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# Implementation of *E.Dulcis* as Albumin-coated Biomaterial Patch and Organic Photosensitizer for Photodynamic Therapy on Melanoma Cell Cancer

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***Abstract*—Photodynamic therapy (PDT) is a treatment modality that relies on photosensitizer administration and light-based activation. The use of chlorophyll as a photosensitizer is due to the abundance of chlorophyll in nature, especially in Indonesia, which is a tropical country. An obstacle to applying chlorophyll as a photosensitizer is the low stability of chlorophyll, so modifications are needed to obtain stable chlorophyll. The coating is a chlorophyll modification method that allows for a more straightforward synthesis and can increase its stability. This research aimed to implement the extracted *Eleocharis dulcis (E.dulcis)* as a chlorophyll source and modify it into an albumin-coated biomaterial as a photosensitizer. The modification results can be applied as patches to obtain albumin-modified biomaterial patches with maximum absorption at wavelengths between 650-750 nm and purity >95%. The photosensitizer chlorophyll coating results from the surface modification method of chlorophyll isolate of *E.dulcis* weed with albumin, which has stable, high hydrophilicity properties and low toxicity. The photosensitizer was then applied to achieve apoptotic melanoma cancer cell death, and red–near-infrared laser exposure was used to get high apoptotic results. In this research, PDT with a laser energy dose of 20 J/cm2, along with the addition of a photosensitizer on melanoma cell cancer, reached the highest percentage of apoptotic cell death for both MCF7 and T47D at 90 ± 0,8 % and 86 ± 0,7 % respectively. The potential of photodynamic therapy based on albumin-coated E.dulcis's chlorophyll as a biomaterial patch and organic photosensitizer in increasing the death of melanoma cell cancer is a significant outcome of this research.**

***Keywords*—*E.dulcis*; chlorophyll; photosensitizer; albumin-coated; photodynamic therapy.**

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## I. INTRODUCTION

Photodynamic therapy (PDT) is a treatment modality that relies on photosensitizer administration and light-based exposure (laser) [1]–[4]. PDT is a relatively new and non-invasive cancer treatment [5]–[8] has a promising therapy due to its efficiency and accuracy [9]. According to Mfouo-Tynga et al. [10], the essential aspects of photosensitizer are high purity and known chemical composition, peak absorption at 600 – 800 nm, high quantum yield in generating singlet oxygen and light-dependent high cytotoxicity, low toxicity, and stability. Meanwhile, Various studies of laser energy dose and cancer targets have been carried out by Al-Khafaji [11]. A combination of He-Ne-PDT with DMF irradiation at a dose of 6 and 12 J/cm$^2$ applied to different concentrations of ALA (1 and 2 mM) has a promising treatment against hormone-unresponsive breast cancer cells. Other studies by Eskiler et al. [12] used 5-aminolaevulinic acid (5-ALA) mediated PDT at an energy dose of 2.8–18.82 J/cm$^2$ significantly reduces the viability of TPC and CS cells. Study by [13] found the best PDT conditions under red light ($\lambda = 660 \pm 20$ nm) at an irradiance of 4.5 mW/cm$^2$ for 667 s (light dose of 3 J/cm$^2$) to treat melanoma cancer cells. Furthermore, the use of various photosensitizers in PDT has shown promising results in various cancers including skin cancer [14].

During 200 years of research on chlorophyll, not only as a basis for photosynthesis but also had additional functions of applications in basic science, medicine, as colorants, and possibly in optoelectronics [15]. Chlorophyll and its derivatives have been widely used in medicinal uses in biomedical applications, such as photodynamic and photothermal therapy, photocatalytic diagnosis [16],

performance as cancer-preventing agents, antimutagens, apoptosis inducers, efficient antioxidants, together with antimicrobial and immunomodulatory molecules [17]. Photosensitizer materials can be inorganic and organic materials. Organic materials used as photosensitizers, such as cyclodextril/chlorophyll [17], and made into supramolecules and Uliana et al. [18] semi-synthesized chlorophyll with cost-effective photosensitizers (PSs) that contain different substituents. Chlorophyll is also used for acne therapy [19] and bioimaging [20]. Protoporphyrin IX and GNPs (Gold nanoparticles) are also used as photosensitizers in cervical cancer and Chlorophyll composites were applied to cancer therapy [21]–[23]. Previously, chlorophyll modification coating methods by Chu et al. [24] with Pluronic as nanocomposite achieved more straightforward synthesis and increase. Zhang et al. [25] used a sodium copper chlorophyllin (SCC) with a porphyrin ring induced Ca–P obtained $(Ca–P/SCC)_{10}$ coating exhibited good corrosion resistance, antimicrobial activity (especially under 808 nm irradiation) and biocompatibility. Moreover, Chu et al. [24] also use a coated surfactant of albumin acting as Pluronic as a nanocomposite since the nature of albumin does not damage chlorophyll.

