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Transformation Chlorophyll a of *Spirulina platensis* to Chlorin e6 Derivatives and Several Applications

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Keywords: *Spirulina platensis*; Chlorin e6 trimethylester; Photodynamic therapy; Hela cell; Antibacterial

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Abstract

BACKGROUND: *Spirulina platensis* contains a large amount of chlorophylls, chlorophyll a, that are starting materials to synthesize functionalized chlorins. Chlorin e6 (Ce6) as well as its derivatives are second generation sensitizers using in photodynamic therapy (PDT) of various cancers. In this study, we transfer chlorophyll a of *S. platensis* to Ce6 derivatives and determine their several applications.

AIM: We aimed to evaluate the effects of Ce6 derivatives to treat cancer cells.

METHODS: Ce6 trimethylester was created from methyl pheophorbide a2 in *S. platensis* provided by the Hidumi Company, Nghe An province, Viet Nam. Hela cells were incubated with Ce6 trimethylester and the irradiated with the diode laser dose of 1.2 J/cm^2/min through the system of filters £ 650 nm. MTT assay and clonogenic assay were used to determine survival rate and cloning efficiency of cells. Antimicrobial effect of Ce6 trimethylester with halogen light were studied with *Propionibacterium acnes* VTCC 0218 and *Staphylococcus aureus* VTCC 0173.

RESULTS: From dry biomass (700 g) of *S. platensis*, after extracting chlorophyll a and methanolysis, 4.2 g of methyl pheophorbide a was obtained. The reaction to give Ce6 trimethylester with 82% yield was performed with potassium hydroxide (KOH) in MeOH/THF/CHCl3. After irradiation with a 650 nm laser at 1.2 J, the cell viability in all samples decreased with Ce6 trimethylester treatment, the survival declining trend of Hela cells treated with Ce6 trimethylester were proportional when concentration of Ce6 trimethylester increased. The rate of colony formation was declined as the concentration of Ce6 trimethylester treated was increased. The growth of both *S. aureus* and *P. acnes* can be inactivated by Ce6 trimethylester PDT. The MIC99 value against *P. acnes* VTCC 0218 and *S. aureus* VTCC 0173 of Ce6 trimethylester with halogen light was 1.25 µg/ml.

CONCLUSION: The Ce6 trimethylester from *S. platensis* cultivated in Viet Nam could be used as a potential photosentizer for photodynamic therapy for treatment of cancer and acne.

Introduction

Spirulina platensis has been used for production nutritional supplements, functional foods, cosmetics as well as other biomaterials [1], [2]. *Spirulina* contains the very large amount of chlorophylls, specially chlorophyll a [3]. Chlorophylls are starting materials to synthesize functionalized chlorins that have the ability to improve the photodynamic activity [4]. Chlorophyll a, as a natural chlorin, is a potential renewable resource to product chlorin photosensitizers [5] which can use for photodynamic therapy to treat cancer. Chlorins, that are a class of tetrapyrroles derived from plant, are potential photosensitizers because they can absorb remarkably strongly in the light with high phototoxic red spectrum [6].

Photodynamic therapy (PDT), is a treatment therapy for cancer, uses some photosensitizers [6]. The photosensitizer molecules have singlet state with two electrons with opposite spins. They are activated by absorption a photon of light with appropriate wavelength to transfer from the excited singlet state to more stable excited triplet state. The photosensitizers in triplet state can participate in photochemical reactions with oxygen to form reactive oxygen species (ROS) which can destroy pathogenic bacteria, tumors,

cancer cells and target tissues [7]. Ce6 that is synthesized readily from natural chlorophyll is a second-generation photosensitizer. It can absorb strongly in the light with red spectrum. Ce6 and its derivatives have the ability to accumulate more effectively in tumors than normal cells, to absorb at longer wavelengths (670 nm) more strongly to be clear faster from organism so they can be used as second generation sensitizers for PDT of various cancers. It was report using in PDT successfully, namely C6-mediated PDT (Ce6-PDT), to treat some various cancer types such as nasopharyngeal cancer, bladder cancer as well as melanoma [8], [9].

Cervical cancer that is caused by infection of the human papilloma virus is the third common cancer in women in the word [10]. This infection of virus persistently causes intraepithelial transformations, following form a cancer if left untreated. The PDT can be an alternative method for the traditional invasive treatment to treat cervical cancer [11], [12].

