component of each stage can be seen in table 1. The higher the ratio of the silica gel to the crude extract resulted in higher squalene purity in NPF stage 1. In the mass ratio of silica gel to crude extract of 4, the highest purity of squalene but with the lowest recovery of squalene was obtained at stage 1, which is in contrast to what happened in the mass ratio of silica gel to crude extract of 1. This indicates that squalene compounds and other non-polar fractions can be adsorbed at a higher ratio of silica gel. The higher ratio of silica gel to crude extract results in a much larger area that can be adsorbed, which results in many non-polar compounds are also adsorbed on the silica gel. This indicates that the concentration of other compounds that are more polar than squalene is also low in the NPF, so that squalene is bound by silica gel.

This also affects the friedelin compound in the higher mass ratio of silica gel to crude extract; it is only detected at the earlier stages. This is comparable to the findings of Fabian et al.,[19] who found that separating non-polar lipid fractions on soybean oil with a mass ratio of silica gel to soybean oil of more than six does not result in successful separation. Squalene and fatty acid steryl esters, for example, were weakly adsorbed onto silica gel and easily dissolved by hexane during the adsorption process. More polar chemicals, such as FFA and TAG, as well as tocopherols and phytosterols, are on the other hand, more firmly adsorbed on silica gel. [20].

However, if in the adsorption process there are no polar compounds, and only non-polar compounds remain, the non-polar compounds will be adsorbed on the silica gel. This causes a decrease in the % purity of squalene in the next stage, after experiencing high purity. Meanwhile, % recovery of squalene, friedelin, and other compounds decreased significantly with the increasing number of stages carried out. This indicates that the increasing number of stages is ineffective because it causes many compounds to be adsorbed onto the silica gel.
Table 1. Composition of non-polar at each stage multistage adsorption-desorption.

| Silica gel to crude extract ratio (g/g) | Stage | Compounds | Squalene | Friedelin | Others |
|----------------------------------------|-------|-----------|----------|-----------|--------|
|                                        |       |           | (g)      | (g)       | (g)    |
| 1:1                                    | 1     | 15.34%    | 3.32%    | 80.34%    | (63.44%)|
|                                        | 2     | 20.32%    | 4.43%    | 75.25%    | (41.03%)|
|                                        | 3     | 23.97%    | 2.89%    | 73.14%    | (24.26%)|
|                                        | 4     | 31.62%    | 2.12%    | 66.26%    | (15.61%)|
|                                        | 5     | 35.96%    | ND       | 64.04%    | (9.45%) |
|                                        | 6     | 31.85%    | ND       | 68.15%    | (9.45%) |
|                                        | 7     | 30.96%    | ND       | 69.04%    | (8.55%) |
|                                        | 8     | 27.23%    | ND       | 72.18%    | (4.97%) |
|                                        | 9     | 15.80%    | ND       | 84.20%    | (3.28%) |
|                                        | 10    | 21.87%    | ND       | 78.13%    | (14.94%)|
| 2:1                                    | 1     | 15.94%    | 5.43%    | 78.63%    | (58.19%)|
|                                        | 2     | 18.32%    | 4.32%    | 77.36%    | (44.16%)|
|                                        | 3     | 20.29%    | 1.78%    | 77.93%    | (35.16%)|
|                                        | 4     | 24.21%    | ND       | 75.79%    | (20.33%)|
|                                        | 5     | 23.82%    | ND       | 76.18%    | (20.84%)|
|                                        | 6     | 21.87%    | ND       | 78.13%    | (14.94%)|
|                                        | 7     | 20.54%    | ND       | 79.46%    | (6.71%) |
|                                        | 8     | 19.21%    | ND       | 80.79%    | (5.38%) |
|                                        | 9     | 16.21%    | ND       | 83.79%    | (4.60%) |
|                                        | 10    | 10.32%    | ND       | 89.68%    | (3.54%) |
| 4:1                                    | 1     | 19.90%    | 3.02%    | 80.10%    | (41.98%)|
|                                        | 2     | 16.98%    | 2.10%    | 80.92%    | (30.94%)|
|                                        | 3     | 11.23%    | ND       | 88.77%    | (29.49%)|
|                                        | 4     | 4.57%     | ND       | 95.43%    | (28.62%)|
|                                        | 5     | 3.60%     | ND       | 96.40%    | (20.20%)|
|                                        | 6     | 3.42%     | ND       | 96.58%    | (14.84%)|
|                                        | 7     | 3.16%     | ND       | 96.84%    | (11.96%)|
|                                        | 8     | 4.80%     | ND       | 95.20%    | (6.72%) |
|                                        | 9     | 4.92%     | ND       | 95.08%    | (4.68%) |
|                                        | 10    | 5.19%     | ND       | 94.81%    | (3.77%) |