Natural products such as Photosensitizers are obtained by partially synthesizing from abundant natural starting compounds and can be isolated at low cost and also in large amounts from plants or algae [26]–[28]. As a tropical country, Indonesia is a photosensitizer source due to the abundance of chlorophyll in nature. One of the plants that can produce quite a lot of chlorophyll as a weed is *Eleocharis dulcis (E.dulcis)*. *E.dulcis* is a typical swamp land plant commonly found in tidal areas with acid-sulfate soils. Extraction results obtained compounds such as cellulose compounds, lignin, and chlorophyll. Moreover, it is known that biological activities credited to chlorophylls and their catabolites comprise cancer prevention, antioxidant and antimutagenic activities, mutagen trapping, modulation of xenobiotic metabolism, induction of apoptosis, antimicrobial properties, and anti-inflammation activity [29]. Study by [30] shows that the dermatological platform comprising Chlorophyll-based extract is the next candidate system for PDT. A recent study displays that chlorophyll potential by conjugating chlorophyll (Chl) to vanadium carbide (V2C) nanosheets for combined photodynamic/photothermal therapy and showed advanced performance in vitro cell line killing and completely ablated tumors in vivo with 100% survival rate under a single NIR irradiation [31]. Currently, scientists encourage to seek natural compounds, including chlorophylls with photosensitizing properties for PDT, which are being discovered and identified with reduced toxicity to healthy tissues and a lower incidence of side effects [8]. For example, chlorophyll derivatives extracted from algae and plants demonstrated that chlorophyllin can localize in lysosomes and mitochondria [26].

In this research, with the potential of *E.dulcis* as a chlorophyll source and its modification of the biomaterial used as a photosensitizer in PDT in the hope of providing apoptotic melanoma cancer cell death, and the use of portable red–-near-infrared laser exposure is also needed to get high apoptotic results. This paper aims to examine the application of PDT on melanoma cancer cells. The albumin-modified biomaterial patch with a red–near infrared laser of 650–750 nm wavelength is applied as an organic photosensitizer for PDT of melanoma cancer cells. Specifically, we have:

a. Chlorophyll photosensitizer coated with albumin has stable properties and high hydrophilicity.
b. Chlorophyll of *E.dulcis* produced has low toxicity.
c. PDT with a laser energy dose of 20 J/cm$^2$, along with the addition of a photosensitizer on melanoma cell cancer, reached the highest percentage of apoptotic cell death for both MCF7 and T47D at 90 ± 0,8 % and 86 ± 0,7 %, respectively.

## II. Materials and Methods

The experiment is given for at least seven steps: production of chlorophyll-coated albumin, modified biomaterial patch manufacturing, cell culturing stage, toxicity test stage, sample preparation stage, population checking stage, and laser exposure. The complete overview of the experiment is illustrated in Fig. 1 below.