Therefore, using PDT with Ce6 photosensitizer can be a potential treatment for cervical cancer. In the present study, Ce6 that was obtained from chlorophylls of *S. platensis* cultured in Viet Nam were used to evaluate the effects in Hela cells. Our findings might provide experimental evidence to support Ce6-PDT in treatment for cancerous cervical tumor.

Materials and Methods

Isolation of methyl pheophorbide a2 and transferred it to Chlorin e6 trimethylester

Chlorophyll was extracted and purified from dried *S. platensis* powder provided by the Hidumi Company, Nghe An province, Viet Nam. Isolation of methyl pheophorbide a2 as a mixture of 13^2R:13^2S diastereomers from *S. platensis* was carried out by the method of Bauer D *et al*., (2019) [5]. *Spirulina* powder (700-800 g) was taken into a Sohxlet thimble (length 32.5 cm, diameter 8 cm). Then add acetone (500 ml) Sohxlet thimble to soaks up all the *Spirulina* powder inside the thimble and add liquid nitrogen (2.5 liters) slowly into thimble. The Soxhlet thimble was put in Soxhlet extractor, and let the thimble returned to 10-20°C. Acetone (2.0 liters) was added into extractor. *Spirulina* powder was extracted 24 hours under an Ar atmossphere, 75 extraction cycles. The acetone extract was filtered and distilled vacuum to recover acetone. Then, add CH_2Cl_2 (200 mL) into the concentrated extract container and continue the vacuum distillation to remove the water in the mixture. Methyl pheophorbide a in the mixture was extracted and purified on chromatographic column. The methyl pheophorbide was crystallized to give dark green crystals.

Methyl pheophobide a was transferred to Ce6 trimethylester by two methods: (1) Methylphephorbide a (2.4 g, 2.8 mmol) was dissolved under an Ar atmosphere in degassed dry THF (25 mL) and $CHCl_3$ (125 mL) was added followed by 1 M KOH in MeOH (12 mL) and (2) methylpheophorbide a (186.5 mg, 0.25 mmol) was dissolved under an Ar atmosphere in THF (12 mL) and then MeOH (125 mL) was added followed by $KOCH_3$ in MeOH (2.5 mL 35% solution). Then, they were treated according to method of Bauer D *et al*., (2019) [5].

Hela cell culture

Hela cell line was purchased from Sigma Chemical Company (St Louis). The cells were seeded in 12-well plates containing E'MEM medium (GIBCO, serum-free medium) supplemented with L-glutamine (200 mM), HEPES (1 M), amphotericin B (0.1%), penicillin-streptomycin 200 x and 10% (v/v) FBS. The cells were cultured under a humidified 5% CO_2; at 37°C to achieve ~90% confluency was used in the photodynamic and laser treatment.

Treatment cells with laser light

The cells were incubated with 0.5, 1, 2.5, 5, 10 µg/ml of Ce6 trimethylester in serum-free E'MEM for 2, 4, 6, 8, 10 hours in dark at 37°C in humidified 5% CO_2. After that, the cells were twice washed with RPMI and irradiated with the diode laser dose of 1.2 J/cm^2/1min through the system of filters £ 650 nm. Control cells were incubated with serum-free E'MEM without Ce6 trimethylester for equivalent times. Treatment cells were trypsinized and washed by PBS for measuring the specific fluorescence intensity of Ce6 trimethylester by flow cytometry, determined the cell survival by MTT assay and colony formation assay to evaluate the long-term proliferative potential of Hela cells following photodynamic therapy.

Flowcytometry

The accumulation and removal of Ce6 trimethylester in the cells was studied with the flow fluorometer FACS Canto II (Becton Dickinson, USA). The cell suspension was omitted at the rate 500 cells/s in the flow solution under the argon laser emitting excitation light at wave length 488 nm. The data was analyzed with the use of FACSDiva verion 2.1 sofware.

MTT assay

The cells were trypsinized after being washed by PBS and then was resuspended in E'MEM medium with 10% FBS. 10 µl MTT solution (2 mg/mL) was added and incubated for 4 hours at

37°C in 5% CO_2. The crystals formed were dissolved within one night by addition 25% SDS solution and using a shaker overnight. The result was recorded by microplate reader at wavelength 540 nm. The rate of cells survival (%) was calculated by formula: [1-(OD of treatment cells/OD of control cells)] x 100.