*a-coumarin, xanthone, and other bioactive compounds that have not been detected in a crude extract
b % wt
c % recovery
d Not Detected

Figure 2 shows an NPF typical GC result at a mass ratio of silica gel to crude extract of 1:1 (g/g). It can be seen that squalene had a wider area or the highest peak up to stage 5. But after the stage was continued, the area of squalene had decreased until the last stage, while there were new peaks that were
not visible in the crude extract. Based on research conducted by Gunawan et al. [20], these new peaks are hydrocarbons. However, it needs to be identified further. The process for the appearance of the hydrocarbons peaks is due to the sufficient time spent during stirring so that it passes the adsorption-desorption equilibrium point. This causes the % recovery from squalene tends to experience a lot of decrease in all mass ratios of silica gel to crude extract. According to the research of Chu et al. [21], the separation of vitamin E from palm fatty acid distillate using the adsorption-desorption process reached equilibrium in 5 min. It was found that the processing time of 30 min is too long for the squalene purification process. In addition to stirring time, the equilibrium in the adsorption process was also influenced by the reaction temperature, agitation rate, and silica mass ratio [21]. Research to increase compounds enriching the content of Vitamin E, phytosterols, squalene, and carotene compounds conducted by Phoon et al. [18] reported that the adsorption process for compounds containing many hydrocarbon chains such as squalene and carotene should be carried out using non-polar adsorbents such as Diaion HP20 and Sepabeads SP 850 because they are more effective than silica gel and Florisil.

![Figure 2](image.jpg)

**Figure 2.** Typical GC analysis on the mass ratio of silica gel to crude extract 1:1 (g/g).

Moreover, % purity and % recovery of squalene were checked with the normal probability plot of residual response shown in figure 3. It can be seen that the points always follow and approach the linear so that the residual value in the linear regression analysis is well fulfilled as for the response that was affected well in the adsorption-desorption process. In % recovery, all interactions between temperature
and stage have a significant effect ($p<0.05$). Meanwhile, for % purity, the only temperature has a significant effect ($p<0.05$), while the stage has no significant effect ($p>0.05$).

![Figure 3](image)

**Figure 3.** Normal probability plot of the residual response of (a) % purity of squalene (b) % recovery of squalene.

Contour plot of mass ratio and number of the stage on % purity and % recovery of squalene is shown in figure 4. The highest value of % purity was obtained at the lowest mass ratio of silica gel to crude extracts of 1 and stage 5 (figure 4a). Although, the lower mass ratio of silica gel to crude extract was not effective, resulting in a higher value % purity than others. Terms of the economy as well, it has a lower cost because of less adsorbent used. However, the % recovery (figure 4b) value is inversely proportional to the ratio of silica gel to crude extract and the number of stages applied. Therefore, with more number stages applied, more squalene compounds were adsorbed on the silica gel.

![Figure 4](image)

**Figure 4.** Contour plot of (a) % purity of squalene vs. ratio and stage (b) % recovery of squalene vs. ratio and stage.
4. Conclusion
The extraction and purification of squalene from *C. Inophyllum* leaves using maceration followed by multistage adsorption-desorption were successfully studied. According to the findings, a larger mass ratio of silica gel to crude extract provides more adsorbent and lowers squalene formation. The stirring time in the adsorption-desorption process should only be carried out until the equilibrium stage is reached so that not many squalene compounds are adsorbed onto the silica gel.

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