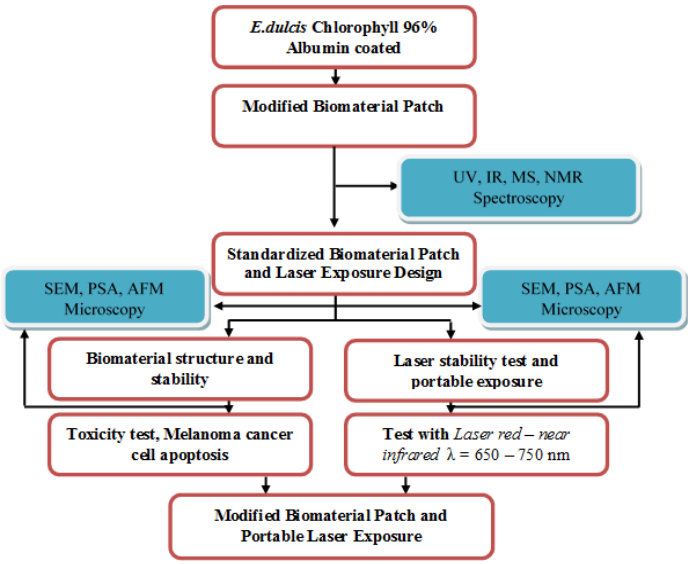



Fig. 1 The proposed schematic diagram of the experiment

Chlorophyll-coated albumin is prepared by dissolving Chlorophyll 1 mg and albumin 1 mg in 0.2 ml chloroform and evaporating on a thin film. The thin film was washed with nitrogen to remove chloroform. Phosphate buffered saline (PBS) (1 mL) was put into the flask and stirred for 30 minutes, followed by sonification for 15-20 minutes, and then the solution was stored at 4 ºC in a dark environment [21].

Manufacturing a modified biomaterial patch begins with preparing the matrix-type transdermal patch consisting of Ethyl Cellulose using a solvent evaporation technique in a petri dish. Ethyl cellulose polymer solution was made in methanol and chloroform with a ratio of 1:1 until a clear solution was formed then added Polyvinyl Alcohol was stirred until homogeneous, then added dibutyl phthalate and extract, stirred homogeneously using a magnetic stirrer rotation 6 for approximately hour so that a final volume of 10 ml was obtained. Then, it was transferred to a 9 cm diameter petri dish covered with aluminum foil and closed on the top of the cup with the funnel upside down. Dry in the

oven at 60°C for one day. Then, put in a desiccator until used. Patch evaluation consists of three types: organoleptic examination, thickness of the resulting patch, and percentage hygroscopic test. Firstly, the organoleptic examination includes observing the resulting patch's shape, color, and smell. Secondly, patch thickness was measured using a micrometer using a 0.01 mm Scrub Micrometer accuracy. Measurements were made at five different places. Thirdly, to check the patch's physical stability under high humidity, the patch was weighed and placed in a desiccator containing a saturated sodium chloride solution for three days. Patches were re-weighed, and moisture percentage and skin Irritation were tested. A skin irritation test was performed on volunteer panelists using the closed patch test method. Patches were used on eight panelists. This patch was affixed to the back of the panelists, and signs of redness, erythema, and edema were observed for 24 hours.

Cancer cell cultures, still in the form of tissue or cell cultures, are cultured to obtain cell lines or single cells. The culture propagation process was conducted in the Immunology Laboratory of Integrated Research and Testing Laboratory, Gadjah Mada University. After being cultured for approximately three weeks, the cell line was ready for harvest, and sample preparation was made. A toxicity test serves to determine the safe dose of the use of a chemical substance to the test organism. This test is left for 24 hours to determine the effect after dosing. Photosensitizer with a concentration of 1 μM – 50 mM in the sample, after being gone for 24 hours, the sample was reacted with MTT and reagent stopper, and then the absorption was read with an ELISA reader. The percentage of cell death calculated by equation 1, photosensitizer safe dose is the concentration where the percentage of cell death because of the dose is still below 50 %.

$$\%\ cell\ death = \frac{Number\ of\ death\ cells}{Number\ of\ whole\ cell} x100\% \qquad (1)$$

The harvested cell lines were divided into 30 parts and placed in a multi-well plate. Each section comprises 1 mm3 cell line or about 1 x 106 cells. In general, the samples were divided into four major groups: the first group was given laser exposure only; the second group was given standard chlorophyll (3 samples); the third group was given chlorophyll, and the fourth group was modified chlorophyll.

The implementation of the apoptosis test, according to Suwito and Xi [32] is as follows: 1 mL of cell culture is inserted into a tissue culture plate in a well and incubated for 24 hours in a $CO_2$ incubator. After that, 1 mL of the test sample solution was added to the cell culture and incubated for 24 hours. The sampled cell cultures are pipetted and put into flow cytometer tube 48 plus 500 LPBS. The mixture was centrifuged (1500 rpm, 5 min, 40 ºC), and the supernatant was discarded. The cell pellet was washed with 500 L annexin and centrifuged again (1500 rpm, 5 min, 40 ºC). The supernatant was discarded, and the pellet was rewashed with annexin. Into the cell pellet, added 5 L of PI, 45 L of annexin, and 5 L of FITC and incubated for 30 minutes. The number of dead cells is calculated by Equation 1 above.