Clonogenic assay

The treatment and trypsinization cells were seed in E'MEM medium with 10% FBS, the experiment was carried out in five replicates. The cells were incubated at 37°C in the environment with 5% CO2 for colony formation. The colonies were dried then stained with Giemsa for 30 minutes; the ones containing more than 50 cells were counted manually. Cloning efficiency was determined by the equation:

$$\text{Relative clone formation rate (\%)} = \frac{\text{colony number of treatment group}}{\text{colony number of control group}} x100\%$$

Antimicrobial effect of TME with halogen light

Propionibacterium acnes VTCC 0218 and *S. aureus* VTCC 0173 from the Vietnam Type Culture Collection were treated with Ce6 and halogen light to determine antimicrobial effect of Ce6. *P. acnes* VTCC 0218 and *S. aureus* VTCC 0173 strains were cultured and treated with TME in condition with or without light irradiation. The bacterial suspensions with concentration 10^6 CFU/ml were added in 96-well microtiter plate containing TME with different concentration and irradiated with halogen light (12 V/50 W; 30,000 lx) for 30 min. Incubation proceeded at 37°C, for 12 h, in optimal aerobic conditions for each bacterial strain. Absorbance at 620 nm of bacterial suspensions after treatment was measured to determine bacterial growth. MIC99 is the TME concentration at which bacterial growth was more than 99% inhibited.

Results

Transformation chlorophyll a of S. platensis to Ce6 derivatives

Ce6 derivatives were obtained by transformation of chlorophyll a using standard protocol described by Bauer D *et al.,* (2019). Briefly, 900 g of dry biomass of *S. platensis* containing more than 0.4% of chlorophyll was subjected into a 10 mm x 38 mm Soxhlet thimble with 400 mL of acetone in addition of nitrogen liquid. The solvent was then refluxed using Soxhlet extractor, under Ar atmosphere for 24 h to ensure 70-75 of deep green chlorophyll extraction cycles. The extract was filtered and acetone was removed by evaporating. The water containing residue was dissolved in 250 mL of CH_2Cl_2 and the water was removed in a rotavapor. The residue was then dried, suspended in 750 mL of dry MeOH and 37.5 mL of H_2SO_4 and stirred at ambient temperature for 20 hours. 500 mL of CH_2Cl_2, 1000 mL of H_2O, 250 mL of saturated NaCl and 350 ml of $NaHCO_3$ were added and the aqueous layer was extracted three times with 250 mL of CH_2Cl_2, washed twice with H_2O, then filtered and evaporated. The red crude extract was dried and dissolved in 35 mL of CH_2Cl_2 and eluted in column chromatography (200 g silica gel, 32–63 mm, 60 Å). The main fraction of methyl pheophorbide a was recovered after elution with CH_2Cl_2 and CH_2Cl_2: acetone (30: 1). The minor fraction of methyl pheophorbide a was obtained by elution with H_2SO_4/MeOH.

The dark green crystal of methyl pheophorbide a was obtained by crystallization from CH_2Cl_2/MeOH (1.5: 10) of the two fractions to form 89: 11 mixture of 132R: 132S diastereomers and the final yield was 4.95 g (0.55% of biomass), mp 226-227°C. TLC (Alox, CH_2Cl_2/Acetone: 10/0.4) Rf = 0.58. IR (KBr): v = 3377 cm^{-1} (w, N-H), 2958 (w), 1734 (s, C- = O, ester), 1701 (s, C = O, ester), 1619 (m, C = C, aromatic), 1556 (w), 1498 (w), 1432 (w), 1365 (w), 1345 (w), 1297 (w), 1213 (s, C-O-C, ester), 1164 (s,br, C-O-C, ester), 1122(w), 1035 (w), 990 (w), 909 (w), 895 (w), 829 (w), 752 (w), 671(w). UV/VIS (THF): λmax (Ɛx10-3) = 321 (21.64), 411 (109.05), 505 (10.39), 535 (9.98), 610 (8.72), 670 (45.55). MS: (EI, 70 eV, direct, T = 200°C): m/z (% rel. intensity) = 606 (100) [M+], 548 (28), 459 (16), 236 (10), 44 (39), 28 (100) [C2H4]+. MS: (ESI, positive, CH_2Cl_2/MeOH 1:10): 607.3 [M + H]+, 629.2 [M + Na]+, 645.2 [M + K]+. MS: (ESI, negative, CH_2Cl_2/MeOH 1:10): 605.2 [M – H]-