## III. Result and Discussion

### *A. Chlorophyll a Extraction and Isolation from E.dulcis*

Extraction and isolation of chlorophyll as a photosensitizer were carried out in the Basic Science Laboratory, Faculty of Mathematics and Natural Sciences, Lambung Mangkurat University, and the MCRPP Laboratory, Ma Chung University. *E.dulcis* leaves cut into small pieces and dried in the open air as much as 250 grams (Fig.2a), were extracted by maceration using methanol for two days. The methanol extract was filtered using a rotary evaporator to obtain a thick extract of 25.1 grams of methanol. The thick methanol extract was diluted with 100 mL of methanol and partitioned using n-hexane with a ratio of up to 1:6. The purpose of this partition was that because chlorophyll tends to prefer non-polar solvents, the non-polar solvent used is n-hexane so that from this process will be easier to separate in vacuum liquid chromatography.

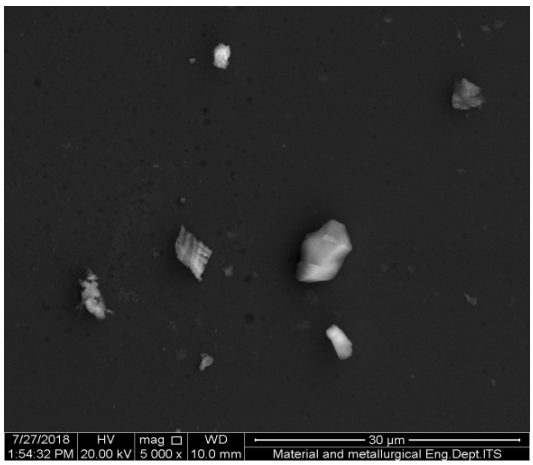
The n-hexane extract was then evaporated using a rotary evaporator to obtain a thick extract of 2.5 grams of n-hexane. The partition process with a separating funnel and evaporation with a rotary evaporator. The viscous extract obtained was then separated by vacuum liquid chromatography method using n-hexane-acetone as eluent [33]. The viscous extract is absorbed on silica gel G60 Merck 7733 and placed on 37 grams of silica gel GF 254 Merck 7730. It is packed in a vacuum liquid chromatography column with a diameter of 10 cm. Elution was carried out every 40 mL by increasing the polarity gradient from 100 % n-hexane to a ratio of n-hexane-acetone 8:2 by column chromatography then the chlorophyll extract was separated to obtain pure chlorophyll a as shown in Fig.2b. Column chromatography results obtained pure chlorophyll with a purity of 96 % as shown in Fig.2c. The isolated chlorophyll has met the requirements as pure chlorophyll since it is more than 96 %, so it fulfilled as a photosensitizer candidate in PDT.


(a) *E.dulcis* leaves (b) Extraction result (c) Chlorophyll column chromatography

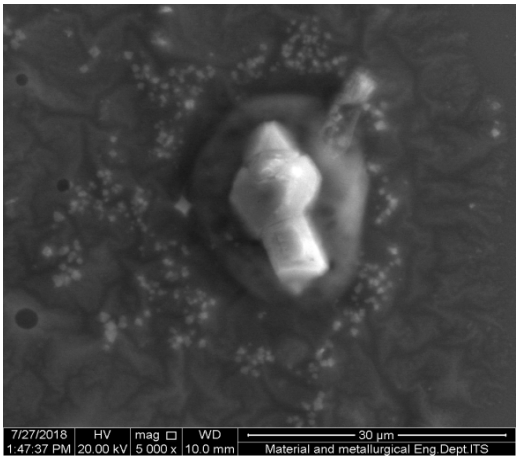
Fig. 2 *E.dulcis* leaves, extracted chlorophyll as a source and its Column chromatography

*B. Chlorophyll Albumin-coated as Biomaterial Patch*

Chlorophyll 0.1 mg and albumin 0.1 mg were dissolved in 0.02 ml chloroform and evaporated on a thin film. The thin film was washed with nitrogen to remove chloroform. Phosphate buffered saline (PBS) (1 mL) was put into the flask and stirred for 30 minutes, followed by sonification for 15-20 minutes. After sonification, the solution was stored at 4 ºC and in the dark [21]. The results obtained from the coating showed that the chlorophyll began to be coated based on observations with SEM in Fig.3. It showed that albumin encloses chlorophyll particles with a size of about 200 nm – 5 µm. This size will affect the cancer system with a core size of 5 µm, so the photosensitizer system will be more accessible to enter to interfere with the existing cancer system. Photosensitizer coated with albumin will increase hydrophilicity due to the lower reaction time with water.