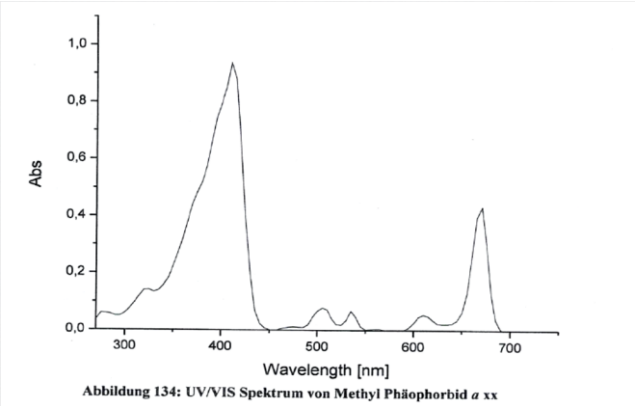

Figure 1: UV/V is spectrum of Methyl pheophobide a in MeOH

The 2.4 g of the first variant (variant A) of methyl pheophorbide a 2 was dissolved in 25 mL dry THF, 125 mL $CHCl_3$ and 12 mL 1 M KOH in MeOH was added. The mixture was stirred and poured into 500 mL ice water. After extraction twice with 100 mL of diethyl ether, the combined organic layer was

washed four times with water and filtered. The solvent was removed and the residue was purified by column chromatography (200 g silica gel, 32 – 63 mm, 60 Å). Blackblue crystals of Ce6 were obtained by crystallization of residue from Acetone/MeOH. Yield 1.84 g (77%), mp 210°C. 186.5 mg of the second variant of methyl pheophorbide a 2 was dissolved in 12 mL of THF, then mixed in dark with 125 mL of MeOH and 2.5 mL of $KOCH_3$ in MeOH 35% for 30 min in ambient temperature. 400 mL of water was added an extraction with 100 mL diethy ether will be carried-out. The organic extracts were washed with water for 4 times and filtered. The solvent was eliminated and the crude product was purified by column chromatography (40 g silica gel, 32–63 mm, 60 Å). The blackblue crystals of Ce6 trimethyester 3 were obtained after crystallization of the fraction from acetone/MeOH. Yield 151 mg (80%), mp (acetone/MeOH) 207 – 208°C. TLC (silica gel, CH_2Cl_2/EtOAc, 7:1): Rf = 0.58. IR (KBr): v = 3298 cm^{-1} (w, N-H), 2953 (m, C-H), 2916 (s, C - H), 1726 (s), 1600 (s), 1440 (m), 1243 (m), 1165 (m), 1063 (m). UV/VIS (MeOH): λmax (ε) = 303 nm (9543), 401 (126760), 501 (21134), 605 (11775), 660 (50134). MS: (EI, 70 eV, T = 200°C): m/z (% rel. intensity) = 638 (100) [M+], 579 (15), 565 (27), 479 (21), 289 (6), 236 (8) MS (DCI, negative, NH_3, 8 mA/sec): m/z (%) 641 (2) 640 (14), 639 (51), 638 (100) MS: (ESI, positive, CH_2Cl_2/MeOH 1:10): 639.2 [M + H]+. MS: (ESI, negative, CH_2Cl_2/MeOH 1:10): 637.3 [M – H]-

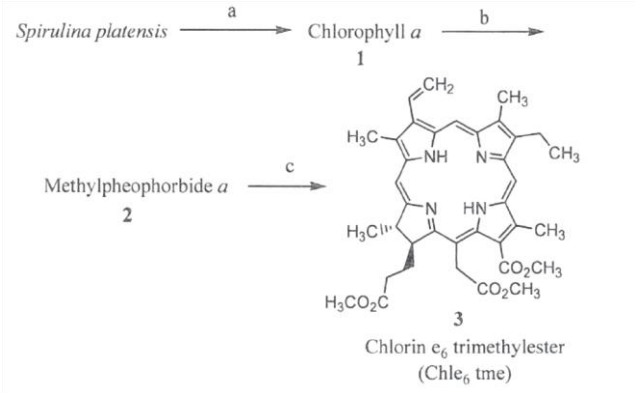

Figure 2: Chlorophyll a extraction from S. platensis and its transformation into Ce6 trimethylester; A) Dry spirulina mass, acetone, N2 (liquid), ca, 60 min, then warm up to room temperature, acetone, reflux, 24 hours; B). H_2SO_4 conc, MeOH, Ar, rt, 20 hours. (c). 1M KOH in MeOH, THF/$CHCl_3$, Ar, rt, 30 min (73%) or KOMe, MeOH, acetone, Ar, rt, 30 min, 85%