(a) Chlorophyll isolate




(b) Chlorophyll coated albumin

Fig. 3 Chlorophyll seen from SEM

*C. Standardized Biomaterial Patch*

*1) Stability and homogeneity:* The test was carried out on samples stored in room conditions with a temperature of 25 ± 2 ºC and Rh 75 ± 5 % to store products with claims of storage at cool temperatures. Finished goods are retained samples for up to one year after expiry [34]. The stability of the chlorophyll coating between 0 and 6 months is persistent; only the absorption intensity decreases very small from 3.2 to 3.0. In the 6th month, the absorption intensity at a wavelength of 658 nm indicates no shift wavelength, so the albumin-modified chlorophyll in the analysis was stable.

The spectra of chlorophyll coating and chlorophyll isolate show that the O-H group contained in the chlorophyll coating is a solvent that does not contain chlorophyll isolate. In addition, N-H absorption was found at 3500 – 3100 $cm^{-1}$. While there is a C=C double bond in the coating, the absorption of 1680-1600 cm-1 is not wide but tapered, which is estimated to be C=C. Differences in interactions between the central atom of the ligand molecule can cause the difference in absorption between coating chlorophyll and isolated chlorophyll. The FTIR results between chlorophyll coating and isolates showed differences, especially at 3500 – 3100 $cm^{-1}$ for isolates. There was no wave number since in a pure state, the OH content was very small for chlorophyll coating at that wave number because there was a solvent, namely distilled water, so suspension between chlorophyll and albumin will be present at that wave number.

*2) Toxicity:* A toxicity test determines the safe dose of using a chemical substance to the test organism. This test was carried out for 24 hours to determine the effect that occurs after dosing. The photosensitizer was used with various concentrations after 24 hours. The sample reacted with MTT and reagent stopper, and then the absorbance was read with an ELISA reader. The percentage of cell death is calculated by equation (1). The safe dose of photosensitizer is the concentration where the percentage of cell death due to the effect of the dose is still below 50 %. OD is the optical density of the ELISA readings.

Table 1 shows that the synthesized photosensitizer has specific properties for normal cells. The photosensitizer from the coating has the lowest concentration compared to isolates and extracts. Based on the type of cell, normal cells are still safe, while the one that gives a significant effect is MCF7, meaning that the synthesized photosensitizer provides a selective effect.

TABLE I
COMPARISON OF NORMAL CELL TOXICITY IC50, MCF AND T47D

| Photosensitizer | Normal Cell (µL/mL) | MCF7 (µL/mL) | T47D (µL/mL) |
|---|---|---|---|
| Isolate | 208.7 | 4.9 | 295.7 |
| Coating | 194.1 | 0.3 | 7.8 |
| Extracts | 704.7 | 10.1 | 96.3 |

*D. Laser test*

To meet its optimum power of 49 mW, a laser test with a wavelength of 654.3 nm was carried out at a distance between 1.5 and 2.0 cm.

*1) Photosensitizer spectrum test:* The photosensitizer absorbance test was carried out using a UV-Vis spectrophotometer. This study carried out the absorbance test on the photosensitizer chlorophyll extraction, chlorophyll a Machung, Chlorophyll isolated from *E.dulcis,* and chlorophyll from the modified product. The photosensitizer material was diluted with DMSO, and the absorption wavelength was measured at 400-700 nm. The results of chlorophyll a's absorbance in Fig. 4 show that the maximum is at the blue and red wavelengths.

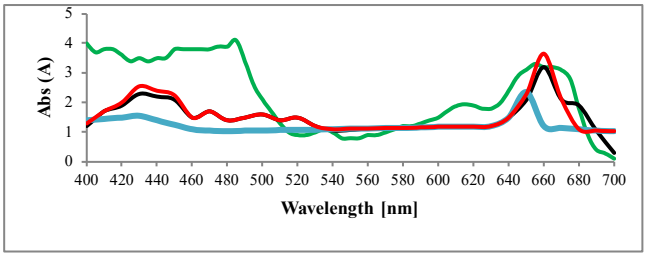



Fig. 4 Absorbance difference of photosensitivity on four types of chlorophyll: green for chlorophyll extract, blue for chlorophyll Ma Chung, black for chlorophyll coating, and red for chlorophyll isolate.