Efficacy of Ce6 trimethylester for photodynamic on Hela cells

Cellular uptake of Ce6 trimethylester following various concentration and different incubation times was analyzed based on the fluorescence of Ce6 trimethylester, using fluorescence microscopy and a flow cytometer. In Figure 3, Ce6 trimethylester treated Hela cells had a strong red color, the fluorescence intensity rapidly increased at the few hours after Hela cells were incubated with various concentration of Ce6 trimethylester and reached a relatively high level at 8 hours after incubation. The result in Figure 2 suggested that 4 hours may be the optimal incubation time of Ce6 trimethylester with Hela cells for our experiments. Then, we varied the concentration of each sample from 0.5; 1.0; 2.5; 5 and 10 µg/mL of Ce6 trimethylester and compared the cellular uptake, the cellular uptake of Ce6 trimethylester increased with the concentration, the cells treated with any of Ce6 trimethylester concentrations had higher intensity than untreated control cells.

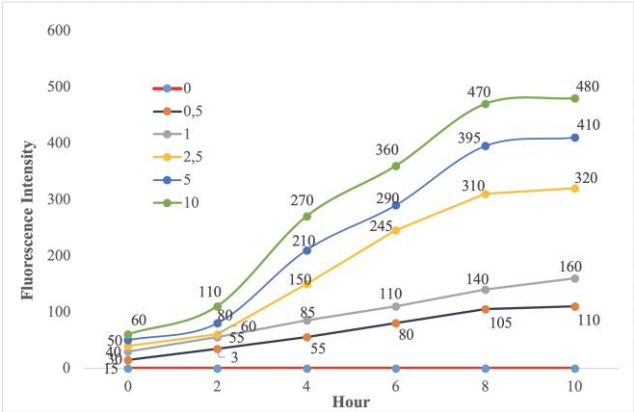

Figure 3: Alterations of Ce6 trimethylester fluorescence intensity in Hela cells at various Ce6 trimethylester concentrations with different incubation time (measured by flow cytometry)

Survival rate of cells was determined by the MTT assay after 8 hours treatment with Ce6 trimethylester (0, 0.5, 1, 2.5, 5, 10 µg/ml, respectively) and the subsequent photodynamic irradiation (Figure 3).

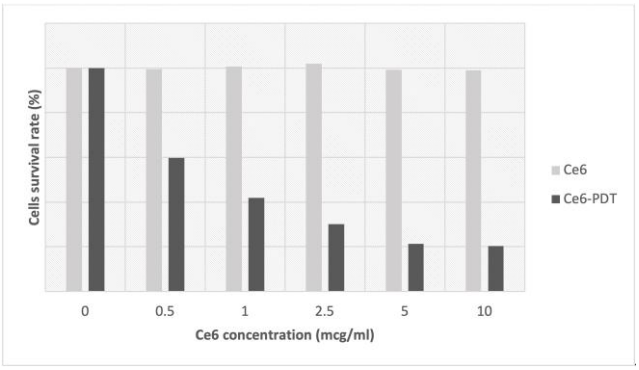

Figure 4: The cell survival rates of Hela cells were assessed by the MTT assay

Hela cells, which was treated with Ce6 trimethylester at corresponding concentrations and without photodynamic irradiation, were used as controls. The results in Figure 3 showed that, different treatment concentrations of Ce6 trimethylester alone could not influence the survival rate of cells. After irradiation with a 650 nm laser at 1.2 J, the cell viability in all samples decreased with Ce6 trimethylester treatment, the

survival declining trend of Hela cells treated with Ce6 trimethylester were proportional when Ce6 trimethylester treatment concentration increased (Figure 4).

Assay of colony formation assay was used to evaluate the ability of proliferation and clonogenicity of single Hela cells following PDT therapy. Following the Ce6 trimethylester treatment at various concentrations, Hela cells without photodynamic irradiation were control groups. The results in Figure 5 showed that the colony formation rate was declined as the Ce6 trimethylester treatment concentration was increased.