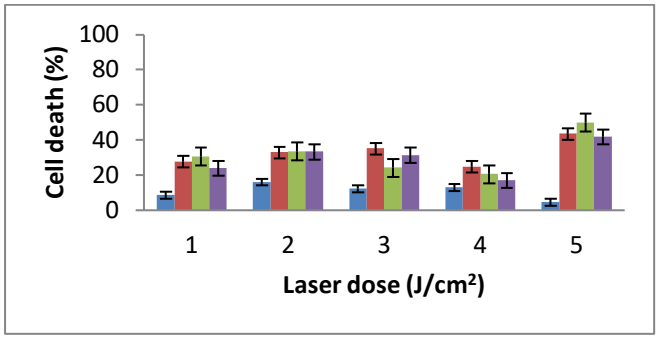
These results are by the research of Milenković et al. [33] which stated that porphyrin-derived chlorophyll has two maximum peaks in the visible region: red (Q band) and blue (B band). The chlorophyll extraction results obtained the same pattern as the literature results, but there were still many other peaks due to the chromophore in other substances contained in the extract. The isolates showed that the maximum peak was at 658 nm, while the other peaks were very small. UV-Vis spectrophotometry results showed a peak at 658 nm, which met the requirements of a photosensitizer. The requirement for a good photosensitizer is to have strong absorption at 600-800 nm [10] compared to previous researchers that the optimum absorption intensity is at 662 nm [34]. These results indicate that there is no significant difference between the results of the study and previous studies.

*2) Quantum Yield and Coefficient of Function:* The lowest exigency coefficient value was 12000 (L/g.cm) for Ma Chung chlorophyll at a wavelength of 632 nm, while the highest was extract at 32300 (L/g.cm) at 658 nm. The requirement for a good photosensitizer is to have an extinction coefficient of 50,000–100,000/M cm [34] the values obtained indicate that the extinction coefficient is still below the given requirements, so additional concentrations or distances are needed to increase the extinction coefficient value.

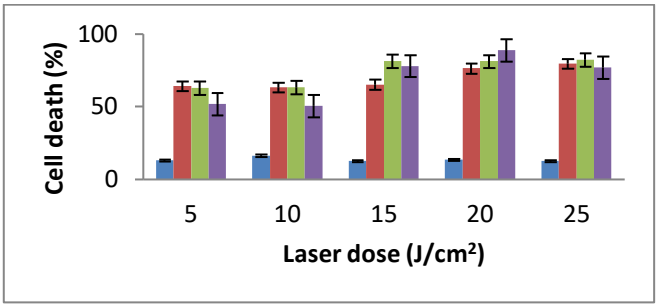
### *E. Laser Exposure to Melanoma Cancer Cell Apoptosis*

Laser exposure on cancer cells begins with placing the laser at a distance of 2 cm according to the optimal laser power characterization and the laser beam corresponding to one of the 96 plates. Then, the tool is set up and cleaned with alcohol before exposure. Laser exposure to cancer cells was carried out in a laminar flow to keep the cells sterile. Cells incubated with chlorophyll photosensitizer were placed in the sample compartment; cells were then exposed to energy doses of 5, 10, 15, 20, and 25 J/cm$^2$ with three energy doses each.

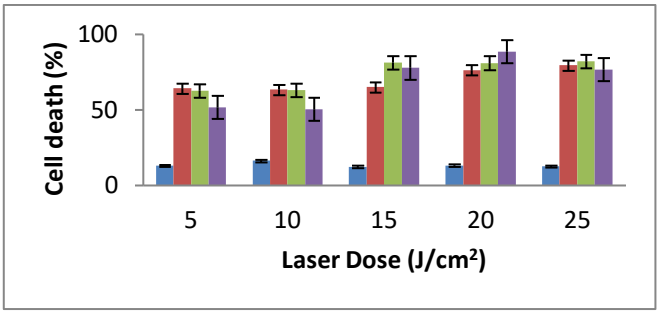
The laser movement is adjusted automatically with variations in the exposure time depending on the laser dose. The Optical Density (OD) measurement results show that using the three photosensitizers in normal cells resulted in relatively low cell death, up to 43 %. This indicates the effect caused by chlorophyll as a photosensitizer is still suitable for normal cells and does not cause harmful effects for normal cells. Meanwhile, the results of OD measurement show that in both MCF7 and T47D cells, using the three photosensitizers, there is high cell death, up to 88 % and up to 85 %, respectively. Both results indicate the effect caused by chlorophyll as a photosensitizer is decent for MCF7 and T47D cells and causes significant cell death (Fig. 6). Fig. 5 shows that the optimum laser dose with the addition of a photosensitizer is at 20 (J/cm$^2$) with the highest percentage of cell death.