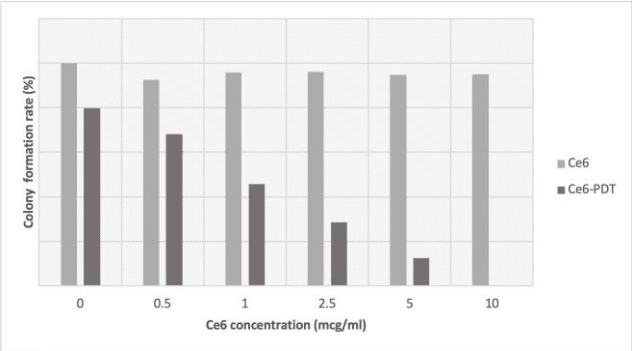

Figure 5: Colony formation assay of Hela after different treatment

Antimicrobial effect of Ce6 trimethylester with halogen light

The antibacterial activity of Ce6 trimethylester with halogen light was tested by the ability of *P. acnes* and *S. aureus* against (Figure 6). TME was prepared at 11 concentrations, include 0.25, 0.5; 0.75; 1.0; 1.25; 1.5; 1.75; 2.0; 2.25; 2.5; 2.75; 3.0 µg/mL. After treatment with TME, halogen light (12 V/50 W, 30000 lx) irradiation had done during 30 min.

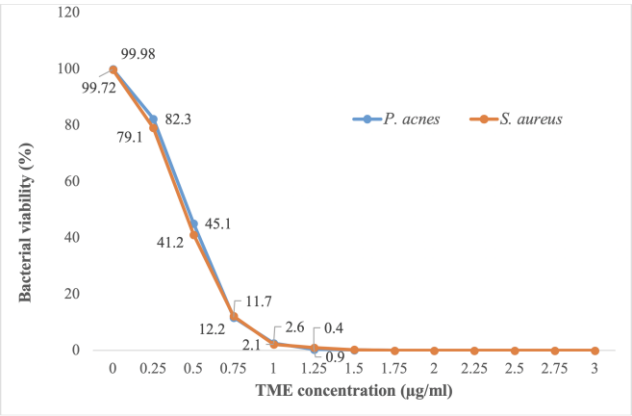

Figure 6: Antimicrobial effect of Ce6 trimethylester using halogen light

The results in Figure 6 indicated that halogen light did not affect the bacterial growth at this condition. The combined treatment TME and halogen light inhibited the growth of bacteria, and this inhibition was dependent on the dose of treatment. The MIC99 value against *P. acnes* VTCC 0218 and *S. aureus* VTCC 0173 of TME with halogen light was 1.25 µg/ml. The results showed that the growth of both *S. aureus* and *P. acnes* can be inactivated by TME PDT.

Discussion

Photodynamic therapy (PDT), which used to treat several diseases (cancers, skin disease, rheumatoid arthritis), includes three essential factors: light, a photosensitizer, and oxygen [13], [14], [15]. These factors are not toxic if they separated; however together they create a photochemical reaction that can generate a highly-reactive product called singlet oxygen [16]. Ce6, as a second generation photosensitizer, is a hydrophilic photosensitizers extracted from porphyrin [17]. It has remarkable characteristics: easy and simple production, selective accumulation ability in target tissues, shorter photosensitizing period,deep penetration into tissues if it is absorbed light having longer wavelength, as well as having minimal side effect [18], [19].

There are three interconnected mechanisms of PDT to treat tumor: (1) direct toxic for the cells of tumors; (2) destroy the vasculature of tumors; and (3) causing a robust inflammatory reaction which can contribute for development of the systemic immunity. These mechanisms have different effects in treatment tumors, which depend on: the dose of photosensitizer used the time interval between photosensitizer administration and light exposure, light flounce rate as well as total light dose. In the present study, the subcellular localization of Ce6 trimethyleste in Hela cells increased with its concentration. These results suggest that the Ce6 trimethylester treatment could inhibit the proliferation of Hela cells as well as decrease the colony formation rate of it, in a dose-dependent manner. In previous report, Ce6 could enhance the production of ROS (reactive oxygen species) and cell apoptosis, inhibit proliferation of the cells, decline abilities of the migration as well as colony formation in SW480 cells. These effects depended on in a Ce6 dose [8].

Antibacterial PDT that used photosensitizer (PS) with laser light may become a potential method to treat acne. Halogen lights that have approximately 35% amount of light emitted falling into the range 600 to 900 nm, emit light spectra similar to sunlight [20] . In this study, we have demonstrated that combination of Ce6 trimethylester and halogen light is a potential method for antibacterial therapy to treat acne. The results showed that Ce6 trimethylester with halogen light has strong antibacterial activity against skin bacteria such as *P. acnes* and *S. aureus in vitro*. With the treatment of 1.25 µg/mL Ce6 with and without irradiation of halogen light, the growth of both *P. acnes* and *S. aureus* were inactivated completely.

In conclusion, the data generated of this study demonstrated that the Ce6 trimethylester obtained from *S. platensis* which was cultivated in Viet Nam could be used as a potential photosentizer for photodynamic therapy for treatment of cancer and acne.

Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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