(a) Normal cell apoptosis percentage




(b) MCF7 cell apoptosis percentage




(c) T47D cell apoptosis percentage

Fig.5 Apoptosis percentage of laser only (blue), laser + chlorophyll coating (red), laser + chlorophyll isolate (green), and laser + chlorophyll extract (violet) on three different types of cells

It also performed eight tests on apoptosis melanoma cells for normal and cancer cells. Those tests are with photosensitizer except for without photosensitizer (TK) and laser exposure without photosensitizer (TK+L). Those with photosensitizer are chlorophyll coating (KC), chlorophyll coating and laser exposure (KC+L), chlorophyll isolate (KI), chlorophyll isolate and laser exposure (KI+L), chlorophyll extract (KE), and the last is chlorophyll extract and laser exposure (KE+L). Test results are detailed in Table 2.

Table 2 demonstrates the effect of exposure to a dose of laser energy on optimum cell death at a dose of 20 J/cm2, where the highest cell death occurs with the addition of a photosensitizer. At least there are three types of PDT treatment that have promising results. Firstly, KI+L (chlorophyll isolate and laser exposure) treatment results for apoptosis MCF7 cell death are 80 ± 0,8 %, and apoptosis

T47D cell death is 77 ± 0,8 %. Secondly, KC (chlorophyll coating) treatment results for apoptosis MCF7 cell death are 89 ± 0,9 %, and apoptosis T47D cell death are 85 ± 0,9 %. Finally, with the best result, KC+L (chlorophyll coating and laser exposure) treatment results for apoptotic MCF7 cell death are 90 ± 0,8 %, and apoptosis T47D cell death is 86 ± 0,7 %. It indicates that PDT containing albumin-coated *E.dulcis* chlorophyll as a biomaterial patch and organic photosensitizer can increase the death of melanoma cell cancer.

TABLE II
APOPTOSIS CELL DEATH PERCENTAGE OF NORMAL CELL, MCF7 CELLS AND T47D CELLS UNDER DIFFERENT TYPES OF TREATMENT

| Treatment types | Normal Cell (%) | MCF7 (%) | T47D (%) |
|---|---|---|---|
| TK | 5 ± 0.1 | 10 ± 0.1 | 12 ± 0.2 |
| TK+L | 25 ± 0.5 | 28 ± 0.5 | 27 ± 0.3 |
| KC | 20 ± 0.6 | 89 ± 0.9 | 85 ± 0.9 |
| KC+L | 25 ± 0.5 | 90 ± 0.8 | 86 ± 0.7 |
| KI | 30 ± 0.8 | 75 ± 0.7 | 75 ± 0.6 |
| KI+L | 35 ± 0.7 | 80 ± 0.8 | 77 ± 0.8 |
| KE | 28 ± 0.5 | 65 ± 0.6 | 64 ± 0.7 |
| KE+L | 35 ± 0.5 | 68 ± 0.6 | 72 ± 0.7 |

## IV. CONCLUSION

The use of chlorophyll extracts, isolates, and surface-modified chlorophyll was tested by the requirements as a new photosensitizer on normal cells, MCF7, and T47D cancer cells. It provided scientific data in the study of surface-modified chlorophyll *E.dulcis* with a percentage of purity up to 96 % as a new photosensitizer candidate for therapeutic applications. Chlorophyll as a photosensitizer coated with the surface modification method of chlorophyll isolate of E. dulcis with albumin has stable properties and high hydrophilicity, as evidenced by the test results in the short term, namely six months. The structure and absorption wavelength are still at 658 nm. The chlorophyll of *E.dulcis* produced has low toxicity. PDT with a laser energy dose of 20 J/cm$^2$, along with the addition of a photosensitizer on melanoma cell cancer, reached the highest percentage of apoptotic cell death for both MCF7 and T47D at 90 ± 0,8 % and 86 ± 0,7 %, respectively. In general, PDT based on albumin-coated E.dulcis‘s chlorophyll as a biomaterial patch and organic photosensitizer is potentially increasing the death of melanoma cell cancer.

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